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Mauro DiNuzzo
Arne Schousboe *Editors*

Brain Glycogen Metabolism

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Mauro DiNuzzo • Arne Schousboe
Editors

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Editors
Mauro DiNuzzo
Center for Basic and Translational Neuroscience
University of Copenhagen
Copenhagen, Denmark

Arne Schousboe
Faculty of Health and Medical Sciences
University of Copenhagen
Copenhagen, Denmark

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Preface

Glycogen is the sole carbohydrate reserve of the brain. Glycogen granules are located in astrocytic processes surrounding neuronal elements, where they can be readily mobilized at times of increased energy need (e.g., during sensory stimulation) or energy failure (e.g., when blood glucose supply is inadequate). Remarkably, the long-held concept that brain glycogen serves solely as an emergency depot has been recently displaced by numerous, unequivocal, and fundamental observations that related its utilization to the support of cognitive functions. Any interference with brain glycogen metabolism affects neurophysiology at multiple hierarchical scales, including cellular (e.g., astrocyte-neuron interactions), network (e.g., neuronal excitability), and system (e.g., learning and memory) levels. Not surprisingly, specific forms of glycogen storage disease are associated with mental retardation, and glycogen has been implicated in aging and several pathological conditions, such as epilepsy, diabetes, and dementia. Glycogen turnover is under dynamic control by neurotransmitters, neuromodulators, and extracellular potassium, or put simply, brain activity.

It goes without saying that this seemed to us a particularly good time to have a book about brain glycogen. While many books have been published about energy metabolism in the central nervous system, none has focused specifically on brain glycogen. Cerebral glycogen metabolism is only briefly touched upon in many neuroscience books, and, with the exception of a couple of special issues, there is a substantial lack of journal titles centered on brain glycogen. It is our opinion that brain glycogen research is more than mature to have a dedicated book. Our idea of such a book has been that of providing an unbiased state-of-the-art summary about the current knowledge of brain glycogen metabolism. The underlying principal aim has been to allow the reader appreciating how the relatively small cerebral glycogen content is so crucial for normal brain function, thereby, fundamentally revisiting the vestigial nature commonly associated with the polysaccharide in the brain. As such, the book fills a gap in current literature. Leading international experts have contributed up-to-date accounts of cerebral glycogen metabolism, covering the most important progresses obtained in the last 25 years. Our intent has been to be as inclusive as possible, especially for the topics that are still without any apparent

consensus. Indeed, while there is no doubt that glycogen is essential to brain function, there are ongoing debates about the relevant biochemical mechanisms. Thus, opposite views normally found as sealed-off compartments in scientific publications finally could coexist in one place. The book chapters accordingly discuss different potential fates for glycogen-derived metabolites and related metabolic pathways that might be implicated in supporting the energetics of neurons and glial cells. Some chapters review the metabolic and functional features of brain glycogen as well as of the brain-specific enzyme isoforms that synthetize and degrade the polysaccharide, which are exquisitely sensitive to small and physiological energy fluctuations. Other chapters cover the localization of glycogen in different brain structures and the relevant metabolic aspects in either white or gray matter. Several chapters describe how physiological and pathological conditions affect glycogen metabolism in the brain. The book also includes historical perspectives that outline how brain glycogen has progressively gained attention as an essential player in cerebral energy metabolism. We believe that the material contained in the book constitutes a good reference about brain glycogen metabolism, which holds a great potential for stimulating further research.

We express our sincere gratitude to all the authors of the book, and we are more than confident that many others will join us, including undergraduate and graduate students as well as researchers and professionals working with or interested in brain glycogen metabolism. All these people will benefit from having the current knowledge of the field at hand. We also thank Springer Science for the continuous support and helpful insights throughout all the stages of putting this work together. Finally, we wish to acknowledge the invited researchers who could not participate to the present endeavor, as many among them have had a pivotal role in developing new ideas and performing critical experiments that advanced the field of brain glycogen metabolism. We hope to have given their work the appropriate credit within the various chapters of the book. One of these researchers is our friend Leif Hertz (1930–2018), to whose memory this book is dedicated.

Copenhagen, Denmark

Mauro DiNuzzo
Arne Schousboe

Contents

Major Advances in Brain Glycogen Research: Understanding of the Roles of Glycogen Have Evolved from Emergency Fuel Reserve to Dynamic, Regulated Participant in Diverse Brain Functions

Gerald A. Dienel and Gerald M. Carlson

Abstract Brain glycogen is extremely difficult to study because it is very labile to physiological status and postmortem autolysis, and glycogen degradative enzymes are rapidly activated by metabolites and signaling molecules. Glycogen is predominantly located within astrocytes in adult brain, and abnormal glycogen metabolism in neurons has lethal consequences. Diverse distribution of glycogen among subcellular compartments suggests local regulation and different functional roles, and recent studies have revealed critically important roles for glycogen in normal brain function and Lafora disease. This brief overview highlights some of the major advances in elucidation of glycogen's roles in astrocytic functions and neurotransmission and the severe consequences of aberrant neuronal glycogen metabolism.

Keywords Astrocyte · Brain · Glycogen · Glycogen debranching enzyme · Glycogen granule · Glycogen phosphorylase · Phosphorylase kinase · Lafora disease (Lafora progressive myoclonic epilepsy, MELF) · Neuron · Neurotransmission

G. A. Dienel (✉)
Department of Neurology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM, USA
e-mail: gadienel@uams.edu

G. M. Carlson
Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA
e-mail: gcarlson@kumc.edu

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Abbreviations

cAMP	cyclic AMP
CMR_{glc}	Cerebral metabolic rate for glucose
CMR_{O2}	Cerebral metabolic rate for oxygen
Glc	Glucose
Glc-6-P	Glucose-6-phosphate
KO	Knockout

1 Introduction

Studies on glycogen metabolism in liver, where it contributes to maintenance of blood glucose, and in muscle, where it supplies energy to sustain contraction, have a long and illustrious history, and many luminaries of science have worked on this polyglucan. Glycogen was discovered in the nineteenth century (1857) by the great physiologist Claude Bernard, who for decades investigated mammalian sugar metabolism (Young 1957). The following year, August Kekulé, a key figure in developing a structural theory of organic chemistry, accurately determined glycogen's empirical formula to be $C_6H_{10}O_5$ (Young 1957), consistent with a polymer of glucose linked via glycosidic bonds. In the twentieth century, work on glycogen metabolism continued, and directly resulted in six Nobel Prizes. At the level of enzymes that act directly on glycogen, Carl and Gerty Cori were awarded (1947) the honor for their work on glycogen phosphorylase, whereas Luis Leloir received the award (1970) for his studies on glycogen synthase. For regulatory aspects of the cascade activation of glycogenolysis, Earl Sutherland was honored (1971) for identifying cAMP as the second messenger linking epinephrine to the activation of glycogen phosphorylase. Finally, their discovery of the stimulation of the catalytic activity of glycogen phosphorylase and phosphorylase kinase through post-translational phosphorylation resulted in the award being presented (1992) to Edmond Fischer and Edwin Krebs. Studies of glycogen metabolism in the twenty first century have been steadily expanding, however, from muscle and liver into the brain, where glycogen seems to play different and sometimes surprising roles.

As an important energy reserve in brain, glycogen is predominantly localized in astrocytes, with very small amounts in neurons. Regulation of astrocytic glycogenolysis by neurotransmitters integrates astrocytic metabolism with neuronal signaling, and dysregulation of glycogen turnover causes severe epilepsy and, ultimately, death of patients with Lafora disease. Glycogen fuels K^+ uptake into cultured astrocytes in the presence of adequate levels of glucose, revealing a role in the energetics of control of the extracellular ionic milieu during neurotransmission. Astrocytic glycogen mobilization during brain activation can also spare blood-borne glucose for use by neurons as energy demand is increased. Glycogenolysis is required for learning and memory consolidation, but the mechanisms remain to be established. Putative functions (that are not mutually exclusive) include fuel for astrocytes,

precursor for astrocytic neurotransmitter glutamate synthesis, fuel for neurons via shuttling of glycogen-derived lactate, and release of lactate to act as a signaling molecule that can regulate neuronal activity (Bergersen 2015; Bozzo et al. 2013; Tang et al. 2014). Our understanding of functions of brain glycogen, its metabolism, and its roles in pathophysiology is far from complete, thus brain glycogen is an important topic for further study.

Characterization of the levels, turnover, location, and functions of brain glycogen actually has a long history marked by major advances in methodology followed by slower but steady progress, with technical difficulties being a persistent problem. Initially, global methods were used in conjunction with chemical analyses, but these procedures were vulnerable to lability of glycogen and impurities in the isolated fractions. Development of microanalytical enzymatic methods and specialized tissue freezing and enzyme inactivation techniques enabled detailed quantification of regional distributions of glycogen and enzymes of glycogen metabolism in relation to those of the glycolytic and oxidative pathways. Use of knockout (KO) mice as models for Lafora disease provided remarkable insights into the roles of glycogen in epilepsy. In spite of many constraints, recent studies provide fascinating perspectives of the participation and regulation of glycogen in normal brain functions and disorders.

This prologue highlights selected discoveries that have had a high impact on brain glycogen research that are discussed in more detail in the chapters in the present volume. Interested readers are also referred to a recent minireview series on brain glycogen in the *Journal of Biological Chemistry* (Prats et al. 2018; Nadeau et al. 2018; Bak et al. 2018; Gentry et al. 2018; Carlson et al. 2018) that also address key aspects of brain glycogen biochemistry and neurobiology.

2 Brain Glycogen as an Emergency Energy Reserve

Glycogen is a macromolecular storage form of glucose, the obligatory fuel for brain, and along with glucose, ATP, and phosphocreatine, glycogen is one of the brain's energy stores. The presence of glycogen in brain was reported in the 1880s, but it was not until the 1930s that brain glycogen was carefully characterized, when classical work was carried out by Stanley E. Kerr and colleagues at the American University of Beirut and published in the *Journal of Biological Chemistry*. Kerr established the requirement for rapid *in situ* freezing of brain to obtain valid values for concentrations of phosphocreatine and lactic acid (Kerr 1935; Avery et al. 1935) and used these procedures to minimize postmortem metabolism of glycogen. He then devised methods to minimize interference from cerebrosides and other contaminates, and reported brain glycogen concentrations (in glucosyl units) of 3.9–5.5 μmol/g (Kerr 1936). Kerr proved that the polysaccharide he purified from brain was, in fact, glycogen, based on elemental composition, optical rotation, quantity of glucose produced by acid hydrolysis, reaction with iodine, melting point of its osazone, and other properties (Kerr 1938). Postmortem metabolism of brain glucose

was found to be more rapid than glycogen, and the concomitant rise in lactate level corresponded to the loss of glucose and glycogen, indicating both to be precursors of lactate (Kerr and Ghantus 1937). Insulin-induced hypoglycemia depleted glycogen in brain, muscle, and liver (Kerr and Ghantus 1936), whereas epinephrine injections, which reduced liver and skeletal muscle glycogen levels, did not affect brain glycogen content (Kerr et al. 1937), nor did short-term exposure to various anesthetics or several convulsion-inducing drugs (Kerr and Antaki 1937). Kerr's pioneering work was the first major technological advance in studies of brain glycogen that had a high impact on the field for more than 40 years. Unfortunately, the necessity to use special procedures to preserve labile metabolites is still not implemented by all researchers.

Rapid depletion of brain glycogen after death led to studies of synthesis and turnover of glycogen, activation of glycogen phosphorylase, and responses of brain glycogen level to various treatments. Re-synthesis of glycogen in brain slices after postmortem autolysis was slow and it could be influenced by experimental conditions, including inhibition by high [K^+] (LeBaron 1955; Kleinzeller and Rybová 1957). Potential physiological roles for glycogen as an *active participant* in brain metabolism were discussed in the early literature, which also reported regional and age-related differences in its concentration (Merrick 1961) and in the levels of enzymes of glycogen metabolism (Breckenridge and Crawford 1961). Studies of glycogen levels and turnover that used acid hydrolysis to degrade polysaccharides and anthrone reagent to assay monosaccharides were sometimes compromised by the presence of non-glucose carbohydrates that turned over more slowly than glycogen (Strang and Bachelard 1971). Metabolic labeling with [^{14}C]glucose has provided considerable information, but rapid turnover of the outer tiers of glycogen influences interpretation of label incorporation or loss in relation to glycogen concentration because degradation coupled with re-synthesis can remove recently-incorporated label and stabilize glycogen concentration. At the local and cellular level in brain, we need to learn considerably more about the control of the number and size of glycogen particles and the regulation of the enzymes involved in glycogenolysis (Nadeau et al. 2018).

A second technological milestone was the enormous improvement in the specificity and sensitivity of quantitative glycogen assays on impure preparations. The Lowry-Passonneau group developed enzymatic methods using phosphorylase (Lowry et al. 1967; Passonneau et al. 1967) or amyloglucosidase (Passonneau and Lauderdale 1974; Lust et al. 1975) to degrade the glycogen, followed by fluorescent-enzymatic assay of glucose-1-phosphate or glucose (Lowry and Passonneau 1972). Their microanalytical analyses of 0.1–1 μg dry-weight samples revealed 300-fold concentration differences in glycogen levels among nine layers of rabbit retina, a much larger range than those of eight other metabolites (Matschinsky et al. 1968). On the other hand, glycogen levels were similar in five layers of mouse cerebral cortex, but higher in subcortical white matter (Folbergrova et al. 1970). After labeling with [^{14}C]glucose, use of phosphorylase, with or without glycogen debranching enzyme, enabled calculation of a 4.4-h turnover time in vivo for total glycogen in normal mouse brain, compared with 0.86 h for the

outer tiers and 8.6 h for limit dextrin (Watanabe and Passonneau 1973). For comparison, the half-life for glucose in non-stimulated rat brain is ~1.5 min (Savaki et al. 1980). Enzymatic assays of glycogen opened the field to a larger group of neuroscientists. Faster turnover of outer tiers raises the possibility that these structural elements are most likely to be involved in moment-to-moment brain functions, and subcellular compartmentation of glycogen in endfeet, perisynaptic structures, and cytosol (see below) suggests localized roles and regulation, but lack of quantitative assays with high temporal-spatial resolution hinders progress.

A third major methodological advance was preservation of labile brain metabolites by new approaches [funnel-freezing (Ponten et al. 1973a; Ponten et al. 1973b), freeze-blowing (Veech et al. 1973), freeze-clamping (Quistorff 1975), and microwave fixation (Medina et al. 1975)], with each procedure having advantages and limitations, including use of anesthesia, stress, and ability to assay regions of interest. It is extremely difficult to fully prevent peri-mortem and postmortem changes in glycogen levels and label content because subject handling, environmental stimuli, and fixation procedures can involve stress- and sensory-related responses that cause glycogenolysis secondary to actions of neurotransmitters, especially norepinephrine released throughout the brain from the locus coeruleus. However, delays in postmortem freezing were useful in revealing the rapid activation of glycogen phosphorylase. For example, 18% of mouse brain glycogen phosphorylase was in the active form (i.e., activity assayed without added AMP as percent of that with 1 mM AMP) in cerebral cortex after in situ freezing through the skull, whereas 70% was in the active form when freezing was delayed for ~1 min (Breckenridge and Norman 1962). Freeze-blowing or freezing through the intact skull also showed ~20% in the active form, whereas it was only 8% after freezing through the intact dura, and it was two-to three-fold higher (~40–60%) after immersion of rats into liquid N_2 or decapitation into liquid N_2 (Lust et al. 1973; Folbergrova et al. 1978). These findings emphasize the significance of Kerr's work and the need for greater understanding of the regulation of brain glycogenolysis.

A fourth methodological advance was the use of ^{13}C NMR to detect glycogen non-invasively, which was first performed in muscle, where the high level of glycogen allows detection of its natural abundance C-1 resonance (Shulman et al. 1990; Jue et al. 1989a, 1989b) Because of the low level of glycogen in the brain this signal is too weak for reliable quantification. Consequently, the glycogen must be isotopically enriched through administration of a labeled glycogen precursor, such as [1-^{13}C]glucose. The tiered structure of glycogen makes labeling of its inner chains somewhat problematic, causing this technique to be better suited for metabolic studies than analysis of concentration. Nevertheless, *in vivo* measurements on rat brain have been performed, and the glycogen reported to be as high as 5.1 μmol/g (Choi et al. 1999), equivalent to the concentrations cited above. A recent study in human brains that utilized prolonged administration of [1-^{13}C]glucose in an attempt to achieve steady-state labeling of glycogen coupled with mathematical modeling based on turnover resulted in an even higher value of 7.8 μmol/g (Öz et al. 2015; DiNuzzo 2013).

Studies in many laboratories over several decades showed: (i) the resting brain glycogen level was relatively stable within the range of ~2–3 μmol/g, i.e., approximating or exceeding brain glucose concentration, with subsequent higher levels of 4–8 μmol/g determined with enzymatic assays in extracts of microwave-fixed brain and *in vivo* ^{13}C NMR assays, (ii) glycogen was consumed during pathophysiological conditions, e.g., ischemia, spreading cortical depression, seizures, and hypoglycemia, and (iii) glycogen levels increased and turnover slowed during prolonged, deep anesthesia. Of note, glycogen alone can support normal brain activity for only a few minutes: the resting rate of glucose (Glc) utilization (CMR_{glc}, Cerebral Metabolic Rate for the substrate designated in the subscript) in rat cerebral cortex is ~1 μmol/g/min (Sokoloff et al. 1977), and glycogen and glucose are depleted in <1 min during ischemia (Lowry et al. 1964). Considered together, the slow turnover under resting conditions, the relative stability to various normal physiological conditions, the rapid activation of phosphorylase, and the fast disappearance of glycogen during energy failure led to the long-held concept that brain glycogen was mainly an *emergency fuel reservoir*.

3 Cellular Localization of Glycogen in Brain

Astrocytes are the predominant, but not exclusive, brain cell type containing glycogen. Early histochemical assays in rapidly-fixed tissue to minimize autolysis (Shimizu and Kumamoto 1952), and early light and electron microscopic studies identified glycogen granules throughout the astrocytic cytoplasm, including their endfeet that surround the vasculature and in perisynaptic regions. Assays also demonstrated that astrocytic glycogen levels increased after exposure to ionizing radiation (Wolfe et al. 1962; Maxwell and Kruger 1965), prolonged barbiturate anesthesia (Phelps 1972), and treatment with the glutamine synthetase inhibitor methionine sulphoximine (Phelps 1975). Ultrastructural studies of glycogen phosphorylase and phosphorylase kinase demonstrated their predominant distribution throughout astrocytic cytosol, endfeet, and perisynaptic processes, with both brain and muscle isoforms of phosphorylase being co-expressed in the same astrocytes, and the brain isoform being expressed in very few neurons (Richter et al. 1996; Psarra et al. 1998; Pfeiffer-Guglielmi et al. 2000, 2003). Neuronal glycogen was usually undetectable, but with a few exceptions, e.g., neuronal dendrites in the vestibular nucleus were packed with mitochondria and glycogen granules (Sotelo and Palay 1968). More recently, through use of very sensitive assays, low levels of glycogen, glycogen phosphorylase, and glycogen synthase were detected in neurons (Saez et al. 2014). *In vitro* studies in brain slices and cultured astrocytes have been particularly important for studies of regulation and function that cannot be carried out *in vivo*, and they identified many compounds that influenced glycogen metabolism, e.g., β-adrenergic blocking drugs, amphetamine-like compounds, norepinephrine, serotonin, histamine, adenosine, vasoactive intestinal peptide, K^+, Ca^{2+}, and various receptor agonists and antagonists e.g., (Quach et al. 1988; Magistretti et al. 1986; Quach et al.

1978, 1980, 1981, 1982; Hof et al. 1988; Magistretti et al. 1981; Subbarao et al. 1995; Zhang et al. 1993; Subbarao and Hertz 1990). These findings led to linkage of astrocytic glycogen metabolism with neuronal signaling (e.g., glycogenolysis triggered by neurotransmitters) and control of the extracellular milieu (via astrocytic K^+ uptake fueled by glycogen). In contrast, functional studies of normal neuronal glycogen metabolism have barely scratched the surface.

A fifth methodological landmark in studies of glycogen was the use of molecular biological techniques to modify levels of glycogen and enzymes of its metabolism. For example, KO of glycogen synthase caused substantial deficits in cognitive ability, clearly demonstrating a requirement for glycogenolysis in learning and memory (Duran et al. 2013, 2019). Glycogen synthesis in neurons is strongly suppressed, but over-expression of protein targeting to glycogen led to neuronal glycogen accumulation and apoptosis (Vilchez et al. 2007). Animal models for Lafora disease have been created using different approaches: (i) a single KO of malin (an E3 ubiquitin ligase) or laforin (a glycogen phosphatase), (ii) a double KO of both malin and laforin, and (iii) a KO of either malin or laforin in conjunction with elimination of either protein targeting to glycogen or of glycogen synthase. These models have been instrumental in major advances to elucidate roles of malin, laforin, and glycogen in severe epilepsy and death (Gentry et al. 2018).

4 A Major Paradigm-Shift: Active Participation of Glycogen in Normal Brain Functions

The first report of a dynamic relationship between sensory stimulation and glycogenolysis in awake rats came from Swanson and colleagues (Swanson et al. 1992). These researchers showed that label is released from pre-labeled glycogen in two specific brain regions in which tactile whisker movement is known to increase neuronal activity: the whisker-barrel field of somatosensory cortex and the ventromedial thalamus. Glycogenolysis also occurs in the cerebral cortex during and after alerting and generalized sensory stimulation (Cruz and Dienel 2002) concomitant with the poorly-understood phenomenon called ‘aerobic glycolysis’ [i.e., the disproportionate increase in glucose utilization compared with oxygen consumption during brain activation (Dienel and Cruz 2016)]. In addition, the resting brain glycogen levels in ethanol extracts (to prevent autolysis that can occur during routine acid extraction) of funnel-frozen brain from very carefully-handled rats were much higher and more variable than generally reported [in the range of 12 μmol/g with coefficients of variation of 30–40% (Cruz and Dienel 2002)]. Similarly-high levels were also detected in microwave-fixed brain (Kong et al. 2002), with considerable regional and subcellular heterogeneity (Oe et al. 2016). If astrocytes contain most of this glycogen and account for ~20–25% of brain volume (Hertz 2011), their glycogen levels may be on the order of ~48–60 μmol/g, close to that in resting gastrocnemius muscle, ~70 μmol/g (Price et al. 1994). Note that this astrocytic volume

fraction is probably overestimated, and may be in the range of 5–10% (Gundersen et al. 2015), with astrocytic glycogen levels being two- to tour-fold higher than calculated above. Brain glycogen levels are strongly influenced by animal handling, stress, and physiological activity, and label release from glycogen during activation of awake rats exceeded the percent decrease in concentration of unlabeled glycogen, suggesting that turnover of the outer tiers is enhanced during functional activity (Dienel and Cruz 2015; Dienel et al. 2007). Furthermore, inhibition of glycogenolysis caused large, regionally-heterogeneous increases in the local CMR_{glc} during sensory stimulation but not during rest, suggesting compensatory responses to lack of glycogen availability (Dienel et al. 2007) and major, unidentified roles for glycogen during brain activation. These findings clearly demonstrate that glycogen utilization in the presence of an adequate glucose supply occurs in living brain and raise the likelihood of functional compartmentation of astrocytic glycolytic pathways. Cultured astrocytes also consume glycogen in the presence of an adequate supply of glucose, e.g., during uptake of K^+ (Subbarao et al. 1995) and in response to oxidative stress (Rahman et al. 2000). Thus, glycogen is not just an emergency energy reserve; it is a dynamic, regulated participant in brain activity.

Liver glycogen has a key role in whole-body glucose homeostasis, and glucose-6-phosphate (Glc-6-P) derived from glycogen is, in part, dephosphorylated by Glc-6-phosphatase to generate glucose that is released to blood (Nordlie and Foster 2010). Muscle has ~1/40th the Glc-6-phosphatase activity of liver (Shieh et al. 2004) and may produce small amounts of glucose from glycogen (beyond the ~10% produced by the debranching enzyme), but most Glc-6-P is probably further metabolized by glycolysis. There are also reports that brain glycogen is degraded to produce glucose, but painstaking analysis of Glc-6-phosphatase activity in living rat brain (Dienel et al. 1988; Nelson et al. 1985, 1986) and in cultured astrocytes (Gotoh et al. 2000) revealed that its activity is negligible, even though the enzyme protein is detectable. In cultured astrocytes glycogenolysis is accompanied by lactate release to the medium (Dringen et al. 1993). Roles of glycogen in brain, muscle, and liver probably overlap somewhat, but are distinctly different with respect to other brain-specific functions and glucose vs. lactate production.

The fates of glycogen-derived Glc-6-P and pyruvate/lactate in brain *in vivo* under different conditions are still important, unresolved issues. When oxidative stress increases, glycogen is consumed and more Glc-6-P enters the pentose phosphate shunt pathway (Rahman et al. 2000). Metabolic modeling of brain activation led to the hypothesis that elevated levels of glycogen-derived Glc-6-P in astrocytes increase feedback inhibition of astrocytic hexokinase, so more blood-borne glucose is available for neurons (DiNuzzo et al. 2010, 2011). Glycolytic metabolism of glycogen-derived Glc-6-P provides 50% more ATP than glucose because the hexokinase step is by-passed, and rapid glycogenolytic generation of ATP may be important for the energetics of membrane transport processes. The pyruvate can be used by astrocytes as oxidative fuel, as an anaplerotic substrate to synthesize glutamate, glutamine, and aspartate, and/or converted to lactate. When glycogen-derived pyruvate is not oxidized, lactate must be released from these astrocytes to regenerate NAD^+ to sustain elevated glycolytic rates. Some lactate may be metabolized by

other brain cells as fuel, but its oxidation is restricted by the accompanying smaller rise in oxygen consumption (CMR_{O2}) compared with blood-borne glucose utilization (and glycogen consumption that is usually not measured) during activation (Dienel and Cruz 2016). In fact, when the ratio of oxygen-to-glucose utilization (CMR_{O2}/CMR_{glc}) is calculated from arteriovenous differences for oxygen, glucose, and lactate, the ratio falls during activation, and if the glycogen consumed is included in the carbohydrate metabolized, the ratio drops further, suggesting that the metabolites of glycogen are not substantially oxidized. Glycogenolysis is claimed to involve astrocyte-neuron lactate shuttling during memory-evoking events and severe hypoglycemia or aglycemia. However, direct proof and quantification of astrocyte-neuron lactate transfer *in vivo* are lacking and the quantitative contribution of shuttled lactate to neuronal metabolism (versus glucose oxidation) is unknown but probably small (Dienel 2017a, b; 2019a; Dienel and Cruz 2016; Diaz-Garcia et al. 2017; Yellen 2018; Diaz-Garcia and Yellen 2019). The available data indicate that most of the lactate derived from the glucose and glycogen consumed in excess of oxygen is released from brain. Efflux of lactate from brain can occur by release to blood (Cruz et al. 1999) and to perivascular fluid (Ball et al. 2010). It has been proposed that brain lactate can be delivered to lymph nodes by perivascular fluid flow (Lundgaard et al. 2017); however, this conclusion is questioned because appropriate steps required to prevent postmortem metabolism (e.g., microwave fixation, freeze-blowing, or funnel freezing) were not carried out. The animals were decapitated and lymph nodes dissected out so that any glucose and glycogen present in the lymph nodes would be converted to lactate during the postmortem ischemic interval prior to tissue freezing. Furthermore, small molecules (molecular weight <500) in perivascular fluid are predominantly released to blood when the cerebral blood vessels traverse through the cribriform plate and the vessels become fenestrated instead of having tight junctions as in the brain (Bradbury and Westrop 1983). These findings indicate that the lactate recovered from the lymph nodes was predominantly from glycolytic metabolism in the node, not trafficking of lactate from the brain to the nodes. In terms of lactate shuttling within brain, if lactate were quantitatively oxidized, there would be stoichiometric equivalence of glucose plus glycogen utilization and oxygen consumption, which is not the case (Dienel 2017a, 2019a, b). Thus, lactate contributes to the manifestation of aerobic glycolysis when incoming blood-borne glucose cycles through glycogen to lactate that is released from brain. Re-synthesis of glycogen also contributes to aerobic glycolysis because glucose is consumed in excess of oxygen.

Lactate trafficking within brain after generation from glycogen and/or glucose also has potentially important consequences. For example, lactate signaling can have metabolic and transcriptional effects arising from shifts in intracellular pH, due to its co-transport with H^+ (the activity of phosphofructokinase is very pH sensitive (Halperin et al. 1969; Trivedi and Danforth 1966) and from changes in the NADH/NAD^+ ratio (Winkler and Hirrlinger 2015; Garriga-Canut et al. 2006; Kumar et al. 2002) via the lactate dehydrogenase reaction. Receptor-mediated signaling actions can modulate excitatory and inhibitory signaling, cAMP levels, and norepinephrine release (Bozzo et al. 2013; Lauritzen et al. 2014; Mosienko et al.

2015; Tang et al. 2014), thereby governing neuronal activity directly and indirectly. Lactate stimulates cerebral blood flow (Choi et al. 2012; Gordon et al. 2008; Hein et al. 2006; Yamanishi et al. 2006), and its release from astrocytic endfeet secondary to glycogenolysis would help increase fuel delivery to activated brain. These are exciting areas of on-going research that open new vistas to functions of glycogen in brain.

5 Concluding Remarks

There is a strong need for improved understanding of glycogen neurobiology: its composition and life cycle in neurons and astrocytes, differential regulation of enzymes involved in glycogenolysis in both major brain cell types, regulation of glycogen utilization during brain activity via intra- and inter-cellular signaling pathways and regulatory molecules, and consequences of inherited defects in neuronal glycogen metabolism. The reviews in this volume constitute a broad knowledge base from which our understanding of the complexity of glycogen turnover in brain activities and diseases can be extended. They should serve to stimulate advancement in this field by increasing research interest in brain glycogen and attracting application of new methodology and novel experimental approaches to study the normal and pathophysiological roles of glycogen in brain.

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Brain Glycogen Structure and Its Associated Proteins: Past, Present and Future

M. Kathryn Brewer and Matthew S. Gentry

Abstract This chapter reviews the history of glycogen-related research and discusses in detail the structure, regulation, chemical properties and subcellular distribution of glycogen and its associated proteins, with particular focus on these aspects in brain tissue.

Keywords Glycogen structure · Glycogen architecture · Polyglucosan · Starch · Amylopectin · Pompe disease · Lafora disease · Glycophagy

Abbreviations

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Atg	Autophagy-related
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CBM	Carbohydrate binding module
CLD	Chain length distribution
CNS	Central nervous system
DP	Degree of polymerization
EM	Electron microscopy
ER	Endoplasmic reticulum
GAA	Acid α-glucosidase
GABARAPL1	GABA type A receptor associated protein like 1
GBE	Glycogen branching enzyme
GDE	Glycogen debranching enzyme
GH	Glycosyl hydrolase

M. K. Brewer · M. S. Gentry (✉)
Department of Molecular and Cellular Biochemistry, Epilepsy and Brain Metabolism Center, Lafora Epilepsy Cure Initiative, and Center for Structural Biology, University of Kentucky College of Medicine, Lexington, KY, USA
e-mail: matthew.gentry@uky.edu

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G_L	Liver-specific glycogen-targeting regulatory subunit of PP1
GlcNAc	N-acetyl-glucosamine
G_M	Muscle-specific glycogen-targeting regulatory subunit of PP1
GNIP	Glycogenin interacting protein
GPCPD1	Glycerophosphocholine phosphodiesterase 1
GSD	Glycogen storage disease
GSK3	Glycogen synthase kinase 3
GYG	Glycogenin
GYS	Glycogen synthase
HPAEC	High performance anion exchange chromatography
kDa	Kilodaltons
LAMP1	Lysosomal-associated membrane protein 1
LB	Lafora body
LC3	Microtubule associated light chain 3
LD	Lafora disease
LSF2	Like Sex Four 2
NADH	Nicotinamide adenine dinucleotide (reduced form)
NMR	Nuclear magnetic resonance
PAS	Periodic acid-Schiff
PASD	Periodic acid-Schiff with diastase
PhK	Phosphorylase kinase
PP1	Protein phosphatase 1
PTG	Protein targeting to glycogen (also known as R5)
PYG	Glycogen phosphorylase
R5	Ubiquitous glycogen-targeting regulatory subunit of PP1 (also known as PTG)
R6	Ubiquitous glycogen-targeting regulatory subunit of PP1
SEX4	Starch Excess 4
Stbd1	Starch binding domain-containing protein 1
TCA	Trichloroacetic acid
UDP	Uridine diphosphate

1 Introduction

In the 1840s, the French physiologist Claude Bernard made an astonishing observation: that sugar could be synthesized *de novo* by the liver of a dog that was exclusively fed meat (Bernard 1850). In 1857, he described the isolation of this sugar-forming substance, which he aptly called *la matière glycogène* (Bernard 1857). Soon afterward, glycogen in muscle was reported, shown to diminish with exercise, and discovered to "ferment" into lactic acid. Over the century that followed, the structure and regulation of glycogen were extensively characterized in the context of liver and muscle, leading to multiple Nobel prizes throughout the twentieth century. Although the mechanisms governing glycogen metabolism have been deeply studied, the work is not yet complete. Meticulous investigations of Nobel

laureates Otto Meyerhof, Carl and Gerty Cori, Luis Leloir, Edwin Krebs and Edmond Fischer, and countless others have paved the way for an even more profound understanding of glycogen metabolism, even in organs where glycogen constitutes a smaller fraction of total weight, such as the brain. Despite glycogen metabolism being a mainstay in textbooks, new aspects of glycogen structure, metabolism and tissue-specific roles are still being defined, aided by modern technologies and the mechanistic investigations of glycogen storage diseases.

This chapter discusses the historical progression of glycogen research and its current condition with particular emphasis on how it has benefitted the overarching field of biochemistry, why the study of brain glycogen has lagged, and what gaps remain in our understanding of glycogen metabolism. There are aspects of glycogen structure and metabolism that are applicable to all types of glycogen, but some aspects are specific to organisms, tissues, and/or nutritional state, and insights can be gleaned through study of the other types of carbohydrates, particularly plant starch. Given the similarities and connections, it is useful to review the literature on glycogen (and starch) to understand what can be extrapolated to brain glycogen. This chapter provides a detailed overview of our current understanding of glycogen structure, architecture, associated proteins, tissue-specific properties and subcellular distribution. Later chapters of this book are devoted to the structure and regulation of glycogen synthase and phosphorylase (Chaps. 3 and 4), so they will not be discussed in depth here. Glycogen storage diseases inform our studies of glycogen in ways that would not otherwise be possible; insights into glycogen metabolism gained from these diseases are discussed in this chapter. The entire disease class has been recently reviewed elsewhere (Adeva-Andany et al. 2016).

2 Discovery of Glycogen and Its Associated Proteins

2.1 Claude Bernard and Early Reports of Brain Glycogen

Glycogen constitutes up to 8% of liver volume and up to 2% of muscle volume, and its content is higher in these tissues than in any other tissue of the adult mammal (Brown 2004). It is not surprising that the fundamental study of its structure and metabolism began in liver and muscle. Claude Bernard, through a series of fundamental experiments, discovered the primary role of the liver in supplying glucose to other organs through the bloodstream (reviewed by Young 1937, 1957; Grmek 1968). In 1848, Bernard noticed that after death, a large amount of glucose was released from the hepatic veins of a dog, even when the dog had not been fed any carbohydrates (Grmek 1968). He concluded that the liver could produce glucose, and by 1857, reported that he could extract glycogen (a term that literally means "sugar-former") by plunging the tissue in boiling water shortly after death and precipitating the material with alcohol (Bernard 1857) (Fig. 1). Through fermentation with yeast and basic chemical tests, he confirmed that its main constituent was glucose (Young 1957). He also noticed that the glycogen yielded a red wine color in the presence of iodine, which facilitated its

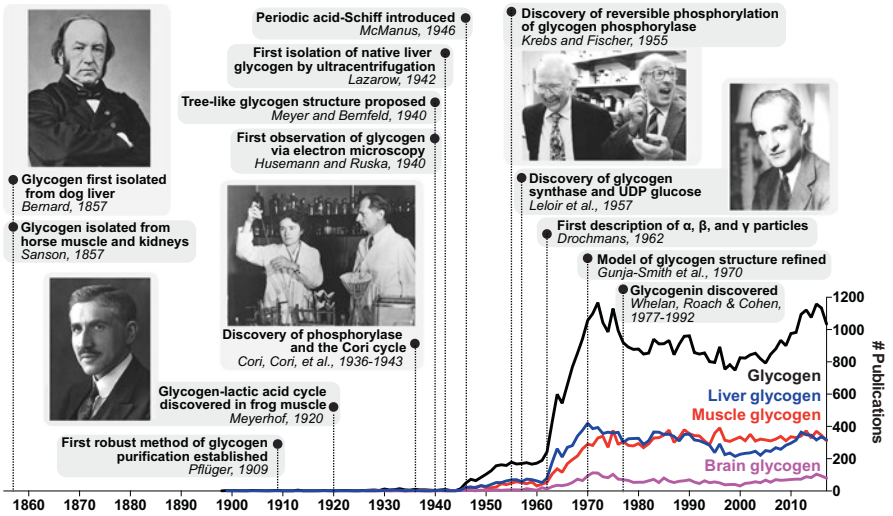

Fig. 1 Timeline of landmark discoveries in the history of glycogen-related research. Notable discoveries and papers are shown. Claude Bernard, the physiologist who discovered glycogen and its function in liver, and Nobel laureates are pictured. PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) searches were performed using the keywords shown (glycogen, liver glycogen, muscle glycogen, or brain glycogen), and the number of publications per year for each set of keywords are shown. Key terms related to glycogen synthase kinase (GSK, GSK3, GSK3 beta) were excluded from search results since much of the GSK-related literature is not directly relevant to glycogen metabolism.

histological observation in other tissues (Foster 1899; Larner 1967). Very shortly afterward, Sanson reported the isolation of "a glycogen-like substance analogous to dextrin" from the spleen, muscle and kidneys of a horse (translated, Sanson 1857) (Fig. 1). By 1875, Bernard and others reported glycogen in mammalian muscle, embryos, and trace amounts in other tissues, but it was difficult to know for certain that those trace amounts were indeed glycogen (Carpenter et al. 1876).

Soon after Bernard's discovery of glycogen, it was noted that glycogen in muscle diminished upon contraction, and Bernard reported that "muscle glycogen always undergoes a lactic acid fermentation, and this is the only change that muscle glycogen ever undergoes, either in the living animal or after death" (Carpenter et al. 1876; Young 1957). In the early twentieth century, a landmark paper from Fletcher and Hopkins demonstrated that lactic acid was formed in muscle under anaerobic conditions (Fletcher and Hopkins 1907). Then, a German physician named Otto Meyerhof published a series of experiments demonstrating that the lactic acid produced by frog muscle was derived from glycogen, and that in the presence of oxygen, it could be either oxidized, or reconverted to glycogen (Meyerhof 1920a, b, c) (Fig. 1). "Meyerhof's brilliant analysis of the glycogen-lactic acid cycle and its relation to respiration explained the course of the heat production and, for the first time, established the cyclic character of energy transformations in the living cell" (Nachmanson et al. 1960). For this discovery and his seminal work on glycolysis, Meyerhof and his colleague A.V. Hill were awarded the 1922 Nobel Prize in Physiology or Medicine.

The German physiologist Eduard Pflüger is credited as the first to establish a method for obtaining pure fractions of glycogen (Fig. 1), and modifications of his method are still used today (Pflüger 1909; Young 1937; Passonneau et al. 1967). While Pflüger, Bernard and most others were unable to detect glycogen in the nervous system, traces of brain glycogen in the normal and diabetic human brain were reported by Cramer in 1880 and Pavy in 1881 (Gage 1917). Similar findings were published in an 1890 edition of *The Lancet*: "Dr. Fütterer has examined various organs of a diabetic person, finding glycogen in the medulla oblongata, spinal cord, and kidney in large quantities, and a little in the liver. A careful examination of the cerebral cortex showed that the vessels were full of glycogen. He concluded that extensive disturbances of nutrition were bound to result from this" (The Lancet 1890).

Although nearly all studies of glycogen in the early 1900s were in the liver and muscle of various organisms, as well as in yeast, a few groups reported glycogen in the central nervous system (CNS) of animals by staining tissues with an iodine solution. Glycogen was observed in the CNS, often in the retina, of the lancelet, lamprey, frog, pigeon, rabbit, and dog (Gage 1917; Holmes and Holmes 1926). Gage asserted its presence in the CNS of lower organisms, in the dorsal root ganglia of the pig, and in the choroid plexus of human embryos (Fig. 2). He validated the identity of the "mahogany-red substance" by incubating the sections with saliva, which con-

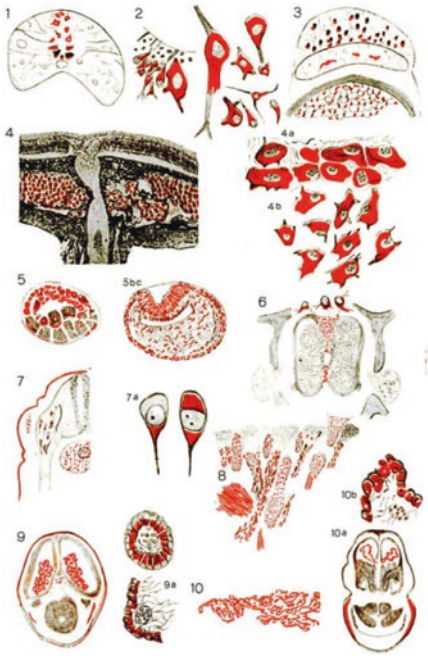

PLATE 1

EXPLANATION OF FIGURES

1 Glycogenated nerve cells of Amphioxus.
2 Nerve cells of Ammocoetes from the brain opposite the eye and ear.
3 Nerve cells in the myel (medulla spinalis) of Ammocoetes with glycogen; also glycogen and fat in the same cells.
4 Retina of an adult Petromyzon showing glycogen in the retinal nerve cells, and the decussation of the optic fibers.
(a) Edge view of glycogenated retinal nerve cells.
(b) Face view of retinal nerve cells.
5 Ova of Petromyzon and Amblystoma (bc) showing the great amount of glycogen in the cells of the animal pole. In the developing nervous system there is much glycogen.
6 Lumbar enlargement of a ten day chick's medulla spinalis showing a prismatic mass of glycogenated cells in the raphé. A similar glycogenated area is present in the medulla oblongata.
7 Spinal ganglion of a 12 mm. pig embryo with glycogenated cells. (a) Some of the cells greatly enlarged.
8 Glycogenated nerve trunks of the brachial plexus of a 30 mm. pig embryo.
9 Glycogenated endymal cells of the brain plexuses in a cat embryo. (a) Endymal cells greatly enlarged.
10 Choroid plexus of a 19 cm. human embryo. (ab) Section through the cerebrum and medulla of a 35 mm. human embryo to show the glycogenated choroid plexus and the cells of the raphé in the medulla.

Fig. 2 Glycogen in the CNS of invertebrates and vertebrates. Drawings illustrating glycogen in the CNS of multiple organisms detected via iodine staining (Gage 1917). Copyright © 2005 John Wiley and Sons, Inc. Used with permission

tained amylases that digested the glycogen so that it no longer stained. “With the higher vertebrates, glycogen in demonstrable amount is not found in the nervous system after the embryonic period, the liver and muscles then assuming the main glycogenic function.” Gage was hopeful that with the proper techniques and material, glycogen would also be found in the adult human CNS (Gage 1917).

With the relationship between glycogen and lactic acid demonstrated in muscle by Meyerhof, a few groups began investigating this relationship in brain. However, brain glycogen and lactic acid remained difficult to isolate and detect, and results were variable. Some groups reported a reduction in glycogen with insulin-stimulated convulsions or anesthetics, while others reported a rise in glycogen after convulsions produced by methylguanidine; Holmes and Holmes in reviewing these studies argued that the low values reported for brain glycogen were within the normal range of variation, and that brain glycogen is unlikely to produce lactic acid under normal conditions (Holmes and Holmes 1926). They astutely commented that “it is possible that the low values which we, in common with other workers, find for the glycogen content of the brain, may be due, not to a slow or inadequate breakdown mechanism, but to an extremely rapid one.” This was indeed the case, and methods for capturing brain glycogen improved over the years; however, it was not until the end of the twentieth century that the most sophisticated methods for measuring brain glycogen emerged. “It seemed to us, therefore, that it is quite possible that glycogen plays a comparatively unimportant (or at least quite obscure) part in the carbohydrate metabolism of mammalian brain” (Holmes and Holmes 1926). This sentiment prevailed for many years, and it did not become clear until the following century that the role of glycogen in brain was more obscure than it was unimportant. Meanwhile, studies on glycogen enzymology had just begun, which led to a series of fundamental discoveries with major impacts on the wider field of biochemistry.

2.2 The Cori Years: Advances in Glycogen Enzymology and Glycogen Structure

Soon after Meyerhof’s Nobel prize was awarded, Carl and Gerty Cori, Czech-born medical doctors who had emigrated to the United States, started working on the effects of insulin and epinephrine on liver glycogen. They reported that the hyperglycemia observed in the blood after epinephrine injections could not be accounted for solely by liver glycogen, and that lactic acid, which would be derived from muscle, could give rise to newly formed liver glycogen (Cori and Cori 1929). They theorized that there was a “cycle of carbohydrates” linking liver glycogen and muscle glycogen, with the intermediates between the two being glucose and lactic acid (Fig. 3a). Their subsequent work demonstrated this theory to be true, and it became known as the Cori cycle.

The Coris made a series of major discoveries in the 1930s (Fig. 1). In 1936 they isolated a novel ester, glucose-1-phosphate, from a mixture of glycogen, inorganic phosphate, and aqueous muscle extract (Cori and Cori 1936). So significant was this

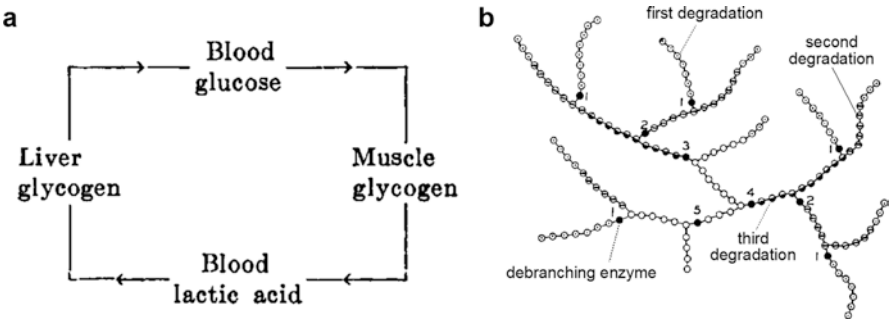

Fig. 3 The Cori cycle and the tree-like structure of glycogen. (**a**) The cycle of carbohydrates between liver and muscle proposed by Cori and Cori (1929) when they observed that ingestion or subcutaneous injection of sodium *d*-lactate led to glycogen deposition in the liver. Their subsequent work validated and elaborated this model, which became known as the Cori cycle. (**b**) A segment of the tree-like structure of glycogen based on Meyer's model and the results of the Cori group. Glucose residues are represented by circles; dotted, bisected, and half-filled circles correspond to glucose residues released by the first, second, and third rounds of degradation with phosphorylase, as indicated. Filled circles represent glucose residues released by α-1,6 glucosidase (i.e. debranching enzyme). Tiers are numbered. Modified from Larner et al. 1952. Copyright © American Society for Biochemistry and Molecular Biology. Used with permission

discovery that the compound became known as the Cori ester (Larner 1967). In subsequent years, the Coris showed that a phosphorylating enzyme found in muscle, heart, liver, brain, and yeast extracts catalyzed the release of glucosyl moieties from glycogen by esterifying them with inorganic phosphate. The resulting glucose-1-phosphate was converted to glucose-6-phosphate by an enzyme they named phosphoglucomutase (Cori et al. 1937, 1938). In a 1939 article in Science, they reported that the enzyme producing the Cori ester (which they aptly named phosphorylase) could also catalyze the reverse reaction, in order to synthesize polysaccharide (Cori et al. 1939). Almost simultaneously, phosphorolytic enzymes catalyzing polysaccharide synthesis were being described in the extracts of peas and potatoes and in yeast (Kiessling 1939; Hanes 1940). In 1943, Cori, Cori and Green crystallized glycogen phosphorylase and published a series of seminal papers on its properties (Cori et al. 1943; Cori and Cori 1943; Cori and Green 1943; Green and Cori 1943). In reward for their pioneering work, the Coris shared the 1946 Nobel Prize in Physiology or Medicine with Bernardo Houssay, who also made important contributions to the field of carbohydrate metabolism through his study of the role of the pituitary gland in hormonal regulation.

The discovery of phosphorylase in mammals, plants and yeast was a major breakthrough in the overarching field of biochemistry (Larner 1967). Phosphorylase was the first enzyme demonstrated to synthesize polysaccharide, and glycogen was the first macromolecule to be synthesized *in vitro* (Manners 1963; Whelan 2007). However, while mammals and yeast utilize glycogen as an energy source, plants synthesize starch, a glucose polymer with very different properties. Investigations of plant starch had been underway years before Bernard's discovery of glycogen

and were progressing in parallel with those of glycogen. By the 1940s it had become clear that starch was composed of two distinct polymeric fractions: amylose, which was composed of long linear chains of glucose and gave an intense blue-black color with iodine, and amylopectin, which was a branched macromolecule and gave a purplish to reddish color (Bates et al. 1943). While the Coris were working out glycogen enzymology, others were studying the polymeric nature of both glycogen and starch. Haworth and Percival established a method for identifying the chemical linkages in glucose polymers through methylation of the free hydroxyls of the glucose polymer prior to hydrolysis with acid (Haworth and Percival 1932). They found that since most of the product had unmethylated hydroxyls at the 1- and 4- positions, the majority of the glucose units in glycogen must be linked by α-1,4 glycosidic bonds. A small proportion of glucose was methylated at the 4-hydroxyl position, which would represent what is called the nonreducing end of a glucose chain (i.e. the end lacking an aldehyde group). Based on the proportion of the methylated products, the average linear chain in glycogen was proposed to contain 12 glucose units. Similar work was done on several varieties of plant starch, and the average chain length in starch was estimated to be 24–30 units (Bawn et al. 1940). In 1947 and 1949, the α-1,6-linked disaccharide isomaltose was isolated from hydrolyzed starch and glycogen, providing definitive evidence for the chemical identity of the branch points (Montgomery et al. 1949; Wolfrom et al. 1951).

For many years it was believed that phosphorylase catalyzed both the synthesis and degradation of glycogen and starch, although synthesis by phosphorylase always required a primer, i.e. the addition of a small amount of carbohydrate upon which phosphorylase could act (Larner et al. 1952; Larner 1967). Curiously, phosphorylase from brain, heart and liver extracts produced branched polysaccharides that yielded a similar color to glycogen when stained with iodine, while the enzyme from potato and muscle extracts synthesized a linear, unbranched polysaccharide resembling amylose (Cori and Cori 1943). It was hypothesized that the former preparations contained a contaminating enzyme that was capable of introducing the α-1,6-linked branch points. A branching enzyme had been identified in potato, and in 1953, Larner characterized the branching enzyme from rat liver and muscle extracts. Using isotopic labeling, he showed that potato, liver and muscle branching enzymes transferred a 1,4-linked chain of 6–11 glucose moieties to create the 1,6-linked branch. Thus, branching enzyme is considered a transglucosidase (Larner 1953). His approach was novel in that he utilized radioactive isotopes rather than measuring branching by a shift in iodine color, susceptibility to phosphorylase, or change in end-group. With the combination of phosphorylase and branching enzyme, glycogen could be synthesized *in vitro*, and for the most part, it resembled the glycogen purified from tissues (Parodi et al. 1969).

Similarly, it was observed that while muscle phosphorylase could completely digest glycogen, recrystallized preparations of muscle phosphorylase and potato phosphorylase degraded glycogen only partially, stopping at the branch points, and leaving what was called a limit dextrin (Cori and Larner

1951). A second enzyme must be present in the crude extracts that could remove the branch points. In 1951, Cori and Larner discovered the contaminating enzyme, showing that it cleaved α-1,6 linkages and released free glucose (Cori and Larner 1951). Walker and Whelan later found that the phosphorylase limit dextrin contained not one, but four glucose units attached to branch points (Walker and Whelan 1960). It was then discovered that the enzyme they had identified, which they called amylo-1,6-glucosidase, also had transglucosidase activity, favoring the transfer of three glucose units from one chain to another (Brown and Illingworth 1962). It is now well established that when phosphorolysis terminates four units away from a branch point, the glycogen debranching enzyme, which possesses both transferase and glucosidase activities, moves three glucose units to another chain and then releases the single branched glucosyl moiety (Huijing 1975).

It appeared at that time that all of the enzymes required for glycogen synthesis and degradation had been identified: glycogen phosphorylase, branching enzyme, and debranching enzyme. Enzymes that were either analogous or distinct to these had also been identified in plants. It became possible to utilize these enzymes to more precisely understand the structure of the glycogen and amylopectin molecules. With such useful tools and insights in common, the fields of starch and glycogen metabolism continued to progress together and overlapped considerably. In 1940 a tree-like structure for glycogen and amylopectin was proposed by Meyer and Bernfeld based on serial degradation with various enzymes (Meyer and Bernfeld 1940) (Fig. 1). This model varied from the prior structures proposed by Staudinger and Haworth, which were based solely on data obtained by chemical methods. The Cori group used stepwise enzymatic degradation of various glycogens and amylopectins with phosphorylase and debranching enzyme to test the various models. It was evident that glycogen was composed of "tiers," and each successive tier could only be accessed by phosphorylase after removal of the branch points by the debranching enzyme; degradation of multiple tiers required alternating treatments of the two enzymes. "The Staudinger and the Haworth models would yield a constant percentage of the total branch points in each tier during successive enzymatic degradation, while the Meyer model would yield a diminishing percentage as one progresses from the outer to the inner tiers.... Only one kind of model could be made to fit this arrangement of branch points; namely, one which represents the polysaccharides as multibranched, tree-like structures" (Larner et al. 1952) (Fig. 3b). It was also noted in this paper that the average chain length of liver and muscle glycogen (15 glucose units) were shorter than those of wheat and corn amylopectin (18 and 24 glucose units, respectively). We now know that chain length is one of the major features that distinguish glycogen from amylopectin. Meyer's model was the accepted model for glycogen and amylopectin until experiments from Whelan's group in 1970 showed the absence of very long chains and refined the model (Fig. 1) (Gunja-Smith et al. 1970, 1971). The currently accepted model for glycogen is based on Whelan's work, but recent data indicates this model requires further refinement (see Sect. 3.1).

2.3 *Emerging Technologies with Staying Power: Periodic Acid-Schiff, the Ultracentrifuge, and the Electron Microscope*

When the Coris began their work, the most common procedure for purifying glycogen was based on the 1909 Pflüger method. The protocol is similar to what Bernard used and involves boiling tissue in hot concentrated alkali and then precipitating the glycogen with alcohol (Pflüger 1909). Modifications of this procedure, especially those of Somogyi (Somogyi 1934), were used by the Coris and are still the most commonly utilized protocols for glycogen extraction. Another frequently used method, introduced in 1934 (Willstätter and Rohdewald 1934), is extraction with cold trichloroacetic acid (TCA); however, it has been observed that not all glycogen can be extracted by this method (see Sect. 6.2), and a slightly different molecular weight is observed for the purified glycogen (Lazarow 1942; Calder 1991). In 1936, the first reliable procedure for extracting brain glycogen was established utilizing Somogyi's method (Kerr 1938). Kerr found that he obtained the highest yields when the brains of dogs were rapidly excised under amytal anesthesia and crushed directly into 60% potassium hydroxide. He also obtained high yields when the brains were frozen *in situ* in liquid air. Importantly, he showed that the purified brain glycogen "is indistinguishable from liver glycogen prepared by Somogyi's method. It dissolves readily in water to form a transparent solution, which is opalescent in reflected light. This when treated with iodine solution gives a Burgundy red color identical with that obtained with a solution of liver glycogen." The similar chemical properties of brain and liver glycogen were important to establish, and made it reasonable to extend the principles of glycogen structure and regulation that were being so thoroughly defined in liver and muscle to brain glycogen.

Quantifications of brain glycogen were now reproducible. Kerr and other groups showed that brain glycogen levels dropped during hypoglycemia in dogs, cats and rabbits (Carter and Stone 1961). Once a robust method for quantifying low levels of brain lactate was also established (Carr 1947), informative experiments on the effects of various stimuli on brain glycogen and lactate could begin. Using a circular saw to rapidly excise the brains of mice, Chance and Yaxley showed that lactate was elevated with both subconvulsive and convulsive levels of various seizure-inducing stimulants, while glycogen was only elevated when a convulsion was reached (Chance and Yaxley 1950). They also carefully defined the rapid decrease of glycogen and lactate levels after death in both normal and convulsed mice, demonstrating that most of the glycogen was lost after 5 min (Fig. 4a). Based on their own measurements and those of their contemporaries, they estimated that "a large number of vertebrates normally possess between 10 and 30 mg of glycogen per 100 g of brain tissue" and that glycogen distribution within the brain was heterogeneous. These values are in agreement with current estimates (Brown 2004). A few groups also began studying glycogen metabolism in isolated cerebral tissues and the effects of sleep on brain glycogen (LeBaron 1955; McIlwain and Tresize 1956; Svorad 1958).

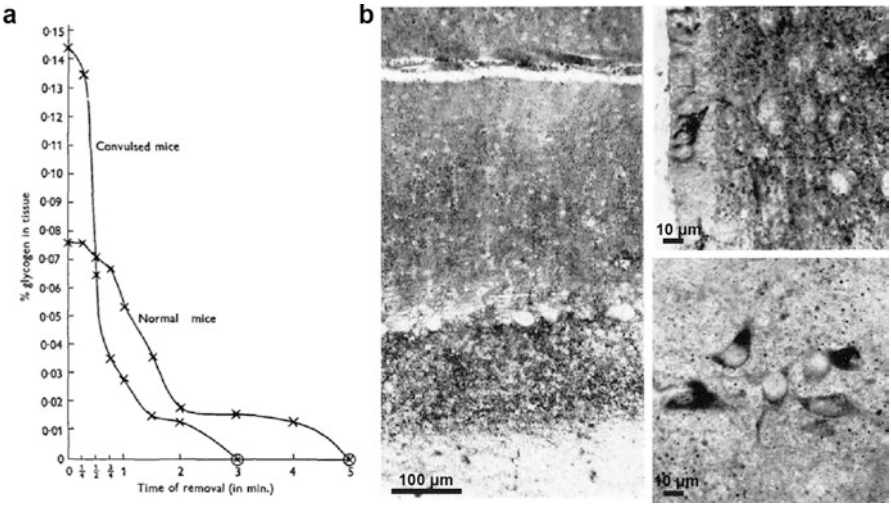

Fig. 4 Early studies of brain glycogen. (**a**) Rapid glycogen loss following extraction from brain of normal and convulsed mice; glycogen was measured using a modification of the method by Kerr (1938) (from Chance and Yaxley 1950). Copyright © Company of Biologists, Ltd. Used with permission. (**b**) Staining of glycogen (dark stain) in the perfused rabbit brain using the lead-tetra-acetate-Schiff method, a modification of PAS (Shimizu and Kumamoto 1952). The identity of stained regions as glycogen was confirmed by salivary digestion of comparable sections. Left: glycogen is abundant in the granular and molecular layers of the cerebellum, but Purkinje neurons lack glycogen. Upper right: ependymal cells of the hypothalamus lining the third ventricle show intense staining for glycogen, and granules are abundant in the neuropil, but nerve cells lack glycogen in this region. Lower right: some small nerve cells in the lateral hypothalamic nucleus appear to contain glycogen. Scale bars have been approximated based on magnification. Copyright © 2004 John Wiley and Sons, Inc. Used with permission

As glycogen quantification methods improved, so did microscopic techniques. Glycogen had long been visualized with iodine using the light microscope, but the stain was not sensitive enough to detect the small amounts found in the normal adult brain. Additionally, alcoholic fixation was also critical, as glycogen would dissolve in aqueous solutions (Gage 1917). Some attempts were made to visualize glycogen with Best's carnitine or the Bauer reaction, but these methods were also criticized for their lack of sensitivity (Kerr 1938; Lillie 1950; Mowry and Bangle 1951). In 1946, McManus introduced the periodic acid-Schiff (PAS) technique as a delicate and convenient way to stain carbohydrates, including glycogen, in tissue sections: the periodic acid reacts with the 1,2 glycol linkage of carbohydrates, producing an aldehyde that can be colored with Schiff reagent (McManus 1946, 1948) (Fig. 1). Although PAS stained most polysaccharides, including glycoproteins and mucins, its specificity for glycogen could be tested by incubation with diastase (a general term for amylase) or saliva (which contains amylase) to digest the glycogen and distinguish it from other types of polysaccharides, which were left intact after digestion (Mowry and Bangle 1951). Using PAS and a modification of this stain (lead-tetra-acetate-Schiff), Shimizu and Kumamoto published a series of papers in the

1950s on glycogen deposition in the normal brain and with pathological insults (Shimizu and Kumamoto 1952; Shimizu 1955; Shimizu and Kubo 1957; Shimizu and Hamuro 1958). Glycogen was detected in the area postrema (an area populated primarily by glial cells) in mammalian brains, particularly around blood vessels, and in ependymal cells of the supraoptic crest in rodents; it could also be detected in nerve cells of the hypothalamic nucleus and other regions when the brain was perfused with appropriate fixatives (Shimizu and Kumamoto 1952) (Fig. 4b). The PAS technique, usually in combination with diastase (PASD), is now the most widely used histochemical stain for glycogen detection in tissues (Bancroft and Gamble 2008).

Another extremely useful technology in visualizing glycogen structure and distribution was born with the invention of the electron microscope in 1931 by Ernst Ruska (Ruska and Knoll 1931). The electron microscope achieved superior resolution to that of the light microscope. In 1934, Marton developed a histological technique that allowed biological specimens to be visualized by the electron microscope without destruction (Marton 1934). With this technique, the fine structure of subcellular organelles could be visualized. The first report of glycogen using electron microscopy (EM) was from Husemann and Helmut Ruska, Ernst Ruska's brother (Husemann and Ruska 1940) (Fig. 1). The spherical particles, which were derivatized to produce better scattering of the x-ray beam, were about 15–30 nm in diameter. But visualization of glycogen was difficult for a number of reasons. Firstly, polysaccharides contain primarily light atoms (carbon, oxygen and hydrogen) and are intrinsically not very electron dense. Secondly, after the identification of ribonucleoprotein particles by EM, particulate components with a diameter of ~150 Å free in the cytoplasm or associated with the endoplasmic reticulum were typically interpreted as ribonucleoprotein (Revel et al. 1960; Revel 1964). Unknown tissue components were sometimes identified by comparing the thin EM sections with thicker, stained sections viewed under the light microscope, but this too had its limits.

The ultracentrifuge, invented by Theodor Svedburg in 1925, became a very powerful tool in the field of biochemistry and allowed glycogen to be isolated its native form. Lazarow showed in 1942 a species which he called "particulate glycogen" could be isolated from liver via ultracentrifugation without the use of harsh chemicals (Lazarow 1942) (Fig. 1). Its sedimentation constant suggested a molecular weight much larger than previous reports for Pflüger-purified glycogen, and the particle, which he showed was made of pure glycogen, could be dispersed by heating, trichloroacetic acid (TCA), or potassium hydroxide treatment. Lazarow concluded that "clearly, then, particulate glycogen is an aggregate of smaller glycogen units." In 1962, Drochmans, combining these two state-of-the art technologies and utilizing a negative staining technique with phosphotungstate, elegantly described the ultrastructure of glycogen particles purified by differential ultracentrifugation (Drochmans 1962) (Fig. 1). He defined three levels of glycogen structure in the rat liver visible by EM: the largest structure was the α particle, which was 60–200 nm in diameter and had a rosette like appearance; the β particle, 20–40 nm in diameter, which associated to form α particles; and the γ particle, which referred to the 3 nm fine filaments comprising the β particle (Fig. 5a, b). The α particles could be

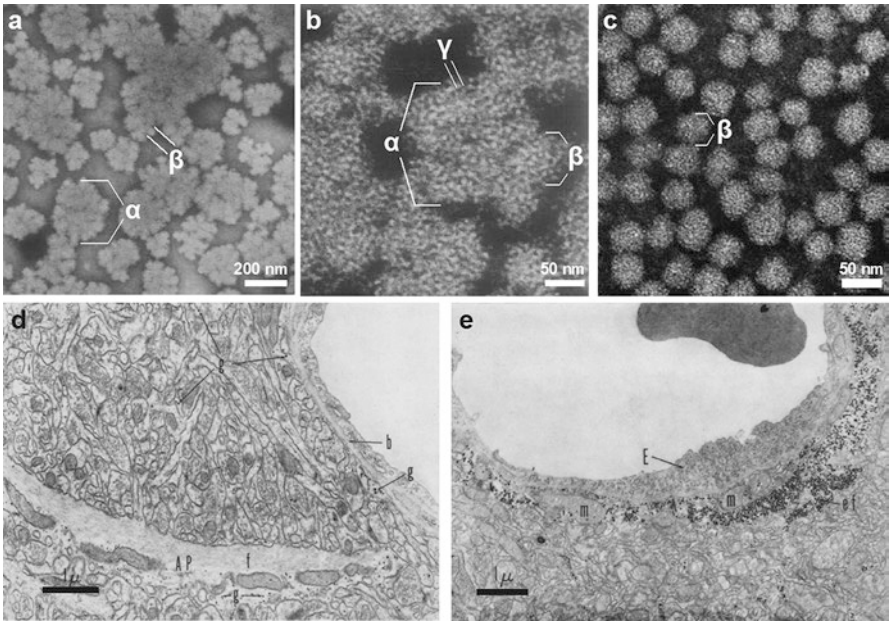

Fig. 5 Glycogen ultrastructure as demonstrated by electron microscopy. (**a**, **b**) Negative staining of natively purified rat liver glycogen from reveals the presence of α, β and γ particles (Drochmans 1962). Copyright © 1962 Elsevier. Used with permission. (**c**) Typically, purified muscle glycogen exists only as β particles (Wanson and Drochmans 1968). Scale bars have been approximated based on magnification. Copyright © Rockerfeller University Press. Used with permission. (**d**, **e**) Glycogen in the rat cerebral cortex visualized by EM (Maxwell and Kruger 1965). (**d**) In sections of normal cortex, glycogen β particles (*g*) can be found in astrocyte processes (*AP*) and astrocytic end feet applied to the basement membrane (*b*). An astrocytic bundle of fibrils (*f*) is labeled. (**e**) One day after irradiation with ionizing particles, end feet (*ef*) are enlarged and contain numerous glycogen granules and mitochondria (*m*). An endothelial cell lining the capillary is also labeled (*E*). Copyright © Rockerfeller University Press. Used with permission

dispersed into β particles in low pH. It became clear that Drochmans's α particle was equivalent to Lazarow's particulate glycogen, and that these particles represent the primary native form of glycogen in the liver. This helped to explain the discrepancies in molecular weights that others had observed for glycogen purified by different methods. In 1968, Wanson and Drochmans also visualized differentially centrifuged glycogen from rabbit skeletal muscle, showing the presence of solely β particles (Wanson and Drochmans 1968) (Fig. 5c). Dozens of EM studies on glycogen in various tissues and organisms followed.

The use of EM on sections of the mammalian brain in the 1960s and later years made it clear that the vast majority of brain glycogen was present in the form of β particles in astrocytic processes (Maxwell and Kruger 1965; Cataldo and Broadwell 1986) (Fig. 5d). Multiple groups showed striking accumulations of glycogen in reactive astrocytes in response to various traumas (Shimizu and Kubo 1957; Shimizu and Hamuro 1958; Maxwell and Kruger 1965) (Fig. 5e). Most groups reported that

in the adult mammalian brain, neurons and microglia contained virtually no glycogen except under certain pathological conditions; however, in a few studies of normal tissue, glycogen was found in nerve cells (reviewed by Koizumi 1974). During this time, numerous groups began using PAS and EM to visualize the microscopic characteristics of polyglucosan bodies, abnormal carbohydrate structures found in a variety of tissues. Polyglucosan bodies were identified in both astrocytes and neurons, in the context of glycogen storage diseases, epilepsy, neurodegenerative disorders, and aging (Cavanagh 1999; Duran and Guinovart 2015). Although glycogen still received little attention in the brain compared to other tissues, its significance was becoming increasingly evident. In 1961, A.W. Merrick stated, "A substantial amount of Russian work within the past decade has been directed toward brain glycogen changes during various function and biochemical states of the animal. The opinion is offered by many of these investigators (several of whom are cited in this paper) that glycogen takes an active part in brain metabolism" (Merrick 1961).

2.4 Sugar Nucleotides, Reversible Phosphorylation, and a Primer for Glycogen Synthesis

In 1950, the Argentinian chemist Luis Leloir discovered the first sugar nucleotide: uridine-diphosphate (UDP) glucose (Caputto et al. 1950). It soon became apparent that the sugar nucleotides were found throughout nature and were essential for the interconversion of carbohydrates, occupying "a central position in carbohydrate metabolism" (Hassid et al. 1959). In 1957, Leloir made the shocking discovery that the true catalyst of glycogen synthesis was not glycogen phosphorylase, but a glucosyl transferase utilizing UDP-glucose as a glucosyl donor, which became known as glycogen synthase (Leloir and Cardini 1957) (Fig. 1). In the words of Larner, "Nature seems to have found a new more powerful glucose donor by making a pyrophosphate derivative of the Cori ester" (Larner 1967). Leloir's group went on to show that glycogen synthesized *in vitro* using glycogen synthase in combination with branching enzyme was identical to natively purified glycogen β particles from muscle; in contrast, glycogen synthesized by phosphorylase and branching enzyme was not exactly equivalent to native glycogen: it differed in its stability and response to various types of degradative treatments (Mordoh et al. 1966; Parodi et al. 1967). These results were confirmed and expanded upon by numerous groups, and Leloir was awarded the 1970 Nobel Prize in Chemistry for his discovery of the sugar nucleotides.

It became apparent that both glycogen phosphorylase and synthase are peculiar enzymes. The Coris observed that glycogen phosphorylase existed in two forms, an active *a* form, and inactive *b* form, which were differentially activated by adenylic acid (Cori and Cori 1946). The Coris had identified another enzyme capable of converting the *a* form to the *b* form, calling this the "prosthetic-group-removing" enzyme (Cori and Green 1943). Kinase activity, the covalent addition of phosphate to one protein by another, was first described in 1954 (Burnett and Kennedy 1954), and in 1955, Fisher and Krebs showed that phosphorylase *a* could be converted to

phosphorylase *b* in the presence of adenosine triphosphate (ATP) by an enzyme from muscle that became known as phosphorylase kinase (PhK) (Fischer and Krebs 1955). Concurrently, Wosilait and Sutherland also demonstrated the presence of an equivalent converting enzyme in liver (Sutherland and Wosilait 1955). These were the first ever demonstrations of reversible phosphorylation, a momentous discovery that has reverberated throughout the life sciences. Fischer and Krebs published a series of papers on the details of this novel mechanism that earned them the 1970 Nobel Prize in Physiology or Medicine, and Sutherland received the 1971 Nobel Prize for his discovery of cyclic adenosine monophosphate (cAMP) and its role in hormonal regulation (Cohen 2002). A few years later, Larner and others showed that glycogen synthase is also subject to allosteric control, that it exists in two forms, and that the interconversion of these forms requires phosphorylation by another kinase (Friedman and Larner 1963). In fact, unlike phosphorylase, which has one phosphosite and one kinase, synthase is hierarchically phosphorylated at multiple sites by multiple kinases, inextricably linking glycogen metabolism to hormonal regulation, energy status, and a milieu of intracellular signals (see Chap. 3) (Roach 1990). One of the kinases discovered to phosphorylate synthase, glycogen synthase kinase 3 (GSK3), was later found to regulate a much greater array of cellular processes and play an important role in many pathologies including cancer, Alzheimer's disease, Parkinson's disease and diabetes (Cohen and Frame 2001; Jope and Johnson 2004). Many of the roles of GSK3 are quite unrelated to glycogen metabolism, leading to an unfortunately misleading keyword in literature searches.

While more pieces in the puzzle of glycogen regulation had fallen into place, other pieces were not even known to be missing. One enzyme must be mentioned at this point that has received insufficient attention despite its discovery in 1963. It was discovered as the deficient enzyme in Pompe disease, one of the glycogen storage diseases (GSDs). GSDs, also known as glycogenoses, are a group of diverse pathologies characterized by glycogen accumulation in various tissues. GSDs were first described in the early 1900s, and over the decades, the enzyme deficiencies causing many of these disorders were identified through biochemical analyses of patient tissues. Typically, patients were lacking one of the basic enzymes involved in glycogen metabolism (reviewed by Huijing 1975). However, in one of the most severe GSDs, Pompe disease, all of the known glycogen-related enzymes had normal activity. Lysosomes had been recently discovered by de Duve with the use of the ultracentrifuge (De Duve et al. 1955), and in 1963, Hers demonstrated that Pompe patients were lacking an enzyme called acid α-glucosidase or maltase, which was found in lysosomes and converts glycogen or maltose to glucose (Hers 1963). He identified Pompe disease as the first of the lysosomal storage diseases, now known to have a combined incidence of 1 in 5000–10,000 (Fuller et al. 2006; Raben et al. 2012). Brown, Brown and Jeffrey demonstrated that the lysosomal enzyme discovered by Hers could cleave both 1,4 and 1,6 glycosidic linkages (Brown et al. 1970; Jeffrey et al. 1970a, 1970b). The role of this enzyme in the degradation of glycogen was unclear, but presumably it was important since its absence resulted in a fatal condition. It is now well established, but not broadly known, that glycogen can be degraded within lysosomes, but the details of this pathway are still being defined (see Sect. 5).

The discoveries of Leloir, Krebs and Fischer spurred a major wave of glycogen-related research in the decade that followed (Fig. 1). By 1970, these fervent investigations were waning, as it seemed to most that the work on glycogen was virtually complete. In 1971, Ryman and Whelan synthesized an exhaustive review of glycogen metabolism, which spanned 158 pages and cited nearly 900 publications. They stated in their introduction:

> "The field of glycogen metabolism is one that to the outside observer has long seemed in a settled condition, with new discoveries being only likely to add gloss to existing facets. This is because it has been possible since the early 1940s to draw metabolic maps that seem to explain the process fully and satisfactorily, these maps being based on highly satisfactory *in vitro* experiments carried out with purified or semi-purified enzymes. This has been the polymer *par excellence* as far as *in vitro* work is concerned.
>
> This apparently settled condition is, in fact, illusory, and this has probably worked to the detriment of progress, since potentially interested investigators have almost certainly turned to other pursuits, feeling that no more major advances would be forthcoming. The true situation is the exact opposite, and the ferment of activity now going on testifies to the growing realization that studies of glycogen metabolism have thrown up key discoveries that have the widest implications throughout biochemistry" (Ryman and Whelan 1971).

One aspect that remained unclear was how glycogen synthesis was initiated *in vivo*. Both the Coris and Leloir observed that *in vitro* glucose polymerization by glycogen phosphorylase or glycogen synthase required the addition of a pre-formed carbohydrate primer (Swanson and Cori 1948; Leloir and Cardini 1957). Some investigators believed that glycogen synthesis could be initiated *in vivo* by glycogen synthase (Salsas and Larner 1975); others reported that a protein acted as the priming factor (Krisman and Barengo 1975). In 1977, Whelan's group discovered a protein covalently bound to glycogen in liver, and later showed that the protein, which became known as glycogenin, was linked to glycogen via a novel tyrosine-glucose linkage (Butler et al. 1977; Rodriguez and Whelan 1985) (Fig. 1). At first, Whelan's discovery was doubted, but his subsequent work and work from the laboratories of Cohen and Roach provided very strong support for this protein acting as the true primer of glycogen biogenesis. These groups established that glycogenin was a glucosyltransferase using UDP-glucose, and that after glycosylating itself, it could also extend the glucose chain to ~8 glucose residues, which would then be acted upon by glycogen synthase and branching enzyme (Lomako et al. 1988, 1990; Pitcher et al. 1988; Viskupic et al. 1992). The initiation of glycogen synthesis by glycogenin is now widely accepted and has been extensively characterized, and similar protein primers for starch synthesis have also been described (reviewed by Roach 2002; D'Hulst and Mérida 2010; Roach et al. 2012).

2.5 *Reviving Interest in Brain Glycogen Metabolism*

In recent years there has been a growing interest in studying brain metabolism, in part due to some important technological advancements. Firstly, in the 1970s, neurochemists began using focused microwave irradiation to rapidly and irreversibly

inactivate enzymes in rodent brains in order to more accurately measure labile metabolites (reviewed by Schneider et al. 1981; Marani 1998). This technique was elegantly applied to determine the regional distribution of glycogen in the rat brain in the 1980s (Sagar et al. 1987; Swanson et al. 1989) and more recently in the mouse brain (Oe et al. 2016). Secondly, after it was suggested that elevated glycogen in a GSD patient could be observed by ^{1}H NMR, Choi et al. introduced the use of NMR spectroscopy to noninvansively measure the turnover of ^{13}C-labelled glycogen *in vivo* in rodents (Salvan et al. 1997; Choi et al. 1999). Öz and colleagues applied this technique to humans, publishing a series of studies on the role of glycogen in normal human brain metabolism and during hypoglycemia (Öz et al. 2003, 2007, 2009).

Another reason for the growing interest in brain glycogen is the emerging theme of metabolic coupling of astrocytes and neurons, particularly involving the transfer of lactate. These interactions are fascinating, complex, and highly debated. They have been recently discussed by multiple excellent reviews (Barros 2013; Dienel and Cruz 2016; Alberini et al. 2018; Bak et al. 2018; Magistretti and Allaman 2018) and in the subsequent chapters of this book. The coupling between astrocytes and neurons is reminiscent of the Cori cycle and the shuttling of lactate between liver and muscle; however, these interactions are more complicated and difficult to study. Glycogen metabolism is intimately linked to granule size, architecture, and its various associated proteins, which are still being investigated. Such topics are not frequently discussed in the context of brain glycogen metabolism. These nuanced aspects of glycogen regulation, in addition to its surprisingly dynamic nature, subcellular distribution, and alternative degradation pathways, are introduced in the next sections.

3 Glycogen Structure

3.1 Basic Structure of Glycogen and Other Glucose Polymers

Glycogen is one of many types of polysaccharides that exist in biology. A polysaccharide refers to any large polymer comprised of many covalently linked monosaccharides; the bonds between them are known as glycosidic linkages. Glycogen and the two primary constituents of plant starch, amylopectin and amylose, are all homopolymers of glucose designed for energy storage in various organisms. We and others refer to these glucose homopolymers as polyglucans. Glycogen and amylopectin contain branches, while amylose is almost exclusively composed of linear chains. The linear chains of glucose are connected by α-1,4 glycosidic linkages, and the branch points are comprised of α-1,6 glycosidic linkages (Fig. 6a). Because of the tree-like arrangement of branching, glycogen and amylopectin contain fewer reducing ends (i.e. ends with a free aldehyde group) than nonreducing ends, which are oriented outward. The α configuration of the glycosidic linkage causes a linear α-1,4 linked chain to twist, and when the chains are long enough, and uninhibited by branch points, they can form single or double helices (Gessler et al. 1999) (Fig. 6b). An alternative β-linked configuration of glucose polymers favors very

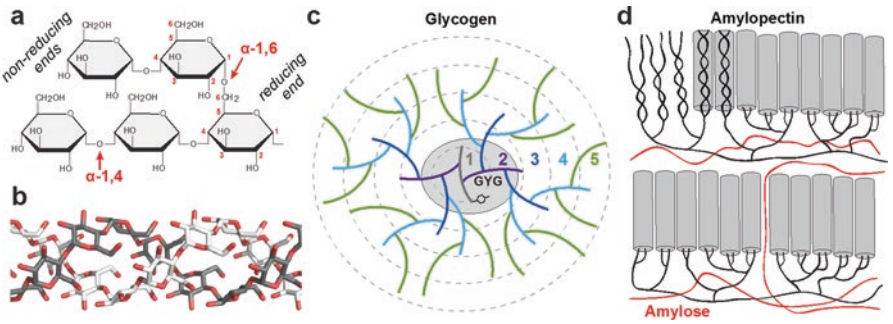

Fig. 6 Structure and synthesis of polyglucans. (**a**) Starch and glycogen contain linear chains of glucose joined by α-1,4 glycosidic linkages, and α-1,6 glycosidic linkages constitute the branch points. The reducing ends (containing an aldehyde group) are oriented near the interior of the polysaccharide molecule, while metabolic enzymes work on the nonreducing ends, which are oriented outward. (**b**) The α conformation of α-1,4 linked polyglucans gives them a propensity to twist, and long unbranched chains can form single or double helices; a model of a double helix is shown. (**c**) A model of the first five tiers of the glycogen particle according to the Whelan model. A tyrosine residue of glycogenin (GYG) is shown, which is covalently linked to the glucan chain making up the first tier. (**d**) A recent model for starch structure according to (Bertoft 2017). In amylopectin, the branched component of starch, branching is clustered, and the long linear chains form double helices (grey cylinders) that make up the crystalline regions of starch. Amylose, the unbranched component of starch, is believed to occupy areas around the amylopectin molecules.

straight chains that can associate into tight fibrils; this is characteristic of structural polyglucans such as cellulose. Mammals do not synthesize or hydrolyze β-glycosidic linkages, so they cannot digest cellulose, but they are well equipped to degrade glycogen and starch (Berg et al. 2002).

Although the models of glycogen and amylopectin structure are both based on the tree-like arrangement originally proposed by Meyer and Bernfeld, the models were individually refined as it became increasingly evident that the arrangement and length of the branches of these glucose polymers were quite different. Glycogen contains a high degree of branching and short linear chains. By enzymatic analyses, there are, on average, 13 glucose units per α-1,4-linked linear chain, and 8% of the total glycosidic linkages are α-1,6 branch points (Cori 1952; Illingworth et al. 1952; Melendez-Hevia et al. 1993; Melendez et al. 1997; Roach et al. 2012) (Fig. 6c). Branching in glycogen is believed to be continuous, meaning the branch points are evenly distributed within the molecule. The presently accepted model for glycogen is based on the revisions of Whelan's group (Calder 1991), but high resolution studies of chain length distribution (CLD) suggests this model may still not be completely accurate (discussed below). Typically, a single glycogen is depicted as originating from a single chain covalently attached to a glycogenin molecule, which gives rise to all subsequent chains (Roach et al. 2012; Prats et al. 2018) (Fig. 6c). Enzymatic experiments indicate 3 to 4 glucose units between each branch point, so each linear chain would yield two branches (Illingworth et al. 1952; Calder 1991). Each new set of branches is referred to as a tier (Fig. 6c), and since the number of linear chains doubles with each succes-

sive tier, the outermost tier would theoretically contain about one-third of the total glucose in the molecule, which is consistent with empirical observations (Larner et al. 1952; Melendez et al. 1997). Mathematical modeling has demonstrated that due to physical constraints, glycogen molecules can theoretically contain up to 12 tiers, corresponding to ~55,000 glucose molecules; beyond this size, the outer chains would become so crowded they would be inaccessible to enzymes (Melendez-Hevia et al. 1993). Indeed, the empirically observed upper limit of glycogen β-particles (44 nm) is consistent with the theoretical maximal diameter of a 12-tiered glycogen molecule (42 nm) (Shearer and Graham 2004; Prats et al. 2018). The continuous branching within glycogen prevents, or dramatically limits, the formation of double helices, which would lead to insolubility of glycogen and inaccessibility of the glucan chains to enzymatic degradation (Emanuelle et al. 2016). Mathematical modeling studies have also demonstrated that the empirically observed average chain length and branching degree are optimal for maintaining solubility and structural homogeneity (Melendez et al. 1998).

Amylopectin is also a branched polyglucan, but it contains longer chains of 20–25 glucose units per chain with infrequent and clustered branching (Manners 1989; Buleon et al. 1998) (Fig. 6d). The clustering of branch points means there are regions of long, unbranched chains that associate to form crystalline, water-excluding double helices (Fig. 6b). How these crystalline regions are arranged is still debated, but the most recent data suggests they are arranged along a polyglucan "backbone" (Bertoft 2017). The alternating crystalline and amorphous layers within starch are advantageous as it allows for very dense glucose packing within starch granules, which can be as large as 100 μm in diameter (Emanuelle et al. 2016). Amylose is believed to be interspersed among amylopectin molecules (Fig. 6d). Glycogen, amylopectin, and amylose have diverse structural properties due to differences in chain length and degree of branching. Glycogen has the shortest average chain length and the highest degree of branching, usually 8%. The degree of branching in amylopectin is nearly half that of glycogen, only 4–6% (Manners 1991; Buleon et al. 1998). Amylose is generally considered to be entirely composed of very long, linear chains, although infrequent branching has been reported (Manners 1989).

3.2 Glycogen Architecture

It is well established that amylopectin and glycogen differ in chain length and degree of branching. But a diverse array of polyglucans exist in nature with structures that vary beyond these two simple parameters. It is becoming increasingly apparent that the distribution of chain lengths and the arrangement of branch points within a polyglucan are variable, producing distinct biological and physiochemical properties. Furthermore, not only do amylopectin and glycogen differ significantly from each other, but each polyglucan comes in a variety of shapes and properties based on species, tissue of origin, and even the nutritional

state of the tissue. Polyglucan diversity in the context of starch has been exhaustively studied and thoroughly parameterized (Pérez and Bertoft 2010; Nakamura 2015). We will use the term "architecture" to refer to the unique chain length distribution, branching frequency and arrangement, and quantity and distribution of non-glucose moieties (notably phosphate) characteristic of a certain type of glycogen or amylopectin.

Since the days of Bernard, investigators have utilized iodine to study polyglucan architecture. Bernard observed in 1877 that newly synthesized glycogen in rabbit muscle following exhaustive exercise gave a bluish coloration with iodine, in contrast to the reddish color of glycogen from rested muscle or well-fed liver (Young 1937). This colorimetric test can be made quantitative by an absorbance spectral scan: the wavelength of maximal absorption (λ_{max}) increases with longer chains and less branching, reflected by the staining color (Swanson 1948; Krisman 1962; Banks et al. 1971). Glycogen from different sources yields a range of colors from yellow to reddish brown with iodine and produces a λ_{max} of 420–490 nm; amylopectin gives a more intense color ranging from red to lavender with λ_{max} of 490–570 nm; and amylose yields a blue to green color with a λ_{max} of 580–640 nm (Archibald et al. 1961; Bailey and Whelan 1961; Krisman and Alfredo 1991). The iodine method is not perfectly quantitative, due to the various effects of the iodine and salt concentrations, temperature, and polysaccharide structure and source (Morris 1946; Archibald et al. 1961). Additionally, although chain length and degree of branching describe different aspects of glycogen architecture, both parameters are related to λ_{max} (Archibald et al. 1961; Bailey and Whelan 1961; Hirai et al. 1994). It is difficult to deconvolve the various architectural parameters of polyglucans using just iodine, but it remains a convenient technique that yields useful information.

High-performance anion exchange chromatography (HPAEC) was introduced in the 1990s to determine the chain length distribution (CLD) of enzymatically debranched polysaccharides with very high resolution (Rani et al. 1992). The CLD for various glycogens is asymmetrically unimodal: in bovine and rabbit liver glycogens, chains ranged from 3 to 35 degrees of polymerization (DP), peaking at DP 6–10 or 7–15, respectively (Rani et al. 1992; Matsui et al. 1993) (Fig. 7a). This range of chain lengths suggests the architecture of glycogen may be intermediary between the Meyer and Whelan models. Amylopectin CLD profiles display a strikingly different bimodal CLD, with distinct groupings of short and long chains, further evidence for amylopectin chains being organized very differently than glycogen (Fig. 7b) (Hanashiro et al. 1996). CLD profiles for skeletal muscle and brain glycogen from mice have also been described recently. The Minassian group showed that the CLD profiles of mouse skeletal muscle and brain glycogen are nearly identical, ranging from DP 2–35 or more, with most chains being DP 4–15 (Nitschke et al. 2013, 2017). Additionally, gradual isoamylolysis of muscle glycogen suggests that the internal chains are longer than the external chains (Nitschke et al. 2013). The Roach group showed a CLD for mouse skeletal muscle glycogen ranging from DP 3–45, with most chains being 7–15 units long (Irimia et al. 2015). After exercise and a 1 h recovery, the CLD shifted toward long chains

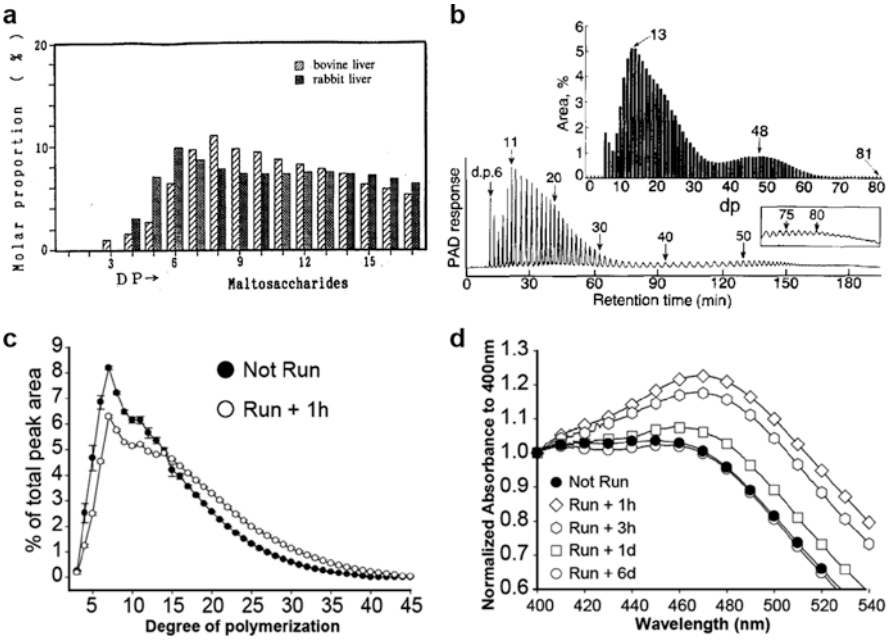

Fig. 7 Chain length distribution (CLD) of glycogen and amylopectin demonstrated by HPAEC. (**a**) CLD of bovine liver and rabbit liver glycogen determined by HPAEC (Matsui et al. 1993). Copyright © 1993 Japan Society for Bioscience and Agrochemistry, reprinted by permission of Taylor & Francis, Ltd. (**b**) HPAEC profile and CLD of potato amylopectin (Hanashiro et al. 1996). Copyright © 1996 Elsevier. Used with permission. (**c**) CLD of skeletal muscle glycogen from rested mice (not run) and 1 h following exhaustive exercise (Irimia et al. 2015). (**d**) Iodine spectra of skeletal muscle glycogen from rested mice and 1 h, 3 h, 1 day, and 6 days post-exercise (Irimia et al. 2015). Copyright © 2015 American Society for Biochemistry and Molecular Biology. Used with permission

(Fig. 7c). Similarly, muscle glycogen had an increased λ_{max} after exercise and recovery, which was gradually restored over the course of 6 days. These data suggest that newly synthesized glycogen is less branched with longer chains that are remodeled over time (Fig. 7d).

It appears that glycogen molecule is not as homogenously structured as the Whelan model suggests, and chain length and branching appear to fluctuate based on nutritional state. Gerty Cori stated in a 1952 lecture that "the relationship of structure to nutritional state has not yet been fully explored, but it seems that glycogen freshly deposited after a fasting period is least branched and has long outer chains whereas the opposite is true of 'old' glycogens" (Cori 1952). Additionally, mammalian and non-mammalian glycogens with similar average chain lengths produce differences in iodine spectra, suggesting that mammalian glycogens contain long chains in the interior of the granule that are not present in non-mammalian glycogens (Manners et al. 1983). Whole glycogen particles also display a continuum of sizes, and their size and ultrastructure vary with tissue type (see Sect. 6.1).

3.3 *Glycogen Structure and Phosphate: Insights from Lafora Disease*

Fontana first demonstrated that [^{32}P]-labeled glycogen could be purified from the livers of rats after the administration of radioactive phosphoric acid (Fontana 1980). Whelan's group subsequently reported that mammalian muscle glycogen contains about 0.064% phosphorus by weight (Lomako et al. 1993b, 1994). However, much like Whelan's reports of a proteinaceous component of glycogen that were later substantiated with the discovery of glycogenin, phosphate was initially treated as a contaminant. The physiological relevance of glycogen phosphate became evident through basic scientific investigations of Lafora disease (LD), a fatal, inherited childhood epilepsy and non-classical glycogen storage disease. LD is characterized by accumulations of abnormal polysaccharides known as Lafora bodies (LBs) that cause neurodegeneration (recently reviewed by Gentry et al. 2018) (Fig. 8a). In the 1960s and 1970s, it was demonstrated that LBs contain high levels of phosphorus and histologically resemble plant amylopectin (Yokoi et al. 1968; Sakai et al. 1970). In 1998 and 2003 the genes associated with the disease were identified, and it is now well established that virtually all LD patients carry recessive mutations in either the *EPM2A* or *EPM2B* gene (Minassian et al. 1998; Serratosa et al. 1999; Chan et al. 2003). *EPM2A* encodes laforin, a glycogen phosphatase (Worby et al. 2006; Tagliabracci et al. 2007), and *EPM2B* encodes malin, an E3 ubiquitin ligase (Gentry et al. 2005; Lohi et al. 2005). *Epm2a−/−* and *Epm2b−/−* mouse models recapitulate the disease with respect to LB formation and neurodegeneration (Ganesh et al. 2002; DePaoli-Roach et al. 2010; Valles-Ortega et al. 2011; Criado et al. 2012; Tiberia et al.

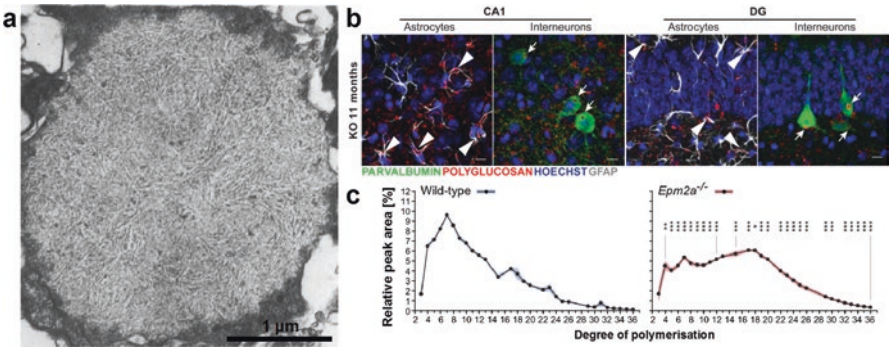

Fig. 8 Insights from Lafora disease, a nonclassical glycogen storage disease. (**a**) A typical LB visualized by electron microscopy in the human retina (Berard-Badier et al. 1980). Scale bar has been approximated based on magnification. Copyright © 1980 Springer Nature. Used with permission. (**b**) Neuronal (arrows) and astrocytic (arrowheads) polyglucosan accumulations visualized by immunostaining in the dentate gyrus (DG) and CA1 of the hippocampus in malin knockout (KO) mice (Valles-Ortega et al. 2011). Scale bars = 10 µm. (**c**) Normal and abnormal CLD of purified polysaccharides from WT and LD (*Epm2a−/−*) mice determined by HPAEC (Nitschke et al. 2017).

2012; Duran et al. 2014) (Fig. 8b). Purified polysaccharides from LD mice have a shifted λ_{max} reminiscent of amylopectin (Valles-Ortega et al. 2011; Brewer et al. 2019), and their CLD profile shows a higher proportion of long chains than wild-type mice, most prominently in brain tissue (Nitschke et al. 2017) (Fig. 8c). Unlike glycogen, purified LBs are quite large (>1 μm in diameter) and insoluble; they are presumed to be aggregates of a polysaccharide with abnormal architecture resulting from aberrant glycogen metabolism (Gentry et al. 2018; Brewer et al. 2019).

Laforin and malin regulate two aspects of glycogen architecture that may be intertwined: chain length and phosphate level. Through studies of its role in LD, laforin was discovered to be the founding member of a class of enzymes known as glucan phosphatases that directly dephosphorylate carbohydrate substrates (Worby et al. 2006; Gentry et al. 2007; Gentry and Pace 2009; Vander Kooi et al. 2010; Meekins et al. 2013, 2015). Laforin is the only known glucan phosphatase in mammals, and decreased laforin activity results in glycogen hyperphosphorylation. Malin ubiquitinates enzymes involved in glycogen metabolism, but the effects of ubiquitination are not clear (see Sect. 5.2). Surprisingly, the absence of malin also leads to hyperphosphorylation, and the absence of either enzyme leads to the accumulation of glycogen with abnormally long chains, which is puzzling (Sullivan et al. 2017). Recent results from mouse models overexpressing a catalytically inactive laforin demonstrate that the inactive laforin rescues the LD phenotype in the *Epm2a*-deficient mouse model (Gayarre et al. 2014; Nitschke et al. 2017). As a result, the relevance of laforin's catalytic activity has been questioned, despite the very high conservation of its catalytic residues and the presence of glucan phosphatases across multiple kingdoms (Gentry et al. 2007; Raththagala et al. 2015). It has also been suggested that laforin and malin form a ubiquitination-targeting complex involved in the disposal of glycogen molecules with aberrantly long chains that could crystallize and cause the molecule to precipitate (Sullivan et al. 2017). A growing body of evidence suggest that laforin and malin are an integral part of an alternative route for glycogen degradation known as glycophagy, involving members of the autophagic pathway and lysosomal α-glucosidase (see Sect. 5.2). However, glycophagy is probably not reserved only for aberrantly structured glycogen molecules, since the accumulated lysosomal glycogen in acid α-glucosidase deficiency (i.e. Pompe disease) is of normal structure based on iodine staining (Levin et al. 1968; Mahler 1969). Additional evidence for a physiological role of glycogen phosphate comes from the Roach lab and a substantial body of work from the field of starch metabolism.

The Roach lab analyzed glycogen CLD and phosphate levels of laforin-deficient mice pre- and post-exercise. When muscle glycogen phosphate was depleted, phosphate levels remained suppressed even after total glycogen and CLD returned to normal (Irimia et al. 2015). Laforin-deficient mice displayed exercise-induced glycogen depletion identical to wild-type mice, but phosphate levels remained elevated and iodine spectra suggested a delay in glycogen remodeling post-recovery. This study demonstrated that laforin dephosphorylates glycogen during exercise-induced cytosolic glycogeolysis, providing strong evidence for its physiological role as a glycogen phosphatase. Whelan suggested in 1994 that phosphate was a marker for the age of a glycogen molecule; the suppression of glycogen phosphate after exhaus-

tive exercise in wild-type mice does indeed suggest that phosphate accumulates with age, possibly from multiple cycles of glycogen degradation and re-synthesis (Lomako et al. 1994; Irimia et al. 2015). The role of phosphate in glycogen metabolism is not clear, but studies from the starch field demonstrate that phosphate plays an important physiochemical and biological role in starch architecture and metabolism (Hejazi et al. 2008; Blennow and Engelsen 2010; Kotting et al. 2010).

In plants, phosphorylation is necessary for the proper synthesis of starch, and reversible phosphorylation is an integral part of starch breakdown. Only amylopectin contains significant amounts of phosphate, which is enriched in the amorphous regions (Takeda and Hizukuri 1982; Blennow et al. 2000). Root and tuber starches, which exhibit less densely packed crystalline helices than other types of starches, have high phosphate levels (Lim et al. 1994; Blennow et al. 1998). Starch phosphate is covalently linked to the C3 and C6 hydroxyls of glucose moieties; about 70–80% of the phosphate is esterified to C6 (Ritte et al. 2006). Phosphate, particularly at the C3 position, disrupts the crystalline helices in amylopectin by introducing steric hindrance, promoting their solubilization (Hansen et al. 2009). Starch degradation is believed to be a cyclic process involving reversible phosphorylation and the concerted action of multiple enzymes: glucan dikinases, amylases, and glucan phosphatases (Emanuelle et al. 2016). Two dikinases that respectively phosphorylate the C6 and C3 position in that order are glucan, water dikinase and phosphoglucan, water dikinase. Phosphorylation promotes solubilization of the glucan chains and facilitates their access by plant amylases. However, the amylases cannot proceed past a phosphate, so phosphate removal is achieved by the glucan phosphatases Starch Excess 4 (SEX4) and Like Sex Four 2 (LSF2), named after the plant phenotype that results from their deficiency and part of the same family as the glycogen phosphatase laforin (Edner et al. 2007; Kotting et al. 2009; Santelia et al. 2011; Gentry et al. 2016; Meekins et al. 2016). Crystal structures and structure-function studies of SEX4 and LSF2 preceded those of laforin (Vander Kooi et al. 2010; Meekins et al. 2013, 2014; Raththagala et al. 2015) and the cooperative study of both systems has led to a wealth of insight about polyglucan architecture and phosphorylation (Gentry et al. 2009, 2016; Emanuelle et al. 2016).

Through studies of LD, the Roach and Minassian groups determined that normal glycogen contains about 1 phosphate moiety per 600–2500 glucose units, depending on species and tissue type (Tagliabracci et al. 2007, 2008; Turnbull et al. 2010; Tiberia et al. 2012; DePaoli-Roach et al. 2015). Phosphates are present as monoesters linked to the C2, C3 and C6 hydroxyls of glucose moieties, in approximately equal quantities (Tagliabracci et al. 2011; Nitschke et al. 2013; DePaoli-Roach et al. 2015). Data from multiple groups using various approaches strongly suggest the phosphate is concentrated at the interior of glycogen molecules (Tagliabracci et al. 2007; Nitschke et al. 2013; Irimia et al. 2015). Liver glycogen contains less phosphate than muscle glycogen, while C6 phosphate has been detected in brain glycogen at similar levels to C6 phosphate in muscle glycogen (Lomako et al. 1994; Tagliabracci et al. 2007; Turnbull et al. 2010; Nitschke et al. 2017). The source of glycogen phosphate remains a mystery: although it has been reported that glycogen synthase can incorporate the β-phosphate of UDP-glucose into glycogen in a rare side reaction, this mechanism could only account for C2 and C3 phosphate, and the

rate of incorporation is very low (1 in 10,000 catalytic cycles) compared to the actual levels observed in glycogen (Tagliabracci et al. 2011; Contreras et al. 2016). Although no glucan dikinase has been identified in mammals, the physiological role of glycogen phosphate will eventually be defined, likely through the continued investigations of starch metabolism and the molecular mechanisms of LD.

4 Glucosamine

Glycogen also contains small amounts of covalent glucosamine. In the 1960s and 70s, Maley et al. reported the incorporation of glycosidically linked [^{14}C]glucosamine into glycogen from [^{14}C]galactosamine, and showed that glycogen synthase could catalyze glucosamine incorporation *in vitro* by using the substrate UDP-glucosamine instead of UDP-glucose (Maley et al. 1966; Tarentino and Maley 1976). Whelan also reported that intraperitoneal injection of [^{14}C]galactosamine led to the incorporation of [^{14}C]glucosamine into glycogen, replacing as much as 10% of the glucose residues (Romero et al. 1980). Covalently linked glucosamine in glycogen apparently would not block phosphorolysis, since it could be released as glucosamine-1-phosphate by phosphorylase. Whelan reported that normal pig and rabbit liver contained 86–266 nmol glucosamine per g glycogen, which was randomly distributed throughout the molecule (Kirkman and Whelan 1986). It is worth noting that an earlier study also demonstrating the incorporation of [^{14}C] into glycogen from injected [^{14}C]glucosamine showed that glucosyl residues were labeled, indicating [^{14}C]glucosamine was converted to [^{14}C]glucose through a deamination reaction prior to glycogen incorporation (Khac et al. 1972).

Additional links between glycogen and glucosamine have been reported. Glycogen synthase can be modified by N-acetyl-glucosamine (GlcNAc), and it has been suggested that glycogenin could also be modified by GlcNAc (Parker et al. 2003; Tavridou and Agius 2003). Furthermore, studies have suggested that glycogen could be a carbohydrate source for protein glycosylation, for which glucosamine is a major precursor, and hypoglycosylation has been reported in GSDs (McMahon and Frost 1996; Hayee et al. 2011; Tegtmeyer et al. 2014; Ondruskova et al. 2018). A physiological role for glycogen in protein glycosylation and the relevance of covalently linked glucosamine await further investigation.

5 Cytosolic Glycogen Synthesis and Degradation

5.1 Glycogenin

Glycogen synthesis begins with glycogenin [UDP-α-glucose:glycogenin α-glucosyltransferase, EC 2.4.1.186], a member of glucosyltransferase family 8 (Campbell et al. 1997) (www.cazy.org). The enzyme possesses two distinct enzy-

matic activities: self-glucosylation and chain elongation, both requiring UDP-glucose as a glucosyl donor and both occurring at the same active site, though the chemistries of the reactions are different (Alonso et al. 1995; Lomako et al. 2004). For this reason the two activities were initially believed to belong to separate enzymes, until Whelan and Cohen discovered that both were accomplished by glycogenin (Lomako et al. 1988; Pitcher et al. 1988). Crystal structures and biochemical studies show that glycogenin functions as an obligate dimer and requires Mn^{2+} for activity (Gibbons et al. 2002; Chaikuad et al. 2011). Autoglucosylation on tyrosine creates a glucose-1-O-tyrosyl linkage, a chemical linkage rarely found in nature, then glycogenin synthesizes a short oligosaccharide primer of at least 7–8 glucose units (Smythe and Cohen 1991; Alonso et al. 1995). Even if this tyrosine is mutated, glycogenin is still capable of glucoslyation, just not of itself (Cao et al. 1993a; Alonso et al. 1994). Whether glucosyl additions occur via intra- or intersubunit reactions has been debated, but recent studies suggest that the first four glucosyl units may be added via an intrasubunit reaction, and longer chains require intersubunit interaction (Chaikuad et al. 2011). It has been suggested that although glycogenin functions as a dimer, at low enough concentrations the two subunits would dissociate after chain initiation, giving rise to two separate glycogen molecules (Lin et al. 1999). The presence of a single chain giving rise to an entire β-particle is supported by structural studies of glycogen (Calder 1991).

In most mammals, there is only one isoform of glycogenin that is widely expressed, but humans have two isoforms: glycogenin-1 (*GYG1*), expressed in all tissues, and glycogenin-2 (*GYG2*) which is primarily expressed only in liver, with minor expression in heart, pancreas, and adipose tissue (Mu et al. 1997; Roach 2002; www.proteinatlas.org). There is some evidence that glycogenin is phosphorylated, but it has not been corroborated (Lomako et al. 2004). Glycogenin can interact directly with glycogen synthase, and a crystal structure of the interaction provides evidence for their cooperation in the initiation of the glycogen granule (Skurat et al. 2006; Zeqiraj et al. 2014). Additionally, insufficiently glucosylated glycogenin does not serve as an efficient primer for glycogen synthase, and phosphorylase can reduce the glucosylation state of glycogenin, making it a less effective substrate for synthase (Cao et al. 1993b; Skurat et al. 1993). Phosphorylase induces the dissociation of glycogen synthase from a proteoglycan fraction in hepatocytes, presumably containing glycogenin (Tavridou and Agius 2003). Thus, priming through glycogenin may be regulated indirectly by the signals that govern glycogen synthase and phosphorylase activity (see Chaps. 3 and 4), rather than by direct regulation of glycogenin. Glycogenin interacting proteins (GNIPs) have been suggested to stimulate the activity of glycogenin (Graham et al. 2010). Paradoxically, glycogenin mutations in humans and mice lead to glycogen accumulation in some tissues and muscle weakness, indicating that without glycogenin, glycogen synthesis can still occur, but in a dysregulated and pathogenic manner (Malfatti et al. 2014; Testoni et al. 2017).

5.2 Glycogen Synthase and Phosphorylase

Glycogen synthase [UDPglucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11] is a member of the glycosyltransferase family 3 that catalyzes the addition of alpha-1,4-linked glucosyl units to a glycogen chain, using UDP-glucose as the donor and releasing UDP as product (www.cazy.com). Two isoforms of synthase exist in mammals: *GYS1*, encoding muscle glycogen synthase, highly expressed in all tissues including brain, and *GYS2,* restricted to liver (Roach et al. 2012) (www.proteinatlas.org). Muscle glycogen synthase has nine phosphorylation sites and was one of the first examples of a hierarchically phosphorylated protein (Roach 1990). Although liver glycogen synthase is also multiply phosphorylated, its activity appears to be regulated by only one phospho-site (Ros et al. 2009). Phosphorylation inhibits synthase activity, although activity can be fully restored in the presence of the potent allosteric activator glucose-6-phosphate. Numerous kinases are responsible for phosphorylating synthase, including protein kinase A, protein kinase C, AMP-activated protein kinase (AMPK), caseine kinase 2, and glycogen synthase kinase 3 (GSK3). Thus, control of glycogen metabolism is inextricably linked to intracellular energy status, glucose homeostasis, insulin signaling, and other external signals through diverse signaling cascades (Roach 1990; Lawrence and Roach 1997; Roach et al. 2012). The structure and regulation of glycogen synthase warrant much attention and are discussed in Chap. 3.

Glycogen phosphorylase [1,4-α-glucan:orthophosphate α-glycosyltransferase, EC 2.4.1.1] is a member of the glycosyltransferase family 35, and catalyzes the transfer of glucose moieties from the glycogen molecule to inorganic phosphate, releasing the product glucose-1-phospate (www.cazy.com). Glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase, facilitating its entry into glycolysis (see Chap. 6). In the presence of excess glucose-1-phosphate, phosphorylase can catalyze the reverse reaction (Cori and Cori 1943). There are three isoforms of phosphorylase corresponding to the tissues in which they are enriched, but not restricted: muscle (*PYGM*), liver (*PYGL*) and brain (*PYGB*) (www.proteinatlas.org). Only the brain isoform is expressed in fetal tissues, and all are expressed to some extent in adult brain (Pfeiffer-Guglielmi et al. 2000). A single site on phosphorylase is phosphorylated by only one kinase: phosphorylase kinase (PhK), a multisubunit enzyme with muscle, liver and brain isoforms. Both phosphorylase and PhK are activated by phosphorylation and allosteric modulators, and an excellent review on their regulation in brain has been published recently (Nadeau et al. 2018). Crystal structures of all phosphorylase isoforms have been determined (Rath et al. 2000; Lukacs et al. 2006; Mathieu et al. 2016). The structure and regulation of brain phosphorylase are also given a comprehensive review in Chap. 4.

5.3 Glycogen Branching and Debranching Enzymes

Like glycogenin, and unlike synthase and phosphorylase, glycogen branching enzyme (GBE) and debranching enzyme (GDE) do not appear to be regulated by phosphorylation or allosteric modulation. There is only one isoform of each, and both are highly expressed ubiquitously (www.proteinatlas.org). Due to their abundance, these enzymes are not considered to be rate-limiting under normal circumstances (Geddes 1986; Melendez et al. 1997; Roach et al. 2012). However, they are clearly essential for maintaining proper glycogen structure, as deficiencies in either enzyme result in GSDs characterized by distinctly abnormal glycogen deposits with pathological consequences (Adeva-Andany et al. 2016). Crystal structures of human GBE and yeast GDE have been determined and the effects of GSD-causing mutations defined, providing molecular level insights into how they affect glycogen structure (Froese et al. 2015; Zhai et al. 2016).

GBE (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, EC 2.4.1.18) belongs to the subfamily 8 of the GH13 family of glucosyl hydrolases. Like other members of this subfamily, it contains a carbohydrate binding module, CBM48 (Janecek et al. 2011; www.cazy.com). Two reaction steps occur successively in its central catalytic core: hydrolysis and transglucosylation (Froese et al. 2015). In the first step, the enzyme cleaves an α-1,4 linkage on a glucan chain, forming a covalent enzyme-glycosyl intermediate; in the second step, an α-1,6 linkage is formed from the same chain or one nearby. The minimum length transferred is 6 or 7 glucosyl units (Brown and Brown 1966; Verhue and Hers 1966), and the average distance between chains is 3 or 4 glucose units (Gibson et al. 1971; Calder 1991). This chain length requirement is supported by the crystal structure of GBE in complex with a heptasaccharide (Froese et al. 2015).

Mammalian GBE can convert amylose or amylopectin to a glycogen-like polysaccharide. Krisman showed that incubation of amylopectin or amylose with GBE purified from rat liver led to a displacement of λ_{max} to a value that coincided with that of glycogen (Krisman 1962). However, GBE did not change the λ_{max} of rabbit liver glycogen, suggesting the molecule already had been maximally branched. Indeed, comparative studies on mammalian and plant branching enzymes indicate that the branching degree in polysaccharides is determined by intrinsic properties of the branching enzyme used to synthesize them (Tolmasky and Krisman 1987; Kuriki et al. 1997). In other words, the optimal structure of the mammalian glycogen molecule may be primarily attributed to the inherent "equilibrium" of the mammalian branching enzyme. Not surprisingly, synthetic glycogens produced *in vitro* by branching enzymes from different organisms have similar, but not identical, structural properties to natural glycogens (Kajiura et al. 2008, 2010; Takata et al. 2009). Perhaps these observations explain the delay in glycogen remodeling reported by the Roach lab after exercise (Irimia et al. 2015): glycogen super-compensation, characterized by high glycogen synthase activity, results in longer chains because branching enzyme has not yet

introduced the appropriate branch points. Only over time does branching enzyme act so that glycogen structure returns to an equilibrium, with CLD and λ_{max} at basal levels. Indeed, it has been suggested that a chronic imbalance of glycogen synthase and GBE activities leads to abnormal polysaccharide formation in pathological conditions (Raben et al. 2001; Tagliabracci et al. 2008; Kakhlon et al. 2013). Also, the ubiquitous expression of a single GBE isoform may help to explain why brain glycogen has a similar CLD to muscle glycogen (Nitschke et al. 2017). Presumably all healthy tissues in a single species would reach the same CLD at "equilibrium."

GDE is required for cytosolic glycogen degradation since phosphorylase terminates four glucosyl residues away from a branch point. Mammalian GDE has two catalytic activities: 4-α-glucanotransferase activity (EC 2.4.1.25) involves transfer of a chain of three glucosyl units from the branch to a nearby nonreducing end, leaving a single α-1,6 glucosyl unit. Its amylo-α-1,6-glucosidase activity (EC 3.2.1.33) follows, and the α-1,6 linkage is hydrolyzed to release free glucose (Ryman and Whelan 1971). The release of free glucose instead of glucose-1-phosphate makes it very convenient to compare the levels of α-1,6 and α-1,4 linkages in polysaccharides. GDE is considered an indirect debranching enzyme; direct debranching enzymes such as isoamylase exclusively cleave α-1,6-linkages and release intact oligosaccharide chains. Direct debranching enzymes occur in plants, bacteria and yeast but not in mammals (Ryman and Whelan 1971). These enzymes are very useful in polysaccharide analysis, especially in determining CLD.

GDE catalytic mutants display a loss of either glucosidase or transferase activity, but not both, strongly suggesting disparate catalytic sites (Nakayama et al. 2001). This was confirmed when the crystal structure of yeast GDE was determined, revealing an elongated structure with catalytic domains at either end of the molecule (Zhai et al. 2016). Structures of both the free wild-type enzyme and a transferase-deficient mutant in complex with maltopentaose were reported. Maltopentaose molecules were bound at both catalytic domains and other sites, illustrating the specificity of GDE transferase activity for a glucan of five units or less and the necessity of non-catalytic binding sites for glycogen association. The data also suggested that the product of the transferase reaction, a chain containing a single α-1,6-linked glucosyl unit, completely dissociates from GDE prior to recruitment to the glucosidase catalytic site. Multiple binding sites are a recurring theme among glycogen- and starch-associated proteins (Baskaran et al. 2010; Meekins et al. 2013; Froese et al. 2015; Raththagala et al. 2015; Zhai et al. 2016). In observing that oligosaccharides were bound both to catalytic and non-catalytic sites in the crystal structure of GBE, Froese et al. stated: "[Non-catalytic binding sites] may provide GBEs the capability to anchor a complex glycogen granule and, as proposed previously, determine the chain length specificity for the branching reaction as a 'molecular ruler.' This agrees with the emerging concept of glycogen serving not only as the substrate and product of its metabolism but also as a scaffold for all acting enzymes" (2015) (See Sect. 7).

6 Glycophagy

6.1 Lessons from Pompe Disease

Phosphorolysis is not the sole means of glycogen degradation in cells. This became evident through studies of Pompe disease, a lysosomal storage disorder characterized by the buildup of vacuole-bound glycogen in virtually all tissues (Hers 1963; Raben et al. 2012). The deficient enzyme in Pompe disease is lysosomal acid α-glucosidase (called GAA or acid maltase) [EC 3.2.1.3] which hydrolyzes both α-1,4 and α-1,6 linkages, optimally at low pH (Hermans et al. 1991). It is a widely expressed enzyme, with a promoter characteristic of a housekeeping gene (Hoefsloot et al. 1990). GAA is synthesized as a precursor protein in the endoplasmic reticulum and undergoes extensive processing before it achieves optimal catalytic activity and reaches its lysosomal destination (Hermans et al. 1991; Wisselaar et al. 1993). It can fully hydrolyze glycogen as well as short oligosaccharides, maltose and other polyglucans to glucose (Hers 1963; Palmer 1971; Matsui et al. 1984). GAA is exo-acting, cleaving single glucosyl units successively from the non-reducing ends of glycogen. However, rather than releasing glucose-6-phosphate like phosphorylase, GAA liberates free glucose, which can translocate into the cytosol via glucose transporters in the lysosomal membrane (Mancini et al. 1990).

How glycogen gets into the lysosome is not well defined, but recent work demonstrates that it involves the autophagic machinery. Autophagy, or 'self-eating,' refers to multiple cellular pathways converging on the lysosomal breakdown of cellular components such as long-lived proteins and organelles (reviewed by Cuervo et al. 2005; Mizushima and Komatsu 2011; Schneider and Cuervo 2014). Autophagy is critical both for general cellular maintenance and for nutrient release in times of energy stress. It is especially critical for turning over worn or damaged cellular components in neurons and other post-mitotic cells (Cuervo et al. 2005; Komatsu et al. 2006; Mizushima and Komatsu 2011). The best characterized autophagic pathway is macroautophagy, activated in response to starvation; 'autophagy' and 'macroautophagy' are frequently used interchangeably. Macroautophagy is a highly conserved pathway in eukaryotes involving a family of autophagy-related (Atg) proteins and the formation of a structure with a characteristic double membrane known as the autophagosome, which engulfs substrates and fuses with the lysosome. Microautophagy and chaperone-mediated autophagy are alternative pathways in which cellular components are targeted directly to lysosomes in the absence of autophagosome formation. In recent years, numerous terms for the selective degradation of specific substrates (via any of the autophagic pathways) have been introduced; e.g. mitophagy (of mitochondria), pexophagy (of perioxisomes), nucleophagy (of nuclear components), reticulophagy (of ER), and xenophagy (of pathogens) (Mizushima and Komatsu 2011; Zhao et al. 2018). In 2011, the Roach group introduced the term 'glycophagy' to refer to the selective autophagic degradation of glycogen (Jiang et al. 2011). Ironically, the term 'glycogenosome' had already been used by some investigators to refer to the glycogen-laden lysosomes found in newborn rat liver, aged neural tissue, and glycogen storage diseases (Iwamasa et al. 1980, 1983; Cavanagh and Jones 2000), but the term did not gain traction.

In addition to glycogenosomes, which have a single membrane characteristic of a lysosome, Pompe (*GAA*−/−) tissues also display accumulated autophagosomes filled with ubiquitinated proteins and cellular debris. In order to study the effect of autophagy on lysosomal glycogen, Raben and colleagues generated a muscle-specific knockout of a critical autophagosome gene, *Atg5*, on the *GAA*−/− background. These mice had just as many glycogenosomes as the *GAA*−/− mice, but they had a reduced number of autophagosomes and no improvement in pathology. Ubiquitinated proteins were increased, but they were not surrounded by the double membrane as in the *GAA*−/− mice (Raben et al. 2008; Lim et al. 2017). The study suggested that the autophagy defect was caused by inefficient fusion of the autophagosomes with the glycogen-laden lysosomes, which formed by an independent pathway. In a later study, the group selectively inactivated a closely related gene, *Atg7*, in fast-twitch *GAA*−/− muscle (Raben et al. 2010). No autophagosomes were formed, glycogenosomes were still present, and there was no change in pathology; however, the glycogen level was reduced. These two studies demonstrate that macroautophagy contributes to some, but not all, of the delivery of glycogen to the lysosome (Raben et al. 2010). The group also illustrated that the muscle pathology of Pompe disease resulted largely from defective autophagic flux. The observation that Atg7, but not Atg5, abrogated glycogen accumulation may be explained by the fact that there are two primary conjugation systems in autophagy, and while Atg5 is only involved in one, Atg7 is required for both (Nakatogawa et al. 2009).

In 2002, Janeček identified a putative CBM20 in a protein of unknown function called starch binding domain-containing protein 1 (Stbd1, also called genethonin 1) (Janeček 2002). The only other proteins in mammals with a CBM20 are laforin and a glycosyltransferase (glycerophosphocholine phosphodiesterase 1, GPCPD1). It was known that the laforin CBM facilitates laforin binding to glycogen (Wang et al. 2002). The Roach group later showed that Stbd1 is highly expressed in glycogen-rich tissues such as muscle, liver, and heart, with trace amounts in brain, kidney and pancreas, and that Stbd1 levels mirrored glycogen levels in fed, fasted and transgenic mice (Jiang et al. 2010). Stbd1 binds glycogen *in vitro* and co-localizes with the lysosomal-associated membrane protein LAMP1 and the Atg8 homolog GABARAPL1 in cell culture, but not with the microtubule-associated light chain 3 (LC3), a marker for auotophagosomes (Jiang et al. 2010, 2011). Demetriadou et al. later showed that when overexpressed in HeLa cells, Stbd1 was N-myristoylated, and this modification facilitates its recruitment with glycogen to subcellular domains associated with autophagy (Demetriadou et al. 2017). The Sun group showed that Stbd1 is elevated in muscle tissue of *GAA*−/− mice, but not in liver or heart, but shRNA-mediated knockdown of Stbd1 did not alter glycogen levels in any tissues (Yi et al. 2013). A full knockout of Stbd1 in the Pompe mice (*Stbd1*−/− *GAA*−/−) did not change lysosomal accumulation of glycogen in muscle or heart tissue, but reduced liver glycogen by 73% (Sun et al. 2016). Glycogen accumulation could be restored by exogenous expression of human Stbd1, but not a mutant lacking the CBM20. Their study demonstrated that Stbd1 may significantly participate in glycophagy in the liver but not in the heart or skeletal muscle, and that the CBM20 is essential for its function.

6.2 Lessons from Lafora Disease

Another link between glycogen and autophagy emerged through studies of LD. Laforin- and malin-deficient mouse models were generated and analyzed by six different groups. Most observed autophagy defects in the brain of these mice (Criado et al. 2012; Puri et al. 2012; Duran et al. 2014; Gayarre et al. 2014). Some groups observed reduced conversion of LC3 from its cytosolic form (LC3-I) to lipidated form (LC3-II), which is associated with its recruitment to autophagosomal membranes (Criado et al. 2012; Gayarre et al. 2014). Both mouse models also displayed an increase in p62 (Criado et al. 2012; Puri et al. 2012; Duran et al. 2014), a protein that normally binds to both ubiquitinated cargo and LC3, is incorporated into the autophagosome, and degraded (Mizushima and Komatsu 2011). Reduced LC3-II and increased p62 levels strongly suggested defects in autophagic flux. Similar defects were also observed in liver of laforin-deficient mice, but not in muscle (Aguado et al. 2010; Irimia et al. 2015). In contrast, the Minassian group did not observe autophagy defects in muscle or brain in either mouse model (Wang et al. 2016; Nitschke et al. 2017).

The Roach, Minassian and Guinovart groups generated LD mouse models with genetically reduced glycogen synthesis and found that LB formation was reduced and neuropathology was abrogated (Turnbull et al. 2011a, 2014; Pederson et al. 2013; Duran et al. 2014). The Guinovart group elegantly demonstrated that the autophagy impairment was secondary to polyglucosan accumulation by crossing malin-deficient mice with mice specifically lacking muscle glycogen synthase in the brain, which eliminated cerebral glycogen synthesis, LB formation, autophagy defects, gliosis, and susceptibility to epilepsy (Duran et al. 2014). These studies indicate that polyglucosan accumulation drives the autophagy impairment and neurological phenotype in LD. Although some groups have argued that laforin and malin regulate autophagy independent of glycogen, this is unlikely to be the case *in vivo* (Gentry et al. 2018). However, as was demonstrated with the Pompe mice, insufficient clearance of glycogen via glycophagy may indeed provoke pathological damage by obstructing overall autophagic flux. This is a very prominent theme among lysosome storage disorders, which frequently have severe neurological phenotypes; this disease class is reviewed in depth elsewhere (Lieberman et al. 2012; Seranova et al. 2017). Corroborating this, recent studies from the Sanz group showed that in LD, mitophagy is impaired due to a global autophagic defect, and endocytic recycling of the astrocytic glutamate transporter GLT1 is altered (Munoz-Ballester et al. 2016; Garcia-Gimeno et al. 2018), likely also resulting from impaired autophagy.

The possibility that laforin and malin are involved in glycophagy builds on work from many labs showing that malin, typically in concert with laforin, ubiquitinates glycogen-associated substrates. Malin is an E3 ubiquitin ligase that was initially demonstrated to bind and ubiquitinate laforin in cell culture (Gentry et al. 2005; Lohi et al. 2005). Malin also promotes the ubiquitination of multiple proteins that bind to glycogen molecules: glycogen synthase, protein targeting to glycogen

(PTG), R6, AMPK and GDE (see Sect. 7). In most of these studies, ubiquitination was shown to require laforin, which is believed to act as a scaffold for malin interaction with its substrate (Cheng et al. 2007; Vilchez et al. 2007; Worby et al. 2008; Moreno et al. 2010; Rubio-Villena et al. 2013). Although ubiquitination led to the degradation of these substrates in cell culture, PTG, R6 and AMPK levels were unchanged in LD mouse models; in contrast, glycogen synthase accumulated in the insoluble glycogen fraction, as did laforin in malin-deficient mice (Wang et al. 2007; DePaoli-Roach et al. 2010; Valles-Ortega et al. 2011; Tiberia et al. 2012). Importantly, whenever the ubiquitin linkages incorporated by malin are defined, they are K63-linked (Moreno et al. 2010; Roma-Mateo et al. 2011a; Rubio-Villena et al. 2013; Sanchez-Martin et al. 2015). K63-linked ubiquitination is a substrate for p62 binding and specifically linked to selective autophagy (Tan et al. 2008; Shaid et al. 2013). The presence of a laforin-malin ubiquitination complex has been highly debated (Gentry et al. 2013, 2018; Sullivan et al. 2017).

Interestingly, the Roach group reported that there was no change in the levels of GABARAPL1 (a member of the Atg8 family and a homolog of LC3; see Lee and Lee 2016) and LAMP1 in laforin-deficient fibroblasts, but these proteins were decreased in malin-deficient fibroblasts and in fibroblasts with both laforin and malin knocked out (Garyali et al. 2014). These results suggest laforin is upstream of malin in the path from glycogen to the lysosome. The Sanz group showed that laforin, malin and p62 form a complex, and that malin mediates the interaction between laforin and p62, likely through ubiquitination (Sanchez-Martin et al. 2015). Additionally, malin and p62 do not co-localize in cells without the expression of laforin. It is well known that laforin binds to and co-localizes with glycogen *in vitro*, in cell culture, and *in vivo*.

A model to explain these studies that is compatible with available data posits: laforin binds to glycogen and recruits malin; the laforin-malin complex promotes K63-linked ubiquitination of various glycogen-bound proteins; p62 binds to K63-linked ubiquitin moieties, recruiting LC3 (or its homolog GABARAPL1) and the autophagosome machinery, which engulfs the glycogen particle and fuses with the lysosome. Since glycogen synthase and laforin expression parallel glycogen levels and these proteins accumulate with glycogen in LD mouse models, they may also be turned over inside the lysosome. The fact that a catalytically inactive form of laforin rescues laforin-deficient mouse models supports this scaffolding role for laforin (Gayarre et al. 2014; Nitschke et al. 2017), but does not exclude the possibility that glycogen dephosphorylation by laforin is still relevant, as during cytosolic glycogenolysis (Irimia et al. 2015). Also, phosphorylation of laforin by AMPK has been shown to enhance the interaction of laforin with malin (Roma-Mateo et al. 2011b); this regulation event could be one way to stimulate glycophagy in times of energy stress, when AMPK becomes activated. Finally, it is well established that laforin preferentially binds to polysaccharides with long chains and localizes to LBs in malin-deficient mice (Chan et al. 2004; Criado et al. 2012; Raththagala et al. 2015). Thus, laforin would selectively recruit the glycophagy machinery to precipitation-prone glycogen molecules with precariously long chains, as has been proposed (Sullivan et al. 2017).

It is worth noting that delivery of glycogen to lysosomes may involve multiple pathways, possibly with cell-specific relevance. Perhaps Stbd1 and laforin, each containing a CBM20, function as distinct receptors for glycophagy. It is interesting that although LBs occupy most tissues in LD, they are enriched in certain cell types within these tissues. For example, in LD mice, 97% of LBs in skeletal muscle are found in fast-twitch type IIb muscle fibers and 2% in slow-twitch type I fibers (Turnbull et al. 2011b). In human skin biopsy, LBs are only found in duct cells, but not secretory cells (Andrade et al. 2003). In human liver, LBs are enriched in the periportal regions, but not the perivenous regions (Carpenter et al. 1974). All of these cell types appear to make glycogen (Montagna et al. 1951; Vøllestad et al. 1984; Quistorff 1985), but the differences in whether or not they make LBs suggest the glycogen is handled differently. Cell-specific glycogen metabolism and polyglucosan body formation are of particular interest in the brain. LBs were first reported in neurons (Lafora 1911), and historically were believed to occupy this cell type almost exclusively (Minassian 2001). However, this observation has been perplexing since the vast majority of brain glycogen is stored in astrocytes. Recent work has demonstrated that neurons possess an active glycogen metabolism (Vilchez et al. 2007; Saez et al. 2014), and LD mouse models accumulate both LBs in neurons and a distinct polyglucosan body known as corpora amylacea in astrocytes (Augé et al. 2018; Rubio-Villena et al. 2018). Early literature on human patients corroborate these findings. Corpora amylacea in addition to LBs have been reported in multiple studies (Van Heycop Ten Ham 1975). Small inclusions, which may also correspond to corpora amylacea, have been identified in astrocytes and oligodendrocytes (Schwarz and Yanoff 1965). Recently, it has been asserted that the striking appearance of neuronal LBs left glial LBs largely overlooked over the years; upon careful review of the literature, small LBs that were assumed to be in neuronal processes are likely to actually be astrocytic (Minassian, personal communication). This topic is further discussed in Chap. 10.

6.3 The benefits of Glycophagy

The lysosomal glycogen pool plays an important role in circumstances when a burst of glucose is needed, such as during the neonatal starvation period (Kotoulas et al. 2004, 2006; Schiaffino et al. 2008). Glycogen-rich autophagosomes have been observed in the neonatal liver, muscle and heart, but they are depleted within hours after birth. A unique role for glycophagy in the heart is also emerging (Reichelt et al. 2013; Delbridge et al. 2015). While liver and muscle normally deplete their glycogen stores with fasting, cardiac tissues accumulate glycogen and upregulate glycogen autophagy under nutrient stress (Reichelt et al. 2013). An advantage of lysosomal glycogen is that it can be mobilized independently or in parallel with cytosolic glycogen by a distinct set of cellular stimuli. For example, glycophagy could be specifically regulated by lysosomal uptake of Ca^{2+}, which enhances GAA

activity (Kotoulas et al. 2006; Delbridge et al. 2015). Very little is known about the role of glycophagy in brain tissue, besides the report of glycogenosomes in the aging brain (Cavanagh and Jones 2000). It has been suggested that glycogen of large molecular weight (i.e. α particles) is specifically degraded in lysosomes, and proceeds by a random, rather than an ordered degradation mechanism (Geddes and Stratton 1977a; Devos and Hers 1980). Based on glycogen degradation patterns it has been speculated that brain glycogen is degraded primarily by phosphorolysis and not by lysosomal hydrolysis (Chee et al. 1983).

7 Glycogen Ultrastructure and Subcellular Distribution

In over a century of glycogen-related research many researchers have debated the existence of multiple forms of glycogen that differ in size, structure, associated proteins, and metabolic activity. The distinction between glycogen *α* and *β* particles came into focus with studies using the electron microscope and ultracentrifugation, and some progress has been made on defining the nature of these particles. Acid-soluble and acid-insoluble fractions of glycogen have also been observed, but the physiological relevance of these distinct pools has been highly debated. These topics, as well as a possible role for glycogen metabolic enzymes in the nucleus, are discussed in the next sections.

7.1 Glycogen α, β, and γ Particles

Drochmans and colleagues were the first to describe the various levels of glycogen structure in native glycogen isolated from liver and muscle, and their observations were corroborated by others (Drochmans 1962; Revel 1964; Wanson and Drochmans 1968; Ryu et al. 2009). The largest glycogen particle, named the α particle, is a rosette-shaped association of several smaller units, known as β particles, that can reach up to 300 nm in diameter and over 10^8 Daltons in molecular weight (Gilbert and Sullivan 2014; Prats et al. 2018). Glycogen β particles typically range in size from 10 nm to 44 nm and have a molecular weight of 10^6–10^7 Daltons (Shearer and Graham 2002; Sullivan et al. 2012). It is generally accepted that a β particle corresponds to a single molecular unit of glycogen that can reach up to 12 tiers as modeled by Whelan and others (See Sect. 3.1) (Fig. 6c). Drochmans designated the 3 nm filaments making up the β particles as γ particles (Drochmans 1962). However, 'γ particle' has also been used to refer to protein-rich subunits on the glycogen β particles (see Sect. 7) (Prats et al. 2018). Generally speaking, glycogen is primarily stored as α particles (or a mixture of α and β particles) in the liver and as β particles in muscle, brain, and other tissues (reviewed below). Recent work has demonstrated a mixture of α and β particles in cardiac tissue (Besford et al. 2012). It is apparent that these norms become disrupted in pathological conditions.

It is estimated that liver α particles contain, on average, 20 to 40 β particle subunits (Devos et al. 1983). Liver glycogen is typically cytoplasmic and often reported to closely associate with smooth endoplasmic reticulum (Bruni and Porter 1965; Cardell and Cardell 1990). In muscle, β particles are associated with sarcoplasmic reticulum, concentrated either in subsarcolemmal regions, between myofibrils, or within the myofibrils; these pools respond differently to metabolic cues (Graham et al. 2010). Aggregates up to 60 nm in diameter, apparently composed of 4–6 β particles, were also observed via EM by Wanson and Drochmans in muscle, and others have reported α particles in rat muscle and insect flight muscle (Wanson and Drochmans 1968; Calder 1991). In skin and adipose tissue, β particles appear as cytoplasmic granules interspersed between ribonucleoprotein particles (Napolitano and Fawcett 1958; Hashimoto and DiBella 1967). In adipose tissue, they are only prominent after refeeding following a fast (Eichner 1984). In leukocytes and thymus, β particles are scattered throughout the cytoplasm and are fairly homogenous in size (Scott and Still 1968; Salmoral et al. 1990). In the retina, cytoplasmic β particles and occasional small α particles (60 nm) have been observed in the cone, but not rod, photoreceptor cells, and in Müller cells (Okubo et al. 1998).

In the brain, glycogen exists primary as cytoplasmic β particles in the cell bodies and processes of astrocytes (Maxwell and Kruger 1965; Sotelo and Palay 1968; Phelps 1975; Cataldo and Broadwell 1986). Normally, they are sparse to nonexistent in neurons, microglia, or oligodendrocytes, but β particles have appeared in neuronal axons and dendrites following fasting or trauma (Phelps 1975; Cataldo and Broadwell 1986). Astroglial β particles become apparent in early embryos and increase in both abundance and diameter with development (Gadisseux and Evrard 1985). Small α particles (80–100 nm in diameter, composed of 4–8 β particle subunits by EM) have also been reported in rat astrocytes (Sotelo and Palay 1968). Biochemical studies of glycogen isolated via a mild cold-water extraction method show significant heterogeneity in molecular weight: a fraction of particles had molecular weights suggestive of small α-particles composed of 3–6 β particle subunits (Chee et al. 1983). In several cases of human glycogenoses, small (60–120 nm in diameter) and large (150–350 nm in diameter) α particles have been reported in astrocytes or neurons (Résibois-Grégoire and Dourov 1966; Kornfeld and LeBaron 1984; Towfighi et al. 1989). Aggregates of α or β particles have also been reported in Alzheimer's disease and the aging brain (Gertz et al. 1985; Mann et al. 1987), which are distinct from polyglucosan bodies (Cavanagh 1999). Reactive astrocytes are known to accumulate glycogen β particles in response to various pathological stimuli, including: brain infarction (Kajihara et al. 2001), methionine sulfoxide (Phelps 1975; Delorme and Hevor 1985), X-ray irradiation (Maxwell and Kruger 1965), and barbiturates (Phelps 1975). Occasionally, intramitochondrial glycogen has also been observed in rat retinal cells (Ishikawa and Pei 1965), in the human and canine myocardium (Buja et al. 1972; Maron and Ferrans 1975), and in astrocytes (Sotelo and Palay 1968; Alaraj et al. 2004).

β particle size exists around a normal distribution, with most granules being 20–30 nm (Calder 1991; Marchand et al. 2002) (Fig. 9a). Since each additional tier is estimated to contribute 3.8 nm to the diameter (Melendez-Hevia et al. 1993;

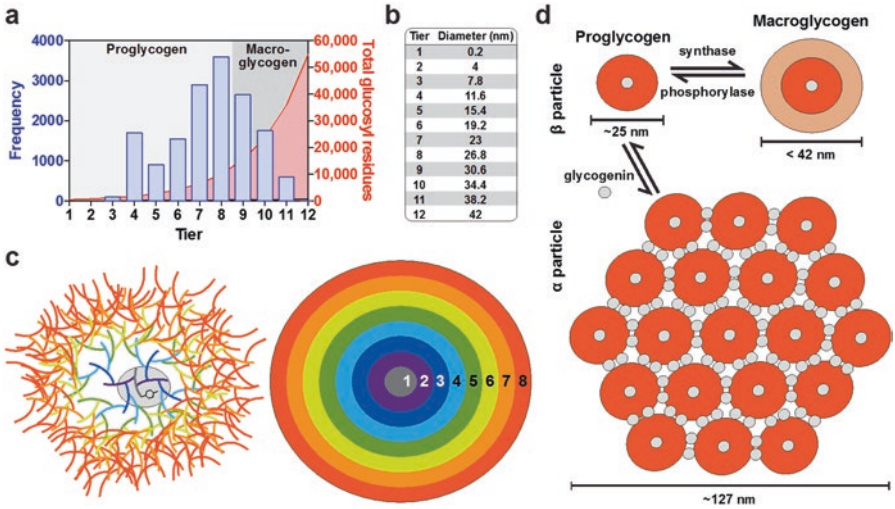

Fig. 9 Size and structure of glycogen α and β particles, proglycogen and macroglycogen. (**a**) Distribution of β glycogen particle size in muscle (Marchand et al. 2002) compared to the estimated number of glucosyl residues per glycogen molecule (Shearer and Graham 2002). The theoretical threshold between proglycogen and macroglycogen is after tier 8. (**b**) Estimated diameter of the tiers of glycogen particles. (**c**) Schematic diagrams of an 8-tiered glycogen molecule based on the studies of Whelan and mathematical modeling of (Melendez-Hevia et al. 1993). (**d**) A possible relationship between proglycogen and macroglycogen and α and β particles and approximated sizes. Grey circles represent glycogenin molecules. Diagram of α particle was adapted from (Tan et al. 2018).

Marchand et al. 2002), the observed size distribution implies that most β particles have only about 7–9 tiers (Fig. 9a, b, c). It is still not clear why most β particles do not reach the maximum diameter, since such large granules can store a significantly greater amount of glucose (Graham et al. 2010). A growing body of evidence suggests that glycogenin regulates β particle size. In cell culture and in tissue, glycogenin does not appear to exist in a free form; it is always attached to glycogen (Alonso et al. 1995; Skurat et al. 1997; Testoni et al. 2017). Overexpression of glycogenin in cell culture did not change total glycogen levels, but it increased the total number of glycogen molecules, which were of a smaller size (Skurat et al. 1997). The Guinovart group showed that mice lacking glycogenin display reduced exercise endurance and an accumulation of glycogen particles in muscle that were larger than those in wild-type mice (Testoni et al. 2017). Also, crystal structures and data from the Sicheri lab suggest glycogen particle size is regulated by the length of the linker between the glycogenin catalytic domain and the region that binds to glycogen synthase; this region is alternatively spliced and varies in length across species (Zeqiraj et al. 2014; Zeqiraj and Sicheri 2015).

In healthy organisms, α particles seem to be a unique feature of the liver. It is well established that total liver glycogen depletes with fasting and exceeds basal levels upon refeeding, a phenomenon known as glycogen super-compensation that

is also observed in muscle and brain (Nilsson and Hultman 1973; Terjung et al. 1974; Matsui et al. 2012). Additionally, hepatic glycogen levels and the activities of glycogen metabolizing enzymes undergo a diurnal rhythm (Halberg et al. 1960; Roesler and Khandelwal 1985). The Gilbert lab analyzed glycogen particles by size exclusion chromatography and EM and proposed a model for diurnal glycogen cycling in liver based on their data and earlier work (Sullivan et al. 2014). Starved livers maintain low glycogen in the form of small β particles. Upon refeeding, glycogen is quickly synthesized on these preexisting β particles, which provide optimal surface-to-volume ratio for rapid resynthesis. Once glycogen levels peak, the β particles are assembled into α particles. The α particles persist as glycogen levels begin to gradually fall, but they are gradually disassembled into β particles, which are preferentially degraded. Thus it appears that the primarily role of α particles is to facilitate a slower release of glucose than β particles (Chandramouli et al. 2015). This indeed seems optimal for the organ whose primary role is to maintain blood glucose homeostasis. Additionally, α particles in diabetic livers are more fragile than those of healthy mice, suggesting they could more easily be broken into β particles and contribute to hyperglycemia (Deng et al. 2015; Hu et al. 2018).

The nature of α particle assembly has remained elusive for decades. Drochmans observed that purified α particles could be dissociated into β particles under acidic conditions (Drochmans 1962). Lazarow, Orrell and Bueding previously showed using ultracentrifugation that acid, alkali and heat drastically changed the sedimentation of liver glycogen, suggesting these treatments disrupt α particle structure (Lazarow 1942; Orrell and Bueding 1964). The Gilbert lab has devoted a significant body of work to elucidating the nature of the α particle bond due to its relevance to diabetes (Sullivan et al. 2010, 2012; Deng et al. 2015, 2016; Tan et al. 2018). The lack of significant dissociation of normal α particles in dimethyl sulfoxide, 2-mercaptoethanol, or sodium dodecyl sulfate suggested the association is not through hydrogen or disulfide bonds or weak protein-protein interactions (Sullivan et al. 2010, 2012). It was reported that unexpectedly high levels of glycogenin were found in a proteomic study of liver glycogen, suggesting that glycogenin was present on the granule surface in addition to the granule core (Stapleton et al. 2010). The Gilbert lab then used proteomics to identify glycogenin as the molecular "glue" holding α particles together (Tan et al. 2018) (Fig. 9d).

7.2 Proglycogen and Macroglycogen

There is a lot of controversy surrounding proglycogen and macroglycogen, which have gone by different terms over the years and have been discussed in great detail by others (Lomako et al. 1993a; Alonso et al. 1995; Rybicka 1996; Shearer and Graham 2002). Although the precise nature of these pools is debated, there does appear to be sufficient evidence for their distinct physiological roles in glycogen turnover (Shearer and Graham 2002; Graham et al. 2010). The relationship between proglycogen/macroglycogen and α/β particles is not completely clear as these dist-

ictions are discussed seperately. However, since they are relevant to tissues generally lacking α particles, proglycogen and macroglycogen likely represent two states of β particle synthesis (Alonso et al. 1995).

When the TCA extraction method was first introduced for the purification of glycogen, it was observed that a portion of the glycogen was resistant to extraction by cold TCA (Willstätter and Rohdewald 1934). It could not be released unless the tissue was treated with heat, alkali, or protease, so it was concluded that this resistant fraction was attached to protein, and the two fractions were called lyo- ("free") and desmo- ("fixed") glycogen (Willstätter and Rohdewald 1934; Van Heijningen and Kemp 1955). Such observations also gave rise to the terms acid-soluble, i.e. extractable, glycogen, and acid-insoluble, i.e. residual, glycogen (Russell and Bloom 1955; Stetten Jr and Stetten 1960). In a 1960 literature review, Stetten and Stetten noted that while the quantity of free glycogen was widely variable with nutritional state, the quantity of fixed glycogen remained constant. However, experiments suggested the latter was more metabolically active, since injected [^{14}C]glucose was more readily incorporated into fixed glycogen (Stetten Jr and Stetten 1960). For decades, researchers continued to debate whether such observations were artefactual, merely reflecting the association of known metabolic enzymes with glycogen (Whelan 1976). Whelan revisited the subject in the late twentieth century while investigating the origin of glycogen synthesis. Using cultured primary astrocytes, his group showed that fixed glycogen, which was re-named proglycogen, contained 10% protein by weight and was rapidly resynthesized upon refeeding after glucose starvation, reaching a size of 400,000 Da. The proglycogen was later converted to macroglycogen, as demonstrated by pulse-chase labeling (Lomako et al. 1993a). The group proposed that proglycogen was an intermediate between glycogenin and the mature glycogen granule (Lomako et al. 1993a; Alonso et al. 1995). Resynthesis of proglycogen prior to macroglycogen following exhaustive exercise was also confirmed *in vivo* in humans (Adamo 1998).

It has been suggested that the upper limit of proglycogen size corresponds to a diameter of ~30 nm, a molecule of approximately 8 tiers; anything larger would be considered macroglycogen (Melendez et al. 1997; Marchand et al. 2002; Shearer and Graham 2002) (Fig. 9a, d). However, Whelan's group estimated that the upper limit of proglycogen was 400 kDa (Lomako et al. 1993a; Alonso et al. 1995), which corresponds to a glycogen molecule with ~2500 glucose residues, i.e. approximately 5 tiers. Whelan's group also suggested that proglycogen and macroglycogen are synthesized by different enzymes, however it may be that the different activities associated with the pro- and macroglycogen fractions both correspond to glycogen synthase. Two independent groups have demonstrated that glycogen synthase in the proglycogen fraction has a higher affinity for UDP-glucose than synthase in the macroglycogen fraction (Curtino and Lacoste 2000; Tavridou and Agius 2003). Curtino and Lacoste proposed that the insolubility of proglycogen in TCA was due to its heavy association with glycogen synthase, not with glycogenin, as Whelan had proposed (Curtino and Lacoste 2000). Tavridu and Agnius showed that the hormone insulin increased the association of glycogen synthase with the proglycogen fraction, and glucagon had the opposite effect (Tavridou and Agius 2003). Insulin is

known to stimulate dephosphorylation (and activation) of glycogen synthase, and the effects of both hormones are well known to regulate glycogen synthesis through signaling cascades, so the difference in synthase activity may be due to its phosphorylation state. Indeed, phosphorylation-dependent stimulation of glycogen synthase and phosphorylase has been shown to induce translocation of these proteins to actin-rich structures proximal to sarcoplasmic reticulum in skeletal muscle (Prats et al. 2005, 2009). In liver, newly synthesized glycogen has also been reported to be associated with a protein backbone (Geddes and Stratton 1977b) and smooth endoplasmic reticulum (Cardell and Cardell 1990). Additionally, glycogenin has been shown to bind to actin (Baqué et al. 1997). *In vitro* studies suggest that glycogen synthase is more active when it is bound to glycogenin (Pitcher et al. 1987, 1988). When glycogen content decreases, glycogen synthase has been shown to translocate from a glycogen-enriched membrane fraction to the cytoskeleton, concurrent with a decrease in activity (Nielsen et al. 2001). In isolated hepatocytes, glucose stimulated the cytoskeleton-dependent translocation of synthase from the cytosol to the plasma membrane, and newly synthesized glycogen molecules always appeared first at the cell membrane, but moved inward as they were replaced by newer molecules (García-Rocha et al. 2001; Fernández-Novell et al. 2002; Ferrer et al. 2003).

Proglycogen may represent a membrane-associated and/or actin-bound form of glycogen that is primed and ready for active anabolism/catabolism. When it grows to a certain size, it becomes macroglycogen, synthesis levels off, and it is released into the cytosol. A similar model has been proposed recently (Prats et al. 2018) and prompts further discussion of glycogen and its associated proteins as a dynamic subcellular entity rather than a static molecule (See Sect. 7) (Rybicka 1996). It is still not clear whether synthesis and degradation can occur simultaneously on the same granule, but proximity of these activities is suggested (Prats et al. 2005). The spatiotemporal regulation of glycogen metabolism is an interesting area of research requiring further elucidation.

7.3 Nuclear Glycogen

Dozens of microscopy studies have reported intranuclear glycogen deposits, most often in pathological conditions of the liver (Chipps and Duff 1942; Sheldon et al. 1962; Tanikawa 1965). In a 1975 case report, Ferrans et al. beautifully reviewed 24 prior studies on intranuclear glycogen either in the context of diabetes, glycogen storage diseases, or as an incidental feature in healthy humans and animals (Ferrans et al. 1975). In these reports, nuclear glycogen was usually in the form of β particles, sometimes dispersed, other times clustered; occasionally a few α particles were reported. The findings of Ferrans et al. were unusual in that nuclear glycogen was observed in the diseased myocardium. Intranuclear glycogen was only found in 6 out of 90 patients with cardiac diseases, and only in a few nuclei per patient. Most of the glycogen was present as β particles, often aggregated and filling the entire

nucleoplasm. The presence of nuclear glycogen was not associated with any cellular damage. The authors proposed three mechanisms for the formation of nuclear glycogen, but shrewdly concluded that only one was possible: (1) there was no ultrastructural evidence supporting phagocytosis or endocytosis of glycogen particles by the nuclear membrane; (2) glycogen particles were too large to pass through the nuclear pore complex; (3) the glycogen must be synthesized *in situ,* which had been previously demonstrated in normal Müller cells of the rat retina (Amemiya 1970) and in Novikoff hepatoma cells (Karasaki 1971). Ferrans et al. proposed that conditions favoring increased permeability of the nuclear membrane facilitated the entry of the metabolic enzymes into the nucleus, but that they became trapped when permeability returned to normal. This implies that atypical trapping of glycogenic enzymes into the nucleus may result in excessive levels of glycogen. Nuclear α and β particles have also been reported in the context of various cancers: human gastric adenocarcinoma (Ohyumi and Takano 1977), sublines of the Ehrlich-Lettré mouse ascites tumor (Kopun et al. 1989), chicken sarcoma (Binggeli 1959) and human and mouse hepatoma (Leduc and Wilson 1959; Ghandially and Parry 1966). Nuclear glycogen synthase activity was also detected in the Ehrlich-Lettré subline (Granzow et al. 1981; Kopun et al. 1982). In the brain, intranuclear glycogen deposits have been observed in the abnormal astrocytes of Alzheimer's disease (Horita et al. 1981; Miyakawa et al. 1982) and in the pituicytes of aged rats (Lafarga et al. 1991). Nuclear polyglucosan was also reported in a neuronal cell culture model of GBE deficiency (Kakhlon et al. 2013).

In more recent years, Guinovart, Gentry and others have shown that nearly all the central glycogen metabolic enzymes can be found in the nucleus: glycogenin (Miozzo et al. 1996; Baqué et al. 1997), glycogen synthase (Ferrer et al. 1997; Cid et al. 2005; Vilchez et al. 2007) glycogen phosphorylase (Sun et al. 2019), and debranching enzyme (Cheng et al. 2007). Guinovart's group published a series of studies demonstrating the translocation of the muscle isoform of glycogen synthase from the cytosol to the nucleus in conditions of glucose deprivation, which required glycogen binding (Ferrer et al. 1997; Cid et al. 2005; Díaz et al. 2011). They also showed that neurons display primarily nuclear localization of glycogen synthase, characteristic of cells lacking glycogen, in contrast to glycogen-rich astrocytes with cytosolic synthase (Vilchez et al. 2007). Additionally, GDE and glucokinase, a key glucose sensing enzyme, also shuttle to the nucleus upon glucose deprivation (Fernández-Novell et al. 1999; Cheng et al. 2007). In contrast, glucose starvation has an opposite effect on the gluconeogenic enzyme fructose bisphosphatase, inducing its translocation to the cytosol (Yáñez et al. 2004). It has been suggested that the nuclear sequestration of these enzymes is yet another way to regulate their activity, preventing them from acting inappropriately in times of energy stress (Jurczak et al. 2008). Laforin and AMPK, which both contain CBMs and bind to glycogen *in vitro* and in cell culture, also translocate to the nucleus with glucose deprivation and glycogen depletion (Cheng et al. 2007; Singh et al. 2012) (Fig. 10a, b).

Nuclear-cytoplasmic shuttling has also been observed for yeast glycogen synthase. In a review discussing these data, it was postulated that "the freeing of glycogen synthase from its cytoplasmic tether to the glycogen particle as these stores

reduced would therefore be a signal that carbon and energy reserves were low. The uptake of glycogen synthase into the nucleus might therefore represent a form of molecular 'fuel gauge.' It is possible that glycogen synthase could regulate transcription in response to energy availability by some as yet undetermined means" (Wilson et al. 2010). 'Moonlighting' of metabolic enzymes in the nucleus is not a new theme. Moonlighting proteins perform multiple unrelated functions, expanding the functional repertoire of the cell without expanding the number of genes, and the first to be described was the lens structural protein ε-crystallin, also known as lactate dehydrogenase (Hendriks et al. 1988). All essential glycolytic enzymes have been observed in the nucleus, as well as some mitochondrial proteins and enzymes involved in methylation and acetylation (Kim and Dang 2005; Boukouris et al. 2016). These energy-sensing proteins have been shown to regulate transcription by diverse means in a "metabolism-epigenetic axis" that is critical for cellular homeostasis (Boukouris et al. 2016). Although the epigenetic role of glycolytic proteins is well described, it is still not clear whether glycolysis occurs in the nucleus, although its products ATP and NADH could certainly be useful. Glycogen-associated enzymes may also have both an epigenetic and energy producing role, and perhaps excessive glycogen deposits result from misregulated nuclear sequestration. AMP-activated protein kinase (AMPK), a master regulator of cellular energy known to bind to and sense glycogen levels (McBride et al. 2009) (see Sect. 7), also translocates to the nucleus and regulates transcription and the circadian clock through phosphorylation (Leff 2003; Lamia et al. 2009).

8 The Glycogen Granule and Its Associated Proteins

In 1968, Scott and Still recognized the dynamic nature of glycogen and introduced the term "glycosome" to refer to the molecule and its associated proteins, stating that the nature of glycogen as a cellular organelle had not been appreciated (Scott and Still 1968). Rybicka reintroduced the term in a detailed historical review of the evidence for such a classification (Rybicka 1996). Unfortunately, the term "glycosome" has been used far more frequently to refer to the glycolytic enzyme-containing peroxisomes of trypanosomatids, a family of unicellular parasites (Michels et al. 2006), which creates confusion. Also, in the strict definition, organelles are membrane-bound entities (Klausner et al. 1992; Mellman and Warren 2000), and glycogen is primarily cytosolic. Perhaps it is safest to simply recognize that a host of proteins are associated with the glycogen granule, its structure is complex and highly regulated, and it is a dynamic participant in cellular metabolism.

The γ particle named by Drochmans was a description of the ramifying fine fibers (3 nm in diameter) comprising β particle structure, likely corresponding to glucan chains (Drochmans 1962). Later authors came to identify protein-rich subdomains on the glycogen molecule as γ particles, which may be a misinterpretation (Prats et al. 2018). Protein is more electron dense than carbohydrate, so with lead and uranyl acetate staining, protein-rich regions appear as darkly stained clusters

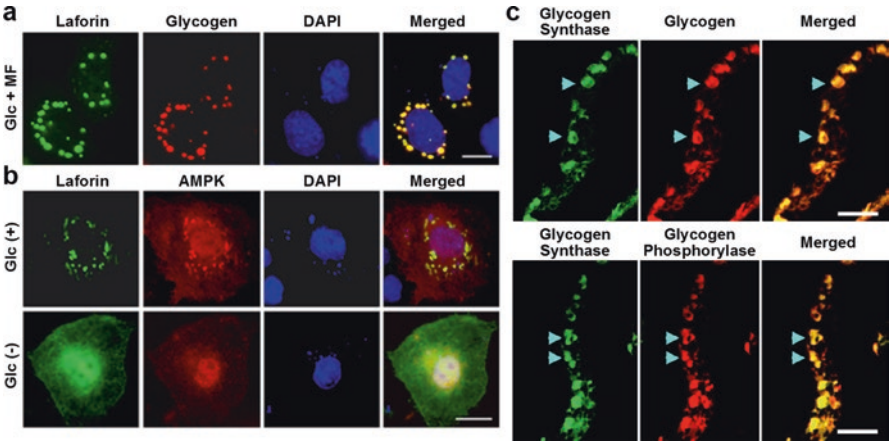

Fig. 10 Nuclear-cytoplasmic shuttling and colocalization of glycogen associated enzymes. (**a**) In media containing glucose and metformin, laforin colocalizes with glycogen in Neuro2A cells (Singh et al. 2012). (**b**) When expressed in COS-7 cells, laforin and AMPK colocalize (with glycogen) in the cytosol in the presence of glucose, and translocate to the nucleus upon glucose starvation (Singh et al. 2012). For (**a**, **b**), scale bars = 10 μm. Copyright © 2012 American Society for Microbiology. Used with permission. (**c**) Immunolabeling of glycogen synthase, phosphorylase, and glycogen itself in tilapia gill sections (Chang et al. 2007). Scale bars = 20 μm. Copyright © Company of Biologists. Used with permission

about 2–3 nm in diameter; alternatively, staining with the histochemical method of Thiéry using silver proteinate results in electron-dense clusters of the same size corresponding to vicinal glycols (Ferrans et al. 1973; Rybicka 1996; Prats et al. 2018). Rybicka refers to the electrodense vicinal glycols as γ particles, which is likely consistent with the meaning originally intended by Drochmans. Due to their very small size, both γ particles and protein-rich subdomains are only visible by EM. It is not yet clear why they exist as discrete, regular entities and which proteins correspond to the protein subdomains. However, it is well established that all of the primary glycogen metabolic enzymes (glycogenin, synthase, phosphorylase, GDE and GBE) bind to and colocalize with glycogen granules in cell culture and *in vivo* (Chang et al. 2007; Cheng et al. 2007; Vilchez et al. 2007; Stapleton et al. 2013; Zhai et al. 2016) (Figs. 10c and 11). In fact, due to its very tight association, glycogen synthase is frequently used as a marker for glycogen and polyglucosan bodies (Criado et al. 2012; Augé et al. 2018). There are a few antibodies that directly bind to the polysaccharide chains of glycogen, but they are not yet commercially available and are still being characterized (Nakamura-Tsuruta et al. 2012; Oe et al. 2016). Other proteins known to closely associate with the glycogen molecule all contain a glycogen-binding CBM: laforin and Stbd1 (Sect. 5), the β-subunit of AMPK, and the glycogen-targeting subunits of protein phosphatase 1 (discussed below) (Fig. 10b, c). Phosphorylase kinase also binds to glycogen (Chebotareva et al. 2009; Nadeau et al. 2018).

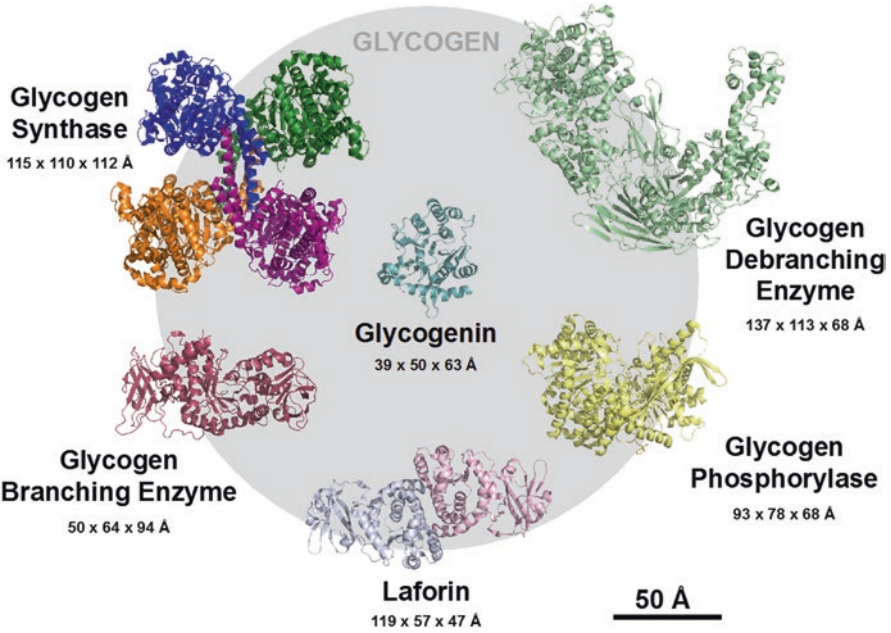

Fig. 11 Crystal structures and relative sizes of glycogen-associated proteins. Crystal structures of yeast glycogen synthase (PDB: 3NAZ), yeast glycogen debranching enzyme (PDB: 5D06), human branching enzyme (PDB: 4BZY), human brain glycogen phosphorylase (PDB: 5IKO), rabbit muscle glycogenin (PDB: 1LL2), and human laforin (PDB: 4RKK). Structures were superimposed manually in PyMol and are shown at equivalent zoom. The size of an average glycogen β particle (diameter = 250 Å) is shown for scale to illustrate the relative sizes of the proteins and glycogen particle. Crystal structure dimensions were calculated based on an inertia axis aligned bounding box in PyMol. In multimeric structures, individual subunits are colored separately

Protein phosphatase 1 (PP1) catalyzes a vast array of dephosphorylation events in cells, regulation of which is achieved by diverse targeting subunits that bring the catalytic subunit (PP1c) into the vicinity of its target (Bollen et al. 2010; Heroes et al. 2013). Seven glycogen-targeting subunits of PP1 have been identified (*PPP1R3A-G*), but four have been most extensively characterized as primary regulators of glycogen synthesis (Newgard et al. 2000; Roach et al. 2012). G_M (also known as R_{GL}, encoded by *PPP1R3A*) expression is restricted to heart and skeletal muscle; G_L (encoded by *PPP1R3B*) is primarily expressed in liver; and Protein Targeting to Glycogen (PTG, also known as R5, encoded by *PPP1R3C*) and R6 (encoded by *PPP1R3D*) are more ubiquitously expressed, with enrichment in glycogen-rich tissues (Roach et al. 2012; www.proteinatlase.org). All these subunits possess both a PP1-binding domain and a putative CBM21 and recruit PP1 to glycogen granules (Newgard et al. 2000; Christiansen et al. 2009; www.cazy.com). Dephosphorylation by PP1 has opposing effects on synthase (activating) and phosphorylase (inhibitory), leading to net glycogen synthesis (Newgard et al. 2000; Greenberg et al. 2006; Vilchez et al. 2007). Overexpression of PTG, G_M, G_L, and R6

leads to glycogen accumulation in cell culture and mouse models (Printen et al. 1997; Gasa et al. 2000; Greenberg et al. 2006; Worby et al. 2008; Montori-Grau et al. 2011; Duran et al. 2014).

AMPK, a cellular energy sensor with multiple intracellular targets, is also considered an important glycogen sensor (Hardie 2014). AMPK is activated by elevated levels of AMP relative to ATP reflecting energy deficits. It is a heterotrimeric complex composed of a catalytic α subunit and regulatory β and γ subunits; the β subunit contains a CBM48 and targets AMPK to glycogen granules (Polekhina et al. 2003; McBride et al. 2009; Janecek et al. 2011). AMPK phosphorylates both isoforms of glycogen synthase, inhibiting its activity (Bultot et al. 2012; Jørgensen et al. 2004). It has been speculated that recruitment of activated AMPK to glycogen granules would lead to decreased glycogen synthesis. There is also significant evidence for regulation of AMPK activity by glycogen via the β subunit (Hardie and Sakamoto 2006). AMPK is particularly inhibited by the presence of a single α-1,6 linked glucose unit or by a glycogen molecule that has been partially degraded by phosphorylase (McBride et al. 2009; Koay et al. 2010). A single glucose unit at a branch point could be exposed during debranching, since GDE is likely to dissociate from glycogen between its transferase and glucosidase activities (Zhai et al. 2016). Thus, glycogen, especially when being actively degraded, is a potent inhibitor of AMPK, and there is likely to be reciprocal regulation between glycogen and AMPK.

Proteomic studies have been performed on glycogen isolated from mouse and rat liver and the mouse adipocyte cell line 3 T3-L1 (Stapleton et al. 2010, 2013). Synthase, phosphorylase, GBE, GDE, and glycogenin were all abundant, as well as PP1 regulatory subunits. Stbd1, laforin, and proteins associated with endoplasmic reticulum were only found in the hepatic proteome; lysosomal α amylase was identified only in the 3 T3-L1 proteome, indicative of cell-specific differences in glycogen metabolism. The lack of AMPK or PhK binding was consistent with earlier studies and attributed to the stringency of the isolation procedure and the nutritional state of the liver (Parker et al. 2007). A number of proteins associated with mitochondria, ribosomes and nuclei were also identified in lower abundance, suggestive of both a heterogeneous subcellular distribution and the link between glycogen and a variety of cellular processes, as has been discussed in previous sections.

It should be noted that many structures of glycogen-associated enzymes have been determined, and they are quite large relative to the size of the glycogen granule (Fig. 11). The dimeric structure of rabbit muscle glycogenin is ~80 kDa in solution and the crystal structure of the monomer is 63 Å at its widest point (Gibbons et al. 2002). This corresponds to one fourth of the diameter of the average glycogen β particle (25 nm, i.e. 250 Å). Yeast glycogen synthase is tetrameric, measuring 115 Å at its widest point, approximately half the diameter of a glycogen granule; each subunit is about 90 Å long (Baskaran et al. 2010). Monomeric human brain phosphorylase is 78 Å long, but it also is known to function as a dimer (Mathieu et al. 2016). Yeast GDE and human GBE monomers are 137 Å and 94 Å long, respectively (Froese et al. 2015; Zhai et al. 2016). The elongated human laforin dimer measures 119 Å in length both in crystals and in solution (Raththagala et al. 2015). PhK and AMPK are both very large multisubunit complexes (Hardie 2014;

Nadeau et al. 2018). Although a complete structure of AMPK is not yet available, structures of the PhK holoenzyme and PhK in complex with phosphorylase determined by cryo-EM reveal that these complexes are 270 Å by 225 Å and 310 Å by 250 Å, respectively, roughly the same dimensions as glycogen β particles (Vénien-Bryan et al. 2009). With this scale in mind, one can begin to envision that the surface of glycogen molecules is likely to be very crowded, and enzymes may compete for access to glucan chains. Molecular crowding has been shown to influence the interaction of PhK with phosphorylase and with the glycogen molecule (Chebotareva et al. 2009). Glycogen and its associated proteins may function as molecular scaffolds, binding to one another and recruiting a variety of enzymes that respond to glycogen levels and regulate energy homeostasis.

9 The future of Brain Glycogen Research

The groundbreaking discoveries of the twentieth century greatly expanded our understanding of glycogen metabolism and monumentally impacted the broader field of biochemistry. As a result, there is a widespread belief that glycogen metabolism is an antiquated area of research that requires no further investigation. However, there is a milieu of interesting questions that remain unresolved regarding glycogen, particularly on its complex yet elusive role in the mammalian brain. Defects in glycogen metabolism are a hallmark of many diseases, including neurodegenerative diseases. The advent of new technologies makes it possible to better study brain glycogen and will most certainly lead to a better understanding of its architecture and metabolism in a variety of contexts.

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Structure and Regulation of Glycogen Synthase in the Brain

Bartholomew A. Pederson

Abstract Brain glycogen synthesis is a regulated, multi-step process that begins with glucose transport across the blood brain barrier and culminates with the actions of glycogen synthase and the glycogen branching enzyme to elongate glucose chains and introduce branch points in a growing glycogen molecule. This review focuses on the synthesis of glycogen in the brain, with an emphasis on glycogen synthase, but draws on salient studies in mammalian muscle and liver as well as baker's yeast, with the goal of providing a more comprehensive view of glycogen synthesis and highlighting potential areas for further study in the brain. In addition, deficiencies in the glycogen biosynthetic enzymes which lead to glycogen storage diseases in humans are discussed, highlighting effects on the brain and discussing findings in genetically modified animal models that recapitulate these diseases. Finally, implications of glycogen synthesis in neurodegenerative and other diseases that impact the brain are presented.

Keywords Glucose metabolism · Glycogen metabolism · Glycogen storage disease · Knockout mice · Transgenic mice

Abbreviations

AD	Alzheimer's disease
Akt/PKB	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
Ap-2	Activator protein 2
ATP	Adenosine triphosphate
bp	Base pairs

B. A. Pederson (✉)
Indiana University School of Medicine-Muncie, Ball State University, Muncie, IN, USA
e-mail: bapederson@bsu.edu

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CA	Corpora amylacea
CAMKII	Ca + 2/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CEBP	CAAT/enhancer binding protein
CK1	Casein kinase 1
CK2	Casein kinase 2
CRE	cAMP responsive element
dpc	Days post coitum
DYRK	Dual-specificity tyrosine-phosphorylated and regulated kinase
EDL	Extensor digitorum longus
EEG	Electroencephalogram
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
Glucose-1-P	Glucose-1-phosphate
Glucose-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
GN	Glycogenin
GS	Glycogen synthase
GSD	Glycogen storage disease
GSK3	Glycogen synthase kinase
GYG1	Glycogenin 1
GYG2	Glycogenin 2
GYS1	Muscle isoform of glycogen synthase
GYS2	Liver isoform of glycogen synthase
HK	Hexokinase
HPLC	High performance liquid chromatography
HRE	Hypoxia response element
kD	Kilodaltons
MEF2	Myocyte enhancer factor 2
MGSKO	Muscle glycogen synthase knockout
MGSKO/GSL30	Muscle glycogen synthase knockout/glycogen synthase line 30
MINI	Muscle initiator
NA	Noradrenaline
Oct1	Octamer binding protein 1
p38beta	Stress-activated protein kinase 2b
PAS	Periodic acid Schiff
PASK	Per/Arnt/Som domain-containing protein kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PGM1	Phosphoglucomutase 1
PhK	Phosphorylase kinase
PI3K	Phosphoinositide 3-kinase
PKA	Proteins kinase A
PKC	Proteins kinase C
PML	Promyelocytic leukemia
PP1c	Protein phosphatase 1

PSSM	Polysaccharide storage myopathy
PTG	Protein targeting to glycogen
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRE	Sterol responsive elements
SSRI	Serotonin reuptake inhibitor
TCA	Tricarboxylic acid
TEF-1	Transcriptional enhancer factor 1
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate
VIP	Vasoactive intestinal peptide

1 Brain Glycogen Measurement

Before delving into the synthesis of glycogen, it is important to discuss the lability of glycogen in the brain. The glycogen concentration in a tissue reflects the net of synthesis of the glucose polymer by glycogen synthase and the degradation by glycogen phosphorylase. Thus, it is important to effectively preserve glycogen in samples. While true for all tissues, brain glycogen in particular is rapidly degraded post-mortem. In adult mouse, brain glycogen levels were reduced ~50% 1 min following decapitation, as compared to mice sacrificed by whole body immersion in liquid nitrogen (Hutchins and Rogers 1970). By 5 min after decapitation, only ~20% of the brain glycogen remained (Hutchins and Rogers 1970). This glycogen remained until 20 min post-decapitation, the last time it was measured. Lowry et al. (1964) reported an even greater loss in brain glycogen (~85%) at 1 min after decapitation in a study where heads were frozen in dichlorodifluoromethane.

Numerous techniques have been used to minimize brain glycogen degradation, including whole body immersion into liquid nitrogen, decapitation into liquid nitrogen or dichlorodifluoromethane, funnel freezing, and head-focused microwave. Decapitation followed by flash freezing of mouse heads in liquid nitrogen preserved brain glycogen to the same extent as when mice were immersed into liquid nitrogen (Hutchins and Rogers 1970). Measuring cooling rates inside mouse brain revealed that it takes 6 s to cool the cortex to 0 °C and 12 s to cool to −30 °C when decapitated heads were immersed in liquid nitrogen (Swaab 1971). In our hands (Canada et al. 2011), when mouse heads were immediately frozen in liquid nitrogen after decapitation or cervical dislocation brain glycogen levels were ~2.5 μmol/g, almost identical to that reported by others (Hutchins and Rogers 1970; Lowry et al. 1964). Two other techniques provide optimal preservation of brain glycogen; freeze-blowing and head-focused microwave. The former technique uses high pressure to expel the brain from the skull cavity onto a metal plate cooled in liquid nitrogen (Ghajar et al. 1982; Veech et al. 1973). This process removes and freezes the brain in less than 1 s. A limitation of this technique is that it is only available for rats and requires that the animal is anesthetized. The latter technique is amenable to both

mice and rats and does not require anesthesia, which increases brain glycogen levels (Morgenthaler et al. 2006; Nelson et al. 1968; Nordstrom and Siesjo 1978). Two limitations of this method are cost of the instrument and the inability to measure enzyme activity in the brain tissue. Head-focused microwave heats brains to ~80 °C in less than 1 s resulting in rapid inactivation of brain enzymes and thus preservation of glycogen and other metabolites. To reach ~85 °C in rat brain, Kong et al. (2002) used 10 kW for 1.2 s. With these parameters, they measured a glycogen concentration of 8 μmol/g in cortex. In comparison, the concentration was ~5 μmol/g and <0.5 μmol/g at 6 kW and 3.5 kW respectively. Subjecting anesthetized C57Bl/6 J mice to a 3.5 kW microwave, which heated the brain to 85 °C in 0.5 s, resulted in brain glycogen levels of ~3 μmol/g (Franken et al. 2003). Higher glycogen levels (>10 μmol/g) were reported in mouse brain when microwave (5Kw) was focused on the brain for ~1 s (Oe et al. 2016). In the author's hands, similar brain glycogen concentrations (~2.5 μmol/g tissue) are obtained when comparing mice sacrificed by microwave (5Kw) with mice sacrificed by cervical dislocation or decapitation when heads are immediately frozen in liquid nitrogen (author's unpublished observations). When studying rodent models, the choice of rodent, the amount of stress the animal experiences, and the age of the animal are important considerations. Rats have the disadvantages of longer times to freeze the brain, due to size, and the elevated glycogen phosphorylase activity in rats (60% active; Lust et al. 1973) as compared to mice (30% active; Lust and Passonneau 1976). Various types of stress and sensory activation are reported to impact brain glycogen levels (Cruz and Dienel 2002). In contrast to the rapid brain glycogen degradation observed in adult rodents, in 10 day old mouse pups, brain glycogen levels were relatively stable for 2 min post-mortem, but after an additional 8 min had dropped to near zero (Lowry et al. 1964).

2 Glycogen Synthetic Pathway

Glucose transport. The pathway for forming glycogen begins with glucose moving from the blood through the blood brain barrier and into astrocytes and neurons (reviewed in McEwen and Reagan 2004; Vannucci et al. 1997) (Fig. 1). This movement of glucose is facilitated by the glucose transporter (GLUT) proteins. While several members of the GLUT family (GLUT1,2,3,4,5,8) are expressed in brain, GLUT1 and GLUT3 appear to be responsible for the majority of glucose uptake. GLUT1 (55kD isoform), which is expressed in the blood-brain barrier (Farrell and Pardridge 1991), serves to transport glucose across this barrier while the 45kD isoform of GLUT1 is expressed in astrocytes (Leino et al. 1997). GLUT3 transports glucose into neurons (Leino et al. 1997). The K_m for glucose of GLUT1 and GLUT3 are ~8.5 mM and 1.4 mM, respectively, giving neurons preference to glucose (Jensen et al. 2014). However, astrocytic end feet completely cover blood microvessels (Mathiisen et al. 2010). The result is that the majority of glucose, and other metabolites, entering the brain will pass through these astrocytic end feet

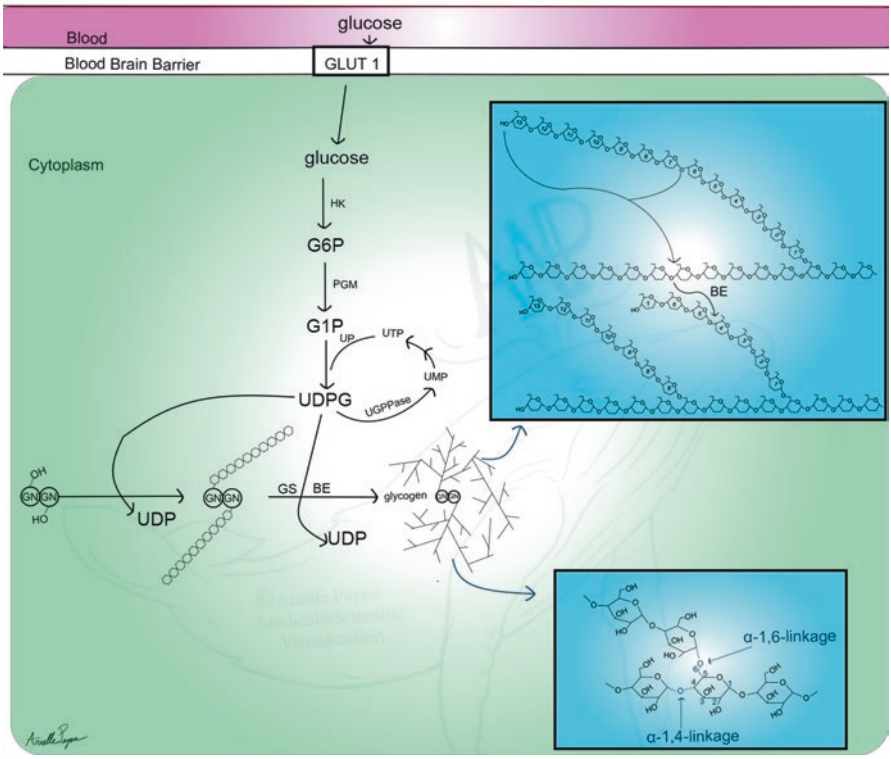

Fig. 1 Glycogen synthetic pathway in astrocytes. *GLUT* glucose transporter, *HK* hexokinase, *G6P* glucose-6-phosphate, *G1P* glucose-1-phosphate, *PGM* phosphoglucomutase, *UDPG* uridine diphosphate glucose, *UTP* uridine triphosphate, *UMP* uridine monophosphate, *UP* UDP-glucose pyrophosphorylase, *UGPPase* UDP-glucose pyrophosphophatase, *GS* glycogen synthase, *BE* branching enzyme, *GN* glycogenin. Hexagons depict glucose molecules. Illustration by Arielle Payne

(Teschemacher et al. 2015). The significance of the other glucose transporters expressed in the brain is still not completely clear, with some of the transporters being expressed in discrete brain regions (reviewed in Choeiri et al. 2002). Although the brain has generally been considered to be insulin-independent, the insulin sensitive glucose transporters GLUT4 and GLUT8 along with the insulin receptor are expressed (reviewed in McEwen and Reagan 2004).

Blood glucose levels tend to correlate with brain glycogen levels (Poitry-Yamate et al. 2009) and incubation of cultured astrocytes in high concentrations of glucose leads to increased glycogen accumulation (Magistretti et al. 1993). Overexpression of GLUT1 in mouse skeletal muscle increased glycogen concentration several fold without effects on glycogen synthase activity, suggesting that glucose transport is limiting in glycogen synthesis, at least in muscle (Ren et al. 1993). However, overexpression of glycogen synthase in muscle also increases glycogen accumulation (Manchester et al. 1996) suggesting that both glucose transport and glycogen

synthase activity are important regulators of glycogen synthesis. Both glutamate and aspartate stimulated glycogen synthesis in cultured astrocytes by increasing glucose uptake (Hamai et al. 1999). However, glycogen content did not increase in neurons when glucose levels were increased (Saez et al. 2014). A 50% reduction of GLUT3 in neurons lead to impaired spatial learning and working memory in a mouse model, but effects on glycogen level were not reported (Zhao et al. 2010b).

Considering differential expression between brain regions, kinetic characteristics, and variable responsiveness to insulin of the GLUT's, further studies should reveal the interplay between glucose transport and glycogen synthesis in specific brain regions and cell types under defined conditions.

Hexokinase. Glucose transporters in the brain are facilitative, thus glucose uptake is connected with the activity of hexokinase. For example, an increase in glycogen accumulation in mouse skeletal muscle by over expression of hexokinase II only occurred when GLUT1 was also overexpressed (Hansen et al. 2000). Glucose taken up into neurons and astrocytes is phosphorylated by the HK1 isoform of hexokinase (Wilson 2003) to form glucose-6-P. This branch point metabolite is an inhibitor of hexokinase, an activator of glycogen synthase, and can serve as a substrate for multiple pathways including glycolysis, the pentose phosphate pathway, hexosamine biosynthetic pathway, and glycogenesis. Under basal conditions, hexokinase is inhibited 90% by glucose-6-P (DiNuzzo et al. 2015). Rather than glucose-6-P proceeding directly through glycolysis, the glycogen shunt was proposed by Shulman and Rothman (Shulman and Rothman 2001). This mechanism was proposed to explain the importance of glycogen in skeletal muscle during exercise. The premise is that instead of glucose-6-P proceeding directly through glycolysis, it is instead used to synthesize glycogen, which is then metabolized to generate ATP for muscle contraction. In support of this concept is the exercise intolerance observed in patients with McArdle disease, caused by a deficiency in muscle glycogen phosphorylase. Also, mice in which the muscle isoform of glycogen synthase (GYS1) is disrupted specifically in the skeletal muscle of adult mice have impaired exercise endurance capacity (Xirouchaki et al. 2016). In contrast, mice with global GYS1 disruption had normal exercise capacity (Pederson et al. 2005a). However, this latter animal model has metabolic changes in the muscle which enhance oxidative capacity. Thus, these adaptations perhaps overcome a normal reliance on the glycogen shunt. To examine the significance of the glycogen shunt in the brain, Walls et al. (Walls et al. 2009) manipulated glycogen metabolism in astrocytes and monitored the TCA cycle and glycolysis. This study indicated a significant role for the glycogen shunt in this cell type. Further studies in the brain will be needed to determine the importance of the glycogen shunt in physiological and pathological conditions.

Phosphoglucomutase. Phosphoglucomutase converts glucose-6-P to glucose-1-P. PGM1 is the main isoform in most tissues and deficiency results in a glycogen storage disease (GSDXIV). The first patient identified had abnormal accumulations of muscle glycogen (Stojkovic et al. 2009). In another study of 19 patients, fibroblast glycogen levels were normal (Tegtmeyer et al. 2014). Apart from one patient with mild intellectual disability (Ondruskova et al. 2014), patients with mutations in

PGM1 do not have adversely affected brains (Tegtmeyer et al. 2014), possibly due to the expression of other isozymes in the brain.

UDP-glucose pyrophosphorylase. Glucose-1-P reacts with UTP producing UDP-glucose through the action of UDP-glucose pyrophosphorylase. In Saccharomyces cerevisiae, disruption of UDP-glucose pyrophosphorylase indicated that this enzyme is essential for viability (Daran et al. 1995). Overexpression of this enzyme in mouse skeletal muscle increased levels of UDP-glucose threefold, but did not impact glycogen levels in muscle, suggesting that it is not rate limiting for glycogen synthesis (Reynolds et al. 2005). Whether this enzyme plays a regulatory role in the brain is not clear. Besides serving as the substrate for glycogen synthase, the UDP-glucose produced by UDP-glucose pyrophosphorylase can be converted to glucose-1-P and UMP through the action of UDP-glucose pyrophosphatase. This protein is expressed in multiple tissues including brain (Heyen et al. 2009) and could serve as a regulator of glycogen synthesis. Though no regulation has yet been demonstrated, the protein ran on SDS-PAGE as a doublet suggesting some form of post-translational modification.

Glycogenin. The glycogenin protein is found as a dimer at the core of glycogen molecules (Fig. 1). This protein is unique in that it serves in the roles of enzyme, substrate, and product. Glycogenin initially self glucosylates, adding a glucose molecule to a tyrosine residue (Tyr195), and then extends this glucose chain to 10–20 residues in length (Roach and Skurat 1997; Smythe and Cohen 1991). After reaching this length, the protein-glucose complex serves as a scaffold for glycogen synthase to continue elongation of the glucose chains. The crystal structure for rabbit muscle glycogenin was solved (Gibbons et al. 2002). It is a member of the GT8 glycosyltransferase family with a single Rossman-fold domain. UDP-glucose binds in a metal-dependent manner in the central beta-sheet structure.

There are two genes, GYG1 and GYG2, encoding glycogenin in humans. GYG2 is expressed in liver, heart, and pancreas (Mu et al. 1997), while GYG1 is more widely expressed, with highest expression in cardiac and skeletal muscle (Barbetti et al. 1996). Imagawa et al. (2014) reported that GYG2, but not GYG1, is expressed in human brain. Consistent with this expression pattern, a patient with an inactivating GYG1 mutation (Thr83Met) was found to have depleted glycogen stores in skeletal muscle, and a switch to slow-twitch oxidative muscle fibers (Moslemi et al. 2010), but no cognitive abnormalities were observed. This mutation prevents the formation of the glucose-O-tyrosine linkage at Tyr195 (Nilsson et al. 2012). In another study, 7 patients with mutations in GYG1 were found to have either depleted glycogenin protein, or in 1 case impaired interaction with glycogen synthase, varying levels of normal glycogen, and an accumulation of polyglucosan in skeletal muscle (Malfatti et al. 2014). This observation challenges the notion that glycogenin is required for the synthesis of glycogen. In contrast, two male siblings with Leigh syndrome, a progressive neurodegenerative disorder, were found to have a hemizygous missense mutation in GYG2 (Imagawa et al. 2014). Both brothers suffered from seizures and neurodegeneration (Imagawa et al. 2014). According to the study's authors, it remains to be established whether the GYG2 mutation is causative of Leigh syndrome in these patients.

In contrast to humans, non-primates appear to only have one gene for glycogenin, GYG. Glycogenin activity was demonstrated in rat brain (Tolmasky et al. 1991). In cultured astrocytes starved of glucose, free glycogenin was present (Lomako et al. 1993). When fed with glucose, a low-molecular weight form of glycogen termed "pro glycogen", was made. However, another study suggested that this pro glycogen may be an artifact of the extraction procedures used (James et al. 2008). Lomako et al. (1993) additionally proposed a "proglycogen synthase" separate from glycogen synthase, but to date this enzyme has not been identified.

In the mouse, glycogenin was identified as a lethal gene (Dickinson et al. 2016). Homozygous null embryos died perinatally. Characteristics included abnormal morphology of brain at E18.5, consistent with neural degeneration. At E15.5, embryos had a flattened forebrain with reduced lateral ventricles. In another study, when GYG was disrupted, approximately 85% of pups lacking glycogenin died shortly after birth, due to cardiopulmonary defects (Testoni et al. 2017). Fetal glycogen levels were not reported, but perhaps the lethality of the disruption is due to the inability to synthesize glycogen, as we observed with the disruption of the GYS1 isoform of glycogen synthase (Pederson et al. 2004a). Surprisingly, surviving GYG knockout mice had normal levels of brain glycogen, despite no detectable expression of GYG in this tissue (Testoni et al. 2017). These findings indicate the importance of glycogenin for survival but challenge the idea that glycogenin is required for the synthesis of glycogen.

Glycogenin associates with glycogen synthase via two mechanisms (Skurat et al. 2006). Namely, glycogen synthase associates with the glucose-primer chain on glycogenin. In addition, there is interaction of glycogen synthase with the C-terminus of glycogenin; the C-terminal 33 amino acids purify glycogen synthase from tissue extracts. Zeqiraj et al. (Zeqiraj and Sicheri 2015; Zeqiraj et al. 2014) solved the structure of *C. elegans* glycogen synthase in complex with a minimal targeting region of glycogenin, finding that glycogenin binds to the first of two Rossman fold domains of glycogen synthase in a region of the protein not involved in tetramer interactions or binding to glucose-6-P or UDP-glucose. When glycogen synthase or glycogenin was mutated in a manner that prevented interaction between the two expressed proteins, glycogen was not synthesized in *S. cerevisiae*, demonstrating the importance of this interaction. The region that separates the glycogen synthase binding domain and the catalytic domain is variable in length between isoforms and species and is a site of alternative splicing in humans (Zhai et al. 2000). Zeqiraj et al. (Zeqiraj and Sicheri 2015; Zeqiraj et al. 2014) suggests that the length of this linker region may control the size of glycogen particles. Glycogenin mRNA levels were increased in cerebral cortex of mice that underwent gentle sleep deprivation (Petit et al. 2010). The significance of glycogenin regulation will have to be borne out by further studies.

Glycogen synthase. The bulk synthesis of glycogen is catalyzed by glycogen synthase (EC2.4.1.11) which forms alpha-1,4 glycosidic linkages using UDP-glucose as the glucosyl donor (Leloir et al. 1959; Villar-Palasi and Larner 1958) (Fig. 1). There are two genes encoding this enzyme in mammals (GYS1 and GYS2). In rat, expression of GYS2 appears to be limited to liver (Kaslow et al. 1985), while

GYS1 is expressed in muscle, heart, fat, kidney, and brain (Kaslow and Lesikar 1984).

Glycogen branching enzyme. The glycogen branching enzyme (amylo-(1,4 to 1,6) transglycosylase) introduces branch points in the glycogen molecule producing chains of glucose that range from 1 to more than 30 glucose residues in both muscle and brain (Nitschke et al. 2017) (Fig. 1). An average chain length of ~13 glucose residues is found in multiple tissues and organisms. Branching increases the solubility of glycogen and provides multiple points of attack for glycogen phosphorylase (Melendez-Hevia et al. 1993). Demonstrating the importance of proper branching, mice that overexpress constitutively active GYS1 in skeletal muscle overaccumulate poorly branched glycogen, i.e., polyglucosan bodies (Pederson et al. 2003). Interestingly, the over expression of glycogen synthase results in increased expression of the glycogen branching enzyme, though not to the extent that it completely compensates for the increase in glycogen synthesis. Complete loss of branching enzyme activity is lethal in utero in a mouse model (Lee et al. 2011) replicating the severe form of glycogen storage disease type IV. There has been limited study of the glycogen branching enzyme in brain, but Tomalsky et al. (1998) characterized the mechanism of action in rat. Mice that are homozygous for a hypomorphic allele of the branching enzyme (GBE1) have a 90% reduction in branching enzyme activity and accumulate polyglucosan bodies in several tissues including brain (Akman et al. 2011). In another mouse model of the most common human GBE1 mutation, Y329S, adult mice had progressive muscular dysfunction, and died prematurely (Orhan Akman et al. 2015). The phenotype of this model was similar to human adult polyglucosan body disease. Polyglucosan accumulates in several tissues in newborn mice, but not in cerebral cortex. As mice age, polyglucosan accumulates in neurons and their axons causing neuropathy. With our collaborators, we found that crossing mice globally heterozygous for GYS1 with hypomorphic GBE1 mutant mice resulted in reduced formation of polyglucosan bodies, improved mobility, and extended lifespan (Chown 2018).

3 Glycogen Synthase Structure

Gene structure. Zhang et al. (1989) determined the sequence of rabbit skeletal muscle glycogen synthase from cDNA clones. A 3.6 kb mRNA was identified that coded for a protein of 734 amino acids and a molecular weight of 83,480. The 5′ untranslated and coding regions were 79% and 90% identical to human muscle glycogen synthase. The 3′ untranslated region was less similar. cDNA for human muscle glycogen synthase was cloned and sequenced by Browner et al. (1989), who found that it encodes a protein of 737 amino acids with a predicted molecular weight of 83,645 daltons. The 3′ untranslated region of the glycogen synthase cDNA is 1.2 kb. Using this cDNA as a probe, mRNA of ~3.5 kb was identified in human fetal and adult heart and skeletal muscle. In rabbit liver and brain, a single hybridizable mRNA of ~2.4 kb was identified, leading Browner et al. to suggest that the liver isoform of

glycogen synthase would be expressed in the brain. The cDNA for mouse glycogen synthase was isolated from a cortical astrocyte cDNA library (Pellegri et al. 1996). The 3534 bp cDNA encoded a protein of 737 amino acids with 87% nucleotide identity and 96% amino acid identity with the human and rabbit muscle glycogen synthase and a 67% nucleotide and 71% amino acid identity with liver glycogen synthase. The cDNA has a 64 bp 5'untranslated region upstream of the first in-frame ATG and a 1259 bp 3'untranslated region. The 2211 bp opens reading frame codes for a protein with a calculated molecular weight of 83,823. These results suggested that the muscle isoform of glycogen synthase (GYS1) is expressed in the brain, and that the small sequence difference between the mouse brain cDNA and human could be a species difference, which indeed appears to the be case (https://www.uniprot.org/uniprot/Q9Z1E4). In humans, the GYS1 gene is located on chromosome 19 (19q13.3) (Groop et al. 1993; Lehto et al. 1993) and spans 32,229 bp with 16 exons. Promoter analysis indicated a number of putative transcription factor binding sites (Fredriksson et al. 2004) (Fig. 2). These included sites for Sp1, activator protein 2 (Ap-2), octamer binding protein 1 (Oct1), myocyte enhancer factor 2

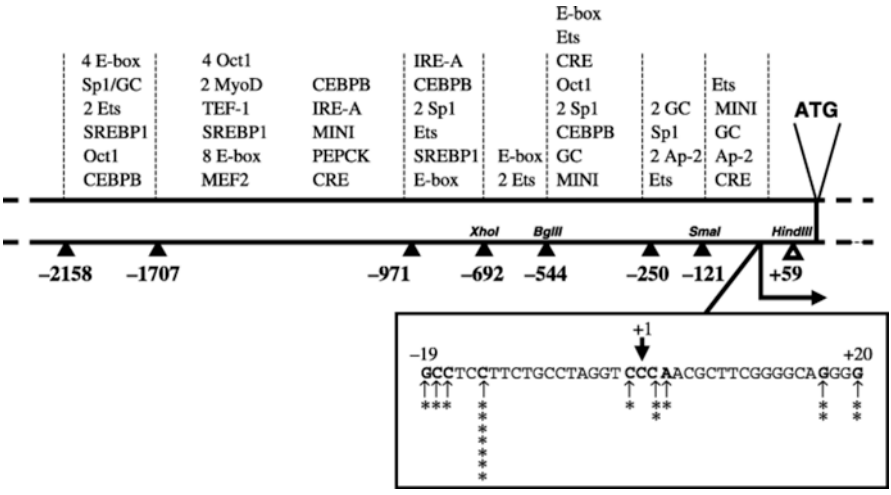

Fig. 2 Glycogen synthase gene (GYS1) promoter constructs and putative transcription factor-binding sites within the constructs. Filled triangles indicate 5′-ends of the different constructs. The −250, −971, −1707 and −2158 constructs were made by introducing a MluI site in a linker primer, whereas the −692, −544, and −121 constructs were created using internal restriction sites. All constructs contained 59 bp of the 5′-untranslated region (open triangles) numbered according to the previously determined transcription initiation site (Bjorbaek et al. 1994; Orho et al. 1995). The major transcriptional initiation site, proposed by seven different clones in the present study, is indicated at position −14 relative to the previously determined single initiation site. Four sites, recognized by one clone each, are not shown in the figure. These sites were located at nt +92, +108, +125 and +168. Ap-2, activator protein 2; *CEBPB* CAAT/enhancer-binding protein beta, *CRE* cAMP responsive element, *IRE-A* insulin response element A, *MEF2* myocyte enhancer factor, *MINI* muscle initiator, *PEPCK* phosphoenolpyruvate carboxykinase, *SREBP* sterol responsive element binding protein, *TEF-1* transcriptional enhancer factor 1. Used with permission from (Fredriksson et al. 2004)

(MEF2), CAAT/enhancer binding protein (CEBP), and enhancer factor-1. In addition, cAMP responsive elements (CRE), muscle initiator sequences (MINI), sterol responsive elements (SRE), E-boxes, Ets-like motifs and phosphoenolpyruvate carboxykinase (PEPCK)-like motifs were found. No TATA or CAAT boxes were identified. The first 250 bp of the 5′ flanking region were determined to account for basal promoter activity and contain elements characteristic of housekeeping genes. More recently, a hypoxia response element (HRE) was identified at position −314 in GYS1 (Pescador et al. 2010).

Protein structure. The human glycogen synthase protein (GYS1) contains 737 amino acids. The molecular weight of the GYS1 holoenzyme, as judged by HPLC gel filtration was 225 kD from brain and 274 kD from muscle, while the molecular weight of the subunit was 88 kD and 87 kD from brain and muscle respectively (Inoue et al. 1987). The reason for these differences in GYS1 between the brain and muscle is not clear. Besides catalytic residues, there are multiple phosphorylation sites in the protein and a region that confers sensitivity to glucose-6-P (Fig. 3). The crystal structure of yeast glycogen synthase, Gsy2p, was solved and found to be a tetramer (Baskaran et al. 2010) (Figs. 4 and 5). Two arginine residues in the C-terminus confer sensitivity to glucose-6-P, which when bound causes translations and rotations among the subunits. This rearrangement permits easier substrate access. Further studies showed that Arg589 and Arg592 are key in allowing the conversion to and from the T state of the enzyme (Mahalingan et al. 2017). Four additional arginine residues in this same helix are involved in the enzyme's response to phosphorylation (Baskaran et al. 2010). The control of the enzyme by glucose-6-P and phosphorylation can be described by a three-state model (Fig. 6) (Baskaran et al. 2010; Pederson et al. 2000). Unphosphorylated glycogen synthase is in an intermediate state (I state or state II). Binding of glucose-6-P converts the enzyme to a high activity state (R state or state III), while phosphorylation converts the

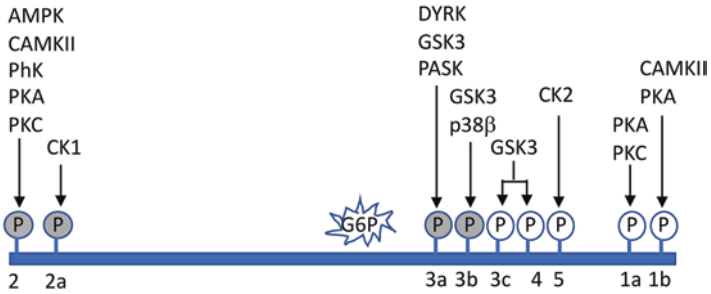

Fig. 3 Glycogen synthase (GYS1) protein structure. Residue 582 in mouse GYS1 is required for glucose-6-P sensitivity. Phosphorylation sites correspond to the following residues in mouse GYS1: site 2, 8; site 2a, 11; site 3a, 641; site 3b, 645; site 3c, 649; site 4, 653; site 5, 657; site 1a, 698; site 1b, 711. *AMPK* AMP-activated protein kinase, *CAMKII* calcium/calmodulin-dependent protein kinase, *CK1* casein kinase 1, *CK2* casein kinase 2, *DYRK* dual-specificity tyrosine-phosphorylated and -regulated kinase, *GSK3* glycogen synthase kinase 3, *p38beta* mitogen-activated protein kinase 11, *PASK* PAS domain-containing serine/threonine kinase, *PhK* phosphorylase kinase, *PKA* protein kinase A, *PKC* protein kinase C

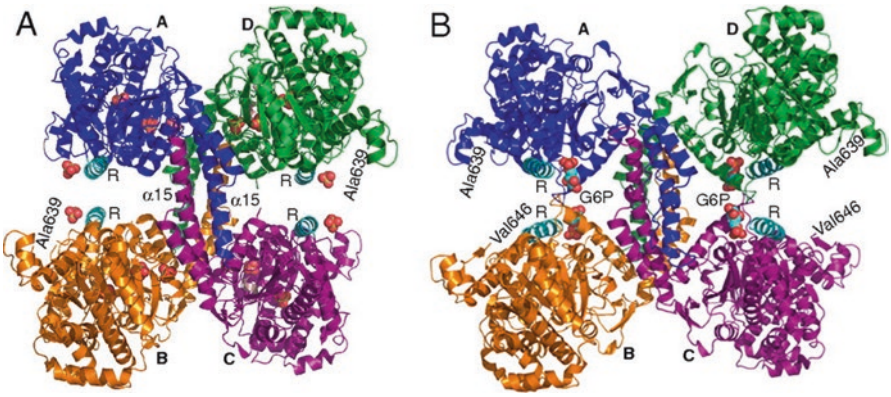

Fig. 4 Glycogen synthase crystal structure. Structures of the basal and activated states of Gsy2p. (**a**) Ribbon diagram of the basal state conformation in which the individual subunits are labeled (A–D) and colored separately. The regulatory helices (α22) are colored cyan and labeled R, while the intersubunit helices are labeled α15 as is the last ordered residue at the **c** terminus of subunits B and D. Ordered sulfate ions in this structure are represented using space filling atoms. (**b**) Ribbon diagram of the activated state of Gsy2p. The color scheme, subunit, and regulatory helix labeling is identical to panel **a**. The bound glucose-6-P molecules at the interface are represented using space filling atoms and labeled G6P. [Produced using Pymol (DeLano 2002) for Windows.] Used with permission from (Baskaran et al. 2010)

enzyme to a low activity state (state I or T state). The low activity state is not as sensitive to glucose-6-P binding, but with saturating levels of this metabolite, glycogen synthase is converted to the high activity state. When the tetrameric structure of the enzyme is taken into account, where hybrid complexes of phosphorylated and unphosphorylated subunits are present, there may be more than three activity states. In addition, while yeast Gsy2p and mammalian GYS1 are both activated by glucose-6-P and inhibited by phosphorylation, the yeast enzyme has 3 phosphorylation sites compared to the 9 found in the mammalian enzyme (Hanashiro and Roach 2002). These additional sites may provide for more complex regulation via phosphorylation/dephosphorylation.

Catalytic mechanism. The catalytic mechanism for glycogen synthase is not well understood. Unlike another glycosyltransferase, glycogenin, glycogen synthase is metal ion- independent, instead using hydrogen bonds to amino acids to stabilize UDP (reviewed in Roach et al. 2012). The particular residues in the yeast glycogen synthase, Gsy2p, implicated in catalysis are Arg320, Lys326, residues 513–521, and Glu509.

In the brain, glycogen synthase was first reported in rat (Leloir et al. 1959) and subsequently partially purified from sheep (Basu and Bachhawat 1961). Kinetic characterization of brain glycogen synthase was conducted with enzyme from rat and pig. In rat, the K_m for UDP-glucose was 0.17 mM and 0.36 mM in the absence and presence of glucose-6-P, respectively (Goldberg and O'Toole 1969). For glycogen synthase partially purified from pig brain, increasing UDP-glucose levels decreased the K_m for glucose-6-P while increased glucose-6-P levels decreased the

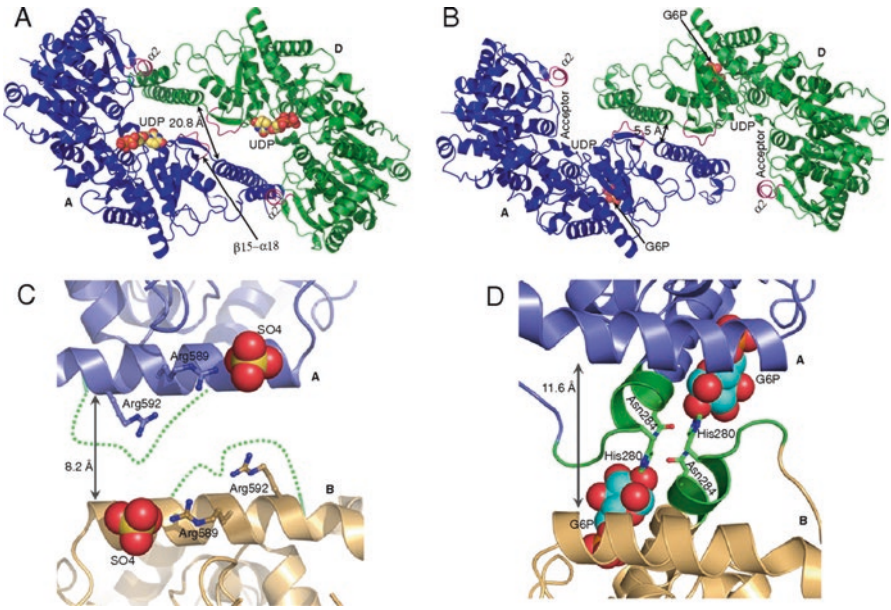

Fig. 5 Comparison of the basal and activated state conformations. (**a**) Ribbon representation of the AD dimer pair in the basal state with bound UDP represented using space filling atoms and ribbon coloring identical to Fig. 4. The loop region β15-α18 and helix α2 are highlighted in purple and labeled. The distance between equivalent positions of the α15 helices is provided. (**b**) Ribbon representation of the AD dimer pair in the activated state using the same coloring scheme as in panel **a**. The glucose-6-phosphate molecules are represented using space filling atoms and labeled, as are the relative positions of UDP and structural elements contributing to glycogen acceptor binding. (**c**) Ribbon representation of the regulatory interface between subunits A and B in the basal state. The individual subunits are indicated with labels and distinct ribbon colors. The bound sulfate molecule near the N terminus of each regulatory helix is displayed using space filling atoms. The disordered region between residues 277–285 is represented as a dashed green line solely to indicate the connectivity. (**d**) Ribbon representation of the regulatory helix interface between subunits A and B in the activated state of the R589A/R592A mutant. The coloring scheme is identical to that in panel **c** and the bound glucose-6-P is displayed using space filling atoms. The residues interacting with glucose-6-P from the 277–285 loop region are represented using green coloring. [Produced using Pymol (DeLano 2002) for Windows.] Used with permission from (Baskaran et al. 2010)

K_m for UDP-glucose for both the dependent and independent forms of the enzyme (Passonneau et al. 1975).

The primary function of glycogen synthase is catalyzing the addition of glucose from UDP-glucose to an elongating glucose chain in a glycogen molecule. However, glycogen synthase also incorporates the beta phosphate of UDP-glucose into glycogen as glucose-6-P (Fig. 7) at a frequency of ~1 phosphate per 10,000 glucose residues (Tagliabracci et al. 2011). This phosphate incorporation into glycogen is one of the mechanisms hypothesized to explain the abnormal glycogen accumulation in Lafora disease (Roach 2015; Tagliabracci et al. 2008). Lafora disease is characterized by myoclonus epilepsy beginning during early adolescence (Lafora and Glueck

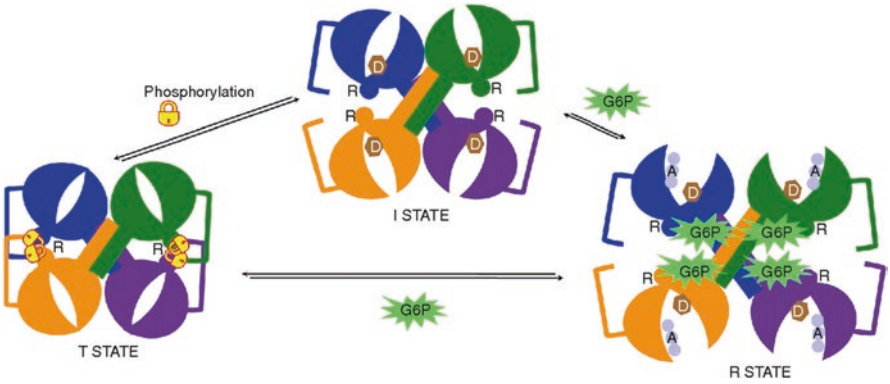

Fig. 6 Glycogen synthase three state model. Schematic representation of the conformational states underlying regulation of Gsy2p activity. The individual subunits are colored according to the coloring scheme in Fig. 4. The regulatory helices containing the arginines are labeled R and the approximate positions of nucleotide-donor sugar (D) and glycogen acceptor (A) are shown for the I- and R-states, respectively. Phosphorylation of Thr668 is shown as "locking" the enzyme in the T-state conformation through intersubunit interactions across the regulatory interface. Glucose-6-P binding frees these constraining interactions to fully activate the enzyme in the R-state conformation. Used with permission from Baskaran et al. (2010)

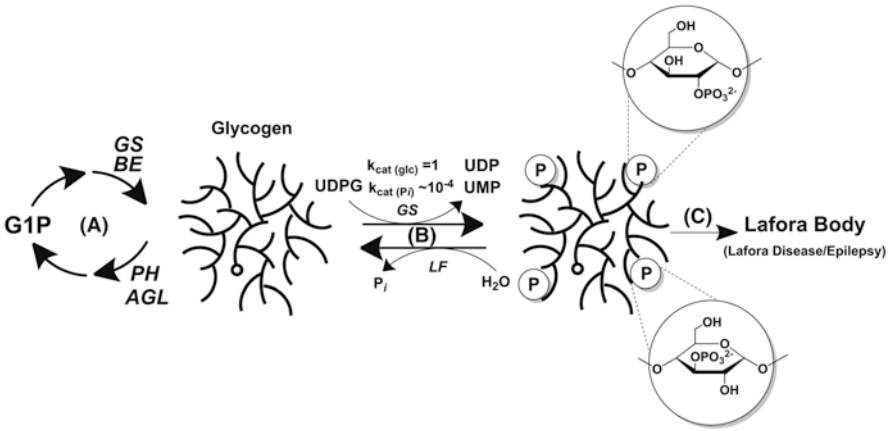

Fig. 7 Model for Glycogen Phosphate Metabolism and Lafora Body Formation. (**a**) Glycogen is synthesized by glycogen synthase (GS) and branching enzyme (BE) and degraded by glycogen phosphorylase (PH) and debranching enzyme (AGL). (**b**) Glycogen synthase infrequently (1 in ~10,000) incorporates phosphate residues into glycogen. The kcat values given simply denote the relative rates of glucose versus glucose phosphate incorporation. Excessive incorporation of phosphate as C2 or C3 phosphomonesters, which disrupts glycogen structure, is normally kept in check by the action of the laforin phosphatase (LF). (**c**) When laforin is defective, excessive phosphorylation results in the formation of Lafora bodies and Lafora disease. Used with permission from Tagliabracci et al. (2011)

1911). Over the course of ~10 years, cognitive function declines, seizure frequency increases, and death typically occurs (Minassian 2001; Serratosa et al. 1999; Turnbull et al. 2016). In these patients, high levels of poorly branched, hyperphosphorylated glycogen, termed polyglucosan or Lafora bodies, accumulate in many tissues including the brain (Minassian 2001; Striano et al. 2008). Neuronal cell bodies and dendrites, but not axons, in all brain regions are affected. Approximately 50% of Lafora disease cases (The Lafora Progressive Myoclonus Epilepsy Mutation and Polymorphism Database; http://projects.tcag.ca/lafora/) are the result of mutations in the *EPM2A* gene which encodes laforin (Minassian et al. 1998; Serratosa et al. 1999). Apart from one patient with a mutation in the *PRMD8* gene (Turnbull et al. 2012), the remainder of Lafora disease cases are due to mutations in the *EPM2B/NHLRC1* gene which encodes malin (Chan et al. 2003), an E3 ubiquitin ligase (Gentry et al. 2005). Laforin, a dual specificity phosphatase, binds to glycogen through a carbohydrate binding domain (Chan et al. 2004; Wang et al. 2002; Wang and Roach 2004) and dephosphorylates glycogen (Tagliabracci et al. 2007). In a mouse model of Lafora disease, created by disruption of Epm2a, muscle glycogen contained ~fivefold higher levels of phosphate as compared to wild type controls (Tagliabracci et al. 2007, 2008). This phosphate is located at the C2-, C3- and C6-positions (DePaoli-Roach et al. 2015). The mechanism for incorporation of phosphate at C2- and C3- can be explained by the action of glycogen synthase, but the mechanism for incorporation at the C6- position is unknown (Chikwana et al. 2013; Contreras et al. 2016; Tagliabracci et al. 2011). Phosphorylation of glycogen decreases branching and solubility, though the mechanism is unclear (Roach 2015). Roach (2011) proposed that the incorporation of phosphate into glycogen may be a catalytic error and that laforin acts as a repair enzyme. An alternative hypothesis for the formation of Lafora bodies is that glycogen synthase activity is increased relative to branching enzyme activity, leading to synthesis of poorly branched glycogen (Vilchez et al. 2007). Both of these hypotheses have recently been challenged. Glycogen synthase activity was normal in both malin and laforin knockout mice (DePaoli-Roach et al. 2010; Tagliabracci et al. 2008) and the overexpression of phosphatase-inactive laforin prevented the formation of Lafora bodies in laforin knockout mice (Gayarre et al. 2014). In this latter model, glycogen remains hyperphosphorylated while the abnormal glycogen chain length pattern characteristic of Lafora bodies is corrected (Nitschke et al. 2017). While the mechanism underlying the formation of Lafora bodies remains an active area of investigation, genetically modified mouse models demonstrate the importance of glycogen synthesis in the pathology of Lafora disease. Disruption of PTG, an activator of glycogen synthase, in a mouse model resulted in a 70% reduction in brain glycogen (Turnbull et al. 2011). When these mice were crossed with a laforin deficient mouse model, formation of Lafora bodies was largely prevented and both myoclonic epilepsy and neurodegeneration were resolved. Similarly, in mice disrupted for both GYS1 and laforin, Lafora bodies and neurodegeneration were absent and seizure susceptibility was normal (Pederson et al. 2013). Also, disrupting GYS1 in malin deficient mice prevented the deleterious effects associate with Lafora disease (Duran et al. 2014). These studies suggest that inhibiting glycogen synthase may be therapeutic for patients with this fatal disease.

4 Glycogen Synthase Localization

Regional localization. Within the mouse brain, glycogen synthase mRNA is expressed widely with the highest levels found in the olfactory bulb, the granular layer of the cerebellum, and the hippocampus (CA1 to CA4 and dentate gyrus) and lower levels in the cerebral cortex and striatum (Pellegri et al. 1996). Likewise, glycogen synthase protein is widely expressed in rat and canine brain with the highest levels observed in hippocampus, cerebral cortex, caudate-putamen, and cerebellar cortex (Inoue et al. 1988). Glycogen synthase enzymatic activity was detected histochemically in both white and gray matter in rat brain (Takeuchi 1965). In humans, glycogen synthase activity in hippocampus was higher than in either white or gray matter, though it should be noted that these measurements were made in tissue from patients with epilepsy (Dalsgaard et al. 2007). Among eight different areas of the rabbit central nervous system, glycogen synthase activity was highest in the molecular layer of the cerebellum (Breckenridge and Crawford 1961). In rat brain, the highest total glycogen synthase activity was measured in cerebellum, and lower levels of activity in hippocampus, medulla oblongata, striatum, midbrain, hypothalamus, and cortex (Knull and Khandelwal 1982). The percent of the glycogen synthase that was in the active form was also highest in the cerebellum. Taken together there is generally an agreement between glycogen synthase expression, glycogen synthase activity, and glycogen content (Oe et al. 2016; Sagar et al. 1987).

Cellular and intracellular localization. Within the brain, glycogen synthase mRNA (Pellegri et al. 1996) and protein (Inoue et al. 1988) were present in both astrocytes and neurons. Higher levels of glycogen synthase mRNA were expressed in astrocytes than neurons (Pellegri et al. 1996). In contrast, higher levels of glycogen synthase protein were expressed in neurons than astrocytes (Inoue et al. 1988). It is not clear what accounts for this apparent discrepancy. Glycogen synthase activity in rat brain was highest in the cytoplasmic fraction, followed by a threefold lower activity in the syntaptoplasmic fraction (Knull and Khandelwal 1982). Much lower activity levels were found in nuclear and mitochondrial fractions. Glycogen synthase mRNA was present in both cytosol and nucleus of cultured astrocytes and neurons from rat hippocampus (Mamczur et al. 2015). Regardless of the activity state of the enzyme, glycogen synthase is associated with glycogen (reviewed in Roach et al. 2012). This occurs despite the lack of a distinct carbohydrate binding motif. The yeast glycogen synthase, Gsy2p, contains a carbohydrate binding site integrated into the catalytic domain (Baskaran et al. 2011). Within neurons, glycogen synthase in mouse embryonic neuronal cultures was present as aggregates in the cytoplasm (Saez et al. 2014). Using fluorescence in situ hybridization (FISH) analysis on cultured cortical neurons, glycogen synthase mRNA was detected in both the soma and processes (Pfeiffer-Guglielmi et al. 2014). In the latter, the mRNA had a granular appearance. The role of glycogen synthase in the neuronal processes is not clear, but has been suggested to perhaps be important in energy demanding processes such as growing, repair, and memory consolidation or potentially in a moonlighting function such as translational control, as proposed by Fuchs et al. (2011).

5 Glycogen Synthase Regulation

5.1 Gene Regulation

Regulation of the glycogen synthase gene has received limited attention. In C2C12 mytotubes, but not HEK293 cells, GYS1 promoter activity was modestly decreased 24 h after insulin treatment (Fredriksson et al. 2004). In cultured cortical astrocytes, but not neurons, glycogen synthase mRNA expression was upregulated by two neurotransmitters, vasoactive intestinal peptide (VIP) and noradrenaline (NA) (Pellegri et al. 1996). This is consistent with the observation that glycogen resynthesis triggered by VIP or NA is dependent on DNA transcription and protein synthesis (Sorg and Magistretti 1992). The neuromodulator, adenosine, accumulates in the extracellular space following increased neuronal activity (Mitchell et al. 1993). Adenosine increased glycogen levels in cultured cortical astrocytes and this effect was blocked when transcription was inhibited (Allaman et al. 2003). Adenosine increased mRNA expression of protein targeting to glycogen (PTG) as well as C/EBP beta and C/EBP delta. Though not examined, the effects of adenosine could work through these mechanisms to potentially affect expression and/or enzymatic activity of glycogen synthase.

5.2 Protein Regulation

The accumulation of glycogen is determined by the balance between synthesis by glycogen synthesis and degradation by glycogen phosphorylase. In rabbit brain, glycogen phosphorylase activity is ~30 fold higher than glycogen synthase (Breckenridge and Crawford 1961), while in mouse brain the concentration of glycogen phosphorylase is 200 fold higher than that of glycogen synthase (Mamczur et al. 2015). These findings highlight the importance of regulation of both of these enzymes. This is especially interesting in light of the unique nature of the brain due to its location within the rigid skull. In contrast, the liver can, and does in certain glycogen storage diseases, expand when it accumulates large amounts of glycogen. This potential for expansion is greatly limited for the brain. The most studied mechanisms for glycogen synthase regulation are activation by glucose-6-P and inhibition by covalent phosphorylation. Glucose-6-P overcomes phosphorylation-induced inactivation and can restore maximal activity. In early studies of the enzyme, the enzyme was classified as either the I form or the D form. The I, independent form, is active without glucose-6-P, while the D, dependent form, is relatively inactive in the absence of glucose-6-P. Taking advantage of this phenomenon, assays to monitor glycogen synthase enzymatic activity are typically conducted in both the presence and absence of added glucose-6-P to provide an index of the phosphorylation state of the enzyme (Roach et al. 2012; Roach and Larner 1977). Alternatively, activity has been measured in the presence of low and high concentrations of

glucose-6-P with a low concentration of the substrate UDP-glucose (Guinovart et al. 1979). When these assays are conducted in cell or tissue extract, activity is interpreted as providing an indication of in vivo glycogen synthase phosphorylation. However, the in vivo activity is affected not only by the phosphorylation state, but also by other factors, including but not limited to, the concentration of glycogen, glucose-6-P, and UDP-glucose.

5.2.1 Activation by Glucose-6-phosphate

Glycogen synthase is allosterically activated by glucose-6-P (Leloir et al. 1959). In the rat brain, glucose-6-P activates glycogen synthase six- to ten-fold (Goldberg and O'Toole 1969). Arginine residues in glycogen synthase that confer glucose-6-P sensitivity were identified in *S. cerevisiae* (Pederson et al. 2000) and rabbit skeletal muscle (Hanashiro and Roach 2002). When three of these residues (579,581, and 582) were mutated to alanine in the yeast glycogen synthase (Gsy2p) and expressed in *S. cerevisiae*, glycogen accumulation was reduced as compared to wild type cells (Pederson et al. 2004b). In a knockin mouse expressing constitutively active glycogen synthase kinase 3 (GSK3), insulin was unable to inactivate muscle GSK3 and promote glycogen synthase desphosphorylation and activation. However, these mice still made normal amounts of glycogen (Bouskila et al. 2008). This suggests a major role for glucose-6-P in regulation of glycogen synthesis. A knockin mouse with Arg 582 of GYS1 modified to alanine expressed glycogen synthase that was insensitive to glucose-6-P, had ~80% reduction in muscle glycogen synthesis induced by insulin, and ~50% reduction in skeletal muscle glycogen levels (Bouskila et al. 2010). These studies demonstrate the importance of glucose-6-P in opposition to phosphorylation control, which was intact in this mutant. Unfortunately, glycogen metabolism in the brain was not examined in the either of these mouse models.

5.2.2 Inhibition by Covalent Phosphorylation

Mammalian glycogen synthase was one of the earliest enzymes found to be phosphorylated at multiple sites (Smith et al. 1971). The much studied muscle isoenzyme has 9 phosphorylation sites, with mutagenesis studies in rabbit GYS1 indicating that sites 2, 2a, 3a, and 3b are most important for inhibiting enzymatic activity (Skurat and Roach 1995; Skurat et al. 1994). Phosphorylation of glycogen synthase at Ser640 (site 3a) and Ser7/10 (sites 2 and 2a) is responsible for keeping neuronal glycogen synthase in its normally inactive state (Vilchez et al. 2007). The concept of hierarchal phosphorylation, where the addition of one phosphate enables the addition of a second phosphate, was also derived from studies on glycogen synthase (Roach 1990, 1991). For instance, casein kinase 2 (CK2) must phosphorylate glycogen synthase prior to subsequent phosphorylation by GSK3. The GSK3 beta isoform is itself regulated by phosphorylation and appears to be the most important

isoform for phosphorylation of GYS1 in muscle (Patel et al. 2008). Additional kinases that phosphorylate glycogen synthase include AMPK, Ca + 2/calmodulin-dependent protein kinase II (CAMKII), phosphorylase kinase (PhK), PKA, proteins kinase C (PKC), casein kinase I (CK1), Per/Arnt/Som) domain-containing protein kinase (PASK), dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK), stress-activated protein kinase 2b (p38beta), (reviewed in Roach et al. 2012) (Fig. 3). AMPK is perhaps one of the most interesting glycogen synthase kinases due to its role as a cellular energy sensor, the presence of a carbohydrate binding domain which binds glycogen, and its proposed role as a glycogen sensor (McBride and Hardie 2009). AMPK has been proposed to be a site 2 kinase (reviewed in Roach et al. 2012). This kinase phosphorylates glycogen synthase in vitro and a knockout of the alpha 2 subunit of AMPK reduces phosphorylation of glycogen synthase at site 2 plus 2a and increases activation of glycogen synthase (Jorgensen et al. 2004). Consistent with a role AMPK acting as glycogen sensor, in skeletal muscle from mice that lack glycogen due to global disruption of GYS1, AMPK phosphorylation is increased, leading to a more active kinase (Pederson et al. 2005b). In contrast, in the brains of mice lacking GYS1 specifically in the central nervous system, no changes in the activation state of AMPK were observed (Duran et al. 2013). Glycogen synthase kinases in the rat brain were found to be largely (~90%) cAMP-independent (Schlender and Reimann 1977). Glycogen synthase purified from canine brain was phosphorylated by brain calmodulin kinase resulting in inactivation of the enzyme (Inoue et al. 1987). Calmodulin kinase was also purified from rat brain which phosphorylated rabbit muscle glycogen synthase at sites 1b and 2 (Schworer et al. 1985) and caused inactivation of the enzyme (Iwasa et al. 1984). Both CK1 and CK2 activities were detected in the soluble fraction from rat brain (Singh and Huang 1985). p38beta bound to glycogen synthase from brain and phosphorylated the enzyme at 4 sites, including 2 sites that were not reported to be phosphorylated by any other kinases (Bouskila et al. 2008). This phosphorylation did not inactivate glycogen synthase. DYRK1A phosphorylated glycogen synthase at site 3 in COS cells resulting in inactivation of the enzyme (Skurat and Dietrich 2004). DYRK1A is expressed in the developing and adult brain (Hammerle et al. 2003). This kinase is overexpressed in Down's syndrome and mice overexpressing DYRK1A have impaired cognitive function (Altafaj et al. 2001).

Dephosphorylation of glycogen synthase results in activation of the enzyme and thus stimulation of glycogenesis. Dephosphorylation is catalyzed by protein phosphatase 1 (PP1c) bound to one of seven glycogen-targeting subunits (PPP1R3A to PPP1R3G, reviewed in Ceulemans and Bollen 2004; Korrodi-Gregorio et al. 2014). Of the seven subunits, PPP1R3D (R6), PPP1R3F (R3F), PPP1R3G, and PPP1R3C (PTG) are expressed in the brain. PPP1R3D is mainly expressed in the brain, particularly in neurons (Rubio-Villena et al. 2013). PPP1R3D interacts with laforin which targets it for degradation resulting in decreased glycogen synthesis. The importance of the regulation is seen in Lafora disease, as discussed above. PPP1R3F is highly expressed in rodent brain and regulates glycogen synthase in astrocytoma cells (Kelsall et al. 2011). Mutant PPP1R3F is unable to bind PP1c, resulting in hyperphosphorylation of Ser640 (site 3a) and Ser644 (site 3b). PPP1R3G mRNA

was detected in human brain (Munro et al. 2005). When disrupted in a mouse model, there was no detectable PPP1R3G protein in the brain, but the effects on brain glycogen metabolism were not described (Zhang et al. 2017). Disruption of PTG resulted in reduced glycogen accumulation in liver and muscle (Zhai et al. 2007) and in the brain (Turnbull et al. 2011, 2014). Overexpression of PTG in cultured mouse astrocytes lead to a large increase in glycogen levels, while down-regulation of PTG via siRNA reduced glycogen levels (Ruchti et al. 2016). PTG mRNA expression is increased in cultured mouse astrocytes by the neurotransmitters noradrenaline and VIP resulting in increased glycogen accumulation (Allaman et al. 2000). This is presumably due to dephosphorylation and activation of glycogen synthase, though this was not measured.

5.2.3 Activation by Insulin

Insulin increased glycogen accumulation in cultured astrocytes (Dringen and Hamprecht 1992) and stimulated glycogen synthase activity (Hamai et al. 1999). These effects were blocked by the phosphoinositide 3-kinase (PI3K) inhibitor, Wortmannin. In another study, the increase in glycogen was shown to occur without an increase in glucose flux across the plasma membrane (Muhic et al. 2015). Studies in skeletal muscle indicated that insulin activates glycogen synthase by promoting dephosphorylation of the protein which reduces the concentration of glucose-6-P required to activate the enzyme (Lawrence 1992). In muscle, phosphorylation at sites 3 (a + b + c) and site 2 and/or 2a are decreased in response to insulin (reviewed in Lawrence and Roach 1997). While early studies suggested that MAPK was important for the insulin stimulated dephosphorylation of glycogen synthase (reviewed in Lawrence and Roach 1997), more recent studies suggest that the process is GSK3-dependent (reviewed in Roach et al. 2012). In vitro, GSK3 phosphorylates sites 3a, 3b, 3c, and 4 (Hemmings et al. 1981). Insulin, acting throughout the protein kinase B (Akt/PKB) pathway promotes phosphorylation of GSK3 at a serine residue that results in inhibition of the kinase (Woodgett 2005). Support for this mechanism in insulin-induced phosphorylation comes from studies in which the beta isoform of GSK3 was either over expressed or disrupted in skeletal muscle. Overexpression led to less active glycogen synthase (Pearce et al. 2004), while disruption resulted in enhanced insulin-stimulated activation of glycogen synthase (Patel et al. 2008). There are two informs of GSK3, both of which are expressed in brain (Yao et al. 2002). In a mouse model where both isoforms of GSK3 had their inactivating phosphorylation site mutated to alanine, GSK3 was constitutively active. In skeletal muscle, this had no effect on the basal activity of glycogen synthase but blunted the insulin-stimulated increase in glycogen synthase activity (McManus et al. 2005). This effect on glycogen synthase activity occurred with the mutation of GSK3 beta but not alpha. The phosphorylation of both GSK3 alpha and beta in mouse cerebral cortex and hippocampus increased with insulin administration (Clodfelder-Miller et al. 2005). Glycogen synthase activity was not assayed in this study. While these studies indicate an important role of GSK3 in

insulin-mediated regulation of glycogen synthase, there may be other kinases and PP1c, in conjunction with glycogen targeting subunits, involved (reviewed in Roach et al. 2012).

5.2.4 Other Covalent Modifications

In addition to covalent phosphorylation, modification of glycogen synthase by O-linked attachment of N-acetylglucosamine was reported to inactivate the enzyme (Parker et al. 2003). This glucosylation of glycogen synthase is increased by high glucose levels in 3 T3-L1 adipocytes and by hyperglycemia in mouse adipose tissue. Therefore, this modification may contribute to the reduced activation of glycogen synthase that occurs in insulin resistance. In addition, acetylation of lysine residues in human liver glycogen synthase has been reported (Zhao et al. 2010a). Neither of these modifications has been reported for glycogen synthase in the brain.

5.2.5 Translocation

Another mechanism by which glycogen synthase is regulated, at least in muscle and liver, is by translocation of the enzyme between different sub cellular compartments. There is evidence for the localization of glycogen synthase to be regulated by the concentration of glycogen, glucose, and glucose-6-P and by phosphorylation. Green fluorescent protein (GFP) labeled GYS1 was localized in the nucleus of C2C12 and COS-1 cells in the absence of glucose while in the presence of glucose the enzyme was localized in the cytosol (Ferrer et al. 1997). In contrast, GYS2 in isolated rat hepatocytes was localized in the cytosol without added glucose and translocated to the cell periphery in response to added glucose (Fernandez-Novell et al. 1997). The changes in cellular localization of GYS2 correlated with intracellular levels of glucose-6-P (Fernandez-Novell et al. 1996). Nielsen et al. (2001) found that GYS1 location is influenced by muscle glycogen content. Glycogen synthase translocated from a glycogen enriched membrane fraction to a cytoskeleton fraction with a decrease in glycogen levels. Immunofluorescence of single EDL fibers indicated that larger aggregates of glycogen synthase protein were present when glycogen levels were high. A role of phosphorylation of glycogen synthase in affecting the subcellular distribution of the enzyme is not clear. In resting rabbit muscle, GYS1 was mainly found in the perinuclear region and myofibrillar cross-striations if GYS1 was phosphorylated at site 1a or at sites 3a and 3b (Prats et al. 2005, 2009). However, if GYS1 was phosphorylated at sites 2 and 2a, localization exhibited a diffuse distribution pattern, not associated with the perinuclear region or cross-striations. In contrast, Cid et al. (2005) reported that phosphorylation of GYS1 is not involved in subcellular distribution. Mutation of all 9 serine residues to alanine did not affect localization of glycogen synthase. However, mutation to alanine of the arginine residues involved in conferring glucose-6-P sensitivity to glycogen synthase, resulted in glycogen synthase moving from the nucleus to the cytoplasm

more quickly that wild type glycogen synthase after addition of glucose. The nuclear accumulation of GYS1 in cultured cells is in the form of spherical aggregates and GYS1 co-localizes with p80-coilin (Cid et al. 2005). P80-coilin associates with promyelocytic leukemia (PML) bodies that have been implicated in several processes including transcriptional regulation (Ruggero et al. 2000). Cid et al. (2005) suggest that glycogen synthase may act as an energy sensor, translocating to the nucleus to perform a moonlighting function when glycogen stores are depleted. A role for GYS1 in translation has also been proposed. GYS1 phosphorylated on Ser640 was preferentially associated with elongating ribosomes and depletion of GYS1 from HeLa cells affected the translation of a subset of mRNA's (Fuchs et al. 2011). The study's authors suggest that GYS1 provides a feedback loop between the energy status of the cell and translation. In the brain, little has been reported regarding translocation of glycogen synthase. In astrocytes and neurons, there was a decrease in the glycogen synthase nucleus/cytosol ratio when astrocytes and neurons were co-cultured (Mamczur et al. 2015). The significance of this is not known and further studies will be required to determine whether translocation of glycogen synthase within astrocytes and neurons occurs in physiological or pathological conditions.

6 Glycogen Metabolism During Development

Embryonic/fetal development. Claude Bernard first isolated and described the distribution of glycogen in fetal tissues in 1859 (Bernard 1859). Most of the early work was histochemical (reviewed in Cremer 1902; Needham 1931) and it was thought that these glycogen stores were essential for growth and differentiation. This idea waned with reports suggesting that the levels of glycogen in embryonic tissue were not higher than in adults (Needham 1931). However, with the advent of biochemical techniques for quantitative analysis of glycogen, the concentration of glycogen has been found to change during fetal development and/or after birth in many tissues, including the brain (Shelley 1961). In the human fetus, brain glycogen levels decreased from 5 μM/g at 9 weeks to <1 μM/g at 22 weeks (Villee 1954). In embryonic chick brain, glycogen changes with developmental age, increasing tenfold from day 6 until days 18 and 20 (Rinaudo et al. 1969). Another increase in brain glycogen concentration occurs from day 20 of development to hatching at 21 days (Edwards and Rogers 1972; Rinaudo et al. 1969). In the rat, the concentration of glycogen increased ~twofold in 18.5 dpc fetuses as compared to 14.5 fetuses (Gutierrez-Correa et al. 1991). Also in rat brain, brain glycogen increased from 5.5 to 7.5 μmol/g tissue in 17 dpc to 20 dpc fetuses, respectively, before dropping to 6.5 μmol/g tissue at term (22 dpc) (Kohle and Vannucci 1977). Histochemically detectable brain glycogen also increased during rat fetal development. Periodic acid-Schiff (PAS) staining for glycogen in developing rat brain revealed regional differences and age dependent changes (Bruckner and Biesold 1981). Glycogen was observed in epithelial cells of the immature chorioid plexus of ventricles I–IV and radial glial cells in the midbrain and medullary raph6 of the brain stem at 14 dpc

(Bruckner and Biesold 1981). As fetal development progressed, these areas maintained glycogen while radial fibers in the brain stem and the cerebral cortex stored glycogen, though to a lesser degree (Bruckner and Biesold 1981). In mouse choroid plexus, glycogen was detected by electron microscopy beginning at 13.5 dpc and is present throughout the remainder of fetal development where it is first located in what Sturrock (1979) referred to as membrane-bound bags, and later was dispersed in the cytoplasm. In the fetal mouse, brain glycogen levels of ~4 mg/g tissue were measured and were not greatly altered in 14.5 to 18.5 dpc UBC swiss mice (Tye and Burton 1980). In C57BL/6 J fetal mouse brain, glycogen levels were measured over this same gestational time with a trend to increase from the 14.5 to 18.5 days of gestation (author's unpublished observations). In general, large glycogen stores accumulate in tissues late in fetal life and have been postulated to have an important function as fuel sources during asphyxia and after the newborn is separated at birth from glucose provided from the placenta (Dawes 1968).

The key enzymes of glycogen synthesis and degradation have been monitored in developing chick and rat. Glycogen synthase activity in the embryonic chick brain doubled from the sixth to 11th day of development, fell to sixth day levels on day 13 and then slowly returned to peak levels at hatching (Rinaudo et al. 1969). Glycogen phosphorylase activity was low at day 6 and rose sharply from day 11 to 15 and then again from day 20 to hatching (Rinaudo et al. 1969). Levels at hatching were 25% below adult levels (Rinaudo et al. 1969). Glycogen synthesis is more active than phosphorylase for the entirety of chick embryonic development (Rinaudo et al. 1969). In another study in chick brain, glycogen synthase and glycogenin activity exhibited more modest changes during fetal development (Carrizo et al. 1997). In the rat, glycogen phosphorylase activity increased and glycogen synthase tended to be less active in 18.5 dpc as compared to 14.5 fetuses (Gutierrez-Correa et al. 1991).

Postnatal development. In postnatal chicks, Rinaudo and Cussotto (1971) reported that brain glycogen levels remain constant for the first 36 h after hatching (during which time the chick does not eat) as well as after 4 days of consuming a normal diet. In contrast, Edwards and Rogers (Edwards and Rogers 1972) reported that glycogen levels fell by 2 days after hatching and continued to decline up to 21 days of age. The decrease from 1 to 2 days of age occurred in all of the brain regions examined, with the largest drop in the cerebellum. Glycogen synthase activity increased almost twofold from hatching to 8 days of age (Rinaudo 1974). Brain glycogen levels (~3 mg/g tissue) were up to ~threefold higher in fetal rabbit than in 5-day old suckling, 15-day old weaning age, and adult rabbit (Curto et al. 1994). Glycogen levels in rat brain were decreased 10 min following vaginal birth but not cesarean section, suggesting that cerebral hypoxia experienced during delivery is the cause of the decrease in brain glycogen (Kohle and Vannucci 1977). Levels rebounded by 30 min after birth and then fell over the next 7 days where they remained constant for 1 week before rising again at 30 days of age. The change in brain glycogen levels from newborn to adult is region- specific in cats and dogs (Chesler and Himwich 1943). In both species, glycogen levels increased in the cortex and decreased in thalamus, cerebellum, medulla, and spinal cord with aging from newborn to adult.

7 Glycogen Synthase Genetic Modifications

Glycogen storage disease type 0. Two families have been identified with loss of function mutations in GYS1. In one of these families, three siblings had a homozygous stop mutation (R426X). The eldest male child died of cardiac arrest at 10.5 years of age. He experienced an episode of tonic-clonic seizures at age 4. Electroencephalogram (EEG) showed bilateral epileptogenic spikes in the parietal and occipital regions. Subsequent EEG's were normal while his gross motor performance was impaired. A brother exhibited heart abnormalities and muscle fatigue at 11 years of age. He had an IQ of 73 (normal is >70). A 2-year-old sister was asymptomatic. The younger siblings exhibited lack of glycogen, predominance of oxidative fibers, and mitochondrial proliferation (Kollberg et al. 2007). An 8-year-old patient from another family was identified with a homozygous two base pair deletion in exon 2 (c.162-163AG) of GYS1, which is expected to produce a protein 162 amino acids in length (Cameron et al. 2009). He collapsed and died during a bout of exercise. In skeletal muscle, glycogen was depleted and there was a total loss of glycogen synthase protein in patient fibroblasts. There was a proliferation of mitochondria and type 1 fiber predominance in skeletal muscle. There were no neurological issues noted. A 12 year-old girl with intellectual disability that suffered from myoclonic epilepsy since infancy was found to have PAS-negative muscle fibers, consistent with depleted glycogen stores, and increased oxidative enzyme activity (Michelson-Kerman et al. 2003). However, whether this patient had a deficiency in glycogen synthase was not determined. The connection between neurological symptoms observed in some of these patients and the presumed inability to make glycogen in the brain are not clear. In regard to a connection between epilepsy and brain glycogen levels, one patient with the inability to make glycogen had epilepsy while 19 patients with epilepsy had elevated levels of glycogen in the hippocampus, as compared to gray and white matter. This finding lead Dalsgaard et al. (2007) to propose that brain glycogen is a prerequisite for sustained neuronal activity as occurs in epileptic seizures. However, in mice lacking GYS1 in the brain, kainate-induced seizure susceptibility was increased as compared to wild type controls (Lopez-Ramos et al. 2015). Regarding, a connection between brain glycogen and cognitive function, patients that presented with cognitive impairment are consistent with studies in rodents where disruption of glycogen synthesis or inhibition of glycogen utilization resulted in learning impairment (Duran et al. 2013; Suzuki et al. 2011). Additionally, it will be interesting to determine whether the lack of glycogen in the brain leads to metabolic changes in this organ as was reported in skeletal muscle from human patients with GYS1 mutations and in a mouse model disrupted for GYS1 (Pederson et al. 2005b).

Animal models with elevated glycogen storage. Animal models with increased glycogen accumulation demonstrate the interplay between the proteins and metabolites involved in glycogen metabolism. A key question regarding glycogen synthesis was the role of glucose availability versus glycogen synthase activity, a push versus pull concept. This concept has not been explored in the brain but has been studied

in skeletal muscle. When a mutant form of GYS1 that cannot be effectively inhibited by phosphorylation was over expressed in skeletal muscle, glycogen accumulation increased up to fivefold along with a decrease in UDP-glucose levels and increase in glucose-6-P levels (Azpiazu et al. 2000; Manchester et al. 1996). There was also a threefold increase in total glycogen phosphorylase activity. GLUT4 levels either decreased or were unchanged as was basal and insulin-stimulated uptake of 2-deoxyglucose (Lawrence Jr. et al. 1997). The glycogen was less branched showing the importance of the ratio of branching enzyme to glycogen synthase (Pederson et al. 2003). An activating mutation in GYS1 (R309H) identified in horses with polysaccharide storage myopathy (PSSM), a neuromuscular disease, leads to glycogen accumulation in skeletal muscle (McCue et al. 2008). This mutation increases activity in the absence of glucose-6-P. Muscle from affected horses paradoxically had increased phosphorylation of sites 2 + 2a, but had a lower K_m for UDP-glucose both in the presence and absence of glucose-6-P (Maile et al. 2017). These findings suggest that the mutation prevents normal inactivating effects of phosphorylation. These studies demonstrate that regulation of glycogen synthase activity impacts glycogen levels. Overexpression of GLUT1 in skeletal muscle increased glucose uptake and glycogen accumulation, without affecting glycogen synthase activity (Ren et al. 1993). This study indicated the importance of glucose uptake for glycogen synthesis. On the other hand, GLUT4 over expression in muscle increased insulin-stimulated glucose uptake but did not affect glycogen levels (Brozinick et al. 1996; Hansen et al. 1995). Another model proposed by Shulman et al. (1995) suggests that glucose transport and phosphorylation by hexokinase controls glycogen synthesis and that the role of phosphorylation control of glycogen synthase is to regulate the concentration of glucose-6-P.

Increased levels of brain glycogen were achieved in rats using an inhibitor of glycogen phosphorylase (Suh et al. 2007). The ~twofold increase in brain glycogen was associated with protection against hypoglycemia-induced neuronal degeneration. Overexpression of constitutively active glycogen synthase specifically in neurons in mouse and Drosophila resulted in glycogen accumulation, loss of neurons, impaired locomotion, and shortened lifespan (Duran et al. 2012). In horses with PSSM, there were no histological abnormalities in brains from horses with this disorder, though it is not clear whether brain glycogen was monitored (Valentine et al. 1997).

Animal models with impaired glycogen synthesis. A global knockout of GYS1 in mice (muscle glycogen synthase knockout MGSKO mice) revealed the importance of glycogen synthase in newborn survival (Pederson et al. 2004a). Approximately ninety percent of newborn pups lacking GYS1 died shortly after birth due to cardiopulmonary defects. Surviving MGSKO animals lack glycogen in tissues where GYS1 is expressed, including brain (Pederson et al. 2005b). Overexpressing GYS1 under the control of the muscle creatine kinase promoter in MGSKO mice resulted in mice (MGSKO/GSL30) with restored glycogen synthesis in cardiac and skeletal muscle, but not in brain (Pederson et al. 2004a). There is no perinatal lethality observed in these mice, indicating that brain glycogen is not required for newborn survival. This conclusion is supported by normal survival in a mouse model

(GYS1Nestin-KO) with central nervous system-specific disruption of glycogen synthase (Duran et al. 2013). In this model, brain glycogen synthase protein and activity, as well as glycogen, were undetectable and brain morphology was normal. The protein expression levels of several enzymes involved in glycogen metabolism were also monitored in the brain. The lack of brain GYS1 did not affect the levels of the brain or muscle isoforms of glycogen phosphorylase, total or phosphorylated GSK3, or glycogenin. Glycogen debranching enzyme expression was decreased and AMPK expression was increased. However, the levels of phosphorylated AMPK were normal. This is in contrast to what was observed in skeletal muscle from MGSKO mice where the levels of AMPK and Acetyl-CoA carboxylase phosphorylation were increased suggesting an increased ability to oxidize fatty acids (Pederson et al. 2005b). Because fatty acids are generally not considered to be a significant fuel for the brain, this difference between skeletal muscle and brain may not be surprising. However, it is also interesting to consider AMPK activity in light of the hypothesis that AMPK is a glycogen sensor (McBride and Hardie 2009). Muscle and brain uptake of glucose was decreased in MGSKO mice after a euglycemic-hyperglycemic clamp suggesting insulin resistance. Numerous other changes in gene expression were found in muscle from MGSKO animals (Parker et al. 2006). It will be interesting to examine what adaptations occur in the brain when the ability to synthesize glycogen has been impaired.

Despite lacking glycogen in skeletal muscle, heart, and brain, MGSKO animals had normal exercise capacity (Pederson et al. 2005a). This is of interest in light of the finding that brain glycogen decreases in rats during exercise and is hypothesized to play a role in central fatigue (Matsui et al. 2011). GYS1Nestin-KO mice were impaired in the Skinner box associative learning task and exhibited decreased long-term potentiation. This is consistent with the memory impairment reported in an inhibitory avoidance test for rats treated with an inhibitor of glycogen phosphorylase (Suzuki et al. 2011). In contrast, MGSKO and MGSKO/GSL30 mice are not impaired in the inhibitory avoidance test (author's unpublished observations). In addition, in the author's hands, learning impairment on this test by mice with central nervous-specific disruption of glycogen synthase is dependent on the strength of the aversive stimulus (author's unpublished observations). These findings provide further impetus for examining adaptations in mice lacking brain glycogen synthase. They also highlight a challenge for interpreting studies in mice that are genetically modified during development. They are beneficial models for genetic diseases but may not be as good a model for examining acquired defects. This has been demonstrated for the disruption of glycogen synthase in muscle. A tamoxifen-inducible model was generated to disrupt of GYS1 specifically in skeletal muscle of adult mice (Xirouchaki et al. 2016). This allowed the examination of the effects of reducing GYS1 while mitigating possible adaptations during development without GYS1. The 85% reduction in GYS1 protein resulted in a 70% reduction in muscle glycogen. The mice displayed a decrease in insulin-stimulated glucose uptake in muscle indicative of insulin resistance, as observed in MGSKO mice. However, in contrast to MGSKO mice, exercise performance was impaired in the tamoxifen inducible model.

Studies by Sinadinos et al. (2014) suggest that glycogen synthesis may contribute to neurological decline with aging. Global disruption of GYS1 in mouse prevents the formation of poorly branched polysaccharide-based aggregates, resembling corpora amylacae (CA) that are normally found in aged human brain. When glycogen synthase was reduced specifically in neurons in drosophila, the result was neurological improvement with age and an extended lifespan.

8 Glycogen Synthase in Disease

Deficiencies in the enzymes involved in glycogen synthesis and utilization result in glycogen storage diseases, some of which are described in this and other chapters of this volume. In addition, glycogen synthesis is affected in several disease states including diabetes, cancer, depression, Alzheimer's disease, and Huntington disease.

Diabetes. Brain glycogen synthesis has been examined in diabetes. Newly synthesized glycogen in the occipital lobe of patients with type 1 diabetes was reduced (Oz et al. 2012), perhaps due to reduced glycogen synthase activity. In cortex and retina, glycogen synthase activity in the presence of glucose-6-P was increased 50–100% in a streptozotocin-induced rat model of type 1 diabetes (Sanchez-Chavez et al. 2008). Brain glycogen supercompensation has been examined as a mechanism for hypoglycemia unawareness that occurs in this disease. A rebound, above normal levels, in brain glycogen levels has been reported after a single bout of hypoglycemia in humans (Oz et al. 2009) and in two rodent studies (Canada et al. 2011; Choi et al. 2003) but not in another rodent study (Herzog et al. 2008). In humans, this was associated with higher levels of newly synthesized brain glycogen after hypoglycemia (Oz et al. 2009). In the Otsuka Long–Evans Tokushima fatty rat model of type 2 diabetes, glycogen synthase protein expression was elevated in the hippocampus, while the phosphorylation state was not affected (Shima et al. 2017). Insulin-induced activation of glycogen synthase was impaired in patients with type 2 diabetes and in subjects with increased risk of developing the disease (reviewed in Fredriksson et al. 2004). Associations between GYS1 and type 2 diabetes have been reported. For example, two polymorphic alleles were found in the glycogen synthase gene, which were enriched in patients with type 2 diabetes (Groop et al. 1993). However, the concentration of the glycogen synthase protein in muscle biopsies was normal indicating that these alleles are markers rather than causes of type 2 diabetes. In another study, insulin-induced GYS1 mRNA expression was blunted in muscle biopsies from diabetic patients versus non-diabetic controls, as was the increased in fractional activity (Huang et al. 2000). These effects were interpreted to be secondary to the chronic hyperglycemia experienced by the patients. However, these and other studies have yielded conflicting results, thus it is not clear whether GYS1 expression is influenced by insulin (reviewed in Fredriksson et al. 2004).

Cancer. High levels of glycogen have been reported in several types of cancer, including brain tumors (reviewed in Cloix et al. 2008). Glycogen synthase activity

was elevated in human glioma tumors (Beckner et al. 2005; Lowry et al. 1983) as was the level of GYS1 mRNA (Kroes et al. 2007). Decreasing glycogen synthase activity via antisense GS cDNA reduced invasiveness of human glioblastoma multiform cells and the tumor size in nude mice (Ardourel et al. 2007).

Depression. In a mouse model of depression, induced by long term exposure to high glucocorticoid levels, both glycogen concentration and glycogen synthase activity were decreased in the hippocampus (Zhang et al. 2015a). The study's authors suggest that this effect of glucocorticoids on glycogen metabolism induces the depression-like behavior in this model. In contrast, rats that were subjected to prenatal acute stress to induce depression (Detka et al. 2014) exhibited increased glycogen levels in the hippocampus and frontal cortex. The selective serotonin reuptake inhibitor (SSRI) fluoxetine, which is used to create depression, increases glycogen content in astrocytes when administered at low doses, but decreases glycogen content at higher doses (Bai et al. 2017). Thus, it is not clear what role brain glycogen may play in this disorder.

Alzheimer's disease. Several pieces of evidence lead to the hypothesis that glycogen synthesis will be inhibited in Alzheimer's disease (AD). GSK3β, which phosphorylates and inactivates glycogen synthase, is upregulated in AD (Llorens-Martin et al. 2014). In addition, there is up regulation of A2A receptors in AD (Gomes et al. 2011). Binding of adenosine to this receptor promotes glycogen breakdown in cultured astrocytes (Xu et al. 2014). Activation of astrocytic insulin receptors promotes glycogen storage (Heni et al. 2011) while insulin resistance occurs in AD. Amyloid-beta (1–42) impairs glycogen storage in human astrocytes (Zhang et al. 2015b), consistent with its effect of increasing GSK3β activity (King et al. 2014), and impairs learning in day-old chicks (Gibbs 2015). Increasing brain glycogen levels with pyruvate supplementation improved spatial learning, but impaired associative learning, in a mouse model of AD (Koivisto et al. 2016), leaving open the question as to whether boosting brain glycogen levels would be beneficial overall. In addition, CA's appear with aging and accumulates to a greater extent in AD (Inoue et al. 1996; Rai et al. 2018; Rohn 2015). Glucose contained in CA's is thought to be relatively unavailable as a fuel source. Whether CA's contribute to the cause or are a result of AD are unknown, but interestingly, in a mouse model unable to synthesize glycogen, these CA's do not appear with aging (Sinadinos et al. 2014). It is not clear whether manipulating glycogen metabolism in the brain would impact the pathology of AD.

Huntington disease. An intriguing role for glycogen synthesis in neurons in the context of Huntington disease was recently proposed (Rai et al. 2018). Overexpression of toxic huntingtin protein induced glycogen synthesis in neurons by activating glycogen synthase. A similar effect was observed after subjection of neurons to oxidative stress or blockage of the proteasome. In each case this increase in glycogen synthase activity was neuroprotective. The increase in glycogen synthase activity induced by the huntingtin protein increased neuronal autophagic flux which removed the cytoxic protein. This study suggests that activating glycogen synthase could be therapeutic in neurodegenerative disorders.

9 Conclusion

Much has been learned about glycogen synthesis since the identification of glycogen synthase more than 60 years ago. The study of this metabolic pathway has largely focused on its role in muscle and liver, with less investigation in the brain. How well findings in these other tissues translate to the brain is in many cases fertile soil for exploration. Recent discoveries showing important roles for brain glycogen make this an exciting time to study the metabolism of this seductive dynamic glucose polymer.

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The Structure and the Regulation of Glycogen Phosphorylases in Brain

Cécile Mathieu, Jean-Marie Dupret, and Fernando Rodrigues-Lima

Abstract Glycogen constitutes the main store of glucose in animal cells. Being present at much lower concentrations in the brain than in liver and muscles, brain glycogen has long been considered as an emergency source of glucose, mobilized under stress conditions (including hypoglyceamia). Nevertheless, over the past decade, multiple studies have shed a new light on the roles of brain glycogen, being notably an energy supply critical for high-cognitive processes such as learning and memory consolidation. Glycogen phosphorylase (GP) is the key enzyme regulating the mobilization of glycogen in cells. It is found in humans as three isozymes: muscle (mGP), liver (lGP) and brain GP (bGP). In the brain, astrocytes express both mGP and bGP while neurons only express the brain isoform. Although GP isozymes are very similar, their distinct regulatory features confer them distinct metabolic functions that are strongly related to the roles of glycogen in different tissues. Here, we provide an overview of the functions, the regulations and the structures of GPs in the brain and their relation to the specific roles of glycogen in astrocytes and neurons. We also discuss novel findings concerning the specific regulations of bGP by oxidative stress, and the potential of these enzymes as therapeutic targets in the brain.

Keywords Glycogen phosphorylase · Energy metabolism · Crystal structure · Allosteric regulation · Redox regulation · Isozyme

C. Mathieu (✉)
Department of Cell & Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN, USA
e-mail: Cecile.Mathieu@StJude.org

J.-M. Dupret · F. Rodrigues-Lima (✉)
Unité BFA, CNRS UMR 8251, Université de Paris, Paris, France

UFR Sciences du Vivant, Université de Paris, Paris, France
e-mail: fernando.rodrigues-lima@univ-paris-diderot.fr

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1 Glycogen and its Metabolism

Glycogen is a globular polymer of glucose which constitutes the only form of glucose storage in animal cells. Discovered by Claude Bernard in 1857, glycogen constitutes the second energy store of human body, after fat tissues (Young 1957; Berg et al. 2002; Adeva-Andany et al. 2016), In human, glycogen is found in three main tissues: the liver, the muscles (including the heart), and to a lesser extent, in the brain (Berg et al. 2002; Adeva-Andany et al. 2016; Roach 2002). In the brain, glycogen is mostly located in the grey matter, in areas rich in synapses, and more particularly in the hippocampus and the *locus coeruleus*, two regions involved in learning and memory (Sagar et al. 1987; Dalsgaard et al. 2007; Kong et al. 2002). Human glycogen is associated to various functions depending on its location. In the liver, it is metabolized to glucose which regulates glycaemia. In muscles, glycogen provides the energy needed for muscle contraction. In the brain, glycogen has long been considered as an emergency store of glucose, mobilized to protect neurons from stress conditions including hypoglycemia or hypoxic stress (Berg et al. 2002; Adeva-Andany et al. 2016; Roach 2002; Obel et al. 2012; Öz et al. 2015).

Yet, it has become clear that cerebral glycogen supports neuronal activity and normal brain functions as it is notably involved in the regulation of awake-sleep cycles as well as in high cognitive processes such as learning and memory consolidation (Kong et al. 2002; Bellesi et al. 2018; Gibbs 2015; Gibbs and Hutchinson 2012; Gibbs et al. 2007) (DiNuzzo et al., this issue). Consequently, the absence or the lack of mobilization of glycogen in brain alter the various processes in which it is involved (Gibbs et al. 2006). On the other hand, glycogen accumulation is also deleterious for cells, and particularly for neurons (Wolfsdorf and Weinstein 2003). These cells are extremely sensitive to the accumulation of glycogen in their cytoplasm, leading readily to their degeneration (Hedberg-Oldfors and Oldfors 2015; Duran and Guinovart 2015). Moreover, disease-related mutations in proteins involved in glycogen metabolism and resulting in the accumulation of glycogen (due to over-synthesis or insufficient degradation), is associated to a broad spectrum of diseases, termed glycogen storage diseases (GSD). These diseases are usually caused by mutations in a single gene and can affect one or several tissues (Wolfsdorf and Weinstein 2003). Lafora's disease and the adult polyglucosan body disease (APBD) are two GSD affecting the brain. They are both characterized by the accumulation of poorly branched glycogen granules (Duran and Guinovart 2015; Roach 2015; Klein 1993; Moses and Parvari 2002). The limited number of ramifications significantly reduces their solubility and favors a fibrillary structure, allowing them to aggregate, but also to be resistant to degradation (Hedberg-Oldfors and Oldfors 2015; Cavanagh 1999). Therefore, a tight regulation of glycogen metabolism is required to ensure the maintenance of a functional but not deleterious pool of glycogen in the cells.

The metabolism of glycogen is the same in all tissues and is ensured by the concerted action of multiple proteins and enzymes. On one hand, glycogen is mainly synthesized from blood-derived glucose. It is successively metabolized into glucose-6-phosphate, glucose-1-phosphate and UDP-glucose, by the hexokinase,

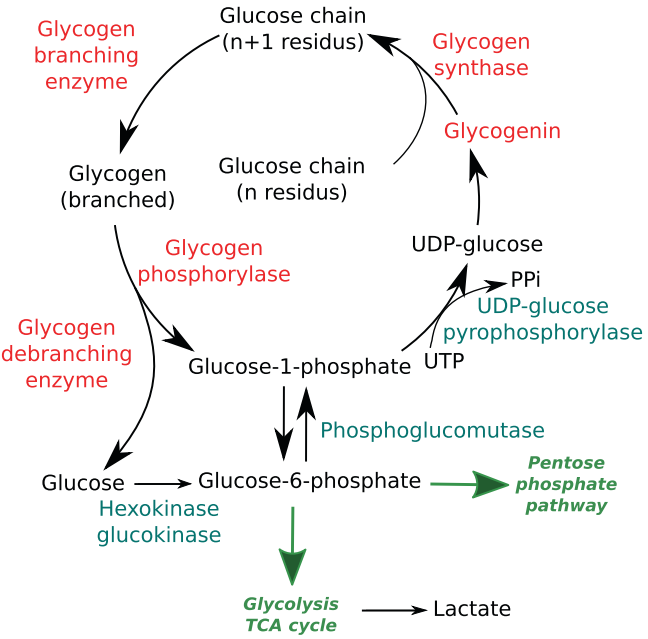

Fig. 1 Metabolism of glycogen. Schematic representation of the metabolism of glycogen and its connection with the different energy metabolism pathways

phosphoglucomutase and UDP-glucose pyrophosphorylase, respectively. In addition, glycogen may also be synthesized from glucose-6-phosphate generated from the gluconeogenesis route, an anabolic pathway that synthesizes glucose from non-carbohydrate precursors including lactate or amico-acid (Pelley 2007). UDP-glucose is then incorporated in the glycogen molecule by the glycogenin and glycogen synthase (GS) in a process is referred as glycogenesis (Fig. 1).

On the other hand, the mobilization of glycogen, or glycogenolysis, into glucose-1-phosphate is under the control of the glycogen phosphorylase (GP) and the glycogen debranching enzyme. The resulting glucose-1-phosphate can then be used in the different energy metabolism pathway, in particular the glycolysis and the TCA cycle, as well as the pentose phosphate pathway (Berg et al. 2002; Adeva-Andany et al. 2016; Roach 2002; Yip et al. 2017) (Fig. 1).

2 The Glycogen Phosphorylases

2.1 The Reaction of Phosphorolysis

At the molecular level, glycogen molecules are composed of branches of glucose subunits linked together by α,1–4 glucosidic bonds. Ramifications are formed through the establishment of α,1–6 glucosidic bonds, resulting in the formation of

glycogen granules (Hedberg-Oldfors and Oldfors 2015; Meléndez-Hevia et al. 1993). GP (EC 2.4.1.1) catalyzes the reaction termed phosphorolysis, which corresponds to the cleavage of the α,1–4 bond linking two subunits of glucose and results in the formation of one subunit of glucose-1-phosphate (Fig. 2). To this end, the enzyme associates with the glycogen molecule through the non-reducing end of one branch (Berg et al. 2002; Newgard et al. 1989). However, when only 4 subunits of glucose separate the extremity of a glycogen branch from a ramification, the presence of an α,1–6 glucosidic bond blocks the degradation of the glycogen molecule by GP. At this stage, the glycogen debranching enzyme ensures the transfer of 3 of the 4 subunits of glucose on an adjacent branch and releases a subunit of glucose through the cleavage of the α,1–6 glucosidic link (Taylor et al. 1975).

Because GP is the only protein capable of degrading the glycogen molecules in cells, this enzyme appears to be a key player in glycogen metabolism. GP has raised a lot of attention in the twentieth century, as the study of this enzyme led to discovery of two important regulatory mechanisms and to the awarding of two Nobel prizes: one to Carl and Gerty Cori, in 1947, for the identification of GP, and the other one to Edmond H. Fischer and Edwin G. Krebs, in 1992, for their discovery of the reversible regulation of GP by phosphorylation. The recent findings of a functional pool of glycogen in the brain raised a new interest for GP, but also for its specific regulation in this tissue.

The phosphorolysis of glycogen also involves a cofactor: the pyridoxal phosphate (PLP). This cofactor is covalently linked to lysine 680 of the active site of GP through a Schiff base. The PLP acts as a proton donor during catalysis. Indeed, the phosphate group of PLP protonates inorganic phosphate, which then protonates the hemiacetal oxygen of the α,1–4 glucosidic bond, leading to the formation of a glucosyl carbocation. The nucleophilic attack of the inorganic phosphate on the carbocation then lead to the formation of glucose-1-phosphate (Berg et al. 2002; Newgard et al. 1989) (Fig. 2). Interestingly, GP is also capable of catalyzing the opposite reaction of synthesis of glycogen from glucose-1-phosphate. Nevertheless, this reaction does not occur *in vivo*, due to the high intracellular ratio of inorganic

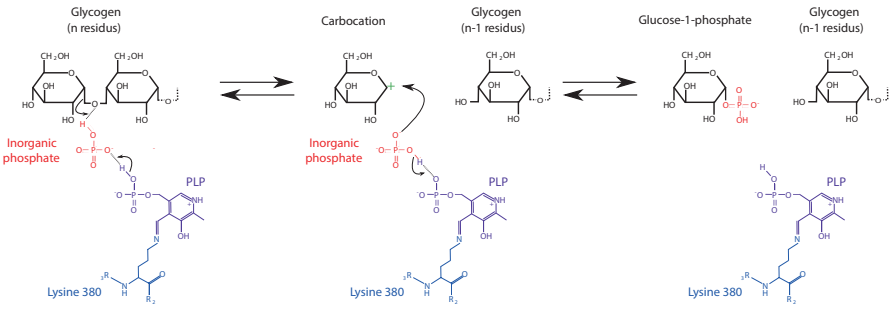

Fig. 2 Phosphorolysis of glycogen. Schematic representation of the reaction catalyzed by GP, involving the PLP as a cofactor

phosphate/glucose-1-phosphate which favors the glycogenolysis (Berg et al. 2002; Newgard et al. 1989).

2.2 The Regulation of Glycogen Phosphorylase Isozymes

In human, GP exists as three isoforms, encoded by three different genes and named after the tissue where they predominate: muscle (GPMM or mGP), liver (GPLL or lGP) and brain GP (GPBB or bGP). These three isoenzymes are highly conserved, sharing between 80 and 85% of sequence identity, as well as close 3D structures (Berg et al. 2002; Adeva-Andany et al. 2016; Newgard et al. 1987, 1988, 1989; Lebo et al. 1984). Nevertheless, despite their high sequence identity, the three isoforms of GP can be distinguished by their structural, regulatory and functional properties. As we will discuss later, both the muscle and the brain isoforms of GP are expressed in the brain, contrary to the liver and muscles that mainly express one isoform (Newgard et al. 1989; Saez et al. 2014; Pfeiffer-Guglielmi et al. 2003). The expression of two isoforms in the brain suggest very complex functions of glycogen in this tissue that are strongly related to the specific regulations of these enzymes.

All three isoforms of GP are allosterically-regulated enzymes, implying that they are found in at least an active state (also named R-state) and an inactive state (T-state) (Roach 2002; Newgard et al. 1989). Two modes of regulation allow the transition between the active and inactive states of the enzyme and can occur simultaneously:

- Phosphorylation of Serine 14
- Binding of allosteric effectors.

2.2.1 The Regulation of GPs by Phosphorylation

In the absence of any allosteric effector, the dephosphorylated form of the GP is inactive (Berg et al. 2002; Newgard et al. 1989; Barford et al. 1991; Barford and Johnson 1989). After phosphorylation of serine 14 by its specific kinase, the phosphorylase kinase (EC 2.7.11.19), the phosphorylated GP switches to its active state. The dephosphorylation and subsequent inactivation of GP, however, is permitted by the protein phosphatase 1 (PP1) associated to specific regulatory subunits. Indeed, PP1 is involved in the regulation of a large diversity of cellular processes, including cell division, metabolism or the regulation of ion pumps, transporter and ion channels (Heilmeyer 1987, 1991; Ceulemans and Bollen 2004). The specific regulation of glycogen metabolism by PP1 is mediated by a glycogen-targeting subunit of the PPP1R3 family. Seven different subunits with various patterns of expression, have been identified in mammals. These subunits allow the differential regulation of glycogen synthase, glycogen phosphorylase and phosphorylase kinase by PP1. Notably, PPP1R3 (also known as PPP1R3A) subunit is mainly expressed in muscles while

PPP1R4 (also known as PPP1R3B) is predominantly found in liver. On the other hand, PPP1R5/PTG as well as PPP1R6 harbor a wide distribution of expression (Doherty et al. 1995, 1996; Printen 1997; Newgard et al. 2000). Interestingly, in the brain, PPP1R6 (also known as PPP1R3D) as well as PPP1R5/PTG (also known as PPP1R3C) subunit have been shown to influence glycogen accumulation (Rubio-Villena et al. 2013; Vilchez et al. 2007).

The phosphorylation of GP is controlled by the glycogenolytic cascade, a phosphorylation cascade activated by the binding of extracellular ligands, such as glucagon or noradrenaline, to their specific receptors. The activation of the glycogenolytic cascade occurs first through the production of cAMP by the adenylate cyclase, and the subsequent activation of PKA. This kinase then initiates the phosphorylation cascade and the activation of the phosphorylase kinase. In addition, extracellular signals triggering calcium release from the endoplasmic reticulum also lead to the activation of the glycogenolytic cascade through the direct interaction of calcium ions with adenylate cyclase and phosphorylase kinase and their subsequent activation (Heilmeyer 1991) (Fig. 3).

Together, these mechanisms allow a regulation of GP according to global energy needs. In the liver, for instance, the regulation of GP by insulin and glucagon controls the release of glucose from liver glycogen and participates in the control of glycemia (van de Werve et al. 1977; Agius 2015). In muscles, the depolarization of the membrane as well as the release of noradrenaline stimulate the glycogenolytic cascade, providing energy to muscle cells during a prolonged exercise (Heilmeyer 1987; Jensen and Richter 2012). Finally, in the brain, extracellular signals including neurotransmitters and neuromodulators such as noradrenaline, serotonin, dopamine, histamine, but also VIP or extracellular variations of potassium allow the mobilization of the astrocytic store of glycogen, in response to brain activity (Allaman et al. 2003; Hutchins and Rogers 1970; Magistretti et al. 1981; Sorg and Magistretti 1991, 1992; Xu et al. 2013, 2014; Coggan et al. 2018).

2.2.2 The Regulation by Allosteric Effectors

GP activity is also regulated through the binding of allosteric effectors including AMP, ATP, glucose or glucose-6-phosphate, which reflect the local energy state of the cell. GP has multiple allosteric sites that can simultaneously bind allosteric effectors with agonistic or antagonistic effects, leading to multiple intermediate activation states, and allowing a fine control of the enzyme activity according to cellular energy requirements (Newgard et al. 1989; Barford et al. 1991; Sprang et al. 1991; Oikonomakos et al. 1995; Lukacs et al. 2006).

As a consequence, a high energy demand with a high AMP/ATP ratio results in the preferential binding of AMP in its allosteric site (the AMP binding site) and the transition/stabilization of GP in its active R-state (Barford et al. 1991; Sprang et al. 1991). The enzyme will then be able to degrade glycogen into glucose-1-phosphate

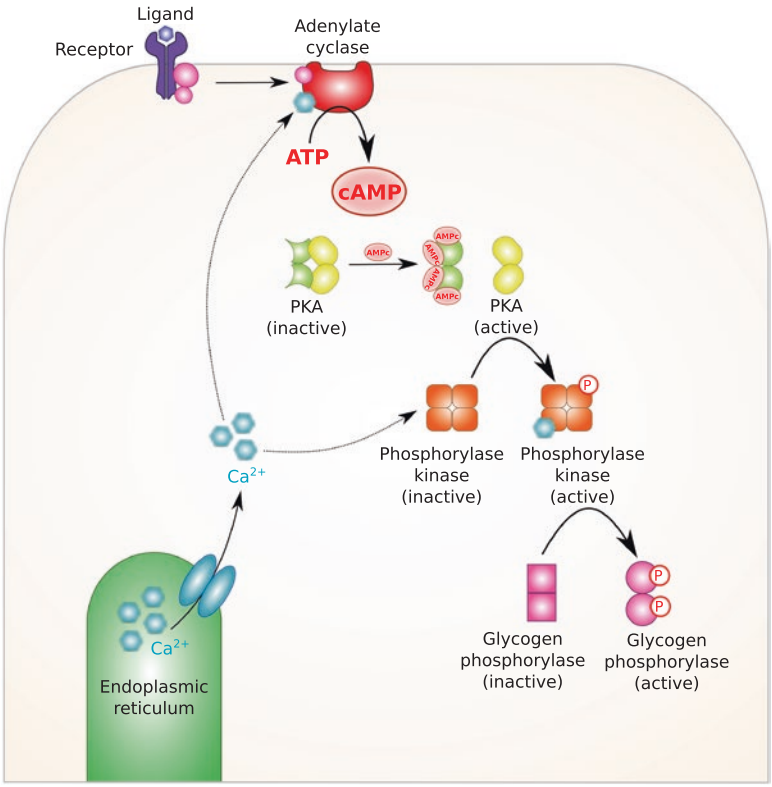

Fig. 3 Glycogenolytic cascade. The activation of GP by phosphorylation is under the control of a phosphorylation cascade, triggered by the binding of extracellular factors, including glucagon and noradrenaline, on their receptor and ultimately resulting in the activation of the enzyme

which is metabolized by the glycolytic pathway and the TCA cycle to produce ATP. On the contrary, ATP or glucose-6-phosphate, which reflect a favorable cellular energy state, can bind the same allosteric site and trigger the inactivation of the enzyme and subsequent replenishment of glycogen stores (Newgard et al. 1989).

Similarly, the binding of glucose in the catalytic site of GP results in the transition/stabilization of the enzyme to its T-state. In addition to its allosteric effect, glucose, but also glucose-1-phosphate, inhibit the activity of GP by competing with glycogen for the binding in the catalytic site (Newgard et al. 1989).

Additionally, as we mentioned above, GP can also catalyze the reaction of glycogen synthesis from glucose-1-phosphate *in vitro*. Although it might be counterintuitive, this reaction is accompanied by the transition of the GP to its R-state, triggered by the binding of glucose-1-phosphate, which acts as the reaction substrate, but also as an allosteric activator. Conversion of GP to its R-state by glucose-1-phosphate allows the activation of the enzyme and the glycogen synthesis (Newgard et al. 1989).

2.3 The Differential Regulation of the GPs

Although all three isoforms of GP are very similar in terms of sequences and structures, they differ in their sensitivity to the different modes of regulation, which appears to be related to the functions of glycogen in the concerned tissues. Indeed, in liver for instance, lGP is poorly sensitive to its regulation by allosteric effectors and responds almost exclusively to phosphorylation (Newgard et al. 1989; Lowry et al. 1964a) (Table 1). As mentioned above, the phosphorylation of GP is in direct link with both insulin and glucagon, and underlines the role of liver glycogen as an energy supply that can be mobilized to provide glucose to the whole body (van de Werve et al. 1977; Agius 2015).

The two isoforms found in the brain, mGP and bGP, are both very sensitive to the binding of the allosteric effectors and are largely regulated by these small molecules, supporting the local function of brain glycogen. Nevertheless, mGP is also strongly activated by phosphorylation, resulting in the over-activation of the enzyme in presence of AMP, while phosphorylation moderately activates bGP (Newgard et al. 1989; Crerar et al. 1995; Müller et al. 2015; Helmreich et al. 1967; Mathieu et al. 2016a). In addition, the behavior of bGP and mGP toward AMP differs. Indeed, as we will describe below, GP is a homodimer. Although the binding of AMP to mGP is cooperative (i.e. the binding of AMP on one monomer of mGP leads to the activation of the second monomer), the binding of AMP to bGP is not (Crerar et al. 1995; Mathieu et al. 2016a). Moreover, it must be pointed out that the affinity of bGP for AMP is higher than that of mGP (Crerar et al. 1995) (Table 1).

Finally, an additional layer of regulation was recently described for the bGP and appears unique to this isoform. Unlike mGP and lGP, this enzyme is highly sensitive to reactive oxygen species (ROS) such as H_2O_2, but also to environmental oxidative compounds, resulting in its reversible inhibition and the redox regulation of the glycogenolysis (Mathieu et al. 2016b, 2017a). This catalytic regulation involves the formation of an intramolecular disulfide bond that prevents the binding of AMP, the allosteric activator, and avoids the subsequent activation of the bGP, without affecting its regulation by phosphorylation. This disulfide bond involves two cysteines residues, Cys318 and Cys326, strongly conserved among mammalian bGPs, the latter being unique to the brain isoform (Mathieu et al. 2016b).

Table 1 Relative activation of the different GP isoenzymes by phosphorylation of Ser14 and AMP (Newgard et al. 1989; Lowry et al. 1964a; Crerar et al. 1995; Helmreich et al. 1967; Mathieu et al. 2016a)

Condition	Liver GP % activity	Muscle GP % activity	Brain GP % activity
Phosphorylation of Ser14	100%	100%	60%
Binding of AMP	10%	80%	100%
Phosphorylation of Ser14 + AMP binding	100%	110%	100%

3 The Structure of the Glycogen Phosphorylase

The first tridimensional structure of a GP isozyme has first been published in 1974, and corresponded to rabbit mGP (Johnson et al. 1974). Over the next two decades, multiples crystal structures of the phosphorylated or non-phosphorylated mGP, as well as crystal structures of the enzyme in complex with different allosteric effectors, led to better understanding of the structural mechanisms underlying the regulation of this GP isozyme (Barford and Johnson 1989; Sprang et al. 1991). The determination of the structure of lGP in 2000, and of bGP in 2016, further allowed a deeper understanding of the specificities of regulation of each isoforms at the structural level (Mathieu et al. 2016a; Rath et al. 2000).

3.1 General Features

The overall structure of GP is very conserved between the different isozymes. The three proteins are homodimeric (2 × 98 kDa), each dimer comprising two faces: the catalytic face and the regulatory face (Newgard et al. 1987; Mathieu et al. 2016a; Johnson et al. 1974; Rath et al. 2000). The catalytic face of GP consists of the glycogen storage site which orientates the substrate to the catalytic site. On the other side, the regulatory face comprises regions involved in the regulation of the activity, including the phosphorylation site, the AMP binding site as well as amino-acids involved in the dimeric interface and responsible for the transmission of the allosteric signal (Barford and Johnson 1989; Sprang et al. 1991; Mathieu et al. 2017a; Rath et al. 2000) (Fig. 4).

In their active state, bGP and mGP dimers may self-associate, leading to the tetramerization of the protein (Barford et al. 1991; Mathieu et al. 2016a, 2017b; Barford and Johnson 1992). This tetramer is inactive because the catalytic site is no longer accessible. Nevertheless, this interaction is very unlikely to occur *in vivo*. Indeed, the binding of glycogen results in the dissociation of the tetramer leading to two active dimers.

3.2 The Structural Basis of mGP and bGP Activation and Inactivation

During activation, GP enzymes undergo structural changes that ultimately lead to the opening of the catalytic site by the gate loop (or 280 loop), which adopts either a close or open conformation, thus regulating the access to the catalytic site (Barford and Johnson 1989; Rath et al. 2000; Mathieu et al. 2017b).

mGP and bGP are believed to share similar structural changes during activation and inactivation. These changes mostly affect the dimeric interface which undergo

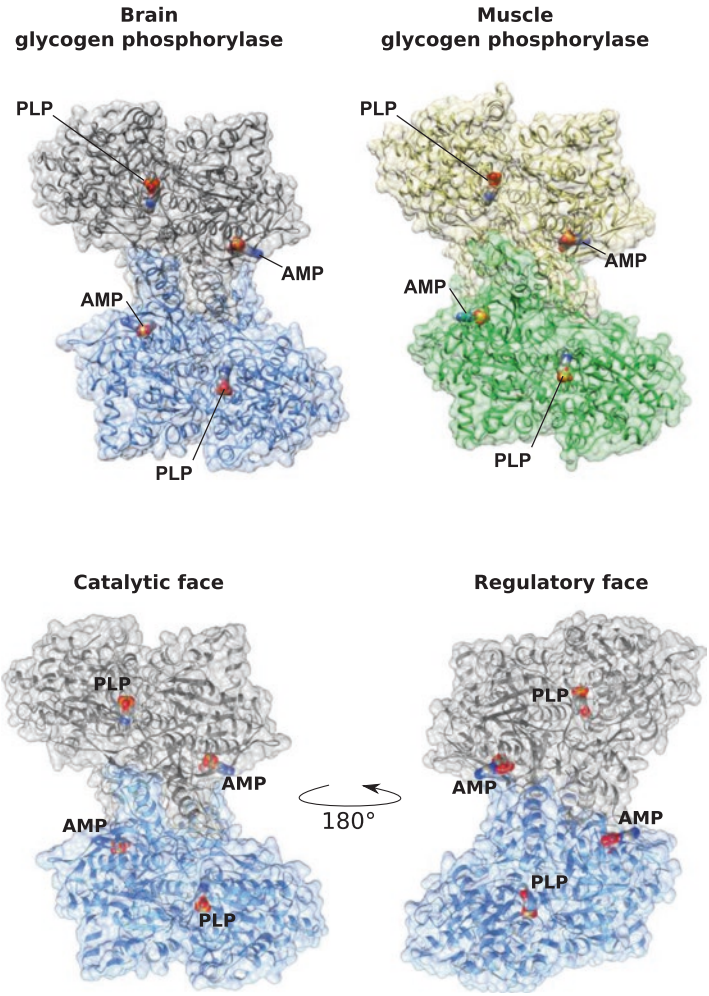

Fig. 4 Overall structures of bGP and mGP. Surface and ribbon representation of the brain (blue and grey) and muscle (green and yellow) GP dimers, showing the strongly conserved overall structures of the two isoforms. The PLP and AMP are shown in their binding site, as surface representation (upper panel). Surface and ribbon representation of the catalytic and regulatory faces of bGP. The catalytic face comprises the catalytic site as well as a glycogen binding site which orientate the substrate to the catalytic site. Regions including the phosphorylation site as well as the AMP-binding site are located on the regulatory face (lower panel)

strong rearrangements, which are particularly evident regarding the relative orientation of the Tower helices. The Tower helices corresponds to the helix 7 of each monomer and are located at the dimer interface, on the catalytic face. They harbor an anti-parallel association and are directly connected to the gate loop of each monomer, thus governing the dimerization of the protein as well as its activation

(Barford and Johnson 1989; Sprang et al. 1991; Mathieu et al. 2016a). In mGP, these two helices undergo a 50° rotation upon activation, resulting in a crossover angle of 75°. The rotation of the Tower helices leads to the destabilization of the gate loop, initially stabilized in a closed conformation, and results in the opening of the catalytic site (Barford and Johnson 1989; Sprang et al. 1991) (Fig. 5). Although the structure of the inactive bGP form is still unknown, the Tower helices of the active bGP have a crossover angle of 85° very similar to mGP and suggest similar activation mechanisms (Mathieu et al. 2016a). Indeed, lGP does not present such rearrangement because of the more rigid body of the dimer. Instead, lGP activation relies on the compaction of the core of the enzyme, comprising notably the Tower helices and the gate loop, but also other secondary structures such as Helix 8, as well as the 250 and 380 loop. The activation of lGP is accompanied with the order to disorder transition of multiple residues in these structures, which results in both a larger dimer interface and the stabilization of the gate loop in open conformation (Rath et al. 2000).

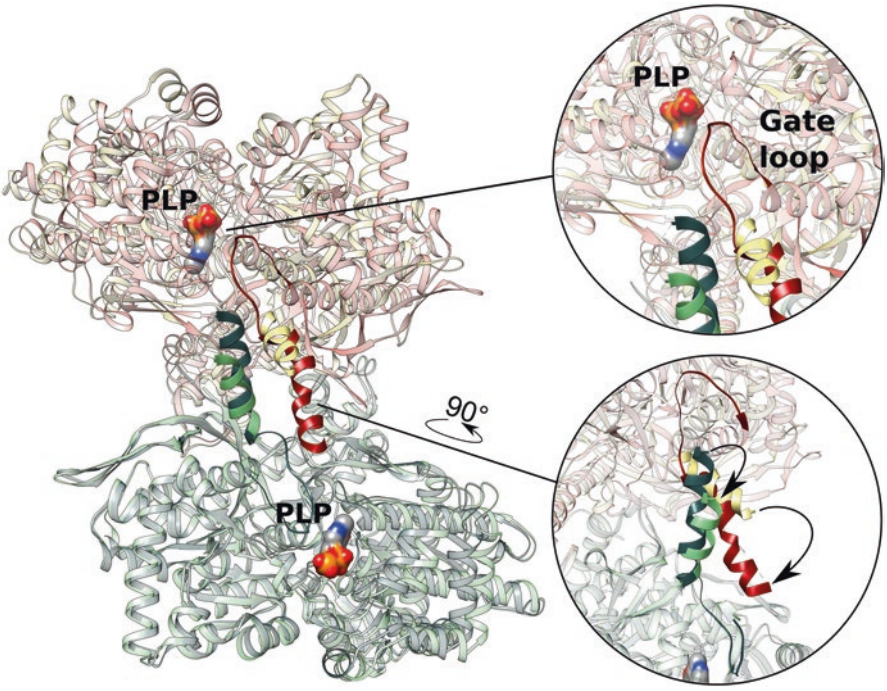

Fig. 5 Structural changes underlying the activation/inactivation of mGP. Overlay of active (yellow and green) and inactive (red and grey) mGP. The rotation of the Tower helices results in the stabilization or the destabilization of the gate loop, promoting respectively the closing and the opening of the catalytic site

3.3 How Structures Give Insight into the Differential Regulation of GP Isozyme

The phosphorylation and AMP binding sites, crucial in GP regulation, are proximal and close to the dimeric interface allowing these effectors to trigger the structural rearrangements responsible for the activation of the enzyme.

3.3.1 The Phosphorylation Peptide

Serine 14 is located on the phosphorylation peptide, a stretch of 21 residues (Young 1957; Berg et al. 2002; Adeva-Andany et al. 2016; Roach 2002, 2015; Sagar et al. 1987; Dalsgaard et al. 2007; Kong et al. 2002; Obel et al. 2012; Öz et al. 2015; Bellesi et al. 2018; Gibbs 2015; Gibbs and Hutchinson 2012; Gibbs et al. 2006, 2007; Wolfsdorf and Weinstein 2003; Hedberg-Oldfors and Oldfors 2015; Duran and Guinovart 2015; Klein 1993; Moses and Parvari 2002; Cavanagh 1999) that is disordered in the inactive state, but becomes stabilized or partially stabilized in mGP upon activation (by either phosphorylation of ser 14 or binding of AMP), through its interaction with the dimeric interface (Barford and Johnson 1989; Sprang et al. 1991). These interactions further stabilize the enzyme in its R-state and remains active (Fig. 6). In bGP, this peptide is not stabilized although the enzyme is in the active state. This may be due to multiple substitution at the dimer interface that may reduce the stabilization of the phosphorylation peptide and could explain, at least in part, the reduced sensitivity of the brain form to phosphorylation (Mathieu et al. 2016a).

3.3.2 The AMP Binding Site

bGP is more sensitive to the activation by AMP and has a higher affinity for this effector, compared to mGP (Crerar et al. 1995). The AMP binding site can be divided into three regions that bind the phosphate, the ribose and the adenine moieties of AMP, respectively (Barford et al. 1991; Barford and Johnson 1989; Mathieu et al. 2016a; Rath et al. 2000). In bGP, the binding of AMP is governed by the phosphate group that establishes most of the contacts with the protein. These contacts include notably a hydrogen bond specific from the brain form of GP, provided by the substitution of Phe196 into a Tyr residue. This brain isozyme-specific substitution allows a direct interaction of AMP with the dimeric interface, which can participate to the high sensitivity and high affinity of bGP for AMP (Mathieu et al. 2016a) (Fig. 6).

The AMP binding site comprises secondary structures of each monomer: the helices 2 and 8, as well as the β4/β5 loop and the adenine loop of one subunit, and the Cap' loop of the other subunit. Each secondary structure performs one or several functions. Thus, the helix 8 connects the AMP binding site to the catalytic site,

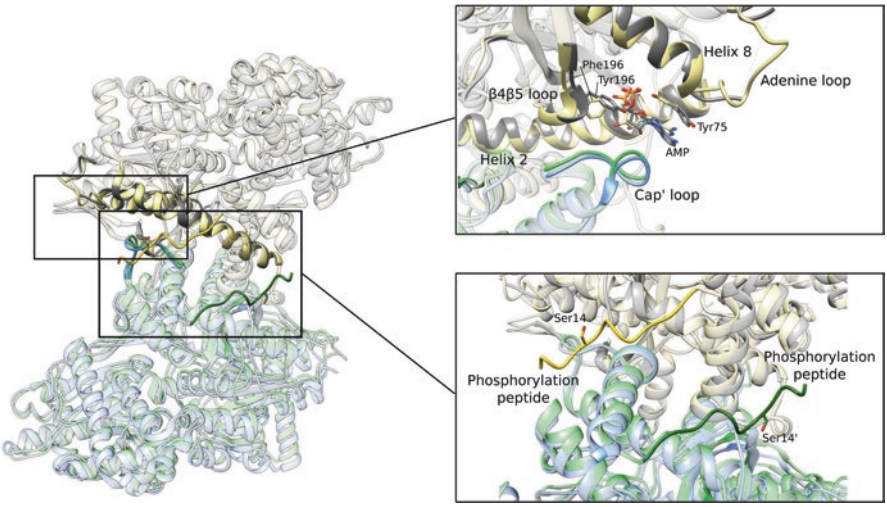

Fig. 6 The AMP-binding site and phosphorylation peptide. The AMP-binding site (upper panel) and the phosphorylation peptides (lower panel) are the two regions of GP involved in the activation of the protein. The AMP-binding site is located at the dimer interface and comprises multiple secondary structures responsible for the binding of the allosteric effector, as well as the transmission of the allosteric signal in the whole dimer. The phosphorylation peptide presents the phosphorylated serine residue. Upon AMP binding, the peptide is stabilized in mGP while it is not in bGP

allowing the transmission of the allosteric signal to the catalytic site. The helix 2 extends across the width of the protein. It ensures the connection between the two AMP binding sites of the dimer and the transmission of the cooperative signal. Moreover, in combination with the adenine loop, the helix 2 allows the transmission of the allosteric signal to the whole dimer. Finally, the concerted action of the helix 2, the Cap' loop and the dimer interface allow the structural rearrangements required for the activation/inactivation process (Barford and Johnson 1989; Sprang et al. 1991; Mathieu et al. 2016a; Rath et al. 2000) (Fig. 6).

In mGP, AMP interacts strongly with the helix 2, particularly through the establishment of a hydrogen bond with the Tyr75, as well as with multiple residues from the adenine loop (Sprang et al. 1991). These interactions are lost in bGP, because of the particular geometry of AMP in its binding site. Indeed, AMP undergoes a 24° rotation of the nucleotide moiety compared to AMP-bound mGP, that results in the different conformation of Tyr75 side chain in the brain enzyme, the loss of hydrogen bonds between AMP and the helix 2, but also precludes the stabilization of the adenine loop along AMP (Mathieu et al. 2016a). The loss of strong interactions between AMP and these secondary structures in bGP might thus explain the non-cooperative binding of AMP to this isoform (Fig. 6).

The adenine loop of bGP is also the central secondary structure responsible for the redox regulation of the enzyme. At the structural level, the formation of a disulfide bridge between cysteines 318 and 326 strongly reduces the mobility of the loop and impairs the binding of AMP, resulting in the insensitivity of the enzyme to its

allosteric activator. However, the high flexibility of the loop allows the enzyme to remain highly flexible. The formation of the intramolecular disulfide bond thus does not interfere with the phosphorylation dependent activation of bGP (Mathieu et al. 2016b).

Interestingly, although AMP-binding site residues are mostly conserved between all three isoforms, many of the interactions with AMP, seen in mGP and bGP, are absent in lGP, primarily because of the more rigid body of this isoform. Moreover, similarly to bGP, the conformation of Tyr75 combined to substitutions in the composition of the adenine loop, preclude its interaction with AMP, probably resulting in the loss of activation of this isoform by AMP and the non-cooperative binding of this effector (Rath et al. 2000; Mathieu et al. 2017b).

4 Two Isoforms for Multiple Functions

It is intriguing to notice that both skeletal muscles and liver mainly express one specific isoform of GP, whereas both muscle and brain isozymes are found in the heart and in the brain (Newgard et al. 1987; Saez et al. 2014; Pfeiffer-Guglielmi et al. 2003, 2014; Schmid et al. 2009). The expression of two isozymes regulated differently in these organs suggests that each isoform fulfills common but also specific functions that can be put in parallel to the functions of glycogen in these tissues. In the brain, glycogen is primarily found in glial cells, especially in astrocytes (Saez et al. 2014; Cataldo and Broadwell 1986; Richter et al. 1996). This almost exclusive localization of glycogen in astrocytic cells combined to its very low amount in brain compared to other tissues has long supported the function of brain glycogen only as an emergency store of glucose. Indeed, early studies revealed that brain glycogen was notably mobilized during ischemia, protecting neurons against irreversible damage due to stress conditions (Sagar et al. 1987; Lowry et al. 1964b). Moreover, other stress conditions including hypoglycemia, physical exercise or hypoxia also results in the mobilization of glycogen store in the brain (Saez et al. 2014; Hossain et al. 2014; López-Ramos et al. 2015; Suh et al. 2007; Wender et al. 2000; Brown et al. 2005; Matsui et al. 2012).

However, more recent studies have put in evidence the presence of an active metabolism of glycogen in neurons, and a critical role of glycogen in normal conditions, including awake-sleep cycles, sensorial stimulation, but also in sustaining high cognitive processes such as learning and memory consolidation (Kong et al. 2002; Obel et al. 2012; Bellesi et al. 2018; Gibbs and Hutchinson 2012; Gibbs et al. 2006; Swanson et al. 1992; Duran et al. 2013) (DiNuzzo et al., this issue). At the cellular level, glycogen is involved in all the steps underlying neurotransmission, being for instance an energy store for neurons, notably through the astrocyte-neuron lactate shuttle, where astrocytes provide energy to neurons in the form of lactate, derived from astrocytic glycogen. In parallel, astrocytic glycogen also provides the energy required for the uptake of glutamate, used as neurotransmitter (Pellerin and Magistretti 2012; Dringen et al. 1993; Suzuki et al. 2011; Newman et al. 2011) (Fig. 7). In addition, astrocytic

glycogen also participates in the maintenance of ions homeostasis, including potassium uptake, and provides the metabolites involved in the synthesis of two neurotransmitters *i.e.* glutamate and GABA (Gibbs et al. 2007; Xu et al. 2013; DiNuzzo et al. 2013; Müller et al. 2014; Sickmann et al. 2012).

However, the function of glycogen in neurons remains poorly understood. Indeed, several publications initially mentioned a limited glycolytic activity in neurons, and the primary use of astrocyte-derived lactate as a source of energy, indicating that glycogen might not primarily serve as a source of energy in these cells (Pellerin and Magistretti 2012; Bouzier-Sore et al. 2003; Bélanger et al. 2011). Yet, recent reports have shown evidence of a glycolytic activity in neurons during stimulation, raising the possibility that glycogen serves also as an energy store in neuronal cells (Díaz-García et al. 2017; Dienel 2017). In addition, neuronal glycogen appears to protect neurons, providing rapidly glucose-6-phosphate and feeding the pentose-phosphate pathway, responsible for the regeneration of reducing power and the detoxification of ROS (Saez et al. 2014; López-Ramos et al. 2015).

These observations clearly highlight the differential roles of astrocytic *versus* neuronal glycogen. Interestingly, astrocytes and neurons do not have the same expression pattern for GP. Astrocytes express both the muscle and the brain forms whereas neurons only express bGP (Saez et al. 2014; Pfeiffer-Guglielmi et al. 2003, 2014). Because of its main regulation by allosteric effectors, bGP appears to predominantly have a local function, allowing the use of glycogen store for the needs of the cell itself, and is consistent with the protective role of neuronal glycogen. On the contrary, mGP, which is also regulated by phosphorylation, also allows the mobilization of glycogen stores in response to neurotransmitters and brain activity

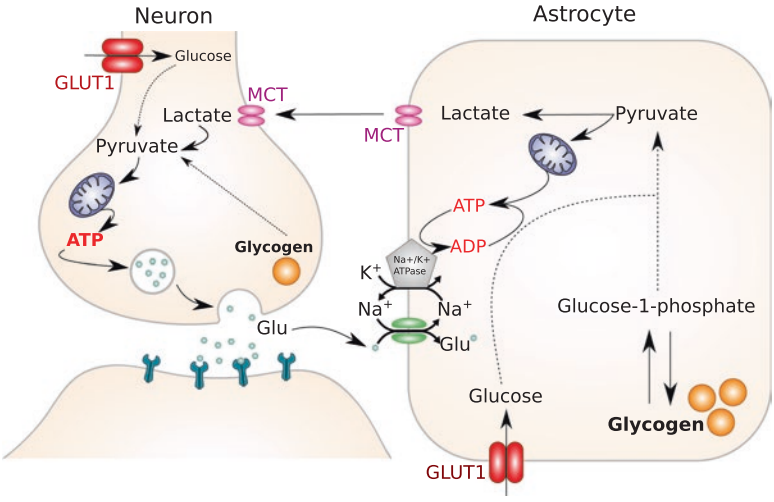

Fig. 7 The role of glycogen in the Astrocyte-Neuron Lactate Shuttle (ANSL). Glycogen stores in astrocytes are metabolized into lactate, which is transferred to neurons and used by neurons as an energy source

(Crerar et al. 1995; Müller et al. 2015; Nadeau et al. 2018). These specificities emphasize the multiple functions of the astrocytic pool of glycogen, being an energy supply for both astrocytes and neurons, but also responding to cerebral activity through the synthesis and the uptake of neurotransmitters as well as the maintenance of ion homeostasis.

5 Concluding Remarks

GPs constitute an extensively studied family of enzymes that play a key role in glycogen metabolism. Because of their differential regulations and particular structural features, both mGP and bGP appear to perform common and but also specific functions in brain glycogen metabolism (Crerar et al. 1995; Müller et al. 2015; Nadeau et al. 2018). Nevertheless, although the allosteric regulation of GPs by allosteric effectors and phosphorylation have been known for decades, the discovery of an additional regulation of bGP by ROS has complexified the understanding of glycogen metabolism in the brain. Indeed, the isoform-specific regulation of bGP by ROS allows the cells to specifically mobilize glycogen in response to extracellular signals without being affected by the cellular energy state (Mathieu et al. 2016b; Nadeau et al. 2018). The exact functions of the redox-regulation of bGP in cells, in normal and pathological conditions, still remain to be elucidated. Previous studies have already pointed an effect of oxidative stress on brain glycogen metabolism, although the exact effects of such stress remains under debate, leading to the activation of glycogen mobilization on one hand, and the accumulation of glycogen on the other hand (Saez et al. 2014; Hossain et al. 2014; Rahman et al. 2000).

Several recent studies shed a new light on glycogen functions in the brain, under both normal and pathological conditions. Cerebral glycogen is crucial for brain functions and cognitive processes such as learning and long-term memory consolidation (Gibbs 2015; Gibbs et al. 2006; Suzuki et al. 2011; Newman et al. 2011). On the other hand, the accumulation of glycogen is particularly deleterious for neurons (Duran et al. 2012, 2014). Glycogen accumulation is observed in a large number of brain diseases such as Alzheimer's disease, Lafora's disease and amyotrophic lateral sclerosis (ALS), as well as in aging (Saez et al. 2014; Sickmann et al. 2012; Sato and Morishita 2015; Dodge et al. 2013; Gertz et al. 1985). This accumulation is suspected of contributing in the loss of cognitive functions associated with these pathologies and the death of neurons. Glycogen metabolism thus appears to be a potential target for the development of therapeutic approaches (Cloix and Hévor 2011). Because of their allosteric regulation, low turnover as well as their key role in glycogen mobilization, GPs are primary targets for the pharmacological control of glycogenolysis (Gaboriaud-Kolar and Skaltsounis 2013). Currently, many studies aim to develop GP effectors in the treatment of diabetes and cancer. To date, most studies have led to the development of GP inhibitors (Gaboriaud-Kolar and Skaltsounis 2013; Zois and Harris 2016). However, restoring the activity of GPs

also offers promises for the treatment of GSD, but also in other pathologies associated with glycogen accumulation.

Determining the structures of mGP and lGP has been crucial in this process as they allowed the identification of binding sites for physiological and pharmacological ligands. The determination of the bGP structure now offers a global and comprehensive view of the structure and regulation of the different isoforms, and provides a molecular basis for the development of isoform-specific therapeutic molecules, thus limiting the unwanted side effects.

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Regional Distribution of Glycogen in the Mouse Brain Visualized by Immunohistochemistry

Yuki Oe, Sonam Akther, and Hajime Hirase

Abstract Considering that the brain constantly consumes a substantial amount of energy, the nature of its energy reserve is an important issue. Although the brain is rich in lipid content encompassing membranes, myelin sheath, and astrocytic lipid droplets, it is devoid of adipose tissue which serves as an energy reserve. Notably, glycogen represents the major energy store in the brain. While glycogen has been observed mainly in astrocytes for decades by electron microscopy, glycogen distribution in the brain has only been partially documented. The involvement of glycogen metabolism in memory consolidation, demonstrated by several research groups, has reiterated the functional significance of this macromolecule and the need for description of its comprehensive distribution in the brain. The combination of focused microwave-assisted brain fixation and glycogen immunohistochemistry permits assessment of glycogen distribution in the rodent brain. In this article, we describe glycogen distribution in the mouse brain using glycogen immunohistochemistry. We find heterogeneous glycogen storage patterns at multiple spatial scales. The heterogeneous glycogen distribution patterns may underlie local energy metabolism or synaptic activity, and its mechanistic understanding should extend our knowledge on brain metabolism in health and disease.

Keywords Glycogen immunohistochemistry · Forebrain · Olfactory bulb · Hippocampus · Cortex · Striatum

Y. Oe (✉)
RIKEN Center for Brain Science, Wako, Saitama, Japan
e-mail: yuki.oe@riken.jp

S. Akther · H. Hirase (✉)
RIKEN Center for Brain Science, Wako, Saitama, Japan

Saitama University Brain Science Institute, Saitama, Japan
e-mail: hajime.hirase@riken.jp

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1 Introduction

The energy consumption of the brain is exceptionally high compared to other organs (Raichle and Gusnard 2002). The fundamental energy substrate—adenosine triphosphate (ATP)—is primarily derived from glucose that is supplied by blood circulation in the brain. According to the astrocyte-to-neuron lactate shuttle hypothesis, glucose from blood circulation is transported to astrocytes and broken down into lactate which, in turn, is shuttled to neighboring neuronal compartments. Inside neurons, lactate is converted into pyruvate and further metabolized by the tricarboxylic acid (TCA) cycle to produce ATP molecules. As in other peripheral organs such as the liver or muscles, a portion of glucose is stored primarily inside astrocytes in the form of glycogen. While described in detail in other chapters of the present volume, brain glycogen metabolism supports: (1) on-demand supply of increased energy and (2) promotion of long-term synaptic plasticity. The degrees to which these functionalities are recruited have not been explicitly described and they likely depend on brain region and state. However, the examination of glycogen distribution will enable us to assess the capacity of astrocytes to execute such functions in distinct brain regions.

There are several ways to determine brain glycogen distribution. First, brain regions of interest can be dissected and biochemical assays can be performed on them. Quantitative biochemical glycogen assays include phenol sulfuric acid and OxiRed fluorescent probe methods which react with the amount of (oxidized) glucose generated by enzymatic breakdown of glycogen. The signal strength reflects the amount of glycosyl units that constitute glycogen molecules in the sample. Although these biochemical methods are quantitative, they require a considerable amount of sample and hence are not suitable for assessing the spatial distribution of glycogen finer than broad brain regions. For instance, the BioVision glycogen assay kit K646–100 recommends 10 mg of tissue. Considering the low glycogen content in the brain (an order of magnitude smaller than the liver or skeletal muscle content of a few 100 μM/g wet tissue), a larger sample amount warrants accurate measurements. Since a mouse brain weighs only about half a gram, dissection and biochemical assay should be performed with great care. In the past, glycogen quantification has been performed with the rat brain which typically weighs over 2 g.

Second, glycogen granules are observed as electron-dense puncta in lead- or osmium-intensified sections by electron microscopy. Therefore, electron microscopy provides a direct assessment of subcellular distribution of glycogen. However, electron microscopy is limited by the size of observable area, which is typically in the range of several micrometers in horizontal dimensions of a thickness of tens of nanometers. Even with the development of serial block-face scanning electron microscopy or automated serial microscopy with ultrathin sections mounted on tapes, the volume is practically limited to a single cortical column. The small volume of observation makes approaches with electron microscopy unrealistic to assess brain-wide glycogen distribution.

Third, nuclear magnetic resonance spectroscopy (MRS) is a method that detects unique resonance frequencies glycogen molecules in a magnetic field. The advantage of MRS is that this method is quantitative and can be readily performed on live subjects. For example, liver and skeletal muscle glycogen levels have been quantified by MRS in human subjects (Cline et al. 1999; Magnusson et al. 1992; Öz et al. 2003). However, MRS is limited by its spatial resolution. Since the brain stores an order of magnitude lower amount of glycogen than the liver or skeletal muscles, a typical voxel used to assess glycogen content is larger than a centimeter in each dimension and requires the summation of scans for extended periods of time to obtain reliable signals (Öz et al. 2007). While this method can possibly address glycogen distribution of a human brain with the coarse resolution, it is not suitable for characterizing the glycogen distribution of individual brain regions or brains of smaller animals.

Fourth, compared with the above methods, histological examination by light microscopy is a suitable approach to characterize the regional, cellular, and subcellular distribution of glycogen. A critical point to consider for the histological approach is how specific glycogen is labeled in brain sections. Glycogen has traditionally been stained by the periodic acid-Schiff (PAS) histochemical staining procedure, particularly for clinical research purposes. PAS staining is used to indicate glycogen in liver samples where hepatocytes store high amounts of glycogen. However, PAS staining also labels glycoproteins and proteoglycans which are abundant in the brain. Therefore, PAS staining is not ideal for the evaluation of brain glycogen distribution in terms of specificity. Immunohistochemistry is a method which achieves relatively high specificity, given a specific primary antibody. Additionally, immunohistochemistry permits labeling of secondary or tertiary molecules which are useful in determining cell types that store glycogen. In this chapter, we describe brain glycogen distribution using two monoclonal antibodies against glycogen.

After describing the procedure for glycogen immunohistochemistry, we describe brain glycogen distribution at three levels: (1) macroscopic distribution by distinct brain region, (2) microscopic (subcellular) distribution within astrocytes, the principal glycogen storage cell type, and (3) mesoscopic distribution in brain areas of high glycogen content.

2 Glycogen Immunohistochemistry

Glycogen is a storage of glucose and its size varies dynamically according to usage or storage of glucosyl units. For instance, when the tissue oxygen level is low, cell energy metabolism shifts to anaerobic metabolism whereby glycogen is broken down into glucose-1-phosphate molecules. Likewise, when excess glucose is available, glycogenesis occurs in cells specialized to store glycogen including hepatocytes, myocytes, and in the central nervous system, astrocytes. Brain glycogen has also been reported to be consumed during prolonged aerobic exercise (Matsui et al. 2011).

Brain immunohistochemistry is typically performed on perfusion-fixed tissue. Perfusion fixation is generally performed in two steps: transcardial perfusion with physiological solution (e.g., saline, phosphate buffered saline, Ringer's solution, etc.), followed by transcardial perfusion of fixative (e.g., 4% paraformaldehyde in 0.1 M phosphate buffer). While the purpose of the first step is to remove blood from the brain, this step typically lasts for a few minutes or longer, which inevitably puts the brain under an anoxic state. As a result, glycogen is vastly degraded and little glycogen remains in the perfusion-fixed brain. A possible way to prepare a fixed brain sample without perfusion is to fix an acutely prepared alive brain slice. Acute brain slice preparation is a common procedure practiced in *in vitro* electrophysiology laboratories and slices are usually recovered in oxygenated artificial cerebral spinal fluid for an hour or so before experiments. Indeed, it has been shown that glycogen in hippocampal acute slices is emptied immediately after slice preparation already (Fiala et al. 2003; Takano et al. 2014). Brain dissection and slice cutting surely induce trauma and resultant extracellular potassium elevation. High extracellular potassium has previously demonstrated to trigger glycogenolysis via activation of soluble adenylyl cyclase (Choi et al. 2012).

Because glycogen is very sensitive to brain insult, a method that instantly preserves the *in vivo* state of metabolism is essential for proper histological visualization of glycogen. Rapid freezing of the brain in liquid nitrogen is a common procedure for biochemistry and in situ hybridization. Focused microwave irradiation is another procedure practiced in biochemical investigation of brain metabolism or protein phosphorylation. The former approach aims to preserve the metabolic state of the brain by halting enzymatic activity by lowering the temperature, whereas the latter approach denatures and inactivates metabolic enzymes by instantaneously heating the sample to 80–90 °C.

Previous biochemical examinations of brain glycogen showed that microwaved preparations preserve significantly higher amounts of glycogen (> ~twice) than rapid freezing (Sagar et al. 1987). More recently, Sugiura et al. (2014) compared the abundance of metabolic substrates in hippocampal samples prepared by post-euthanasia whole-brain freezing, in situ freezing, or focused microwave irradiation. Accordingly, they found that focused microwave irradiated samples contained higher amounts of ATP, adenosine diphosphate (ADP) and pyruvate compared with the other methods, which had more degraded purine and lactate levels. This result shows that focused microwave irradiation preserves the aerobic respiratory state of the brain (which is the default *in vivo* respiration mode) and is adequate for glycogen immunohistochemistry.

While brain samples prepared by focused microwave irradiation have been used for biochemical evaluation of glycogen content (Sagar et al. 1987) and PAS staining (Kong et al. 2002), glycogen immunohistochemistry has not been performed until recently. Here we summarize the glycogen immunohistochemistry procedures used in our laboratory. Currently, two mouse monoclonal IgM antibodies have been verified to be suitable for glycogen immunohistochemistry (Fig. 1). The ESG1A9 antibody (ESG) is raised against enzymatically synthetized glycogen (hence the name ESG), and it tends to have higher affinity to large glycogen molecules (Nakamura-Tsuruta et al. 2012). The IV58B6 antibody (IV) has affinity for glycogen

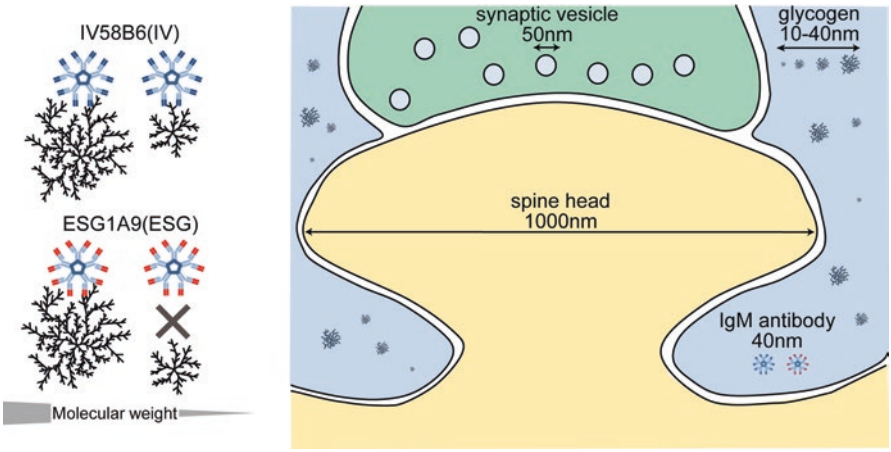

Fig. 1 Consideration of spatial scales in glycogen immunohistochemistry. Left panel: The IV58B6 antibody is considered to have an affinity for glycogen of all sizes, whereas the ESG1A9 antibody has an affinity for large glycogen macromolecules. Right panel: Sketch of a synapse and perisynaptic astrocytic processes incorporating realistic spatial scales

of all sizes (Baba 1993). The exact manner in which these two antibodies express the differential affinity patterns has not been identified. It is supposed that enzymatically synthesized glycogen has a different branching pattern from that of natural glycogen, and the epitopes are associated with the branching pattern.

Adult mice are euthanized by focused microwave irradiation using a MMW-05 microwave fixation system (Muromachi Kikai, Tokyo, Japan). Considering circadian modulation of astrocytic and metabolic functions, it is a reasonable idea to sacrifice mice in a defined time of the day. For instance, all mice studied in our recent study were sacrificed between 6:00 and 8:00 p.m., i.e., within 2 h before the end of diurnal period (Oe et al. 2016). Briefly, a mouse was confined in a specialized tubular animal holder with a hollow wall filled with water (WJM-24 or WJM-28, Muromachi Kikai). The holder with the animal is then placed inside MMW-05. High-energy microwave (5 kW) was focused at the head of the mouse for 0.94–1.05 s. Immediately after focused microwave euthanasia, the brain was removed from the skull and immersed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer) for overnight at 4 °C.

Brain sections of 60 μm thickness were prepared in PB using a microslicer (Pro-7 Linear Slicer, DSK, Japan). After washing in phosphate buffered saline (PBS), the sections were incubated in PBS containing 0.1% Triton X-100 and primary antibodies for 24 hours at 4 °C while gently shaking. The concentrations of ESG and IV antibodies are 1:50 (15 μg/ml) and 1:300 (30 μg/ml), respectively. As mentioned previously, both ESG and IV antibodies are IgM which are several times larger than IgG in molecular size. Therefore, penetration of the primary antibodies should be performed extensively. The authors are not aware of an IgG antibody for glycogen that can be used for immunohistochemistry at this time. The sections were then

washed three to five times in Tris-buffered saline and incubated with fluorescent secondary antibodies (1:1000 in PBS containing 0.1% Triton X-100, Alexa Fluor 488 or 594, Life Technologies). Brain slices were mounted on slide glasses and coverslipped with PermaFluor mounting medium (Thermo Scientific).

3 Glycogen Distribution in Major Brain Regions

Based on the immunohistochemistry protocol described in the previous section, glycogen distribution was visualized in coronal and sagittal sections of the mouse brain. These preparations allow the evaluation of glycogen distribution in macroscopic scales. As shown in examples in Fig. 2, the IV (pan-glycogen) antibody labels the brain fairly extensively with regional heterogeneity. For example, the hippocampus generally has high-intensity signals while the principal cell body layers of the hippocampus are low in signal intensities. Moreover, the white matter has been found to be generally low in signal intensity. Accordingly, the corpus callosum, hippocampal fimbria-fornix, medial forebrain bundle, and arbor vitae of the cerebellum are distinguished as low-intensity structures. Moreover, stripe patterns

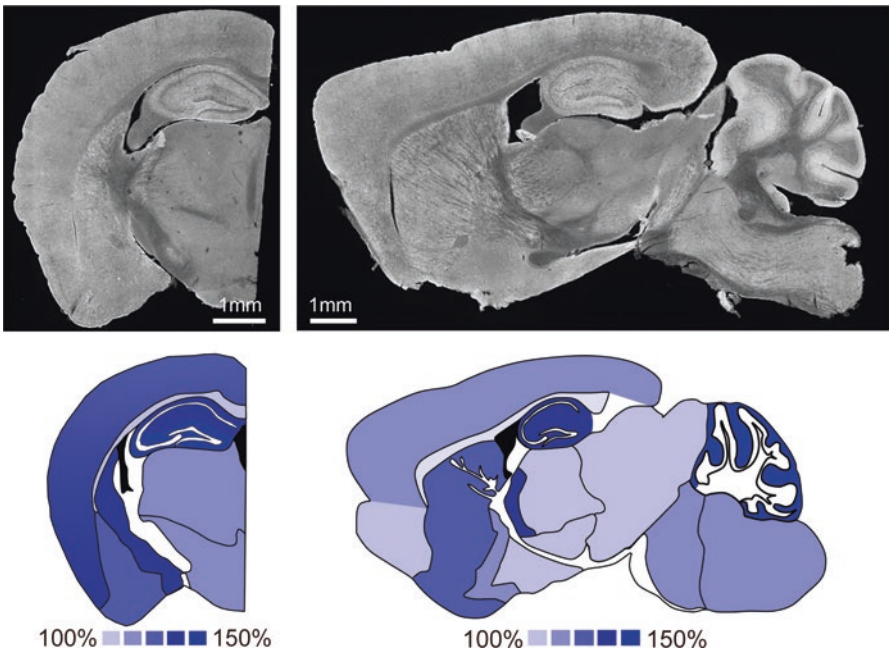

Fig. 2 Glycogen immunohistochemistry on microwave-fixed mouse brain at microscopic scale using IV58B6. Upper panel: Examples of glycogen immunohistochemistry of coronal (left) and sagittal (right) sections. Lower panel: Relative glycogen immunohistochemical signal averaged over 9 mice. Reproduced from Oe et al. (2016)

of axon bundles labeled in low intensity are apparent in sagittal sections of the striatum. Amongst major gray matter structures, the cerebellar cortex, hippocampus, thalamic reticular nucleus, striatum, globus pallidus, substantia nigra (pars reticulata), and pons are labelled well with IV. On the other hand, subcortical structures including the dorsal thalamus and hypothalamus generally were found to be low in IV signals.

The ESG antibody labels the brain rather modestly and signals are barely discernible from intrinsic fluorescence in many brain areas where green-fluorescent secondary antibody is used for visualization. However, where signals were found high with the IV antibody, ESG signals are also detected with high intensity. For instance, ESG signals are easily detected in the hippocampus, striatum and enriched in some parts of allocortex (e.g., entorhinal, perirhinal, and olfactory cortices). In the neocortex and cerebellar cortex, sparse labeling patterns on superficial layers are observed. We note that substantia nigra pars reticulata and ventral tegmental area, the two main dopaminergic brain areas, are labeled in high intensities.

It is imperative to verify the signal strength of microwave fixation-assisted glycogen immunohistochemistry with the actual glycogen content. To this end, we have measured the glycogen contents in the microwave-treated cortex, striatum, hippocampus and cerebellum using a biochemical assay kit (K646–100, BioVision). As a result, we found a reasonable correspondence between glycogen content and relative immunohistochemical signal, except for the cerebellum (Oe et al. 2016). The cerebellar glycogen content was measured to be lower than relative immunohistochemical signals, most likely because the biochemical cerebellar samples included a large proportion of the white matter (arbor vitae) compared with other forebrain structures. Multiple studies have addressed glycogen content by brain region using biochemical assays on microwave fixed samples. We have rearranged published results from rats (Kong et al. 2002; Matsui et al. 2011; Sagar et al. 1987) and mice (Oe et al. 2016) in Fig. 3. These results agree well and the mouse glycogen immunohistochemistry data show that the hippocampus, cerebellum, and the cortex store relatively high levels of glycogen. Interestingly, striatal glycogen is found to be relatively low in both studies, which differs from our mouse study. It is possible that species differences exist in the storage and usage of glycogen. Additionally, regional glycogen storage may depend on metabolic states of the brain, including sleep cycle and circadian rhythm.

4 Astrocytes Store Glycogen in the Forebrain

The forebrain is the most recently developed division of the vertebrate brain and is responsible for higher order functions, such as perception, memory, and most likely, consciousness. The forebrain includes cortical structures and the basal forebrain, basal ganglia, thalamus, and hypothalamus. While there are regional variabilities in glycogen storage within the forebrain, glycogen signals are predominantly found in astrocytes in all areas of the forebrain. As such, IV glycogen immunohistochemistry results in astrocytic patterns. Neurons and microglia are predominantly devoid of IV

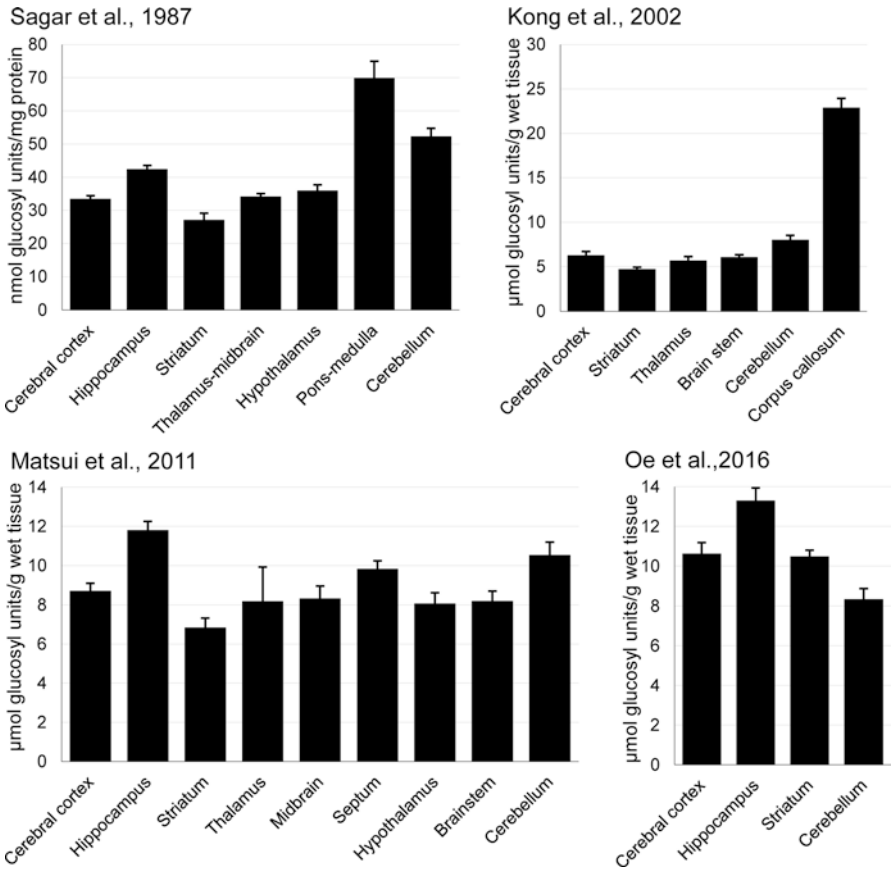

Fig. 3 Biochemical assessment of glycogen content by major brain region. Four published studies are presented. These measurements are made using microwave-fixed brains. Measurements are from rats for Sagar et al. (1987), Kong et al. (2002), and Matsui et al. (2011) while measurements by Oe et al. are from mice. Bar graph are replotted from respective original publications with bar orders modified for comparison purposes

signals. Astrocytic IV signals are characterized by high-intensity and dense punctate patterns (Fig. 4). Less intense and relatively smooth signals are observable in astrocytic cytosol in between IV puncta. By contrast, ESG signals are exclusively punctate and there is hardly any discernible signals outside puncta (Oe et al. 2016). Whether the punctate patterns labeled by IV and ESG overlap has not been addressed; however, these punctate patterns are emphasized in astrocytic microprocesses (Fig. 4a) and endfeet (Fig. 4b), rather than somata or primary branches. The distinct punctate distribution pattern supports the supposed role of glycogen as intermediate energy storage and the source of lactate for long-term synaptic plasticity. Moreover, enriched accumulation of glycogen in perisynaptic microprocesses and endfeet has been confirmed by a recent study using serial block-face scanning electron microscopy (Calì et al. 2016).

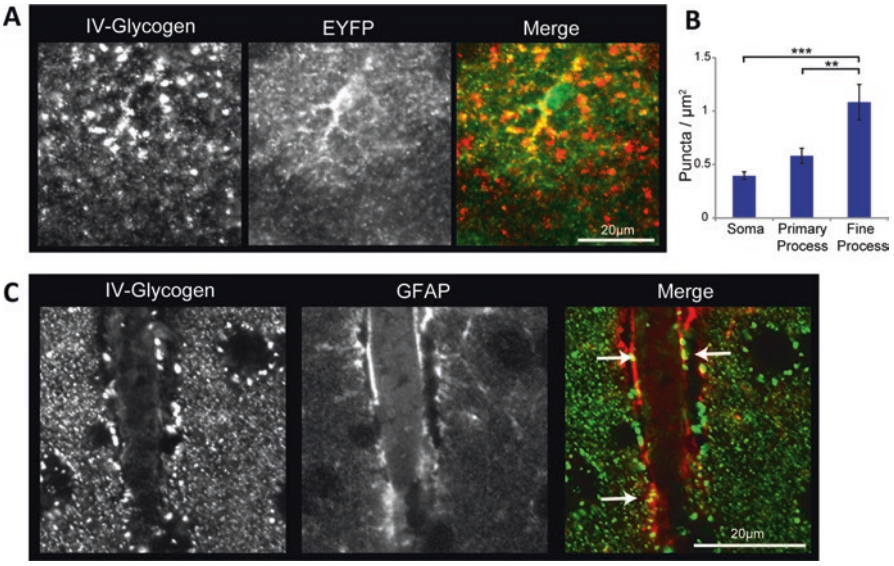

Fig. 4 Glycogen distribution in the cerebral cortex gray matter of the mouse. (**a**) Double immunohistochemistry showing IV glycogen antibody-labeling and YFP-labeled single astrocyte morphology. IV glycogen immunohistochemistry shows that glycogen is preferentially localized in fine processes of astrocytes. (**b**) Quantification of immunolabeled puncta shows preferential localization of glycogen in fine process of astrocytes. (**c**) Perivascular glycogen distribution. Large and intense glycogen puncta are observable along the vasculature. GFAP immunohistochemistry labels astrocytic endfeet which delineate the vasculature. Arrows indicate large and intense glycogen puncta that overlap with GFAP signal. Reproduced from Oe et al. (2016). GFAP: glial fibrillary acidic protein

Glycogen macromolecules are known to be organized in multiple levels of complexity; beta particles which are essentially single-core glycogen molecules, and alpha particles which are an aggregation of multiple glycogen molecules. Each beta particle has a dimer of glycogenin as the core where glycogen chains are originated. Beta particles have a maximum diameter of ~50 nm when glucose is fully stored, whereas alpha particles can be as large as a few hundred nanometers and observable in the liver (for concise reviews, Obel et al. 2012; Prats et al. 2018). Interestingly, a recent report showed that glycogenin is not a requirement of glycogen particle formation and paradoxically, glycogenin deficiency results in higher glycogen accumulation with larger particles (Testoni et al. 2017). It remains unknown if all brain glycogen particles are strictly associated with glycogenin.

An important point to consider is that both ESG and IV antibodies are IgM type and hence have a diameter of 40 nm, a size similar to a medium-size beta glycogen particle (Fig. 2). Puncta signals obtained by glycogen immunohistochemistry has aggregated appearances hinting at the existence of beta particle aggregates. Indeed classical papers by Phelps and Koizumi exemplify astrocytic glycogen particle aggregates in their electronmicrographs (Koizumi 1974; Phelps 1972). Whether such aggregates qualify to be

alpha particles is an open question. It is further tempting to speculate that the epitope for the ESG antibody is somewhat associated with a conformation of beta particle aggregates. Further structural studies are required to reveal the true nature of antibody affinity.

Classical electron microscopy studies have additionally identified electron-dense glycogen granules in pericytes, which are scaffold cells of cerebral capillaries and venules (Cataldo and Broadwell 1986). IV glycogen immunohistochemistry shows strong condensation of particles around blood vessels (Fig. 4b). A majority of perivascular glycogen signals are co-localized with GFAP-positive processes, indicating astrocytic endfeet. Co-registration of electron microscopy and ^{13}C-labeled glucose by ultra-high spatial resolution ion microprobe imaging (NanoSIMS) showed incorporation of glucose to glycogen in endfeet and other parts of astrocytes (Takado et al. 2015), though the amount of de novo glucosyl unit incorporation is 25 times smaller than that of hepatocytes in fasted mice. Additionally, there are scattered perivascular signals, which may be of pericyte origin. Pericytic glycogen distribution has not been characterized in detail, possibly because there is not an antibody that uniquely labels the morphology of pericytes. Fortunately, the proteoglycan NG2 promoter-driven transgenic mouse expresses DsRed in both oligodendrocyte precursor cells (so-called NG2-positive glia) and pericytes (Zhu et al. 2007). Future studies should be able to address pericytic glycogen distribution with this mouse strain.

5 Glycogen Distribution in Distinct Mouse Brain Regions

5.1 Cerebral Cortex

As stated previously, the total amount of cerebral cortical glycogen is relatively high compared with diencephalic structures such as the thalamus and hypothalamus. Moreover, large glycogen puncta are observable by ESG glycogen immunohistochemistry. Generally, the temporal lobe accumulates higher amounts of glycogen than other lobes. Within the temporal lobe, the piriform cortex stands out in glycogen content. Outside the temporal lobe, glycogen levels are modestly rich and regional variability across different areas of the cortex is modest.

A common characteristic of cortical glycogen distribution is layer dependency. Generally, superficial layers accumulate more glycogen than deep layers. In particular, layer 1 shows the most intense glycogen immunohistochemistry signal with large glycogen puncta which are also labeled by the ESG antibody (Fig. 5a). This may be related to the fact that synapse density is the highest in layer 1 and decreases with depth in the barrel cortex of developed mice (Chandrasekaran et al. 2015), in particular the synapse density of asymmetrical (excitatory) synapses (DeFelipe et al. 2002). Similarly, glycogen signal decreases with cortical depth and is the lowest in layer 6 (Fig. 5b), where the lowest synapse density within the cortical

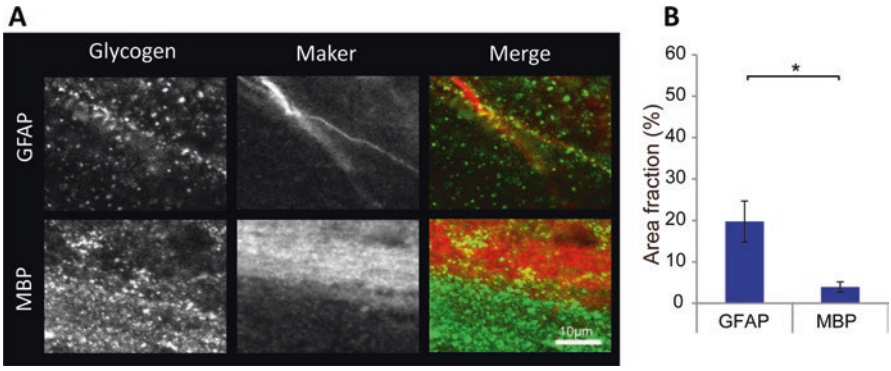

Fig. 5 Glycogen distribution in the cerebral cortex white matter. (**a**) In the upper panel, the corpus callosum is immunolabeled for glycogen (IV) and GFAP. Glycogen puncta are observed along the GFAP positive structure, suggesting astrocytic localization of glycogen. The lower panel represents the corpus callosum/alveus and hippocampal gray matter border. Strong myelin basic protein (MBP, oligodendrocyte marker) signals are seen in the cortical white matter, where glycogen signals are generally low. (**b**) Quantification of glycogen puncta by confocal microscopy indicates a preferential localization in astrocytes to oligodendrocytes (reproduced from Oe et al. (2016))

parenchyma has been reported (Chandrasekaran et al. 2015; DeFelipe et al. 2002; Schuz and Palm 1989). The positive correlation between glycogen storage and synapse density supports the idea that neuronal synapses are the consumers of glycogen metabolites (e.g., lactate). It is imperative to note however, that the synaptic organization of the mouse neocortex is considerably different from that of the rat or the human (Anton-Sanchez et al. 2014; DeFelipe et al. 2002; Palomero-Gallagher and Zilles 2017). Likewise, the periallocortex (e.g. entorhinal cortex) may have a different profile due to its distinct synaptic organization.

A natural question that arises is if the layer-dependent glycogen storage reflects the metabolic rate of a given layer. In an attempt to seek the relationship between constitutive metabolic activity and cortical layer, aerobic metabolism capability was visualized by cytochrome-c oxidase staining. Cytochrome-c oxidase signals are classically known to be obtained where energy demand is supposedly high, such as in visual areas projected from the intact eye after monocular deprivation (Wong-Riley 1979) and in layer 4 barrels of the whisker somatosensory cortex (Wong-Riley and Welt 1980). Comparison of cytochrome-c oxidase and glycogen signals in consecutive cortex sections, however, did not appear to be closely related. Rather, glycogen signals appeared relatively independent of cytochrome-c oxidase signals (Oe et al. 2016). For instance, the barrel structures were hardly discernible from IV glycogen immunohistochemistry. Therefore, it is reasonable to postulate that *constitutive* metabolic activity in the cortex might not rely heavily on glycogenolysis and is supported by glucose supply that comes from the dense network of blood circulation. Nevertheless, it is noted that *evoked* intense neuronal activity by sensory stimulation leads to local glycogenolysis in the corresponding somatosensory area, as shown by ^{14}C(3,4)glucose autoradiography (Swanson et al. 1992). One model that has gained experimental support is that glycogenolysis, and the resultant metabolite shuttle to

nearby synapses, is a key mediator of synaptic plasticity and learning (Gibbs et al. 2006; Newman et al. 2011; Suzuki et al. 2011; Wang et al. 2017). According to this view, glycogen is consumed in an on-demand manner and can be regarded as a reserve metabolite storage rather than constant supply of energy substrate.

Relatively large immunolabeled puncta (>0.5 μm diameter) are observable with the ESG antibody, especially in superficial layers. Large ESG puncta are distributed in a pattern with relatively busy clusters and sparse areas. This patchy trend is more obvious in the hippocampus and striatum as described in the next sections. Meanwhile, it is noteworthy that superficial layers, especially layer 1, show patchy (though less pronounced) patterns. Of note, experience-dependent synaptic plasticity has been observed in the adult brain in layer 1 (Trachtenberg et al. 2002).

The cortical white matter—the corpus callosum—shows glycogen immunoreactivity, albeit at lower levels than the gray matter. We present both IV and ESG immunoreactivity in the corpus callosum and hippocampal alveus in Fig. 6. As analyzed

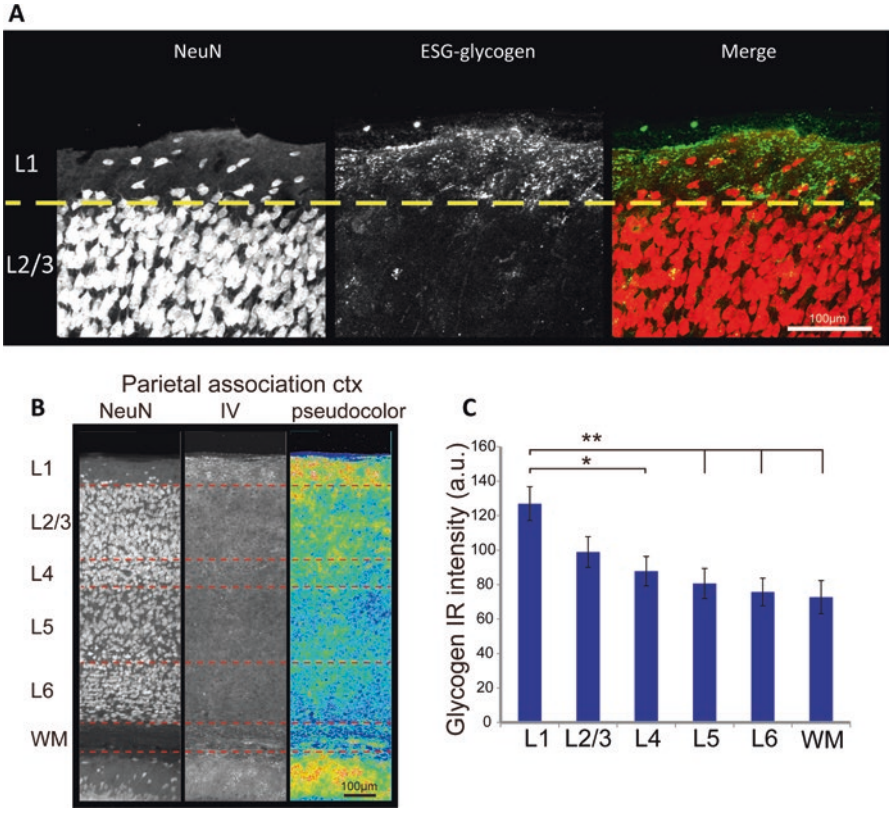

Fig. 6 Layer-dependent glycogen distribution in the mouse cortex. (**a**) ESG glycogen immunohistochemistry shows an enriched condensation of glycogen in layer 1. (**b** and **c**) IV glycogen immunohistochemistry shows a layer-dependent glycogen distribution pattern where glycogen signals are higher in superficial layers (reproduced from Oe et al. (2016))

by Oe et al. (2016), the majority of glycogen signals are detected in astrocytes. Oligodendrocytic localization of glycogen particles has been evaluated in myelin basic protein-positive structures, showing an order of magnitude smaller signal strengths, which may be signal leak from white matter astrocytes due to the resolution limit of confocal microscopy. Indeed, published electron microscopic observations have reported lack of glycogen in cortical and callosal oligodendrocytes (Caley and Maxwell 1968; Mori and Leblond 1969) while callosal astrocytes store abundant amounts of glycogen (Mori and Leblond 1969). However, in peripheral nerves, myelin-forming Schwann cells have been reported to store glycogen (Brown et al. 2012). The functional significance of white matter glycogen has been established as energy source mobilized during energy-scarce conditions (e.g., anoxia and hypoglycemia) in the optic nerve (Brown et al. 2003; Wender et al. 2000).

5.2 Hippocampus

The hippocampus is an allocortical structure known for its critical role in episodic memory formation. Hippocampal glycogenolysis has been shown to be crucial for long-term plasticity and consolidation of memory in the recent decade by pharmacological inhibition of glycogen phosphorylase using 1,4-dideoxy-1,4-imino-d-arabinitol (Newman et al. 2011; Suzuki et al. 2011). Glycogenolysis and the downstream metabolism have been suggested to be promoted by the beta-2 noradrenergic receptor (Gao et al. 2016). Hippocampal glycogen levels decrease by prolonged exhaustive exercises, during which multiple major monoamine levels (dopamine, noradrenaline, serotonin) are elevated (Matsui et al. 2011). These monoamines are released by volume transmission and activate astrocytic metabotropic pathways via respective G protein-coupled receptors (for a review, Hirase et al. 2014). Of note, recent papers suggest co-release of noradrenaline and dopamine from classical noradrenergic fibers originating in the locus coeruleus (Kempadoo et al. 2016; Takeuchi et al. 2016). Given the involvement of glycogen in synaptic plasticity and memory consolidation, its distribution in the hippocampus is an important topic and gains further insights into how glycogen metabolism is organized in behaving animals.

The hippocampus is a glycogen-rich structure and the IV antibody labels both the hippocampus proper and dentate gyrus intensely. Glycogen amount has a layer dependence. Principal cell body layers are low in glycogen content most likely due to the highly condensed neuronal somatic presence and hence relatively scarce astrocytic somata and processes (Ogata and Kosaka 2002). By contrast, layers populated by principal cell dendrites have high glycogen levels. These layers include strata oriens, radiatum, lucidum (CA3 only), and lacunosum-moleculare of the hippocampus proper; stratum moleculare and hilus of the dentate gyrus. Glycogen distribution appears seamless across CA1, CA2, and CA3. There is an impression that the CA3 stratum lucidum has subtly lower glycogen signals than other neuropil layers.

Remarkably, the ESG antibody labels the hilus more intensely than all other layers. The functional significance of high glycogen signals in the hilus remains elusive, yet there is an intriguing coincidence that the hilus receives the most intense noradrenergic innervation amongst all hippocampal layers (Loy et al. 1980), hinting at a noradrenergic drive of glycogen metabolism. The hilus is also known as the polymorphic layer and contains unmyelinated axons from dentate granule cells and various types of GABAergic interneurons. Hilar interneurons have been reported to discharge at high rates during dentate spikes—spontaneous, massive, synchronized neuronal activities originating in the dentate gyrus (Bragin et al. 1995). Moreover, neurogenesis continues to occur in the subgranular zone in adulthood, making the hilus subject to axonal proliferation which accompanies ATP-dependent actin polymerization. These features are suggestive of high energy demands in the hilus, and glycogen storage may guarantee sustained energy supply.

Similar to the cortex, IV antibody shows high-intensity punctate patterns with smooth low-intensity signals in the cytosol, the former presumably labeling beta glycogen aggregates. ESG glycogen immunohistochemistry shows a striking pattern in young adult mice in that large puncta are distributed in a "patchy" pattern consisting of puncta-busy and puncta-sparse areas (Fig. 7). The patchy patterns are present in the neuropil areas of the hippocampus proper and dentate gyrus, while patchy patterns are more obvious in the hippocampus proper. The patches are delineated by the morphology of individual astrocytes: there are astrocytes with dense ESG-labeled glycogen puncta and another population of astrocytes devoid of ESG labeling (Oe et al. 2016). Whether these glycogen-puncta-rich and -poor astrocytes comprise two molecularly distinct astrocyte populations or the glycogen storage difference is induced by local environmental factors is not known. Interestingly, the patchy glycogen pattern generally disappears in aged mice. IV glycogen immunohistochemistry shows more uniform distribution in the hippocampus of aged mice, whereas ESG glycogen immunohistochemical signals become low with sporadic high-signal spots reminiscent of polyglucosan bodies. Such alterations in glycogen IHC signals in the aged mouse brain could reflect the known aging-induced increase in *corpora amylacea*.

5.3 Striatum

The striatum, a major subcortical structure in rodents, is known for its function in motor executive functions, reward cognition, reinforcement learning, and motivational salience. Two major afferents to the striatum are the glutamatergic and dopaminergic projections from the cortex and substantia nigra, respectively. The striatum is distinct from other forebrain structures in that the principal cells are GABAergic. In fact, GABAergic medial spiny neurons represent >95% of the neuronal population. Medial spiny neurons are classified into D1 and D2 dopamine receptor-dominant types, which form the direct and indirect afferent pathways, respectively. There does not seem to be a strict spatial organization of D1 and D2 medial spiny neurons and they

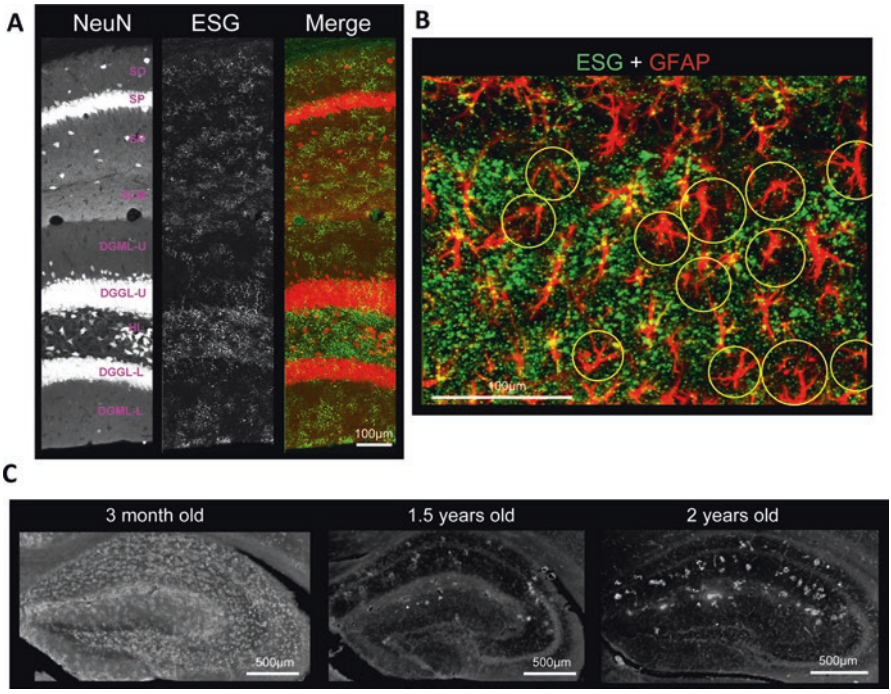

Fig. 7 Patchy glycogen distribution in the mouse hippocampus. (**a**) ESG glycogen immunohistochemistry across hippocampal layers. ESG signals are clustered in patches in dendritic layers (SO, SP, SR, SLM) of dorsal CA1. SO: stratum oriens, SP: stratum pyramidale, SR: stratum radiatum, SLM: stratum lacunosum-moleculare, DGML-U/L: dentate gyrus molecular layer upper/lower blade, DGGL-U/L: dentate gyrus granular layer upper/lower blade, HL hilus. (**b**) Double immunohistochemistry of ESG-labeled glycogen (green) and GFAP (red) shows that patchy glycogen distribution is organized by individual astrocytes. Yellow circles indicate astrocytes that comprise off-patch areas. The sample is from CA1 SP (upper ~15%) and SR (lower ~85%). (**c**) Patchy distribution disappear in aged mice and sporadic high-signal-intensity spots are evident. Reproduced from Oe et al. (2016) with modifications

appear to be homogeneously intermingled. Striatal cytoarchitecture is characterized by the frequent presence of myelinated fiber bundles of cortical and thalamic origins that pierce through the neuropil in stripes. In addition to the white/gray matter segregation, the striatum has histologically identified blobs (e.g., by μ-opioid receptor expression) called the striosomes (also known as the "patches," but here we avoid this term—not to be confused with the patchy glycogen distribution), while the remaining part is referred to as the matrix. In mice coronal sections, the striosomes appear as irregularly shaped areas of diagonal size 100–300 μm and are 300–600 μm apart.

Despite the vastly different cytoarchitecture, mouse striatal glycogen distribution share similar patterns to that of hippocampus proper neuropil. The neuropil is labeled well by IV and ESG antibodies, whereas the fiber bundle white matter yields relatively low glycogen signals. Remarkably, patchy glycogen puncta distributions

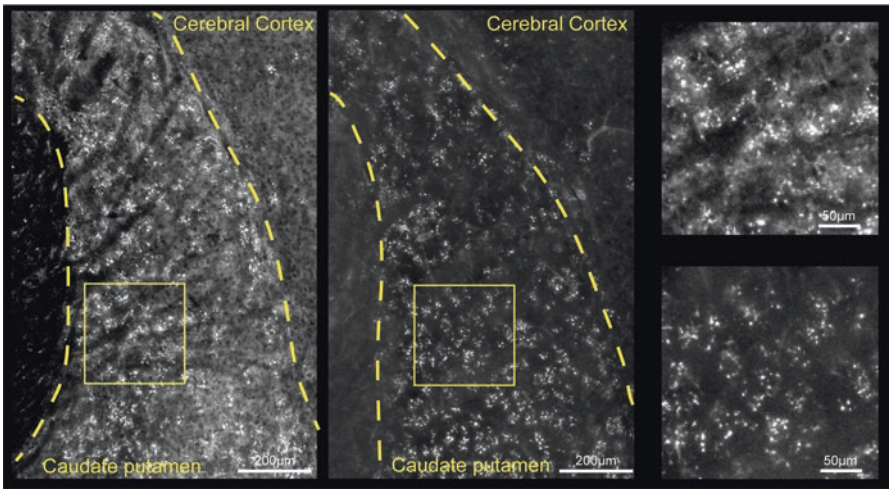

Fig. 8 Glycogen distribution in the striatum. IV (left) and ESG (center) glycogen immunohistochemistry in the mouse striatum (caudate putamen). Dashed lines indicate the borders with the internal capsule (to the left, basal ganglia white matter) and cerebral cortex (to the right). IV glycogen immunohistochemistry shows that striatal fiber bundles (white matter) are devoid of glycogen signals. Patchy patterns are observable with ESG signals. Areas marked by yellow squares are magnified in the right panels

are observed throughout the extent of striatal neuropil. As in the hippocampus, the patchy distribution is organized by intermingled distribution of ESG-immunoreactive and relatively ESG-free astrocytes (Fig. 8). Striatal patchy patterns are organized with a patch size of 50–150 μm, which is smaller than striosomes. Whether the glycogen patches are related to the distribution of D1 and D2 medial spiny neurons remains to be addressed. However, since medial spiny neurons are fairly densely distributed (< 50 μm apart) and their dendrites do not distinguish territories, heterogeneous astrocytic glycogen amounts are not deemed to be defined by proximity to D1 or D2 medial spiny neurons. A recent study suggested that striatal astrocytes are functionally classified into subtypes depending on their preference to signal to either medial spiny neuron subtype (Martin et al. 2015). It remains possible that this astrocytic diversity is related to the patchy glycogen pattern.

5.4 Olfactory Bulb

The olfactory bulb is a specialized allocortical structure that processes odor information. It is stratified with seven cytoarchitecturally distinct layers, namely: olfactory nerve layer; glomerular layer, external plexiform layer; mitral cell layer; internal plexiform layer; granule cell layer; and subependymal layer. It receives projections from the olfactory epithelium that terminate in the glomerular layer via the olfactory nerve layer.

The principal cells of the olfactory bulb are mitral and tufted cells whose dendrites comprise glomeruli, the characteristic neuropil-rich structures that receive olfactory nerve projections. Each glomerulus represents an elementary feature of a smell, and the combination of glomerular output is considered to encode complex olfactory information that is passed onto the olfactory cortex.

Overall, the olfactory bulb glycogen signal level is comparable to that in the cerebral cortex according to the strength of IV signal. IV glycogen immunohistochemistry delineates cytoarchitectural characteristics of the olfactory bulb, which is an indication that the storage of glycogen depends on cell type. Evidently, the mitral cell layer is virtually devoid of glycogen signals and glomeruli are outlined with a strip of low signal intensity area that coincide with the territory of juxtaglomerular (aka periglomerular) neurons. The granule cell layer is generally low in glycogen levels except for a mesh-like structure presumably representing astrocytic syncytium. Unlike the hippocampus, patchy patterns are not observed in the neuropil of external and internal plexiform layers or within a glomerulus. Glomeruli are easily distinguishable because of their relatively high glycogen content compared to external plexiform layer neuropil (Fig. 9). These relative glycogen levels are analogous to the expression of glycogen phosphorylase activity observed in the young rat (Coopersmith and Leon 1987). The olfactory bulb receives heavy neuromodulatory projections including noradrenergic, serotonergic, and cholinergic fibers. In addition, some 10% of juxtaglomerular neurons are dopaminergic. Noradrenergic activity-induced glycogenolysis in the rat olfactory bulb has been demonstrated using a biochemical glycogen assay (Coopersmith and Leon 1995). According to this work, beta adrenergic receptor activation is the main trigger of glycogenolysis while alpha adrenergic receptor (presumably alpha-1) activation becomes gradually dominant as the rat matures.

5.5 Non-forebrain Areas

Outside the forebrain, the cerebellum and brain stem are high in glycogen storage. Neuronal localization of glycogen in non-forebrain areas has been reported previously. For example, in the facial nucleus of the rat brain stem, glycogen immunohistochemical puncta are mainly found in motor neurons as identified by the expression of NR3B (NMDA receptor subunit 3B) cells (Takezawa et al. 2015). The same study also reports relative scarceness of astrocytic glycogen, although we shall note that glycogen immunohistochemistry was performed on perfusion-fixed brains. Also, we find that brain stem neurons with large somata (>30 μm diameter) tend to accumulate visible amounts of glycogen puncta.

Most literature agrees that the cerebellum is a rich, if not the richest, site of glycogen storage. The cerebellar cortex consists of three layers: the molecular, Purkinje, and granular layer. The Purkinje layer is the cell body layer of Purkinje cells (the GABAergic principal output cells) and Bergmann glia that enwrap the synapses of Purkinje cells in full (Grosche et al. 1999). Purkinje cells' dendrites are formed in

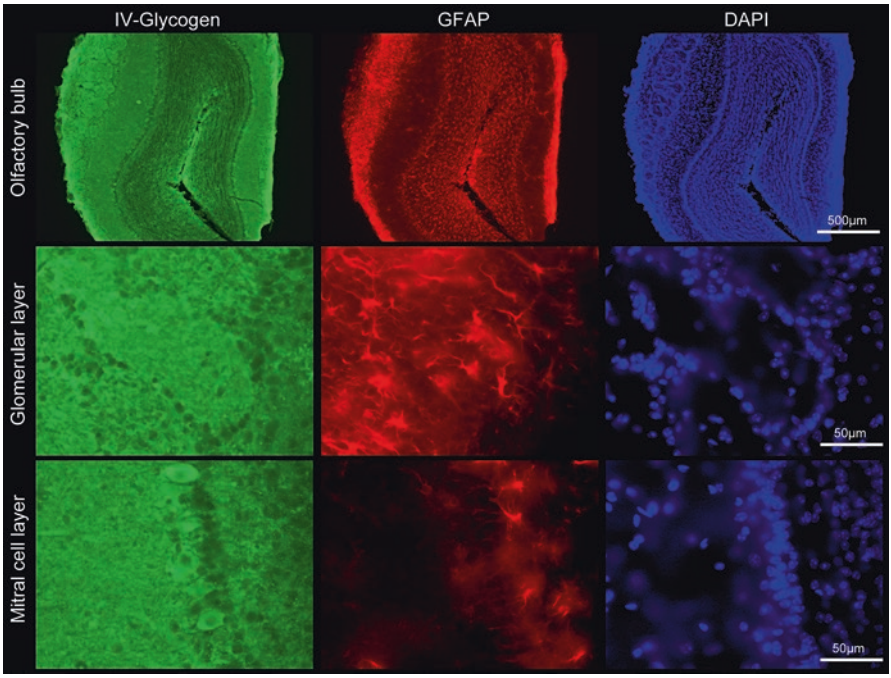

Fig. 9 Glycogen distribution in the olfactory bulb. IV glycogen immunohistochemistry (left) was performed together with GFAP immunohistochemistry (center) and DAPI staining (right). Glomerular and mitral cell layers are magnified in middle and bottom panels, respectively (the olfactory nerve layer is to the left). Glycogen distributions show a characteristic layer-dependent pattern. Glomeruli show high glycogen signals. Whereas periglomerular somata are low in glycogen signal. Mitral cell somata are low in glycogen, however, a population of neuron-like somata show visible green fluorescence

the molecular layer which is superficial to the Purkinje layer. A Purkinje cell has up to 200,000 spines that form synapses with parallel fibers (Napper and Harvey 1988), projecting axons from granule cells that run perpendicularly to the Purkinje cell dendrite plane. In addition, each Purkinje cell is innervated by a climbing fiber which provides powerful excitatory input. Finally, the granular layers are where glutamatergic granule cell bodies are located. Instead of Bergmann glia, the granular layer is populated with so-called velate astrocytes, a kind of protoplasmic astrocytes that ensheath granule cells. Glycogen immunohistochemistry reveals that the molecular layer of the cerebellar cortex has the most intensive glycogen immunohistochemical signals in the mouse brain. Moreover, the granular layer shows scattered distribution of punctate glycogen signals, showing signs of velate astrocytic localization. Bergmann glia accumulate glycogen as evidenced by IV and ESG signals from relatively small somata located in between Purkinje cells. Further characterization of glycogen distribution in the cerebellar cortex is desired and we are currently conducting a more detailed examination of cerebellar glycogen distribution in the mouse.

6 Concluding Remarks

In this chapter, we have described murine brain glycogen distribution according to glycogen immunohistochemistry. Glycogen immunohistochemistry allowed the assessment of regional variability. As a result, we were able to find layer differences in cortical structures and patchy patterns in the hippocampus, striatum, and to a smaller extent, superficial layers of the cortex.

An important issue to remember is that glycogen is a dynamic molecule whose size depends on the metabolic state, which is most likely governed by brain states. Brain glycogen immunohistochemistry data presented here and in Oe et al. (2016) are from mice sacrificed at the end of the light phase of 12/12 h light/dark housing condition. Glycogen distribution may differ in animals prepared at different hours, behavioral conditions, and levels of maturity. In fact, we found that the patchy ESG-labeled glycogen patterns disappear in aged mice. Instead, the ESG antibody labels much larger clots, presumably polyglucosan bodies, that appear frequently (5–30 per hippocampal slice depending on age) in the hippocampus. A previous report suggested that such polyglucosan accumulation is observed in neurons (Sinadinos et al. 2014). More recently, proteomic analyses in the hippocampus revealed a shift of glycogen metabolism enzymes' expression from astrocytes to neurons as mice age (Drulis-Fajdasz et al. 2018). Such a change in metabolism may underlie the disappearance of patchy pattern in aged mice, while functional significances of patchy glycogen distribution remain elusive. Another situation where glycogen storage is altered in astrocytes is inflammation (Shimizu and Hamuro 1958). An electron microscopy study reported that astrocytes accumulate higher amounts of glycogen after radioactive irradiation (Maxwell and Kruger 1965). Further investigations are needed to identify the dynamic nature of brain glycogen distribution in health and disease.

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Technical and Comparative Aspects of Brain Glycogen Metabolism

Long Wu, Nicholas J. M. Butler, and Raymond A. Swanson

Abstract It has been known for over 50 years that brain has significant glycogen stores, but the physiological function of this energy reserve remains uncertain. This uncertainty stems in part from several technical challenges inherent in the study of brain glycogen metabolism, and may also stem from some conceptual limitations. Factors presenting technical challenges include low glycogen content in brain, non-homogenous labeling of glycogen by radiotracers, rapid glycogenolysis during postmortem tissue handling, and effects of the stress response on brain glycogen turnover. Here, we briefly review aspects of glycogen structure and metabolism that bear on these technical challenges, and discuss ways these can be overcome. We also highlight physiological aspects of glycogen metabolism that limit the conditions under which glycogen metabolism can be useful or advantageous over glucose metabolism. Comparisons with glycogen metabolism in skeletal muscle provide an additional perspective on potential functions of glycogen in brain.

Keywords 2-deoxyglucose · Brain · Carbon-13 · Glycogen · Immunohistochemistry · Microwave fixation · Radioisotope

1 Introduction

It has been known for over 50 years that brain has significant glycogen stores, but the physiological function of this energy reserve remains uncertain. This uncertainty stems in part from several technical difficulties inherent in the study of brain glycogen metabolism, and may also stem from some conceptual limitations. This chapter will briefly outline basic structural and bioenergetic aspects of glycogen metabolism, discuss how these lead to certain technical challenges for the study of brain glycogen, and compare what is known about glycogen metabolism in brain with skeletal muscle.

L. Wu · N. J. M. Butler · R. A. Swanson (✉)
Department of Neurology, University of California, San Francisco,
and San Francisco Veterans Affairs Health Care System, San Francisco, CA, USA
e-mail: Raymond.swanson@ucsf.edu

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2 Glycogen Structure and Distribution

Glycogen is a large, branched polymer of glucose. The polymer form eliminates the high osmolarity that would result from an equimolar concentration of free glucose. It also protects the otherwise free "reducing" ends of glucose from auto-oxidation (Fig. 1), and provides a means of intracellular localization of the energy store. Glycogen polymers are roughly spherical and organized into concentric tiers, with the inner tiers composed by chains that normally contain two branches, and the

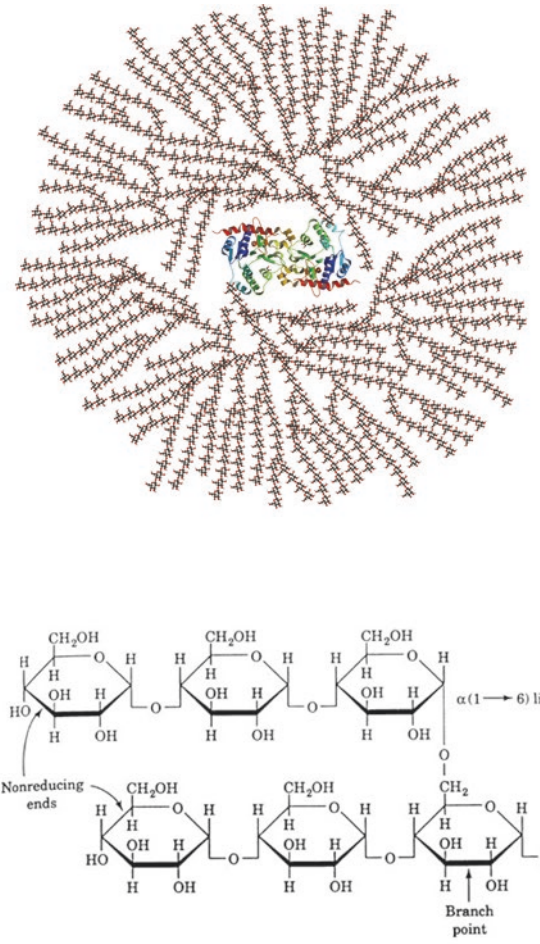

Fig. 1 Schematic two-dimensional cross-sectional view of glycogen. A core protein of glycogenin is surrounded by branches of glucose units. The entire globular granule may contain around 30,000 glucose units. The individual glucose moieties of glycogen are linked by α-1, 4-glycosidic bonds, with branch points at approximately every 12 glucose residues linked by α-1, 6-glycosidic bonds. The exposed ends of all glycogen chains are non-reducing. Image from Haggstrom (2014)

outer tiers composed of unbranched chains (Gunja-Smith et al. 1970). The linear chains of glucose residues are joined by α-1,4-glycosidic bonds, with branch points at approximately every 10–14 glucose residues linked by α-1,6-glycosidic bonds (Calder 1991). The exposed ends of all glycogen chains are non-reducing. Mathematical modeling suggests that the size of a glycogen molecule is limited to 12 tiers, which means a total of approximately 53,000 glucose residues and a radius of 21 nm. In accordance with this modeling, the glycogen particles found in the mouse brain and resting human skeletal muscle ranged from 10 to 44 nm in diameter (Cataldo and Broadwell 1986; Wender et al. 2000; Marchand et al. 2002). The average diameter of these glycogen particles is 20–30 nm, which is estimated to be 7–8 tiers (Goldsmith et al. 1982; Melendez-Hevia et al. 1993).

Glycogen polymers ("granules") are present in the cytosol, endoplasmic reticulum (ER)/ sarcoplasmic reticulum (SR) and lysosomes (Cardell Jr. 1977; Stapleton et al. 2010; Geddes et al. 1992). In liver cells, approximately 10% of all glycogen particles are found in lysosomes (Jiang et al. 2010), where they undergo slow degradation by acid maltase. Almost all tissues contain some detectable glycogen, but the relative amounts of glycogen vary enormously. Glycogen levels in the adult rat under physiological conditions are as follows: liver > > skeletal muscle > cardiac muscle > brain > kidney (Table 1). Glycogen in mammalian brain is localized primarily to astrocytes (Cali et al. 2016; Cataldo and Broadwell 1986; Wender et al. 2000; Koizumi 1974), but much smaller amounts are also found in meningeal cells,

Table 1 Glycogen content in different tissue types

Tissue	Glycogen (mg/g tissue)	Citation
Liver	30.23 ± 2.5	Vissing et al. (1989)
Liver	43.12 ± 5.5 *	Kusunoki et al. (2002)
Liver	32.3 ± 2.0	Khandelwal et al. (1979)
Heart	4.51 ± 0.33	Vissing et al. (1989)
Heart	4.16 ± 0.23	Conlee et al. (1989)
Kidney	0.06 ± 0.01	Khandelwal et al. (1979)
Kidney	0.03 ± 0.01 *	Nannipieri et al. (2001)
Muscle (GPS) [c]	5.94 ± 0.25 [a]	Baker et al. (2005)
Muscle (white gastrocnemius)	5.79 ± 0.72	Garetto et al. (1984)
Muscle (white gastrocnemius)	7.01 ± 0.17	Vissing et al. (1989)
Brain (cortex)	0.68 ± 0.03 [b]	Sagar et al. (1987)
Brain (cortex)	0.60 ± 0.03	Kong et al. (2002)
Brain (cortex)	2.09 ± 0.27 *	Cruz and Dienel (2002)
Brain (whole brain)	2.06 ± 0.21	Oe et al. (2016)

Glycogen content in adult rat or mouse tissues under physiological conditions were shown in the table. Data are reported as means ± SD (*) or means ± SEM

[a]Reported as mmol/kg dry weight; converted here using values of 160.3 mg /mmol as molecular weight of glycogen and 76% as the water content of muscle (Pivarnik and Palmer 1994)

[b]Reported as mmol/mg protein; converted here using values of 122 mg protein/gram wet weight in brain (Banay-Schwartz et al. 1992)

[c]*GPS* gastrocnemius-plantaris-soleus muscle complex

endothelial cells, and other cell types. Neurons contain appreciable amounts of glycogen during development, but this falls to very low levels in the mature brain except in certain brainstem neurons (Koizumi 1974; Ibrahim 1975; Cavalcante et al. 1996; Saez et al. 2014; Cataldo and Broadwell 1986; Borke and Nau 1984; Oe et al. 2016).

3 Glycogen Metabolism

The synthesis of a *de novo* glycogen granule is thought to be initiated by glycogenenin, although recent studies with glycogenin deficient mice indicate other mechanisms of glycogen initiation are likely possible (Testoni et al. 2017). Glycogenin glycosylates itself at a tyrosine residue and catalyzes the extension of glucan chains (Smythe and Cohen 1991; Cao et al. 1993). The glucan chains serve as primer for glycogen synthetase, which catalyzes the formation of α-1, 4-glycosidic linkages of glycogen. Glycogen synthesis requires uridine diphosphate glucose (UDP-glucose) as a substrate, which is formed from uridine triphosphate and glucose 1-phosphate by UDP-glucose pyrophosphorylase (Fig. 2). α-1, 6-glycosidic branch points are subsequently produced by glycogen branching enzyme (1,4-alpha-glucan-branching enzyme) at approximately every 12 glucose residues.

Glycogenolysis is mediated by glycogen phosphorylase, which hydrolyzes glucose residues at α-1, 4 linkage points to generate glucose 1-phosphate. Glycogen debranching enzyme linearizes glycogen chains near the α-1, 6 branch points to provide linear substrate for glycogen phosphorylase (Nakayama et al. 2001). Glycogen phosphorylase is thought to be the rate limiting enzyme in glycogen breakdown. The activity of glycogen phosphorylase is regulated by changes in energy state through allosteric actions of AMP, which accelerates activity; and by ATP and glucose-6-phosphate, which slow enzymatic activity. Glycogen phosphorylase is also regulated by its phosphorylation state, through the action of glycogen phosphorylase kinase. Glycogen phosphorylase kinase is in turn regulated by a variety of signaling pathways through phosphorylation and allosteric interactions. These regulatory actions provide a mechanism for "anticipatory" glycogen mobilization to prevent any actual decline of cellular energy state. For example, glycogen phosphorylase kinase is activated by epinephrine through epinephrine-induced elevations in cAMP and activation of protein kinase A. Glycogen phosphorylase kinase can also be partly activated by elevated levels of Ca^{2+}, via binding to its calmodulin subunit. This mechanism is particularly important in skeletal muscle where muscle contraction triggers the release of Ca^{2+} from the sarcoplasmic reticulum.

Glycogen phosphorylase liberates glucose residues from glycogen as glucose-1-phosphate, which is freely converted to glucose-6 phosphate (Fig. 2). The shuttling of each glucose moiety on and off glycogen requires the net consumption of one ATP equivalent. This bioenergetic expense has implications for the potential physiological functions of brain glycogen, as discussed later in this chapter. It may also be significant that formation of glucose-6 phosphate from glycogen does not consume ATP, unlike the initial hexokinase step of glycolysis.

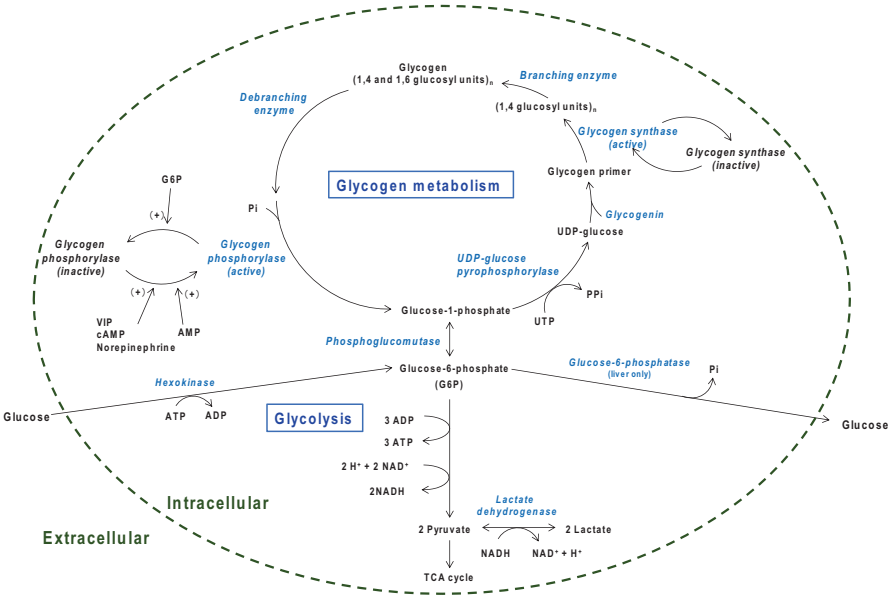

Fig. 2 Bioenergetics and regulation of glycogen metabolism. Glycogen synthase extends an existing glucosan chain of α-1, 4-glycosidic linkages using UDP glucose as substrate. Glycogen branching enzyme subsequently forms α-1, 6-glycosidic bonds to create branch points. Glycogen degradation is mediated by debranching enzyme and glycogen phosphorylase. Glycogen phosphorylase is regulated allosterically in response to hormones, e.g. norepinephrine and vasoactive intestinal peptide (VIP); by changes in energy state (AMP, glucose-6-phosphate (G6P), and others), and by second messengers such as cAMP. The immediate product of glycogen phosphorylase is glucose 1-phosphate, which is freely converted to glucose-6-phosphate. Hepatocytes (but not other cell types) can rapidly dephosphorylate glucose-6-phosphate to generate free glucose for export. There is a net cost of one ATP per molecule of glucose-6-phosphate that is cycled onto and off of the glycogen polymer, as 2ATP equivalents are consumed in forming UDP glucose from glucose-1-phosphate and 1 ATP equivalent is gained back at the formation of glucose-1-phosphate from a glucose residue and inorganic phosphate (Pi) at the glycogen phosphorylase step

4 Brain-Specific Aspects of Glycogen Metabolism

Although neurons are thought to be the primary energy consuming cells in brain, astrocytes contain the vast majority of brain glycogen. Electron microscopy identifies glycogen granules throughout astrocyte cell bodies and processes, particularly near axonal boutons and dendritic spines (Cali et al. 2016). Glutamate uptake is an energy-intensive astrocyte function, and interestingly glycogen phosphorylase has been found to be associated with the astrocyte glutamate transporter, GLT-1 (Genda et al. 2011). Astrocyte glycogen is quickly degraded under conditions of energy failure (Swanson et al. 1989; Suh et al. 2007a; Lowry et al. 1964), as would be expected. Glycogen metabolism in astrocytes is also induced by a several neurotransmitters and other signaling molecules, including vasoactive intestinal peptide (VIP), noradrenaline, arachidonic acid, glutamate, cAMP, and K^+ (Magistretti

1988; Sorg et al. 1995; Cummins et al. 1983; Cambray-Deakin et al. 1988a, b; Subbarao et al. 1995; Subbarao and Hertz 1990; Walls et al. 2009). These signaling molecules serve to couple astrocyte glycogen metabolism to neuronal activity. For VIP and noradrenaline in particular, the anatomical organization of these inputs provides a framework for coordinated signaling. The narrow radial pattern of arborization of intracortical VIP neuron and the tangential intracortical trajectory of the noradrenergic fibers suggests that these two systems may function in a complementary fashion: VIP regulating energy metabolism locally, within individual columnar modules, and norepinephrine exerting a more global effect (Magistretti et al. 1981).

Direct autoradiographic and biochemical measures of glycogen turnover show it to be increased by sensory neuron stimulation in the awake rat (Swanson et al. 1992; Dienel et al. 2007). Conversely, conditions causing focally or globally reduced neuronal activity lead to a corresponding local or global increases in glycogen content, suggesting reduced glycogen utilization (Swanson 1992b). These conditions include focal brain injury, anesthetics, slow wave sleep, and hibernation (Pudenz et al. 1975; Nelson et al. 1968; Phelps 1972; Watanabe and Passonneau 1973; Swanson 1992b).

5 Experimental and Technical Considerations in Study of Brain Glycogen

Low glycogen content in brain. Several aspects of glycogen structure and regulation in brain pose unique challenges for experimental observation. First among these is the relatively low concentration of glycogen in brain. Many of the classical histochemical methods for detecting glycogen were developed using liver or muscle tissues, in which glycogen content is far higher than in brain. For example, the periodic acid schiff (PAS) method of staining polysaccharides works well in liver and muscle, but it in brain the low glycogen content and relatively higher content of glycoproteins and glycolipids, which also react with PAS, makes this approach less useful, even when coupled with dimedone blocking of aldehydes (Cammermeyer and Fenton 1981). Immunohistochemical methods using glycogen antibodies are far more sensitive (Oe et al. 2016). Nonspecific glycogen labeling by either of these methods can be identified as label that persists in tissues after digestion with amylase or rendered ischemic for a few minutes prior to fixation. Low glycogen levels similarly pose a challenge for magnetic resonance studies of glycogen, as recently reviewed (Soares et al. 2017).

Use of radiotracers to assess glycogen turnover. A challenge common to many studies of metabolism is that there is no fixed relationship between the metabolite flux and measured metabolite concentrations. Glycogen turnover in particular—i.e. the breakdown and resynthesis of individual glycogen molecules—may accelerate, decelerate, or stop altogether with little or no change in net glycogen content (Fig. 3). Radiolabeled or isotope enriched substrates are therefore widely used for assessments of metabolic rates. However, accurate quantification by these

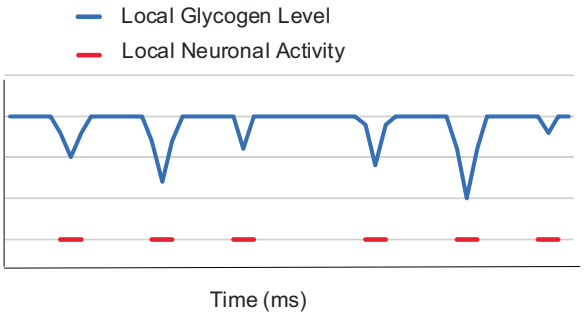

Fig. 3 Schematic relationship between neuronal activity and astrocyte glycogen metabolism. Short bursts of neuronal activity induce mobilization of astrocyte glycogen. The glycogen is resynthesized between bursts, resulting in glycogen turnover with no net change in glycogen content over time. It has been proposed that glycogenolysis is triggered by neuronal activity to provide substrate for increased glycolytic demand over intervals too short to be met by changes in local blood flow

approaches requires that the labeled molecules be homogenously distributed among the unlabeled molecules. This is difficult to achieve in glycogen because individual glucose moieties are sequentially added to and removed from the outer glycogen tiers, and have widely variable dwell times in the glycogen polymer (Youn and Bergman 1987; Elsner et al. 2002). The relationships between rates of glycogenolysis and label release therefore depend upon the patterns of glycogen synthesis and breakdown at the level of individual glycogen granules (as diagrammed in Fig. 4). These limitations do not render assessments of glycogen turnover impossible, but they do limit the precision of these measures and often require certain assumptions. For example, very prolonged administration of tracers can be used to improve homogeneity of glycogen labeling, and this can be further refined by modeling patterns of glycogen turnover (DiNuzzo 2013; Oz et al. 2015; Soares et al. 2017).

The use of radiolabeled glucose to monitor glycogen metabolism is also complicated by the fact that normal glucose metabolism rapidly distributes the label to amino acids, lipids and many other cell constituents other than glycogen. This problem can be surmounted in part by physical isolation of glycogen prior to isotope analysis, or by the use of 2-deoxyglucose, which is incorporated into and released from glycogen but is much more slowly metabolized to other molecules (Nelson et al. 1984; Kai Kai and Pentreath 1981). Glucose labeled at the 3 or 4 carbon positions also mitigates this problem (Swanson et al. 1992), because glucose entering the tricarboxylic acid cycle (from which most other metabolites are formed) loses the 3 and 4 carbons in the pyruvate dehydrogenase reaction. In vivo magnetic resonance spectroscopy studies can in some cases use native ^{13}C abundance to assess brain glycogen concentrations, but the low abundance of ^{13}C coupled with low concentrations of glycogen in brain currently limit the temporal and spatial resolution of this approach (Soares et al. 2017).

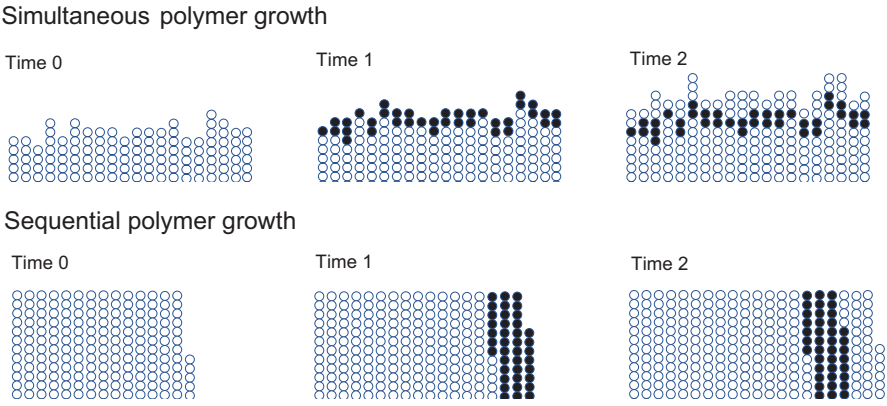

Fig. 4 Results of radiolabeling tracer experiments are influenced by the mode of glycogen polymer growth and breakdown. Schematic diagram showing distribution in glycogen of labeled glucose (filled circles) injected early during glycogen synthesis. Each column of circles represents an individual glycogen polymer. In one scenario, all polymers simultaneously add glucose moieties. In the opposite scenario, each polymer is synthesized to its maximum size before a second one begins to expand. Many other intermediates or more complex patterns are also possible, as are differing patterns of glycogen polymer breakdown. These differing patterns produce a different relationship between rates of glycogen turnover and rates of label release. Redrawn from Youn and Bergman (1987)

Rapid post-mortem glycogenolysis. Glycogenolysis is triggered by very small elevations in AMP, befitting its role as an emergency energy store. This process begins almost instantly with cessation of blood flow, and continues until the brain is frozen, acidified, or otherwise treated to inactivate glycogen phosphorylase. (For frozen brains, the process resumes upon thawing unless other measures are also taken.) This presents a major challenge to histochemical detection of glycogen, particularly with the low concentration of glycogen in brain. Post-mortem glycogenolysis can be prevented by "funnel freezing" with direct application of liquid nitrogen to the animal skull (Ponten et al. 1973; Dienel et al. 2002), or more conveniently by high-energy microwave fixation of brain *in situ*, in which brain temperature is elevated to levels that denature glycogen metabolizing enzymes within 1 s or less (Medina et al. 1975; Sagar et al. 1987). The microwave approach has limitations, however. The high heat induced by and required for microwave fixation can reduce the water content of the brain, thus artificially elevating the glycogen content expressed per gram wet weight. This can be avoided by normalizing to mg protein rather than to wet weight, but special care must be taken to ensure that the fixed (denatured) protein is fully solubilized for protein determination. Failure to correct for brain dehydration or fully solubilize proteins leads to erroneously elevated glycogen measurements in microwave fixed brain (Swanson and Sagar, unpublished observations). A second limitation is that the heating and water vapor formation often distort normal cell architecture and destroy immunoreactivity of many anti-

gens; however, these problems can be overcome by careful titration of microwave power (Oe et al. 2016; Oe et al., this volume).

It is often useful to express glycogen measurements in terms of glucose molecule equivalents. The molecular weight of glucose is 180.16 g/mol; however, the molecular weight of each glucose moiety in glycogen is somewhat less, because the glycosidic linkages between the glucose moieties in glycogen each subtract the equivalent of one H_2O from their molecular weight. There is one α-1,4 glycosidic bond between each glucose moiety in the linear chains, and there is in addition one α-1,6 glycosidic bond at each branch point, which occurs approximately every tenth glucose residue. The calculated molecular weight for glycogen can thus be estimated to be 160.3 g/mol glucose equivalent. The true value will vary slightly depending upon the degree of polymer branching.

Hormonal and neurotransmitter effects on glycogenolysis. This aspect of glycogen metabolism may be uniquely problematic for studies of glycogen in brain because it involves sensory experience of the subject. There is a generally inverse relationship between neuronal activity and glycogen levels, as evidenced by elevated brain glycogen levels during anesthesia, hibernation, and slow wave sleep. Conversely, as outlined above, glycogen phosphorylase is activated by a number of signaling molecules to initiate glycogenolysis in anticipation of actual energy demand. For example, epinephrine and norepinephrine released during the stress response are potent glycogenolytic signaling molecules. It follows that animals that are stressed during the interval before brain harvest may have regionally lower brain glycogen levels than non-stressed animals. Experimental evidence supports this concern (Cruz and Dienel 2002). This observation suggests that, like glucose utilization, glycogen levels and turnover rates are likely influenced in a regionally discrete manner by the subject experience and response near the time of the observation.

6 Physiological Functions of Glycogen in Brain

Function under energy failure conditions. There is no question that glycogen can serve as an energy reserve in brain. Electrophysiology experiments using optic nerve and corpus callosum have shown that astrocyte glycogen can sustain axon function during glucose deprivation or during high frequency stimulation (Wender et al. 2000; Brown et al. 2005, 2012). These and other studies also suggest that lactate or pyruvate derived from astrocyte glycogen can be used by neurons for oxidative metabolism (Dringen et al. 1993; Poitry-Yamate et al. 1995; Pellerin et al. 1998; Tekkok et al. 2005). Other studies have demonstrated that increased glycogen in astrocytes improves survival of neurons under conditions of oxygen deprivation or glucose deprivation, both in culture and *in vivo.*

The total glycogen store in brain is approximately 3 mM (as expressed in glucose equivalents). Given estimates of basal brain glucose utilization rate (Sokoloff et al.

1977), this amount of glycogen would be expected to fuel brain metabolism for less than 15 min if it were the only energy supply available. However, there is a compensatory reduction in the rate of glucose utilization and synaptic activity during energy compromise (Suda et al. 1990). In addition, it is rare to have a complete cessation of glucose delivery to brain. During severe insulin-induced hypoglycemia, for example, the flux of glucose from blood to brain is reduced but not zero, and brain glycogen is consumed very gradually (Ratcheson et al. 1981; Choi et al. 2003). A quantitative analysis suggests that normal levels of astrocyte glycogen should be able to support brain metabolism for about 100 min during hypoglycemia (Gruetter 2003). In agreement with this assessment, an experimental study demonstrated that an 80% increase in rat brain glycogen at the onset of severe hypoglycemia extended the period of brain electrical activity by 90 min (Suh et al. 2007b). The rats with elevated initial brain glycogen concentrations also had improved neuronal survival.

Glycogen is far less useful as an energy store in brain under ischemic conditions, in which only anaerobic (glycolytic) energy metabolism is possible. Under these conditions each glucose molecule stored in glycogen can generate only 3 ATP, as opposed to the 34–36 ATP generated under aerobic conditions. Accordingly, glycogen stores are very rapidly depleted (within 3 min) during complete ischemia (Lowry et al. 1964). Moreover, there is no possibility of transferring usable energy substrates from astrocytes to neurons under anoxic conditions because substrates that can exit from astrocytes, such as lactate and pyruvate, can only be metabolized aerobically. However in focal ischemia (as results from occlusion of a cerebral artery), there is often a border zone area with partially preserved blood supply in which energy supply is barely adequate to maintain cell viability. Spreading depression from the ischemic core can strikingly increase energy demands in this "penumbra" area and exacerbate ischemic injury (von Bornstadt et al. 2015). Astrocyte glycogen stores are rapidly consumed during spreading depression (Feuerstein et al. 2016), and increased glycogen stores delay its advancing wave front (Seidel and Shuttleworth 2011). A study of focal brain ischemia showed reduced infarct size in rats that had elevated brain glycogen content (Swanson et al. 1990).

While the aggregate evidence that glycogen can serve as an emergency energy store in brain is convincing, it nevertheless does not establish this as the physiological, evolutionarily directed function of brain glycogen. (As an analogy, the catabolism of skeletal muscle to support nutrition during extreme starvation does not demonstrate this as the primary function of muscle.) In fact, several aspects of brain glycogen are poorly explained by this interpretation. These include the continuous turnover of brain glycogen under normal conditions; the complex neuromodulatory influences that promote astrocyte glycogenolysis in the absence of energy failure; and perhaps most fundamentally, the location of glycogen in astrocytes rather than in neurons.

Given the energetic cost of shuttling glucose into and out of glycogen polymers, it is useful to delineate the conditions under which this might carry advantages over direct glucose utilization. One obvious condition would be to provide buffering for transient, local insufficiencies in glucose supply, as might happen for example in the

brief interval between a burst of neuronal activity and subsequent increase in local blood flow (Fig. 3). This idea was proposed by Swanson (Swanson 1992a) and more formally developed by Shulman and colleagues as the "glycogen shunt" hypothesis (Shulman et al. 2001; Shulman and Rothman 2001). A related idea proposes that astrocyte glycogenolysis occurs preemptively in activated tissues as a glucose sparing mechanism, to prevent reduction in available glucose that would otherwise occur (Dienel and Cruz 2015; DiNuzzo et al. 2010). Although these concepts have gained wide support, there is uncertainty as to when or if such a transient glucose insufficiency occurs under physiological conditions. Glucose concentration will not become rate-limiting for energy metabolism unless it falls near the glucose Km of hexokinase, which is less than 0.1 mM (Thompson and Bachelard 1977). Glucose concentration in normal rodent brain is approximately 2–2.5 mM, which can also be taken as the approximate concentration of glucose in the intracellular space. (Values for humans are about 30% lower). Can neuronal activity outstrip supply to the extent that local glucose concentrations fall near the hexokinase Km value? The classic study by Silver and Ericińska (Silver and Erecinska 1994) showed that spreading depression, which causes nearly simultaneous bursting of neurons at its wave front, reduced extracellular glucose concentrations from 2.4 mM to 2 mM, a value well above the hexokinase Km. Similarly, studies of brain energy metabolites during pentylenetetrazole-induced status epilepticus showed reductions in brain glucose concentration to no lower than 0.9 mM (McCandless et al. 1987; Folbergrova et al. 1985; Ingvar et al. 1984), excluding settings in which cerebral blood flow was impaired. Though not definitive, these results obtained using spreading depression and seizure activity, both of which massively increase brain energy demands, cast some doubt on the concept that changes in brain glucose utilization resulting from normal activity could require buffering by local mobilization of glycogen stores.

7 Comparisons to Liver and Skeletal Muscle

Assessment of glycogen functions in other tissues may suggest alternative possible roles for glycogen utilization in brain. Liver has a specialized role as a glycogen storage depot for systemic use. Hepatocytes express high concentrations of glucose-6-phosphatase for liberating free glucose into the blood circulation for use by other organs. However, astrocytes do not have high levels of glucose-6-phosphatase, and it follows that astrocytes do not have a role analogous to hepatocytes as a store of free glucose for use by other cells. Skeletal muscle contains glycogen levels intermediate between liver and brain, and like brain the stored glycogen in muscle undergoes continuous turnover and is not released to the systemic circulation. Skeletal muscle fiber types can be classified as Type 1 "red" or Type 2 "white", with the red fibers containing far more mitochondria and white fibers containing far more glycogen (Nielsen et al. 2011). The Type 2 white fibers contract more quickly but fatigue more rapidly than Type 1 red fibers. Muscle glycogen turnover increases during exercise (Nielsen et al. 2011; Shulman and Rothman 2001), similar to the glycogen

turnover increase induced by neuronal activity in brain. However, contracting muscle has repetitive intervals of ischemia corresponding to muscle contractions, during which glycolytic metabolism is essential for ATP production, and this does not have a correlate in brain. Moreover, glycogen in muscle is localized to the contacting myocytes themselves, and there is no evidence that glycogen is metabolized in one cell to fuel activity in another cell as has been proposed to occur in brain.

Muscle fatigue correlates with glycogen depletion (Allen et al. 2008; Matsui et al. this volume), but, surprisingly, the mechanistic link between these events has not been established (Allen et al. 2008; Ortenblad et al. 2013). This point is exemplified by observations in McCardle's disease, an inherited condition caused by a genetic deficiency in muscle glycogen phosphorylase. Individuals with MCardle's disease are generally well, but develop early fatigue with exercise. This occurs even though myocyte glucose levels *increase* with exercise (MacLean et al. 1999; Sahlin 1990). Moreover, muscle performance in McCardle's disease is not significantly improved by hyperglycemia, as should occur if glycogen serves only to buffer intervals of reduced glucose availability. The biochemical defect in these muscles appears not to be a simple lack of glycolytic substrate, but rather a reduced capacity to sequester inorganic phosphate along with other changes that reduce the free energy ($\Delta G'$) provided by ATP hydrolysis (Malucelli et al. 2011).

8 Summary

Studies of brain glycogen metabolism are complicated by factors that stem directly from specific structural and regulatory features of glycogen. These factors must be addressed and considered in interpreting study results. Bioenergetic aspects of glycogen metabolism place certain constraints on the settings under which glycogen utilization may be advantageous to brain. These aspects, in conjunction with some aspects of muscle glycogen metabolism, suggest that current concepts of brain glycogen physiology may need to be expanded.

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Metabolism of Glycogen in Brain White Matter

Angus M. Brown, Laura R. Rich, and Bruce R. Ransom

Abstract Brain glycogen is a specialized energy buffer, rather than a conventional reserve. In the rodent optic nerve, a central white matter tract, it is located in astrocytes, where it is converted to lactate, which is then shuttled intercellularly from the astrocyte to the axon. This basic pathway was elucidated from non-physiological experiments in which the nerve was deprived of exogenous glucose. However, this shuttling also occurs under physiological conditions, when tissue energy demand is increased above baseline levels in the presence of normoglycemic concentrations of glucose. The signaling mechanism by which axons alert astrocytes to their increased energy requirement is likely to be elevated interstitial K^+, the inevitable consequence of increased neuronal activity.

Keywords Lactate · Glycogen · Glucose · Optic nerve · Compound action potential

1 General Energy Requirements of the Brain

All living cells share the requirement for energy. For our single celled ancestors who emerged from the sea they evolved in, this was a relatively straightforward process, as they were bathed in a soup of easily accessible energy substrates. However, as multi cellular organisms evolved, more complex methods of delivering energy to cells had to adapt to this increased morphological complexity, since not all cells were in direct contact with the sea (Lane 2015). When life moved from the sea, which could be considered a soup of metabolic substrates, onto land, thus, limiting the availability of easily accessible energy sources, mechanisms had to evolve to

A. M. Brown (✉) · B. R. Ransom
School of Life Sciences, University of Nottingham, Nottingham, UK

Department of Neurology, University on Washington, Seattle, WA, USA
e-mail: ambrown@nottingham.ac.uk

L. R. Rich
School of Life Sciences, University of Nottingham, Nottingham, UK

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deliver energy to every cell in the body. The energy burden placed on complex multi-cellular organisms facilitated the development of mitochondria (Davies 1998), which increased the energy yield by adapting to oxidative phosphorylation, driven by the increase in atmospheric oxygen resulting from the emergence of blue green algae about 2 billion years ago (Gale 2009). The emergence of multi-cellular land dwelling organisms required an efficient means by which to deliver oxygen and energy substrate to all cells. The development of the cardiovascular system in which the lungs, heart and blood vessels worked in concert to transfer atmospheric oxygen to the blood exploited the avid reaction between oxygen and iron, contained in haemoglobin in red blood cells (Boron and Boulpaep 2009). The heart pumps blood to all tissues, thus, the system may be simplistically viewed as delivery of glucose as the prime substrate to tissues, with oxygen employed to ensure that the glucose efficiently metabolised (Boron and Boulpaep 2009). In humans the increase in brain volume requires an exponentially greater blood flow since maintaining the transmembrane gradients that underlie electrical conduction (Attwell and Laughlin 2001), coupled with the additional computing power (Wang et al. 2008) came at a high metabolic cost (Karbowski 2007). The dogmatic view of brain energy metabolism is that the systemic blood supplies the brain with oxygen and glucose, the cerebrospinal fluid glucose concentration being about two thirds that of the systemic concentration (Boron and Boulpaep 2009). The cerebrospinal fluid contacts the interstitial fluid via the Virchow Robin space (Boron and Boulpaep 2009), with interstitial glucose concentrations determined by glucose delivery and localised energy requirement. This complex balance can be regarded as a flux between delivery, which is governed by, but not limited to, systemic glucose concentration, density of glucose transporters, interstitial volume, rate of cerebrospinal fluid flow, and glucose uptake. The interstitial glucose is taken up by all cells in the brain to fuel their individual energy requirements, which superficially at least appears logical, since there is no dispute that interstitial glucose can be in the millimolar range and bathes every cell in the brain (Hu and Wilson 1997a).

2 Consequences of Shortfall in Glucose Delivery to the Brain

From the whole organ point of view the brain must be considered unique as it receives a fixed proportion of cardiac output (20%) irrespective of its immediate requirements (McKenna et al. 2006). Thus, unlike other organs, such as muscle and gut, which can temporarily increase their blood flow tenfold (Pocock and Richards 2006), the brain cannot increase the volume of blood it receives, although it should be borne in mind that the brain receives ten times more blood than it contributes to body weight, and at any instant the adult human brain contains about 2 pints of blood. Given this fixed delivery of blood flow it is vital that the brain receives an excess of substrate relative to its needs, i.e. supply of substrate must always exceeds demand. The consequences of failing to meet this demand can be appreciated by considering two scenarios. For example, standing up suddenly from a sitting

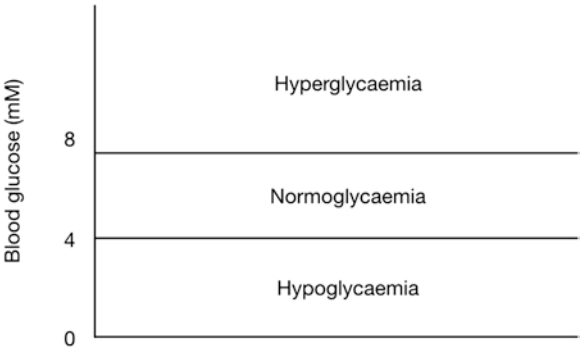

Fig. 1 Blood glucose concentrations associated with hypoglycaemia. The normoglycaemic range is 4.0–7.2 mM. Above this lies the hyperglycaemic range associated with diabetes. Below 4.0 mM lies the hypoglycaemic range, with decreasing blood glucose concentrations evoking the following responses. 3.8 mM; counter regulatory hormone release of glucagon and adrenaline. 3.8–3.2 mM; onset of autonomic and neuroglycopenic symptoms. 3.0–2.4 mM; neurophysiological dysfunction. 3.0 mM; widespread EEG changes. 2.8 mM; cognitive dysfucniton. <1.5 mM; reduced consciousness, convulsions and coma. Adapted from Fig. 6.1 (Frier et al. 2014)

position, may result in a temporary feeling of 'light-headedness', and an impending feeling of faintness. This is due to low blood pressure known as orthostatic hypotension, resulting in a case of insufficient blood flow to the brain leading to temporary mismatch between demand and supply (Silverthorn 2007). A more extreme example occurs during insulin-induced hypoglycaemia (IIH) experienced by type 1 diabetic patients who mismatch prevailing glucose levels with injected insulin to the extent that blood glucose levels are driven to hypoglycaemic levels resulting in inadequate supply of glucose to the brain, the consequences being altered brain function involving loss of consciousness, seizure, coma and even death (Frier and Fisher 2007). The case of IIH is informative as it indicates there are no substrates equivalent to glucose present in the systemic circulation that can substitute for glucose in the event of systemic hypoglycaemia. However, events within the brain at a cellular level are more controversial (Fig. 1).

3 Metabolic Glia-to-Neurone Lactate Shuttling

It was originally thought, and universally accepted, that each cell within the brain, be it neurone or glial cell, directly took up glucose from the interstitial space. This was supported by the distribution of glucose transporters, whereby all cells studied expressed at least one subtype of glucose transporter, as did the blood brain barrier (BBB) to facilitate uptake of glucose from the systemic circulation into the brain parenchyma (Vannucci et al. 1997). A general conclusion reached was of neuronal GLUT3 and astrocytic GLUT1 expression (Vannucci et al. 1997). GLUT1 and GLUT3 are insulin independent and have a high affinity for glucose, and are thus

responsible for continuous uptake of glucose (Vannucci et al. 1997). However, this simplistic arrangement has been questioned in recent decades (Thorens and Mueckler 2010). Initial studies relating glucose uptake with oxygen consumption were inconsistent with complete glucose oxidation, with the ratio found to be in the region of 5.5 at rest, rather than 6 expected from compete oxidation (Dienel et al. 2009). The implied inefficiency with regard to glucose oxidation was at odds with the high demand for energy which is a key feature of the brain. However, this phenomenon is supported by the presence of significant interstitial lactate (Mangia et al. 2009a). Lactate has long been viewed as a toxic waste compound resulting from incomplete glucose oxidation, its accumulation, particularly in the brain, an indicator of pathology (Tymoczko et al. 2015). However, in muscle, during intense exercise, the consumption of glucose is not matched by the uptake of oxygen required to fully metabolise all of the lactate produced, leading to an accumulation of lactate and a build up in systemic circulation of up to ten times that of resting levels (Tymoczko et al. 2015). A more productive role for lactate was proposed in the mid 1990s by Pellerin and Magistretti, who linked increases in synaptic activity with increased astrocytic metabolism, in a scheme where increased synaptic activity leads to increased interstitial glutamate, which is buffered by Na^+-coupled glutamate uptake in astrocytes. This leads to increased glucose uptake into the astrocytes, which is glycolytically converted to lactate. The two molecules of ATP thus produced fuel the Na^+ pump required to restore the trans-membrane Na^+ gradient, and convert glutamate to glutamine. The glutamine and lactate are separately shuttled to the neurones where the glutamine is converted to glutamate and the lactate is used as a substrate for oxidative metabolism (Pellerin and Magistretti 1994). Such a scheme is stoichiometrically attractive but has met with fierce criticism: a representative critique of both sides of the argument are summarised in the following reviews (Pellerin and Magistretti 2012; Dienel 2012; DiNuzzo et al. 2010; Mangia et al. 2009b; Korf 2006). The scheme proposed by Pellerin and Magisteretti makes several important assumptions, the first of which is that neurones can use lactate as a substrate, which presumes that neurones can take up, metabolise and efficiently metabolise lactate. It has been presumed that the BBB offers an efficient barrier to lactate uptake into the brain (Simpson et al. 2007). However, whole body studies have suggested that significant lactate can cross the BBB and be used by the brain (Dalsgaard 2006; Dalsgaard et al. 2004).

4 The Rodent Optic Nerve Model of Central White Matter

For the scheme i.e. lactate transfer between glia and neurones, to be accepted it must be viewed on a more general level i.e. what are the advantages of lactate transfer between the cell types? Numerous papers have been published that demonstrate lactate release from astrocytes and lactate uptake by neurones (Dringen et al. 1993, 1995). The controversial issues arise in inconsistencies of not what can happen, but what does happen, under physiological conditions. These considerations have led to

a polarisation of the field with opinions divided as to whether the lactate shuttle operates under physiological conditions. These problems arose mainly from the experimental models used to illustrate lactate fluxes. Culture models are accompanied by the legitimate argument that they exist under non *in vivo* conditions (Dringen et al. 1993, 1995), of particular concern the infinitely large interstitial space that separates cells, which under *in vivo* conditions would be closely apposed, separated by a narrow interstitial gap (Ransom 2009). Even certain *in vitro* models are less than ideal as stimuli may induce non-physiological behaviour. Additional complications derive from consideration of the source of lactate, which may be glucose, glycogen or fructose (Dringen et al. 1993; Meakin et al. 2007; Magistretti and Allaman 2018; Pellerin et al. 2002). For the purposes of this review we will focus on the role of glycogen in the CNS white matter. The presence of glycogen in the brain has been known since the invention and utilisation of electron microscopy (Koizumi 1974; Koizumi and Shiraishi 1970a, b; Phelps 1972, 1975) and its measurement by biochemical assay championed by Oliver Lowry (Passonneau et al. 1967). However, these initial exploratory studies were not followed up by any functional studies. The reasons for this are likely related to the paucity of glycogen present in the brain being considered insignificant as an energy store in the manner it is in liver and skeletal muscle (Nelson et al. 1968). It is perhaps useful at this point to describe the established roles of glycogen present in liver and skeletal muscle. In skeletal muscle glycogen acts as a localised energy reserve for immediate use upon increased work by muscles. Its breakdown, particularly in response to increased energy demand, can lead to increased lactate production due to incomplete oxidative metabolism of the glycogen (Stryer 1995). Liver glycogen acts as an energy reserve to sustain normoglycaemic blood glucose. Falls in systemic glucose are compensated for by glycogen breakdown and release of glycogen-derived glucose into the systemic circulation (Stryer 1995). Thus, although it may be viewed that the role for liver glycogen is to supply all tissue with glucose, since the brain is more sensitive to shortfalls in glucose that other organs, liver glycogen's principle role is to supply the brain with glucose—it is thus prescient to ask 'what then is the role of brain glycogen?'

Glycogen in the adult mammalian brain is located almost exclusively in astrocytes (Cataldo and Broadwell 1986). Although glycogen related enzymes are found in neurones (Vilchez et al. 2007) and axons (Pfeiffer-Guglielmi et al. 2006, 2007), glycogen itself is expressed in neurones only under pathological conditions such as Lafora's Disease (Vilchez et al. 2007), thus, we can safely assume that in the adult mammalian brain glycogen is exclusively present in astrocytes in both grey and white matter. Such a location immediately prompts the key question, why is the glycogen not present in the neurones since they consume more energy than astrocytes? In addition, the molecular apparatus required to transport the lactate between cells must be present and functioning. The most plausible reason for the astrocytic location of glycogen is to relocate some energy burden away from neurones and onto astrocytes. This has a precedent with regard to glutamate uptake. Whereas for neurotransmitters such as serotonin, the reuptake mechanism occurs in the presynaptic terminal once the neurotransmitter has been released from the post-synaptic

receptor (Siegel et al. 2006), and is the target of selective serotonin reuptake inhibitors that are at the core of the treatment of depression (Ferguson 2001), glutamate is taken up into the astrocyte via Na^+-coupled transporters (Sonnewald et al. 1997). It is subsequently processed in the astrocyte to glutamine prior to release as an inactive compound into the interstitial fluid for uptake and regeneration into glutamate in the neurones (Schousboe et al. 1997). This moves the metabolic burden from oxidative neurones to glycolytic astrocytes (Tymoczko et al. 2015) as the Na^+ influx that accompanies the glutamate movement must be re-equilibrated via the ATP dependent Na^+ pump, and the enzyme glutamine synthase, which converts glutamine to glutamate, requires ATP.

It is known that the majority of glucose uptake in the brain occurs in astrocytes, yet the majority of energy produced is via neurones (Nehlig et al. 2004; Chuquet et al. 2010). These apparently dichotomous findings can be allied if we accept that lactate transfer between astrocytes and neurones occurs.

5 Establishing the Presence and Elementary Role of Glycogen in Rodent Optic Nerve

We first started investigating glycogen in white matter over 20 years using the rodent optic nerve as our model system. The *in vitro* rodent optic nerve lacks the complications of glutamatergic synapses and cell bodies and as such is a relatively simple model of central white matter consisting of axons, astrocytes and oligodendrocytes (Ransom and Orkand 1996). Recordings of the stimulus evoked compound action potential (CAP) can be used to assess the conduction of the nerve (Stys et al. 1991), since the CAP consists of the summed contribution of all conducting axons (Cummins et al. 1979), thus, a decrease in CAP area is indicative of a loss of contributing axons (Stys et al. 1991). The investigations were prompted by the following experimental evidence. In the *in vitro* rodent model of central white matter, in which the tissue is maintained in a superfusion chamber perfused with oxygenated aCSF at 37 °C (Stys et al. 1991), replacement of oxygen with nitrogen or adding cyanide (Stys et al. 1992) causes a rapid failure of the CAP within a few minutes, indicating the inability for CAP conduction to persist in the absence of oxidative phosphorylation. However, when glucose was withdrawn, but with oxygen still present, the CAP took up to 30 min before it began to fail after which time it was rapidly abolished (Ransom and Fern 1997). There were several possible explanations for this observation: (1) the tissue survives on the available glucose-derived metabolites present in the tissue, (2) the tissue survives on glucose present in the intra- and extracellular milieu, (3) there is an endogenous energy substrate present that supports the tissue temporarily. The third scenario appears most likely based on the following evidence. Survival of cultured neurones was improved if they were co-cultured with astrocytes, although at the time of the study, the nature of this support afforded by the astrocytes was not investigated (Whatley et al. 1981). Subsequent

studies demonstrated similar findings but revealed that the key factor that determined neuronal survival was the presence of glycogen in astrocytes, since depleting astrocytes of glycogen attenuated neuronal survival (Swanson and Choi 1993). Subsequent tissue culture studies demonstrated that astrocytes release lactate when supplied with glucose, a property not demonstrated by neurones (Dringen et al. 1995). With this backdrop we proceeded in our studies on rodent optic nerve to understand the role of glycogen in central white matter tissue. Due to historical precedent our first paper was carried out in rat optic nerve (RON) (Wender et al. 2000), but all subsequent studies were carried out on mouse optic nerve (MON). The presence of glycogen in the RON was confirmed using both EM and biochemical assay (Wender et al. 2000), demonstrating a low concentration of glycogen in comparison to those in the liver or skeletal muscle. The ability of glycogen to support axon conduction was monitored as its ability to support the stimulus evoked CAP in the absence of exogenously applied glucose, with any loss of the CAP area being considered as a loss of conducting axons (Stys et al. 1991). The glycogen content was measured at about 10 pmol ug protein^{-1}. Removal of glucose caused a reduction in glycogen content to a nadir of about 2 pmol ug protein^{-1}. This temporally correlated with the failure of the CAP after about 30 min. This encouraging information prompted us to progress with a more detailed assessment of glycogen. The lability of glycogen content was investigated by incubating the tissue with 10 mM glucose, 25 mM fructose or 1 mM noradrenaline plus 1 mM glucose. These were chosen for the following reasons, since we would anticipate that 25 mM glucose should increase glycogen, but equivalent fructose should have no effect on glycogen since it does not contribute to glycogen content (Champe and Harvey 2008). Glucose surplus to immediate requirements of astrocytes and axons is converted to glycogen in the astrocytes to form a readily available pool of lactate. Noradrenaline previously has been shown to deplete glycogen (Sorg and Magistretti 1992). The results were as expected, demonstrating that glycogen can be up or down regulated rapidly. As well as the glycogen content being measured under such circumstances the latency to CAP failure was measured, as this would give an indication as to whether there were any functional consequences to manipulating glycogen levels.

6 Glycogen-Derived Lactate Supports Axon Conduction in Rodent Optic Nerve

The latency to CAP failure increased from 30 to about 40 min after pre-incubation for 2 h in 25 mM glucose, but with 25 mM fructose there was no effect. This was confirmation that loading the interstitial space with utilisable substrates did not augment latency to CAP failure. Noradrenaline led to a significant decrease in the latency to CAP failure, thus, these results were indicative of a role for glycogen in supporting CAP conduction in the absence of exogenously applied glucose (Wender

et al. 2000). Since glycogen is too large a molecule to be transported between astrocytes and axons it must first be converted to a transportable conduit in astrocytes prior to shuttling to axons. The most likely candidate for this is lactate, as it is considered the end product of glycolysis (Schurr and Payne 2007) and it is the most visible substrate to be released by glycogen-rich astrocytes (Dringen et al. 1993). If the conduit is lactate, then exogenously applied lactate in the absence of glucose should support axon conduction, and this is what we found, 20 mM lactate supporting the CAP for over 2 h. As a logical consequence of this there must exist transport mechanisms for lactate efflux from astrocytes and uptake into axons, and this is most likely to be via monocarboxylate transporters (MCTs), which are inhibited by various compounds, including quercitin, CIN and pCMBS. Quercetin, related to saffron, accelerated the CAP failure during aglycemia but did not affect the ability of CAP conduction in the presence of 20 mM lactate suggesting it blocks lactate efflux from astrocytes, but does not affect lactate influx into axons. The addition of CIN or PCMBs accelerated CAP failure during aglycemia and partially inhibited the CAP supported by 20 mM lactate, suggesting they block lactate uptake into axons. This introductory study established important benchmarks with regards to CNS white matter glycogen, which can be summarised as follows; (1) Glycogen is present in CNS white matter astrocytes, (2) Glycogen content can be up and down regulated, thereby providing a labile pool from which lactate can be released, (3) Glycogen content at the onset of aglycaemia determines latency to CAP failure, (4) Lactate can support CAP conduction, which is blocked if lactate transport is interrupted (Wender et al. 2000).

7 Establishing Role of Glycogen during Hypoglycaemia and Increased Energy Demand in Rodent Optic Nerve

These intriguing results encouraged us to carry out a more detailed study on the role of CNS white matter glycogen, but we chose the mouse as our model for the following reasons; investigations into the effects of anoxia on rat optic nerve (RON) led us to conclude that the diffusion distance to the centre of the RON under our experimental conditions, where the nerve is placed in a superfusion chamber, was too great for adequate perfusion of glucose, and the centre of the tissue is almost certainly ischaemic (Baltan Tekkök et al. 2003). The smaller MON, and its smaller diffusion distance bypasses this problem, as well as offering the advantage of being compatible with the development of transgenic models, which are almost exclusively murine in origin. The MON CAP displayed a qualitatively similar trend to that of the RON in that on withdrawal of glucose it failed, but failure was accelerated compared to the rat at 20 versus 30 min, with the glycogen content correspondingly lower at 8 pmol ug protein^{-1} (Brown et al. 2003). A period of aglycemia depleted glycogen, which could be replenished when the nerve was subsequently exposed to glucose-containing aCSF, but not lactate-containing, as shown by a

vastly accelerated CAP failure following a period of aglycaemia after which 20 mM lactate aCSF was added to rescue the CAP. The glycogen content was shown to be labile as it was affected by bathing the tissue for 2 h in various concentrations of glucose from 2 to 30 mM, with the higher the ambient glucose, the higher the glycogen content. Subsequent exposure of MONs treated with increasing glucose concentrations led to increased latency to CAP failure following introduction of aglycaemia, with the higher the ambient glucose, and thus, the higher glycogen levels, the longer the latency to CAP failure. Plotting glycogen content prior to aglycaemia versus latency to CAP failure resulted in a linear relationship: the importance of this relationship is that knowing the glycogen content immediately prior to aglycemia allows one to predict with a high degree of accuracy the latency to CAP failure. Translating this to the clinical environment, where type 1 diabetic patients who mismatch insulin administration relative to ambient glucose can lead to hypoglycaemic episodes (Frier and Fisher 2007), implies that increasing the glycogen content in these patients would extend the period of time before brain disruption and could potentially stave off brain damage and allow the patient and clinical staff additional time to intervene (Fig. 2).

Thus, far the role of glycogen that has been deduced from these experiments is to support axon conduction in the absence of glucose, which is admittedly an artificially experimental environment. However, this information is useful in establishing key roles for glycogen and the associated mechanisms involved (Brown et al. 2003). However, we next sought to determine if glycogen played a role under more physiological conditions. Thus, we investigated the contribution of glycogen to supporting CAP conduction in hypoglycaemic concentrations of glucose. Superfusion of CSF containing 2 mM glucose supported the CAP for 2 h, which was an unexpected result given that 2 mM glucose in the systemic circulation is considered hypoglycaemic (Frier and Fisher 2007) and incapable of supporting brain function. However, in our MON model, depleting glycogen with a brief period of aglycemia revealed

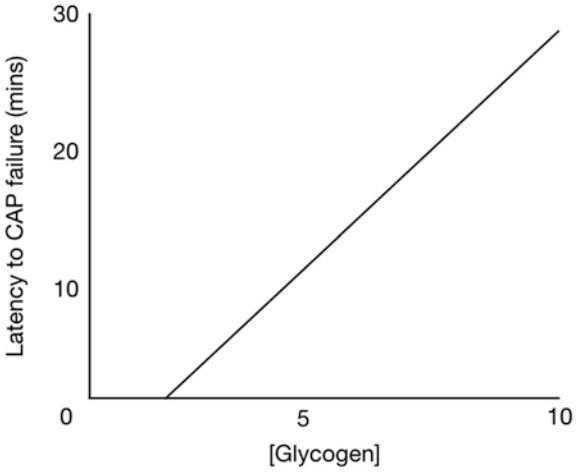

Fig. 2 Relationship between latency to CAP failure after onset of aglycaemia and concentration of glycogen (pmol μg protein^{-1}) present in the MON. Adapted from Fig. 2D (Brown et al. 2003)

that 2 mM glucose could no longer support the CAP, a result reinforced by the ability of CIN to reduce the CAP area when superfusing 2 mM glucose. The conclusion from these experiments was that 2 mM glucose alone is insufficient to support the CAP and is supplemented by glycogen, however, depleting glycogen or interfering with lactate transport results in loss of the CAP, confirming a supporting role for glycogen in the presence of hypoglycaemic concentrations of glucose, where glycogen is metabolised to provide supplemental lactate to ensure the energy demands of the tissue are met by the supply of glucose and lactate (Frier and Fisher 2007). These results suggested that glycogen can play a role in brain function under more physiological conditions, and this was further explored by imposing high frequency stimulus on the MON in order to increase the energy demand of the tissue to determine how this was met with regards to substrate supply. Imposing 100 Hz stimulus for 4 min caused an elongation of the CAP profile, no doubt due to the resulting perturbation of trans-membrane ion gradients affecting Na^+ influx (Stys and Lopachin 1996), but the CAP was maintained above baseline levels. However, the addition of CIN caused a rapid fall in the CAP, as did quercetin, supportive of glycogen metabolism being used to provide supplemental lactate to the axons in order to meet this increased energy demand. Addition of CIN with 10 mM glucose under control conditions of 1 stimulus every 30 s had no effect on the CAP. This role for glycogen in supporting increased energy demand in the presence of normoglycaemic concentrations of glucose has profound implications in proposing a physiological role(s) for glycogen. Imposing 100 Hz stimulus for periods of time ranging from 1 to 4 min prior to exposing the tissue to aglycemia under baseline stimulus protocol resulted in accelerated CAP failure, with the degree of attenuation directly correlated to the duration of high frequency stimulus (Fig. 3).

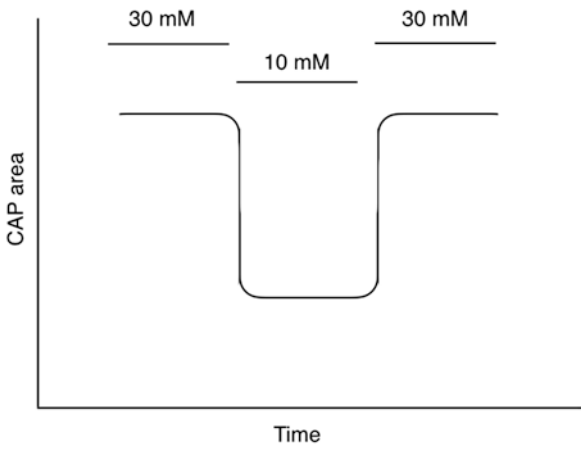

Fig. 3 CAP area is affected by substrate concentration during high frequency stimulus. The CAP area is maintained in 30 mM glucose but falls when exposed to 10 mM glucose in an MON exposed to 100 Hz. Adapted from Fig. 4C (Brown et al. 2003)

8 Inhibiting Glycogen Metabolism and Blocking Lactate Uptake

Whereas glycogen in the liver and skeletal muscle act as a reservoir of energy substrate that can be used for long term sustenance e.g. liver glycogen is reported to be exhausted after 24 h of starvation (Stryer 1995), and muscle glycogen can support extended duration physical exercise such as marathon running (Tymoczko et al. 2015), the brains glycogen must be viewed as fulfilling a fundamentally different role. We have already discussed that the role of liver glycogen is to maintain normal glycaemic concentrations of glucose in order to supply the brain with adequate glucose. The feeding of the other organs/tissues is a consequence of a systemic blood supply. Thus, the role of brain glycogen is not as one would expect to supply the brain with glucose but to act as a short-term energy buffer to deliver energy substrate in the form of lactate during periods of need such as a/hypoglycaemia and increased energy demand under normal glycaemic conditions (Fig. 4).

The development of compounds that inhibit glycogen phosphorylase, the key enzyme that initiates glycogen metabolism, allowed further investigation into the role of brain glycogen. Although predominately of a confirmatory nature such data provided a strong foundation from which established roles of glycogen emerged. Addition of DAB (Andersen et al. 1999; Fosgerau et al. 2000; Walls et al. 2008) or isofagomine (Andersen et al. 2001; Waagepetersen et al. 2000), compounds that inhibit glycogen phosphorylase, resulted in an accelerated latency to CAP failure in MONs exposed to aglycemia (Brown et al. 2005). The effect of these compounds was independent of the glycogen content, and indeed in the presence of DAB the glycogen content increased significantly but could not be utilised. This provided an indirect indication as to the relative turnover rate of glycogen, since application of DAB for 2 h increased glycogen content by over a third (Walls et al. 2008). This suggests an active turnover of glycogen in the MON (see later). The compound CIN is known to inhibit pyruvate uptake into mitochondria, which could cloud results, so

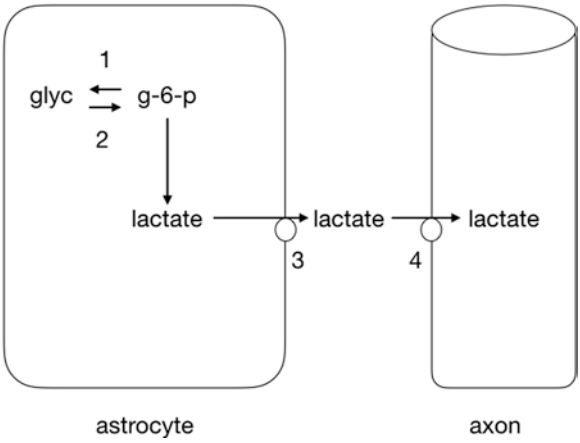

Fig. 4 Glycogen-derived lactate fuels axon conduction during aglycaemia. In the absence of exogenously applied glucose astrocytic glycogen is metabolised to lactate prior to shuttling to the axon via monocarboxylate transporters (3 and 4). Glycogen synthase controls glycogen synthesis (1) and glycogen phosphorylase its metabolism (2)

we used D-lactate, the inactive stereoisomer of L-lactate, which acts as a competitive compound at the MCT but is not metabolised intracellularly, to block lactate uptake into axons. D-lactate at 20 mM had no effect on the CAP area when administered with 10 mM glucose, but accelerated latency to CAP failure at the onset of aglycaemia supporting a role for lactate transfer (Tekkok et al. 2005). Such transfer must require the appropriate transporters to shuttle lactate between cells, plus there must be the capability to transport lactate out of astrocytes and into axons. Immunohistochemical studies using antibodies specific for MCT subtypes showed that the MCT1 was present on axons, since these were co-labeled with the axon specific antibody neurofilament, whereas the MCT 2 was co-localised with astrocyte specific stain GFAP (Tekkok et al. 2005). The MCT co-transports lactate with H^+ thus, lactate movement is associated with alterations in pH. We investigated these phenomena in a study in which we determined the injury mechanisms incurred by the MON on exposure to aglycemia (Yang et al. 2014). Lactate biosensors recorded the lactate concentration on the periphery of the pial-glia boundary, and thus did not measure interstitial lactate, and pH sensitive microelectrodes were used to construct a scheme following exposure of the MON to aglycemia. At the onset of aglycemia there was a rapid and complete fall in lactate, supporting a flux of lactate from astrocytes to axons, which is abolished at the onset of aglycemia, since the axons are without glucose and take up all available lactate. The pH results were more difficult to interpret. The baseline pH under superfusion with 10 mM glucose is 7.25, considerably more acidic then the pH of the perfusing aCSF, at 7.45. This is likely due to the hydrogen ions accompanying a tonic release of lactate by the astrocytes. Upon introduction of aglycaemia there was a two-step process with an initial rapid pH jump to about 7.4 while the CAP was maintained. However, coinciding with the subsequent CAP failure, which signifies the exhaustion of glycogen, the pH jumped to 7.45 i.e. equivalent to bath pH. We interpret this as signifying the glycogen is exhausted and thus no more lactate is produced or transported, and hence there is no outward hydrogen flux to acidify the interstitial fluid (Yang et al. 2014) (Fig. 5).

9 Lactate Biosensors Reveal Tonic Lactate Efflux from Rodent Optic Nerve

The lack of effect of CIN on the CAP in the presence of 10 mM glucose and limiting uptake of lactate into axons has no significant effect on axon conduction, which can be supported without the need of lactate transfer from astrocytes, whether the source is glucose or glycogen (Brown et al. 2003). However, the introduction of lactate sensors into our technical armoury used to study the roles of glycogen revealed a finding that is consistent with previous reports, namely that there exists in the interstitial space significant concentrations of lactate (Yang et al. 2014). Since the lactate sensor is placed outside of the nerve at the pia glial border, a stable signal actually indicates a steady efflux from the tissue. The reason why there is a tonic lactate efflux from the tissue in the presence of normoglycaemia is consistent with a

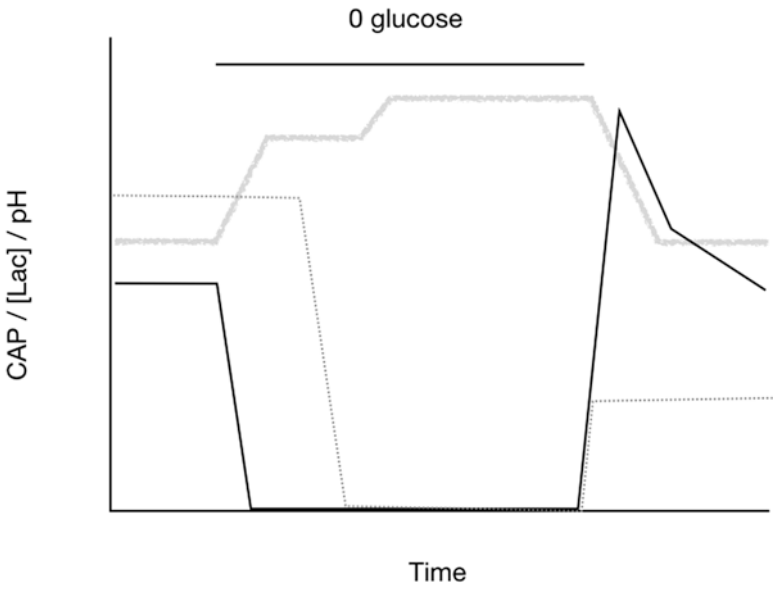

Fig. 5 Relationship between pH, $[Lac]_o$ and CAP during aglycaemia. Resting pH (grey line) was about 7.2 under baseline conditions. At the onset of aglycaemia there was a two-stage alkalization of pH, the first stage coincided with a decrease in [Lac] (black line) measured at the pial glial border, and the second phase coincided with the loss of the CAP (dotted line). Adapted from Fig. 4C (Yang et al. 2014)

glucose utilisation/oxygen uptake ratio of less than 6, but the fundamental consequences are unknown. Is it the case that there is a degree of versatility in the substrates used by axons under baseline conditions, and that blocking the route of lactate uptake results in a corresponding increase in glucose uptake? In addition, the fact that we record lactate at the outer boarders of the nerve is key evidence that it is not utilised and may be considered surplus to immediate requirements. This is supported by data recorded during aglycaemia when the lactate levels are abolished rapidly (Yang et al. 2014), even although electrophysiological evidence points to the fact that lactate is being transferred from astrocytes to axons within the nerve, but in the absence of exogenously applied glucose all lactate present in the interstitial fluid is avidly taken up by axons leaving none to move from the interstitial compartment to the outer border of the nerve.

10 Lactate Release from Astrocytes

Using state of the art FRET sensors the release of lactate from astrocytes has been studied in both in vitro and in vivo conditions. Lactate pools are present in astrocytes and are released through lactate channels as well as via the conventional MCT

(Sotelo-Hitschfeld et al. 2015). The lactate pool is estimated to be about 1.5 mM, thus, a substantial reserve of lactate is present for immediate release. The channel is not dependent upon the balance of trans-membrane lactate and H^+ concentrations, thus, lactate efflux occurs even when it is electrochemically unfavourable. The release of lactate is dependent upon astrocyte membrane potential, depolarisation favouring release. Thus, computational studies that show a near instantaneous increase in interstitial lactate in response to stimulus support the presence of a large, rapidly available pool of intracellular lactate (Aubert et al. 2007, 2005).

11 Astrocyte Heterogeneity

In studies of this kind astrocytes are considered a homogeneous cell population sharing identical functions. However, it should be borne in mind that there are specialised subtypes of astrocytes e.g. Muller cells and Bergmann glia (Farmer and Murai 2017). In addition, the heterogeneous nature of astrocytes is related to the brain region in which they reside, their phenotype determined, in part, by developmental cues (Farmer and Murai 2017). Although in its infancy recent studies are attempting to relate astrocyte phenotype with function suggesting at least three main astrocyte subpopulations (Chia-Ching et al. 2017). Also of relevance is the fact that virtually all brain tumours are of glial origin, the malignant phenotype contributing to the pathology (Sontheimer 2008).

Given the reliance on the astrocyte membrane potential sensitivity to K^+, which acts as a sensor of interstitial K^+ and hence axonal activity (Ransom and Goldring 1973), one may expect that astrocytes would express similar resting membrane potentials, but this was not found to be the case in both grey and white matter. In hippocampus recorded using patch pipettes there was a wide range of resting membrane potentials, which may have its origins in the method of recordings. High resistance sharp microelectrodes tended to record more hyperpolarised potentials, whereas patch pipettes recorded more depolarised potentials (McKhann 2nd et al. 1997). However, in MON a bipolar distribution of membrane potential was recorded using sharp microelectrodes, with the morphology of the recorded cells falling into two categories (Bolton et al. 2006). Thus, there does not appear to be a uniform astrocyte resting membrane potential close to E_K, but rather there is a range of RMPs, which may signify different functions of astrocytes. Although currently not known one would suspect that astrocytes which do not show a Nernstian response to K^+ i.e. astrocytes that also show a permeability to Na^+ and/or Cl^- (Kandel et al. 2013) would not be as sensitive in their response to neuronal activity, thus, the role of responding to physiological elevations in K^+ and subsequently supplying lactate to neurones may be the responsibility of astrocytes that are exclusively permeable to K^+.

12 Implications from Other Models on the Functions of Central White Matter Glycogen

In the last decade or so there have been many studies investigating the role of glycogen in the nervous system, although most were carried out in grey matter. Nonetheless some of the data reported, and conclusions reached, can be extrapolated to white matter to reveal potential roles of glycogen and lactate shuttling that await experimental confirmation.

Computational studies, which are based on experimentally derived data, are important in yielding information that may be experimentally very difficult to obtain. A multi compartment model assembled using the MATLAB programming environment has been used to investigate how compartmentally segregated lactate concentrations are affected under physiologically relevant conditions. The initial model showed a significant resting lactate concentration in the interstitial fluid. On introduction of stimulus there was transient decrease in this lactate concentration before it recovered and steadily increased until cessation of the stimuli (Aubert et al. 2005). This was a critical finding in that it reproduced experimental data obtained under in vivo conditions using lactate sensors to record interstitial lactate (Hu and Wilson 1997b). The significance of the transient decrease is that it indicates that at the onset on stimulus the energy requirement of the axons is met by uptake of interstitial lactate, and crucially that astrocytic production of the lactate lags behind the axonal requirement. The transient decrease, in the order of between 10 and 15 s (Fig. 1, Hu and Wilson 1997b), rapidly reverses, an indication that whatever signalling mechanisms that exists between axons and astrocytes has commenced, and suitable delivery of lactate in excess of immediate axonal requirements is being delivered to the interstitial space. A clue to the signalling mechanism was revealed in a further experiment in which repetitive stimuli were imposed, and the lactate concentration increased in an attenuating manner (Aubert et al. 2005), similar to the effect of repetitive stimuli on the *Necturus* glial cell membrane potential in Kuffler's classic experiments (Orkand et al. 1966). Efflux of K^+ from axons during stimulus was hypothesised (Hodgkin and Huxley 1952) and subsequently measured (Hodgkin and Huxley 1953) in experiments on squid axons, and is universally accepted as a consequence of axonal action potential firing (Hille 2001), thus, there is convincing evidence that the signalling pathway whereby neurones/axons signal astrocytes during periods of increased activity is via increased interstitial K^+ (Choi et al. 2012), to which astrocytes are exquisitely sensitive. The higher the degree of neuronal activity the greater amount of lactate is shuttled from astrocytes to neurones/axons (Fig. 6).

13 Effects of Pathology on Glycogen Content

A variety of pathological conditions are known to affect glycogen content in grey matter, a consequence that may have implications to white matter function. The maturation of astrocytes is temporally correlated with expression of glycogen such that undifferentiated neural stem cells do not express glycogen (Brunet et al. 2010),

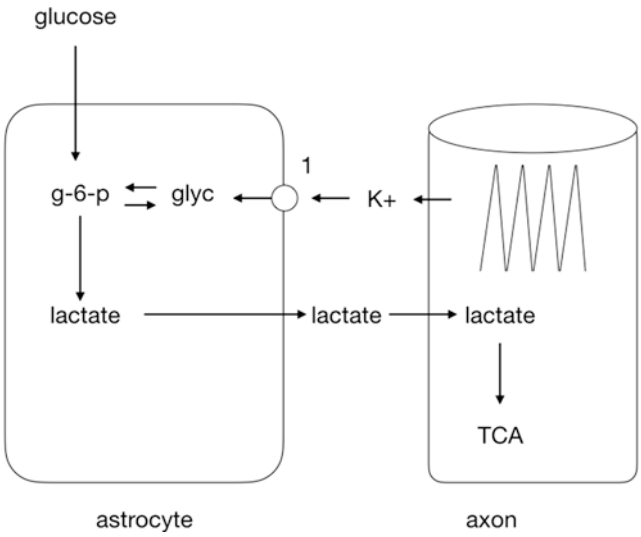

Fig. 6 K^+ stimulated glycogenolysis. Axonal action potentials cause release of K^+ to the interstitium, which act upon the Na^+ coupled bicarbonate transporter (1) leading to intracellular alkalization of the astrocyte. pH sensitive adenyl cyclase activates glycogen phosphorylase promoting glycogen metabolism, ultimately leading to lactate transfer to axons. Adapted from Fig. 7 (Choi et al. 2012)

whereas mature astrocytes do, thus, the expression of glycogen can be used as an indicator of astrocyte differentiation. In addition, stress in known to be a factor that regulates glycogen expression in hippocampus. Stress causes a reshaping of astrocyte morphology and a subsequent decrease in glycogen expression, where glycogen degradation outweighs glycogen synthesis despite reports that stress increases expression of both glycogen synthase and phosphorylase (Zhao et al. 2017).

There is a connection between glycogen expression and the migraine associated with cortical spreading depression (CSD). Glycogen metabolism is required by astrocytes to maintain low interstitial K^+, a vital buffering function that maintains the trans-membrane ion gradients optimal for neuronal excitability and action potential firing. It has been reported that sleep deprivation causes glycogen synthesis rather than degradation, which would interfere with K^+ buffering. An increase in K^+ can result in activation of Pannexin-1 channels, whose activation is thought to underlie the migraine aura that accompanies CSD. Inhibition of glycogen metabolism causes activation of Pannexin-1 channels with sleep deprivation causing Pannexin-1 channel opening and reduced CSD threshold. The reduced threshold could be reversed by application of glucose or lactate implying glycogen metabolism is vital to maintaining K^+ homeostasis, with disruptions of this vital function leading to defined pathological consequences (Kilic et al. 2018).

14 Conclusions

In the MON glycogen is present in astrocytes where its ability to support axons during periods of aglycaemia led to defining its physiological role as providing supplemental lactate to axons during periods of increased energy demand under normoglycaemic concentrations of glucose. However, since glycogen reserves are limited the tissue benefits only for a short period of time. It appears that astrocytic glycogen may play a vital role in the metabolic glia to axon signalling that occurs during periods of increased neuronal activity, when K^+ released from active axons acts as an indicator of increased activity, to which astrocytes are exquisitely sensitive in their response, supplying glycogen derived lactate to the hungry axons. Unresolved issues remain - under what physiological conditions is lactate required by axons and how is this signalled and translated into action by the astrocytes. Such issues may be addressed with higher resolution measurements of real time interstitial lactate. From the pathological viewpoint intercellular lactate shuttling may underlie diabetic neuropathy, memory impairment, migraine and age related neurovascular disorders.

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Glycogenolysis in Cerebral Cortex During Sensory Stimulation, Acute Hypoglycemia, and Exercise: Impact on Astrocytic Energetics, Aerobic Glycolysis, and Astrocyte-Neuron Interactions

Gerald A. Dienel and Douglas L. Rothman

Abstract Most glycogen in cerebral cortex is located in astrocytes, and the importance of glycogenolysis for critical functions, including neurotransmission and memory consolidation, is strongly supported by many studies. However, specific mechanisms through which glycogen sustains essential functions remain to be established by rigorous, quantitative studies. Cerebral cortical glycogen concentrations are in the range of 10–12 μmol/g in carefully-handled animals, and the calculated rate of glycogenolysis ($CMR_{glycogen}$) during sensory stimulation is approximately 60% that of glucose utilization (CMR_{glc}) by all cells, with lower rates during acute hypoglycemia and exercise to exhaustion. $CMR_{glycogen}$ is at least fourfold higher when the volume fraction of astrocytes is taken into account. Inclusion of glycogen consumed during sensory stimulation in calculation of the oxygen-glucose index ($OGI = CMR_{O2}/CMR_{glc}$, which has a theoretical maximum of 6 when no other substrates are metabolized) reduces OGI from 5.0 to 2.8. Thus, at least 53% of the carbohydrate is not oxidized, suggesting that glycogen mobilization supports astrocytic glycolysis, not neuronal oxidation of glycogen-derived lactate that would cause OGI to exceed 6. Failure of glycogenolysis to dilute the specific activity of lactate formed from blood-borne [6-^{14}C]glucose indicates compartmentation of glycolytic metabolism of glucose and glycogen and the rapid release from cerebral cortex of glycogen-derived lactate. Together, these findings invalidate the conclusion by others that glycogen-derived lactate is a major fuel for neurons during

G. A. Dienel (✉)
Department of Neurology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM, USA
e-mail: gadienel@uams.edu

D. L. Rothman
Magnetic Resonance Research Center and Department of Diagnostic Radiology, Yale University, New Haven, CT, USA
e-mail: douglas.rothman@yale.edu

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neurotransmission, memory consolidation, and exercise to exhaustion. Alternative mechanisms, including glucose sparing for neurons, are presented as testable explanations for data interpreted as lactate shuttling.

Keywords Astrocyte · Cerebral cortex · Brain activation · Exercise · Glucose utilization · Glucose sparing · Glycogen · Glycogen shunt · Glycogen turnover · Hypoglycemia · Lactate · Lactate shuttling · Oxygen consumption · Neuron · Neurotransmission · Memory consolidation · Sensory stimulation

Abbreviations

$(A\text{-}V)_{substrate}$	Arteriovenous difference across the brain for the identified substrate
Asp	Aspartate
BAY U6751	4-(2-Chlorophenyl)-L-ethyl-1,4-dihydro-6-methyl-2,3,5-pyridinetricarboxylic acid 5-isopropyl ester disodium salt hydrate
CBF	Cerebral blood flow rate
CMR	Cerebral metabolic rate for substrate of interest = $CBF(A\text{-}V)_{substrate}$
CMR_{glc}	Cerebral metabolic rate for glucose = $CBF(A\text{-}V)_{glc}$
$CMR_{glycogen}$	Cerebral metabolic rate for glycogen = Δ[glycogen]/time
CMR_{O2}	Cerebral metabolic rate for oxygen $CBF(A\text{-}V)_{O2}$
CP-316,819	[R-R∗,S∗]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-L-(phenylmethyl)propyl]-1H-indole-2-carboxamide
DAB	1,4-Dideoxy-1,4-imino-D-arabinitol
DG	2-deoxy-D-glucose
DMSO	Dimethyl sulfoxide
FDG	2-fluoro-2-deoxy-D-glucose
Glc	Glucose
Glc-6-P	Glucose-6-phosphate
Gln	Glutamine
Glu	Glutamate
GLUT	Glucose transporter; GLUT1 in vascular endothelium and astrocytes, and GLUT3 and GLUT4 in neurons
GPR81	G-protein-coupled lactate receptor81, also known as HCAR1
HCAR1	Hydroxycarboxylic acid receptor1 also known as GPR81
KO	Knockout
Lac	Lactate
LTP	Long-term potentiation
MCT	Monocarboxylic acid transporter; MCT1 and MCT4 isoforms are mainly astrocytic, whereas MCT2 is predominantly neuronal
NMDA	*N*-Methyl-D-aspartate

OCI	Oxygen carbohydrate index = $CMR_{O2}/[CMR_{glc} + 0.5CMR_{lac} + CMR_{glycogen}] = (A\text{-}V)_{O2}/((A\text{-}V)_{glc} + 0.5(A\text{-}V)_{lac} + \Delta[\text{glycogen}])$, where lactate and [glycogen] are expressed in glucosyl units (2Lac = 1Glc)
OGI	Oxygen-glucose index = $CMR_{O2}/CMR_{glc} = (A\text{-}V)_{O2}/(A\text{-}V)_{glc}$ (CBF cancels out). This calculation assumes no other substrates are oxidized
PAPs	Peripheral astrocytic processes
RSA	Relative specific activity (SA) = ratio of the SA of a compound of interest to the SA of a reference compound, e.g., SA lactate/SA glucose
SA	Specific activity (dpm/μmol)
TCA	Tricarboxylic acid

1 Introduction

A range of studies have shown the importance of glycogenolysis for critical brain functions, including neurotransmission and memory consolidation (reviewed by (Duran and Guinovart 2015; Bak et al. 2018)). However, the understanding of how glycogenolysis supports specific brain functional processes is complicated by its compartmentation primarily in astrocytes (Cataldo and Broadwell 1986), with small amounts in neurons (Saez et al. 2014; Pfeiffer-Guglielmi et al. 2003). In other organs with high transient increases in energy demand, such as skeletal muscle and heart, glucose-6-phosphate (Glc-6-P) from glycogenolysis directly provides energy via glycolytic ATP to all of the cells. In contrast, ATP from glycolytic metabolism of astrocytic glycogen cannot directly support neuronal energy demands. In the cerebral cortex the fraction of total energy demand from neurons is approximately 2–3 times greater than astrocytes (Yu et al. 2018; Lanz et al. 2013), yet the glucose is stored as glycogen mainly in astrocytes and its consumption is triggered by specific neurotransmitters and other compounds related to neuronal signaling activity (Obel et al. 2012). Among its functions, glycogen can preferentially fuel ATPases for pumping of K^+ (Hertz et al. 2015a, b) and Ca^{++} (Müller et al. 2014) that probably contribute to its roles in higher brain functions.

1.1 Energetic Advantage of Glycogenolysis

Glc-6-P is a ‘branch-point’ metabolite that can either continue down the glycolytic pathway, enter the pentose phosphate shunt pathway, or be stored by its incorporation into glycogen, predominantly in astrocytes (Fig. 1). Glycogen synthesis involves several enzymatic steps: the phosphoglucomutase reaction transforms Glc-6-P to Glc-1-P which is then converted to UDP-glc by UDP-glc pyrophosphorylase prior to incorporation of the glucosyl group into glycogen by glycogen synthase.

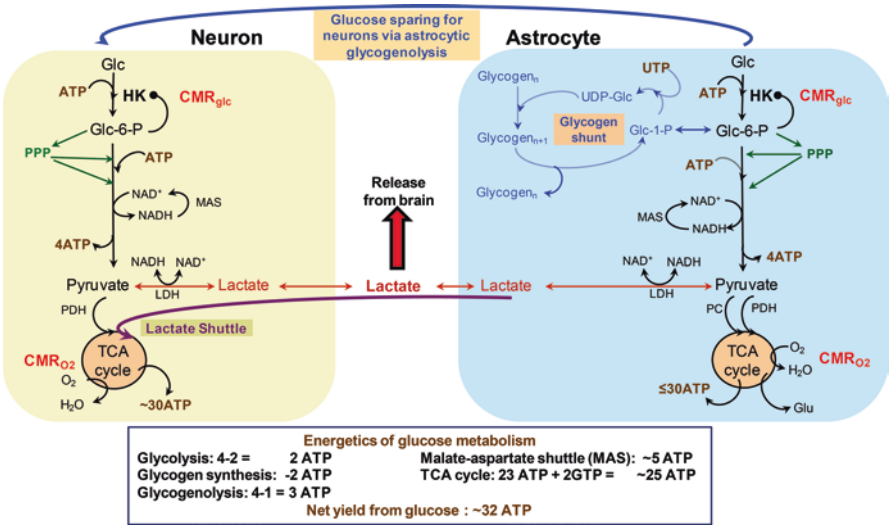

Fig. 1 Models for the glycogen shunt and fate of glycogen carbon. Glucose (Glc) is metabolized to form pyruvate via the glycolytic pathway with a net yield of 2 ATP, and another 30 ATP are produced by the oxidative pathway and the malate-aspartate shuttle (MAS) that transfers NADH reducing equivalents from the cytoplasm to mitochondria. MAS activity is required to produce pyruvate as an oxidative and anaplerotic substrate because regeneration of NAD^+ by the lactate dehydrogenase (LDH) reaction causes loss of pyruvate as lactate, which must be released from the cell for glycolysis to continue. The hexokinase (HK) reaction is the first irreversible step in the glycolytic pathway, and it is feedback inhibited by glucose-6-phosphate (Glc-6-P) (denoted by the 'ball'). The flux through the hexokinase step represents the total rate of glucose utilization (CMR_{glc}) that can be compared with the rate of oxygen consumption (CMR_{O2}). The stoichiometry of oxygen-to-glucose utilization can be quantified by the oxygen-glucose index (OGI = CMR_{O2}/CMR_{glc}, according to the reaction $1\ Glc + 6O_2 \rightarrow 6\ CO_2 + 6H_2O$). As long as no other substrates are metabolized the theoretical maximal OGI is 6. Some Glc-6-P can enter the pentose phosphate shunt pathway (PPP), and in normal adult brain, most of the carbon re-enters the glycolytic pathway at the fructose-6-P and glyceraldehyde-3-P steps. Most glycogen in brain is located in astrocytes, although very small amounts are present in neurons (not shown). Glycogen synthesis requires 1 ATP equivalent as UTP, so incorporation of 1 glucosyl unit into glycogen ($glycogen_n \rightarrow glycogen_{n+1}$) uses 2 ATP. Glycogenolysis has a net yield of 3 ATP, for an overall yield of 1 ATP per turnover of 1 glucosyl unit. The advantage of storage of glucose in glycogen is that synthesis can be carried out when energy demand is lower and degradation can quickly fulfill increased ATP demand. The yield from pyruvate oxidation via the pyruvate dehydrogenase (PDH) reaction in astrocytes is the same as neurons, but if some pyruvate enters the TCA cycle via the pyruvate carboxylase (PC), the ATP yield is reduced because 1 ATP is used for CO_2 fixation, and the resulting oxaloacetate serves as a catalytic or anaplerotic compound. The anaplerotic capability of astrocytes to synthesize glutamate de novo from glucose requires the PC reaction to make oxaloacetate and the PDH reaction to add one acetyl CoA to oxaloacetate to make a 'new' 6-carbon compound, citrate, that is metabolized further to α-ketoglutarate and transaminated to form a new molecule of glutamate (Glu). The lactate shuttle is proposed to transfer glucose- and/or glycogen-derived lactate from astrocytes to neurons where it can be oxidized. This process requires stoichiometric balance of CMR_{O2} with total CMR_{glc} plus $CMR_{glycogen}$, which is not observed experimentally (see Sect. 3.1). An alternative model proposed by DiNuzzo et al. (2010a) is that glycogenolysis reduces astrocytic demand for blood-borne glucose during brain activation, sparing an equivalent amount of glucose for neuronal utilization (see Sect. 4). This model is evaluated in our companion paper (Rothman and Dienel 2019). Modified from Fig. 9 in Dienel (2019a) © American Physiological Society, with permission

Glycogen is degraded by phosphorylase to produce Glc-1-P, followed by its conversion to Glc-6-P, then metabolism by glycolysis or the pentose shunt. Glycogen has the energetic advantage that its synthesis can occur during periods of lower ATP demand and its mobilization does not require the ATP to generate Glc-6-P, thereby providing a net yield of 3 glycolytic ATP per glucosyl moiety compared with 2 ATP from glucose. Thus, the cost of 1 ATP plus 1 UTP required to incorporate one glucosyl unit into glycogen is 'pre-paid', and glycogenolysis quickly generates Glc-6-P for metabolism via energy- or NADPH-producing pathways when local demand is accelerated. Cycling of Glc-6-P through glycogen instead of its direct entry into downstream pathways is called the glycogen shunt (Shulman et al. 2001; Shulman and Rothman 2001) (Fig. 1).

1.2 Roles of Glycogen in Astrocytic and Neuronal Glycolysis

Insight into the mechanism by which glycogenolysis may additionally support neuronal energetics comes from consideration of the two pathways of astrocytic Glc-6-P formation, hexokinase acting on glucose and glycogen phosphorylase acting on glycogen. Hexokinase is the first irreversible enzymatic step of the glycolytic pathway (Fig. 1), and Glc-6-P regulates hexokinase activity by noncompetitive feedback inhibition (Crane and Sols 1954). Hexokinase type I is the predominant isoform in brain, with some type II, and little of the major liver isozyme, type III hexokinase or glucokinase (Grossbard and Schimke 1966). The type III isoform has been detected in nuclear periphery of brain cells, including cerebellar Purkinje neurons (Wilson 2003; Sebastian et al. 1999; Preller and Wilson 1992). Hexokinase Type I is 50% inhibited by Glc-6-P in the range of 0.02–0-.07 mM for soluble, particulate, and homogenate preparations (Newsholme et al. 1968; Wilson 2003). Normal brain Glc-6-P concentration is about 0.1–0.2 mM (Veech et al. 1973), and brain hexokinase activity in vivo is only ~3% of its maximal activity assayed in vitro, due mainly, but not exclusively, to inhibition by Glc-6-P (Lowry and Passonneau 1964; Bachelard and Goldfarb 1969).

Inhibition of hexokinase by Glc-6-P may be important for two reasons. First, passage of Glc-6-P through astrocytic gap junctions is highly restricted, in contrast to glyceraldehyde-3-P, lactate, NADH, NADPH, anionic fluorescent dyes, amino acids, and various electrolytes that can readily diffuse through the syncytium ((Gandhi et al. 2009b) and references cited therein). Retention of Glc-6-P in the astrocyte where it was produced from glucose or glycogen can contribute to regulation of the rate utilization of blood-borne glucose (CMR_{glc}) in each astrocyte by governing its hexokinase rate. Second, DiNuzzo and colleagues (2010b, 2011, 2012) proposed the novel hypothesis based on metabolic modeling that Glc-6-P produced from glycogen during brain activation may further inhibit hexokinase, thereby reducing astrocytic utilization of blood-borne glucose and sparing an

equivalent amount of this glucose for use by neurons. In this model, glycogenolysis bypasses the hexokinase step and provides the Glc-6-P for glycolytic metabolism in astrocytes, whereas blood-borne glucose fuels the incremental rise in glycolytic flux in activated neurons (see Sect. 4).

1.3 Glc-6-P and Regulation of Glycogen Phosphorylase Isoforms in Brain Cells

Glycogen phosphorylase exists as three major isoforms, liver, muscle, and brain. Most studies of the complex regulation of these enzymes by small ligands and phosphorylation are based on muscle and liver phosphorylase, and much less is known about regulation of brain phosphorylase (Nadeau et al. 2018). Muscle phosphorylase b (AMP-dependent, non-phosphorylated, inactive state) is activated by AMP (as well as Ca^{++} and phosphorylation), and Glc-6-P inhibits this activation by competing with AMP for the same binding site, whereas Glc-6-P has little effect on muscle phosphorylase a (AMP-independent, phosphorylated, active state) (Griffiths et al. 1976; Danchin and Buc 1973; Morgan and Parmeggiani 1964). In hepatocytes, regulation by Glc-6-P is more complex, and it can govern the subcellular distribution of phosphorylase a and act in conjunction with glucose to inhibit phosphorylase a (Aiston et al. 2003, 2004).

The brain and muscle glycogen phosphorylase isoforms are more similar to each other than to the liver isoform (Hudson et al. 1993). Both the brain and muscle isozymes are expressed in astrocytes (Pfeiffer-Guglielmi et al. 2000, 2003), whereas only the brain isoform is in neurons (Pfeiffer-Guglielmi et al. 2003; Saez et al. 2014). The brain and muscle (and liver) isoforms differ in terms of their activation by phosphorylation and AMP and other properties (Crerar et al. 1995; Mayer et al. 1992)}, as well as their responses to norepinephrine vs. AMP (Müller et al. 2015) and to glycolytic supercompensation (Jakobsen et al. 2017). Brain phosphorylase a is inhibited by Glc-6-P with a K_i of ~5 mM, and its kinetics with brain phosphorylase b are complex, showing activation by Glc-6-P up to 0.5 mM and inhibition at concentrations >2 mM (Mayer et al. 1992). These findings suggest that any increases in brain Glc-6-P concentration during glycogenolysis may preferentially further suppress hexokinase activity, as opposed to inhibiting phosphorylase, but many factors govern phosphorylase activity. To our knowledge, changes in Glc-6-P concentration in astrocytes have not been evaluated during glycogen mobilization, and future studies to evaluate the DiNuzzo hypothesis require detailed metabolic analyses in astrocytes after the onset of glycogenolysis, including hexokinase activity and changes in the concentrations of Glc-6-P and Glc.

1.4 Technical Difficulties in Evaluating Glycogen Level and Turnover in Brain Tissue

Measurements of glycogen concentration, glycogen turnover, and the magnitude of the glycogen shunt in living brain are extremely difficult for many reasons. First, glycogenolysis is activated by various neurotransmitters, especially norepinephrine, when subjects experience stress, alerting, handling, sensory stimulation, and other situations. Second, phosphorylase is very rapidly activated by increased energy demand during normal brain activation, as well by hypoglycemia, hypoxia, anoxia, and ischemia. Assays of glycogen concentration and labeling, therefore, require rapid enzyme inactivation (e.g., by in situ freezing or microwave fixation) in conjunction with appropriate handling care and extraction and analytical procedures. Third, glycogen synthesis can help maintain overall glycogen concentration in tissue during activation, and glycogen shunt flux can minimize net incorporation of label into glycogen due to greater turnover of the outer tiers compared with limit dextrin (Watanabe and Passonneau 1973). Fourth, complexity of glycogen turnover also arises from the possibility that any single glycogen molecule may turn over independently of other glycogen granules in the same cell, and both synthesis and degradation of glycogen in different regions (e.g., endfeet, cytosol, large processes, and perisynaptic peripheral astrocytic processes [PAPs]) of one astrocyte may take place simultaneously. Also, glycogen turnover in different astrocytes in the same brain region of interest may be governed by conditions in each cell as well as by neurotransmitters. Thus, regulation of glycogen level and turnover can be governed simultaneously at global, local, and cellular levels.

1.5 Glycogenolysis in Activated Cerebral Cortex

This review focuses on selected, quantitative studies of glycogen in cerebral cortex of awake rats to emphasize the importance of further studies of the poorly-understood roles of glycogen in astrocytic energetics in living brain. Evidence is presented for high rates of glycogenolysis, on-going turnover during activation, and predominant conversion to lactate that is released from cerebral cortex. Glycogen consumption during acute hypoglycemia serves to compensate for the glucose supply-demand mismatch, rates are lower than during sensory stimulation or exhaustive exercise. The notion of glycogen-derived lactate shuttling to neurons (Fig. 1) to maintain neurotransmission and memory consolidation or to fuel the brain during exhaustive exercise is challenged, and alternative mechanisms are presented to explain reported data. Strong emphasis is placed on the importance of comprehensive, quantitative assays of interrelated metabolic fluxes and testing of predicted stoichiometries of models for roles of glycogen in brain functions. The following sections describe glycogen utilization during activation, evidence for glycolytic metabolism of

glycogen, release of glycogen-derived lactate from cerebral cortex, the concept of glucose sparing for neurons by astrocytic glycogenolysis, roles for glycogen in neurotransmission and learning/memory, and evaluation of glycogenolysis rates during acute hypoglycemia and exercise to exhaustion. Because aspects of these discussions require background in metabolism to appreciate the technical complexities of analysis and interpretation of roles of glycogen in brain function, the introductory and summary material is intended to highlight 'take-home' concepts for non-experts.

2 Glycogen Level, Glycogenolytic Rate, and Turnover During and After Sensory Stimulation of Awake Rats

Glycogen is an important storage site for glucose in brain, and its utilization in astrocytes during physiological activation is a major component of overall brain energetics.

2.1 Mobilization of Unlabeled Glycogen

Comparison of the net rate of glycogenolysis ($CMR_{glycogen}$) in cerebral cortex with CMR_{glc} during and after sensory stimulation of awake rats reveals that $CMR_{glycogen}$ is a large fraction of the rate of utilization of blood-borne glucose by all cells. In two independent experiments carried out a year apart, the mean concentration of glycogen in resting cerebral cortex of very carefully-handled awake rats was 12.4 μmol/g (Table 1), similar to levels reported in other studies (Kong et al. 2002; Oe et al. 2016) and several fold higher than reported in earlier studies (see references cited in (Cruz and Dienel 2002)). It was further shown that the lower values in earlier studies could be quantitatively explained by losses that occurred in the extraction procedures used (Cruz and Dienel 2002).

After 5–6 min of generalized sensory stimulation, glycogen concentration fell 23%, and decreased further at 15 min recovery for a net decrease of 4 μmol/g, or 32% (Table 1). CMR_{glc} was determined in the Dienel et al. (2002) study by measuring the rate of incorporation of plasma [6-^{14}C]glucose into total metabolites. CMR_{glc} increased 26% during stimulation and normalized within 15 min after termination of stimulation. However, this value for calculated CMR_{glc} during activation is probably underestimated due to rapid efflux of [^{14}C]lactate from tissue (Cruz et al. 1999, 2007; Adachi et al. 1995) and because it was based on label accumulation mainly in TCA cycle derivatives when only one triose was labeled by [6-^{14}C]glucose. If instead [1,6-^{14}C]glucose were used as the precursor, labeling of derivatives of pyruvate and calculated CMR_{glc} would be about twice as high.

Table 1 CMR_{glc}, glycogen levels, and calculated $CMR_{glycogen}$ in cerebral cortex of awake rats during rest, sensory stimulation, and recovery

Condition	CMR_{glc} (μmol/g/min)	Unlabeled glycogen (μmol/g)	Mean change in [glycogen] during activity interval (μmol/g)	[a]$CMR_{glycogen}$ (μmol/g/min) [%CMR_{glc}]	[b]Unlabeled glycogen if astrocytes 25% of brain mass (μmol/g/min)	$CMR_{glycogen}$ if astrocytes 25% of brain mass (μmol/g/min) [%CMR_{glc}]
Rest	0.74[c]	12.3[c], 12.5[d] (12.4)	—	–	49.6	–
Activation, 5 min	0.93[c]∗	9.6[c], 9.6[d] (9.6)	2.8	0.56 [60%]	38.4	2.24 [241%]
Recovery, 15 min	0.73[c]	8.2[c], 8.5[d]∗ (8.4)	1.2	0.08 [11%]	33.4	0.32 [44%]

∗$p < 0.05$ vs. rest

[a]$CMR_{glycogen}$ was calculated by dividing the mean net change in glycogen concentration during the indicated experimental interval by the duration of the interval. [b]Average tissue volume fraction occupied by astrocytes was stated by Hertz and colleagues (Hertz et al. 2007) to vary regionally and estimated to be *at most* 20–25% (for further discussion and cited references, see the section in Hertz et al. 2007 entitled, "High oxidative rates in astrocytes in the brain in vivo"); also see Sect. 2.1 and (Gundersen et al. 2015). Mean values in awake rats are from [c](Dienel et al. 2002) that used a 5 min sensory stimulation duration and metabolic labeling with [6-^{14}C]glucose, and [d](Cruz and Dienel et al. 2002) that analyzed samples from our previous study (Madsen et al. 1999) that used a 6 min sensory stimulation duration. Means of the two studies are indicated in parentheses and used to calculate changes in [glycogen] during activity intervals

$CMR_{glycogen}$ during activation and recovery was 0.56 and 0.08 μmol/g/min, equivalent to 60% and 11%, respectively, of calculated CMR_{glc} in all cerebral cortical cells using [6-^{14}C]glucose (Table 1). For comparison, glycogenolysis rates determined in earlier literature under various conditions were lower and ranged from 0.003 to 0.1 μmol/g/min, but increased to ~2–3 μmol/g/min during ischemia (see Table 3 in (Dienel 2012)).

Based on an estimated astrocytic volume fraction in cerebral cortex of *at most* 20–25% (Hertz et al. 2007), resting glycogen concentration in astrocytes would be at least fourfold higher, ~50 μmol/g (Table 1), approaching that in resting gastrocnemius muscle, ~70 μmol/g (Price et al. 1994). When an estimated astrocytic mass of 25% is taken into account, $CMR_{glycogen}$ exceeds calculated CMR_{glc} by 2.4-fold during activation, and it rises to 44% of CMR_{glc} during recovery (Table 1). Use of ~25% mass fraction is conservative, and $CMR_{glycogen}$ per unit astrocytic mass may be much higher because Gundersen et al. (2015) stated in their "Section A. Neuroglia" that the glial (mostly astrocytic processes) contribution to gray matter neuropil in CA1 hippocampus ranges from 4% to 6% in rat and human, and rarely exceeds 20%. These remarkably-high rates of glycogenolysis at the cellular level during activation and recovery indicate that glycogen fulfills important, unidentified functions. Furthermore, they suggest that glycolytic ATP from glycogen derived Glc-6-P

may be the major energy source for astrocytes during intense transient increases in metabolic demand associated with neuronal activation.

2.2 Estimated Glycolytic ATP Production Rates from Glycogenolysis

The rate of glycolytic ATP generation from $CMR_{glycogen}$ during sensory stimulation (Table 1) is 0.56 μmol/g/min × 3 ATP/glucosyl moiety = 1.68 μmol ATP/g/min in cerebral cortex, and it is at least 3 × 2.24 = 6.72 μmol ATP/g/min in astrocytes based on their estimated mass fraction. For comparison to overall ATP production from glucose during stimulation, the OGI fell from about 5.5 to 5 (see Sect. 3.1.1 and Table 4), indicating that 5/6 or 83% was oxidized and 17% metabolized glycolytically. CMR_{glc} = 0.93 μmol/g/min and oxidative ATP production rate is 0.93 × 0.83 × 30 μmol ATP/glc = 23.2 μmol/g/min, whereas glycolytic ATP rate is 0.93 × 0.17 × 2 ATP/glc = 0.32 μmol/g/min, for a total of 23.5 μmol ATP/g/min. Thus, estimated astrocytic glycolytic ATP production rate from glycogen is ~5–21 times higher than glucose-derived glycolytic ATP formation in all cells (i.e., 1.68/0.32 or 6.72/0.32), and it corresponds to ~7–29% to total ATP production from blood-borne glucose during activation. These numbers are regarded as tentative because the moment-to-moment metabolic rates and the true $CMR_{glycogen}$ in astrocytes are not known, but they provide an initial data set to evaluate the importance of glycogenolysis to astrocytic energetics.

2.3 Metabolic Labeling Reveals that the Glycogen Shunt is Active During Sensory Stimulation of Awake Rats

The glycogen shunt (Shulman et al. 2001; Shulman and Rothman 2001) was proposed to explain why astrocytes use glycogen during activation even though adequate glucose supplies are available. In analogy to muscle, glycogenolysis is used when there are brief needs for rapid ATP production during high levels of neuronal activity (Fig. 1). In between these periods glucose is shunted from Glc-6-P to resynthesize lost glycogen. Evidence for shunting of Glc-6-P through glycogen during activation can be obtained by comparison of the fractional release of label from pre-labeled glycogen with the magnitude of change in total glycogen concentration. When glycogen was pre-labeled with [1-^{14}C]glucose followed by multi-modal sensory stimulation involving gentle brushing of the whiskers and body along with acoustic and visual stimulation of awake rats, the percent decrease in labeled glycogen exceeded that of the net decrease in unlabeled glycogen concentration in three brain regions (Table 2). Label release was detectable in cerebral cortex, inferior colliculus, and superior colliculus, whereas glycogen concentration fell only in

Table 2 Release of label from glycogen exceeds fall in concentration of unlabeled glycogen during activation

Brain region	Unlabeled glycogen (μmol/g)			Labeled glycogen (nCi/g)		
	Rest	Activation	% change	Rest	Activation	% change
Cerebral cortex	4.6	4.0∗	−13	2.3	1.75∗	−24
Inferior colliculus	4.1	4.0	−2	2.4	2.0∗	−17
Superior colliculus	3.7	3.7	0	2.0	1.8∗	−10

∗$p < 0.05$ vs. rest. From Fig. 1 of (Dienel et al. 2007a). The amount of ^{14}C-label recovered in purified glycogen from each brain was normalized to its respective integrated specific activity of arterial plasma glucose to take into account any differences in precursor availability during the labeling period

cerebral cortex. Thus, glycogen synthesis was on-going even as it was degraded, and synthesis matched degradation in the two colliculi. Net incorporation of [6-^{14}C] glucose into cerebral cortical glycogen during rest, activation, and recovery, i.e., 0.003, 0.002, and 0.005 μmol/g/min, respectively, confirmed the continuous but low net rates of incorporation of blood-borne glucose into glycogen in cerebral cortex during all stages of the activity cycle (Dienel et al. 2002).

Label release exceeded concentration change by 10–17%, and using mean glycogen level of ~4 μmol/g (Table 2) the outer tier degradation rate during the 10 min stimulation interval is estimated to be 0.04–0.07 μmol/g/min (i.e., (4 μmol/g) × (0.1–0.17)/10 min), a rate that can be compared with CMR_{glc} in these three regions of ~0.9–2 μmol/g/min during activation (Dienel et al. 2002, 2007a). As a very rough estimate, glycogen shunt flux in this experimental paradigm, based on outer tier turnover, is ~20–40 times higher than net incorporation of label from blood-borne glucose into glycogen during activation, but it is a small fraction (<5–10%) of glucose utilization by all cells. However as pointed out by Öz, DiNuzzo, and colleagues (Öz et al. 2015, 2017; DiNuzzo 2013) the rate of glycogen synthesis may be substantially greater than estimated from total label incorporation due to the complexity of the glycogen turnover process which does not homogeneously incorporate label across the glycogen particles present.

2.4 Compensatory Increases in CMR_{glc} When Glycogenolysis is Inhibited

The significance of glycogenolysis to energetics of sensory stimulation is underscored by the regionally-heterogeneous increases in utilization of blood-borne glucose in brain of awake rats when glycogenolysis was inhibited with CP-316,819 (Dienel et al. 2007a). This study used the protocol of Suh et al. (2007) who showed an 88% increase in glycogen level in vivo after treatment with CP-316,819 treatment. This protocol also prolonged EEG activity and reduced neuronal death during severe hypoglycemia, consistent with release of phosphorylase inhibition

by CP-316,819 that requires glucose concentrations in the normoglycemic range (see discussion and references cited by Suh et al.). In this experiment it was hypothesized that if glycogenolysis were activated by sensory stimulation, blocking glycogen phosphorylase would result in its breakdown being replaced by an equivalent amount of glucose consumption above the level measured in control animals without the inhibitor. As illustrated in Table 3, sensory stimulation caused especially-large responses in layer 4 plus adjacent tissue of parietal and sensory cortex, with net rises in CMR_{glc} above resting rates of 0.9 and 1.37 μmol/g/min, respectively. These net increases approximate or exceed their resting CMR_{glc}, and are 2.9- and 1.8-fold higher than the respective responses in vehicle-treated rats. Furthermore, the net compensatory increases in CMR_{glc} in layer 4 of sensory and parietal cortex over and above those in vehicle-treated rats during stimulation (~0.60 μmol/g/min, Table 3) are similar to $CMR_{glycogen}$, 0.56 μmol/g/min, for the entire dorsal cerebral cortex and correspond to 50–55% of resting CMR_{glc} by all cells (Table 3). If the regional differences are set aside, these findings suggest that most of the compensatory increases in CMR_{glc} may occur mainly in astrocytes to replace the energy that in control animals was supplied by glycogenolysis. Furthermore, as discussed in Sect. 4, the large increase in glucose consumption when glycogen phosphorylase is inhibited supports the hypothesis that astrocytic glycogenolysis spares glucose during intense activity for the neuron.

2.5 Summary: Contribution of Glycogen in Energetics

Glycogen is present in cerebral cortex at high levels, its utilization rate during sensory stimulation is a large fraction of total carbohydrate consumption (blood-borne glucose utilization plus glycogenolysis) by all cells. Glycolytic ATP production rate by glycogenolysis exceeds the amount of glycolytic ATP generated from blood-borne glucose by all cells, glycogen synthesis and degradation take place continuously but at rates that vary with physiological state. Consistent with its proposed key energetic role inhibition of glycogenolysis causes a large increase in utilization of blood-borne glucose.

3 Fate of Glycogen Carbon Skeleton

Glycolytic metabolism of glycogen produces pyruvate in astrocytes that can have various alternative metabolic fates: oxidation via the TCA cycle, transamination to alanine, carboxylation to oxaloacetate by pyruvate carboxylase, and reduction to lactate that can be released from the activated tissue to blood or perivascular fluid or shuttled to neurons for oxidation as supplemental fuel. Detailed analyses of metabolite concentration changes, metabolic pathway fluxes, and metabolic

Table 3 Selected compensatory increases in CMR_{glc} when glycogenolysis in awake rats is inhibited with CP-316,819

Brain region	CMR_{glc} in vehicle-treated rats (μmol/g/min)			CMR_{glc} in CP-316,819-treated rats (μmol/g/min)			Net increase CP-316,819 vehicle	Net increase (CP—vehicle)	Net increase % of resting CMR_{glc}
	Rest	Activation	Net increase	Rest	Activation	Net increase			
Sensory cortex	1.06	1.66	0.60	1.01	2.02	1.01	1.68	0.41	38.7
Layer 4 + surround	1.20	1.97	0.77	1.01	2.38	1.37	1.77	0.60	50.0
Parietal cortex	0.99	1.34	0.35	0.96	1.56	0.60	1.71	0.25	25.3
Layer 4 + surround	1.06	1.37	0.31	0.99	1.89	0.90	2.90	0.59	55.7

Data are from (Dienel et al. 2007a) where CMR_{glc} in many brain regions are reported. Rats were pre-treated with an inhibitor of glycogenolysis (CP-316,819) or vehicle, then CMR_{glc} was assayed during a 30 min interval in both groups with [^{14}C]deoxyglucose during rest and generalized sensory stimulation. Cortical layer 4 is the location of the thalamo-cortical input that is highly activated in the whisker-to-barrel cortex when the whiskers are brushed

labeling patterns described in the following sections provide strong evidence that most glycogen is not oxidized in astrocytes or neurons and is instead released from activated tissue.

3.1 Glycogen-Derived Pyruvate/Lactate is Not Completely Oxidized: Oxygen Consumption is Too Low

3.1.1 Oxygen-Glucose Index (OGI) Falls During Activation

Evidence for oxidation of glycogen-derived pyruvate in living brain is very difficult to obtain and distinguish from glucose oxidation. However, an indirect approach is to evaluate the oxygen-to-carbohydrate stoichiometry during activation and recovery. We previously determined the oxygen-glucose index (OGI), the ratio of the rate of oxygen utilization (CMR_{O2}) to CMR_{glc} ($OGI = CMR_{O2}/CMR_{glc} = CBF(A\text{-}V)_{O2}/CBF(A\text{-}V)_{glc} = (A\text{-}V)_{O2}/(A\text{-}V)_{glc}$, where CBF = cerebral blood flow rate) by measuring the arteriovenous differences (A-V) for oxygen, glucose, and lactate (lac) in the same blood samples during rest, activation, and recovery (Madsen et al. 1999). OGI fell from 5.5 to 5.0 during activation and rose to 7.2 during recovery (Table 3). When no other substrates are metabolized, the theoretical maximum for OGI is 6.0 because 6 O_2 are required for complete oxidation of one glucose molecule ($6\ O_2 + 1\ Glc \rightarrow 6\ CO_2 + 6\ H_2O$).

3.1.2 Inclusion of Glycogen Reduces Oxygen-Carbohydrate Index (OCI) Below OGI

To assess whether CMR_{O2} can fully support glycogen oxidation, the amount of glycogen expressed in glucosyl units consumed during a 1-minute interval (corresponding to the duration of the (A-V) sampling interval) and lactate expressed in glucose equivalents was included in the OGI calculation. The resulting oxygen-carbohydrate index ($OCI = CMR_{O2}/(CMR_{glc} + (CMR_{lac}/2) + CMR_{glycogen}) = (A\text{-}V)_{O2}/((A\text{-}V)_{glc} + [(A\text{-}V)_{lac}/2] + \Delta[\text{glycogen}]$ in 1 min) fell from 4.95 to 2.80 during stimulation and from 7.2 to 6.1 during recovery (Table 3). Note that inclusion of three major carbohydrate pools in the OCI calculation (Glc, Lac, glycogen) brings the ratio closer to 6.0 during rest and recovery. However, the glycogen level is still subnormal after 15 min recovery and replenishment will consume glucose without oxygen to depress OGI (see Sect. 3.2). In addition to these carbohydrates, about 20% of the glutamate taken up into astrocytes during excitatory neurotransmission is oxidized (Hertz and Rothman 2017). Based on the rate of the glutamate-glutamine cycle (neurotransmission rate, V_{NT}) in awake rats of 0.57 μmol/g/min (Lanz et al. 2013), astrocytic glutamate oxidation would be $0.57 * 0.2 = 0.11$ μmol/g/min, and

Table 4 Influence of glycogenolysis on magnitude of fall in oxygen-glucose index (OGI) during sensory stimulation and recovery and duration of reduced glycogen concentration during recovery

Stage of activity cycle	[a]Glycogen concentration (μmol/g)	Δ[glycogen] per min (μmol/g)	[b]$(A\text{-}V)_{O2}$ (μmol/g)	[b]$(A\text{-}V)_{glc}$ (μmol/g)	[b][$(A\text{-}V)_{lac}$]/2 (μmol/g)	[b]OGI	[c]OCI	Time to restore initial glycogen level (hours)
Rest	12.3	–	3.75	0.68	−0.04	5.51	5.86	
Activation	9.6	0.45	2.97	0.60	0.01	4.95	2.80	
Recovery	8.5	0.07	3.82	0.53	−0.025	7.21	6.11	
Net consumed	3.8							
Estimated glycogen synthesis rate (μmol/g/min)								
0.005[d]								12.6
0.037[e]								1.7
0.073[e]								0.9

[a] From (Cruz and Dienel et al. 2002) that used a 6 min sensory stimulation duration, and glycogen was measured in ethanol extracts of the same brains of animals in which [b] mean arteriovenous differences (A-V) for oxygen (O_2), glucose (glc), and lactate (lac) were determined by (Madsen et al. 1999). These mean values were used to calculate the oxygen-glucose index as OGI = $(A\text{-}V)_{O2}/(A\text{-}V)_{glc}$ (Note: these values differ somewhat from the means of the individual OGIs determined in each animal) and the [c]oxygen-carbohydrate index, OCI = $(A\text{-}V)_{O2}/[(A\text{-}V)_{glc} + ((A\text{-}V)_{lac})/2 + \Delta[\text{glycogen/min}])$, where the lactate was converted to glucose equivalents by dividing by 2 and glycogen consumed during the 1 min interval of the arteriovenous sample was calculated as 1 min × (Δ[glycogen]/assay interval duration); the interval was 6 min for activation and 15 min for recovery. [d]Measured rate of net incorporation of blood-borne glucose into glycogen during recovery from a 5 min stimulation, i.e., 0.005 μmol/g/min (Dienel et al. 2002). [e]Estimated as either 5 or 10% of measured CMR_{glc} (i.e., 0.73 μmol/g/min (Dienel et al. 2002)) during recovery (i.e., 0.037 or 0.073 μmol/g/min)

inclusion glutamate oxidized (as glucose equivalents: 0.11∗[5/6]) in 1 min would further reduce OCI to 2.6 during activation (ignoring the complexities of truncated vs. complete oxidation of glutamate and conversion to glucose equivalents).

If all of the glycogen consumed during 1 min of activation (0.45 μmol/g) were oxidized, 2.70 μmol O_2/g would be required (6 × 0.45). However, only a small excess of this amount, 2.97 μmol O_2/g, was actually taken up into during activation and cannot support complete oxidation of the glucose taken up (Table 4), indicating that lactate is probably generated from both glucose and glycogen. Based on an OCI of 2.8, about 47% of the total carbohydrate was oxidized (2.8/6). The partitioning between oxidation of glucose and glycogen cannot be determined in this study, but

if glycogenolysis is assumed to replace all of the astrocyte's oxidative ATP from glucose (basal plus activated) the following calculation can be made. Based on the calculations in Sect. 3.3.2 (second paragraph), if glycogen fully replaces oxidation of glucose, then the rate of lactate production from glycogen in astrocytes is 0.4 μmol/g/min (i.e., 2∗[0.54–0.34]). From Table 5, the net rise in cerebral cortical lactate level during activation is 1.1 μmol/g divided by 5 min equals a rate of 0.22 μmol lactate/g/min. Because glycogen-derived lactate does not dilute blood-borne glucose-derived lactate (see below, Sect. 3.3.2 and Table 5), most of the lactate from glycogen must be quickly released at a rate about twice that (0.4/0.22) of lactate accumulation in cerebral cortex that presumably occurs mainly in neurons.

3.1.3 Glycogen Carbon Utilization to Synthesize Amino Acids and Other Compounds

Alanine was not included in the above calculations because its cellular distribution is not known, the increase in its concentration during activation was small (0.2 μmol/g), and, although its labeling doubled, it was a minor fraction of the total metabolite pool; serine level and labeling were unchanged from rest to activation (Dienel et al. 2002). Pyruvate carboxylase activity was not measured, but is typically small compared to glucose oxidation in neurons and astrocytes (Yu et al. 2018; Lanz et al. 2013). Mass balance calculations based on changes in concentrations of measured metabolites and the number of carbons per compound indicates that the fall in glycogen carbon greatly exceeded the sum of the incremental changes in the other metabolites retained in brain (Dienel et al. 2002), consistent with metabolite release from cerebral cortex.

In day-old chick brain, strong circumstantial evidence supports the conclusion that glycogen is a precursor for glutamate synthesis during specific stages of memory consolidation (Gibbs and Hertz 2005; Gibbs et al. 2007; Hertz and Gibbs 2009; Gibbs 2016), indicating that some glycogen-derived pyruvate enters the anaplerotic astrocytic oxidative pathway via pyruvate carboxylase and pyruvate dehydrogenase (Fig. 1). However, the actual quantity of glycogen carbon used for glutamate synthesis in chick brain needs to be determined. In view of the much higher glutamate levels in neurons compared with astrocytes (Ottersen et al. 1992; Storm-Mathisen et al. 1983), the relatively small perisynaptic compartments that are associated with glutamate-glutamine fluxes, and the oxidation of an estimated 20% of the glutamate upon its uptake into astrocytes (McKenna et al. 1996; Hertz and Rothman 2017), the replenishment of transmitter glutamate associated with memory stages with carbon from glucose and/or glycogen probably involves small pools and needs to be examined in more detail.

Table 5 Influence of glycogenolysis on lactate specific activity in cerebral cortex during sensory stimulation

Compound	Concentration (μmol/g)		Specific activity (SA) (dpm/μmol)		Lactate SA relative to Glucose SA (RSA)		Lactate SA if glycogen → Lac	Lactate RSA if glycogen → Lac
	Rest	Activation	Rest	Activation	Rest	Activation	Activation	Activation
Glucose	1.9	2.0	88	78	1.0	1.0		
Lactate	0.6	1.7	46	46	0.55	0.59		
Glycogen	12.3	9.6	0.29	0.27	0.003	0.003		
Δ[Glycogen] → Lac								
Total		2.7 → 5.4 Lac					11	0.14
Amount in 1 min		0.54 → 1.08 Lac					28	0.36

Mean values are from Tables 3 and 5 of (Dienel et al. 2002) that used a 5 min stimulation duration in which brain metabolites were labeled by intravenous injection of [6-^{14}C]glucose for 5 min. Because [6-^{14}C]glucose produces one labeled and one unlabeled lactate, the specific activity (SA) of lactate would be half that of glucose if there were no dilution or loss because 1 mole of glucose generates 2 moles of lactate with the same dpm as glucose; a relative specific activity (RSA) of 0.5 is the theoretical maximum. The calculated lactate SAs and RSAs that include the lactate equivalents of glycogen consumed are the values that would be expected if all of the glycogen-derived lactate were retained in the brain or if the amount of lactate produced in one min were retained in the brain. Calculated dilutions would occur if the lactate remained in astrocytes throughout the experimental interval, then diluted the blood-borne glucose-derived [^{14}C]lactate upon homogenization of tissue, or if glycogen-derived lactate continuously mixed with and diluted the blood-borne [^{14}C]glucose-derived [^{14}C] lactate

3.2 Glycogen Resynthesis During Recovery Contributes to Low CMR_{O2}/CMR_{glc}

Aerobic glycolysis is the preferential upregulation of carbohydrate utilization compared with oxygen consumption even though oxygen level and delivery to brain is normal (Dienel and Cruz 2016), and aerobic glycolysis can be quantified by the extent of the fall in OGI or OCI. Not only does glycogenolysis substantially increase the magnitude of aerobic glycolysis during activation, glycogen level is still below normal after the 15 min recovery interval (Table 4). All of the stimulus-evoked changes in concentrations of ^{14}C-labeled and unlabeled cerebral cortical metabolites except glycogen normalized within 15 min of recovery from sensory stimulation (Dienel et al. 2002). Re-synthesis of 4 μmol/g glycogen during the ensuing recovery period consumes glucose without oxygen, causing aerobic glycolysis to become manifest and persist. The time required to replace glycogen was first estimated using the measured net rate of incorporation of glucose into glycogen during recovery, and it would take nearly 13 h (Table 4). If glycogen synthesis subsequently increased to 5 or 10% of glucose utilization, restoration of glycogen content would still take about 2 or 1 h, respectively. Thus, if subjects are stressed or activated prior to or during metabolic assays, glycogen will be consumed and replaced, enhancing the magnitude of and prolonging the duration of aerobic glycolysis.

3.3 Lactate Production from Glycogen and Its Release from Cerebral Cortex

Section 3.1 presented evidence that the oxygen consumed during activation cannot support oxidation of all of the glucose taken up, let alone the glycogen metabolized. Glycogen-derived lactate shuttling to neurons involves oxidation and is therefore ruled out as being a major pathway. Based on mass balance of major metabolites, net conversion of total pyruvate carbon (from glucose and glycogen) to other compounds is small compared to utilization of glycogen. This leaves lactate as the most likely end product of glycogenolysis, with its release to blood and perivascular fluid. The following sections present detailed analyses of metabolite concentrations and metabolic labeling that support the conclusion that lactate production with release is a major fate of glycogen.

3.3.1 Glycogen-Derived Lactate Release from Cultured Astrocytes

Dringen et al. (1993) showed that stimulation of glycogenolysis in cultured astrocytes is accompanied by release of lactate to the culture medium in amounts nearly stoichiometric with glycogen concentration. In addition, their demonstration that

release of glucose to the medium could not be detected was very important because it revealed a major difference in the fate of glycogen carbon in astrocytes compared with hepatocytes that supply glucose to blood. However, the amount of lactate released from cultured cells is probably inflated due to the equilibrative lactate dehydrogenase and lactate transport reactions. The released lactate is continuously diluted by large culture medium volume compared with intracellular volume, thereby 'pulling' lactate from cells into the medium and enhancing glycolytic flux (Hertz and Dienel 2005).

3.3.2 Glycogen-Derived Unlabeled Lactate Does Not Dilute Lactate Labeled by Blood-Borne Glucose or Accumulate in Cerebral Cortex

Analysis of labeling of rat cerebral cortical glucose, lactate, and glycogen by an intravenous injection of [6-^{14}C]glucose during rest and activation provides evidence for compartmentation of glycolytic metabolism of glucose and glycogen and for rapid release of glycogen-derived lactate. The concentration of glucose did not change from rest to activation, indicating supply-demand coupling, whereas lactate level rose almost threefold, and glycogen content fell 22% (Table 5). The specific activity (SA = dpm/μmol) of cerebral cortical lactate was about half that of glucose, i.e., equivalent to the theoretically-maximal relative SA (RSA = $SA_{lactate}/SA_{glucose}$). Metabolism of [6-^{14}C]glucose to lactate produces two molecules, only one of which is labeled, causing lactate SA to fall by 50%. Glycogen labeling was very low and its SA was more than 100-fold lower than that of lactate (Table 5). The high RSA of lactate indicates that essentially all of the lactate retained in the cerebral cortex was derived from blood-borne glucose and that dilution by glycogenolysis was negligible.

If all of the glycogen consumed during activation were converted to lactate (12.3–9.6 = 2.7 glucosyl units × 2 = lactate equivalents), 5.4 μmol/g lactate would be produced, an amount 4.9 times the measured rise in cerebral cortical lactate level of 1.1 μmol/g (Table 5). Thus, even if all of the lactate retained in the cerebral cortex were considered to be from glycogen (which can't be the case, based on lactate RSA), more than 80% of the glycogen-derived lactate would have to have been oxidized and/or released from cerebral cortex. The calculations in Sect. 3.3.3 indicate that efflux from cerebral cortex must be the predominant fate of the glycogen-derived lactate, not oxidation in neurons.

3.3.3 Compartmentation of Glycogen and Glucose Glycolytic Fluxes

If all of the glycogen-derived lactate rapidly and completely mixed with the labeled lactate formed from labeled glucose, the resulting dilution of the lactate SA and RSA can be calculated by adding the 5.4 μmol/g lactate equivalents of glycogen

consumed to the measured cerebral cortical lactate concentration during activation. The recalculated lactate SA would fall from 46 to 11 (75% dilution of the SA) and the RSA from 0.59 to 0.14 (Table 5). The time course of glycogenolysis is not known, but if assumed to be constant, the amount of lactate equivalents formed during the last minute of activation just prior to tissue harvest (1.08 μmol/g) would still reduce the lactate SA and RSA to 28 (39% dilution of SA) and 0.36, respectively. Thus, using either the total lactate or the lactate equivalents per minute, the lactate SA would be diluted well below the observed values if glycogen-derived lactate were retained in tissue and mixed with that from glucose.

Another approach to evaluate dilution of lactate by glycogenolysis is to consider cerebral cortical lactate as a single pool that arises from rapid lactate exchange across all cellular membranes so that total astrocytic and neuronal glycolytic fluxes continuously contribute to it. Based on Fig. 8 of Lanz et al. (Lanz et al. 2013) the total astrocytic oxidative consumption of glucose in resting awake brain can be calculated as Vpc + Vg/2, where Vpc is the rate of the pyruvate carboxylase reaction (with units of μmol glucose/g/min) and Vg is the rate of the astrocytic TCA cycle (with units of μmol triose/g/min; dividing Vg by two converts the rate to units of glucose oxidation). $CMR_{glc\text{-}oxidation\text{-}astrocyte}$ = 0.14 + (0.4/2) = 0.34 μmol/g/min. $CMR_{glycogen}$ = 2.7 μmol/g/5 min (Table 3) = 0.54 μmol/g/min. If glycogen fully replaces glucose oxidation in astrocytes during activation, 63% (0.34/0.54) of the glycogen would be oxidized (with no net change in oxygen consumption, only substituting the source of pyruvate). An increase in astrocytic oxidation rate during activation is neglected in this calculation, but it may be about 20% based on the rise in acetate oxidation during photic stimulation of awake rats (Dienel et al. 2007b). The rate of astrocytic lactate production from glycogen can then be calculated by difference: 2∗(0.54–0.34) = 0.4 μmol/g/min. In the 5 min stimulation interval, this would produce 2 μmol/g leaving 3.5 μmol/g (5.4–2) or 65% of the lactate unaccounted for in the absence of its efflux from tissue.

Lactate dilution by fluxes of glucose and glycogen into the total lactate pool is calculated as follows. From the total rate of glucose utilization of 0.93 μmol/g/min (Table 1), total pyruvate/lactate production from blood-borne glucose would be 2∗0.93 = 1.86 μmol/g/min. For one lactate pool fed by glucose and glycogen, the dilution of lactate would be 1-(1.86/[1.86 + 0.4]) = 16% dilution, which could have been detected experimentally. On the other hand, if none of the glycogen were oxidized, the rate of lactate production from glycogen would be 2∗0.54 = 1.08 μmol/g/min. Then the dilution would be 1-(1.86/[1.86 + 1.08]) = 37%, a value equivalent to that calculated above by adding the glycogen-derived lactate to the lactate recovered in cerebral cortex and recalculating the specific activity (1-[28/46]) = 39%. One of the uncertainties in the above analysis is that the fraction of glycogen oxidized or converted to lactate has not been measured, and this is an important issue for future work.

Failure of glycogenolysis to dilute lactate SA and RSA during a 5 min stimulation indicates (1) lactate recovered in cerebral cortical extracts was derived from

blood glucose, and (2) products of glycolytic metabolism of blood-borne glucose and glycogen are segregated. Lactate compartmentation is not due to retention of all or the per minute amount of glycogen-derived lactate in astrocytes during activation because SA dilution would have occurred when the tissue was homogenized and extracted. Most of the astrocytic glycogen-derived lactate must, therefore, have been either oxidized, most likely replacing glucose as an oxidative source for the astrocytes due to hexokinase inhibition, and/or released from cerebral cortex, presumably via perivascular endfeet, without mixing with [^{14}C]glucose-derived lactate. Oxidation was ruled out by assay of oxygen consumption, and efflux needs to be examined directly.

The basis for compartmentation remains to be established, but one possibility is that most or all of the [^{14}C]glucose-derived lactate is in neurons and unlabeled glycogen-derived lactate in astrocytes. This means that shuttling of glycogen-derived lactate to neurons where it would mix with and dilute the neuronal pyruvate/lactate pools (Fig. 1) must be a relatively small flux compared with efflux of lactate away from the activated region and from cerebral cortex. Conceivably, the smaller fractional volume of astrocytes could result in a higher intracellular lactate concentration in astrocytes during rapid glycogenolysis that would favor its release from endfeet to blood as an infinite sink. In contrast, at the cerebral cortical lactate level of 1.7 μmol/g (Table 5) lactate uptake into neurons would be 71% of Vmax (assuming Michaelis-Menten kinetics, v = Vmax S/[Km + S], where S = substrate concentration, v = transport rate, Vmax = maximal transport rate) because the Km of neuronal monocarboxylic acid transporter, MCT2, for lactate is about 0.7 mmol/L, compared with about 3.5 and 28 mmol/L for the astrocytic MCT1 and MCT4, respectively (Manning Fox et al. 2000). Also, *net* neuronal uptake would be governed by its oxidation rate that is much slower than transport (Hertz and Dienel 2005; Dienel and Hertz 2001) whereas release to blood would be favored due to its dilution (arterial plasma lactate level was about 0.7 mmol/L in the same animals (Dienel et al. 2002) and high blood flow rate that increased from 1.1 mL/g/min at rest to 1.8 mL/g/min after 6 min of sensory stimulation (Madsen et al. 1998).

Previous work from our laboratory has demonstrated rapid release of glucose-derived labeled lactate to cerebral venous blood during spreading depression (Cruz et al. 1999) and lactate release to perivascular fluid and meninges during acoustic stimulation (Ball et al. 2010). However, efflux of unlabeled glycogen-derived lactate to these two drainage systems has not, to our knowledge, been directly demonstrated. This is even more technically difficult than assays of labeled lactate release because it would be necessary to measure the lactate specific activity in the drainage fluid and compare it to lactate SA in the source tissue and show a dilution by unlabeled glycogen-derived lactate that could be prevented with a phosphorylase inhibitor.

3.4 The Majority of Lactate Elevation During Brain Activation is Most Likely Due to Neuronal Nonoxidative Glycolysis and Not Astrocytic Glycolysis/Glycogenolysis

The increase in brain lactate level during activation is often *assumed* (based on tissue culture assays) to be produced by astrocytes from glucose and/or glycogen, but this notion has never been directly evaluated and proven. Indeed, at least some, and possibly the majority, of the lactate is probably of neuronal origin. The findings that [^{14}C]lactate increases in direct proportion to unlabeled lactate and the relative specific activity (RSA: SA = dpm/μmol, and RSA = $SA_{lactate}/SA_{[6\text{-}14C]glucose}$) of cerebral cortical lactate is ~0.5 (because one lactate is labeled and one is unlabeled giving the same dpm as glucose but 2 moles of lactate/mole glucose) during rest, activation, and recovery in normal cerebral cortex and during spreading cortical depression (Dienel and Cruz 2009) indicate that virtually all lactate recovered in extracts is derived from blood-borne [^{14}C]glucose. These data are most simply explained if lactate is generated in stimulated neurons where it is not diluted by unlabeled glycogen-derived lactate.

Neuronal metabolism of most of the blood-borne glucose during activation is predicted by the DiNuzzo hypothesis (see Sect. 4) that glycogenolysis spares blood glucose for neurons during activation (Fig. 1), and supported by a recent study demonstrating increased neuronal glycolysis with lactate production and probable export during activation (Yellen 2018; Diaz-Garcia et al. 2017). Further support for increased neuronal glycolysis during activation comes from rapid mobilization of the glucose transporter GLUT4 from intracellular membrane stores to the hippocampal synaptic plasma membrane (within seconds after triggering action potentials) to support synaptic vesicle cycling (Ashrafi et al. 2017; Ashrafi and Ryan 2017) and hippocampal memory formation (Pearson-Leary et al. 2018; Pearson-Leary and McNay 2016). Also, glycolytic enzymes are mobilized at presynaptic sites of *C. elegans* during hypoxia-induced energy stress, and disruption of assembly of the glycolytic metabolon blocks synaptic vesicle cycling and synaptic recovery, and disrupts locomotion (Jang et al. 2016). Collectively, these independent metabolic studies experimentally support an increase in neuronal glycolysis during activation and are consistent with glucose sparing for neurons by glycogenolysis. Clearly, more work is required to directly measure the cellular contributions to total glucose utilization during activation.

3.5 Summary: Major Metabolic Products of Glycogen

The fate of glycogen carbon is especially difficult to evaluate in living brain. However, indirect evidence from parallel, quantitative metabolic labeling studies and analysis of metabolite concentration changes indicates that most of the glycogen cannot be oxidized. Incorporation of glycogen carbon into amino acids and

oxaloacetate is also small. Lactate production must, therefore, be the predominant fate of glycogen, with rapid removal from the region of generation.

Two independent lines of evidence, no dilution of lactate SA and further reduction of OCI by glycogenolysis during activation, support the following inferences: (1) glycogen-derived lactate is rapidly released from activated cerebral cortex, (2) the proportions of glycogen oxidized and converted to lactate are estimated to be 60/40, (3) based on the limiting supply of oxygen during activation, glycogen oxidation would require that an equivalent amount of blood-borne glucose be converted to lactate and released instead of being oxidized, (4) little, if any, glycogen-derived lactate is shuttled to neurons and oxidized, and (5) a major function of glycogenolysis is rapid production of oxidative and glycolytic ATP for use by astrocytes. Obviously, more work is required to evaluate these conclusions, but they underscore the importance of glycogen as an active participant in energetics of astrocytic activation. An important issue is the degree to which glycogen totally or partially replaces glycolytic and oxidative metabolism of glucose in astrocytes, and in doing so how much blood-borne glucose is spared for neurons.

4 Two Aspects of Glycogenolysis: Fueling Astrocytes and Sparing Glucose for Neurons

Glycogen is located mainly in astrocytes where its utilization can provide ‘fast’ glycolytic ATP for ion pumping and at the same time replace astrocytic glycolytic metabolism of blood-borne glucose. The shift from glucose to glycogen could spare an equivalent amount of blood-borne glucose for neurons.

4.1 Glucose Sparing by Glycogen

Based on metabolic modeling, DiNuzzo and colleagues proposed that Glc-6-P generated glycogenolysis increases feedback inhibition of astrocytic hexokinase, thereby reducing the amount of blood-borne glucose consumed by astrocytes and sparing an equivalent amount of glucose for neurons. In this model, Glc-6-P derived from glycogen in astrocytes would support some or all of fuel the incremental increase in flux through astrocytic glycolytic pathway when glycogenolysis is activated, not blood glucose. On the other hand, blood glucose would be the source for the increase in glucose utilization during activation when glycogen is consumed. This means that the increase in the metabolism of labeled glucose during the interval of intense glycogenolysis would be predominantly in neurons, consistent with increased labeling of the large glutamate pool located in neurons when glycogen is consumed (Dienel et al. 2002), and suggesting that most or all of the labeled lactate recovered in cerebral cortex and released from cerebral cortex would be of neuronal origin.

A shift in utilization of blood-borne glucose from being the almost exclusive energy source in astrocytes and neurons under resting fed conditions (Nehlig et al. 2004) to potentially predominantly neuronal utilization (based on the glucose-sparing hypothesis) has important implications for interpretation of metabolic brain images assayed with [^{14}C]deoxyglucose (DG) and [^{18}F]fluorodeoxyglucose-positron emission tomography (FDG-PET) (Reivich et al. 1979; Sokoloff et al. 1977). The increase in CMR_{glc} reflected by brain images during activation would reflect neuronal, not astrocytic, glucose utilization, with the inference that small increases in CMR_{glc} in diseased compared with normal brain may predominantly reflect neuronal deficits. This is a very important issue because the cellular basis of brain images is not known, and most previous reports have ascribed the images to astrocytic glucose uptake that fuel the neurons through the astrocyte-neuron lactate shuttle.

The astrocyte-neuron lactate shuttle model is based on the premise that glutamate uptake into astrocytes stimulates astrocytic glycolysis, with lactate shuttling to neurons for oxidation (Pellerin and Magistretti 1994). Compartmentation of glycolysis in astrocytes was suggested to be the basis for DG and FDG-PET images (Magistretti and Pellerin 1996), and a subsequent study claimed that the FDG-PET images are driven by glutamate uptake (Zimmer et al. 2017). However, the stoichiometry of metabolism related to glutamate uptake in astrocytes does not support the lactate shuttle model (Dienel 2017a), there are many other serious flaws in the model, including failure of the rise in CMR_{O2} to match $CMR_{glc + glycogen}$, that invalidate its translation from tissue culture to in vivo situations (reviewed in (Dienel 2019a)), and the FDG-PET images assigned to glutamate uptake are not valid because the method does not have cellular resolution and contributions of neuronal activation were not taken into account (Dienel et al. 2018). Careful evaluation of the hypothesis by DiNuzzo and colleagues (see our companion paper, Rothman and Dienel 2019) is important for understanding of cell-cell interactions in activated brain and for interpretation of metabolic brain images in human brain.

4.2 Tentative Experimental Support for the Glycogen Glucose-Sparing Model

To our knowledge, there is no direct evidence supporting the DiNuzzo model. However, this hypothesis is supported by data obtained by Prebil et al. (2011) who reported that adrenergic treatment of cultured astrocytes evoked an increase in cytosolic glucose concentration. The rise in glucose level was blocked by inhibition of glycogenolysis, consistent with suppression of glucose utilization secondary to glycogen breakdown and an increased concentration of Glc-6-P. Reduced hexokinase activity would cause intracellular glucose level to rise as it re-equilibrated with medium glucose because the intracellular glucose level is the net result of the rate of influx, efflux, and metabolism, and it will rise when metabolism falls. This experimental paradigm could be used in future studies with [^{14}C]deoxyglucose assays to

measure medium glucose utilization at the hexokinase step as glycogenolysis is triggered and to evaluate the net change in Glc-6-P and intracellular Glc concentrations after glycogen is mobilized.

Another line of evidence for lactate in brain tissue being derived from non-glycogen sources comes from the study of Fray et al. (1996). Extracellular glucose and lactate were monitored by microdialysis in freely-moving rats when glycogenolysis was elicited by a β-adrenergic agonist or a second messenger, a cAMP analog, both of which increased microdialysate glucose level with no change in lactate level. Treatment with a β-adrenergic antagonist blocked the agonist-evoked rise in glucose level. Also, a tail pinch stimulated increases in both glucose and lactate levels, but only the glucose increase was blocked by a β-adrenergic antagonist, indicating differential regulation of glucose and lactate levels. An inference is that glucose level rose due to glucose sparing by glycogenolysis, whereas the lactate was derived from blood-borne glucose. These possibilities need to be tested and can be distinguished by metabolic labeling assays.

4.3 Summary: Astrocytic Glycogenolysis Can Free Up Glucose for Neurons

The model for glucose sparing for neurons by glycogenolysis in astrocytes does not involve astrocyte-neuron shuttling of lactate or other metabolites. Instead, hexokinase activity is further suppressed by glycogen-derived Glc-6-P, allowing unmetabolized blood-borne glucose to diffuse within the interstitial fluid after its uptake into the basal lamina (Mangia et al. 2009, 2011; Simpson et al. 2007) and to diffuse throughout the astrocytic syncytium formed by gap junction-coupled astrocytes to neurons (Gandhi et al. 2009a).

5 Summary of Major Attributes and Functional Implications of Glycogenolysis in Cerebral Cortex During Physiological Stimulation of Awake Rats

Rodent cerebral cortical glycogen concentrations are much higher (10–12 μmol/g) than historically recognized (2–6 μmol/g) when the animals are sequestered or very carefully handled to avoid alerting, stress, and sensory-motor stimulation that occurs when more than one animal is in a cage, cages are transported, and animals are handled prior to tissue harvest. This two- to six-fold difference in concentration probably represents the very labile fraction of the outer tiers of glycogen, since >20% of the glycogen was consumed within 5–6 min of sensory stimulation at a rate 60% of CMR_{glc} by all cells. The outer tiers are estimated to turn over via the glycogen shunt at a rate ~20–40 times the rate of net incorporation of [^{14}C]glucose

into glycogen. Remarkably, when the approximate volume fraction of astrocytes is taken into account, cerebral cortical glycogen level approaches that in resting muscle, and $CMR_{glycogen}$ is at least twice CMR_{glc}. The ~1.8–2.9-fold compensatory increases in utilization of blood-borne glucose in the activated sensory and parietal cortex support the conclusion that glycogenolysis makes a major contribution to astrocytic energetics even in the presence of adequate glucose levels and glucose delivery to the brain.

Lack of dilution of cerebral cortical lactate by glycogenolysis reveals compartmentation of glycolytic metabolism of glucose and glycogen, and indicates that glycogen-derived lactate must be quickly released from cerebral cortex without shuttling to and oxidation in neurons or mixing with lactate labeled by blood-borne [^{14}C]glucose. Inclusion of glycogen carbon in the oxygen-carbohydrate index substantially reduces its value, and glycogen re-synthesis substantially prolongs the duration of aerobic glycolysis. Thus, most of the glycogen-derived pyruvate that is not oxidized in astrocytes must be converted to lactate and rapidly discharged from cerebral cortex.

There may also be increased entry of some glycogen-derived Glc-6-P into the pentose shunt pathway (PPP) to produce NADPH during activation. PPP flux from glycogen increases in cultured astrocytes to detoxify peroxides even in the presence of glucose in the medium (Rahman et al. 2000). The glutathione peroxidase/glutathione reductase system consumes NADPH to detoxify hydrogen peroxide produced by monoamine oxidase or superoxide dismutase during brain activation (Dringen et al. 2005, 2007), and during acoustic stimulation, PPP flux in vivo increases 3.5-fold in the major tonotopic band in the inferior colliculus (Dienel 2012). Carbon one of Glc-6-P is decarboxylated in the PPP, and, in normal adult brain, most of the carbon probably re-enters the glycolytic pathway at the fructose-6-P or glyceraldehyde-3-P steps, with generation of glycolytic ATP as the major outcome of glycogenolysis, along with some NADPH production.

Two major points strongly challenge the notion that shuttling of glycogen-derived lactate to neurons as fuel is required to support neuronal energetics of neurotransmission, memory consolidation, and exercise to exhaustion: (1) the quantity of oxygen consumed during activation cannot support complete oxidation of all of the glycogen and glucose consumed, indicating that increased quantities of lactate must be produced and released from cerebral cortex, and (2) lack of lactate specific activity dilution. More work is required to test directly whether these characteristics of sensory stimulation apply to other experimental paradigms, including neurotransmission, learning/memory and exercise.

6 Roles of Brain Glycogenolysis in Neurotransmission and Memory Consolidation and Brain Slices

Knockout (KO) of brain glycogen synthase impairs memory consolidation and hippocampal long-term potentiation (LTP), clearly proving a major role for glycogen in cognitive activities (Duran et al. 2013). Because neurons have very low levels of

glycogen (Saez et al. 2014) the effects of glycogen synthase KO may be ascribed mainly to astrocytic glycogen. However, a fascinating recent discovery presented by Jordi Duran) at the International Conference in Brain Energy Metabolism (Valdivia, Chile, March, 2018) was that KO of *neuronal* glycogen synthase also disrupts memory and hippocampal LTP, indicating that the effects of glycogen on cognitive functions are quite complex and involve actions in both astrocytes and neurons (Duran et al. 2019).

Shuttling of glycogen-derived lactate from astrocytes to neurons as fuel has been proposed to be necessary to maintain excitatory, glutamatergic neurotransmission in vitro and memory consolidation in vivo. However, based on the above sensory stimulation studies, it is highly unlikely that much, if any, glycogen-derived lactate was transported to neurons and oxidized. Furthermore, none of the studies that claimed that lactate shuttling fuels neurons actually measured glycogen, astrocyte-neuron lactate shuttling, neuronal lactate metabolism, lactate transport or its inhibition, or demonstrated that the all of the rise in extracellular lactate was actually derived from glycogen. Instead, indirect assays were used, including inhibition of glycogenolysis, extremely high-dose lactate injections to rescue memory, and impairment of lactate transport with inhibitors or monocarboxylic acid transporter (MCT) knockdown. These issues are discussed in detail by Dienel (2019a, b) and in recent reviews (Dienel 2017a, b; Dienel and Cruz 2016). A brief synopsis of key issues with testable, alternative explanations for observed findings is provided below.

6.1 Disruption of Glycogenolysis Impairs Neurotransmission in Cultured Cells

6.1.1 Electrophysiological and Developmental Considerations

A number of studies have shown that inhibition of glycogen phosphorylase with different compounds interferes with glutamatergic neurotransmission. For example, when miniature excitatory postsynaptic currents (mEPSC) were measured in neuronal monocultures and astrocyte-neuron co-cultures, the amplitudes of the currents were twice as high in the co-cultures and reduced by 40% by treatment with the glycogen phosphorylase inhibitor 4-(2-Chlorophenyl)-1-ethyl-1,4-dihydro-6-methyl-2,3,5-pyridinetricarboxylic acid 5-isopropyl ester disodium salt hydrate (BAY U6751) in the co-cultures but not neuronal cultures (Mozrzymas et al. 2011). In hippocampal slices from 1-month-old rats, BAY U6751 disrupted the late phase of long-term potentiation (LTP) whereas in slices from 20- to 22-month-old rats there was a tendency for enhancement of LTP (Drulis-Fajdasz et al. 2015). These effects were suggested as possibly due to age-dependent lactate shuttling and neuronal fueling by other pathways (Drulis-Fajdasz et al. 2018). However, lactate shuttling was not measured and other explanations are likely.

6.1.2 Vesicular Glutamate Release and Glutamate Re-uptake in Co-cultures

Neurotransmission was evaluated in cerebellar astrocyte-neuron co-cultures by pre-loading the neurons with tracer amounts of D-[^{3}H]aspartate (D-Asp) as non-metabolizable analog to track vesicular glutamate release and re-uptake during superfusion with a glucose-containing medium and brief pulses of *N*-methyl-D-aspartate (NMDA), glycine, and K^+ (Sickmann et al. 2009). Inhibition of glycogenolysis with [R-R∗,S∗]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide (CP-316,819) impaired D-[^{3}H]Asp release and reuptake, whereas glycogenolysis blockade with 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) *did not* alter release and reuptake. Inclusion of 1 mmol/L D-lactate along with CP-316,819 to inhibit neuronal uptake of lactate via MCT2 enhanced the observed effects, leading to the authors' conclusion that lactate transfer to neurons was necessary for energetics of excitatory neurotransmission. However, it was not established why only CP-316,819 has these effects and not DAB, weakening this conclusion. Furthermore, previous work, most from the same lab, demonstrated that cerebellar neurons in monoculture are highly glycolytic, releasing about half of the glucose metabolized as lactate under resting and depolarizing conditions (Waagepetersen et al. 2000; Jekabsons et al. 2017; Gebril et al. 2016). In addition, pulses of NMDA stimulate cerebellar neuronal monocultures to release D-Asp, increase deoxyglucose phosphorylation (i.e., CMR_{glc}), enhance lactate release to the medium, and preferentially oxidize glucose, not lactate (Bak et al. 2006, 2009, 2012). It is not clear why there should be neuronal dependence on lactate shuttling and oxidation to sustain neurotransmission in co-culture when lactate oxidization is not upregulated during NMDA pulses in monoculture. Alternative mechanisms and explanations for the observed findings include the following:

Disruption of Ca^{++} Homeostasis by DAB: Gliotransmitter Release

When DAB is used to inhibit glycogenolysis in cultured astrocytes, regulation of intracellular Ca^{2+} homeostasis is disrupted (Fig. 2a). Ca^{2+} dysregulation is due to glycogen being the preferred substrate (in the presence of glucose in the medium) for the ATPase that pumps Ca^{2+} into the endoplasmic reticulum (Müller et al. 2014). The resulting elevated [Ca^{2+}] can cause release of the gliotransmitter glutamate from astrocytes to alter neuronal activity (Araque et al. 1998a, b; Zorec et al. 2012; Parpura et al. 1994), thereby decreasing glutamate and D-[^{3}H]Asp release. It is not known if CP-316,819 blockade of glycogenolysis has the same effect on the calcium pump as DAB, but if it does, reduced D-[^{3}H]Asp release may be the consequence of gliotransmitter action on neurons, not absence of lactate shuttling.

Disruption of K^+ Uptake and Astrocyte Signaling by DAB

The extent of glycogenolysis is directly proportional to the concentration of K^+ in the medium of mouse cortical slices (Hof et al. 1988), and K^+ uptake is inhibited by DAB, thereby interfering with clearance of K^+ from extracellular fluid during neurotransmission (Xu et al. 2014). In addition, vesicular release of ATP from astrocytes is stimulated by glutamate, adenosine, or elevated [K^+], and this release is abolished by DAB, demonstrating a role for glycogenolysis in astrocytic signaling (Xu et al. 2014). The consequences of disruption of K^+ clearance to prolong its extracellular concentration during neuronal signaling (Fig. 2a) and ATP signaling on excitatory neurotransmission remain to be evaluated.

Secondary Effects of Blockade of Neuronal Lactate Transport: Inhibition of Mitochondrial Pyruvate Transport and HCAR1 Activation

D-Lactate may not only impair lactate transport through MCT2, but also inhibit pyruvate uptake into mitochondria (Fig. 2b), lowering oxidative ATP production in both neurons and astrocytes, and altering energy-dependent processes related to neurotransmission, including synaptic vesicle cycling to interfere with glutamate and D-[^{3}H]Asp release. If oxidative metabolism is depressed by 1 mmol/L D-lactate, a compensatory stimulation of glycolysis is anticipated, with increased lactate release from astrocytes to suppress neuronal firing via the G_i-protein-coupled lactate receptor (HCAR1 or GPR81; Fig. 2b). Also, release of increased amounts of L-lactate generated within neurons when pyruvate oxidation is depressed may be impaired by D-lactate blockade of neuronal MCT2, perhaps inhibiting glycolysis, leading to energy deficiency in neurons (Fig. 2b). In contrast, this concentration of D-lactate would not inhibit astrocytic MCT1 and MCT4 that have higher Km values, and increased astrocytic glycolysis in astrocytes and enhanced lactate release may further disrupt neuronal energetics and glutamate and D-[^{3}H]Asp release via HCAR1.

Disruption of Movements of Peripheral Astrocytic Processes

Glutamate exposure of cultured astrocytes elicits [Ca^{2+}] elevations and stimulates extension of peripheral astrocytic processes (PAPs) mediated by actin cycling (Cornell-Bell et al. 1990a, b). The fuel for activity-driven PAP movements is not known, but they are packed with glycogen (Oe et al. 2016), enriched with metabotropic glutamate receptors, glutamate transporters and glutamine synthetase (Derouiche and Frotscher 1991; Lavialle et al. 2011; Derouiche and Rauen 1995), and contain small mitochondria (Derouiche et al. 2015). PAPs are dynamic structures that advance and retract to and from synapses (Reichenbach et al. 2010), and

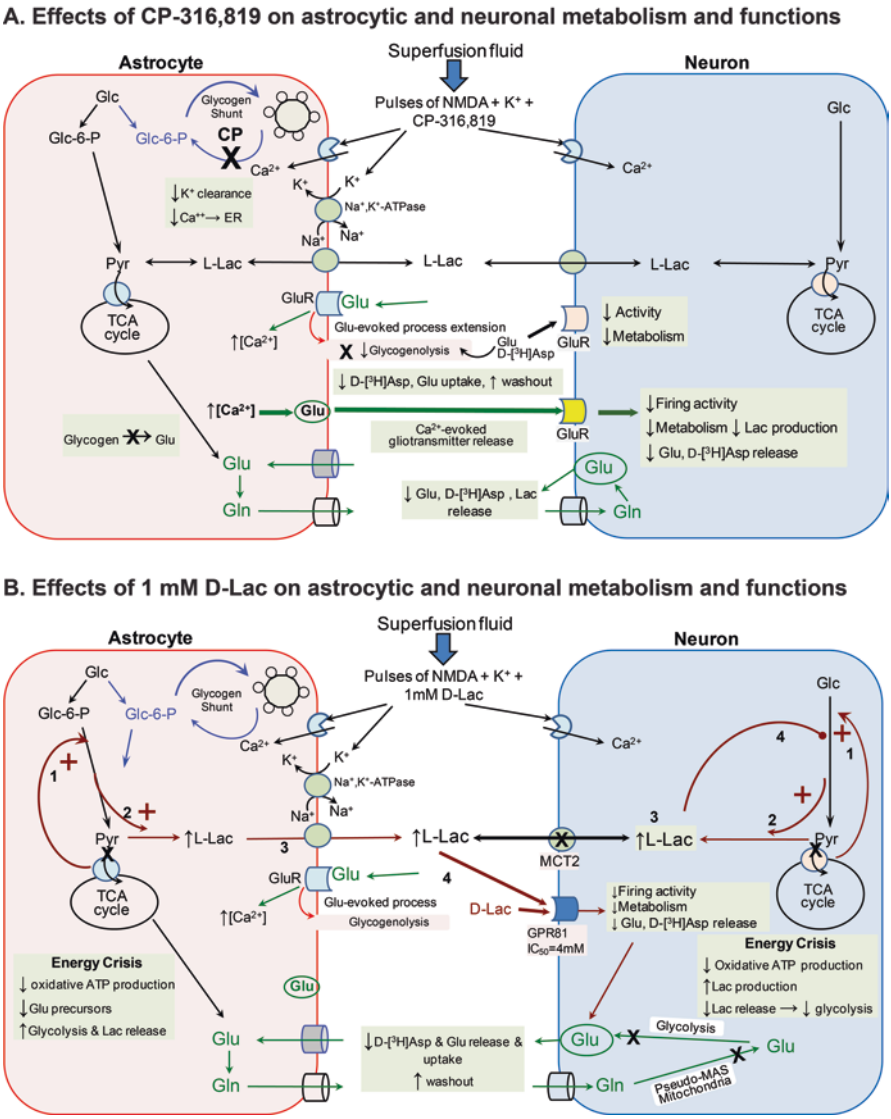

Fig. 2 Alternative mechanisms that may help explain consequences of inhibition of glycogenolysis without astrocyte-neuron lactate shuttling**.** There are testable alternative mechanisms that may contribute to or explain observed experimental findings without involving lactate shuttling if effects of impairing astrocytic processes that are preferentially fueled by glycogen or to side effects of lactate uptake inhibitors are taken into account. (**a**) In the study by Sickmann et al. (2009), cerebellar astrocyte-neuron co-cultures were used to evaluate glutamatergic neurotransmission (see Sect. 6.1.2) by using D-[^{3}H]Asp, a glutamate analog that is used to track neuronal vesicular Glu release and (mainly astrocytic) Glu reuptake. To depolarize cells and cause glutamate and D-[^{3}H]Asp release, the co-cultures were superfused with pulses of NMDA + glycine + KCl with or without CP-316,819 (CP) to inhibit glycogenolysis and affect different processes (denoted by X). Glycogenolysis preferentially fuels Na^+,K^+-ATPase and Ca^{2+} ATPase that pumps Ca^{2+} into the

their movements are regulated (reviewed by (Jackson and Robinson 2018)). Closer proximity of PAPs to active synapses may facilitate K^+ and glutamate uptake into astrocytes from extracellular fluid. A testable hypothesis is that inhibition of glycogenolysis impairs fueling of astrocytic PAP advances toward synapses releasing glutamate, thereby reducing uptake of glutamate and D-[^{3}H]Asp and increasing D-[^{3}H]Asp washout in the superfusate (Fig. 2a). Prolonged elevation of extracellular glutamate may also contribute to suppression of presynaptic glutamate (and D-[^{3}H]Asp) release via metabotropic glutamate receptors (Moussawi et al. 2011; Scanziani et al. 1997).

Fig. 2 (continued) ER. K^+ uptake into astrocytes after release from neurons would be impaired, prolonging the extracellular rise in [K^+] during neurotransmission. Intracellular Ca^{2+} concentration elevations cause gliotransmitter glutamate release, leading to its interactions with neuronal glutamate receptors, thereby altering neuronal firing activity, reducing Glu and D-[^{3}H]Asp release, and secondarily altering neuronal metabolism and neuronal lactate production and release. If glutamate-evoked movements of peripheral astrocytic processes toward active synapses are fueled by glycogenolysis that is inhibited, astrocytic uptake from extracellular fluid of glutamate, D-[^{3}H]Asp, and K^+ may be reduced, causing increased washout of D-[^{3}H]Asp in the superfusate. In addition, reduced glutamate uptake from the synaptic cleft may allow its interaction with glutamate receptors to downregulate neuronal activity and metabolism, also reducing neuronal lactate release. These secondary consequences of glycogenolysis inhibition may explain reduced D-[^{3}H]Asp release and less reuptake with greater washout. Blockade of glycogenolysis may also deprive the astrocytes of precursor for anaplerotic synthesis of glutamate (see Sect. 3.1.3), interfering with neurotransmission. Major consequences of inhibition of glycogenolysis are indicated by text in green highlights and may also contribute to abnormal electrophysiological responses observed in co-cultures and brain slices (see Sect. 6.1.1). (**b**) The co-cultures were superfused in the presence or absence of 1 mM D-lactate to block neuronal uptake of putative glycogen-derived lactate via MCT2 without impairing astrocytic lactate fluxes through MCT1 and MCT4. However, D-lactate probably also inhibits mitochondrial pyruvate uptake in all cells, suppressing ATP production by the oxidative pathways, and causing an energy crisis that triggers a compensatory rise in glycolysis in all cells (response #1, brown arrow). Astrocytes will produce (arrow #2) and release (arrow #3) lactate that interacts with neuronal GPR81 (arrow #4) to reduce neuronal firing, glutamate and D-[^{3}H]Asp release, and neuronal metabolism. The metabolic crisis may also reduce reuptake of D-[^{3}H]Asp into astrocytes and provision of glutamate precursors. In neurons, the compensatory rise in glycolysis (#1, brown arrow) will increase production of L-lactate (arrow #2) that now accumulates (#3) due to D-lactate-mediated inhibition of efflux to the medium via MCT2. Lactate accumulation would eventual inhibit neuronal glycolysis (line #4, with ball at end), exaggerating neuronal energy failure, causing functional decline. Interference with oxidative metabolism and glycolysis in neurons may also disrupt conversion of glutamine to glutamate via the pseudo-malate-aspartate shuttle (MAS) (Hertz and Chen 2017) and with vesicular packaging of glutamate (Ueda 2016) (see Sect. 6.1.2.3). Major potential consequences of D-lactate treatment are indicated by text in green highlights. Experiments using CP-316,819 plus 1 mM D-lactate would exhibit the combined effects illustrated in panels **a** and **b**. Reproduced from Fig. 2 of (Dienel 2019b) [©2019 Wiley Periodicals, Inc., with permission] that was modified from Fig. 9B of (Dienel 2019a) © American Physiological Society, with permission. **Abbreviations**: *Glc* glucose, *Glc-6-P* glucose-6-phosphate, *Pyr* pyruvate, *Lac* lactate, *TCA* tricarboxylic acid, *Glu* glutamate, *GluR* glutamate receptor, *Gln* glutamine, *NMDA N*-methyl-D-aspartate, *Asp* aspartate, *ER* endoplasmic reticulum, *CP* CP-316,819, *DAB* 1,4-dideoxy-1,4-imino-D-arabinitol, *GPR81* G-protein-coupled lactate receptor81 also known as *HCAR1*, hydroxycarboxylic acid receptor1, IC_{50} concentration at which the response is reduced by half, *MAS* malate-aspartate shuttle, *MCT* monocarboxylic acid transporter

6.1.3 Summary: Roles of Glycogen in Neurotransmission

Disruption of Ca^{2+} and K^+ homeostasis, gliotransmitter release, filopodial movements, and oxidative metabolism secondary to inhibition of glycogenolysis and MCT2 may explain or contribute to the observed interference with glutamate release and reuptake without glycogen-derived lactate shuttling to neurons. These consequences may also contribute to electrophysiological abnormalities in astrocyte-neuron co-cultures and brain slices treated with BAY U6751, but secondary effects of BAY U6751 on astrocytes have not, to our knowledge, been evaluated to the same extent as DAB and CP-16,819. Furthermore, the differential effects of DAB and CP-16,819 on co-cultures underscore the importance of using more than two glycogen phosphorylase inhibitors that may have different, unrecognized side effects. Sickmann et al. (2009) did take into account the poor cell permeability of DAB that was previously evaluated in detail by Walls et al. (2008), but effects of CP-16,819 on K^+ and Ca^{2+} homeostasis need to be confirmed. A unique aspect of glycogen phosphorylase inhibition by CP-16,819 is that it requires normoglycemic glucose levels, and inhibition is released under hypoglycemic conditions, an important attribute for its potential use in diabetic patients (see (Suh et al. 2007) and cited references). Thus, if this inhibitor is used when glucose supply is reduced or demand markedly increased, glycogenolysis must be assessed.

6.2 Disruption of Glycogenolysis Impairs Learning/Memory-Related Activities in Rodents In Vivo

Memory-evoking situations transiently increase extracellular lactate concentrations in microdialysates by ~20% to ~2-fold. Inhibition of glycogenolysis with DAB prevented the rise in lactate level and impaired memory consolidation and LTP, leading to the *assumption* that the lactate was astrocytic glycogen-derived and lactate transfer to neurons was required for memory and LTP (Newman et al. 2011; Zhang et al. 2016; Alberini et al. 2018; Gao et al. 2016; Steinman et al. 2016; Suzuki et al. 2011). However, examination the experimental findings indicates that the data supporting the conclusion that lactate fuels neurons are incomplete and inconclusive, and alternative mechanisms may contribute to or explain the findings.

As discussed in Sect. 6.1, disruption of astrocytic intracellular Ca^{2+} homeostasis when glycogenolysis is inhibited by DAB is anticipated to cause release of gliotransmitter glutamate and alter neuronal activity (and secondarily neuronal metabolism and lactate production), potentially impairing the early stages of memory consolidation (Fig. 3a). Also, movements of PAPs to and from active synapses, glutamate and K^+ uptake into astrocytes, and glutamate-glutamine cycling during neuronal signaling (Fig. 3a), as well as astrocytic ATP release, are probably disrupted by DAB treatment, potentially altering the functional sequelae initiated by a memory-triggering event.

6.2.1 Rescue by Massive Doses of L-Lactate

There are important differences in the experimental design of the in vivo studies cited above, but memory could be 'rescued' (i.e., its consolidation was preserved as opposed to memory that was lost and regained) by microinjection of 0.5 μL of 50 but not 30 mmol/L L-lactate (Newman et al. 2011), and memory and LTP rescued by 1 μL of 100 but not 10 mmol/L L-lactate (Suzuki et al. 2011; Zhang et al. 2016). In the Suzuki et al. (2011) study, the volume of distribution of L-lactate was estimated by the hippocampal volume labeled by diffusion of Chicago Sky blue at 1 h after a 1 μL microinjection, and the average tissue concentrations for 10 and 100 mmol/L L-lactate were estimated to be 0.5 and 5 mmol/L. If this 20-fold decrease also applies to D-lactate, the 20 mmol/L injection may fall to a mean level of 1 mmol/L. However, the anionic blue dye is probably restricted to extracellar space whereas D- and D-lactate can enter and leave cells and be discharged to blood via MCTs, so the dye diffusion volumes are unlikely to represent those of lactate. The actual concentration gradients and time courses of lactate clearance from tissue are unknown. This is important because for several reasons:

Unidentified Thresholds and Role(s) for Lactate in Memory Consolidation and Its Rescue

Normal resting brain lactate concentration is in the range of 0.5–1 mmol/L, and, if the above tentative average tissue concentration based on dye labeling is accepted, injection of the 10 and 30 mmol/L L-lactate doses that were *ineffective* in memory rescue cause increases in brain lactate level by 0.5 and 1.5 mmol/L, respectively, i.e., 50–300% increases, depending on the initial baseline value. These percent increases are comparable to the rise in extracellular lactate in microdialysate observed during memory-evoking events, suggesting that (a) the rise in lactate concentration during the memory-evoking event is not sufficient to contribute to memory formation, and (b) the massive, pathological dose of 100 mmol/L lactate has effects unrelated to the true extracellular lactate concentration during and shortly after a memory-evoking event.

The extremely high doses of lactate could be expected to cause intracellular acidification and increase the NADH/NAD^+ ratio, both of which should impair glycolysis in neurons and astrocytes (Fig. 3b). Because neuronal vesicular loading of glutamate preferentially uses glycolysis (Ueda 2016), excitatory neurotransmission could be altered. Similarly, astrocytic energetics that depend on glycolysis may be disrupted. Consequences of massive lactate doses are much more complex than simply providing oxidative fuel for neurons.

Receptor-Mediated Inhibition of Neuronal Firing by L- and D-Lactate

Treatment of cultured excitatory and inhibitory neurons with L- or D-lactate inhibits firing via HCAR1 with an IC_{50} of 4.2 mmol/L (Bozzo et al. 2013), indicating that the estimated average hippocampal lactate level of ~5 mmol/L after injection of

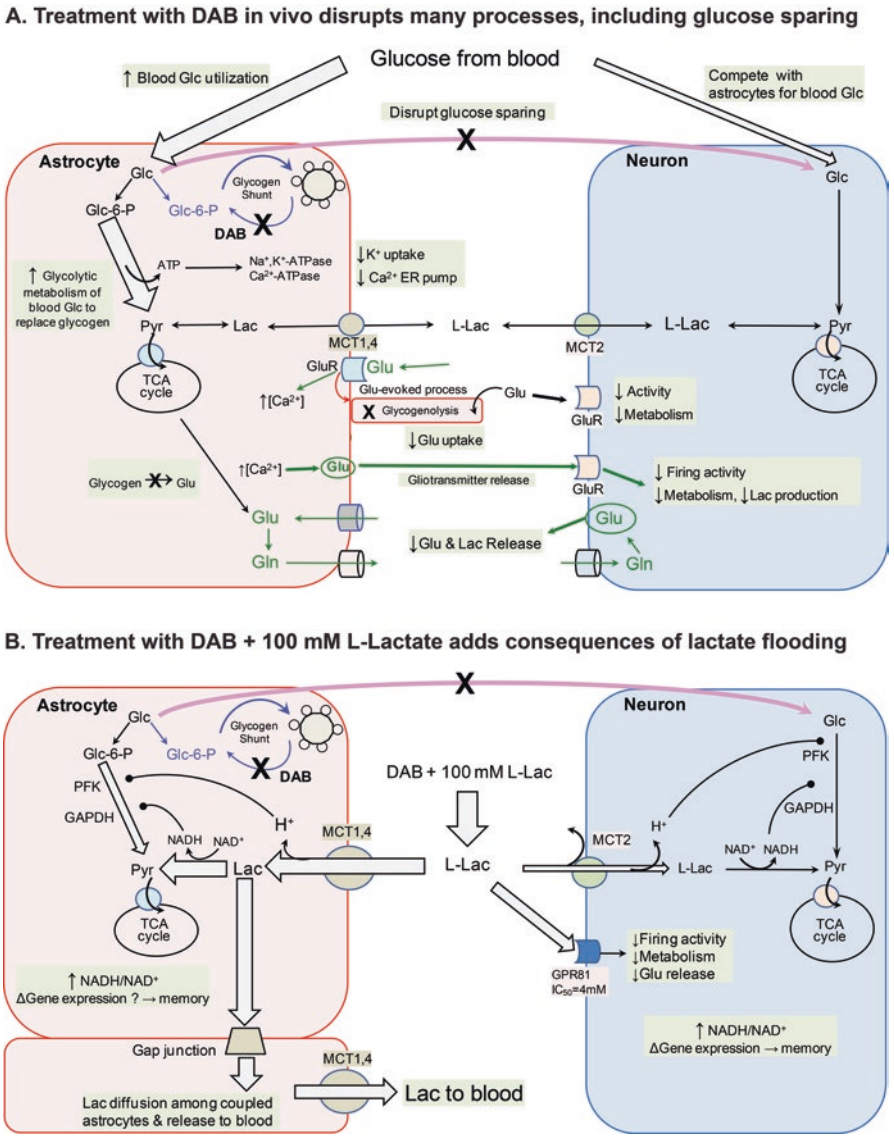

Fig. 3 Alternative mechanisms to explain impairment of memory consolidation in rats by inhibition of glycogenolysis or lactate transport and memory rescue by massive lactate doses. Three interventions were used to evaluate memory formation, impairment, and rescue in rat brain (see Sect. 6.2): (1) DAB was injected into the brain to inhibit astrocytic glycogen phosphorylase (denoted by X); (2) DAB plus an extremely large amount of sodium lactate was injected into brain with the goal of replacing glycogen-derived lactate and increase its oxidative metabolism in neurons; and (3) 20 mM D-Lactate or 60 μM α-cyano-4-hydroxycinnamate (4-CIN) was injected to block neuronal L-lactate uptake via MCT2. Notably, these procedures have additional consequences that confound data interpretation (figure text in green highlights). Consequences of blockade of MCT2-mediated lactate transport with impairment of mitochondrial pyruvate uptake are

100 mmol/L (Suzuki et al. 2011) can cause 50% inhibition of hippocampal firing, with more suppression in neurons closer to the injection site (Fig. 3b). Furthermore, in vivo administration of L-lactate via microdialysis probes to give an extracellular level of 12.5 mmol/L caused a reversible 88% inhibition of hippocampal pyramidal neuron firing (Gilbert et al. 2006). HCAR1 is enriched in pre- and postsynaptic membranes of excitatory synapses in the hippocampus and cerebellum (Lauritzen et al. 2014) and is in a position to influence energetics at the synapse. It is, therefore, important to establish the magnitude and duration of the lactate concentration gradient from the injection site and to evaluate receptor-mediated inhibition of neuronal function with high-dose lactate injections. This information would help evaluate whether LTP can be evoked in the presence of high lactate concentrations or whether

Fig. 3 (continued) similar to those described for 1 mM D-lactate in astrocyte-neuron co-cultures (see Fig. 2b), but probably greater in magnitude due to higher concentration of D-lactate and the very low K_i of 4-CIN for mitochondrial pyruvate transport (6.3 μM (Halestrap 1975)). (**a**). Inhibition of glycogenolysis is also expected to disrupt glucose sparing and cause a compensatory rise in astrocytic glycolytic metabolism of blood-borne glucose. Activated astrocytes and neurons now compete for the glucose entering brain. Because endfeet surround the vasculature, astrocytes have greater access to the incoming glucose, potentially depriving neurons of fuel. In addition, inhibition of glycogenolysis with DAB in vivo would impair the Na^+,K^+-ATPase and Ca^{2+} ATPase, to cause the same effects described in Fig. 2a for CP-316,819 and highlighted in green text boxes. Impairment of glutamate-evoked movements of peripheral astrocytic processes would reduce astrocytic uptake of glutamate and K^+ during neurotransmission. Elevated glutamate levels in the synaptic cleft may activate glutamate receptors to reduce neuronal activity that would secondarily reduce metabolism and neuronal lactate release. (**b**). DAB and a massive dose of sodium L-lactate was injected into brain with the intention of replacing putative glycogen-derived lactate as neuronal fuel (see Sect. 6.2.1). Increased extracellular lactate levels would reduce neuronal activity via GPR81, thereby suppressing metabolism and potentially reducing glutamate and lactate release from neurons. Lactate influx into all cells is concentration-driven and is accompanied by a proton to cause intracellular acidification, inhibit phosphofructokinase (PFK) and reduce glycolytic flux (inhibition denoted by black lines with ball at end). Massive lactate influx would also cause a large increase in the NADH/NAD^+ ratio and reduce NAD^+ availability for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also impairing glycolytic flux. The increase in NADH/NAD^+ ratio may also induce expression of memory-related genes. Because astrocytic MCT1 and MCT4 have higher Km values for lactate than neuronal MCT2 (3.5–7, 28–35, and 0.7 mM, respectively (Manning Fox et al. 2000; Bröer et al. 1999)) more lactate will enter astrocytes, diffuse throughout gap junction-coupled cells, followed by discharge from endfeet to blood. Near-saturation of MCT2 by high lactate concentrations (denoted by black arrow returning to interstitial fluid) should maintain lactate influx into neurons at near-maximal velocity. High lactate through-flux via astrocytes to blood means that astrocytes handle most of the removal of the injected lactate. Reproduced from Fig. 4 of (Dienel 2019b) [©2019 Wiley Periodicals, Inc., with permission] that was modified from Fig. 9B of (Dienel 2019a) © American Physiological Society, with permission. **Abbreviations**: *Glc* glucose, *Glc-6-P* glucose-6-phosphate, *Pyr* pyruvate, *Lac* lactate, *MCT* monocarboxylic acid transporter, *DAB* 1,4-dideoxy-1,4-imino-D-arabinitol, *4-CIN* α-cyano-4-hydroxycinnamate, *TCA* tricarboxylic acid, *Glu* glutamate, *GluR* glutamate receptor, *Gln* glutamine, *ER* endoplasmic reticulum, *GPR81* G-protein-coupled lactate receptor81 also known as *HCAR1*, hydroxycarboxylic acid receptor1, IC_{50} concentration at which the response is reduced by half, *MCT* monocarboxylic acid transporter, *PFK* phosphofructokinase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

lactate clearance from tissue had already occurred within the 30 min interval between 100 mmol/L lactate injection and high-frequency stimulation to evoke LTP (Suzuki et al. 2011). The role(s) of transient HCAR1-mediated inhibition of neuronal activity on brain function and memory consolidation remain to be established.

Redox Shifts Caused by Extremely-High Lactate Levels

Another consequence of pathologically-high injections of L-lactate is a large shift in the intracellular NADH/NAD^+ ratio (Fig. 3b) due to mass action-driven entry of lactate into all brain cells to push the lactate dehydrogenase reaction towards pyruvate plus NADH. Yang et al. (2014) showed that immature cultured neurons grown in high-glucose media and exposed to extremely-high lactate levels express memory-related genes, indicating that responses evoked by large redox changes can be unrelated to true memory-triggering events yet give the appearance of 'rescue'. Notably, the values for the control NADH/NAD^+ ratio of 55.97 (highly reduced) and the 250% rise upon addition of 20 mmol/L L-lactate reported by Yang et al. (2014) are much too high and cannot be correct (see the Methods in Yang et al.). These abnormally-high redox ratios in cell extracts contrast sharply with NADH/NAD^+ of about 0.3 (oxidized) in extracts of cultured astrocytes (see (Wilhelm and Hirrlinger 2011) and discussion and cited references therein).

Of related interest, exercise causes modest increases in mouse hippocampal lactate concentration (but it is not clear whether brain enzymes were inactivated by in situ freezing or microwave fixation) that leads to induction of brain-derived neurotrophic factor (BDNF) by a sirtuin 1 (SIRT 1) deacetylase-dependent pathway and improves memory/learning (El Hayek et al. 2019). Intraperitoneal lactate injections had similar effects, suggesting that a rise in hippocampal lactate level is sufficient to improve memory and expression of memory-related genes. The implication is that aerobic glycolysis and intracerebral lactate production during brain activation in association with a memory evoking test (e.g., footshock or maze) or lactate injections can initiate this or related memory consolidation processes independent of lactate serving as fuel.

Lactate as Fuel and Anaplerotic Precursor

If lactate-derived pyruvate is used as oxidative fuel in neurons and astrocytes after lactate flooding injections, some may also be used to synthesize glutamate during memory processing in rats, as in day-old chick brain (Gibbs and Hertz 2005; Gibbs et al. 2007; Hertz and Gibbs 2009; Gibbs 2016). Conceivably, very high doses of lactate may substitute for glycogen-derived pyruvate and contribute to 'rescue' of memory consolidation (see Sect. 3.1.3).

6.2.2 Putative Inhibition of Glycogen-Derived Lactate Shuttling to Neurons

D-Lactate (20 mmol/L), α-cyano-4-hydroxycinnamate (4-CIN, 6, 20, or 60 μmol/L), or MCT knockdown experiments were used to try to interfere with transfer of glycogen-derived lactate from astrocytes to neurons. As mentioned above (Sect. 6.1.2.3), 1 mmol/L D-lactate could be expected to inhibit MCT2 and pyruvate oxidation, with the same outcomes as described for in vitro experiments (Fig. 2b). At the doses used, 4-CIN is likely to have similar effects as D-lactate. A caveat is that 4-CIN was dissolved in 1% dimethyl sulfoxide (DMSO) (Newman et al. 2011), and propranolol and co-injected drugs were dissolved in 10% DMSO (Gao et al. 2016). DMSO is a solvent that has significant effects on metabolism and glutamatergic neurotransmission over a very wide range of concentrations, 0.000025% to 0.25% (see (Nasrallah et al. 2008) and cited references), and data obtained with DMSO must be interpreted with caution.

Injection of antisense oligodeoxynucleotides to knockdown specific MCT isoforms caused about a 50% decrease in total transporter protein level at 12 h after the injection (given 1 h before training), but lactate transport was not measured and proven to be inhibited. If the fall in MCT protein amount is linear with time, the rate would be ~4%/h, for a decrease of 8% by1h after the memory-evoking event. This decrement is unlikely to disrupt lactate transport because transport capacity exceeds its metabolism by >5-fold in cultured neurons and by >50-fold in cultured astrocytes (Dienel and Hertz 2001).

6.3 Summary: Many Potential Roles for Glycogen in Learning/Memory

There is no question that glycogen has complex roles in neurotransmission, LTP, and cognition, and that inhibition of glycogenolysis and deletion of glycogen synthase has profound effects on brain function. However, the simple conclusion that glycogen-derived lactate fuels neuronal energy demands is quite unlikely to be correct or quantitatively important. The bottom line is that regardless of the approaches used, lactate transport, inhibition, and oxidation were never measured and quantified. In fact, the origin of extracellular lactate has not been proven to be from astrocytic glycogen, although this notion has been asserted as fact. The mechanisms remain to be established and alternatives that do not involve lactate shuttling need to be tested experimentally. If glycogen is not used as a major oxidative fuel, what are its contributions to astrocytic energetics? These issues are discussed in our companion paper (Rothman and Dienel 2019).

7 Brain Glycogenolysis During Acute Hypoglycemia and Exercise to Exhaustion

To this point, this chapter has emphasized glycogenolysis in cerebral cortex in normal, awake, normoglycemic rats that had been sequestered in a shelter for about 3 h before being abruptly exposed to the laboratory environment and given sensory stimulation. Alerting and attentiveness to a novel situation probably activated widespread noradrenergic signaling throughout the brain via the locus coeruleus to trigger glycogenolysis, followed by cellular metabolic responses to increased signaling activities in response to sensory stimulation. Three important points need to be taken into account when interpreting the functions and fate of glycogen in the presence of adequate supply and delivery of glucose to the brain. First, about 25% of the basal resting glycogen concentration was rapidly consumed (within 5–6 min) during sensory stimulation at rates that are about 60% of CMR_{glc} (Table 1). Second, inclusion of glycogen in the oxygen-carbohydrate index causes the ratio to fall further, indicating that most of the glycogen (plus glucose) is not oxidized (Table 4). Third, glycogenolysis does not dilute the specific activity of brain lactate that is derived almost exclusively from blood-borne labeled glucose (Table 5). These properties indicate that glycogenolysis probably mainly satisfies the energetic demands of astrocytes, and glycogen-derived lactate is rapidly released from brain (because it does not accumulate in brain) without mixing with lactate formed from blood-borne glucose. This section evaluates rates of glycogenolysis during acute hypoglycemia and during exhaustive exercise that causes hypoglycemia and increases release of neurotransmitters that stimulate glycogenolysis.

7.1 Acute Hypoglycemia Triggers Glycogenolysis

When the arterial plasma glucose concentration of α-chloralose-anesthetized rats was clamped at <2 mM during an intravenous infusion of [^{13}C]glucose, the brain [^{13}C]glucose concentration gradually fell from an initial level of 3.2 μmol/g because metabolic demand for glucose in brain exceeded its supply (Choi et al. 2003). The brain [^{13}C]glucose level approached the limit of detection by MRS (~0.25–0.5 μmol/g) within about an hour, indicating that most of the glucose entering brain was phosphorylated immediately after its uptake. Brain [^{13}C]glycogenolysis commenced when brain [^{13}C]glucose was 0.1 μmol/g, and during the following hour when brain [^{13}C]glucose was ‘zero’ (i.e., not detectable) [^{13}C]glycogen was consumed at a rate of 0.04–0.05 μmol/g/min (Table 6), corresponding to about 8–11% of cerebral cortical CMR_{glc} in non-stimulated, similarly-anesthetized but normoglycemic rats (Nakao et al. 2001) (Table 6). Choi et al. (2003) suggested that glycogenolysis accounted for most or all of the deficit in glucose supply to brain during hypoglycemia.

Table 6 Effects of acute hypoglycemia and treadmill running exercise on brain glycogen and glucose utilization rates

Physiological state	Ref.	Glycogen concentration (μmol/g)	$CMR_{glycogen}$[a] (μmol/g/min)	Plasma or blood glucose level (μmol/ml)	Brain glucose level (μmol/g)	Brain lactate level (μmol/g)	Ref.	Plasma glucose level (μmol/ml)	CMR_{glc} (μmol/g/min)	
		Initial → final		Initial → final	Initial → final	Initial → final			Rest	Run
α-chloralose anesthesia, acute hypoglycemia	Choi et al. (2003)	5 → 1.8	Cx 0.04–0.05[b]	Plasma 10 → <2	3.2 → ~0 → limit of detection =0.25–0.5		Nakao et al. (2001)[c]	6.5	Whisker barrel Cx 0.45	
Awake, acute hypoglycemia	Herzog et al. (2008)	5.4 → 2	Parietal Cx 0.18[d]	Plasma 10 → 1.9	2 → 0.4		Suda et al. (1990)[e]	2.4	Parietal Cx 0.91	
Awake, running 28 m/min ~85% V_{O2Max}							Vissing et al. (1996)[f]	9–10	Cx 1.21 Hip 0.87	1.51 1.05
Awake, running 20 m/min ~50–70% V_{O2Max} Untreated	Matsui et al. (2011) (Figure 2, Table 1)[g]	60 → 120 min Cx 9.5 → 5 Hip14.5 → 8	60 → 120 min 0.08 0.11	Blood 60 → 20 min Glc 6 → 3.8 Lac 2 → 5.5	60 → 120 min Cx 3.2 → 1.3 Hip 3.5 → 1.6	60 → 120 min Cx 1.9 → 2.5 Hip 2.0 → 2.7				
Awake, running 20 m/min ~50–70% V_{O2Max} Untreated	Matsui et al. (2017) (Figures 1 and 3)[h]	0 → 84 min Cx 8.2 → 2 Hip 14 → 4	60 → 84 min 0.26 0.42	Blood 0 → 84 min Glc 5.5 → 1.8 Lac 0.5 → 13	0 → 84 min Cx 3.1 → 0.5 Hip 3.2 → 0.4	0 → 84 min Cx 2.1 → 3 Hip 2.6 → 3.1				

(continued)

Table 6 (continued)

Physiological state	Ref.	Glycogen concentration (μmol/g)	$CMR_{glycogen}$[a] (μmol/g/min)	Plasma or blood glucose level (μmol/ml)	Brain glucose level (μmol/g)	Brain lactate level (μmol/g)	Ref.	Plasma glucose level (μmol/ml)	CMR_{glc} (μmol/g/min)	
		Initial → final		Initial → final	Initial → final	Initial → final			Rest	Run
Awake, running 20 m/min ~50–70% V_{O2Max} Vehicle control	Matsui et al. (2017) (Figures 5, S2, and S3)[i]	60 → 90 min Cx 11 → 4 Hip 15 → 7	60 → 90 min 0.23 0.27	Blood 60 → 90 min Glc 7 → 1.8 Lac 4 → 13		60 → 90 min Cx 1.2 → 2.2 Hip 1.5 → 2.4				
Awake, running 20 m/min ~50–70% V_{O2Max} + DAB	Matsui et al. (2017) (Figures 5, S2, and S3)[j]	60 → 73 min Cx 11 → 4 Hip 15 → 11	60 → 73 min 0.54 0.31	Blood 60 → 73 min Glc 5.5 → 2 Lac 4 → 11		60 → 73 min Cx 1.2 → 1.8 Hip 1.5 → 2				

Values were taken from tabular data or estimated from graphical presentations in the cited references; the latter values are considered approximate

[a]$CMR_{glycogen}$ was calculated by dividing the net change in glycogen concentration by the experimental duration

[b][^{13}C]Glycogenolysis started when brain (mainly cerebral cortex [Cx]) [^{13}C]glucose level in α-chloralose anesthetized rats approached zero (i.e., the limit of detection by MRS, which is about 0.25–0.5 μmol/g). $CMR_{glycogen}$ was estimated over about a 1 h interval during which brain [^{13}C]glucose levels were not detectable. There was, however, continuous influx of glucose into brain from blood (See Table 8), and $CMR_{glycogen}$ was about 9–11% of CMR_{glc} determined in normoglycemic rats by Nakao et al. (1999)

[c]Glucose utilization rates (CMR_{glc}) were measured in non-stimulated whisker barrel (somatosensory) cortex

[d]Glycogen concentrations in parietal cortex fell during the 3 h hypoglycemic episode and correspond to about 18% of CMR_{glc} under similar hypoglycemic conditions determined by Suda et al. (1990). Plasma glucose levels were not reported but were calculated to be about 10 mM, based on brain/plasma distribution ratio for glucose of 0.2 (Holden et al. 1991) and brain glucose concentration of 2 μmol/g

[e]Regional CMR_{glc} was measured in awake normo- and moderately-hypoglycemic rats Hypoglycemia caused modest reductions in cortical CMR_{glc} ranging from −3 to −20%, with a non-significant decrease in parietal cortex

[f]Rats were first trained on a treadmill at 28 m/min running speed for 35 min/day for 7 days. CMR_{glc} was assayed during running for 30 min, and the arithmetic average of regional cerebral cortical CMR_{glc} rose 26%, and hippocampal (Hip) CMR_{glc} increased 23% during exercise

[g]Rats were trained on a treadmill at increasing running speeds from 5 to 25 m/min for 30 min/day in five sessions over 6 days. Experiments were carried out 2 days after training with fasting for 3 h and running at 20 m/min for different durations. Because brain glycogen, glucose, and lactate levels were similar at 0, 30, and 60 min running time and their levels changed at 120 min, values for the 60 and 120 min intervals are tabulated, and $CMR_{glycogen}$ calculated using 60 min (60–120) as the experimental duration. It is not clear when or if the rats in this study became exhausted, and the actual duration for change of metabolite levels is uncertain. Exhaustion in normal or vehicle-injected controls occurred at 80–90 min in the Matsui et al. (2017) study, suggesting that the rats at 120 min in the Matsui et al. (2011) study became exhausted, then recovered somewhat at 120 min, consistent with the higher blood glucose and lower blood lactate at 120 min compared to values at exhaustion in the Matsui et al. (2017) study

[h]Rats were trained as above, and exercised at 20 m/min to exhaustion when they could not keep the pace, lay flat on the treadmill, and stayed in place. Brain glycogen, glucose, and lactate levels were similar at 0, 30, and 60 min running time Matsui et al. (2011), and exhaustion occurred at 84 min, so the 24 min interval (60–84) was used as the duration to calculate $CMR_{glycogen}$

[i]Rats were treated as above, given vehicle injections, and exercised to exhaustion that occurred at about 90 min. Blood glucose levels were stable at 30 and 60 min, then fell at 90 min. A 30 min interval (60–90) was used for calculation of $CMR_{glycogen}$

[j]Rats were treated as above and given intracerebroventricular injections of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) to inhibit glycogen phosphorylase. Rats were exercised to exhaustion that occurred at about 73 min. Blood glucose levels were stable at 30 and 60 min, then fell at 73 min, and a 13 min interval (73–60) was used for calculation of $CMR_{glycogen}$

When awake rats were rendered hypoglycemic by an insulin injection to reduce arterial plasma glucose level from 10 to 1.9 mM, the rate of glycogenolysis in parietal cortex over a 3 h interval was 0.18 μmol/g/min (Herzog et al. 2008), or 20% of parietal CMR_{glc} in awake, acutely hypoglycemic rats (Suda et al. 1990) (Table 6).

Together, these two sets of findings demonstrate that hypoglycemia reduces delivery of glucose from blood to brain and decreases glucose utilization rate due to supply-demand mismatch. As a compensatory measure, consumption of glycogen, the brain's stored glucose, increases. Most of the brain's glycogen is in astrocytes, and $CMR_{glycogen}$ in hypoglycemic astrocytes ranged from ~10–20% of utilization of blood-borne glucose by all brain cells determined in separate studies under similar conditions (Table 6). The glycogenolytic response to hypoglycemia is a much smaller fraction of CMR_{glc} than during sensory stimulation of awake, normoglycemic rats (60%, Table 1).

7.2 Exercise to Exhaustion

7.2.1 Treadmill Running Increases Glucose and Glycogen Utilization in Rat Brain

Vissing et al. (1996) showed that 30 min of treadmill running at a rate corresponding to about 85% of maximal oxygen utilization capacity (V_{O2Max}) increased CMR_{glc} throughout the brain, with a 23–26% average increases in regions of cerebral cortex and hippocampus (Table 6) and 50–100% and 2-fold increases in regions of the thalamus and cerebellum, respectively (not shown).

A series of very interesting and important studies by Matsui and colleagues (Matsui et al. 2011, 2012, 2017) established that glycogen consumption also increased in all brain regions during running to exhaustion. The high baseline glycogen levels obtained in these reports are similar to those obtained in earlier studies in rats (Dienel et al. 2002; Cruz and Dienel 2002; Kong et al. 2002; Oe et al. 2016) and humans (Öz et al. 2015). In these studies, the treadmill speed was 50–70% V_{O2Max}, and $CMR_{glycogen}$ in cerebral cortex and hippocampus ranged between 0.08–0.42 μmol/g/min in untreated or vehicle-treated rats (Table 6). The lowest rates were in the Matsui et al. (2011) study that were calculated based on a 60 min duration (from 60–120 min running). However, these rates have the highest uncertainty because the rats in subsequent studies (Matsui et al. 2012, 2017) became exhausted at 80–90 min, suggesting that the rats became exhausted and recovered somewhat between 90 and 120 min. Partial recovery may account for the higher blood and brain glucose levels at 120 min compared with the values at exhaustion at 80–90 min (see Table 6 and its legend).

The glycogenolysis rate of about 0.25 μmol/g/min is 17% of mean cerebral cortical CMR_{glc} in rats running for 30 min at a higher speed, and hippocampal rates of 0.27 and 0.42 μmol/g/min correspond to about 25–40% of CMR_{glc} in that structure (Table 6). In these experiments, brain glucose, lactate, and glycogen concentrations

were stable at 30 and 60 min of running, and after 60 min when plasma glucose fell towards 2 mM (secondary to depletion of glycogen in liver and muscle), glucose supply to the brain became inadequate, cortical and hippocampal glucose levels fell from ~3 to ~0.5 μmol/g, and glycogen was consumed during the interval between 60 min and onset of exhaustion. Blood and brain glucose levels were positively correlated with regional glycogen level and negatively correlated with lactate concentration in most regions (Matsui et al. 2011).

The exercising rats were previously habituated to running on the treadmill over a 6 day period, so they are unlikely to experience alerting and activation of locus coeruleus noradrenergic signaling as were the sheltered rats with abrupt exposure to the environment prior to sensory stimulation. These factors may contribute to the rapid, twofold higher $CMR_{glycogen}$ in stimulated rats (Table 1) compared with the smaller, delayed rise in exercising rats (Table 6). However, $CMR_{glycogen}$ during exercise may be underestimated because the actual time course of glycogen consumption during the 60–90 min interval is not known.

At the point of exhaustion, brain ATP concentrations were normal, but levels of its metabolites increased indicating higher turnover. Also, brain levels of metabolites of noradrenaline and serotonin rose during exercise and were negatively correlated with brain glycogen level in cortex (Matsui et al. 2011), indicating higher release and metabolism of these neurotransmitters that are known to stimulate glycogenolysis. Thus, there are two triggers to enhance glycogen mobilization during exercise to exhaustion, hypoglycemia and neurotransmission. The rates of glycogenolysis in exercising rats are higher than in sedentary hypoglycemic rats that had lower brain glucose levels (Table 6), presumably due to stimulatory effects of monoamine neurotransmitters and other complex metabolic changes in brain and peripheral organs during exercise to exhaustion.

7.2.2 Exercise Increases Blood and Brain Lactate Levels and Estimated $CMR_{lactate}$

Arterial plasma lactate levels are relatively low (0.5–1 mM) prior to sensory stimulation of relatively sedentary rats, and they increase by a small amount (0.5–1.5 mM) when the animals are stimulated and move around (Dienel et al. 2002, 2007a; Madsen et al. 1999). In sharp contrast, exercise to exhaustion in humans causes very large increases in arterial plasma lactate level (from 0.7–1.1 mM up to 15–21 mM) due to lactate release from muscle; these high concentrations drive lactate down its concentration gradient into brain and provide supplemental oxidative fuel (van Hall et al. 2009; Quistorff et al. 2008). During human exercise to exhaustion, the oxygen-carbohydrate index ($OCI = [A\text{-}V]_{O2}/([A\text{-}V]_{glc} + 0.5[A\text{-}V]_{lac}$) falls from close to 6 at rest to as low as 1.7–3 when determined by arteriovenous (A-V) differences for oxygen, glucose and lactate, indicating that 50–72% of the glucose plus lactate taken up across the blood-brain barrier is not oxidized (Quistorff et al. 2008). Consumption of endogenous brain glycogen during exercise to exhaustion is not detected by A-V difference, and if glycogen consumed were included in the

calculation the OCI would fall (e.g., from 4.95 to 2.8 during sensory stimulation, Table 4) below 1.7–3 because measured net $[A\text{-}V]_{O2}$ accounts for oxidation of all substrates.

In exercising rats in the Matsui et al. (2017) study, blood lactate levels rose (non-significantly) from about 0.5 mM at rest to 2–4 mM at 30 and 60 min of running, then increased to about 13 mM at the point of exhaustion (Table 6). At the same time, the brain lactate increased by very modest amounts (<1 μmol/g). When converted to hexose units (divide by two), the rise in brain lactate level corresponded to 10–20% of the fall in brain glucose level and 4–7% of the fall in brain glycogen concentration (Table 6).

Because blood lactate concentration greatly exceeded that in brain after 60 min of running (Table 6), calculated influx of lactate into cerebral cortex during the 60–90 or 60–120 min interval increased by an estimated 1.2–2-fold at exhaustion, depending on the values used for V_{max} and K_T for lactate transport (Table 7). Calculated $CMR_{blood\ lactate}$ in cerebral cortex at exhaustion (73–90 min) was about 75–85% of the rate of unidirectional lactate uptake (Table 7), indicating that most of the incoming lactate is oxidized. If the rate of unidirectional uptake in excess of oxidation rate between 60 and 90 min is 0.05 μmol/g/min (Table 7), lactate influx in excess of metabolism during this 30 min interval could contribute 1.5 μmol lactate/g, which exceeds the net rise in brain lactate concentration of 0.5–1 μmol/g lactate at exhaustion (Table 6). Blood lactate influx is, therefore, the source of some or all of the rise in brain lactate level.

Calculated $CMR_{blood\ lactate}$ rose up to about twofold from 60 min to exhaustion (depending on the values of V_{max} and K_T), and after conversion to hexose units, the rate is about 0.1–0.3 μmol/g/min (Table 7), equivalent to about 50–100% of $CMR_{glycogen}$ in the same animals (Table 7), and about 10–20% of CMR_{glc} during shorter, more vigorous exercise (Table 6). For comparison, the cumulated uptake of lactate into human brain during exhaustive exercise was about 20% of cumulated glucose uptake, but at the point of exhaustion $[A\text{-}V]_{lac}$ was a higher fraction of $[A\text{-}V]_{glc}$ compared with earlier times even though most of the glucose plus lactate is not oxidized (Quistorff et al. 2008) (see above, fall in OCI in exercising humans). Thus, as in humans, blood lactate supplements glucose as oxidative fuel in brain of exercising rats, particularly between 60 min and exhaustion, but blood lactate is also consumed at low rates once its level exceeds that in brain.

Neuronal MCT2 has a Km for lactate of 0.7 mM (Bröer et al. 1999), and based on brain lactate levels of 2–3 mM at exhaustion (Table 6), it can be calculated using simple Michaelis-Menten kinetics ($v = V_{max}\ S/(K_T + S)$ instead of reversible kinetics because intracellular and extracellular levels in different cell types are not known), neuronal lactate transport rate is 75–80% of V_{max}. Near saturation of MCT2 may underlie its 50% increase in levels during exhaustive exercise (Matsui et al. 2017) to raise neuronal lactate transport capacity. Because the trans-membrane lactate gradients are not known, the direction of lactate fluxes cannot be determined. Conceivably, neuronal lactate oxidation could spare neuronal glucose for glycolysis (and lactate production and release), since glycolysis is the preferred source of ATP for loading glutamate into synaptic vesicles (Ueda 2016). In resting humans, blood-borne

Table 7 Comparison of calculated lactate influx rate into cerebral cortex and cortical $CMR_{lactate}$ with cortical $CMR_{glycogen}$ during exercise to exhaustion

Experimental condition	Lactate influx (μmol/g/min) (Triose units)	$CMR_{lactate}$ (μmol/g/min) (Triose units)	$CMR_{glycogen}$[a] (μmol/g/min) (Hexose units)
Matsui et al. (2011) Untreated	60 → 120 min 0.09 → 0.17[b] 0.23 → 0.39[c]	60 → 120 min 0 → 0.09[b] 0.01 → 0.21[c]	60 → 120 min 0.08
Matsui et al. (2017) Untreated	0 → 84 min 0.03 → 0.25[b] 0.07 → 0.53[c]	0 → 84 min −0.08 → 0.19[b] −0.24 → 0.41[c]	60 → 84 min 0.26
Vehicle-control	60 → 90 min 0.16 → 0.26[b] 0.39 → 0.55[c] 0.46 → 0.61[d]	60 → 90 min 0.11 → 0.21[b] 0.27 → 0.46[c] 0.37 → 0.55[d]	60 → 90 min 0.23
DAB-treated	60 → 73 min 0.16 → 0.25[b] 0.39 → 0.54[c]	60 → 73 min 0.11 → 0.21[b] 0.27 → 0.45[c]	60 → 73 min 0.54

Lactate influx rates from blood into cerebral cortex and cortical $CMR_{lactate}$ were calculated from data tabulated in Table 6 using reversible Michaelis-Menten kinetics as described by Boumezbeur et al. (2010) and Benveniste et al. (2018):

$V_{in} = V_{max}$ [Lac]b / (K_T + [Lac]b + [Lac]Cx), and

$CMR_{lactate} = V_{in} - V_{out} = V_{max}$ ([Lac]b − [Lac]Cx)/(K_T + [Lac]b + [Lac]Cx),

where V_{in} is the influx rate, V_{out} is the efflux rate, V_{max} is the maximal transport rate, K_T is the concentration at half-maximal transport, [Lac]b and [Lac]Cx are lactate concentrations in blood and cerebral cortex, respectively. Calculated rates correspond to the time intervals in the exercise studies, where 0 represents the resting state, 60 min is the time after which blood and brain glucose begin to fall and blood lactate increases sharply, and the longest times (84, 90, and 73 min) are the points of exhaustion. Negative values for $CMR_{lactate}$ indicate efflux from brain to blood. Note that lactate influx rate and $CMR_{lactate}$ must be divided by two to convert the triose units to hexose units for comparison to $CMR_{glycogen}$ and CMR_{glc}

[a]Values for $CMR_{glycogen}$ are from Table 6

[b]V_{max} and K_T were taken to be 0.4 μmol/g/min and 5.1 mM, respectively, as reported for adult human brain with reversible kinetics (Boumezbeur et al. 2010)

[c]We are unaware of determination of K_T and V_{max} in awake rats using reversible kinetics in the literature. To evaluate the impact of different values for kinetic constants, rates were calculated using unpublished preliminary data for V_{max} = 0.75 μmol/min/g and K_T = 2.5 mM

[d]Blood and plasma lactate levels are equal in resting humans, but plasma lactate levels rise more than that in whole blood during exercise, and when blood lactate increased to 4 mM, plasma was 6 mM, and when blood lactate was 10 mM, plasma was 16 mM (Hildebrand et al. 2000). These plasma/blood ratios were used to convert the blood levels reported in Fig. S2B of Matsui et al. (2017) to estimated plasma levels, and calculate lactate influx and CMR_{lac} in one example to illustrate the effects of higher lactate levels in plasma. These corrections yielded plasma lactate levels of 6 and 20.8 mM from reported blood levels of 4 and 13 mM (Table 6)

lactate is oxidized in astrocytes and neurons in the same proportion as glucose, but the relative proportions are not known during exercise. Conceivably, glycolytic metabolism of glycogen in astrocytes to generate lactate may raise intracellular lactate level and reduce its uptake from interstitial fluid, which could spare blood-borne lactate for oxidation in neurons, secondarily sparing neuronal glucose for the glycolytic pathway. These are intriguing issues that require further study.

Note that the calculated rates of lactate influx and $CMR_{lactate}$ are not as accurate as desired because the kinetic constants (V_{max} and K_T) in awake adult rat brain determined with more accurate reversible Michaelis-Menten kinetics are not known. As a first approximation, we used values determined in human brain (V_{max} = 0.4 μmol/g/min and K_T = 5.1 mM) (Boumezbeur et al. 2010) and preliminary unpublished estimates of values for rats (V_{max} = 0.75 μmol/g/min and K_T = 2.5 mM; see legend to Table 7). Note that the rates are directly proportional to V_{max} and inversely related to K_T plus the sum of blood and brain lactate levels (Table 7, legend), so the V_{max} ratio of 0.75/0.4 = 1.9 is the main driving force to increase the rates. Another issue is that Matsui et al. (2011, 2017) reported blood lactate levels, and plasma lactate is the source for uptake into brain. At rest, human blood and plasma lactate levels are equal, but during exercise plasma levels rise higher than blood levels (Hildebrand et al. 2000). Conversion of blood to estimated plasma levels increased influx and $CMR_{lactate}$ rates even more (Table 7). In spite of uncertainties in the absolute values for these rates, the trends for each set of kinetic constants are similar, and relative differences among the groups and time intervals are considered reasonably predictive.

The origin of the increase in brain lactate during exercise cannot be determined from the data in Table 6 because the elevated brain lactate is due, at least in part, to uptake from blood (Table 7), and some may also be derived from glycolytic metabolism of glucose and/or glycogen. The negative correlations between brain glycogen and brain lactate concentrations (Matsui et al. 2011) could be due to either or both glycogenolysis and lactate uptake into brain from blood, which increases as blood and brain glucose fall (Tables 6 and 7). Although metabolic pathway fluxes and compartmentation probably differ during exhaustive exercise and sensory stimulation, the failure of glycogen consumed to accumulate in brain as lactate (the mean change in cortical glycogen level in the Matsui et al. (2011, 2017) was 6.2 μmol/g (Table 6), equivalent to 12.4 μmol/g lactate, whereas lactate level rose <1 μmol/g), and failure to be oxidized during sensory stimulation suggests that the exercise-induced rise in brain lactate is predominantly from brain metabolism of blood-borne glucose and from lactate taken up from blood. Future metabolic labeling studies are required to evaluate the sources of brain lactate during exhaustive exercise.

7.2.3 Functions of Glycogenolysis During Exhaustive Exercise

Is Glycogen-Derived Lactate Shuttled to Neurons as Oxidative Fuel?

Neurons do not take up glucose- or glycogen-derived lactate when activated in brain slices incubated in 10 mM glucose or in normoglycemic animals in vivo; instead, they increase glycolytic metabolism of glucose (Yellen 2018; Diaz-Garcia and Yellen 2019; Diaz-Garcia et al. 2017). However, metabolic demands and fuel usage probably differ when blood lactate is substantially elevated and the brain is hypoglycemic to limit neuronal glycolysis.

Detailed analysis of metabolic changes in brain, muscle, and liver by Matsui et al. (2017) included demonstration that levels of the neuronal lactate transporter, MCT2, in cerebral cortex and hippocampus increased by about 50%, and the glucose transporter GLUT1 level increased in astrocytes and endothelium by 25% and 50%, respectively, in cortex but not in hippocampus. Also, the neuronal glucose transporter GLUT3 level increased 60% in cortex with no change in hippocampus. Thus, glucose transport capacity across the blood brain barrier and across plasma membranes of astrocytes and neurons is increased during exhaustive exercise. Based on these findings and literature studies that claimed shuttling of glycogen-derived lactate during memory-evoking events (Suzuki et al. 2011; Zhang et al. 2016; Newman et al. 2011), Matsui et al. (2017) suggested that glycogen-derived lactate may fuel neurons during exhaustive exercise. Instead, however, under conditions with high blood lactate, enhanced lactate influx and $CMR_{lactate}$, and very low brain and blood glucose (Tables 6 and 7), MCT2 probably facilitates entry of plasma-derived lactate into neurons for oxidation to compensate for inadequate glucose supply.

Furthermore, none of the above-cited memory studies that concluded there was shuttling of glycogen-derived lactate to neurons actually measured lactate transport from astrocytes to neurons, none identified the metabolic and cellular origin of the lactate in extracellular fluid, and alternative interpretations of observed data that do not involve astrocyte-neuron lactate shuttling (Dienel 2019b) were not tested and ruled out (see Sect. 6 and Figs. 2 and 3). It is clear from the Matsui et al. series of studies that glycogen is an important fuel for brain during exhaustive exercise, but its cellular roles and metabolic fate remain to be established. Liver and muscle glycogen is also consumed during exercise, but the reported concentrations in these organs (Matsui et al. 2011, 2012, 2017) were probably underestimated due to postmortem ischemia. In contrast, postmortem levels of glycogen and other labile brain metabolites were appropriately preserved by microwave fixation to inactivate brain enzymes.

Roles of Glycogenolysis and MCT2 in Exercise Endurance

The hypothesis that glycogen mobilization contributes to endurance and may have a role in central fatigue was tested by Matsui et al. (2017) by pre-treating rats with an intracerebroventricular (icv) injection of an inhibitor of glycogen phosphorylase, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) 15 min prior to exercise. Hippocampal (but not cortical) glycogenolysis was impaired and the rise in lactate level was reduced by a small amount (~0.4 μmol/g, Table 6), ATP concentration fell by about 12% at exhaustion, and time to exhaustion was accelerated to about 73 min vs. 90 min with vehicle injection.

To evaluate whether lactate transport was involved, the rats were given an icv injection of an MCT2 inhibitor, α-cyano-4-hydroxycinnamate (4-CIN), which reduced hippocampal ATP level by 30% and the time to exhaustion by 35% from 80

to about 50 min. These findings were interpreted as both glycogenolysis and neuronal lactate transport being key factors in fatigue and in brain energetics during exhaustive exercise.

Interpretive Caveats with DAB and 4-CIN Treatments

The icv injections consisted of 10 μl volumes of 150 mM DAB in 0.9% saline or 36 mM 4-CIN in 40% dimethylsulphoxide (DMSO) in 0.9% saline (Matsui et al. 2017). The authors assumed a 100-fold dilution of these injectant concentrations in brain to give estimated final concentrations of 1.5 mM DAB, 0.36 mM 4-CIN, and 0.4% DMSO. The distribution an icv injectant in brain was visualized with Chicago sky blue, which appeared to label mainly the perivascular space in the major blood vessels (Fig. S4 of Matsui et al. (2017)), a labeling pattern similar to that of Evans blue-bound albumin, amyloid-β, glucose, lactate, and other metabolites (Dienel 2012; Ball et al. 2010), suggesting that much of the injectant in the cerebrospinal fluid may have been cleared from the tissue via the perivascular-lymphatic drainage system without mixing with interstitial fluid.

Inhibition of glycogenolysis in cultured astrocytes by pretreatment with 300 μM DAB for 60 min impaired fueling of the ATPase involved in Ca^{++} uptake into the endoplasmic reticulum by glycogen (Müller et al. 2014) (see Sect. 6.1.2.1 and Fig. 2a). Also, DAB (10 mM for 2 min) interferes with K^+ clearance from extracellular fluid and ATP release by cultured astrocytes (Hertz et al. 2015a; Xu et al. 2013, 2014) (see Sect. 6.1.2.2). Dysregulation of Ca^{++} and K^+ homeostasis can have secondary effects involving gliotransmitter release to alter neuronal activity and metabolism (reviewed in (Dienel 2019a, b)), perhaps contributing to changes in hippocampal lactate and ATP levels and fatigue, independent of any lactate shuttling to neurons as oxidative fuel (see Sect. 6 and Figs. 2 and 3).

4-CIN (estimated 360 μM) not only inhibits MCT2 (IC_{50} = 24 μM (Bröer et al. 1999)), but it is also a potent inhibitor of pyruvate transport into mitochondria (K_i = 6.3 μM (Halestrap 1975)). 4-CIN can enter erythrocytes, it blocks transport of pyruvate into and out of isolated mitochondria, and in perfused rat heart that has MCT1 and other MCTs (Halestrap and Price 1999) 200 μM 4-CIN increased the output of lactate and pyruvate, indicating inhibition of mitochondrial pyruvate transport and oxidation enhances glycolysis (Halestrap and Denton 1975; Halestrap 1975). Thus, 360 μM 4-CIN is likely to block oxidative metabolism of pyruvate *in all brain cells* within its distribution zone after icv injection, causing energy failure and contributing to the observed fall in ATP concentration and accelerated onset of fatigue (see Sects. 6.1.2.3 and 6.2.2 and Fig. 2b). This conclusion is supported by findings of McKenna et al. (2001) who showed that 250 μM 4-CIN reduced oxidation of glucose to CO_2 by 45–60% in cultured astrocytes and neurons without affecting glucose uptake from the medium. Another confound is that 0.4% DMSO modulates brain metabolism of glucose and pyruvate (Nasrallah et al. 2008), and its effects on metabolism during exhaustive exercise, while taken into account by vehicle treatments, are not known.

A major effect of 4-CIN treatment during exhaustive exercise is probably inhibition of oxidation of glycogen- and glucose-derived pyruvate, with additional effects on neuronal plasma membrane lactate fluxes. If neuronal pyruvate oxidation is impaired, neuronal glycolysis should be stimulated to provide more ATP. If the efflux of lactate from these neurons is impaired by 4-CIN, intracellular lactate would accumulate, eventually suppressing neuronal glycolysis and enhancing neuronal energy failure and onset of fatigue. On the other hand, enhanced glycolysis in astrocytes would lead to lactate efflux to blood and perivascular fluid as well as release to extracellular fluid via MCT1 and MCT4, where it could interact with the neuronal lactate receptor HCAR1 to suppress neuronal firing (Bozzo et al. 2013; Lauritzen et al. 2014) and secondarily reduce neuronal metabolism. For a more detailed discussion of these complex issues, see Sect. 6 and (Dienel 2019a, b). Inhibition of mitochondrial pyruvate transport and oxidation by 4-CIN, besides MCT2 inhibition, could explain the observed fall in ATP concentration and faster onset of fatigue without any lactate shuttling to fuel neurons.

7.3 Summary

Brain glycogen is consumed during hypoglycemia and prolonged exercise to exhaustion and contributes to cellular energetics and time of onset of fatigue. MCT2-mediated lactate transport may also be involved, but the directions and magnitudes of cellular lactate fluxes remain to be established. As in the studies of sensory stimulation and exhaustive exercise in humans where the oxygen-carbohydrate index falls to a very low value, the likelihood is that most glycogen is metabolized glycolytically to support astrocytic energetics, with lactate release to blood and perivascular-lymphatic drainage systems. There is currently no compelling evidence that glycogen-derived lactate fuels brain during exhaustive exercise. Alternative explanations and mechanisms for roles of glycogen and the sources of brain lactate need to be tested and evaluated.

8 Concluding Comments

Glycogen is clearly a very important active participant in astrocytic energetics, with glycolytic ATP production being important for support of different energy-dependent processes during activation. Na^+,K^+-ATPase activity for K^+ uptake from extracellular space is a major energy demand for astrocytes and neurons (DiNuzzo et al. 2017) that can be preferentially satisfied by glycogenolysis, even in the presence of glucose (Xu et al. 2013). In addition, glycolytic ATP generated by glycogenolysis may be utilized to maintain Ca^{++} homeostasis and fuel its pumping into the endoplasmic reticulum (Müller et al. 2014), and it may help fuel filopodial movements during neurotransmission (Reichenbach et al. 2010). Sparing of blood-borne glucose for

use by neurons when glycogen is mobilized in astrocytes (DiNuzzo et al. 2010b) is a novel alternative to the notion of glycogen-derived lactate shuttling. A stoichiometric model for the role of the glycogen shunt for glucose sparing during sensory stimulation and intense activation is evaluated in our companion paper (Rothman and Dienel 2019). This review has emphasized the roles of glycogen in the functions and energetics of cerebral cortex, and there are probably regional differences in functions of glycogen (e.g., in hippocampus and other structures involved in learning/memory), as well as differences between gray and white matter. Future quantitative studies of roles of glycogen in these processes and related metabolic pathway fluxes are required to better understand the functions of glycogen in living brain.

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State-Dependent Changes in Brain Glycogen Metabolism

Mauro DiNuzzo, Anne B. Walls, Gülin Öz, Elizabeth R. Seaquist, Helle S. Waagepetersen, Lasse K. Bak, Maiken Nedergaard, and Arne Schousboe

Abstract A fundamental understanding of glycogen structure, concentration, polydispersity and turnover is critical to qualify the role of glycogen in the brain. These molecular and metabolic features are under the control of neuronal activity through the interdependent action of neuromodulatory tone, ionic homeostasis and availability of metabolic substrates, all variables that concur to define the state of the system. In this chapter, we briefly describe how glycogen responds to selected behavioral, nutritional, environmental, hormonal, developmental and pathological conditions. We argue that interpreting glycogen metabolism through the lens of brain state is an effective approach to establish the relevance of energetics in connecting molecular and cellular neurophysiology to behavior.

Keywords Glycogen β-particle · Brain state · Astrocyte-neuron interactions · Ion homeostasis · Metabolic compartmentation

M. DiNuzzo (✉)
Center for Basic and Translational Neuroscience, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: mauro.dinuzzo@neuroenergetics.org

A. B. Walls · H. S. Waagepetersen · L. K. Bak · A. Schousboe
Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

G. Öz
Center for Magnetic Resonance Research, Department of Radiology, University of Minnesota, Minneapolis, MN, USA

E. R. Seaquist
Department of Medicine, University of Minnesota, Minneapolis, MN, USA

M. Nedergaard
Center for Basic and Translational Neuroscience, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Center for Translational Neuromedicine, University of Rochester Medical School, Rochester, NY, USA

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Abbreviations

GABA	γ-Aminobutyric acid
GP	Glycogen phosphorylase
GS	Glycogen synthase
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
PAP	Peripheral astrocytic process
REM	Rapid eye movement
TCA	Tricarboxylic acid
VIP	Vasoactive intestinal peptide

1 Introduction

The brain incessantly undergoes spontaneous fluctuations in regional cortical activity, neuronal excitability, neuron-glia communications and metabolism that reflect excitatory and inhibitory inputs updating the brain about the internal and external environment as well as plasticity changes coordinating functional connectivity and information processing (Bergmann 2018; Lee and Dan 2012; Quilichini and Bernard 2012; Zagha and McCormick 2014). All these energy-consuming transactions feed a number of recurrent loops between cortical and subcortical structures whose ultimate goal is to make behavioral responses appropriate for adaptation and survival (DiNuzzo and Nedergaard 2017). In the present context, we focus on two main aspects characterizing brain state, namely neuronal excitability (i.e. the capability of responding to incoming stimuli with appropriate behavioral responses) and synaptic plasticity (i.e. the capability of modifying neuronal excitability according to the relevance and information content of incoming stimuli). The brain is an open system and as such it works by exchanging energy with its surroundings. In particular, the metabolism of a living cell, tissue or organism is not separable from its interactions with the environment, i.e. what is commonly referred to as function. Brain state alters tissue energy metabolism in terms of metabolic rates, pathways and substrates (Aalling et al. 2018). Most importantly, not only does cerebral metabolism adapt to specialized cellular energy demands, but metabolic products exert critical signaling functions that in turn can modulate brain activity (DiNuzzo 2016).

The first demonstrations that brain glycogen level parallels a number of altered behavioral states date back to the 1950s (Chance and Yaxley 1950; Kubo and Shimizu 1957; Svorad 1958a, b). Since then, many experimental investigations carried out in humans and other mammals (some of which we review in this chapter) have unequivocally shown that glycogen actively supports brain function in a state-dependent fashion. In the following, we first summarize the metabolic features of brain glycogen that are fundamental to understand its role as glucose reserve. We

then enumerate the experimental findings supporting a close relationship between brain state and glycogen metabolism. Our aim is to illustrate the complexity of such relationship, although the literature is not complete under many viewpoints and our discussion is thus necessarily incomplete. Finally, we speculate about the potential relevance of glycogen to the basic neurophysiological mechanisms that concur to shape brain states.

2 Molecular and Metabolic Features of Brain Glycogen

2.1 Structure

Glycogen is a homogeneously branched polysaccharide that has evolved to store large amounts of readily-releaseable glucose residues while circumventing osmolarity issues (Melendez et al. 1997). The structural features of glycogen need to be taken into account in order to understand the biochemical properties underlying its synthesis and degradation patterns (Roach 2002). Briefly, glycogen biosynthesis is thought to start from an initial glucose chain derived from the self-glucosylating protein glycogenin (but see Testoni et al. 2017). An individual glucose chain grows linearly by polymerization through α-1,4-glycosydic linkages and branches out every 4–5 residues through α-1,6-glycosydic linkages. This gives rise to a mature glycogen granule (or β-particle) consisting of 12 concentric tiers of chains accommodating 13 glucose residues on average. Having a branching degree of 2, each successive tier contains twice the amount of glucose of the preceding ones. Cessation of biosynthesis of both the single chain and the whole molecule occurs due to steric factors hindering the catalytic activity of glycogen synthase (GS) and possibly glycogen branching enzyme (Melendez-Hevia et al. 1993; Melendez et al. 1998). Although particle size is thought to be a self-limiting factor, other yet unknown mechanisms might contribute to control the size of individual β-particles. Notably, many unphysiological conditions are associated with excessive elongation of glucosyl chains that might result in a bigger molecular radius of glycogen molecules not necessarily accompanied by higher glucose content. As an illustration, using computational modeling it has been shown that the loss of affinity of GS for inner chains yields glycogen particles with a substantially bigger size compared with “normal” mature particles with the same number of glucose units (DiNuzzo 2013).

Much like synthesis, degradation of glycogen by glycogen phosphorylase (GP) can only occur at the surface of the glycogen granule, i.e. enzymes do not have free access to the bulk of glucose residues. Moreover, GP can exclusively proceed from glucose chains longer than 4 residues, at which point maximally trimmed chains must be transferred to adjacent chains by glycogen debraching enzyme. Therefore, in principle only 34.6% (9/13 of 50%, i.e. last tier content) of the total glucose contained in the glycogen molecule can be mobilized before any debranching occurs. In fact, the effective concentration that GP can “see” at any moment is a complex

function depending on the molecular location of the enzyme (DiNuzzo 2013). Glycogen structure is especially relevant to labeling experiments, because the dynamics of labeled glucose cannot be thought as a random process and standard kinetic modeling might provide incorrect results (Oz et al. 2015).

Another fundamental and somewhat overlooked consequence of glycogen structure is the accessibility to degradation. Aberrant hyperphosphorylated glycogen granules called polyglucosans as well as glycogen-based aggregates, such as Lafora bodies and corpora amylacea, are synthesized under both physiological (e.g., aging) and pathological (e.g., epilepsy) conditions (DePaoli-Roach et al. 2015; Roach 2011; Sinadinos et al. 2014; Tagliabracci et al. 2011). Polyglucosans are characterized by excessive chain length and inhomogeneous branching, which makes these compounds water-insoluble and thus unable to undergo enzymatic degradation. There is evidence that insolubility of glycogen is independent of incorporation of C2 and C3 phosphate monoesters by GS and instead largely depends on dysfunctional chain-length regulation (Sullivan et al. 2017, 2019). Overall, the levels of glycogen in the brain are not the sole variable to consider, because the underlying glycogenolytic capacity critically depends on whether the polysaccharide is accessible to degradation. Unfortunately, at present there is considerable lack of experimental tools for the measurements of glycogen structure (e.g., homogeneity and branching). In principle, excessive chain length can be revealed by measuring the glucose released during alternating treatments with glycogen phosphorylase (yielding a limit dextrin) and glycogen debranching enzyme. In addition, comparison of the total glucose released by the latter procedure with that released by lysosomal acid α-glucosidase could possibly reveal the fraction of glycogen resistant to hydrolysis (DiNuzzo et al. 2015b). The presence of phosphate esters can be measured from digested glycogen using inorganic phosphate assay after, for example, recombinant laforin treatment (Tagliabracci et al. 2007). Specific phosphate esters can be identified through matrix-assisted laser desorption ionization time-of-flight mass spectrometry eventually combined with nuclear magnetic resonance (NMR) spectroscopy (Tagliabracci et al. 2011).

2.2 Concentration

Glycogen concentration is commonly determined by ex vivo amyloglucosidase-based assay. Glycogen levels from rodent brain are reported to be between 3 and 12 μmol/g (Dienel and Cruz 2015). Such a wide range reflects the exquisite sensitivity of glycogen upon experimental parameters. Animal protocols suffer from several important experimental issues, such as high lability of glycogen and its rapid post-mortem degradation (depending on tissue fixation, e.g., liquid nitrogen versus focused-beam microwave irradiation), stress-induced glycogenolysis due to animal handling before euthanasia, nutritional history (e.g., overnight fasting prior to

experiments) and time of the day (predominant sleep or wake periods), among others (Cruz and Dienel 2002; Dienel 2019b). The conditions under which glycogen concentrations are measured in human brain by in vivo ^{13}C magnetic resonance (^{13}C-NMR) spectroscopy are also stressful and require continuous labeled glucose infusion protocols and long acquisition periods (>8 h) (Oz et al. 2007). Values obtained by ^{13}C-NMR spectroscopy may be biased by partial volume effects, for example if the spectroscopic voxel contains several structures known to have different glycogen content. Using ^{13}C-NMR spectroscopy, an average value of nearly 8 μmol/g for glycogen level has been recently reported in the occipital lobe of healthy human subjects (Oz et al. 2015).

Under physiological conditions, glycogen and glycogen-metabolizing enzymes are almost exclusively confined to astrocytes, although neurons also store very low amounts of glycogen (about 10- to 30-fold less than in astrocytes) (Saez et al. 2014). Assuming a volume fraction of astrocytes in cortical gray matter to be in the range 10–15% across species [mouse cerebral cortex <11% (Braitenberg and Schüz 1998); rat hippocampus 8 ± 4% (Mishchenko et al. 2010); cat motor cortex 11.4% (Barron et al. 1988); cat frontal cortex 15.5% (Williams et al. 1980); rabbit visual cortex 12.7 ± 2.2% (Schmolke and Schleicher 1989); human parietal cortex 11.4% (Virgintino et al. 1997)] and a basal glycogen level in the gray matter of mammalian brain of 6–10 μmol/g (Cruz and Dienel 2002; Oz et al. 2015), the glycogen concentration in terms of glucose equivalents in astrocytes would be 40–100 mmol/L or at least >30-fold of the free glucose concentration at euglycemia (~1.2 mmol/L). Even though the "effective" glycogen concentration available to enzyme action is actually lower, the amount of glucose that can be released by glycogenolysis is very large, especially considering the rapidity of glycogen breakdown (Obel et al. 2012b). Most of brain glycogen resides in perineuronal peripheral astrocytic processes (PAPs) and in perivascular endfeet, while a minor fraction is found in cell soma and primary astrocytic processes (Oe et al. 2016). In particular, PAPs include a huge number of filopodia and lamellipodia having very small intracellular volume, high cytosolic tortuosity and restricted diffusion (Hertz et al. 2007). Thus, glycogen might be particularly important in cellular regions with high surface-to-volume ratio, a feature that has been suggested to identify a role for glycogenolysis in the control of ion homeostasis and glucose channeling (DiNuzzo 2019).

A poorly understood phenomenon of glycogen metabolism is glycogen supercompensation, a rebound of glycogen synthesis above basal levels occurring whenever glycogen is degraded. Glycogen supercompensation occurs in brain as well as in other tissues (see for example Matsui et al. 2012) in response to both physiological (e.g., physical exercise) or pathological (e.g., hypoglycemia) energy challenges. In the brain, given the high degree of polydispersity (see below) and the relative abundance of immature glycogen particles, it is difficult to predict whether supercompensation takes place in the same particles that underwent partial degradation or also involves recruitment of new granules. Furthermore, it is presently unknown whether glycogen supercompensation is epiphenomenic or functionally meaningful.

2.3 Polydispersity

In the brain, glycogen is almost exclusively found in the form of β-particles. These are quasi-spherical molecules having a theoretical maximal diameter of ~46 nm, corresponding to a molecular weight of ~10^7 Da and a total number of glucose residues of about 53,000 (13 residues per chain times 2^{12} chains, where 2 is the branching degree and 12 the total number of tiers) (DiNuzzo 2013). Glycogen synthesis is thought to occur in discrete steps with associated enzymology, leading first to the formation of proglycogen (i.e. a ~400 kDa polysaccharide, acid-insoluble fraction) and then macroglycogen (i.e. the mature glycogen molecule, acid-soluble fraction) (Lomako et al. 1991, 1993). However, the existence of well-defined glycogen species is presently questioned and there is strong evidence of continuous polydispersity in vivo (Katz 2006; Roach 2002). Glycogen β-particle size is best seen using three-dimensional serial block electron microscopy. Using this method, recent glycogen particle counting from mouse primary motor cortex showed a very broad distribution of molecular size spanning the range 8–58 nm (Bellesi et al. 2018), similar to that reported from human vastus lateralis muscle biopsies (10–44 nm) (Marchand et al. 2002). The distribution of glycogen size observed in the brain was somewhat bimodal, with most of the particles having a diameter of >22 nm and a small but separable peak around 16–19 nm. Notably, this size corresponds to a molecular weight of 200–400 kDa, but whether it indeed represents a distinct glycogen pool cannot be presently established. The study also found an average granule diameter of 27 nm in cortical astrocytes, with few particles exceeding 40 nm (Bellesi et al. 2018), essentially the same value reported in human skeletal muscle fibers (mean diameter 26 nm) (Marchand et al. 2002) or rabbit retina Müller gial cells (mean diameter 23 nm) (Magalhaes and Coimbra 1970). It should be realized that these studies suffer from rapid post-mortem (or post-extraction) glycogenolysis, which most likely shifted the glycogen size distribution towards smaller values. Further ultrastructural electron microscopy experiments employing focused-beam microwave irradiation are required.

In addition to ultrastructural studies employing electron microscopy, estimates of glycogen polydispersity in brain tissue can be obtained through immunohistochemistry, although more knowledge is needed about the affinity of specific antibodies for glycogen particles of different size (Hirase et al. 2019; Oe et al. 2016). Size distribution of purified glycogen molecules in solution can be measured by dynamic light scattering (Zhang et al. 2001), refractive index-based size exclusion chromatography (Deng et al. 2016; Izumi et al. 2013; Sullivan et al. 2014) and time-resolved acid hydrolysis (Deng et al. 2016).

2.4 Turnover

Glycogen turnover is the simultaneous insertion and release of glucose residues in the β-particle, which can lead to steady-state (i.e. no change in glycogen levels) when the underlying reaction rates are identical, and otherwise to net synthesis or

net degradation. While in principle glycogen turnover only applies at the level of individual glycogen granule, the situation in vivo is complicated by the fact that different granules can be found in different states of synthesis and degradation. The distribution of glycogen granule size suggests that the fraction of mature β-particles is relatively small. The high degree of polydispersity is consistent with the notions that (1) GP operates very rapidly by targeting the small pool of mature β-particles (i.e. those exhibiting the highest glycogenolytic capacity), and (2) GS is considerably slower, yet it operates on the large pool of incomplete β-particles. The activity of GP or GS can be measured by a spectrophotometric method using the enzyme purified from the brain. This approach employs coupled enzyme systems to measure the optical properties associated with the activity-dependent production of a specific substrate, commonly NAD(P)H. However, this approach only measures enzyme capacity (e.g., sensitive to enzyme phosphorylation state), and it does not take into account other in vivo conditions that critically depend on neuronal activity (e.g., level of allosteric effectors such as AMP).

As mentioned above, glycogen β-particles exhibit highly non-homogeneous turnover. Indeed, synthesis and degradation occur simultaneously at the molecular surface of glycogen. Thus, the "effective" concentration of glycogen changes with the size of the molecule. It is, however, possible to define a molecular turnover that is independent of both the size of individual particles and the total number of particles. By optimizing the fit between measured and predicted time-course of glycogen labeling experiments, it has been estimated that human brain glycogen turns over with a rate of 40 residues per minute under basal conditions (Oz et al. 2015). In order to obtain the turnover rate in the brain, the molecular turnover value needs to be multiplied by the tissue glycogen content, which gives a turnover of 6 nmol/(g min) for a concentration of 8 μmol/g. This number represents the activity of GS and GP averaged over long time periods and over many glycogen granules. Therefore, turnover per se is not informative on the frequency and rate of glycogenolytic bursts.

The concept of glycogen shunt takes into account the temporal separation between glycogen synthesis and degradation (Shulman et al. 2001a, 2001b; Shulman and Rothman 2001, 2015). As an illustration, let us consider a glycogen turnover of 6 nmol/(g min) or 0.1 pmol/(g s). When examined at a temporal resolution of 1 ms, this turnover can be explained by either a continuous process involving 0.1 fmol/(g ms) or a series of discrete bursts, for example lasting 1 ms and occurring at a frequency of 10 Hz, each involving 10 fmol/(g ms), i.e. a rate 100-fold higher than the continuous case. Thus, a rapid burst of net glycogen breakdown for glycolytic ATP is followed by a longer period of net glycogen resynthesis (in the above example having a duration of 99 ms). Overall, such process does 'shunt' glucose through glycogen, which is important as glycogen can meet large and rapid increases in glucose demand that could not be supplied by glucose transport and phosphorylation. These considerations are critical to understand the role of glycogen in the brain, but at present there are no experimental tools to measure glycogen shunt in astrocytes, i.e. glycogenolysis at high spatiotemporal resolution.

Glycogen turnover is commonly determined in vivo or in vitro using labeling experiments, e.g. radioassays or NMR spectroscopy employing ^{14}C- or ^{13}C-labeled glucose, respectively. Compared to similar labeling techniques, ^{13}C-NMR

spectroscopy has two important advantages. First, it does not require the delicate and time-consuming process of post-mortem tissue extraction/fixation and glycogen isolation. Second, it allows to follow the full temporal dynamics of label incorporation, retention and washout in the same animal/subject in vivo. On the other side, it also has disadvantages: it requires high-tech equipment, whole body labeling with [^{13}C]glucose is expensive, sensitivity may be an issue, and time frames for analysis of changes in glycogen level or labeling differ from other methods. Other promising (though only qualitative) approaches that have been used in other tissues include real-time optical methods, such as 2-NBDG pre-loading (Louzao et al. 2008), although in the cerebral cortex and hippocampus this glucose analogue predominantly labels neurons in situ (unpublished observation).

3 State-Related Factors Affecting Synthesis and Degradation of Brain Glycogen

3.1 Behavioral Factors

3.1.1 Rest or Quiet Waking

Uniquely defining basal activity for the "restless" brain (Raichle 2011) is a difficult, if not impossible, task. Many experimental studies assume that different sub-states of wakefulness can be studied in relative terms, i.e. by examining changes between them. Although this assumption is not inherently incorrect, it is de facto impractical because wakefulness depends on a large number of variables and the actual baseline state, which is only incompletely defined, may significantly alter the functional and metabolic response to a given stimulus (Scholvinck et al. 2010; Shulman et al. 2009, 2007). In the present section, we discuss the relevance of some of these variables in the context of brain glycogen metabolism. In experiments involving humans, rest is defined as a state in which the subjects commonly lie supine, voluntarily abstain from (or substantially reduce) movements and maintain a low vigilance level (i.e. mind-wandering). This state is substantially different from its animal counterpart, where rest does not necessarily involve inactive immobility, and when it does it is normally associated with high levels of restraint-associated stress and/or discomfort. These considerations indicate that the concept of "quiet waking" (reduced locomotion and absence of sensory/cognitive stimulation) is perhaps more appropriate in order to attempt generalizing findings obtained in humans and animals.

Glycogen concentration in resting healthy human subjects is about 6–8 μmol/g (Oz et al. 2015), which is close to the mid-range value of glycogen levels reported in rat brain (4–12 μmol/g) (Cruz and Dienel 2002; Garriga and Cusso 1992; Kong et al. 2002; Swanson et al. 1989a, 1990) and mouse brain (3–14 μmol/g) (Nelson et al. 1968; Oe et al. 2016; Watanabe and Passonneau 1973). The corresponding turnover times are nearly 22 h in humans (Oz et al. 2015, 2007), 7–10 h in rats (Choi and Gruetter 2003) and 4–5 h in mice (Watanabe and Passonneau 1973) (although

some reports vary substantially, see for example Prokhorova and Tupikova 1958). These numbers are consistent with turnover rates of 0.35 μmol/(g h) in humans (Oz et al. 2015, 2007), 0.5 in rats (Choi et al. 1999) and 0.6–0.9 in mice (Watanabe and Passonneau 1973), which roughly correspond to 1–2% of cerebral metabolic rates of glucose in these species (Dienel 2019a).

3.1.2 Stimulation or Active Waking

Similar arguments of those mentioned above can be adduced to use the concept of "active waking" instead of "stimulation". Indeed, the latter can involve one or more sensory modalities with different impact on stimulus perception and/or cognitive stimulus processing, which might or might not result in specific behavioral responses (e.g., increased locomotion). Moreover, stimulation consists of different stages (alerting, onset, progression, recovery) that are normally associated with changes in neuromodulatory tone inducing corresponding changes in brain metabolism (Dienel and Cruz 2016). Locomotion per se is not correlated with changes in brain glycogen content (Hutchins and Rogers 1970).

Ex vivo ^{14}C autoradiography demostrated a ~20% label loss upon 10–20 min (but not 2–5 min) of whisker stimulation in rat somatosensory cortex (Swanson et al. 1992) (Fig. 1). Similarly, 5–6 min or 30 s of generalized sensory stimulation in rats decreased the amount of ^{14}C from prelabeled glycogen by 33% or 16%, respectively (Cruz and Dienel 2002). Notably, 5–6 min (but not 30 s) of stimulation were required to observe a decrease (about 22%) in total glycogen concentration, suggesting that the glycogenolytic response to stimulation involves only the metabolically active (i.e. where labeled residues first disappear) surface of the molecule. Moreover, glycogen turnover is thought to increase much more than net degradation during stimulation, which implies concomitant resynthesis (Dienel et al. 2007; Dienel and Cruz 2015). These notions are somewhat contradictory with the finding that no change of ^{13}C-glycogen was found upon 20 min of visual stimulation in the occipital cortex of human subjects when previously-labeled glycogen was allowed to turn over in the presence of unlabeled glucose (DiNuzzo 2013; Oz et al. 2007). It should also be noted that the human studies measure signal from a relatively large volume-of-interest and may have been confounded by partial volume effects. Hence, glycogenolysis may have not been detectable within the sensitivity of the technique due to predominant label loss from the outier tiers of glycogen prior to stimulation in a fraction of the volume. Interestingly, inhibition of glycogenolysis in rats had no effect on resting glucose consumption, but resulted in a regional compensatory rise in glucose consumption during generalized sensory stimulation (about 25–50% above the respective value in stimulated vehicle-treated controls) (Dienel et al. 2007). Overall, glycogen is mobilized upon stimulation with a glycogenolytic rate approaching half of cerebral glucose utilization, or a 25- to 50-fold above the turnover during unstimulated conditions (for an in-depth discussion, see Dienel 2019a). This notion is in agreement with the very large enzymatic capacity of both GS and GP relative to their basal reaction rates. For example, in rat brain maximal activities

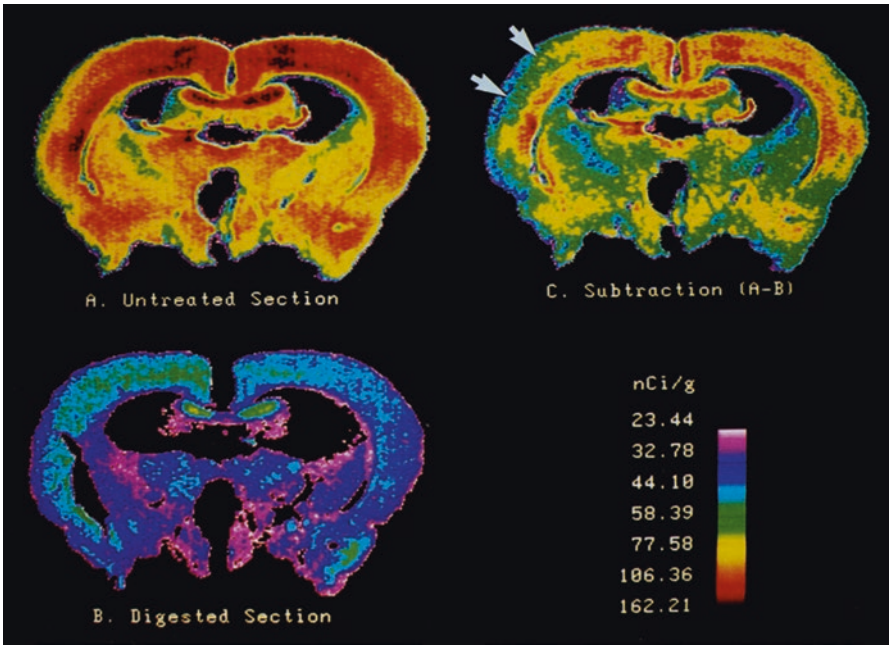

Fig. 1 Glycogen utilization in rat brain during unilateral whisker stimulation. Representative coronal sections including somatosensory cortex showing ^{14}C-labeled glycogen before (**a**) and after (**b**) amyloglucosidase-based glycogen digestion. The difference image (**c**) shows side-to-side ^{14}C activity changes in the barrel field region, indicating stimulation-induced glycogenolysis. Adapted from (Swanson et al. 1992) (with permission)

of GP and GS have been reported to be as high as 300 μmol/(g h) and 26 μmol/(g h), respectively (see Nelson et al. 1968 and references therein). There is some evidence that glycogen supercompensation might occur in the brain after sensory stimulation (Abdul-Ghani and Nahas 1990).

3.1.3 Anesthesia

The effect of anesthesia is generally characterized by a dramatic increase of brain glycogen levels, which is almost independent of body temperature and anesthesia-induced hyperglycemia (Nelson et al. 1968). Changes in brain glycogen metabolism associated with anesthesia are secondary to depression of neuronal activity (i.e. glutamergic and monoaminergic neurotransmission), as anesthetics are without effect when applied directly in astrocytic cultures (Swanson et al. 1989b). Since anesthesia affects energy metabolism much more in gray matter than white matter (McKenna et al. 2012), it is expected that anesthesia-induced changes in glycogen content are correspondingly more elevated in gray matter regions. Accordingly, prolonged (8 h) anesthesia has been found to increase glycogen levels in all cortical

layers (200% in layer I and 30–40% in layers II–VI) but not in subjacent white matter of mouse brain (Folbergrova et al. 1970).

Anesthesia-induced rise in brain glycogen content (~20%) has been reported in mice during ether vapor exposure (1 h at 4.5%) (Estler and Heim 1960). Halothane (2 h at 1.5–2.5%) has been observed to increase glycogen concentration by ~40% in rat cerebral cortex and cerebellum with bigger changes in adult compared with young animals (Gip et al. 2002). Similar values have been reported in mouse brain for halothane (50% or 150% increase at 2 or 4 h, respectively) and other volatile anesthetics (up to 200%) (Brunner et al. 1971). Isoflurane (105 min at 2%) has been reported to increase brain glycogen content by 20% in rat brain (Morgenthaler et al. 2006). Pentobarbitone (40 mg/Kg) anesthesia has been found to increase glycogen content in rat cerebral cortex, cerebellum, brainstem and spinal cord (range 50–120%) (Nahas and Abdul-Ghani 1989). Similarly, rats given pentobarbitone (50 mg/Kg) had a 35% increase in whole-brain glycogen content (assayed at 15–25 min) (Dalsgaard et al. 2007). Phenobarbitone (150 mg/Kg) has been reported to increase glycogen concentration in mouse cerebral cortex, hippocampus, cerebellum and brainstem (range 15–50% assayed at 60 min) (Estler 1961; Gatfield et al. 1966). Similarly, mice anesthetized with phenobarbitone (150 mg/Kg) had whole-brain glycogen increasing progressively with time (60–250% during 24 h) and glycogen turnover decreasing to one-half of control animals (Watanabe and Passonneau 1973) (Fig. 2). The effect of phenobarbitone on mouse brain glycogen (assayed at 8 h) has been shown to be dose-dependent (60%, 190% and 220% for 75, 170 and 240 mg/Kg) (Nelson et al. 1968). In another study, phenobarbitone (250 mg/Kg) failed to alter glycogen in rat brain (8% increase was not significant; assayed at 75 min), although it immediately halved glycogen turnover (Strang and Bachelard 1973). An ultrastructural study of the effect of phenobarbitone (150 mg/Kg) on mouse brain glycogen demonstrated substantial accumulation of the polysaccharide in hippocampus and frontal cortex, in large part due to the formation of aggregates of β-particles often included in autophagic vacuoles (Phelps 1972) (Fig. 3). Tranquillizers and other substances depressing noradrenergic signaling have also been associated with increased brain glycogen levels (Hutchins and Rogers 1970; Nelson et al. 1968; Svorad 1959). Overall, the above-mentioned results (often affected by postmortem glycogen degradation to variable extent) demostrate that anesthesia produces a substantial rise of glycogen content and a decline of glycogen turnover in the brain.

3.1.4 Sleep

Replenishment of brain glycogen stores consumed during waking is the key mechanism underlying the so-called energy hypothesis of sleep (Benington and Heller 1995; Scharf et al. 2008). Initial observations for diurnal changes of brain glycogen content were obtained in rats, whereby lower values were observed during the predominantly-wake compared with the predominantly-sleep period of the day (Hutchins and Rogers 1970; Pronaszko-Kurczynska 1976; Svorad 1958b) (Fig. 4). Thereafter, rapid (i.e. minutes) and substantial (i.e. 70% above basal levels) glycogen accumulation upon natural slow-wave sleep onset and likewise rapid return to

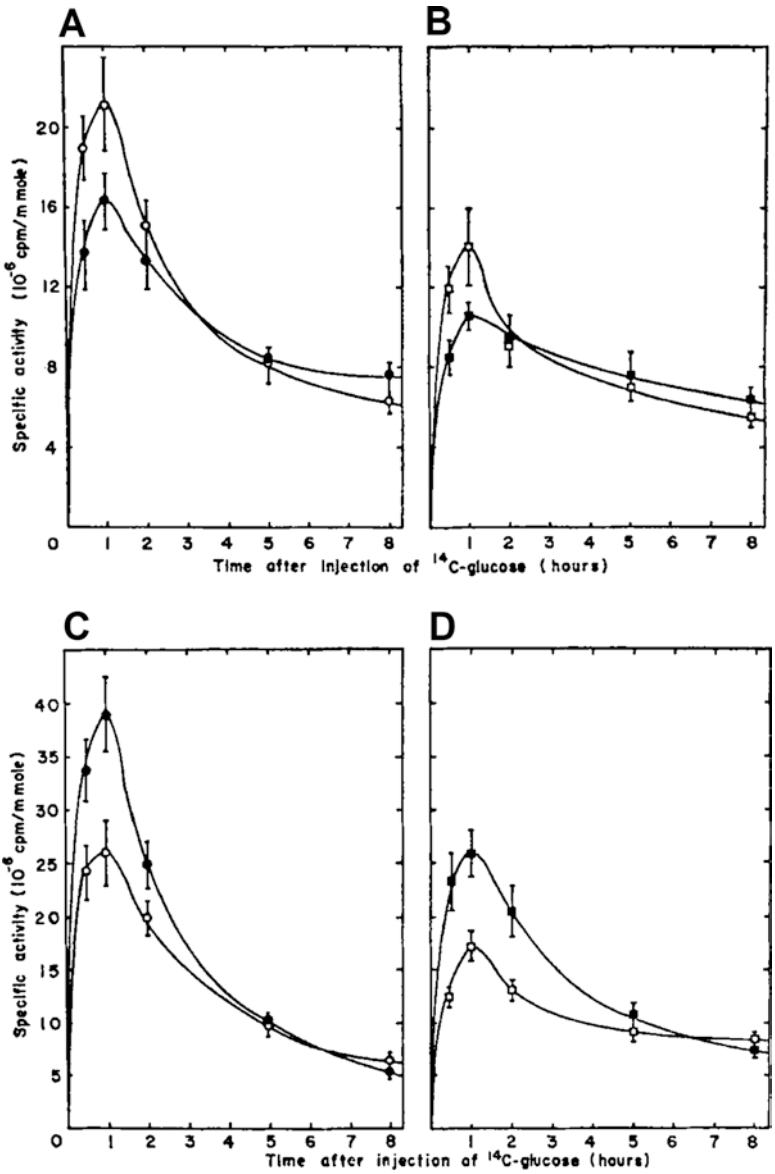

Fig. 2 Incorporation of labeled glucose into mouse brain glycogen. (**a**, **b**) [U-^{14}C]glucose glucose (10 μCi/20 g) was given 5 h after either saline (control, open symbols) or phenobarbital (150 mg/Kg) anesthesia (filled symbols). Label incorporation during phenobarbital anesthesia is showed for glycogen (**a**) and limit dextrin (**b**). (C,D) Labeled glucose was given 5 h after either saline (control, open symbols) or hydrocortisone (25 mg/Kg) anesthesia (filled symbols). Label incorporation during hydrocortisone treatment is showed for glycogen (**c**) and limit dextrin (**d**). The corresponding glycogen turnover decreased in anesthetized animals and increased in hydrocortisone-treated animals, yet both drugs brought about a substantial increase in total glycogen concentration. Adapted from (Watanabe and Passonneau 1973) (with permission)

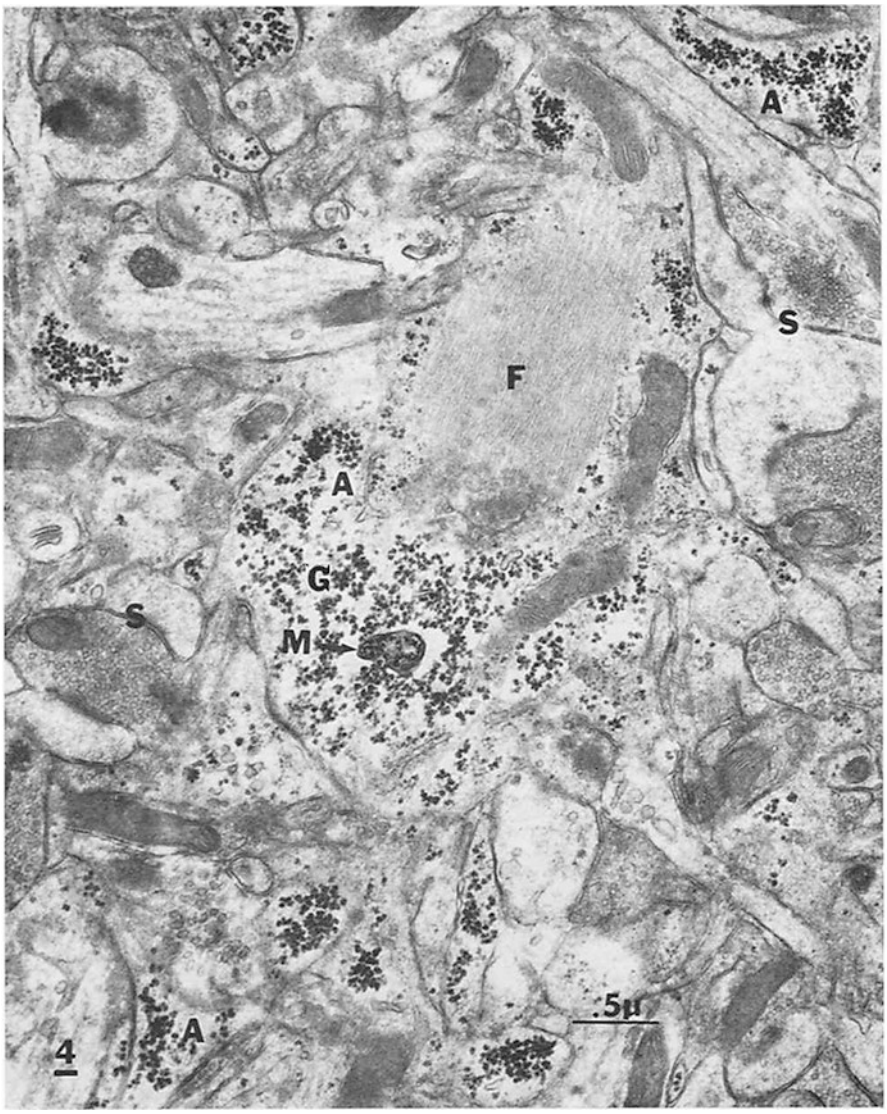

Fig. 3 Glycogen granules in the dentage gyrus of mouse hippocampus after anesthesia. Six hours after phenobarbital (250 mg/Kg) injection glycogen granules (G) were diffusely present in the cytoplasm of peripheral astrocytic processes (A), the latter interspersed among axo-dendritic synapses (S). Several glycogen granules were contained in a membrane-bound organelle (M). Large astrocytic processes containing gliofilaments (F) are also visible in this electromicrograph. High density of glycogen β-particles is a hallmark of the anesthetized brain and is not observed under wakefulness, natural sleep or sleep deprivation (compare with Fig. 5c). Scale bar: 0.5 μm. Adapted from (Phelps 1972) (with permission)

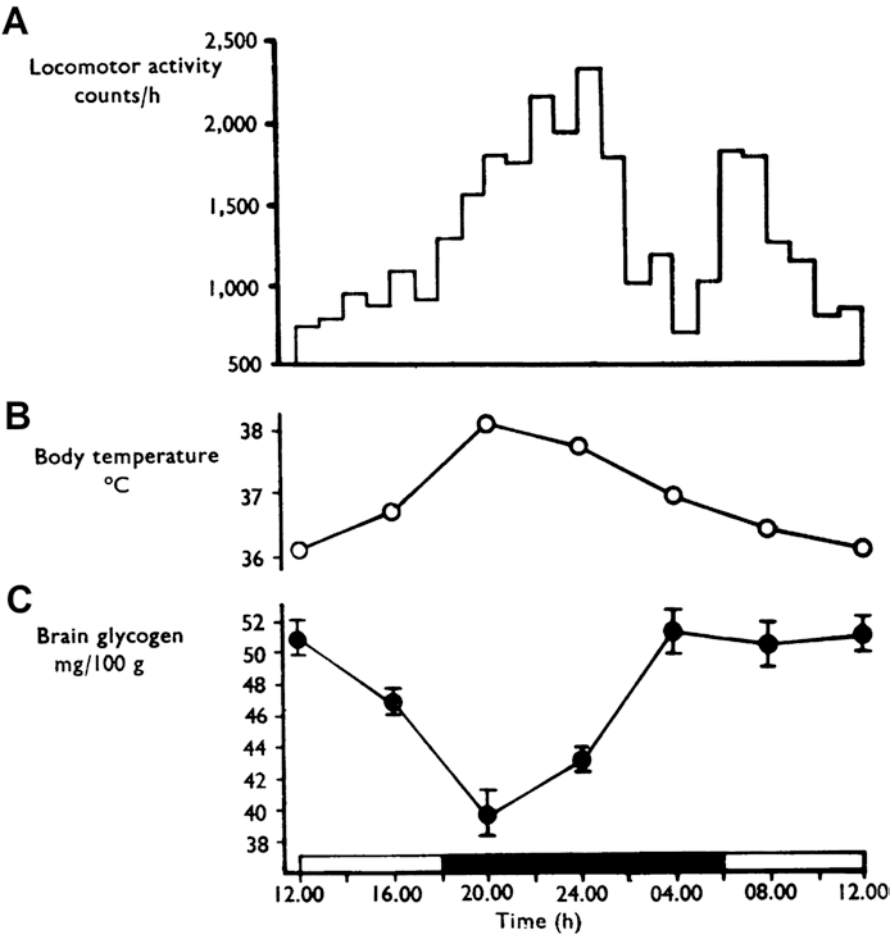

Fig. 4 Typical circadian changes in locomotor activity, body temperature and brain glycogen content in mice. (**a**) Locomotion increased progressively during the dark period (predominantly awake), but animals moved substantially even during the light period (predominantly sleep). Therefore, there was no clear correlation between locomotor activity and either body temperature (**b**) or brain glycogen (**c**). In particular, changes in brain glycogen content (mean ± SEM) appeared to anticipate the light-dark transitions, although the statistical power of the study was limited (3 trials). Adapted from (Hutchins and Rogers 1970) (with permission)

baseline upon awakening were reported in rats (Karnovsky et al. 1983). Notably, these changes in cerebral glycogen content parallel sleep- and wake-related alterations in brain extracellular glucose and lactate concentrations, consistent with reduced glucose utilization and lactate production taking place during sleep (reviewed in DiNuzzo and Nedergaard 2017). Moreover, the rapidity of changes of brain glycogen in following sleep-wake cycle is at odds with recovery of brain glycogen content after sleep deprivation (2 h) or anesthesia (3–9 h) (Choi and Gruetter 2003; Franken et al. 2006; Karnovsky et al. 1983; Watanabe and Passonneau 1973),

which evidences the profound differences between these brain states. The role of glycogen resynthesis in sleep is presently debated (Petit et al. 2015). However, a recent electron microscopy study investigating glycogen granules in mouse cerebral cortex during wakefulness and natural sleep strongly supports the notion that brain glycogen is closely linked to the sleep-wake cycle (Bellesi et al. 2018). Specifically, sleep has been associated with reduction in granule number as well as concomitant increase in granule size and distance from the synaptic cleft, resulting in an overall increase of cortical glycogen levels by ~6% during sleep compared with wakefulness (Bellesi et al. 2018) (Fig. 5). As stated above, this study is limited by post-mortem degradation (Lowry et al. 1964) and the sleep-related change in brain glycogen content might be inaccurate. Nonetheless, the sleep-related alteration of glycogen localization serves to illustrate the notion that concentration changes do not exhaust the possible modifications of brain glycogen metabolism taking place under different states. For example, rearrangement of glycogen granules in different subcellular domains of astrocytes might alter the functional relevance of glycogen synthesis and degradation under different brain states. In addition, it has been suggested that sleep might serve to restore glycogen structure (eventually channeling aberrant mocules to lysosomal degradation or lymphatic drainage) rather than to replenish glycogen levels in the brain (DiNuzzo et al. 2015b).

Glycemic levels are unlikely to play a role in the changes of brain glycogen content during the sleep-wake cycle, because the diurnal variations of blood glucose are opposite to those of brain glycogen (Ahlersova et al. 1980). The pattern of brain glycogen oscillations is also reversed compared to what happens in muscle, whereby glycogen is increased during (sedentary) wakefulness and decreased during sleep (although there is also a purely circadian component; see Takahashi et al. 2015 and references therein).

3.1.5 Sleep Deprivation

Sleep deprivation is sometimes assumed to be an extension of waking. However, such assumption is wrong, if anything because external interventions (i.e. stressors) are required to maintain wakefulness (McEwen 2006). A study on Drosophila Melanogaster demonstrated that sleep deprivation is associated with elevated stress levels, which recapitulated the observed whole-body metabolic changes even in the absence of sleep loss (Harbison and Sehgal 2009). The difficulty in separating the effect of stress from sleep loss is evidenced by the finding that glucocorticoids strongly influence brain glycogen levels during sleep deprivation protocols in rats (Gip et al. 2004). Accordingly, gentle sleep deprivation elicts a nearly twofold increase in plasma corticosterone levels (Petit et al. 2010). Although some animal studies have incorporated control stress groups (e.g., see Karadzic and Mrsulja 1969a), it is still questionable whether such controls are appropriately established (Karnovsky et al. 1983).

The complex effects of stressors as well as other variables on brain glycogen metabolism can explain the high degree of discrepancy between different studies (Petit et al. 2015). It has been reported that REM (or paradoxical) sleep depriva-

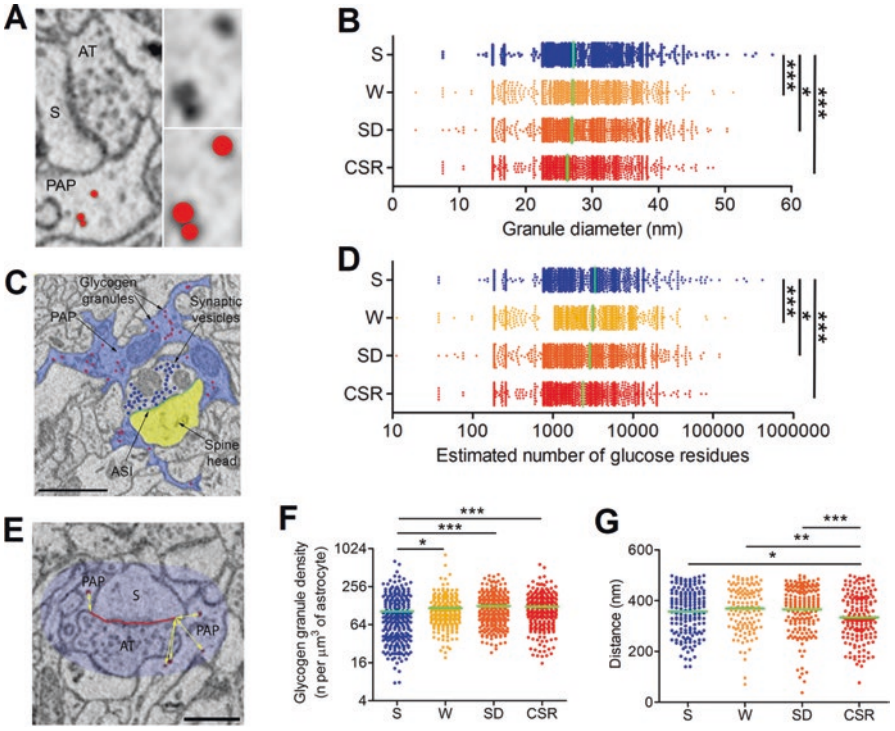

Fig. 5 Brain glycogen granule distribution and subcellular localization in the cerebral cortex of mouse under different states. (**a**, **c**, **e**) Glycogen segmentation (red circles) from a representative electron micrograph of mouse primary motor cortex (layers II/III) containing a peripheral astrocytic process (PAP) surrounding a synapse (dendritic spine, S; axon terminal, AT). Segmentation of cellular elements for exclusion of synaptic vesicles (blue circles) and identification of axon-spine interface (ASI). (**b**) Distribution of granule diameter under sleep (S), wake (W), sleep deprivation (SD, 8 h) or chronic sleep restriction (CSR, 4.5 days). (**d**) Number of glucose units in cortical glycogen estimated from granule diameter and corresponding density of glycogen granules (**f**) and distance from ASI (**g**). Regardless the limitations of the study (see text) it is apparent that brain state affects several features of glycogen, namely granule density and localization, in addition to granule size and turnover. Adapted from (Bellesi et al. 2018). Copyright © 2018 Bellesi, de Vivo, Koebe, Tononi and Cirelli

tion reduces brain glycogen levels in rats and cats (Karadzic and Mrsulja 1969b). Specifically, decreases in glycogen content in hippocampus and subcortical structures (but not in the cerebral cortex) were observed during REM sleep deprivation (Karadzic and Mrsulja 1969a; Mrsulja et al. 1967). Similarly, total sleep deprivation resulted in a decrease of glycogen content in rat hippocampus (9%) and cerebellum (23–24%) but not in cortex at 6 h, while cortical glycogen actually increased after 12 h of sleep deprivation (Gip et al. 2002, 2004). The same outcome was observed in mouse cerebral cortex, with a monotonic glycogen increase during sleep deprivation up to 6 h (Franken et al. 2006). Quite opposite

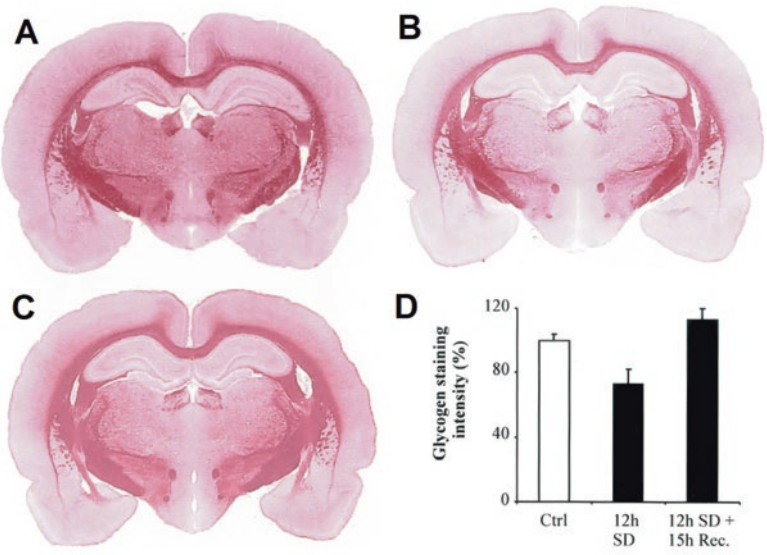

Fig. 6 Effect of sleep deprivation and recovery sleep on rat brain glycogen. Glycogen was histochemically determined in control animals (**a**) as well as in rats sleep deprived for 12 h (**b**) and after 15 h of recovery sleep (**c**). (**d**) Brain glycogen content (mean ± SEM) decreased during sleep deprivation and returned to basal levels after recovery sleep. Adapted from (Kong et al. 2002). Copyright © 2002 Society for Neuroscience

changes were reported in rat brain (excluding cerebellum and brainstem), where glycogen content decreased after 12–24 h but not 6 h of sleep deprivation (Kong et al. 2002) (Fig. 6). No or inconsistent changes in glycogen levels at the end of 6 h of sleep deprivation or sleep fragmentation have been observed in the cerebral cortex of several mouse strains (Baud et al. 2016; Franken et al. 2003; Petit et al. 2010). A recent electron microscopy study revealed a reduction of glycogen in layers II/III of mouse frontal cortex after either 7–8 h of forced wake or 4.5 days of sleep restriction (glycogen decreased by 15% or 29%, respectively) (Bellesi et al. 2018) (Fig. 5), suggesting that changes in cortical glycogen levels might have a regional and/or laminar localization.

Notably, sleep deprivation induces a substantial rise in protein targeting to glycogen, which results in increased activity of both GS and GP (Petit et al. 2002, 2010) and thus affects brain glycogen turnover but not necessarily glycogen levels (Morgenthaler et al. 2009). Therefore, concentration alone could be insufficient to reveal changes in brain glycogen metabolism during sleep deprivation (Gip et al. 2002). The fact that either GP inhibition or sleep loss are associated with inefficient reuptake of extracellular K^+ and increased susceptibility to cortical spreading depression strongly supports the notion that sleep deprivation affects brain glycogen metabolism regardless of any effect on its tissue concentration (Kilic et al. 2018).

3.1.6 Physical Exercise

Glucose consumption and glycogenolysis are both increased during exercise in the brain, liver and muscle (see Matsui et al. 2011 and references therein). Exercise-induced glycogen mobilization in multiple areas of rat brain is mainly due to monoaminergic signaling, while only marginally affected by hypoglycemia after prolonged exhaustive exercise (Matsui et al. 2015, 2011). In particular, noradrenergic and serotonergic tone increases in the brain during exercise and the ensuing rise in glycogenolysis is hypothesized to be implicated in central fatigue (Dalsgaard and Secher 2007; Matsui et al. 2011; Nybo and Secher 2004). Recovery from exercise is associated with supercompensated glycogen levels in both brain and skeletal muscle (6–24 and 24–48 h, respectively), with rates of resynthesis correlated with rates of previous degradation (Matsui et al. 2012). Interestingly, in cultured astrocytes acutely exposed to norepinephrine glycogen is initially degraded and subsequently (observed at 9 h) resynthesized to highly supercompensated levels (Sorg and Magistretti 1991, 1992).

3.2 Nutritional Factors

3.2.1 Glycemia

Under physiological conditions blood glucose concentration always exceeds cerebral demand (Mergenthaler et al. 2013) and mild variations in systemic glucose concentration have normally minor effects on brain glycogen content (Hutchins and Rogers 1970). During severe (e.g., cerebral glucose approaching Km of hexokinase, or 0.05 mmol/L) and prolonged hypoglycemia brain glycogen stores are almost depleted (see Hutchins and Rogers 1970 and references therein). Brain glycogen is also degraded during acute hypoglycemia (Canada et al. 2011; Choi et al. 2003; Herzog et al. 2008; Oz et al. 2009; Suh et al. 2007). This condition leads to initial glycogen degradation followed by its supercompensation (Canada et al. 2011) (but see Herzog et al. 2008). Glycogen supercompensation was proposed as part of a counter-regulatory response to delay energy depletion during subsequent hypoglycemic or hypoxic events. In the context of recurrent hypoglycemia, e.g., in diabetic patients under insulin therapy, such neuroprotective effect would also carry the risk of failure in detecting systemic hypoglycemia, possibly resulting in hypoglycemia unawareness (Choi et al. 2003; Oz et al. 2009). However, ^{13}C-NMR spectroscopy in healthy humans revealed a clear glycogen supercompensatory response following acute but not recurrent hypoglycemic episodes, which suggest that glycogen supercompensation is not involved in hypoglycemia unawareness (Oz et al. 2017). On the other side, prolonged hyperglycemia results in increased brain glycogen content (Nelson et al. 1968; Watanabe and Passonneau 1973), whereas acute (up to 120 min) hyperglycemia has been reported to produce either a fall (Hutchins and Rogers 1970) or a rise (Goldberg and O'Toole 1969) in cerebral glycogen. Glycogen

content in cultured astrocytes strongly covaries with ambient glucose (Cummins et al. 1983; Swanson et al. 1989b). Yet, the concentration changes of brain glycogen in vivo are possibly quite small (i.e. in the order of 10-15%) during physiological fluctuations of systemic glucose levels.

3.2.2 Diet

Fasting has been found to reduce brain glycogen (e.g., 25% after 24 h in rats) (Prasannan et al. 1963). The effect of starvation upon brain glycogen is transient (<72 h in rats) and probably dependent on the concomitant decrease of brain glucose, as glycogen turnover appears to be unchanged (Garriga and Cusso 1992; Visweswaran et al. 1969). Normalization of brain glycogen levels during prolonged starvation probably involves gluconeogenesis from amino acids. Preconditioning to gluconeogenesis has been indeed suggested to explain resistance to hypoxia and high rates of glycogenesis occurring in body tissues of rats on high fat diet (Purshottam et al. 1978). Interestingly, ketogenic diet is known to reduce susceptibility to epileptic seizures (Lutas and Yellen 2013) and it has been shown to improve cognitive performances in rats (Murray et al. 2016) and preserve memory in aging mice (Newman et al. 2017) (but see Zhao et al. 2004 for possible detrimental effects of ketogenic diet during development), all features also associated with brain glycogen metabolism. Unfortunately, glycogen levels in the brain during ketogenic diet have not been investigated. However, there are indirect findings supporting a role for β-hydroxybutyrate in stimulating glycogen accumulation, conceivably because of inhibition of glycolysis induced by metabolism of ketones (Matsui et al. 2017; Thurston et al. 1980). Conversely, carbohydrate intake does not affect brain glycogen content, while it significantly correlates with glycogen levels in both muscle and liver (Soya et al. 2018).

3.3 Environmental factors

3.3.1 Temperature

Brain glycogen normally changes in opposite fashion compared to the circadian variation of body temperature (Hutchins and Rogers 1970) (although REM sleep is associated with a small increase in brain temperature; see Wehr 1992) (see Fig. 4). The attenuation of brain activity accompanying hibernation or hypothermia is likewise associated with increases in cerebral glycogen content (Al-Badry and Taha 1983; Koizumi 1974; Lust et al. 1989). This finding could simply reflect the decrease in cerebral blood flow and metabolism during systemic as well as brain-selective hypothermia (Okubo et al. 2001; Walter et al. 2000). Yet, exposure of awake mice to either low (8–10 °C) or high (30–32 °C) ambient temperature significantly decreased brain glycogen content by 15–20% after 4–8 h (Hutchins and Rogers 1970). A caveat is that

glycogenolysis may have been stimulated by stress and/or discomfort of the animals under both conditions (Cruz and Dienel 2002; Zhao et al. 2017). Accordingly, it is possible that systemic effects are responsible for ambient temperature-induced changes in brain glycogen concentration and not brain temperature per se. In comparison, muscle glycogen metabolism is largely dependent on environmental temperature. For example, during hypothermia or acute exposure to cold, muscle glycogen is readily depleted (Fuhrman and Fuhrman 1966) and high ambient temperature (32–35 °C) is detrimental to glycogen synthesis, especially during recovery from exercise (Blackwood et al. 2019; Naperalsky et al. 2010). It is noted that fluctuations in brain temperature do normally occur even in constant ambient temperature and are primarily due to intracerebral heat production (Kiyatkin 2010).

3.3.2 Altitude

The brain relies on an uninterrupted supply of oxygen from the bloodstream. Hypobaric and hyperbaric conditions are associated with hypoxemia and hyperoxemia, respectively. Both acute and chronic exposure of rats to hypobaric hypoxia (i.e. high altitude; approximate range 3000–8000 m) leads to a reduction in brain glycogen concentration (Chapler and York 1972; Harik et al. 1995; Purshottam et al. 1977; Woolley and Timiras 1963) followed by supercompensation (Brucklacher et al. 2002; Canada et al. 2011; Cui et al. 2001; Lopez-Ramos et al. 2015), while normobaric or hyperbaric hyperoxia steadily augments brain glycogen deposition (Bronovitskaya and Shapovalova 1957; Dydyk et al. 1978).

3.3.3 Hydrostatic Pressure

For humans and non-acquatic mammals hydrostatic pressure only becomes relevant at depths of more than 100 m or rates >100 m/min, because mechanical compression on nervous system can lead to overstimulated and/or uncoordinated nerve conduction. Specifically, hyperbaric pressure-induced hyperexcitability is thought to depend on NMDA receptor activation, possibly through reduction of Mg^{2+} blockade efficacy (Mor and Grossman 2007, 2010). Thus, any pressure-induced change in brain glycogen is secondary to neuronal hyperexcitation and nonetheless difficult to disentangle from the oxygen toxicity and nitrogen narcosis associated with such extreme conditions.

3.3.4 Ionizing Radiation

Exposure of the head of mice, rats and monkeys to electromagnetic radiation or alpha-particles (helium-4 nuclei) has been repeatedly reported to result in accumulation of glycogen within the whole cerebral cortex or close to the exposed brain area, respectively (Ibrahim et al. 1970; Klatzo et al. 1961; Miquel and Haymaker

1965; Miquel et al. 1963; Wolfe et al. 1962). In rat brain, the X-ray or γ-ray radiation-induced rise in brain glycogen content was found to increase monotonically with the exposure (in the range 0.3–5 C/Kg) and glycogen elevation persisted for up to 96 h after irradiation (Miquel et al. 1966). The effect of ionizing radiation on brain glycogen has been suggested to be a consequence of damage to astrocytic mitochondrial oxidative metabolism (Miquel and Haymaker 1965), increased glucose uptake and phosphorylation (Ibrahim et al. 1970) or selective impairment of glycogen degrading enzymes (Lundgren and Miquel 1970).

3.4 Hormonal Factors

3.4.1 Sex

Sex-related differences in brain glycogen metabolism have been poorly investigated. A single report about diurnal changes of glycogen content in the pineal gland of mouse brain showed no difference between males and females during the estrous cycle (Kachi et al. 1973), although this might be region-dependent. It is interesting to notice that in Drosophila Melanogaster sleep patterns are genetically correlated with glycogen metabolism in males and with triglyceride metabolism in females, perhaps reflecting sex-specific metabolic needs (Harbison and Sehgal 2008). Since gonadal steroids affect brain and body bioenergetics (including glucose uptake, glycolysis, tricarboxylic acid cycle, mitochondrial oxidative phosphorylation and oxidative stress; reviewed in (Rettberg et al. 2014), it is likely that brain glycogen metabolism is sexually dimorphic (Tamrakar et al. 2014). Notably, susceptibility to epileptic seizures, which is affected by brain glycogen metabolism (Lopez-Ramos et al. 2015), also appear to differ in males and females (Giorgi et al. 2014; Veliskova and Desantis 2013).

3.4.2 Stress

Exposure to stressors causes the activation of central stress response through rapid (i.e. seconds) release of norepinephrine to multiple brain areas followed by delayed (i.e. minutes) release of glucocorticoids from adrenal cortex to the bloodstream (reviewed in Pearson-Leary et al. 2016). Both acute and chronic stress has been associated to decrements in brain glycogen content (Hashiguchi et al. 1998; Zhao et al. 2017). Yet, the effect of systemic administration of adrenal corticosteroids, which readily cross the blood-brain barrier, was earlier reported to increase the deposition of glycogen in the brain as in other tissues (Abood and Kocsis 1950; Gordan 1956; Gordan et al. 1951; Kosmina 1967; Prasannan et al. 1964; Timiras et al. 1956) (see Fig. 2), possibly via gluconeogenesis from amino acids (Herbet et al. 2019; Woodbury 1972). In vivo and in vitro studies reported increased, decreased or unchanged brain glycogen levels after glucocorticoids administration

(Ibrahim 1975; Tombaugh et al. 1992; Zhang et al. 2015). The exact mechanisms by which chronic stress affects glycogen synthesis and degradation in the brain need to be further investigated.

3.5 Developmental Factors

3.5.1 Early Development

Aerobic glycolysis is a crucial metabolic pathway during development, whereby it provides substrate for biosynthetic reactions that are critical for growth and structural maintenance of brain tissue (Bauernfeind and Babbitt 2014; Caravas and Wildman 2014). The involvement of glycogen in neurogenesis/synaptogenesis and early brain development and maturation has not been thoroughly investigated. However, a strong developmental increase of hippocampal and cerebellar glycogen content (peaking at P34 and slowly declining thereafter, i.e. after the switch from cerebral utilization of ketone bodies to carbohydrates) has been reported in rat brain (Gip et al. 2002). A similar decrease of glycogen content in cerebral cortex, cerebellum and diencephalon has been reported in mice after P16 (Schreiber 1981). The glycogenolytic response to norepinephrine is accordingly absent in mouse primary astrocytic cultures at early developmental age (10–14 days) but progressively present thereafter (>18 days) (O'Dowd et al. 1995).

3.5.2 Aging

In both neurons and astrocytes, aging is associated with important reorganizations of major energy metabolic pathways, including glycolytic, glycogenolytic and mitochondrial oxidative metabolism (Drulis-Fajdasz et al. 2018; Ivanisevic et al. 2016). In particular, a global age-related decrease in energy metabolism has been reported in human brain, with glucose uptake decreasing more than oxygen utilization (Goyal et al. 2017) (but see Dienel 2019a for a discussion of the limitations of this study). At least part of the ensuing reduction in aerobic glycolysis can be explained by a decrease in glycogen metabolism due to cerebral accumulation of unmetabolizable corpora amylacea and/or polyglucosans (Duran and Guinovart 2015; Mrak et al. 1997). Interestingly, inhibition of glycogenolysis has been reported to disrupt long term potentiation in hippocampal slices from young mice and enhance it in aged animals (Drulis-Fajdasz et al. 2015), possibly after a shift in the localization of glycogen-metabolizing enzymes from astrocytes to neurons (Drulis-Fajdasz et al. 2018). Appearance of neuronal glycogen seems to be indeed associated with physiological aging (Sinadinos et al. 2014), but the consequences of glycogen synthesis in neurons are controversial (Duran et al. 2012; Saez et al. 2014).

3.6 Pathological Factors

3.6.1 Glycogen Storage Diseases

The inherited deficiency or mutation of specific enzymes underlying the metabolic pathways for glycogen synthesis and degradation (or regulation thereof) lead to severe pathological phenotypes involving skeletal muscle, liver, hearth, kidney, brain and spinal cord (Ellingwood and Cheng 2018; Hicks et al. 2011; Ozen 2007). Although the principal clinical outcomes are related to hepatic and muscular function, the relevance of disorders of glycogen metabolism to the central nervous system is apparent in Type II glycogen storage diseases. These diseases are associated with accumulation of glycogen in all body tissues due to the loss-of-function of the lysosomal enzyme α-glucosidase (Pompe disease) or lysosome-associated membrane protein (Danon disease). Depending on the extent of glycogen accumulation in cerebral cortex, Type II glycogen storage diseases can be accompanied by mental retardation, learning difficulties and intellectual disability (Boucek et al. 2011; Kashio et al. 1991; Spiridigliozzi et al. 2017; Teng et al. 2004), although how exactly the impaired lysosomal disposal of glycogen granules impact on brain function is unknown.

3.6.2 Dementia

Recent work in laboratory animals demonstrated that brain glycogenolysis is necessary for memory formation and retention (Duran et al. 2013; Gibbs et al. 2006; Newman et al. 2011; Suzuki et al. 2011; Zhang et al. 2016). Cerebral metabolic changes during sleep and sleep sub-stages are also associated with synaptic plasticity and neuronal firing rate homeostasis (reviewed in DiNuzzo and Nedergaard 2017), possibly subserving memory consolidation (e.g., corticalization of hippocampus-dependent memory) (Almeida-Filho et al. 2018; Puentes-Mestril et al. 2019). Given the involvement of brain glycogen in learning and memory as well as in the effects of sleep and sleep loss, it is plausible that glycogen metabolism plays a role in memory impairment during dementia. Remarkably, Alzheimer's disease-related, age-related as well as vascular dementia are all associated with an increased cerebral accumulation of corpora amylacea, glycoproteinaceous waste aggregates primarily containing glucose polymers (e.g., polyglucosans) (Augé et al. 2018; Hoyaux et al. 2000; Mann et al. 1987; Pisa et al. 2018, 2016; Rohn 2015; Snow et al. 1988). Although the precise role of corpora amylacea in the brain is debated (Augé et al. 2017), their abundance during memory impairment and/or hyperexcitability has been hypothesized to induce dysfunctional glycogen metabolism (not necessarily associated with changes in brain glycogen levels) (DiNuzzo et al. 2015b). Notably, hyperexcitability characterizes the early stage of Alzheimer's disease progression (i.e. mild cognitive impairment) and it has been even suggested to be a primary mechanism contributing to cognitive decline (Palop and Mucke

2009). When one excludes the accumulation of glycogen in the form of corpora amylacea (or similar aggregates), the concentration and/or turnover of brain glycogen in dementia can be hypothesized to be reduced due to the upregulation of glycogen synthase kinase 3β and ensuing inhibition of GS (see Bak et al. 2018; Bass et al. 2015 and references therein).

3.6.3 Lafora Disease and Epilepsy

Lafora disease is the sole pathological condition that unquestionably relates aberrant brain glycogen structure and metabolism with epilepsy and neurodegeneration (although Lafora disease is also associated with alterations in glucose transport, glutamate uptake, autophagy, oxidative stress and inflammatory reactions) (Duran and Guinovart 2015; García-Gimeno et al. 2018; Gentry et al. 2018; Nitschke et al. 2018; Rubio-Villena et al. 2018). Accordingly, glycogen is the key target for treatment, as knockout of GS or depletion of protein targeting to glycogen in animal models of Lafora disease rescued several features of the disease, such as formation of Lafora bodies, neurodegeneration and susceptibility to epileptic seizures (Duran et al. 2014; Pederson et al. 2013; Turnbull et al. 2011, 2014). However, the absence of glycogen synthesis in these animal models is expected to carry important side-effects, including memory impairment and hyperexcitability (Duran et al. 2013). Indeed, glycogen is critical for neurotransmitter and ion homeostasis, thus any disturbance in glycogen synthesis and degradation is expected to affect neuronal excitability and synaptic plasticity (see below). Interestingly, animal models of Lafora disease exhibit episodic memory deficits increasing with age (Criado et al. 2012; Garcia-Cabrero et al. 2012), consistent with the observation that in Lafora disease patients the appearance of epileptic seizures coincides with a progressive decline in intellectual functions (Cukiert et al. 1990; Singh and Ganesh 2012). Aberrant glycogen structure has been suggested to be implicated in epileptogenesis due to impairment of astrocytic glycogenolysis and corresponding deficits in K^+ reuptake and glutamate metabolism (DiNuzzo et al. 2014, 2011).

3.6.4 Traumatic Brain Injury

The effect of traumatic injury on brain glycogen metabolism resembles that of hypoglycemia or hypoxia-ischemia. In particular, in animal models of brain trauma glycogen content decreased and turnover increased within minutes in the involved regions (Watanabe and Passonneau 1974), while a decrease in glycogen turnover and glycogen supercompensation occurred at 24 h after injury (Otori et al. 2004; Watanabe and Passonneau 1974). The trauma-induced supercompensated glycogen levels increase tolerance to ischemia and thus are likely neuroprotective (Otori et al. 2004). Interestingly, sex hormones influence the recovery from injury (Bramlett and Dietrich 2001), although the involvement of sex-specific differences in brain glycogen metabolism during traumatic injury has not been investigated.

4 Relevance of Glycogen to Neuronal Excitability and Synaptic Plasticity

4.1 Ion and Transmitter Homeostasis

Glycogen in astrocytes actively contributes to modulate the electrochemical activity of neurons. A critical function of glial astrocytes is the control of neuroactive compounds in the extracellular space (Verkhratsky and Nedergaard 2018) and glycogen is implicated in regulating the levels of both K^+ and glutamate. Specifically, not only does glycogen-derived energy support K^+ homeostasis (Choi et al. 2012) but active uptake of excess extracellular K^+ after neuronal stimulation is impaired when glycogenolysis is inhibited, even in the presence of glucose (Kilic et al. 2018; Xu et al. 2013). In addition, glycogen-derived pyruvate is a preferred precursor of glutamate through astrocytic pyruvate carboxylation (see below) and glutamate can be an anaplerotic substrate of glycogen through astrocytic gluconeogenesis (Hevor et al. 1986). The roles of glycogen in active K^+ reuptake and glutamate/GABA cycling have direct relevance to neuronal excitability, as confirmed by the increased susceptibility to epileptic seizures (Lopez-Ramos et al. 2015) and to cortical spreading depression (Kilic et al. 2018) upon inhibition of glycogenolysis. Glycogen is also exquisitely sensitive to neuromodulators (reviewed in DiNuzzo et al. 2015a), which is important because the glycogenolytic response to these substances can dissociate glycogen metabolism from underlying neuronal activity (i.e. extracellular K^+ and/or glutamate). For instance, glycogenolysis might represent a novel pathway by which monoaminergic signaling regulates Na^+/K^+-ATPase activity (DiNuzzo 2019) and the ensuing interstitial K^+ concentration in a state-dependent manner (Ding et al. 2016). Rapid glycogen breakdown following monoaminergic signaling has been also suggested to have profound repercussions on the channeling of glucose to neurons, which might be critical for supporting neuronal activity and associated energy demand (Dienel and Cruz 2015; DiNuzzo et al. 2015a).

While studying the role of glycogen in relation to memory formation in a chick learning model (Gibbs et al. 2006, 2007; Hertz et al. 2003) it was found that glycogen may function as an important substrate for de novo synthesis of glutamine and subsequently glutamate serving as a neurotransmitter. This involves degradation of glycogen to lactate/pyruvate via the concerted action of glycogenolysis and glycolysis, as explained in detail elsewhere (Bak et al. 2018), followed by pyruvate carboxylation and synthesis of α-ketoglutarate in the tricarboxylic acid (TCA) cycle. The α-ketoglutarate will act as substrate for aspartate aminotransferase forming glutamate which is subsequently converted to glutamine. Since glycogen is preferentially if not exclusively located in astrocytes (Cataldo and Broadwell 1986; Ibrahim 1975) and the two enzymes pyruvate carboxylase and glutamine synthetase are likewise exclusively expressed in astrocytes (Norenberg and Martinez-Hernandez 1979; Yu et al. 1983), the cellular location of the synthetic machinery required for glycogen acting as a precursor for glutamine is astrocytic. Moreover, glutamine synthesized in astrocytes will act as precursor for neuronal synthesis of

the two amino acid neurotransmitters glutamate and GABA since glutamine transporters located in the plasma membranes of astrocytes and neurons will facilitate transfer of glutamine from astrocytes to neurons (see Leke and Schousboe 2016). The finding that an obese rat model exhibiting hampered glycogen metabolism in combination with a lowered cerebral glycogen content had reduced glutamate-glutamine cycle activity (Sickmann et al. 2010) clearly supports the functional importance of astrocytic glycogen for optimal glutamatergic activity. This is in keeping with the notion that filling of the vesicles in glutamatergic neurons is dependent on the cytosolic glutamate concentration which, in turn, depends on an adequate supply of glutamine from surrounding astrocytes. Moreover, it has been demonstrated that inhibition of glycogen-shunt activity results in significant reduction in glutamatergic activity in neuronal/astroglial cultures from mouse brain as well as in normal Sprague-Dawley and diabetic rats (Sickmann et al. 2012, 2009).

Glycogen could also be involved in cortical information processing, although such possibility has hitherto never been directly explored. Specifically, vasoactive intestinal peptide (VIP) is a powerful glycogenolytic agent (Sorg and Magistretti 1991 1992). VIP-positive interneurons mediate the disinhibition of pyramidal neurons in many areas of the cerebral cortex, which is thought to subserve gain modulation in a brain state-dependent manner (Neske and Connors 2016; Pi et al. 2013). Thus, by controlling local energy metabolism via glycogen mobilization, VIP-positive interneurons might transiently alter extracellular levels of K^+, glutamate/GABA, lactate (Magistretti 1990; Magistretti et al. 1998, 1984) and in turn synaptic plasticity rules (DiNuzzo and Nedergaard 2017). The regulation of extracellular K^+ by glycogenolysis might also give astrocytes the control of subthreshold neuronal oscillations and possibly brain state transitions (Bellot-Saez et al. 2018; Ding et al. 2016; Kjaerby et al. 2017).

4.2 Metabolic Compartmentation

Glucose is metabolized through glycolysis to form pyruvate and/or lactate and further to carbon dioxide in the TCA cycle (for details, see Schousboe et al. 2015). This is also the case for glucose or glucose-1-phosphate generated from glycogen by glycogenolysis, but the pools of pyruvate and lactate may not be identical in two scenarios. It has been shown that astrocytes may generate a large amount of lactate which may be derived from both glucose and glycogen (Dringen et al. 1993; Schousboe et al. 1997; Waagepetersen et al. 2000b; Walz and Mukerji 1988). Using cultured astrocytes from mouse cerebral cortex or cerebellum and [U-^{13}C]glucose to label the glucose and glycogen pools it has been demonstrated that lactate originating from glucose and glycogen, respectively, was not present in the same metabolic pool (Sickmann et al. 2005). This underlines the complexity of astrocytic metabolism being compartmentalized not only considering the glycolytic and glycogenolytic processes but also the TCA cycle (Sickmann et al. 2005; Waagepetersen et al. 2001, 2003). This is in keeping with the demonstration of compartmentalized

pyruvate metabolism in astrocytes (Obel et al. 2012a; Waagepetersen et al. 2000a). Further in vivo labeling studies of rat brain [6-^{14}C]glucose metabolism during rest, stimulation and recovery showed very high specific activity of recovered lactate, indicating a strong metabolic compartmentation between blood-borne glucose and endogenous glycogen (for details, see Dienel 2019b).

The metabolic basis for pyruvate compartmentation is not known. It has been suggested that pyruvate generated from glycolytic processing of blood-borne extracellular glucose takes place predominantly in neurons, while glycogenolysis-derived pyruvate and its clearance from brain tissue as lactate occurs in astrocytes (Dienel 2019b). This scenario entails that at any given instant an individual astrocyte metabolizes either glucose or glycogen, yielding a corresponding compartmented pool of intracellular glucose-6-phosphate and downstream metabolites, something that has long been discussed in the biochemical literature (see DiNuzzo 2019 and references therein). A complete switch between glucose and glycogen metabolism in astrocytes can be explained by the so-called glucose-sparing mechanism involving glucose phosphates (DiNuzzo et al. 2011). Specifically, glycogenolysis induces a transient elevation in intracelullar glucose-6-phospate (and possibly glucose-1,6-bisphosphate), which inhibits glucose phosphorylation and channels glycogen-derived glucose-1-phosphate into astrocytic metabolism (DiNuzzo et al. 2010, 2011).

As glycogen is essentially restricted to astrocytes, both glucose-sparing and glycogen-derived lactate production endows these cells with the ability to control the extent to which neurons can increase glycolysis or oxidative phosphorylation, respectively. As stated above, metabolism is inextricably linked with function and astrocytic glycogen breakdown may have direct effects on neurotransmission. For example, glycolysis in neurons supports presynaptic vesicular glutamate loading and glutamate exocytotic machinery (Hinckelmann et al. 2016; Ikemoto et al. 2003; Ishida et al. 2009; Zala et al. 2013). In turn, glutamatergic and monoaminergic neurotransmission stimulates filopodial astrocytic movements, possibly fueled by glycogen (Dienel 2019b), that bring fine processes of these cells closer to neuronal synapses (Bellesi et al. 2015). Such a mechanism would configure a metabolic counterpart of astrocytic support to changes in synaptic strength. In particular, the degree of glucose-sparing for neurons by astrocytic glycogenolysis together with participation of glycogen in the glutamate-glutamine cycle would modulate glutamate quantal content as well as release and uptake rates. At the same time, transient NADH changes might be produced in dendritic spines by either increased neuronal glycolysis or neuronal uptake of astrocytic glycogen-derived lactate and subsequent conversion to pyruvate by lactate dehydrogenase. Alternatively, or in addition, lactate taken up by dendritic spines can also be derived from glycolysis in presynaptic terminals (DiNuzzo and Giove 2012). Whatever the mechanism, the ensuing change in redox state is known to be involved in control of gene expression, notably of plasticity-related genes (Yang et al. 2014). Although the functional advantage of compartmentation between glucose and glycogen metabolism is presently unknown, it is intriguing to speculate that the metabolic effects of glycogenolysis in astrocytes might modulate synaptic transactions and plasticity.

5 Concluding Remarks

In summary, glycogen cannot be fully characterized by its tissue concentration, because structural integrity, particle size distribution and turnover also play critical roles for its metabolism. Nonetheless, studies that measured brain glycogen levels clearly indicated that this polysaccharide actively participates in many basic as well as higher brain functions. In particular, neuronal excitability is affected by glycogen metabolism, as glycogen appears to be necessary for ion and transmitter homeostasis and inhibition of glycogenolysis is associated with increased susceptibility to epileptic seizures and cortical spreading depression. Glycogen is required for astrocytic potassium uptake and it is involved in glutamate/GABA synthesis. Moreover, glycogen is implicated in synaptic plasticity, as inhibition of glycogenolysis or glycogenesis impairs learning and memory. Neuronal excitability and synaptic plasticity largely concur to define brain state, as the ability of responding to external stimulations and to make new memories are state-dependent (e.g., consciousness versus sleep/anesthesia), and so is brain glycogen metabolism. Given the multifaceted effects of glycogen synthesis and degradation to brain function in health and disease, it can be predicted that glycogen research will be at the frontiers of neuroscience in the years to come.

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Glycogen in Astrocytes and Neurons: Physiological and Pathological Aspects

Jordi Duran, Agnès Gruart, Juan Carlos López-Ramos, José M. Delgado-García, and Joan J. Guinovart

Abstract Brain glycogen is stored mainly in astrocytes, although neurons also have an active glycogen metabolism. Glycogen has gained relevance as a key player in brain function. In this regard, genetically modified animals have allowed researchers to unravel new roles of this polysaccharide in the brain. Remarkably, mice in which glycogen synthase is abolished in the brain, and thus devoid of brain glycogen, are viable, thereby indicating that the polysaccharide in this organ is not a requirement for survival. While there was growing evidence supporting a role of glycogen in learning and memory, these animals have now confirmed that glycogen participates in these two processes.

The association of epilepsy with brain glycogen has also attracted attention. Analysis of genetically modified mice indicates that the relation between brain glycogen and epilepsy is complex. While the formation of glycogen aggregates clearly underlies epilepsy, as in Lafora Disease (LD), the absence of glycogen also favors the occurrence of seizures.

LD is a rare genetic condition that affects children. It is characterized by epileptic seizures and neurodegeneration, and it develops rapidly until finally causing death. Research into this disease has unveiled new aspects of glycogen metabolism.

J. Duran
Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology, Barcelona, Spain

Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

A. Gruart · J. C. López-Ramos · J. M. Delgado-García
Division of Neurosciences, Pablo de Olavide University, Seville, Spain

J. J. Guinovart (✉)
Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology, Barcelona, Spain

Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

Department of Biochemistry and Molecular Biomedicine, University of Barcelona, Barcelona, Spain
e-mail: guinovart@irbbarcelona.org

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Animal models of LD accumulate polyglucosan bodies formed by aberrant glycogen aggregates, called Lafora bodies (LBs). The abolition of glycogen synthase (GS) prevents the formation of LBs and the development of LD, thereby indicating that glycogen accumulation underlies this disease and the associated symptoms, and thus establishing a clear relation between the accumulation of glycogen aggregates and the incidence of seizures.

Although it was initially accepted that LBs were essentially neuronal, it is now evident that astrocytes also accumulate polyglucosan aggregates in LD. However, the appearance and composition of these deposits differs from that observed in neurons. Of note, the astrocytic aggregates in LD models show remarkable similarities with corpora amylacea (CA), a type of polyglucosan aggregate observed in the brains of aged mice and humans. The abolition of GS in mice also impedes the formation of CA with age and at the same time prevents the formation of a number of protein aggregates associated with aging. Therefore CA may play a role in age-related neurological decline.

Keywords Learning · Memory · Long-term potentiation · Epilepsy · Lafora disease · Corpora amylacea · Hypoxia

Abbreviations

ANLS	Astrocyte-neuron lactate shuttle
APBD	Adult polyglucosan body disease
CAL	Corpora amylacea-like
DAB	1,4-dideoxy-1,4-imino-D-arabinitol
EPM2	Progressive myoclonic epilepsy 2
GBE	Glycogen branching enzyme
GP	Glycogen phosphorylase
GS	Glycogen synthase
HFS	High-frequency stimulus
LBs	Lafora bodies
LD	Lafora disease
LFP	Local field potential
LGS	Liver glycogen synthase
LTP	Long-term potentiation
MGS	Muscle glycogen synthase
PGBs	Polyglucosan bodies
PP1	Protein phosphatase 1
PTG	Protein targeting to glycogen
SAMP8	Senescence accelerated mouse prone 8

1 Brain Glycogen in Learning and Epilepsy

Glycogen is produced by GS—the only enzyme able to synthesize glucose polymers in mammals—and degraded by glycogen phosphorylase (GP) in the cytosol and by alpha-glucosidase in the lysosome. Mammals express two isoforms of GS encoded by *GYS1* and *GYS2*. The latter encodes the hepatic isoform (LGS), whose expression is restricted to the liver, while the former encodes the muscle isoform (MGS), which is widely expressed in all organs except the liver (Kaslow et al. 1985). MGS is regulated by phosphorylation at multiple serine residues located in the amino- and carboxy-terminal domains of the enzyme. Phosphorylation by several kinases, including GSK3, induces the inactivation of the enzyme (Roach et al. 1998). Dephosphorylation is facilitated by scaffolding proteins, such as Protein Targeting to Glycogen (PTG), which bring the catalytic subunit of protein phosphatase 1 (PP1) into contact with GS on the glycogen particle, thus causing its dephosphorylation and consequent activation (Vilchez et al. 2007). GS is also allosterically activated by glucose-6-phosphate (G6P) in the brain (Goldberg and O'Toole 1969) and in other tissues (Bouskila et al. 2010; von Wilamowitz-Moellendorff et al. 2013). Glycogen is a branched molecule, which is a crucial property that confers solubility in water. Therefore, the synthesis and degradation of this polysaccharide requires two additional enzymes, namely glycogen branching enzyme, which generates the branching points during the synthesis, and glycogen debranching enzyme (GBE), which removes them during degradation.

Glycogen concentration in the brain is much lower than in the liver or muscle, thus explaining why its function in brain has been largely overlooked. However, a growing body of evidence has accumulated in the last decade indicating that brain glycogen plays an important role in memory formation and learning, in susceptibility to epilepsy, and in many other brain functions. These roles have been elucidated mainly by experiments in which glycogen usage has been impeded by means of specific metabolic inhibitors of GP and more recently confirmed with the use of genetically modified animal models.

The role of glycogen in memory formation was first demonstrated in young chicks with bead discrimination experiments, in which chicks are trained to avoid beads of a specific color (Gibbs et al. 2006). Intracranial injection of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), a GP inhibitor, or 2-deoxyglucose, which blocks glycolysis, impaired learning of this task. The authors of that study thus concluded that glycogenolysis and aerobic glycolysis are necessary for memory formation (reviewed in (Gibbs 2015)). Other studies performed in rats using DAB injection or knocking down lactate transporters in astrocytes or neurons are consistent with the idea that the mobilization of astrocytic glycogen to generate lactate to feed neurons is critical for memory formation (ANLS hypothesis, see below) (reviewed in (Alberini et al. 2018)).

Epilepsy results from a sudden synchronization of the activity of a group of neurons. During epileptic seizures, neurons and astrocytes dramatically increase their energy consumption. Therefore, the availability of glycogen in this context could be

of great importance. In this regard, high glycogen content has been found in biopsies of the brains of epileptic patients (Dalsgaard et al. 2007). Moreover, in the methionine sulfoximine epileptic model, an increase in brain glycogen content has been reported (Phelps 1975; Hevor et al. 1985), and susceptibility of two inbred mouse strains to methionine sulfoximine inversely correlates with their capacity to accumulate glycogen in the brain (Bernard-Helary et al. 2000). Furthermore, the amount of glycogen in epileptic foci is reduced after seizures induced by kainate (Walls et al. 2014) and other epileptogenic agents or situations (Lopez-Ramos et al. 2015). These series of studies have shown a correlation between glycogen content and epilepsy. However, they do not categorically prove that glycogen is directly involved in the disorder.

The use of genetically modified animals in the study of the physiological and pathological aspects of brain glycogen presents clear advantages over methods based on drug treatments, which are hampered by non-specificity and uncontrolled side effects. Furthermore, deletion of specific genes only in the brain of these animals ensures that the results reflect the importance of glycogen exclusively in this organ.. Furthermore and in contrast to pharmacological approaches, genetic manipulation allows researchers to increase the amount of glycogen in specific cell types.

In this regard, the generation of a mouse model that lacks GS specifically in the nervous system ($GYS1^{Nestin\text{-}KO}$), and thus depleted of glycogen in the brain, has shed light on the long-standing question regarding the role of glycogen in this organ (Duran et al. 2013; Lopez-Ramos et al. 2015). The first conclusions drawn from this model is that the animals are viable and that they have a normal lifespan. The same is true for mice lacking GYS1, which are depleted of glycogen in the whole body except the liver (Raben et al. 2001). These results support the notion that brain glycogen is not an absolute requirement for life and that the brain can survive on free glucose. However, $GYS1^{Nestin\text{-}KO}$ animals are not normal, as they present deficiencies in learning and memory and an increased susceptibility to epilepsy (Duran et al. 2013; Lopez-Ramos et al. 2015).

$GYS1^{Nestin\text{-}KO}$ mice show a significant deficit in capacity to learn an instrumental conditioning task (Fig. 1a). They also show deficits in concomitant activity-dependent changes in synaptic strength, the most striking of which is the almost complete absence of Long-Term Potentiation (LTP), the long-lasting increase in the strength of excitatory synapses after a high-frequency stimulation protocol (Duran et al. 2013) (Fig. 1b). LTP is believed to be the cellular process that underlies information storage within neural systems (Martinez and Derrick 1996; Gruart et al. 2006). These observations clearly demonstrate the key role of brain glycogen in the proper and timed acquisition of relatively difficult associative learning tasks. The characterization of $GYS1^{Nestin\text{-}KO}$ mice has also contributed to shedding light on the relation between brain glycogen and epilepsy. Animals lacking brain glycogen are more susceptible to hippocampal seizures after the administration of kainate, a drug widely used to induce epilepsy (Lopez-Ramos et al. 2015) (Fig. 2). This observation thus indicates that glycogen availability contributes to the maintenance of the proper equilibrium between excitatory and inhibitory neurotransmission.

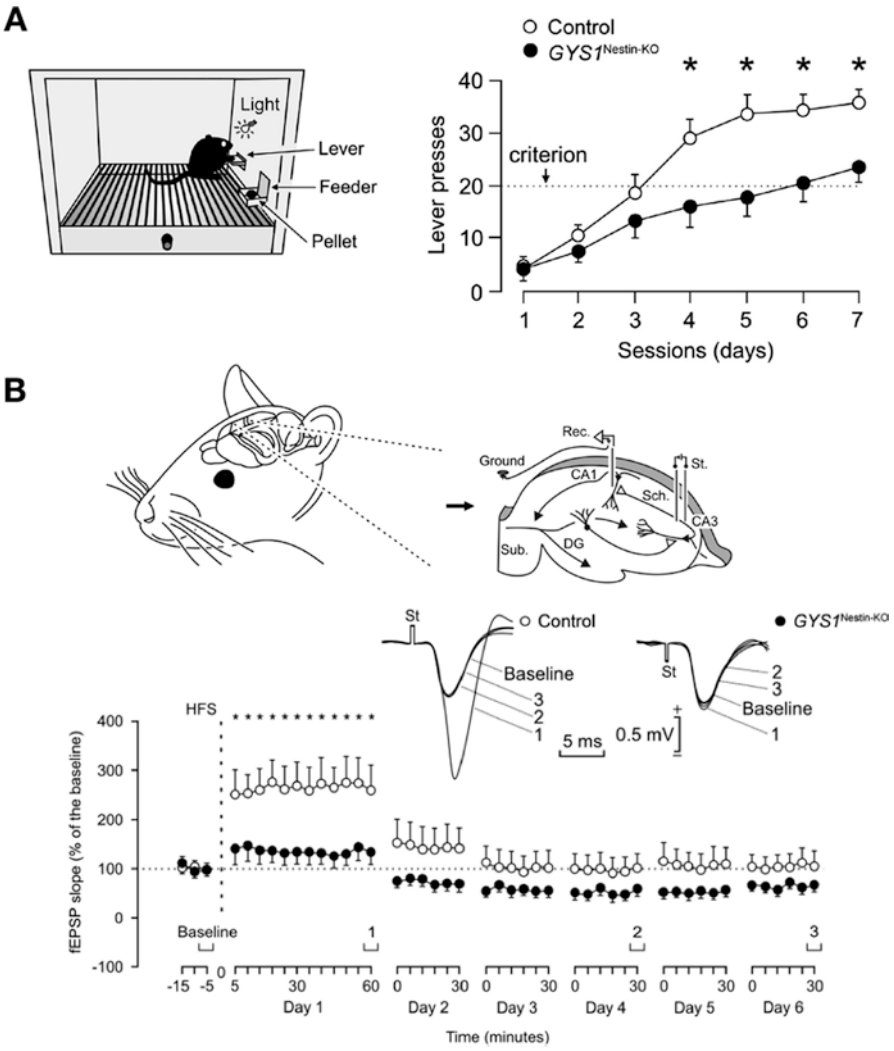

Fig. 1 Adapted from (Duran et al. 2013). (**a**) Impaired performance of GYS1$^{Nestin\text{-}KO}$ mice in an operant conditioning task. Mice were trained in a Skinner box to press a lever to obtain a food pellet (left). Lever presses in the first 7 days of training of task (right). Dotted line corresponds to criterion. (**b**) Animals were chronically implanted with stimulating electrodes in the hippocampal Schaffer collaterals and with a recording electrode in the ipsilateral pyramidal CA1 area. An extra wire was attached to the bone as ground (Top) (DG, dentate gyrus; Sub., subiculum). Time course of long-term potentiation (LTP) evoked in the CA3-CA1 synapse after a high-frequency stimulation (HFS) session (bottom) (mean ± s.e.m. fEPSP slopes given as a percentage of values collected during baseline recordings (100%)). Representative examples of fEPSPs collected at the indicated times are plotted at the top. ∗Statistically significant ($P < 0.05$) differences between the two groups

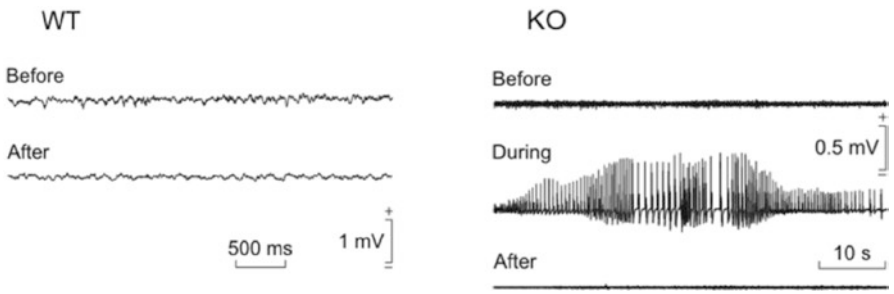

Fig. 2 Adapted from (Lopez-Ramos et al. 2015). Effects of kainate injection on spontaneous electric potential generated by neurons (Local Field Potentials, LFPs) recorded in the hippocampus of behaving mice. Representative examples of LFPs recorded from a wild-type (WT) and a $GYS1^{Nestin\text{-}KO}$ (KO) mouse before and 30 min after a kainate injection (8 mg/kg,i.p.)

2 Astrocytes Vs. Neurons

A further complication in the study of brain glycogen arises from the fact that the polysaccharide is present mainly in astrocytes. Therefore, in brain function, all the physiological roles of glycogen as an energy reserve or contributing to the consolidation of memory have been essentially attributed to astrocytic glycogen. It has been hypothesized that astrocytes accumulate glycogen for the benefit of neurons. However, since astrocytes do not have glucose-6-phosphatase they cannot release free glucose to be taken up by neurons. Therefore, the question as to how neurons can benefit from glycogen stored in astrocytes is puzzling. A simple explanation is that astrocytes use their own glycogen in times of high energy demand, sparing interstitial glucose for neurons. A more sophisticated mechanism implies that astrocytes degrade glycogen to lactate, which is released, and taken up and consumed by neurons—a hypothesis known as the astrocyte-neuron lactate shuttle (ANLS). This hypothesis continues to be debated (reviewed in (Waitt et al. 2017)).

Another difficulty encountered when studying brain glycogen arises from its rapid degradation in post-mortem conditions. This explains why glycogen is difficult to detect in cell types with a low glycogen content, such as neurons, and why its role in these cells has been overlooked (Hertz and Chen 2018). Localization studies performed in microwave-fixed brains (a procedure that stops enzymatic activity immediately and thus preserves the metabolic state) indicate that neurons also contain glycogen, although at much lower concentrations than astrocytes (Oe et al. 2016). Furthermore, the presence of glycogen deposition in neurons in certain conditions such as LD (see below) demonstrates that these cells do synthesize glycogen. In this regard, it has been shown that primary cultured neurons accumulate glycogen, although in small amounts (Fig. 3a), and express GS and GP, thus indicating that these cells have the capacity to synthesize and degrade this polysaccharide (Vilchez et al. 2007; Saez et al. 2014). The availability of glycogen in these neurons is relevant for their tolerance to hypoxia (Fig. 3b). Therefore, a pressing question was to dissect the relative roles of neuronal and astrocyte glycogen in

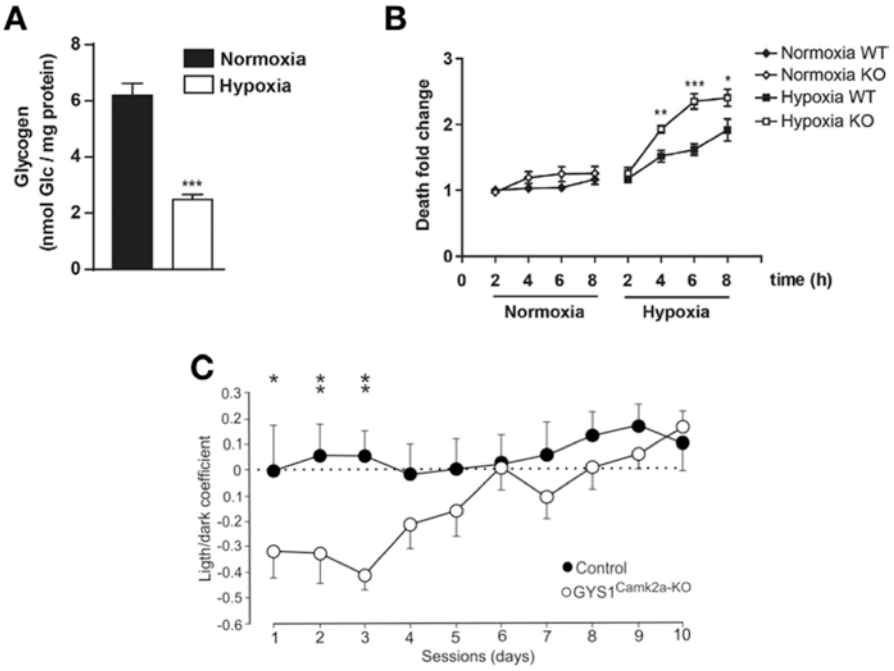

Fig. 3 (**a**) Adapted from (Saez et al. 2014). Neurons contain glycogen, which is mobilized in hypoxia. Glycogen content was determined in control (Normoxia, exposed to environmental 21% O2) and treated neurons (Hypoxia, exposed to 1% O2) and represents the mean ± s.e.m. (**n.**7). ***$\mathbf{P <}$ 0.001 versus Normoxia. (**b**) Adapted from (Saez et al. 2014). Glycogen synthase (GS) has a protective role in neurons under hypoxia. Death fold change after increasing exposure to hypoxia in GS wild-type (WT) (black) and knockout (KO) (white) neurons. (**c**) Adapted from (Duran et al. 2019). Performance of control and $GYS1^{Camk2a\text{-}KO}$ mice was studied in an operant conditioning task. Mice were placed in a Skinner box and trained to press a lever to obtain a pellet only when a light bulb was switched on. Control mice outperformed $GYS1^{Camk2a\text{-}KO}$ mice [$F_{(9,225)} = 2.82$; $P = 0.01$]

learning and epilepsy. The characterization of a mouse model depleted of glycogen specifically in neurons of the forebrain ($GYS1^{Camk2a\text{-}KO}$) involved in memory and learning has shed light on this long-standing issue (Duran et al. 2019). As in the case of $GYS1^{Nestin\text{-}KO}$ animals, in which GS is depleted from both neurons and astrocytes, $GYS1^{Camk2a\text{-}KO}$ mice showed decreased LTP evoked in the hippocampal CA3-CA1 synapse and a significant deficiency in the acquisition of an instrumental learning task (Fig. 3c). In contrast, they did not present the greater susceptibility to hippocampal seizures and myoclonus observed in the $GYS1^{Nestin\text{-}KO}$ model. These results unequivocally demonstrate the presence of an active glycogen metabolism in neurons in vivo and its fundamental role in the proper acquisition of new motor and cognitive abilities and in the changes in synaptic strength underlying such acquisition.

A conclusion drawn from these studies is that neuronal glycogen is responsible for some of the roles previously attributed exclusively to astrocytic glycogen (Hertz and Chen 2018). Therefore the relevance of neuronal glycogen for brain function should be reconsidered.

3 Lafora Disease

A rare genetic disease, Progressive Myoclonic Epilepsy 2 (EPM2), also known as Lafora disease (LD), has greatly contributed to enhancing our knowledge of glycogen metabolism in the brain and its relation to epilepsy. This disease is an invariably fatal epilepsy that affects both genders equally. Its onset occurs during adolescence, in apparently healthy children, causing absence seizures and/or visual auras. Patients then typically experience generalized tonic-clonic seizures and insidious decline in cognitive function. LD at onset is difficult to distinguish from idiopathic generalized epilepsies. Myoclonic seizures, staring spells, and generalized convulsions follow and escalate over time. LD patients also develop epileptic and non-epileptic visual hallucinations (Gentry et al. 2018; Nitschke et al. 2018).

LD is caused by mutations in either *NHLRC1*, which encodes malin, an E3-ubiquitin ligase, or *EPM2A*, which encodes laforin, a serine-threonine phosphatase. Patients carrying loss-of-function mutations in either of these two genes are indistinguishable. Genetically modified animal models, i.e. the malin knockout (malin^{KO}) (DePaoli-Roach et al. 2010; Valles-Ortega et al. 2011) and the laforin knockout mice (Ganesh et al. 2002), are ideal systems in which to study the disease.

The hallmark of LD is the presence of large inclusions of glycogen aggregates known as polyglucosan bodies (PGBs) or, more specifically, Lafora bodies (LBs), in the brain (Fig. 4) and in other tissues such as muscle and heart (Cavanagh 1999). LBs were traditionally considered to be neuronal inclusions that occur concomitantly with neurodegeneration and epilepsy and that are related to the inexorable worsening of the condition until death in early adulthood (Machado-Salas et al. 2012). However, studies with LD mouse models have demonstrated the presence of PGB also in astrocytes (Valles-Ortega et al. 2011; Auge et al. 2018; Rubio-Villena et al. 2018). Thus, malin^{KO} mice present two types of PGB in the brain, one affecting neurons and the other astrocytes. These two types differ in size, distribution and presence of specific neo-epitopes that are recognized by natural antibodies present in the sera of mammals (Auge et al. 2018). Interestingly, astrocytic, but not neuronal, PGBs also appear in aged control animals, in animal models of accelerated aging and in animals with enhanced glycogen synthesis in the brain (Auge et al. 2018). This observation indicates that the absence of malin triggers the formation of LBs in neurons and enhances the formation of PGBs in astrocytes. Therefore, only the PGBs present in the neurons are specific to LD, while those in astrocytes can be found in other conditions. Given their similarity to corpora amylacea (see below), these astrocytic PGBs could be referred to as corpora amylacea-like granules (CAL) (Fig. 4a).

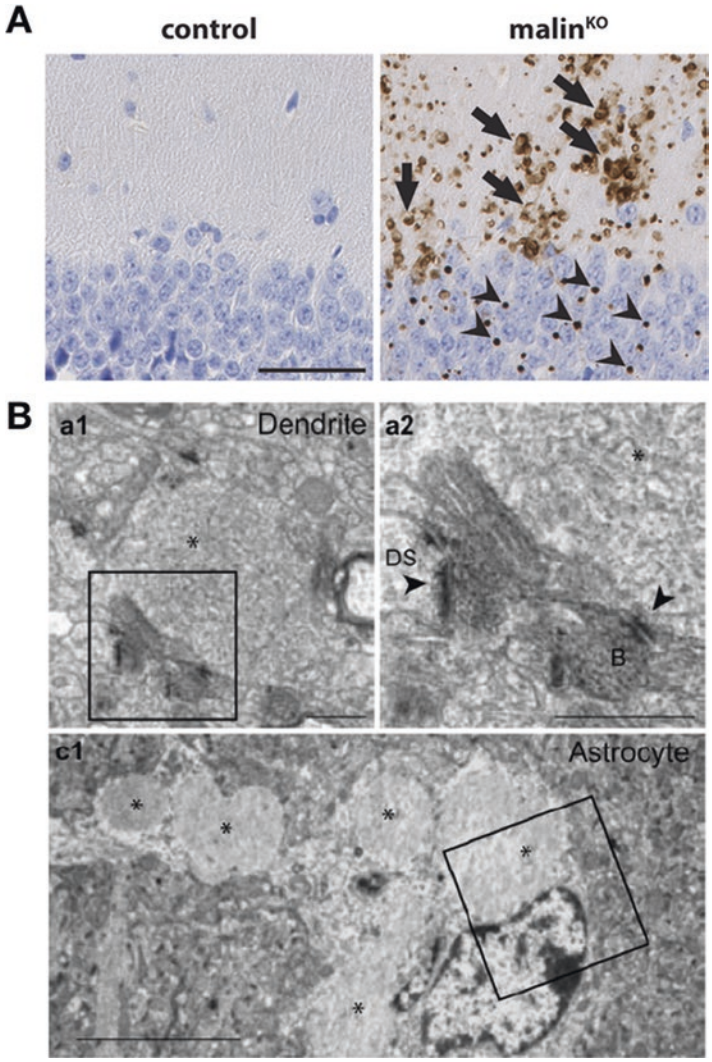

Fig. 4 Lafora bodies in malin^{KO} brain. (**a**) GS immunostaining of control and malin^{KO} brains. Arrows indicate clusters of CAL inclusions, arrowheads indicate nLBs. (**b**) Adapted from (Valles-Ortega et al. 2011). Electron microscopy images of CA1 region. Micrographs depict the presence of LBs and glycogen granules in dendrites (a1, a2) and in astrocytes (c1). ∗, Lafora Body; black arrowhead: postsynaptic density; B: synaptic bouton; DS: dendritic spine. Scale bars are 5 μm in c1 and 0.5 μm in a1 and a2

The mechanism by which these aggregates form remains unclear. However, given that laforin is able to remove phosphate from glycogen, it has been proposed that the accumulation of phosphorylated glycogen is the main underlying factor involved. This increased phosphate would underlie a change in glycogen structure,

decreasing its degree of branching and making it less soluble (Gentry et al. 2009). However, this does not explain the similar accumulation of LBs in the absence of malin, a situation marked by an increase in laforin (Duran et al. 2014). Furthermore, the phosphatase activity of laforin does not appear to be required to prevent LD (Gayarre et al. 2014), and glycogen hyperphosphorylation does not cause the formation of LBs (Nitschke et al. 2017). As mentioned above, while CAL granules are also formed in aging and other conditions in which glycogen synthesis is increased, neuronal LBs appear only in models lacking malin or laforin. However, the presence of high numbers of CAL granules in the malin^{KO} mouse model suggests that the lack of malin alters glycogen metabolism not only in neurons but also in astrocytes. Nevertheless, there is an important difference. While the absence of malin (or laforin) is a requirement to trigger the formation of neuronal LBs, it is not a prerequisite to generate astrocytic CAL granules. However, the absence of malin clearly enhances the formation of these aggregates.

Since the malin–laforin complex had been described to have functions other than that of regulating glycogen synthesis, such as the control of autophagy, the role of glycogen accumulation in the etiopathology of LD was a matter of debate. In this regard, autophagy impairment was hypothesized to underlie neurodegeneration in LD (Criado et al. 2012; Knecht et al. 2012). A first indication that glycogen accumulation is the direct cause of the neurodegeneration and functional impairments seen in LD was that the deletion of PTG prevents the manifestation of LD in the malin^{KO} model (Turnbull et al. 2011). These animals showed reduced glycogen accumulation and the resolution of neurodegeneration and myoclonic epilepsy. However, since PTG is a regulatory protein of PP1, which has many targets, it could not be ruled out that the observed effects were due to changes in the phosphorylation of other targets.

A direct demonstration that glycogen accumulation induces neurodegeneration came from experiments in which a mutant form of MGS in which 9 regulatory serine residues were mutated to alanine, rendering MGS that is not unactivatable by phosphorylation (MGS-9A), was specifically expressed in Purkinje neurons of mice (Duran et al. 2012). Glycogen accumulation in these neurons resulted in a time-dependent loss of these cells by apoptosis and the associated motor impairment (Fig. 5a). Similarly, overexpression of MGS-9A in fly (*Drosophila*) neurons leads to the accumulation of glycogen, reduced lifespan and locomotion defects (Fig. 5b).

However, final evidence of the causal role of glycogen accumulation in the etiopathology of LD was provided by experiments that showed that not only the double laforin-MGS KO (Pederson et al. 2013) and double malin-MGS KO animals (Duran et al. 2014) do not show the accumulation of brain glycogen (Fig. 6a, b) but also that the neurological alterations inherent to LD are rescued. In this regard, a malin-deficient mouse that cannot synthesize glycogen in the brain (malin^{KO} + MGS^{KO}) does not show the increase in markers of neurodegeneration seen in the malin^{KO} model (Fig. 6c). Furthermore, the partial reduction of MGS expression by deleting only one of the GYS1 alleles (malin^{KO} + MGS^{het}) is sufficient to decrease the levels of glycogen (Fig. 6b) and the neurodegeneration markers (Fig. 6c) almost to the levels of control animals (Duran et al. 2014). The increase in LTP of hippocampal synapses and the susceptibility to kainate-induced epilepsy seen in the malin^{KO} model

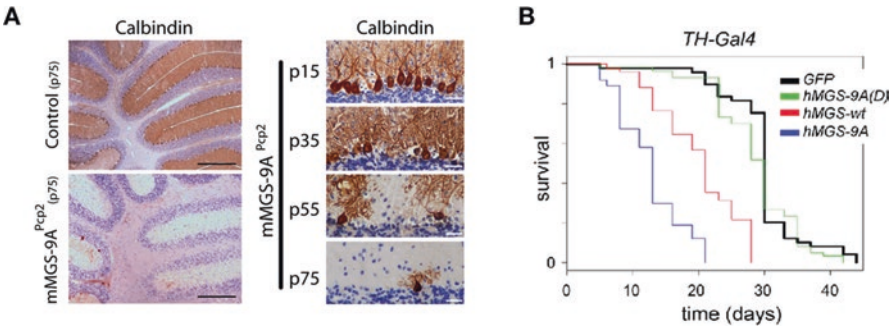

Fig. 5 Adapted from (Duran et al. 2012). (**a**) Progressive loss of Purkinje neurons in animals overexpressing MGS-9A in these cells (mMGS-9A^{Pcp2}). Cerebellar sections of p15-p75 mMGS-9A^{Pcp2} animals labelled to visualize Purkinje cells by Calbindin protein expression (brown). Sections were also labelled with haematoxylin (blue). Scale bars, 500 μm (left) and 30 μm (right). (**b**) Survival assay of control (GFAP) or flies overexpressing wild type MGS (hMGS-wt), MGS-9A (hMGS-9A) or a catalytically inactive form of MGS-9A (hMGS-9A(D)) specifically in neurons

are also reversed in these malin^{KO} + MGS^{het} animals (Fig. 6d). These observations thus indicate that the malin^{KO} mice are rescued from their neurological dysfunctions when GS is diminished. This finding gains relevance in the context of the treatment of LD, as it implies that partial inhibition of GS activity may be sufficient to prevent the progression of the disease. This notion has fueled interest in identifying inhibitors of GS or oligonucleotides able to block its expression in the CNS, as potential treatments for the disease.

At this point, it is interesting to draw attention to a paradox, namely that both the absence and the accumulation of brain glycogen (MGS$^{Nestin\text{-}KO}$ and malin^{KO}, respectively) induce increased susceptibility to epilepsy (Duran et al. 2014; Lopez-Ramos et al. 2015). This paradox could be attributed to non-degradable nature of the glycogen accumulated in LD, thus bringing about a similar situation to that found when there is a lack of glycogen. In this regard, it has been proposed that abnormal glycogen structure contributes to susceptibility to epilepsy (DiNuzzo et al. 2015). In contrast, while the accumulation of brain glycogen in malin^{KO} brains generates an increase in LTP in the CA3-CA1 synapse, the absence of brain glycogen in the MGS$^{Nestin\text{-}KO}$ model results in the loss of LTP in the same synapse (Duran et al. 2013, 2014). Thus, in this case, the increase in LTP in malin^{KO} mice is not a consequence of an incapacity to mobilize glycogen.

As mentioned before, autophagy impairment had been hypothesized to underlie neurodegeneration in LD. Interestingly, autophagy impairment is rescued in malin^{KO} + MGS^{KO} animals, and partially rescued in the malin^{KO} + MGS^{het} animals (Fig. 7a). These observations thus demonstrate that the accumulation of glycogen is not a consequence of autophagy impairment, but rather the cause of it, and show that glycogen accumulation precedes autophagy and not vice versa. To reinforce this idea, other models of glycogen accumulation in the CNS in which malin levels have not been modified (namely animals overexpressing 9A-MGS or PTG) also show impaired autophagy (Duran et al. 2014) (Fig. 7b).

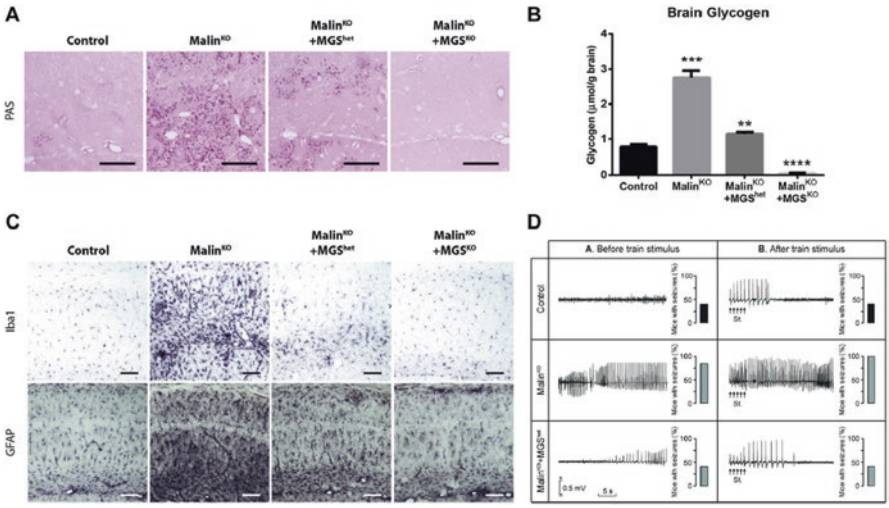

Fig. 6 Adapted from (Duran et al. 2014). (**a**) MGS is indispensable for the formation of LBs. PAS staining shows that LBs accumulated in malin^{KO} brains are absent in malin^{KO} + MGS^{KO} and greatly reduced in malin^{KO} + MGS^{het} brains. (**b**) Graph represents biochemical determination of brain glycogen concentration. Malin^{KO} + MGS^{KO} brains are devoid of glycogen, and the concentration of the polysaccharide is clearly reduced in malin^{KO} + MGS^{het} brains. (**c**) Neurodegeneration is rescued in malin^{KO} mice that cannot synthesize glycogen in the brain. GFAP and Iba1 stainings, as markers of neurodegeneration, show increased staining in the malin^{KO} mice with respect to the controls, thus indicating neurodegeneration. This staining is normalized in the malin^{KO} + MGS^{KO} animals and partially normalized in the malin^{KO} + MGS^{het} animals. (**d**) Increased susceptibility to kainate-induced epilepsy is also dependent on MGS. Representative hippocampal local field potential (LFP) recordings were carried out 30 min after kainic acid injection before and after train stimulation. The percentage (%) of mice presenting seizures within each group is shown to the right of the LFP recordings. Malin^{KO} animals show enhanced susceptibility, which is normalized in malin^{KO} + MGS^{het} animals

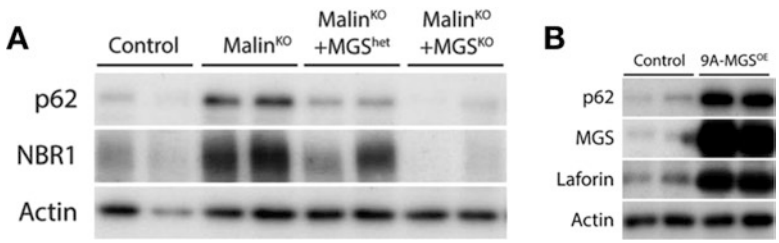

Fig. 7 Adapted from (Duran et al. 2014). (**a**) Analysis of autophagy markers. Accumulation of p62 and NBR1 in malin^{KO} brains was dependent on the expression of MGS. Brain extracts from 11-month-old mice were analyzed by western blot with antibodies against p62 and NBR1. Actin was used as loading control. (**b**) Brain extracts from 3-week-old 9A-MGSOE and littermate controls were analyzed as in **a**

The extensive neuronal loss and severe neuropathological phenotypes observed in patients and animal models of LD suggest that neurons are particularly vulnerable to excess glycogen accumulation (Delgado-Escueta 2007; Valles-Ortega et al. 2011; Duran et al. 2014; Lopez-Gonzalez et al. 2017). This evidences the need for tight control of glycogen synthesis in neurons since an excess of the polysaccharide results in the death of this cell population by apoptosis (Vilchez et al. 2007; Duran et al. 2012). However, the aforementioned accumulation of glycogen in astrocytes may also contribute to the physiopathology of LD.

Nevertheless, LD is not the only condition in which glycogen is deposited in an abnormally manner in nervous tissue. In other diseases like adult polyglucosan body disease and diabetic neuropathy, but also in normal aging, glycogen accumulates in the form of PGBs in neural tissue.

4 Adult Polyglucosan Body Disease

Adult Polyglucosan Body Disease (APBD) is a rare progressive neurodegenerative disorder caused by mutations in (GBE). The lack of GBE produces abnormal glycogen with low solubility due to the lack of branching, thereby leading to the accumulation of PGBs. Loss-of-function mutations of the enzyme cause glycogen storage disease type IV, also known as Andersen disease, a condition that affects neonates and children. In this condition, glycogen accumulates most severely in liver, cardiac and muscle cells, resulting in cirrhosis and death within 5 years. APBD, the adult-onset form of the disease, results from the partial loss of activity of the enzyme (Bruno et al. 1993). It is characterized by the deposition of PGBs in nervous tissue, which induces severe leukodystrophy and atrophy of the spine and medulla (Lossos et al. 1998). The accumulation of glycogen in astrocytes is sufficient to cause the disease (Dainese et al. 2013).

5 Diabetic Neuropathy and Diabetic Retinopathy

Diabetic neuropathy and diabetic retinopathy are two common complications of diabetes mellitus, affecting approximately 60% and 30% of diabetic patients respectively (Vincent and Feldman 2004; Simo et al. 2014). Peripheral nerves are surrounded by the perineurium, which, although acting as a diffusion barrier, is not as efficient as the blood–brain barrier. Consequently, peripheral nerves are in a less well-regulated microenvironment compared to the CNS. In normal conditions, nerve glycogen is present mainly in Schwann cells. However, glycogen accumulation has been described in the axons of peripheral nerves in diabetic patients (Yagihashi and Matsunaga 1979; Mancardi et al. 1985) and in several animal models of diabetes (Moore et al. 1981; Zotova et al. 2008). In fact, intra-axonal glycogen deposition is one of the parameters used to assess diabetic neuropathy (Orloff and Greenleaf 1990).

This glycogen is accumulated in large spherical deposits similar to LBs (Powell et al. 1977). In the retina, glycogen is present in Müller cells and in various types of neuron, especially those of the inner retina (Rungger-Brandle et al. 1996). Although the retina has one of the highest metabolic demands of any tissue, retinal oxygen tension is relatively low, especially in the inner layers. This conditioning might explain why retinal neurons accumulate more glycogen than brain neurons in normal conditions. Interestingly, several reports describe an increased deposition of glycogen in the retina of animal models of diabetic retinopathy (Sosula et al. 1974; Sanchez-Chavez et al. 2008; Osorio-Paz et al. 2012). In the light of the discovery of the toxic role of glycogen accumulation in LD, it is reasonable to hypothesize that glycogen accumulation in the nerves and retinas of diabetic patients could contribute to the physiopathology of diabetic neuropathy and diabetic retinopathy.

6 Aging

The accumulation of PGBs, known as corpora amylacea (CA), has also been observed in the aged human brain (Cavanagh 1999). CA share multiple histological and biochemical characteristics with LBs, including a composition of insoluble, poorly branched polysaccharide, resistance to digestion by amylase, and minor protein content.

Remarkably, the significance of PGB accumulation with regard to aging has been largely overlooked, and only a few authors have proposed that CA have a relevant role in neurodegeneration (Singhrao et al. 1993). Although human brain CA are formed mainly by polyglucosan, the presence of waste elements is a recurrent feature of these structures. This observation suggests that that they are involved in trapping and sequestering potentially hazardous products (Cavanagh 1999; Pirici et al. 2014; Rohn 2015). CA contain a number of neo-epitopes—specific epitopes that are not present in healthy brain structures but appear in situations of cellular stress and tissue damage (Binder 2010; Auge et al. 2017). The neo-epitopes in CA are recognized by natural IgM antibodies, thus revealing the potential role of the natural immune system in the removal of these aggregates (Auge et al. 2017).

In the same way in which CA accumulate with age in the human brain, CAL granules progressively appear in the aging mouse brain. These granules are present in a wide range of mouse strains but are particularly abundant in the senescence-accelerated mouse prone 8 (SAMP8) model (Manich et al. 2016). This model is a non-genetically modified strain of mice with an accelerated aging process and it shares characteristics with aged humans, such as a reduced lifespan, lordosis, hair loss, and reduced physical activity (Takeda 2009). In these animals, CAL granules appear in various regions of the brain as early as 3 months of age, and their number increases faster than in other strains (Jucker et al. 1994a, b; Del Valle et al. 2010). Remarkably, glycogen also accumulates in the brain of aged flies (*Drosophila*). This has relevant consequences for neurological function in vivo. If endogenous glycogen synthase (dGS) is knocked down by RNAi using neuron-specific drivers, glycogen

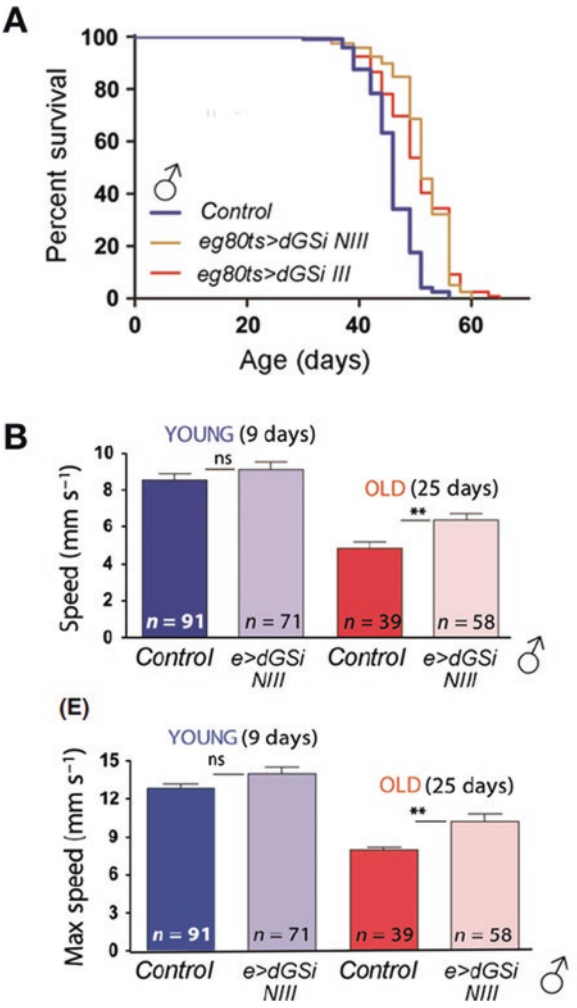

Fig. 8 Adapted from (Sinadinos et al. 2014). Functional consequences of reduced GS in the nervous system of aging Drosophila. (**a**) Survival curves of control and flies with reduced expression of GS (eg80ts > dGSi NIII and eg80ts > dGSi III). (**b**) Average (top graph) maximum (bottom graph) climbing speed of young (9 d, blue) and old (25 d, red) male control flies and flies with reduced expression of GS (e > dGSi NIII)

deposition in the brain is reduced. Adult males expressing dGS-RNAi have a significantly longer median (log-rank test) and maximum lifespan than driver-only-expressing controls (Fig. 8a). Young males expressing dGS-RNAi show a normal climbing response, with no significant change in climbing speed relative to control flies. In contrast, aged males showed a higher average and maximum climbing speed than controls (Fig. 8b). Interestingly, these changes are not observed in female flies.

As indicated earlier, neo-epitopes are present on CAL granules in mouse brains and on CA in human brains (Manich et al. 2016; Auge et al. 2017). Although the composition of CA must be re-examined due to the possible false positive staining in immunohistochemical studies (Auge et al. 2017), there is wide consensus that CA contain waste products (Cavanagh 1999; Pirici et al. 2014) and ubiquitin (Cisse and Schipper 1995; Wilhelmus et al. 2011; Pirici et al. 2014). It has been proposed

that the presence of neo-epitopes on CA is related to the removal of these bodies via the natural immune system after their extrusion (Auge et al. 2017). In this regard, CAL granules contain p62 protein, which has a ubiquitin-binding domain and is involved in the sequestration of ubiquitinated proteins and organelles (Liu et al. 2016). As expected, CAL granules are not present in any brain region of MGS^{KO} mice, thereby confirming that glycogen is an essential component of the aggregates. Interestingly, aggregates of alpha-synuclein, hsp70, and ubiquitin, which normally can all be found in the brains of aged controls, are also absent in MGS^{KO} mouse brains (Sinadinos et al. 2014). This observation indicates that glycogen is involved in the formation of protein-based aggregates, such as age-dependent accumulations of aggregation-prone or stress-response proteins. The increased accumulation of these markers in the brains of young $malin^{KO}$ mice is consistent with this proposal.

Collectively, these findings suggest that the progressive accumulation of glycogen aggregates in aged brains is a widespread phenomenon that contributes to neurological decline and that mutations in malin and laforin in LD dramatically increase the rate of this process. These observations thus point to glycogen synthesis as a promising target for reducing the age-related deterioration of the nervous system.

In summary, new evidence, mainly from genetically modified animals, has shed light on the role of glycogen in both astrocytes and neurons. It is now acknowledged that glycogen is required for normal functioning of the brain, but that its overaccumulation induces neurodegeneration, as demonstrated in Lafora disease.

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Endurance and Brain Glycogen: A Clue Toward Understanding Central Fatigue

Takashi Matsui, Mariko Soya, and Hideaki Soya

Abstract Brain glycogen stored in astrocytes produces lactate as a neuronal energy source transported by monocarboxylate transporters (MCTs) to maintain neuronal functions, such as hippocampus-regulated memory formation. Although exercise activates brain neurons, the role of astrocytic glycogen in the brain during exercise remains unknown. Since muscle glycogen fuels active muscles during exercise, we hypothesized that astrocytic glycogen plays an energetic role in the brain during exercise to maintain endurance capacity through lactate transport. To explore this hypothesis, we have used a rat model of prolonged exercise, microwave irradiation for the accurate detection of brain glycogen, capillary electrophoresis-mass spectrometry-based metabolomics, and inhibitors of glycogenolysis (1,4-dideoxy-1,4-imino-D-arabinitol; DAB) and lactate transport (α-cyano-4-hydroxycinnamate; 4-CIN). During prolonged exhaustive exercise, muscle glycogen was depleted and brain glycogen decreased when associated with decreased blood glucose levels and increased serotonergic activity known as central fatigue factors, suggesting brain glycogen decrease as an integrative factor for central fatigue. Prolonged exhaustive exercise also increased MCT2 protein in the brain, which takes up lactate in neurons, just as muscle MCTs are increased. Metabolomics revealed that brain but not muscle adenosine triphosphate (ATP) was maintained with lactate and other glycogenolytic and glycolytic sources. Intracerebroventricular (icv) injection of DAB suppressed brain lactate production and decreased hippocampal ATP levels at exhaustion. An icv injection of 4-CIN also decreased hippocampal ATP, resulting in lower endurance capacity. Our findings provide direct evidence that astrocytic glycogen-derived lactate fuels the brain to maintain endurance capacity during exhaustive exercise. Brain ATP levels maintained by glycogen might serve as a possible defense mechanism for neurons in the exhausted state.

Keywords Endurance exercise · Central fatigue · Brain glycogen · Lactate · Metabolomics

T. Matsui (✉) · M. Soya · H. Soya (✉)
Sport Neuroscience Division, Faculty of Health and Sport Sciences, Advanced Research Initiative for Human High Performance (ARIHHP), University of Tsukuba, Ibaraki, Japan
e-mail: matsui.takashi.ga@u.tsukuba.ac.jp; soya.hideaki.gt@u.tsukuba.ac.jp

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Abbreviations

4-CIN	α-Cyano-4-hydroxycinnamate
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)
AAA	Aromatic amino acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
DAB	1,4-Dideoxy-1,4-imino-D-arabinitol
F1-6P	Fructose-1, 6-bisphosphate
fMRI	Functional magnetic resonance imaging
GLUT	Glucose transporter
icv	Intracerebroventricular
IMP	Inosine monophosphate
MCT	Monocarboxylate transporter
MHPG	Methoxyhydroxyphenylglycol
MI	Microwave irradiation
NA	Noradrenaline
PCr	Phosphocreatine
TCA	Tricarboxylic acid

1 Introduction

Glucose derived from the blood is the primary energy source for generating adenosine triphosphate (ATP) in the brain (Sokoloff et al. 1977), but an important brain energy reserve is glycogen synthesized from glucose in astrocytes (Belanger et al. 2011). Astrocytic glycogen is broken down into lactate as a neuronal energy substrate transported by monocarboxylate transporters (MCTs) (Machler et al. 2016). Indeed, brain glycogen decreases during memory tasks (O'Dowd et al. 1994) and in some physiologically exhaustive conditions, such as sleep deprivation (Kong et al. 2002) and hypoglycemia (Herzog et al. 2008). The genetic and/or pharmacologic inhibitions of glycogenolysis and/or lactate transport impair neuronal survival under severe hypoglycemia, axonal transmission, and hippocampus-related memory formation (Swanson and Choi 1993; Newman et al. 2011; Suzuki et al. 2011). Therefore, astrocytic glycogen-derived lactate is a critical energy source for meeting brain energy demands for neuronal functions and/or survival.

Physical exercise activates brain neurons and increases brain energy demand, although to a lesser degree than what occurs in muscles during exercise (Secher et al. 2008). Blood glucose and lactate contribute to brain energetics during moderate or intense exercise (Vissing et al. 1996; Ide et al. 2000; Larsen et al. 2008). Muscle glycogen is an important energy source for maintaining muscle contraction during endurance exercise (Gollnick et al. 1974); however, the role of brain glycogen

during exercise remains uncertain. In this article, we describe our recent studies testing the hypothesis that astrocytic glycogen plays an energetic role in the brain during exercise to maintain endurance capacity through lactate transport.

2 Glycogen: The Sole Energy Storage in the Brain

The astrocyte-neuron lactate-shuttle hypothesis proposes that lactate released from astrocytes into the extracellular space is metabolized by neurons (Pellerin and Magistretti 1994; Matsui and Soya 2013) (Fig. 1). To date, it has been thought that the only energy source for the brain is blood-borne glucose. However, previous studies have suggested that lactate released from astrocytes is associated with neuronal activation (Tsacopoulos and Magistretti 1996), and that this lactate is transported to be metabolized predominantly in active neurons via MCTs (Chuquet et al. 2010). Furthermore, culture experiments have revealed that glycogen localized in astrocytes is degraded into lactate by excitatory neurotransmitters, such as noradrenaline (NA) and serotonin (5-hydroxytryptamine; 5-HT) (Magistretti 1988).

Not so long ago, it was believed that brain glycogen levels were so low that they could not play a significant role in cerebral metabolism during several physiological stimulations, including exercise. This is because there were technical difficulties in

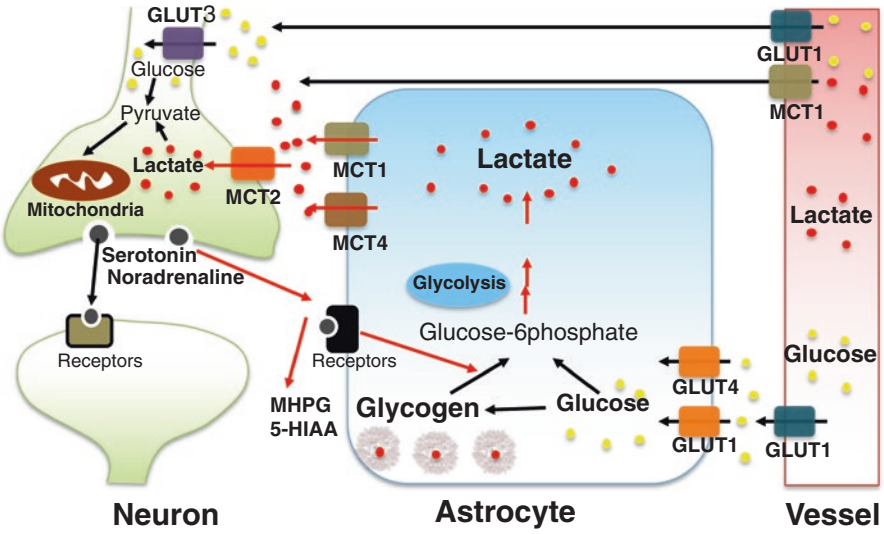

Fig. 1 Glycogen metabolism in the brain: contribution to the astrocyte–neuron lactate shuttle. G-6-P, glucose-6-phosphate; GLUT, Glucose transporter; MCT, monocarboxylic acid transporter. Energy sources for neurons include not only blood glucose but also lactate. Astrocytic glycogen is synthesized from blood glucose and degraded into lactate by excitatory neurotransmitters, such as NA and 5-HT. Lactate is taken up by neurons and changed to pyruvate, which is used for ATP synthesis in the mitochondria. Reproduced from Matsui and Soya (2013) with modifications

determining the post-mortem brain glycogen levels, which was due to the rapid breakdown of brain glycogen following death. A current technique resolves this issue by using high-power (10 kW) microwave irradiation (MI) to inactivate enzymes mediating glycogen metabolism (Kong et al. 2002; Matsui et al. 2011). Kong et al. (2002) indicated that using high-power MI for about 1 s inhibits glycogen metabolism by elevating the brain temperature to approximately 90 °C, which allows the determination of accurate brain glycogen levels.

Animal studies using MI have shown that astrocytic glycogen is degraded into lactate to provide fuel for neurons during hypoglycemia and sleep deprivation (Kong et al. 2002; Herzog et al. 2008). Interestingly, the inhibition of hippocampal glycogen degradation in rats with 1,4-dideoxy-1,4-imino-D-arabinitol (DAB, a glycogen phosphorylase inhibitor) prevented long-term memory formation (Suzuki et al. 2011). Furthermore, hyper brain glycogen contributes to maintaining neuronal activation during hypoglycemia (Suh et al. 2007a). These studies suggest that astrocytic glycogen is an important energy storage for producing lactate for neurons when the glucose provision from blood is insufficient and when there are sudden increases in energy demands during neuronal activation.

3 Brain Glycogen Decreases During Prolonged Exercise

Exercise increases brain energy demand through neuronal activation, and prolonged exercise induces hypoglycemia, leading us to postulate that brain glycogen decreases during exercise. To address this issue, we exercised male Wistar rats on a treadmill for different durations (30–120 min) at moderate intensity (20 m/min) and measured their brain glycogen levels by amyloglucosidase-based assay following MI.

At the end of 30 and 60 min of running, blood glucose levels did not decrease compared with those of pre-exercise, but at the end of 120 min, blood glucose was 46% lower than pre-exercise levels (Fig. 2). After 30 and 60 min, brain glycogen levels remained unchanged from resting levels, but liver and muscle glycogen decreased. After 120 min, brain glycogen levels decreased significantly by an average of 34–60% in five discrete brain loci (the cerebellum 60%, cortex 48%, hippocampus 43%, brainstem 37%, and hypothalamus 34%) compared to those of pre-exercise levels (Fig. 2). The brain glycogen levels after running in all five regions were significantly correlated with the respective blood glucose (positive) and with brain lactate (negative) levels after running (data not shown). Furthermore, in the cortex, the levels of metabolites of NA (methoxyhydroxyphenylglycol; MHPG) and 5-HT (5-hydroxyindoleacetic acid; 5-HIAA), which are potentially involved in the degradation of brain glycogen, increased during prolonged exercise and were negatively correlated with glycogen levels (Fig. 3). These findings indicate that brain glycogen decreases when associated with decreased blood glucose levels and increased serotonergic activity, both of which are known as central fatigue factors (Matsui et al. 2011).

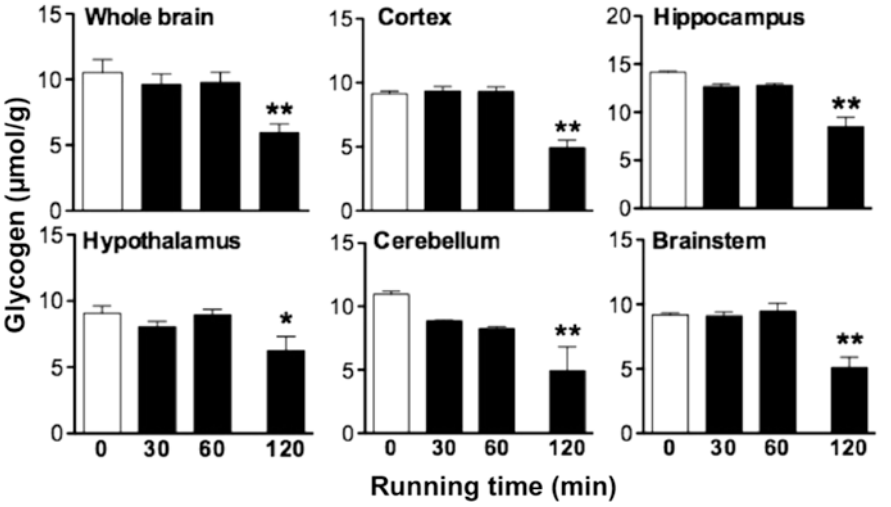

Fig. 2 Brain glycogen decreases during prolonged exercise. The data represent the mean ± SEM (n = 5–6 rats). $^{*}P < 0.05$; $^{**}P < 0.01$ compared to pre-exercised rats (Dunnett's *post hoc* test). Reproduced from Matsui et al. (2011) with modifications

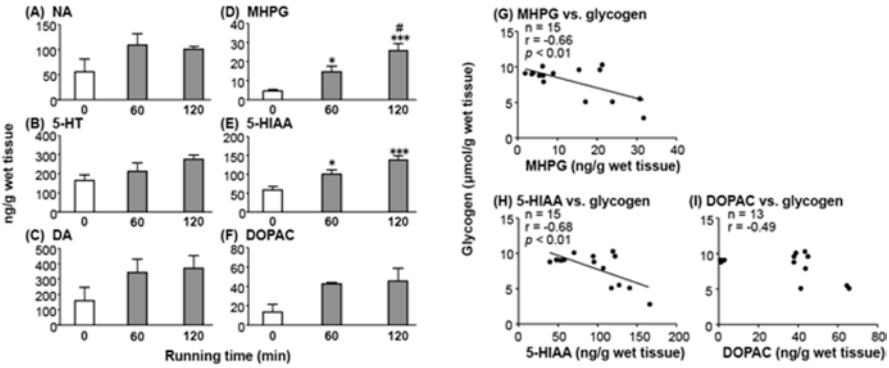

Fig. 3 Monoaminergic activity in the cortex during prolonged exercise. Data represent the mean ± SEM (n = 4–6 rats). (**a**) NA; (**b**) 5-HT; (**c**) dopamine (DA); (**d**) methoxyhydroxyphenylglycol (MHPG); (**e**) 5-hydroxyindoleacetic acid (5-HIAA); and (**f**) 3,4-dihydroxyphenylacetic acid (DOPAC), $^{*}P < 0.05$; $^{***}P < 0.0001$ compared to pre-exercise, $^{\dagger}P < 0.05$ compared to 60 min (Tucky's post hoc test). (**g**) correlation between MHPG and glycogen levels; (**h**) correlation between 5-HIAA and glycogen levels; and (**i**) correlation between DOPAC and glycogen levels (Pearson's product–moment correlation test). Reproduced from Matsui et al. (2011) with modifications

4 Brain Glycogen Decrease: A Possible Integrative Factor of Central Fatigue

A decrease in brain glycogen may be an integrative factor of central fatigue during prolonged exercise. To date, hypoglycemia with liver glycogen depletion, an increase in brain 5-HT (serotonin hypothesis), and a high brain temperature (hot brain) have been recognized as central fatigue factors during prolonged exercise (Newsholme et al. 1992; Nybo and Secher 2004). These factors are interesting because they are induced from peripheral tissues and have an influence on central fatigue. In addition, hypoglycemia and 5-HT are not only central fatigue factors but also activators for glycogenolysis in astrocytes. Thus, brain glycogen decreases are a possible integrative factor for the development of central fatigue during exercise (Fig. 4).

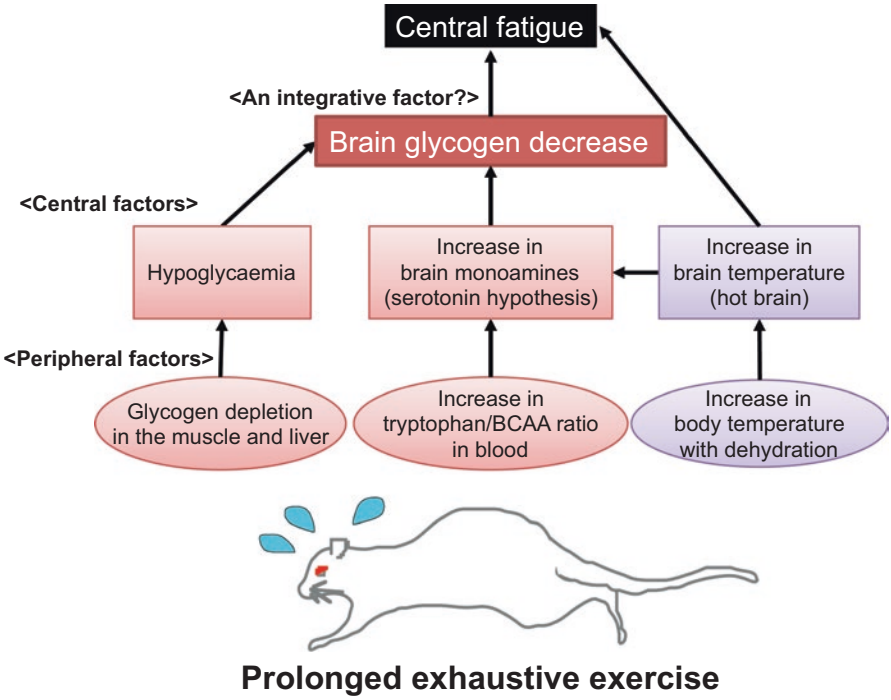

Fig. 4 Hypothetical diagram showing brain glycogen decrease as an integrative factor of central fatigue during prolonged exercise. Prolonged exercise induces glycogen depletion in the muscles and liver, as well as hypoglycemia, which causes peripheral fatigue. Hypoglycemia elicits energy shortages in the brain and likely induces central fatigue. Increase in brain 5-HT due to a rise in the ration of tryptophan to branched-chain amino acid (BCAA) in the blood also induces central fatigue by eliciting lassitude (serotonin hypothesis). Furthermore, increases in body and brain temperature attributed to dehydration induce central fatigue directly and/or indirectly through increases in brain NA and 5-HT. Hypoglycemia and 5-HT are not only inducing factors of central fatigue but also enhancing factors of astrocytic glycogen degradation. Indeed, we observed that brain glycogen levels after running were correlated with the respective blood glucose and increased 5-HT metabolism (Matsui et al. 2011). A decrease in exercise-induced brain glycogen could be an integrative factor of central fatigue. Reproduced from Matsui and Soya (2013)

5 Prolonged Exercise Not Only Decreases Glycogen But Also Increases MCT2 Protein in the Brain

We next examined the effect of exercise on the MCT proteins in the brain (Matsui et al. 2017). Rats were exercised on the treadmill until exhaustion (20 m/min; time to exhaustion 84.4 ± 2.9 min). Blood lactate was significantly increased and glucose levels were significantly decreased compared with the sedentary group ($P < 0.01$). Blood ketone body (β-hydroxybutyrate) levels increased ($P < 0.01$). Exhaustive exercise also caused a depletion (decrease by 97.3%) of muscle glycogen levels ($P < 0.01$). Brain glycogen levels in the cortex and hippocampus were decreased by 75.1 and 66.3%, respectively ($P < 0.01$), but the depletion in the brain did not occur to the same extent as that seen in the skeletal muscles. Concomitantly, MCT2 protein levels in the cortex and hippocampus increased with exhaustive exercise ($P < 0.05$), similar to the increases in MCT proteins observed in the skeletal muscles (Figs. 5a, c, e). Glucose transporter (GLUT) 1 and 3 protein levels increased only in the cortex, and GLUT4 increased in muscles (Figs. 5b, d, f, g). These results imply that MCT2 and GLUT1 and 3 in the brain are very rapidly upregulated with acute prolonged exercise, which is similar to the MCTs in the skeletal muscles (Coles et al. 2004).

6 Metabolomics of the Brain with Exhaustive Exercise

In the next step, we used capillary-electrophoresis-mass-spectrometry-based metabolomics to observe comprehensive energetics of the brain (cortex and hippocampus) and muscle (plantaris) (Matsui et al. 2017). Metabolomics measured 159, 183, and 182 metabolites and revealed that 76, 79, and 72 metabolites were changed significantly in the plantaris muscle, cortex, and hippocampus, respectively, with exhaustive exercise. Principal component analysis and hierarchical cluster analysis clearly indicated the difference in metabolic profiles between sedentariness and exhaustion in all tissues.

The glycolysis map of the plantaris muscle after exhaustive exercise showed depletion of glycogen and glucose and almost total depletion of glycolytic sources and lactate ($P < 0.05$) (Fig. 6a). However, tricarboxylic acid (TCA)-cycle sources increased ($P < 0.05$) (Fig. 6b), suggesting the contribution of β-oxidation of lipids lacking in the brain. Maps of the cortex and hippocampus revealed a decrease in glycogen, glucose, and upstream glycolytic metabolites, including fructose-1, 6-bisphosphate (F1-6P) ($P < 0.05$), but none of these metabolites were depleted. Downstream metabolites, such as F1-6P, together with TCA-cycle sources, were sustained, and the lactate level was increased ($P < 0.01$) (Fig. 6c–f).

Metabolomics-produced purine and pyrimidine maps of the muscle and brain showed that ATP and phosphocreatine (PCr) decreased significantly in the exercise-exhausted group compared with the sedentary group ($P < 0.05$) (Fig. 7a) but that brain

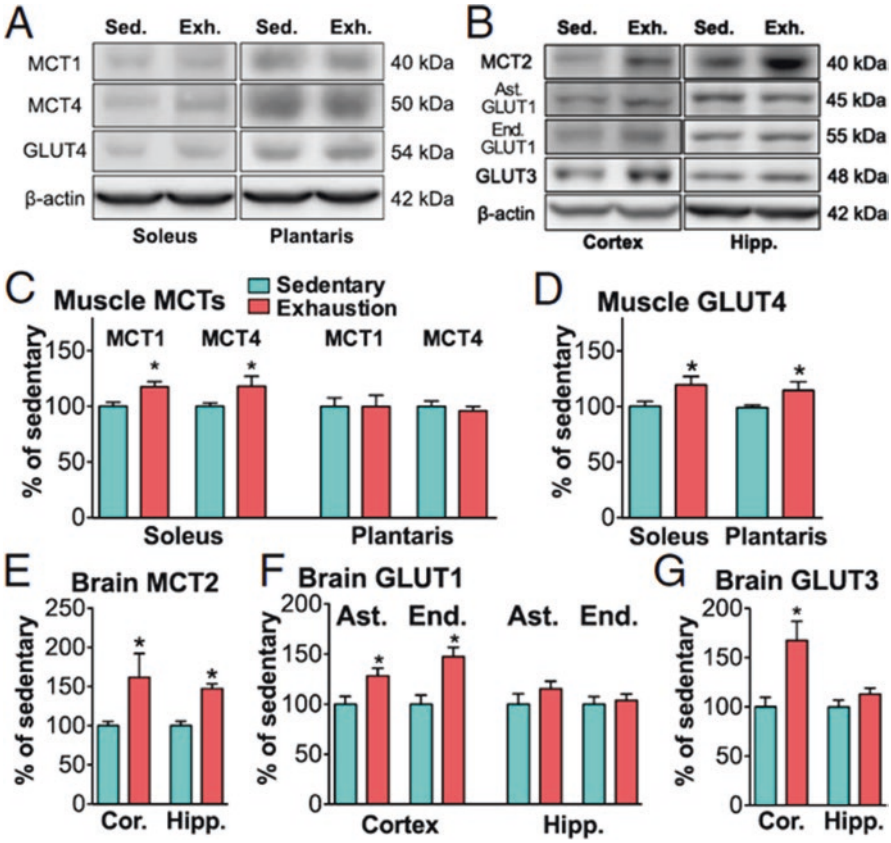

Fig. 5 Exhaustive exercise increases MCT and GLUT protein in muscles and the brain. (**a**) Typical photos of Western blotting bands for MCT1, MCT4, GLUT4, and β-actin in muscles. Exh., exhaustion; Sed., sedentary. (**b**) Typical photos of Western blotting bands for MCT2, astrocytic (Ast.) GLUT1, endothelial (End.) GLUT1, GLUT3, and β-actin in the brain. (**c**) Muscle MCT protein. (**d**) Muscle GLUT4 protein. (**e**) Brain MCT2 protein in the cortex and hippocampus. (**f**) Brain GLUT1 protein. (**g**) Brain GLUT3 protein. Data are expressed as mean ± SE (n = 5 per group). *$P < 0.05$. Reproduced from Matsui et al. (2017)

ATP and PCr levels were unchanged after exhaustive exercise (Fig. 7b, c). Several downstream sources of purine metabolism, such as adenosine monophosphate (AMP), inosine, inosine monophosphate (IMP), hypoxanthine, and uric acid, increased in both the muscle and brain of exercise-exhausted animals ($P < 0.05$) (Fig. 7). These data are direct evidence that ATP consumption is increased in both the brain and muscles but that only brain ATP levels are maintained during exhaustive exercise.

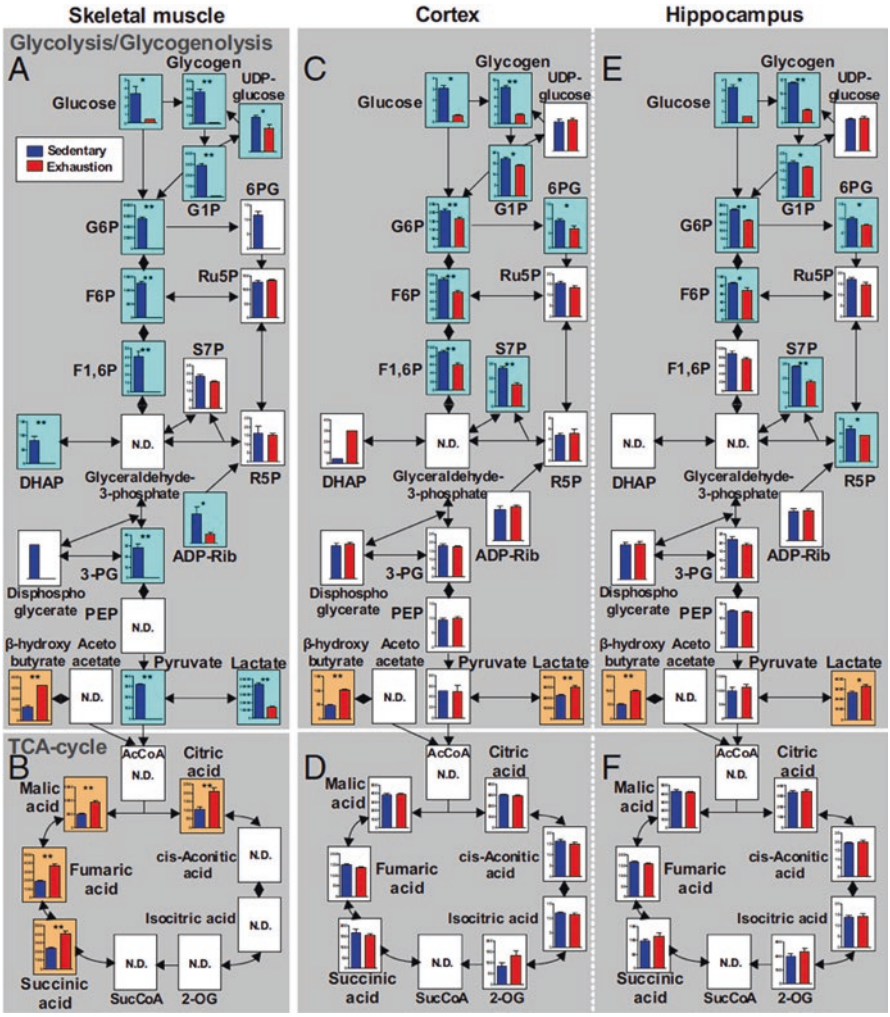

Fig. 6 Lactate increases in the brain but not in muscles during prolonged exhaustive exercise. Glycolytic pathways measured by metabolomics in the (**a**) plantaris muscle, (**c**) cortex, and (**e**) hippocampus. TCA-cycle pathways in the (**b**) plantaris muscle, (**d**) cortex, and (**f**) hippocampus. Glucose and glycogen results are inserted from results of glycogen assays. Data are expressed as mean ± SE (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus sedentary group (Student's t test); N.D., not determined. Blue backgrounds indicate significantly decreased sources, and orange backgrounds imply significantly increased sources with exhaustive exercise. Graphs with a y-axis show absolute detected amounts (nmol/g wet tissue), and graphs without a y axis show relative levels. The map of the plantaris muscle after exhaustive exercise shows a depletion of glycogen and glucose and almost total depletions of glycolytic sources, including lactate. Maps of the cortex and hippocampus reveal a decrease in glycogen and glucose and upstream glycolytic metabolites, including F1-6P, but none of these metabolites were depleted. Downstream metabolites, such as 3-PG and pyruvate, together with TCA-cycle sources, were sustained and lactate was increased. Reproduced from Matsui et al. (2017)

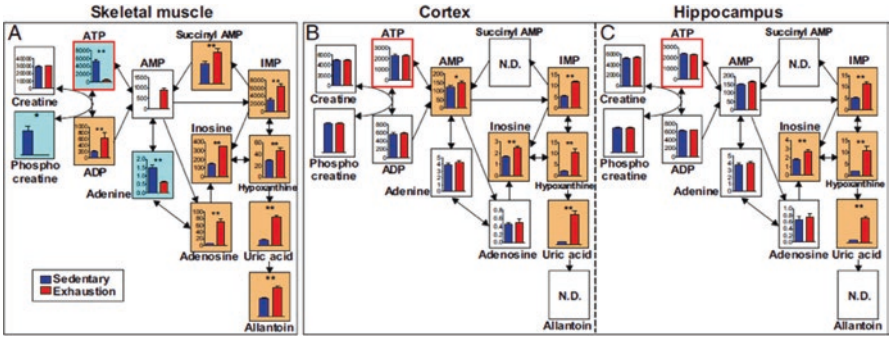

Fig. 7 ATP is maintained in the brain but not in muscles during prolonged exhaustive exercise. Purine metabolism pathways measured by metabolomics in the (**a**) plantaris muscle, (**b**) cortex, and (**c**) hippocampus. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the sedentary group (Student's t test). Blue backgrounds indicate significantly decreased sources, and orange backgrounds imply significantly increased sources following exhaustive exercise. Graphs with a y-axis show absolute detected amounts (nmol/g wet tissue), and graphs without a y-axis show relative levels. Data are expressed as mean ± SE (n = 5 per group). The abbreviated metabolite names are defined in Table S1. Muscle and brain maps show that ATP and PCr were decreased significantly in the exercise-exhausted group compared with the sedentary group ($P < 0.05$), but brain ATP and PCr were unchanged after exhaustive exercise. Several downstream sources of purine metabolism, such as AMP, inosine, IMP, hypoxanthine, and uric acid, were increased in both the muscle and brain of exercise-exhausted animals. Reproduced from Matsui et al. (2017)

7 Energetic Role of Brain Glycogen During Exhaustive Exercise

The reduction of brain ATP induces neuronal death (Suh et al. 2007b; Sugiura et al. 2011). However, we revealed with metabolomics that brain ATP but not muscle ATP levels are maintained with residual glycogen during prolonged exhaustive exercise (Fig. 7). These findings indicate that the brain, rather than muscle, is protected energetically, likely avoiding neuronal death or dysfunction during exhaustive exercise, supporting the "selfish brain" theory regarding energy competition among organs (Peters et al. 2004).

A localized blockade of brain glycogen breakdown (glycogenolysis) using DAB inhibited the increases in hippocampal lactate and decreased hippocampal ATP during exhaustive exercise (Fig. 8a–d). A localized disruption of the MCT2 protein using (α-cyano-4-hydroxycinnamate; 4-CIN) also disrupted the maintenance of hippocampal ATP (Fig. 8f, g). These data indicate that glycogen-derived lactate transported by increased MCT2 is required for brain ATP maintenance during exhaustive exercise, at least in the hippocampus, which is direct evidence for the energetic importance of brain glycogen during endurance exercise. This evidence provides new insight into the strategies that protect and promote brain functions related to performance in animals (e.g., endurance capacity and/or cognitive functions).

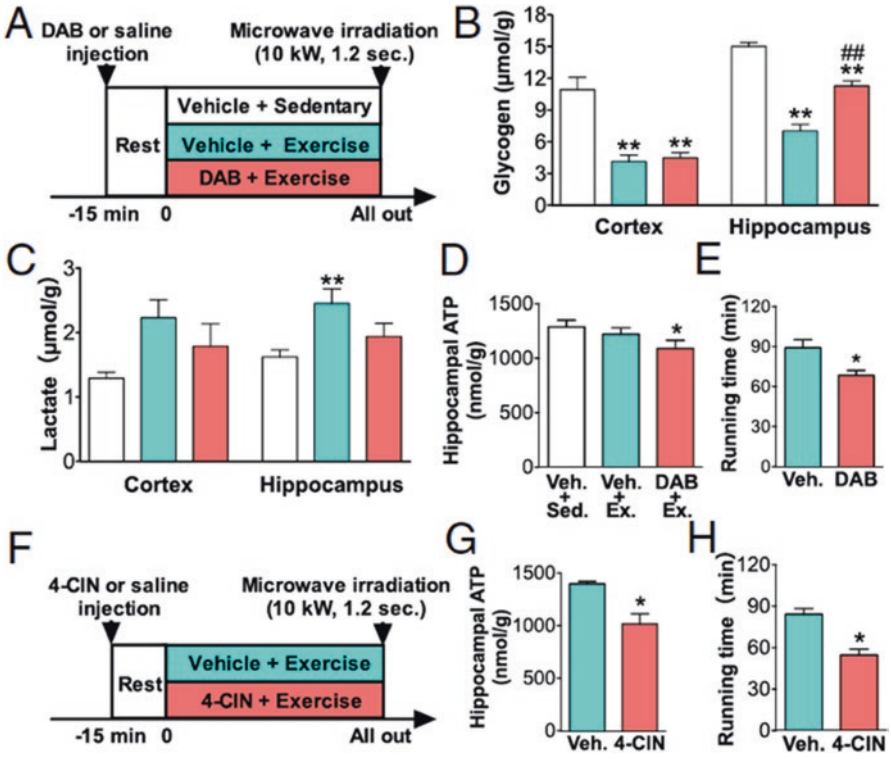

Fig. 8 Blockade of brain glycogenolysis and MCT2 decreases endurance capacity associated with brain ATP. Data are expressed as mean ± SE (n = 5–7 per group). (**a**) Experimental design for exhaustive prolonged exercise with DAB icv injection in rats. (**b**, **c**) Glycogen (**b**) and lactate (**c**) in the cortex and hippocampus. $^{**}P < 0.01$ versus vehicle (Veh.) + sedentary group; $^{\#\#}P < 0.01$ versus Veh. + exercise (by one-way ANOVA with Tukey's post hoc tests). (**d**) ATP in the hippocampus. (**e**) Running time to exhaustion with DAB icv injection. (**f**) Experimental design for exhaustive prolonged exercise with 4-CIN icv injection in rats. (**g**) ATP in the hippocampus. (**h**) Running time to exhaustion with 4-CIN icv injection. $^{*}P < 0.05$ versus vehicle group (by Student's t test). Reproduced from Matsui et al. (2017)

8 Brain Glycogen and Endurance Capacity

The blockade of brain glycogenolysis and lactate transport via MCT2 accelerated exhaustion during prolonged exercise (Fig. 8). These data support the hypothesis that brain lactate derived from astrocytic glycogen plays a role in endurance capacity because of its energetic contribution (Nybo and Secher 2004; Larsen et al. 2008; Matsui et al. 2011). Although DAB affected hippocampal glycogen content, it did not affect cortical glycogen with exhaustive exercise (Fig. 8b). However, intracerebroventricular (icv) inhibition of glycogen phosphorylase blocked glycogen depletion in the hypothalamus, brainstem, and cerebellum, although it only significantly reduced lactate levels in the hippocampus, raising the question of which brain sites signal exhaustion.

DAB inhibited the decrease of glycogen in the hippocampus caused by exhaustive exercise (Fig. 8b). Because hippocampal glycogen-derived lactate acts in memory function (Suzuki et al. 2011), the decreased glycogen utilization in the exhausted hippocampus might be a cause of exercise-induced cognitive fatigue. However, there is methodological difficulty in detecting cognitive functions of exercise-fatigued animals because, due to fatigue, they cannot move when given memory tasks. In humans, exhaustive exercise decreases cognitive functions, including working memory (Perciavalle et al. 2015), but it is unknown whether or not this reflects hippocampus-based cognitive decline. A new system for determining the effects of moderate exercise on pattern separation, a dentate gyrus-specific ability (Suwabe et al. 2017), can be applied together with functional magnetic resonance imaging (fMRI) analysis for fatigue research on this important topic.

Hippocampal neurons also play an important role in the onset of locomotion and exhibit locomotion velocity-dependent firing with theta oscillation (Fuhrmann et al. 2015). Although untested, glycogen-derived lactate might be a contributor to locomotion-dependent hippocampal firing. This postulation could provide novel insight into the significance of the hippocampus not only for memory but also for exercise capacity, implicating the underlying positive relationship between aerobic fitness and cognitive function (Kramer et al. 1999; Erickson et al. 2009; Hyodo et al. 2016).

In addition, the glycogen decrease in the hypothalamus, cerebellum, and brainstem due to exhaustive exercise was inhibited with DAB icv injection. Hypothalamic lactate is an important factor in counterregulation during hypoglycemia (Chan et al. 2013), and brainstem lactate controls of arousal through stimulation of noradrenergic neurons (Tang et al. 2014). Therefore, the blockade of brain glycogenolysis and lactate transport would result in a lower endurance capacity through suppression of brain region-specific functions (e.g., hippocampus: locomotion; hypothalamus: regulation of energy metabolism; cerebellum: motor control; brainstem: arousal control; etc.).

9 Biochemical Insight into the Development of Exhaustion and Central Fatigue during Prolonged Exercise

Fatigue induced by prolonged exercise is separated into muscle and central (brain) factors (Nybo and Secher 2004). Our metabolomics provide insight into the biochemistry behind fatigue during prolonged exercise (Fig. 9). In the muscles of exercise-exhausted rats, ATP, PCr, and glycogen were significantly decreased, whereas hypoxanthine levels were increased due to purine metabolism (Figs. 3 and 4). These findings are consistent with studies on exhausted skeletal muscles (Sahlin et al. 1999). Although undetected in the present study, purine metabolism generates ammonia, which is a known muscle fatigue factor (Broberg and Sahlin 1989). Therefore, the depletion of energy sources and accumulation of inhibitors of muscle contraction (e.g., ammonia) are factors in muscle fatigue (failure of muscle contraction).

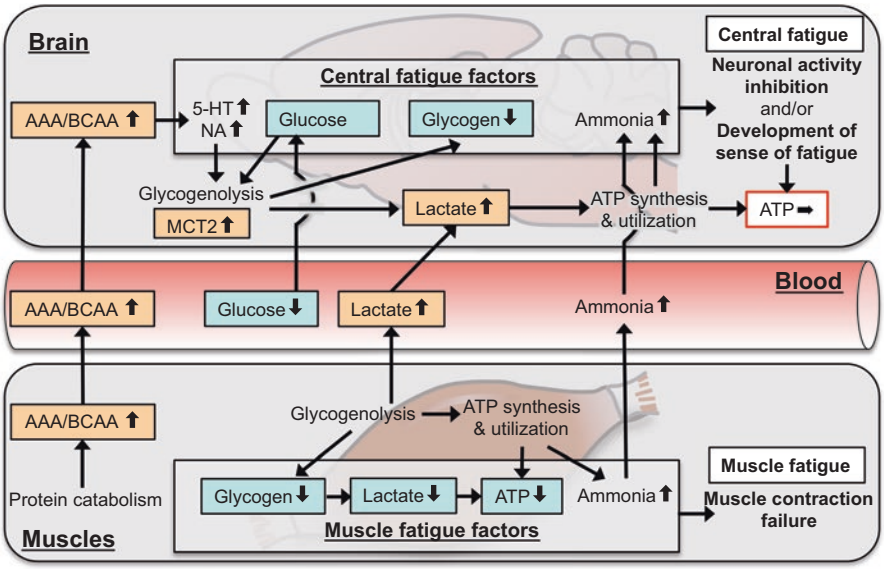

Fig. 9 Schematic figure for muscle–brain coupling in the development of physiological fatigue during exhaustive exercise. In the exhausted skeletal muscle, ATP decrease is associated with glycogen depletion, whereas ammonia production via purine metabolism is increased. These are muscle fatigue factors for the failing muscle contraction. In the exhausted brain, increased uptake of muscle-derived tryptophan as a 5-HT precursor induces a sense of fatigue (tryptophan–serotonin hypothesis). Hypoglycemia, induced together with muscle glycogen depletion, causes a decrease in brain glycogen that is probably compounded by increased NA and 5-HT. In addition, although undetected in the present study, endogenous production of ammonia via purine metabolism in the muscles and brain is probably increased and contributes to inhibition of neuronal activity. Overall, physiological fatigue in the exhausted state is regulated via complex muscle–brain coupling and serves primarily to maintain brain ATP as a biological outcome for neuronal protection. Reproduced from Matsui et al. (2017)

In the brain, ammonia, which is essentially detoxified by astrocytes through the glutamate–glutamine cycle, increases derived from exercising muscles and/or the brain itself and inhibits neuronal activity (Nybo and Secher 2004). The uptake of tryptophan is also increased due to the elevated ratio of branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) in the blood, which are precursors of NA and 5-HT. Increased brain tryptophan induces elevated 5-HT levels, promoting a "sense of fatigue" and inhibiting neuronal activity (the tryptophan–serotonin hypothesis) (Cotel et al. 2013). Further, hypoglycemia induced by depletion of liver and muscle glycogen creates a lack of energy that further inhibits neuronal activity. NA, 5-HT, and hypoglycemia are also strong activators of astrocytic glycogenolysis. Indeed, increased AAAs, which are likely converted to NA and 5-HT, are correlated with decreased brain glycogen. These factors are also induced not only during fatigue but also by sleep deprivation (Lopez-Rodriguez et al. 2003) and hypoglycemia (Heyes et al. 1990; Herzog et al. 2008), conditions that decrease brain glycogen. Thus, the decrease in brain glycogen is a possible common mechanism for central fatigue.

Central fatigue factors, such as ammonia, 5-HT, NA, and/or their precursors, are derived from muscles and reach the brain via the bloodstream. Ammonia and 5-HT appear to suppress ATP and glycogen consumption through the development of a sense of fatigue and neuronal inhibition. NA and 5-HT activate glycogenolysis and MCT expression for lactate synthesis and transport, thereby maintaining ATP synthesis. These factors would function to maintain brain ATP as the primary outcome through muscle–brain metabolic coupling in exhaustion, implicating central fatigue as a defense mechanism for brain neurons (Fig. 9).

10 Concluding Remarks

In this article, we have shown evidence from animal studies for the energetic role of lactate derived from astrocytic glycogen in the brain during prolonged exercise, thereby contributing to endurance capacity, in keeping with the known role of astrocytic glycogen in memory formation involving the hippocampus (Newman et al. 2011; Suzuki et al. 2011). Shedding light on the mechanism of the positive relationship between endurance and memory, our metabolomics analysis also revealed that the decrease in brain glycogen is a possible factor for exercise-induced central fatigue, which involves muscle–brain metabolic crosstalk. Importantly, the ATP maintenance contributed by brain glycogen likely serves as a neuroprotective mechanism at exhaustion.

Recent human studies using nuclear magnetic resonance (NMR) have also detected brain glycogen metabolism in healthy people and type I diabetes patients (Oz et al. 2009, 2012, 2017). If NMR can be applied to exercise and sports, it may soon be possible to propose new concepts targeting brain glycogen with exercise and/or nutrition to mitigate central fatigue during exercise.

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Role of Brain Glycogen During Ischemia, Aging and Cell-to-Cell Interactions

Chinthasagar Bastian, John Quinn, Christine Doherty, Caroline Franke, Anna Faris, Sylvain Brunet, and Selva Baltan

Abstract The astrocyte-neuron lactate transfer shuttle (ANLS) is one of the important metabolic systems that provides a physiological infrastructure for glia-neuronal interactions where specialized architectural organization supports the function. Perivascular astrocyte end-feet take up glucose via glucose transporter 1 to actively regulate glycogen stores, such that high ambient glucose upregulates glycogen and low levels of glucose deplete glycogen stores. A rapid breakdown of glycogen into lactate during increased neuronal activity or low glucose conditions becomes essential for maintaining axon function. However, it fails to benefit axon function during an ischemic episode in white matter (WM). Aging causes a remarkable change in astrocyte architecture characterized by thicker, larger processes oriented parallel to axons, as opposed to vertically-transposing processes. Subsequently, aging axons become more vulnerable to depleted glycogen, although aging axons can use lactate as efficiently as young axons. Lactate equally supports function during aglycemia in corpus callosum (CC), which consists of a mixture of myelinated and unmyelinated axons. Moreover, axon function in CC shows greater resilience to a lack of glucose compared to optic nerve, although both WM tracts show identical recovery after aglycemic injury. Interestingly, emerging evidence implies that a lactate transport system is not exclusive to astrocytes, as oligodendrocytes support the axons they myelinate, suggesting another metabolic coupling pathway in WM. Future studies are expected to unravel the details of oligodendrocyte-axon lactate metabolic coupling to establish that all WM components metabolically cooperate and that lactate may be the universal metabolite to sustain central nervous system function.

Keywords D-lactate · White matter · Axon · Ischemia · Ageing · Corpus callosum · End-feet

C. Bastian · J. Quinn · C. Doherty · C. Franke · A. Faris · S. Brunet · S. Baltan (✉)
Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA
e-mail: baltans@ccf.org

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1 Introduction

Energy homeostasis in the brain is maintained primarily by oxidative metabolism of glucose to fulfill the energy demands associated with ionic gradients in neurons and astrocytes. The metabolic rate of white matter (WM) is about 50% of the rate of gray matter on the basis of oxygen consumption (Nishizaki et al. 1988), and axons rather than glial cells are the main energy-consuming components of WM (Attwell and Laughlin 2001). It is important to recognize that axon energy metabolism is independent of the neuronal cell body. Because axons extend for great distances from their neuronal cell bodies, they are dependent upon local energy production to maintain ionic homeostasis in order to maintain electrical excitability. Consequently, axons may suffer from energy deprivation independent of the energy status of neuronal cell bodies. The cellular and anatomic organization of astrocytes determine their role in emerging as the main provider to maintain axon function during energy deprivation. Glycogen and glycogen phosphorylase activity are confined to astrocytes (Phelps 1972; Pfeiffer-Guglielmi et al. 2003), and astrocytes are strategically positioned to uptake glucose from the blood circulation via glucose transporter 1 (GLUT 1) (Vannucci et al. 1997; Maher et al. 1994; Simpson et al. 1994) (Fig. 1). Moreover, astrocytic end-feet take up glucose and store it as glycogen

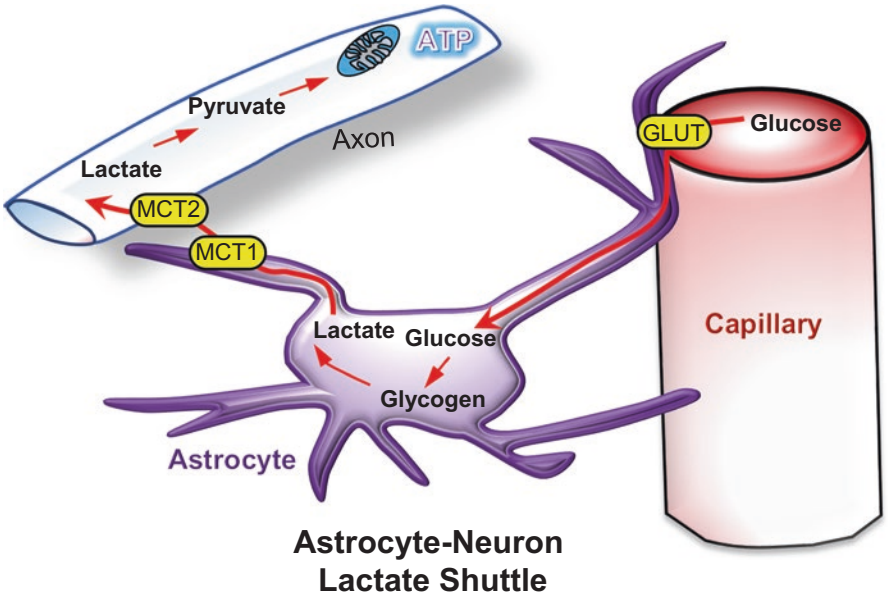

Fig. 1 Schematic diagram of structural and functional components of the astrocyte-neuron lactate shuttle transport (ANLST) in WM. Astrocytes (purple) enwrap capillaries (red) with their end-feet to take up glucose via glucose transporter 1 (GLUT) to store it as glycogen and convert it to lactate via monocarboxylate transporter 1 (MCT1) when ambient glucose levels are low. Axons (blue) take up lactate via MCT2 to derive energy using their mitochondria

(Chih et al. 2001) (Fig. 1). Indeed, astrocytes are the only cells in the mammalian brain that contain significant amounts of glycogen (Cataldo and Broadwell 1986). Based on these properties, astrocytes are the principal cells that are uniquely equipped to maintain energy homeostasis (Magistretti and Pellerin 1996), even for axons extending long distances from their neuronal soma (Brown et al. 2003; Baltan et al. 2008). During increased activity or glucose depletion, glycogen in astrocytes is converted to lactate and extruded via astrocyte monocarboxylate transporter 1 (MCT1) to axons. Axons take up the lactate via monocarboxylate transporter 2 (MCT2) (Fig. 1). Thus, the astrocyte-neuron lactate transfer shuttle (ANLS) efficiently supports axon function. In this paradigm, glycogen serves as a glucose reserve and substrate buffer for local energy demand. The regulation of glycogen in astrocytes is under the control of enzymatic machinery that is exquisitely sensitive to cell energy status. Furthermore, at the cellular level, the sodium-potassium pump (Na^+/K^+ pump) plays a key role in neuron-astrocyte metabolic interactions that form the basis for coupling between axonal activity and energy demands (Munzer et al. 1994; Hertz et al. 2007). The astrocytic Na^+/K^+ pump is intimately sensitive to increases in extracellular K^+ (Henn et al. 1972; Hajek et al. 1996; Honegger and Pardo 1999), and as a result, astrocytes act as sensors during axonal activity when extracellular K^+ is elevated particularly when other ATP-dependent pumps are activated and consume energy during increased axonal activity.

The first established physiological role of brain glycogen proved to be challenging and conflicting. Although Kuffler (Kuffler and Nicholis 1964; Kuffler and Potter 1964; Kuffler and Nicholls 1966; Kuffler et al. 1966) proposed the idea that glial cells participate in maintaining neuronal energy metabolism, it was Tsacopoulus (Tsacopoulos et al. 1994) who established substrate transfer from glial cells to neuronal cells while working with a honeybee retina model system. However, since then, accumulating evidence has shown that there is a functional ANLS in multiple regions of the mammalian brain (Magistretti et al. 1994; Pellerin et al. 2002; Wender et al. 2000). The human brain evolved such that there are equal volumes of glia and neurons and phylogeny indicates that glial density increases with brain complexity and evolution. Therefore, it is no surprise that astrocyte glycogen, which is the sole energy store in brain, can be shared with axons (Brown et al. 2003; Baltan et al. 2008; Tekkok et al. 2005). Faithful conduction of WM axons is crucial for maintaining or regaining brain function after acute or chronic injury. WM tracts comprise axons of various sizes and lengths with varying degrees of myelination. Due to the challenge of meeting metabolic needs of transport and signal conduction in these axons, lactate emerges as a critical energy substrate in WM tracts. Chapter "Metabolism of Glycogen in Brain White Matter" from this book by Ransom and Brown provides the proof-of-concept studies that established the principles of ANLS during glucose deprivation or high-intensity activity in WM. Of particular interest is whether this support system is impaired during aging, which could contribute to age-dependent neurodegenerative diseases. These questions currently remain under investigation. This chapter focuses on (a) whether glia-derived lactate transfer to axons in the optic nerve, a pure WM tract, remains operational to benefit axon function recovery [Figure 2a, Compound Action Potential (CAP), optic nerve]

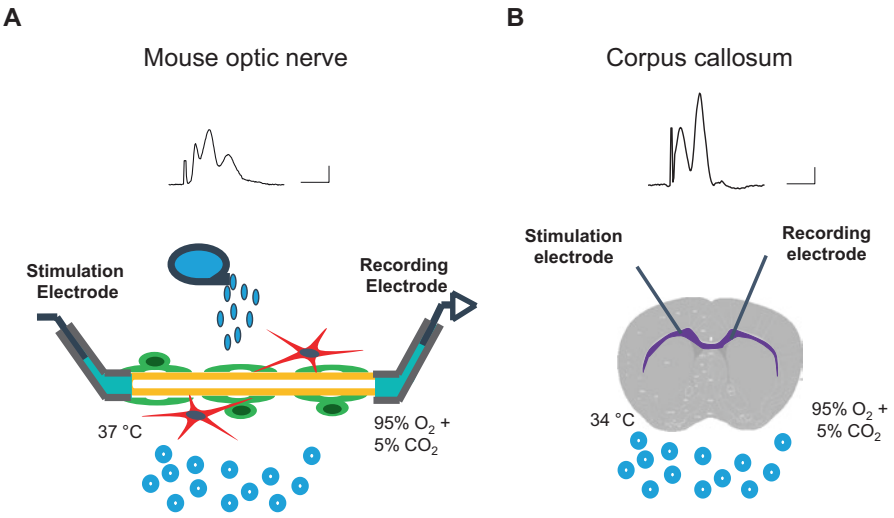

Fig. 2 Electrophysiological recording configuration of axon function. (**a**) Evoked compound action potentials (CAPs) in mouse optic nerve using suction electrodes display a typical three-peaked CAPs at 37 °C. Peaks denote most myelinated and faster-conducting (P1) to lightly myelinated, slow-conducting axons in order (P3). (**b**) Evoked CAPs in corpus callosum show two peaks using extracellular recording configuration at 34 °C. The first peak (P1) represents myelinated, while the second peak (P2) represent the unmyelinated axon response. Recording chambers were oxygenated with 95% O_2 + 5% CO_2

after other metabolic insults such as ischemia induced by combined glucose-oxygen deprivation; (b) whether lactate supports axon function in different WM tracts that consist of myelinated and unmyelinated axons (Fig. 2b, CAP, corpus callosum); and (c) whether aging alters astrocytes and vulnerability of aging axons to glucose deprivation. This chapter will also interrogate whether the glia-axon lactate support mechanism is unique to astrocytes by reviewing evidence supporting oligodendrocyte glycolysis as a potential source of lactate to support axon function in CNS WM. This chapter provides selected published data, while also providing our new and unpublished evidence to focus on the role of lactate as a universal energy buffer for myelinated and unmyelinated axons in young and aging WM.

2 Role of Astrocyte Glycogen During Ischemia in WM

Brain energy metabolism is interrupted in many neurological disorders. Energy failure is the central cause of loss of cerebral function during a hypoglycemic episode or during ischemia. Extensive axonal energy demand and physiological maintenance of glial cells place WM tracts under metabolic challenge, even under physiological conditions (Ransom and Orkand 1996; Harris and Attwell 2012). Moreover, due to their unique vascular network that is less dense and more sparse compared to

gray matter, WM components and glial cells have relatively restricted access to energy substrates (Moody et al. 1990). The morphology of WM structure and components further confound energy metabolism because oligodendrocytes wrap myelin around axons to facilitate axon transport, but this inevitably forms a barrier around axons (see Sect. 5 for oligodendrocyte-axon lactate support system) that restricts extracellular metabolites from gaining access to axons (Nave 2010a). Expediently, morphological evidence shows that astrocytes contact axons at nodes of Ranvier (Black and Waxman 1988), presumably to deliver energy substrate to axons via MCT1 (Pellerin et al. 1998). This shuttle system in principle proposes that capillary glucose taken up by astrocyte end-feet is stored as glycogen in astrocytes, to be delivered to axons as lactate on demand (Dringen et al. 1993). Naturally, the first experiments assessed the efficiency of ANLS in WM during glucose depletion. Studies using mouse optic nerve (MON) established a direct correlation between astrocyte glycogen content and axon function (Brown et al. 2003) and further suggested a link between astrocyte-derived lactate and axonal metabolic coupling (Tekkok et al. 2005) during aglycemia. Whether the principles of this shuttle apply to WM during different metabolic insults such as ischemia was investigated. Evidence from neuronal studies showed that glutamate-mediated cortical injury was attenuated by lactate and that neurons preferred lactate as an energy source during excitotoxicity (Alessandri et al. 1996; Ros et al. 2001). However, glycogen-derived lactate under ischemic conditions failed to confer protection to axon function or to improve recovery (Fig. 3a, b, green). After incubating optic nerves in high (30 mM) glucose to upregulate glycogen levels, oxygen and glucose deprivation (OGD) indeed caused a more rapid reduction in axon function following high glucose incubation (Fig. 3b, c, purple), followed by a comparable level of recovery observed

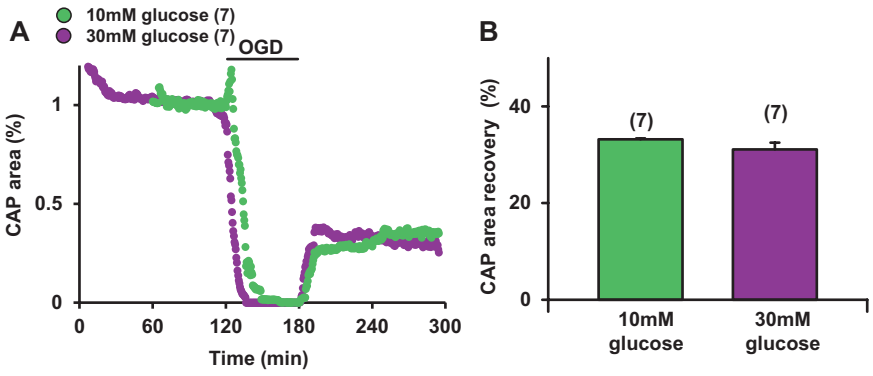

Fig. 3 Lactate cannot be used as a substrate during ischemia. (**a**) Time course of compound action potential (CAPs) area shows that following oxygen glucose deprivation (OGD), axon function recorded in MONs incubated in high (30 mM, purple) glucose declines faster than in MONs incubated in normal (10 mM, green) glucose, but both groups recover to similar levels following 60 min of OGD. (**b**) Histograms show that CAP area recovery is similar between the two groups and is independent of lactate availibility during OGD (60 min). *Figure reproduced from Baltan 2015, Met. Brain Dis*

under normoglycemic conditions (10 mM glucose). In *in vitro* preparations, removal of oxygen and glucose is an accepted form of ischemia and mimics the interruption of blood supply to parts of the brain upon formation of a thrombus. Unlike aglycemia, oxidative phosphorylation is inhibited during ischemia. WM and axon function show an unusual resilience to even prolonged durations of anoxia (Tekkok et al. 2003), such that a subgroup of axons sustain their function solely on glucose. However, during ischemia, glycogen-derived lactate cannot be used as a substrate to support axon function. In contrast, glycogen indeed becomes the preferred metabolite for neuronal elements in the peri-infarct area and neurons benefit from lactate to survive in this region following a stroke (Alessandri et al. 1996, Ros et al. 2001). Studies in the penumbra region after a WM stroke have not yet provided an explanation for this metabolic preference.

3 Lactate Supports Axon Function in Different WM Tracts

The optic nerve consists of fully myelinated axons; therefore, initial experiments inevitably considered ANLS function only in myelinated fibers, raising the subsequent question as to whether this cell-cell interaction is equally important for providing metabolic support to unmyelinated axons. Studies on mammalian peripheral nerves have suggested that Schwann cells operate ANLS to support large myelinated fibers akin to astrocytes in the CNS (A fibers), while glycogen does not benefit the slow-conducting, small diameter unmyelinated axons (C fibers) (Brown et al. 2012). However, lactate is as effective as glucose in supporting sciatic nerve function. We reassessed this phenomenon in corpus callosum (CC), which is a WM tract that consists of a mixture of myelinated and unmyelinated nerves (Fig. 4). Axon function in CC is more sensitive to any form of injury compared to optic nerve. For

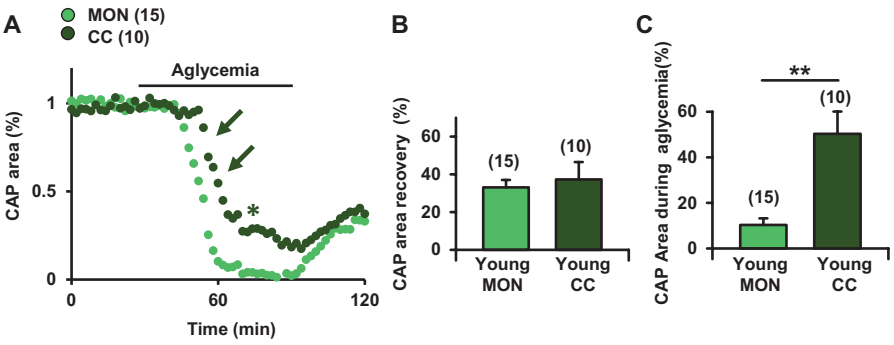

Fig. 4 CC axons are more resilient to removal of glucose than MONs. (**a**) Exposing MONs (light green) and CC (dark green) to aglycemia (0 mM glucose for 60 min) shows a more rapid (green arrows) loss of axon function in MONs compared to CC (**b**), although axon function recovers to similar extent after aglycemia. (**a**, **c**) Interestingly, greater number of axons remain functional during aglycemia in CC (dark green histogram in **c** and asteriks in (**a**)). $^{**}p < 0.01$

instance, exposure to transient OGD for 30 min results in irreversible loss of axon function in CC, while this duration of OGD in optic nerve is followed by ~75% recovery (Tekkok et al. 2007). To our surprise, axon function in CC displayed an unexpected level of resistance to removal of glucose (Fig. 4a, dark green line) compared to optic nerve axons (Fig. 4a, light green line). In addition to a notable delay in axon function loss during aglycemia (Fig. 4a, dark green arrows), a substantial portion of axons sustained their function during glucose deprivation (Fig. 4a, dark green asterisk and c). Moreover, axon function in optic nerve and CC showed identical recovery following 60 min of aglycemia (Fig. 4b). It is interesting to contemplate whether myelinated or unmyelinated axons exhibit this resilience to removal of glucose and whether the subsequent recovery is primarily due to rescue of function of myelinated or unmyelinated axons. Typical evoked compound action potentials (CAPs) in CC exhibit two peaks, one for myelinated axons with a shorter conduction time and a second one for unmyelinated axons with a slower conduction time (Fig. 4b). During aglycemia, the first peak showed an immediate sensitivity to glucose deprivation and was lost first, while the remaining CAP area was mainly due to the second peak. Though the second peak also declined in amplitude over time, a prominent portion was sustained, even at the end of 60 min of aglycemia. Likewise, the recovery was mainly attributable to the second peak. These observations can be interpreted as unmyelinated nerves being more resistant to aglycemia; however, it is also plausible that myelin is the immediate target, so therefore axons become demyelinated and contribute to the unmyelinated group. Further histological and ultrastructural studies are underway to conclusively answer these questions. It is important to note that there is a greater resistance of CC axons to anoxia compared to optic nerve, suggesting that unmyelinated axons and/or the smallest axons with the thinnest myelin sheaths are resistant to anoxia (Baltan 2006). Collectively, these results reveal that CNS WM is remarkably tolerant to glucose deprivation when lactate is available, although there is regional variability in their ability to function and survive this metabolic switch. To achieve optimal protection of the CNS in various neurological diseases, it is critical to understand the properties of regional energy metabolisms and injury mechanisms for successful therapeutic approaches.

4 ANLS Supports Axon Function in Aging WM

What happens to astrocyte structure and function with age? Recent studies performing RNA sequencing of astrocytes from different regions of the brain across the lifespan of the mouse identified age-related transcriptional changes that could contribute to cognitive decline (Clarke et al. 2018). Astrocytes have distinct region-dependent transcriptomal identities and they age in a region dependent manner. For instance, astrocytes in hippocampus and striatum undergo more dramatic transcriptional changes compared to cortical astrocytes. As a result, aged astrocytes in hippocampus or striatum express more reactive neuroinflammatory and ischemia-

specific genes together with an activated morphology. On the other hand, the top-down regulated genes in aging astrocytes are involved in mitochondrial function, energy production, and anti-oxidant defence, suggesting that aging astrocytes have impaired energy capacity and low anti-oxidant ability. Since astrocytes are the most abundant class of glial cells, their RNA sequencing profiles may provide clues to a better understanding of the aging brain and age-related changes specific for the blood-brain barrier, for metabolic regulation, and for cognitive function. Because neuron-specific gene expression patterns change relatively little in comparison to astrocytes (Soreq et al. 2017), major changes to astrocytes with age suggest these glial cells are involved in various age-related diseases such as dementia, Alzhemier's disease, Parkinson's disease, as well as various neurodegenerative diseases such as amyotrophic lateral sclerosis and multiple sclerosis. Drastic changes in aging astrocytes also raise the question as to whether these neurodegenerative diseases are neuronal or glial in origin and onset.

Astrocytes are arranged in a specific manner among myelinated axons. While maintaining 3-dimensional cell-to-cell interactions with other glia, astrocytes extend their processes perpendicular to axons and traverse them, interweaving in a bundle of myelinated structures in young WM (Fig. 5a, top row, green for GFAP (+) astrocytes and blue for Sytox (+) nuclei; "magnified" images show cell bodies chosen from "merged" images as indicated by white dotted squares). This architectural organization between astrocytes and axons is such that the tips of astrocyte processes reach in close proximity to nodes of Ranvier, presumably to monitor levels of function, energy demand, and maintenance of ionic homeostasis. This anatomical precision supports the concept of ANLS function. Interestingly, aging causes visible anatomical changes in WM astrocytes in addition to the above transcriptional changes. Aging astrocytes show a prominent increase in soma size and a remarkable increase in the diameter of their processes (Fig. 5a, bottom row, green for GFAP (+) astrocytes and blue for Sytox (+) nuclei; "magnified" images show cell bodies chosen from "merged" images as indicated by white dotted squares). Moreover, these thick processes change their vertical orientation to parallel and follow aging axons along their length. This orientation change of aging astrocyte processes seem to correlate with alterations in Aquaporin 4 (AQP-4) expression. AQP-4 is expressed in the end-feet of young astrocytes, but becomes dispersed throughout the cytoplasm of aging astrocytes (Baltan, data not shown). Loss of perivascular AQP-4 expression is accepted as a factor that renders the aging brain vulnerable to neurodegenerative conditions (Zeppenfeld et al. 2017). Therefore, it is plausible that these structural changes in size and alignment may also imbalance the nutritive function of astrocytes in WM. Indeed, when aging axons (obtained from 12-month-old mice) were deprived of ambient glucose (10 mM), they showed less recovery of CAP area compared to young axons (obtained from 8-week-old mice) following a period of aglycemia (60 min), suggesting that aging axons undergo a metabolic switch to become more dependent upon aerobic metabolism. Indeed, axons undergo complex and profound changes with aging, characterized by an increased diameter enwrapped in thicker myelin and populated with longer and thicker mitochondria that produce less ATP but increased reactive oxidative stress markers (Stahon et al. 2016).

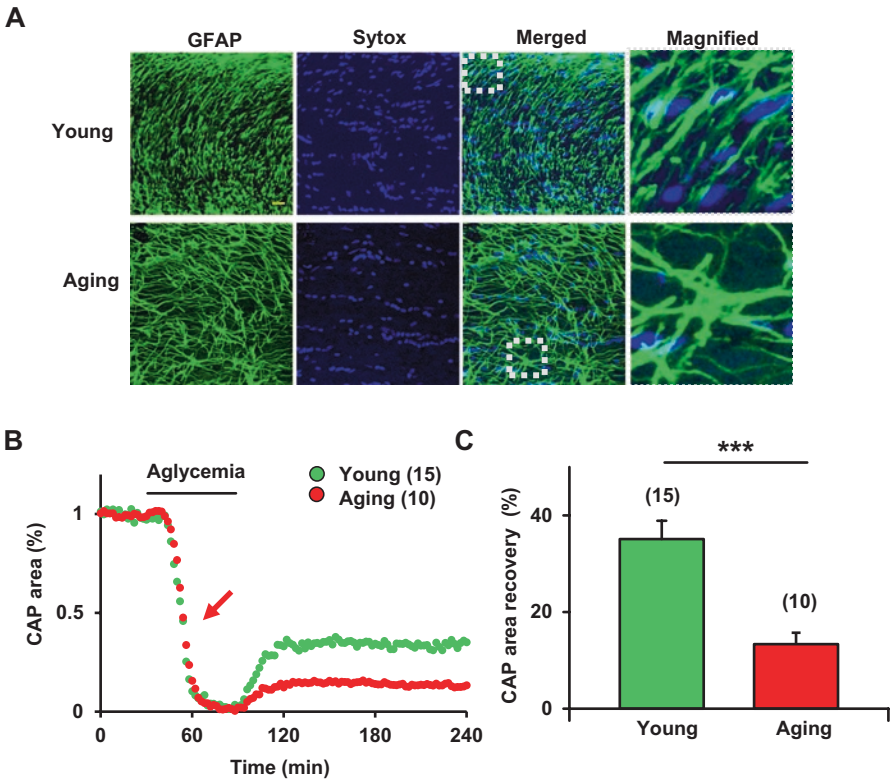

Fig. 5 Aging causes increase in astrocyte size and increased vulnerability to aglycemia in axon function. (**a**) GFAP (+) astrocytes (green) increase in size and display a horizontal orientation with age, while their nuclei remain the same size (Sytox (+) blue). Note the magnified images for compasion between young and aging astrocytes. (**b**, **c**) Aging axons (red line and red histogram) become more vulnerble to removal of glucose compared to young axons (green line and green histograms). $^{***}p < 0.001$

Mitochondrial dysfunction in aging axons may be the reason why aging axons become more dependent upon glucose (Stahon et al. 2016; Baltan 2014). This metabolic switch may contribute to the increased susceptibility of aging WM to aglycemia. It also suggests that lactate may not be the preferred energy substrate for aging axons, or that lactate delivery to axons may be compromised. Either a decline in MCT2 expression levels by aging axons or reduced availability of lactate provided by aging astrocytes are likely to be the main contributors to dysfunctional ANLS in aging WM. To directly test whether aging axons efficiently take up and use lactate as an alternative energy source, lactate was added (20 mM – carbon equal of glucose) during aglycemia (0 mM). This approach completely disguises the effects of aglycemia in young WM (Fig. 6a, b, green trace and histogram) so that lactate equally maintains axon function (Fig. 6a, b, blue trace and histogram). Surprisingly, lactate equally sustained aging axon function when glucose was

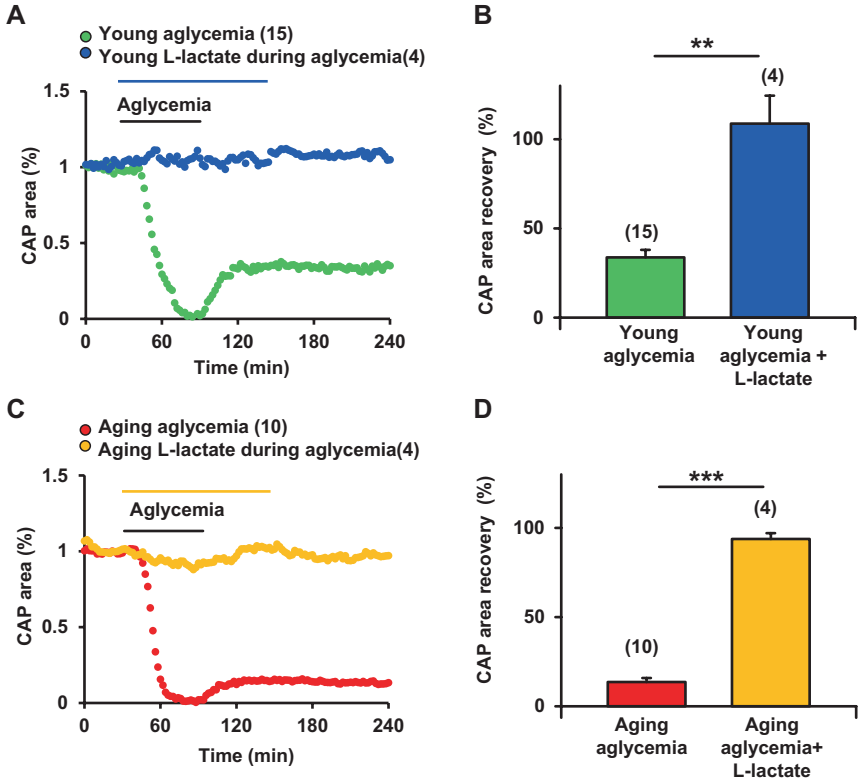

Fig. 6 Aging axons use lactate as an alternative energy substrate as efficiently as young axons. (**a**, **b**) Replacing glucose (10 mM) with lactate (20 mM, carbon equivalent) fully sustains young axon function during aglycemia (blue line and blue histogram) compared to aglycemia (green line, green histogram). (**c, d**) Aging axons use lactate (yellow line, yellow histogram) when glucose is not available to fully maintain their function as opposed to aglucose (red line and red histogram). $^{**}p < 0.01$, $^{***}p < 0.001$

removed (Fig. 6c, d, yellow trace and histogram compared to red aglycemia trace and histogram), confirming that aging axons can use lactate interchangeably with glucose to maintain full functionality. This ability of aging axons indicate that MCT2 expression and function are maintained in aging axons. Furthermore, when glucose is removed, the rates of loss of young and aging axon function remain identical (Fig. 5b, green trace for young and red trace for aging axon function; red arrow indicates the identical rate of loss of axon function), further verifying that aging axons maintain their molecular machinery relevant to ANLS and can use lactate despite major changes in their mitochondrial structure and function.

Notably in this approach, lactate is provided externally, replacing glucose to verify axonal uptake of lactate is functional. Whether aging astrocytes take up glucose efficiently, store it as glycogen, and convert it to lactate to sustain aging axons is another question. A practical way of testing this ability of astrocytes is to incubate

MONs in 30 mM glucose to upregulate astrocyte glycogen levels and then subject MONs to aglycemia. In young MONs, this experimental design delays loss of axon function during aglycemia and prevents loss of axon function at the end of aglycemia (60 min) due to increased availability of lactate, followed by an improved recovery after aglycemia (Brown et al. 2003; Baltan et al. 2008; Tekkok et al. 2005). A similar approach using aging MONs showed an identical delay in the loss of axon function during aglycemia, preservation of axon function at the end of aglycemia, and improved recovery of axon function following aglycemia. Indeed, aging axons incubated in high glucose before aglycemia recovered better compared to young axons, suggesting that aging astrocytes are able to take up glucose, regulate their glycogen stores, and deliver lactate to axons as efficiently as young astrocytes. In this aspect, aging does not impair the beneficial role of astrocytes or ANLS despite prominent anatomical and transcriptomic alterations. On the other hand, it is plausible that astrocytes alter their shape, size, and orientation to continue to efficiently support ANLS in aging WM. However, a recent study indicates a shift of glycogen metabolism enzymes in their localization from astrocytes to neurons in hippocampus, suggesting that neurons become independent of astrocytes and ANLS during aging (Drulis-Fajdasz et al. 2018). Therefore, aging breaks down the linkage between glycogen and the neural plasticity involved in learning and memory, and aging is associated with cognitive disturbances that are partly attributable to brain metabolic deficiency (Korol and Gold 1998; Abdelhafiz et al. 2015). Likewise, aging astrocytes *in vitro* demonstrate decreased glucose uptake and glutamine synthase activity (Souza et al. 2015). It is interesting that this crosstalk between astrocytes and axons remain undisrupted in aging WM. These findings emphasize the differences in cell-cell interactions between gray matter and WM in young and aging brain, and increases our understanding of how mechanisms of interaction between glia and neurons vary between different brain regions.

5 Is ANLS Unique to Astrocytes?

Oligodendrocytes display metabolic diversity similar to neurons and can utilize both glucose and lactate to extract energy and to synthesize lipids to deposit as myelin along axons (Sanchez-Abarca et al. 2001). Oligodendrocytes contact multiple axons and oligodendroglial cytoplasm extends into multiple myelinating shafts that connects the periaxonal space, establishing a network of access to axons. During the process of myelination, each oligodendrocyte produces large amounts of myelin, making them metabolically demanding during development. Once myelination is complete, preserving the intricate composition of myelin remains as a metabolic challenge due to the slow and challenging process of myelin delivery. This morphological organization is now recognized as the basis of a metabolic collaboration between oligodendrocytes and axons that is more intricate than our current understanding (Funfschilling et al. 2012). Oligodendrocytes are charged with regulating the efficiency of signal propagation through specific myelination of

axons as the activity and metabolic needs of the CNS change. It is proposed that glucose supply at the nodes of Ranvier may be adequate to maintain mitochondrial ATP production under steady-state conditions without any glial cell support. On the other hand, with increased activity in longer and larger fibers, both oligodendrocytes and astrocytes supply axons with pyruvate/lactate as the need arises (Lee et al. 2012; Funfschilling et al. 2012; Nave 2010a, b). This supports the suggestion that the lactate shuttle is not restricted to astrocytes and axons, but rather that another specialized lactate shuttle exists between oligodendrocytes and their myelinated axons (Amaral et al. 2013). Recent genetic studies propose that oligodendrocytes sustain axonal energy metabolism by performing aerobic glycolysis and delivering lactate to the myelinated compartments via MCTs (Funfschilling et al. 2012). This metabolic support depends upon non-compacted regions of myelin that connect oligodendrocyte soma to the periaxonal cytosolic space under the myelin sheath. These tube-like cytosolic compartments called myelinic channels run parallel to axons, facing the axonal internode, and assist metabolic exchange via MCTs. Alterations in metabolic support by oligodendrocytes is now accepted to underlie various diseases, ranging from amyotrophic lateral sclerosis to psychiatric diseases (Nave and Ehrenreich 2014).

6 Concluding Remarks

Astrocytes in WM store glycogen that is converted to lactate when glucose concentration is low or when there is increased energy demand under normoglycemic conditions such as increased activity, confirming that CNS WM glycogen plays a crucial role as an energy reserve and buffer store (Brown et al. 2003). Therefore, astrocyte glycogen content governs neuronal and axonal function (Swanson and Choi 1993) and when glycogen content is depleted during prevailing hypoglycemia, brain function fails and suffers irreversible injury (Auer 1986; Frier and Fisher 1999). Glycogen content present in the tissue at the onset of aglycemia determines the duration of axon function (Brown et al. 2003), a relationship that may have profound implications for patients that suffer from type-1 diabetes mellitus and experience frequent silent WM infarcts leading to declining cognitive function. Similarly, axon function at the presynaptic input dictates synaptic transmission and synaptic plasticity underlying learning and memory-related events that decline with age and in neurodegenerative diseases. Therefore, all evidence points toward astrocyte-derived lactate as a crucial substrate and this metabolic coupling is validated in both gray matter and WM portions of the brain. Astrocytes transporting glucose from the vascular side via end feet to neuronal and glial cells also highlights an important mission of the neurovascular unit (NVU). Oligodendrocytes, microglia, oligodendrocytes and vascular cells consisting of endothelial cells, smooth muscle cells, pericytes and extracellular matrix (ECM), astrocytes form a complex intricate arrangement. The dynamic interactions among components of the NVU affect the clinical outcome of numerous central nervous system (CNS) diseases. For instance, in addition to its importance in the field of stroke, NVU dysfunction is now accepted

as the cause or the result of other CNS diseases including Alzheimer's disease, vascular dementia, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Arai et al. 2011). Furthermore, the diverse cells comprising the NVU are heterogeneous in developmental origin, physiological properties and function while they respond to injury, maintaining their interaction with each other. Therefore ANLS is an important part of the cell-cell signaling mechanism that contributes to multi-complex functions of NVU under physiological and pathological conditions.

Furthermore, a recent concept that oligodendrocytes support axon function by delivering lactate proposes another metabolic coupling in WM, suggesting that astrocytes are not alone in this nurturing role. The circumstances that trigger this oligodendrocyte-axon lactate shuttle system and its signaling mechanisms are of great interest, particularly because oligodendrocytes aim to cooperate with NVU to repair WM after injury such that injured axons either undergo degeneration or become resistant (Hamanaka et al. 2018). Therefore, the impaired oligodendrocyte-axon lactate shuttle is proposed to contribute to chronic demyelinating and psychiatric diseases (Nave and Ehrenreich 2014). Future studies are expected to unravel the details of oligodendrocyte-axon lactate metabolic coupling to establish that all WM components metabolically cooperate and that lactate may be the universal metabolite to sustain CNS function.

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Dysregulation of Glycogen Metabolism with Concomitant Spatial Memory Dysfunction in Type 2 Diabetes: Potential Beneficial Effects of Chronic Exercise

Mariko Soya, Subrina Jesmin, Takeru Shima, Takashi Matsui, and Hideaki Soya

Abstract Cognitive dysfunction is one of the comorbidities of diabetes mellitus, but hippocampus-dependent learning and memory, a component of cognitive function, shows particular decline in type 2 diabetes, suggesting an increased risk for dementia and Alzheimer's disease. Cognitive function is related to dysregulated glucose metabolism, which is the typical cause of type 2 diabetes; however, hippocampal glycogen and its metabolite lactate are also crucial for hippocampus-dependent memory function. Type 2 diabetes induced hippocampus-dependent learning and memory dysfunction can be improved by chronic exercise and this improvement may possibly mediate through an adaptation of the astrocyte-neuron lactate shuttle (ANLS). This chapter focuses on the dysregulation of hippocampal glycometabolism in type 2 diabetes examining both existing evidence as well as the potential underlying pathophysiological mechanism responsible for memory dysfunction in type 2 diabetes, and showing for the first time that chronic exercise could be an effective therapy for type-2-diabetes-induced hippocampal memory decline.

Keywords Type 2 diabetes mellitus · Hippocampus-dependent learning and memory · Hippocampal glycometabolism · Monocarboxylate transporter 2

M. Soya · S. Jesmin · T. Matsui · H. Soya (✉)
Sport Neuroscience Division, Advanced Research Initiative for Human High Performance (ARIHHP), Faculty of Health and Sport Sciences, University of Tsukuba, Tsukuba, Japan
e-mail: soya.hideaki.gt@u.tsukuba.ac.jp

T. Shima
Laboratory of Exercise Biochemistry and Neuroendocrinology, Faculty of Health and Sport Sciences, University of Tsukuba, Tsukuba, Japan

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Abbreviations

ANLS	Astrocyte-neuron lactate shuttle
BDNF	Brain-derived neurotrophic factor
DAB	1,4-Dideoxy-1,4-imino-$_{D}$-arabinitol
GLUT	Glucose transporter
GSK-3β	Phosphorylated-GS kinase-3β
LETO	Long-Evans Tokushima Otsuka
LTP	Long-term potentiation
MCT	Monocarboxylate transporters
MHPG	NA metabolites
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
OLETF	Otsuka Long-Evans Tokushima Fatty
pCofilin	Phosphorylated-Cofilin
pCREB	Phosphorylated-cAMP-response-element-binding protein
PGK	Phosphoglycerate kinase
PTG	Protein targeting to glycogen
STZ	Streptozotocin
VIP	Vasoactive intestinal peptide
ZDF	Zucker diabetic fatty
ZL	Zucker lean
ZO	Zucker obese

1 Introduction

Diabetes mellitus is a metabolic disorder that is associated with a number of serious complications. The International Diabetes Federation reported 425 million persons as having diabetes mellitus in 2018, and this number is estimated to increase to 629 million by 2045. The World Health Organization (WHO) ranked diabetes mellitus among the top 10 causes of the 56.9 million deaths worldwide in 2016, indicating that diabetes mellitus is a common and serious disorder worldwide warranting global attention.

A complication of diabetes mellitus that has received considerable attention in recent years is hippocampal cognitive dysfunction, which is a recognized risk factor for dementia and Alzheimer disease (Heijer et al. 2003; Cukierman et al. 2005; Mccrimmon et al. 2012; Umegaki et al. 2013). Learning and memory deficits in cognitive components are more profound in patients with type 2 diabetes than in those with type 1 diabetes (Biessels et al. 2006; Biessels and Despa 2018; Mccrimmon et al. 2012), and this could be associated with hippocampal atrophy (Gold et al. 2007; Mccrimmon et al. 2012). The American College of Sports Medicine (ACSM) and the American Diabetes Association (ADA), based on a number of studies, recommend regular physical activity for improvement of diabetes

and indicate that chronic exercise has health benefits that go beyond blood glucose control, weight control, and a reduction of the risk of well-known complications of type 2 diabetes although its effects are yet to be investigated for hippocampal memory dysfunction (Mu et al. 2001; Holloszy 2005; O'Gorman et al. 2006; Sriwijitkamol et al. 2007; Colberg et al. 2010; Lee et al. 2011; Jenkins et al. 2012).

At present, no consensus has been reached regarding the underlying mechanism of memory dysfunction in type 2 diabetes, including hippocampus-based memory decline, but dysregulation of glucose utilization in the brain (Sickmann et al. 2010), brain-derived neurotrophic factor (BDNF) (Stranahan et al. 2009), neuroinflammation (Whitmer 2007), and oxidative stress have all been associated with memory dysfunction in patients with type 2 diabetes (Whitmer 2007; Stranahan 2015). Interestingly, one metabolic adaptation shown in type 2 diabetes is elevated glycogen deposition in the heart (Bhattacharjee et al. 2006; Shearer et al. 2011). This suggests that a metabolic adaptation involving glycogen might also occur in the hippocampus, a crucial site for memory formation, and lead to memory dysfunction in type 2 diabetes as hippocampal glycogen-derived lactate in astrocytes and its transport into neurons plays a crucial role in hippocampus-dependent memory formation (Newman et al. 2011; Suzuki et al. 2011).

Chronic exercise, which positively affects hippocampus-dependent memory function in animals (van Praag et al. 1999; Brown et al. 2003; Liu et al. 2009; Creer et al. 2010), also increases hippocampal glycogen levels in normal rats (Matsui et al. 2012) and lactate transporter levels (monocarboxylate transporters; MCT) in type 1 diabetic rats (Aveseh et al. 2014), suggesting that chronic exercise could potentially ameliorate type-2-diabetes–related memory dysfunction through an adaptation of hippocampal glycometabolism which means glycogen metabolism (metabolism of sugars and other carbohydrate). In this chapter, we summarize the evidence for an association between brain glycometabolism dysregulation and cognitive decline in type 2 diabetes and the effects of chronic exercise on hippocampus-dependent memory dysfunction.

2 Cognitive Decline and Dysregulated Hippocampal Glycometabolism in Type 2 Diabetes

There are two types of diabetes mellitus: type 1 tends to develop in childhood and is characterized by a lack of insulin due to destruction of the βcells of the islet of Langerhans in the pancreas. By contrast, obesity and lack of physical activity are the crucial risk factors for type 2 diabetes, a disease condition with reduced insulin sensitivity and insulin resistance. Both type 1 and type 2 diabetes are associated with high blood glucose levels (hyperglycemia), which is the cause of numerous complications including cognitive dysfunction (Mccrimmon et al. 2012). Furthermore, insulin resistance is also a risk factor for cognitive dysfunction (Geroldi et al. 2005). In human studies, neuroimaging provides evidence that

diabetes affects the brain, as adverse effects are evident in the cortical and subcortical regions, such as the medial temporal lobe (hippocampus and amygdala) (Heijer et al. 2003; Manschot et al. 2006), which are related to cognitive impairment associated with attention, executive function, information processing speed, and memory in patients with type 2 diabetes (Manschot et al. 2006). In these patients, the hippocampus may be the first site affected by type 2 diabetes (Gold et al. 2007). The neuropathological changes seen in the hippocampus with type 2 diabetes are also characteristic of Alzheimer's disease, but the same changes are not seen in patients diagnosed with dementia.

Conversely, in rodents, cognitive function tested using the T-maze and Morris water maze shows a decline in streptozotocin (STZ)-induced type 1 diabetic mice (Flood et al. 1990; Biessels et al. 1996; Kamal et al. 1999). In terms of alterations related to learning and memory at the cellular level, N-methyl-d-aspartate (NMDA)-dependent long-term potentiation (LTP) in the CA1 and NMDA-independent LTP in the CA3 fields of the hippocampus are also impaired in STZ-induced diabetic rats, and this impairment is correlated with the severity of hyperglycemia (Biessels et al. 1996; Chabot et al. 1997; Tekkök and Krnjevic 1999). Based on this research, type 2 diabetes could have a similar type of impact on cognitive function, particularly through its effects on the hippocampus. Much information is available regarding the effects of glucose metabolism on cognitive function, but the role of glycometabolism on cognitive deficits in type 2 diabetes is not yet clearly understood. Recent findings suggest that hippocampal glycogen plays an important role in memory processing (Newman et al. 2011; Suzuki et al. 2011), and that hippocampal glycometabolism could be altered/dysregulated in type 2 diabetes (Fig. 1).

2.1 Hippocampal Glycometabolism and Cognitive Function

The energy requirements of the brain are high, and brain energy metabolism changes depending on neuronal activity. Pioneering work on brain energy metabolism using the 2-deoxyglucose (2-DG) autoradiographic technique demonstrated that blood glucose is metabolized in the brain by neuronal activity, suggesting a metabolic coupling between neuronal activity and blood vessels (Newman et al. 2011). Furthermore, activation of glutamatergic neurons causes the release of glutamate into the intercellular space, and glutamate is a trigger that induces glycolysis in astrocytes (Magistretti and Pellerin 1999). The glutamate released from glutamatergic synapses is co-transported with Na^+ into astrocytes, which express the glutamate transporters GLAST and GLT1. This co-transport activates the astrocytic Na^+/K^+-ATPase, and glycolysis is subsequently stimulated in the astrocytes through activation of phosphoglycerate kinase (PGK) (Magistretti and Pellerin 1999). The lactate produced through glycolysis in the astrocytes is transported to the neurons via MCT (astrocyte: MCT1 and MCT4; neuron: MCT2) for use as an energy substrate, and this metabolic coupling between these two cell types has led to the proposal of a hypothetical 'astrocyte-neuron lactate shuttle' (ANLS) (Pellerin et al. 1998). In the

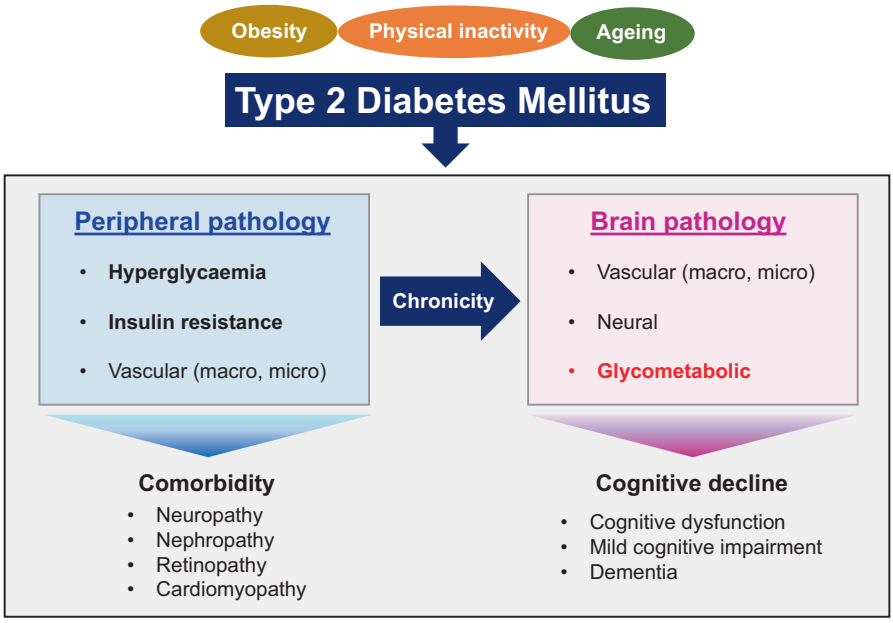

Fig. 1 Proposed pathophysiological mechanisms in type 2 diabetes. In contrast to type 1 diabetes, which is caused by insulin deficit due to the immune-mediated βcell destruction of pancreatic cells in the islets of Langerhans, type 2 diabetes occurs through insulin resistance and reduced insulin sensitivity that presents as hyperglycemia and is induced by genetic susceptibility, several environmental, lifestyle factors, such as obesity, physical inactivity, and ageing, leading to various comorbidities. The chronicity of these symptoms on the peripheral tissues affects the central nervous system and causes cognitive decline, which has been recently recognized as one of the comorbidities of diabetes. Brain glycometabolism is associated with cognitive function, particularly in the hippocampus, so dysregulated glycometabolism could be the underlying mechanism leading to the dysfunction of memory in type 2 diabetes

case of high or long-lasting neuronal activity, the glucose supply from the blood vessels to neurons becomes insufficient, thus, the contribution of glycogen stored exclusively in astrocytes is important for sustaining neuronal activity and maintaining a high rate of glycolysis (Swanson et al. 1992).

Astrocytic glycogen degradation (via glycogenolysis) is stimulated by noradrenaline (NA), vasoactive intestinal peptide (VIP), adenosine, and serotonin resulting in the release of lactate that is transported to neurons in the same manner as lactate derived from glycolysis (Magistretti et al. 1981; Hof et al. 1988; Sorg and Magistretti 1991, 1992; Dringen et al. 1993; Choi et al. 2012; Matsui et al. 2015). Interestingly, the tight coupling between neuronal activity and energy metabolism suggests that 'metabolic plasticity' underlies neuronal plasticity (Magistretti 2006; Choi et al. 2012). Indeed, metabolic adaptation occurs via glycogen metabolic processes under *in vitro* conditions. As mentioned above, neurotransmitters (namely NA, VIP, and adenosine) and neuromodulators induce glycogenolysis in a short time (seconds to minutes); however, NA has long-term effect in that it can stimulate a transcriptionally

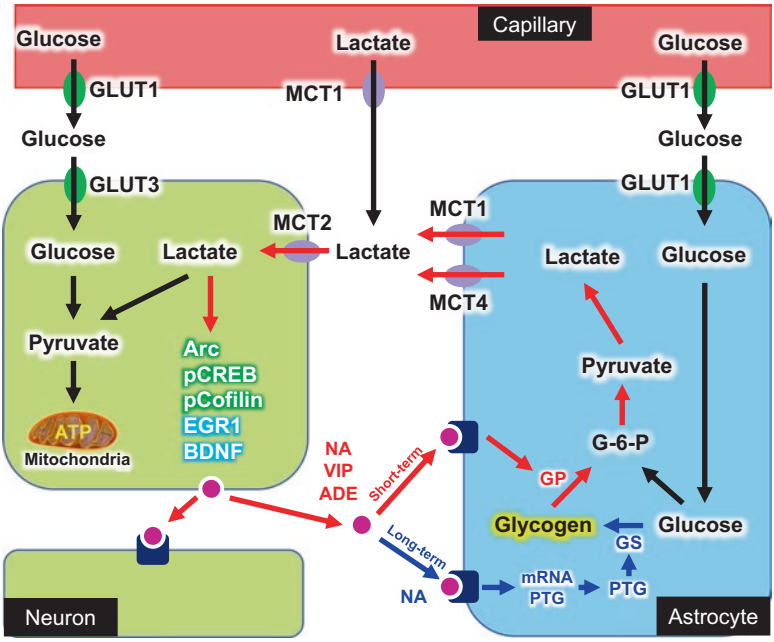

Fig. 2 Simplified illustration of the astrocyte-neuron lactate shuttle: Contribution of glycogen-derived lactate to cognitive function. During high or long-lasting neuronal activity, brain glycogen-derived lactate becomes an important energy source to satisfy neuronal energy demand. Glycogen degradation is induced by noradrenaline (NA), vasoactive intestinal peptide (VIP), and adenosine (ADE), resulting in lactate production. Particularly in the hippocampus, lactate is transported via monocarboxylate transporters (MCT) and serves both as an energy substrate and in memory processing through induction of protein expression related to neural plasticity, such as Arc, phosphorylated-cAMP-response-element-binding protein (pCREB), and phosphorylated-Cofilin (pCofilin). Early growth response protein 1 (EGR1) and brain-derived neurotrophic factor (BDNF) are also induced by lactate in *in vitro* experiments. These lactate effects are suppressed by blocking MCT2 or inhibiting glycogen degradation, suggesting that glycogen-derived lactate is crucial for memory processing and neuronal plasticity. NA induces both glycogenolysis as a short-term effect and glycogen synthesis as a long-term effect by inducing protein targeting to glycogen (PTG) and glycogen synthase (GS)

regulated action that results in glycogen synthesis (Allaman et al. 2000, 2003). The long-term effects of NA include NA-triggered cyclic-AMP production, which increases the expression of the transcriptional factor CCAAT/enhancer-binding protein (C/EBP), glycogen synthase, and protein targeting to glycogen (PTG) (Cardinaux and Magistretti 1996; Ruchti et al. 2016) (Fig. 2).

Recent studies have demonstrated that L-lactate is used as an energy substrate for neurons (Bélanger et al. 2011), and it also acts as a signaling molecule to induce the expression of neural plasticity related genes (i.e., activity-regulated cytoskeleton-associated protein [Arc], c-Fos, and Zif268) in cultured neurons (Yang et al. 2014). These are immediate early genes (IEGs), which are induced during both long-term memory and long-term plasticity (Alberini 2009; Bramham et al. 2010; Caroni et al.

2012). These effects of L-lactate are mediated by NMDA receptor activity in neurons and its downstream signaling Erk1/2 cascade, which means that L-lactate potentiates glutamatergic neuronal currents by ensuring an increase in intracellular calcium through NMDA receptor activation and changes in the cellular redox state, and that it acts as a neuromodulator to induce plasticity-related gene expression (Yang et al. 2014). Although most of these observations about ANLS were elucidated *in vitro* experiments using cortical cultured neurons or astrocytes, the most important current work on lactate effects associated with learning and memory in the hippocampus has shown that lactate derived from astrocytes is essential for long-term memory formation (Suzuki et al. 2011). Hippocampus-dependent long-term memory formation (measured using an inhibitory avoidance task) and LTP were inhibited when lactate production from astrocytes was blocked using DAB (1,4-dideoxy-1,4-imino-D-arabinitol). By contrast, co-injection of DAB and L-lactate rescued inhibited memory consolidation and LTP (Suzuki et al. 2011).

The underlying mechanisms by which glycogen-derived lactate induces memory formation could be associated with the induction of Arc, phosphorylated-cAMP-response-element-binding protein (pCREB), and phosphorylated-Cofilin (pCofilin), which are related to synaptic plasticity (Suzuki et al. 2011) (Fig. 2). A similar observation relating hippocampal glycogen to working memory has also been made with another behavioral paradigm (closed plus maze) (Newman et al. 2011). Little *in vivo* evidence supports a role for brain glycogen in memory function, but the blockade of glycogenolysis with DAB impaired a discrimination avoidance learning task in chicks (Gibbs et al. 2006). Similarly, the lack of brain glycogen generated by knockout of glycogen synthase specifically (GYS1) in the nervous system impaired memory in an operant conditioning task and LTP (Boury-Jamot et al. 2015).

2.2 Dysregulated Hippocampal Glycometabolism and Cognitive Decline in Type 2 Diabetes

A number of studies have shown that the central nervous system is affected by diabetes, and that diabetes-related cognitive decline is an independent risk factor for dementia and Alzheimer's disease (Biessels et al. 2006; Biessels and Despa 2018; Mccrimmon et al. 2012). These diseases are accompanied by morphological changes and metabolic alteration/dysregulation in the hippocampus and cortex, which are regions associated with cognitive function (Heijer et al. 2003; Sickmann et al. 2012; Biessels and Despa 2018). The hippocampus, in particular, shows atrophy in the initial stage of these neuro-degenerative disorders (Heijer et al. 2003; Gold et al. 2007). The energy supply to the brain depends strongly on glucose supply from blood vessels; thus, impairment of glucose metabolism by insulin deficiency, as commonly occurs in diabetes, could be one of the risk factors for impaired cognitive function. Moreover, hippocampal glycogen-derived lactate metabolism associated with cognitive function could also be affected by diabetes. However, little research

has examined the relationship between hippocampal glycometabolism and cognitive decline/dysfunction in diabetes.

Previous studies examining the glycogen levels in the hippocampus and/or cortex in diabetic rats have demonstrated that STZ-induced type 1 diabetic rats had increases in glycogen levels in the cortex depending on the STZ treatment duration (Plaschke and Hoyer 1993). Conversely, in type 2 diabetes models, Zucker obese (ZO) rats had hyper-levels of hippocampal and cortical glycogen than did their counterpart Zucker lean (ZL) genetic control rats, whereas Zucker diabetic fatty (ZDF) rats had lower levels of cortical glycogen compared with control ZDF lean rats (Sickmann et al. 2010). One possible explanation for the conflicting results between these two studies is that the methodology used for brain tissue preparation was neither optimal nor standard/validated for glycogen measurement (Swanson et al. 1989; Dringen et al. 1993). Brain glycogenolysis degraded rapidly by ischemia; therefore, microwave irradiation can inactivate glycogenolysis enzymes needed for the accurate measurement of brain glycogen (Kong et al. 2002).

Our laboratory has endeavored to elucidate the glycometabolism pattern in type 2 diabetic rats by measuring glycogen and MCT levels in the hippocampus using microwave irradiation, with consideration to the issues mentioned above. For three reasons we used Otsuka Long-Evans Tokushima Fatty (OLETF) rats as our type 2 diabetes model: (a) OLETF rats exhibit hyperphagia; (b) they have a dysregulated sympathoadrenal response and show a decline in executive function (Suge et al. 2012); and (c) the onset of diabetes takes time, in agreement with human type 2 diabetes (Kawano et al. 1992). Type 2 diabetic rats exhibit hyper-glycogen levels and diminished MCT2 protein levels in the hippocampus when compared with their counterpart genetic control rats Long-Evans Tokushima Otsuka (LETO), and a negative relationship exists between the two components of the glycometabolism system in neurons (Shima et al. 2016a) (Fig. 3). Notably, the negative correlation exhibited in type 2 diabetic rats has been observed only in the hippocampus, and not in the hypothalamus and the cortex, even though these regions exhibit changes similar to those seen in the hippocampus, namely, glycogen (hypothalamus and cortex) and MCT2 (hypothalamus) increases, implying that the hippocampus may be the first region affected by dysregulation of glycometabolism induced by type 2 diabetes (Shima et al. 2016a). The significance of hyper-hippocampal glycogen in type 2 diabetic rats may indicate metabolic compensation for the decreased lactate utilization via MCT2 and this consequence may have linkage with ANSL although concrete evidence demands. Indeed, the same phenomenon was reported in the hearts of patients with type 2 diabetes (Bhattacharjee et al. 2006; Shearer et al. 2011). Notably, MCT1 and MCT4 are expressed in astrocytes and have a similar transport roles in astrocytes in all three brain regions (hippocampus, hypothalamus and cortex) as stated above (Shima et al. 2016a). Thus, collectively the findings observed in the study by Shima et al. at the progressive stage of type 2 diabetic hippocampus in concern to glycometabolism and the associated molecular mechanism warrant future studies to be conducted at pre diabetes or at the early stage of diabetes for the advancement of clinical improvement of diabetic complications.

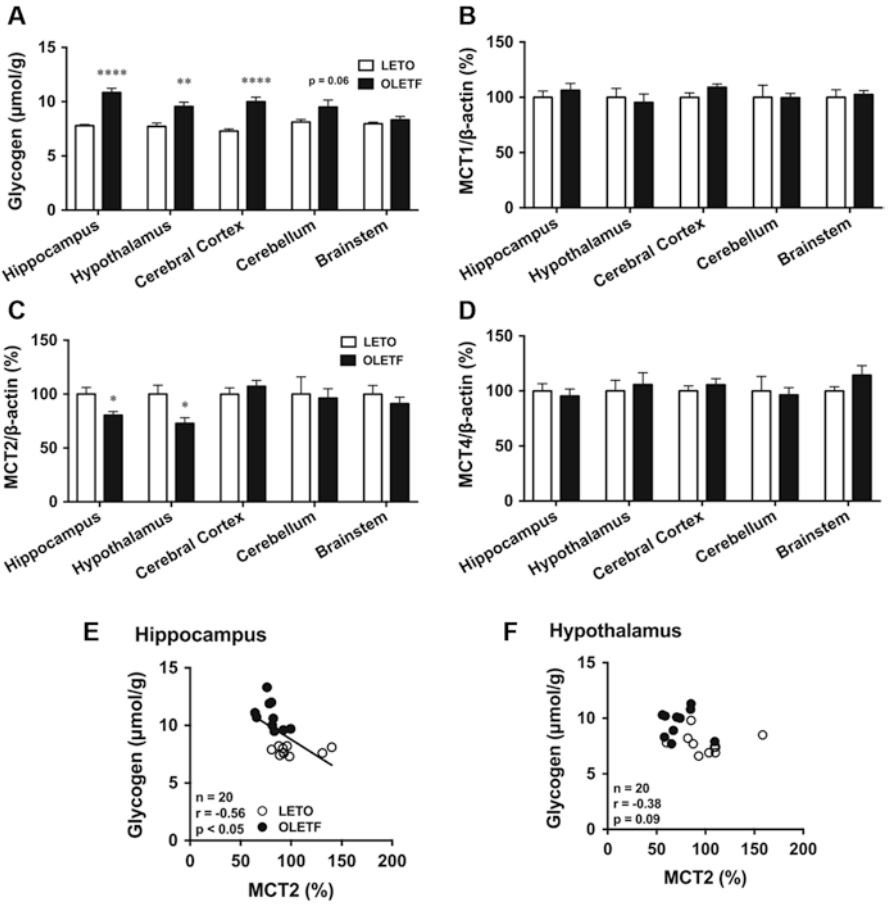

Fig. 3 Dysregulated hippocampal glycometabolism in a rat model of type 2 diabetes. Glycogen levels in the hippocampus, hypothalamus, and cerebellum are higher in the OLETF type 2 diabetes rat model than in control LETO rats. Conversely, MCT2 expression in the hippocampus and hypothalamus is lower in OLETF rats than in LETO rats, even though MCT1 and MCT4 levels show no significant differences between the LETO and OLETF rats. However, a significant correlation exists between MCT2 and glycogen levels, but only in the hippocampus, suggesting that memory dysfunction in OLETF rats could be caused by dysregulated hippocampal glycometabolism. (**a**) Glycogen levels in the hippocampus, cortex, hypothalamus, cerebellum, and brainstem, (**b**) MCT1 levels, (**c**) MCT2 levels, (**d**) MCT4 levels, (**e**) Correlation between MCT2 and glycogen levels in the hippocampus, (**f**) Correlation between MCT2 and glycogen levels in the hypothalamus. (Referenced by Shima et al., *J Physiol Sci,* 2016)

3 Chronic Exercise Effects on Dysregulated Hippocampal Glycometabolism and Cognitive Decline in Type 2 Diabetes

Chronic exercise can normalize blood glucose level control by enhancing glucose uptake in the peripheral tissues in an insulin-independent manner and is an effective therapy for type 2 diabetes complications such as neuropathy (Winder et al. 1987; Mu et al. 2001; Balducci et al. 2006). Cognitive decline is one of the complications of type 2 diabetes (Gispen and Biessels 2000; Whitmer 2007), with hippocampal dysfunction being a particularly serious disability associated with the development of dementia and Alzheimer's disease (Pouwer et al. 2003; Witting et al. 2006; Ahtiluoto et al. 2010). Since a number of studies have demonstrated an improvement of cognitive function, such as hippocampus-dependent memory (van Praag et al. 1999; Liu et al. 2009; Creer et al. 2010; Bolz et al. 2015), with chronic exercise, this type of exercise could have the potential to affect hippocampal dysfunction in type 2 diabetes in a beneficial manner. Recently, our laboratory identified a chronic exercise regimen effective for hippocampal dysfunction in type 2 diabetes (Shima et al. 2016b); thus, the effects of acute and chronic exercise on hippocampal glycometabolism in normal and type 2 diabetic rats will be reviewed next.

3.1 Hippocampal Glycometabolism During Acute Exercise in Normal Rats

Exercise requires mobilization of every system of the body, including muscles and the brain (Vissing et al. 1996; Secher et al. 2008). Glucose molecules stored as glycogen play a crucial role in the immediate energy supply needed by exercising muscles to maintain muscular contraction because depletion of muscle glycogen during exercise causes a decline in endurance performance (Secher et al. 2008). Muscle glycogen decreases in an activity-dependent manner (Gollnick et al. 1974), and depletion of muscle and liver glycogen during hypoglycemia occurs when rats are subjected to prolonged exhaustive exercise (Winder et al. 1987). Exercise also affects the brain and increases neuronal activity (Saito and Soya 2004; Ohiwa et al. 2006, 2007, Soya et al. 2007a, b); furthermore, glucose is utilized as an energy source by neurons during exercise (Vissing et al. 1996). However, whether brain glycogen, and particularly hippocampal glycogen, decreases in a similar manner to that seen for muscle glycogen following prolonged exhaustive exercise remains an unanswered question.

In one previous experiment, glycogen levels in muscles and the liver were depleted after exhaustive exercise (treadmill running), which induced hypoglycemia (Matsui et al. 2011). Hippocampal glycogen also decreased with acute moderate exhaustive exercise (20 m/min, until exhaustion) (Matsui et al. 2011). Furthermore, levels of methoxyhydroxyphenylglycol (MHPG) and

5-Hydroxyindoleacetic acid (5-HIAA), which are the respective metabolites of NA and serotonin (5-TH), were increased in cerebral cortex after exercise, and their levels were negatively correlated with a decrease in cortical glycogen, suggesting that NA and 5-HT could be part of an underlying mechanism of exercise-induced brain glycogen degradation (Matsui et al. 2011).

Based on these results, next we will discuss exercise-induced hippocampal glycogen dynamics, especially in the recovery phase of glycometabolism. Muscle glycogen decreases with exhaustive exercise and then returns to above basal levels (supercompensation) 24 h after exercise (Bergström and Hultman 1966). Surprisingly, brain glycogen, including hippocampal glycogen, also exhibits supercompensation 6 h after exhaustive exercise (Matsui et al. 2012); furthermore, the rate of brain glycogen supercompensation depends on the rate of glycogen decrease during exercise, similar to the situation seen in muscles (Gaesser and Brooks 1980) and consistent with studies demonstrating brain glycogen supercompensation at 4–7 h after insulin-induced hypoglycaemia (Choi et al. 2003; Canada et al. 2011).

An adaptation to chronic exercise occurs, as indicated by glycogen supercompensation following exhaustive exercise (James and Kraegen 1984), as 4 weeks of chronic moderate exercise resulted in an increase of basal hippocampal and cortical glycogen levels in normal rats (Matsui et al. 2012). The underlying mechanisms of hippocampal glycogen synthesis is not clear, but exercise is reported to activate NA neurons (Kitaoka et al. 2010), and NA metabolites, such as MHPG, increase following exhaustive exercise and are negatively correlated with brain glycogen decreases (Matsui et al. 2011), suggesting that NA-induced glycogen synthesis might be involved (Allaman et al. 2000; Crosson et al. 2003; Ruchti et al. 2016) (Fig. 4).

3.2 Hippocampal Glycometabolism with Acute Exercise in Type 2 Diabetes Rats

A number of studies have shown that exercise is effective in restoring dysregulated glucose control in type 2 diabetes (Sigal et al. 2007; Ruchti et al. 2016); however, the most effective/beneficial exercise conditions and exercise effects on hippocampal glycometabolism remain unestablished. To address this, we have to use a relatively identical exercise intensity protocol for both non-diabetic rats and diabetic rats. Exercise increases blood lactate level which depends on the exercise duration and intensity; the exercise intensity at which blood lactate level begins to exponentially increase is called lactate threshold. Stress responses (plasma ACTH and adrenaline release) are also taken place at lactate threshold (Soya et al. 2007a), thus it is useful to determine the relatively same exercise intensity protocol based on the lactate threshold for the study rats to be compared. We used an acute moderate exercise protocol similar to a moderate exercise intensity based on the lactate threshold of type 2 diabetic rats (OLETF: 22.6 ± 0.3 m/min) and genetic control rats (LETO: 28.3 ± 1.8 m/min) (Shima et al. 2016b), and we then examined the effect of acute

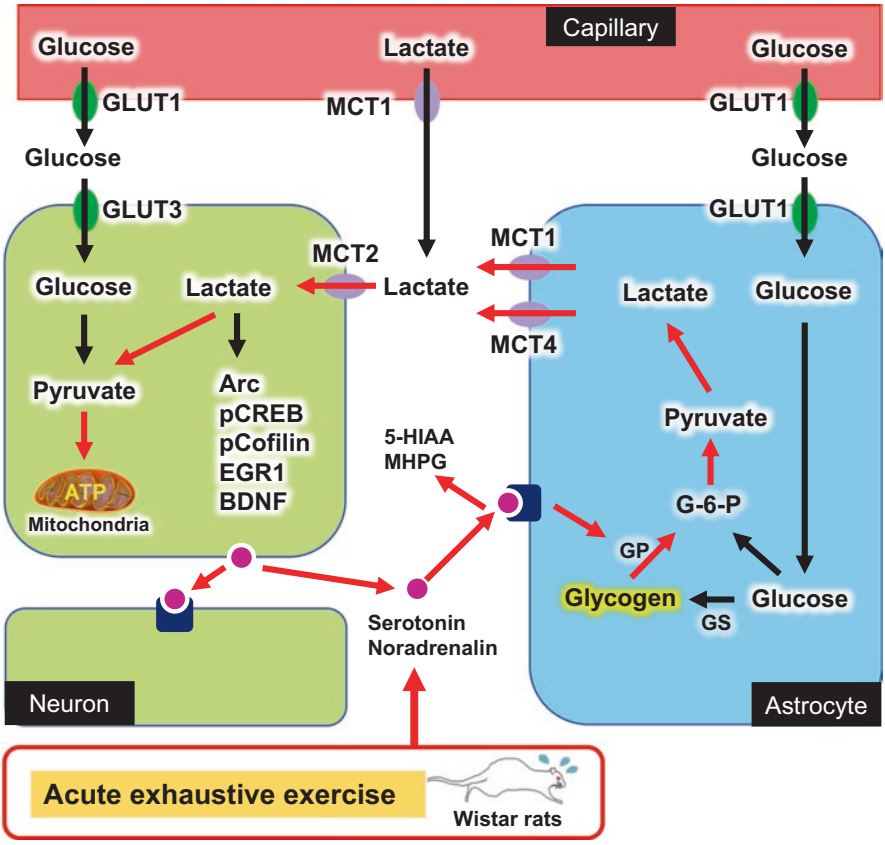

Fig. 4 Brain glycometabolism during acute exhaustive exercise. Brain glycogen levels decrease in the hippocampus, cortex, hypothalamus, cerebellum, and brainstem following acute exhaustive treadmill running exercise, while increases in 5-HIAA and MHPG, which are metabolites of serotonin (5-HT) and NA, respectively, occur after exercise. The decrease in glycogen levels in the cortex during exercise was negatively correlated with increased 5-HIAA and MHPG levels in the cortex, suggesting that 5-HT and NA may be involved in glycogenolysis during exercise. (Summarized figure based on Matsui *et al., J Physiol,* 2011)

moderate exercise on hippocampal glycometabolism (Shima et al. 2016b). We first noted a decrease in hippocampal glycogen following 30 minutes of acute moderate exercise in both OLETF and LETO rats that was positively correlated with the hippocampal lactate increase (Shima et al. 2016b). Diabetes patients exhibit hyper-glycogen in the heart (Bhattacharjee et al. 2006), and the OLETF rats exhibited hyper-glycogen in the hippocampus, which could be a result of adaptation to declining lactate utilization due to the downregulation of MCT2 (Shima et al. 2016a, b). These results suggest that hippocampal glycometabolism although dysregulated in type 2 diabetic rats is activated with acute moderate exercise and this activation with acute exercise is also observed in normal rats hippocampus.

3.3 Effects of Chronic Exercise on Type-2-Diabetes-Induced Dysregulation of Glycometabolism Accompanied with Cognitive Decline

Regular exercise is an effective therapy for type 2 diabetes because it leads to improvement of glycometabolism in peripheral organs (Mu et al. 2001; Holloszy 2005; O'Gorman et al. 2006; Sigal et al. 2007). In addition, chronic exercise not only enhances hippocampus-dependent memory but it also increases hippocampal glycogen levels in normal rats and MCT expression in both peripheral organs and brain tissues (hippocampus and cortex) in diabetes (van Praag et al. 1999; Juel et al. 2004; Liu et al. 2009; Creer et al. 2010; Nikooie et al. 2013; Aveseh et al. 2014; Bolz et al. 2015), suggesting that chronic exercise could be an appropriate exercise therapy for ameliorating hippocampal memory function. OLETF rats exhibited significant hippocampal-dependent memory dysfunction when compared to LETO rats, but, surprisingly, after four weeks of chronic moderate exercise, which is reported to increase hippocampal glycogen levels, memory function was also ameliorated in the OLETF rats (Shima et al. 2016b) (Fig. 5). Chronic exercise induced hyperglycogen levels in the hippocampus of normal rats (Matsui et al. 2012) as well as in OLETF rats with concomitant normalization of downregulated MCT2 levels in neurons, increased glycogen synthase (GS) levels, and also ameliorated glucose transporter 1 (GLUT1) expression in astrocytes in the hippocampus without any significant alteration in the expression of GLUT3, MCT1 and MCT4 (Shima et al. 2016b). Interestingly, chronic exercise increased BDNF levels in the hippocampus of LETO rats, whereas no change in BDNF expression levels occurred in OLETF rats; furthermore, expression of other proteins associated with the downstream BDNF signaling, such as tyrosine receptor kinase B (TrkB), pCREB, and phosphorylated-GS kinase-3β(GSK-3β), were not changed by chronic exercise in either LETO or OLETF rats, implying that normalization of MCT2 levels and further increases in glycogen levels in the hippocampus could be an adaptation in response to chronic exercise that could contribute to the amelioration of hippocampus-dependent memory dysfunction in OLETF rats (Fig. 6).

A dysregulation of MCT2-mediated lactate uptake in neurons may be a novel etiology of memory dysfunction in type 2 diabetes, especially in the context of the hippocampus (Shima et al. 2016b). However, four weeks of chronic exercise did not improve well-known peripheral symptoms/abnormalities of type 2 diabetes, such as alterations in circulatory glucose, insulin and HbA1c levels, which means that more long-term exercise is needed to simultaneously ameliorate the pathology concerned not only with cognitive dysfunction but also with dysregulated glucose control at the peripheral level. Nevertheless, the amelioration of memory dysfunction induced with chronic (four weeks) exercise was exhibited earlier than other pathologies in the peripheral organs. Based on our preliminary findings, abnormal hippocampal glycometabolism and memory dysfunction are already evident at the pre-type2diabetes stage, so the nervous system may require more rapid adaptation to type 2 diabetes than do other organs. These findings suggest that conventional

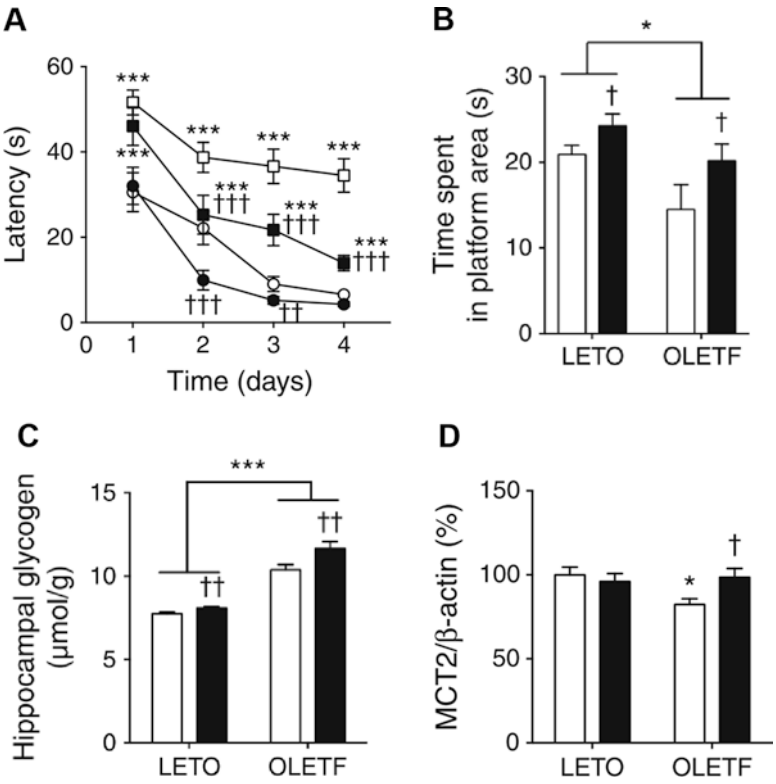

Fig. 5 The effects of chronic exercise on hippocampus-dependent memory and dysregulated hippocampal glycometabolism in type 2 diabetes. The escape latency during learning test of OLETF rats was shortened by 4 weeks of moderate exercise, the time spent in platform area during probe test was increased after 4 weeks of exercise. Furthermore, hippocampal glycogen levels were increased in exercised group of both LETO and OLETF rats, and MCT2 levels in the hippocampus were normalized in exercised OLETF rats group, suggesting that4 weeks of moderate exercise ameliorated hippocampus-dependent memory via improvement of MCT2-mediated lactate uptake into the neurons in OLETF rats (**a**) Escape latency, (**b**) Time spent in platform area during probe test, (**c**) Hippocampal glycogen levels, (**d**) Hippocampal MCT2 levels. Circles, LETO rats; squares, OLETF rats; white symbols and bars, sedentary rats; black symbols and bars, exercised rats, n = 6–8 rats per group. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs LETO rats; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs sedentary rats (Referenced by Shima *et al., Diabetologia,* 2016)

clinical diagnostic indices or criteria for type 2 diabetes, such as glucose and HbA1c levels, may not reflect the earliest organ involvement, which would be the brain with functional hippocampal impairment, as is evident in our unpublished observations and in other studies during the early phase of pre-type-2 diabetes (Soares et al. 2013). Thus, new diagnostic criteria might be necessary to prevent or delay the onset of type 2 diabetes with an urgent focus on the initiation and progression of brain memory dysfunction. Further, it is important to innovate an appropriate exercise regimen either from pre-diabetes or early diabetes, and even starting at advanced

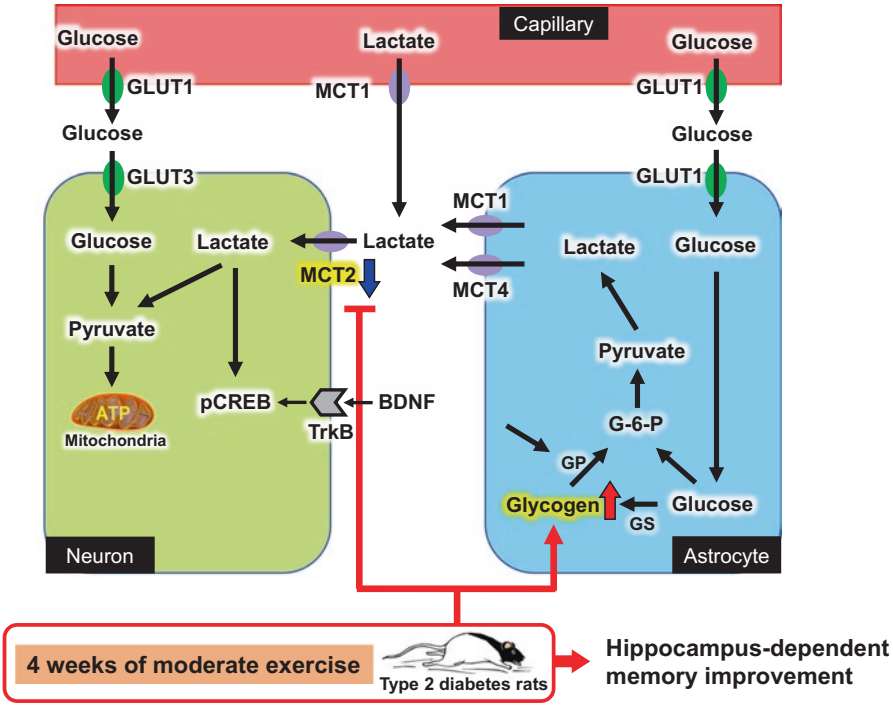

Fig. 6 Conceptual diagram of the chronic exercise effect on ameliorating hippocampus-dependent memory in type 2 diabetes. A 4-week regimen of moderate exercise improves memory dysfunction in type 2 diabetes via amelioration of hippocampal glycometabolism dysregulation. The dysregulated hippocampal glycometabolism and its relationship to lactate transport might be one of the possible etiologies of memory dysfunction in type 2 diabetes. (Summarized figure based on the Shima *et al., Diabetologia,* 2016)

stages, which would clinically improve peripheral diabetic or pre-diabetic symptoms with exerting a beneficial and effective positive role on memory dysfunction through in depth comprehensive future studies.

4 Concluding Remarks and Future Direction

The rapid increase in the number of patients with type 2 diabetes worldwide is a great global problem; hence, a strategy is needed apart from pharmacotherapy such as insulin treatment or other established anti-diabetic drugs, to treat or solve this problem. Cognitive decline is a particularly devastating complication of type 2 diabetes, as hippocampal dysfunction is a serious disability that is associated with dementia, Alzheimer disease, and depression (Pouwer et al. 2003; Ahtiluoto et al. 2010) ultimately causing a remarkable morbidity in social life. Although there are to date still very few studies focusing on this area, in this chapter we largely reviewed

the ameliorating effects of chronic exercise on memory dysfunction in type 2 diabetes at the advanced stage observed in our recent findings. The dysregulation of hippocampal MCT2-mediated lactate uptake into neurons could be the etiology of hippocampus-dependent memory dysfunction in type 2 diabetes, and this is a completely novel addition to this field. Although we do not have any clear and concrete evidence linking our results to ANLS, we assume that the exercise induced amelioration of dysregulated glycometabolism in hippocampus together with cognitive impairment may take place through the adaptation of ANLS although future studies should clarify this speculation through extensive in vivo and in vitro investigations. The current findings have potential implications for translation to human subjects, especially in the context of exercise intensity and duration, as lactate threshold has been a crucial determinant for the exercise models in our diabetes studies. Further investigation is needed to determine the optimal exercise condition to ameliorate type-2-diabetes-induced memory dysfunction together with beneficial effects on peripheral diabetic symptoms and complications, even with advanced diabetes, from the perspective of lifestyle-based therapeutic strategies and intervention. Indeed, we are currently on the way to explore the best exercise regimen for diabetes and diabetes-induced organ complications both in terms of prevention and therapeutics through a multi-disciplinary and multi-faceted translational research approach.

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Development of a Model to Test Whether Glycogenolysis Can Support Astrocytic Energy Demands of Na^+, K^+-ATPase and Glutamate-Glutamine Cycling, Sparing an Equivalent Amount of Glucose for Neurons

Douglas L. Rothman and Gerald A. Dienel

Abstract Recent studies of glycogen in brain have suggested a much more important role in brain energy metabolism and function than previously recognized, including findings of much higher than previously recognized concentrations, consumption at substantial rates compared with utilization of blood-borne glucose, and involvement in ion pumping and in neurotransmission and memory. However, it remains unclear how glycogenolysis is coupled to neuronal activity and provides support for neuronal as well as astroglial function. At present, quantitative aspects of glycogenolysis in brain functions are very difficult to assess due to its metabolic lability, heterogeneous distributions within and among cells, and extreme sensitivity to physiological stimuli. To begin to address this problem, the present study develops a model based on pathway fluxes, mass balance, and literature relevant to functions and turnover of pathways that intersect with glycogen mobilization. A series of equations is developed to describe the stoichiometric relationships between net glycogen consumption that is predominantly in astrocytes with the rate of the glutamate-glutamine cycle, rates of astrocytic and neuronal glycolytic and oxidative metabolism, and the energetics of sodium/potassium pumping in astrocytes and neurons during brain activation. Literature supporting the assumptions of the model is discussed in detail. The overall conclusion is that astrocyte glycogen metabolism is primarily coupled to neuronal function via fueling glycolytically pumping of Na^+ and K^+ and sparing

D. L. Rothman (✉)
Magnetic Resonance Research Center and Department of Radiology, Yale University, New Haven, CT, USA
e-mail: douglas.rothman@yale.edu

G. A. Dienel
Department of Neurology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM, USA

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glucose for neuronal oxidation, as opposed to previous proposals of coupling neurotransmission via glutamate transport, lactate shuttling, and neuronal oxidation of lactate.

Keywords Astrocyte · Brain · Brain activation · Glucose utilization · Glucose sparing · Glutamate-glutamine cycle · Glycogen · Glycogen shunt · Glycogen turnover · Lactate · Lactate shuttling · Oxygen consumption · Neuron · Neurotransmission · Potassium · Sodium/potassium ATPase

Abbreviations

$(A\text{-}V)_{substrate}$	Arteriovenous difference across the brain for the identified substrate
ANL shuttle	Astrocyte-neuron lactate shuttle
Asp	Aspartate
CBF	Cerebral blood flow rate
CF	Correction factor
CMR	Cerebral metabolic rate for substrate of interest = $CBF(A\text{-}V)_{substrate}$
CMR_{glc}	Cerebral metabolic rate for glucose = $CBF(A\text{-}V)_{glc}$
$CMR_{glc\text{-}nonox}$	Rate of non-oxidative metabolism of glucose
$CMR_{glc\text{-}nonox\text{-}A\text{-}b}$	Cerebral metabolic rate of nonoxidative metabolism of glucose in the astrocyte (A) under baseline conditions
$CMR_{glc\text{-}nonox\text{-}N\text{-}b}$	Cerebral metabolic rate of nonoxidative metabolism of glucose in the neuron (N) under baseline conditions.
$CMR_{glc\text{-}ox}$	Rate of oxidative metabolism of glucose
$CMR_{glc\text{-}ox\text{-}A\text{-}b}$	Cerebral metabolic rate of glucose oxidation in the astrocyte (A) under baseline conditions
$CMR_{glc\text{-}ox\text{-}N\text{-}b}$	Cerebral metabolic rate of glucose oxidation in the neuron (N) under baseline conditions.
$CMR_{glc\text{-}tot}$	Total rate of glucose utilization (i.e., at the hexokinase step)
$CMR_{glycogen}$	Cerebral metabolic rate for glycogen = Δ[glycogen]/time
CMR_{O2}	Cerebral metabolic rate for oxygen = $CBF(A\text{-}V)_{O2}$
CP-316,819	[R-R∗,S∗]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide
DAB	1,4-dideoxy-1,4-imino-d-arabinitol
DG	2-deoxy-D-glucose
DG-6-P	DG-6-phosphate
FDG	2-Fluoro-2-deoxy-D-glucose
FDG-6-P	FDG-6-phosphate
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
Glc	Glucose

Glc-6-P	Glucose-6-phosphate
Gln	Glutamine
Glu	Glutamate
Lac	Lactate
LDH	Lactate dehydrogenase
MAS	Malate-aspartate shuttle
MCT	Monocarboxylic acid transporter; MCT1 and MCT4 isoforms are mainly astrocytic, whereas MCT2 is predominantly neuronal
MRS	Magnetic resonance spectroscopy
NAA	N-Acetylaspartate
NAL shuttle	Neuron-astrocyte lactate shuttle
OCI	Oxygen carbohydrate index = $CMR_{O2}/[CMR_{glc} + 0.5CMR_{lac} + CMR_{glycogen}] = (A\text{-}V)_{O2}/((A\text{-}V)_{glc} + 0.5(A\text{-}V)_{lac} + \Delta[\text{glycogen}])$, where lactate and [glycogen] are expressed in glucosyl units (2Lac = 1Glc)
OGI	Oxygen-glucose index = $CMR_{O2}/CMR_{glc} = (A\text{-}V)_{O2}/(A\text{-}V)_{glc}$ (CBF cancels out) This calculation assumes no other substrates are oxidized.
PAG	Phosphate-activated glutaminase
PPP	Pentose phosphate shunt pathway
TCA	Tricarboxylic acid
V_{ATP}	Rate of ATP production
V_{cycle}	Rate of the glutamate-glutamine cycle
$V_{cycle\text{-}b}$	Baseline rate of the glutamate glutamine cycle
V_{efflux}	Rate of lactate efflux
$V_{glu\text{-}ox\text{-}b}$	Baseline rate of astrocytic glutamate oxidation
$V_{PC\text{-}b}$	Baseline rate of pyruvate carboxylase
$V_{PDH\text{-}A\text{-}b}$	Baseline rate of pyruvate dehydrogenase in the neuron (N)
$V_{PDH\text{-}N\text{-}b}$	Baseline rate of pyruvate dehydrogenase in the astrocyte (A)
V_{shunt}	Rate of glycogenolysis = $CMR_{glycogen}$
ΔV_{cycle}	Incremental change in V_{cycle}

1 Introduction

1.1 Major Questions in Neuroenergetics

The last 20 years have seen substantial progress in understanding the energy requirements to support brain neuronal signaling. Among other discoveries, the major findings have been the measurement of the relationships between glutamate (Glu) and γ-aminobutyric acid (GABA) neurotransmitter cycling between neurons and astrocytes and cell-specific energetics and metabolic fluxes. Studies have found linear relationships between the glutamate-glutamine (Gln) and GABA-glutamine cycles, anaplerotic glutamine synthesis, and neuronal and glial glucose oxidation

(Yu et al. 2018; Lanz et al. 2013; Patel et al. 2010; Sonnay et al. 2016; Duarte et al. 2011). In the resting awake state almost 80% of total cerebral cortex energy consumption supports functional processes in neurons and glia. These experimental findings are consistent with energy budgets constructed from energy requirements determined experimentally from single cells (Yu et al. 2018; Harris et al. 2012; Howarth et al. 2012; Attwell and Laughlin 2001). Despite this progress several important questions remain unanswered regarding functional neuroenergetics including:

- **Why does the brain have an obligatory requirement for glucose consumption?** Many studies have shown that alternative substrates can be used by brain, but insulin-induced hypoglycemic coma in vivo is quickly reversed only by glucose (or maltose that is converted to glucose), not many other substrates including lactate, acetate, and β-hydroxybutyrate that are supplemental oxidative fuels in vivo (Clarke and Sokoloff 1999; Sokoloff 1960). Studies dating back to the 1960s have found that, even under conditions of extended fasting, brain glucose oxidation can only be partially replaced by fuels such as ketone bodies (Cahill Jr. 2006). More recently, it was shown that while the entire nonsignaling-coupled cerebral cortical energy requirements can be replaced by ketones, whereas only approximately 50% of the energetics used to support signaling can be replaced (Chowdhury et al. 2014) and metabolism of tracer amounts of β-[^{14}C]hydroxybutyrate does not rise during acoustic stimulation (Cruz et al. 2005). Similarly, studies in which lactate was infused or generated by exercise to produce high plasma levels have been unable to demonstrate complete suppression of brain glucose uptake in vivo (Quistorff et al. 2008; Smith et al. 2003).
- **Why is there a near-stoichiometric relationship between increments in glucose oxidation and glutamate/GABA neurotransmitter cycling?** As initially found by Sibson et al. (1998), there is close to a 1:1 molar relationship between increments in the GABA/glutamate/glutamine neurotransmitter cycle (V_{cycle}) and neuronal glucose oxidation. When the functional component of glial glucose oxidation is included, a 1:1 relationship is achieved (Yu et al. 2018; Lanz et al. 2013; Patel et al. 2010; Sonnay et al. 2016; Duarte et al. 2011). Figure 1a shows the slopes and intercepts of these relationships based upon recent meta-analyses. As discussed below, this relationship has been interpreted as implying the existence of a molecular

Fig. 1 (continued) pyruvate/lactate, with most of the lactate being shuttled to neurons as oxidative fuel. Numerical values are pathway fluxes in glucose equivalents normalized to $CMR_{glc\text{-}ox\text{-}N}$ = 1 μmol/g/min and the ATP yields from these fluxes. From Fig. 5 of (Hyder et al. 2006) © 2006, SAGE Publications with permission. **Abbreviations**: $CMR_{glc\text{-}ox\text{-}tot,}$ total rate of cerebral glucose oxidation by all brain cells; $CMR_{glc\text{-}ox\text{-}N,}$ rate of cerebral glucose oxidation in neurons; $CMR_{glc\text{-}ox\text{-}A,}$ cerebral metabolic rate of glucose oxidation in astrocytes; V_{PC}, rate of pyruvate carboxylase in astrocytes; V_{cycle}, rate of glutamate-glutamine cycle; Glu, glutamate; Gln, glutamine; Glc, glucose; Pyr, pyruvate; Lac, lactate; OAA, oxaloacetate; GABA, γ-aminobutyric acid; Mal, malate, Suc, succinate; α-KG, α-ketoglutarate; Cit, citrate

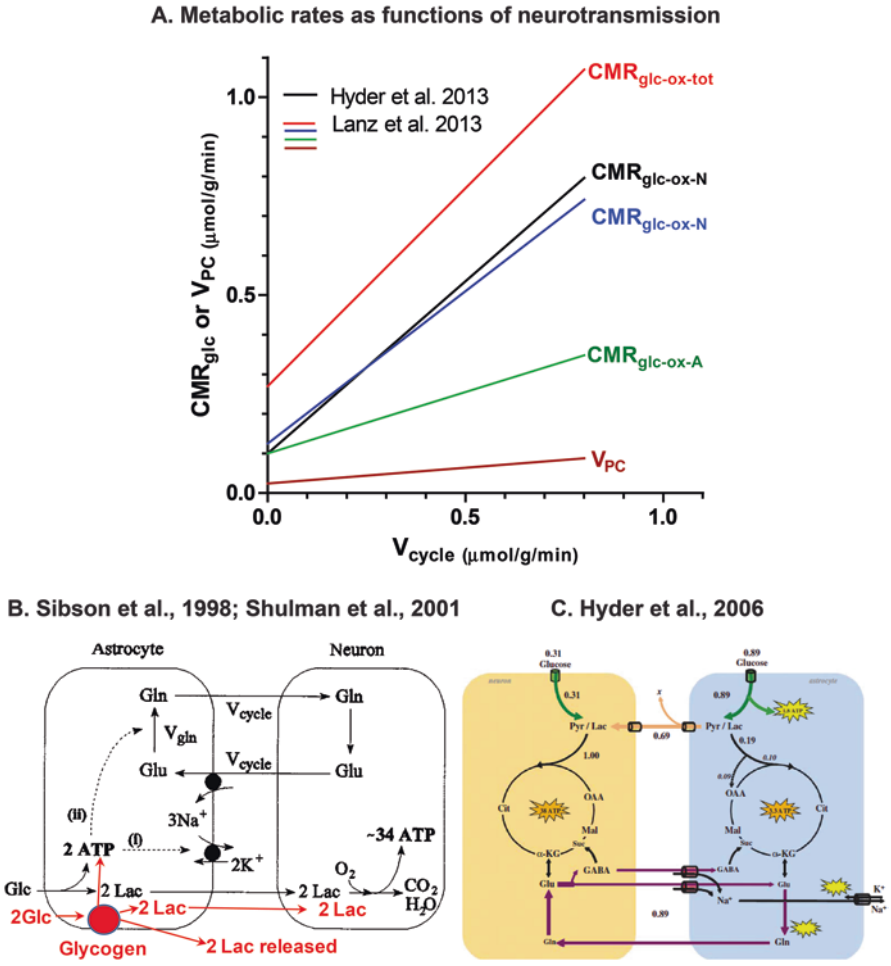

Fig. 1 Neuronal and glial metabolic fluxes and early models to explain the 1:1 relationship between neuronal glucose oxidation and glutamate-glutamine cycle rate. (**a**). Meta-analyses of the rates of glucose oxidation in neurons and astrocytes and pyruvate carboxylation in astrocytes determined by magnetic resonance spectroscopy (MRS) reveal linear relationships to the rate of the glutamate-glutamine cycle over a wide range of activities, from isoelectric to awake resting states. The y-axis intercept represents the metabolic rate at isoelectric EEG, which is interpreted as the energetics of nonsignaling brain activities. Increases in metabolic rate arise from functional signaling activities, with graded rates between isoelectricity and the awake resting state obtained by use of different anesthetics. Lines were plotted from the equation of the best-fit regression line in Fig. 2A of (Hyder et al. 2013) and from interpolated values calculated from the regression lines in Fig. 8 of (Lanz et al. 2013). (**b**) The black line drawing is Fig. 4 of (Sibson et al. 1998) © (1998) National Academy of Sciences, with permission of the authors. The red diagram added to the Sibson model is based on Fig. 1 of (Shulman et al. 2001a). Note that astrocyte-neuron lactate shuttling provides neuronal oxidative fuel in both models, but they differ in the source of lactate, either glucose or glycogen, and the stoichiometry. (**c**) Updated model in which glucose utilization is partitioned between astrocytes and neurons, with higher glycolytic activity in astrocytes to produce

mechanism coupling glycolytic ATP in the glia to the Na^+, K^+-ATPase activity associated with supporting glutamate transport into the astrocytes, with the associated transport of Na^+ ions into the cell requiring pumping out. However, no direct in vivo evidence has been found supporting this hypothesis.

- **Why is there an increase in nonoxidative glucose metabolism during intense sensory stimulation?** In the resting awake state, almost all of the ATP produced by the brain comes from glucose oxidation, with an oxygen glucose index ($OGI = CMR_{O2}/CMR_{glc}$, where CMR is cerebral metabolic rate for the subscripted substrate) ranging from the theoretical maximum of 6 to 5, depending on species and methodology (Hyder et al. 2006). Lear and Ackermann (1989) recognized that glycolysis is preferentially upregulated during stimulation and this increased flux causes a significant drop in the OGI and greater production of brain lactate (reviewed in Dienel 2019a, 2012; Dienel and Cruz 2016). Nevertheless, from an energetic standpoint, the extra ATP being generated during the activation by nonoxidative glycolysis remains a small fraction of the total ATP needs, suggesting that it is fueling specific processes. These specific processes and what cell types they occur in remain unidentified.

1.2 Early Mechanistic Models for Astrocyte-Neuron Energetics and Neurotransmission

Several models have been proposed to address the question of what is the mechanism that explains the measured relationships between neuronal and astrocytic glucose metabolism and neurotransmitter cycling. These models can be separated based on whether or not the coupling between glutamate/glutamine cycling and glucose consumption is via the astrocyte-neuron lactate shuttle (ANL shuttle), which is to be distinguished from the neuron-astrocyte lactate shuttle, NAL shuttle (Mangia et al. 2009, 2011; DiNuzzo et al. 2010a). In the ANL shuttle-based models, the glucose needed to fuel functional processes is taken up in astrocytes and converted to lactate. Once converted to lactate the majority is then transported out of the astrocyte to be taken up and oxidized by neurons. The ANL shuttle was proposed initially based on astrocyte cell culture studies to explain the elevation in lactate observed during sensory stimulation studies (Pellerin and Magistretti 1994). In these cell culture studies, it was found that added glutamate led to an increase in 2-deoxy-D-[^{3}H] glucose (DG) phosphorylation (Pellerin and Magistretti 1994). It was proposed that the nonoxidative glycolysis was due to the need for glycolytic ATP to fuel the Na^+,K^+-ATPase needed to pump the Na^+ co-transported with glutamate out of the astrocyte and to convert glutamate into glutamine, and that this process could explain in vivo nonoxidative glycolysis during sensory activation. In 1998 Sibson and coworkers initially measured the ~1:1 in vivo relationship between the glutamate/GABA/glutamine cycle and neuronal glucose oxidation. They further showed

it was consistent with an ANL shuttle-based model, shown in Fig. 1b, in which astrocytic metabolism of glucose provided the glycolytic ATP needed to fuel the transport of glutamate into the astrocyte. The 1:1 stoichiometry arose because per glutamate molecule transported 1 ATP was needed to restore Na^+ and K^+ balance using the Na^+, K^+-ATPase and another ATP was needed to convert glutamate to glutamine. The use of glycolytic ATP was rationalized based on both of these events being rapid processes (Magistretti et al. 1999; Sibson et al. 1998).

Although the modified ANL shuttle model quantitatively predicted the in vivo stoichiometry between GABA/glutamate/glutamine neurotransmitter cycling and neuronal glucose oxidation, it provided no predictions of the amount that net non-oxidative glycolysis would increase as a function of GABA/glutamate/glutamine cycling. Shulman et al. (2001a) addressed this limitation by proposing that astrocytic glucose metabolism during intense activation was coupled to the glycogen shunt, a pathway originally described in exercising muscle to explain aerobic lactate generation (Shulman and Rothman 2001) and also proposed by Swanson (1992), which is shown schematically in the red text in Fig. 1b. In the brain, glycogen is almost entirely localized to astrocytes making it an ideal candidate for rapid lactate generation and transfer to neurons (Dringen et al. 1993). In the glycogen shunt, some of the astrocytic glucose uptake, instead of directly entering glycolysis, is first stored as glycogen. The glycogen is then broken down during intense bursts of activity to fuel the Na^+, K^+- ATPase and glutamine synthesis with glycolytic ATP. Due to the loss of 1 ATP in the synthesis of glycogen (relative to direct glycolysis), the net stoichiometry of the shunt would be 2 glucose per glutamate, with the lactate equivalents of 1 glucose transferred to the neuron for oxidation and the other equivalents being released into the blood. This stoichiometry was shown to agree with measurements during seizure. The shunt model was later modified by Hyder and coworkers (2006) to take into account astroglial glucose oxidation as shown in Fig. 1c.

Soon after the ANL shuttle was proposed it was criticized on a variety of grounds, including glutamate-evoked glycolysis not being a robust finding in many astrocyte cultures, it did not include glutamate oxidation upon its uptake, and the predicted stoichiometries of astrocytic lactate production and transfer did not match experimental results (reviewed in Dienel 2017, 2019a). To date, direct in vivo evidence of net transfer of lactate between astrocytes and neurons has not been demonstrated. In 2014, Patel and coworkers directly tested whether the increment in glucose uptake during seizure would be astrocytic at the rate relative to the GABA/glutamate/glutamine cycle predicted by the ANL shuttle-based glycogen shunt models shown in Fig. 1b, c (Patel et al. 2014). In the Patel study, the neuronal contribution was measured by extracting synaptosomes from animals infused with 2-fluoro-2-deoxy-D-glucose (FDG) and comparing the ratio of FDG-6-phosphate (FDG-6-P) to N-acetylaspartate (NAA) under control and seizure conditions. They found that neuronal glucose phosphorylation by hexokinase was proportional to total glucose phosphorylation from which they concluded that, within the accuracy of the study, there was little or no ANL shuttle activity.

As an alternative to ANL shuttle-based models, several models to explain mechanistic coupling of neuron-astrocyte energetics with neurotransmission have been proposed based on the localization of neuronal phosphate-activated glutaminase (PAG) in the inner membrane of mitochondria near presynaptic terminals. As a consequence of this localization, in order to transport a molecule of glutamate made from glutamine by PAG to the cytosol where it can be pumped into vesicles for use in neurotransmission, the malate-aspartate shuttle (MAS) needs to be used, as shown in Fig. 2a. The partial or pseudo-MAS requires an NADH from glycolysis which is subsequently transferred to the electron transport chain. The predicted stoichiometry of this mechanism is 1 glucose per 2 glutamates which is approximately half the experimentally-measured slope. However, as discussed in Sect. 2 the original proposals for this mechanism did not take into account that approximately half of neuronal glucose consumption is in the presynaptic terminals (Yu et al. 2018) where the majority of neurotransmitter glutamate will be synthesized, which changes the predicted stoichiometry to approximately 1:1. An additional potential coupling mechanism is due to pumping of glutamate into vesicles (Fig. 2b) via a glutamate-proton antiporter which requires an ATP per glutamate. This ATP appears to be preferentially provided by glycolysis (Ueda 2016) which also predicts an approximately 1:1 relationship when the distribution of glucose consumption between pre- and postsynaptic neuronal regions is taken into account (Sect. 2). These mechanisms are shown together in Fig. 2b. Although the proposed neuronal-based mechanisms (Fig. 2a, b) can explain the experimentally-measured neuronal stoichiometry between $CMR_{glc\text{-}ox\text{-}N}$ and V_{cycle} they do not explain the astroglial stoichiometry nor how neuronal and astroglial functional metabolism is coupled.

Fig. 2 (continued) glutamate that is loaded into synaptic vesicles. The pseudo-MAS model was based on studies in cultured neurons in which the NADH was derived from glycolytic metabolism of glucose. However, in brain in vivo, it is possible that metabolism of extracellular lactate could provide NADH for the pseudo-MAS. Modified from Fig. 5 of (Hertz and Chen 2017) ©2017 Hertz and Chen, open access with permission, that was derived from Fig. 3 of (Palaiologos et al. 1988) ©1988, John Wiley and Sons, with permission. (**b**) Energetics of conversion of glutamine into vesicular neurotransmitter glutamate. Under conditions when all glucose in the presynaptic terminal is oxidized, the 2 NADH produced by glycolytic metabolism of one glucose molecule can participate in the 'true' MAS and pseudo-MAS to support the glutamate-glutamine cycle via conversion of glutamine to glutamate and vesicular packaging of glutamate. From Fig. 4D of (Dienel 2019a), © 2019 American Physiological Society, with permission. **Abbreviations**: Glu, glutamate; $Glu_{vesicle}$, vesicular glutamate; Gln, glutamine; PAG, phosphate-activated glutaminase; Glc, glucose; Pyr, pyruvate; Pyr_{ox}, pyruvate oxidized; Lac, lactate; OAA, oxaloacetate; Asp, aspartate; ETC, electron transport chain; CMR_{O2}, cerebral metabolic rate of oxygen; mal, malate, Suc, succinate; αKG, α-ketoglutarate; GAP, glyceraldehyde-3-phosphate; GAPDH, GAP dehydrogenase; 1,3PG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; TCA, tricarboxylic acid

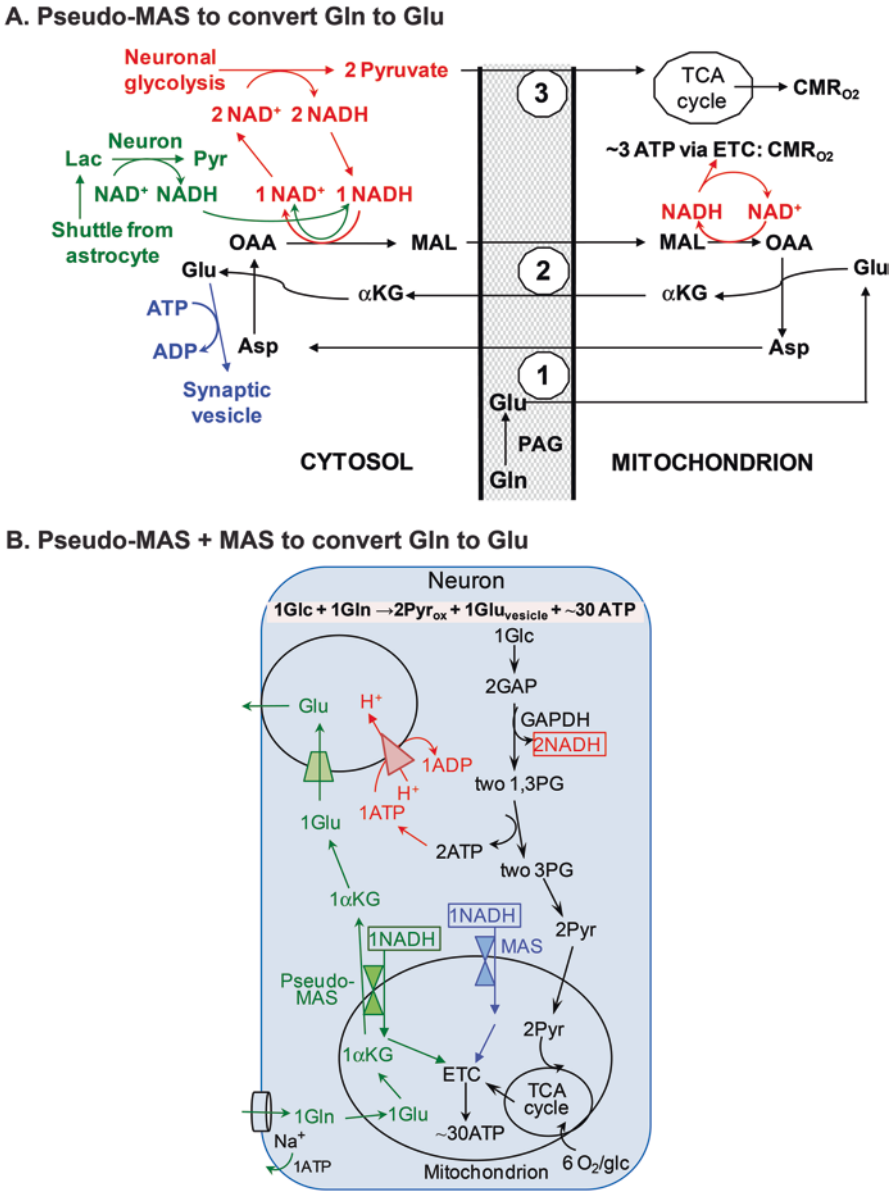

Fig. 2 Roles of the pseudo-malate-aspartate shuttle (MAS) and 'true'-MAS in Glu-Gln cycling. (**a**) Conversion of glutamate to glutamine requires the pseudo-MAS. The MAS transfers reducing equivalents from cytosol to mitochondria, enabling pyruvate to be taken up into mitochondria by its carrier (reaction 3) and oxidized. The pseudo-MAS differs from the 'true' MAS in that the glutamate is generated from the action of phosphate-activated glutaminase on glutamine derived from the glutamate-glutamine cycle that is taken up into neurons. The glutamate is transported into the matrix by the aspartate-glutamate carrier (aralar, reaction 1). Transamination produces α-ketoglutarate that is exported to the cytosol by the ketodicarboxylic acid carrier (reaction 2), where it is transaminated to produce

1.3 A New Stoichiometric Model for Roles of Glycogenolysis in Coupling of Astrocyte-Neuron Energetics with Neurotransmission in Activated Brain

Over the last two decades there has been increasing evidence for the importance of glycogen for brain functions, such as learning and memory (Hertz et al. 2013; Duran et al. 2013, 2019; Newman et al. 2011; Zhang et al. 2016; Suzuki et al. 2011). In addition, improvements in brain glycogen isolation and visualization (Cruz and Dienel 2002; Swanson 1992), as well as noninvasive magnetic resonance spectroscopic (MRS) methods have shown that brain glycogen concentrations are several-fold higher than previously reported (Öz et al. 2017; Öz et al. 2015; DiNuzzo 2013; Cruz and Dienel 2002; Oe et al. 2016; Kong et al. 2002), with proportional increases of the amount of glycogenolysis measured during intense neuronal activity (Dienel et al. 2002, 2007a). Given the important role of glycogen to provide rapid ATP without exhaustion of glucose levels during high energy demand in tissue such as muscle, it is surprising that glycogen is unable to indirectly play a similar role for neurons, which have 2 to 3 times higher energy requirements than astrocytes.

In this paper, we address the above questions regarding functional glucose metabolism by proposing a new glycogen shunt model that is shown in Fig. 3. There are three primary differences from previous work. First, as opposed to an ANL shuttle, astrocytic glycogen consumption effectively transfers glucose carbon to the neuron by reducing total and incremental astrocytic glucose uptake, thereby sparing an equivalent amount of blood-borne glucose for neuronal metabolism; there is no cell-cell substrate transfer. The sparing of glucose is based on the proposal by Swanson (1992) and metabolic modeling by DiNuzzo and coworkers (2010a, b, 2012). Second, the glycogen shunt model incorporates as a mechanism the proposal of DiNuzzo and colleagues (DiNuzzo et al. 2013, 2017; Mangia et al. 2013; Xu et al. 2013) and earlier work (Hof et al. 1988; Hertz and Peng 1992; Rosenthal and Sick 1992; Quach et al. 1978; Swanson 1992) that glycolytic ATP from glycogenolysis supports the pumping of K^+ released by neuronal activity, thereby linking astrocyte glycogen (and glucose) metabolism to neuronal activity. Third, while the relationship between the neuronal malate-aspartate shuttle and neuronal glutamate synthesis from glutamine proposed by Hertz and Chen (2017) (Fig. 2a) is incorporated into the model, it extends this model through the coupling mechanisms between neuronal and astrocytic glucose and glycogen metabolism.

From the glycogen shunt model, stoichiometric relationships can be derived between the rate of net glycogenolysis (V_{shunt}) and several fluxes including neuronal total (tot-N) and oxidative (ox-N) glucose consumption ($CMR_{glc\text{-}tot\text{-}N}$, $CMR_{glc\text{-}ox\text{-}N}$), the GABA/glutamate/glutamine cycle (V_{cycle}), and the relative neuronal and astrocytic functional neuroenergetic costs (as a function of V_{cycle}). We end the chapter by reviewing predictions of the model that can be tested experimentally.

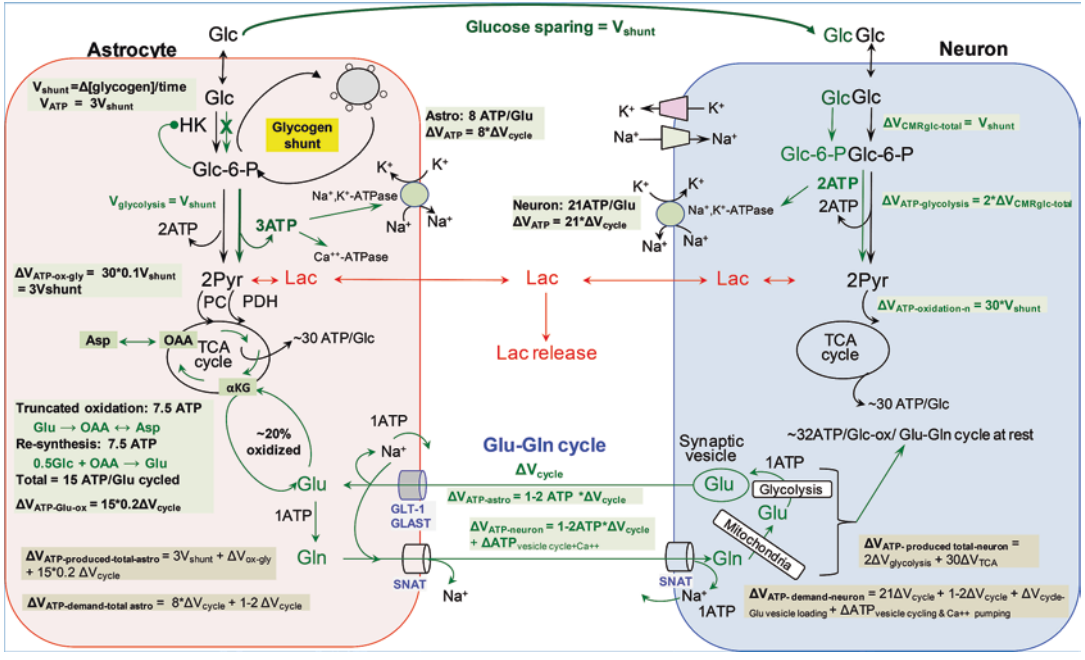

Fig. 3 The revised, updated glycogen shunt model. The glycogen shunt model shows the relationships between the rate of glycogenolysis during activation, glucose sparing for neurons, and metabolic pathway fluxes. In brief, during activation glycogenolysis in astrocytes replaces the incremental rise in astrocytic blood-borne glucose utilization, sparing an equivalent amount of glucose for neuronal glycolytic and oxidative metabolism. The model can be tested by evaluating the ability of glycogenolysis to satisfy the energetic demands of Na^+, K^+-ATPase and glutamate-glutamine cycling in astrocytes and neurons. The stoichiometric relationships derived from the model are given in Sect. 2 and the assumptions used in formulating the model and their justifications are discussed in detail in Sect. 3. The ATP requirements for Na^+, K^+-ATPase in neurons and astrocytes are taken from Fig. 2 of DiNuzzo et al. (2017). **Abbreviations**: Glu, glutamate; $Glu_{vesicle}$, vesicular glutamate; Gln, glutamine; Glc, glucose; Glc-6-P, glucose-6-phosphate; Pyr, pyruvate; Lac, lactate; OAA, oxaloacetate; Asp, aspartate; αKG, α-ketoglutarate; V_{shunt}, rate of glycogen utilization; V_{cycle}, rate of Glu-Gln cycle; $V_{glycolysis}$, glycolytic rate; GLT-1 and GLAST, glutamate transporters (co-transport of Na^+ with Glu); SNAT, solute neutral amino acid transporter (Na^+-linked Gln transporter); TCA, tricarboxylic acid; V_{TCA}, rate of TCA cycle; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; V_{ATP}, rate of ATP production from the indicated sources; $V_{ATP\text{-}demand}$, rate of ATP consumption for the indicated cell type and processes; Δ, incremental change from baseline to activation of the indicated rate; V_{cycle}, rate of glutamate-glutamine cycle; Truncated Glu oxidation, entry of Glu into the TCA cycle at the αKG step and retention in the cycle or interconversion of OAA with Asp such that the 4-carbon backbone of Glu is used to re-synthesize Glu. See text for definitions of other fluxes

2 Description of the Glycogen Shunt Model

In this section, the glycogen shunt model is derived along with the predicted relationships between the rate of net glycogen breakdown (V_{shunt}), and the increments in Vcycle (ΔV_{cycle}), and neuronal total and oxidative glucose uptake, as well as the relative functional energetics of neurons and astroglia.

2.1 Definition of the Glycogen Shunt Versus Glycogen Turnover

The difference between the glycogen shunt and glycogen turnover is that turnover can be just due to low level parallel activity of glycogen synthase and glycogen phosphorylase involved in particle remodeling, but not contributing much to overall energetics. In liver and resting, unstimulated brain (e.g., as shown by low label incorporation and slow label release with no change in concentration Watanabe and Passonneau 1973), this is most likely what is happening. The caveat is that simply cycling of a glucose molecule into and out of glycogen costs 1 ATP, whereas net degradation of pre-formed glycogen yields 3 ATP per glucosyl unit compared with 2 ATP from glycolytic metabolism of glucose. Conversely, the cost of net synthesis can be paid during times of low energy demand and adequate glucose supply.

In the glycogen shunt there is also turnover, but net glycogen synthesis and net degradation are temporally separated such that there is net degradation or net synthesis of glycogen (i.e., substantial concentration changes along with label incorporation or label loss, e.g., Dienel et al. 2007a, 2002). A rapid burst of glycogen breakdown for glycolytic ATP (primarily) is followed by a longer period during which glycogen is re-synthesized. Because the necessary energy could have been supplied directly by glucose this process represents a ‘shunt’ of glucose through glycogen. The most likely purpose of the shunt, as others have pointed out, is to maintain homeostasis of glucose-6-phosphate and glucose levels. Brain glucose transport activity can be maintained relatively low due to having glycogen as a reserve for sudden large increases in glucose demand beyond what transport can supply at the onset of activation.

The glycogen shunt differs from the traditional view of glycogenolysis and glycogen synthesis in that in the traditional view the organ synthesizes glycogen and stores it for extended periods until needed, such as when the glycolytic flux is much higher than could be supplied from the blood (e.g., muscle glycogenolysis during a sprint). Alternatively, the glycogen shunt can work the other way in which a rapid burst of synthesis is followed by a longer period of slow breakdown such as in yeast where the role of glycogen in homeostasis of glucose and particularly glucose-6-phosphate is very clear.

Further discussion of these points is presented in the following papers (Shulman and Rothman 2001, 2015, 2017; Shulman et al. 2001a, b), while noting that the

present report revises and updates the concept of the glycogen shunt in brain compared with in these earlier publications and the new model separates the glycogen shunt from involvement in the ANL shuttle model, as discussed above.

2.2 *Explanation of the Model*

Figure 3 shows pathway diagrams of the coupling between glucose metabolism and GABA/glutamate/glutamine cycling between neurons and astrocytes under nonactivated baseline and activated conditions. Because excitatory synapses are believed to account for the large majority of energy consumption during activation an excitatory neuron is diagrammed. However, the V_{cycle} flux in the relationships is the sum of the glutamate-glutamine and GABA-glutamine cycles and neuronal glucose metabolism, and neuronal energetics is the sum of GABAergic and glutamatergic neurons.

Based on meta-analyses of in vivo MRS studies (see Fig. 1a), the metabolic rates in the diagram can all be expressed as functions of the baseline (subscript b) rate of the GABA/glutamate/glutamine cycle ($V_{cycle\text{-}b}$) with intercepts at isoelectricity ($V_{cycle\text{-}b} = 0$) according to the following equations where $CMR_{glc\text{-}ox}$ is the cerebral metabolic rate of glucose oxidation, $CMR_{glc\text{-}ox\text{-}N}$ is the cerebral metabolic rate of neuronal glucose oxidation, $CMR_{glc\text{-}ox\text{-}A}$ is the cerebral metabolic rate of astrocytic glucose oxidation, V_{PC} is the flux through pyruvate carboxylase in the astrocyte, and $V_{glu\text{-}ox}$ is the flux through the astrocyte glutamate oxidation pathway. At steady state $V_{PC} = V_{glu\text{-}ox}$. The slopes and intercepts used in the equations are derived from studies primarily using ^{13}C-MRS of rat cerebral gray matter. However, as shown in Fig. 1a, results from humans are consistent with these relationships, as are recent results from other species including the tree shrew, rat, and mouse (Lanz et al. 2013; Sonnay et al. 2017b; Hyder et al. 2013). Equations (1)–(5) present the quantitative relationships of the above pathway fluxes to V_{cycle}, as shown in Fig. 1a.

$$CMR_{glc-ox} = 1.0\, V_{cycle} \tag{1}$$

$$CMR_{glc-ox-N-b} = 0.9\, V_{cycle} \tag{2}$$

$$CMR_{glc-ox-A-b} = V_{PC} + 0.5\, V_{PDH-A} = 0.3\, V_{cycle} \tag{3}$$

$$V_{PC} = 0.2\, V_{cycle} \tag{4}$$

$$V_{glu-ox} = 0.2\, V_{cycle} \tag{5}$$

Figure 3 also shows the incremental fluxes predicted by the model during activation when glycogenolysis (V_{shunt}) increases as a function of the increment in V_{cycle} (ΔV_{cycle}). Incremental neuronal glucose consumption is limited to the amount of glucose spared due to the astrocyte switching from glucose to glycogen consumption

to produce glycolytic and oxidative ATP. The rate of V_{cycle} is determined by the rate of glucose oxidation in the presynaptic terminals via the pseudo-malate-aspartate shuttle mechanism (Hertz and Chen 2017), which is approximately ½ of total presynaptic neuronal glucose oxidation (Fig. 2). The rest of the neuronal glucose consumption is nonoxidative and fuels processes that use glycolytic ATP, such as glutamate pumping into synaptic vesicles (Ueda 2016). Note that, for simplicity, the GABA-glutamine cycle is not shown in the diagram but its contributions to V_{cycle} and $CMRg_{lc\text{-}ox\text{-}N}$ are included.

Based on the pathway diagrams in Fig. 3 and mass balance, the following steady state flux relationships in Eqs. (6)–(11) are derived for the incremental fluxes during activation. V_{shunt} is not given as an increment because under nonactivated conditions glycogen levels are constant so that there is no net flux, although turnover can occur, e.g. (Watanabe and Passonneau 1973; Dienel et al. 2002):

$$\Delta CMR_{glc-tot-N} = V_{shunt} \tag{6}$$

$$\Delta CMR_{glc-ox-N} = 0.5\, V_{shunt} \tag{7}$$

$$\Delta CMR_{glc-nonox-N} = 0.5\, V_{shunt} \tag{8}$$

$$\Delta V_{cycle} = 0.5\, V_{shunt} \tag{9}$$

$$\Delta V_{cycle} = \Delta CMR_{glc-ox-N} \tag{10}$$

$$\Delta CMR_{glc-tot-A} = -KV_{shunt} \tag{11}$$

The constant K in Eq. (11) ranges from 0 (in which case only incremental astrocytic glucose consumption is spared by V_{shunt}) to 1 (up until $V_{shunt} > CMR_{glc\text{-}A\text{-}b}$), in which case total incremental glucose consumption is reduced to 0.

As shown in these equations, the glycogen shunt model predicts several relationships between V_{shunt} and neuronal glucose metabolism and V_{cycle} that can be tested experimentally. Additional predictions regarding the coupling of glucose metabolism between neuronal and astrocytic energetics are derived in Sect. 2.3.

2.3 *Coupling Between Neuronal and Astrocytic Energetics in the Glycogen Shunt Model*

Figure 3 shows the predicted energetics of the glycogen shunt model relative to V_{shunt} and the increment in V_{cycle} (ΔV_{cycle}) and their coupling to Na^+ and K^+ pumping in the neurons and astrocytes. Based on the glycogen shunt model, the incremental ATP ($\Delta V_{ATP\text{-}A}$) generated by the astrocytes is given by Eq. (12) in which the first term is the glycolytic contribution from glycogenolysis and the second term is from glutamate

oxidation and oxidation of lactate/pyruvate generated from glycogenolysis. Based on reports of no or little increase in anaplerosis during sensory stimulation or seizure (Sonnay et al. 2017a, b; Patel et al. 2004) partial glutamate oxidation as opposed to complete glutamate oxidation (which requires anaplerosis) is assumed.

$$\Delta V_{\mathrm{ATP-A}} = 3\,\mathrm{V}_{\mathrm{shunt}} + 15\,\Delta V_{\mathrm{glu}} \;=\; 3\,\mathrm{V}_{\mathrm{shunt}} + 15 * 0.1 * \mathrm{V}_{\mathrm{shunt}} = 4.5\,\mathrm{V}_{\mathrm{shunt}} \quad (12)$$

Based on glucose sparing, the glycolytic and oxidative ATP generated by the neuron is also determined by the glycogen shunt with the first term from glycolysis and the second from oxidative phosphorylation derived from the glycolytic pathway and the TCA cycle.

$$\Delta V_{\mathrm{ATP-N}} = \mathrm{V}_{\mathrm{shunt}} + 15\,\mathrm{V}_{\mathrm{shunt}} = 16\,\mathrm{V}_{\mathrm{shunt}} \quad (13)$$

The ratio of the functional ATP consumption of astrocytes and neurons in the cerebral cortex is determined by the ratio of Eqs. (12) and (13). It is assumed in the glycogen shunt model that this ratio remains constant under conditions when the shunt is not active in order to maintain fidelity of synaptic transmission which can be tested by comparison with experimental measurements performed under nonactivated conditions. Due to the neuron shifting away from nonoxidative glycolysis under nonactivated conditions the ratio for comparison becomes.

$$\Delta V_{\mathrm{ATP-N}} / \Delta V_{\mathrm{ATP-A}} \;= 16 / 4.5 = \;3.56 \quad (14)$$

As with the glucose flux relationships (Eqs. 1–11), this assumption can be tested by comparison with experimental measurements of ATP production as a function of $\Delta \mathrm{V}_{\mathrm{cycle}}$.

It is also assumed in the glycogen shunt model that all of the ATP generated by glycogenolysis is coupled to incremental Na^+ and K^+ pumping by the astrocytes to restore K^+ taken up by the astrocytes during activation back to the extracellular space; the ATP costs per glutamate cycled shown in Fig. 3 are from Fig. 2 of (DiNuzzo et al. 2017). Based upon this assumption and the calculation by DiNuzzo and colleagues regarding the fraction of total astroglial $\mathrm{V}_{\mathrm{ATP\text{-}A}}$ devoted to ion pumping the incremental Na^+ and K^+ fluxes are given by

$$\Delta V_{\mathrm{K+-A}} = 2\left(\Delta V_{\mathrm{ATPshunt-A}} \;+\; (2/3)\,\Delta V_{\mathrm{ATP-glu-ox-A}}\right) = 8\,\mathrm{Vshunt} = 16\,\Delta V_{\mathrm{cycle}} \quad (15)$$

$$\Delta V_{\mathrm{Na+-A}} = 3\,\Delta V_{\mathrm{ATPshunt-A}} + (2/3)\,\Delta V_{\mathrm{ATP-glu-ox-A}} = 12\,\mathrm{V}_{\mathrm{shunt}} = 24\,\Delta V_{\mathrm{cycle}} \quad (16)$$

Based on cellular measurements and energy budget calculations (Yu et al. 2018) approximately 90% of incremental oxidative ATP generated by the neuron ($\Delta \mathrm{V}_{\mathrm{ATP\text{-}ox\text{-}N}}$) is used to fuel Na^+ and K^+ pumping ($\Delta \mathrm{V}_{\mathrm{K+\ or\ Na+^{-}N}}$).

$$\Delta V_{\mathrm{K+-N}} = 0.9 * 2 * \Delta V_{\mathrm{ATP-ox-N}} = 28.8\,\mathrm{V}_{\mathrm{shunt}} = 57.6\,\Delta V_{\mathrm{cycle}} \quad (17)$$

$$\Delta V_{\mathrm{Na+-N}} = 0.9 * 3 * \Delta V_{\mathrm{ATP-ox-N}} = 43.2\, \mathrm{V_{shunt}} = 86.4\, \Delta V_{\mathrm{cycle}} \quad (18)$$

2.4 Experimental and Theoretical Bases of the Shunt Model

In the following sections, the experimental and theoretical bases of the flux and energetic relationships (Eqs. 6–18) in the model are provided. Potential caveats and justifications are discussed in more detail in Sect. 3.

2.4.1 Glucose Sparing ($\Delta CMR_{glc\text{-}tot\text{-}N} = V_{shunt}$)

The relationships between V_{shunt} and changes in neuronal and astrocyte glucose consumption are derived from the concept of glucose sparing introduced by Swanson (1992) and formalized by DiNuzzo et al. (2012, 2010b, a). During activation, the use of glycogenolysis by the astrocyte reduces its incremental, and potentially total glucose consumption, relative to baseline, thereby sparing the equivalent amount of glucose for the neuron. In the glycogen shunt model, the extreme case of glucose sparing is assumed – in which the only incremental glucose available to the neuron is due to the glucose spared by glycogen consumption in the astrocytes. Although under fed conditions this limitation is unlikely to occur, in Sect. 3 we show that glucose transport can potentially limit brain neuroenergetics even under physiological fasting in the absence of the glycogen shunt. Although this limitation would not occur under nonactivated levels of activity, we predict that the relationship is maintained throughout the neuronal activity range in order to maintain a constant activity independent energetic cost of signaling per glutamate.

2.4.2 V_{cycle} Coupled to the Presynaptic Malate-Aspartate Shuttle and Glucose Oxidation ($\Delta CMR_{glc\text{-}ox\text{-}N} = \Delta V_{cycle}$)

As described in more detail in Sects. 3.6 and 3.7, based on cellular and enzyme localization studies, the maximum rate of glutamate synthesis from glutamine in the neuron is limited by the flux of the pseudo-MAS that removes newly-synthesized glutamate by PAG from the mitochondria (see Fig. 2a, b). The pseudo-MAS requires one reducing equivalent from glycolysis per glutamate removed. The pseudo-MAS model was developed from results of studies in cultured cerebellar glutamatergic neurons, and the NADH was derived from neuronal glycolysis. However, as shown in Fig. 2a, it is also glycogen-derived lactate shuttling in vivo that could potentially provide some NADH for the pseudo-MAS; this possibility is, however, considered highly unlikely and of very low magnitude, if any (see Sect. 3.8). When glucose is the oxidative substrate, the stoichiometry between glutamate synthesis from

glutamine ($V_{glutamate}$) and neuronal glucose oxidation is 0.5 (Hertz and Chen 2017), and, with the further assumption of metabolic steady state, $V_{PAG\text{-}N} = V_{cycle}$:

$$2\,\Delta CMR_{glc-ox-N-presynaptic} = \Delta V_{PAG-N} = \Delta V_{cycle} = 0.5\,V_{shunt} \tag{19}$$

The majority of PAG activity is believed to be localized on the presynaptic side of the neuron. Based on theoretical and experimental measurements (reviewed in Yu et al. 2018), neuronal glucose oxidation is approximately equal between the pre- and postsynaptic regions in the neuron. Substituting equation (19) into Eq. (9) (i.e., $\Delta V_{cycle} = 0.5\,V_{shunt}$) yields Eq. (10) (i.e., $\Delta V_{cycle} = \Delta CMR_{glc\text{-}ox\text{-}N}$).

This relationship is in good agreement with the experimentally-measured relationships between the GABA/glutamate/glutamine cycle and neuronal glucose oxidation (Yu et al. 2018; Lanz et al. 2013) ranging from approximately 0.75 to 0.9 $\Delta V_{cycle.}$ Since the distribution between pre- and postsynaptic glucose oxidation is only approximately known we could choose one of these measured values but have avoided doing so in order that our calculations are independent of previous measurements of these fluxes in nonactivated states. We note that the meta-analyses published to date report the combined neurotransmitter cycling and glucose oxidation fluxes of glutamatergic and GABAergic neurons. A more precise testing of the glycogen shunt model would involve assessing the fluxes in each cell type separately.

As with the relation between V_{shunt} and neuronal glucose uptake and oxidation, we predict this relationship is maintained under non-shunt conditions in order to maintain the fidelity of neurotransmission, as it ensures a constant relationship between the number of glutamate molecules released and energy available from glucose oxidation to support ion pumping and other functional neuronal processes.

Note that the rate of mitochondrial glutamate production and ΔV_{cycle} could be lower than given in Eq. (20), since in the glycogen shunt model the relationship between neuronal and astrocytic functional glucose metabolism is primarily determined by K^+ and Na^+ flows across the cell membranes, as opposed to astrocyte glutamate uptake and lactate release, as in prior ANL shuttle models. Although the reason why the maximum amount of neurotransmitter glutamate is synthesized is not known, there is evidence that the number of glutamate molecules released per vesicle is required to ensure synaptic transmission fidelity with lower amounts leading to reductions in synaptic strength (Scimemi and Beato 2009).

2.4.3 Glycolytic ATP from Glycogenolysis Supports Astrocyte K^+ and Na^+ Pumping (ΔK^+-A = 2 $\Delta V_{ATP\text{-}shunt\text{-}A}$ = 6 V_{shunt})

This assumption in the glycogen shunt model is based upon a significant body of literature in cell and brain slice models as well as inhibitor studies in vivo (see discussion in Sect. 3.9). In addition, DiNuzzo et al. (2017) have estimated the K^+ and Na^+ fluxes across astrocyte membranes as a function of V_{cycle}, allowing direct comparison with the experimentally-measured rates of V_{shunt} and ΔV_{cycle}.

2.4.4 Constancy of the Ratios Between Neuronal and Astrocytic Glucose Metabolism and GABA/Glutamate/Glutamine Neurotransmitter Cycling Across the Full Range of Neuronal Activity

It is assumed in the glycogen shunt model that the metabolic relationships determined by the limiting case of the shunt model are maintained at all levels of neuronal activity. The basis of this assumption is that the membrane depolarizations associated with signaling events, associated with synaptic strength, depend on transmembrane ion flows that are directly coupled to energy metabolism via the Na^+/K^+ and other ATPases. These energy costs account for the very large majority of energy required to sustain brain function (Yu et al. 2018; Harris et al. 2012). Therefore, a change in the ratio between energy production from glucose metabolism and GABA/glutamate/glutamine cycling as a function of activity would mean that synaptic strength would be activity-dependent, impairing the fidelity of signaling.

2.4.5 Approximately-Equal Increment in Oxidative and Nonoxidative Neuronal Glucose Consumption During the Shunt ($\Delta CMR_{glc\text{-}ox\text{-}N} = \Delta CMR_{glc\text{-}nonox\text{-}N} = 0.5\ V_{shunt}$)

At present, the processes that lead to the experimentally-observed relationships between the increment in oxidative and nonoxidative glucose consumption during intense sensory activations are not known. However, a candidate process, which is incorporated into the model (Figs. 2b and 3), is the pumping of glutamate (and GABA) into presynaptic vesicles which requires 1 ATP per glutamate pumped (Ueda 2016). Evidence suggests that this ATP is preferentially derived from glycolysis (Ueda 2016). The stoichiometry of this process is given by:

$$\Delta V_{Glu-vesicle} = 2\,\Delta CMR_{glc-nonox-N-presynaptic} \qquad (20)$$

where $\Delta V_{Glu\text{-}vesicle}$ is the incremental rate of glutamate transport into vesicles, which at steady state is equal to ΔV_{cycle}.

However, since only approximately ½ of $\Delta CMR_{glc\text{-}ox\text{-}N}$ is presynaptic, Eq. (20) can be rewritten as

$$\Delta V_{Glu-vesicle} = \Delta CMR_{glc-nonox} = \Delta V_{cycle} \qquad (21)$$

Under conditions of lower activity where the shunt is not active there presumably is sufficient ATP from oxidative glucose metabolism and glycolysis to supply the energetic needs for this process. The remainder of nonoxidative neuronal glycolysis associated with intense activation would be due to yet unidentified processes requiring rapid ATP availability (e.g., synaptic vesicle recycling (Ashrafi et al. 2017; Ashrafi and Ryan 2017; Rangaraju et al. 2014) and energetics of postsynaptic spines that have few, if any, mitochondria (reviewed in Dienel 2019a).

To summarize, Eqs. (1)–(21) are based on assumptions derived from experimental measurements, mass balance, and flux analyses illustrated in Figs. 1–3. They represent a first approximation of the energetic relationships and can be refined in future studies in which the relationships are tested against data in which the rate of glycogenolysis, total glucose consumption, and total oxidative glucose consumption are measured under a variety of conditions.

3 Discussion of Evidence for and the Impact of the Glycogen Shunt Model: Justifications and Potential Caveats

The glycogen shunt model provides a formal, stoichiometric structure for quantitatively testing experimental data from both brain activation studies and measurements made under nonactivated conditions to investigate the role for astroglial glycogenolysis in determining the relationships between neuronal and astroglial glucose metabolism and function. Of particular importance will be the development of methods that allow real-time measurements of V_{shunt}, V_{cycle}, and pathways of cerebral glucose metabolism simultaneously under activated conditions in each subject.

3.1 The Glycogen Shunt Model Can Explain the Obligatory Need of the Brain for Glucose to Support Functional Processes and the Stoichiometry of Neuronal and Astroglial Glucose Metabolism with GABA/Glutamate/Glutamine Cycling

In this section, we discuss how the glycogen shunt can provide a quantitative explanation for the outstanding questions about the relationship between brain glucose metabolism and function summarized in the Introduction.

3.1.1 The Role of the Glycogen Shunt in the Obligatory Requirement of the Brain for Glucose as a Fuel During Nonactivated and Activated Conditions

Studies have shown that the brain requires glucose to support functional activity with the percentage increasing at higher levels of brain activity (Cremer et al. 1983, 1988; Hargreaves et al. 1986; Cunningham et al. 1986; Cunningham and Cremer 1981). Based on the analysis of experimental data with the shunt model, there are two major components of energetics that require glycolytic ATP from glucose (or

glycogen in the astroglia). In the astroglia, the main process is the restoration of Na^+/K^+ and other ionic gradients via ATPases. In the neurons there is an increase in nonoxidative glucose consumption of ½ V_{shunt}, which we assign to supporting the pseudo-MAS and glycolytic ATP for glutamate and GABA transport into vesicles. These fluxes cannot be replaced by non-glucose oxidative fuel sources, such as lactate or ketones, and they help explain the preferential upregulation of glycolysis during activation (Dienel and Cruz 2016).

The need for rapid glycolytic ATP in the astroglia can partially explain the increase in lactate reported during activation (review of early studies by Korf and de Boer 1990; Korf 1996; other examples in Cruz et al. 2007; Fray et al. 1996; Dienel et al. 2002; Madsen et al. 1999) and under glycogen-depleted conditions some of the increase in nonoxidative glucose metabolism. However, based on the analysis there is also a requirement in the neuron for nonoxidative glycolysis during intense activation. The increase in nonoxidative glucose metabolism would also contribute to the reported increase in lactate levels in the above-cited studies. The findings of Dienel et al. (2002) that after 5 minutes the elevated lactate has the same ^{14}C fractional enrichment as glucose further supports an increase in neuronal nonoxidative glycolysis of blood-borne glucose. As described in Fig. 2 and discussed in more detail below, half of the measured neuronal nonoxidative glycolysis can be attributed to the need for glutamate pumping into vesicles, which has been shown experimentally to require ATP from glycolysis (Ueda 2016). The other processes that contribute to the requirement for neuronal glycolytic ATP at present are not known but could be related to the physical distance between mitochondria and postsynaptic spines and dendrites due to the small size of these structures. Energy budget models suggest that a considerable fraction of functional ATP consumption takes place in these structures in order to support the ion pumping associated with excitatory postsynaptic potentials. Even though, based on a ~1:1 split between neuronal oxidative and neuronal nonoxidative glycolysis, the glycolytic ATP contribution to this process will still be relatively small compared to ATP from glucose oxidation (unlike in the astroglia) it could play a critical role in making up for a short term energy gap at the initiation of the process.

Under nonactivated conditions fuel sources other than glucose can only replace 50% of functional neuronal glucose oxidation (Chowdhury et al. 2014; Quistorff et al. 2008; Smith et al. 2003). The remaining requirement for glucose can be explained by the continued need for glycolytic ATP and NADH production in order to maintain the GABA/glutamate/glutamine cycle through the pseudo-MAS and vesicle filling with glutamate and GABA (see Figs. 2 and 3 and sections below describing the mechanism). The stoichiometry of both of these processes is two glutamates per glucose undergoing oxidative glycolysis resulting in a minimum rate of glucose metabolism of $0.5*V_{cycle}$ as has been pointed out in several publications (see Hertz and Chen 2017).

3.1.2 The Glycogen Shunt and Glucose Sparing, *Not Glutamate Uptake into Astrocytes*, Determine the Relationship Between Astroglial and Neuronal Functional Energetics

As shown in Fig. 1 and Eqs. (1)–(5), over the last two decades experimental measurements, largely using MRS, have found a linear relationship between neuronal and astroglial glucose metabolism and GABA/glutamate/glutamine cycling. The linear relationship has close to 1:1 stoichiometry with total cerebral glucose oxidation (Fig. 1a) which led to earlier proposals, reviewed in the Introduction, that glutamate itself was responsible for the coupling via the need for glycolytic ATP in order to provide energy for its transport into the astroglia after its release from neurons (Fig. 1b). The glucose taken up by the astroglia to provide this ATP would subsequently be released as lactate and taken up by the neurons to provide oxidative ATP production.

In the revised glycogen shunt model (Fig. 3) proposed here, in contrast to glycolytic ATP requirements for astroglial glutamate transport being the coupling mechanism, the coupling is due to the requirement of glycolytic ATP from glycogenolysis to provide ATP to support close to the full incremental astroglial ion pumping requirements. The glucose spared by the use of glycogen instead of blood-borne glucose determines the maximum energy available to the neuron to support function. The proposal that glycogenolysis could help support neuron energetics via glucose sparing was originally put forth by Swanson (1992) and formalized with metabolic modeling by DiNuzzo, Mangia, and colleagues (DiNuzzo et al. 2010b, 2012), as was the proposal that total ion fluxes could couple neuronal and glial functional metabolism (DiNuzzo et al. 2013, 2017; Mangia et al. 2013).

When glycogen cannot be used, which physiologically could happen if glycogen stores are depleted, the astroglia replace it with glucose uptake and nonoxidative metabolism at approximately 3/2 times (i.e., to compensate for less ATP yield by glycolytic metabolism of glucose *vs*. glycogen) the rate of neuronal glucose consumption. The increased demand on brain glucose supplies would result in conditions where neuronal glucose oxidation was not sustainable. The neuron could then sacrifice nonoxidative glycolysis, which fuels processes mainly related to glutamate synthesis and vesicle packing which the cell could go without for an extended time due to the large reserve in the cytosolic glutamate and glutamate vesicle pools (Ottersen et al. 1990a, b, 1992), while protecting the much larger ATP yield from oxidative glycolysis which supports the ion pumping needed to sustain functional activity.

3.1.3 The Glycogen Shunt in Combination with the Pseudo-MAS Mechanism Determines the Stoichiometry Between Cerebral Glucose Metabolism and GABA/Glutamate/Glutamine Neurotransmitter Cycling

In the previous models of the glycogen shunt the coupling of the GABA/glutamate/glutamine cycle to glucose uptake was via astroglial glutamate uptake and glutamine synthesis (Hyder et al. 2006; Shulman et al. 2001a). Neuronal and astroglial

glucose metabolism is then coupled via the ANL shuttle (Pellerin and Magistretti 1994). In the present model the coupling between neuronal and astroglial glucose metabolism is primarily through the relative ATP available under activated conditions for ion pumping. The relationship between V_{cycle} and $CMR_{glc\text{-}ox}$ is through the pseudo-MAS mechanism proposed by Hertz and Chen (2017) and described in Sects. 1 and 2, and discussed in more detail in Sects. 3.6 and 3.7, as opposed to direct astroglial coupling based on glutamate uptake (Pellerin and Magistretti 1994).

The pseudo-MAS mechanism predicts that the transport of a glutamate molecule derived from glutamine from the neuronal mitochondria to the cytosol requires an NADH reducing equivalent from *neuronal* glycolysis (see Sect. 3.8 that discusses why glycogen-derived lactate cannot support this process due to insufficient oxygen consumption to support oxidation of this lactate in neurons). The reducing equivalent via coupling to glucose oxidation and the electron transport chain drives the MAS with glutamate being transported out of the mitochondria for every NADH-derived reducing equivalent transported inward (see Fig. 2a, b). Based on the measured relationship between incremental neuronal glucose oxidation and GABA/glutamate/glutamine cycling (Eq. (2)), close to the maximum rate achievable by the pseudo-MAS mechanism is used. The high rate of synthesis is consistent with studies that have shown reducing cytosolic GABA and glutamate concentration results in impaired synaptic signaling (for a review see Scimemi and Beato 2009).

3.2 The Glycogen Shunt Helps Maintain Brain Glucose Homeostasis

The need for glucose sparing by the glycogen shunt appears inconsistent with the high amount of brain glucose delivery. Cerebral blood flow increases the same or more than glucose metabolism during sensory stimulation and seizure and as a result glucose delivery is always sufficient to meet the demands for both glycolytic and total ATP. However the brain also needs to maintain a low concentration of glucose relative to the blood as well as maintain homeostasis of glycolytic intermediates during large changes in glycolysis. As discussed in recent publications (Shulman and Rothman 2015, 2017), a key role of the glycogen shunt is to maintain low levels of intracellular glucose and glycolytic intermediates despite large increases in the glycolytic rate. By either glycogen synthesis taking up glucose or glycogenolysis releasing glucosyl units, glucose transport can be optimized to meet the average needs of the cell with the glycogen acting as a buffer which provides energy in the place of glucose during high activity or takes up excess glucose entering the brain during low metabolic activity or high postprandial plasma glucose levels (also see Swanson 1992). As a consequence, brain glucose transport activity can be reduced so that, under average activity, brain glucose concentrations are ~20% of those in the blood. The advantage of maintaining a low glucose level is due to the high reactivity of the glucose molecule as shown nonspecific glycation reactions being a major cause of

cellular damage in diabetes (Giacco and Brownlee 2010; Rowan et al. 2018). Brain glucose levels in diabetic animal models rise from a control level of ~2 to ~7 μmol/g when the corresponding plasma levels are ~9 and 27 mM, respectively (Gandhi et al. 2010), indicating that brain glucose levels approaching 75–80% of the normal plasma level would cause diabetic complications.

Because of glycogen being predominantly localized to the astrocytes, its role as a buffer for providing energy to the neuron when overall glucose transport is inadequate is complicated relative to muscle and other tissues where glycogen is found in all cells. In the glycogen shunt model, the mechanism of energy provision is by glucose sparing with the astroglia obtaining their incremental, and under extreme conditions, possibly total energy needs largely from glycogenolysis. However, this mechanism assumes that there is an actual need for glucose sparing. In order to test this possibility, we calculated the maximum glucose transport under euglycemic and physiological hypoglycemic conditions in awake humans to assess the maximum increase in glucose consumption that can be supported with and without the glycogen shunt. In addition, we review studies that support the ability of the brain to sustain the increased electrical response to continuous activation is impaired under mild to moderate hypoglycemia.

3.2.1 Calculation of the Maximum Activation Sustainable in the Absence of the Glycogen Shunt

The transport parameters used for the calculation were taken from the ^{1}H MRS study of de Graaf et al. (2001), who separately measured these parameters for gray and white matter. These parameters were similar to those reported in other human studies (Gruetter et al. 1996, 1998; Seaquist et al. 2001; de Graaf et al. 2001). Parameters from human studies were used because they were measured in the awake state. The majority of studies on rodents have been performed under anesthesia. About 40% of the endothelial glucose transporter molecules are localized in the cytoplasmic space, providing a mechanism for upregulation of transport capacity across the blood-brain barrier (Farrell and Pardridge 1991). Due to glucose transport recruitment with increased activity (Hargreaves et al. 1986; Cremer et al. 1983; Cunningham and Cremer 1981), studies performed under conditions of low neuronal activity may underestimate transport capacity in the awake state.

The reversible two state Michaelis-Menten model was used for the calculations of glucose transport. Although a four state model more accurately describes the molecular mechanism of glucose transport, it has been shown that the two state model accurately reproduces the kinetics of glucose transport (Duarte and Gruetter 2012; Duarte et al. 2009; Simpson et al. 2007). Maximum glucose transport capacity was calculated using the approach of de Graaf et al. (2001) for calculating total glucose uptake capacity but without including hexokinase kinetics (equivalent to assuming K_m of hexokinase ~ 0) (Eq. 22). The parameters in the model are the volume of distribution of glucose (V_d), the half maximum transport constant (K_t), and the ratio of the maximum unidirectional rate of transport (V_{max}) over the rate of total

glucose metabolism ($CMR_{glc\text{-}tot}$). For a given concentration of plasma glucose (G_o), the maximum net transport rate occurs when intracellular glucose (G_i) is at 0 concentration

$$G_i = -Vd\left[\left(\left(V_{max} / CMR_{glc-tot}\right)-1\right)G_o - K_t\right] / \left[\left(V_{max} / CMR_{glc-tot}\right)+1\right] \quad (22)$$

Setting G_i in Eq. (22) equal to 0 and rearranging yields the following expression

$$CMR_{glc-tot-max} = V_{max} / \left[\left(K_t / G_o\right)+1\right] \quad (23)$$

In the majority of MRS glucose transport studies, the ratio ($V_{max}/CMR_{glc\text{-}tot\text{-}b}$) (where the subscript b denotes baseline) has been measured as opposed to V_{max} directly. To incorporate this ratio, Eq. (23) can be rearranged into the following expression with F_{max} given by the fractional increase in $CMR_{glc\text{-}tot}$ above the baseline value.

$$\left(1+F_{max}\right) = \left(V_{max} / CMR_{glc-tot-b}\right) / \left[\left(K_t / G_{o)}\right)+1\right] \quad (24)$$

Using the transport constant values reported by de Graaf and coworkers for human gray matter (K_t = 1.1 mM, $V_{max}/CMR_{glc\text{-}tot\text{-}b}$ = 3.2), the maximum sustainable fractional increase in glucose transport is F_{max} = 0.88 for a plasma glucose concentration under fed conditions of 5.5 mM (Hwang et al. 2019) and a value of F_{max} = 0.58 for a plasma glucose concentration of 3.5 mM which can occur under physiological conditions such as a 2 day fast (Rothman et al. 1991).

Under physiological hypoglycemia the maximum sustainable activation value is just above that reported from PET studies during sensory stimulation of 0.2 – 0.5 (see Table 1 in (Shulman et al. 2001b) during sensory stimulation.

However, in the absence of availability of glycogen, the astroglia need to increase their glucose consumption to meet the demand for glycolytic ATP. Due to the 2/3 lower efficiency of net ATP production per plasma glucose molecule versus glycogenolysis for a given increment in activity incremental glucose consumption would increase by

$$CMR_{glc-tot-no\,shunt} = CMR_{glc-tot\,shunt} + \left(3/2\right)V_{shunt} \quad (25)$$

From Eq. (25) it can be seen that in the absence of glycogenolysis the rate of glucose consumption during activation could exceed glucose transport capacity in human cerebral cortex. Furthermore, similar larger increases in functional glucose metabolism have been measured in animal models (e.g., Collins et al. 1987; Dienel et al. 2007b; Dienel et al. 2007a; Cruz et al. 2007; Cruz et al. 2005; Nakao et al. 2001). In addition, based on high resolution [^{14}C]DG autoradiography sub-regions within the sensory cortices (e.g., layer 4 and regions such as visual cortex columns) below the resolution of PET may have considerably higher increases in sustained glucose metabolism (Sokoloff 1981).

To summarize, glucose transport can potentially become limiting for brain metabolism during activation under hypoglycemic conditions. Although glucose transporter upregulation can occur after a lag, during the transient period at the start of an activation the switch of the astrocyte to glycogenolysis may spare sufficient glucose for the neuron to maintain functional activity. More work is required to establish the time courses and magnitudes during activation of glycogenolysis and glucose transporter mobilization at the blood brain barrier and in brain cells (also see Sect. 3.9.2).

3.2.2 Evidence that Intense Activation Cannot Be Sustained for Extended Periods Under Mild to Moderate Hypoglycemic Conditions in the Absence of Glycogen

It is well established in animal and human studies that sustained moderate hypoglycemia leads to an impairment of stimulation-induced increases in cortical activity, as measured through evoked potentials in humans and local field potentials and multi-unit activity in animal studies (Herzog et al. 2013; Amiel et al. 1991). Unfortunately, a direct measurement of the impact of blocking the glycogen shunt on the maximum supportable activation has not yet been performed. However, Swanson and coworkers (Suh et al. 2007) have shown that increasing brain glycogen concentration by 88% using a displaceable phosphorylase inhibitor (CP-316,819) increased the ability of the rats in the awake state to maintain brain electrical activity by 91 minutes under hypoglycemic conditions, consistent with the glycogen shunt sparing glucose consumption. Similar results have been reported by Gruetter, Öz, and coworkers when glycogen was super-compensated by pre-depletion and infusion of glucose and insulin (Duarte et al. 2017; Öz et al. 2012, 2017; Choi et al. 2003).

In addition to physiological stimulation, Blennow and coworkers looked at the impact of physiological hypoglycemia induced by 24 h of fasting, on the ability of rats to sustain seizure activity, as assessed by EEG (Blennow et al. 1977a, 1979). After the induction of seizure, both the increase in $CMR_{glc\text{-}ox}$ (measured by A-V difference for O_2) and electrical activity was the same in the fasted and fed groups. Consistent with glucose sparing by the glycogen shunt, after depletion of glycogen, the EEG of the fasted rats with plasma glucose levels below 3 mM ceased showing the burst and suppress pattern characteristic of bicuculline induced seizure (which was replaced by single spikes) and CMR_{O2} returned to pre-seizure values. Burst and suppress EEG activity could be restored with an injection of glucose but ceased after several minutes. Additional evidence for the importance of the glycogen shunt in sustaining seizure activity is that the level of lactate in fasted versus fed animals at 5 minutes differed by only approximately 1.5 μmol/g (~8.5 versus 7 μmol/g; see Fig. 4 in (Blennow et al. 1979)) despite the average brain glucose level during the first 5 minutes of seizure being approximately three-fold higher in fed rats (3 versus 1 μmol/g). If lactate were a major fuel that is rapidly and preferentially consumed compared with glucose during intense activity, its concentration should have been

depleted, particularly in the fasted animals. Burst and suppress electrical activity in the fasted rats could be transiently restored by injection of glucose, underscoring the importance of glucose compared with lactate.

3.3 *Factors Determining the Relative Rates of Oxidative and Nonoxidative Neuronal Glucose Consumption*

Half of the measured increment of nonoxidative glucose consumption could be explained based on studies that have shown that the vacuolar H^+-ATPase that pumps protons into synaptic vesicles requires an estimated 1 ATP per 2 H^+ in order to maintain the proton gradient in vesicles; glycolytic ATP is preferentially used to fuel the proton pump, but the stoichiometry between glutamate pumping by VGLUT to load the vesicles and H^+, and Cl^- is not fully understood (Ueda 2016). The stoichiometric relation to nonoxidative glycolysis of this mechanism has been discussed in detail by Hertz and Chen (2017).

Hertz and Chen (2017) have also proposed that an additional need for nonoxidative glycolysis may be due to the need for redox equivalents to move glutamate out of the mitochondria using the pseudo-MAS (see Sects. 3.6 and 3.7, below). If these processes were separate, meaning that the ATP generated from glycolysis used to produce reducing equivalents was not involved in vesicle glutamate transport, they would explain the total measured nonoxidative glycolysis. However, given the close spatial proximity in the presynaptic terminals, complete separation is unlikely. In addition, the MAS mechanism can only operate transiently without an equal increase in the TCA cycle (and oxidative glucose and oxygen consumption) to remove the remove accumulating redox equivalents via the electron transport chain. As illustrated in Fig. 2a, b, if all of the NADH produced by glycolysis were solely used by the pseudo-MAS and the mitochondrial NADH were oxidized in the electron transport chain, pyruvate would accumulate in the cytoplasm. The pyruvate could not be converted to lactate because NADH is not available for the lactate dehydrogenase (LDH) reaction, and it would have to be oxidized via the TCA cycle or exported from the cell.

3.4 *Mechanism(s) of Coupling Between Neuronal Glutamatergic Activity and Glycogenolysis*

Relationships between glycogenolysis and neuronal activity are technically-difficult to study and are poorly understood. A number of reports using glycogen phosphorylase inhibitors or glycogen synthase knockout have implicated glycogen in neurotransmission and long-term potentiation in vitro and in vivo (Sickmann et al. 2009; Suzuki et al. 2011; Duran et al. 2013, 2019; Drulis-Fajdasz et al. 2015; Mozrzymas

et al. 2011). However, the mechanisms by which glycogenolysis blockade affects neuronal functions remain to be established. A number of astrocytic processes are preferentially fueled by glycogen, and their disruption could secondarily affect neuronal activities, as discussed in detail elsewhere (Dienel 2019a, b; Dienel and Rothman 2019). Some possibilities include glucose supply deficits when glucose sparing is impaired, slower K^+ uptake from the synaptic cleft during high synaptic activity, abnormal astrocytic Ca^{++} homeostasis and gliotransmitter release to alter neuronal firing, reduced filopodial extensions from astrocytes to neurons to impair efficiency of K^+ and glutamate uptake from the synaptic cleft that may result in glutamate spillover and its interaction with presynaptic receptors to reduce firing. These and other possibilities remain to be evaluated experimentally.

Magistretti and colleagues reported that exposure of brain slices to glutamate does not stimulate glycogenolysis (Magistretti et al. 1981), whereas glycogenolysis is stimulated by K^+ (Quach et al. 1978) and it is linearly related to extracellular K^+ concentration over the range of 5–12 mM (Hof et al. 1988). Details of the signaling pathways involved in astrocytic K^+ uptake and glycogenolysis were worked out by Hertz and colleagues (Xu et al. 2013) when they followed up the hypothesis by Mangia, DiNuzzo and colleagues that K^+ uptake is fueled by glycogen (DiNuzzo et al. 2013, 2012, 2014; Mangia et al. 2013). Hertz and colleagues subsequently reviewed the details of linkage of Na^+,K^+-ATPase activity, astrocytic K^+ uptake, and glycogen mobilization (Hertz et al. 2015a, b, c). Clearly, ion fluxes associated with neurotransmission couple astrocytic glycogen mobilization with neuronal signaling.

Sickmann et al. (2009) evaluated effects of inhibition of glycogenolysis on glutamatergic neurotransmission in cerebellar astrocyte-neuron co-cultures, and concluded that glycogen-derived lactate shuttling to neurons is required to maintain excitatory neurotransmission. In brief, they depolarized the neurons and measured vesicular D-[^{3}H]aspartate (Asp) release from neurons and its reuptake mainly by astrocytes as representative of glutamate transmission. Inhibition of glycogenolysis with CP-316,819, but not with 1,4-dideoxy-1,4-imino-d-arabinitol (DAB), reduced release and re-uptake, and inclusion of 1 mM D-lactate to block L-lactate uptake into neurons further impaired release and reuptake.

Failure of DAB to impair neurotransmission seriously weakens the argument that lactate derived from glycogen is a necessary neuronal fuel. Also, D-lactate is likely to inhibit pyruvate uptake into mitochondria, reducing oxidative production of ATP and creating an energy crisis in both astrocytes and neurons that will stimulate glycolysis and lactate production (Dienel 2019a, b). Lactate released from astrocytes (their lactate transporters are not affected by 1 mM D-lactate) in higher amounts may interact with the neuronal lactate receptor HCAR1 to reduce excitatory and inhibitory firing (Bozzo et al. 2013) and interfere with neurotransmission. HCAR1 is mainly localized at pre- and postsynaptic sites of excitatory neurons (Lauritzen et al. 2014). In contrast, upregulation of glycolysis and lactate production in neurons will result in intracellular lactate accumulation due to the low K_m of MCT2 and its inhibition by D-lactate. The combination of CP-316,819 + D-lactate treatment during excitatory conditions deprives astrocytes of an important fuel and causes neuronal energy failure that will depress neurotransmission and glutamate uptake without

lactate shuttling. With the popularity of the notion of astrocyte-neuron lactate shuttling, whether from glucose or glycogen, many authors interpret various findings as supportive of the idea without providing any direct experimental evidence. While there could conceivably be some glycogen-derived lactate shuttling, oxygen consumption is much too low to oxidize all carbohydrate consumed during activation (Dienel and Rothman 2019).

3.5 Evidence for Glucose Sparing Versus the ANL Shuttle as the Mechanism by Which Astroglia Increase Neuronal Fuel Availability During Activation

The hypothesis by Swanson (1992) and DiNuzzo and colleagues (2012; 2011; 2010a, b) that glycogenolysis spares glucose for neurons has not, to our knowledge, been tested in vitro or in vivo. This is an extremely difficult undertaking because it requires rigorous, quantitative procedures to measure the quantity of glycogen consumed, the amount of extracellular glucose metabolized by astrocytes, and the additional glucose consumed by neurons. Use of the glycogen shunt model developed herein can be used to test the ability of glycogen to satisfy the energy demands of Na^+-K^+ fluxes and V_{cycle} in astrocytes and to show that the equivalent amount of glucose consumed by neurons is sufficient for ATP expenditures for these processes in neurons, consistent with glucose sparing.

If, as proposed by DiNuzzo et al. (cited above), glycogenolysis increases the concentration of glucose-6-phosphate (Glc-6-P) in astrocytes to further inhibit hexokinase to an extent such that extracellular glucose is not further metabolized, the intracellular glucose concentration should rise. Indeed, when Prebil et al. (2011) treated cultured astrocytes with adrenaline or noradrenaline to trigger glycogenolysis, the cytosolic glucose level rose by 55%. This increase was prevented by DAB, consistent with reduced glucose utilization when glycogen is mobilized. The glucose is not produced by the action of Glc-6-phosphatase because cultured astrocytes do not release glucose to the culture medium when glycogen is degraded (Dringen et al. 1993). Although very low Glc-6-phosphatase activity is detectable in brain and brain cells, it does not convert Glc-6-P back to glucose in detectable amounts in vitro or in vivo (Gotoh et al. 2000; Dienel et al. 1988; Nelson et al. 1985, 1986). The limiting factor for phosphatase activity is extremely slow transport of Glc-6-P into the endoplasmic reticulum (compared with glycolytic rate) where it can be dephosphorylated by phosphatase (Dienel 2019c).

Fray et al. (1996) reported that extracellular lactate and glucose concentrations determined in microdialysates from awake rats are differentially regulated. Stimulating glycogenolysis with isoprenaline, a nonspecific β-adrenergic agonist, caused the glucose level to rise while lactate level was unchanged. Treatment with propranolol, a nonspecific β-adrenergic antagonist, blocked the rise in glucose level evoked by isoprenaline. A tail pinch caused both glucose and lactate levels to rise, and propranolol

blocked the increase in glucose but not lactate. Failure of propranolol to abolish the rise in lactate level yet prevent the increase in glucose is consistent with the rise in glucose level secondary to astrocytes consuming less glucose when glycogen is mobilized. These findings also suggest that the lactate is not derived from glycogen. Instead, it comes from (perhaps neuronal) metabolism of blood-borne glucose. In both of these two examples, further work is required to evaluate the sources of glucose and lactate, but carefully-designed metabolic labeling studies can advance the field.

There is a fundamental conflict between the models in which glycogen spares glucose and shuttling of glycogen-derived lactate fuels activated neurons. In conjunction with the lactate shuttling concept, lactate has often been called the preferred brain fuel over glucose. Lactate can indeed be a supplemental brain fuel (and reduce glucose utilization by feedback mechanisms) when its plasma concentration rises during infusion or vigorous exercise. However, oxidation of available lactate does not substantially increase when present with glucose at normal concentrations in activated brain cells during intense activity. For example, cultured glutamatergic neurons do not increase lactate oxidation when stimulated in the presence of glucose plus lactate; instead, more glucose is oxidized (Bak et al. 2006, 2009, 2012). During seizures, glycogen is degraded, glycolysis is upregulated, and lactate accumulates to high levels in brain; both glucose and oxygen consumption rise substantially but OGI falls indicative of enhanced glycolysis (Chapman et al. 1977; Borgström et al. 1976). If lactate produced within the brain were the preferred oxidative fuel compared with glucose, OGI should be stable or increase above 6 if glycogen is oxidized (but not included in the OGI calculation). In starved rats subjected to seizures, the brain glucose level falls and lactate concentration rises to a level similar to that in fed, seizing rats (Blennow et al. 1977a, b). If lactate were an important neuronal fuel, especially when glucose supply is inadequate, lactate should be avidly consumed, substantially reducing or preventing its accumulation in brain. This is not, however, the observed experimental outcome.

3.6 Evidence that the MAS Is Required for Neuronal Oxidation of Glucose

Glycolytic metabolism of glucose consumes NAD^+ and produces NADH at the glyceraldehyde-3-phosphate dehydrogenase step. Because NAD^+ and NADH are present in brain in low concentrations (Medina et al. 1980), these compounds serve a catalytic role. The MAS regenerates cytoplasmic NAD^+ by transferring reducing equivalents from NADH in the cytosol to mitochondria so that pyruvate is available as an oxidative fuel. The MAS involves two shuttle systems across the mitochondrial membrane, one exchanges glutamate for aspartate and the other exchanges malate for α-ketoglutarate (as illustrated in Fig. 2a, except that the glutamate is from the cytoplasmic pool, not from glutamine as shown). The regulatory step is electrogenic influx of glutamate into the mitochondrial matrix along with a proton (LaNoue and Tischler 1974). NAD^+ can

also be regenerated by the cytoplasmic LDH reaction to produce lactate that, when it diffuses from the cell, eliminates pyruvate as an oxidative fuel.

Many studies have shown that the MAS is essential for glucose oxidation in brain in vivo, in brain slices, and in isolated presynaptic endings (synaptosomes). In all three model systems, inhibition of the MAS substantially reduces glucose oxidation, increases the lactate/pyruvate ratio, stimulates lactate efflux, and reduces phosphocreatine (PCr) and ATP concentrations (Fitzpatrick et al. 1983; Kauppinen et al. 1987; Cheeseman and Clark 1988; McKenna et al. 1993). Similarly, respiration in cultured cortical neurons is reduced by half when the aspartate-glutamate transporter component of the MAS is knocked out (Llorente-Folch et al. 2013). Furthermore, L-lactate can rescue glutamate toxicity in wildtype neurons but not in those in which the MAS was knocked out (Llorente-Folch et al. 2016). Thus, MAS flux is a required pathway that is tightly integrated with brain and neuronal glucose oxidation.

Some authors (e.g., Schurr 2014) believe that lactate is the final product of glycolysis and that lactate is transported into mitochondria where it is oxidized by mitochondrial LDH to pyruvate that enters the TCA cycle. However, if lactate were the primary (or exclusive) neuronal oxidative substrate, it should have been able to support the rise in ATP demand caused by glutamate exposure, and it did not (Llorente-Folch et al. 2016). As noted above, partial inhibition of the MAS also led to glycolytic upregulation, lactate accumulation and release, and ATP deficits. These findings demonstrate that direct mitochondrial uptake and oxidation of lactate that accumulates when the MAS is impaired cannot support brain and neuronal energetics. Another strong argument against lactate being the major brain oxidative fuel is that LDH is long-considered to be a cytoplasmic marker in brain subcellular fractionation studies (Johnson and Whittaker 1963; Tamir et al. 1972). Analysis of the enzymatic composition of purified brain mitochondria demonstrated that <1% of the total LDH activity in the whole homogenate is recovered in mitochondria (Lai et al. 1977; Lai and Clark 1976). It is, therefore, not clear how such a small fraction of the total LDH can metabolize all of the lactate generated in the cytoplasm. The concept of lactate being the predominant oxidative fuel in brain is highly unlikely to be correct, and if it occurs, it must be a minor flux.

Ignoring the semantic argument whether pyruvate or lactate is the final product of glycolysis, the brain lactate/pyruvate ratio is about 10-14 to 1 (Veech et al. 1973; Siesjö 1978; Nordström 2010). This high ratio indicates that lactate rapidly equilibrates with pyruvate and raises the possibility lactate may be a dynamic buffer or reservoir for pyruvate, since α-ketoacids are chemically less stable than carboxylic acids. Lactate does serve as an overflow mechanism when glycolytic rate exceeds that of the MAS. The low K_m (0.7 mM) for the neuronal lactate transporter MCT2 (Bröer et al. 1999) restricts neuronal lactate influx and efflux when its concentration increases, and reduced lactate efflux during high glycolytic activity was proposed to help maintain a high intracellular substrate levels for neuronal oxidation (Chih and Roberts Jr 2003; Chih et al. 2001).

3.7 *Involvement of the pseudo-MAS for Conversion of Glutamine to Transmitter Glutamate*

Studies by the Hertz-Schousboe group elucidated mechanisms for conversion of glutamine to transmitter glutamate in cultured cerebellar glutamatergic granule neurons (reviewed in Schousboe et al. 2013). The phosphate-activated glutaminase (PAG) is thought to be located on the outer surface of the inner membrane of mitochondria, and the glutamate produced by PAG could either be released to the cytoplasm and pumped into synaptic vesicles, or it could enter the mitochondria, be metabolized and exit via the pseudo-MAS prior to vesicular loading (Fig. 2a). Their work demonstrated that inhibition of aspartate aminotransferase and the ketodicarboxylic acid carrier reduced transmitter glutamate release from cultured glutamatergic neurons (Palaiologos et al. 1988, 1989; Bak et al. 2008; Ziemińska et al. 2004). Based on these and related findings, Hertz and Chen (2017) formulated the model illustrated in Fig. 2a to explain half of the stoichiometric relationship between glucose oxidation and glutamate cycling.

MRS studies revealed a linear 1:1 relationship between neuronal glucose oxidation and V_{cycle} (Fig. 1a), and involvement of the pseudo-MAS in transmitter glutamate production can explain half of this relationship, i.e., 0.5 glucose oxidized per glutamate cycled, and all of it when presynaptic glucose oxidation is taken as half of total neuronal oxidation. In this model, based on data obtained in cultured neurons, the NADH that participates in the pseudo-MAS must have been produced by neuronal glycolysis. Under resting conditions or under anesthesia when the oxygen-glucose index is close to 6 (i.e., nearly all of the glucose is oxidized), the 'true' MAS could oxidize the second NADH produced by glycolysis so that the presynaptic glucose oxidation:V_{cycle} stoichiometry is 1:1 (Fig. 2b). As long as the rates of the 'true' MAS and pseudo-MAS are matched, the 1:1 relationship could be maintained in presynaptic nerve endings even if there is disproportionate upregulation of glycolysis compared to oxidative metabolism. Other indentified mechanisms may also contribute to the 1:1 relationship.

3.8 *Evidence Against Glycogen-Derived Lactate Shuttling Coupled with Neuronal Lactate Oxidation to Support the pseudo-MAS for Conversion of Glutamine to Transmitter Glutamate*

An alternative in vivo mechanism to provide the NADH for the pseudo-MAS (instead of or supplementing neuronal glycolysis) is influx of extracellular lactate and its oxidation to pyruvate + NADH by LDH (Fig. 2a). In other words, glycogen-derived lactate shuttling from astrocytes to neurons may be coupled with the glutamate-glutamine cycle via the pseudo-MAS and serve as the mechanism linking glycogenolysis to

glutamatergic neurotransmission. However, there is no compelling experimental evidence for this possibility. Shuttling has never been experimentally demonstrated to take place, and nearly all supportive evidence for lactate shuttle models is circumstantial and most is derived from immature cultured cells. In contrast, there is very strong in vivo evidence against lactate shuttling and use of lactate as major fuel during activation (reviewed in Dienel 2012, 2017, 2019a; Dienel and Cruz 2016).

In brief, the major arguments against lactate shuttling are as follows: Glutamate-evoked production of lactate is not a robust characteristic of cultured astrocytes, and many labs reported either no effect of glutamate treatment on astrocytic glycolysis or an inhibition of glycolysis (Dienel 2012; Dienel and Cruz 2004). Furthermore, the stoichiometry required by the glutamate-stimulated glycolysis shuttle model was not satisfied (Dienel 2017) in the original publication of the model by Pellerin and Magistretti (1994). The model has never been quantitatively validated and lactate shuttling has never been directly demonstrated. More important, lactate shuttling tightly coupled with its oxidation requires a parallel, quantitative rise in oxygen consumption (CMR_{O2}). However, measured increases in CMR_{O2} cannot support the oxidation of the carbohydrate consumed during activation, and when glycogen is included in the OGI calculation, the discordance increases substantially, as discussed in our companion article (Dienel and Rothman 2019). Metabolic studies have demonstrated that labeled products of ^{14}C labeled glucose are not quantitatively retained in activated brain tissue. This is important because label retention would occur if lactate were oxidized and the label incorporated into the large TCA cycle-derived amino acid pools. Detailed analysis of the basis for label loss identified lactate as the predominant, diffusible metabolite that carries label and carbon from the brain, explaining the fall in OGI, at least in large part. Upregulation of the pentose phosphate shunt pathway (PPP) during activation with recycling of the fructose-6-phosphate back into the PPP can also make a large contribution to the fall in OGI due to loss of labeled and unlabeled carbon one from blood-borne [1-^{14} or 1-^{13}C]glucose. Label is lost from carbon one in the first pass through the PPP and with extensive recycling, the resulting unlabeled fructose-6-P that that re-enters the PPP as glucose-6-P due to high isomerase activity will lose unlabeled CO_2 from carbon one, contributing to the fall in OGI to an extent varying with magnitude of recycling. The bottom line is that coupling of glycogen-derived lactate shuttling with neuronal transmitter glutamate synthesis via the pseudo-MAS is not a viable explanation for a critical role of glycogenolysis in the glutamate-glutamine cycle.

The following examples explain why it is highly unlikely for glycogen-derived lactate to be shuttled to neurons and metabolized as a major oxidative fuel using either the ‘true’ MAS to transfer the reducing equivalents produced by lactate dehydrogenase or to provide NADH for the pseudo-MAS to power the conversion of glutamine to glutamate and generate neurotransmitter glutamate.

- In the studies by Madsen et al. (1998, 1999), the respective metabolic rates during rest and activation for CMR_{O2} were 4.13 and 5.35 μmol/min/g and for CMR_{glc} were 0.75 and 1.08 μmol/min/g, giving incremental increases of 1.22 and

0.33 μmol/min/g, respectively. If all of this glucose were oxidized, it would consume 1.98 μmol O_2/min/g (6∗0.33), more than that of the oxygen actually utilized. Oxygen cannot support complete oxidation of the glucose taken up into brain, let alone the glycogen consumed.

- V_{shunt} in the same brains of the above Madsen studies calculated from net glycogen consumption in 6 min was 0.42 μmol/min/g (12.1-9.6 = 2.5 μmol/g; 2.5/6 = 0.42) (Cruz and Dienel 2002). Thus, the total incremental rate of carbohydrate (glucose plus glycogen) consumed is 0.33 + 0.42 = 0.75 μmol/g/min, the oxidation of which would require 4.5 μmol O_2/g/min. The incremental CMR_{O2} accounts for only 27% of the required amount to oxidize the carbohydrate, ignoring the oxygen consumed by transmitter glutamate oxidation. The fractions of each fuel oxidized in neurons and astrocytes remains to be established, but it is clear that most of the carbohydrate consumed cannot be oxidized.
- In the Madsen study, ΔV_{cycle} was calculated to be 0.2 μmol/g/min (on-going unpublished analysis of these data), which corresponds to 48% of V_{shunt} (0.2/0.42). From Eq. (7) of the glycogen shunt model, $\Delta CMR_{glc\text{-}ox\text{-}N} = 0.5\, V_{shunt} = 0.21$ μmol/g/min, the calculated neuronal rate of oxidation of glucose spared by glycogenolysis. $\Delta CMR_{glc\text{-}ox\text{-}N}$ would consume 1.26 μmol O_2/g/min, which is essentially equal to the measured incremental rise in CMR_{O2} of 1.22 μmol O_2/g/min. If all of the glycogen consumed were converted to lactate and shuttled to neurons for oxidation, it would require 2.52 μmol O_2/g/min (0.42∗6). In other words, after neuronal oxidation of the half of spared glucose there is no oxygen left over to oxidize lactate produced from glycogen in astrocytes.
- V_{cycle} consumes 1 NADH per glutamate inserted into the neurotransmitter pool from glutamine. If shuttled lactate provided all of this NADH, the lactate shuttle plus oxidation rate would be equal to the incremental rise in V_{cycle}, or 0.2 μmol/g/min (in hexose units). This would require 1.2 μmol O_2/g/min (6∗0.2), equal to all of the incremental rise in CMR_{O2}.This would mean that neurons could not have an incremental rise in glycolysis to satisfy the processes fueled by glycolytic ATP, e.g., vesicular glutamate packaging (Ueda 2016) and to oxidize the generated pyruvate to fuel other processes via ATP from the TCA cycle.
- Diaz-Garcia et al. (2017, 2018) clearly demonstrated that extracellular lactate derived from astrocytic glucose or glycogen was *not* consumed by hippocampal neurons during electrical stimulation or in whisker barrel cortex in vivo during whisker stimulation. This finding goes against the possibility that glycogen-derived lactate shuttling to provide NADH for the pseudo-MAS and conversion of glutamine to transmitter glutamate.
- The alternative mechanism proposed in the present study and supported by data from studies using a variety of experimental conditions strongly support the hypothesis that glycogenolysis fuels astrocytic energy demands related to Na^+,K^+-ATPase and Glu-Gln cycling, and glucose spared for neurons also accounts for the ATP required for these processes.

3.9 *Evidence for Astrocytic and Neuronal Processes Requiring Glycolytic ATP*

3.9.1 Astrocytic Processes Requiring Glycolytic ATP

The glycogen shunt model emphasizes the need for glycolytic ATP to support astrocytic processes that require a 'fast' supply of ATP, especially the Na^+,K^+-ATPase. On the other hand, oxidative ATP from glycogen-derived pyruvate oxidation and glutamate oxidation upon its uptake could fuel the much smaller, slower energy demands of the glutamate-glutamine cycle and larger, prolonged demands of other processes (Fig. 3). Preliminary unpublished calculations based on the glycogen shunt model are in excellent agreement with the conclusion that the 3 ATP produced per glucosyl unit by glycolytic metabolism of glycogen-derived Glc-6-P are sufficient to satisfy the energy demands of Na^+ and K^+ pumping. In addition, the excess ATP produced from glycogenolysis and oxidation can be used for Ca^{++} pumping and filopodial movements to and from active synapses to optimize clearance of K^+ and glutamate from the synaptic cleft during prolonged, intense activity. Thus, the glycogen shunt and glycolytic metabolism of glycogen can provide most of the ATP required for ion pumping during intense neuronal activity while sparing an equivalent amount of glucose for neurons. Further testing of available data sets is in progress.

Additional, indirect evidence supports the conclusion that glycogen is metabolized predominantly by glycolysis with release of lactate from brain. As noted above, the amount of oxygen consumed during activation in the Madsen et al. (1999) study cannot support the complete oxidation of the amount of glucose consumed, let along the glycogen metabolized. While partitioning of breakdown of these carbohydrates between the glycolytic and oxidative pathways requires additional experiments, an important clue is that glycogenolysis does not dilute the specific activity of brain lactate derived from blood-borne [6-^{14}C]glucose (Dienel and Cruz 2009) and the lactate equivalents of glycogen are not retained in the brain (Dienel et al. 2002; Cruz and Dienel 2002). If glycogen-derived lactate were retained in brain, the brain lactate level would have increased substantially, the lactate specific activity determined in homogenates would have been considerably diluted, and, if oxidized, oxygen consumption would have markedly increased. These finding indicate compartmentation of glycolytic metabolism of glucose and glycogen, with rapid release from brain of unlabeled lactate derived from glycogen (and potentially some oxidation of glycogen in the astrocyte which would further decrease astrocyte glucose phosphorylation). This phenomenon might be readily explained by predominant metabolism of glycogen in astrocytes and uptake and oxidation of blood-borne glucose by neurons, such that the labeled lactate recovered in brain during activation has neuronal origin, which is predicted by the glucose sparing component of the glycogen shunt model (Fig. 3).

At present we do not know the contributions of different molecular mechanisms that activate glycogenolysis in the astrocytes. Quach et al. (1978) showed that the glycogenolytic effect of noradrenaline in brain slices was evoked by a much lower concentration than the stimulation by cAMP accumulation (EC_{50} = 0.49 and 8.58 μM, respectively). In cultured astrocytes, the increases in cAMP level preceded the rise in intracellular glucose concentration after adrenergic stimulation (Prebil et al. 2011), and phasic increases in intracellular [Ca^{++}] mediated by α_1-adrenergic receptors were four-fold faster than cAMP/protein kinase A activation (Horvat et al. 2016). These findings are relevant to the sensory stimulation studies in which V_{shunt} can be determined (Dienel et al. 2002, 2007a; Madsen et al. 1999) because startling and alerting, a component of the sensory stimulation protocol, cause activation of the locus coeruleus noradrenergic system upon removal of the shelter in which the animal was sequestered prior to sensory stimulation. Hof et al. (1988) later showed that K^+-evoked glycogenolysis depends on influx of extracellular Ca^{++}. Hertz and colleagues using differentiated, cultured astrocytes revealed that activation of glycogenolysis is complex but starts with small increases in extracellular [K^+]. Through L-channel opening, the extracellular rise in [K^+] leads to elevation of intracellular [Ca^{++}], which then activates phosphorylase kinase. Phosphorylase kinase activates phosphorylase thereby stimulating glycogenolysis. Other pathways such as through cAMP play a minor role in activity coupled glycogenolysis (Hertz et al. 2015b, c; Xu et al. 2013, 2014). Thresholds for triggering glycogenolysis are most easily determined in cultured cells and brain slices, but must also be established in living brain.

The roles of glycogen in K^+ clearance from extracellular space also have important implications in brain disorders. Knockout of glycogen synthase causes mice to have greater susceptibility to hippocampal seizures during stimulation of Schaffer collaterals or treatment with kainate (Lopez-Ramos et al. 2015). Lafora disease is characterized by abnormal glycogen structure and severe epilepsy, ultimately leading to death (Gentry et al. 2018; DiNuzzo et al. 2014, 2015). These findings underscore the importance of improved understanding the functions of glycogen in normal brain function.

Not only does extracellular K^+ stimulate glycogenolysis, it can also have additional metabolic effects. For example, treatment of cultured astrocytes with high (>15 mM) concentrations of K^+ stimulates oxygen consumption in cultured astrocytes (Hertz and Kjeldsen 1985; Hertz et al. 1973), whereas physiological concentrations increase pyruvate carboxylase activity, with a 50% rise in the rate of CO_2 fixation when [K^+] was increased from 2 to 10 mM (Kaufman and Driscoll 1992). The muscle isoform of pyruvate kinase is predominant in brain and in cultured astrocytes (Farrar and Farrar 1995; Tolle et al. 1976; Terlecki 1989; Ngo and Ibsen 1989), and the muscle isoform has a requirement for and is activated by K^+ (Oria-Hernandez et al. 2005; Kachmar and Boyer 1953; Nowak and Suelter 1981). Thus, K^+ can stimulate anaplerosis and glycolysis to an extent that may vary with local [K^+].

3.9.2 Neuronal Processes Requiring Glycolytic ATP

Neurons are highly dependent on oxidative metabolism of glucose to satisfy their energy demands, with oxidation rates being directly proportional to V_{cycle} over a wide range (Fig. 1a). However, neurons also require glycolytic metabolism to fulfill critical processes. There is a long history of reports (often contradictory) indicating that glucose is required for normal neuronal function and that alternative substrates can only partially replace glucose (reviewed in Dienel 2019a). Many of these functions remain to be identified and quantified, but recent studies have demonstrated that upregulation in neuronal glycolytic metabolism of glucose is essential for synaptic function (reviewed by Ashrafi and Ryan 2017; Yellen 2018; Diaz-Garcia and Yellen 2018). Previous studies by Bachelard and colleagues also established that oxidative substrates cannot fully replace glucose to support evoked potentials (Cox and Bachelard 1982, 1988a, b; Cox et al. 1985; Bachelard et al. 1984). Subsequent critical and detailed reviews evaluated discordant results in which glucose replacement with lactate did or did not sustain neuronal functions; one reason was speed of brain slice cutting, with slower procedures enabling lactate to support evoked potentials (Chih and Roberts Jr 2003; Chih et al. 2001; Okada and Lipton 2007).

In addition, a significant finding is that the capacity for presynaptic glucose transport increases with higher activity. For example, treatment of cultured neurons with glutamate or N-methyl-D-aspartate (NMDA) increased plasma membrane localization of the glucose transporter GLUT3 (Ferreira et al. 2011; Weisova et al. 2009). When cultured neurons were stimulated during firing of 600 action potentials, an AMP-activated protein kinase-mediated mechanism triggered a ~three-fold rise in the expression of glucose transporter GLUT4 at the presynaptic membrane within about a minute (Ashrafi et al. 2017). Also, during memory consolidation in vivo, GLUT4 is upregulated, and memory is impaired when GLUT4 is inhibited (Pearson-Leary et al. 2018; Pearson-Leary and McNay 2016). Thus, presynaptic glucose transport capacity increases as a result of ATP-demanding activities, including cognitive processing. AMP-activated protein kinase is also necessary for the energetics of excitatory synaptic activation in cultured neurons, as well as regulation of expression of early intermediate genes that are involved in learning and memory; inhibition of hippocampal AMP kinase in vivo prior to footshock impairs long term avoidance memory when tested 24 h later (Didier et al. 2018; Marinangeli et al. 2018).

Not only does transport capability increase with neuronal activation, but glycolytic upregulation is necessary to generate ATP when energy demand rises. Endocytosis and recycling of synaptic vesicles, including vesicular acidification, were identified as a major energy cost to presynaptic terminals of cultured hippocampal cells (presumably astrocyte-neuron co-cultures), exceeding that of Na^+,K^+-ATPase (Rangaraju et al. 2014). In this study, blockade of glycolysis with deoxyglucose (but without an alternative substrate) or inhibition of oxidative phosphorylation with oligomycin severely impaired synaptic function. Inhibition of myosin light chain kinase reduced mobilization of the reserve pool of synaptic vesicles, but not the immediately releasable pool (Ryan 1999). The cost of reserve vesicle processing is not known.

Diaz-Garcia et al. (2017) provided compelling evidence that neurons upregulate glycolytic metabolism during electrical stimulation of hippocampal slices in vitro and during stimulation of whiskers to activate the barrel cortex in vivo. Additional evidence that glycolysis is essential for neurotransmission includes accumulation of glycolytic enzymes at presynaptic sites during metabolic stress (Jang et al. 2016) and localization of glycolytic enzymes to synaptic vesicles (reviewed by Ashrafi and Ryan 2017; Ueda 2016).

The evidence above strongly supports an important role for non-oxidative glycolysis in neuronal metabolism during activation. Emphasis on the ANL shuttle in the literature has limited the study of these mechanisms. We hope that this paper and the studies cited above will help stimulate future work to evaluate essential roles of glucose in neuronal functions.

3.10 Neuronal Activity Requirements for the Glycogen Shunt to Be Used

Evaluation of the glycogen shunt model requires confining the analysis to the early phase of intense brain activation during which there is net consumption of glycogen. One of the limitations is that cycling of glucose-6-phosphate through glycogen cannot be measured with current technology, and the roles of continuous glycogenolysis and glucose sparing without net consumption under resting conditions cannot be determined. The likelihood of a threshold for net glycogenolysis is suggested by the study of Cruz and Dienel (2002) in which rats were given 30 seconds of sensory stimulation. Unlabeled glycogen levels were unchanged after the brief stimulation, whereas labeled glycogen was 16% lower (not quite reaching statistical significance, $p = 0.059$), suggesting net release of newly-synthesized outer tiers precedes consumption of unlabeled inner tiers.

Given the high amount of brain signaling activity in the resting awake state (which is several fold higher than the increments in signaling in even intense sensory activation) it is unclear why there appears to be a rapid transition to use of the glycogen shunt above this level of activity. Studies have shown that the total increment in signaling activity across the ensemble is proportional to the increment in glucose oxidation, consistent with the findings relative to V_{cycle} (Maandag et al. 2007) . However, the distribution of signaling frequency changes, with much of the increment being due to large percentage increases in the signaling frequency of a subset of neurons which have been called rapid signaling neurons (RSN, Maandag et al. 2007). The signaling frequencies of the RSN can be over five-fold higher than the overall ensemble average, and are likely associated with large release of K^+ which triggers glycogenolysis.

Even during resting awake and anesthetized conditions, there are still neurons that transiently signal at very high rates relative to the average resting awake value, although a smaller percentage. Evidence of continued activity of glycogen turnover

related to the shunt, presumably in microdomains, is provided by measurements using ^{14}C and ^{13}C-labeled glucose of glycogen turnover (Watanabe and Passonneau 1973; Choi and Gruetter 2003; Choi et al. 1999). Recent analysis by DiNuzzo and coworkers (Öz et al. 2015, 2017; DiNuzzo 2013) suggest that the rate of turnover may be several fold higher than previously reported due to not taking into account the dynamics of glycogen particle synthesis and breakdown properly. Although outside of the scope of the present paper it would be of value to calculate from the turnover measurements what fraction of astroglia are involved in the glycogen shunt and compare the number to electrical recordings of the fraction of rapidly signaling neurons under those conditions.

4 Concluding Comments and Future Studies

A direct outcome of the glycogen shunt model development, its critical evaluation with respect to the literature, and its initial testing is that the glycogen shunt in combination with the pseudo-MAS governs the stoichiometry between glucose metabolism and GABA/glutamate/glutamine neurotransmitter cycling. Glutamate uptake into astrocytes linked to astrocyte-neuron lactate shuttling is *not the coupling mechanism*, and glycogen- and glucose-derived lactate are *not major oxidative fuels for neurons* although lactate can supplement glucose in both astrocytes and neurons.

Future fully-quantitative studies are needed to evaluate cellular and subcellular energetics in astrocytes and neurons and to identify functions served by ATP derived from glycolysis and oxidative metabolism. Among the key measurements that need to be performed to test and extend the model are the following:

- **Comparison between theoretical neuronal and astroglial glucose and glycogen metabolic fluxes and ΔV_{cycle} with experimental measurements during brain activation**. The flux relationships are tested against in vivo experimental data in which glycogenolysis, total and oxidative glucose uptake, and V_{cycle} are measured. In addition, the theoretical neuronal and astrocytic ATP production ($V_{ATP\text{-}N}$, $V_{ATP\text{-}A}$) relative to ΔV_{cycle} must be calculated and compared with experimental measurements.
- **Comparison of theoretical flux relationships between neuronal and astroglial energetics and ΔV_{cycle} and the values measured under nonactivated conditions**. In order to test whether the energetic relationships determined by the glycogen shunt are maintained at lower levels of brain activity the theoretical relationships between neuronal and astroglial ATP production and ΔV_{cycle} must be compared with the relationships determined by meta-analysis of experimental measurements done under different levels of brain activity from awake to isoelectricity, as shown in Fig. 1.
- **Comparison of the theoretical increment in glucose consumption when the shunt is blocked with experimental measurements**. In order to examine whether the glycogen shunt is able to spare glucose for neuronal consumption

the relationship between glucose consumption and ΔV_{cycle} when the shunt is blocked must be calculated and shown that it leads to an increase in incremental glucose requirements. This prediction is tested by comparing experimental measurements of the ratio between $CMR_{glc\text{-}tot}$ and $CMR_{glc\text{-}ox}$ with the shunt active with measurements performed when the shunt is either blocked by a glycogen phosphorylase inhibitor or through experimental conditions.

Along with methods development, studies are required to establish the thresholds and mechanisms for triggering glycogenolysis, the magnitudes, time courses, and durations of responses, the relationship between glucose sparing and upregulation of glucose transport capacities across the blood-brain barrier and plasma membranes of astrocytes and neurons, and the functions of glycolytic ATP in activated neurons.

Testing of the glycogen shunt model presented in this article has been initiated using the limited available literature in which sufficient quantitative measurements have been made. Initial results show excellent agreement between the quantitative predictions of the shunt model and the stoichiometric relationships of glycogenolysis rate with glutamate-glutamine cycle rate, fueling of astrocytic ion pumping by glycogen-derived ATP, and fueling of neuronal ion pumping and other neuronal energy demands by glycolytic and oxidative metabolism of the spared glucose. These results should stimulate development of new methods to quantify glycogenolysis in real time in living brain under different conditions in conjunction with the major pathway fluxes in astrocytes and neurons and rates of glutamatergic and GABA-ergic neurotransmission.

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