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The Complementary Roles of Glucose and Lactate in Meeting Neuronal Energetic Needs: An Investigation into the Modulation of Cortical Metabolism and Extracellular Metabolite Pools in the Mouse

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List of Abbreviations

ANLS: Astrocyte-to-Neuron Lactate Shuttle
AMP: Adenosine monophosphate
ASD: Autism spectrum disorder
ATP: Adenosine triphosphate
BBB: Blood-brain barrier
BHB: β–hydroxybutyrate
BDNF: Brain-derived neurotrophic factor
BOLD: Blood oxygenation level dependent
CBF: Cerebral blood flow
CMRGlc: Cerebral metabolic rate of glucose
CMRO2: Cerebral metabolic rate of oxygen
CNS: Central nervous system
COX IV: Cytochrome c oxidase
FDG: 18F-fluorodeoxyglucose
FFA: Free fatty acids
FG: Fast glycolytic muscle
fMRI: Functional magnetic resonance imaging
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GLUT: Glucose transporter (isoform 1 and 3)
GSH: glutathione (neutralizing agent for ROS)
G6P: Glucose-6-phosphate
HCAR1: Hydroxycarboxylic acid receptor 1
HK: Hexokinase
H_2O_2: Hydrogen peroxide
I.P.: Intraperitoneal
kDa: Kilodaltons
KATP: ATP-sensitive potassium transporters
LDHB: Lactate dehydrogenase enzyme favoring the pyruvate to lactate conversion
LDHA: Lactate dehydrogenase enzyme favoring the lactate to pyruvate conversion
MCT: Monocarboxylate transporter (isoform 1, 2 and 4)
M1: Primary motor cortex
Na^+/K^+-ATPase: Sodium-potassium pump
NAD: Nicotinamide adenine dinucleotide (oxidized)
NADH: Nicotinamide adenine dinucleotide (reduced)
NADPH: Nicotinamide adenine dinucleotide phosphate
NALS: Neuron-to-Astrocyte lactate shuttle
OGI: Oxygen glucose index
OXPHOS: Oxidative phosphorylation (TCA & mitochondrial respiration)
PET: Positron emission tomography
PPP: Pentose phosphate pathway
PFA: 4% paraformaldehyde/0.2% picric acid solution
PFK: Phosphofructokinase-1
PK: Pyruvate kinase
ROS: Reactive oxygen species
SEM: Standard error of the mean
S.C.: Subcutaneous
SO: Slow oxidative muscle
TCA: Tricarboxylic acid cycle
V1: Primary visual cortex
4-CIN: Alpha-cyano-4-hydroxycinnamate (MCT blocker)

Abstract

Our knowledge of cerebral metabolism is shifting towards an understanding and appreciation of non-neuronal and non-glucose contributions to meeting the brains high energetic needs. Specifically, astrocytes are hypothesized to provide metabolites that sustain neuronal energy needs as well as modulate neuronal signalling. This metabolic collaboration between cellular compartments occurs in the extracellular space of the brain where astrocytes can provide endogenously created metabolites or regulate cerebral blood flow and subsequently metabolite delivery. The work presented herein investigated the contribution of the blood brain barrier and non-glucose fuels (e.g. lactate) in the maintenance of the extracellular glucose and lactate pools. The transport of blood-borne alternative fuels across the blood-brain barrier, namely lactate, are essential to maintaining extracellular metabolite pools and thus play an important role in the metabolic cooperation between the various compartments of the brain (i.e. neurons and astrocytes) and body (i.e. central and peripheral systems). Through observations of preferential upregulation of monocarboxylate pathways, we hypothesize a larger scale collaboration in which the body metabolize various fuels towards common downstream products (i.e. glucose and lactate), enabling the brain to optimize but a few pathways while reaping benefits from a wide variety of sources. Commonly observed metabolic dysregulation in brain pathologies may underscore an inability to flexibly adapt these pathways to changing environmental bioenergetic challenges. The role of peripheral metabolites (i.e. lactate) in maintaining extracellular metabolite pools is an important consideration in the on-going debate about the neuronal use of astrocyte derived lactate and relevant context for future modeling studies.

Chapter I: Introduction

The Brain: An Energetically Demanding Organ

The brain is an energetically demanding organ. When compared to other organs, the metabolic rate of the brain is second only to that of the heart and kidneys (Wang et al., 2010). Despite contributing to approximately 2% of the human body weight, the brain consumes approximately 20% of the available energy, as estimated by oxygen consumption (Attwell & Laughlin, 2001). This significant energy consumption is not limited to moments of intense cerebral activation. Rather, measurements of *whole brain* metabolism and cerebral blood flow (CBF) during brain activation have consistently failed to reveal any significant changes at the whole-organ level (Ide & Secher, 2000; Mintun et al., 2004; Sokoloff et al., 1955), suggesting that the relatively small and localized increases in CBF and metabolism do not influence whole brain neuroenergetics (see section entitled *Plasticity of Neuroenergetics Processes During Neuronal Activation*). Despite the brain's high baseline need for energy, it possesses an extremely limited internal store, in the form of glycogen (see section entitled *Glycogen: Glucose Storage in the Brain*) (Brown, 2004; Duran & Guinovart, 2015), capable of sustaining the brain's energetic need for mere minutes on its own (Brown & Ransom, 2015). Thus, the brain not only requires large amounts of energy but requires this energy to be continuously and consistently delivered. Though these statements have become practically cliché in the field of neuroenergetics, they properly put into perspective the brain's high metabolic rate while highlighting the importance and reliance on peripheral metabolite uptake.

Delivery of Metabolic Substrates Across the Blood-Brain Barrier

As an added complexity to the brain's dependence on continuous metabolite delivery from circulation, the brain has to be protected from pathogens and neurotoxic plasma

components (Sweeney et al., 2019). The prenatal development of the blood-brain barrier (BBB) is a protective mechanism by which the flow of molecules, both in and out of the brain, is tightly regulated through the development of a highly selective membrane. Transport across this membrane is regulated through chemical and physical process, some of which are explored below. Consequently, a leaky BBB, a condition in which molecules can more readily diffuse to the brain due to an altered BBB, is associated with serious disease states (Profaci et al., 2020; Rosenberg, 2012).

Cellular Composition of the BBB

The composition of the BBB can vary slightly between arterioles and capillaries. However, they are generally composed of endothelial cells, glial cells (most commonly astrocytes) and mural cells (pericytes or smooth muscle cells) (see **Figure 1**). Given the presence of key transporters in endothelial cells and the increasing recognition of astrocytic involvement in cerebral and neuronal bioenergetics, the subsequent sections will focus more closely on these two elements of the BBB.

Endothelial cells composing the brain's vasculature distinguish themselves from peripheral endothelial cells primarily through the development of tight junctions. Tight junctions are endothelial junctional structures that form a continuous and sealed membrane through which molecules cannot easily diffuse, thereby allowing the regulation of molecule entry into the brain. Tight junctions are absent in peripheral vasculature, resulting in easier diffusion of various molecules into and out of the blood vessels.

Combined with neurons, these cells (i.e. endothelial cell, glial cell and mural cells) make up the neurovascular unit. The complex interactions between these cells regulate various functions, including control of CBF and BBB integrity (Yu et al., 2020). Indeed, co-culturing

pluripotent stem cells with astrocytes, neural stem cells and pericytes is required to mimic BBB functionality (Appelt-Menzel et al., 2017). The precise molecular mechanisms by which these interactions result in the development and maintenance of the BBB is beyond the scope of this work but have been extensively reviewed elsewhere (Bernard-Patrzynski et al., 2019; Daneman & Prat, 2015; Gastfriend et al., 2018; Lécuyer et al., 2016; Profaci et al., 2020). However, it is relevant to note that the distinguishing features of the brain's vasculature require the close interaction between endothelial cells and other brain cells, including astrocytes. The emerging role of astrocytes in bioenergetics will be further discussed below (see section entitled *Astrocyte-to-Neuron Lactate Shuttle Hypothesis*).

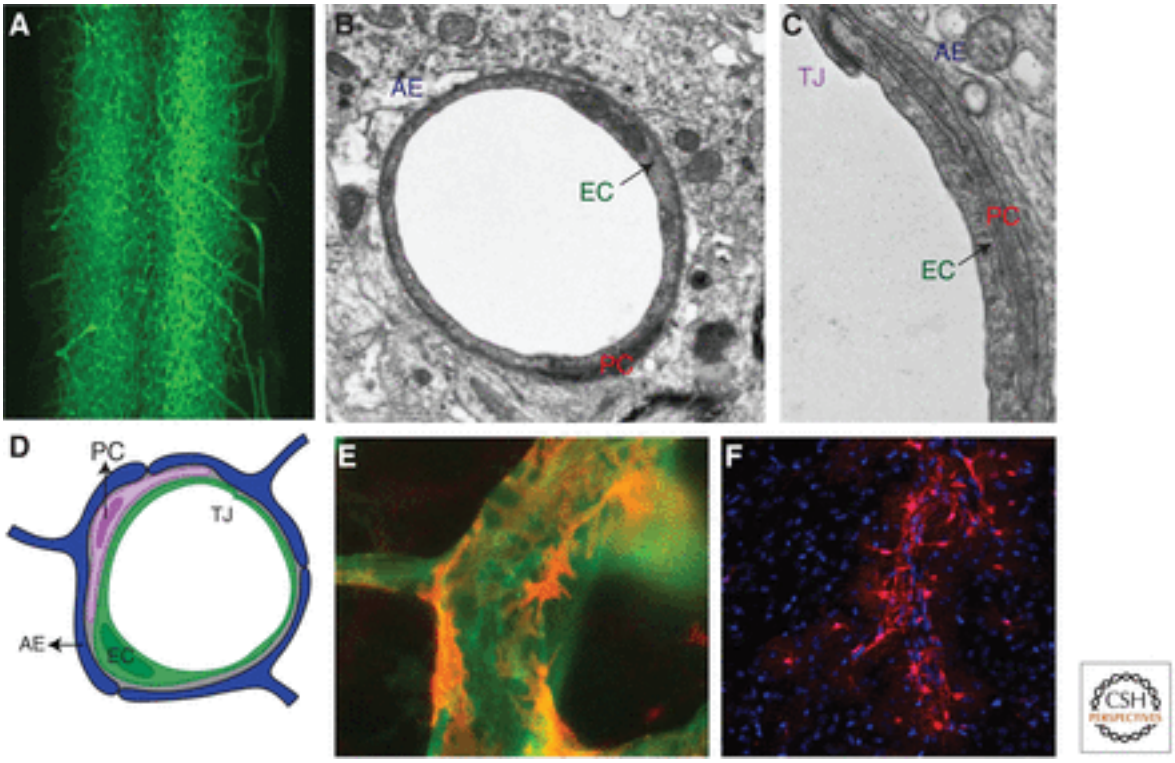

Figure 1 | Schematic representation of the BBB composition
Components of the BBB. (A) Vascular cast of a spinal cord showing density of the central nervous system (CNS) vascular network. (B) Electron micrograph (EM) of a cross section of a CNS vessel depicting a relationship among endothelial cells (ECs), pericytes (PCs), and astrocytes. (C) Magnified EM of ECs depicting a relationship among ECs (with tight junctions [TJ]), PCs, basement membranes (BMs), and astrocyte endfeet (AE). (D) Schematic representation of the cell types within the neurovascular unit. (E) Immunofluorescence micrograph depicting relationship of PCs (red) with ECs (green). (F) Micrograph depicting relationship of astrocytes (red-labeled with GFAP-cre; Rosa-tdTomato) with blood vessels (unstained). Astrocytes extend processes that ensheath the blood

vessels, such that the outline of the blood vessels can be visualized by the endfeet of these processes. Figure is reproduced (Daneman & Prat, 2015), with permission, and provided courtesy of Matthew Boisvert and Nicola Allen.

Metabolite Transport Across the BBB

The expression distribution of glucose and lactate transporter isoforms are summarized in **Figure 2**, as well as detailed in the sections below.

Hexose (glucose) transport in endothelial cell. Tight regulation of transport across the BBB includes the passage of necessary substrates for energy production, such as glucose, across endothelial cell membranes. Passive diffusion across the endothelial membranes is severely limited by the presence of tight junctions and given glucose's hydrophilic nature, it cannot readily diffuse through the fatty cell membranes (Mikitsh & Chacko, 2014; Pardridge, 1991). Thus, glucose is transported from the blood to the brain through facilitative transmembrane glucose transporters embedded in the endothelial cells of the BBB (Holman, 2020). Specifically, glucose transport across the endothelial cell membranes of the BBB is mediated by isoform-1 of the glucose transporters (GLUT1).

The GLUT1 isoform possesses a higher affinity for glucose than other hexoses (e.g. fructose) (Gould et al., 1991) and is by and large the primary GLUT expressed in the endothelial cells of the BBB (Patching, 2017). Glucose uptake in the brain is directly related to the level of GLUT1 expression at the BBB (Zeller et al., 1997). Despite this strong correlation, there is debate as to whether glucose transport is a rate limiting processes for glucose metabolism. While some argue that the transport, and therefore availability, of glucose is what limits its metabolic rate within the brain (Barros et al., 2005; Vannucci et al., 1998), others state that glucose transport across the BBB through GLUT1 is only rate limiting during extreme conditions when

the brain metabolic requirements (e.g. intense exercise) or the substrate availability (e.g. hypoglycaemia) are significantly altered (McKenna et al., 2012).

GLUT transport (including GLUT1) is passive, meaning that the substrates (e.g. glucose) are transported down their concentration gradient. Given that brain glucose levels are approximately 3-times lower than blood levels (Abi-Saab et al., 2002; Ances et al., 2008a; Gruetter et al., 1998; Kemppainen et al., 2005; Monsod et al., 2002; Pouwels & Frahm, 1998), the direction of endothelial glucose transport using GLUT1 is typically from blood to brain. This concentration gradient is not only maintained on a more macro level between the blood and the brain, but also appears to be maintained on a more micro level within the endothelial cell. GLUT1 is differentially expressed on the luminal (blood facing side of the vessel) and abluminal (brain facing side of the vessel) membranes of the endothelial cells (Cornford & Hyman, 2005; Farrell & Pardridge, 1991; Kubo et al., 2015; McAllister et al., 2001; Simpson et al., 2001), with a greater luminal density of GLUT1. The exact ratio of the distribution is debated. To further favor this micro asymmetry of glucose concentration within the endothelial cells, hexokinase (the enzyme required for the first step in glucose metabolism) is preferentially expressed at the abluminal side of the endothelial membrane (McAllister et al., 2001). Hexokinase conversion of glucose to glucose-6-phosphate (G6P) causes a dip in glucose concentration at the abluminal membrane. Combined with the greater uptake of glucose at the luminal membrane, this creates a concentration gradient that pushes glucose towards the abluminal transporters and into the brain.

Monocarboxylate (lactate) transport in endothelial cell. Endothelial cells possess the necessary machinery to transport many other substances beyond glucose. Relevant to the work presented here is the capacity to transport monocarboxylates, chiefly lactate. Lactate transport

across the BBB is achieved through the ubiquitous presence of the first isoform of the monocarboxylate transporters (MCT1).

Like the facilitative glucose transporters, monocarboxylate transporters are also bi-directional and transport monocarboxylates down their concentration gradient (Hertz & Dienel, 2005; Pérez-Escuredo et al., 2016; Pierre & Pellerin, 2009). However, unlike glucose, there is debate as to whether, under normal conditions, lactate concentration in the blood is consistently above or below levels found in the brain in humans (Abi-Saab et al., 2002; Detre et al., 1991; Dienel, 2012; Ide et al., 2000; Kemppainen et al., 2005; Merboldt et al., 1992; Pouwels & Frahm, 1998; Prichard et al., 1991; Rosdahl et al., 1993). This spurred the debate as to whether the brain in a net importer or exporter of lactate (Aubert et al., 2005; Bergersen, 2015; Boumezbeur et al., 2010a; Díaz-García et al., 2017a; Jha & Morrison, 2020; Li et al., 2022; Proia et al., 2016; Riske et al., 2017). It is our opinion that the brain is likely both, depending on blood and brain levels, but that lactate is primarily imported to sustain cerebral bioenergetics. Though this will be the underlying assumption throughout our thesis, it should be made clear that there are diverging perspectives particularly for the human brain (e.g. Díaz-García et al., 2017). In contrast, rodents appear to provide a clearer picture of peripheral to central lactate gradients. In rodents, blood lactate levels are consistently above those observed in the brain (Abi-Saab et al., 2002; Allard et al., 2013; Chen et al., 2014; Chen et al., 2009, 2009; Duarte et al., 2014; Elferchichi et al., 2010; Folbergrová et al., 1969; Gatfield et al., 1966; Hallström et al., 1989; Horn & Klein, 2013; Kramer et al., 1993; Lin et al., 2014; Matsui et al., 2011; Mayman et al., 1964; Muñoz et al., 2010; Pederson et al., 2005, 2005; Rocchitta et al., 2013; Ross et al., 2010), thereby suggesting net import of lactate into the brain. Regardless of the species, however, there is strong evidence that net import of lactate into the brain can occur when lactate levels are either artificially (e.g.

injections) (De Feyter et al., 2013; Dienel & Hertz, 2001; Herzog et al., 2013; Huang et al., 2021; Lezi et al., 2013; Miller et al., 1984; Mintun et al., 2004; Nemoto et al., 1974; Qu et al., 2000; Smith et al., 2003; van Hall et al., 2009; Wiegers et al., 2016, 2019; Wyss et al., 2011) or naturally elevated (e.g. exercise) (Dembitskaya et al., 2022; Fellows et al., 1993; Fray et al., 1996; Hu & Wilson, 2002; Ide et al., 2000, 2000; Ide & Secher, 2000; Kuwabara et al., 1995; Maddock et al., 2011; McNay et al., 2013; Overgaard et al., 2012; Prichard et al., 1991; Sappey-Marinier et al., 1992; Secher & Quistorff, 2005; Siebenmann et al., 2021, 2021; Urrila et al., 2003, 2004; van Hall, 2010).

Cerebral Metabolite Transport

Hexose (glucose) transport in astrocytes. Given that the BBB is composed of several cell types (e.g. endothelial cells, pericytes, astrocytes), metabolites, including glucose, must therefore travel across multiple membranes before reaching the interstitial fluid of the brain, to then be taken up by neurons. Following transport across endothelial cells, glucose molecules must travel through the astrocytic endfeet that surround most of the brain vasculature (Mathiisen et al., 2010)[1]. Glucose transfer though astrocytes is also facilitated by the ubiquitous GLUT1 (Simpson et al., 2007), though through a differentially glycosylated isoform. Within astrocytes, the 45kDa isoform of GLUT1 is predominant, whereas the 55kDa isoform is predominant in endothelial cells (Devraj et al., 2011; Maher et al., 1994; Morgello et al., 1995; Simpson et al., 2007; Yu & Ding, 1998). The different conformations (45kDa vs 55kDa GLUT1) appear to be

[1] The exact coverage of the brain vasculature by astrocytic endfeet has been questioned. Korogod and colleagues have found that when brains are not fixed in formol, a substances known to shrink tissue, the astrocytic coverage of the brain vasculature was smaller indicating that formol-induced shrinkage artificially increased the overall coverage of endfeet on vasculature (Korogod et al., 2015).

the result of differential phosphorylation, but no functional difference has yet to be found (Devraj et al., 2011).

Monocarboxylate (lactate) transport in astrocytes. Monocarboxylate transport, into or out of the brain, must similarly cross the many layers of the neurovascular unit. Like endothelial cells, astrocytes express the high affinity MCT1. However, they also express MCT4's (Debernardi et al., 2003; Hanu et al., 2000; Pellerin et al., 2005; Pierre & Pellerin, 2005, 2009). Interestingly, within astrocytes, the spatial distribution of transporters appears to vary with the isoform. MCT1 expression is rather ubiquitous, whereas MCT4 expression appears to be primarily limited to astrocytic processes (Pierre & Pellerin, 2005).

When compared to other MCT isoforms, MCT4 has a relatively low affinity for common monocarboxylates (e.g. lactate, pyruvate). However, MCT4's affinity for lactate still exceeds that of other monocarboxylates (e.g. 22-28 mM for lactate vs 153 mM for pyruvate) (Pérez-Escuredo et al., 2016). It is assumed that these characteristics allow for astrocytic lactate uptake through MCT1 and significant astrocytic export of lactate through MCT4 (Contreras-Baeza et al., 2019; Dimmer et al., 2000), while retaining other substrates (such as pyruvate) within the cell to help maintain appropriate redox states though inter-conversion of pyruvate and lactate which restores reduced nicotinamide adenine dinucleotide (NADH; see section entitled *Glycolysis*). The NADH regenerative function of lactate as its converted back to pyruvate is an important consideration in the hypothesis of a non-glucose centric view of brain metabolism that will be presented later (see section entitled *Astrocyte-to-Neuron Lactate Shuttle* Hypothesis).

Hexose (glucose) transport in neurons. After crossing the BBB and astrocytes, glucose can then be released into the interstitial fluid to be taken up by neurons, assuming it has not yet been consumed by the glycolytic endothelial cells or astrocytes (see section entitled *Metabolic*

Pathways for more details). Neurons express GLUT3, an isoform similar to GLUT1 with the exception of two characteristics: a) it does not possess a secondary glycosylated isoform and b) it exhibits a higher affinity and greater transport capacity for glucose than GLUT1 (Simpson et al., 2008). As a reminder, at each subsequent transport step, the quantity of freely available glucose decreases (from blood to endothelial cell to astrocyte), therefore the availability of glucose in the interstitial fluid is but a fraction of what was available in the blood, endothelial cells and astrocytes. Thus, the prevailing premise is that the presence of this higher affinity and capacity transporter is necessary to provide neurons with reliable access to the limited residual circulating glucose in the interstitial fluid (Arnhold, 2020; Simpson et al., 2008).

Monocarboxylate (lactate) transport in neurons. Consistent with the expression of GLUT3, neurons express a monocarboxylate transporter that is not (significantly) present in any other central nervous system cell type: MCT2 (Hanu et al., 2000; Pierre et al., 2002; Pierre & Pellerin, 2009). This transporter also possesses the highest affinity for its respective monocarboxylate substrates (Pérez-Escuredo et al., 2016). Similar to the premise of neurons requiring high affinity glucose transporters, we hypothesize that neurons express this high-affinity transport to take up the residual lactate that has crossed the BBB (or was produced and then exported by astrocytes as will be detailed in the section *Astrocyte-to-Neuron Lactate Shuttle Hypothesis*). It is important to note that there is a competing hypothesis which suggested that the presence of monocarboxylate in neurons serve to remove lactate build up from within during times of increased neuronal activity (Neuron-to-Astrocyte Lactate Shuttle Hypothesis (NALS)). Regardless, it is worth noting that other cell types, such as slow twitch muscles (also known as oxidative or red muscles), known for their lactate import capabilities preferentially express MCT2 (as well as MCT1) (Bergersen, 2007; Brooks, 2009a; Hashimoto et al., 2005; Juel, 1997;

McCullagh et al., 1996; Todd, 2014), and that MCT2 expression in neurons is particularly enriched in the energetically demanding post-synaptic spines (Pérez-Escuredo et al., 2016).

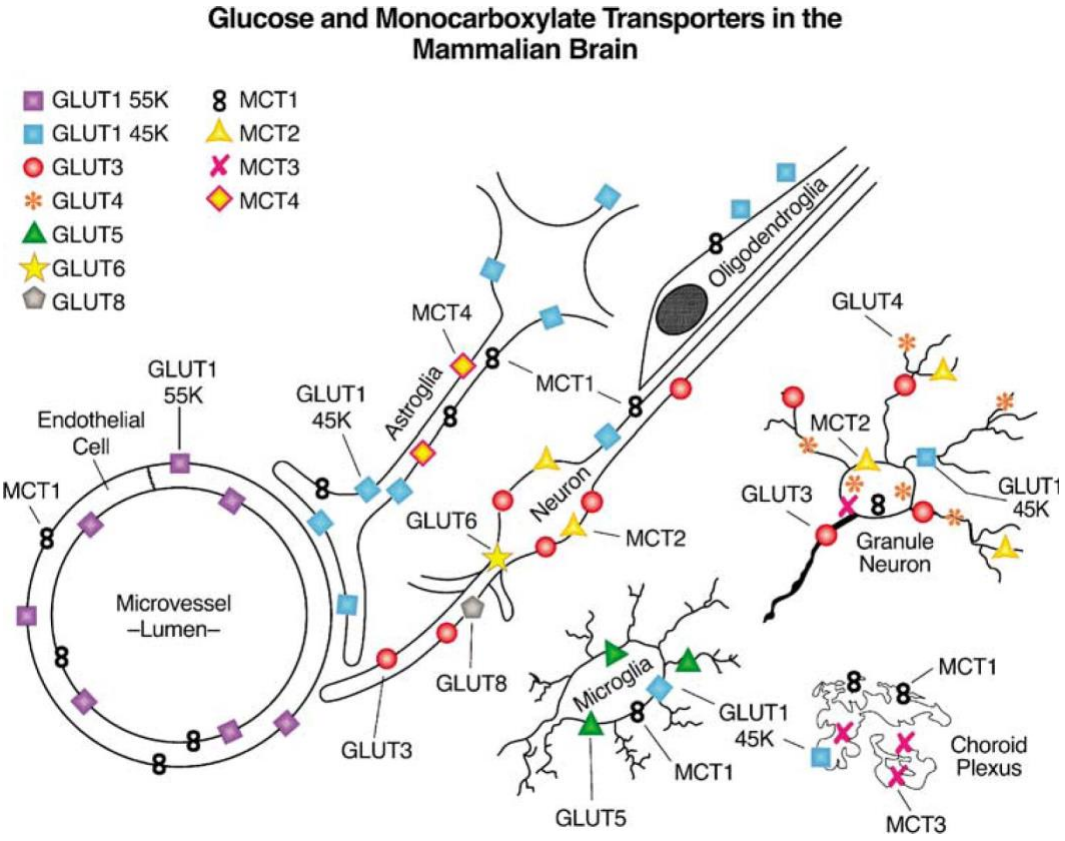

Figure 2 | Schematic representation of the differential transporter expression within brain cells
A schematic representation of the cellular localization of glucose transporter (GLUTs) and monocarboxylate transporters (MCTs) in mammalian brain. Figure is reproduced with permission per SAGE publication copyright regulations (Simpson et al., 2007).

Glycogen: Glucose Storage in the Brain

As mentioned, the brain is almost entirely dependent on peripheral blood circulation to provide the metabolic substrates required to meet its energetic needs. The brain does store a limited amount of energy, in astrocytes, by converting a portion of available glucose into glycogen, a process known as glycogen synthesis or glycogenesis (Brown, 2004; Brown & Ransom, 2007; Falkowska et al., 2015). Contrary to the rest of the body, the brain's limited

energy storage can only sustain its neuroenergetic needs for a few minutes (Brown, 2004; Brown & Ransom, 2007; Falkowska et al., 2015).

Glycogen has been hypothesized to serve as a metabolic buffer between acute changes in neuroenergetic needs and the mechanisms in place to increase delivery of substrates, which include increased cerebral blood flow (Drake & Iadecola, 2007; Paulson et al., 2010), translocation of transporters (Medina & Owen, 2002; Uemura & Greenlee, 2006), hepatic production of glucose and insulin (Rojas & Schwartz, 2014) as well as modulation of metabolic rates by influencing key steps in metabolic pathways (Caesar et al., 2008). In such a view, glycogen serves as a short-lived energy buffer until compensatory mechanisms, induced by elevated neuronal activity, can be engaged. Astrocytic glycogen can indeed support the neuroenergetic needs of surrounding neurons through glycogenolysis, either by supporting the energetic needs of the astrocytes through glucose-6-phosphate (G6P) production thereby decreasing competition for glucose, or by production and shuttling of lactate to surrounding neurons (Coggan et al., 2018; Deitmer et al., 2019; Dienel & Rothman, 2019) (more details on the function of lactate in neuroenergetics are provided in the section entitled *Astrocyte-to-Neuron Lactate Shuttle*).

The temporary metabolic buffer provided by glycogen appears to have a significant behavioral impact. Glycogen, and its by-products, is essential for memory formation. Without glycogen, long-term potentiation in neurons, the mechanism believed to underlying memory formation, is severely hindered consequently impacting memory formation (Calì et al., 2019; Descalzi et al., 2019; Waitt et al., 2017). Although it merits consideration and understanding in the broader context and discussion of neuroenergetics, the precise role of glycogen in learning,

memory and metabolic support is outside the scope of this work and has been extensively reviewed elsewhere (Alberini et al., 2018; Duran et al., 2019; Rich et al., 2019).

Metabolic Pathways

To sustain its large energetic needs, the brain uses well established metabolic pathways to create the universal energy currency of the brain adenosine triphosphate (ATP). Once glucose reaches the brain, it is integrated into one of two main energy producing pathways: glycolysis or oxidative phosphorylation (OXPHOS). Given that these pathways have been studied and discussed at length (Ainscow & Brand, 1999a, 1999b; Fernie et al., 2004), the following paragraphs will only highlight key points and differences, which are further summarized in **Table 1**.

Glycolysis

The initial steps of glycolysis are the (almost) mandatory gateway for energy producing metabolism using glucose. For glucose to enter any subsequent metabolic pathways, it must first be converted to G6P via hexokinase – the initial step of glycolysis. From there, it can be a) converted to stored energy as glycogen (glycogenesis) via glycogen synthase, b) enter the pentose phosphate pathway (PPP) to create reducing agents (synthesis of nicotinamide adenine dinucleotide phosphate (NADPH)) or precursors for the synthesis of nucleotides for homeostatic functions allowing the maintenance of the redox state and hexosamine biosynthesis, respectively or lastly c) continue through the glycolysis pathway to be converted to either pyruvate or lactate (later sections will provide an overview of the metabolic fate of lactate). The following paragraphs will focus on the continuation of G6P through glycolysis, and subsequent sections will explore the complementary role of glycolysis to OXPHOS through its supply of

intermediates and support of homeostatic redox functions, as well as the PPP. **Figure 3** illustrates the interactions between these main pathways for glucose consumption.

Unsurprisingly, being the universal energy currency of the body, ATP production and use is crucial for organismal function. As an energy producing pathway, glycolysis only yields a net 2 ATP per glucose molecule, which is comparatively minuscule when compared to the 30-32 ATP yielded by OXPHOS (Pfeiffer et al., 2001). Despite its limited capacity for ATP production, the glycolytic pathway does have the advantage of being a much faster process than OXPHOS as well as not being an oxygen dependent process. In cancerous cells, glycolysis can be upregulated to processes glucose over 100 times faster than OXPHOS (Epstein et al., 2017). In healthy cells, overactivation of the glycolytic pathway can lead to detriments such as cellular acidification (through the overproduction of lactate) and Ca^{2+} efflux (which can signal apoptosis) (Chan et al., 2018). However, the anaerobic (oxygen-independent) nature of glycolysis gives it the advantage of not creating any detrimental reactive oxygen species (ROS).

The sustenance of neuroenergetic needs appears to be more complex than choosing the pathway that produces the most ATP. Both cancer cells (Shiratori et al., 2019; L. Yu, Chen, et al., 2017) and endothelial cells (Leung & Shi, 2022) favor the upregulation of glycolysis as a metabolic pathway for proliferation, presumably due to its ability to support biosynthesis (Ganapathy-Kanniappan, 2018; Lunt & Vander Heiden, 2011; Orang et al., 2019; Polet & Feron, 2013; Tennant et al., 2010; Verdegem et al., 2014). What's more, glycolysis appears to sustain one of the highest energy demanding processes in the brain: the sodium-potassium pump (Na^+/K^+-ATPase) (Lipton & Robacker, 1983; Okamoto et al., 2001; Raffin et al., 1988; Sepp et al., 2014). Glycolysis occurs in the cytoplasm, versus OXPHOS which takes place in the mitochondria. Perhaps the proximity of glycolytic enzymes to the Na^+/K^+-ATPase in the cellular

membranes is one of the considerations for the use of glycolysis to maintain ion gradients through this pump.

OXPHOS and Regulation of ROS

For the purposes of this work, OXPHOS will refer to both the citric acid (TCA) cycle and mitochondrial respiration via the electron transport chain, as illustrated in **Figure 3**. As previously mentioned, the glycolytic end products (pyruvate or lactate) are integrated at the beginning of this process to eventually produce 30-32 ATP.

Beyond its location (mitochondria), speed (slower than glycolysis) and higher production of ATP (30-32 ATP vs 2 ATP), OXPHOS distinguishes itself from glycolysis by being an oxygen dependent pathway. Importantly, this means that OXPHOS results in the production of ROS, such as $O_2^{\bullet -}$ (Liemburg-Apers et al., 2015; Mailloux, 2020; Murphy, 2009). ROS production is positively correlated with glucose consumption (Beckhauser et al., 2016; Halliwell, 1992; Liemburg-Apers et al., 2015; Shah et al., 2013), meaning the more this pathway is used, the more ROS is produced. This is important because ROS production must be delicately balanced. Excess ROS, either produced through increases glucose use or defective/inefficient processes, causes DNA damage (Guo et al., 2013; Indo et al., 2007; Nissanka & Moraes, 2018) but too little interferes with the signalling properties of ROS (D'Autréaux & Toledano, 2007; Sinenko et al., 2021), including its impact on metabolism (Forrester et al., 2018).

Regulation of glycolysis by OXPHOS products. Increased production of ROS and ATP (two products of OXPHOS) inhibit *glycolytic* enzymes (Larsson et al., 2000; Liemburg-Apers et al., 2015; Mullarky & Cantley, 2015; Shi et al., 2009). ATP allosterically inhibits a rate-limiting step of glycolysis by binding to phosphofructokinase-1 (PFK) (Cole & Eastoe, 1988; Pelley, 2007). ROS inhibits enzymes downstream of PFK, such as glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) & pyruvate kinase (PK) (Anastasiou et al., 2011; Ghanbari Movahed et al., 2019; Mullarky & Cantley, 2015; Muronetz et al., 2019; Ramzan et al., 2013). Ultimately, these enzymatic inhibitions limit the production of final glycolytic intermediates (where that final intermediate is pyruvate or lactate is debated (Rogatzki et al., 2015; Schurr & Payne, 2007) and lead to an accumulation of intermediate substrates (e.g. glyceraldehyde 3-phosphate), thus creating a negative feedback loop for the entire glycolytic pathway. Interestingly, ATP is also an important (but not exclusive (Balaban, 1990; Hüttemann et al., 2007)) regulator of OXPHOS (Ainscow & Brand, 1999b; Berg et al., 2002). More specifically, when ATP levels dip, the decreasing ATP/ADP ratio induces elevated rates of OXPHOS until the ATP/ADP ratio is restored.

In sum, OXPHOS products (ATP and ROS) provide two distinct inhibitory mechanism for glycolysis, and a decrease in the availability ATP increases OXPHOS. This complementary regulation and intertwined feedback loops carefully alter cellular metabolism through these pathways and maintains homeostasis to thwart the many detrimental events, including cell death (Redza-Dutordoir & Averill-Bates, 2016), associated with ROS production.

Pentose phosphate pathway

Limiting glucose flux though glycolysis via inhibition of downstream products (e.g. GAPDH) and rate-limiting enzymes (e.g. PFK) serves to maintain cellular homeostatic redox levels in part by diverting glucose to the pentose-phosphate pathway (PPP) (Cherkas et al., 2020; Mullarky & Cantley, 2015). Through the PPP, glucose regenerates the necessary nicotinamide adenine dinucleotide phosphate (NADPH) stores required to maintain important antioxidants such as glutathione (GSH) to neutralize ROS (Chandel, 2021; Ge et al., 2020) as well produces essential nucleotide building blocks (Kruger & von Schaewen, 2003). Put together, these

observations show the mutual regulation and complementary role of the PPP, glycolysis and OXPHOS (Conley et al., 2001; Liemburg-Apers et al., 2015; L. Yu, Lu, et al., 2017). These pathways are intimately linked by the brain's need for high ATP production and the homeostatic need to control the consequent creation of ROS.

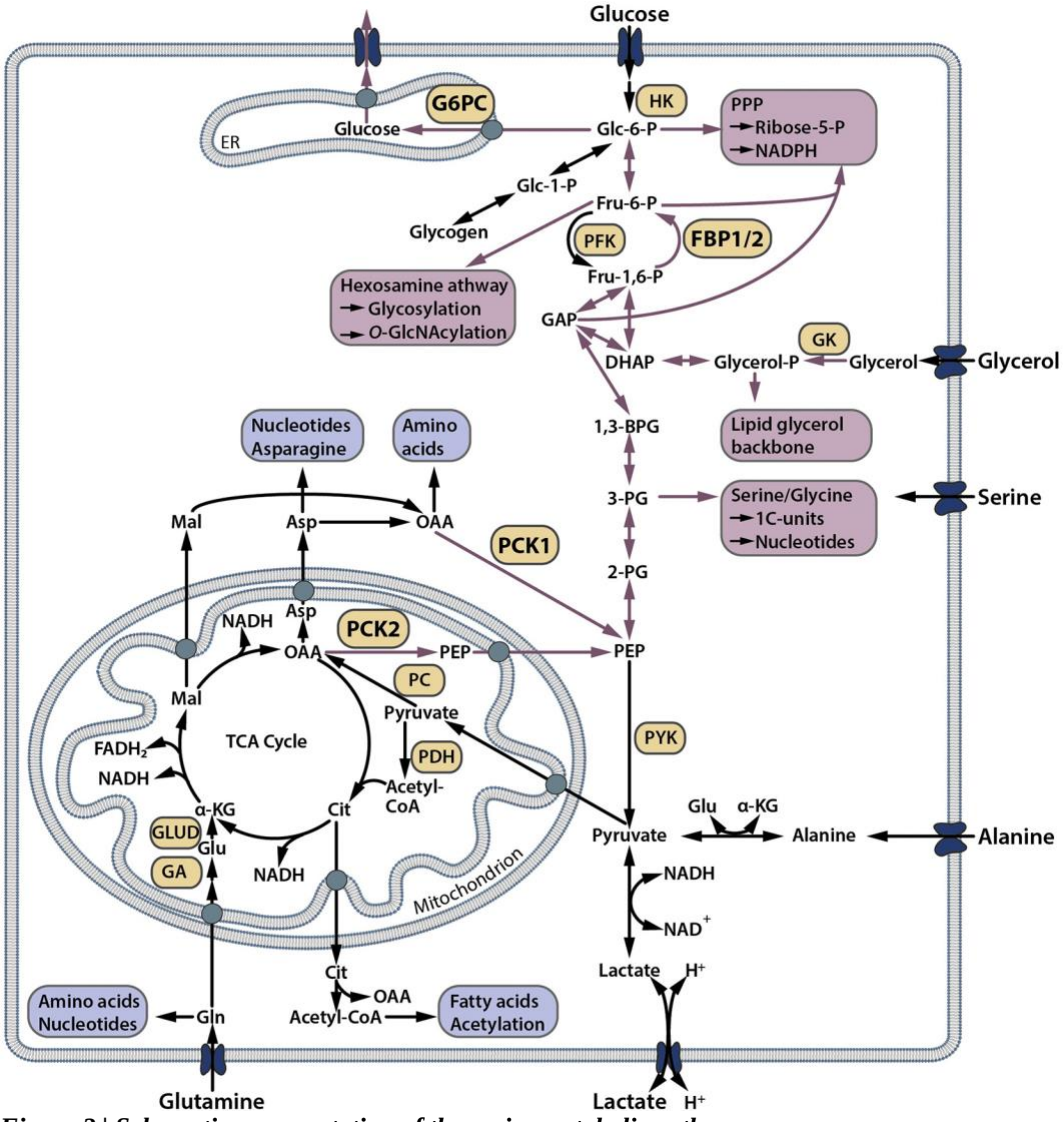

Figure 3 | Schematic representation of the major metabolic pathways
Summary of critical metabolic pathways and they key steps and products, including glycolysis, the PPP, glycogenosis and OXPHOS. The figure was reproduced from (Grasmann et al., 2019), published under a Creative Commons user license.

Table 1 | Summary and comparison of the glycolytic and oxidative metabolic pathways

Factor	Glycolysis	Oxidative Phosphorylation
Speed	Fast	Slow
Net Energy Production	2 ATP	30-32 ATP
Role of Oxygen	Can occur aerobically or anaerobically	Only occurs aerobically
Location	Cytoplasm	Mitochondria
Regulation/Rate Limiting Steps	Hexokinase Phosphofructokinase-1 Pyruvate kinase	Phosphate ADP
Reactive Oxygen Species Production	Low	High
End product	Two pyruvate or lactate molecules	CO_2 and H_2O
Primary users	Endothelial cells Astrocytes	Neurons

Table 1 | Summary and comparison of the glycolytic and oxidative metabolic pathways
This table provides a summary of the key differences between oxidative phosphorylation, also known as the Krebs or TCA cycle, and glycolysis. This information is a summary from various sources (Ainscow & Brand, 1999a, 1999b; Berg et al., 2002; Bose et al., 2003; Cox, 2005; Fernie et al., 2004).

Plasticity of Neuroenergetics Processes During Neuronal Activation

Apart from epileptic seizures, the brain's basal metabolic rate is high enough that neuronal stimulation rarely alters measures of whole brain metabolism. Rather, stimulation induces localized acute and chronic changes within the brain. Acute changes allow the brain to adapt to sudden bioenergetic challenges and chronic changes prepare brain regions to respond to these bioenergetic challenges more efficiently in the future. Some of the relevant changes are highlighted below.

Cerebral Blood Flow (functional hyperemia)

The primary contributor to both the high overall metabolism of the brain and the regional variations following activation is the high metabolic needs of synaptic transmission, more specifically the energy-dependent maintenance of membrane gradients through the reversal of ion fluxes (Attwell & Laughlin, 2001; Clark et al., 2011; Harris et al., 2012; Lee et al., 2004). Thus, elevated signalling, due to activation, increases the energetic needs of regional brain tissue, resulting in a greater need for metabolite (e.g. glucose) availability. Given the brain's limited capacity for energy storage, reactive regional blood flow and healthy and plastic vasculature is necessary to meet these acute bioenergetic challenges and maintain normal function (Caesar et al., 2008; Muthukumaraswamy et al., 2009).

In stimulated regions of the brain, cerebral blood flow rapidly increases, peaking within seconds. If stimulation is sustained, cerebral blood flow decreases slightly before plateauing (Ances et al., 2001; Buxton, 2010; Fantini et al., 2016; Kim et al., 1999; Mehler et al., 2019; Paulson et al., 2010). This response is largely modulated by astrocytes (Gu et al., 2018; Lind et al., 2013; MacVicar & Newman, 2015; Marina et al., 2020; Rosenegger et al., 2015; Straub & Nelson, 2007; Watts et al., 2018). Activation of astrocytic neurotransmitter receptors (e.g. glutamate) evoke increases in intracellular calcium (Ca^{2+}). This increase in calcium induces the release of vasoactive factors such as potassium, arachidonic acid metabolites and nitric oxide from astrocytic endfeet that surround the cerebral vasculature. These activation signals propagate across astrocytes through gap junction and pannexin or connexin channels, encouraging further nitric oxide production and subsequently vascular dilation (Muñoz et al., 2015). This is one illustration of the importance and cooperation of the so-called neurovascular unit in maintaining normal brain function.

Transporter Expression

In addition to increasing the availability of metabolites through increased blood flow (see above) and hepatic gluconeogenesis (Hyun & Sohn, 2022; Pocai, Obici, et al., 2005; Thorens, 2022), neuronal activity can also increase the membrane expression of transporters, thus enabling greater uptake to match energetic demands.

GLUT. Neuronal activation increases GLUT1 expression in capillary endothelial cells as well as astrocytes (Koepsell, 2020). Neuronal activity may also increase neuronal membrane expression of GLUT3 and GLUT4, but these findings are inconsistent (Koepsell, 2020). Regular exercise, which has been shown to result in neuronal activity (Machado et al., 2016; Soya et al., 2011), increases cortical GLUT1 (Takimoto & Hamada, 2014) expression and can regulate both GLUT1 and GLUT3 expression in a mouse model of Alzheimer's disease (Pang et al., 2019).

GLUT expression is regulated through various functions and feedback loops. An exhaustive review is outside the scope of this work, but to provide the reader with an appreciation for the breadth of mechanisms that can influence glucose transporter expression, we note the following: transcriptionally and post-transcriptionally, hypoxia increases GLUT1 mRNA transcription and enhances its stability (Bruckner et al., 1999), translation of the GLUT mRNA can be increased by insulin, and post-translational changes can impact the stability of the transporter protein, thus increasing transporter expression by extending its half-life (Duelli & Kuschinsky, 2001).

GLUT transporter expression can be altered by conditions other than neuronal activation. For example, seizures, glucose depletion and insulin have been shown to impact endothelial glucose transport (Koepsell, 2020). Insulin treatment (hypoglycemia) induced mobilization of GLUT4 transporters to the neuronal membrane in an AMPK dependent process (Ashrafi et al.,

2017) as well as a moderately increased GLUT3 (Duelli & Kuschinsky, 2001) and GLUT1 protein and mRNA expression (Sajja et al., 2014; Simpson et al., 1999). In cerebral endothelial cells, the hypoglycemic increase in GLUT1 was greater at the luminal membrane (Simpson et al., 1999), favoring uptake of glucose from circulating blood. Conversely to hypoglycemia, chronic hyperglycemia results in a moderate decrease in GLUT1 (Duelli & Kuschinsky, 2001; Leão et al., 2020) (but not in endothelial cells Simpson et al., 1999). The impact of hyperglycemia on GLUT3 is unclear (Leão et al., 2020). Chronic nicotine exposure, a substance known to regionally increase glucose utilisation, has been shown to proportionally increase GLUT1 and GLUT 3 transporter density (Duelli, Staudt, et al., 1998). In contrast, chronic visual deprivation, which limit regional glucose utilization, has been shown moderately decrease GLUT1 and GLUT3 expression in primary visual structures (Duelli, Maurer, et al., 1998). Despite a comparable reduction in glucose utilization to chronic nicotine exposure (14% vs 15%), the decrease in GLUT1 expression following visual deprivation was modest, perhaps pointing to high metabolic rate of the brain requiring a certain abundance of transporters.

MCT. Less is known about the regulation and adaptation of MCT expression in the central nervous system following neuronal activation. This lack of information is interesting given the shuttling of lactate to neurons from astrocytes, through MCT's, has been frequently demonstrated as essential for memory forming processes (Descalzi et al., 2019; Netzahualcoyotzi & Pellerin, 2020). Endothelial cell expression of MCT1 appears to be partially regulated by cAMP signalling which modulates the vesicular trafficking of the protein (Smith et al., 2012; Uhernik et al., 2014). Much more is known about the regulation and expression of MCT's in the muscle (Pérez-Escuredo et al., 2016), but it is unclear at this time if the same mechanisms impact MCT expression in the central nervous system following neuronal activation.

There is, however, evidence that both glutamate, the ubiquitous excitatory neurotransmitter of the brain released following neuronal activation, and the well-known brain derived neurotrophic factor (BDNF), both of which are released during or following neuronal activation (Pierre et al., 2009), are involved in the successful trafficking of MCT2 from an intracellular pool to neuronal membranes (Pérez-Escuredo et al., 2016). Other modulating proteins are insulin-like growth factor 1 which can increase the translation activity of MCT2 through PI3K-Akt–mTOR pathway (Debernardi et al., 2003; Pierre et al., 2003; Chenal et al., 2007; Chenal et al., 2008). Acute exercise increased cortical MCT1, MCT2 and MCT4 expression within hours and last up to 24 hours. In the hippocampus, MCT2 expression was immediately increased following exercise in a BDNF dependent manner (Robinet et al., 2010; Robinet et al., 2011; Takimoto & Hamada, 2014). These effects are similar to what can be observed in skeletal muscles (Coles et al., 2004).

Similarly to GLUT's, MCT expression can be altered by conditions that impact metabolite use and availability. Genetically obese or diabetic mice often exhibit elevated levels of cortical MCT1, MCT2 and MCT4 (Pierre et al., 2007). There are, however, contrasting results such as the decreased hippocampal MCT2 expression in diabetic Long-Evans Tokushima rats (Shima et al., 2018). Work by Canis and colleagues (Canis et al., 2009) confirms that hyperglycemia increases MCT1 expression specifically in endothelial cells and astrocytes. Food deprivation, and thus hypoglycemia, can increase MCT2 mRNA expression (Pérez-Escuredo et al., 2016). Hypoxia, in the form of ischemia, can increase MCT expression in neurons and astrocytes (Rosafio and Pellerin 2014) and can enhance transcription of MCT4 mRNA (Pérez-Escuredo et al., 2016).

Metabolite fluctuations

Glucose, lactate and oxygen. During neuronal stimulation, four general observations can be made regarding the main energy metabolites glucose, lactate and oxygen. **First**, there is a local increase in cerebral glucose uptake (Lundgaard et al., 2015; Patel et al., 2004; Sampol et al., 2013). As mentioned above, this net elevated uptake following neuronal activation is comprised of: a) increased blood flow which increases metabolite availability, b) increased expression of glucose transporters which enhances the capacity of various cellular comportments to take up glucose and c) increased glucose utilization in neuronal (and other) cells which increases the disparity between intra- and extracellular metabolite pools driving uptake through diffusion principles. **Second**, this increased uptake of glucose within neurons (and other cells) depletes the interstitial glucose pool found in the extracellular space of the brain (Béland-Millar & Messier, 2018, 2022; Hu & Wilson, 1997b; Li & Freeman, 2015; Lourenço et al., 2017; Patel et al., 2004; Smith et al., 2018). **Third**, this dip in extracellular glucose is accompanied by a significant increase in extracellular lactate (Béland-Millar & Messier, 2018, 2022; Hu & Wilson, 1997b; Li & Freeman, 2015; Lourenço et al., 2017; Patel et al., 2004; Sampol et al., 2013; Smith et al., 2018). Below we will explore the controversies regarding the origin of this increase in extracellular lactate (neuronal or astrocytic), but this increase in lactate is broadly considered to be the result of glycolysis outpacing OXPHOS. The decreases and increases in extracellular glucose and lactate, respectively, progress generally throughout stimulation but plateau and return to baseline rather quickly once the stimulation has ended (Li & Freeman, 2015; Patel et al., 2004). The magnitude of the extracellular fluctuations as well as the latency to return to baseline are dependent on the intensity and frequency of the stimulation (Korf, 2006; Smith et al., 2018). The observed changes cited above are closely matched to measured electrical activity (a proxy of neuronal activity) (Lourenço et al., 2017; Patel et al., 2004). **Finally**, a decrease in

local oxygen concentration is observed (Hu & Wilson, 1997b). Interestingly, this decrease in oxygen is much smaller than expected given the concomitant rise in glucose consumption.

Glycogen. Significant and immediate changes in metabolite consumption during high intensity neuronal activation requires a sufficient local pool of metabolites. Functional hyperemia eventually provides an additional influx of blood-borne metabolites, but the temporal correlation between the onset of neuronal activity and increased cerebral blood flow can be delayed by seconds, if not minutes (Brown & Ransom, 2015; Li & Freeman, 2015). This points towards another important player in metabolic cooperation: glycogen. Indeed, astrocytic glycogen content decreases quickly with neuronal activation (Brown, 2004; Kong et al., 2002; Matsui et al., 2011; Öz et al., 2007) and plays an important role in memory consolidation (Gibbs, 2016).

When there is a surplus, glucose is converted to glycogen (glycogenesis) in an energy (ATP) dependent process. During times of increased metabolic demand, such as neuronal activation, glycogen is converted to G6P (glycogenolysis). Production of G6P through glycogenolysis rather than glycolysis has some advantages. First, additional G6P (glycolysis + glycogenolysis) provides additional substrates to be metabolized. Second, if neurons do indeed exclusively consume glucose (as will be discussed below), production and use of G6P in astrocytes through glycogenolysis would provide a glucose-sparing effect for neurons through its inhibitory feedback loops on astrocytic hexokinase (Liu et al., 1999; Newsholme et al., 1968; Tornheim, 2018). Perhaps most importantly, glycogenolysis in itself is not an energy dependent process. Though ATP is consumed in the production of glycogen, no ATP is consumed by the glycogen phosphorylase and phosphoglucomutase enzymes responsible for the conversion of glycogen to G6P (Nadeau et al., 2018). Additionally, glycogen conversion is also considered to be faster than glycolysis, whose pace is set by hexokinase (Brown & Ransom, 2015). Together,

despite requiring additional ATP at the onset (glycogenesis), glycogenolysis provides a quick metabolic buffer that can both replace and spare glucose use. Importantly, this glycogen phosphorylase is highly sensitive to changes in energy shifts (AMP) (Brown & Ransom, 2015).

Regulation of metabolic pathways during activation

Regional changes in energy consumption during neuronal activation involve the (up)regulation of the main energy producing pathways, OPXHOS and glycolysis. The primary points of regulation for these pathways (e.g. PFK) have already been discussed. What remains is the extent to which and the cellular compartment in which these pathways are upregulated in response to the bioenergetic challenge that is neuronal stimulation.

Unfortunately, there is no clear consensus as to proportional increase, the timescale scale of these changes or relative increase in respective cell (e.g. neurons vs astrocytes) types for these energy producing pathways during activation. What is known is that both astrocytes and neurons increase glycolysis as well as OXPHOS in response to neuronal activation (Dienel, 2019a; Vaishnavi et al., 2010), and that upregulation of glycolysis outpaces that of OXPHOS (hence the lactate accumulation) (Dienel, 2019a). An expected consequence of this outpacing is the production of lactate in excess of the glucose and oxygen consumed during neuronal stimulation (Fox & Raichle, 1986). Some authors suggest that neural activity inititally upregulates neuronal OPXHOS, followed shortly thereafter by astrocytic glycolysis and glycogenolysis (Kasischke et al., 2004; Pellerin & Magistretti, 1994). If neuronal OXPHOS is immediately elevated following neural activity, lactate dynamics, as measured extracellularly via biosensors and intracellularly via genetically encoded sensors for lactate (laconic), suggests that the first seconds of neuronal OXPHOS would be maintained through its own glycolysis or internal pyruvate stores (Zuend et al., 2020).

Although this introduction will not resolve this long-debated issue (Yellen, 2018), we can nonetheless appreciate the complexity of the interactions between this so-called neurovascular unit. Indeed, regulation of these pathways and key enzymes within are regulated by a plethora up and downstream products. The production of glycogen creates G6P which can inhibit hexokinase. The ascorbic acid release from astrocytes following glutamate stimulation can inhibit glucose uptake within neurons (Castro et al., 2009). In addition to modulating glycolysis (Zhang et al., 2019), lactate can modulate spiking activity in cerebral cortex neurons (Karagiannis et al., 2021) as well as disturb electrical oscillations in hippocampal networks and attenuate synaptic transmission in excitatory pyramidal cells (Hollnagel et al., 2020) through its impact on neurotransmitter release in pre-synaptic neurons. All of these factors influence the metabolic requirements and adaptions of individual cells.

Uncoupling of Oxygen and Glucose Consumption During Cerebral Activation

Key insights from neuroimaging. The development of non-invasive methods has been critical in expanding our understanding of *in vivo* mammalian neuroenergetics. In this respect, two particular methods stand out: positron emission tomography (PET) and fMRI (functional magnetic resonance imaging). Though a full review of the function and insights these techniques allow is outside the scope of this work, a key contribution to our understanding of human brain metabolism will be highlighted.

With the use of fMRI, oxygen consumption in the brain is estimated using the blood-oxygen-level dependent (BOLD) signal, as well as other vascular and energetic parameters (Ances et al., 2008; Davis et al., 1998; Leontiev & Buxton, 2007; Sicard & Duong, 2005). Oxygen consumption and delivery are considered reliable estimates of the brain's neuroenergetic needs given that synaptic activity is the primary consumer of ATP, and oxidative

phosphorylation (an oxygen dependent pathway) is considered to be the primary source of ATP production. This approach can identify, with great spatial and temporal accuracy, regions of brain activity through changes in the BOLD signal (Mier & Mier, 2015; Purnell et al., 2011; Rodgers et al., 2016). However, it is not particularly informative with regards to precise metabolic parameters. For this reason, BOLD signals are often complemented with measures of cerebral metabolic rate of oxygen consumption (CMRO2) to estimate cerebral energy consumption more accurately (Ances et al., 2008; Davis et al., 1998; Leontiev & Buxton, 2007; Sicard & Duong, 2005), using the same basic neuroenergetic assumptions above. PET can collect absolute quantitative CMRO2, as well as cerebral metabolic rate of glucose (CMRGlc), at a reduced spatial and temporal resolution. Though PET lacks the spatial and temporal resolution of fMRI, it can more accurately quantify substrate uptake (e.g. oxygen, glucose). Unlike fMRI which utilizes the natural oxygenation of the blood to measure changes in blood flow to infer brain activity, PET utilizes radioactive tracers that, when metabolize, emit signals that can be quantified.

These approaches (PET and fMRI) were combined by Peter Fox and Marcus Raichle in the late 1980's to provide a controversial and insightful piece of information (Fox et al., 1988; Fox & Raichle, 1986) that has since been reliably replicated (Benveniste et al., 2018; Frahm et al., 1996; Lowry & Fillenz, 1997; Vaishnavi et al., 2010). They reported an uncoupling between the magnitude of the increases in CBF and oxidative metabolism (CMRO2) during stimulation. In other words, when stimulated, the increase in CBF was not proportional to the increase in CMRO2. The increase in oxygen consumption (CMRO2) during stimulation was smaller than what the large increase in CBF may have suggested. Studies further quantified this difference by calculating the ratio of glucose to oxygen consumption, also known as the oxygen-glucose index

(OGI) (Mergenthaler et al., 2013). Stoichiometrically, for full oxidation of every (1) glucose molecule, 6 molecules of oxygen should be consumed. At resting state, this ratio can vary from 4.1:1 to 6.2:1 (Dienel, 2017; Fromm & Hargrove, 2012; Saraste, 1999), suggesting that at rest, glucose is largely consumed via oxidative pathways (OXPHOS). During activation this ratio is often reduced, anywhere from 2.7:1 to 6.3:1, depending on the experimental conditions (Dienel, 2017). This reduction in the oxygen-glucose index (OGI) implies that during activation, an increasing portion of glucose is being consumed non-oxidatively. Consequently, activation-induced increases in energetic demands likely require the use of glucose in a non-oxygen dependent pathway. As to which pathways, and which subcellular compartments this glucose is being consumed in non-oxidatively was unclear at the time and remains the topic of debate which has crystallized around the Astrocyte-to-Neuron Lactate Shuttle (ANLS) hypothesis proposed by Pellerin and Magistretti in 1994 (Pellerin & Magistretti, 1994).

Astrocyte-to-Neuron Lactate Shuttle Hypothesis

Briefly, the ANLS hypothesis posits that glutamate release following neuronal activation is taken up by surrounding astrocytes via the Na^+-dependent glutamate transporter. This sudden increase in intracellular Na^+ activates the Na^+/K^+ pump in order to restore intracellular Na^+ levels. The Na^+/K^+ pump is energy (ATP) dependent as it transports Na^+ against a concentration gradient. To compensate for this increased energetic demand in response to neuronal signalling, the proponents of the ANLS hypothesis suggest that astrocytes upregulate their glycolytic capacity in order to fuel the increased activity of the Na^+/K^+ pump. The increase in glycolysis results in additional intracellular pyruvate. Within the astrocytes, a portion of the pyruvate is then converted to lactate via the lactate dehydrogenase B (LDHB) enzyme and subsequently transported out of the astrocytes and into the extracellular space via MCT1/4. This extracellular

lactate would be taken up by surrounding neurons via MCT2. Once in the intracellular space of the neurons, lactate would be converted back to pyruvate via the equilibrative lactate dehydrogenase A (LDHA) enzyme (Elustondo et al., 2013; Glancy et al., 2021; Lemire et al., 2008). The glucose molecule, initially metabolized in the astrocyte, can now enter the OXPHOS pathway within the neuron as pyruvate to produce maximal ATP. In summary, glucose is taken up by astrocytes and converted to pyruvate (glycolysis) and subsequently lactate to be shuttled off to the surrounding neurons, where is it converted back to pyruvate to enter OXPHOS. It is important to note that this hypothesis does not suggest that this astrocyte-derived lactate is the only source of neuronal energy, simply that it is an important one. In this hypothesis, neurons remain capable of metabolize glucose through their own internal pathways.

Though the ANLS hypothesis has gained a lot of momentum (Bouzier-Sore et al., 2003, 2006; Pellerin, 2008; Wyss et al., 2011), there are proponents of a more traditional and neuro-glucose centric perspective on the role of lactate in brain metabolism (Dienel, 2012; Dienel & Hertz, 2001; Mangia et al., 2009). This opposing perspective can be summarised by the Neuron-to-Astrocyte Lactate Shuttle (NALS) hypothesis. The primary distinction to be made between these two theories is the directional flux and use of lactate. The NALS hypothesis posits that neural activation induces increased neuronal consumption of glucose, and not astrocyte-derived lactate. This additional glucose consumption would be the result of elevated glycolysis (for metabolite intermediates), PPP (for antioxidants) and OXPHOS (for ATP production) pathways. Elevated glycolysis would in turn result in accumulated lactated which would then be shuttled to the surrounding astrocytes as a metabolic waste-product. Astrocytes would transfer this neuronal lactate to the surrounding vasculature to be discarded or used elsewhere in the body. **Table 2** summarizes key arguments and findings core to these hypotheses. An in-depth review of the

merits of these arguments will not be conducted as this have been done numerous times and would fall outside the scope of this work which has as its objective to add fundamental information to our knowledge of mammalian brain metabolism and contribute to this discussion without intention of proving or disproving. It is, however, worth noting that many of the arguments made in favor of the NALS, or more specifically against the ANLS, are primarly based on modelling studies and theoretical considerations of thermodynamics. Though the ANLS hypothesis initially lacked *in vivo* evidence, there has been substantial recent *in vivo* and *in vitro* work providing compelling evidence for this theory (non-exhaustive list: Cai et al., 2022; Roumes et al., 2021, Bouzier et al., 2000; Wyss et al., 2011; Zimmer et al., 2017; Netzahualcoyotzi & Pellerin, 2020; Mächler et al., 2016).

Table 2 | Supporting evidence for the ANLS and NALS hypothesis

Factor	ANLS	NALS
Astrocyte Morphology	1. Endfeet surrounding blood vessels and perisynaptic processes at synapses (Halassa et al., 2007; Kacem et al., 1998; Oberheim et al., 2009; Witcher et al., 2007) 2. Nonoverlapping and connected astrocytic processes (Bernardinelli et al., 2004; Rash et al., 2001) 3. Energy stores are found exclusively in astrocytes (Brown, 2004; Meurant, 2012; Vilchez et al., 2007) 4. Larger number of astrocytes than neurons in the brain (Nedergaard et al., 2003)	1. Challenges with cryopreservation (Korogod et al., 2015) 2. Lack of mitochondria in dendritic spines (Bergersen et al., 2001, 2002) 3. Presence of mitochondria in astrocytes (Lavialle et al., 2011; Lovatt et al., 2007; Pardo et al., 2011)
Glucose Transport	1. Increased astrocytic glucose consumption following stimulation (Jakoby et al., 2014; Pellerin & Magistretti, 1994; Zimmer et al., 2017) 2. Glutamate inhibits transport of glucose in neurons (Porras et al., 2004)	1. Neurons possess a higher capacity glucose transport system (DiNuzzo et al., 2010a; Mangia et al., 2009; Simpson et al., 2007)
Lactate Transport	1. As with muscles, MCT4 export from astrocytes and MCT2 import for neurons (Pierre & Pellerin, 2005) 2. Evidence of mitochondrial lactate oxidation (Hashimoto et al., 2008)	1. Lactate is preferentially exported from the brain (Ball et al., 2010; Blomqvist et al., 1990; Cruz et al., 1999; Gandhi et al., 2009; Hawkins et al., 1973; Madsen et al., 1995) 2. Blocking lactate transport only increases neuronal glucose consumption by 10% (Dienel, 2012)
Glycolysis Upregulation	1. Down regulation of PFKFB3 in neurons (Almeida et al., 2004; Herrero-Mendez et al., 2009) 2. Activity related ions increase astrocytic glycolysis (Bittner et al., 2011; Lerchundi et al., 2015; San Martín et al., 2017)	1. Activation stimulates neuronal glycolysis rather than astrocytic glycolysis (Dienel, 2017) 2. Neurons are capable of increasing their glucose consumption, transport and glycolysis (Dienel, 2012) 3. Inhibition of astrocytic glycolysis by through glycogenolysis (DiNuzzo et al., 2010b)
Cell-Specific Metabolic Profiles	1. Inhibition of mitochondria is innocuous for astrocytes and inhibition of glycolysis is innocuous for neurons (Fünfschilling et al., 2012; Supplie et al., 2017; Volkenhoff et al., 2015) 2. High phosphorylation of the PDH enzyme (Halim et al., 2010; Itoh et al., 2003) and poor coupling of the	1. Astrocytes are also capable of metabolizing lactate (Zielke et al., 2007, 2009) 2. Both astrocytes and neurons release lactate under various conditions (Walz & Mukerji, 1988)

	mitochondrial complex (Lopez-Fabuel et al., 2016) in astrocytes 3. High rate of neuronal oxidative phosphorylation (Boumezbeur et al., 2010; Bouzier-Sore et al., 2006; Itoh et al., 2003) 4. High rate of astrocytic glycolysis (Bittner et al., 2010; Herrero-Mendez et al., 2009; Itoh et al., 2003) 5. Neuronal ability to metabolise lactate (Boumezbeur et al., 2010; Bouzier et al., 2000; Qu et al., 2000; Schurr et al., 1997; Serres et al., 2005)	3. Not all astrocyte cultures release lactate following glutamate stimulation (Dienel, 2012; Hertz et al., 1998) 4. Stimulation activates both glycolysis and oxidative phosphorylation in astrocytes (Hertz et al., 2007) 5. Glycogenolysis in astrocytes maintains high levels of G6P to inhibit hexokinase and save glucose for neuronal use (Dienel & Cruz, 2016)
Methodological Limitations	1. In vitro experiments done separately 2. Inferring feasibility rather than proving it 3. Difficulty in proving essential stochiometric (Dienel, 2017) 4. Challenges in adequate quantification of metabolite use with labelled substrates (Adachi et al., 1995; Cruz et al., 1999; Dienel, 2012; Dienel & Cruz, 2009) 5. The ability of lactate to sustain evoked potentials is a result of slice preparation (Lajtha et al., 2007)	1. No abnormalities in GLUT3 haploinsufficient mice (Schmidt et al., 2008; Stuart et al., 2011; Wang et al., 2006)
LDH	1. Differential expression of LDH isoform (as well as other enzymes) (Berkich et al., 2007; Bittar et al., 1996; Hamberger & Hyden, 1963; Hyden & Lange, 1962; Laughton et al., 2000, 2007; Ramos et al., 2003; Zhang et al., 2014)	1. Differential expression not always corroborated 2. Distinct distribution of LDH does not predict cellular production or consumption of lactate (Bak & Schousboe, 2017; Quistorff & Grunnet, 2011; Ross et al., 2010) 3. Comparative measurements of enzyme presence in astrocytes and neurons were done in developing brains (Dienel, 2017)
Resting State Metabolism	1. Neuronal preference for lactate (Bouzier-Sore et al., 2003, 2006; Itoh et al., 2003) 2. Astrocytes maintain higher levels of intracellular lactate and NADH/NAD^+ than neurons (Mächler et al., 2016; Mongeon et al., 2016) 3. Astrocytic lactate impacts neuron polarisation through lactate/pyruvate conversion (Sada et al., 2015) 4. At rest, astrocytes are responsible for half of the glucose uptake (Nehlig, 2004), and more during active (Chuquet et al., 2010)	1. Abnormally elevated NADH redox ratio by using pure lactate (Dienel, 2012) 2. Basal extracellular lactate levels would have to be much higher to serve as an efficient, long term fuel (Dienel, 2012)

	5. Labelled lactate is metabolized in a pyruvate carboxylase free comportment (neurons) (Bouzier et al., 2000)	
Active State Metabolism	1. Blocking lactate transport with exogenous lactate inhibits changes in redox state (Díaz-García et al., 2017; Mongeon et al., 2016) 2. Activity dependent decreases in neuronal glucose consumption when lactate is available (Mächler et al., 2016; van Hall et al., 2009) 3. Stimulation induces a decrease (Hu & Wilson, 1997b; Mangia et al., 2003; Mangia et al, 2007) followed by a sustained increase (Bouzier-Sore et al., 2003, 2006; Klepper & Voit, 2002; Pellerin & Magistretti, 1994; Mangia et al., 2007) in brain lactate, mirrored by redox changes (Kasischke et al., 2004) 4. Stimulation induced astrocytic glucose uptake (Barros & Deitmer, 2010; Chuquet et al., 2010; Pellerin et al., 2007) and inhibits neuronal glucose uptake (Castro et al., 2009; Porras et al., 2004) 5. Astrocyte glutamate transport regulates, at least partially, FDG glucose uptage (Zimmer et al., 2017)	1. Neurons metabolise glucose in an activity-dependent manner (Bak et al., 2006; Díaz-García et al., 2017b; Lundgaard et al., 2015; Patel et al., 2014) 2. Blocking lactate transport during neuronal stimulation did not inhibit changes in redox state 3. Neurons are capable of producing lactate (Bak et al., 2009) 4. Lactate oxidation would require stochiometric increase in oxygen consumption (Dienel, 2017) 5. Lactate accumulation is much smaller than pyruvate (Dienel et al., 2007)
Functional Data	1. Cognitive ysfunction caused by inhibition of astrocytic lactate transport, astrocytic glycogen production or attenuated astrocytic glucose uptake can be rescued by extracellular lactate or other monocarboxylates (Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011; Descalzi et al., 2019; Alberini et al., 2017; Zhang et al., 2022; Roumes et al., 2021; Netzahualcoyotzi & Pellerin, 2020) 2. Astrocytic lactate is capable of sustaining neuronal function (Dringen et al., 1993; Morgenthaler et al., 2006; Nagase et al., 2014; Nehlig, 2004; Parsons & Hirasawa, 2010; Rouach et al., 2008; Sickmann et al., 2009) 3. Astrocyte glutamate uptake necessary for glucose brain uptake (Cholet et al., 2001; Chuquet et al., 2010; Herard et al., 2005; Sokoloff et al., 1977; Voutsinos-Porche et al., 2003; Zimmer et al., 2017)	1. Lactate is not sufficient to sustain evoked potentials (Allen et al., 2005; Dienel & Hertz, 2005) 2. Astrocytic vascular control could be to export lactate to be used by the muscles 3. Lactate cannot fulfill many glucose functions (Dienel, 2012) 4. Lactate is exclusively an opportunistic fuel (Dienel, 2012) 5. Energy produced by glycolysis would not be sufficient to sustain the energetic needs of the astrocyte (Dienel, 2012) 6. Astrocytes do not metabolise sufficient glucose to provide adequate fuel to neurons (Dienel & Hertz, 2001)

4. Astrocytic vascular control (Attwell et al., 2010; Carmignoto & Gómez-Gonzalo, 2010; Gordon et al., 2008; Iadecola & Nedergaard, 2007; Takano et al., 2006)
5. Astrocytes, lactate and LDHA regulate neuronal activity (Magistretti & Allaman, 2018; Herrera-López & Galván, 2018; Yao et al., 2023; Skwarzynska et al., 2022; Beard et al., 2022; Bonvento & Bolaños, 2021)
6. Lactate transporters modulate BOLD fMRI signals (Roumes et al., 2021)
7. Lactate injections rescue the loss in voltage-sentive dye signals during hypoglycemia (Wyss et al., 2011)

Table 2 | Supporting evidence for the ANLS and NALS hypothesis
This table provides a summary of key pieces of evidence that support or contradict the competing metabolic theories.

Summary

The brain is an energetically demanding organ. Despite this, it possesses an extremely limited energy store (glycogen). Thus, the brain is heavily reliant on peripheral production and delivery of metabolites for optimal neuroenergetics. The delivery of metabolites to the brain involves the exchange of metabolites across cellular compartments, including endothelial cells and astrocytes. This exchange relies on differentially expressed transporters (e.g. GLUTs and MCTs). In addition to transporter expression, exchange of metabolite across cellular compartments, and therefore fluctuations of extracellular metabolite pools, are heavily influenced by concentration gradients. This includes concentration gradients between peripheral (blood) and central (brain) metabolite pools, as well as cortical gradients between intra- and extracellular metabolite pools (e.g. glucose concentration within a neuron vs glucose concentration in the cerebrospinal fluid). In turn, concentration gradients of these metabolic substrates are primarily regulated by their processing through the major metabolic pathways (glycolysis, OXPHOS, and PPP) – all of which have their own purpose.

Taken together, two things become clear. Firstly, that the exchange of metabolites across cellular compartments, including neurons and the BBB, is critical for cerebral metabolic health. Secondly, that there is an inherent interconnectedness between peripheral and central metabolite pools, as well as intra- and extracellular cerebral metabolite pools. Despite this interconnectedness, investigations of cerebral energetics seldom integrate a system wide perspective into their studies. What is less certain is the glucose-centric perspective of neuronal metabolism which has been called into question. A paradigm shifting hypothesis (ANLS) has suggested that neurons may utilize, with some going as far as to say preferentially so, lactate to meet their energetic needs. The central change brought upon by this hypothesis is the directional

flow of lactate – to neurons, rather than from. Naturally, transfer of metabolites across the extracellular space for metabolic purposes would impact the extracellular metabolite pools. Thus, we endeavored to contribute to this on-going debate and bridging knowledge gaps by taking a broader systems perspective in investigating how peripheral, environmental factors and genetic factors may influence the exchange and use of metabolites.

Results

When the peripheral availability of various metabolic fuels was altered, we uncovered a seemingly universal response pattern where blood lactate increased but cortical extracellular glucose was increased (see **Chapter II**; Béland-Millar et al., 2017). We therefore hypothesized a coordinated effort between central and peripheral mechanisms to coordinate the breakdown of various substrates towards a universal down-stream metabolite (lactate) in the liver. Lactate production would therefore spare glucose use and enable an optimized and simplified central metabolism that would favor limited number of metabolic pathways. This hypothesis was supported by findings of enhanced monocarboxylate (lactate) transport and metabolism in the brain of rats chronically exposed to a high-fat diet (see **Chapter III**; Béland-Millar et al., 2020) as well as the attenuation of the extracellular glucose rise following pharmacological inhibition of peripheral monocarboxylate transporters (see **Chapter VI**).

We subsequently confirmed extracellular glucose and lactate uncoupling in behavioral and visual paradigms of neuronal activation (see **Chapter IV and V**, respectively; Béland-Millar & Messier, 2018, 2022). Interestingly, these studies hinted at the dependency of these extracellular metabolite fluctuations on the intensity of the induced neuronal activation (and consequently novelty) as well as metabolite availability. Additional peripheral lactate prevented a dip in cortical extracellular glucose (see **Chapter V**; Béland-Millar & Messier, 2018),

suggesting a glucose sparing effect. Pharmacological inhibition of monocarboxylate transporters also attenuated the expected rise in behavior-induced extracellular lactate (see **Chapter VI**), further emphasizing the role of peripheral metabolite pools, and specifically lactate, on acute cerebral energetics. Pharmacological inhibition of glucose and lactate transporters also highlighted the immense speed at which endothelial cells adapt their transporter expression in response to bioenergetic challenges (see **Chapter VI**).

Finally, using the same biosensor approach in combination with other techniques (e.g. positron emission tomography), we demonstrated an altered cerebral metabolic profile in an autism mouse model (see **Chapter VII**; Béland-Millar et al., 2023). This model also presented with attenuated motor-induced extracellular cortical lactate, as well as elevated cerebral glucose uptake and stable oxygen levels. Taken together, this suggests that this ASD mouse model compensates for aberrant cerebral metabolism by shuttling glucose through non-oxidative pathways such as glycolysis or PPP to meet its energetic needs to combat inefficient mitochondrial respiration.

Chapter II: Effects of Systemic Metabolic Fuels on Glucose and Lactate Levels in the Brain Extracellular Compartment of the Mouse

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Abstract

Classic neuroenergetic research has emphasized the role of glucose, its transport and its metabolism in sustaining normal neural function leading to the textbook statement that it is the necessary and sole metabolic fuel of the mammalian brain. New evidence, including the Astrocyte-to-Neuron Lactate Shuttle hypothesis, suggests that the brain can use other metabolic substrates. To further study that possibility, we examined the effect of intraperitoneally administered metabolic fuels (glucose, fructose, lactate, pyruvate, ß-hydroxybutyrate, and galactose), and insulin, on blood, and extracellular brain levels of glucose and lactate in the adult male CD1 mouse. Primary motor cortex extracellular levels of glucose and lactate were monitored in freely moving mice with the use of electrochemical electrodes. Blood concentration of these same metabolites were obtained by tail vein sampling and measured with glucose and lactate meters. Blood and extracellular fluctuations of glucose and lactate were monitored for a 2-h period. We found that the systemic injections of glucose, fructose, lactate, pyruvate, and ß-hydroxybutyrate increased blood lactate levels. Apart for a small transitory rise in brain extracellular lactate levels, the main effect of the systemic injection of glucose, fructose, lactate, pyruvate, and ß-hydroxybutyrate was an increase in brain extracellular glucose levels. Systemic galactose injections produced a small rise in blood glucose and lactate but almost no change in brain extracellular lactate and glucose. Systemic insulin injections led to a decrease in blood glucose and a small rise in blood lactate; however brain extracellular glucose and lactate monotonically decreased at the same rate. Our results support the concept that the brain is able to use alternative fuels and the current experiments suggest some of the mechanisms involved.

Keywords*: brain metabolism, blood-brain barrier, galactose, insulin, pyruvate,* ß-*hydroxybutyrate, fructose, astrocytes*

Introduction

The study of mammalian brain bioenergetics has centered on the transport of glucose and its metabolism in the presence of oxygen. This view is based on the inescapable fact that the brain eventually ceases to function in the complete absence of either glucose or oxygen. The various aspects of the brain's metabolism of glucose have been extensively studied and form the basis of our understanding regarding how the brain uses energy. The possibility that the brain could use different metabolic substrates or that intermediary metabolic substrates could play a major role in brain metabolism has been suggested at various times in the history of brain metabolism research (Schurr, 2014). The revival of some earlier hypotheses have crystalized around the astrocyte-lactate shuttle hypothesis. Modeled from the lactate shuttle in muscles (Brooks, 1986; Brooks et al., 1999; Brooks, 2000; Giugliano et al., 2008), the Astrocyte-to-Neuron Lactate Shuttle (ANLS) hypothesis was first proposed by Pellerin and Magistretti (Pellerin & Magistretti, 1994). This hypothesis postulates that in times of increased neuronal activity, and thus energy demand, astrocytes take up blood glucose via their particularly well-positioned endfeet on capillaries and convert this glucose to lactate. Lactate is then shuttled to surrounding neurons for oxidative phosphorylation, avoiding the glycolytic steps involved in the full metabolism of glucose.

Support for the ANLS hypothesis comes from a variety of research fields. Some key findings center on molecular studies that have identified a mitochondrial redox complex comprised of a monocarboxylate transporter (MCT1) and lactate dehydrogenase (LDH) enzyme in neurons (Brooks et al., 1999; Hashimoto et al., 2008; Hashimoto & Brooks, 2008; Passarella et al., 2008). Consequently, neuronal mitochondria would appear to possess the ability to take in lactate and convert it to pyruvate in order to sustain oxidative metabolism. Secondly, studies

relating to memory performance in chicks (Baker & Edwards, 2007; Gibbs & Hertz, 2008) and rats (Newman et al., 2011) demonstrate the importance of lactate transfer for memory consolidation. Lastly, in vitro rat hippocampal slices prove to be quite efficient at metabolizing lactate (Brooks, 2007; Schurr et al., 1988, 1997). For a more complete presentation of the evidence supporting this hypothesis, readers are directed to the following reviews (Bouzier-Sore & Pellerin, 2013; Pellerin, Pellegri, Bittar, et al., 1998; Pellerin et al., 2007; Pellerin, 2008; Pellerin & Magistretti, 1996; Schurr et al., 1999; Schurr, 2006). Additionally, for a review of the similarities between the muscular lactate shuttle and the cerebral ANLS hypothesis, see Brooks (Brooks, 2002, 2009) and Gladden (Gladden, 2004).

A competing hypothesis, the neuron-to-astrocyte lactate shuttle (NALS) (Mangia et al., 2009), is based on a number of arguments that center on the different characteristics of GLUT1, the glucose transporter found on the epithelial cells of blood vessels and on astrocytes, and GLUT3, the transporter found mainly on the neuropil of neurons (Chih et al., 2001; Chih & Roberts, 2003; Mangia et al., 2009; Massucci et al., 2013). GLUT1 has a high affinity for glucose (Heyes, 2010) and is also capable of transporting other hexose at a much-reduced rate (Gould et al., 1991). GLUT1 appears to be more prevalent on the luminal membrane of the vascular epithelium compared to the abluminal side (Cornford & Hyman, 2005; Farrell & Pardridge, 1991; Kubo et al., 2015; Simpson et al., 2001), though the exact ratio and distribution is debated (Akiyoshi et al., 1999; Farrell & Pardridge, 1991). This asymmetrical distribution of GLUT1 and the presence of hexokinase within the cells suggest that glucose is mostly transferred unidirectionally from blood to brain even though, given greater brain glucose concentrations, glucose could be transported from brain to blood. Based on its transport kinetics, the ability the transporter GLUT3 to transfer glucose is thought to be greater than GLUT1,

making glucose entry into neurons more efficient (Simpson et al., 2007, 2008). However, the necessity for glucose to diffuse through endothelial cells, pericytes, astrocytes as well as the extracellular space can limit neuronal access suggesting that high affinity transporters may be essential to utilizing any residual glucose found in the extracellular space following its transport through the numerous membranes.

Similar to glucose, lactate transport between blood and brain appears to be bidirectional and favors the use of the monocarboxylate transporter MCT1. MCT1 transport of lactate begins with a proton binding and ends with the dissociation of the proton and the lactate ion on the opposite side of the membrane (Halestrap & Price, 1999). MCT1 is also capable of transporting other naturally-occurring monocarboxylates, such as pyruvate, lactate, acetoacetate, and ß-hydroxybutyrate. The transport of these other monocarboxylates, however, occurs at a lower rate (Halestrap, 2012; Halestrap & Price, 1999; Juel & Halestrap, 1999). In contrast to GLUT1, MCT1 appears to be equally distributed between the luminal and the abluminal membrane of epithelial cells (Kubo et al., 2015; Leino et al., 1999).

Beyond examining the impact of systemically administered nutrients on brain extracellular lactate and glucose, we wanted to revisit the mechanisms involved in the memory-facilitating effects of sugars (Gold, 1995; Messier, 2004). Glucose has been shown to improve memory in rats, mice, pigeons, and humans (Messier et al., 1998, 1999; Messier & Destrade, 1988; Parkes & White, 2000; Parsons & Gold, 1992) while fructose effect has been demonstrated in rats (Messier & White, 1987; Rodriguez et al., 1994). Both systemic and intracerebral injections of glucose improve memory performance (McNay et al., 2000; Messier & White, 1987). Additionally, intra-hippocampal injections of lactate are also capable of improving memory. In contrast, the intra-hippocampal injection of the MCT2 (the predominantly neuronal

monocarboxylate transporter) blocker alpha-cyano-4-hydroxycinnamate (4-CIN) impairs memory performance (Newman et al., 2011), hinting toward an important role of lactate transport in memory. As a first step, we examined the effects of the systemic injection of alternative fuels on systemic and extracellular glucose and lactate fluctuations in order to better understand the mechanisms underlying brain energetics.

Methods

Animals

Sixty one, 15–20 week old, male CD1 mice (Charles River Canada, St-Constant, Québec, Canada) were individually housed and maintained on a reverse 12-h night/day cycle with light on at 7 p.m. Mice had ad libitum access to standard chow (Teklad Global 18% Protein 2018, Teklad Lab Animal Diets, Envigo, Mississauga, Canada) and water, unless otherwise specified. All testing was conducted during the night phase of the cycle with the use of dim red lighting. All procedures in this study adhere to guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Surgical procedure

Pre-surgical treatment included a 0.05 mg/kg subcutaneous (s.c.) injection of buprenorphine hydrochloride (Reckitt Benckiser Healthcare, Hull, North Humberside, UK) as well as 1 ml of 0.9% saline (Hospira, Montreal, Canada).

Immediately prior to surgery, mice were anesthetised using 4–5% isoflurane (Fresenius Kabi Canada Ltd., Richmond Hill, ON) then secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Mice were maintained under anesthesia during the surgery with 1–2.5% isoflurane and kept warm with a heated pad (TP650, Gaymar Industries, Orchard Park, NY, USA). Based on the stereotaxic atlas of Franklin and Paxinos (Franklin & Paxinos, 2008), two guide cannulas (BASi cannulas Bioanalytical Systems, West Lafayette, IN) were positioned above the left and right primary motor cortex. The target coordinates, from Bregma, were ± 0.18 mm lateral and +1 mm anterior. The cannulas were positioned just beneath the skulls surface so that the sensing cavity (1 mm) of the electrochemical electrode (inserted later, immediately prior to testing) would be situated within the primary motor cortex of the mouse

(**Figure1**). The guide cannulas were fixed using a UV-polymerized compound (CG3 & CG4, Clear Cure Goo, Southlake, TX, USA) and four 0.10″-inch screws positioned anterior and posterior to the two guide cannulas.

Post-surgical analgesia included application of transdermal bupivacaine (Chiron Compounding Pharmacy Inc., Guelph, ON, Canada) and s.c. injections of buprenorphine hydrochloride twice daily for 3 days. Mice were given 7–8 days to recover before testing.

Electrodes

The use and details of these commercially available electrodes have been extensively detailed elsewhere (Kiyatkin et al., 2013; Kiyatkin & Wakabayashi, 2015; Wakabayashi & Kiyatkin, 2012; Wilson & Gifford, 2005). Briefly, extracellular brain levels of glucose and lactate were monitored using two platinum enzyme-linked electrodes (7005-Glucose-C and 7005-Lactate-C; Pinnacle Technology Inc., Lawrence, KS, USA). Sensors were prepared from Pt-Ir wire of 180 µm diameter, wrapped concentrically with an AgCl reference electrode. Near the tip of the electrode, a 1 mm section is coated with either glucose or lactate oxidase. These enzymes oxidize the analytes of interest, resulting in an equivalent release of hydrogen peroxide (H_2O_2). H_2O_2 is subsequently detected and recorded as an amperometric oxidation current by the electrode (Hu & Wilson, 1997a, 1997b). Data was collected at 1 Hz, transmitted from a potentiostat to a computer and recorded with the help of Sirenia Acquisition Software (Pinnacle Technology Inc., Lawrence, KS, USA). In addition to the oxidase enzymes, the metabolite sensitive surface is also coated with a series of membranes to increase the specificity and selectivity of the electrodes. For example, the potential contribution of ascorbic acid is reduced with the presence of ascorbic acid oxidase. These particular enzymes convert the electroactive

ascorbate, which has the potential to interfere with the electrodes recordings, to non-electroactive dehydroascorbate and water (Hu et al., 1994).

Calibration. Immediately prior to and following in vivo testing, the electrodes were calibrated according to factory recommendations. Briefly, the electrodes were gently placed in 100 mM PBS (pH 7.4) at 23°C (pre-testing) or 37°C (post-testing) and tested for metabolite sensitivity by adding glucose (1 mM) and lactate (0.1 mM) to the PBS solution, as well as selectivity with the use of a single ascorbic acid (0.25 mM) addition.

Pre-calibration curves demonstrated high sensitivity with incremental linear increases for both glucose (average sensitivity 3.32 nA/1 mM) and lactate (average sensitivity 3.40 nA/0.1 mM) electrodes at 23°C. Both glucose and lactate electrodes also exhibited high levels of selectivity by demonstrating minimal sensitivity to ascorbic acid (average sensitivity 0.18 nA/ 0.25 mM and 0.75 nA/0.25 mM, respectively). The post-calibration curves, obtained at 37°C, demonstrated similar results with linear increases for both glucose (average sensitivity 5.14 nA/1 mM) and lactate (average sensitivity 4.70 nA/ 0.1 mM) electrodes. Post calibration curves for ascorbic acid suggested a certain level of wear on the electrodes, most likely due to the insertion and removal process of these sensitive testing instruments (average sensitivity 0.79 nA/0.1 mM and 4.21 nA/0.1 mM for glucose and lactate, respectively).

Brain extracellular testing procedure

Following electrode calibration, mice were lightly anesthetised with isoflurane and the electrodes were inserted into the motor cortex through the surgically implanted guide cannulas and attached to the pre-amplifier. Mice were tested in a custom-made square polymethyl methacrylate-testing cage with standard bedding (Teklad 7097 Corncob bedding, Envigo, Mississauga, Canada) and water. A mounted camera (Pinnacle Technologies) above the cage

recorded the duration of the testing and was synchronized with the electrode sampling. Mice had free access to water but chow was removed 2 h prior to injection and returned 2 h post-injection.

Approximately 2 h following insertion, when the electrodes returned stable readings, mice received a 2 g/kg intraperitoneal (i.p.) injection of either glucose (Dextrose Anhydrous, EMD Chemicals, Gibbstown, NJ, USA; n = 4), lactate (Lactic Acid, Acros Organics, NJ, USA; n = 10), fructose (D-Fructose, Fisher Scientific, Fair Lawn, NJ, USA; n = 6), pyruvate (Sodium Pyruvate, Fisher Scientific, Fair Lawn, NJ, USA; n = 6), galactose (Sigma Aldrich, St. Louis, MO, USA; n = 6), ß -hydroxybutyrate (Sigma Aldrich, St. Louis, MO, USA; n = 5), or insulin at 1.2 I.U. (Novo Nordisk Canada Inc., Mississauga, ON; n = 7). Saline (0.9%; Hospira, Montreal, Canada; n = 4) injections were used as a control group. This particular dosage was chosen based on previous results that demonstrated the ability of 2 g/kg of sugar to improve memory (Messier & White, 1987). Additionally, this dosage also corresponds to the amount of sugar these animals readily ingest (Messier & White, 1984). Extracellular data was collected for 2 h post-injection. When the testing was complete, mice were immediately perfused. Animals that were used to test the effects of systemic injections on brain extracellular levels of lactate and glucose were different than those used in subsequent tests on blood glucose and lactate measurements.

Blood tests

Blood samples were obtained by gently piercing the dorsal tail vein with a 23 gauge needle (Becton, Dickinson and Company, Franklin Lakes, NJ) and massaging the tail for blood extraction. To avoid measuring pooled blood, the first blood drop was wiped before collection. Plasma glucose and lactate concentrations (mmol/L) were measured with a glucose (Accu Chek, Aviva, Roche Diagnostics, Mannheim, Germany) or lactate meter (StatStrip Xpress, Nova Biomedical, Innovation House, Runcorn, Cheshire, UK). Prior to testing, mice were transferred

to the testing room within their respective testing cage for 1 h in order to acclimate. A single glucose and lactate baseline measure was taken at the end of the acclimatization period. Afterwards mice received a 2 g/kg i.p. injection of either glucose (n = 4), lactate (n = 4), fructose (n = 4), pyruvate (n = 4), galactose (n = 4), ß-hydroxybutyrate (n = 4), or insulin at 1.2 I.U. (n = 4). Saline (0.9%; n = 4) injected mice were used as the control group. Subsequently, glucose and lactate measures were taken at 30, 60, 90, and 120 min post-injection. This testing procedure was replicated for another saline group under two additional conditions in order to estimate the effect of stress. These groups were sampled every 5, rather than 30 min with half of them undergoing 15 min daily manipulation for 2 weeks (n = 4) while the other half did not (n = 4).

Histology

Following testing, mice were lightly anesthetised using isoflurane and the electrodes gently removed for post-calibration. Following calibration, the mice received an i.p. injection of sodium pentobarbital (65 mg/kg; MERK, Kirkland, QC, Canada) before being transcardially perfused with a 4% paraformaldehyde/0.2% picric acid solution (PFA). Brains were then postfixed in the same PFA solution for 1 h at 4°C, then transferred to 10% sucrose overnight. The following day, the brains were frozen with CO_2 and stored at −80°C for later cryostat sectioning at 14 µm. Electrochemical electrode placement within the primary motor cortex was confirmed with the use of the stereotaxic atlas of Franklin and Paxinos (Franklin & Paxinos, 2008).

Statistical analysis

Glucose and lactate fluctuations observed in the periphery and extracellular space following various metabolic challenges were analyzed separately. A 2-h time frame was used because the observed metabolites usually returned to baseline or stabilized within this time.

Relative measures. All data were standardized by dividing the readings, in nA, by their respective pre-event baseline and multiplying the result by 100 to obtain relative percentage change from baseline. For electrochemical electrode values, each baseline was individually calculated with an average of 10 s before injection and every 120 consecutive measurements were averaged in order to obtain a mean value every 2 min.

Glucose and lactate were analyzed separately with a between-within (split-plot) mixed ANOVA ($\alpha = 0.05$), and the data obtained for each injection was compared to the saline injection group as well as baseline. Unless otherwise stated, the data met the respective assumptions of the analysis. If sphericity or homogeneity was violated, a Greenhouse-Geisser correction was applied. Due to the a priori decision and interest in differences across groups, post-hoc pairwise comparison tests ($\alpha = 0.05$) were computed in order to compare the experimental groups to the saline controls as well as compare the experimental groups amongst themselves. All statistical analyses were performed using SPSS v. 23 (IBM).

Results

Table 1 presents a summary of blood and brain extracellular levels of glucose and lactate following systemic injections.

Effect of stress on peripheral values of glucose and lactate

It has been previously established that stress can increase systemic levels of glucose and lactate in a variety of species (Adolphs et al., 1999; Gruber et al., 2010; Hall & Van Ham, 1998; Nagamatsu et al., 1994; Rand et al., 2002; Silbergeld, 1974; Wells et al., 1986), including rodents (Abel, 1994; Barnett et al., 1960; Sim et al., 2010; Vachon & Moreau, 2001) and humans (McCowen et al., 2001; Munck et al., 1984; Sharda et al., 1975). Unsurprisingly, taking tail vein blood in awake mice can cause significant stress. Because we were testing the impact of substances that influence blood glucose and lactate, which can in turn have an impact on brain extracellular glucose and lactate levels, we used a sampling procedure that would minimize the effect of stress while still providing useful data. We tested a number of alternatives. **Figure 2** illustrates the fluctuations of blood lactate and glucose following sampling conducted every 5 min without (2A) and with a 2-week habituation/handling period (2B). **Figure 2C** presents the results using a 30-min sampling interval. Split-plot mixed ANOVA did not reveal any significant effect of group for either blood glucose [$F(2, 9)$ 3,87, $p = 0.061$, Observed Power = 0.545] or lactate [$F(2, 9)$ 0,285, $p = 0.759$, Observed Power = 0.083] values. However, pairwise comparisons for glucose did indicate a significant between group difference when comparing the 5 min sampling paradigm to the 30 min sampling paradigm ($p = 0.023$) but not when comparing the 30 min sampling paradigm and the 5 min sampling paradigm with prior habituation ($p = 0.107$). Therefore, sampling every 30 min produced similar values as those obtained following a 2-week habituation period. Pairwise comparisons did not reveal statistically significant

differences between the lactate levels of the different sampling paradigms. In summary, regular manipulation and habituation results in similar levels of physiological stress responses to the 30-min sampling paradigm. Thus, the following peripheral data were obtained with a 30-min sampling paradigm.

Intraperitoneal injection of alternative fuels increases systemic lactate

Figures 3 A–E shows increased systemic lactate above saline values after each of the injected metabolites, while systemic glucose remained mostly unchanged (with the obvious exception of the glucose injection). The insulin injection (**Figure 3G**) reduced blood glucose values but did not change blood lactate levels except for the first 30-min sample, most likely a consequence of the i.p. injection and the related stress. The analysis of peripheral glucose and lactate indicated a significant effect of time [$F(4, 96)$ 4.59, $p = 0.002$, $\eta 2 = 0.160$; $F(2.39, 57.45)$ 60.26, $p < 0.000$, $\eta 2 = 0.715$] and injected substance [$F(7, 24)$ 4.10, $p = 0.004$, $\eta 2 = 0.545$; $F(7, 24)$ 3.52, $p = 0.010$, $\eta 2 = 0.507$], respectively. Pairwise comparisons were conducted to determine which group(s) significantly differed from control (saline) values as well as determine the precise times at which these values differed from both saline and baseline levels (as indicated on **Figures 3 A–G**). Post-hoc analyses demonstrated that the only injection capable of raising systemic glucose above saline values was, unsurprisingly, the glucose injection. Also, the injection of galactose slightly raised blood glucose above baseline values at 30 and 90 min post-injection. Pairwise comparisons confirmed that lactate, pyruvate, and ß-hydroxybutyrate injections increased blood lactate levels above saline values. The percent average blood lactate increase following the injection of glucose ($p = 0.563$), fructose ($p = 0.290$), galactose ($p = 0.449$), pyruvate ($p = 0.267$), and ß-hydroxybutyrate ($p = 0.244$) was not statistically different from that observed after a lactate injection. However, the injection of glucose, fructose and

galactose produced smaller blood lactate increases and the comparison with saline injection values were only significant for glucose and fructose at the 30-min sample.

Systemic injection of alternative fuels increases glucose and decreases lactate in the primary motor cortex extracellular space

Figure 4A shows the effect of a saline injection on extracellular glucose and lactate content in the primary motor cortex. Of note is the initial, significant transitory rise in extracellular lactate (30%), which returns to baseline 10–15 min post-injection. As this rise in extracellular lactate is consistently observed in all conditions immediately following injections, we suggest that it is a result of restraining of the mice and subsequent motor behavior. Indeed, injecting the mice require their restraint which results in struggling during the injection procedure. Immediately following the injection, mice typically engaged in enhanced grooming, and running behavior for approximately 10 min. Although we did not quantify motor activity during the post-injection period, the video recordings were annotated to show bouts of running or intense grooming. In general, the level of activity appeared higher in the saline group and was mostly similar across experimental groups except for mice receiving lactate, which were less active. Also, the changes in extracellular lactate and glucose associated with discrete motor activity were much less than the changes associated with the systemic injections and were not found to be a significant contributing factor in the present experiments. Finally, this small lactate increase associated with the initial injection occurred for all substances injected except for lactate; following a lactate injection, the injection-associated rise is greater (50%) and returns to baseline 30 min later. Additionally, toward the end of the 2-h testing period, there is a small steady decline in glucose level that is likely associated with the 4-h fasting.

Figures 4B–G show that the systemic injection of alternative fuels significantly increased extracellular glucose levels, beyond what was observed following a saline injection. Extracellular glucose but not lactate showed a significant effect of substance injected [glucose: $F(7, 40)$ 6.62, $p < 0.000$, $\eta 2 = 0.537$; lactate: $F(7, 40)$ 2.09, $p = 0.067$, $\eta 2 = 0.268$], and a significant effect of time [glucose: $F(59, 2360)$ 30.66, $p < 0.000$, $\eta 2 = 0.434$; lactate: $F(59, 2360)$ 44.37, $p < 0.000$, $\eta 2 = 0.526$], respectively. We were interested in examining the differences between saline and each substance as well the significance of the differences between the baseline values and values after injections. On each panel of **Figure 4**, we indicated the significance of pairwise comparison between the values obtained after the saline injection and those observed after each substance. The thin horizontal lines below each graph represent significant tests for extracellular glucose values while the thicker horizontal line indicates significant tests for extracellular lactate values.

When the effects of alternate fuels on extracellular glucose are examined, all substances except galactose produce a rise in extracellular glucose. This difference between galactose and other metabolic substrate will be addressed later in the discussion. Among these significant effects, there are similarities and differences in the slope of the initial increase in extracellular glucose and the time period during which extracellular glucose remains elevated. First, the extracellular glucose rates of rise following glucose, lactate and ß-hydroxybutyrate injections are very similar. Also, extracellular glucose rates are almost identical when comparing the effect of fructose and pyruvate injections. Possibly, the rapid rise in extracellular glucose observed after the systemic injection of glucose, lactate and ß-hydroxybutyrate is associated with their greater ability to cross the blood brain barrier or the ease with which they are systemically metabolized. In contrast, the delayed rise in extracellular glucose observed after the systemic injection of

fructose, pyruvate and galactose may reflect the slower transport or the requirement of additional metabolic steps to transform fructose, pyruvate, and galactose into easily transportable substrates. Finally, we observed that the effect of the injection of lactate on extracellular glucose levels is much more variable than what was found for other injections. We could not identify any mediating variable for this variability in the present results.

The effects of alternate fuels on brain extracellular lactate present a different pattern altogether. First, the systemic injection of fructose, pyruvate, and ß-hydroxybutyrate all led to significant decreases in brain extracellular lactate. This decrease was absent following the systemic injection of glucose, lactate and galactose. We also noted that all systemic injections, including saline, briefly increased extracellular lactate levels but this increase was greater and more sustained after the systemic injection of lactate. We now turn to the effect of systemic insulin on extracellular glucose and lactate.

Systemic insulin decreases glucose and lactate in the primary motor cortex extracellular space

The decrease in extracellular glucose following systemic insulin has been described previously (McCall et al., 1986) and is thought to be associated with the decreased blood glucose levels and the proportionally reduced gradient-dependent transport from blood to brain. Systemic insulin also reduced extracellular lactate at exactly the same rate and extent, as was extracellular glucose. The 40% decrease in extracellular lactate was greater than the one that followed the systemic injection of fructose (20%), pyruvate (20%), and ß-hydroxybutyrate (30%).

Comparisons of trends between blood and brain extracellular levels of glucose and lactate following systemic injections

If the brain-blood barrier transport of glucose and lactate are solely taken into account, the brain extracellular levels of glucose and lactate should be proportional. For glucose, previous

data has described a relation between blood and brain glucose that is usually described as brain glucose being 20–30% of blood glucose (Silver & Erecińska, 1994). This relation is maintained during fasting as well following insulin injections (Dunn-Meynell et al., 2009). In the present set of results, this relationship was observed following glucose and insulin systemic injections. However, the systemic injection of pyruvate and ß- hydroxybutyrate both led to a decrease in blood glucose and a rise in brain extracellular glucose. Conversely, the systemic injection of fructose and lactate did not raise blood glucose levels but led to an increase in brain glucose levels.

Finally, lactate transport is thought to be directional and dependent, among other factors, on concentration gradient across the blood-brain barrier (Figley, 2011). In the present experiments, only the systemic injection of lactate slightly increased brain extracellular lactate levels. Two other metabolic substrates, ß-hydroxybutyrate, and pyruvate, that increased blood lactate to similar levels as the systemic injection of lactate were associated with decreased brain extracellular lactate levels. Systemic injections of glucose, fructose and galactose all led to equivalent increased blood lactate levels but glucose and galactose did not produce lower brain extracellular lactate levels while fructose did. Together these observations suggest that blood and brain extracellular levels of glucose and lactate do not follow a simple distribution based on blood brain transport and concentration gradient between blood and brain.

In summary, the systemic injection of various metabolic substrates in awake and freely moving mice led to variations in brain extracellular lactate levels in the motor cortex (see **Table 1** for a summary). These metabolic substrates were injected in quantities consistent with typical food ingestion or exercise and were not supra-physiological. Insulin injections did not impair the animals' movement and did not induce loss of consciousness. Although the experimental

conditions are obviously artificial, we nonetheless devised test conditions that are relevant for normal physiological states. Finally, the animals across groups were comparable regarding their motor activity except the animals in the lactate group that tended to be less active. During their random bouts of activity, the extracellular glucose and lactate changes observed in the motor cortex of all mice were much smaller than that produced by the systemic injections. Also, the motor activity was randomly distributed throughout the test session with the exception of the period immediately following the systemic (i.p.) injection when the animals struggled briefly and moved about the cage after being released from the hold of the experimenter. Within the limits of the present experimental design, the present results provide new information on how metabolic fuels are transferred and used by the brain and also raise a number of questions that need to be addressed.

Discussion

Methodological considerations

Before we discuss the results of the present experiments, a number of aspects of the techniques used and the nature of the sampling have to be stated. First, we measured glucose and lactate extracellular levels in the motor cortex of freely moving mice. Although mice could move around the testing enclosure, the amount of activity was limited to a few bouts randomly spread out during the 2 h of observation. Second, the brain extracellular milieu is a site of bilateral exchange between the brain parenchyma and blood circulation. The electrochemical electrodes that we inserted in the motor cortex measure the content of glucose and lactate in that milieu (net flux) but give no information as to the directional flux (influx vs. efflux) of the metabolites. Thus, the brain extracellular milieu is like an antechamber where people go through to enter a room. Figuratively, our measures give information on the number of people transiting in the antechamber at any given time but no information on whether they are going into the room or leaving it.

We used percentage of change over baseline to present data to allow interindividual and group comparisons. In theory, the use of pre- and post-calibration could allow us to provide absolute values. However, a number of limitations make this difficult. First, pre-calibration of the electrodes provides the values that allow the direct conversion between current recorded and concentration of glucose or lactate. When the electrodes are inserted into the living brain, the physical electrode damages brain tissue and initiates the usual responses to insult, including inflammation and microglial activation. The adaptation period we used here (about 2 h) appears to lead to the stabilization of the recorded current at the electrode, which likely corresponds to the end of the acute response to tissue damage following electrode insertion. Following that

period, the electrodes appear to react to brain activation and systemic injection rapidly and consistently. There is a small consistent and mostly linear decline in current over 24 h that is likely due to biofouling, the accumulation of biological material or cells on the electrode surface. Post-calibration, in theory, measures the electrode sensitivity at the end of the experiment. However, it has been a challenge to remove electrodes through the guide cannula holding the electrode in place without any damage to the membranes so as to provide reliable measurements across all animals.

The main concern with comparing percentage of change over baseline measures of glucose and lactate is their respective difference in absolute concentration in the brain. As calculated with the use of our electrode calibration, average baseline brain extracellular values of glucose and lactate were 1.4 ± 0.59 and 0.9 ± 0.56 mmol/L, respectively. Average blood values of glucose and lactate ranged from 8 to 10 mmol and 1.7 to 3.1 mmol/L, respectively. The observed brain extracellular glucose values fell within the expected 20% of blood concentration; however, brain extracellular lactate values were closer to 35–40% of blood values. Additionally, when glucose is metabolized through the glycolytic pathway, each molecule of glucose gives rise to two lactate molecules. As a consequence, analysis of relative changes of these metabolites in the brains' extracellular space, or the periphery, may lead to an overestimation of the absolute contribution these metabolites to brain energetics. For example, if blood lactate rises 400%, blood lactate concentration rises from 2.5 to 10 mmol/L. This near 400% rise in blood lactate is regularly observed in exercise studies, reaching values close to or above 10 mmol/L (Meek et al., 2009; Petrosino et al., 2016; Wasserman, 1967). In contrast, if blood glucose only rises 10%, blood concentration goes from 9 to 9.9 mmol/L. Thus, a 400% rise in lactate depicted in a graph may give the illusion that blood lactate levels greatly exceed that of glucose and these results

interpreted as lactate having a greater relative contribution. With these caveats in mind, we now turn to possible hypotheses that can be used to account for our observations. These hypotheses are presented as possibilities and not as a conclusive interpretation of our results.

Peripheral increases in lactate mediate some of the brain's use of systemic metabolic fuels

The primary finding culminating from this work is the observation of an uncoupling occurring between extracellular and blood values of our two metabolites of interest. Indeed, we observed a robust and long lasting increase of blood lactate following the systemic injection of glucose, fructose, lactate, pyruvate, and ß-hydroxybutyrate that was accompanied by maintained (ex: fructose) or even decreased (ex: ß-hydroxybutyrate) blood glucose levels. In contrast, brain extracellular levels of these two metabolites follow opposing trends with glucose increasing and lactate maintaining either a steady-state or decreasing. As such, individually, it would appear that these metabolites are uncoupled between the periphery and the brain.

Though these metabolites appear to be individually uncoupled (e.g., blood lactate levels not predicting brain lactate levels), the data suggest that they do not act independently of one another. A possible interpretation of the observed pattern of these metabolites and how they may influence each other would be that these metabolic substrates (except, of course, lactate) are first metabolized to lactate in the liver. The lactate produced by the liver then enters the brain and used either as a signaling molecule (Barros, 2013; Mosienko et al., 2015; Tang et al., 2014) or for oxidative metabolism (Kasischke et al., 2004; Serres et al., 2003; Waagepetersen et al., 1998a, 1998b). Thus, metabolic substrates would first be transformed into lactate before the brain can use them. This view is supported by the negligible rise in blood glucose following the injection of most alternative fuels (except, of course, glucose) and the high rate of conversion of these substrates to lactate, including glucose (Kreisberg et al., 1970). The liver is capable of metabolizing various

substrates to lactate. Indeed, pyruvate can be easily converted to lactate with the ubiquitous presence of LDH5 that favors the conversion of pyruvate to lactate (Fritz et al., 1969; Wroblewski & Gregory, 1961). Fructose can be converted to lactate through fructokinase, aldolase B and triokinase: enzymes that allow fructose to enter the glycolytic pathway as well as undergo gluconeogenesis (Exton & Park, 1968; Mayes, 1993). ß-hydroxybutyrate can be converted to aceto-acetyl CoA and enter the TCA cycle for oxidative metabolism (Armulik et al., 2010; E. N. Bergman, 1971; Robinson & Williamson, 1980) or, alternatively, be converted into acetone and enter the methylglyoxal pathway where it can ultimately be converted to lactate (Armulik et al., 2010; Glew, 2010). Thus, fructose, pyruvate and ß-hydroxybutyrate, after conversion to lactate, can enter the TCA cycle for oxidative metabolism without being first converted to glucose. Furthermore, some of these substrates have been shown to decrease the contribution of glucose carbon into CO2 (Ide et al., 1969; Rolleston & Newsholme, 1967) and hepatic glucose uptake (Davis et al., 1984; Larrabee, 1995). In addition to the liver's ability to convert most substrates to lactate, endothelial cells of blood vessels also exhibit a highly glycolytic nature that commonly metabolizes glucose, or other substrates, to lactate which hints toward favorable export of lactate to surrounding organs (De Bock et al., 2013; Eelen et al., 2015; Mann et al., 2003). The injection of glucose, fructose, lactate, pyruvate, and ß-hydroxybutyrate have been shown to raise blood lactate (Abi-Saab et al., 2002; Davis et al., 1984; Hallström et al., 1989; Huckabee, 1958; Kaye et al., 1958; Kohler et al., 2007; Pan et al., 2000; Revelly et al., 2005; Sahebjami & Scalettar, 1971) however this is the first demonstration and comparison of the similarity with which they seem to affect two major metabolic fuels (i.e., glucose and lactate).

Despite the liver's ability to convert these substances to lactate and lactate's ability to cross the blood-brain barrier and act as an alternative fuels, it is important to note that lactate, as a signaling molecule, may also regulate pathways involved in memory formation and (unsurprisingly) homeostatic metabolic properties of cells and organs (Barros, 2013; Boury-Jamot et al., 2016). In the brain, recent evidence has demonstrated presence of the HCAR1 gene in neurons (Bergersen & Gjedde, 2012). In adipocytes, this gene codes for a Gi-coupled lactate receptor capable of inhibiting adenylyl cyclase (Lovatt et al., 2012). Though the elucidation of the functional role of HCAR1 is still lacking, the presence of such a receptor may further emphasize the role of lactate as a signaling molecule and its role in maintain metabolic homeostasis. Indeed, Barros (Barros, 2013) states the limitations of pure neuronal glucose use and discusses the ways in which astrocytic lactate could be used. Of particular interest is their discussion of "metabolic recruitment by lactate" which illustrates how lactate could participate in maintaining metabolic homeostasis by means other than acting as an alternative fuel. For example, lactate could stimulate inhibitory GABAergic neurons (Shimizu et al., 2007) and promote lateral inhibition leading to increased metabolic availability of substrates to the remaining active neurons. Also, as demonstrated by Rovetto (Rovetto et al., 1975) and others (Gilbert et al., 2006; Ruusuvuori et al., 2010; Shimizu et al., 2007), lactate could increase the acidification of cells and therefore decrease their spontaneous activity. Interestingly, lactate may account for the observations that astrocytes decreased their use of glucose and glycogen in active regions of the brain (Bittner et al., 2011; Loaiza et al., 2003; Pellerin & Magistretti, 1994; Porras et al., 2004, 2008; Zhao et al., 2011) while decreasing their glucose use in less active brain regions (Sotelo-Hitschfeld et al., 2012), thus facilitating the diffusion of glucose from areas of low activity to areas of high activity.

In the next section, we explore three hypotheses that could explain the metabolic uncoupling observed in our data. We explore these options within a perspective of lactate as a metabolic fuel. However, it is crucial to remember that this is a limited perspective of how the brain may be adapting to these changes in the systemic availability of metabolites. Any changes or adaptations occurring could be the downstream consequence of the initial rise in extracellular lactate.

Extracellular brain increases in glucose: gluconeogenic hypothesis

This hypothesis is based on the long-held belief that glucose is the preferred and principal brain fuel (Levine et al., 1998; Lipton & Robacker, 1983). The blood lactate increase following the systemic injection of glucose, fructose, lactate, pyruvate and ß-hydroxybutyrate is consistent with previous observations. However, explaining and understanding the increase in brain extracellular glucose following these systemic injections requires more thought. The hypothesis that lactate is a gluconeogenic precursor (Bergman et al., 2000; Kreisberg et al., 1970; Ross et al., 1967; Struck et al., 1965) and that the increase in brain extracellular glucose results from excess peripheral lactate being converted to glucose faces a number of objections. Although there has been indication of increased gluconeogenesis at the blood-brain barrier (Daneman et al., 2010), it is highly unlikely that this conversion would occur in endothelial cells at a rate significant enough to raise brain extracellular glucose levels by 30% as endothelial cells are highly glycolytic. Gluconeogenesis has been shown to occur in astrocytes (Wiesinger et al., 1997), however this would again imply that the lactate crosses the blood-brain barrier and is converted to glucose while no brain extracellular lactate increase is detected by the lactate biosensor. Indeed, our present data show that brain extracellular lactate decreases below baseline 20 to 40 min after the injection of fructose, pyruvate and ß-hydroxybutyrate. If we interpreted

these observations using this hypothesis, it would imply that the rate of gluconeogenesis greatly exceeds the rate of lactate entry. This seems unlikely as astrocytes appear to favor the release of lactate and not glucose (Dringen et al., 1993). In addition, sustaining such a high rate of gluconeogenesis would be extremely costly as gluconeogenesis is an energy dependent process (Katz, 1985).

Glucose sparing hypothesis

This hypothesis suggests that the increased brain extracellular glucose is the result of decreased glycolysis since, as we previously indicated, gluconeogenesis is a costly, energy-dependent process (Katz, 1985). In this hypothesis, astrocytes take up lactate and later shuttle it to surrounding neurons using the astrocytes' fine processes that ensheath synaptic sites. Similarly to the postulated ANLS, this alternative hypothesis would suggest that increased extracellular cerebral glucose is a consequence of increased lactate use and shuttle to neurons, thus limiting the need for glucose-based glycolysis and increasing lactate-based oxidative metabolism in neurons. Consequently, favoring lactate-based oxidative metabolism and decreasing glucose-based glycolysis would lead to the eventual accumulation of glucose in the brain extracellular space. In vitro work supports this notion as lactate has been shown to decrease glucose use in neuronal and astrocytic cell cultures (Schurr et al., 1999; Schurr & Gozal, 2011; Tabernero et al., 1996). Further evidence supporting the lactate-shuttling capabilities of astrocytes is the presence of lactate transporters that favor lactate-export (De Bock et al., 2013; Eelen et al., 2015; Mann et al., 2003) as well as the differential expression of LDHs in neurons and astrocytes. Neurons preferentially present with higher levels of LDH1, which favor the lactate to pyruvate reaction, while astrocytes preferentially present with higher levels of LDH5, which favor the pyruvate to lactate conversion (Bittar et al., 1996; Laughton et al., 2000; Venkov et al., 1976). In other

words, astrocytes can convert the glycolytic by-product pyruvate to lactate and export it into the extracellular space.

However, the ability to export lactate is not sufficient to explain the rise and subsequent decrease in extracellular glucose. Indeed, if the brain extracellular glucose increase is the result of lack of use, transport kinetics and concentration gradient should counter that accumulation. However, as previously mentioned, lactate function in the brain is not limited to its metabolic properties but can also function as a signaling molecule capable of regulating pathways involved in memory formation and (unsurprisingly) homeostatic metabolic properties of cells and organs (Barros, 2013; Boury-Jamot et al., 2016). Enzyme assays in other tissues, such as the liver and kidney, have demonstrated the ability of acute and chronic lactate incubation to inhibit hexokinase (and other important glycolytic regulating enzymes) by regulating its association to mitochondria, thus rendering it more susceptible to inhibition (Leite et al., 2011), suggesting the possibility that high lactate transport could inhibit glucose transport. Additionally, heart perfusion techniques in rats demonstrated that one of the leading causes of decreased glycolysis in hypoxic hearts is a decrease in pH caused by the accumulation of lactate (Rovetto et al., 1975). Thus, large lactate accumulations appear capable of decreasing glycolytic glucose use in other organs. In contrast, another heart perfusion study in the rat demonstrated the ability of lactate to increase the translocation of GLUT1 to the plasma membrane (Medina et al., 2002). Interestingly, this increased transporter translocation did not lead to increased phosphorylation of glucose analogs but rather to a decrease of their phosphorylation. These results lead us to think that lactate may have similar effects in the mouse brain, resulting in decreased hexokinase activity and thus glucose use, but possibly increasing transport through increased GLUT1 externalization. In conclusion, the hypothesis that the brain extracellular glucose rise we

observed after systemic injection of a number of metabolic substrates could be explained by the preferential use of lactate and the subsequent accumulation of glucose.

Opportunistic hypothesis

A final consideration is the potential ability of the brain to use metabolic substrates that are in highest concentration in the blood, a capacity that may have a significant evolutionary advantage for bioenergetics. In this hypothesis, glucose is most commonly used as its blood concentration is usually significantly higher than other substrates. Indeed, all of the metabolic substances we injected systemically presumably raised their blood concentration and could cross the blood-brain barrier through GLUT1 or MCT1, albeit at variable rates (Gould et al., 1991; Halestrap, 2012; Halestrap & Price, 1999; Juel & Halestrap, 1999). In addition, the brain possesses the necessary enzymes to metabolize these various substances (Diggle et al., 2010; Funari et al., 2007; Laughton et al., 2000; Majumder & Eisenberg, 1977; McKenna, 2012; Ning et al., 2001; Venkov et al., 1976). Finally, GLUT1 or MCT1 transporters and the enzymes associated with the transport of these metabolic substrates have been shown to be upregulated following chronic exposure to a specific metabolic substance (Adelman et al., 1966; Corring, 1980; Moore et al., 1976). Although it is not known whether the brain is using these substrates directly in vivo, some of the pre-requisite elements are present in the brain to allow this. Because we did not measure the blood content of each metabolic substance apart from glucose and lactate, it is difficult to evaluate the merit of this hypothesis to explain the present results.

Galactose

Galactose was observed to significantly raise blood glucose and blood lactate but to a smaller degree than glucose. The liver is capable of metabolizing galactose using the Leloir Pathway that converts galactose to glucose-6-phosphate, the first metabolic product of glycolysis

(Cohn & Segal, 1973; Holden et al., 2003; Isselbacher, 1959). In our experiments, there was almost no effect of systemic galactose on brain extracellular glucose and lactate. Based on the facilitated transport of glucose, we should have observed some increase in extracellular glucose with the caveat that galactose may have decreased brain glucose uptake by competing with glucose for its transport (Baur & Heldt, 1977) or inhibiting its phosphorylation by modifying hexokinase availability (Knull et al., 1973; Park & Johnson, 1955). Similarly, according to the ANLS corollary noted above, the modest increase in blood lactate could have led to an increased accumulation of brain extracellular glucose—an effect not observed in our experiments.

A likely explanation for the different systemic and extracellular responses to this particular substrate may lie in the rate of its metabolism. Fructose, pyruvate and β-hydroxybutyrate are all quickly metabolized or excreted. This is illustrated by studies evaluating blood content or carbon label excretion of various metabolites. These studies highlight the mouse's ability to excrete or metabolize over 80% of the injected metabolites within 2 h post-injection (Cha et al., 2008; Eiger et al., 1980; Guggenheim & Mayer, 1952; Miller et al., 1952; Wang et al., 2009). Particularly, fructose (4 g/kg) and β-hydroxybutyrate (12 mg) are capable of returning to blood baseline levels 30 min following their respective injections (Cha et al., 2008; Eiger et al., 1980). In contrast, within a 2 h window, one experiment showed that mice are only capable of metabolizing approximately 30% of injected galactose (Ning et al., 2001). In that experiment, the cell concentration of galactose was <10% of their blood concentration, indicating a slow uptake, likely a consequence of the competition between glucose and galactose transport. In addition to this slow rate of influx, it appears the metabolic rate of galactose is even slower indicating that its metabolism is not a function of its concentration-dependent transport but rather its limited enzymatic activity (Baur & Heldt, 1977). However, the galactose intermediate,

galactose-1-phosphate, has been identified in the mouse brain, but galactose itself was not (Ning et al., 2001). These observations suggest that either the small amount of galactose that can cross the blood-brain barrier is quickly metabolized, or that the brain is capable of endogenously producing galactose-1-phosphate. Our observations that galactose produces little change in brain extracellular glucose and lactate could be solely ascribed to its limited rate of absorption. This conclusion has to be tempered with our observation that blood glucose and lactate were elevated following galactose injection. Perhaps blood levels of glucose and lactate have to reach a higher level to influence brain extracellular glucose and lactate.

Insulin

The effect of systemic insulin injections is equally intriguing as it illustrates the impact of reducing blood glucose levels with only a small increase in blood lactate levels. The effect of insulin has to be contrasted with the effect of pyruvate and ß-hydroxybutyrate that both reduced blood glucose levels while increasing lactate levels. Following the systemic injection of pyruvate and ß-hydroxybutyrate, brain extracellular glucose levels are elevated in the face of decreasing blood glucose levels. Following an insulin systemic injection, there is a small transient increase in blood lactate levels accompanied by a concurrent decrease in blood glucose. Under these conditions, brain extracellular lactate and glucose monotonically decrease in unison. Together the effects of pyruvate and ß-hydroxybutyrate on blood and brain glucose suggest that high blood lactate levels can sustain high brain extracellular glucose levels even when blood glucose levels are low. Conversely, low blood glucose together with lower blood lactate levels cannot sustain high brain extracellular glucose levels and brain extracellular lactate cannot compensate for this decrease. In a preliminary experiment (data not shown) we observed that increasing blood lactate, 2 h after an insulin injection, led to an increase in brain extracellular glucose levels up to

50% above pre-insulin baseline suggesting that blood lactate can reverse the decrease in brain extracellular glucose levels produced by systemic insulin injections.

Conclusion

We are aware that our observations, as they stand, cannot be used to establish the relative prominence of the hypotheses we entertained in the discussion because we did not measure glucose or lactate utilization within the different brain metabolic pathways nor did we measure the brain levels of most of the systemically injected substances. However, our results provide new and useful *in vivo* information obtained under physiological conditions. The inescapable fact that the brain eventually ceases to function in the complete absence of either glucose or oxygen has focussed the theories of neuroenergetics toward the oxidation of glucose as being the prime mechanism for energy acquisition of the mammalian brain. However, the ability of the brain to adapt to low glucose in insulin-injecting diabetes patients (Maran et al., 2000), to low oxygen in people at high altitude (Hochachka et al., 1996) and increased ketone body in diet (Murray et al., 2016; Roy et al., 2015) suggests that the brain makes use of more than one mechanism to acquire energy. These alternate mechanisms can compensate for decreased glucose and oxygen but can never completely replace oxygen- and glucose-dependent metabolic activities. This limitation suggests that some brain physiological activity is uniquely dependent on those particular sources of energy. One possible candidate would be the sodium/potassium pump that appears to be highly dependent, at least in part, on the glycolytic pathway (Lipton & Robacker, 1983; Mercer & Dunham, 1981; Pellerin & Magistretti, 1996; Raffin et al., 1992; Sepp et al., 2014; Silver & Erecińska, 1997). Our in vivo results are consistent with the hypothesis that alternate metabolic substrates can either be used by the brain and/or reduce the need for glucose under physiological conditions.

Author contributions

AB planned and performed experiments, completed the data analysis and wrote the manuscript. JL, JC, and TY performed and analyzed preliminary experiments and helped refine experimental protocols and data analysis. CM planned experiments and contributed to the data analysis and writing of manuscript.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1 | Summary of glucose and lactate trends following injections

	Glucose		Lactate	
Injection	**Brain**	**Blood**	**Brain**	**Blood**
Saline	➔(Baseline)	➔(Baseline)	➔(Baseline)	↑(~200%)
Glucose	↑(~180%)	↑(~180%)	➔(Baseline)	↑(~400%)
Fructose	↑(~165%)	➔(Baseline)	↓(~80%)	↑(~400%)
Lactate	↑(~175%)	➔(Baseline)	➔(Baseline)	↑(~500%)
Pyruvate	↑(~155%)	↓(~80%)	↓(~80%)	↑(~550%)
ß-Hydroxybutyrate	↑(~185%)	↓(~70%)	↓(~70%)	↑(~600%)
Galactose	➔(Baseline)	↑(~120%)	➔(Baseline)	↑(~350%)
Insulin	↓(~60%)	↓(~50%)	↓(~60%)	↑(~280%)

Illustration of the metabolite trend (glucose or lactate) in their respective compartments (blood and extracellular space of the brain) following the systemic injection of metabolic fuels. Arrows indicate whether the metabolite increase (↑), decreased (↓) or remained at baseline (➔). Values depict the approximate zenith or nadir achieved by these metabolites, in relation to their baseline (i.e. glucose decreased up to 80% its baseline value in the blood following a pyruvate injection).

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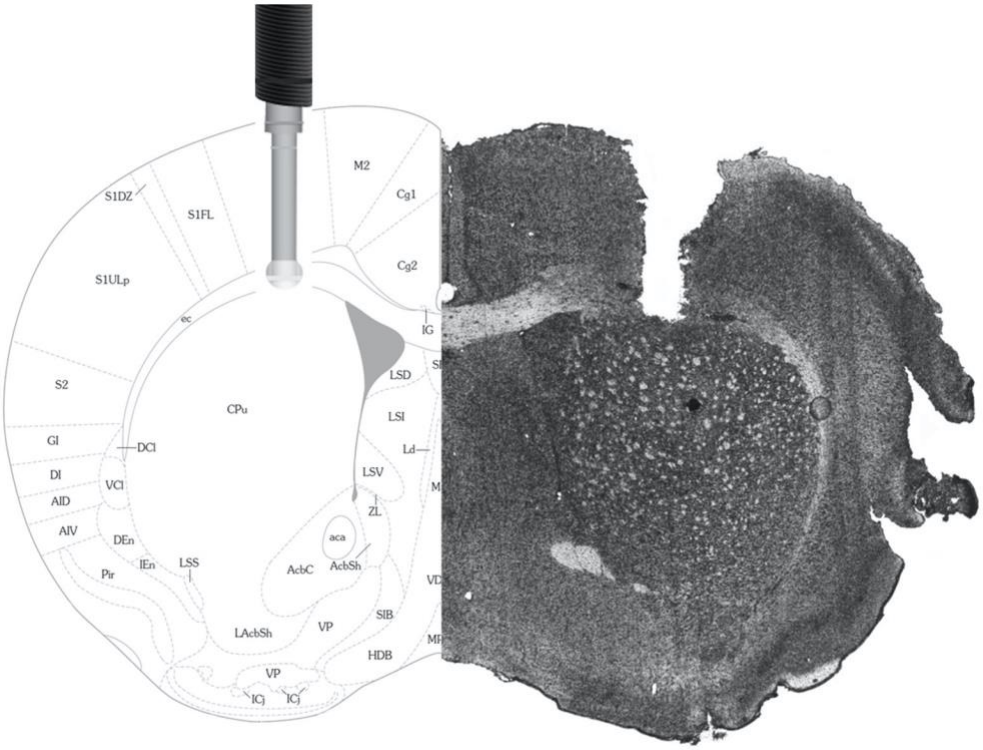
Figure 1 | Histology and Stereotaxic Location of Electrodes



On the right is a picture of a 14 μm mouse brain slice taken following the implantation of an electrode in the primary motor cortex (M1). For comparison, on the left is a picture from the Franklin and Paxinos (2008) Mouse Brain Atlas used to determine stereotaxic coordinates and confirm electrode placement in the primary motor cortex.

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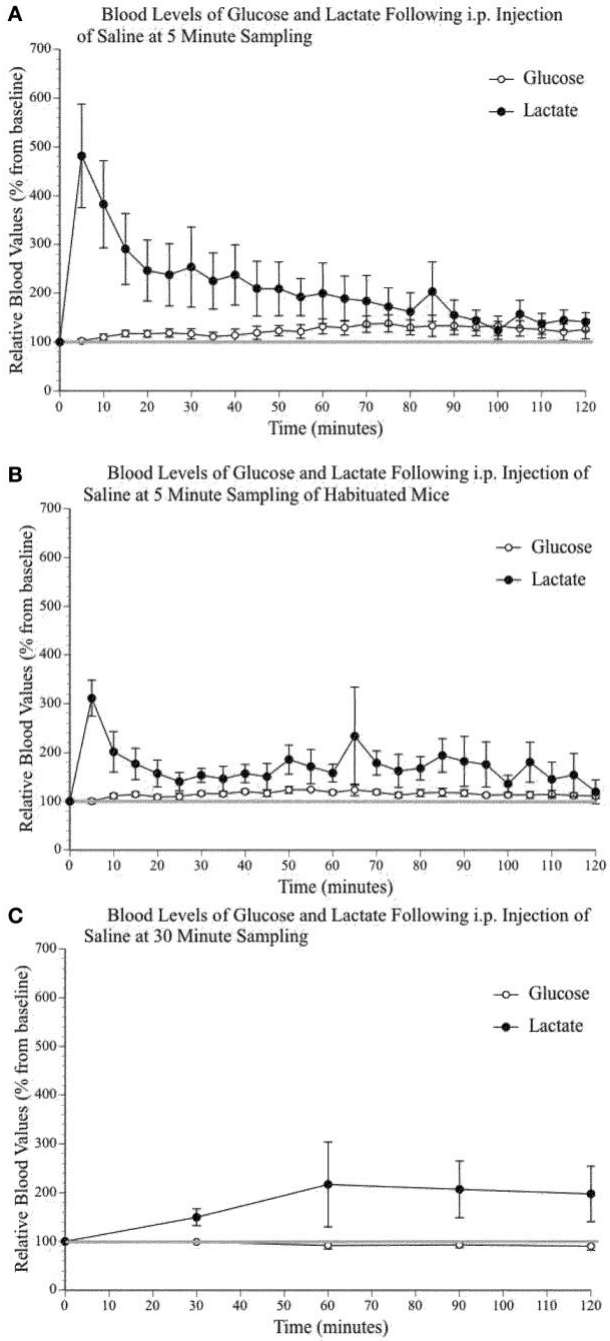
Figure 2 | Relative blood concentration of glucose and lactate following a 0.9% i.p. injection of saline



*Relative blood concentration of glucose and lactate following a 0.9% i.p. injection of saline **(A)** at 5 min sampling interval in naïve mice (n = 4); **(B)** at 5 min sampling interval in habituated mice (n = 4); **(C)** and at 30 min sampling interval in naïve mice (n = 4). Values are expressed as a percent of their baseline value (taken prior to injection) and error bars represent the standard error of the mean.*

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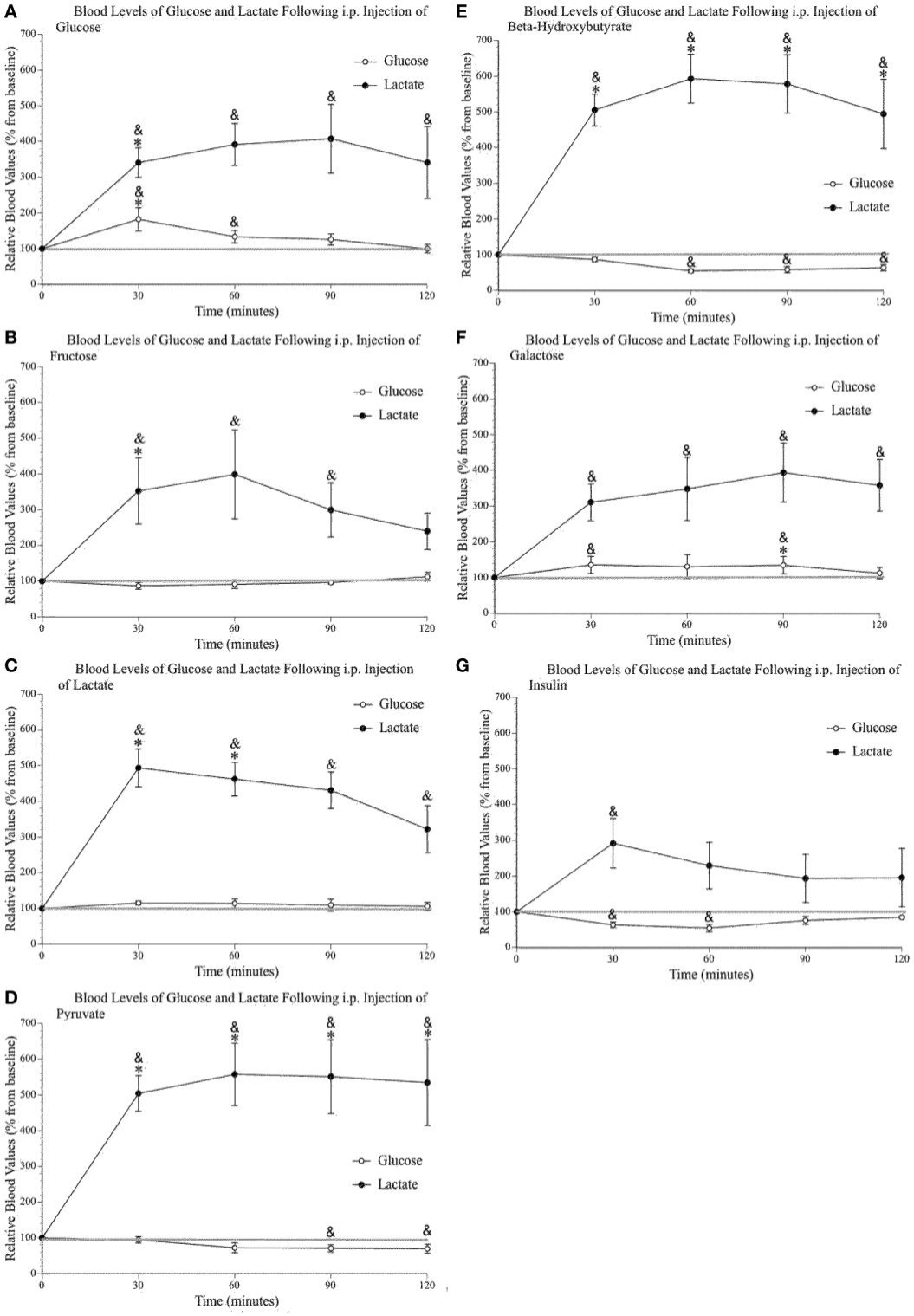
Figure 3 | Relative blood concentration of glucose and lactate following a 2 g/kg i.p. injection of glucose, fructose, lactate, pyruvate, β–hydroxybutyrate and galactose



Relative blood concentration of glucose and lactate following a 2 g/kg i.p. injection of ***(A)*** *glucose (n = 4);* ***(B)*** *fructose (n = 4);* ***(C)*** *lactate (n = 4);* ***(D)*** *pyruvate (n = 4);* ***(E)*** *β–hydroxybutyrate (n = 4);* ***(F)*** *galactose (n = 4).* ***(G)*** *Relative blood concentration of glucose and lactate following a 1.2 I.U. i.p. injection of insulin (n = 4). Values are expressed as a percent of their baseline value (taken prior to injection) and error bars represent the standard error of the mean.* ******* *= significant difference when compared to control (saline) values ($p < 0.05$) & = significant difference when compared to baseline ($p < 0.05$).*

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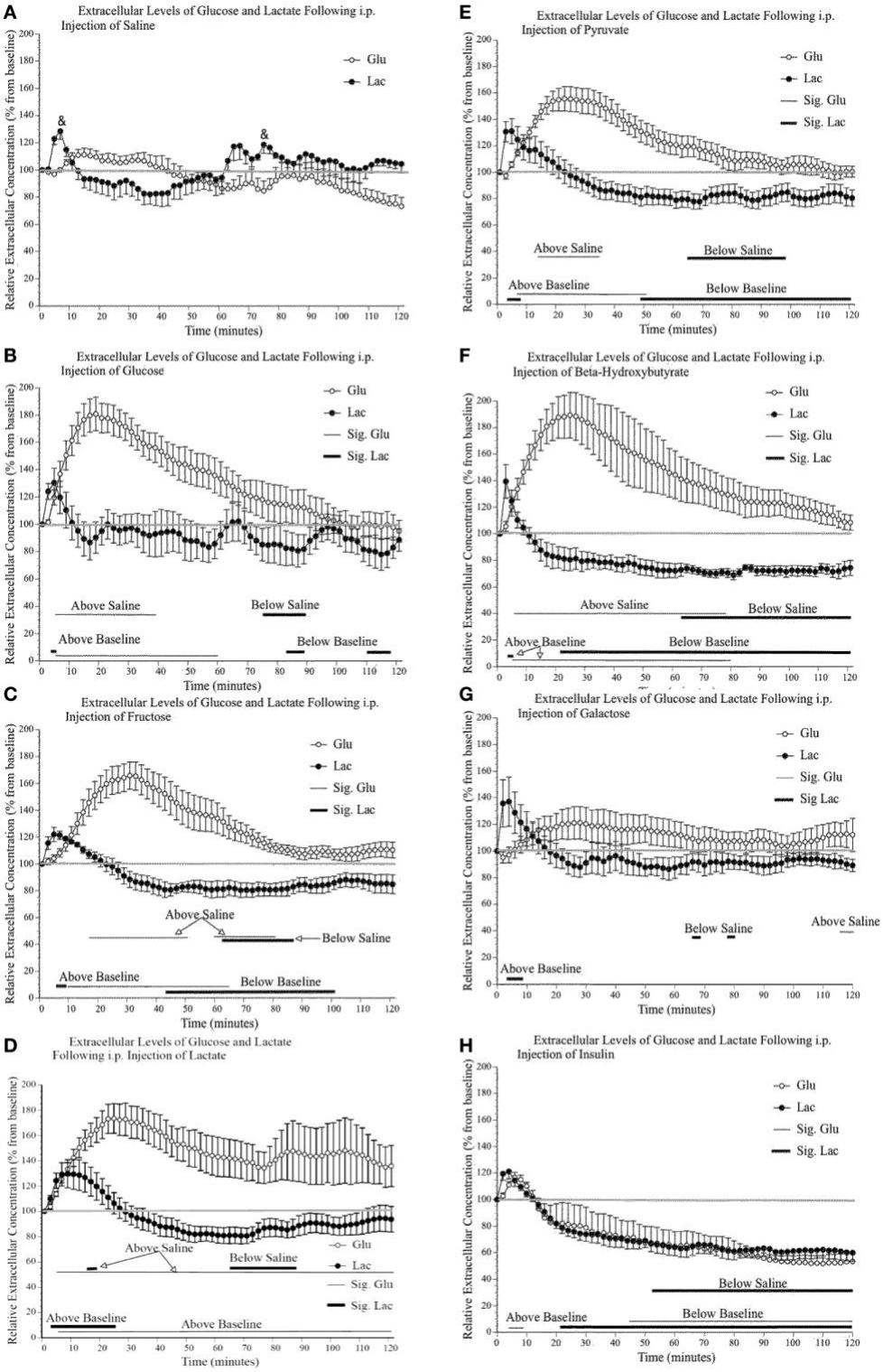
Figure 4 | Relative extracellular concentration of glucose and lactate



*Relative extracellular concentration of glucose and lactate following a **(A)** 0.9% i.p. injection of saline (n = 4), a 2 g/kg i.p. injection of **(B)** glucose (n = 4); **(C)** fructose (n = 6); **(D)** lactate (n =*

*10); **(E)** pyruvate (n = 6); **(F)** β–hydroxybutyrate (n = 5); **(G)** galactose (n = 6), as well as **(H)** a 1.2 I.U. i.p. injection of insulin (n = 7). Values are expressed as a percent of their baseline value (taken prior to injection) and error bars represent the standard error of the mean. The thin horizontal lines below each graph represent significant tests for extracellular glucose values while the thicker horizontal line indicates significant tests for extracellular lactate values ($p \leq 0.05$). Significant tests for comparisons between saline values are indicated separately from the comparison with baseline values.*

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Chapter III: Brain and muscle adaptation to high-fat diets and exercise: Metabolic transporters, enzymes and substrates in the rat cortex and muscle

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Abstract

There is evidence suggesting that the effects of diet and physical activity on physical and mental well-being are the result of altered metabolic profiles. Though the central and peripheral systems work in tandem, the interactions between peripheral and central changes that lead to these altered states of well-being remains elusive. We measured changes in the metabolic profile of brain (cortex) and muscle (soleus and plantaris) tissue in rats following 5-weeks of treadmill exercise and/or a high-fat diet to evaluate peripheral and central interactions as well as identify any common adaptive mechanisms. To characterize changes in metabolic profiles, we measured relative changes in key metabolic enzymes (COX IV, hexokinase, LDHB, PFK), substrates (BHB, FFA, glucose, lactate, insulin, glycogen, BDNF) and transporters (MCT1, MCT2, MCT4, GLUT1, GLUT3). In the cortex, there was an increase in MCT1 and a decrease in glycogen following the high-fat diet, suggesting an increased reliance on monocarboxylates. Muscle changes were dependent muscle type. Within the plantaris, a high-fat diet increased the oxidative capacity of the muscle likely supported by increased glycolysis, whereas exercise increased the oxidative capacity of the muscle likely supported via increased glycogen synthesis. There was no effect of diet on soleus measurements, but exercise increased its oxidative capacity likely fueled by endogenous and exogenous monocarboxylates. For both the plantaris and soleus, combining exercise training and high-fat diet mediated results, resulting in a middling effect. Together, these results indicate the variable adaptions of two main metabolic pathways: glycolysis and oxidative phosphorylation. The results also suggest a dynamic relationship between the brain and body.

Keywords: *Glucose transporter, Monocarboxylate transporter, β-hydroxybutyrate, Phosphofructokinase, Lactate dehydrogenase B, COXIV*

1. Introduction

In addition to general health benefits (Ekblom-Bak et al., 2014; Janssen & LeBlanc, 2010; Kokkinos, 2012; Lee et al., 2014; Matthews et al., 2007; Noel-weiss et al., 2008; Paffenbarger et al., 1986, 1993; Paffenbarger et al., 1994; Stessman et al., 2009) physical activity also has beneficial effects on brain functions such as memory (Khabour et al., 2010; Stroth et al., 2009; Van der Borght et al., 2007), cognitive functions in general (Bherer et al., 2013; Kamijo et al., 2009; Nanda et al., 2013; Radak et al., 2001), mood (Byrne & Byrne, 1993; Peluso & Andrade, 2005; Steptoe et al., 1989) as well as synaptic and neural plasticity (Bekinschtein et al., 2011; Cotman & Berchtold, 2002; Molteni et al., 2002; Pereira et al., 2007). Similarly, changes in diet can also have a positive impact on mood (McMillan et al., 2011; Spencer et al., 2017), cognition (Féart et al., 2013; Spencer et al., 2017; Whalley et al., 2004), synaptic plasticity (Gómez-Pinilla, 2008) as well as longevity and overall health (Black et al., 1994; Hession et al., 2009; Li, 2014; Martínez-González et al., 2015; Samaha et al., 2003; Savica et al., 2010; Sofi et al., 2010; Yancy et al., 2004). Both diet and exercise are important in slowing age-associated cognitive decline (Head, 2007; Lee et al., 2010; Lövdén et al., 2013; Miller et al., 2012; Ngandu et al., 2015; Williams & Kemper, 2010). Since the effects of exercise and diet on body and brain appear intermingled, we examined the impact of both in the same animals on muscle and brain metabolic markers.

1.1. Exercise induced changes in metabolism

1.1.1. Muscle. Within muscles, regular exercise increases transport of glucose (Perseghin et al., 1996), oxygen (Gavin et al., 2007) and alternative fuels such as ketone bodies or lactate (Coles et al., 2004), as well as mitochondrial density (Hood, 2001; Spina et al., 1996) and associated ATP enzymes (Holloszy, 1967). These changes results in muscles that express a

higher capacity for glycolysis (Tremblay et al., 1994), oxidative phosphorylation (Burgomaster et al., 2005; Egan et al., 2011; Holloszy, 1967; Short et al., 2003) and lipid metabolism (Talanian et al., 2007, 2010; Tunstall et al., 2002). Egan and Zierath (Egan & Zierath, 2013) suggest that metabolic changes facilitate the maintenance of substrate homeostasis and the regulation of metabolic pathways such as glycogenolysis and glycolysis during and after exercise. In the liver, mRNA levels of gluconeogenesis promoting-genes are increased, whereas genes associated to oxidative phosphorylation either remain the same or are downregulated (Lezi et al., 2013). These authors suggest that these opposing changes facilitate the hepatic production of glucose, for muscle use, via blood lactate without the competition of its use within the hepatic oxidative phosphorylation pathway.

1.1.2. Brain. In contrast to muscles, the brain exhibits fewer metabolic changes in response to regular exercise. Many authors report increased function and density of mitochondria (Bayod et al., 2011; Marques-Aleixo et al., 2014; Steiner et al., 2011), though this is not a universal finding with some authors reporting no change in the biochemical components of oxidative phosphorylation (Lezi et al., 2013). There has also been evidence of increased GLUT1 expression in brain structures associated with movement following 48 h access to a running wheel (Allen & Messier, 2013).

1.2. Diet induced changes in metabolism

1.2.1. Muscle. A common metabolic alteration found in skeletal muscles following a high-fat diet is insulin resistance (Kraegen et al., 1991) which is accompanied by a decreased glucose uptake, though this decrease uptake does not seem to be the result of altered GLUT4 expression (Hansen et al., 1997). Ciapaite and colleagues demonstrated increased fatty acid accumulation, ß-oxidation and oxidative phosphorylation in the fast-twitch extensor digitorum

longus muscle (Ciapaite et al., 2015) with two different high-fat diets being consumed for 5 weeks. Messa and colleagues, on the other hand, demonstrated increased oxidative capacity of both the slow-twitch soleus muscle and the fast-twitch extensor digitorum longus muscle following both 4 weeks and 8 weeks of high-fat diet (Messa et al., 2020). In rats, a short 2-week high-fat diet induced changes in lipid metabolism for the slow-twitch soleus muscle but not the fast-twitch extensor digitorum longus muscle (Andrich et al., 2018). These distinctions may be the result of the use of different strains (C57 vs CD-1), species (mice vs rats), sex or exact composition of the high-fat diet (67% vs 45% fat vs 37% fat). The precise requirements and pathways for these modifications are not yet known, however work by Gandara and colleagues would suggest that these changes do not occur as a result of changes in muscle mitochondrial content (Gandara et al., 2019).

1.2.2. Brain. Functionally, a high-fat diet impairs cognitive performance (Pistell et al., 2010) and exacerbates cognitive aging (Amin et al., 2011; Morrison et al., 2010). High-fat diets that impair cognitive performance has been associated with increased glial reactivity and cerebral inflammation (Pistell et al., 2010). High-fat diets, as well as a fructose-rich diet (Agrawal & Gomez-Pinilla, 2012), promote a shift away from oxidative phosphorylation towards lipid peroxidation (Agrawal & Gomez-Pinilla, 2012; Amin et al., 2011). The lipid peroxidation metabolic pathway results in higher production of reactive oxygen species through its secondary and tertiary oxidative modifications. Increased reactive oxygen species lead to increased inflammation (Morrison et al., 2010). Lipid peroxidation alters the expression of key membrane proteins such as glucose transporters and insulin receptors and deficiencies in insulin transport, signalling or sensitivity are key elements of diet-induced impairments in the brain (Agrawal & Gomez-Pinilla, 2012).

1.2.3. Diet and exercise induced changes in metabolism. There is little information on the combined or additive effects of a sedentary lifestyle and a high-fat diet on slow twitch vs fast twitch muscle metabolism. In the soleus, a month-long high-fat diet can impair both the insulin dependent and independent glucose uptake following exercise (Tanaka et al., 2007), and regular aerobic exercise can improve the high-fat diet induced insulin-resistance (Maeda et al., 2015). Though both exercise and a high-fat diet are capable of increasing lipid metabolism individually, perhaps through PDK4 inhibition of glycolysis (Rinnankoski-Tuikka et al., 2012), there is an additive effect on the capacity of skeletal muscles to metabolize lipids when both treatments are combined (Yun et al., 2020). This same study demonstrated that a high-fat diet alone decreased glycogen synthase activity, without changes GLUT4 expression or hexokinase activity. In contrast, 3 weeks of swimming increased glycogen synthase activity, as well as GLUT4 and hexokinase expression. Interestingly, when exercise and a high-fat diet were combined, glycogen synthase, GLUT4 and hexokinase expression all increased (Kim et al., 2000). However, this study did not differentiate between changes in slow-twitch or fast-twitch muscles. This distinction is important due to the difference in functionality, fiber composition and metabolic profile that exists between various muscle types. For example, the soleus muscle is a slow-oxidative (SO) muscle (Philippi & Sillau, 1994), primarily composed of type I fibers (Cornachione et al., 2011; DeNies et al., 2014; Gollnick et al., 1974) and metabolic enzymes that favor oxidative phosphorylatio which together sustain endurance activity (i.e. marathon running). In contrast, the plantaris is a fast-glycolytic (FG) muscle (Ishihara et al., 1991), primarily composed of Type II fibers (Cornachione et al., 2011; DeNies et al., 2014) and metabolic enzymes that favor glycolysis as an ATP producing mechanisms to engage in short bursts of motor behavior, more specifically proprioception for the plantaris (Spina, 2007). Although these

muscle types interact closely, with metabolic cooperation being observed by shuttling glycolytic end product from fast-glycolytic to slow-oxidative muscles to undergo oxidative phosphorylation (Brooks, 1986, 2018), they are fundamentally different.

1.3. Objective

In the present experiment, we examined the changes in metabolic pathways, transporters and enzymes in both muscle and brain to test whether there are common mechanisms operating in both brain and muscle when diet and exercise are manipulated.

2. Results

Fig. 1 presents a summary of the results

2.1. Blood

Fig. 2 illustrates the relative concentrations of various plasma energy substrates. The high-fat diet significantly increased blood levels of ß-hydroxybutyrate ($F(1,20) = 12.91$, $p = 0.002$) and free fatty acids ($F(1,30) = 28.19$, $p < 0.001$), irrespective of exercise condition. Exercise significantly increased plasma lactate ($F(1,35) = 8.42$, $p = 0.006$). This exercise-induced increase in blood lactate, when compared to their diet-respective controls, was larger in the high-fat group ($p = 0.005$) than the normal chow group ($p = 0.294$).

2.2. Cortical measures: High-fat diet decreases cortical glycogen and increases MCT1 transporter expression

Fig. 3, **Fig. 4**, **Fig. 5** illustrate the substrate and protein expressions measured in the rat cortex. Exercise did not significantly alter any of the substrates measured. However, the high-fat diet significantly decreased cortical glycogen ($F(1,29) = 5.79$, $p = 0.023$) and increased the expression of the monocarboxylate transporter 1 ($F(1,28) = 5.00$, $p = 0.033$).

2.3. Muscle measures

Fig.6, **Fig. 7** illustrate the relative expression of the various proteins and enzymes measured in the rat plantaris and soleus muscle. The observed changes are detailed below.

2.3.1. Combination of high-fat diet and exercise increases MCT1 in muscles. Within the high-fat group, exercise significantly increased monocarboxylate transporter 1 (MCT1) expression in the plantaris muscle ($p = 0.014$) when compared to its respective high-fat diet/sedentary control. The soleus muscle, however, demonstrated an interaction effect

(Diet*Muscle; F(1,28) = 5.71, p = 0.24). Both exercise (p = 0.046) and diet (p = 0.008) significantly increased MCT1 expression in the soleus. Indeed, the combination of a high-fat diet and treadmill running increased MCT1 soleus expression above exercise/normal chow controls as well as sedentary/high-fat controls. These results indicate that rats who were exercised on a high-fat diet had higher levels of MCT1in muscles.

2.3.2. High-fat diet increases MCT2 in the plantaris muscle. A high-fat diet increased monocarboxylate transporter 2 (MCT2) expression within the plantaris almost two-fold (F(1,23) = 48.76, p < 0.001), but not within the soleus (F(1,23) = 0.19, p = 0.664). To a lesser degree, exercise was also capable of significantly increasing MCT2 expression (p = 0.022) within the soleus muscle of the rats fed the normal chow, which was not significantly observed in the plantaris muscle. These results indicate that MCT2 expression is strongly increased in the plantaris by a high-fat diet, and slightly increased by exercise in the soleus muscle of rats eating a standard chow.

2.3.3. Exercise increases and a high-fat diet decreases MCT4 expression in the soleus. When compared to their sedentary controls, exercise significantly increased monocarboxylate transporter 4 (MCT4) expression two-fold in the soleus (F(1,29) = 15.32, p = 0.001) but not the plantaris (F(1,29) = 0.05, p = 0.821). Additionally, within the soleus, the high-fat diet significantly reduced MCT4 expression when compared to its normal-chow controls (F(1,29) = 13.18, p = 0.001). In other words, MCT4 expression in the soleus increased with exercise and decreased with a high-fat diet, leading to an average effect when both treatments were combined.

2.3.4. Exercise-induced increases in cytochrome c oxidase are attenuated by a high-fat diet. Analysis of cytochrome c oxidase (COX IV) revealed a three-way interaction between exercise, diet and muscle type ($F(1,19) = 18.83$, $p < 0.001$). Exercise increased COX IV expression across diets and muscle types, though this effect was more prominent in the plantaris (10-fold increase vs 5-fold increase). This exercise-induced increase in COX IV was attenuated by the high-fat diet. Rats undergoing treadmill running *and* the high-fat diet expressed significantly lower quantities of COX IV when compared to their normal-chow controls, within both the soleus ($p = 0.003$) and the plantaris ($p < 0.001$) muscle.

2.3.5. Exercise increase plantaris but not soleus expression of hexokinase. A two way interaction revealed that, when compared to their sedentary controls, exercise significantly increased hexokinase expression within the plantaris ($F(1,29) = 11.92$, $p = 0.002$), but not the soleus ($F(1,29) = 0.48$, $p = 0.493$) muscle.

2.3.6. Plantaris expression of lactate dehydrogenase B is increased by both exercise and a high-fat diet. Observed changes in lactate dehydrogenase B (LDHB) were significantly more pronounced in the plantaris muscle ($F(1,32) = 6.07$, $p = 0.019$). Indeed, within the plantaris muscle, both exercise ($p = 0.028$) and a high-fat diet ($p = 0.009$) were capable of increasing LDHB expression when compared to their sedentary normal-chow controls. However, their combined effects were not additive.

2.3.7. High-fat diet increases phosphofructokinase expression in the plantaris muscle. The high-fat diet significantly increased phosphofructokinase (PFK) within the plantaris muscle ($F(1,28) = 19.81$, $p < 0.001$), but not the soleus muscle ($F(1,28) = 0.20$, $p = 0.658$).

3. Discussion

3.1. Summary

The aim of this experiment was to examine the concurrent effect on brain and muscle of two "lifestyle" changes thought to have an impact on both brain function and muscle metabolism. The results show that the impact of these changes on the metabolic profile is more prominent in muscle. The main finding in the cortex was that high-fat diet decreased glycogen and increased monocarboxylate transporter 1 (MCT1) expression. These results suggest that high-fat diets produce a shift away from glucose and glycogen-based glucose storage and promote the use of ketone bodies or lactate as metabolic fuel.

In the skeletal muscles, exercise and diet had clear and distinctive effects on metabolic profiles. Whereas both exercise and diet appeared to increase the capacity for oxidative phosphorylation, it would seem that this ATP producing pathway is most likely supported by glycogen synthesis and alternative fuels following exercise, whereas a high-fat diet likely supports oxidative phosphorylation through aerobic glycolysis. Interestingly, the combination of the high-fat diet and exercise appeared to create a metabolic profile somewhere in between those of exercise and high-fat diet. There were fewer effects on the soleus than the plantaris, perhaps because more challenging training conditions are needed to induce changes given the oxidative nature of the soleus (Gandara et al., 2019).

3.2. Cortical changes in metabolite transporters

High-fat diet increased cortical MCT1 expression while exercise had no effect on cortical expression of GLUT or MCT transporters. Pierre and colleagues showed that mice fed a high-fat diet for 6-12 weeks had increased cortical MCT2 and MCT1 expression starting 6 weeks after the initiation of the high fat diet (Morrison et al., 2010). This suggests that changes in other

monocarboxylates transporters, specifically MCT2, are greater after 6+ weeks access to a high-fat diet compared to a 4-week in mice (Agrawal & Gomez-Pinilla, 2012) and rats (Tanaka et al., 2007).

What is perhaps more surprising is the lack of change in other transporters following exercise, specifically GLUT1 and MCT2. Both acute and chronic exercise have demonstrated the ability to increase cortical GLUT1 (Maeda et al., 2015; Rinnankoski-Tuikka et al., 2012; Tunstall et al., 2002) and acute exercise also seems to increase MCT2 (Brooks & White, 1978; Kim et al., 2000; Yun et al., 2020). The time frame during which GLUT1 and MCT2 expression is significantly altered by exercise may have been missed as there is evidence that change in expression is no longer observable 24h after exercise (Brooks & White, 1978; Kim et al., 2000; Rinnankoski-Tuikka et al., 2012).

A similar study to the present one reported a cumulative effect of diet and exercise on hippocampal MCT1, as well as a non-cumulative effect both exercise and high-fat diet on MCT4 expression (Pifferi et al., 2007). Within the cortex, only MCT1 was altered, with no cumulative effect of exercise, suggesting a region-specific effect, similar to previously described differential patterns of protein expression in the cortex and hippocampus following acute exercise (Brooks & White, 1978; Yun et al., 2020).

3.3. High-fat diet decreased cortical glycogen

The observed decrease in cortical glycogen may indicate detrimental effects of the high-fat diet on glucose metabolism. High-fat diets have been shown to decrease glycogen synthesis (Hayashi et al., 1997) without any changes in glycolytic or oxidative processes (Brechue et al., 1994), all while transiently affecting BBB transport and uptake of glucose (Berg et al., 2002).

Low cerebral glycogen has also been interpreted as an attempt by the brain to exhaust all other possible metabolic resources (Davidson et al., 2006).

In our experiment, the lack of apparent changes in the capacity for glucose transport and availability, combined with the increased cortical MCT1 expression as well as increased blood levels of BHB and FFA (monocarboxylates transporter across the BBB through MCT1), suggests the dynamic and flexible nature of cortical neuroenergetics. The brain may easily adapt to alternative sources of energy, such as ketone bodies, when they are available in large and consistent quantities (Moore et al., 1976). Within the brain, MCT1 and MCT4 are most commonly expressed in astrocytes, whereas as MCT2 is almost exclusively found in neurons (Masino & Niño-Zarazúa, 2016; Pierre et al., 2007; Pifferi et al., 2011). The observation that only the astrocytic MCT1 was modulated by the high-fat diet is consistent with the astrocyte-to-neuron lactate shuttle (Forero-Quintero et al., 2017). Increased cortical MCT1 expression is interpreted as a likely sign of increased monocarboxylate uptake, which would only be necessary if it was also accompanied by increased use by the brain. The increase in MCT1 specifically suggests that the modulation of monocarboxylate transport occurs either at the endothelial cells or the astrocytes. Previous work (Moore et al., 1976) demonstrated that elevated blood monocarboxylates (specifically lactate) is associated with elevated extracellular glucose levels and that inhibition of both GLUT1 and MCT1 transport at the blood-brain barrier elicits a stronger compensatory increase in MCT1 than GLUT1 (unpublished data). We propose that most metabolic processes converge towards the creation of either glucose or lactate and the brain adapts to the availability of glucose or monocarboxylates.

3.4. Plantaris

We now turn to the discussion of our observations in muscles. There is a vast literature on the impact of exercise and diet on muscle physiology and function. However, we will limit our discussion to the points that are most relevant to our comparison of cortical and muscle adaptation to exercise and a high-fat diet. Because the brain uses both the glycolytic and oxidative phosphorylation pathways, we will also highlight the differences between the two muscle types that favour one or the other.

3.4.1. Exercise increases oxidative phosphorylation capacity are supported through exogenous metabolites and glycogen formation, without upregulating glycolytic capacity within the plantaris. Exercise increases the oxidative capacity of the typically glycolytic plantaris, as evidenced here by increases in COX IV and demonstrated previously through observations of increased mitochondrial biogenesis (de Assis et al., 2016; Um et al., 2008), changes in fiber composition (Van der Borght et al., 2009) and heightened oxidation of alternative fuels such a free fatty acids (de Assis et al., 2016) as well as glucose (Matsui et al., 2017). This increase in the capacity for oxidative phosphorylation is accompanied by an increased expression of LDHB but not PFK, the main metabolic control of glycolysis (Portela et al., 2017; Takimoto et al., 2013). Given that exercise is also historically associated with an increase in glycogen content (Ke et al., 2011; Schasfoort et al., 1988), the authors propose that increases in oxidative phosphorylation in the plantaris are met through elevated glycogen content (replenished by hexokinase) or import (MCT1) and conversion (LDHB) of circulating lactate to pyruvate, subsequently entering the OXPHOS pathway.

3.4.2. High-fat diet increases plantaris capacity for glycolysis and oxidative phosphorylation. Though high-fat diets are generally known to increase lipid oxidation at the cost of oxidative phosphorylation (Lezi et al., 2013; Messa et al., 2020), recent evidence suggest

that glycolytic muscles may be less susceptible to this effect (Lezi et al., 2013). This could be due to the lack of change in muscle fiber composition induced by high-fat diets (Baxter et al., 2006; McNeilly et al., 2012), though this is not a universal finding (Bough et al., 2006). These studies are limited by the rarely homogenous fiber composition of muscles, such as the gastrocnemius muscle, which is composed of both glycolytic and oxidative fibers (Jais et al., 2016). Glycolytic and oxidative fibers can react differently to environmental stimuli and the choice of muscle as well as careful consideration during interpretation are necessary.

In stark contrast to the exercise induced changes, we determined for the first time that a high-fat diet increased PFK concentration within the plantaris. High-fat diets increase fatty acid oxidation and limit carbohydrate use through the inhibition of the glycolytic pathway at the PFK step as a result of increased citrate synthase activity (Lark et al., 2012; Wapnir et al., 1973). Consequently, high-fat diets are typically associated with no change, or decreased expression of PFK; a phenomenon observed in rat skeletal and heart muscles (Hou et al., 2003; Ivy, 1991; Takahashi et al., 2019). Combined with the changes in MCT2 and LDHB, it would appear that increases in OXPHOS capacity are met through the upregulation of two distinct pathways. OXPHOS may be supplied through endogenous means with an increased capacity for glycolysis (increase in PFK) (Hargrave et al., 2015; Pifferi et al., 2007; Sandoval et al., 2018), as well as through exogenous means by increasing the capacity for influx of monocarboxylates found in the blood via the known lactate importer, MCT2 (Béland-Millar et al., 2017; Pierre & Pellerin, 2009), and their subsequent conversion to pyruvate via LDHB.

3.4.3. Effects of combined treatments on plantaris metabolic profile. The combined treatment of treadmill and a high-fat diet was the only condition capable of significantly increasing hexokinase, MCT1 expression, and COX IV (but with a modulating effect of diet)

while maintaining similarly elevated levels of LDHB, PFK and LDHB. We interpret these changes as a suggestion that the plantaris adapts to diet and exercise by adopting an averaged position in many of the metabolic parameters. When increased activity and a high-fat diet are combined, the capacity for the OXPHOS pathway is upregulated, but so is lipid utilisation (Andrich et al., 2018). Given the increased availability of lipids and ketone bodies due to the high-fat diet, the shuttling of metabolites to the OXPHOS pathway appears to be attenuated to favor more lipid oxidation than would normally be the case with exercise alone (Messa et al., 2020). However, there is still a significant increase in OXPHOS capacity that would likely be met with the import and conversion of external metabolites, such as lactate, as is evidenced by an increase in MCT1 (and MCT2) as well as LDHB. The upregulation of PFK and the increase in hexokinase may increase glycolytic capacity or lead to additional glycogen conversation. This hypothesis is consistent with the observation that a combined exercise regimen and high-fat diet increase glycogen synthase (Andrich et al., 2018).

It would seem that the plantaris readily adapts to the metabolic environment by selectively adapting both of its primary metabolic pathways in response to factors such as circulating metabolites (diet) and chronic increases in metabolic demand (exercise).

3.5 Soleus

3.5.1. Exercise increases MCT2 and MCT4 soleus expression with little indication as to the pathway supporting the increase in oxidative phosphorylation capacity. Exercise increased the oxidative capacity (COX IV) of the oxidative soleus muscle (Amin et al., 2011; Pierre & Pellerin, 2005). Given that oxidative muscles typically rely on increased glucose conversion to meet exercise requirements, it is surprising that no changes in PFK or hexokinase were observed (Leino et al., 2001). Perhaps this is the result of the soleus' already large capacity

for transport of glucose (Hargrave et al., 2015) or the insufficient ability of our exercise regimen to increase oxidative demand enough to impact glucose transport.

The exercise-induced increase in MCT2 and MCT4 expression rather than MCT1 is an uncommon observation (Jais et al., 2016; Little et al., 2018; Martin, 1980). Acute exercise studies have often shown a transient effect of a single exercise bout on all isoforms of MCT within muscles (Brooks & White, 1978; Lövdén et al., 2013; Yun et al., 2020), but studies evaluating the impact of long term exercise on other MCT isoforms are lacking due to previous evidence of its low expression (Bloemberg & Quadrilatero, 2012; Ferraro et al., 2014). Recent evidence, however suggest a higher content of MCT2 than initially thought (Hyatt et al., 2015).

As a result of exercise training, it appears that the soleus muscle is capable of increasing its oxidative capacity without the need to alter the expression of key enzymes in primary metabolic pathways. Despite this lack of change in key enzymes, monocarboxylate transport appears very plastic, suggesting a starting point in future research investigating how the soleus muscle sustains its increased oxidative capacity following exercise training.

3.5.2. Diet has no effect on transport or enzyme concentration in the soleus. Unlike the plantaris, we observed no effect of a high-fat diet on the measured enzymes and proteins in the soleus. This is contrary to previous evidence indicating that a high-fat diet decreased glucose oxidation and increased lipid oxidation (Ciapaite et al., 2015; Lezi et al., 2013; Messa et al., 2020; Talanian et al., 2007; Yasuda et al., 2006), as well as altered fiber composition (Baxter et al., 2006) specifically within the soleus. However, a substantial number of experiments report different outcomes. Suwa and colleagues found no change in fiber composition of the soleus following a 6-week high-fat (60%) diet (McNeilly et al., 2012). Work conducted by Gandara and colleagues found no effect of a 14-week high-fat diet on mitochondrial content or biogenesis

(Steiner et al., 2011), but the fat content of their diet was significantly lower than 60%. Finally, altered fiber composition are typically found in experiment that exposed animals to diets for a longer time than the one used in this study. For example, in mice, changes in fiber composition were observed in high-fat diets lasting 8-16 weeks (Lezi et al., 2013), or even a year (Baxter et al., 2006) – with no observed changes in a 6-week diet exposure (McNeilly et al., 2012). Changes in lipid and glucose oxidation, however, seem to appear more quickly. Evidence of increase lipid consumption and decreased glucose oxidation are found as early as 2 to 4-weeks into a high-fat diet (Lezi et al., 2013; Messa et al., 2020; Talanian et al., 2007). The oxidative profile of the soleus muscle appears to be more immediately susceptible to exercise rather than diet-induced changes. Future work will have to unravel the specific conditions underlying the variable effects of diet on the soleus.

3.5.3. Concurrent exercise and high-fat diet on soleus: MCT1 as a potential indicator of increased lipid metabolism. As we found in the plantaris, the concurrent exercise and high-fat diet treatment was the only condition to increase both MCT1 and COX IV and attenuate the exercise-induced increase in MCT4. MCT1 expression in skeletal muscle tissue has been frequently associated to its oxidative capacity (Berg et al., 2002; Brechue et al., 1994; Hayashi et al., 1997) as well as the development of a metabolic profile more adapted to lipid use (Davidson et al., 2006; Messa et al., 2020).

Just as changes in MCT1 expression can be indicative of a switch to a more lipid-based metabolic profile, changes in MCT4 expression can be an indicator of anaerobic stress (Baldwin et al., 1976). Increasing intracellular lactate alters the intracellular redox ratio (Hou et al., 2003; Ivy, 1991), as well as result in an accumulation of downstream glycolytic end-products which act as a negative feedback loop, favoring the shuttling of glucose-derived carbon through the

pentose-phosphate pathway rather than glycolysis (Takahashi et al., 2019). The pentose-phosphate pathway, in turn, results in the production of glutathione, an antioxidant important for combatting metabolic stress (McCullagh et al., 1996). Increases in MCT4 expression during exercise may reflect the need to control intracellular lactate levels, either endogenously produced by upregulated glycolysis or influx through MCT1/MCT2, so as to avoid toxicity (Dubouchaud et al., 2000). The necessity of this adjustment is mitigated when exercise is combined with a high-fat diet. We propose that this is because increased OXPHOS capacity is met, at least in part, by alternative metabolites circulating in the blood (i.e. elevated BHB and FFA) imported via increases in MCT1, and increased reliance on lipid oxidation which leads to less taxed glycolytic and OXPHOS pathway.

3.6. Conclusion

The current experiment demonstrated that under the same diet and exercise regimen, the expression of monocarboxylate transporter 1 is the main cortical adaptation observed while muscles present with a more varied response that is dependent on muscle type. MCT1 is most commonly expressed in astrocytes at the blood-brain barrier. This relatively limited influence of exercise and diet on the brain (increase in MCT1 and decrease in glycogen as a result of diet) is consistent with the hypothesis that the brain takes advantage of the body's versatile ability to metabolise and convert varied metabolic fuels to lactate as we have previously discussed (Moore et al., 1976). This ability allows the brain to maximise its metabolic efficiency by maintaining at least two metabolic fuel paths that are modulated according to the conditions in the rest of the body. Future research should focus on the collaborative nature of the body and brain metabolism to elucidate on how these compartments interact to improve cognitive function.

4. Experimental Procedure

4.1. Animals

Three-week-old (~40g) male Wistar rats were purchased from Crea Japan (Tokyo, Japan). The animals were individually housed in metal cages in an animal room maintained between 22–24°C with a 12:12-h light-dark cycle. Unless otherwise specified, the rats had *ad libitum* access to both water and standard chow or the high-fat diet. The rats were fed either a standard rodent chow diet (10% calories from fat, D12450J; Research Diets, New Brunswick, NJ, U.S.A) or a high-fat diet (60% calories from fat, D12492; Research Diets). A high fat-diet was chosen due to its ability to increase fatty acids and ketone bodies as an alternative fuel source in order to more readily identify switches in metabolic profiles. This research was approved by the Ethics Committee of Animal Experimentation at Osaka University of Health and Sport Sciences.

4.2. Experimental procedure

The rats were fed their respective diet for 5 weeks during which time they underwent 5 weeks of treadmill training. The exercise group was subjected to exercise bouts 5 times a week consisting of running on a treadmill for 1hr at a moderate intensity (20 m/min, 8% grade), previously shown to be equivalent to 50–70% $\dot{V}O_2$ max for rats (Brooks & White, 1978). The rats were motivated to continue running for the duration of the testing via mild electric shocks and gentle prodding when necessary. Prior to the treadmill exercise experiment, all rats were familiarized with treadmill at running speeds of 5–20 m/min on an 8% grade for ~10 min/day for 5 days. Age-matched sedentary control rats for all experiments in this study were kept in cages until they were euthanized.

4.3. Tissue collection

Twenty-four hours following the last exercise bout, and subsequent to a 2 hour fast, the rats were euthanized by cerebral-focused microwave irradiation (5 kW, 1.7 s; MMW-05; Muromachi Kikai, Tokyo Japan). Immediately following the irradiation, mice were decapitated, and their trunk blood extracted. Plasma was immediately isolated by spinning trunk blood at 5200 g and stored at −80°C until analysis. During the isolation of the plasma, the rats were placed on an ice-cooled surface and the skeletal muscles (plantaris and soleus) were extracted from the hind limbs. Subsequently the cortex, was extracted immediately after the skeletal muscles. Each collected tissue was immediately frozen in dry ice-cooled isopentane and stored at −80°C until analysis. The entire procedure, from the start of the irradiation to cortex extraction, took approximately 6-7 minutes.

4.4. Western blot

Sample preparation and Western blot analysis were performed according to the protocol described in previous studies (Enoki et al., 2006; Takimoto et al., 2013; Takimoto & Hamada, 2014). The tissue samples were homogenized in buffer A (210 mM sucrose, 30 mM HEPES, 10 mM EDTA, 2 mM EGTA, 40 mM NaCl, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4). The protease inhibitors used were EDTA and PMSF. Then, 400 μl of buffer B (1.167 M KCl and 58.3 mM tetrasodium pyrophosphate) was added, mixed briefly, and placed on ice for 15 min. After centrifugation at 200,000 g for 75 min at 4°C (Himac CS100GXII; Koki Holdings, Tokyo, Japan) the supernatant was discarded and the pellet was washed thoroughly with buffer C (10 mM Tris·HCl base and 1 mM EDTA, pH 7.4). The pellet was then homogenized in 500 μl of buffer C. After mixing 200 μl of 16% SDS, the samples were vortex-mixed and centrifuged at 1,100 g for 10 min at room temperature (MX-160; TOMY SEIKO, Tokyo, Japan). The resulting

supernatant was solubilized in Laemmli sample buffer and boiled. We used the Lowry protein assay (Bio-Rad, Hercules, CA) to measure protein concentrations.

The brain homogenate samples (20 μg of protein) were separated by SDS-PAGE using either a 12% polyacrylamide gel for MCTs, GLUTs, LDHB, and GAPDH, a 12.5% gel for BDNF and COX IV or a 10% gel for PFK and hexokinase. The proteins were then transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) at 100 V for 1 h. The membranes were incubated overnight at 4°C with primary antibody [GLUT1 antibody (7903; Santa Cruz Biotechnology, Santa Cruz, CA), GLUT3 (30107; Santa Cruz Biotechnology), BDNF (546; Santa Cruz Biotechnology), COX IV (4850; Cell Signaling Technology, Beverly, MA), GAPDH (2118; Cell Signaling Technology), hexokinase (2867; Cell Signaling Technology), MCT1 (AB3540P; Merck KGaA, Darmstadt, Germany), MCT2 (AB3542; Merck KGaA), MCT4 (AB3314P; Merck KGaA), LDHB (100775; Santa Cruz Biotechnology) and PFK (377436; Santa Cruz Biotechnology)]. All primaries were incubated at a 1/1000 concentration, with the exception of MCT2 which was incubated at a 1/2000 dilution. The membranes were incubated in blocking solution [Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and 5% non-fat dry milk] for 1 h at room temperature and washed with TBST. The membranes were then incubated for 1 h at room temperature with either anti-rabbit (GE Healthcare, Buckinghamshire, UK) or anti-mouse (GE Healthcare) IgG antibody at a 1/2500 dilution. The antibody-bound protein was reacted with enhanced chemiluminescence (ECL prime; GE Healthcare), and the blot signal was detected by a short exposure to blue light-sensitive film (Konica Minolta Medical & Graphic, Tokyo, Japan). The intensity of the bands was quantified by densitometric analysis using Scion Image software (Scion). The immunoblot samples were adjusted to equal 1.0, and

each sample value was expressed relative to the adjusted mean value for the sedentary normal chow control group.

4.5. Substrate Analysis

All experimental protocols were completed using the same initial tissue. However, subsequent manipulations for the respective substrate measurement varied.

4.5.1. Cortical lactate. The lactate measurements were performed according to the method described by Passonneau and Lauderdale (Passonneau & Lauderdale, 1974). Briefly, samples were added to 100 µl of ice-cold 3 M perchloric acid, homogenized using a homogenizer (PT3100; Polytron, Kinematica, Bohemia, NY, U.S.A.), and then centrifuged at 1,000 g for 5 min at 4°C. The resulting supernatant was mixed with buffer containing glycine, hydrazine, and nicotinamide adenine dinucleotide to which lactate dehydrogenase was subsequently added. The fluorescence measurements were taken at 350 nm excitation and 450 nm emission. The lactate concentration was calculated from a standard curve.

4.5.2. Cortical glucose and glycogen. Briefly, samples were homogenized with 500 µl of ice-cold 0.3 M perchloric acid. Samples were then added to an AMG (0.2U/ml glucose amylase, 1mM of sodium acetate pH 4.8, 0.3 mM bovine serum albumin) or NaAc (100 mM sodium acetate pH 5.5, 0.3 mM bovine serum albumin) buffer for glycogen and glucose analysis, respectively and left to react at room temperature for 90 minutes. Following the room temperature reaction, the samples were centrifuged at 1,000 g for 10 minutes at 4°C. The supernatant was reacted at room temperature for 30 minutes with a glucose reagent (0.9 mM bovine serum albumin, 0.1 M Tris-HCL pH 8.1, 0.4 mM nicotinamide adenine dinucleotide phosphate oxidized form, 1mM adenosine triphosphate, 0.6. mM dithiothreitol, 2mM magnesium

chloride 440U/ml, 0.025 mM glucose-6-phosphate, 57U/ml hexokinase). Subsequently, the fluorescence measurements were taken at 350 nm excitation and 450 nm emission with a fluorometer (RF5300PC; Shimazu, Kyoto, Japan). The glucose and glycogen concentrations were calculated from a standard curve.

4.5.3. Cortical β-hydroxybutyrate. The level of β-hydroxybutyrate, a ketone body, was measured using a β-hydroxybutyrate colorimetric assay kit (BioVision, Milpitas, CA). Samples preparation and experimental procedures were executed following manufacturer recommendations.

4.5.4 Plasma free fatty acids. Plasma concentrations of free fatty acids (FFA) were measured using a commercially available assay kit (NEFA-C kit; Wako, Osaka, Japan).

4.5.5. Plasma β-hydroxybutyrate. Plasma concentrations of β-hydroxybutyrate (BHB) were measured using a commercially available assay kit (β-hydroxybutyrate colorimetric assay kit; BioVision).

4.5.6. Plasma insulin. Plasma concentrations of insulin were measured using an insulin ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

4.5.7. Blood glucose and lactate. Blood glucose and lactate concentrations were measured using automated glucose (Glutest Every; Sanwa Kagaku Kenkyusho, Nagoya, Japan) and lactate (Lactate Pro; Arklay, Kyoto, Japan) analyzers.

4.6. Statistical analyses

Data are expressed as means ± SE. Data was analysed using a two-way ANOVA and the differences between groups were considered statistically significant at $p < 0.05$. Interaction

effects are detailed in the results section, main effects and post-hoc pairwise comparisons are illustrated on the bar graphs with the letters “e”, “d” or “m”. These letters indicate significant ($p < 0.05$) differences when comparing across diets (d), exercise (e) conditions or muscle (m) types.

Funding

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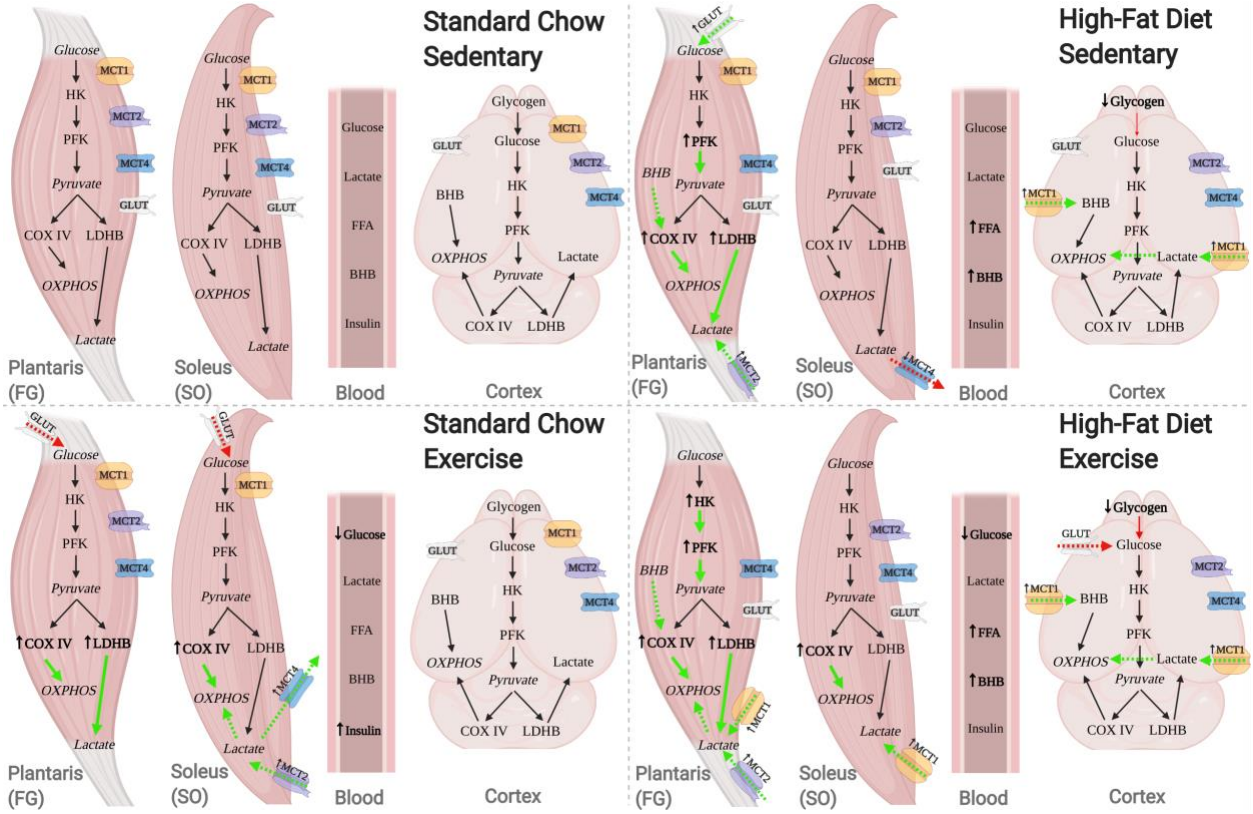
CRediT authorship contribution statement

Alexandria Beland-Millar: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. Masaki Takimoto: Data curation, Formal analysis, Methodology. Taku Hamada: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision. Claude Messier: Formal analysis, Funding acquisition, Supervision, Writing - review & editing**.**

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure 1 | Summary of measured changes



This figure presents the changes in substrates and proteins measured, respective to each exercise, diet and tissue condition. From left to right, the tissues represented in each panel is the plantaris (FG: Fast-twitch Glycolytic skeletal muscle), the soleus (SO: Slow-twitch Oxidative skeletal muscle), the plasma (trunk blood) and cortex.

The substrates and proteins measured are identified within a simplified metabolic pathway. Substrates and proteins necessary for appropriate illustration but not measured are italicized (i.e. glucose in skeletal muscles). Changes that are significantly different from <u>control</u> (standard chow, sedentary), based on post-hoc pairwise comparisons, are bolded. The direction of the change is indicated by a black arrow preceding the substrate or protein. Arrows below significant changes in protein identify possible pathways that are increased (green) or decreased (red) as a result of the changes in expression of these key proteins. Lastly, dashed-arrows represent hypothetical changes in directional fluxes of monocarboxylates (lactate, BHB, pyruvates) as a result of changes in blood concentration or transporter expression.

HK: Hexokinase
PFK: Phosphofruktokinase
LDHB: Lactate Dehydrogenase B
COX IV : cytochrome c oxidase
OXPHOS: Oxidative phosphorylation (TCA cycle + mitochondrial respiration)
MCT: monocarboxylate transporter (isoform 1,2,4)

GLUT: glucose transporter (due to minimal changes in expression, GLUTs are lumped together)
FFA: Free Fatty Acids
BHB: ß-hydroxybutyrate

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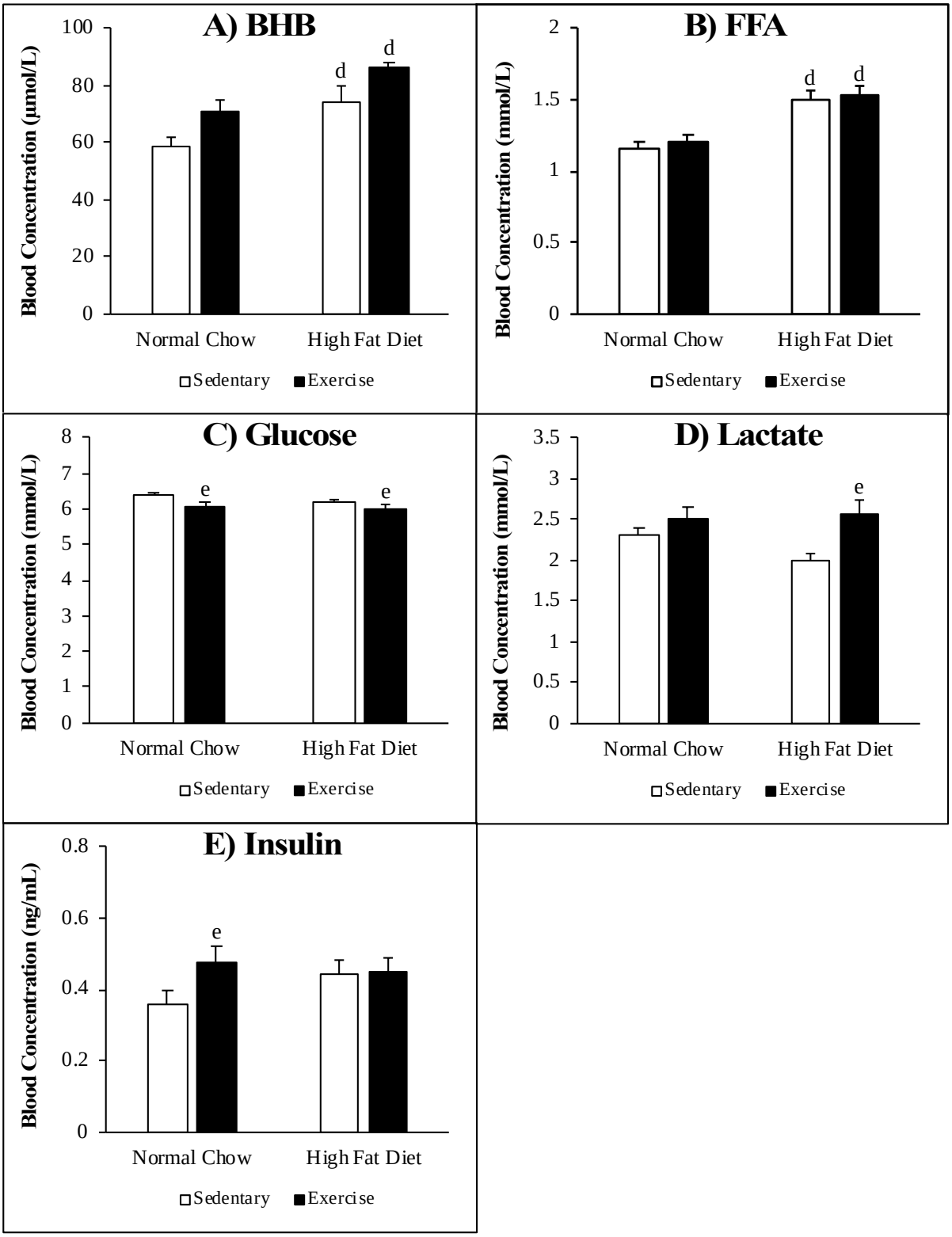
Figure 2 | Blood substrate concentration



*This figure presents the plasma concentration of **A)** β-hydroxybutyrate (BHB ; n=4-7 per group), **B)** free fatty acids (FFA ; n=8-10 per group), **C)** glucose (n=6-9 per group), **D)** lactate (n=9-10 per group) and **E)** insulin (n=9-10 per group) in sedentary or active rats fed with either normal chow or a high-fat diet. The letters above the bars represent significant ($p \leq 0.05$) differences between diet (d) or exercise (e) groups based on both the omnibus ANOVA and post-hoc pairwise comparisons.*

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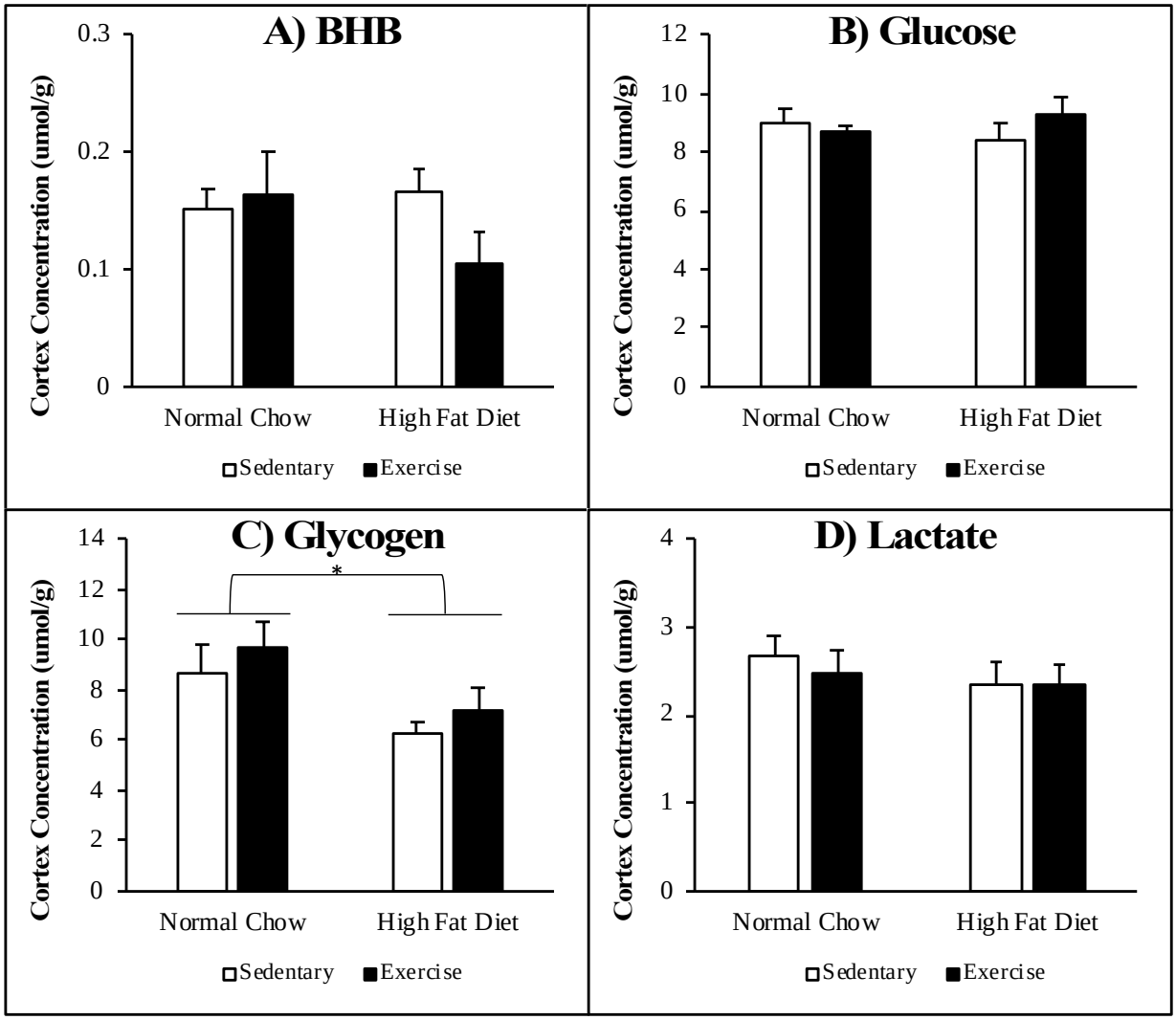
Figure 3 | Cortex substrate concentration



*Cortical concentration of **A)** β-hydroxybutyrate (BHB ; n=5-8 per group), **B)** glucose (n=7-9 per group), **C)** glycogen (n=7-10 per group) and **D)** lactate (n=7-10 per group) in sedentary or active rats fed with either normal chow or a high-fat diet. The only significant difference was found for glycogen expression between normal and high-fat diet.*

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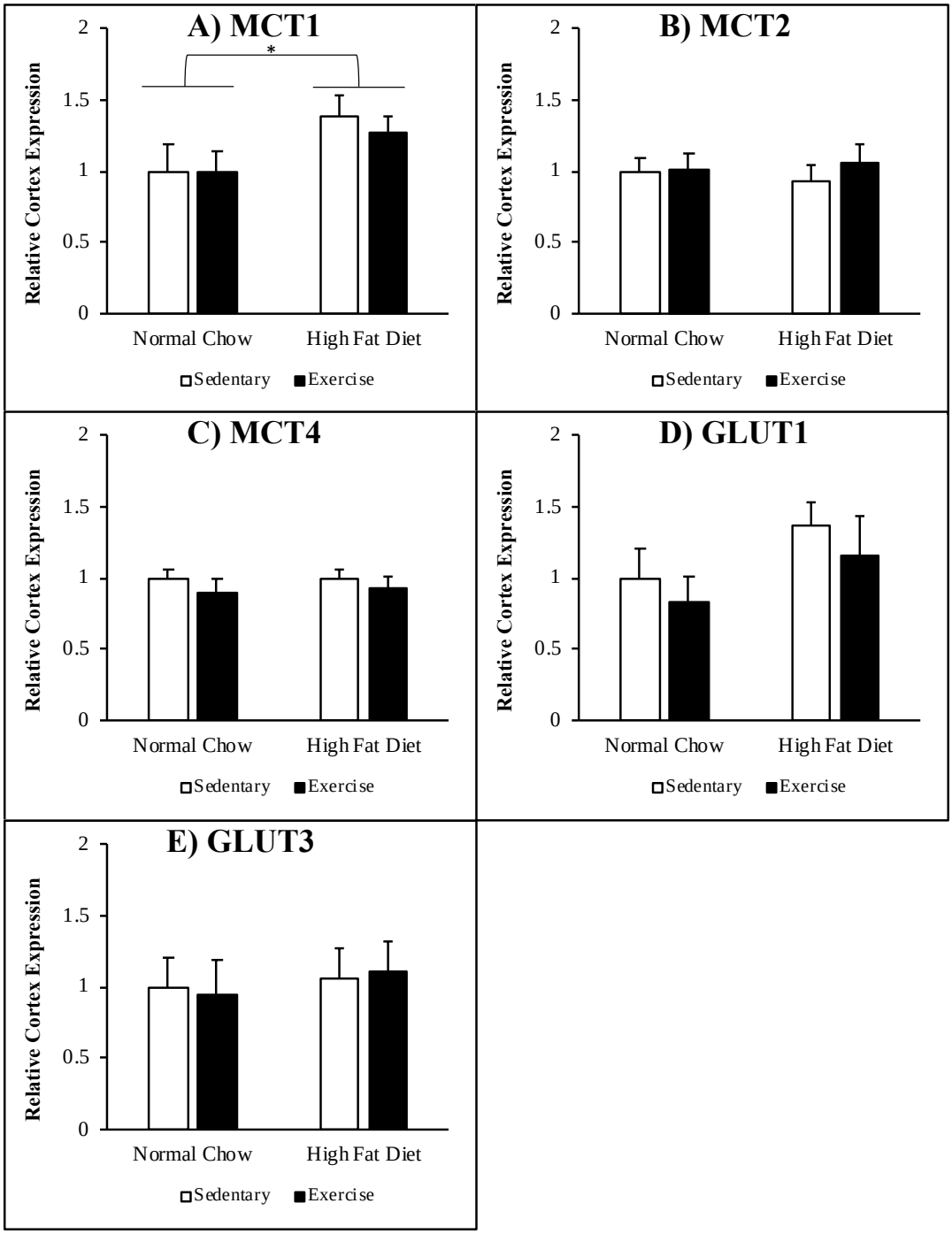
Figure 4 | Cortical transporter expression



*Relative cortex expression of **A)** monocarboxylate transporter 1 (MCT1 ; n=8 per group), **B)** monocarboxylate transporter 2 (MCT2 ; n=10 per group), **C)** monocarboxylate transporter 4 (MCT4 ; n=6-8 per group), **D)** glucose transporter 1 (GLUT1 ; n=6-7 per group) and **E)** glucose transporter 3 (GLUT3 ; n=9-10 per group) in sedentary or active rats fed with either normal chow or a high-fat diet. The results are expressed as a relative measure of the sedentary*

rats fed normal chow. The only significant difference was found for the MCT1 transporter expression between normal and high-fat diet.

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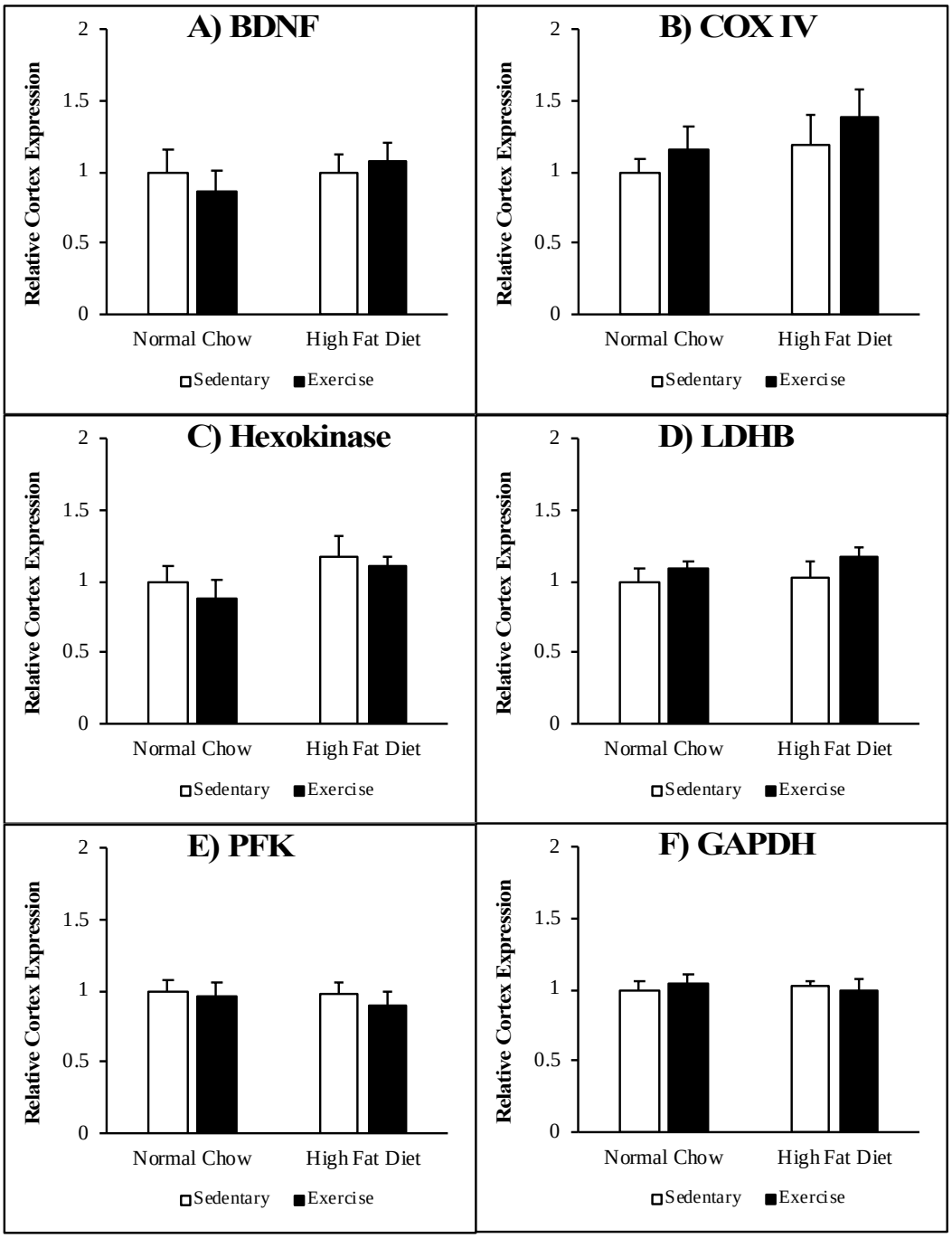
Figure 5 | Cortical protein and enzyme expression



*Relative cortical expression of **A)** brain-derived neurotrophic factor (BDNF ; n=8-9 per group), **B)** cytochrome c oxidase (COX IV ; n=7-9 per group), **C)** hexokinase (n=7-9 per group), **D)** lactate dehydrogenase B (LDHB ; n=8-10 per group), **E)** phosphofructokinase (PFK ; n=7-9 per group) and **F)** glyceraldehyde 3-phosphate dehydrogenase (GAPDH ; n=9-10 per group) in*

sedentary or active rats fed with either normal chow or a high-fat diet. The results are expressed as a relative measure of the sedentary rats fed normal chow. No significant differences were found.

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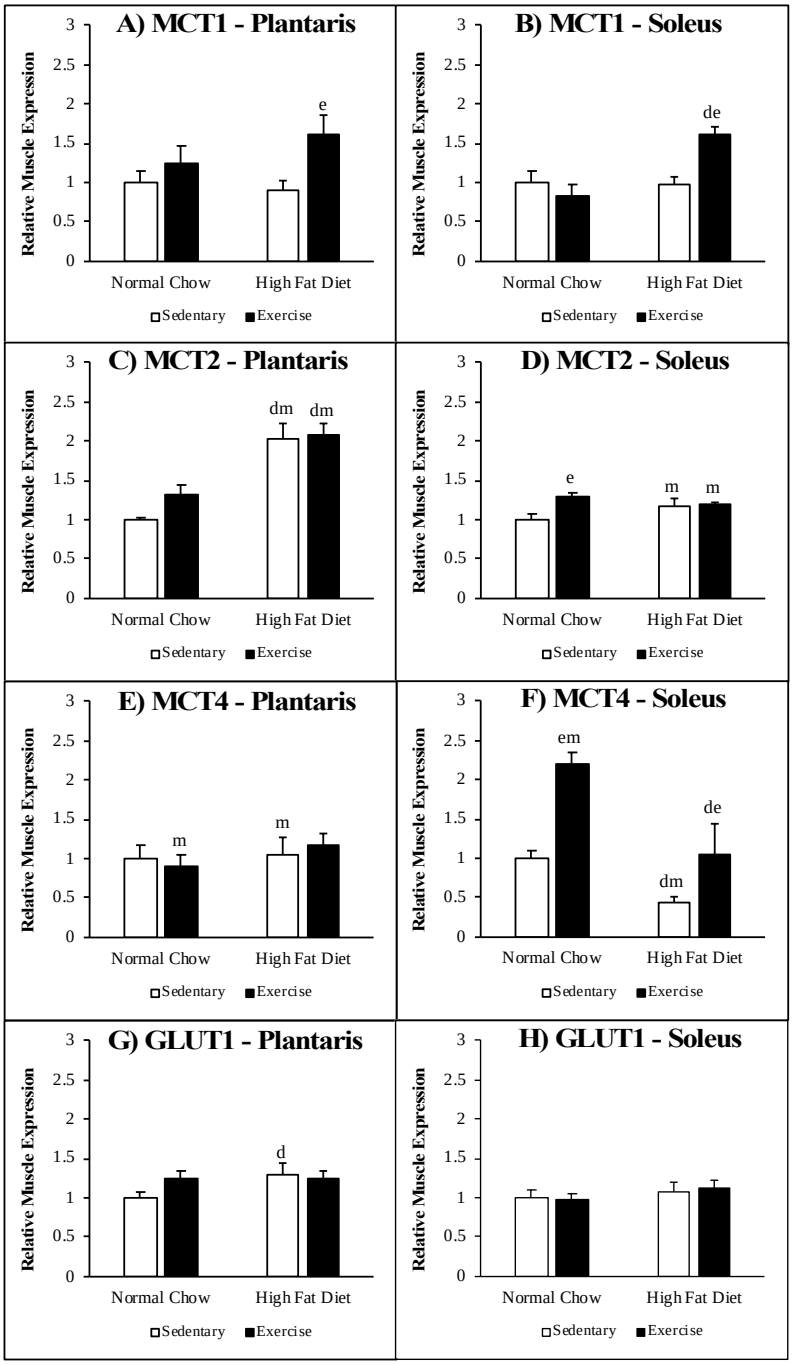
Figure 6 | Muscle transporter expression



*Relative plantaris or soleus muscle expression of **A-B)** monocarboxylate transporter 1 (MCT1 ; n=3-6 per group), **C-D)** monocarboxylate transporter 2 (MCT2 ; n=2-6 per group), **E-F)** monocarboxylate transporter 4 (MCT4 ; n=2-6 per group) and **G-H)** glucose transporter 1 (GLUT1 ; n=3-5 per group) in sedentary or active rats fed with either normal chow or a high-fat diet. The results are expressed at a relative measure of the sedentary rats fed normal chow. The*

letters above the bars represent significant ($p \leq 0.05$) differences between diet (d), exercise (e) or muscle type (m) groups.

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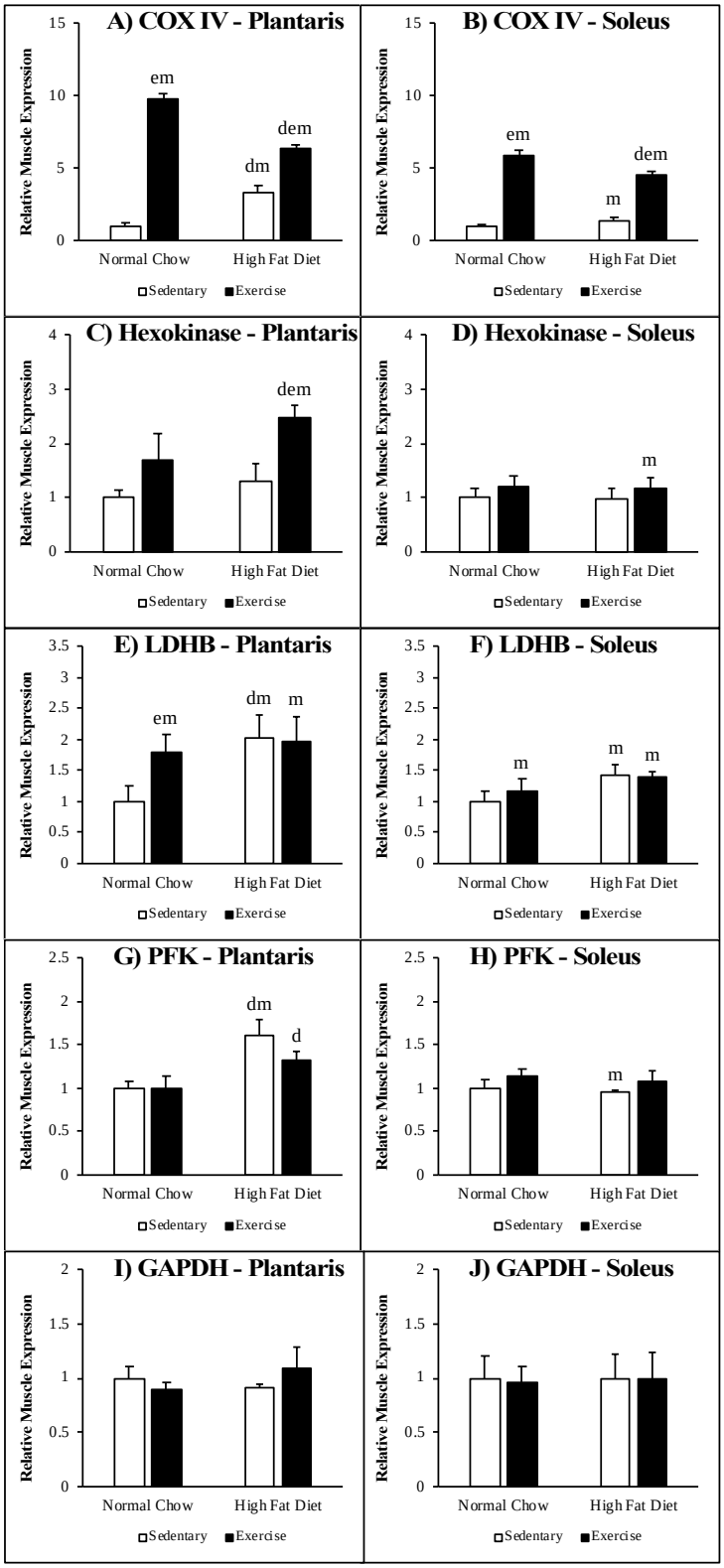
Figure 7 | Muscle protein and enzyme expression



*Relative plantaris or soleus muscle expression of **A-B)** cytochrome c oxidase (COXIV ; n=2-5 per group), **C-D)** hexokinase (n=4-6 per group), **E-F)** lactate dehydrogenase B (LDHB ; n=4-6 per group), **G-H)** phosphofructokinase (PFK ; n=3-6 per group) and **I-J)** glyceraldehyde 3-phosphate dehydrogenase (GAPDH ; n=2-5 per group) in sedentary or active rats fed with either normal chow or a high-fat diet. The results are expressed as a relative measure of the sedentary rats fed normal chow. The letters above the bars represent significant ($p \leq 0.05$) differences between diet (d), exercise (e) or muscle (m) groups.*

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Chapter IV: Voluntary Behavior and Training Conditions Modulate in vivo Extracellular Glucose and Lactate in the Mouse Primary Motor Cortex

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Abstract

Learning or performing new behaviors requires significant neuronal signaling and is metabolically demanding. The metabolic cost of performing a behavior is mitigated by exposure and practice which result in diminished signaling and metabolic requirements. We examined the impact of novel and habituated wheel running, as well as effortful behaviors on the modulation of extracellular glucose and lactate using biosensors inserted in the primary motor cortex of mice. We found that motor behaviors produce increases in extracellular lactate and decreases in extracellular glucose in the primary motor cortex. These effects were modulated by experience, novelty and intensity of the behavior. The increase in extracellular lactate appears to be strongly associated with novelty of a behavior as well as the difficulty of performing a behavior. Our observations are consistent with the view that a main function of aerobic glycolysis is not to fuel the current neuronal activity but to sustain new bio-infrastructure as learning changes neural networks, chiefly through the shuttling of glucose derived carbons into the pentose phosphate pathway for the biosynthesis of nucleotides.

Keywords: *primary motor cortex, running wheel, biosensor, glucose, lactate*

Introduction

Glucose is generally seen as the primary, if not sole, metabolic fuel for the brain (Berg et al., 2002). The importance of glucose in the central nervous system is supported by the cognitive deficits and loss of consciousness that accompany hypoglycemia and reduced glucose transport to the brain (Abdul Muneer et al., 2011; Mergenthaler et al., 2013; Patching, 2017) as seen in the GLUT1 deficiency syndrome: a genetic disorder characterized by a deficiency in the facilitative glucose transporter 1 (GLUT1) responsible for shuttling glucose across the blood-brain barrier (Heilig et al., 2003; Marin-Valencia et al., 2012; Yu et al., 2020). GLUT1 deficiency syndrome can be debilitating, but is rarely lethal (Di Vito et al., 2018). The early introduction of a ketogenic diet can prevent epilepsy and many of the deficits associated with GLUT1 deficiency syndrome (Brockmann, 2009; Friedman et al., 2006; Kass et al., 2016; Klepper, 2012; Klepper et al., 2004; Klepper & Leiendecker, 2013). These clinical effects indicate that the brain is capable of retaining its ability to use alternative fuels such as ketone bodies, and that it is not limited to the use of glucose to meet its high energetic needs.

The use of alternative energy sources and pathways for the production of cerebral energy has led to the controversial topic of lactate as a metabolic substrate for energy production. Briefly, the debate centers around the directional flux of lactate, reflecting its role either as a by-product or an energy source. The astrocyte-to-neuron lactate shuttle (ANLS) hypothesis stipulates that the sodium-dependent astrocytic uptake of surrounding glutamate following neuronal activation results in elevated glycolysis within astrocytes, as a response to the energy requirements of the astrocytic sodium-potassium ion pump. This elevated glycolysis leads to an accumulation of lactate, which is then shuttled to surrounding neurons and utilized within the oxidative phosphorylation pathway (selected reference Magistretti, 2006; Pellerin & Magistretti, 1994,

2012). The most direct evidence for this hypothesis comes from in vitro studies. On the other hand, the neuron-to-astrocyte lactate shuttle (NALS) hypothesis stipulates that increased neuronal activity upregulates glycolysis within the neurons, resulting in elevated intracellular lactate as a downstream product. This lactate is then shuttled to the extracellular space to be taken up and exported from the central nervous system via astrocytes and their intimate connection with the surrounding vasculature. Though this theory is more in line with the glucose-centric view of brain energetics, evidence for this hypothesis mostly relies on modeling approaches (selected references Díaz-García et al., 2017; Dienel, 2017; Simpson et al., 2007). A full review of the merits and pitfalls of each hypothesis is outside the scope of this work and have been extensively reviewed elsewhere (Dienel, 2012; Jolivet et al., 2010; Magistretti & Allaman, 2018; Mangia et al., 2011; Mason, 2017).

Meeting the Metabolic Needs of the Brain

The brains energetics needs are met primarily through the function and collaboration of the glycolytic and oxidative phosphorylation (OXPHOS) pathways. For the sake of brevity, we will consider both the tricarboxylic acid (TCA) cycle and mitochondrial respiration (electron transport chain) when discussing OXPHOS. OXPHOS is the primary ATP producing pathway and is considered to occur mostly in neurons (Dienel, 2019a; Magistretti & Allaman, 2015) as the majority of cerebral energy goes toward maintaining Na+/K+ membrane potential following signaling (Berg et al., 2002). Glycolysis, on the other hand, produces fewer ATP molecules but has the capacity to occur at a much faster rate (Bui & Thompson, 2006) and feeds the OXPHOS pathway with necessary intermediates, such a pyruvate.

We can consider the possibility that these intermediates are exclusively produced internally if the upregulation of glycolysis within neurons following activation is equivalent to, or surpasses,

the demand imposed by OXPHOS. This view is supported by work conducted by Hall (Hall et al., 2012) and Díaz-García (Díaz-García et al., 2017). Alternatively, we can consider that these intermediates may be provided by surrounding cells, in concert with neurons. Many brain cells, such as astrocytes (Bolaños et al., 2010; Dienel & Hertz, 2001), endothelial cells (Culic et al., 1997; Eelen et al., 2018; Yetkin-Arik et al., 2019), and activated microglial (Ghosh et al., 2018) are considered to be primarily glycolytic, or even provide storage in the form of glycogen, such as in astrocytes. Therefore, these cells could reasonably be expected to produce glycolytic products in excess of any OXPHOS needs, and as such, provide a portion of the required substrates for neuronal OXPHOS.

This cooperative organization and distribution of the hypothesized metabolic specialty across neurons and astrocytes resembles the muscular lactate shuttle proposed by Brooks (Brooks, 1985, 1998, 2009) in which fast-glycolytic-muscles supply slow-oxidative muscles with lactate. Of note, both the ANLS and the shuttle proposed by Brooks are developed to explain energy consumption and exchange in conditions of activation and not resting state. These phenomena are therefore expected to be observed during increased neuronal activity within the brain, as a compensatory and homeostatic mechanism countering the large energetic demand of re-polarizing membranes (Wang et al., 2017) and re-supplying of neurotransmitter vesicles in neurons (Rangaraju et al., 2014). Thus, in response to neuronal activity, OXPHOS and glycolysis are upregulated in both neurons and astrocytes (Ivanov et al., 2014), in addition to increased blood flow required to support enhanced rates of metabolism (Chaigneau et al., 2007; Iadecola, 2017; Mintun et al., 2001). Though both pathways are upregulated in both neurons and astrocytes following stimulation, OXPHOS appears to be preferentially upregulated in neurons whereas glycolysis appears to be upregulated globally within many cell types (Dienel, 2019a). There

remains controversy as to whether the upregulation of glycolysis occurs primarily in astrocytes or neurons. As this debate resides outside the scope of this article, we refer the readers to other papers (Díaz-García et al., 2017; Magistretti & Allaman, 2015).

Metabolite and Metabolism Fluctuations Following Activation

Fox and Raichle (subsequently confirmed by various researchers) first brought to light the local de-coupling between glucose and oxygen following neuronal activation (Fox & Raichle, 1986; Paulson et al., 2010; Watts et al., 2018). These extracellular measures completely disrupted our understanding of metabolism. Their observation of a fall in the stochiometric ratio between glucose and oxygen consumption during activation points to an important rise in non-oxidative glucose consumption. In other words, during activation, not all glucose entering the brain is fated to undergo OXPHOS, which begs the question: where does this additional glucose go? The non-oxidative consumption of glucose has three likely fates: (1) efflux out of the central nervous system in the form of lactate as a glycolytic end-product, resulting from an increase in glycolysis that surpasses the immediate capacity for OXPHOS, (2) entering the pentose phosphate pathway for antioxidant and nucleotide production or (3) glycogenesis, the non-oxygen requiring glycogen synthesis pathway.

One of the many tools used to elucidate the flux and fate of metabolites within the brain is the observation of extracellular fluctuation. A robust observation that has emerged from this research is the extracellular decrease in glucose and increase in lactate following and during neuronal activation. These findings have been replicated in different models and under varying conditions (Béland-Millar & Messier, 2018; Frahm et al., 1996; Kiyatkin & Lenoir, 2012; Madsen et al., 1995; McNay et al., 2006; McNay & Sherwin, 2004; Sampol et al., 2013). However, debate remains as to the particular origin of influx and efflux of these metabolites.

The homeostatic metabolic responses engaged following activation (neurotransmitter release), that is to say increased blood flow, decoupling of glucose-oxygen use increased OXPHOS and glycolysis, somehow result in increased extracellular lactate and decreased extracellular glucose. Interestingly, however, is that this trend in extracellular metabolite fluctuation is highly susceptible to training/practice and novelty. Habituation, in the form of repeated stimuli, such as in the novel object task (Bauch et al., 2017; Béland-Millar & Messier, 2018), or repeated behavior, such as wheel running (Fisher et al., 2016; Grill-Spector et al., 2006), has been shown to decrease both the signaling and consequently the metabolic requirements of the activated cortical area(s). Historically this has been attributed to the development and increased efficiency of the neural networks.

From the Where to the What of Energy Metabolism?

The ANLS and NALS hypotheses focus and differ primarily on where glucose and lactate are produced and consumed, with little regard to the function they serve beyond energy production. A complementary hypothesis has recently emerged, focusing on the function of these various products and metabolic pathways, suggesting diverging functions for energy derived from aerobic glycolysis (glycolysis terminating in lactate despite sufficient oxygen levels) and OXPHOS. This hypothesis proposes that much of the energy derived from aerobic glycolysis is primarily used to sustain the energy requirements for the physical building of neurites and dendritic spines accompanying new learning (Bauernfeind et al., 2014; Fellows et al., 1993; Goyal et al., 2014; Shannon et al., 2016; Zhou et al., 2019). This hypothesis is consistent with the observations of decreased extracellular lactate (Béland-Millar & Messier, 2018; Takita et al., 1992), signaling (Fisher et al., 2016; Grill-Spector et al., 2006) and altered metabolism (Gonzalez-Lima et al., 1989; Toga & Collins, 1981) following habituation.

The studies herein were conducted to validate a motor task in the study of brain metabolism. In the present experiment, we examined the effect of short- and long-term wheel-running training, as well as climbing and spinning, on changes in extracellular glucose and lactate using micro biosensors inserted in the primary motor cortex. We aimed to confirm if the tasks result in the expected metabolite fluctuations in other behavioral tasks and stimulation. Validating these new behavioral tasks was important to the authors as many common behavioral assessments are incompatible with tethered systems that measure metabolite fluctuations. In addition, many tasks rely on the assumption that the rodents are engaging with their environment and learning but these behaviors are limited in their ability to be observed and measurable. Motor behaviors that would elicit similar and robust responses are easier to assess, either in terms or speed or distance, as well as easier to determine when the mice are or are not engaging in said behavior.

Experimental Procedures

Animals

Thirty-six, 14- to 16-week old, male CD1 mice (Charles River Canada, St-Constant, QC, Canada) were individually housed and maintained on a reverse 12-h night/day cycle with lights on at 7 p.m. Mice had ad libitum access to standard chow (Teklad Global 18% Protein 2018, Teklad Lab Animal Diets, Envigo, Mississauga, Canada) and water, unless otherwise specified. All testing was conducted during the night phase of the cycle with the use of dim red lighting. All procedures in this study followed the Canadian Council on Animal Care guidelines and were approved by the University of Ottawa Animal Care Committee.

Surgery

Extracellular glucose and lactate measures were obtained with the use of biosensors inserted into the primary motor cortex. The surgical procedures used are identical to those detailed in previous work (Béland-Millar et al., 2017; Béland-Millar & Messier, 2018; Newman et al., 2011; Scavuzzo et al., 2021). Briefly, under anesthesia (Isoflurane, Fresenius Kabi Canada Ltd., Richmond Hill, ON, Canada) and analgesia (0.05 mg/kg subcutaneous buprenorphine hydrochloride, Reckitt Benckiser Healthcare, Hull, North Humberside, United Kingdom), a guide cannula (Pinnacle Technology, KS, United States) was placed above the right and left primary motor cortex (M1; from Bregma, ±1.8 mm lateral and +1.1 mm anterior) just below the skulls surface, according to the stereotaxic atlas of Franklin and Paxinos (Franklin & Paxinos, 2008). The cannulas were held in place with the use of 0.10-inch screws (Pinnacle Technology, KS, United States) and a UV-polymerized compound (UV Clear Fly Finish, Ashland, OR, United States).

Testing Procedures

After a week of recovery, two electrochemical electrodes (7004-Glucose-C and 7004-

Lactate-C; Pinnacle Technology Inc., Lawrence, KS, United States) were inserted into the guide cannula so that the 1 mm sensing cavity of the electrode protruded from the guide cannula and rested in the primary motor cortex. The characteristics of these commercially available electrodes have been presented previously (Béland-Millar et al., 2017; Kiyatkin et al., 2013; Kiyatkin & Wakabayashi, 2015; Wakabayashi & Kiyatkin, 2012; Wilson & Gifford, 2005). The mice were then placed in their testing cage with water and a free-standing running wheel. The mice were left to acclimate to their testing cage and running wheel for 2 h while the electrodes stabilized. Running events occurring during this 2-h stabilization were not included in the analysis. Otherwise, all running events occurring during the testing period (between 10 am and 5 pm) were include in the analysis. The running wheel was intentionally included during the acclimation so that the mice could habituate and start to learn how to use it. Preliminary experiments showed that initial interactions with the running wheel were clumsy and brief, however the 2-h calibration window was sufficient for the mice to start readily engaging with and using the running wheel for longer periods of time. In addition to these running bouts, the mice were tested for effortful behaviors (vertical hold and spin, see below) twice a day, for 2 days, with 20-min intervals between the behaviors and in a counterbalanced fashion. The length of the manipulation and interval was established during preliminary experiments. This chosen interval was sufficient for extracellular metabolite levels to return to baseline levels. See **Figure 1** for an illustration of the testing timeline.

Voluntary Wheel Running

The mice had unlimited access to a running wheel (Low-Profile Wireless Running Wheel for Mouse, Med Associates, VT, United States) during testing procedures. The number of rotations/minute was recorded by the Running Wheel Manager Data Acquisition Software through a wireless hub (Med Associates Inc., VT, United States) and was used to compare run speed where

appropriate.

Because mice were free to use the running wheels at any time, there were differences in running speed and duration within and across mice. In previous projects with the same procedure, we had observed that the amplitude of change in extracellular glucose and lactate in the motor cortex correlated with the length of time the mice ran in a single running bout. These observations and the uneven distribution of running bout lengths within each animal led us to analyze the running bouts across animals as a function duration in order to extract the effects of running bout length on extracellular glucose and lactate. Bout length was measured in seconds, representing the duration in which the mouse engaged in running behavior on the running wheel. Bout speed was measured by dividing the number of wheel rotations by the bout length. We grouped runs based on their duration: 30–60, 60–120,120–180, 180–240, and 240+ seconds. For example, the graph depicting 30-s events includes the running events from all animals that lasted at least 30 s but did not exceed 59 s.

We also evaluated running bouts as a function of experience. Short-term experience was evaluated with 24 h of prior access to the running wheel. These mice underwent the usual surgery and testing schedule. Mice who had long-term exposure to the running wheel were given access to it in their home cages, 4 weeks prior to testing. On the third week of their access, mice underwent surgery and were implanted with the guide cannulas (see above). They then underwent the week of recovery and their 4th week of access to the running wheel. Following the 4th week, they were brought in for testing and the electrodes were inserted. For both of these conditions (short-term and long-term experience), only runs of 240 s or longer were retained and analyzed due to larger and more consistent change in glucose and lactate extracellular levels produced. This allowed a better comparison with the less experienced mice (see section entitled *Results*).

Effortful Behaviors

Pilot data showed that the voluntary wheel running induced changes in extracellular glucose and lactate that were smaller in magnitude than those produced by electrical stimulation (Hu & Wilson, 1997b). Moreover, the magnitude of these changes in extracellular metabolite levels were inconsistent, both across and within mice, but appeared to be anecdotally related to the duration and speed of the running event. We therefore hypothesized that the differences we observed with running bouts were proportional to the effort mice exerted while running. We tested this hypothesis by adding what we estimated to be motor tasks that are more challenging than voluntary wheel running, with the prediction that greater extracellular glucose and lactate changes in the motor cortex would be observed. These behaviors were coined "effortful behaviors." Pilot experimentation showed that the two motor tasks that were compatible with the tethered electrode set-up and elicited strong glucose and lactate changes were the Vertical Hold and Spinning motor tasks described in the next section. These tasks used the propension of mice to re-orient themselves toward the top of a vertical plane and climb toward it. Typical tasks, such as the rotarod, could not be utilized with this tethered experimental setup as the fall risks pulling the electrodes out.

Vertical Hold. In this task, the mouse was placed on a vertical metal mesh (10 × 17 inches) with half-inch square holes. The mesh was held vertically and still as the mouse was free to explore for 2 min. This was initially used a control for the following behavior in which the mesh was moved. As the mice habituated to this task, they tended to orient and move toward the top of the mesh and spend more time there.

Spin. The mouse was placed on the same mesh as previously described but for this task, the mesh is slowly spun around in the vertical plane which led the mice to re-orient themselves toward the top of the mesh. A mouse was initially spun at the rate of 1 full rotation per 4 s. After

completing four full rotations, the mouse was then rotated counterclockwise at the same rate. Once another four rotations have been completed, the mouse was once again spun clockwise – however, this time a full rotation is completed every 2.5 s for another four rotations before completing the same conditions counter-clockwise. In summary, the rotations were done in sets of 4; the first two sets being slower, the last two being faster and switching between clockwise and counterclockwise rotations between each set of four rotations. This pattern was repeated for 2.5 min after which the mouse was returned to its cage.

Those particular speeds were chosen following pilot experimentation. At slower speeds, most mice were able to continue their normal pattern of behavior and exploration, seemingly undisturbed by the spinning and reaching the top of the mesh without issue. At higher speeds, most of the mice fell off of the mesh, unable to hold on. During the slower testing (4 s per full rotation), mice attempted to re-orient themselves to face the top of the mesh and a few even explored the mesh further, managing to reach the top. However, during the faster spinning (2.5 s per full rotation), the mice continued to attempt to reorient themselves toward the top of the mesh but were often unable to do so effectively. They remained in the middle of the mesh, with little exploration toward the top but did not typically fall.

Statistical Analysis and Relative Measures

All extracellular data were standardized by dividing the readings with their respective pre-event baseline[2] and multiplying the result by 100 to obtain relative percentage change from baseline. When necessary, the data was averaged every few seconds. Glucose and lactate were

[2] Baseline was calculated by averaging 10 s of data prior to each individual event. These 10 s of data were taken 20 s prior to the event to avoid any drift in electrode sensitivity that can occur over hours of testing, and to avoid artificial noise that may occur a few seconds prior to manipulating the mouse (e.g., any accidently jostling of the electrodes when the mouse is picked up).

analyzed separately with a between-within (split-plot) mixed ANOVA ($\alpha = 0.05$), and the data obtained for each injection was compared to a control injection group as well as baseline. Unless otherwise stated, the data met the respective assumptions of the analysis. If sphericity or homogeneity was violated, a Greenhouse–Geisser correction was applied. Due to the a priori decision and interest in differences across groups, post hoc pairwise comparison tests ($\alpha = 0.05$) were computed in order to compare the experimental groups to the saline controls as well as compare the experimental groups amongst themselves. All statistical analyses were performed using SPSS v. 23 (IBM).

To facilitate visualization of data, significant differences ($p < 0.05$) from baseline are illustrated directly on the graphs with the use of horizontal lines below the data. Significant differences from baseline for extracellular lactate are indicated via a thick line, whereas significant baseline differences for extracellular glucose are indicated via a thin line. Differences from respective control groups are indicated by a gray shaded rectangle around the respective data points. Where applicable, data typically considered to be "non-significant" (probability greater than 0.05) may still be discussed in order to provide a more complete understanding of the results[3].

[3] "Practices that reduce data analysis or scientific inference to mechanical "bright-line" rules (such as "$p < 0.05$") for justifying scientific claims or conclusions can lead to erroneous beliefs and poor decision making. A conclusion does not immediately become "true" on one side of the divide and "false" on the other" (Wasserstein & Lazar, 2016).

Results

Extracellular Lactate Rises and Extracellular Glucose Decreases in the Motor Cortex During Voluntary Wheel Running

Voluntary wheel running in mice who have not been previously exposed to the running wheel showed different patterns of extracellular metabolite fluctuations based on the length of their run. Wheel running behavior that occurred for at least 2 min (120 s) was associated with increased levels of cortical extracellular lactate as well as decreased levels of extracellular cortical glucose. In these longer runs (120 s or more), the rise in extracellular lactate occurred approximately 20 s following the start of the behavior (**Figure 2**). Interestingly, this rise in extracellular lactate did not appear to occur in the shortest run duration. In 30 s runs, neither extracellular glucose nor lactate significant varied from baseline. In 60 s runs, only lactate began to increase beyond baseline levels. When compared to the 30-s runs, extracellular lactate rose to significantly higher levels for the 120- ($p = 0.007$), 180- ($p = 0.002$), and 240-sec runs ($p < 0.001$). Despite lactate increasing within 20 s of the start of the running behavior in longer bouts, this increase in lactate was not observed in the 30 s runs, perhaps suggesting some sort of physiological preparation.

Decreases in cortical extracellular glucose from baseline appeared later than the extracellular rise in lactate, significantly dropping below baseline levels no earlier than 50-s or as late as 110-s following the start of the voluntary wheel running, as illustrated in **Figure 2**. When compared to the 30-s runs, the decrease in extracellular glucose was only significantly different for the 240-s runs ($p = 0.026$). This seems to indicate that compensatory mechanisms are capable of maintaining extracellular glucose levels at a steady state for some time. These mechanisms likely include increases in blood flow and the use of internal glycogen stores to compensate for

the heightened metabolic rate.

Effect of Experience: 4-Week Prior Access to a Running Wheel Attenuates the Rise in Extracellular Lactate and Hastens the Decrease in Extracellular Glucose

To facilitate the investigation of the effect of training on extracellular metabolite fluctuations, the longer (240 s) bouts of running were used as they most clearly demonstrated the diverging extracellular glucose and lactate patterns. When mice were given free-access to a running wheel 4 weeks prior to testing, the exercise-induced increase in extracellular lactate was attenuated ($p < 0.001$), while the decrease in extracellular glucose occurred earlier ($p < 0.034$) when compared to naïve mice (see **Figures 2E,F**). **Figure 2F** shows that the decrease in extracellular glucose occurred approximately 40 s earlier (thin line indicating significant different from baseline), and that this decrease was more pronounced (shaded gray rectangle indicate significant difference form control). Extracellular lactate almost never varied from baseline and was significantly different from the naïve counterpart for the duration of the running behavior.

Effortful Behaviors Have a Larger Impact on Extracellular Lactate Than Voluntary Wheel Running

When compared to 2 min (120 s) of wheel running (**Figure 2C**), both the vertical hold and the spinning conditions (**Figure 3**) significantly increased extracellular lactate ($p = 0.007$ and $p < 0.001$, respectively), with no significant impact on extracellular glucose. In fact, extracellular lactate during effort behaviors was significantly higher than the levels observed during the 120 s wheel running for almost the entire duration of the testing (shaded rectangles in **Figure 3**). However, neither extracellular glucose nor lactate significantly differed between the two effortful conditions.

Effect of Novelty: Gained Ability vs. Habituation

To distinguish between the effect of novelty and improved ability due to prior exposure, we also compared extracellular glucose and lactate on the first and second day of testing for both running wheel (240 s bouts) and spinning. Day 1 of testing represented novel manipulations (spinning) and first access to the running wheel whereas day 2 represented either two prior occurrences of the spinning task or a 24-h prior exposure to the running wheel.

When compared to the first day of wheel running, running behavior in mice who were exposed to the running wheel for 24-h trended toward increased extracellular lactate beyond the levels observed on day 1 ($p = 0.057$). This was particularly evident during the second half of the running bout (**Figures 4A,B**). Comparing the speed (number of wheel rotations/second) of these 240 s runs revealed that mice ran faster on the second day ($p = 0.012$; Day 1, Mean = 0.66, SEM = 0.018; Day 2, Mean = 1.07, SEM = 0.043). This suggests that, between the first and second day of exposure, the mice gained proficiency with the running wheel, and were able to exert more effort. The second day of testing may more accurately reflect normal brain activation due to exercise rather than the novelty/habituation of day 1.

In contrast, comparing the spinning events on the first and second day of testing (**Figures 4C,D**) revealed an attenuation in extracellular lactate ($p = 0.017$) and a trend toward further decreases in extracellular glucose ($p = 0.055$). These results are similar to those observed following the 4 weeks of habituation to the running wheel which suggests that the mice either quickly habituate to this manipulation or that activation on day 1 is more representative of novelty than motor activation as a result of the behavior in which they are engaged. Altogether, these results suggest that novelty and experience have differential short-term effects on extracellular lactate fluctuation.

In summary, long-term exposure and training with a running wheel attenuates the rise in extracellular lactate while simultaneously hastening the decrease in extracellular glucose. Short-term (24 h) exposure and training with a running wheel increases extracellular lactate: This could likely be the result of an improved ability to use the running wheel, subsequently allowing more effort to be exerted. Lastly, results from the more passive behavior (being spun rather than choosing to run) indicates that the novelty induced increase in extracellular lactate is short lived. This latter observation is corroborated with our previous findings with the novel object task, where fluctuations in the primary visual cortex were quickly attenuated upon a second presentation of the object (Béland-Millar & Messier, 2018).

Discussion

Technical Limitations of the Biosensors

When interpreting the current results, it is important to consider the technical limitations of the methodology used. Though extremely sensitive with high temporal resolution, the inserted biosensors measure relative changes in metabolite concentrations within the extracellular space. Techniques, such as microdialysis, can provide more accurate measurements of absolute values, however they lack the temporal resolution opted for in these experiments. Once inserted, the electrodes undergo gradual "bio-fouling" as various biological materials attach themselves to the electrodes. This results in an extremely small, gradual but constant decline in the signal. This signal decreases progressively in hours rather than minutes and do not have a direct impact on our acute extracellular sampling during manipulations, as has been previously demonstrated (Béland-Millar et al., 2017). However, this gradual decline and the slight variations in electrode manufacturing, results in variability of the absolute values measured, both across and within (from one day to another) mice. In order to be able to compare the values both within and across mice, the observed fluctuations are more accurate as measures relative to pre-event baselines. The data was transformed from the raw nA values to relative changes in extracellular fluctuations, as described in the methods, to allow for comparison within and across mice.

In addition to the slight decline in sensitivity, electrode insertion damages tissues resulting acute and chronic immunological responses (Kozai et al., 2015). In the short term (minutes to hours) electrode insertion may impact regional blood flow through the creation of vascular occlusion or effects on the BBB. Within hours, glial activation is induced which can, over the course of days, encapsulate the electrode. This encapsulation eventually results in the primary measurement of reactive tissue or may cause a slight delay in measuring extracellular metabolites

as they cross this additional barrier. These impacts are mediated by insertion of the electrode immediately prior to the testing, rather than at the time of surgery, and by limiting our continuous measurements to 2 days. In lab testing demonstrated that extracellular measurements were robust and replicable across this timeline, but occasionally began to differ on the third day of continuous sampling. Unaveraged data also reveal changes within a second of manipulation or behavior, suggesting that the impacts of bio-fouling on the sampling delay is likely less than the sampling speed of the electrodes (1 s) (**Figure 5**).

Since the electrodes sample the extracellular space, they measure net metabolite fluctuations in the extracellular space of the motor cortex without providing information on the direction of the flux. Therefore, the glucose and lactate measures obtained with the electrodes reflect the net result of blood-brain barrier (endothelial) transport both in and out of the brain, as well as astrocytic and neuronal influx and efflux of these same metabolites within the brain parenchyma. For example, an increase in extracellular lactate could represent either increased transport of lactate across the blood-brain barrier into the extracellular space, increased neuronal glycolysis resulting in lactate export or lactate shuttling from astrocytes into the extracellular space, among other possibilities. Our strategy in the present experiment and previous ones (Béland-Millar et al., 2017; Béland-Millar & Messier, 2018) has been to measure extracellular glucose and lactate in many different situations together with peripheral metabolic challenges to derive a number of hypotheses on the movement of glucose and lactate under various conditions of brain activity and metabolic status. Our hypotheses also rely on previous experiments that have examined glucose and lactate metabolism either in vivo or more often in cell cultures and brain slices preparations.

Electrical vs. Motor Stimulation

In the present study and our previous ones, we examined the effect of in vivo behavioral or

sensory stimulation on brain extracellular glucose and lactate. There is evidence that in vivo wheel running is sufficient to induce neuronal activity in the primary motor cortex, and that this neuronal activity fluctuates with the speed of the wheel running (Fisher et al., 2016). Our results confirmed a comparable impact of voluntary wheel running on extracellular metabolite fluctuations as those obtained either by discrete stimulation (Hu & Wilson, 1997a) or alternative behavioral testing (McNay et al., 2006).

Interestingly, if we compare our behavioral results to those of discrete electrical stimulations, such as the work done by Hu and Wilson, we observe a similar pattern. Hu and Wilson measured extracellular glucose, lactate and oxygen in the dentate gyrus of anesthetized rats following performant path stimulation (Hu & Wilson, 1997a). Two patterns of response were observed by Hu and Wilson: an immediate short-term pattern and a longer term one over the course of repeated stimulations. The immediate pattern was a sharp 10% decrease in glucose and oxygen, and a 25–35% decrease in lactate followed by a quick rebound that overshot briefly the pre-stimulation values. The overlaid long-term pattern was a progressive increase in extracellular lactate (up to 200% baseline), a moderate decrease in extracellular glucose (80% of baseline) and a very small (5%) increase in oxygen. When the stimulations ended, glucose and oxygen returned to baseline within 5 min, while lactate was still at 20% above baseline 20 min later. In a previous dataset, we observed that the initiation of any movement was associated with a brief (3 s) 13% decrease in extracellular glucose and a 22% decrease in lactate in the motor cortex, which are comparable in size but much briefer than those observed following electrical stimulation. In the present experiment, sustained running produced a similar de-coupling in extracellular glucose and lactate. However, the increase in lactate and decrease in glucose observed here were much smaller than those observed with electrical stimulation. The results suggest that neuronal activation

induces two different processes. The first process, occurring within seconds of stimulation (Hu & Wilson, 1997a) as well as subsequently to motor initiation (prior data set), appears to be a rapid and short-term depletion of glucose and lactate from the extracellular space followed by a longer-term and progressive accumulation of lactate accompanied by a progressive decrease in extracellular glucose that continues as long as the behaviors are occurring. At face value, these results suggest that immediately after brain stimulation, extracellular glucose and oxygen are depleted. This depletion is likely the result of increased oxidative phosphorylation of glucose. As discussed in the introduction, neuronal activation is an energetically costly process, and most energy in the brain is provided through oxidative phosphorylation of glucose. The short-term decrease in extracellular lactate that occurs simultaneously suggests that lactate is also used as metabolic fuel during this early activation either by neurons or other cells. Running, and general motor behavior, would increase circulating lactate, and given that lactate transport is dependent upon a concentration gradient, decreased extracellular suggests lactate is entering brain cells not exiting the brain. However, our previous experiment examining the impact of visual stimulation on lactate and glucose in the visual cortex found a similar lactate increase in the visual cortex. Therefore, this increase is unlikely to be due exclusively to lactate released by muscle activity entering subsequently the brain. Extracellular lactate produced from glycogen breakdown may be a source of metabolic support, enabling appropriate brain function (Brown & Ransom, 2015; Matsui et al., 2017; Suh et al., 2007) but recent evidence suggests that glycogen may support astrocytic, rather than neuronal energy needs, thus sparing substrates (e.g., lactate, glucose) for neuronal use (Dienel & Cruz, 2015; Dienel & Rothman, 2020; DiNuzzo et al., 2010b).

The secondary process, represented by the longer-term accumulation of lactate in the extracellular space and the continued decrease in extracellular glucose, suggests elevated glucose

consumption. Elevated glucose consumption, accompanied with elevated extracellular lactate, points to a significant portion of the metabolized glucose (or glycogen) being converted to lactate and shuttled out of the cell. Importantly, the type of cell producing this lactate has been extensively debated (Díaz-García & Yellen, 2019; Fernández-Moncada et al., 2018; Yellen, 2018), and it is unclear if this lactate is shuttled out of the cell as waste product or shuttled out of the astrocytes as a potential energy source for neurons.

The hypothesis that OXPHOS and aerobic glycolysis both occur during neuronal stimulation is consistent with the well-described decoupling between glucose utilization and oxygen consumption measured by Positron Emission Tomography during brain activation (Fox & Raichle, 1986). If the increase of extracellular lactate is directly related to neuronal oxidative glycolysis, then the examination of the time course of extracellular glucose decrease and extracellular lactate may provide additional information. Although brain electrical stimulation is a useful tool to examine neuronal activation, it does not reproduce the varied pattern of neuronal activation produced by in vivo non-anesthetized sensory stimulation or motor activity. In the next section we examine the temporal pattern of extracellular glucose and lactate produced in the motor cortex by different behaviors.

Progressive De-Coupling of Extracellular Glucose and Lactate During Motor Behavior

We showed that as wheel running continues, extracellular lactate continues to rise, and glucose continues to decrease. We previously demonstrated that this same de-coupling pattern of occurs in the visual cortex of freely moving mice when visually stimulated (Béland-Millar & Messier, 2018). Our observations are also compatible with the reduction in extracellular glucose (McNay et al., 2000; McNay & Gold, 2001; Newman et al., 2011) and increase in extracellular lactate (McNay et al., 2006; Newman et al., 2011) levels in the hippocampus during spatial

working memory testing.

As we have found, the magnitude of the decrease appears to be proportional to the cognitive demands of the tasks (McNay et al., 2000, 2001; McNay & Gold, 2001). Interestingly, others have found that the decrease in extracellular glucose during cognitive testing is magnified in older animals and is accompanied by impaired performance (McNay & Gold, 2001). However, the fact that this performance impairment can be alleviated by an ip or intra-cranial injection of glucose, suggests that the large decrease in extracellular glucose in older animals may be due to a mismatch between the neuronal availability and demand of glucose. Together these results support the importance of glucose availability and is consistent with the observation that new training produces an increase in GLUT1 glucose transporter expression in the hippocampus for memory training (Choeiri et al., 2005) and in the sensorimotor, motor cortex and cerebellum in mice given 48-h access to a running wheel (Allen & Messier, 2013). In that last experiment, running wheel access also increased the expression of beta-tubulin III, a neuron-specific microtubule, which suggested that the increased GLUT1 expression was also associated with neuronal remodeling (Allen & Messier, 2013).

A second observation is the temporal de-coupling between lactate and glucose. When the animal starts running, the decrease in extracellular glucose appears to lag from 10 to 90 s behind the increase in extracellular lactate. We propose that this delay represents the cells initial reliance on astrocytic glycogen stores to meet energetic demands following activation (Brunet et al., 2010). This buffer provided by the internal glycogen stores limits the need for cells to use intracellular glucose pools (Dienel, 2019a), sparing extracellular glucose. Subsequently, when the initial local supply of glycogen is reduced, intracellular glucose pools are more heavily relied upon, thereby reducing extracellular glucose. The increased reliance on astrocytic glycogen may result in

elevated extracellular lactate, as suggested by Dienel (Dienel, 2019b). There is, however, additional controversy as to whether glycogen is converted to lactate to support surrounding neurons or whether glycogen is used glycolytically within astrocytes to spare extracellular glucose for surrounding neurons (Brown & Ransom, 2015; Duran et al., 2019; Matsui et al., 2017; Newman et al., 2011; Rich et al., 2019; Suh et al., 2007; Suzuki et al., 2011; Swanson, 2020). Regardless, increases in extracellular lactate are likely at least partially the result of glycogenolysis. Combined with the glycogen stores, it is also likely that glucose uptake from blood may be able to transiently compensate for the increased demand, thereby resulting in relatively stable extracellular glucose vales.

This work extends previous research and demonstrates that neuronal activation leads to a quick increase in extracellular lactate and more subtle decrease in extracellular glucose within the primary motor cortex of free moving and awake mice engaging in voluntary motor behavior. Additionally, it demonstrates, in awake animals, the additive effect of continuous activation.

Effort Magnifies Extracellular Metabolite Fluctuations

Previous research has demonstrated that increasing task difficulty increases neuronal demand, which in turn leads to increased cortical activation (Adler et al., 2001; Bokde et al., 2005; Chen et al., 2008; Gevins et al., 1997; Siciliano et al., 2017; Winstein et al., 1997) as well as increased cerebral blood flow (Winstein et al., 1997), oxygen (Causse et al., 2017), glucose uptake (Díaz-García et al., 2017; Lundgaard et al., 2015; Matsunami et al., 1989; Picard et al., 2013; Sampol et al., 2013) and increased extracellular lactate (Sampol et al., 2013; Urrila et al., 2003). We observed an amplification of the metabolite de-coupling during effortful behaviors, when compared to the running behavior. If we consider decreases in extracellular glucose and increases in extracellular lactate as indicators of increased metabolism, effortful behaviors appear to amplify

metabolic cortical demand.

There was no additive effect of the spinning behavior on glucose and lactate fluctuations, beyond what was observed in the vertical hold, suggesting that both the spinning and vertical hold conditions similarly engaged the primary motor cortex or that the differences in neural activation across these two conditions did not lie within the primary motor cortex. In muscle, the rate of glycogenolysis can increase with workload (Hearris et al., 2018). Therefore, amplified increases in extracellular lactate produced by effortful behaviors may be the result of increased aerobic glycolysis or elevated rates of glycogenolysis, most likely some combination of the two. Altogether, we propose that the vertical hold paradigm is an appropriate measure of behavior induced neural activation and a useful paradigm for future investigation of awake, freely moving mice, with the advantage over the spinning paradigm that it less likely to result in damaging sensitive equipment.

Novelty and Experience Differentially Impact Extracellular Fluctuations

In contrast to increases in neuronal activation during challenging tasks, the repetition of a novel or challenging tasks reduces neural activation/firing (Apšvalka et al., 2015; Gevins et al., 1997; Maccotta & Buckner, 2004; Raichle et al., 1994; Satpute et al., 2016; van Mulukom et al., 2013; Yamaguchi et al., 2004) as well as cerebral blood flow (Apšvalka et al., 2015; Landau et al., 2004; Tsujimoto et al., 2000) and metabolic activity (Béland-Millar & Messier, 2018; Haier et al., 1992; Picard et al., 2013). Specifically, Fisher et al. (Fisher et al., 2016) demonstrated that stereotypic running, following 2 weeks of access to a running wheel, reduced neuronal firing. Zhou et al. (Zhou et al., 2019) demonstrated that motor learning occurred in two phases. Initial learning and rapid changes in neural activity occurred swiftly after the new behavior, lasting anywhere from a few minutes to a few hours, during which motor errors decreased. Subsequently, substantial

cortical reorganization occurred within several weeks of training and was associated with more efficient control of movement and behavior. This bi-phasic response appears to be observed in our results. Long term training/habituation (4 weeks) to a running wheel abolishes the rise in extracellular lactate, suggesting decreased metabolic activity as a result of neuronal efficiency. Short-term habituation (24 h) had the opposite effect, resulting in extracellular lactate levels that surpassed the levels observed on day 1. This contrasted pattern of metabolic activation is similar to what was observed by Sif et al. (Sif et al., 1991) during training of a new task. Incomplete training leads to high level of metabolic activity lasting 24 h in relevant brain regions, while mastered training does not (Sif et al., 1991). Similarly, repeated training sessions produce lasting metabolic increases during the first days of training but when the task is mastered, the metabolic activation produced by the trained behavior is limited to less than 5 min (Bontempi et al., 1996).

This bi-phasic response appears to be restricted to behaviors that have a longer learning curve or require active, rather than passive, participation in learning the behavior. In the novel object task, a relatively passive paradigm, when mice are exposed to a previously novel stimulus for the second time the fluctuations in extracellular metabolites that were observed during the initial presentation are completely absent (Béland-Millar & Messier, 2018). We observed this same trend for behaviors that are not initiated, or controlled, by the mouse (effortful behaviors). During the first exposure (day 1) to the effortful behaviors, the expected de-coupling pattern between extracellular glucose and lactate is observed. This response is significantly altered on day 2. This abolished response is not only different than the trends observed with 24-h of wheel-running exposure (increase in extracellular lactate on day 2) but is similar to the results observed following 4 weeks of exposure to the running wheel.

Beyond novelty and habituation, stress and fitness may also play a role in the observed

fluctuations. For the most part, though not controlled, fitness was assumed to be similar given the identical housing and manipulation of the mice. A notable exception, of course, is the group of mice receiving 4-weeks of prior access to the running wheels. On average, these mice ran 14 times further in a day than the mice who just received access to their running wheel, and ran faster (see section entitled *Results*). Alongside the neuronal restructuring that occurs with learning and practice, fitness may have played a role in limiting the rise in extracellular lactate during wheel running with decreased peripheral (blood) lactate production (Favier et al., 1986). Though stress is unlikely to play a significant role in the wheel running data given the habituation and voluntary nature of the behavior, it may have played a role in the testing of the effortful behaviors given the manipulation required to place the mouse on the grid. The impact of stress is challenging to untangle from the impact of the behavior given that both motor behavior and stress are known to increase cerebral lactate (Favier et al., 1986). However, in rats, stressors such as tail pinch and social interactions have resulted in decreased extracellular glucose (Kiyatkin & Lenoir, 2012), which is not observed here.

Link to Cortical Metabolism

Beyond the ANLS and NALS, one hypothesis focuses on another functional role of lactate production through aerobic glycolysis. It has been suggested that aerobic glycolysis, that is to say glycolysis terminating in the production of lactate rather than pyruvate even in the presence of sufficient oxygen levels, is used to fuel biosynthesis and development and or plasticity of neural networks to support “learning” (Bauernfeind et al., 2014; Fellows et al., 1993; Goyal et al., 2014; Shannon et al., 2016). Intracellular lactate production (or import) alters the redox ratio by increasing intracellular NADH through the equilibrative LDH enzyme. This altered intracellular redox ratio decreases the production of glycolytic intermediates destined for oxidative

phosphorylation through negative feedback loops due to the accumulation of various downstream products. This accumulation, in turn, increases the shuttling of the glycolysis/glucose derived carbon through the pentose phosphate pathway. Processing of glucose derived carbon through the pentose phosphate pathway results in the synthesis of various proteins, lipids and nucleotides, necessary for the biosynthesis and development of neural network infrastructure (Raichle, 2015). This is further supported by evidence indicating the need for glycolysis and lactate during memory formation (Harris et al., 2019; Steinman et al., 2016; Suzuki et al., 2011).

The observations presented here are in agreement with this hypothesis, in so much that aerobic glycolysis, and therefore extracellular lactate, would be increased when the behavior is novel as well as when the behavior is being learned (i.e., learning to use the running wheel); and as the behavior becomes "learned" the necessary neural pathways have been built or made more efficient, thus reducing neuronal activation, consequently (according to this hypothesis) diminishing the need for aerobic glycolysis, and therefore extracellular lactate, to support the development of new neural network infrastructure. Indeed, with training, such as in wheel running, vascular GLUT1 expression is increased as well as indicators of neuronal plasticity in the motor cortex and cerebellum (Allen & Messier, 2013). This suggests a strong link between metabolic and cortical reorganization as well as a possible shift to more OXPHOS-oriented metabolism following the development of neural networks and infrastructure. At this juncture, we consider this to be the most likely hypothesis to explain the decrease in extracellular lactate following habituation. However, we cannot discount other hypotheses (e.g., ANLS, NALS) or possibilities such as improved removal or neuronal consumption of lactate, either in neurons or astrocytes. Evidence of increase glucose transporter expression following exercise training could indicate improved glucose oxidation, limiting the need for transfer of lactate, either from glycogen or

aerobic glycolysis.

Although our results are not able to distinguish and determine which hypothesis is correct (ANLS, NALS or aerobic glycolysis for biosynthesis), our observed data fit well with the possibility that aerobic glycolysis occurs to fuel biosynthesis. The large increases in extracellular lactate observed during the learning of the wheel running or the first day of spinning would indicate large levels of aerobic glycolysis as the mice engage in and learn new behaviors. Subsequently, the abolished rise in extracellular lactate following training and familiarity would indicate absent, or low levels, of aerobic glycolysis following exposure to these behaviors. Interestingly, the hastened decrease in extracellular glucose following chronic exposure to the running wheel may indicate that extracellular lactate, either blood-borne or as a result of aerobic glycolysis, may have, in part, sustained neuronal metabolic activity.

Conclusion

This work has extended the observation that neuronal activity results in increases in extracellular lactate and decreases in extracellular glucose within the primary motor cortex. This work has also identified and compared observable voluntary and imposed behaviors as a method of motor cortex activation for use with these sensors. It has demonstrated how these fluctuations can vary with experience, novelty and intensity of motor behaviors. Although our observations do not directly demonstrate the hypothesis, they are nonetheless compatible with the notion that a main function of aerobic glycolysis is not to fuel the current neuronal activity but to sustain new bio-infrastructure as neural networks develop, chiefly through the shuttling of glucose derived carbons into the pentose phosphate pathway for the biosynthesis of nucleotides.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by Animal Care Committee University of Ottawa.

Author Contributions

AB-M: conceptualization, data curation, formal analysis, writing – original draft, and writing – review and editing. CM: formal analysis, funding acquisition, supervision, writing – original draft, and writing – review and editing. Both authors: contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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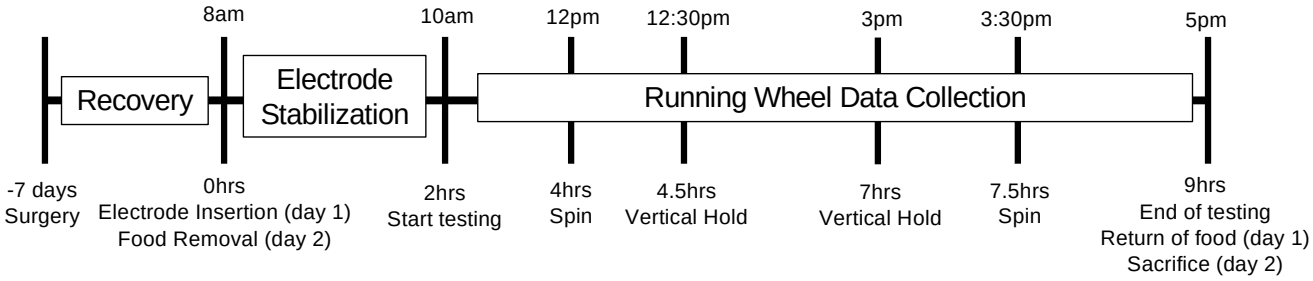
Figure 1 | Illustration of the testing timeline



This figure illustrates the testing timeline. The mice were acclimated to the facility for 3 weeks prior to testing. Seven days prior to testing, the mice underwent surgery for cannula implantation. On the morning of the testing (day 1), the mice were lightly anesthetized, and the electrodes were inserted following calibration. The mice were then placed into their testing cages with access to water, bedding and a running wheel. The electrodes were left to stabilize for 2 hours while the mice acclimated to their testing cage and familiarized themselves with the running wheel. Following this acclimation and stabilisation period, spontaneous and voluntary running events were recorded for the next 7 hours. Twice during this testing window, the mice were also subjected to our effortful behaviors (spin and vertical hold) in a counterbalanced order. Once the testing period concluded, food was returned to the cage. Once the following day, testing procedures were identical with the exception of food removal in the morning (replacing the electrode insertion), and calibration of the electrodes at the end of the day, after which the mice were euthanised. The group of mice receiving access to a running wheel 4 weeks prior to testing is the only variation to this timeline. Surgery for this group of mice still occurred 1 week prior to testing (3 weeks into their exposure to the running wheel).

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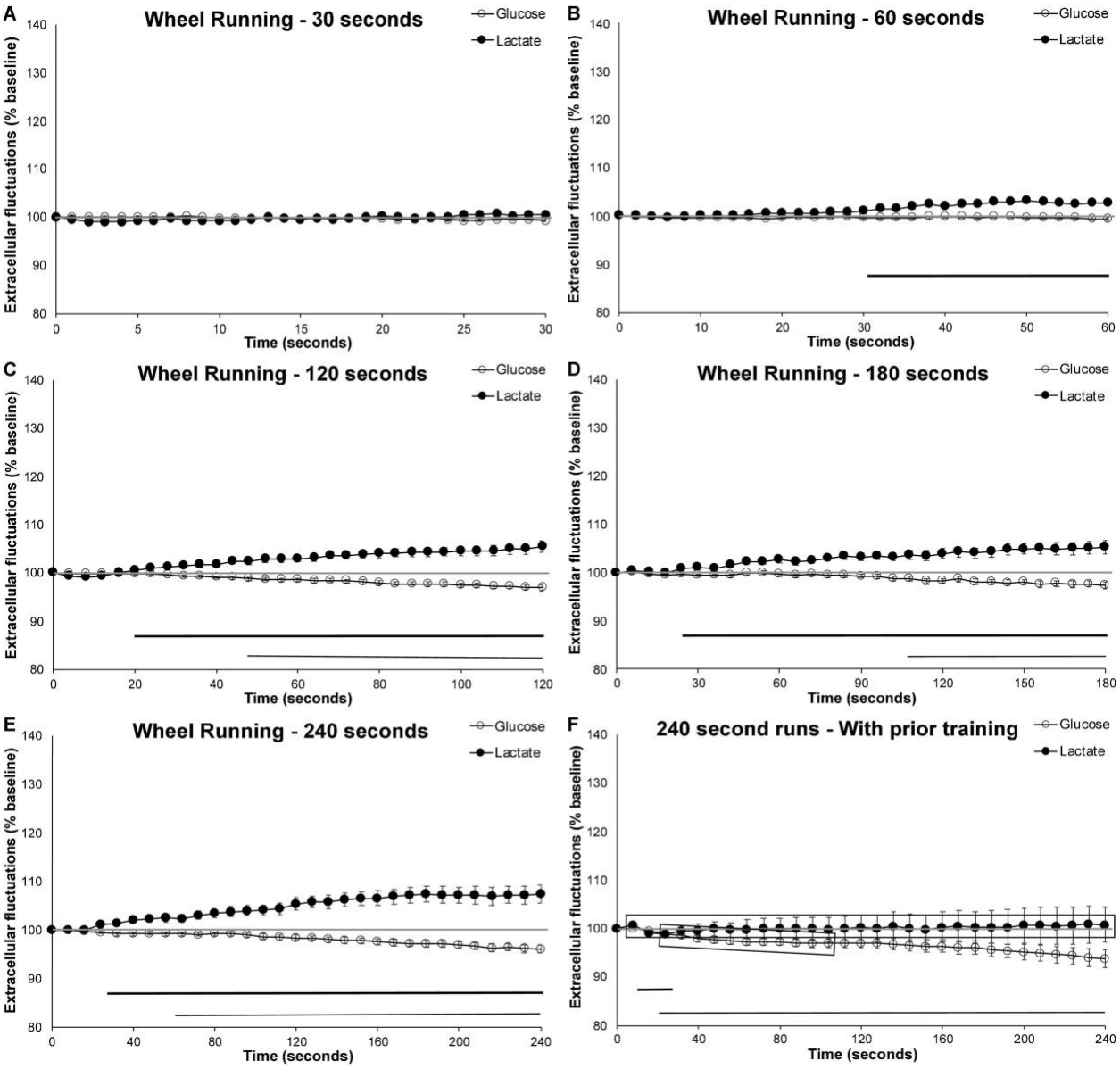
Figure 2 | Extracellular glucose and lactate fluctuations during voluntary wheel running



*Extracellular cortical glucose (open circle) or lactate (filled circle) during voluntary wheel running for individual running events lasting between **(A)** 30–59 s (n events = 32, 10 mice), **(B)** 60–119 s (n event = 58, 10 mice), **(C)** 120-179 s (n events = 35, 10 mice), **(D)** 180–239 s (n events = 31, 10 mice), or **(E)** 240 + seconds (n events = 36, 10 mice). **(F)** Running events of at least 240 s taken from mice (n = 4) who were previously exposed to the running wheel for 4 weeks (n events = 29). Time zero corresponds to the mouse initiating running. Values are expressed as a percent of their baseline taken prior to activity and the error bars indicate the standard error of the mean. The mid-graph gray line shows baseline (100%) value. Horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line).*
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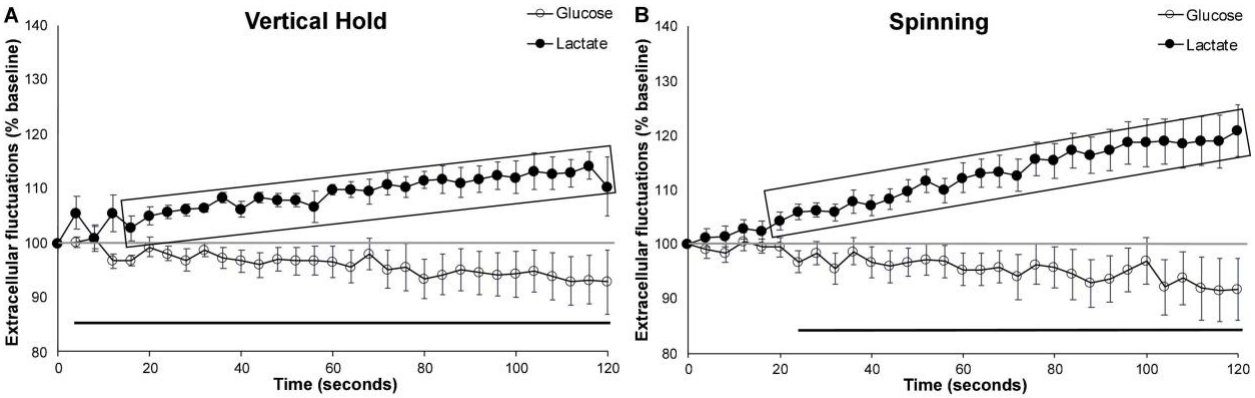
Figure 3 | Effortful behaviors



*Average extracellular cortical glucose (open circle) or lactate (filled circle) during **(A)** all vertical holds events (n = 7), **(B)** all spinning events (n = 10). Gray-shaded rectangles surrounding the glucose (open circles) and lactate (filled circles) curves indicate significant differences from respective controls (120 s runs). Extracellular glucose and lactate did not significantly differ between the vertical hold and spinning conditions. Horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). The mid-graph gray line shows baseline (100%) value. Gray-shaded rectangles surrounding the glucose and lactate curves indicate significant differences from the running wheel (120 s), for both extracellular glucose (open circle) and lactate (filled circle).*

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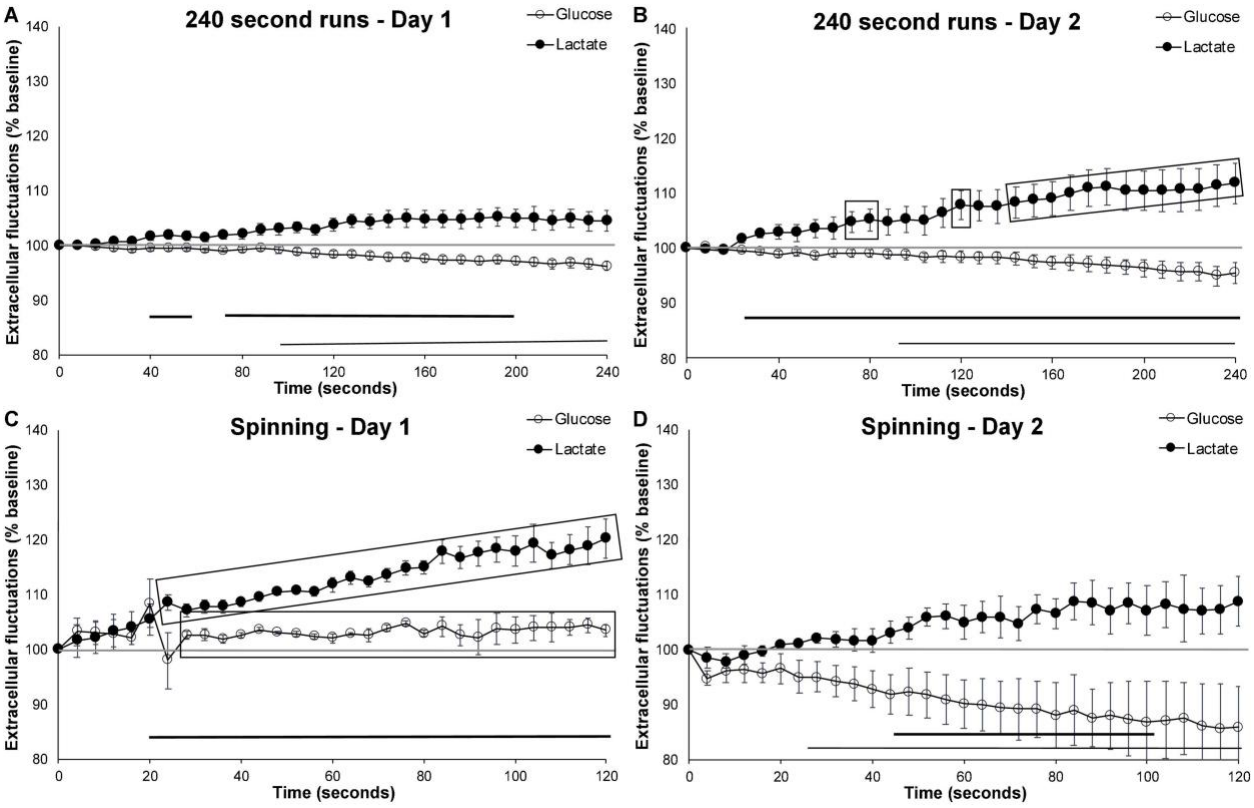
Figure 4 | Effect of novelty on extracellular glucose and lactate



Comparing extracellular cortical glucose (open circle) or lactate (filled circle) on day 1 and day 2 of testing. ***(A)*** *Running events of at least 240 s on the first day of running wheel access (n events = 23, n mice = 10).* ***(B)*** *Running events of at least 240 s on the second day of running wheel access (n events = 12, n mice = 10).* ***(C)*** *Spinning events on the first day of testing (n = 3) and* ***(D)*** *spinning events on the second day (n = 4). Gray-shaded rectangles surrounding the glucose and lactate curves indicate significant differences from day 1 and day 2 of testing, for both extracellular glucose (open circle) and lactate (filled circle). Horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). The mid-graph gray line shows baseline (100%) value.*

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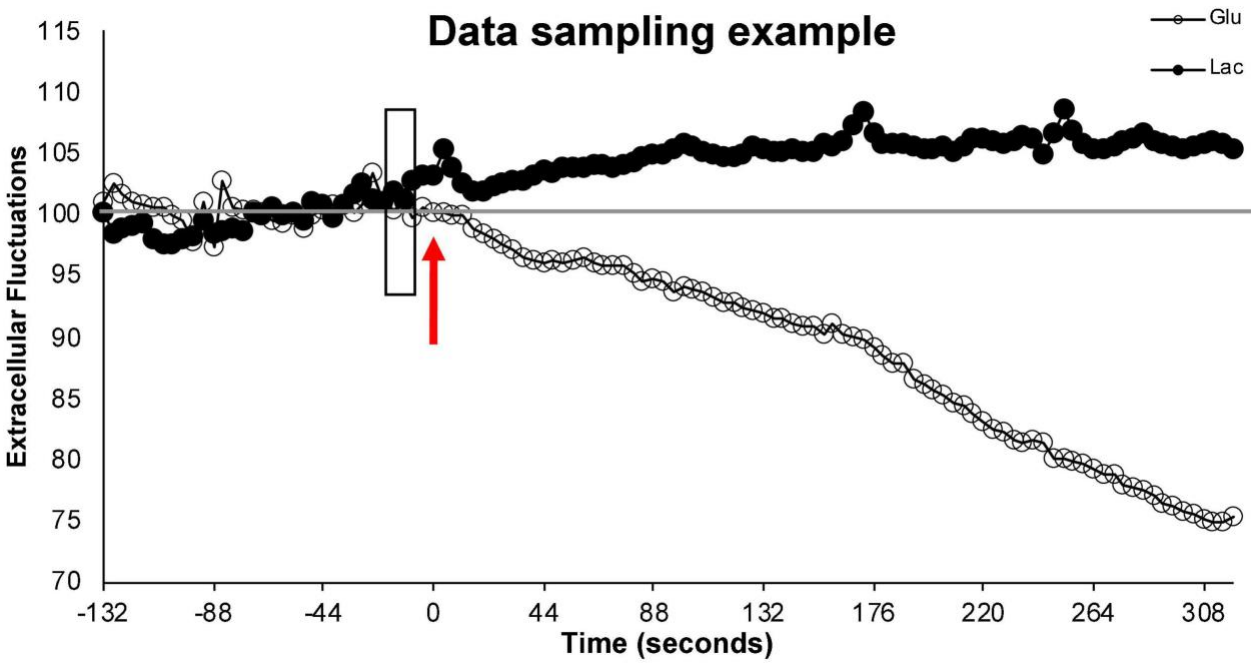
Figure 5 | Illustration of data sampling



This figure illustrates a single run and demonstrates the sampling stability of the electrodes prior to behavior. Time 0 and the arrow (red) represents the start of the run. The 2+ minutes of extracellular values (% from baseline) prior to the start of the run (left of the arrow) represent fluctuations in extracellular glucose (open circle) and extracellular lactate (filled circle) during normal (non-running) activity in the testing cage. These values remain close to baseline. The shaded rectangle represents the 10 s of data that is averaged to create the individualized baseline with which the relative extracellular values during this run will be calculated. The extracellular values (% from baseline) to the right the arrow represent the relative extracellular glucose and lactate values during the running behavior. To simplify the representation, data points were averaged every 4 s.

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Chapter V: Fluctuations of extracellular glucose and lactate in the mouse primary visual cortex during visual stimulation

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Abstract

We measured the extracellular glucose and lactate in the primary visual cortex in the CD-1 mouse using electrochemical electrodes. To gain some additional information on brain metabolism, we examined the impact of systemic injections of lactate and fructose on the brain extracellular glucose and lactate changes observed during visual stimulation. We found that simple stimulation using a flashlight produced a decrease in visual cortex extracellular glucose and an increase in extracellular lactate. Similar results were observed following visual stimulation with an animated movie without soundtrack or the presentation of a novel object. Specificity of these observations was confirmed by the absence of extracellular glucose and lactate changes when the mice were presented a second time with the same object. Previous experiments have shown that systemic injections of fructose and lactate lead to an increase in blood lactate but no change in blood glucose while they both increase brain extracellular glucose but they do not increase brain extracellular lactate. When mice were visually stimulated after they had received these injections, we found that lactate, and to a slightly lesser degree fructose, both reduced the amplitude of the changes in extracellular glucose and lactate that accompanied visual stimulation. Thus, neural activation leads to an increase in extracellular lactate and a decrease in extracellular glucose. Novelty, attentional resources and availability of metabolic fuels modulate these fluctuations. The observations are consistent with a modified view of brain metabolism that takes into account the blood and brain glucose availability.

Keywords: *Brain metabolism, Visual cortex, Glucose, Lactate, Fructose, Novel object, Visual stimuli*

1. Introduction

Compared to other organs, the brain specific resting metabolic rate appears to be only second to that of the heart and kidneys, and greater than the liver, muscles and adipose tissue (Kojo et al., 2016; Sedlock et al., 1989; Wang et al., 2010). Some questions remain as to the extent to, and the means by which the brain modulates its metabolic rate in response to increased neuronal activity as well as the metabolites it uses to sustain this activity. During physical exercise, there are significant increases in muscle metabolic use of both glucose and alternative substrates such as lactate (Ahlborg et al., 1974; Bangsbo et al., 1996; Biensø et al., 2015; Fink et al., 1975; Hermansen & Stensvold, 1972; Sahlin, 1990). Unfortunately, the same pattern is more difficult to ascertain in the brain (Gibson, 2007; Messier, 2004; Raichle & Gusnard, 2002; Sokoloff et al., 1955).

Similar to muscles, an increase in neuronal activity is expected to generate localised increases in metabolic demand and therefore consumption of metabolites[4] (Caesar et al., 2008; Muthukumaraswamy et al., 2009). A vast body of literature supports this assumption. Notably, there are important increases in cerebral blood flow following passive and active stimulation (Caesar et al., 2008; Fox et al., 1988; Fox & Raichle, 1986; Gur et al., 1982; Paus et al., 1998). The increase in cerebral blood flow (CBF) raises the availability of glucose, alternative substrates and oxygen in active neuronal circuits – possibly increasing their consumption as a result of their increased perfusion. Research using fluorodeoxyglucose and positron emission tomography (FDG-PET) in humans demonstrated an increase in glucose uptake in auditory cortex following auditory stimulation (Mazziotta et al., 1982) and verbal fluency testing (Parks et

[4] Though neural activity can induce localized increases in metabolism, these changes are rarely large enough to induce changes in whole brain metabolism. This is due to the combined effect of an extremely metabolically active organ possessing a high metabolic resting rate and an efficient and flexible metabolic organ capable of reducing metabolic in one region to favor another (Dienel, 2017).

al., 1988). A negative correlation between metabolic indices and performance is often reported indicating that better performance is also associated with either more efficient metabolism or more energetically efficient strategies. In vivo microdialysis in rat Purkinje cell layers demonstrated an increase in both extracellular lactate and glucose following stimulation (Caesar et al., 2008). Interestingly, the changes in extracellular glucose, lactate and cerebral blood flow were all attenuated when blocking AMPA receptors, suggesting a unique and connected pathway for the regulation and use of both glucose and lactate during activity.

The high energetic cost of neuronal activity is commonly believed to be a result of stimulation-induced increases in action potentials, membrane repolarisation and neurotransmitter release (Clark et al., 2011; Lee et al., 2004). These energetically costly processes (Harris et al., 2012) account for a large portion of increases in local cerebral metabolism and changes in cerebral blood flow during stimulation. Mechanisms associated with action potentials and neurotransmitter release are estimated to account for ~80% of the total energy required for excitatory signalling and that the increase of one action potential will raise "oxygen consumption by 145 mL/100 g grey matter/h" (Attwell & Laughlin, 2001).

The visual cortex is no exception to the stimulation-induced increases in energy demand: increases in blood flow and metabolism are observed in the visual cortex when stimulated. Multiple techniques, such as NIRS (Kato et al., 1993), FDG PET (Kushner et al., 1988; Vlassenko et al., 2006) and fMRI (Moradi & Buxton, 2013), have all demonstrated increased metabolism and blood flow in the visual cortex following visual stimulation. This increase in CBF and glucose metabolism appears to be accompanied by a 2 to 3-fold increase in cerebral lactate (Prichard et al., 1991; Sappey-Marinier et al., 1992). Cerebral lactate appears to be closely linked to neuronal activity as its fluctuations are least partially regulated by

neurotransmitter release (Caesar et al., 2008). Its cerebral increase has been suggested to be a result of increased glycolysis (Gjedde & Marrett, 2001; Sappey-Marinier et al., 1992). The stimulation-induced increase in metabolic activity of the visual cortex appears to be dependent on the complexity (Phelps et al., 1981) as well as the subjects' expectation (Kastner et al., 1999) of the stimuli.

The use of biosensors also demonstrated that visual stimulation induced increases in cerebral lactate (Fray et al., 1996; Li & Freeman, 2015). However, the fate of cerebral glucose following stimulation is slightly more complex, with some results demonstrating an increase and others a decrease, likely due to the methodological differences between the studies (Fray et al., 1996; Li & Freeman, 2015). Thus, it would appear neuronal activity increases glucose metabolism, as well as cerebral lactate. What still remains a question of interest in the field of cerebral metabolism is how this local increase in blood flow and nutrient delivery during increased stimulation is translated to functional use and transfer of metabolites.

Metabolite flux can occur in one of two ways: influx and efflux. However, the analysis of their relative contributions is challenging for a number of reasons. First, the fluctuation of various metabolites can vary independently of one another because they are modulated and influenced by a variety of external factors. For example, increasing extracellular glucose could be interpreted as either an increase in cellular/vascular efflux (cells exporting glucose or increased cerebral blood flow delivering more glucose), a decrease in cellular/vascular influx (cells could be importing less glucose thus causing an accumulation) or a variable combination of both. At this point, there is an agreement that during increased neuronal stimulation, there is a local increase in glucose use and lactate shuttling. There is, however, divergence of opinion when it comes to the directional flux of these metabolites. One position, the Astrocyte-to-Neuron

Lactate Shuttle hypothesis (ANLS), proposes that glucose undergoes glycolytic metabolism within astrocytes and lactate issued from glycolysis is shuttled to activated neurons (Pellerin & Magistretti, 1994, 2012). The opposing Neuron-to-Astrocyte Lactate Shuttle hypothesis (NALS), proposes that active neurons metabolize glucose and transfer the resulting lactate to astrocytes for disposal (Dienel, 2012; Mangia et al., 2009).

The approach used in the present report is to examine the impact of visual stimulation on extracellular glucose and lactate simultaneously when blood concentrations of metabolic substrates (lactate and fructose) are increased in the peripheral circulation. We then measured the resulting changes in extracellular glucose and lactate with biosensors when mice are presented with visual stimuli that vary in their complexity and nature. The present report is an extension of our finding that an increase in peripheral levels of lactate produced by fructose, lactate, beta-hydroxybutyrate and pyruvate all increase blood lactate but not blood glucose. These metabolites also all produce a significant increase in brain extracellular glucose suggesting that these metabolites can contribute to brain metabolism (Béland-Millar et al., 2017).

2. Materials and methods

2.1. Animals

Twelve, 18-week old, male CD1 mice (Charles River Canada, St-Constant, Québec, Canada) were individually housed with standard bedding (Teklad 7097 Corncob bedding, Envigo, Mississauga, Canada) and maintained on a reverse 12-h night/day cycle with lights on at 7 p.m. Mice had ad libitum access to standard chow (Teklad Global 18% Protein 2018, Teklad Lab Animal Diets, Envigo, Mississauga, Canada) and water, unless otherwise specified. All testing was conducted during the dark (active) phase of the cycle with the use of dim red lighting. All procedures in this study adhere to guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

2.2. Surgical procedure

Pre-surgical treatment included a 0.05 mg/kg subcutaneous (s.c.) injection of buprenorphine hydrochloride (Reckitt Benckiser Healthcare, Hull, North Humberside, UK) as well as 1 ml of 0.9% saline (Hospira, Montreal, Canada). Immediately prior to surgery, mice were anesthetised using 4–5% isoflurane (Fresenius Kabi Canada Ltd., Richmond Hill, ON) then secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA).

Mice were maintained under anesthesia during the surgery with 1–2.5% isoflurane and kept warm with a heated pad (TP650, Gaymar Industries, Orchard Park, NY, USA). Based on the stereotaxic atlas of Franklin and Paxinos (2008), two guide cannulas (BASi cannulas Bioanalytical Systems, West Lafayette, IN) were positioned above the left and right primary visual cortex. The target coordinates, from Bregma, were ± 0.25 mm lateral and −3 mm anterior. The cannulas were positioned 0.25 mm beneath the skull's surface so that the sensing cavity (1 mm) of the electrochemical electrode (inserted later, immediately prior to the testing phase)

would be situated within the primary visual cortex of the mouse (**Fig. 1**). The guide cannulas were fixed using a UV-polymerized compound (CG3 & CG4, Clear Cure Goo, Southlake, TX, USA) and four 0.10-inch screws positioned anterior to the two guide cannulas. Post-surgical care included application of transdermal bupivacaine (Chiron Compounding Pharmacy Inc., Guelph, ON, Canada) and s.c. injections of buprenorphine hydrochloride (0.05 mg/kg) twice daily for 3 days. Mice were given 7–8 days to recover before testing.

2.3. Electrodes

The electrodes have been previously extensively described (Kiyatkin et al., 2013; Kiyatkin & Wakabayashi, 2015; Wakabayashi & Kiyatkin, 2012; Wilson & Gifford, 2005). Briefly, extracellular brain levels of glucose and lactate are monitored using two platinum enzyme-linked electrodes (7005-Glucose-C and 7005-Lactate- C; Pinnacle Technology Inc., Lawrence, KS, USA). Sensors are prepared from Pt-Ir wire of 180 μm in diameter, wrapped concentrically with an AgCl reference electrode. Near the tip of the electrode, a 1 mm section is coated with either glucose or lactate oxidase. These enzymes oxidize the analytes of interest, resulting in a release of hydrogen peroxide (H_2O_2). H_2O_2 is subsequently detected and recorded as an amperometric oxidation current by the electrode (Hu & Wilson, 1997a). The data was collected at 1 Hz, transmitted from a potentiostat to a computer and recorded with the Sirenia Acquisition Software (Pinnacle Technology Inc., Lawrence, KS, USA). In addition to the oxidase enzymes, the metabolite sensitive surface is also coated with a series of membranes to increase the specificity and selectivity of the electrodes. For example, the potential contribution of ascorbic acid is reduced with the presence of ascorbic acid oxidase. Ascorbic acid oxidase converts the electroactive ascorbate, which can interfere with the electrodes recordings, to non-electroactive dehydroascorbate and water (Hu et al., 1994).

Immediately prior to, and following in vivo testing, the electrodes were calibrated according to manufacturer's recommendations. Briefly, the electrodes were gently placed in 100 mM PBS (pH 7.4) at 23 °C (pre-testing) or 37 °C (post-testing) and metabolite sensitivity was measured with four additions of both glucose (1 mM) and lactate (0.1 mM) to the PBS solution. Selectivity was also measured with a single addition of ascorbic acid (0.25 mM), mid-way through the calibration.

Pre-calibration curves demonstrated high sensitivity with incremental linear increases for both glucose (average sensitivity 3.53 nA/1 mM) and lactate (average sensitivity 3.99 nA/0.1 mM) electrodes at 23 °C. Both glucose and lactate electrodes also exhibited high levels of selectivity by demonstrating minimal sensitivity to ascorbic acid (average sensitivity 0.43 nA/ 0.25 mM and 0.38 nA/0.25 mM, respectively). The post-calibration curves, obtained at 37 °C, demonstrated similar results with linear increases for both glucose (average sensitivity 4.33 nA/1 mM) and lactate (average sensitivity 8.03 nA/ 0.1 mM) electrodes. Post calibration curves for ascorbic acid suggested a certain level of wear on the electrodes, most likely due to the insertion and removal process of electrodes as well as biofouling (average sensitivity 0.86 nA/0.25 mM and 1.5 nA/0.25 mM for glucose and lactate, respectively). Using these calibration values, basal extracellular glucose and lactate levels were estimated to be 2.29 mM and 0.32 mM, respectively, at rest (measured during a 10 min resting period where the mouse was inactive).

2.4. Brain extracellular testing procedure

Following electrode calibration, mice were lightly anesthetised with isoflurane and a glucose electrode was inserted into the right primary visual cortex while a lactate electrode was inserted in the left primary visual cortex. Mice were tested in a custom-made square polymethyl

methacrylate-testing cage with standard bedding and water. A camera (Pinnacle Technology Inc., Lawrence, KS, USA) mounted above the cage recorded the animal's behaviour and Sirenia synchronized the video recording with the readings obtained for both electrodes. Mice had free access to water but chow was removed 2 h prior to injection and returned 3 h post-injection.

Approximately 2 h following electrode insertion, when they returned stable readings, mice received a 2 g/kg intraperitoneal (i.p.) injection of either lactate (Lactic Acid, Acros Organics, NJ, USA) or fructose (d-Fructose, Fisher Scientific, Fair Lawn, NJ, USA). Saline (0.9%) injections were used as a control. This particular dosage was chosen based on previous results that demonstrated the ability of 2 g/kg of glucose or fructose to improve memory (Messier & White, 1987). Additionally, this dosage also corresponds to the amount of sugar these animals readily ingest (Messier & White, 1984). Extracellular fluctuations of glucose and lactate were observed for 2 h post-injection during which time animals received 22 min of various visual stimulation tasks with pauses between each stimulation task (see **Fig. 2**). When the post-calibration was complete, mice were immediately perfused for histology to determine electrode placement.

Mice were subjected to the visual stimulation conditions in the test cage using a counterbalanced order, starting 15 min post-injection as injection-induced stress briefly influences extracellular metabolite levels (Béland-Millar et al., 2017). Each of the visual stimulation trial was separated from the next one by a no-stimulation 10–25 min period. The impact of visual stimulation on extracellular glucose and lactate in the primary visual cortex was observed after the saline injection and subsequently compared to the effect of these same stimulation following the systemic administration of either lactate or fructose. **Fig. 2** presents an example of the experimental design timeline.

2.4.1. Flashlight stimulation

A flashlight (BYB Super Bright 9 LED Mini Aluminum Flashlight; ~400 lx) was used to stimulate both eyes with random patterns of motion and speed for 2 min. The flashlight was situated 15–20 centimetres from the mouse's eyes. Electrode current was sampled every second but averaged every 5 s for data analysis.

2.4.2. Animated film (with or without soundtrack)

The mice were presented with a complex stimulus in the form of an animated film entitled "The Secret of NIMH[5]". An iPad air (model MD786C/A; screen size: 7.75 × 5.75 in.) was horizontally inserted into the testing cage and mice were exposed to the first 10 min of the animated film (300–600 lx) with (~60 decibels) or without soundtrack condition. The data was sampled every second, but averaged every 15 s. The iPad was wiped with alcohol pads (Webcol Alcohol Preps, Coviden, Mansfields, MA, USA) before and after each use.

2.4.3. Object recognition

The mice were presented with an object they had never encountered, (a large yellow Lego Block) for 5 min by placing the object in the center of their cage. The object was returned to the cage 25 min after the initial presentation for an additional 5 min. During each presentation of the object, the time the mice spent interacting with that object was calculated. The mouse was considered to be interacting with the object when it was physically touching the object, either with its nose or paws. The extracellular data was sampled every second, but averaged every 15 s. The objects were wiped with alcohol pads before and after each use.

[5] The secret of NIMH is an animated movie, a story about mice and rats. In the movie, experimental rats escape from the National Institute of Mental Health laboratories. The experiments boosted their intelligence, enabling them to escape, as well as extending their lifespans. We thought this story would be of interest to the mice in our experiment.

2.5. Histology

Following testing, mice were lightly anesthetised using isoflurane and the electrodes gently removed for post-calibration. Mice were then euthanized with an i.p. injection of sodium pentobarbital (65 mg/kg; MERK, Kirkland, QC, Canada) and transcardially perfused with a 4% paraformaldehyde/0.2% picric acid solution (PFA). Brains were post-fixed in the same PFA solution for 1 h at 4 °C, and then transferred to a 10% sucrose solution overnight. The following day, brains were frozen with CO2 and stored at −80 °C for later cryostat sectioning at 14 μm. Electrochemical electrode placements within the primary visual cortex were confirmed with the use of the stereotaxic atlas of Franklin and Paxinos (Franklin & Paxinos, 2008).

2.6. Statistical analysis

All data were standardized by dividing the readings, in nA, by their respective baselines and multiplying the result by 100 to obtain relative percentage change from baseline. The respective baseline levels were individually calculated by averaging 10 consecutive nA measurements, recorded by the micro-electrodes in the visual cortex, prior to injection or visual stimulation with an average of 10 s before injection or respective visual stimulation. To facilitate the visual representation of the results, every 5, 15 or 120 consecutive measurements were averaged in order to obtain a mean value.

To examine the impact of injections, extracellular glucose and lactate were analysed separately using a repeated measures ANOVA followed by pairwise comparisons. Changes in glucose and lactate estimates were analyzed separately using a between-within (split-plot) mixed ANOVA ($\alpha = 0.05$). The ANOVA was followed up with contrasts comparing the observed values to their respective baseline and to the other conditions. Unless otherwise stated, the data met the respective assumptions of the analysis. If sphericity or homogeneity was violated, a

Huynh-Feldt correction was applied. Due to the interest in differences across groups, post-hoc pairwise comparison tests ($\alpha = 0.05$) were computed in order to compare the experimental groups to the controls as well as compare the experimental groups amongst themselves, whether omnibus test was significant or not. All statistical analyses were performed using SPSS v. 20 (IBM). We report power analysis by stating the $\eta 2$ statistic for significant results and observed power statistic for non-significant results.

3. Results

3.1. Surgical electrode insertion

Fig. 1 shows the coordinates for the tip of the electrodes within the primary visual cortex. The sensing cavity extended 1 mm above the tip of the electrode.

3.2. The effect of visual stimulation on extracellular glucose and lactate following a saline injection

The saline injection (**Fig. 3**) produced a significant but transient rise in both extracellular glucose (35% above baseline; $F(4.77, 14.31) = 3.4$, $p = 0.032$, $\eta 2 = 0.539$) and lactate (15% above baseline ; $F(26.08,78.23) = 2.9$, $p < 0.000$, $\eta 2 = 0.493$). Glucose and lactate values returned to baseline levels 22 and 8 min after the injection, respectively. Changes in the first 15 min may be associated with the stress response produced by the injection. We observed similar changes in the motor cortex and they appear to be modulated by prior handling. Also, contrary to the present results, we had observed a small monotonic decrease in the observed extracellular glucose levels throughout the two-hour observation period but mice in that previous experiment had access to a running wheel that they readily used during the testing period which may explain the decrease in both brain extracellular glucose and blood glucose (Béland-Millar et al., 2017).

3.3. Visual stimulation induced a rise in lactate and a decrease in extracellular glucose in the primary visual cortex

The analyses presented in this section concern the impact of visual stimulation on extracellular glucose and lactate following a control injection. The visual tasks were presented in a counterbalanced order so that the effects of the visual stimulation, described later, are not dependent on specific extracellular glucose and lactate levels at various time points after the injections. Because of this experimental design, the effects of the visual stimulation at various

time points are intertwined with the effects of the injections. The flashlight stimulation (**Fig. 4A**. Glucose: $F(1.42,11.36) = 1.60$, $p = 0.241$, Observed Power = 0.239 ; lactate: $F(2.79,22.34) = 5.36$, $p = 0.007$, $\eta 2 = 0.401$), the movie presentation without soundtrack (**Fig. 4B**. Glucose: $F(3.99,27.93) = 6.98$, $p = 0.001$, $\eta 2 = 0.499$; lactate: $F(7.75,54.25) = 8.63$, $p < 0.000$, $\eta 2 = 0.552$) and the introduction of a novel object (**Fig. 4D**. Glucose: $F(4.50,36.01) = 4.20$, $p = 0.005$, $\eta 2 = 0.344$; lactate: $F(4.24,33.95) = 6.31$, $p = 0.001$, $\eta 2 = 0.441$) all generated significant decreases in brain extracellular glucose (about 10%) while simultaneously producing increases in brain extracellular lactate (15–20% increase) – with the exception of the decrease in extracellular glucose during the flashlight stimulation which did not reach significance.

Compared to the silent condition, the addition of the soundtrack to the movie presentation did not produce a significant decrease in extracellular glucose ($F(1.98,9.91)$ 0.93, $p = 0.424$, Observed Power = 0.168) but still produced a significant but smaller increase of extracellular lactate ($F(11.27,78.92)$ 7.98, $p < 0.000$, Observed Power = 0.533). Inspection of **Fig. 4B and C** shows that the addition of the soundtrack to the film reduced the number of time points during which glucose and lactate levels significantly differed from baseline. However, despite these differences, the post-hoc analyses to our repeated measures ANOVA did not reveal a significant difference between the presence or absence of the soundtrack for either brain extracellular glucose ($p = 0.791$) or lactate ($p = 0.258$). An independent t-test did not reveal any significant differences in the time spent observing or interacting with the video ($p < 0.654$), whether the movie did (Mean: 257 ± 54 s) or did not (Mean: 285 ± 34sec) have a soundtrack.

Also, the observed extracellular metabolite fluctuations do not appear to be the result of electrode drift or random neuronal activity as very little extracellular fluctuations of glucose and lactate are observed in a 10-min period with no experimental visual stimulation (**Fig. 8**)

3.4. Familiarity reduces fluctuations of brain extracellular glucose and lactate

The second presentation of the same object did not produce significant changes in either extracellular glucose ($F(7.07,56.57)$ 1.29, $p = 0.271$, Observed Power = 0.505) or lactate ($F(2.66, 21.27)$ 1.16, $p = 0.345$, Observed Power = 0.254). The comparison of **Fig. 4D and E** shows that extracellular glucose and lactate (glucose: $p = 0.024$; lactate $p = 0.025$) in the visual cortex were significantly attenuated during the second presentation of the object compared to the initial presentation. Thus novelty appears to be important for the extracellular response observed in the primary visual cortex following the introduction of a new object. A possible contributor to the observed differences between the initial and second presentation of an object is induced arousal. Following a systemic saline injection, exploration of a novel object decreased by more than half when presented a second time (see **Table 1**). However, as described in the next section, animals injected with lactate and fructose spent less time exploring the novel object.

3.5. Systemic administration of fructose and lactate

Because we were interested in examining the interaction between the extracellular changes in glucose or lactate produced by visual stimulation and the impact of a systemic injection of either fructose or lactate on these changes, the following statistical analyses include substance injected as a factor. We first examined the overall effect of fructose and lactate on brain extracellular glucose and lactate for the duration of the experiment. The analysis of brain extracellular glucose fluctuations following the different injection conditions (**Figs. 3** and **5 A and B**) revealed no main effect of substance injected ($F(2,11) = 3.22$, $p = 0.079$, Observed Power = 0.493) but did reveal a significant effect of time ($F(3.50,38.45) = 11.30$, $p < 0.000$, $\eta 2 = 0.507$) and an interaction between substance injected and time ($F(6.99,38.45) = 3.04$, $p = 0.012$, $\eta 2 = 0.356$). The respective graphs (**Figs. 3** and **5A & B**) show an increase in

extracellular glucose for both fructose and lactate with systemic lactate producing the greatest extracellular glucose rise followed by systemic fructose.

The analysis of brain extracellular lactate fluctuations following the different injections conditions (**Figs. 3** and **5A and B**) revealed no main effect of substance injected ($F(2,11) = 1.38$, $p = 0.291$, Observed Power = 0.237), a significant effect of time ($F(12.38,136.16) = 8.46$, $p < 0.000$, $\eta 2 = 0.435$) but no interaction between substance injected and time ($F(24.76,136.16) = 0.62$, $p = 0.916$, Observed Power = 0.504). These results suggest that there were no overall significant difference in the effect of all three injections on brain extracellular lactate.

3.6. Interaction between systemic fructose and lactate injections with brain extracellular glucose or lactate changes during visual stimulation

The effect of the flashlight stimulation on extracellular glucose was significantly modified by the substance injected ($F(2,19)$ 3.74, $p = 0.043$, $\eta 2 = 0.282$), but the ANOVA reveal no effect of time ($F(2.08,39.51) = 2.10$, $p = 0.134$, Observed Power = 0.414) nor interaction ($F(2.08,39.51) = 0.70$, $p = 0.599$, $\eta 2 = 0.211$). Post-hoc comparisons revealed that the observed decrease in extracellular glucose during flashlight stimulation following the saline injection (**Fig. 4A**) was abolished following the systemic fructose (**Fig. 6A**; $p = 0.048$) or lactate injections (**Figs. 7** and **8A** ; $p = 0.024$).

There was no significant effect of substance injected for extracellular lactate following flashlight stimulation ($F(2,19)$ 1.42, $p = 0.267$, Observed Power = 0.266) but there was a significant effect of time ($F(2.97,56.50) = 4.65$, $p = 0.006$, $\eta 2 = 0.197$) and an interaction between time and injections ($F(5.95,56.50) = 2.70$, $p = 0.023$, $\eta 2 = 0.221$). Follow-up ANOVAs showed that there was a significant effect of time for saline ($F(2.45,19.62) = 3.92$, $p = 0.030$, $\eta 2 = 0.329$),

but not for fructose ($F(8.24,49.44) = 0.75$, $p = 0.648$, Observed Power = 0.313) or for lactate ($F(2.45,12.25) = 0.72$, $p = 0.535$, Observed Power = 0.152). Taken together, these results show that the systemic injection of fructose or lactate significantly attenuated the decrease in extracellular glucose and the increase in extracellular lactate following the flashlight stimulation.

When the mice were presented with a movie without soundtrack, extracellular glucose did not statistically differ as a function of systemic injections, and there were no significant time or interaction (systemic injection factor: $F(3,20)$ 2.33, $p = 0.105$, Observed Power = 0.499; time: $F(3.22,64.42)$ 2.49, $p = 0.064$, Observed Power = 0.613; interaction: $F(3.66,64.42)$ 1.30, $p = 0.252$, Observed Power = 0.600). The same could be said for the novel object presentation (injection factor: $F(2,16)$ 1.51, $p = 0.251$, Observed Power = 0.274 ; time: $F(3.71,59.35)$ 1.59, $p = 0.192$, Observer Power = 0.444 ; interaction: $F(7.42,59.35)$ 1.07, $p = 0.397$, Observer Power = 0.432).

When the mice were presented with a movie without soundtrack, extracellular lactate did not statistically differ as a function of the injected substance (systemic injection factor: $F(3,20)$ 0.67, $p = 0.581$, Observed Power = 0.165). The same could be said for the novel object presentation (systemic injection factor: $F(2,16)$ 0.99, $p = 0.395$, Observed Power = 0.191). There was an effect of time for both the presentation of the movie without soundtrack ($F(8.36,167.15)$ 9.83, $p < 0.000$, $\eta 2 = 0.329$) and the novel object ($F(4.92,78.72)$ 10.15, $p < 0.000$, $\eta 2 = 0.388$), but not of interaction (movie presentation: $F(25.07,167.15)$ 1.38, $p = 0.122$, Observed Power = 0.930; novel object: $F(9.84,78.72)$ 1.30, $p = 0.244$, Observer Power = 0.619). Despite a trend suggesting decreased exploratory behaviour, the time spent exploring the object did not significantly differ between its first and second introduction in the fructose or the lactate injection condition.

Inspection of **Figs. 4**, **6** and **7** demonstrates that the injection of both fructose or lactate substantially decreased, by over 50%, the number of time points at which the extracellular values of either glucose or lactate varied from baseline – suggesting that the systemic injection of fructose and lactate did interact with extracellular fluctuations of glucose and lactate in the visual cortex during visual stimulation. Pairwise comparisons between the extracellular glucose levels observed during the film presentations without soundtrack statistically confirm this observation. The systemic injection of lactate abolished the fall in extracellular glucose levels during the movie presentation (without soundtrack) when compared to either saline ($p = 0.024$) or fructose ($p = 0.044$). However systemic injection of lactate did not produce extracellular lactate levels different from saline ($p = 0.511$) or fructose ($p = 0.453$). The systemic injection of fructose did not significantly modulate extracellular glucose ($p = 0.998$) or lactate ($p = 0.844$) levels, when compared to saline.

4. Discussion

The present results show that visual stimulation increases lactate extracellular levels and decreases glucose levels in the mouse visual cortex. Simple light stimulation, complex visual stimulation and the presentation of a new object all produced similar effects. The addition of sound to the movie presentation and the second presentation of an object were associated with smaller changes in glucose or lactate suggesting that attention and novelty modulate the changes in extracellular lactate and glucose in the visual cortex, possibly through changes in induced arousal and exploratory behaviour. These results also replicate the previously observed increases in brain extracellular glucose produced by systemic injections of lactate and fructose in the motor cortex (Béland-Millar et al., 2017). Finally, we demonstrated that the changes of extracellular glucose and lactate in the visual cortex associated with visual stimulation were attenuated by the systemic injection of fructose and lactate. These results raise a number of issues that we address in the next sections.

4.1. Extracellular metabolite coupling following visual stimulation in the mouse primary visual cortex

The present results confirm the association of sustained neuronal stimulation with a simultaneous increase in extracellular lactate and a decrease in extracellular glucose (Caesar et al., 2008; Kuhr & Korf, 1988; Li & Freeman, 2015; Mangia et al., 2007; McNay et al., 2000; McNay & Gold, 2001; Newman et al., 2011, 2017; Schurr et al., 1997). Certain similarities can even be observed within the current and previous findings, such that the relative rise in brain extracellular lactate is higher than the relative decrease in glucose. This typical[6] result is

[6] This pattern of response is not found everywhere in the brain. Kiyatkin and Lenoir (Kiyatkin & Lenoir, 2012) found that the same stimuli induced a rapid glucose increase in the nucleus accumbens but a decrease in the substantia nigra pars reticulata in which cells tend to have a high activity in the unstimulated state.

interesting given that glycolysis produces two lactate molecules from one glucose molecule. When electrical neuronal activity is simultaneously monitored with extracellular glucose and lactate, a very tight coupling is found between neuronal activity, glucose and lactate (see Fig. 2 of Li & Freeman, 2015).

Although it is difficult to dismiss the mounting evidence indicating a large degree of metabolic coupling between extracellular glucose, lactate and sustained neuronal activity, there is still debate regarding the use and directional flux of lactate in cerebral metabolism. The measure of brain extracellular levels of lactate and glucose cannot inform about the directionality of glucose and lactate fluxes. Thus, the technique cannot distinguish between the lactate being transported out of the brain as metabolic by-product from the lactate being shuttled from astrocytes to neurons as metabolic fuel.

4.2. The role of attentional resources in neural activation

A less contradictory topic is that of cognitive work load being negatively correlated with extracellular glucose concentration (McNay et al., 2000) and positively correlated with neural activation (Culham et al., 2001; Maximo et al., 2016; Rao et al., 1993). Simply put, past research has demonstrated that the more complex a task, the more neural activity it engages, and consequently the more it increases the brain metabolic consumption of glucose. The increased metabolic requirement of a complex task is further supported by the facilitation observed following systemic or brain administration of glucose (Gold et al., 2013; Messier, 2004; Smith et al., 2011). This facilitation of the task performance is not observed in easier tasks, which is consistent with the involvement of an increasing number of brain areas as task difficulty increases and consequently increased metabolic requirement (Culham et al., 2001; Maximo et al., 2016).

We propose that this absence of difference between the simple and complex stimulation conditions is a result of attention. Flashlight stimulation of the visual cortex is not dependent on the mouse's attentional processes as the light is shone directly onto their eyes; therefore the visual stimulation is passive. However in the movie presentation, the mice could move around and choose to orient towards the iPad screen that likely involved more attentional processing. Indeed, visual cortex activation is correlated with attentional load (Culham et al., 2001), and attention has been suggested to modulate activity in the primary visual cortex, subsequently influencing task performance (Ress et al., 2000).

The observation that the second presentation of an object was associated with attenuated changes in extracellular lactate and glucose is consistent with the hypothesis that the attentional resources and metabolic requirements devoted to a stimulus are increased by novelty. Novelty appears to be an important factor in determining where attentional resources are allocated (Coull, 1998; Luck et al., 2000; T. Moore & Zirnsak, 2017). Also, neural firing and patterns are altered between familiar and unfamiliar stimuli presentation, frequently presenting with lower activity levels and thus energy use when familiar stimuli are presented (Fried et al., 1997; Grill-Spector et al., 2001; Hawco & Lepage, 2014; Supp et al., 2007; Tanaka & Curran, 2001). In addition to these brain-related factors, arousal also modifies levels of blood metabolites (Coco et al., 2009; Flint, 2004; Grego et al., 2004; Scholey et al., 2001). These changes are important to consider since brain levels of metabolites are considered to be proportional to those observed in the blood (Mergenthaler et al., 2013; Oldendorf, 1971). Since the observed varying levels of arousal, measured here by the exploratory behaviour of the mice in response to an object being placed in their cage, were diminished upon the second introduction of this same object we cannot exclude

the possibility that these observations are in part a result of fluctuations in blood levels or glucose and lactate.

4.3. Sensory modality interactions

The observation that the removal of the movie soundtrack led to greater fluctuations of lactate and glucose can be explained in a number of ways. First, albino mice such as the CD-1 strain we use, might favour auditory information over visual ones when both are present while attending more to visual information when there is no auditory information available due to their limited visual acuity. Second the interaction between auditory and visual stimulation could be related to the complexity of the task and their relative associated attentional load. An auditory stimulation, combined with simple tasks may increase awareness. However, in a more complex situation that includes moving and changing visual stimuli (as in a movie), attentional resources diverted to the auditory stimuli may take away from the cognitive resources devoted to the visual information and modulate performance (Smucny et al., 2013). The present results show the importance of considering the involvement of all senses in a task because information garnered through different modalities can influence and interact with one-another in the brain (Shimojo & Shams, 2001). These cross-modalities interactions are known to affect cellular activity (Meredith & Stein, 1983) as well as performance (Bolli et al., 1983; Romei et al., 2007).

4.4. The effect of metabolite availability on extracellular glucose and lactate fluctuations during visual stimulation

In the present experiments, we found that the increased extracellular lactate and decreased extracellular glucose produced in the visual cortex by visual stimulation is attenuated by the systemic administration of fructose and lactate (**Figs. 4B & D**, **5B & D**). Systemic injections of both fructose and lactate increase blood lactate levels but have no effect on blood

glucose levels (Béland-Millar et al., 2017). These same injections increase brain extracellular glucose levels but have little effect on brain extracellular lactate (Béland-Millar et al., 2017). The relative effectiveness of systemic fructose and lactate injections to attenuate the rise and fall of brain extracellular lactate and glucose, respectively, were not equivalent. Systemic administration of lactate, which induced a much more sustained increase in brain extracellular glucose, produced a stronger attenuation of extracellular fluctuations in all visual tasks. Thus, the present results suggest that the ability of a systemically administered metabolic fuel to alter the observed changes in brain extracellular lactate and glucose is dependent on how much it raises and sustains higher levels of extracellular glucose. It could be argued that the attenuation in extracellular fluctuation of both glucose and lactate is the result of diminished attentional resources, or diminishes arousal, associated with the visual stimulation. However, given that were no significant decrease in the time spent interacting with the object during its first or second presentation (after lactate or fructose injections) and that this pattern of blunted glucose and lactate fluctuations is also observed in passive stimulation (i.e. flashlight stimulation), it would appear that the observed attenuated metabolite fluctuations are modulated by the systemic availability/injection of these alternative fuels.

A large number of experiments have shown the ability of systemic injections of glucose (McNay et al., 2000; McNay & Gold, 2001; Messier, 2004), fructose (Messier & White, 1987) and lactate (Newman et al., 2011) to improve cognitive tasks. These three metabolic substrates share the ability to increase in brain extracellular glucose while producing a rise in blood lactate. A reasonable argument can be made that an increase in brain extracellular glucose is the proximal cause of the functional improvement observed after the systemic injection of glucose, lactate and fructose. Interestingly, other metabolic substrates such as pyruvate (Krebs & Parent,

2005; Ragozzino et al., 1995) and beta-hydroxybutyrate (Reger et al., 2004; Zou et al., 2009) that also increase blood lactate and brain extracellular glucose (Béland-Millar et al., 2017) also facilitate cognitive tasks. These results suggest that the more distal cause of the cognitive facilitation of the various metabolites cited above is the increase in blood lactate. This hypothesis could be further tested by examining the effect of galactose that also raise blood lactate but only produces a small rise in brain extracellular glucose (Béland-Millar et al., 2017). Galactose has mostly been studied in the context of its long-term effects on memory, inducing deficits following chronic administration (Chen et al., 2006).

An alternate hypothesis is that the rise in brain extracellular glucose observed after the systemic injections of various metabolites is due to the transport of these metabolic substrates into the brain, their use in alternate metabolic pathways that would eventually lead to the sparing of glucose and its accumulation in the extracellular space. This hypothesis is constrained by data showing that glucose, fructose and lactate are not transported at equal rates across the blood brain barrier (Hellmann et al., 1982; Kuhr et al., 1988; Nemoto et al., 1974; Oldendorf, 1971) and that the observed temporal rise in brain extracellular glucose is closely matched following systemic injections of these metabolic substrates (Béland-Millar et al., 2017). Considering that both fructose and lactate can sustain activity within hippocampal slices (Wada et al., 1998), there is the possibility that both lactate and fructose transport into the brain contribute to the rise in extracellular brain glucose. However, fructose is mostly transported into the brain through the GLUT5 transporter. GLUT5 is not widely distributed at the blood brain barrier (Choeiri et al., 2002) and is not substantially upregulated by high-fructose diets (Messier et al., 2007), which suggests that fructose can be transported into the brain but at a much lower rate than glucose or lactate.

The observation that the systemic administration of many metabolites (glucose, lactate, fructose, pyruvate, beta-hydroxybutyrate) increases blood lactate (but not brain extracellular lactate), suggest the possibility that the brain can increase its use of the newly and abundantly available lactate to sustain its metabolic requirements. Indeed, brain lactate use is widely debated. Proponents of the Astrocyte-to-Neurons lactate shuttle would likely propose that lactate is directly taken up by neurons to undergo oxidative phosphorylation, thus sparing glucose and resulting in its accumulation in the extracellular space. Conversely, proponents of the opposing Neuron-to-Astrocyte Lactate shuttle would likely propose that the increased extracellular glucose is a result of lactate being shuttled to through the gluconeogenic pathway resulting in increased glucose production. Whether lactate undergoes direct (i.e. lactate being metabolised by neurons) or indirect (i.e. lactate undergoing gluconeogenesis) metabolism is currently debated. As of yet, there has been no clear in vivo results establishing whether this increase in extracellular glucose, following the systemic administration of metabolic substrates, is a consequence of an increase in availability and use of blood lactate or increased cerebral gluconeogenesis.

Can the ANLS or NALS theories explain the attenuation of the activity-induced increase in lactate and decrease in glucose when either blood glucose or lactate levels are elevated? The ANLS proposes that, when neurons are activated, astrocytes take up glucose, transform it through glycolysis into lactate that is then taken up by neurons and used as additional metabolic fuel. Although the present experiment does not provide information on metabolic fluxes, the absence of activity-induced changes in the extracellular compartment when brain extracellular glucose levels are increased through systemic injections would suggest that the astrocytic step could be bypassed when extracellular glucose is plentiful. This, in turn, would indicate that the ANLS describes a phenomenon associated with lower blood (and brain) glucose as would be

found in the fasting state but not in the post-prandial state or, far that matter, in uncontrolled diabetic states where blood and brain glucose are elevated.

The NALS proposes that glucose is taken up by neurons, transformed through glycolysis into lactate that is eventually transported out of the brain without further metabolism. The absence of activity-induced changes in the extracellular compartment (both lactate and glucose) when brain extracellular glucose levels are high would suggest that neurons no longer use glucose in the glycolytic process since no additional lactate appears in the extracellular space when neurons are active and extracellular glucose is elevated. That observation is contrary to predictions of the theory but could be explained if, again, the glycolytic pathway is used mostly when extracellular brain glucose levels are high. Thus, the present results do not resolve the controversy between the ANLS and NALS theories but rather suggest that the brain may be preferentially using different metabolic pathways depending on the blood and brain levels of glucose. The glycolytic pathway would be preferentially used when there is a surplus of glucose such as during the post-prandial period while glucose phosphorylation pathway which produces more energy with each glucose molecule would be the preferred pathway when blood and brain glucose levels are lower, as in the fasting state. This would be consistent with the observation that the glycolytic pathway can rapidly produce ATP but with each glucose molecule yielding only two ATP: in the presence of an abundance of glucose, this pathway can be efficient. Conversely, in conditions where glucose is less abundant, the phosphorylation pathway that yields 30 ATP but is potentially less time efficient would be preferred. In these lower-glucose conditions, neuronal activity would produce a measurable decrease in brain extracellular levels. There are no a priori difficulties with the possibility that the brain uses a mixture of the two pathways in response to substrate availability or levels of neuronal firing.

4.5. Conclusions

In summary, visual stimulation leads to an increase in extracellular lactate and a decrease in extracellular glucose in the visual cortex. Novelty, attentional resources and availability of metabolic fuels modulate these fluctuations. The observations are consistent with a modified view of brain metabolism that takes into account the blood and brain glucose availability but does not resolve the controversy between the ANLS or NALS theories.

Acknowledgements

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Table 1 | Effect of novelty and systemic injection on time spent exploring a novel or previously novel object

Condition	Saline		Lactate		Fructose	
	Novel	Old	Novel	Old	Novel	Old
Mean Exploration Time (seconds)	106.4	43.4*	26.3&	15.5	29.8&	10.5
SEM	24.1	14.3	13.3	8.3	4.1	3.6

Mean exploration time (sec ± SEM) of the novel (white) or previously novel/old (black) object following a saline, lactate or fructose injection.
** Indicates significant ($p < 0.005$) difference between in the first (new) presentation of the object, when compared to the second presentation (old).*
& Indicates significant ($p < 0.05$) difference compared to the saline condition.

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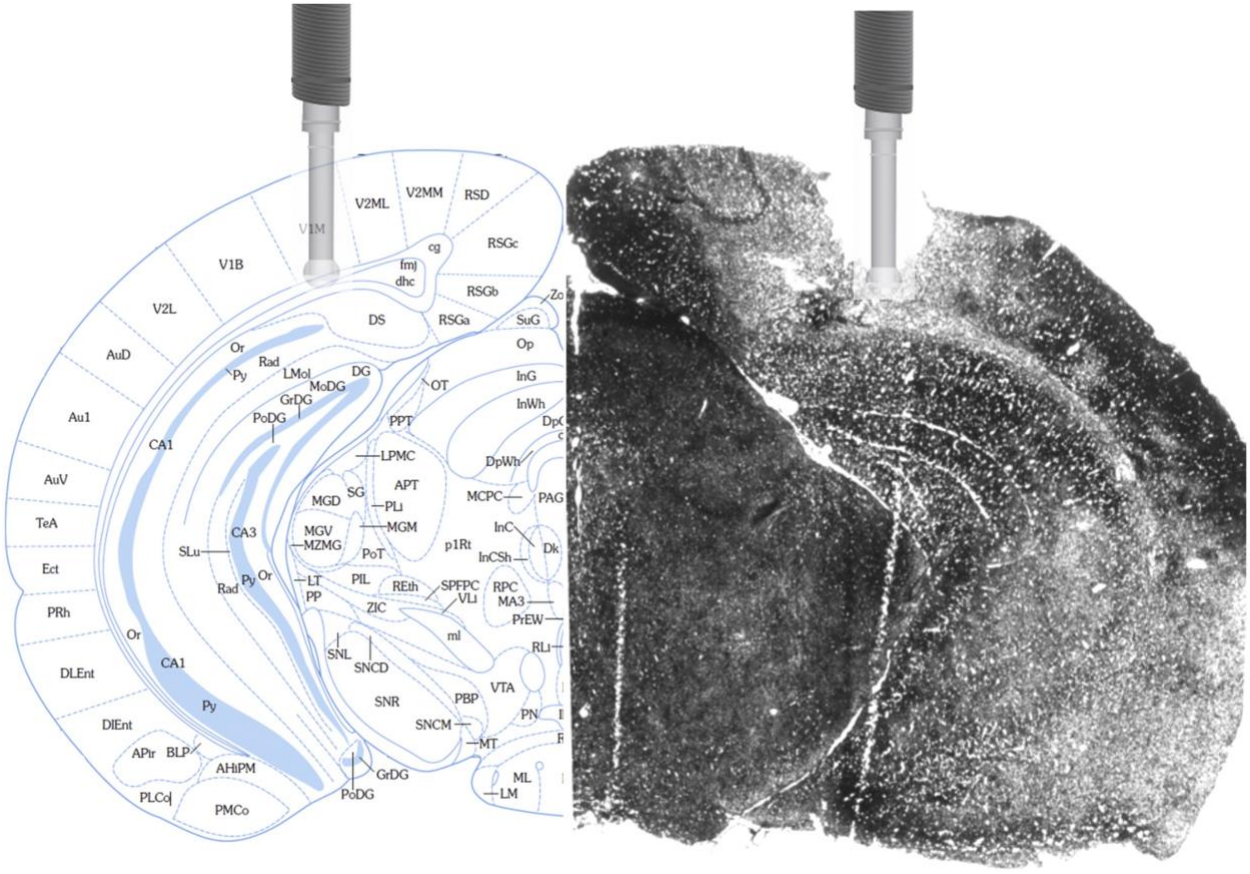
Figure 1 | Electrode localization



The right panel shows a 14 µm mouse brain slice after the insertion of an electrode in the primary visual cortex (V1). The left panel is a drawing from the Franklin and Paxinos (2008) Mouse Brain Atlas showing the approximate placement of the electrode in the primary visual cortex (V1). The light grey cylinder is the 1mm analyte sensitive portion of the electrode.

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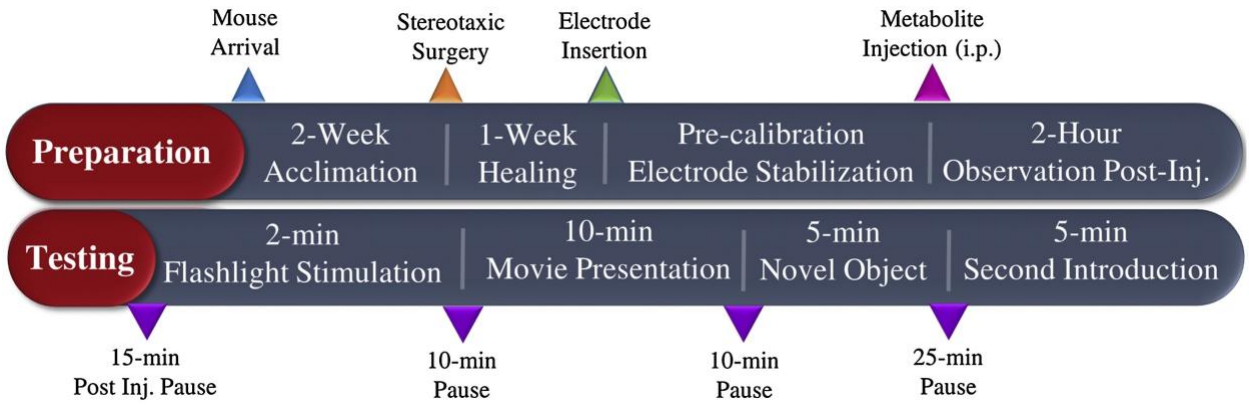
Figure 2 | Timeline



Protocol timeline: Testing conditions and injection order was counterbalanced within each injection group.

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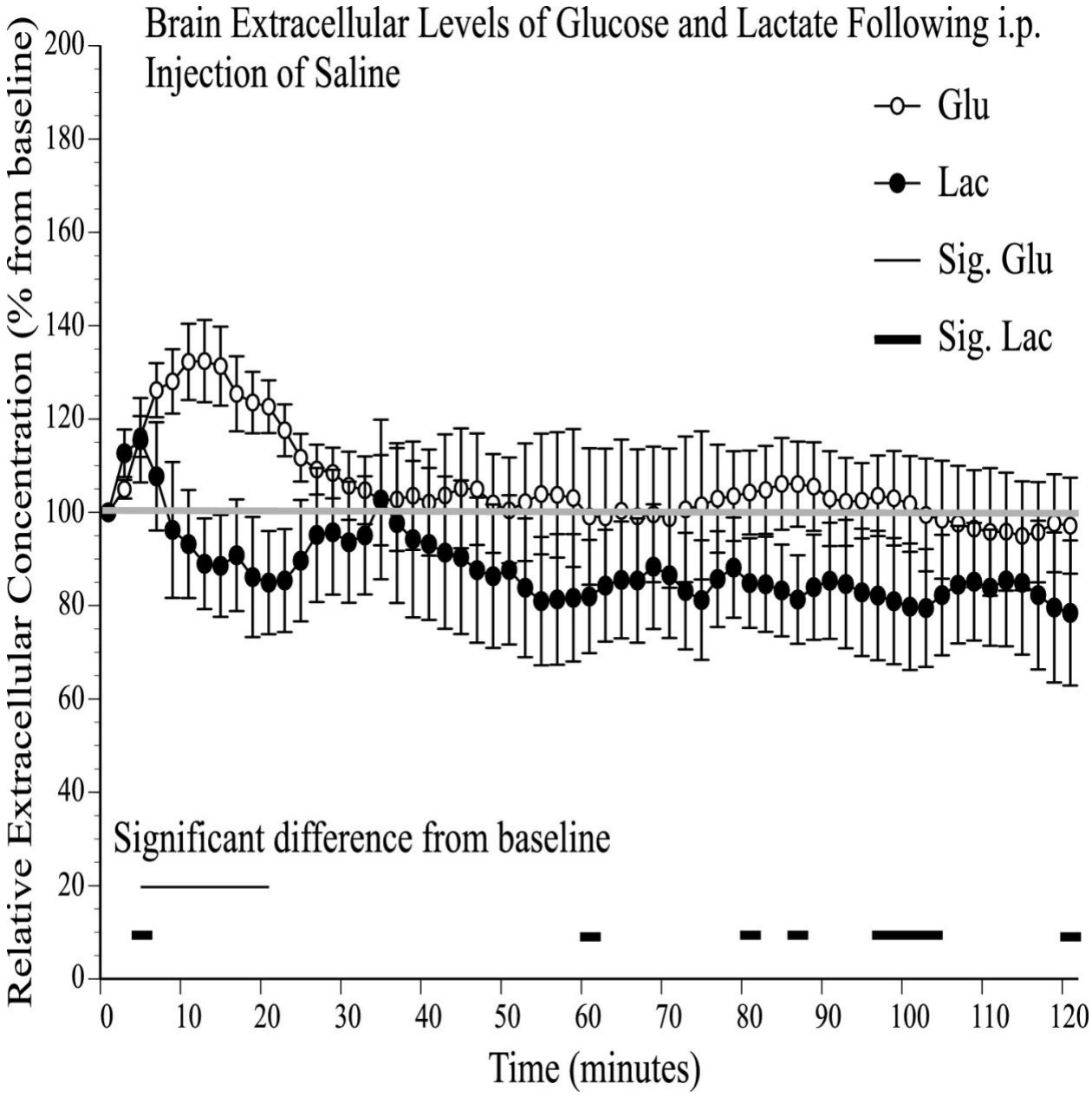
Figure 3 | Brain extracellular glucose and lactate fluctuations following a saline injection



The figure shows the relative changes in extracellular brain glucose and lactate following an i.p. injection of saline 0.9% (n = 4). Values are expressed as a percent of their baseline prior to injection. The bars on each point indicate the standard error of the mean. Time zero corresponds to the i.p. injection. The horizontal lines below the graph represent significant ($p \leq 0.05$) differences for extracellular glucose (thin) and lactate (thick) when compared to their corresponding baselines.

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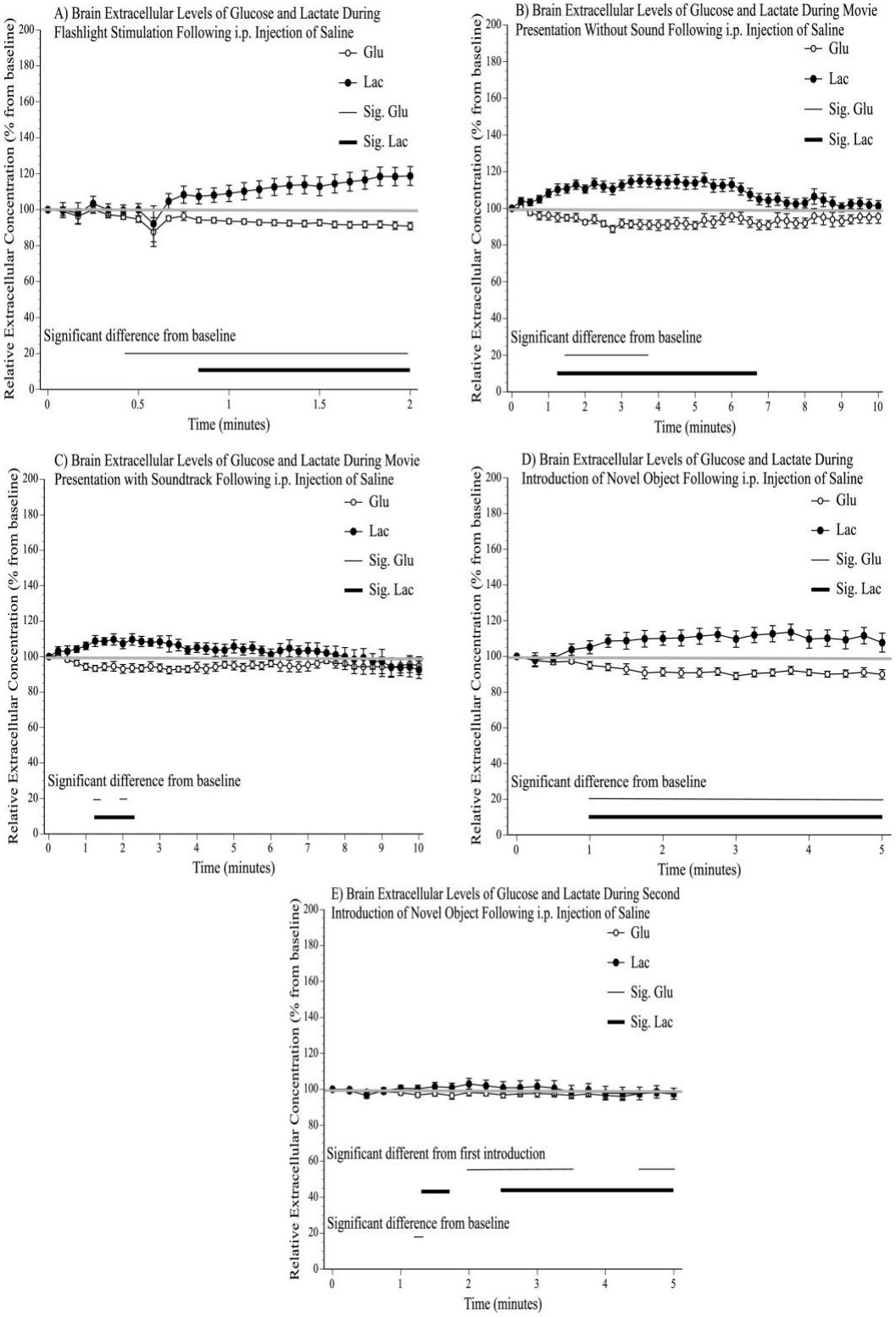
Figure 4 | Brain extracellular glucose and lactate fluctuations during visual stimulation following a saline injection



Following an i.p. saline injection, the panels show the relative changes in extracellular brain glucose during a ***(A)*** *2 min flashlight stimulation (n = 10),* ***(B)*** *10 min presentation of the movie "The secret of NIMH" without soundtrack (n = 9),* ***(C)*** *10 min presentation of the movie "The secret of NIMH" with soundtrack (n = 5),* ***(D)*** *5 min presentation of a novel object (n = 9) and* ***(E)*** *5 min reintroduction of the previously novel object 25 min later (n = 9). Values are expressed as a percent of their baseline taken prior to introduction of the visual stimulation. The bars on each point indicate the standard error of the mean. Time zero is the start of the visual stimulation (Panel A, B and C) or introduction of the object in the testing cage (Panel D and E). The horizontal lines below the graph represent significant ($p \leq 0.05$) differences for extracellular glucose (thin) and lactate (thick) when compared to their corresponding baselines or previous introductions.*

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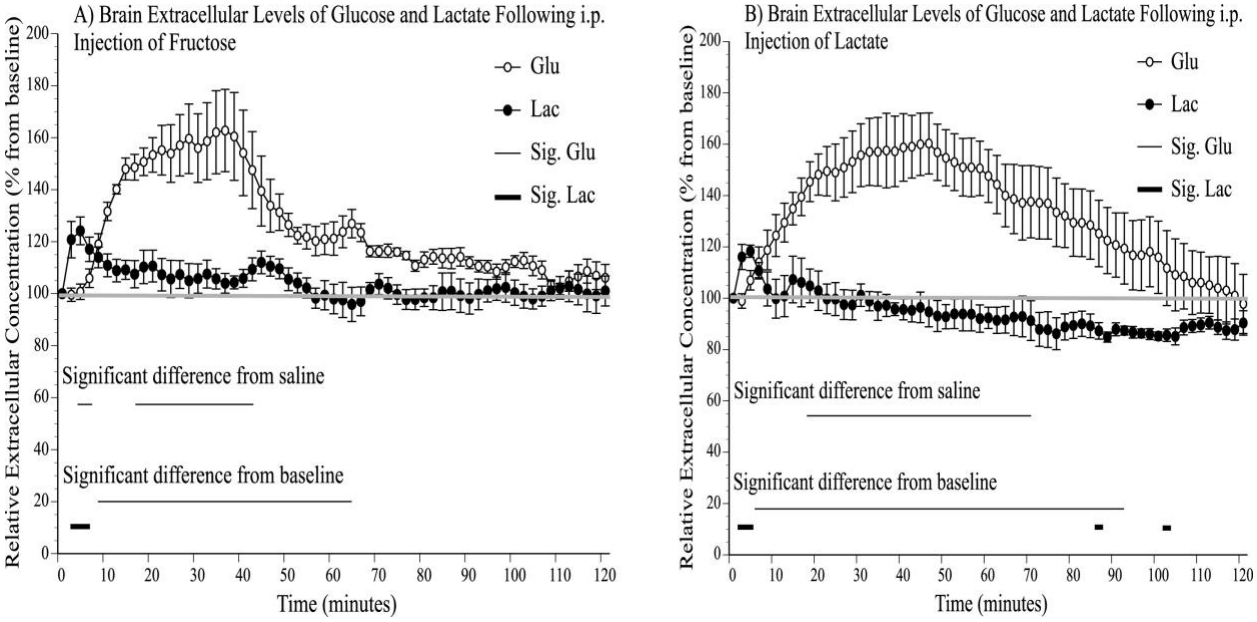
Figure 5 | Relative changes in extracellular brain glucose and lactate following a fructose or lactate injection



Relative changes in extracellular brain glucose and lactate following an i.p. injection of ***(A)*** *2 g/kg of fructose (n = 5) or* ***(B)*** *2 g/kg of lactate (n = 5). Values are expressed as a percent of their baseline taken prior to injection. The bars on each point indicate the standard error of the mean. Time zero corresponds to the end of the i.p. injection. The horizontal lines below the graph represent significant ($p \leq 0.05$) differences for extracellular glucose (thin) and lactate (thick) when compared to their corresponding baselines or the saline injection.*

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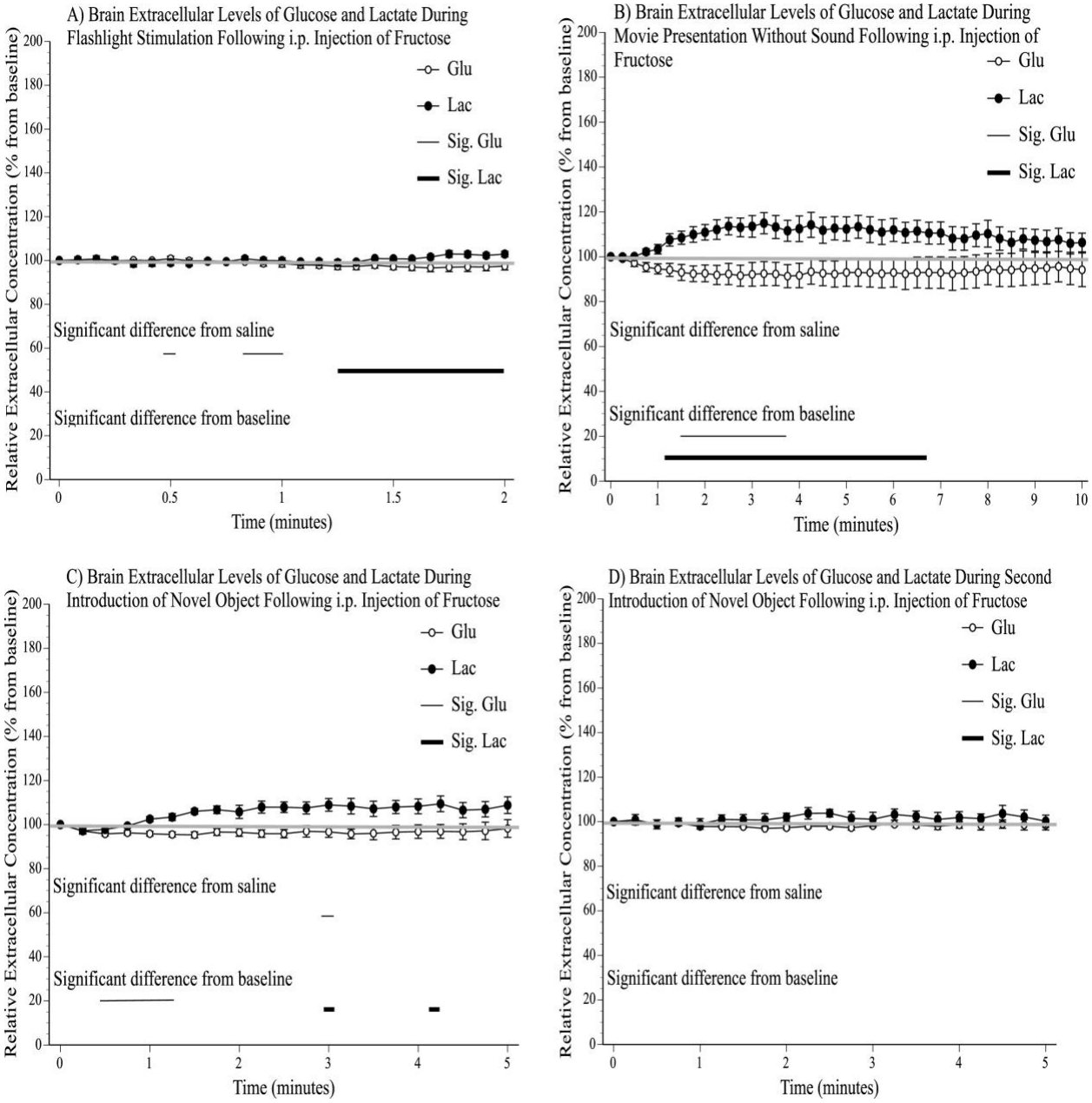
Figure 6 | Brain extracellular glucose and lactate fluctuations during visual stimulation following a 2g/kg i.p. fructose injection



Brain extracellular glucose and lactate fluctuations during visual stimulation following a 2g/kg i.p. fructose injection. The panels show the relative changes in extracellular brain glucose during ***(A)*** *a 2 min flashlight stimulation (n = 7),* ***(B)*** *a 10 min presentation of the movie "The secret of NIMH" without soundtrack (n = 5),* ***(C)*** *a 5 min presentation of a novel object (n = 5) and* ***(D)*** *a 5 min reintroduction of the previous object 25 min later (n = 5). Values are expressed as a percent of their baseline taken prior to introduction of the visual stimulation. The bars on each point indicate the standard error of the mean. Time zero corresponds to the start of the*

visual stimulation (Panel A and B) or introduction of the object in the testing cage (Panel C and D). The horizontal lines below the graph represent significant ($p \leq 0.05$) differences for extracellular glucose (thin) and lactate (thick) when compared to their corresponding baselines or saline conditions.

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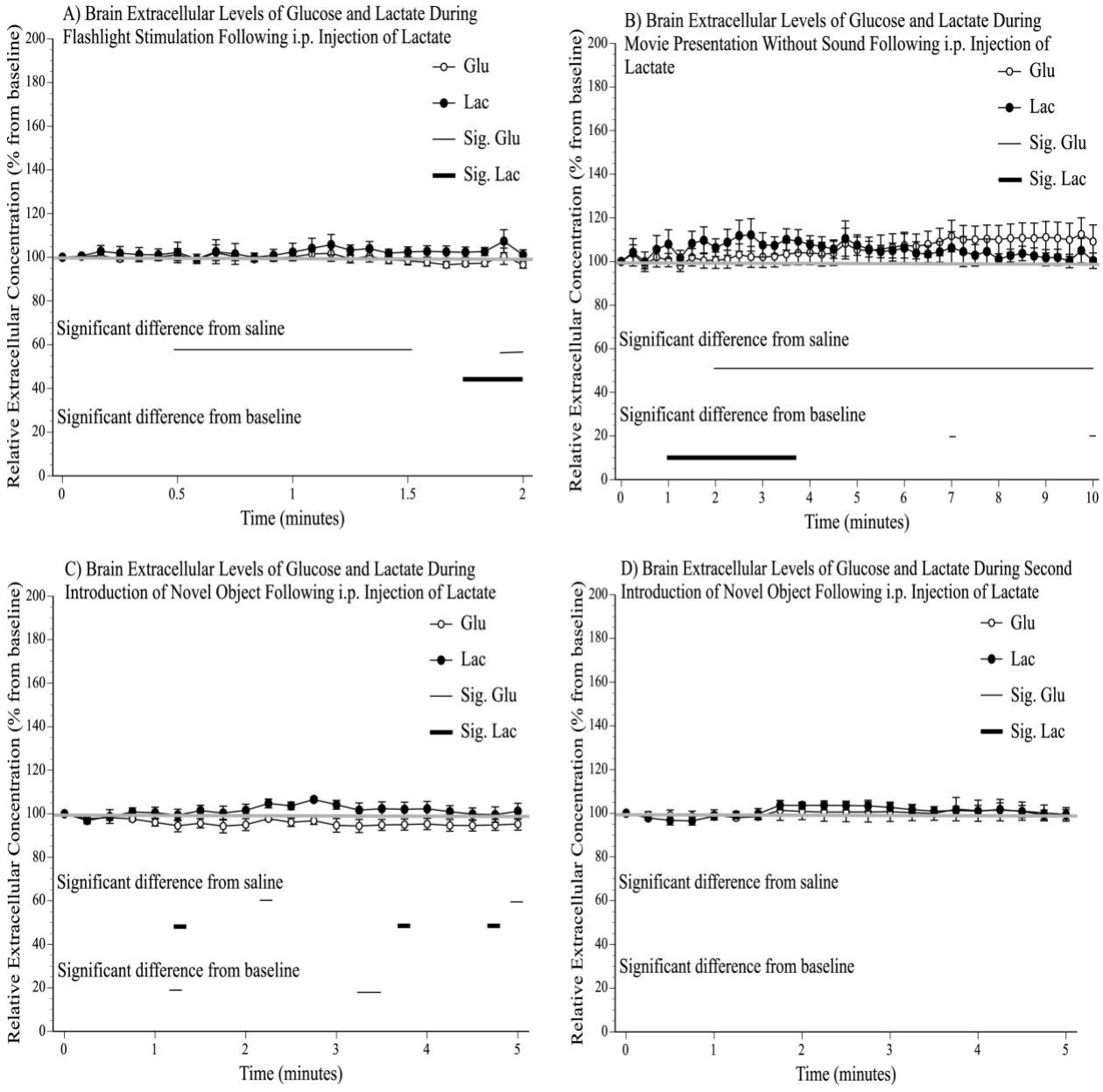
Figure 7 | Brain extracellular glucose and lactate fluctuations during visual stimulation following a 2 g/kg i.p. lactate injection



Brain extracellular glucose and lactate fluctuations during visual stimulation following a 2 g/kg i.p. lactate injection The panels show the relative changes in extracellular brain glucose during ***(A)*** *a 2-min flashlight stimulation (n = 7),* ***(B)*** *a 10-min presentation of the movie "The secret of NIMH" without soundtrack (n = 5),* ***(C)*** *a 5-min presentation of a novel object (n = 5) and* ***(D)*** *a 5-min reintroduction of the previous object 25 min later (n = 5). Values are expressed as a percent of their baseline taken prior to introduction of the visual stimulation. The bars on each point indicate the standard error of the mean. Time zero is the start of the visual stimulation (Panel A and B) or introduction of the object in the testing cage (Panel C and D). The horizontal*

lines below the graph represent significant ($p \leq 0.05$) differences for extracellular glucose (thin) and lactate (thick) when compared to their corresponding baselines or saline conditions.

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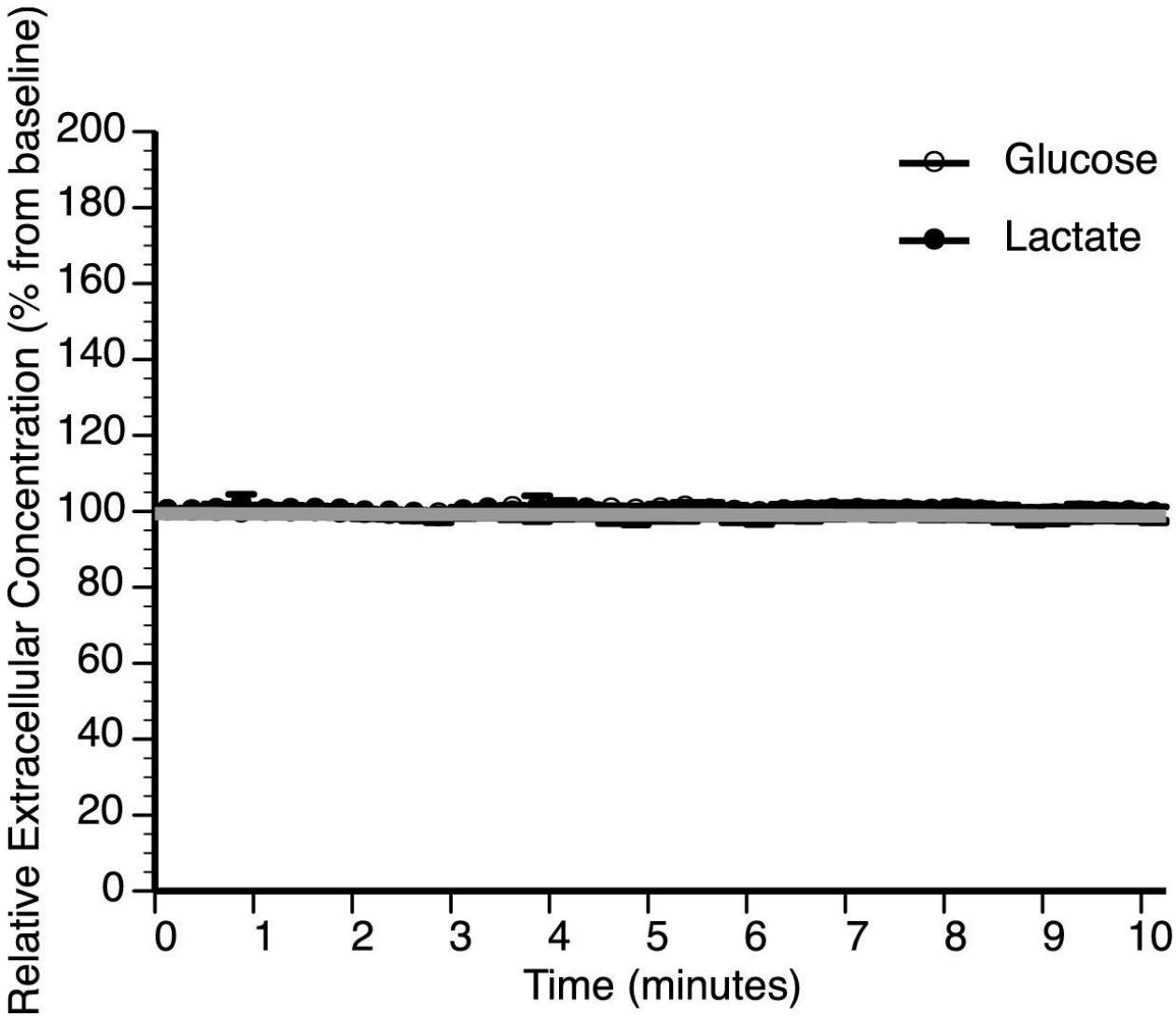
Figure 8 | Relative changes in brain extracellular glucose and lactate fluctuations in the visual cortex during one of the pause between visual stimulation conditions



Relative changes in brain extracellular glucose and lactate fluctuations in the visual cortex during one of the pauses between visual stimulation conditions.

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Chapter VI: Impact of Peripheral Glucose and Monocarboxylate Transporter Inhibition on Mouse Cortical Extracellular Glucose and Lactate

Author contributions: AB-M conceptualization, data curation, formal analysis, writing – original draft, writing – review and editing. CM formal analysis, funding acquisition, supervision, writing – original draft, writing – review and editing.

Abstract

Due to its extremely finite energy reservoir, the brain is heavily reliant on blood-borne metabolites and their delivery through the blood-brain barrier to meet its regional and transient bursts of energetic needs. Despite this reliance, few studies investigating the controversial question of neuronal lactate use measure the contribution of peripheral metabolites. In this study, we investigated the impact of peripheral transporter inhibition on cortical extracellular metabolite fluctuations. The glucose (GLUT1; WZB117) and monocarboxylate (MCT1; AZD3965) transporter inhibitor, were administered intraperitoneally to adult male mice. Subsequently, cortical extracellular glucose and lactate in the primary motor cortex were monitored in awake mice with the use of biosensors. Extracellular metabolite fluctuations were assessed following motor behaviors and metabolic challenges (intraperitoneally administered glucose, lactate or fructose). Dual inhibition of both GLUT1 and MCT1 resulted in an immediate increase in endothelial MCT1 protein expression, elevated blood lactate and extracellular brain glucose. MCT1 inhibition reduced extracellular lactate during motor behaviors, implicating peripheral lactate and blood-brain barrier transporter in activity-induced increases in cortical lactate. Finally, western-blot measures revealed that endothelial MCT1 and GLUT1 expression can be altered within minutes of inhibition. These findings suggest an important contribution of endothelial MCT1 and blood lactate to extracellular glucose pools and possible glucose-sparing mechanisms as well as hint to preferential upregulation of monocarboxylate use and transport during times of reduced transport capacity or metabolite availability. Overall, this work highlights the highly adaptable nature of the blood-brain barrier metabolite transport systems.

Keyword: *AZD3965, WZB117, Biosensor, Western Blot, Brain, Blood, Glucose, Lactate, Fructose, Primary Motor Cortex, Endothelial Cell, Blood Brain Barrier*

Introduction

Blood-brain barrier transporters: Connecting cerebral need with vasculature

The brain is a metabolically demanding organ, in large part due to the energetic requirements of signaling (Howarth et al., 2012), and possesses extremely limited internal energy stores (Rich et al., 2019). Combined, this makes the brain's biological and cognitive functions particularly sensitive to altered metabolite availability and energy deficits (Veys et al., 2020). Thus, to meet its global as well as regional and transient metabolic needs (Palombit et al., 2022), the brain must receive an uninterrupted and modulable supply of energy. This is achieved through the closely interconnected neurovascular unit where byproducts of signaling quickly induce changes in local cerebral blood flow and delivery of metabolites (Feng et al., 2004; Harders et al., 1989). Among other mechanisms, this activation-induced signaling modulates the blood-brain barrier (BBB) (Bélanger et al., 2011; Kaplan et al., 2020; Mason, 2017).

The BBB, through its endothelial tight junctions that make up the vascular wall, strictly regulates the movement of most substances, including metabolic substrates, from the blood to the brain (Reese & Karnovsky, 1967; Zhao et al., 2015). Therefore, metabolite transport across the BBB requires high endothelial expression of different transporter proteins to facilitate selective movement of substrates into and out of the central nervous system (Abbott et al., 2010; Ayloo & Gu, 2019; Zhao et al., 2015). Specifically, dedicated transporters are required to facilitate the movement of glucose and lactate, two important energy sources (Karagiannis et al., 2021), into the brain (Adijanto & Philp, 2012; Halestrap, 2012; Holman, 2020; Mueckler & Thorens, 2013; Pierre & Pellerin, 2005; Yan, 2017). Endothelial cells predominantly express the first isoform of the glucose (GLUT1) and monocarboxylate (MCT1) trans-membrane transporter proteins. GLUT1 and MCT1 transport glucose and lactate, respectively, along their concentration gradient

(Halestrap & Wilson, 2012; Brown, 2000). Due to this restrictive passage and the brain's high metabolic rate, blood levels of both glucose and lactate typically exceed that of the brain, setting a gradient that favors transport of these substrates from blood to the brain (van Hall et al., 2009; Duelli & Kuschinsky, 2001).

Endothelial transporter plasticity

To meet the fluctuating metabolic needs of the brain, metabolite delivery to the brain must be adaptable. This can take many forms, including increased hepatic glucose production Frizzell et al., 1993; Marty et al., 2007; Pocai, Lam, et al., 2005; Pocai, Obici, et al., 2005; Sprague & Arbeláez, 2011; Winnick et al., 2016), flexibility in the relative contribution of various metabolic pathways to energy production (Bruce et al., 2017; Mattson et al., 2018; Monsorno et al., 2022; Pellerin & Magistretti, 2004; Schurr et al., 1997) and, importantly for this work, by altering transporter expression (Allen & Messier, 2013; Cura & Carruthers, 2010; Gerhart et al., 1997; Matsui et al., 2017; Pellerin, Pellegri, Martin, et al., 1998; Pifferi et al., 2007; Takimoto & Hamada, 2014). Endothelial GLUT1 and MCT1 transporters are highly plastic and responsive to events such as metabolic stress and diet (Cura & Carruthers, 2010; Gerhart et al., 1997; Pifferi et al., 2007; Düking et al., 2022), circulating glucose (Devaskar et al., 1991) and cellular pH concentrations (Uhernik et al., 2011). Within the brain, the transporter changes in response to varying conditions are not exclusive to the endothelial cells (Macheda et al., 2005; Zhang et al., 1999; Pellerin, Pellegri, Martin, et al., 1998; Allen & Messier, 2013; Matsui et al., 2017; Takimoto & Hamada, 2014; Düking et al., 2022).

Metabolite transport in the brain

Through endothelial BBB transporters, metabolites either cross into the extracellular space of the brain or into astrocytic end-feet (see recent evidence suggesting that astrocytic

endfeet are not as ubiquitously present on brain capillaries as previously thought (Korogod et al., 2015). Once in the extracellular space of the brain, metabolites are taken up by local cells, namely neurons and astrocytes, through their own respective glucose (GLUT3, neurons; GLUT1, astrocytes) and lactate (MCT2, neurons; MCT1 & MCT4, astrocytes) transporters (Halestrap & Wilson, 2012; Mueckler, & Thorens, 2013).

The extracellular metabolite pools are derived primarily from blood-borne glucose (Barros & Deitmer, 2010) as well as blood-borne and endogenously created lactate (Boumezbeur et al., 2010; Dienel, 2012; Roumes et al., 2021). Previously, we demonstrated that altering the systemic (blood) availability of various metabolites (i.e. glucose, lactate, fructose, β-hydroxybutyrate) resulted in a consistent pattern of metabolite fluctuation: elevated blood lactate and elevated extracellular brain glucose (Béland-Millar et al., 2017). In this work, we proposed possible hypotheses to explain this de-coupling between the peripheral (blood) and central (brain) metabolites. One such hypothesis was the glucose sparing hypothesis wherein we hypothesized that the elevated blood lactate was taken up and consumed by the brain, thereby sparing extracellular glucose use and resulting in its extracellular accumulation. Central to this idea is the contribution of blood-borne extracellular metabolites, namely lactate, to neuroenergetics, which is a contentious topic in it of itself (Aubert et al., 2005; Deitmer et al., 2019; Díaz-García & Yellen, 2019; Dienel, 2017, 2019b; Netzahualcoyotzi & Pellerin, 2020; Pellerin & Magistretti, 2012; Tang, 2018).

In the present experiments, we test this hypothesis and contribute to the on-going debate of lactate as a metabolic fuel. We measure the impact of pharmacological inhibition of GLUT1 and MCT1 on endothelial transporter expression as well as blood and extracellular brain glucose and

lactate during rest, behavioral tasks and following increased systemic availability of metabolites (i.e. glucose, lactate and fructose).

Materials and methods

Animals

One-hundred and sixty-two 14- to 16-week-old, male CD1 mice (Charles River Canada, St-Constant, QC, CAN) were individually housed and maintained on a reverse 12-h night/day cycle with lights on at 7 p.m. Mice had *ad libitum* access to standard chow (Teklad Global 18% Protein 2018, Teklad Lab Animal Diets, Envigo, ON, CAN) and water, unless otherwise specified. All testing was conducted during the night phase of the cycle with the use of dim red lighting. All procedures in this study adhered to Canadian Council on Animal Care guidelines and were approved by the University of Ottawa Animal Care Committee.

Extracellular Measurements of Cortical Metabolites

The general timeline for extracellular testing is summarized in **Figure 1A**.

Surgery. Pre-surgical treatment included a 0.05 mg/kg subcutaneous (s.c.) injection of buprenorphine hydrochloride (Reckitt Benckiser Healthcare, Hull, North Humberside, UK) and 1 ml of 0.9% saline (Hospira, QC, CAN). Immediately prior to surgery, mice were anesthetized using 4-5% isoflurane (Fresenius Kabi Canada Ltd., ON, CAN) then secured to a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Mice were maintained under anesthesia during the surgery with 1-2.5% isoflurane and kept warm with a heated pad (TP650, Gaymar Industries, Orchard Park, NY, USA). Based on the stereotaxic atlas of Franklin and Paxinos (2008), two guide cannulas (BASi cannulas Bioanalytical Systems, West Lafayette, IN) were positioned above the left and right primary motor cortex. The target coordinates, from Bregma, were ± 1.8 mm lateral and +1.1 mm anterior. The cannulas were positioned just beneath the skull surface so that the sensing cavity (1mm) of the electrochemical electrode would protrude, when inserted at the time of testing, and be situated within the primary motor cortex of the mouse. The

guide cannulas were fixed using a UV-polymerized compound (UV Clear Fly Finish, Ashland, OR, USA) applied around four 0.10-inch skull screws positioned anterior and posterior to the two guide cannulas. Post-surgical analgesia included application of 0.1 ml of transdermal bupivacaine (Chiron Compounding Pharmacy Inc., ON, CAN) around the incision area and s.c. injections of buprenorphine hydrochloride twice daily for 3 days. Mice were given 7-8 days to recover before testing.

Electrodes. The use and details of these commercially available electrodes have been extensively presented elsewhere (Kiyatkin et al., 2013; Kiyatkin & Wakabayashi, 2015; Wakabayashi & Kiyatkin, 2012; Wilson & Gifford, 2005). Briefly, extracellular brain levels of glucose and lactate were measured using two platinum enzyme-linked electrodes (7004-Glucose-C and 7004-Lactate-C; Pinnacle Technology Inc., Lawrence, KS, USA). Sensors were prepared from Pt-Ir wire of 180 µm diameter, wrapped concentrically with an AgCl reference electrode. Near the tip of the electrode, a 1 mm section is coated with either glucose or lactate oxidase. These enzymes oxidize the analytes of interest, resulting in a proportional release of hydrogen peroxide (H_2O_2). H_2O_2 is subsequently detected and recorded as an amperometric oxidation current at the electrode (Hu & Wilson, 1997b). Data was collected at 1 Hz, transmitted from a potentiostat to a computer and recorded using the Sirenia Acquisition Software (Pinnacle Technology Inc., Lawrence, KS, USA). In addition to the oxidase enzymes, the metabolite sensitive surface is also coated with a series of membranes to increase the specificity and selectivity of the electrodes. For example, the potential contribution of ascorbic acid is reduced with the presence of ascorbic acid oxidase. These particular enzymes convert the electroactive ascorbate, which has the potential to interfere with the electrode's recordings, to non-electroactive dehydroascorbate and water (Hu et al., 1994).

Calibration. Immediately prior to and following *in vivo* testing, the electrodes were calibrated according to factory recommendations. Briefly, the electrodes were gently placed in 100 mM PBS (pH 7.4) at 23 °C (pre-testing) or 37 °C (post-testing) and tested for metabolite sensitivity by adding glucose (1 mM) and lactate (0.1 mM) to the PBS solution, as well as selectivity with the use of a single ascorbic acid (0.25 mM) addition. Pre-calibration curves demonstrated high sensitivity with incremental linear increases for both glucose (average sensitivity 0.22 nA/1 mM), and lactate (average sensitivity 0.024 nA/0.1 mM) electrodes. Both glucose and lactate electrodes also exhibited high levels of selectivity by demonstrating minimal sensitivity to ascorbic acid (average sensitivity 0.32 nA/ 0.25 mM and 0.85 nA/0.25 mM, respectively). The post-calibration curves suggested a certain level of wear on the electrodes, most likely due to the insertion and removal process of these sensitive testing instruments. Similar results were obtained with linear increases for both glucose (average sensitivity 0.44 nA/1 mM) and lactate (average sensitivity 0.03 nA/ 0.1 mM) electrodes. Post calibration curves for ascorbic acid registered increased sensitivity (average sensitivity 3.42 nA/0.25 mM and 11.34 nA/0.25 mM for glucose and lactate, respectively).

Histology. At the end of testing, mice were lightly anesthetized using isoflurane and the electrodes carefully removed for post-calibration. Following calibration, the mice received an ip injection of sodium pentobarbital (65 mg/kg; MERK, Kirkland, QC, CAN) before being transcardially perfused with a 4% paraformaldehyde/0.2% picric acid solution (PFA). Brains were then post-fixed in the same PFA solution for 1 hour at 4 °C, then rinsed twice for an hour with a 10% sucrose solution. After the final rinse, the brain remained in 10% sucrose overnight and then was rinsed a final time 1 hour prior to freezing. The brains were frozen with the use of CO_2 and stored at −80 °C for later cryostat sectioning at 14 μm. Electrochemical electrode

placement within the primary motor cortex was confirmed with the use of the stereotaxic atlas of Franklin and Paxinos (2008).

Testing Procedures

Electrode Insertion. Following electrode calibration, mice were briefly anesthetized with 1.5-2% isoflurane and the electrodes were inserted into the motor cortex through the surgically implanted guide cannulas and attached to the pre-amplifier. Mice were tested in a custom-made square polymethyl methacrylate-testing cage with standard bedding (Teklad 7097 Corncob bedding, Envigo, ON, CAN) and water. A camera (Pinnacle Technologies) mounted above the cage recorded throughout the duration of the testing and was synchronized with the electrode sampling. Mice had free access to water, but chow was removed 2 hours prior to injection to ensure similar fasting states between the mice and returned following the last manipulation.

Injections. Approximately 2 hours following insertion, when the electrodes returned stable readings, mice received a 0.1 ml intraperitoneal (ip) injection of either saline (0.9%; Hospira, Montreal), vehicle (1:1 DMSO and dH_2O solvent; Sigma, MO, USA), glucose transporter 1 inhibitor (WZB117; 20 mg/kg; MedChemExpress, NJ, USA), monocarboxylate transporter 1 inhibitor (AZD3965; 30 mg/kg; MedChemExpress, NJ, USA), or both inhibitors together. Three hours following the injection of the vehicle or inhibitors, the mice received a 2 g/kg ip injection of either glucose (Dextrose Anhydrous, EMD Chemicals, Gibbstown, NJ, USA), lactate (Lactic Acid, Acros Organics, NJ, USA) or fructose (D-Fructose, Fisher Scientific, Fair Lawn, NJ, USA). Extracellular data was collected for three days. Each testing day consisted of two injections, 3 hours apart, and two sets of behavioral manipulations 2 hours after each

injection. Mice always received the same inhibitor treatment; however, the metabolite injections were counterbalanced.

A 50% DMSO/dH_2O solution was chosen as a vehicle as it was the only solvent capable of effectively dissolving both transporter inhibitors and was only efficient in doing so at a 50% dilution. The GLUT1 inhibitor (WZB117) dose (20 mg/kg) was chosen to emulate the concentration used in other studies that demonstrated its effectiveness to inhibit glucose uptake in cancer tumors. The 20 mg/kg dose used in this study was higher than the doses reported in other studies (Liu et al., 2012; Suzuki et al., 2018; Wei et al., 2018) but did not exceed the highest *in vivo* use of this GLUT1 inhibitor (Shibuya et al., 2014). Given how well this inhibitor was tolerated in previous studies when injected daily, we felt comfortable with the higher dosage. Mice receiving the GLUT1 inhibitor WZB117 received a single injection due to its irreversible nature (Liu et al., 2012). Unlike the GLUT1 inhibitor, the MCT1 inhibitor (AZD3965) is typically administered orally at a concentration of 100mg/kg (Bola et al., 2014; Guan et al., 2019; Guan & Morris, 2019; Noble et al., 2017; Polański et al., 2014). For consistency and given that the tethered experimental set up did not allow for gavage, we opted for an intraperitoneal injection. Since the quantity of drugs reaching the blood is typically much higher following an intraperitoneal injection than oral gavage, we used a lower dosage (30 mg/kg). Due to its reversible nature (Guan et al., 2019), the MCT1 inhibitor AZD3965 was re-injected daily. The doses used for the ip injection of metabolites (glucose, lactate and fructose) was chosen based on previous results that demonstrated the ability of 2 g/kg of sugar to improve memory (Messier & White, 1987). Additionally, this dosage corresponds to the amount of sugar these animals readily ingest (Messier & White, 1984).

Behavioral Testing. The impact of the transporter blockers on the extracellular glucose and lactate changes produced in the motor cortex by two behaviors was measured. We have previously observed that extracellular lactate levels increased, and glucose levels decreased when mice engaged in sustained motor behavior (i.e. running wheel). However, running behavior and the induced fluctuations in extracellular glucose and lactate were inconsistent, both within and across mice and the relative effects were often small (Béland-Millar & Messier, 2022). We developed and used two motor behaviors that induced larger extracellular lactate and glucose changes, could be better controlled and were compatible with the tethered equipment: the vertical hold and spinning behaviors, detailed below.

Vertical Hold. In this task, the mouse was placed on a vertical metal mesh (10 x 17 inches) with half-inch square holes. The mesh was held vertically and still as the mouse was free to explore for 2 minutes. This was initially used a control for the following behavior in which the mesh was moved. As the mice habituated to this task, they tended to orient and move towards the top of the mesh and spend more time there.

Spinning. The mouse was placed on the same mesh as previously described but for this task, the mesh is slowly spun around in the vertical plane which led the mice to re-orient themselves towards the top of the mesh. A mouse was initially spun at the rate of 1 full rotation per 4 seconds. After completing 4 full rotations, the mouse was then rotated counterclockwise at the same rate. Once another 4 rotations have been completed, the mouse was once again spun clockwise – however, this time a full rotation is completed every 2.5 seconds for another 4 rotations before completing the same conditions counter-clockwise. In summary, the rotations were done in sets of 4; the first two sets being slower, the last two being faster and switching

between clockwise and counterclockwise rotations between each set of four rotations. This pattern was repeated for 2.5 minutes after which the mouse was returned to its cage.

Those particular speeds were chosen following pilot experimentation. At slower speeds, most mice were able to continue their normal pattern of behavior and exploration, seemingly undisturbed by the spinning and reaching the top of the mesh without issue. At higher speeds, most of the mice fell off the mesh, unable to hold on. During the slower testing (4 seconds per full rotation), mice attempted to re-orient themselves to face the top of the mesh and a few even explored the mesh further, managing to reach the top. However, during the faster spinning (2.5 seconds per full rotation), the mice continued to attempt to reorient themselves towards the top of the mesh but were often unable to do so effectively. They remained in the middle of the mesh, with little exploration towards the top but did not typically fall.

Grip-Strength. During task development trials, mice that received the combined glucose and lactate transport inhibitor injections were more likely to fall from the mesh, particularly during the spinning. It was hypothesized that the inhibition of glucose and lactate transporters resulted in an energy deficiency, possibly leading to muscle fatigue and/or poor muscular performance. We tested this theory by using a grip strength meter (model # 1027SM; Columbus Instruments, Columbus, OH) following the injection of the inhibitors. The grip-strength meter measured kilograms of force following fore and hind limb pull. Two hours after each injection, the mice were tested 5 times and the results were averaged to a single value to counteract small variations in placement, pull speed, etc. Contrary to our hypothesis, grip-strength performance was increased for all inhibition groups when compared to controls (**Figure 2**).

Blood Sampling

To limit the likelihood of damaging the tethered equipment if the mice struggled during blood sampling, we measured blood levels of glucose and lactate in different mice than those used to measure extracellular cortical glucose and lactate. The measures were taken under the same conditions used to measure brain extracellular glucose and lactate. Each mouse was also tested for three days with an identical timeline for the inhibitor and metabolite injections, the latter being counterbalanced. The general timeline for blood sampling is summarized in Figure 1B.

Prior to testing, all mice were transferred to a reverse light cycle testing room to acclimate for two hours. Food was also removed at this time. Blood samples were obtained by gently piercing the dorsal tail vein with a 23-gauge needle (Becton, Dickinson and Company, Franklin Lakes, NJ) and massaging the tail for blood extraction. To avoid measuring pooled blood, the first blood drop was not used. Plasma glucose and lactate concentrations (mmol/L) were measured with a glucose (AccuChek, Aviva, Roche Diagnostics, Mannheim, Germany) and lactate (StatStrip Xpress, Nova Biomedical, Innovation House, Runcorn, Cheshire, UK) meter, respectively. A single glucose and lactate baseline measure was taken at the end of the acclimatization period, after which the mice received a 0.1 ml ip injection of either saline (0.9%; Hospira, Montreal), DMSO vehicle (1:1 DMSO and dH_2O solvent; Sigma, MO, USA), glucose transporter 1 inhibitor (WZB117; 20 mg/kg; MedChemExpress, NJ, USA), monocarboxylate transporter 1 inhibitor (AZD3965; 30 mg/kg; MedChemExpress, NJ, USA), or both inhibitors. Mice receiving the glucose transporter inhibitor (WZB117) received only one injection because it has been shown to be an irreversible transporter blocker (Liu et al., 2012), whereas the monocarboxylate transporter inhibitor (AZD3965) was re-injected daily as it is considered a reversible blocker (Guan et al., 2019; Guan & Morris, 2019). Three hours after the injection of

the vehicle or inhibitors, the mice received a 2 g/kg ip injection of either glucose (Dextrose Anhydrous, EMD Chemicals, Gibbstown, NJ, USA), lactate (Lactic Acid, Acros Organics, NJ, USA) or fructose (D-Fructose, Fisher Scientific, Fair Lawn, NJ, USA). Blood glucose and lactate samples were taken 15, 30, 60, 90 and 120 minutes after the injections.

Western Blot Procedures

Tissue Preparation. Mice were injected with a transport inhibitor(s) either 5 mins , 4 h or 24 h prior to prior to sodium pentobarbital injection. Following the sodium pentobarbital injection, mice were transcardially perfused through the left ventricle with ice-cold saline (0.9%). Brains were rapidly removed, and the cortex isolated on ice. Mice either received DMSO vehicle (1:1 DMSO and dH_2O solvent; Sigma, MO, USA), AZD3965 (monocarboxylate transporter 1 inhibitor, 30 mg/kg, MedChemExpress, NJ, USA), WZB117 (glucose transporter 1 inhibitor, 20 mg/kg, MedChemExpress, NJ, USA) or both inhibitors.

Immediately after isolation of the cortex, tissue was homogenized using a combination of mechanical dissociation with a pipette and the use of a neural dissociation kit (Miltenyi Biotec, California, USA, # 130-092-628). The cell suspension was then depleted of myelin (Miltenyi Biotec, California, USA, #130-096-733) and CD45+ (Miltenyi Biotec, California, USA, #130-052-301) cells with the use of magnetic microbeads, following manufacturer instructions. Subsequently, CD31-positive cells (endothelial; Miltenyi Biotec, California, USA, #130-097-418) were isolated from this cell suspension using magnetic microbeads following manufacturer instructions. The isolated CD31+ cells were stored in PBS at -80 °C until further processing.

The CD31+ cells were isolated by centrifugation (2000g for 10 minutes at 4 °C; Sorvall RC-6+, Thermo Fisher, NC, USA, #46910) and the pellet resuspended in RIPA buffer with protease inhibitors (1:100; Protease Inhibitor Cocktail, Sigma, MO, USA, #P8340). The cell

suspension was homogenized via ice-cold sonication (Ultrasonic Cleaner, Codyson, China, model #CD-4820), maintained on ice for 10 minutes and then the proteins were extracted from the supernatant following centrifugation (19,000g for 20 minutes at 4 °C). The protein concentration of samples was quantified using Pierce BCA Protein assay (kit #23227, Thermo Scientific, Illinois, USA). The general timeline for tissue preparation is summarized in Figure 1C.

Western Blot. Approximately 3 µg of protein were loaded into the wells of SDS-acrylamide gels (GX Stain-free FastCast 12% Biorad gels, Bio-Rad, ON, CAN, #1610184) and separated by a constant current of 100 V, cooled to 15 °C (ThermoCube, Ted Pella, Inc., CA, USA, model #50062), for 120 minutes (mini-PROTEAN 3 Dodeca Cell, Bio-Rad, ON, CAN, #165-4100). Proteins were then transferred to a nitrocellulose membrane (0.2 µm, Bio-Rad, ON, CAN) overnight at 10 V (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, ON, CAN). After blocking with 5% skim milk in 10 mM PBS for 1 hour at room temperature, membranes were incubated with primary antibodies in TBST (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20) overnight at 4 °C. The following morning, membranes were washed with TBST and incubated at room temperature for 1 hour with the appropriate secondary antibodies, also diluted in TBST. After a final wash, membranes were imaged using an Odyssey Imaging system (Li-Cor, Nebraska, USA, model #9120). For appropriate relative quantification of the bands, total protein quantification was calculated using UV light activation (Chemi Doc XRS, Bio Rad, ON, CAN, model Universal hood II) of the Bio-Rad stain-free gels following the electrophoresis stage.

Antibodies. Primary antibodies were a rabbit anti-GLUT1 (1/5000, Millipore, #07-1401, lot #3167016) and a rabbit anti-MCT1 (1/200, Alomone, #AMT-011, lot #AU0202). A rabbit anti-vascular endothelial growth factor receptor 2 (VEGFR-2, Cell Signaling, catalogue #55B11)

was used as a positive control for endothelial cells, whereas a mouse anti-GFAP (Millipore, 1/4000, #MAB360, lot #3244400) was used as a negative control for purity of the extraction. A mouse (Invitrogen, #825464) or rabbit (LI-COR, #B80731-02) secondaries was used.

Statistical Analysis & Relative Measures

All extracellular and blood (glucose and lactate) data were standardized by dividing the readings by their respective pre-event baseline and multiplying the result by 100 to obtain relative percentage change from baseline. For extracellular glucose and lactate, consecutive measurements were averaged to obtain a mean value every 2, 4, 6 or 8 seconds for behaviors (running and effortful behaviors) as well as every 2 minutes for the 2-hour post-injection readings. Glucose and lactate were analyzed separately with a between-within (split-plot) mixed ANOVA ($\alpha = 0.05$), and the data obtained for each injection was compared to the control groups as well as baseline. Unless otherwise stated, the data met the respective assumptions of the analysis. If sphericity or homogeneity was violated, a Greenhouse-Geisser correction was applied. Due to the a priori decision and interest in differences across groups, pairwise comparison tests ($\alpha = 0.05$) were used in order to compare the experimental groups to controls as well as compare the experimental groups amongst themselves despite non-significant omnibus results.

Grip-strength results were analyzed using a one-way ANOVA. Unless otherwise stated, the data met the respective assumptions of the analysis. Significant omnibus results were followed up with pairwise comparisons ($\alpha = 0.05$).

Western blot images were analyzed with the Odyssey Li-Cor software. The bands were normalized across gels using the pre-stained protein ladder (Chameleon® Duo, Li-Cor, USA).

Western blot measures of GLUT1 and MCT1 were analyzed using a mixed ANOVA, following up significant main effects with pairwise comparisons ($\alpha = 0.05$).

Though only results below the significance threshold ($p < 0.05$) will be considered "significant", other results near the significance threshold may be presented in order to better illustrate the impacts of this study[7]. All statistical analyses were performed using SPSS v. 23 (IBM).

[7] "Practices that reduce data analysis or scientific inference to mechanical "bright-line" rules (such as "$p < 0.05$") for justifying scientific claims or conclusions can lead to erroneous beliefs and poor decision making. A conclusion does not immediately become "true" on one side of the divide and "false" on the other."(Wasserstein & Lazar, 2016)

Results

Endothelial transporter expression

DMSO decreases endothelial GLUT1. Figure 3 presents the results of the western blot measurements of MCT1 and GLUT1 expression in cortical endothelial cells. Intraperitoneal DMSO injections progressively decreased the expression of both GLUT1 and MCT1 protein in cortical endothelial cells. The 0.1ml injection of a 50% DMSO solution decreased GLUT1 (p = 0.001) and MCT1 (p = 0.072) endothelial cell expression by approximately 50% within 24 hours. This decrease was significant as early as 4 hours post-injection for GLUT1 (p = 0.007). This was an unexpected finding that makes it more difficult to interpret the remaining results. However, in view of the absence of adequate inert vehicle solution, we continued experiments and considered this effect when interpreting the remaining results. Therefore, comparisons to DMSO were made to the time-respective condition (i.e. 4hr GLUT1 inhibition is compared to 4hr DMSO).

Given that DMSO has been shown to increase BBB permeability (Kleindienst et al., 2006) (though older studies would contradict this finding (Keane et al., 1977; Ziylan et al., 1988) as well as increase mitochondrial respiration (Nasrallah et al., 2008), the authors propose that this initial trend towards decreasing the expression of GLUT1 and MCT1 is a protective mechanism engaged by feedback loops to limit overactive mitochondrial respiration and consequently the damaging over production of reactive oxygen species.

MCT1 inhibition transiently decreased GLUT1 expression in cortical endothelial cells. MCT1 inhibition led to an immediate (time=0, ie, 5 minutes post-injection) 50% decrease in GLUT1 endothelial cell expression when compared to DMSO 0-hr (p = 0.001; **Figure 3B**), but subsequent increase 4- and 24-hrs post inhibitor injection (4 hours, p = 0.016; 24 hours, p = 0.005), when considered DMSO time-matched analyses. Though the mechanisms underlying the

initial decrease remain to be elucidated, we consider that MCT1 inhibition resulted in an eventual increase in endothelial GLUT1 expression to compensate for the reduced uptake of circulating monocarboxylates.

MCT1 inhibition had no observable effect on the MCT1 expression in cortical endothelial cells. Visual analysis of **Figure 3D** would suggest that MCT1 inhibition led to a moderate and immediate decreased in MCT1 endothelial cells expression, but this effect was not significant.

GLUT1 inhibition temporarily increased GLUT1 expression in cortical endothelial cells. GLUT1 inhibition increased endothelial GLUT1 expression for at least 4 hours post injection (0 hours, $p < 0.000$; 4 hours, $p = 0.025$; **Figure 3B**). The amplitude of the increased endothelial GLUT1 expression was attenuated through time (2-fold increase at 0hr, 1-fold increase at 4hr and 0.5-fold increase at 24hr), with levels at 24 hours post-inhibitor injection being comparable to the DMSO-attenuated expression.

GLUT1 inhibition had no statistically significant effect on the MCT1 expression in cortical endothelial cells. Though a visual analysis of **Figure 3D** would suggest that GLUT1 inhibition led to an immediate increase in MCT1 endothelial cells expression, this effect was not statistically significant.

Combined GLUT1 and MCT1 transporter inhibition results in stable GLUT1 but a 4-fold increase MCT1 expression in cortical endothelial cells. The inhibition of both MCT1 and GLUT1 transporters immediately increased MCT1 expression in endothelial cells (0hr, $p < 0.001$; **Figure 3D**) almost 4-fold. Following this initial increase, MCT1 endothelial cell expression returned to and remained at DMSO time-matched levels (4hr, $p = 0.508$; 24hr, $p = 0.203$).

GLUT1 endothelial cell expression, on the other hand, was not immediately increased by the combined inhibition of GLUT1 and MCT1 transporters (**Figure 3D**) but remained significantly above time-matched DMSO levels 4- ($p = 0.026$) and 24-hours ($p = 0.003$) post-injection, suggesting a delayed effect of this dual inhibition on GLUT1.

Summary. DMSO had an unexpected effect of reducing GLUT1 expression in endothelial cells. MCT1 blockade increased GLUT1 expression as early as 4 hours, while it had little effect on MCT1 transporter expression. GLUT1 blockade appeared to increase GLUT1 expression for at least 4 hours. The combined blockade of GLUT1 and MCT1 increased GLUT1 expression as early as 4 hours and for up to 24 hours while the same combined blockade produced a large but short-lived increase in MCT1 expression.

Effect of saline, DMSO and transport inhibitor injections on blood and extracellular glucose and lactate

Primary Motor Cortex.

Saline. The control saline injection (**Figure 4A**) induced an early rise in both extracellular lactate and glucose. We have observed these effects previously and have demonstrated they are associated with the injection procedure (Béland-Millar et al., 2017). Following the initial rise, there was a gradual and continuous return to baseline for both brain extracellular glucose and lactate.

DMSO. The overall effect of DMSO (**Figure 4B**) was not significantly different from the effect of the saline injection (**Figure 4A**), for either extracellular lactate ($p = 0.358$) or glucose ($p = 0.267$). However, when compared to the saline injection, DMSO produced a longer increase in brain glucose and a longer decrease in lactate from baseline.

Inhibitors. The injection of the MCT1 inhibitor, either alone (MCT1, $p = 0.009$) or combined with the GLUT1 inhibitor (MCT1 and GLUT1, $p = 0.024$), increased brain extracellular lactate above the values observed for the DMSO control (**Figure 4C** and **Figure 4E** - shaded squares). The increase in extracellular lactate above baseline levels occurred approximately 35 minutes later in the dual inhibition condition. MCT1 inhibition also decreased extracellular glucose ($p = 0.065$; **Figure 4C** – shaded squares) when compared to DMSO, resulting in a dip below extracellular values approximately 50 minutes post-injection. No other inhibitor condition reduced extracellular glucose values below baseline.

Inhibition of GLUT1 transporters did not significantly impact extracellular glucose or lactate levels when compared to DMSO levels (**Figure 4D**).

Individual MCT1 or GLUT1 inhibition reduced or abolished the initial increase in extracellular glucose induced by DMSO (**Figure 4**) with dual inhibition increasing extracellular glucose level beyond those observed in the MCT1 ($p = 0.003$) or GLUT1 ($p = 0.043$) inhibition.

Summary – inhibitor impact on extracellular glucose and lactate levels. When compared to the DMSO vehicle, MCT1 inhibition increased brain extracellular lactate (independently or combined with GLUT1 inhibition) and MCT1 inhibition reduced extracellular glucose below baseline and below levels observed following a vehicle injection.

Blood. Figure 5 and **Table 1A** present and summarize blood glucose and lactate levels following control, vehicle and inhibitor injections. The control DMSO injection induced a large, immediate and sustained rise in blood lactate as well as a smaller, early and sustained decrease in blood glucose (**Figure 5B**). When compared to the saline group (**Figure 5A**) the DMSO injection did not significantly alter blood glucose levels ($p = 0.118$) but did significantly increase blood lactate ($p = 0.033$).

All transport inhibitor conditions (**Figure 5**) attenuated both the DMSO-induced rise in blood lactate (MCT1, $p = 0.001$; GLUT1, $p = 0.019$; MCT1 & GLUT1, $p = 0.017$ – shaded squares) and the decrease in blood glucose (MCT1, $p = 0.076$; GLUT1 $p < 0.001$; MCT1 & GLUT1, $p = 0.082$ – shaded squares). GLUT1 inhibition increased blood glucose values above baseline levels, as well as above the levels observed in both other inhibition conditions (MCT1, $p = 0.011$; MCT1 & GLUT1, $p = 0.010$).

Summary – inhibitor impact on blood glucose and lactate levels. Transporter inhibition attenuates both the DMSO induced rise in blood lactate and decrease in blood glucose, and GLUT1 inhibition increases blood glucose levels above baseline levels.

Summary – integrated overview. As a result of the gradient dependent transport of metabolites across the blood-brain barrier, brain and body compartments of glucose and lactate are not independent. Thus, the observations described in the preceding sections need to be interpreted together. Interestingly, of the inhibitors, the GLUT1 inhibition condition induced highest blood glucose and lactate levels, but also resulted in the most stable (least amount of variation from baseline) brain extracellular levels of both metabolites (**Figure 4D & Figure 5D**). Despite inducing higher levels of blood glucose than DMSO, MCT1 inhibition resulted in brain extracellular glucose values below baseline and control (DMSO) values (**Figure 4C**), even in the presence of elevated GLUT1 expression (**Figure 3B**). These results may point to the role of monocarboxylates in cerebral metabolism and glucose sparing. Lastly, dual inhibition (**Figure 4E**) induced the highest extracellular glucose levels of the inhibitor conditions, despite comparable blood levels of both glucose and lactate. Here, endothelial GLUT1 expression was elevated to similar levels as those observed in MCT1 inhibition (**Figure 3B**), but one must assume a higher degree of transporter inhibition (GLUT1 and MCT1 inhibition vs just MCT1

inhibition). A characteristic difference was the elevation of endothelial MCT1 expression (**Figure 3D**), further supporting the role of monocarboxylates in cerebral energetics and glucose-sparing and perhaps even hinting towards the preferential use of monocarboxylates in times of energetic challenges.

Intraperitoneal Glucose Injection

Primary Motor Cortex. Figure 6 and **Table 1B** present and summarize the extracellular lactate and glucose changes following a glucose injection with and without prior transport inhibition. Expectedly, glucose injections increased brain extracellular glucose concentrations to levels higher than those observed following saline or DMSO injections (**Figures 4A&B** and **Figure 6A**). Following a glucose injection (without prior transport inhibition), the initial transient increase in extracellular lactate produced by the stress of the injection procedure is still observed, as well as the slower and more gradual decline in both glucose and lactate.

Pairwise comparisons indicated that the dual transporter inhibition (MCT1 & GLUT1; **Figure 6D**) amplified both the increase in extracellular glucose (Glucose injection, $p = 0.044$; MCT1 inhibition, $p = 0.008$) as well as the decrease in extracellular lactate (Glucose injection, $p = 0.065$; MCT1 inhibition, $p = 0.050$) when compared to both the control glucose injection and the solo MCT1 inhibition condition, respectively. Though not enough to indicate a significant change in the overall trend, GLUT1 inhibition did increase extracellular glucose above control levels for the first 15 minutes of testing (**Figure 6C** – shaded square). Finally, each inhibitor condition attenuated the initial increase in extracellular lactate produced by the stress of the injection procedure (**Figures 6 B-D** – shaded squares).

Time-dependent differences from baseline revealed that a 2 g/kg ip injection of glucose raised extracellular glucose levels above baseline for approximately 40 minutes (**Figure 6A**).

This increase in extracellular glucose above baseline levels lasted 50 (MCT1, **Figure 6B**), 60 (GLUT1, **Figure 6C**) and even 80 (MCT1 and GLUT1, **Figure 6D**) minutes under the various transporter blockade conditions. Additionally, GLUT1 inhibition, individually or combined with MCT1 inhibition, hastened the decrease in brain extracellular lactate. Extracellular lactate dropped below baseline 20 (GLUT1, **Figure 6C**) to 65 (GLUT1 and MCT1, **Figure 6D**) minutes earlier than what was observed following the control injection (**Figure 6A**).

Summary. Following a 2 g/kg systemic glucose injection a) GLUT1 inhibition increased peak brain extracellular glucose, extended the amount of time brain extracellular glucose remained above baseline as well as hastened the decrease in brain extracellular lactate, and b) these effects were even more pronounced following a combined inhibition of both MCT1, namely an amplified increase in brain extracellular glucose and a more pronounced decrease in brain extracellular lactate.

Blood. **Figure 7** and **Table 1B** present and summarize the blood lactate and glucose changes following a glucose injection with and without prior transport inhibition. The glucose injection (without inhibition) increased blood glucose levels above baseline for the first 30 minutes, and increased blood lactate levels above baseline for the duration of the testing, peaking at 90 minutes (**Figure 7A**).

When compared to the control condition, all inhibition conditions magnified the increase in blood glucose and attenuated rise in blood lactate (**Figure 7** – shaded squares). The magnified increase in blood glucose was particularly prevalent following the MCT1 inhibition, whether injected individually or combined with GLUT1 inhibition (MCT1, $p = 0.010$, GLUT1, $p = 0.040$, MCT1 & GLUT1, $p = 0.020$). The reduced blood lactate was present in all conditions, but less

obvious in the combined inhibition condition (MCT1, $p = 0.019$, GLUT1, $p = 0.003$, MCT1 & GLUT1, $p = 0.076$).

Summary – integrated overview. The dual transporter inhibition (GLUT1 & MCT1) led to the highest extracellular glucose, despite comparable blood glucose levels to that of the other inhibition conditions. The dual inhibition condition (GLUT1 & MCT1) did, however, induce a greater and more sustained increased in blood lactate, suggesting that elevated blood lactate may have played a role in the amplified and sustained increased in extracellular glucose. These results continue to support the importance of monocarboxylate transport and use in modulating and maintaining extracellular metabolite pools.

Intraperitoneal Lactate Injection

Primary Motor Cortex. Figure 8 and **Table 1C** present and summarize the extracellular lactate and glucose changes following a lactate injection with and without prior transport inhibition. The systemic injection of 2 g/kg lactate (without prior inhibition) resulted in extracellular glucose rising quickly, peaking at about 25 minutes post injection, and then slowly returning to baseline. Lactate was transiently increased as a result of the injection, after which it declined steadily, reaching a nadir 40 minutes post injection and stabilizing at around 80% of baseline values (**Figure 8A**).

Pairwise comparisons of metabolite trends revealed the following. MCT1 inhibition reduced the rise in brain extracellular glucose ($p = 0.087$, **Figure 8B**) when compared to control, but not the other inhibition conditions (GLUT1, $p = 0.664$; GLUT1 and MCT1, $p = 0.303$). GLUT1 inhibition abolished the extracellular dip observed in the control lactate injection and induced a rise in extracellular lactate that was significantly different from all other inhibition conditions (MCT1, $p = 0.018$; MCT1 and GLUT1, $p = 0.002$), as well as control ($p = 0.087$,

Figure 8C). Although the combined inhibition of MCT1 and GLUT1 attenuated the initial rise in extracellular glucose (**Figure 8D** – shaded square), there were no overall differences from control ($p = 0.557$).

Both MCT1 and GLUT1 inhibition hastened the return of extracellular glucose to baseline levels. Following MCT1 inhibition, brain extracellular glucose only increased above baseline between 5- and 10-minutes post-injection. Following the GLUT1 inhibition, extracellular glucose returned to baseline levels approximately 40 minutes earlier than control (60 mins vs 100 minutes post injection).

Summary. In summary, a) inhibition of GLUT1 prevented the decrease in brain extracellular lactate levels that followed an ip injection of lactate, b) MCT1 inhibition, individually or combined with GLUT1, blunted the peak and slope of the rise in brain extracellular glucose while maintaining extracellular lactate levels below baseline.

Blood. Figure 9 and **Table 1C** present and summarize the blood lactate and glucose changes following a lactate injection with and without prior transport inhibition. In the control condition, peripheral lactate injections (without prior transport inhibition) produced sustained and elevated blood lactate levels that did not return to baseline within the 2 hours of testing. Despite its large impact on blood lactate, a 2 g/kg ip injection of lactate had no impact on blood glucose (**Figure 9A**).

Overall, the general pattern of stable blood glucose and large increases in blood lactate was observed in all conditions (**Figure 9**), with the exception of MCT1 inhibition (**Figure 9B**) significantly magnifying the increase in blood lactate when compared to control ($p = 0.004$, **Figure 9A**) and temporarily raising blood glucose levels above baseline 60 minutes post-injection.

Summary – integrated overview. Despite elevated blood lactate and relatively stable blood glucose, MCT1 inhibition prior to the lactate injection resulted in attenuated extracellular glucose and GLUT1 inhibition elevated extracellular lactate – even in the presence of stable or elevated endothelial GLUT1 expression. Measuring glucose and lactate fluctuations in both the blood and the brain allowed us to observe that MCT1 transport, and to a lesser extent GLUT1 transport, is essential for the quick and large increase in extracellular glucose following a lactate injection.

Intraperitoneal Fructose Injection

Primary Motor Cortex. Figure 10 and **Table 1D** present and summarize the extracellular lactate and glucose changes following a fructose injection with and without prior transport inhibition. Following an ip fructose injection (without prior transport inhibition), we observed the typical stress-related rise in extracellular lactate immediately after the injection and a fructose-induced increase in extracellular glucose (**Figure 10A**), followed by a gradual and steady decline in extracellular glucose and lactate.

Our analysis revealed no overall differences for either extracellular glucose or lactate between the transport inhibitor conditions and control, or between transport inhibitor conditions. However, there were some time-specific differences. All transport inhibition conditions hastened the return of brain extracellular glucose to baseline. In control conditions, extracellular glucose remained above baseline levels for approximately 1-hour post-fructose injection. After transport inhibitor injection, brain extracellular glucose returned to baseline approximately 30 minutes post-fructose injection. The combined transport inhibition of GLUT1 and MCT1 not only hastened the return of brain extracellular glucose to baseline but also attenuated its post-injection rise, flattening out the inverted U trend typically observed (**Figure 10D**). MCT1 inhibition

delayed the significant decrease in extracellular lactate below baseline levels by 30 minutes when compared to control.

***Summary*.** In summary, a) the inhibitor conditions did not induce an overall change in the fluctuation trends of extracellular glucose or lactate, b) the inhibition of MCT1 delayed the dip in extracellular lactate below baseline and c) transporter inhibition, in particular the combined GLUT1 and MCT1 inhibition, decreased the amount of time extracellular glucose remained above baseline.

Blood. **Figure 11** and **Table 1D** present and summarize the blood lactate and glucose changes following a fructose injection with and without prior transport inhibition. In the control condition, peripheral fructose injections (without prior inhibition injections) trended towards raised blood lactate but had no effect on blood glucose (**Figure 11A**).

Pairwise comparisons indicated that only the combined inhibition of MCT1 and GLUT1 significantly elevated blood glucose levels when compared to control ($p = 0.014$) or other inhibitor conditions (MCT1, $p = 0.023$; GLUT1, $p = 0.050$).

Summary – integrated observations. Comparing extracellular and blood metabolites suggests that the inhibitor-induced reduction in the time extracellular glucose remained above baseline was the not the results of diminished blood glucose.

Motor Behavior

DMSO. Figure 12 presents extracellular lactate and glucose changes during motor behaviors following a DMSO injection. Previously, we showed that neural activation of the primary motor cortex by voluntary wheel running induced an increase in extracellular lactate, and sometimes induced a concomitant smaller decrease in extracellular glucose. This pattern,

particularly that of the rise in extracellular lactate, was replicated and amplified by both "effortful" behaviors: vertical hold and spinning (Béland-Millar & Messier, 2022).

We examined the impact of the same behaviors following a DMSO injection. We found that, overall, neither brain extracellular glucose or lactate significantly varied from their saline counterpart (Béland-Millar & Messier, 2022) during the vertical hold (glucose, $p = 0.756$; lactate, $p = 0.237$) or spinning condition (glucose, $p = 0.761$; lactate, $p = 0.241$).

Extracellular lactate during the spinning condition did increase above the levels observed in the vertical hold ($p = 0.011$), but extracellular glucose did not vary between the two conditions ($p = 0.865$).

Summary. In summary, the overall impact of DMSO on extracellular glucose and lactate during the vertical hold or spinning behaviors does not differ than their saline counterparts. Though extracellular glucose does not vary between the conditions, the spinning behavior induces a larger increase in extracellular glucose.

Effect of Transport Inhibition on Behavior Induced Extracellular Glucose and Lactate Fluctuations.

Vertical hold. Pairwise comparisons indicated that none of the inhibition conditions altered the overall trend of extracellular glucose (MCT1, $p = 0.622$; GLUT1, $p = 0.170$; GLUT1 & MCT1, $p = 0.423$) or lactate (MCT1, $p = 0.983$; GLUT1, $p = 0.443$; GLUT1 & MCT1, $p = 0.815$) beyond what was observed in the DMSO vertical hold. However, time-specific analyses revealed that GLUT1 inhibition, whether administered alone or in combination with MCT1 inhibition, was capable of reducing extracellular glucose levels below baseline levels (**Figure 12** – left panels), unlike the DMSO condition.

Spinning. Pairwise comparisons indicated that the inhibition of MCT1, whether administered alone or in combination with GLUT1 inhibition, attenuated the rise in extracellular lactate (MCT1, $p = 0.037$; GLUT1, $p = 0.665$; GLUT1 & MCT1, $p = 0.003$; **Figure 12** – right panels). There was no impact of the inhibition conditions on extracellular glucose when compared to DMSO (MCT1, $p = 0.691$; GLUT1, $p = 0.933$; GLUT1 & MCT1, $p = 0.692$). Time-specific analysis revealed that GLUT1 inhibition was the only condition in which the spinning behavior significantly decreased extracellular glucose below baseline levels and that the dual inhibition of GLUT1 & MCT1 delayed the rise in extracellular lactate by over 20 seconds.

Summary. GLUT1 inhibition appears to accentuate or induce a decrease in extracellular glucose during motor behaviors, while MCT1 inhibition appears to diminish the rise in extracellular lactate during the more effortful behavior, spinning.

Discussion

Transporter Plasticity

Altogether, this work supports the view that cortical uptake of circulating metabolites is highly and quickly adaptable. Indeed, endothelial and cortical cells can upregulate their transporter expression as a result of various conditions, such as altered metabolite availability (Canis et al., 2009; Leino et al., 2001; Pifferi et al., 2007; Portela et al., 2017), hypoxia (Feng et al., 2004; Yeh et al., 2008) as well as cognitive (Choeiri et al., 2005) and physical (Allen & Messier, 2013; Portela et al., 2017; Takimoto & Hamada, 2014) workloads. It also further emphasizes caution when utilizing DMSO as a vehicle, given the long-lasting impacts observed on transporter expression.

Interestingly, and to our knowledge, this is the first demonstration of MCT1 inhibition resulting in an immediate and acute decrease in endothelial GLUT1 expression, ultimately resulting in an overshoot in GLUT1 expression 4 and 24 hours post-MCT1-inhibitor-injection. Lactate, through mechanisms such as the hypoxia-inducible factor (HIF), has been shown to increase GLUT1 expression (Huang et al., 2012; Lee et al., 2015). Diminished transport of lactate from the periphery to the central nervous system may have impacted downstream mechanisms (e.g. HIF) resulting in altered cerebral uptake and a compensatory increase in GLUT1.

Cortical endothelial expression of MCT1 proved to be extremely consistent, transiently varying from time matched control levels only during the dual inhibition of both MCT1 and GLUT1. The seemingly immediate preferential upregulation of MCT1 over GLUT1 when both transporters are pharmacologically inhibited suggests a certain priority in rescuing lactate transport. This preferential upregulation of MCT1, in light of MCT1 and GLUT1 inhibition, may

support the view of a cortex that favors lactate consumption, as suggested by Bouzier-Sore and colleagues (Bouzier-Sore et al., 2006). Alternatively, the seeming prioritization of MCT1 expression during inhibited metabolite transport may be to ensure that there is no interruption in lactate efflux from the cells and avoids potential cell toxicity (Norenberg et al., 1987). Regardless, studies such as these as well as studies by others, such as Canis and colleagues (Canis et al., 2009) who demonstrated that hyperglycemic states in rats can lead to the widespread increase of MCT1, support the notion of monocarboxylate (MCT) transporter modulation is a mechanism to achieve metabolic balance.

Contribution of Lactate to the Brain

Prior evidence has established that extracellular values, including metabolites, can be a reflection of intracellular states and needs (Fellows et al., 1992; Mookerjee et al., 2017). Though this work does not resolve the on-going debate surrounding the relative and compartmental use of lactate as a metabolic substrate for the brain, it does paint an interesting picture of collaboration across the metabolites and their transporters to maintain extracellular metabolite pools. Both GLUT1 and dual GLUT1 and MCT1 inhibition raised extracellular glucose above controls following an ip glucose injection. Interestingly, this increased in extracellular glucose above control levels was not observed following the MCT1 inhibition – despite comparable endothelial GLUT1 expression and blood glucose levels. When compared to the impact of the other inhibition conditions, and when we consider the magnified impact of the dual inhibition, these results may indicate the contribution of lactate and monocarboxylate transports in maintaining extracellular glucose levels. Indeed, the magnified impact of the dual inhibition on extracellular brain glucose may be the result of elevated blood lactate and a high recycling rate of MCT1, as observed by the 4-fold increase following the initial inhibition. Indeed, Pellerin and

colleagues suggested that MCT recycling may be more easily adapted than glucose transporters (Pellerin, 2010).

The currently contentious topic surrounding the directional flow and use of lactate (Barros & Weber, 2018; Dienel, 2017; Drulis-Fajdasz et al., 2018; Mächler et al., 2016; Mangia et al., 2009; Patel et al., 2014; Patsatzis et al., 2019; Tang, 2018) most regularly refers to conditions of neuronal activation and not rest. Indeed, at rest, the brain is considered a net exporter of lactate (van Hall et al., 2009). This is further supported with our findings of elevated extracellular brain lactate immediately following the inhibition of MCT1 (alone or combined with GLUT1 inhibition). Elevated extracellular brain lactate suggests that some of the glycolytic by-product, lactate, was unable to leave the extracellular space, thereby accumulating. However, work by van Hall suggests that despite the net export of lactate, the brain still utilizes the lactate it imports across the BBB (van Hall et al., 2009), therefore playing an essential part in brain energetics. The work goes on to conclude, as other have, that exercise increases the brain's import and use of lactate. Indeed, exercise induces neural activity (Brümmer et al., 2011; Hyland et al., 2019; Lulic et al., 2017; Perrey, 2013; Singh & Staines, 2015; Winstein et al., 1997), subsequently increasing neuronal demand for energy (Dirks et al., 1980; Ide & Secher, 2000; Shetty et al., 2012; Sibson et al., 1998) and cerebral blood flow to the activated areas (Ogoh & Ainslie, 2009; Perrey, 2013) to deliver additional oxygen and metabolites to meet the elevated need.

MCT1 inhibition during the spinning effortful behavior attenuated the rise in extracellular lactate, suggesting that the behavior-induced rise extracellular lactate is intrinsically linked to blood-borne lactate. This is in contrast with what was observed at rest, where MCT1 inhibition increased extracellular lactate. This goes to further the notion that the brain is a net importer of

lactate during neuronal activation, but an exporter of lactate during rest. Interestingly, this only occurred during the (assumed) more challenging spinning behavior. An interesting picture starts to appear when we combine this observation with that of the GLUT1 inhibition only decreasing extracellular glucose during the vertical hold behavior. Why would brain extracellular glucose only decrease during the allegedly simpler behavior? We propose that, similarly to the observation that brain glucose consumption is negatively correlated with exercise intensity/blood lactate (Smith et al., 2003), that elevated cortical uptake of lactate during the more complex spinning behavior compensates for a portion of the glucose use, therefore effectively "rescuing" the extracellular brain glucose. The higher reliance on blood lactate during this specific behavior condition (spinning), results in an attenuated rise in extracellular lactate when MCT1 is pharmacologically blocked. Altogether, this points to lactate as a partial metabolic fuel during neuronal activity, but that a significant contribution to brain energetics during neuronal activity is only reached when a certain threshold is attained. It remains to be seen if this threshold refers to neuronal states, such as metabolic need, or peripheral states, such as elevated lactate availability and how this may impact metabolite delivery through changes in cerebral blood flow.

Technical limitations and considerations

Use of DMSO as a vehicle.

Toxicity. Despite the fact that DMSO is not an inert substance, it is a commonly used as a vehicle due to its highly polar domain and two apolar methyl groups making it an excellent solvent for water-insoluble drugs (Blevins et al., 2002; Fossum et al., 2008; Nasrallah et al., 2008; Santos et al., 2003). DMSO has been demonstrated to impact various functions, ranging from improving cognitive function in Alzheimer's mouse models (Penazzi et al., 2017) to modulating biological processes such as gene expression (Moskot et al., 2019). Beyond these

side-effects, there is also controversy surrounding DMSO's toxicity. Work by Blevins and colleagues (Blevins et al., 2002) have concluded that small volumes (0.3ul) of relatively pure DMSO (75%) are safe to inject directly into the brain. There is even evidence of increased cell growth following the incubation of ovarian carcinoma cells in DMSO (Rodríguez-Burford et al., 2003). In contrast, Hanslick and colleagues (Hanslick et al., 2009) demonstrated that intraperitoneal injection of DMSO could induce small amounts of neuronal apoptosis with doses as low as 0.3 ml/kg, however this impact becomes much more evident at 3 and 10 ml/kg doses. In larger doses (10 ml/kg), the impact of DMSO on activated caspase-3 immunoreactivity was particularly prominent within the cortex. Zhang and colleagues (C. Zhang et al., 2017) not only demonstrated the dose dependent impact of DMSO on neuronal viability but demonstrated the relative vulnerability of neurons to DMSO when compared to astrocytes. Cultures containing 0.5-1% DMSO saw a reduction in neuronal viability and NeuN expression. However, astrocyte cultures containing 1% DMSO for 24 to 48 hours revealed increases GFAP expression and proliferation. Astrocyte cultures with over 5% DMSO concentration, however, reduced both the GFAP expression and proliferation. Not to mention that preparing and storing drugs in DMSO may alter their activity and potency (Peters et al., 2002). Though the use of DMSO was unavoidable, it is critical to be aware of these effects. In an attempt to mitigate the impact of DMSO on our observations, metabolite injections and behavioral testing were conducted 3 hours following any DMSO injection, and the daily injected volume of DMSO fell within reasonable ranges (0.1ml or approximately 2.5ml/kg).

Blood-brain barrier integrity. Beyond the off-target effects of DMSO stated above, it may also impact blood-brain barrier (BBB) permeability and metabolism. Research has demonstrated that the administration of DMSO can cause its accumulation in brain slices

(Nasrallah et al., 2008) as well as alter BBB permeability (Broadwell et al., 1982; Kleindienst et al., 2006). This altered BBB permeability has been confirmed with Evans Blue as well as horseradish peroxidase, both of which were not able to enter the brain without the addition of DMSO. Notably, this effect is transient, with BBB permeability returning to normal 2 hours post-injection (Broadwell et al., 1982). Though a review of the evidence suggests it is unlikely that the inhibitors cross the BBB (Benyahia et al., 2021; Guan et al., 2019; Ojelabi et al., 2016), we unfortunately did not have the means to measure the BBB permeability to these specific substances.

DMSO and metabolism. Relatively low concentrations of DMSO (0.000025-0.25%) is capable of increasing the metabolic rate of brain slices, likely by stimulating respiration and shuttling additional pyruvate to the Krebs cycle (Nasrallah et al., 2008). Combined with DMSO's ability to cross the BBB, the necessity of this substance as a vehicle imposes the need for careful consideration during interpretation.

AZD3965 and WZB117 as transport inhibitors. To limit the impact of DMSO and the injection procedure on our extracellular readings following metabolite injections, the inhibitors were injected 3 hours prior to the metabolite injection, at which time extracellular metabolite fluctuations had stabilized. The GLUT1 inhibitor, WZB117, is capable of inhibiting glucose transport within a minute following administration (Liu et al., 2012), and has been demonstrated to have long lasting effects. Indeed, WZB117 inhibition has been shown alter metabolic enzyme expression within 6 hours of administration (Liu et al., 2012). These cascading inhibitory effects have been demonstrated to last anywhere between 48 hours (Liu et al., 2012) and 9 days (Li et al., 2019) in cell cultures. This inhibitor was thought to be irreversible (Liu et al., 2012), though this assumption was recently questioned (Ojelabi et al., 2016). As such, one must consider not

only the downstream effects of WZB117 on metabolic enzymes, but its potentially reversible nature – not to mention transporter plasticity.

The MCT1 inhibitor, AZD3965, also appears to inhibit uptake within minutes of administration (Guan et al., 2019) with maximal inhibition 6 hours following administration (Curtis et al., 2017). AZD3965 continues to inhibit MCT1 transport for up to 12 hours following its removal from cell cultures (Guan et al., 2019). When peripherally administered, the inhibitor remains minimally detectable in the blood for as little as 12 hours (Guan & Morris, 2019) or as long as 72 hours (Curtis et al., 2017).

Lastly, there are the ever-present challenges with pharmacological inhibition of labile transporters. It is ever uncertain, at any point in time, exactly how many transporters are inhibited and how quickly the transporters may be recycled. Secondly, there is an abundance of possible second-hand effects from off target or systemic inhibition of these transporters.

Electrochemical biosensors. When interpreting these results, it is important to consider the technical limitations of the employed methodology. Though extremely sensitive, the inserted biosensors measure relative changes in metabolite concentrations within the extracellular space, meaning that their primary function is not to measure absolute metabolite values. Once inserted, the electrodes undergo gradual "bio-fouling" as various biological materials attach themselves to the electrodes. This results in an extremely small and gradual decline in the signal. The signal is affected in a measure of hours not minutes, and as such should have no direct impact on our acute extracellular sampling (Kozai et al., 2015). However, this bio-fouling and slight manufacturer variations creates variability which requires the use of relative (to pre-event baseline) values to allow for comparisons within and across mice.

The extracellular nature of the measures obtained with these electrodes further complicates their interpretation. The electrodes measure net metabolite fluctuations in the extracellular space of the motor cortex, and as such the measures reflect the net result of: blood-brain barrier (endothelial) transport both in and out of the brain, as well as astrocytic and neuronal influx and efflux of these same metabolites, not to mention the impact of other surrounding cells such as oligodendrocytes and microglia. Therefore, an increase in extracellular lactate, for example, could represent increased transport of lactate across the blood-brain barrier into the extracellular space, increased neuronal glycolysis resulting in lactate export or even lactate shuttling from astrocytes into the extracellular space – minus any lactate being shuttled out of the central nervous system or being taken up by surrounding cells.

Conclusion

This study has contributed to the evidence that the brain is extremely plastic in its ability to modulate the relative use of different fuels, such as glucose and lactate, to produce its required energy. This plasticity appears to be, at least in part, underlined by rather immediate and long-term changes in the expression of relevant transporters in cortical endothelial cells as well as the need to meet a certain threshold with regards to the intensity of the behavior-induced neural activation. At its core, this study was meant to answer a question revealed in our previous work (Béland-Millar et al., 2017) that left us wondering if the similar brain extracellular pattern observed following various peripheral metabolite injections were all due to extracellular lactate. Though this work demonstrates that lactate likely plays a part in the observed extracellular metabolite fluctuations, the mechanisms underlying these are (unsurprisingly) complex and nuanced. To further our understanding of the data presented herein, future work should consider elucidating the implications of these transporter inhibitors on functional behavior, such as

memory, as well as explore the intracellular metabolite fluctuations with positron emission tomography of fluorodeoxyglucose (FDG).

Table 1 | Summary of the extracellular and blood glucose and lactate changes from baseline

Table 1a | Summary of glucose and lactate fluctuations after transport blocker injection

	Glucose		Lactate	
	Brain	Blood	Brain	Blood
Saline	-	-	-	-
50% DMSO	↑	↓	↓	↑↑
MCT1	↑	-	↑	↑
GLUT1	-	↑	↓	↑
MCT1 & GLUT1	↑	↓	↑	↑

Table 1b | Summary of glucose and lactate fluctuations following blockers and glucose injection

	Glucose		Lactate	
	Brain	Blood	Brain	Blood
50% DMSO	↑	↑	↓	↑↑
MCT1	↑	↑↑↑	-	↑
GLUT1	↑↑	↑↑	↓	↑
MCT1 & GLUT1	↑↑	↑↑↑	↓↓	↑↑

Table 1c | Summary of glucose and lactate fluctuations following blockers and lactate injection

	Glucose		Lactate	
	Brain	Blood	Brain	Blood
50% DMSO	↑↑	-	↓	↑
MCT1	↑	↑	↓	↑↑
GLUT1	↑	↑	-	↑
MCT1 & GLUT1	↑	-	↓	↑

Table 1d | Summary of glucose and lactate fluctuations following blockers and fructose injection

	Glucose		Lactate	
	Brain	Blood	Brain	Blood
50% DMSO	↑↑	-	↓	↑
MCT1	↑↑	-	↓	↑
GLUT1	↑	↑	↓	↑
MCT1 & GLUT1	↑	↑	↓↓	↑

*These tables summarize significant changes in extracellular and blood glucose and lactate from baseline under following **A)** the control and inhibitor injections, **B)** glucose injections, **C)** lactate injections, or **D)** fructose injections. Up arrows indicate that the respective metabolite increased above baseline values whereas down arrows indicate a decrease in the respective metabolite below baseline levels. Additional arrows, either up or down, indicate that a certain condition had a larger effect than others on the net fluctuation of the respective metabolite.*

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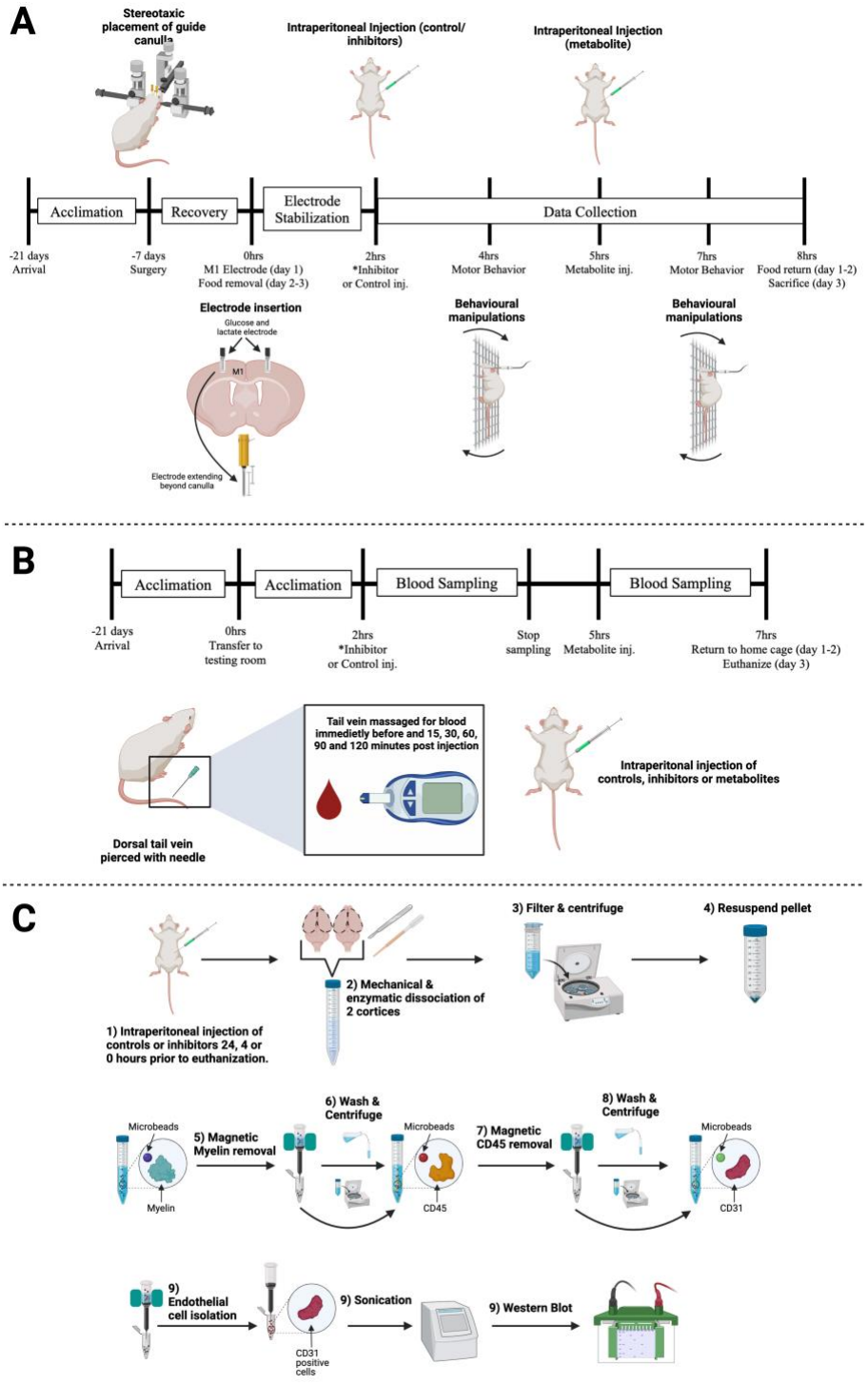
Figure 1 | Testing timelines



Representation of the procedure timelines for testing the three cohorts. ***A)*** *Mice intended for extracellular measurements of glucose and lactate underwent surgery to implant guide cannulas over the primary motor cortex (M1). Following a week of recovery, mice were brought to the testing chambers and the electrodes implanted through the guide cannulas. Once the electrodes stabilized (2 hours), mice were injected with either the control solutions (DMSO or saline) or the inhibitors (AZD3965 and/or WZB117). Two hours later, mice were manipulated (vertical hold, spin and grip strength) with 10-minute breaks in between each manipulation, which were presented in a counterbalanced order. Three hours after the control or inhibitor injections, mice received a metabolite injection. Two hours after this metabolite injection, mice were once again manipulated (vertical hold, spin and grip strength) with 10-minute breaks in between each manipulation, which were presented in a counterbalanced order. After this manipulation, mice were given access to food (day 1-2). Day two and three of testing were identical, with the exception that at 0hrs only the food was removed (electrodes were already inserted) and on day 3 the mice were euthanized after the final manipulation rather than returned to their testing cage with food.* ***B)*** *Blood values of glucose and lactate were measured in a separate group of mice, to avoid injury to both the mice and the sensitive testing equipment. Prior to testing, the mice were acclimated to their testing room and cages for 2 hours, with access to water. To draw blood, the dorsal vein of their tails was pierced with a 23-gauge needle and then massaged for blood. After the baseline reading, mice received an ip injection of either the control solutions (DMSO or saline) or the inhibitors (AZD3965 and/or WZB117). Blood to measure glucose and lactate was then withdrawn 15, 30, 60, 90, and 120 minutes post injection. An hour later, another baseline measure was taken and mice received an ip metabolite injection (glucose, lactate or fructose). Blood samples were once again obtained 15, 30, 60, 90, and 120 minutes post injection. Once completed, the mice were returned to their home cages. To mirror the brain extracellular sampling protocol, mice were similarly tested twice more (day 1-3). To limit stress, no restraints were use during blood sampling.* ***C)*** *A third cohort of mice was used to exclusively measure the impact of the control (saline and DMSO) and inhibitor (AZD3965 and/or WZB117) injections on endothelial cell transporters (GLUT1 and MCT1). These mice received their respective injections 24, 4 or 0 (5-10 minutes) hours prior to perfusion and endothelial cell extraction. Following a transcardial saline perfusion, cortices were extracted and mechanically (pipette) and enzymatically (neural dissociation kit) dissociated. Following manufacturer instructions, the tissue was then filtered, centrifuged and the supernatant removed. The pellet was resuspended with magnetic microbeads for myelin and CD45 for removal. The sample was filtered through a magnetic, trapping the myelin and CD45 positive cells. Cells were washed between steps. Subsequently, magnetic beads for CD31 positive cells were added to the sample., trapping the CD31 positive cells with the magnet. Cells were then extracted, sonicated and processed for a traditional western blot.*

All mice acclimated to the facility for 3 weeks prior to experimental manipulations. Counterbalanced for the metabolite injections.

**GLUT1 inhibitor (WZB117) is an irreversible inhibitor and therefore was only injected on day 1. MCT1 inhibitor (AZD3965) is a reversible inhibitor, therefore requiring injections on each testing day.*

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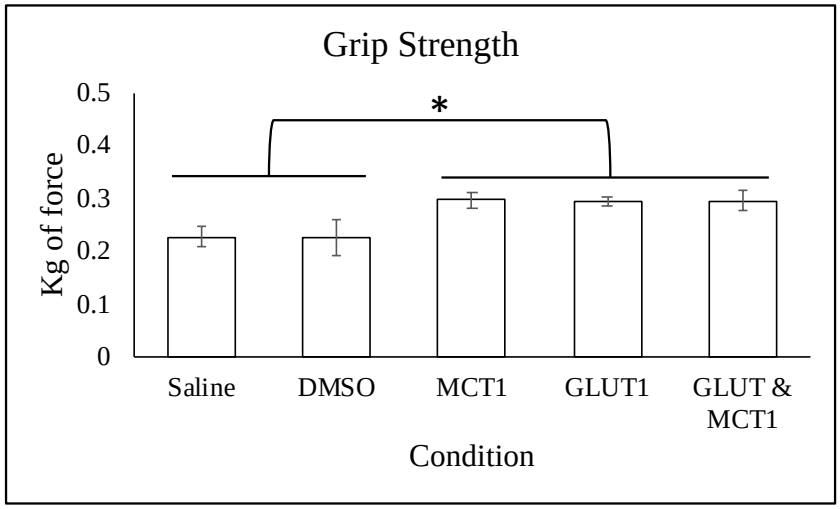
Figure 2 | Grip Strength



Strength of fore and hind limbs, measured in kilograms of force, 2 hours following an ip injection of 0.1 ml of 0.9% saline (n=5), 0.1 ml of 50% DMSO/dH$_2$O solution (n=5), 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=15), 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=8), or 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=11). All inhibition condition significantly increased ($p < 0.05$) grip strength when compared to either control conditions (saline or DMSO).

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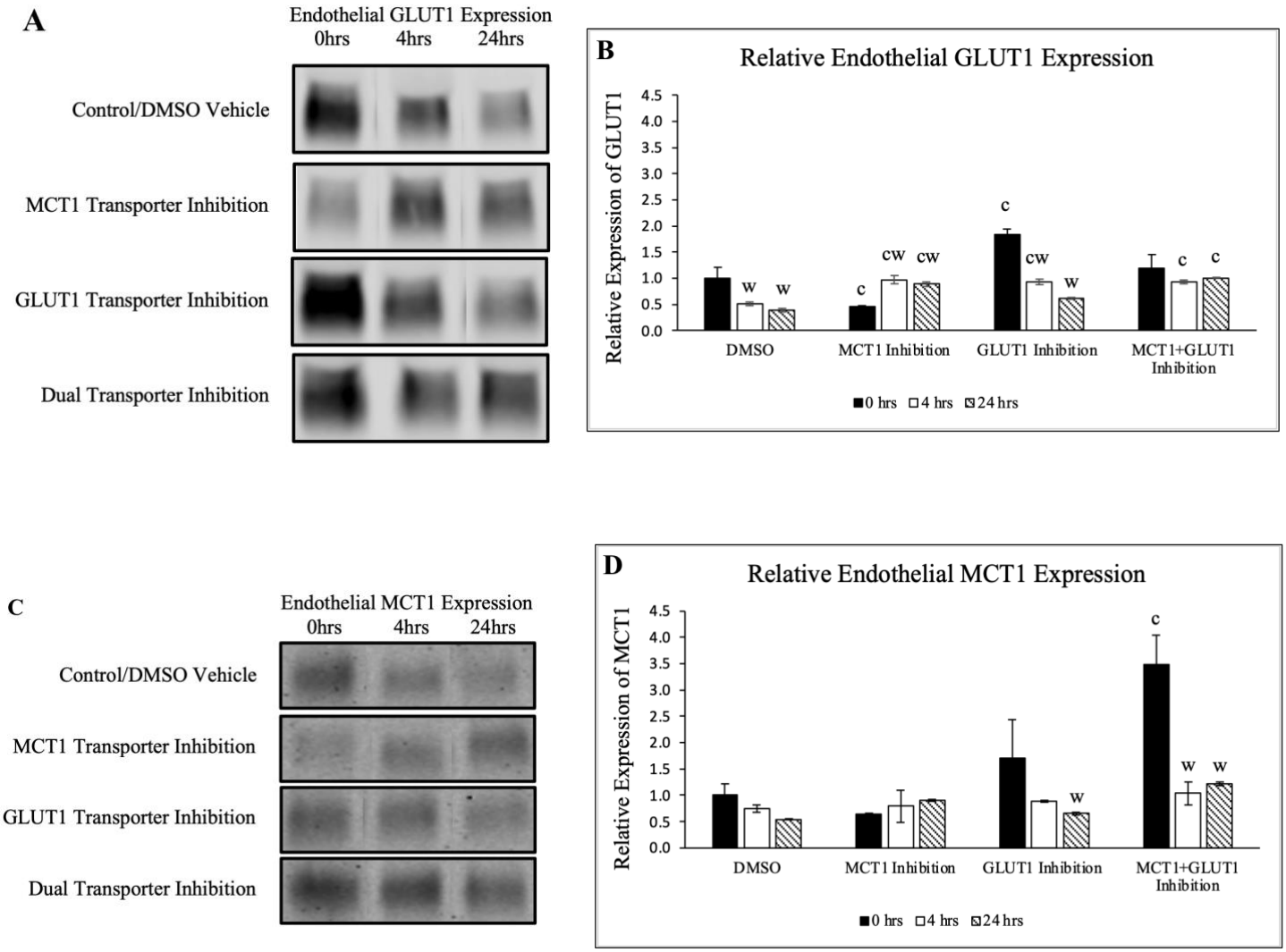
Figure 3 | Endothelial Expression of Metabolite Transporters Following Pharmacological Inhibition



A & C) *Representative immunoblots of MCT1 and GLUT1 transporter expression in brain endothelial cells following peripheral pharmacological inhibition of MCT1 (30 mg/kg of AZD3965), GLUT1 (20 mg/kg of WZB117) or combined inhibition of both transporters for either 5-10 minutes (0hr), 4- or 24-hours.* ***B & D)*** *Relative densities of MCT1 and GLUT1 transporters were normalised to the total protein load and values were expressed as relative to the control (DMSO 0hr) condition. Significant ($p < 0.05$) differences from control across conditions (i.e. DMSO 0hr vs MCT1 0hr, or DMSO 4hrs vs combined inhibition 4hrs) are illustrated with the letter "c" for control. Significant ($p < 0.05$) differences within groups from their respective 0hr baseline (i.e. GLUT1 0hr vs GLUT1 4hrs) are illustrated with the letter "w" for within-group. Error bars represent the standard error of the mean.*

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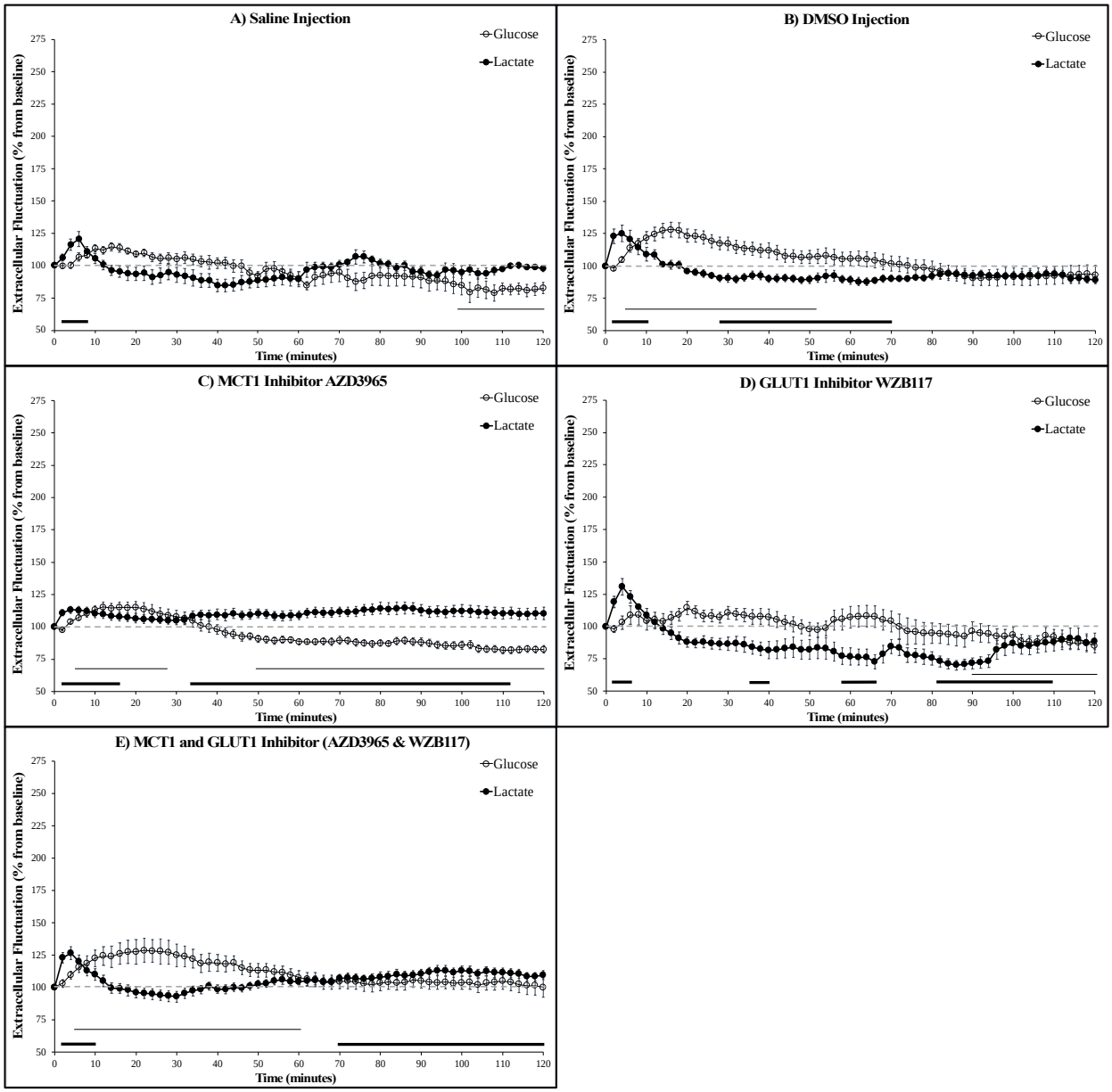
Figure 4 | Brain extracellular glucose and lactate fluctuations following intraperitoneal vehicle and transport inhibitor injections



*Motor cortex extracellular glucose (open circle) or lactate (filled circle) following an intraperitoneal injection of either **A)** 0.1 ml of 0.9% saline (n=7), **B)** 0.1 ml of 50% DMSO/dH$_2$O solution (n=15), **C)** 30mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=23), **D)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=9), or **E)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=8). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and horizontal lines below the data indicates significant ($p < 0.05$) differences*

from baseline for both extracellular glucose (thin line) and lactate (thick line). Shaded rectangles surrounding the data points indicate significant differences from control (DMSO), for both extracellular glucose (open circle) and lactate (filled circle).

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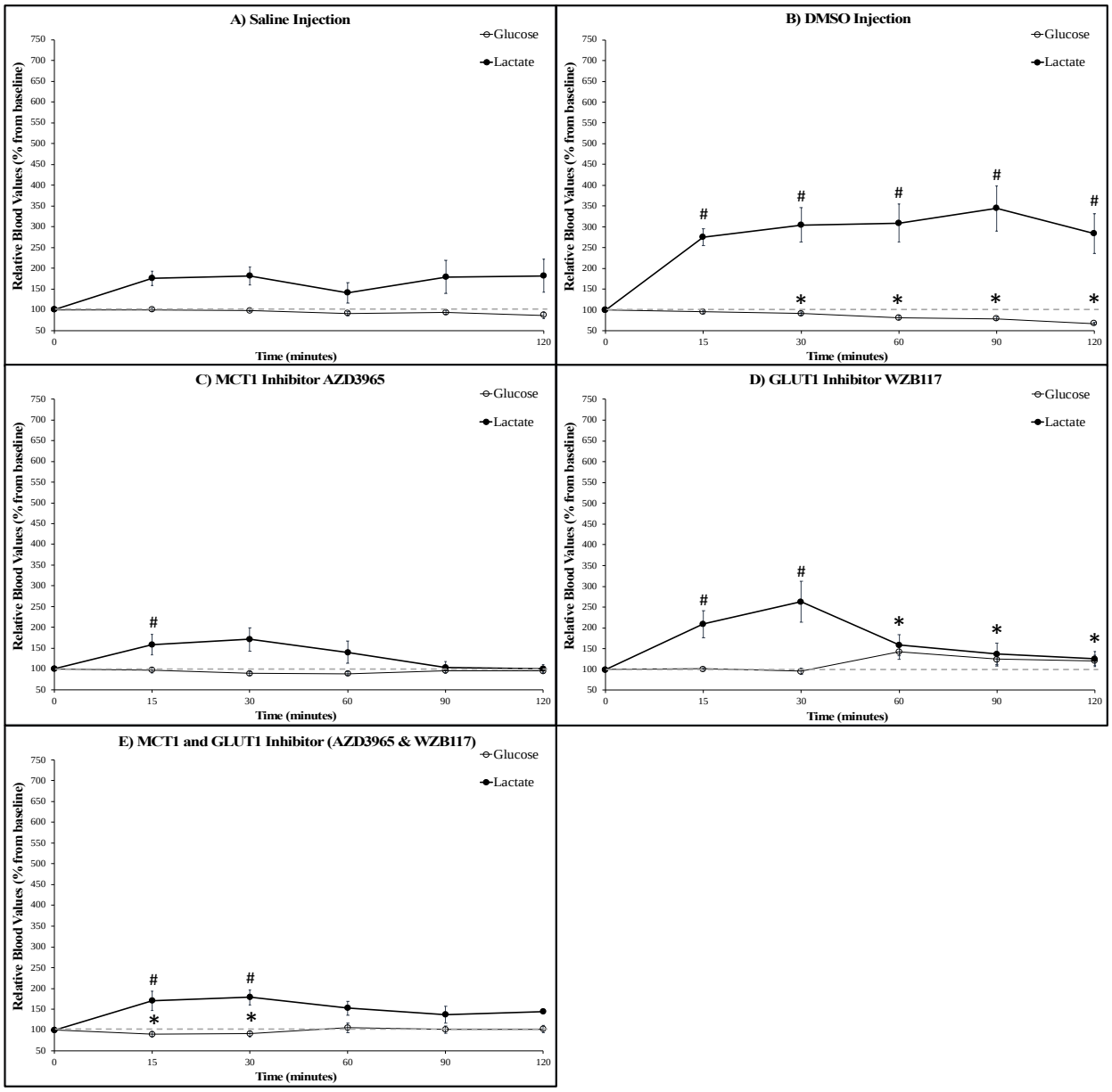
Figure 5 | Blood glucose and lactate fluctuations following intraperitoneal injections of vehicle and transport inhibitor



*Blood glucose (open circle) or lactate (filled circle) following an intraperitoneal injection of either **A)** 0.1 ml of 0.9% saline (n=5), **B)** 0.1 ml of 50% DMSO/dH₂O solution (n=16), **C)** 30mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1ml of a 50% DMSO/dH₂O solution (n=8), **D)** 20mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH₂O solution (n=7), or **E)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH₂O solution (n=8). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and*

the symbols indicate significant ($p < 0.05$) differences from baseline for extracellular glucose (*) or lactate (#). Shaded rectangles surrounding the data points indicate significant differences from control (DMSO), for both extracellular glucose (open circle) and lactate (filled circle).

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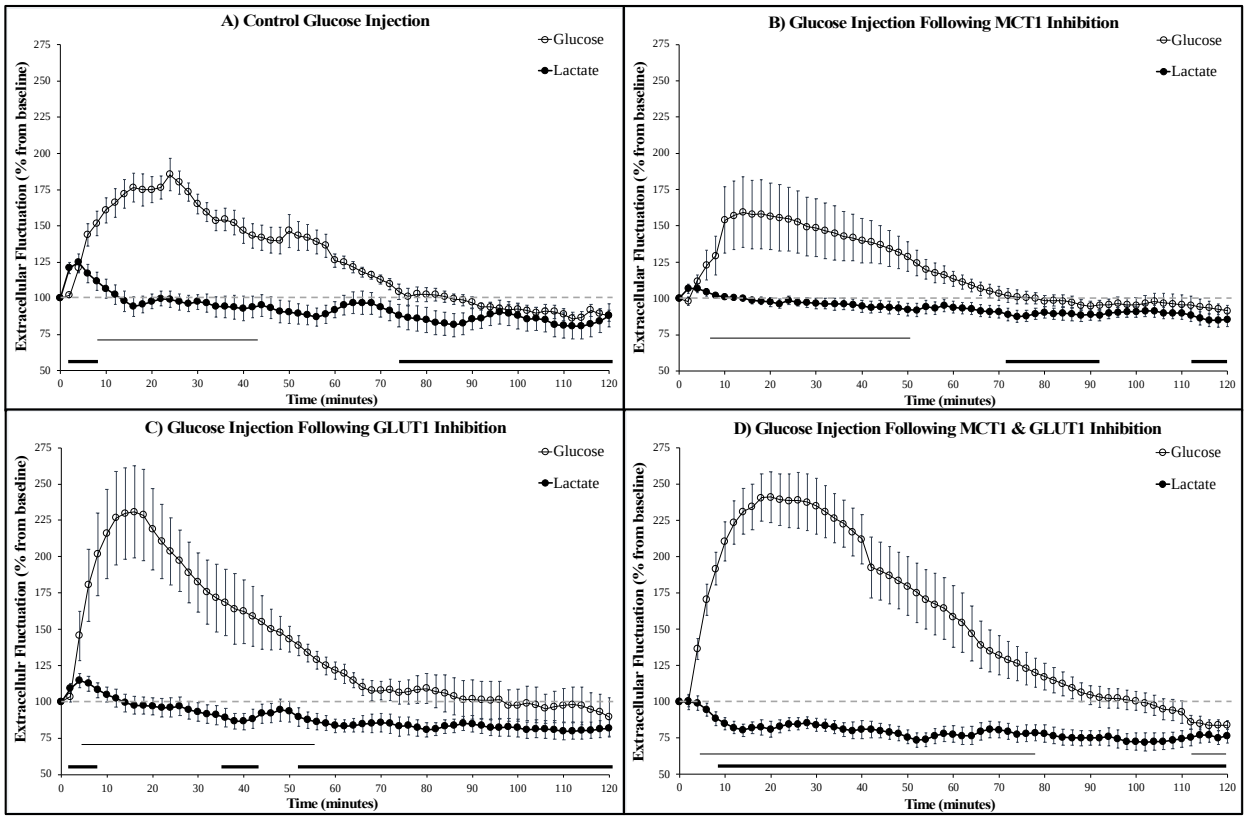
Figure 6 | Brain extracellular glucose and lactate fluctuations following an intraperitoneal glucose injection



*Extracellular cortical glucose (open circle) or lactate (filled circle) following an intraperitoneal glucose injection (2 g/kg). Three hours prior to this injection, the mice received either **A)** no treatment (n=7), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=7), **C)** 20 mg/kg of GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=6), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=6). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). Shaded rectangles surrounding the data points indicate significant differences from control (control glucose injection), for both extracellular glucose (open circle) and lactate (filled circle).*

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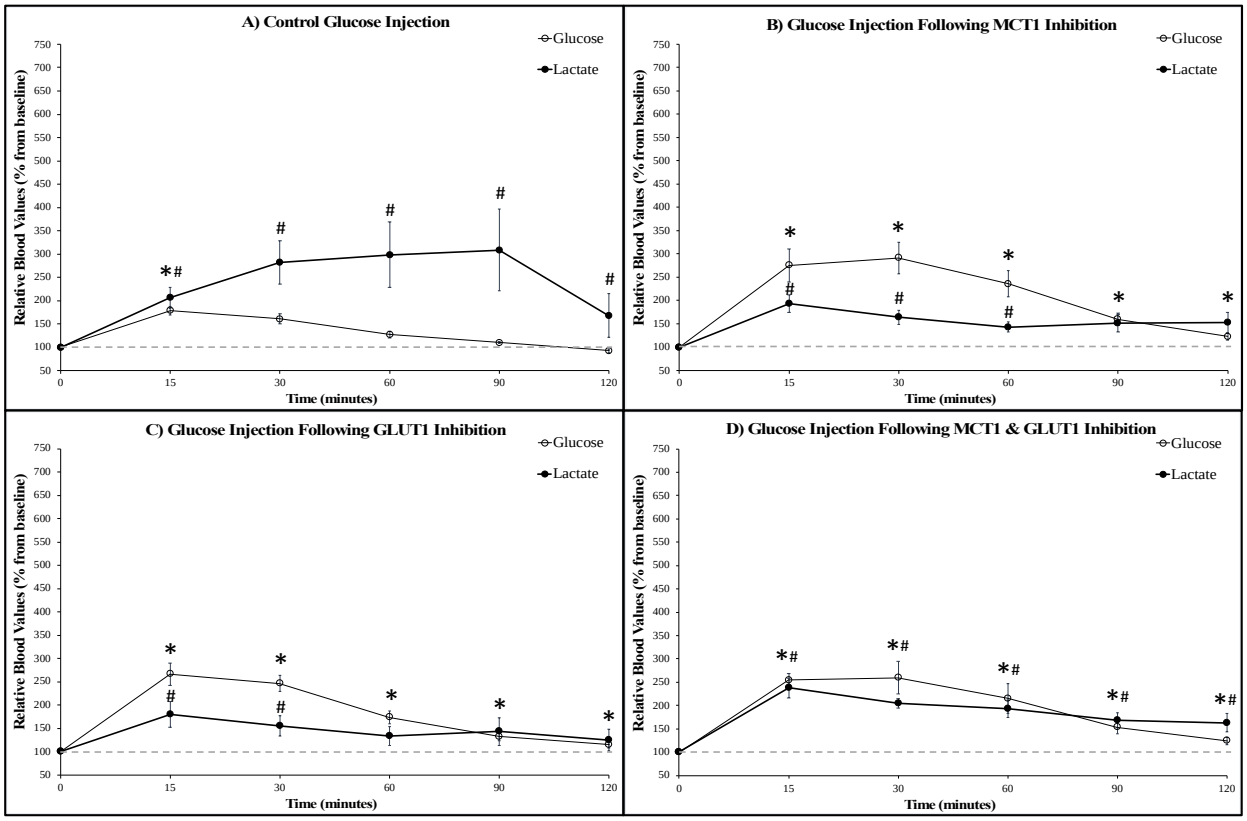
Figure 7 | Blood glucose and lactate fluctuations following an intraperitoneal injection of glucose



*Blood glucose (open circle) or lactate (filled circle) following an intraperitoneal glucose injection (2 g/kg). Prior to this injection, the mice received either **A)** no treatment (n=5), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH_2O solution (n=7), **C)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH_2O solution (n=6), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH_2O solution (n=8). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and the symbols indicate significant ($p < 0.05$) differences from baseline for extracellular glucose (*) or lactate (#). Shaded rectangles surrounding the data points indicate significant differences from the control glucose injection, for both extracellular glucose (open circle) and lactate (filled circle).*

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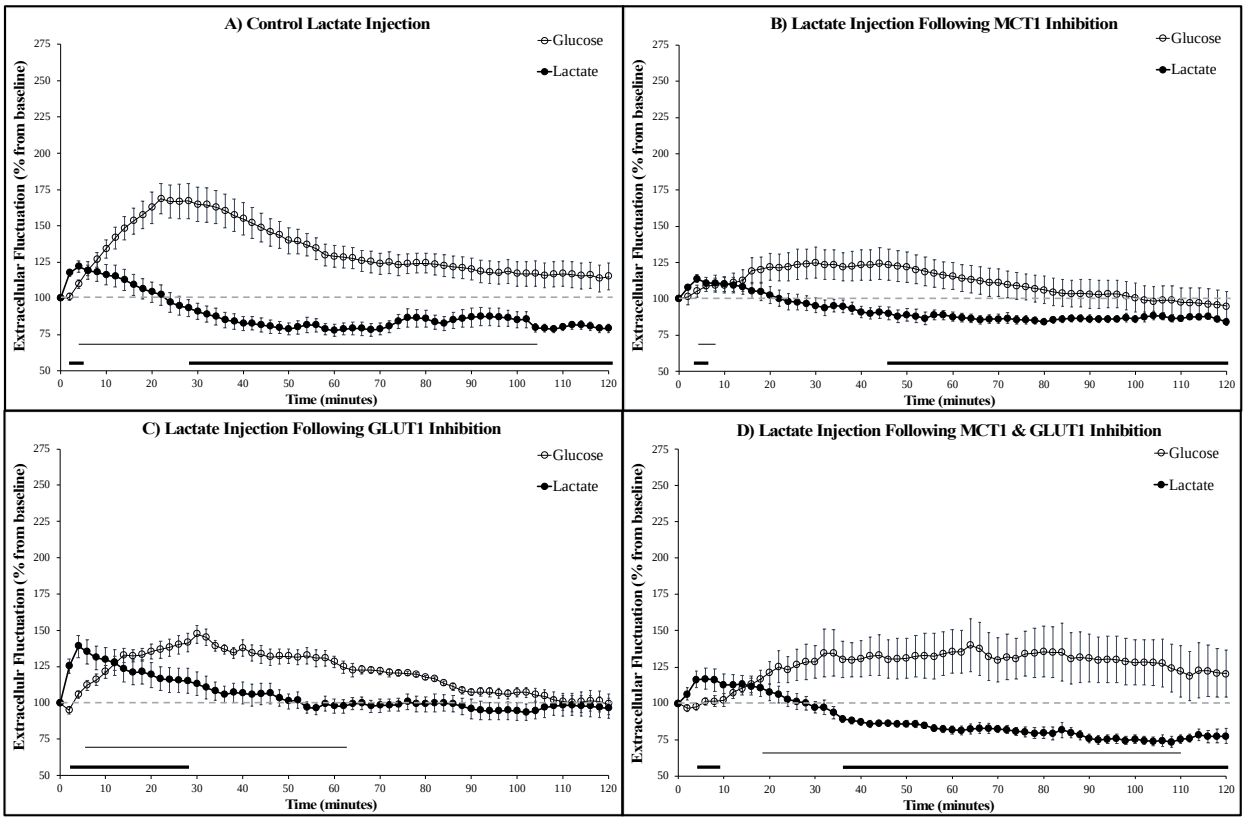
Figure 8 | Brain extracellular glucose and lactate fluctuations following an intraperitoneal lactate injection



*Extracellular cortical glucose (open circle) or lactate (filled circle) following an intraperitoneal lactate injection (2 g/kg). Three hours prior to this injection, the mice received either **A)** no treatment (n=13), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=9), **C)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=7), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=7). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). Shaded rectangles surrounding the data points indicate significant differences from control (control lactate injection), for both extracellular glucose (open circle) and lactate (filled circle).*

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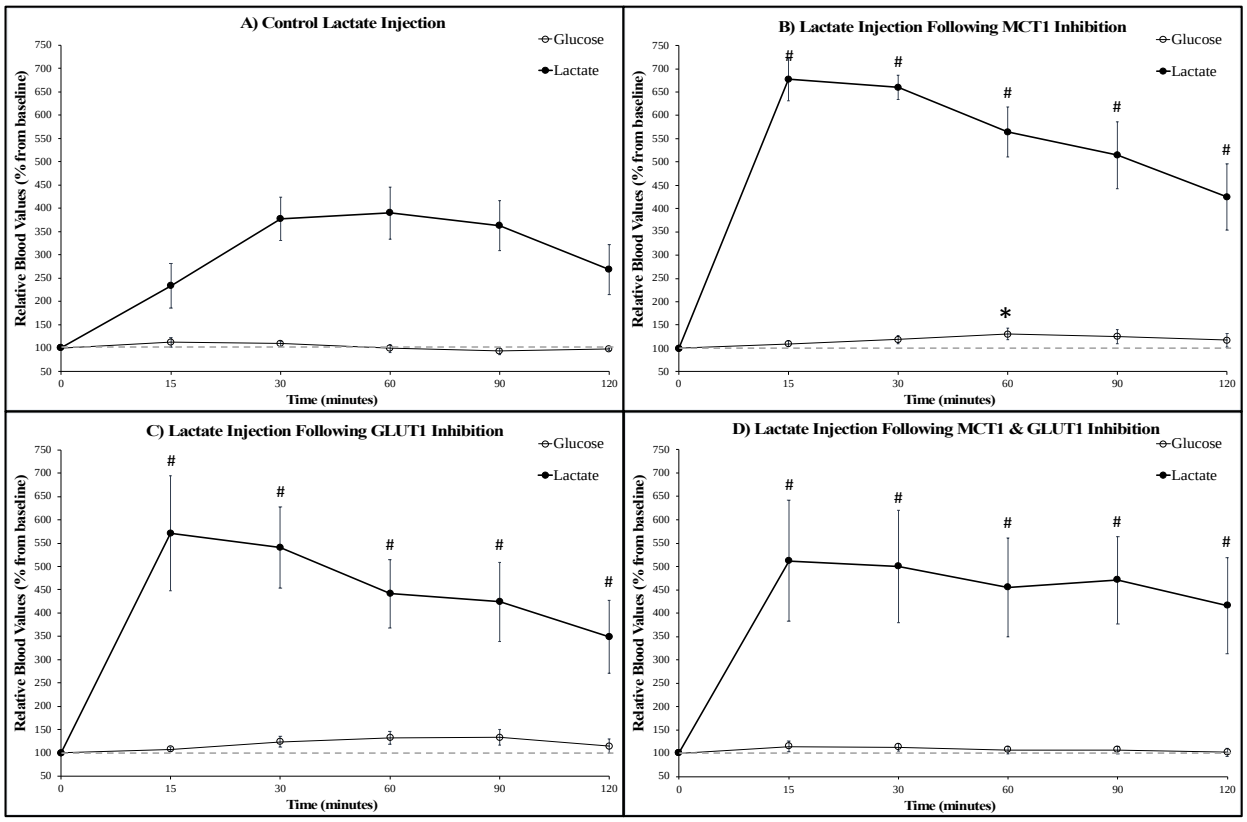
Figure 9 | Blood glucose and lactate fluctuations following an intraperitoneal injection of lactate



*Blood glucose (open circle) or lactate (filled circle) following an intraperitoneal lactate injection (2 g/kg). Prior to this injection, the mice received either **A)** no treatment (n=6), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=8), **C)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1ml of a 50% DMSO/dH$_2$O solution (n=7), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=6). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and the symbols indicate significant ($p < 0.05$) differences from baseline for extracellular glucose (*) or lactate (#). Shaded rectangles surrounding the data points indicate significant differences from the control lactate injection, for both extracellular glucose (open circle) and lactate (filled circle).*

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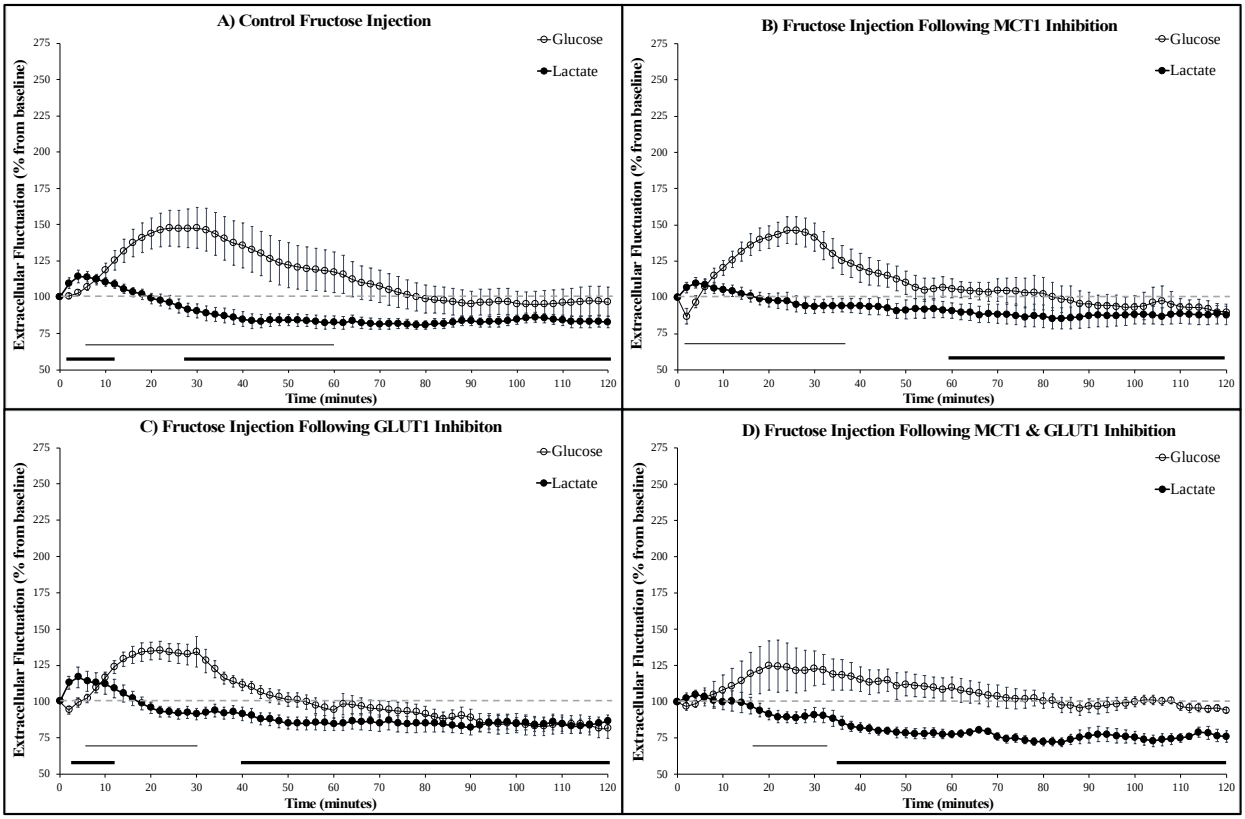
Figure 10 | Brain extracellular glucose and lactate fluctuations following an intraperitoneal fructose injection



*Extracellular cortical glucose (open circle) or lactate (filled circle) following an intraperitoneal fructose injection (2 g/kg). Three hours prior to this injection, the mice received either **A)** no treatment (n=8), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=5), **C)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=8), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=5). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). Shaded rectangles surrounding the data points indicate significant differences from control (control fructose injection), for both extracellular glucose (open circle) and lactate (filled circle).*

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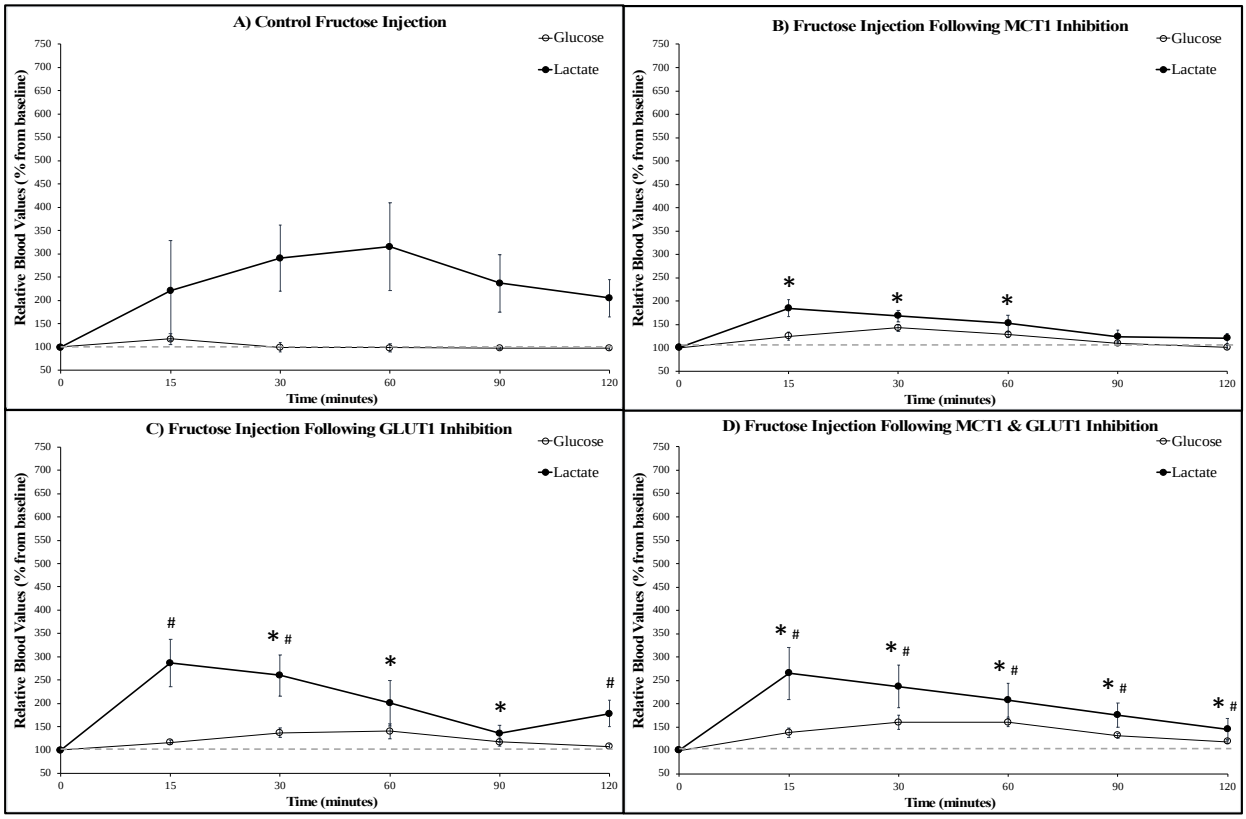
Figure 11 | Blood glucose and lactate fluctuations following an intraperitoneal injection of fructose



*Blood glucose (open circle) or lactate (filled circle) following an intraperitoneal fructose injection (2g/kg). Prior to this injection, the mice received either **A)** no treatment (n=4), **B)** 30 mg/kg of the MCT1 transport inhibitor AZD3965 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=4), **C)** 20 mg/kg of the GLUT1 transport inhibitor WZB117 in 0.1 ml of a 50% DMSO/dH$_2$O solution (n=5), or **D)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH$_2$O solution (n=6). Time zero corresponds to the end of the injection. Values are expressed as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The dashed line is a visual reference for baseline (100%) values and the symbols indicate significant ($p < 0.05$) differences from baseline for extracellular glucose (*) or lactate (#). Shaded rectangles surrounding the data points indicate significant differences from the control fructose injection, for both extracellular glucose (open circle) and lactate (filled circle).*

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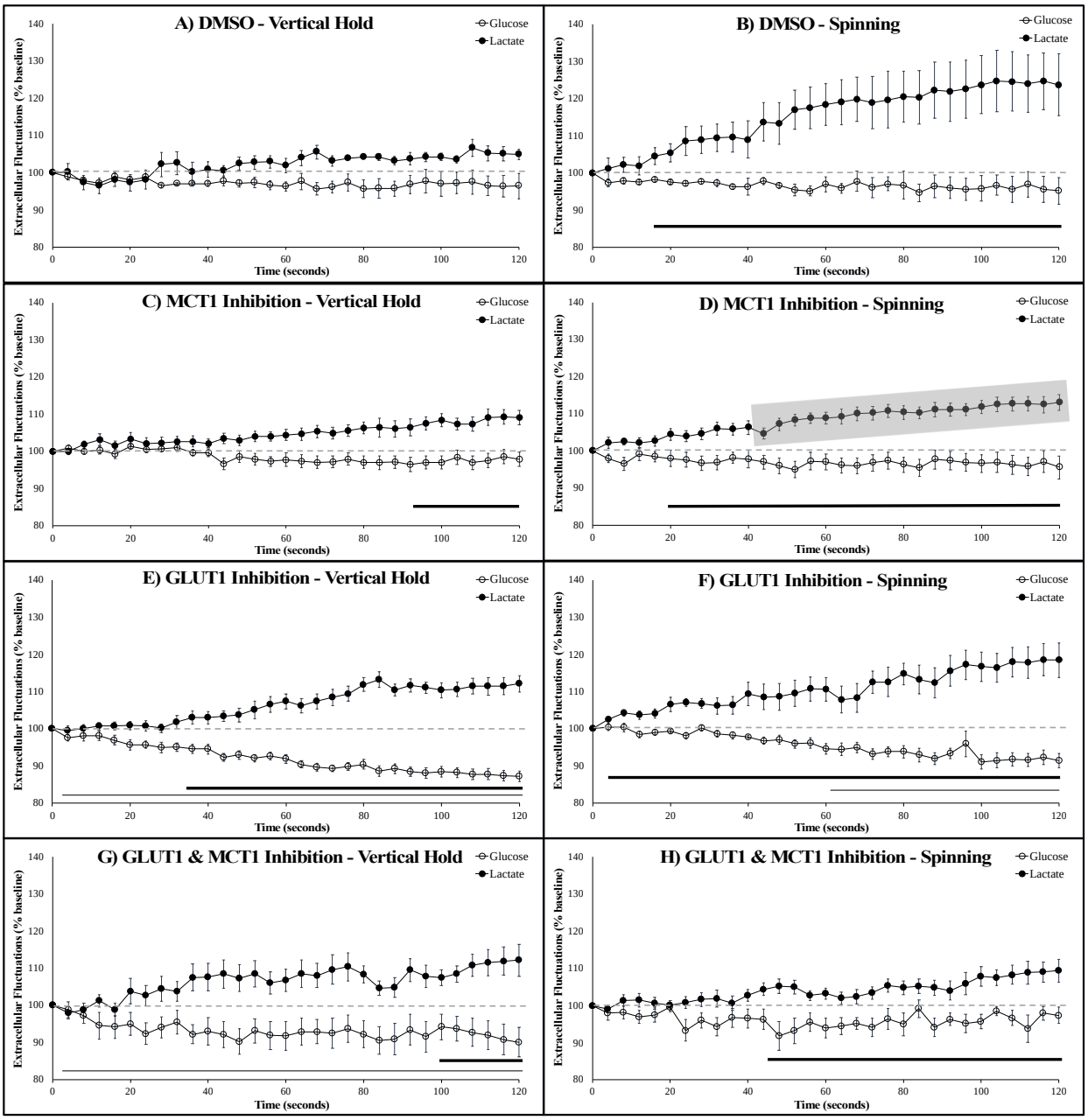
Figure 12 | Extracellular glucose and lactate fluctuations during vertical hold and spinning manipulations



*Extracellular cortical glucose (open circle) or lactate (filled circle) during vertical hold (A, C, D, G) and spinning (B, D, F, H) manipulations following an ip injection of either **A-B)** 0.1 ml of 50% DMSO/dH₂O solution (Hold, n=5; Spinning, n=8), **C-D)** 30 mg/kg of AZD3965 in 0.1 ml of a 50% DMSO/dH₂O solution (Hold, n events=14, n mice = 7; Spinning, n events=11, n mice=7), **E-F)** 20 mg/kg of WZB117 in 0.1 ml of a 50% DMSO/dH₂O solution (Hold, n events=14, n mice=6; Spinning, n events=13, n mice=7), or **G-H)** 30 mg/kg of AZD3965 and 20 mg/kg of WZB117 in 0.2 ml of a 50% DMSO/dH₂O solution (Hold, n events=15, n mice=7; Spinning, n events=14, n mice=7). Time zero corresponds to start of the vertical hold. Values are expressed*

as a percent of their baseline taken prior to injection and the error bars indicate the standard error of the mean. The mid-graph dashed line shows baseline (100%) value. Horizontal lines below the data indicates significant ($p < 0.05$) differences from baseline for both extracellular glucose (thin line) and lactate (thick line). Shaded rectangles surrounding the data points indicate significant differences from the respective DMSO control, for both extracellular glucose (open circle) and lactate (filled circle).

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Chapter VII: Altered brain metabolism in adult 16p11.2 haplodeficient male mice

Béland-Millar, A., Kirby, A., Truong, Y., Ouellette, J., Yandiev, S., Bouyakdan, K., Pileggi, C., Naz, S., Yin, M., Carrier, M., Kotchetkov, P., St-Pierre, M. K., Tremblay, M. È., Courchet, J., Harper, M. E., Alquier, T., Messier, C., Shuhendler, A. J., & Lacoste, B. (2023). 16p11.2 haploinsufficiency reduces mitochondrial biogenesis in brain endothelial cells and alters brain metabolism in adult mice. *Cell reports, 42*(5), 112485. https://doi.org/10.1016/j.celrep.2023.112485

Author contributions: The end of the article summarizes the broader author contributions. Relevant to this thesis, AB-M conducted, collected and analysed the western blot and electrochemical biosensor data, as well as wrote the manuscript (original and subsequent edits), in collaboration with BL.

Abstract

Neurovascular abnormalities in mouse models of 16p11.2 deletion syndrome are reminiscent of alterations reported in murine models of glucose transporter deficiency, including reduced brain angiogenesis and behavioral alterations. Yet, whether neurovascular alterations affect brain metabolism in *16p11.2*$^{df/+}$ mice is unknown. Here, we report that anesthetized *16p11.2*$^{df/+}$ mice displayed elevated brain glucose uptake, a phenomenon recapitulated in mice with endothelial-specific 16p11.2 haplodeficiency. Awake *16p11.2*$^{df/+}$ mice displayed attenuated relative fluctuations of extracellular brain glucose following systemic glucose administration. Targeted metabolomics on cerebral cortex extracts measured enhanced metabolic responses to systemic glucose in *16p11.2*$^{df/+}$ mice that also displayed reduced mitochondria number in brain endothelial cells. This was not associated with changes in mitochondria fusion or fission proteins, but *16p11.2*$^{df/+}$ brain endothelial cells lacked the splice variant NT-PGC1α, suggesting defective mitochondrial biogenesis. We propose that altered brain metabolism in *16p11.2*$^{df/+}$ mice is compensatory to endothelial dysfunction, shedding light on previously unknown adaptative responses.

Keywords: *Autism spectrum disorders, 16p11.2 deletion, metabolism, brain, glucose, lactate, mouse, mitochondrion*

Introduction

The brain is highly vulnerable to defects in energy intake, production and utilization, highlighting the importance of the interplay between vascular, glial and neuronal systems for proper function (Andreone et al., 2015; Sonnay et al., 2017; Carmeliet & Jain, 2011). We recently revealed a causal relationship between endothelial deficits and neuronal abnormalities in a mouse model of the 16p11.2 deletion syndrome (*16p11.2*$^{df/+}$ mice) (Ouellette et al., 2020), a common mutation linked to autism spectrum disorders (ASD) (Rylaarsdam & Guemez-Gamboa, 2019).

Copy number variations associated with psychiatric conditions are frequently found in the human 16p11.2 locus (Kumar et al., 2008; Weiss et al., 2008; Steinberg et al., 2014). Among genes found at the 16p11.2 locus (7qF3 in mice), *AldoA* regulates glycolysis and gluconeogenesis, while *Mapk3* and *Maz1* control vascular health (Andrikopoulos et al., 2017; Smits et al., 2012; Yu et al., 2016). Interestingly, *16p11.2*$^{df/+}$ mice display striking similarities with mouse models of glucose transporter-1 (GLUT-1) deficiency syndrome (Ouellette et al., 2020; Futuse et al., 2019; Veys et al., 2020; Tang et al., 2021). Despite these similarities, whether brain metabolism is altered in *16p11.2*$^{df/+}$ mice is still unknown, and the question remains whether 16p11.2-associated neurovascular alterations lead to changes in brain metabolism.

Growing evidence implicates metabolic contributions to the pathogenesis of ASD. It is generally accepted that these metabolic discrepancies in ASD relate to mitochondrial dysfunction (Citrigno et al., 2020; Frye et al., 2021), and we have recently shown that *16p11.2*$^{df/+}$ mice display alterations in energy expenditure and concentration of plasma metabolites related to the mitochondrion (Menzies et al., 2021). While metabolic disturbances were also identified in the

brain of ASD patients (Anil Kumar et al., 2017; Cheng et al., 2017; Gu et al., 2013; Jann et al., 2015; Rossignol & Frye, 2012), the cellular and molecular mechanisms underlying these alterations are unknown. Here, we investigated key metabolic players in the cerebral cortex of adult male *16p11.2*$^{df/+}$ mice that displayed significant neurovascular deficits compared to females (Ouellette et al., 2020), and their wild-type (WT) littermates in order to determine whether the previously-observed neurovascular deficits are associated with and/or lead to changes in brain metabolism.

Results

Anesthetized 16p11.2df/+ male mice display elevated brain glucose uptake

As glucose is a major fuel for brain cells, we first assessed glucose entry by means of positron emission tomography (PET) of [^{18}F]-2-fluorodeoxyglucose (FDG) uptake. FDG-PET signals revealed a significant increase in resting-state glucose uptake in the brain of anesthetized adult *16p11.2*$^{df/+}$ males compared to age-matched WT littermates (**Figure 1A-C**). This phenotype appeared throughout the central nervous system (CNS) of mutant animals (**Figure S1A**) and was more pronounced in fasted mice (**Figure 1C** and **Figure S1B**). Postmortem autoradiographs confirmed that increased FDG-PET signals were due to increased FDG accumulation in brain tissue (**Figure S1C**). Furthermore, increased parenchymal FDG was not attributed to compromised cerebrovascular permeability, as demonstrated by the absence of Evans blue dye leakage in *16p11.2*$^{df/+}$ mice (**Figure 1D**). As glucose is most efficiently used when it undergoes oxidative phosphorylation, we also assessed whether elevated glucose uptake in *16p11.2*$^{df/+}$ mice correlates with increased oxygen extraction/utilization. When quantifying cerebral blood oxygen saturation by photoacoustic imaging (**Figure S1D-G** and **Figure S2**), we found no global difference in blood oxygen saturation between male *16p11.2*$^{df/+}$ and WT littermates, suggesting that increased oxidative phosphorylation may not drive elevated FDG-PET signals in *16p11.2*$^{df/+}$ male mice.

Awake 16p11.2df/+ male mice display brain-specific alterations in glucose metabolism

To determine whether extracellular metabolite fluctuations are impacted by constitutive 16p11.2 (7qF3) haploinsufficiency, we measured extracellular glucose and lactate in the primary motor (M1) cortex of awake, freely moving adult male *16p11.2*$^{df/+}$ mice and WT littermates using electrochemical biosensors. We altered the systemic availability of different metabolites

with various energy-producing capacities (i.e., glucose, lactate, fructose and β-hydroxybutyrate-BHB) through intraperitoneal (i.p.) injections and measured relative fluctuations of glucose and lactate in the extracellular space (**Figure 1G-L** and **Figure S3D-H**). Extracellular brain lactate was monitored in addition to glucose as a known by-product of glycolysis which can be used as an indicator of non-oxidative glucose consumption (Kiyatkin & Lenoir, 2012). We found that systemic glucose injection failed to induce the expected surge in relative extracellular glucose above baseline in *16p11.2*$^{df/+}$ mice (**Figure 1J**). Measurement of peripheral glucoregulatory responses in *16p11.2*$^{df/+}$ and WT mice via i.p. glucose and insulin tolerance tests (**Figure 1E,F** and **Figure S3A-C**) found no difference between genotypes, ruling out altered peripheral glucose availability as a root cause for the lack of net brain extracellular glucose increase in mutant mice. Growing evidence shows that the brain can meet a portion of its energetic needs by using alternative fuels such as ketone bodies or lactate, transported into the brain by the first isoform of the proton-coupled monocarboxylate transporter (MCT-1) (Jensen et al., 2020). Here, we find that increases in systemic lactate, fructose and BHB via i.p. injections are all capable of increasing extracellular brain glucose to comparable levels in *16p11.2*$^{df/+}$ mice and WT controls (**Figure 1L** and **Figure S3D,F**). However, 16p11.2 haploinsufficiency abolished the accumulation of extracellular brain lactate following an i.p. lactate injection (**Figure 1K**).

Finally, to assess whether extracellular glucose or lactate accumulation varied during physical activity (i.e., M1 cortex activation), mice underwent a behavioral task to activate the motor cortex (Béland-Millar & Messier, 2022) (**Figure 1M**). For both mutant and WT mice, extracellular glucose in M1 cortex remained relatively stable during this task (**Figure 1N**). However, only WT mice exhibited the expected rise in extracellular lactate, a normal consequence of neuronal activation (Béland-Millar & Messier, 2018) (**Figure 1O**).

Normal levels of transporter and canonical glycolytic pathways in the brain of male 16p11.2df/+ mice

One possible explanation for increased brain glucose uptake may be an elevated expression of cortical transporter proteins. Western blot measurements of cortical GLUT-1, GLUT-3 and MCT1 levels revealed no difference between *16p11.2*$^{df/+}$ mice and WT littermates (**Figure S4A-C**). Our previous mouse cerebral cortex endothelial cell RNAseq database (Ouellette et al., 2020) also showed normal expression of endothelial *Scl2a1* (GLUT-1) and *Scl16a1* (MCT-1) in *16p11.2*$^{df/+}$ mice (**Figure S4A,C**). Immunohistochemistry also revealed comparable levels of endothelial GLUT-1 in *16p11.2*$^{df/+}$ mice and WT littermates (**Figure S4D**). Thus, elevated glucose uptake and attenuated extracellular lactate response to an i.p. lactate injection in *16p11.2*$^{df/+}$ mice may not result from changes in transporter levels. Increased brain glucose uptake, unchanged transporter expression and stable extracellular glucose pools suggests rapid glucose utilization in *16p11.2*$^{df/+}$ mice and/or saturated glucose transport at the blood-brain barrier. Stable blood oxygen saturation would indicate that glycolytic upregulation may be a likely mechanism supporting elevated glucose utilization. However, we find that protein levels of key enzymes involved in glycolysis are unchanged in the cerebral cortex of *16p11.2*$^{df/+}$ mice, including hexokinases 1&2, glycogen synthase kinase 3-beta (GSK3-β), as well as 5' AMP-activated protein kinase (AMPK) and its phosphorylated form (**Figure S4E**).

Adult male 16p11.2$^{df/+}$ *mice display enhanced metabolic responses to systemic glucose in the cerebral cortex*

To better understand the kinetics of altered brain glucose metabolism in adult *16p11.2*$^{df/+}$ male mice following increases in systemic glucose, we performed targeted metabolomics on cerebral cortex extracts to assess changes in metabolite profiles after i.p. glucose injections. We

matched the timing of metabolite extraction to the time course of extracellular glucose assessment (see **Figure 1J**), as follows: after a PBS injection (equivalent to baseline); 5 min after a glucose injection (rising slope); and 15 min after a glucose injection (maximal values preceding falling slope). Cortical metabolome of PBS-injected WT and *16p11.2*$^{df/+}$ littermates demonstrated distinct profiles, as shown by cluster separation of datasets by partial least squares-discriminant analysis (PLS-DA), confirming altered basal brain metabolism in *16p11.2*$^{df/+}$ mice compared to WT littermates (**Figure 2A**). From the top 25 altered metabolites (that include amino acids and nucleotides), deoxycytidine 5-monophosphate, inosine 5-monophosphate and malic acid were found most significantly increased in *16p11.2*$^{df/+}$ compared to WT mice, indicative of changes in DNA biosynthesis, purine metabolism and TCA cycle (**Figure 2A**). Metabolites measured 5 min (**Figure 2B**) and 15 min (**Figure 2C**) after i.p. glucose injection also demonstrated distinct profiles between *16p11.2*$^{df/+}$ and WT littermates (see also **Figure S5**). Overall, peripheral glucose injections in *16p11.2*$^{df/+}$mice resulted in increased abundance of metabolites involved in energy metabolism, including trans-aconitic acid, an intermediate metabolite in the TCA cycle; carnitine, required for energy production and fatty acid metabolism; citramalic acid, involved in pyruvate metabolism; and fructose 1,6-biphosphate, a high-energy intermediate of glycolysis (**Figures 2B,C**). These metabolite alterations differ from those found at baseline (**Figure 2A**), supporting our evidence of increased brain glucose metabolic rates in *16p11.2*$^{df/+}$ mice. We also identified time-dependent changes in metabolic responses to increased systemic glucose availability between WT and *16p11.2*$^{df/+}$ mice (**Figure 3** and **Figure S5**). In particular, time course heat maps for either group averages (**Figure 3A**) illustrate distinct dynamic responses to glucose availability between genotypes. Altogether, these

findings demonstrate that *16p11.2*$^{df/+}$ mice display a shift in brain glucose metabolism, which varies in function of glucose availability.

Endothelial-specific 16p11.2 haploinsufficiency leads to elevated brain glucose uptake

Our previous work demonstrated that endothelium-specific 16p11.2 deletion (*16p11.2*$^{\Delta EC}$ mice) partially recapitulated neurovascular and behavioral abnormalities observed in constitutive *16p11.2*$^{df/+}$ mutants (Ouellette et al., 2020). Therefore, we opted to look more closely at the role of the endothelium in 16p11.2 deletion-induced metabolic dysregulation. Similar to anesthetized *16p11.2*$^{df/+}$ male mice, *16p11.2*$^{\Delta EC}$ males displayed a significant increase in resting-state brain FDG uptake compared to age-matched *Cdh5-Cre*$^{Tg/+}$ control littermates (**Figure 4A**). This phenotype also appeared throughout the CNS of mutant animals (**Figure 4B**). These data demonstrate that endothelial 16p11.2 deficiency is sufficient to drive a CNS-wide increase in glucose uptake.

Brain endothelial cells from adult 16p11.2*$^{df/+}$ *male mice possess less mitochondria

The observations above led us to test whether the metabolic machinery is altered in brain endothelial cells from constitutive *16p11.2*$^{df/+}$ mutants *in vivo*. Analysis of transmission electron micrographs revealed a significant reduction in the number of mitochondria within brain capillary endothelial cells (**Figure 4C**), but not in surrounding astrocytic endfeet (**Figure S6A**), in *16p11.2*$^{df/+}$ mice compared to WT littermates. There was, however, no impact of the 16p11.2 deletion on mitochondria ultrastructure (**Figure 4C**). Fewer endothelial mitochondria in *16p11.2*$^{df/+}$ mice may imply a reduced capacity for oxidative phosphorylation, further supporting that the observed metabolic changes are unlikely to stem from elevated oxidative phosphorylation/mitochondrial glucose use. To assess whether reduced mitochondria number in *16p11.2*$^{df/+}$ brain endothelial cells was caused by altered mitochondria dynamics, we measured

protein levels of key regulators of mitochondria fusion and fission, including Optic Atrophy 1 (OPA1), dynamin-related protein 1 (DRP1), and Mitofusins-1&2 (MFN1, MFN2). None of these proteins appeared altered in *16p11.2*$^{df/+}$ brain endothelial cells (**Figure 4D** and **Figure S6B**). To test the possibility of dysregulated mitochondrial biogenesis in *16p11.2*$^{df/+}$ brain endothelial cells, we quantified protein levels of the nuclear transcription factor peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), as well as its truncated splicing variant N-terminal (NT)-PGC-1α which orchestrate the expression of genes involved in mitochondrial biogenesis by activating additional transcription factors (Chang et al., 2018). While protein levels of PGC-1α did not appear significantly reduced in *16p11.2*$^{df/+}$ brain endothelial cells compared to WT cells (**Figure S6C**), a most striking finding was the absence of NT-PGC-1α in 16p11.2-deficient brain endothelial cells (**Figure 4E** and **Figure S6D**). These data suggest impaired mitochondrial biogenesis as a potential source of reduced mitochondria number and ensuing endothelial dysfunction in *16p11.2*$^{df/+}$ mice.

Discussion

Here, we demonstrate that glucose uptake was increased in a mouse model of the 16p11.2 deletion syndrome. While, FDG uptake has been shown to mostly depend on astrocytes (Zimmer et al., 2017; Zimmer et al., 2022; Rocha et al., 2022), we have previously shown that, at least structurally, astroglial and microglial lineages develop normally in *16p11.2*$^{df/+}$ mice (Ouellette et al., 2020). In addition, we provide evidence of normal mitochondria in astrocytic endfeet of *16p11.2*$^{df/+}$ mice. Increased brain glucose transport, measured by FDG, has also been demonstrated in a mouse model of GLUT1 deficiency (Furuse et al., 2019). In this GLUT1 deficiency model, glucose influx into the brain was expectedly reduced, but intracellular phosphorylation was increased, resulting in elevated deoxyglucose accumulation (Furuse et al., 2019). Thus, demonstrating means by which brain glucose uptake can take place without proportionate increases in transport (Leybaert et al., 2007). Despite all the normal parameters observed in the 16p11.2 haplodeficient mice (e.g., oxygen extraction, transporters, permeability, hexokinase), elevating blood glucose concentration did not result in net elevations of extracellular brain glucose in 16p11.2 haplodeficient mice, suggesting that glucose transport is saturated in order to meet heightened brain glucose use through non-oxidative pathways. Elevated extracellular glucose following systemic injections of lactate, fructose and BHB further supports the notion of saturated vascular glucose transport as these substrates enter the brain through alternative transports (MCT1 and GLUT5) and, as alternative fuels, can spare glucose use (Svart et al., 2018; LaManna et al., 2009; van Hall et al,. 2009; Wyss et al., 2011).

In line with previous reports of mitochondrial dysregulation in neurodevelopmental disorders, including ASD (Graham et al., 2020; Yardeni et al., 2021), we have demonstrated a decreased mitochondria number in 16p11.2-deficient capillary endothelial cells, consistent with

absence of transcription factor NT- PGC1α regulating mitochondrial biogenesis. This finding is particularly interesting considering that endothelial-specific 16p11.2 deletion recapitulates many of the observations made with constitutive 16p11.2 haplodeficiency, including cerebrovascular and behavioral abnormalities (Ouellette et al., 2020). Since endothelial cells are glycolytic (Leung & Shi, 2022; Groschner et al., 2012; Eelen et al., 2015), the energy producing capacity of endothelial mitochondria is secondary to its signaling properties in maintaining proper health and function, such as vasodilation (Eelen et al., 2015), blood-brain barrier integrity (Doll et al., 2015; Eelen et al., 2018), and endothelial proliferation (Leung & Shi, 2022).

Neuronal activation, including during behavior, increases extracellular lactate through what is thought to be a glycolysis (Pellerin & Magistretti, 1994) or glycogen (Newman et al., 2011) dependent process. Here, 16p11.2 haplodeficiency abolished this behavior-induced rise in extracellular lactate in M1 cortex, suggesting reduced glycolytic capacity. This is in contrast to multiple reports of increased glycolysis in ASD (Pecorelli et al., 2020; Rose et al., 2017), and our observation of elevated inosine 5-monophosphate, which has been associated with elevated glycolysis in rabbit heart (Lewandowski et al., 1991). Shortly after the metabolic challenge (glucose injection), trans-Aconitic acid was upregulated in the cerebral cortex of *16p11.2*$^{df/+}$ mice, which may have inhibited oxidative phosphorylation (OXPHOS) (Weinhouse et al., 1951). Subsequently, carnitine and fructose 1,6-bisphosphate were upregulated. Carnitine is essential for long-chain Acyl-CoA transport into the mitochondria, and thus beta-oxidation, likely representing an increase in fatty acid oxidation (Virmani & Cirulli, 2022). Moreover, trends towards increased p-AMPK/AMPK ratios may indicate a subtle reduction in glycogen production (Jeon, 2016) and a shift towards β-oxidation (Huang et al., 2017), respectively. Fructose 1,6-bisphosphate is capable of both reducing neuronal excitability (Shao et al., 2018)

and shifting glycolytic products away from OXPHOS and towards the pentose phosphate pathway (PPP) (Lian et al., 2007). The results further nuance the altered metabolic profile of the ASD model, suggesting a shift away from glucose-centric ATP producing pathways towards lipid metabolism and shunting of glucose towards the PPP. The PPP uses glucose to maintain antioxidant capacity in cells (Cherkas et al., 2020). Enhanced processing of glucose through the PPP may be required in *16p11.2*$^{df/+}$ mice to compensate for enhanced ROS production that accompanies the often-observed dysfunctional mitochondria (Napoli et al., 2013; Rose et al., 2014; Rose et al., 2017). Given that the PPP is not an oxygen dependent pathway, shunting additional glucose taken up by the *16p11.2*$^{df/+}$ mouse brain towards this pathway may provide an explanation for both comparable oxygen saturation and elevated glucose uptake.

Altogether, we raise the question of how additional glucose taken up by the brain is metabolized, and for what purpose. Our findings show that constitutive 16p11.2 (7qF3) hemizygosity leads to a shift in brain glucose metabolism. We propose a new model whereby *16p11.2*$^{df/+}$ male mice modulate their glycolytic pathway to shunt additional glucose towards the PPP caused by dysfunctional endothelial cells that possess less mitochondria. Consistent with observations of dysregulated signaling, hypermetabolism, and benefits of altered diets in ASD (Eltokhi et al., 2020; Castro et al., 2017), this model suggests that the resulting energy deficit is mediated by increased mitochondrial fatty acid oxidation to meet the brain's elevated energetic demands. However, alternative possibilities cannot be ruled out. During development, glycolysis is increased in correlation with moments of high synaptic growth. Lactate (a glycolytic by product) has been shown to act as a signaling molecule capable of potentiating angiogenesis and neurogenesis (Oyarzábal et al., 2021) as well as regulate neuronal activity (Karagiannis et al., 2021; Skwarzynska et al., 2022; Hollnagel et al., 2020) and synpatic plasticity (Margineanu et

al., 2018; Herrera-López et al., 2020). Recent hypotheses suggest a main function of glycolysis, and its by-products, is to support biosynthesis (Bauernfeind et al., 2014; Goyal et al., 2014; Shannon et al., 2016; van Heerden et al., 2015). Therefore, altered glycolysis may represent an energetic adaptation to deficient mitochondria, or a biosynthetic compensatory mechanism to support the additional bioinfrastructure required due to unregulated growth within the ASD brain. Future work may wish to investigate mechanisms underlying the metabolic dysfunction induced by this mutation and the dependence of these dysregulations on endothelial dysfunction with respect to ROS production and the PPP.

Limitations of the study

The results presented herein lead us to hypothesize a causal relationship between endothelial abnormalities and CNS-wide altered metabolic profile in *16p11.2*$^{df/+}$ male mice. This hypothesis is, however, based on assumptions that should be verified in future work. The tools and methods enabled inferences regarding the impact of this mutation on metabolic pathways, which will need to be confirmed with more direct measures. Chiefly, we recognize that measures of protein expression do not equate enzymatic or transporter activity and acknowledge that the methodologies used (e.g., FDG-PET scans, biosensors, photoacoustic imaging, metabolomics) to infer activity of various metabolic pathways (OXPHOS, glycolysis, PPP) provide an incomplete snapshot. Biosensors measure net-fluxes of metabolites without representing the various influxes and effluxes, the metabolomics and measures of protein expression are not specific to cellular or sub-cellular compartments. Future studies may wish to include measures of mitochondrial respiration that enable the location of dysfunction, if any, at specific complexes (e.g, inhibition of Complex II by oxaloacetate), as well as measure direct oxygen use and energy production. Nuclear magnetic resonance spectrometry coupled with ^{13}C-labeled glucose (^{13}C NMR) would

allow for a more comprehensive understanding of substrate fate through the complementary pathways discussed (glycolysis, OXPHOS, PPP). Finally, this work focuses on the *metabolic* contributions of these substrates to the observed results. However, it does not assess or disentangle the impact that these substrates have on other processes through their signaling properties (e.g., effect of lactate on neuronal activity). Despite these limitations, we provide initial evidence of an altered CNS metabolic profile in a mouse model of a common mutation linked to ASD.

Experimental model and subject details

Animals

All animal procedures were approved by the University of Ottawa Animal Care Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines.

Mouse husbandry. All mice were bred in house and housed maximum five per cage with free access to water and food. Mice subjected to awake monitoring of brain metabolites were kept on a reverse light cycle. Males *16p11.2*$^{df/+}$ (Jackson laboratory, stock #013128; mixed B6/129 background) were crossed with WT females of the same background (Jackson laboratory, stock #101043) to obtain hemizygous *16p11.2*$^{df/+}$ offspring as well as WT littermates. As recommended by Jackson Laboratory to improve *16p11.2*$^{df/+}$ pup survival, breeding cages (both constitutive and conditional mutants) were supplemented with breeding chow (#2019, Envigo Teklad) and DietGel (#76A, ClearH_2O) up to weaning age[6]. Testing procedures were conducted with mice aged P>50.

Genotyping. *16p11.2*$^{df/+}$ and control littermates (WT) mice were genotyped using the following primers: 5'-CCTCATGGACTAATTATGGAC-3' (forward) and 5'-CCAGTTTCACTAATGACACA-3' (reverse) with a PCR product of 2.2Kb for *16p11.2*$^{df/+}$ mice.

Method details

FDG-PET scan

Mice were fasted for 8 hrs prior to imaging and anesthetized by isoflurane. A tail vein cannula was inserted prior to being placed in a custom 4-position multi-animal hotel secured to the gantry of an Inveon D-PET scanner (Siemens). Mice were kept warm during the duration of the PET scan, which was followed by a 10 min transmission scan acquired under standard conditions. Once aligned in the PET field of view, a 45-minute acquisition protocol in list mode format was initiated and approximately 200 μCi [^{18}F]-2 fluoro-D-deoxyglucose (FDG) was injected *intravenously* exactly 30 sec afterwards. Iterative reconstruction was performed using 3D ordered-subsets expectation maximisation (3D-OSEM) followed by fast maximum *a posteriori* (fastMAP) with the following parameters: MAP OSEM iterations, 2; MAP subsets, 16; MAP iterations, 18. Under anesthesia, mice were immediately transferred to a 3.0 T pre-clinical MRI (MR Solutions Inc.), and a T_2-weighted, axially sectioned, respiratory-gated Fast Spin Echo image of the head was acquired with the following parameters: 31 slices; 0.5 thickness and 0.1 spacing; FOV = 30 with FOV ratio = 0.75; TR=4800, TE=68, nex=4. VivoQuant was used for visualization of radiotracer uptake in the brain, to align PET with MRI images, and to segment the brain into individual brain regions using a proprietary mouse brain atlas algorithm (VivoQuant v. 4.0, InviCRO, LLC). The count densities were averaged for all volumes of interest at each time point to obtain a time *versus* activity curve for the entire brain, and average count densities were averaged for each brain region analysed for a bin spanning 40 to 45 min post-FDG injection. All quantified data were normalized to injected dose, measured by a CRC-15 PET dose calibrator (Capintec Inc), and expressed as percentage injected dose per mL of tissue (%I.D./mL).

For *in situ* autoradiography, fasted mice were administered 500 μCi of FDG by *intravenous* bolus injection under isoflurane anesthesia, followed by euthanasia by exsanguination *via* cardiac puncture 45 min after radiotracer injection. Cardiac perfusion was performed with saline to clear blood from tissues, and brains were resected and frozen in optimal cutting temperature medium compound (Tissue-Tek) on dry ice. Frozen blocks were equilibrated to -20°C for 30 min prior to sectioning. Tissue 12 μm-thick sections were mounted onto microscope slides, air dried for 5 min, and exposed to a [^{18}F]-sensitive storage phosphor screen (Perkin Elmer) overnight at 4°C. Screens were scanned on a Cyclone Storage Phosphor System (Perkin Elmer), and image data was processed with ImageJ.

Extracellular brain glucose and lactate measurements

Surgery. Pre-surgical treatment included a 0.6 mg/kg subcutaneous (s.c.) injection of slow-release buprenorphine (Chiron Compounding Pharmacy Inc, Guelph, ON, CAN) and 1 ml of 0.9% saline (Hospira, QC, CAN). Immediately prior to surgery, mice were anesthetized using 4-5% isoflurane (Fresenius Kabi Canada Ltd., ON, CAN) then secured to a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Mice were maintained under anesthesia during the surgery with 1–2.5% isoflurane and kept warm with a heated pad (TP650, Gaymar Industries, Orchard Park, NY, USA). Based on the stereotaxic atlas of Franklin and Paxinos (2008), two guide cannulas (BASi cannula Bioanalytical Systems, West Lafayette, IN) were positioned above the left and right primary motor cortex. The target coordinates, from Bregma, were ± 1.8 mm lateral and +1.0 mm anterior. The cannulas were positioned just beneath the skull surface so that the sensing cavity (1 mm) of the electrochemical electrode would protrude, when inserted at the time of testing. These guide cannulas were fixed using a UV-polymerized compound (UV Clear Fly Finish, Ashland, OR, USA) applied around four 0.10″-inch skull screws positioned anterior and posterior to the two

guide cannulas. Post-surgical analgesia included application of 0.1 ml of transdermal bupivacaine (Chiron Compounding Pharmacy Inc., ON, CAN) around the incision area. After surgery, the mice were individually housed for 7 days to allow recovery and limit possible incidents with the newly inserted cannulas.

Electrodes. Briefly, extracellular brain levels of glucose and lactate were measured using two platinum enzyme-linked electrodes (7004-Glucose-C and 7004-Lactate-C; Pinnacle Technology Inc., Lawrence, KS, USA). Sensors were prepared from Pt-Ir wire of 180 µm diameter, wrapped concentrically with an AgCl reference electrode. Near the tip of the electrode, a 1 mm section is coated with either glucose or lactate oxidase. These enzymes oxidize the analytes of interest, resulting in an equivalent release of hydrogen peroxide (H_2O_2). H_2O_2 is subsequently detected and recorded as an amperometric oxidation current at the electrode. Data was collected at 1 Hz, transmitted from a potentiostat to a computer and recorded using the Sirenia Acquisition Software (Pinnacle Technology Inc., Lawrence, KS, USA). In addition to the oxidase enzymes, the metabolite sensitive surface is also coated with a series of membranes to increase the specificity and selectivity of the electrodes. For example, the potential contribution of ascorbic acid is reduced with the presence of ascorbic acid oxidase. These particular enzymes convert the electroactive ascorbate, which has the potential to interfere with the electrode's recordings, to non-electroactive dehydroascorbate and water.

Calibration. Immediately prior to and following *in vivo* testing, the electrodes were calibrated according to factory recommendations. Briefly, the electrodes were gently placed in 100 mM PBS (pH 7.4) at 23°C (pre-testing) or 37°C (post-testing) and tested for metabolite sensitivity by adding glucose (1 mM) and lactate (0.1 mM) to the PBS solution, as well as selectivity with the use of a single ascorbic acid (0.25 mM) addition. Pre-calibration curves demonstrated high

sensitivity with incremental linear increases for both glucose (average sensitivity 0.314 nA/1mM) and lactate (average sensitivity 0.023 nA/0.1 mM) electrodes. Both glucose and lactate electrodes also exhibited high levels of selectivity by demonstrating minimal sensitivity to ascorbic acid (average sensitivity 0.143 nA/0.25 mM and 0.206 nA/0.25 mM, respectively). The post-calibration curves suggested a certain level of wear on the electrodes, most likely due to the insertion and removal process of these sensitive testing instruments. Similar results were obtained with linear increases for both glucose (average sensitivity 0.447 nA/1mM) and lactate (average sensitivity 0.068 nA/ 0.1 mM) electrodes. Post calibration curves for ascorbic acid found increased sensitivity to ascorbic acid for the lactate electrodes (average sensitivity 0.038 nA/0.25 mM and 2.159 nA/0.25 mM for glucose and lactate, respectively).

Testing Procedure. Seven days following surgery, and immediately after electrode calibration, the mice were briefly anesthetized with 1.5-2% isoflurane and the electrodes were inserted into the motor cortex through the surgically implanted guide cannulas and attached to the pre-amplifier. Mice were tested for two days in a custom-made square polymethyl methacrylate-testing cage with standard bedding (Teklad 7097 Corncob bedding, Envigo, ON, CAN) and water. A mounted camera (Pinnacle Technologies) above the cage recorded the duration of the testing and was synchronized with the electrode sampling. Mice had free access to water, but chow was removed 2 hours prior to injection to ensure similar fasting states between the mice and returned after the last manipulation. Given that previous work demonstrated that extracellular brain concentrations of glucose and lactate stabilize within 2 hours post-injection and that the intracortical electrodes reliably measured extracellular concentrations for at least 48 hours, mice received 2 injections a day (10am and 1pm) for 2 days. The mice received a 2 g/kg intraperitoneal (i.p.) injection of either of either glucose (Dextrose Anhydrous, EMD Chemicals, Gibbstown, NJ,

USA), lactate (Lactic Acid, Acros Organics, NJ, USA), fructose (D-Fructose, Fisher Scientific, Fair Lawn, NJ, USA) or β-hydroxybutyrate, BHB (Sigma Aldrich, St. Louis, MO, USA) in a counterbalanced order.

Two hours after each injection, the mice were placed on a vertical metal mesh (10 x 17 inches) with half-inch square holes. The mesh was held vertically while the mouse was left free to explore for 2 minutes. This approach was chosen following internal testing. Other tasks proved either proved too challenging with sensitive testing equipment (e.g., rotarod or treadmill) or unreliable in terms of measurements or mouse behavior. For example, using a running wheel within the caged resulted in irregular running bouts that were not consistent in intensity or duration, making comparisons challenging. In addition, during our trials we observed the well-documented phenomenon of metabolic efficiency that comes with practice. To avoid missing metabolite fluctuations as a result of habituation, we opted for an ecological behavior that it did not require training but result in robust metabolite fluctuations. These preliminary efforts indicated that the grid climb was a behavior the mice readily engaged in with no prior training while resulting in regular and robust fluctuations of extracellular brain glucose and lactate that were similar to the documented pattern expected to occur following neuronal activation.

Photoacoustic imaging

Imaging was performed using the Vevo LAZR-X high-frequency ultrasound and photoacoustic imaging (PAI) system (FUJIFILM VisualSonics, Toronto, Canada). All images were acquired using the MX250 21MHz linear array transducer, fitted with medium sized optical fibers. Animals were anesthetized with a xylazine/ketamine cocktail (0.1 ml/100 g). The head of each animal was secured and stabilized using a stereotactic frame attached to the imaging platform. Key physiological parameter such as heart rate, respiration rate and temperature were monitored

throughout the imaging session. B-mode imaging was used for region of interest selection, followed by PAI mode. 2-D and 3-D PAI images were acquired under Oxy-Hemo Mode, using dual-wavelength imaging, alternating between 750nm and 850nm, for real-time display of oxygen saturation (sO_2). All 3D data were acquired by moving the transducer with a stepper motor, at a step size of 254µm.

Peripheral measurements of glucose metabolism

Body composition analysis. Total fat and lean mass were assessed using a nuclear echo magnetic resonance imaging (MRI) whole-body composition analyzer.

Glucose tolerance test. Experimental mice were food-deprived during 4 h with *ad libitum* access to water. A bolus of glucose (1.5 g/kg of body weight or lean body weight) was administered via an intraperitoneal (i.p.) injection and glycaemia was measured from blood sampled at the tail vein using a Accu-chek Performa glucometer at T0 (before injection), 15, 30, 45, 60, 90 and 120 min. Tail vein blood samples were collected via a capillary for insulin assays at T0, 15 and 30 min.

Insulin tolerance. Experimental mice were food-deprived during 4 h with *ad libitum* access to water. A bolus of insulin (0.2U/kg of body weight) was administered via an intraperitoneal (i.p.) injection and glycaemia was measured from blood sampled at the tail vein using a Accu-chek Performa glucometer at T0 (before injection), 15, 30, 45, 60, 90 and 120 min.

Western blot on cerebral cortex or brain endothelial cell lysates

Tissue Preparation. All mice were euthanized by cervical dislocation. For cerebral cortex proteins, the brains rapidly removed in cold 1M PBS and the cerebral cortex was then microdissected and kept on ice. The cortex was then mechanically dissociated in RIPA buffer (NaCl 150mM, Sodium Deoxycholate 12mM, SDS 3.5mM, Tris 50mM, Triton x-100 1%v/v, pH

8.0) with protease and phosphatase inhibitors. The cell suspension was homogenized via ice-cold sonication, mixed at 4°C for 15 minutes then centrifuged (13,000rpm for 20 minutes at 4°C). The protein concentration of supernatant was quantified using Pierce BCA Protein assay (kit #23227, Thermo Scientific, Illinois, USA). For endothelial proteins, the cerebral cortex was dissected in cold HBSS without calcium and magnesium using autoclaved tools submerged in 100% ethanol for 30 minutes prior dissections. The cortex was minced in 2-3 mm pieces and dissociated in Neural Tissue Dissociation Kit P compounds (Miltenyi Biotec, 130-092-628) to obtain a cell suspension. Cell isolation procedures were completed according to the manufacturer's instructions. The cell suspension was incubated with CD31-coated magnetic microbeads (Miltenyi Biotec, 130-097-418) and placed on a magnetic MACs separator to isolate endothelial cells (ECs). ECs isolated from one mouse were seeded in 2 wells of a 6-well plate coated with attachment factor protein 1X (ThermoFisher Scientific, S006100). For a pure EC population, ECs were cultured in an EC specific medium (Lonza, CC-3202) which was replaced 48 hours post-seeding and every 48 hours until 100% confluency was reached for subsequent protein extraction. To lyse cells, 50uL of RIPA buffer (NaCl 150mM, Sodium Deoxycholate 12mM, SDS 3.5mM, Tris 50mM, Triton x-100 1%v/v, pH 8.0) with protease and phosphatase inhibitors was added to each well and left on ice for 30 minutes. The supernatant was transferred to a 1.5mL tube and then centrifuged at 13,000rpm for 10 minutes. The protein concentration of the collected supernatant was quantified using Pierce BCA Protein assay (kit #23227, ThermoScientific, Illinois, USA).

Immunoblotting. Fifteen (15) µg of protein were loaded into the wells of SDS-acrylamide gels (GX Stain-free FastCast 12% Biorad gels, Bio-Rad, ON, CAN, #1610184) and separated by a constant current of 100 V for 120 minutes in running buffer (SDS 35mM, Tris 250mM, Glycine 1865mM). After UV activation of the gels, the proteins were then transferred to a nitrocellulose

membrane in ice-cold transfer buffer (Tris 48mM, Glycine 38mM, methanol 20% v/v) for 30 minutes at 100 V. After the transfer, the nitrocellulose membranes and gels were imaged to quantify the total protein transferred. The membranes were then blocked with 5% skim milk in 1M PBS for 1 hour at room temperature and incubated with primary antibodies raised again either MCT1 (1:200, Alomone, #AMT-011), GLUT1 (1:1000, Novus-Bio, #NB110-39113), GLUT3 (1:2000, ProteinTech, 20403-1-AP), hexokinase-1 (1:1500, Abcam, ab150423), hexokinase-2 (1:1500, Abcam, ab20839), GSK3β (1:3000, Cell Signaling, 12456), AMPK (1:1000, Cell Signaling, 2532), phospho-AMPK (1:800, Cell Signaling, 2535), DRP1 (1:2000, BD Biosciences, 611113), MFN1 (Abcam, ab126575, 1:1000), MFN2 (1:1000, Abcam, ab56889), OPA1 (1:2000, Abcam, ab42364), tubulin (1:5000, Sigma, T6199), PGC1α (1:1500, Millipore, ST1202), GAPDH (1:10000, ProteinTech, 60004-1-Ig) or Vinculin (1:5000, Abcam, ab129002) in TBST (Tris 50mM, NaCl 150mM, Tween-20 1% v/v) overnight at 4°C. The membranes were then washed with TBST (3 x 10 minutes) and incubated at room temperature for 1 hour with the secondary antibody (1/10,000, Fisher Scientific, #PR-W4011), also diluted in TBST. After a final wash (TBST, 3 x 10 minutes), the proteins were detected by enhanced chemiluminescence (ECL; ¼, Fisher Scientific, #34579) and imaged with the Odyssey Imaging system. The samples were normalized to the total protein content (GLUT1, GLUT3, MCT1, hexokinase-1&2, GSK3β and AMPK) or to a loading control (OPA1, DRP1, MFN1&2, PGC1α) and calculated as relative to group average.

Immunofluorescence

All mice were euthanized by cervical dislocation. Whole brains were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. All brains were washed in PBS, submerged in 15% sucrose overnight, followed by 30% sucrose overnight, then embedded in OCT medium.

Brains were cut coronally into 25-µm-thick serial sections using a cryostat (HM525 NX, ThermoScientific), and finally mounted on charged glass slides. Sections were blocked using a solution containing 10% donkey serum, 0.5% Triton X-100 in PBS (0.5% PBT) and 0.5% cold water fish skin gelatin, and incubated overnight at 4 °C with primary antibodies anti-CD31 (1/200, BD Pharmingen, Cat# 553370), and anti-GLUT1 (1/200, Novus-Bio, Cat# NB110-39113). The following day, sections were washed with 0.5% PBT and incubated with species-specific AlexaFluor secondary antibodies (donkey anti-rat IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor 488, 1/300, Invitrogen, Cat# A-21208 and donkey anti-rabbit IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor 568, 1/300, Invitrogen, Cat# A-10042) for 2 hours at room temperature. Sections were washed with 0.5% PBT and PB then coverslipped in Fluoromount G (EMS). Immunostained sections were examined using a Zeiss Axio Imager M2 microscope equipped with a digital camera (Axiocam 506 mono) and the Zeiss ApoTome.2 module for optical sectioning. Nine 20-µm-deep z-stacks were acquired at 20X per mouse (three images per brain section, three sections per brain). GLUT1 distribution was determined using Fiji-Image J. Images were converted to 8-bit, running the Z-Project with the Max Intensity setting to obtain a 2D image. The image threshold was determined using the Huang pre-set to acquire a binary image. Analyze Particles, with size set to 5-infinity, was used to find the proportion of the image containing the signal of interest. GLUT1 distribution on cortical vessels was quantified by analyzing the surface ratio of the total CD31-positive vessel surface area also positive for GLUT1.

Systemic glucose injections and tissue collection for metabolomics

Prior injections, mice had free access to water while chow was removed for 2 hours to ensure similar fasted state. Groups of five (5) mice received an intraperitoneal (i.p.) injection of either 1x sterile 50mM PBS pH 7.4 (baseline group) or 2g/kg of glucose (Dextrose Anhydrous,

EMD Chemicals, Gibbstown, NJ, USA) for a final 1% body weight injection volume. All mice were euthanized by cervical dislocation at 5 min (PBS, glucose) or 15 min (glucose) following i.p. injection. The brain was extracted from the skull from which the cerebellum and brain stem were removed. The cerebral hemispheres were bisected along the midline for removal of the striatum, hippocampus and thalamus. After which, the intact cortex was further dissected to isolate ~20mg of tissue per sample. The cortex was then flash-frozen and stored at -80°C before further processing for metabolite extraction (performed simultaneously for all mice).

Targeted metabolomics

Sample temperature was maintained on ice or dry ice where possible, and all solvents were MS grade and pre-equilibrated to -20°C. On the day of extraction, frozen brain tissue samples were grounded into powder using a mortar and pestle on liquid nitrogen. 20 mg tissues weighed in to a cold 2mL bead beater tube containing 4 ceramic beads (2.8 mm) and 50% methanol. Bead beating was done to homogenize the tissue sample with the addition of acetonitrile. Samples were then incubated with a 2:1 dichloromethane:water solution on ice for 10 minutes. The polar and non-polar phases were separated by centrifugation at 4000g for 10 minutes at 1°C. The upper polar phase was dried using a refrigerated CentriVap Vacuum Concentrator at -4°C (LabConco Corporation, Kansas City, MO). Samples were resuspended in water and run on an Agilent 6470A tandem quadruple mass spectrometer equipped with a 1290 Infinity II ultra-high performance LC (Agilent Technologies) utilizing the Metabolomics Dynamic MRM Database and Method (Agilent), which uses an ion-pairing reverse phase chromatography (https://www.agilent.com/cs/library/applications/5991-8073EN.pdf). This method was further optimized for phosphate-containing metabolites with the addition of 5 µM InfinityLab deactivator (Agilent) to mobile phases A and B, which requires decreasing the backflush acetonitrile to

90%. Multiple reaction monitoring (MRM) transitions were optimized using authentic standards and quality control samples. Metabolites were quantified by integrating the area under the curve of each compound using external standard calibration curves with Mass Hunter Quant (Agilent). No corrections for ion suppression or enhancement were performed, as such, uncorrected metabolite concentrations are presented. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. P values <.05 were considered statistically significant. Data for group comparisons are presented as box and whisker plots and statistical analysis was performed by an unpaired t test. The box represents the 25-75 interquartile range, and the horizontal line represents the median value. The box plot analysis was performed using Prism 7 (GraphPad Software, La Jolla, CA, USA). Multivariate statistical modelling was performed on log-transformed, mean-centered and pareto-scaled data using MetaboAnalyst 5.0 (www.metaboanalyst.ca). Before multivariate analysis, missing values were replaced with 1/3 of the limit of detection of each metabolite. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed on the identified metabolites. The variables highly contributing to the group separation were selected with a variable importance in projection (VIP)≥1. The clustering analysis and pathway analysis were performed as well using MetaboAnalyst 5.0.

Transmission electron microscopy (TEM)

Mice were anesthetized with a ketamine/xylazine cocktail (1mg/kg, intraperitoneally) and perfused through the aortic arch with 3.5% acrolein and 4% paraformaldehyde. Fifty-micrometer-thick coronal sections of the brains were cut in sodium phosphate buffer (PBS 50 mM, pH7.4) using a Leica VT1000S vibratome (Leica Biosystems) and stored at -20ºC in cryoprotectant until further processing. Sections were post-fixed and embedded using variations of the protocol by

Deerinck et al. (https://ncmir.ucsd.edu/sbem-protocol). In brief, the sections were washed 3 times in PBS for 10 min and incubated in 1.5% potassium ferrocyanide and 2% aqueous osmium tetroxide in 0.1M phosphate buffer (pH 7.4) for 1 hour at room temperature. The sections were subsequently washed 5 times with double-distilled water (ddH_2O) for 3 minutes, then incubated 20 minutes in a fresh solution of thiocarbohydrazide (1% w/v) at room temperature. Sections were washed again 5 times with ddH_2O for 3 minutes, incubated 30 minutes in 2% aqueous osmium tetroxide, and washed 5 times with ddH_2O for 3 minutes. Sections were dehydrated using increasing ethanol concentrations followed by propylene oxide, and then embedded in Durcupan resin (Sigma-Aldrich) between ACLAR sheets at 55°C for 3 days. Ultrathin sections were generated at ~65 nm using a Leica UC7 ultramicrotome. Imaging was performed in the antero-frontal (2.8 to 1.98mm Bregma) and parieto-somatosensory (-0.70 to -1.82mm Bregma) areas. In each region, 10 capillaries were randomly photographed per mouse, ranging in diameter between 3 and 6 μm, using a FEI Tecnai Spirit G2 transmission electron microscope operating at 80kV and equipped with a Hamamatsu ORCA-HR digital camera (10 MP). For each capillary, an image at 4,800X was acquired, in addition to 1-3 images at 13,000X to generate a high-resolution mosaic of the capillary. Metrics included i) number of mitochondria normalized to cytoplasm area ($1/\mu m^2$); ii) total mitochondrial area normalized to cytoplasm area (%); iii) average size (nm^2) of individual mitochondria; iv) average maximal length (nm) of individual mitochondria; as well as v) aspect ratio of individual mitochondria (ratio between the major and minor axes of a mitochondrion); and vi) proportion of mitochondria display one or more cristae. All metrics were quantified manually from micrographs, using Polygon and Freehand Selection tools (mitochondrial and cytoplasmic area) or Straight Line (mitochondrial width, length), followed by the Measure function.

Photothrombotic (PT) stroke and Evans blue dye (EBD) leakage assay

To compared BBB integrity between WT and *16p11.2*$^{df/+}$ mice, we quantified of the parenchymal extravasation of EBD (70kDa) in cortical tissue lysates following retroorbital injection of the dye. In brief, mice were anesthetized with an i.p. injection of ketamine/xylazine cocktail (1 mg/kg). 2% Evans Blue Dye (EBD) solution was injected (3 ml/kg, 60 mg/kg) retro-orbitally. Body temperature was maintained during surgery at 37 ± 0.5°C using a heating pad. 1 hour after EBD circulation, the mice were transcardially perfused with cold PBS. The cerebral cortex was isolated, weighed and homogenized in a sevenfold volume of 50% trichloroacetic acid (Sigma-Aldrich, T0699). After centrifugation (10,300 rpm for 20 min at 4°C), the supernatants were collected and then diluted fourfold with 100% anhydrous ethanol. Diluted samples were loaded into a 96-well plate. Fluorescence intensity was measured using a microplate fluorescence reader (620 nm excitation, 680 nm emission; Synergy H1 Hybrid Reader, BioTek). The extravasated dye was quantified using a standard curve and expressed as ng.µg^{-1}/mg of tissue. As a positive control to assess significant EBD leakage, we performed cortical focal PT stroke in WT mice. In brief, mice received an i.p. injection of light-sensitive dye Rose Bengal (RB, 100mg/kg) while placed in a stereotaxic frame. RB will be allowed to circulate for 5 min, and a laser (530nm, 20mW) was placed 3 cm above the skull at coordinates used for targeting the M1 cortex. The laser remained ON for 10 min, activating RB and causing platelet aggregation and blood clot formation. The EBD leakage assay was performed as described above 24 hrs after stroke on cortical tissue extracts comprising the stroke core and peri-infarct region.

Quantification and statistical analyses

No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications (Ouellette et al., 2020; Ruskin et al., 2017). All

animal numbers in this study are in line with well-accepted standards from the literature for each method. All data presented in this work were obtained from experimental replicates (i.e., multiple animal cohorts from different litters). All attempts of replication were successful. All data analysis was conducted blind to genotype/experimental condition. Groups were reassembled upon completion of data analysis according to genotype, age, sex. Randomization of individual samples/animals was performed by numbering. All statistical tests were performed using GraphPad Prism 9.0 Software. Data distribution was assumed to be normal, but this was not formally tested. Unpaired Mann-Whitney U test or t test (each animal being considered as a sample) was used for two-group comparisons between *16p11.2*$^{df/+}$ mice and control littermates for metrics such as average FDG uptake, oxygen saturation, total hemoglobin content, quantification of GLUT1 and MCT1, metabolite abundance, etc. A 2-way ANOVA (e.g. 'genotype x time') and Sidak's *or* Tukey's *post-hoc* test was used for kinetics of FDG uptake, oxygen saturation, extracellular brain metabolite fluctuations, etc. $P < 0.05$ was considered significant. Statistical details of each experiment can be found in figure legends.

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Author contributions

A.B., A.K., Y.T., J.O., S.Y., K.B., C.P., S.N., P.K., M.Y., T.A., and A.J.S. performed experiments. M.C. and M-K. St-P. provided technical support for TEM tissue processing. A.B., J.O., S.Y., K.B., C.P., S.N., P.K., M.Y., T.A., and A.J.S. analyzed data. B.L. conceived and led the project. A.B. and B.L. wrote the manuscript with input from all co-authors.

Competing interests

The authors declare no competing interests.

Data availability statement

All data and protocols are available from the corresponding author upon request.

Graphical abstract

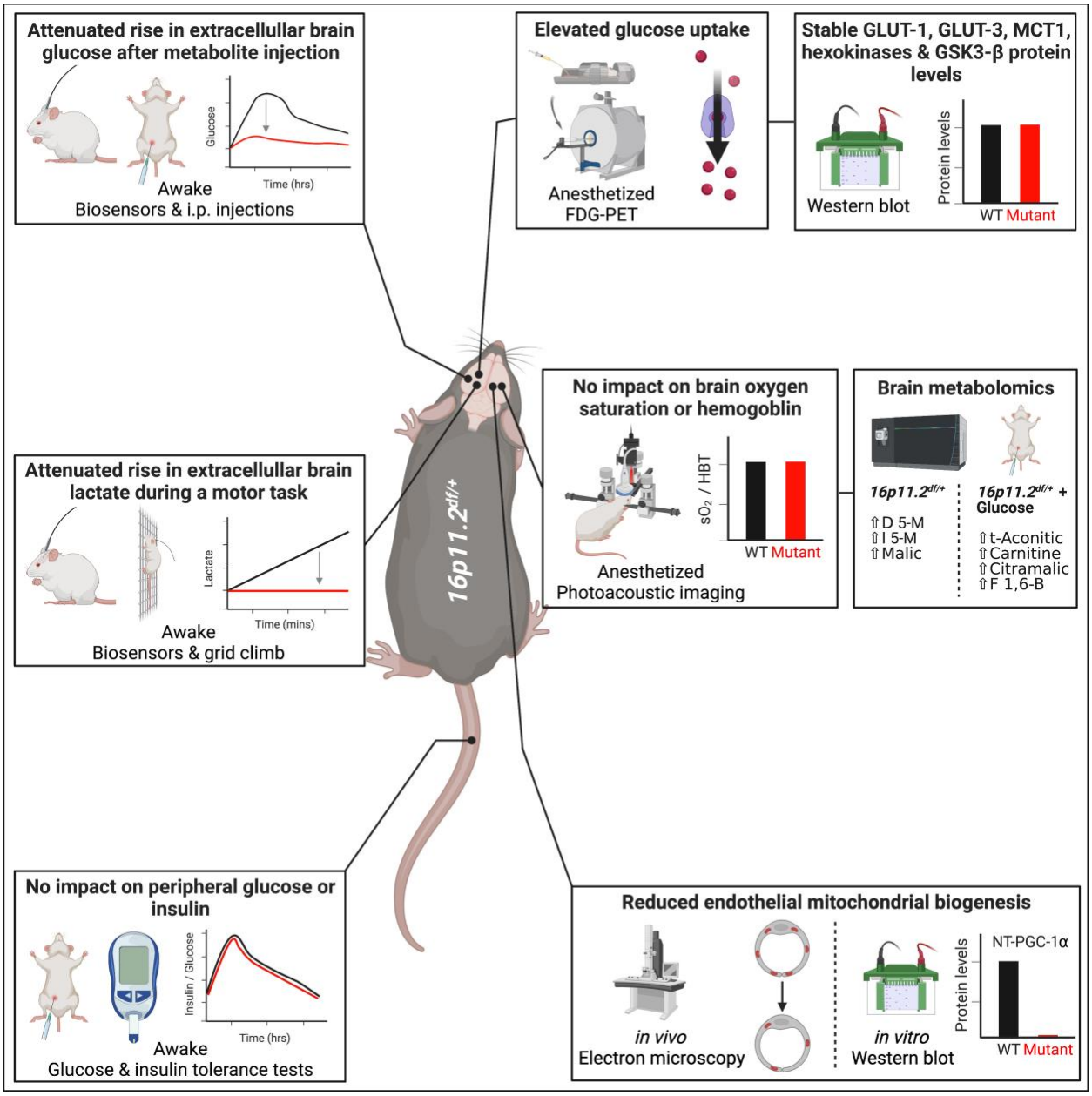
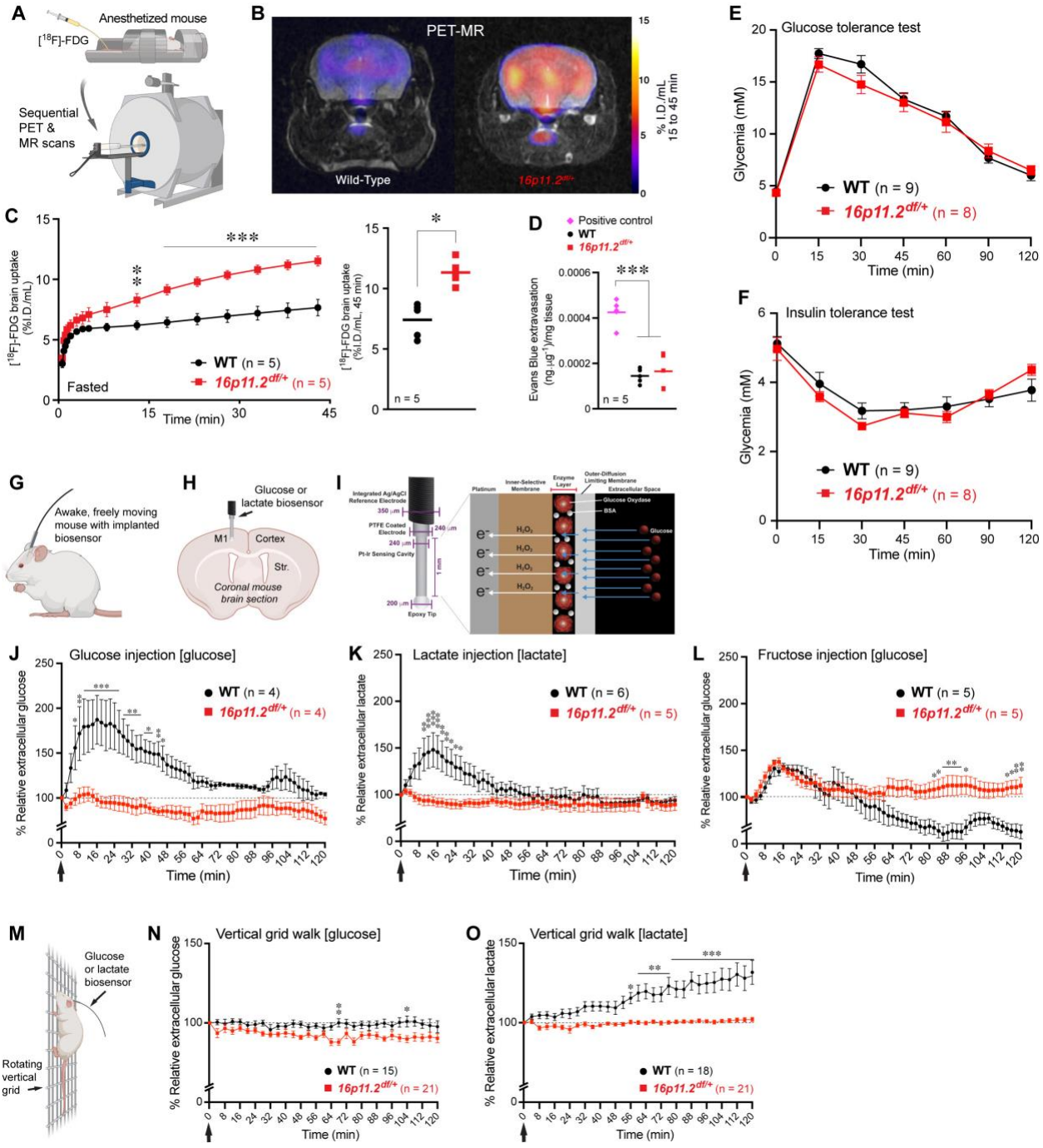


Figure 1 | Brain-specific alteration of glucose metabolism in adult *16p11.2$^{df/+}$* male mice



(A) *Administration of [^{18}F]-2 fluoro-D-deoxyglucose (FDG) tracer using positron emission tomography (PET) superimposed with magnetic resonance imaging (MR).*
(B) *Representative PET-MR scans acquired between 15- and 45-min post-tracer injection. I.D./mL, injected dose per mL of tissue.*

(C) *FDG uptake measured in fasted and anesthetized male 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates.*
(D) *Quantification of microvascular integrity (Evans blue dye extravasation) in male 16p11.2$^{df/+}$ (n = 5) and WT littermates (n = 5). Positive control: Evans blue dye extravasation 24 hours after a photothrombotic stroke in adult male WT mice.*
(E, F) *Measurement of peripheral glycemia during a glucose tolerance test (E) or insulin tolerance test (F), with doses normalized to body weight, in 16p11.2$^{df/+}$ (n = 8) and WT (n = 9) littermates.*
(G) *Illustration of an implanted biosensor for in vivo measurements of extracellular metabolites in the primary motor (M1) cortex of awake mice.*
(H) *Location for biosensor implantation. Str., striatum; M1, primary motor cortex.*
(I) *Illustration depicting the enzyme-mediated process underlying biosensor electrode function.*
(J) *Measurement of extracellular glucose in M1 cortex following a 2g/kg intraperitoneal (i.p.) injection of glucose in male 16p11.2$^{df/+}$ (n = 4) and WT (n = 4) littermates.*
(K) *Measurements of extracellular lactate in M1 cortex following a 2g/kg i.p. injection of lactate in male 16p11.2$^{df/+}$ (n = 5) and WT (n = 6) littermates.*
(L) *Measurement of extracellular glucose in M1 cortex following a 2g/kg i.p. injection of fructose in male 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates.*
(M) *Depiction of a vertical climbing task for measuring relative fluctuations of extracellular glucose or lactate in activated M1 cortex.*
(N) *Measurements of extracellular glucose in M1 cortex during the vertical grid climbing task in 16p11.2$^{df/+}$ (n = 21 trials) and WT (n = 15 trials) littermates.*
(O) *Measurements of extracellular lactate in M1 cortex during the same vertical grid climbing task in 16p11.2$^{df/+}$ (n = 21 trials) and WT (n = 18 trials) littermates.*
*Data are mean ± s.e.m. (C, left and E-O) or mean with individual values (C, right, and D). *P<0.05, **P<0.01, ***P<0.001 by a 2-way repeated measure ANOVA and Sidak's post-hoc test (C, left and J-O), a 1-way ANOVA and Tukey's post-hoc test (D), or a two-tailed Mann-Whitney test (C, right). n = number of animals per group (A-L) or number of trials (N, O).*

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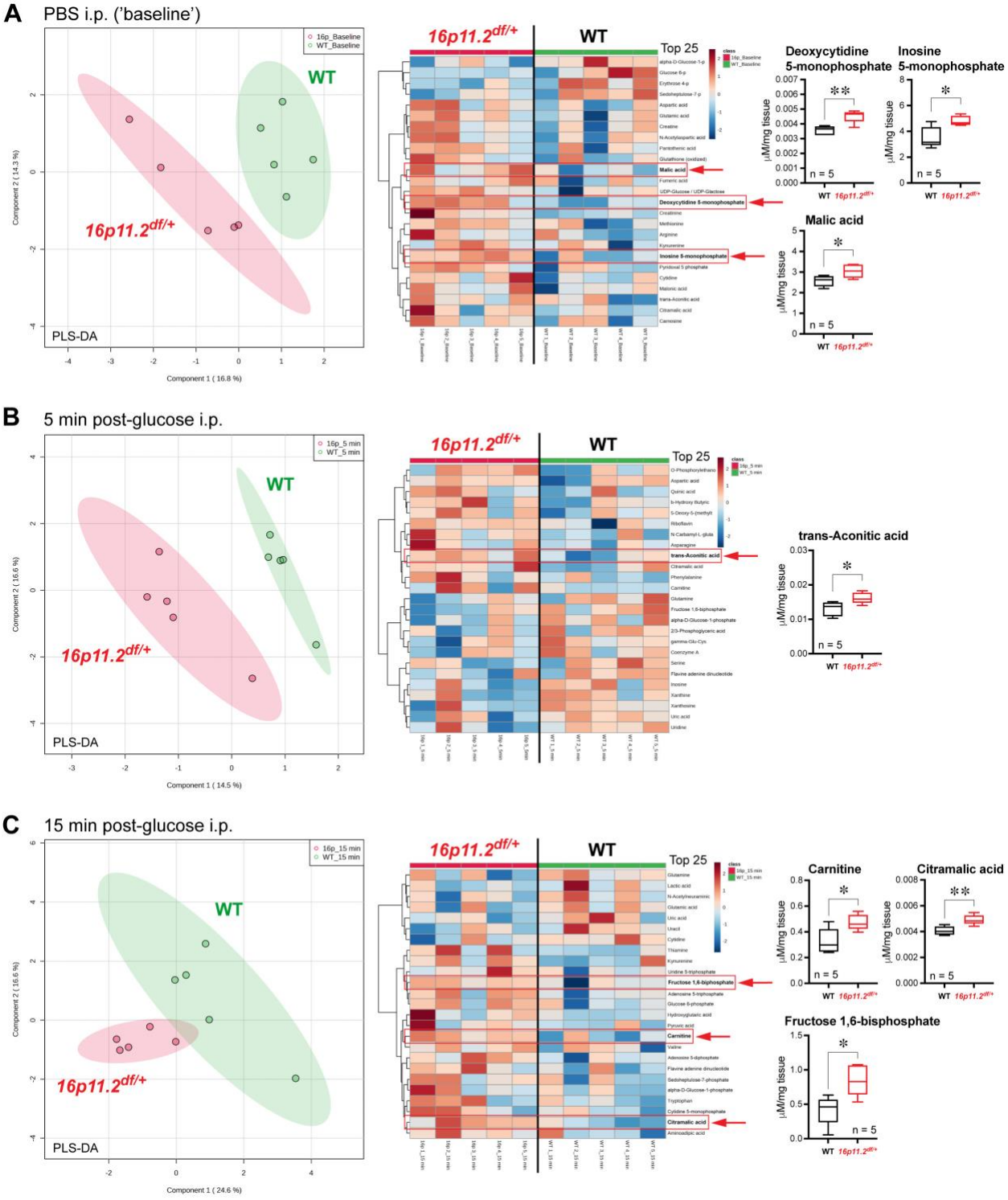
Figure 2 | Enhanced metabolic response to peripheral glucose in the cerebral cortex of *16p11.2$^{df/+}$* male mice



***(A)** Left, Partial Least Squares-Discriminant Analysis (PLS-DA) of metabolomics data from cerebral cortex of 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates 5 min after an intraperitoneal*

(i.p.) injection of 50 mM sterile phosphate buffer saline (PBS, 'baseline'). Each data point represents one mouse. Middle, Heat map (multivariate statistical analysis) displaying the top 25 metabolites significantly altered by the genotype at baseline for all samples. Right, Metabolites detected as most significantly altered by univariate statistical analysis.
***(B)** Left, PLS-DA of metabolomics data from cerebral cortex of 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates 5 min after a 2 g/kg i.p. glucose injection. Each data point represents one mouse. Middle, Heat map (multivariate statistical analysis) displaying the top 25 metabolites significantly altered by the genotype 5 min after glucose administration for all samples. Right, Metabolites detected as most significantly altered by univariate statistical analysis.*
***(C)** Left, PLS-DA of metabolomics data from the cerebral cortex of 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates 15 min after a 2 g/kg i.p. glucose injection. Each data point represents one mouse. Middle, Heat map (multivariate statistical analysis) displaying the top 25 metabolites significantly altered by the genotype 15 min after glucose administration for all samples. Right, Metabolites detected as most significantly altered by univariate statistical analysis.*
*Data in right-most panels are whisker boxes (min to max, center line indicating median). *P<0.05, **P<0.01 by a two-tailed unpaired t test. n = number of animals per group.*

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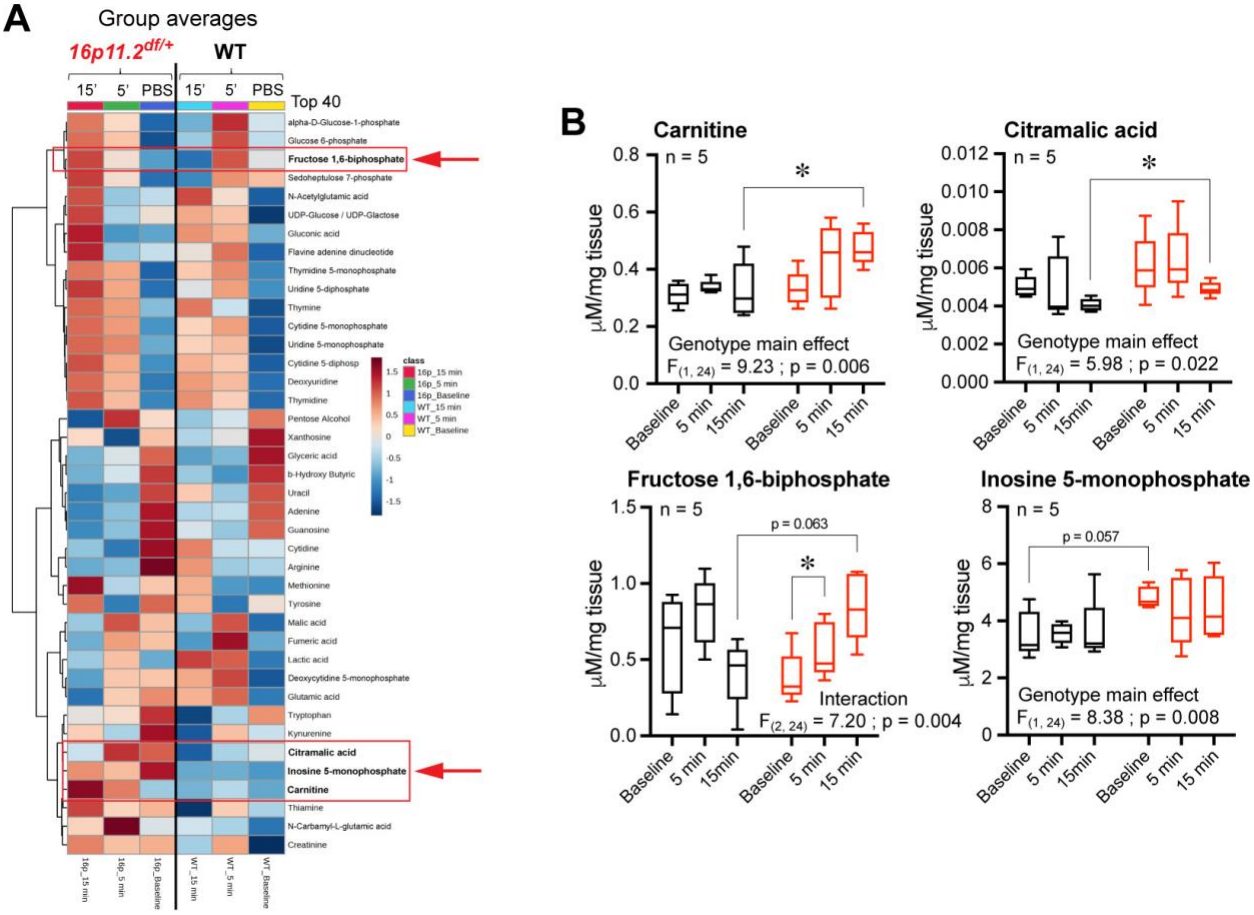
Figure 3 | Time course of cerebral metabolic response to peripheral glucose in *16p11.2$^{df/+}$* and WT male mice



***(A)** Heat map (multivariate statistical analysis) displaying group averages for the top 40 metabolites significantly altered by genotype and/or glucose administration.*
***(B)** Metabolites detected as most significantly altered by multivariate statistical analysis. Data in B are whisker boxes (min to max, center line indicating median). *P<0.05 by 2-way ANOVA and Tukey's post-hoc test. n = number of animals per group.*

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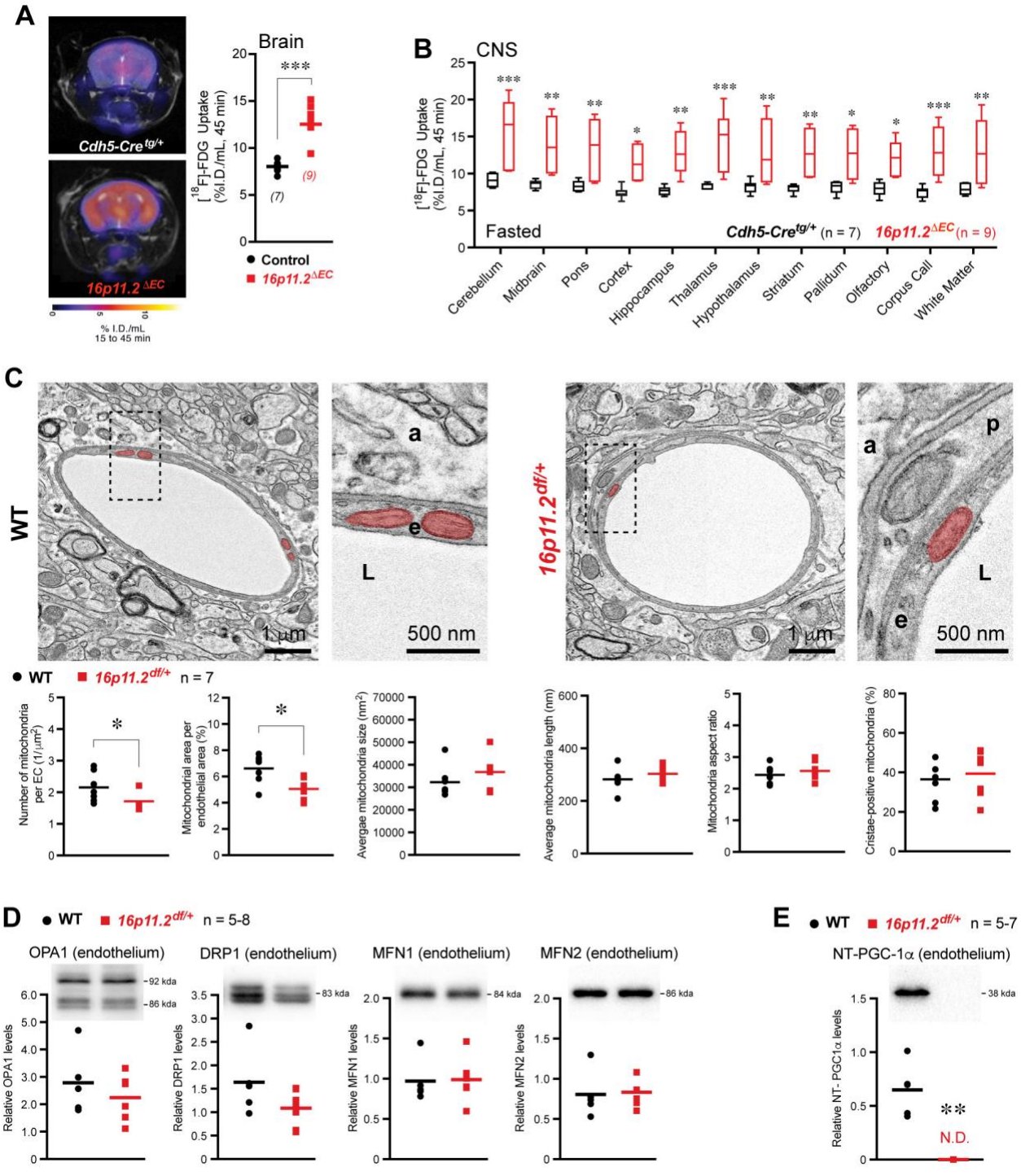
Figure 4 | Reduced mitochondrial number in 16p11.2-deficient brain endothelial cells



(A) *Left, Representative image of positron emission tomography-magnetic resonance imaging (PET-MR). FDG, [^{18}F]-2 fluoro-D-deoxyglucose; I.D./mL, injected dose per mL of tissue. Right, Acute brain FDG uptake quantified by PET imaging 45 min post-tracer injection in the*

brain of fasted, anesthetized male mice with endothelium-specific 16p11.2 deletion (16p11.2$^{\Delta EC}$, n = 9) and control littermates (Cdh5-Cre$^{tg/+}$, n = 7).
***(B)** Acute regional FDG uptake quantified by PET-MR imaging 45 min post-tracer injection in the central nervous system (CNS) of fasted, anesthetized male 16p11.2$^{\Delta EC}$ mice (n = 9) and control littermates (n = 7).*
***(C)** Top panels, Representative images of mitochondrial structure assessment in cortical capillaries from WT or mutant mice by transmission electron microscopy. a, astrocyte; e, endothelium; L, lumen; p, pericyte. For clarity, endothelial mitochondria were pseudocoloured in red. Bottom graphs, Quantitative assessment of mitochondria structure in cortical endothelial cells from 16p11.2$^{df/+}$ mice (n = 7) compared to WT littermates (n = 7).*
***(D)** Western blot quantification of relative levels of proteins associated with mitochondria fusion or fission (OPA-1, DRP1, MFN1&2) in primary mouse brain endothelial cells (n = 5-7) isolated from male 16p11.2$^{df/+}$ and WT littermates.*
***(E)** Western blot quantification of relative N-terminal (NT)-PGC-1α protein levels in primary brain endothelial cells from male 16p11.2$^{df/+}$ (n = 7) and WT (n = 5) littermates. N.D., not detected.*
*Data are mean with individual values (A, C-E) or whisker boxes in B (min to max, center line indicating median). *P<0.05, **P<0.01, ***P<0.001 by a two tailed Mann-Whitney test (A, C-E) or a 2-way ANOVA and Tukey's post-hoc test (B). n = number of animals per group.*

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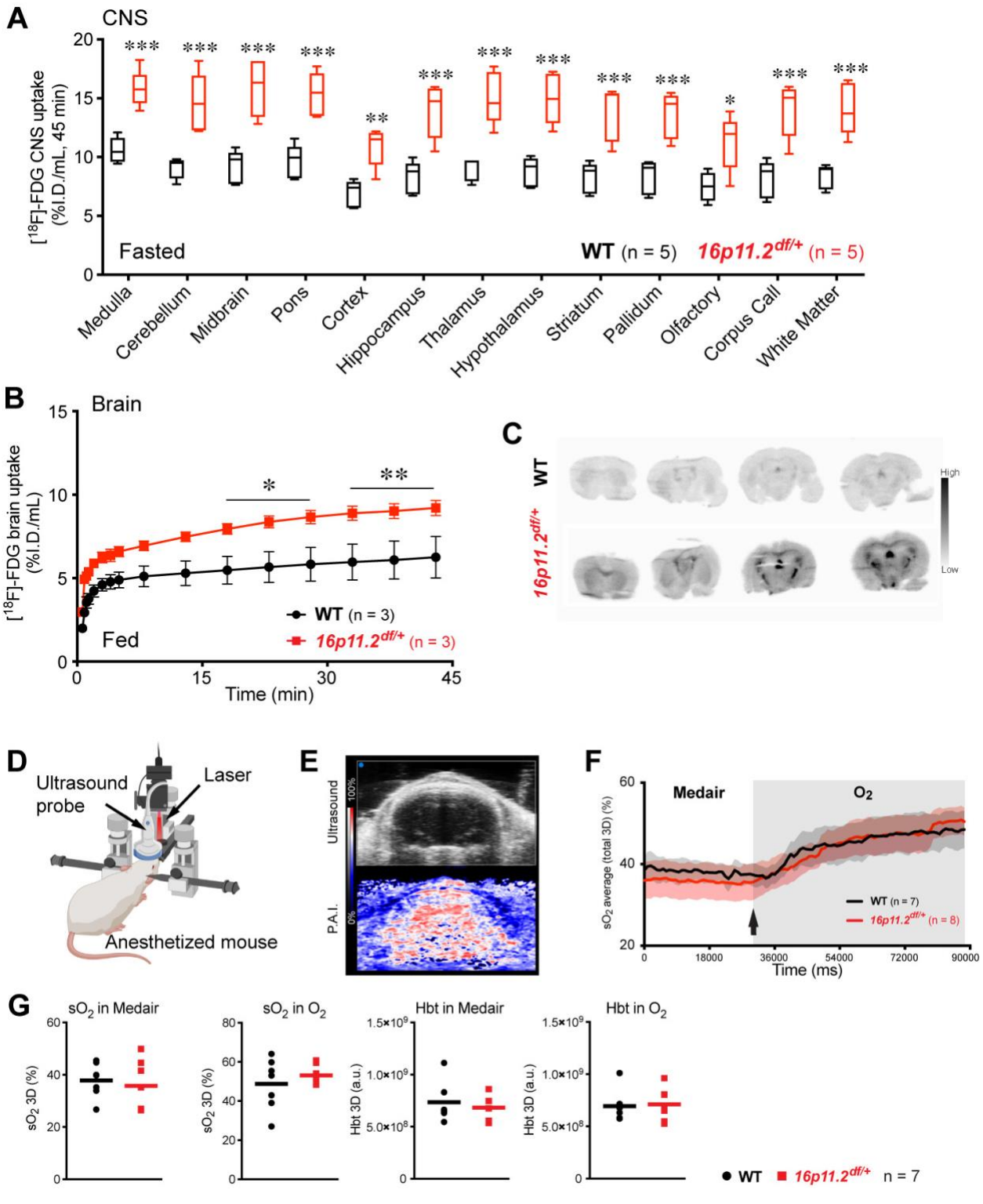
Supplemental Figure 1 | Additional details on brain metabolism in vivo. Related to Figure 1.



(A) *Acute and regional FDG uptake, quantified by PET imaging 45 min post-tracer injection in fasted, anesthetized male 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates for all central nervous system (CNS) regions. [^{18}F]-FDG, [18F]-2 fluoro-D-deoxyglucose; I.D./mL, injected dose per mL of tissue.*
(B) *FDG uptake is significantly elevated throughout a 45 min PET scan in fed, anesthetized male 16p11.2$^{df/+}$ mice (n = 3) compared to WT littermates (n = 3).*
(C) *Postmortem autoradiographs show elevated FDG levels within brain tissue after perfusion of male 16p11.2$^{df/+}$ and WT littermates.*
(D and E) *Depiction (D) and representative image (E) of the ultrasound and photoacoustic imaging (P.A.I.) system used to quantify circulating oxygen saturation (sO_2) and total hemoglobin (Hbt) in the cerebral cortex when using regular air (Medair) or pure oxygen (O_2) delivered through the anesthesia mask.*
(F) *Dynamics of cerebrovascular oxygen saturation during the transition from Medair to pure O2. in male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates.*
(G) *Quantifications of sO_2 and Hbt in Medair or O_2 in male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates. See also Figure S2.*
*Data are whisker boxes in A (min to max, center line indicating median), mean ± s.e.m. (B, F), or mean with individual values (G). *P<0.05, **P<0.01, ***P<0.001 by a 2-way ANOVA and Tukey's post-hoc test in (A) or a 2-way repeated measure ANOVA and Sidak's post-hoc test in (B). n = number of animals per group.*

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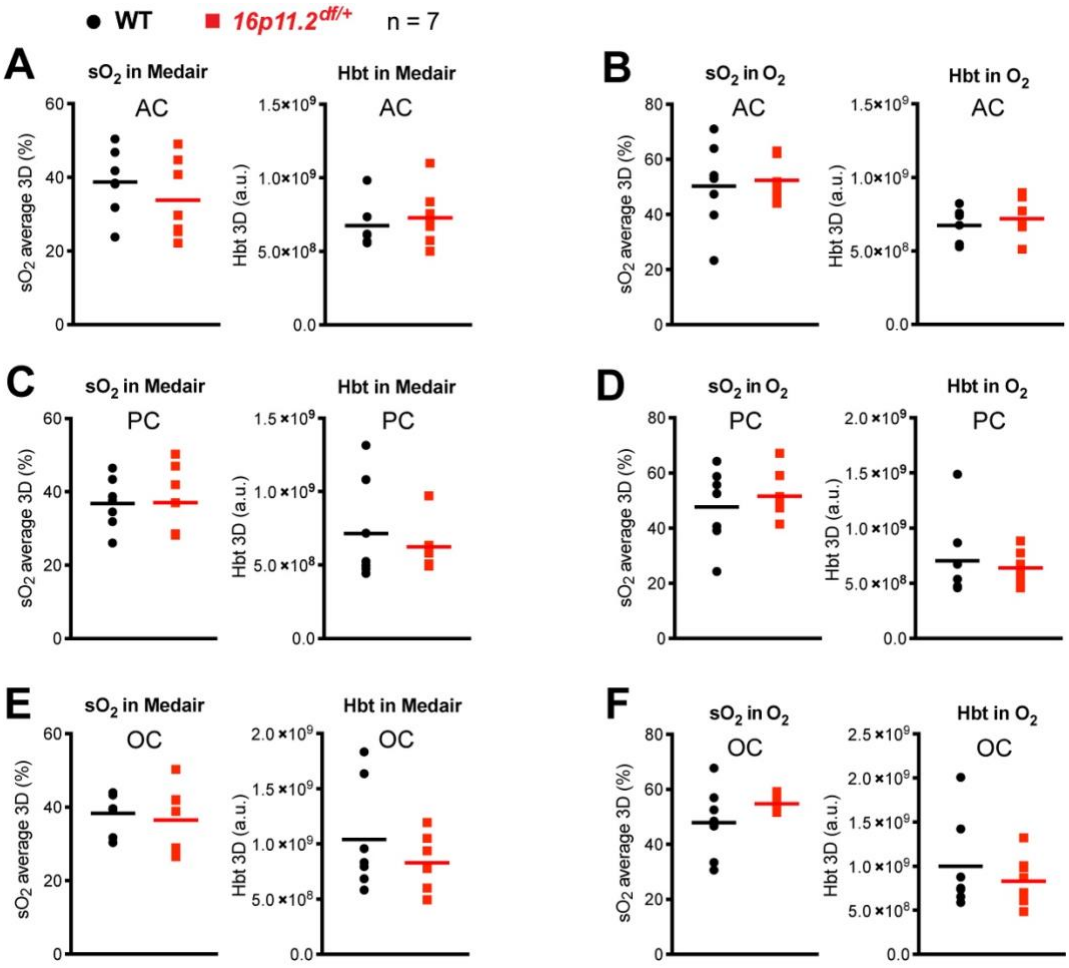
Supplemental Figure 2 | Cortical circulating oxygen saturation and total hemoglobin in normal air or pure oxygen acquired by photo acoustic imaging. Related to Figure 1.



(A, B) *Quantifications of circulating cortical oxygen saturation (sO_2) and total hemoglobin (Hbt) in normal air (Medair in A) and pure oxygen (O_2 in B) in the anterior subdivision of the cerebral cortex from male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates.*
(C and D) *Quantifications of circulating cortical oxygen saturation (sO_2) and total hemoglobin (Hbt) in normal air (Medair in C) and pure oxygen (O_2 in D) in the parietal cortex from male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates.*
(E and F) *Quantifications of circulating cortical oxygen saturation (sO_2) and total hemoglobin (Hbt) in normal air (Medair in E) and pure oxygen (O_2 in F) in the occipital cortex from male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates.*
All data are mean with individual values. AC, anterior cortex; OC, occipital cortex; PC, parietal cortex. n = number of animals per group.

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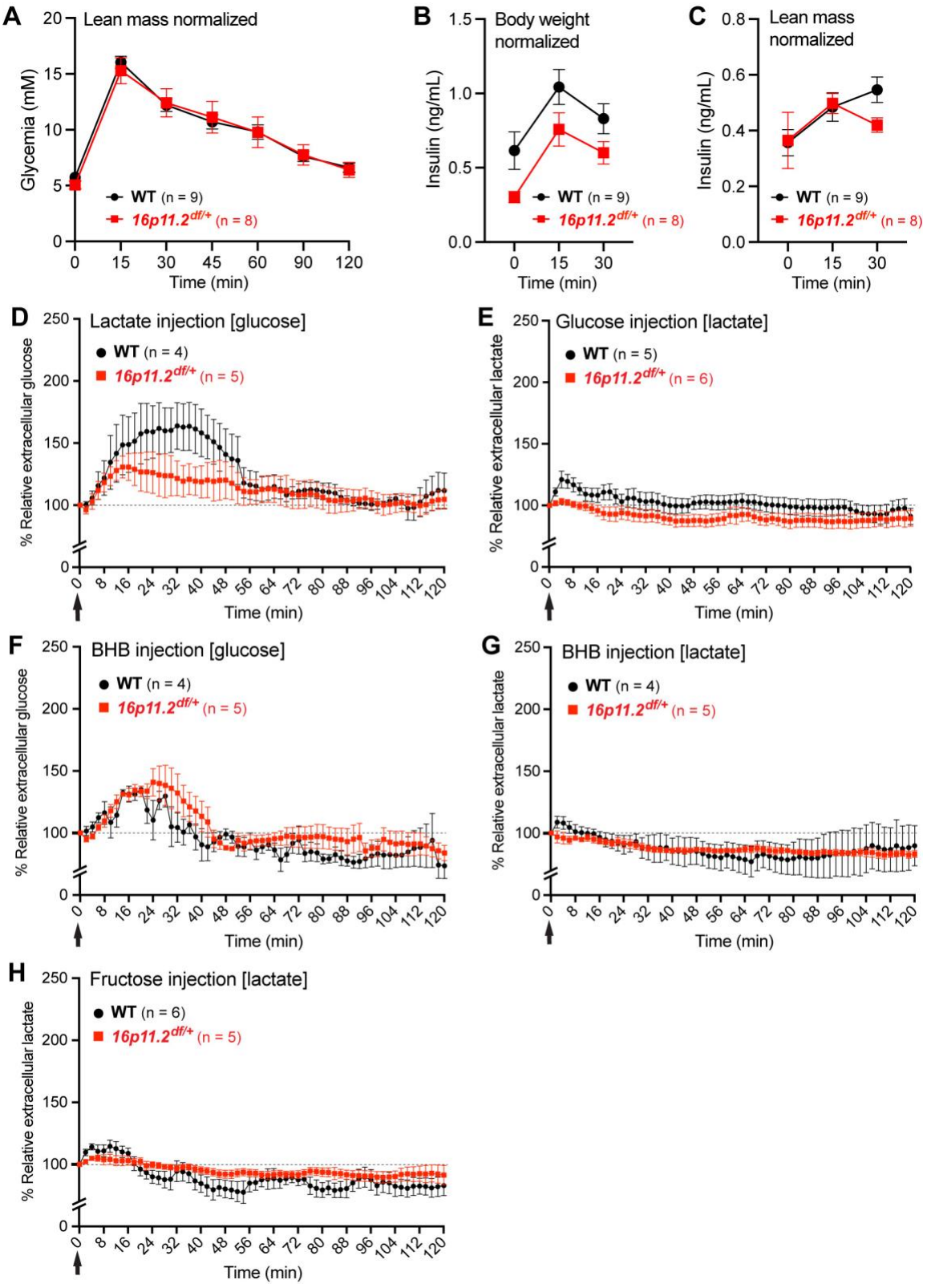
Supplemental Figure 3 | Additional details on brain glucose metabolism in awake *16p11.2*$^{df/+}$ mice. Related to Figure 1.



(A) *Measurement of peripheral glycemia using a glucose tolerance test, with glucose dose normalized to lean mass in male 16p11.2$^{df/+}$ (n = 8) and WT (n = 9) littermates.*
(B) *Measurement of blood insulin levels, during the glucose tolerance test (normalized to body weight) in male 16p11.2$^{df/+}$ (n = 8) and WT (n = 9) littermates.*
(C) *Measurement of blood insulin levels during the glucose tolerance test (normalized to lean mass) in male 16p11.2 $^{df/+}$ (n = 8) and WT (n = 9) littermates.*
(D) *Measurements of extracellular glucose in M1 cortex following a 2g/kg intraperitoneal (i.p.) injection of lactate in male 16p11.2 $^{df/+}$ (n = 5) and WT (n = 4) littermates.*
(E) *Measurement of extracellular lactate in M1 cortex following a 2g/kg i.p. injection of glucose in male 16p11.2 $^{df/+}$ (n = 6) and WT (n = 5) littermates.*
(F, G) *Measurement of extracellular glucose (F) or lactate (G) in M1 cortex following a 2g/kg i.p. injection of β-hydroxybutyrate (BHB) in male 16p11.2 $^{df/+}$ (n = 5) and WT (n = 4) littermates.*
(H) *Measurement of extracellular lactate in M1 cortex following a 2g/kg i.p. injection of fructose in male 16p11.2 $^{df/+}$ (n = 5) and WT (n = 6) littermates.*
*All data are mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001 by a 2-way repeated measure ANOVA and Sidak's post-hoc test. Time 0 in D-H (small arrow) represents the end of the i.p. injection. n = number of animals per group. Thin dashed lines in D-H represent baseline (100%).*

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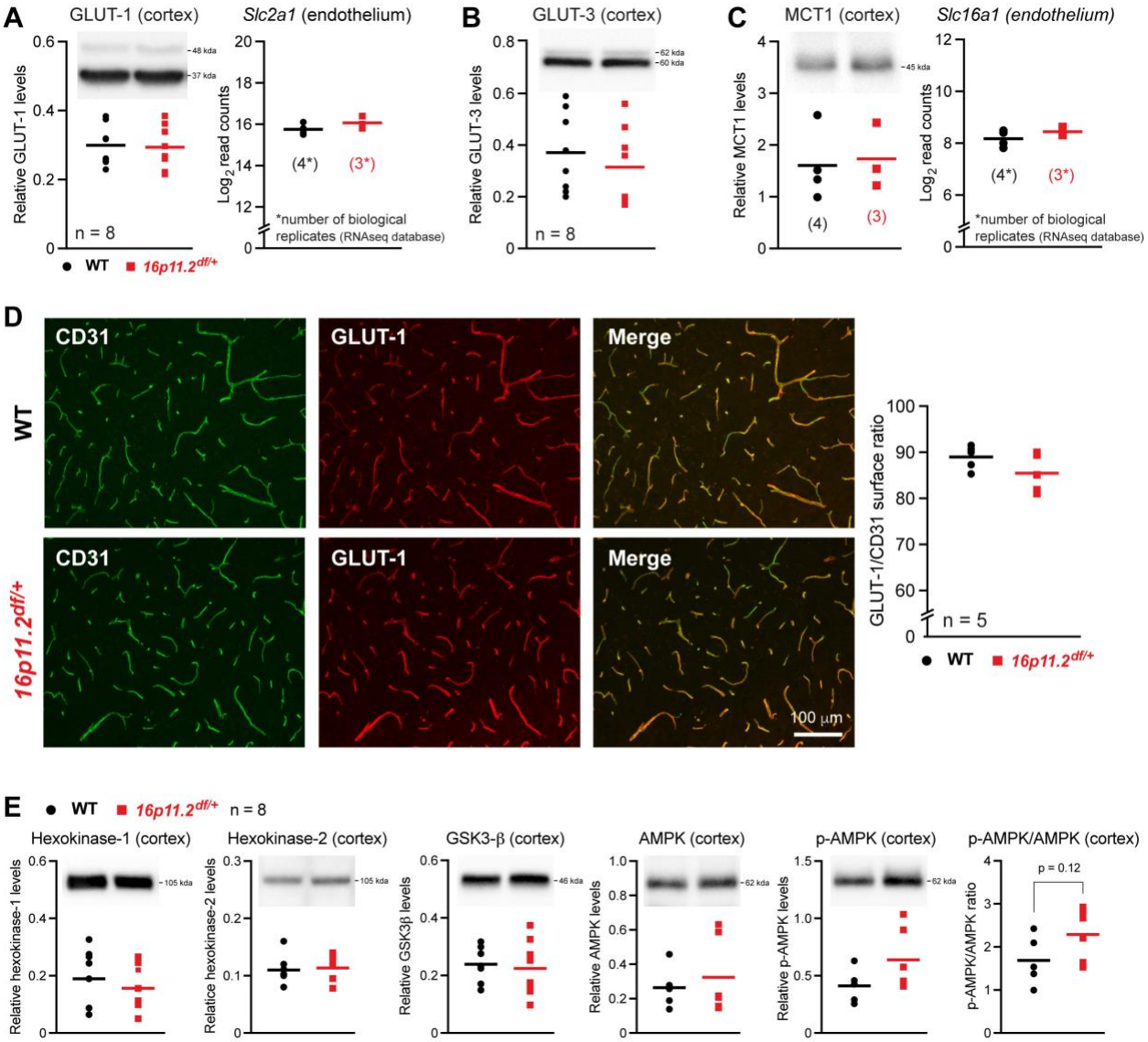
Supplemental Figure 4 | Glucose transporter levels and glycolytic pathways are unchanged in the cerebral cortex of *16p11.2*$^{df/+}$ mice. Related to Figure 1.



(A) *Left, Western blot quantification of relative GLUT-1 protein levels in cortical samples from male 16p11.2*$^{df/+}$ *(n = 8) and WT (n = 8) littermates. Right, Expression of Slc2a1 gene (encoding GLUT-1) in cerebral cortex endothelial cells (*RNAseq data from our published database).*
(B) *Western blot quantification of relative GLUT-3 protein levels in cortical samples from male 16p11.2*$^{df/+}$ *(n = 8) and WT (n = 8) littermates.*
(C) *Left, Western blot quantification of relative MCT1 protein levels in cortical samples from male 16p11.2*$^{df/+}$ *(n = 8) and WT (n = 8) littermates. Right, Expression of Slc16a1 gene (encoding MCT1) in cerebral cortex endothelial cells (*RNAseq data from our published database).*

(D) *Representative images (left) and quantification (right) of GLUT-1 immunoreactivity (red) over CD31-positive cortical endothelium (green) on brain sections from male 16p11.2$^{df/+}$ (n = 5) and WT (n = 5) littermates.*
(E) *Western blot quantification of relative protein levels of Hexokinases-1&2, GSK3- β, AMPK and p-AMPK in cortical samples from male 16p11.2$^{df/+}$ (n = 8) and WT (n = 8) littermates.*
All data are mean with individual values. n = number of animals per group.

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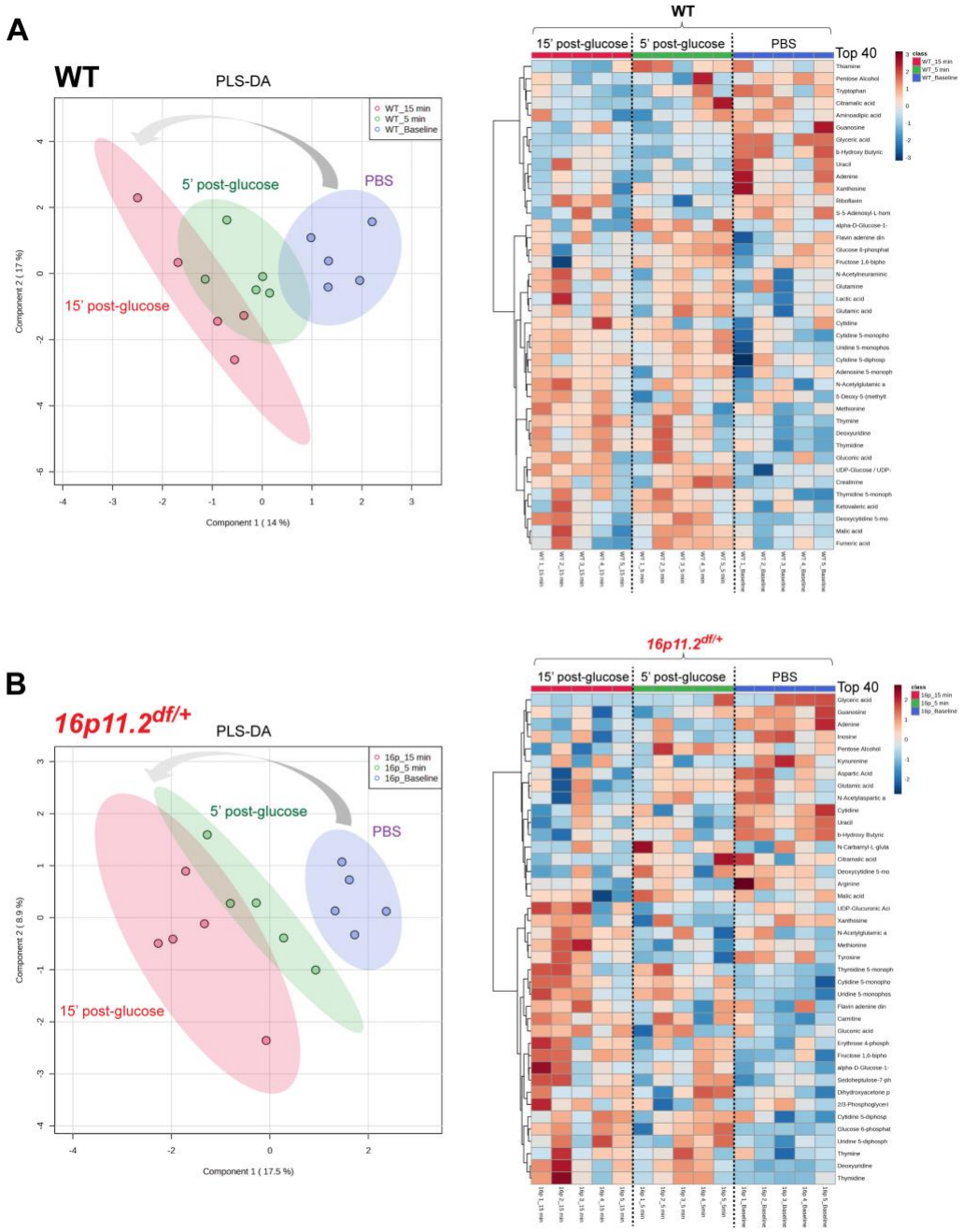
Supplemental Figure 5 | Additional details on brain metabolomics. Related to Figures 2 and 3.



(A) *Left, Partial Least Squares-Discriminant Analysis (PLS-DA) of metabolomics data from cerebral cortex of WT mice (n = 5) 5 min after an intraperitoneal (i.p.) injection of 50mM sterile phosphate buffer saline (PBS, 'baseline'); 5 min after a 2 g/kg i.p. glucose injection (n = 5); or 15 min after a 2 g/kg i.p. glucose injection (n = 5). Each data point represents one mouse. The light grey curved arrow indicates direction of changes between time points. Right, Heat map*

(multivariate statistical analysis) displaying the top 40 metabolites most significantly altered by glucose administration in WT mice.
(B) *Left, PLS-DA on metabolomics data from cerebral cortex of 16p11.2$^{df/+}$ mice (n = 5) 5 min after a i.p. injection of 50mM PBS ('baseline', n=5), 5 min after a 2 g/kg i.p. glucose injection (n = 5), or 15 min after a 2 g/kg i.p. glucose injection (n = 5). Each data point represents one mouse. The light grey curved arrow indicates direction of changes between time points. Right, Heat map (multivariate statistical analysis) displaying the top 40 metabolites most significantly altered by glucose administration in 16p11.2$^{df/+}$ mice.*

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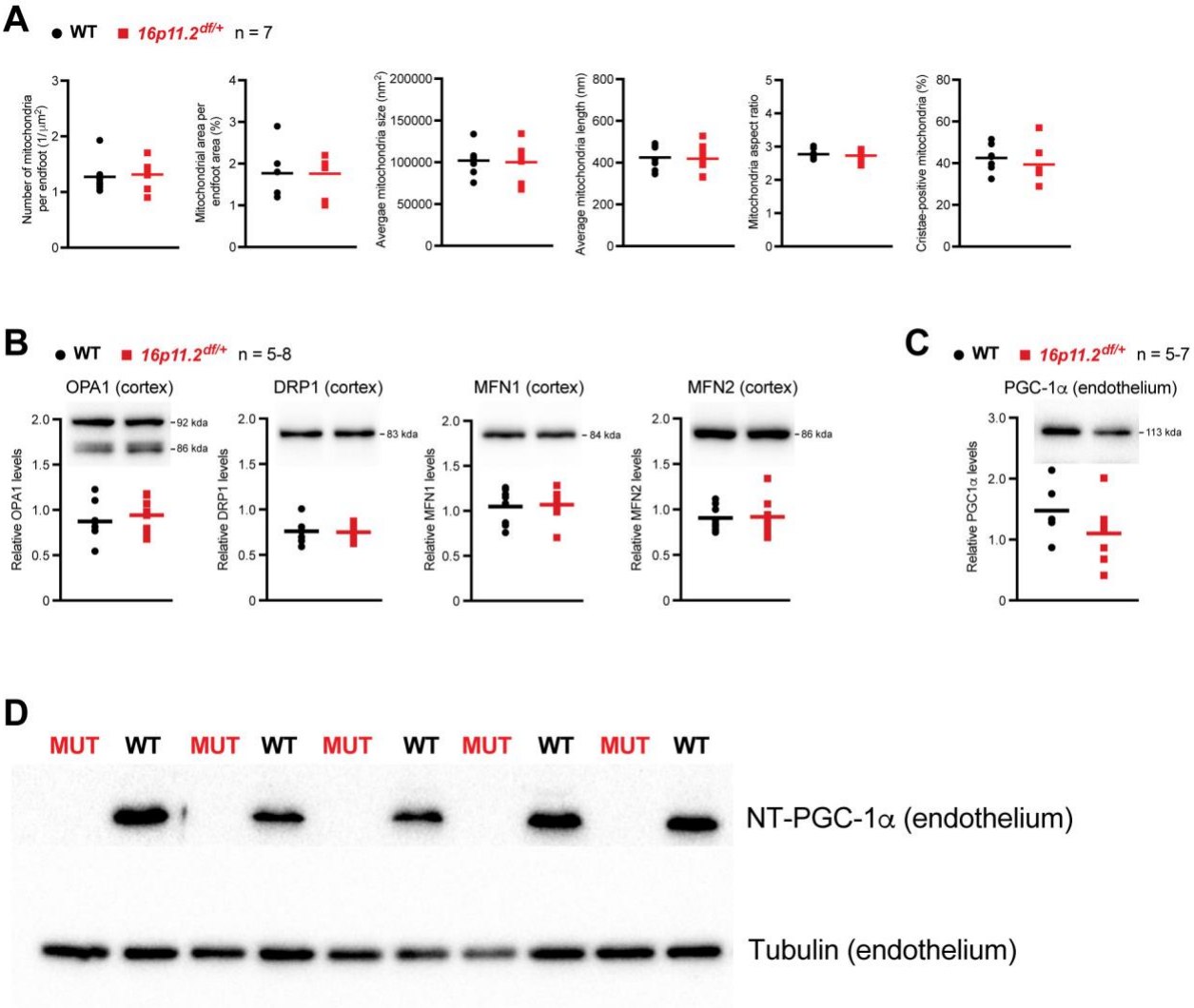
Supplemental Figure 6 | Additional details on mitochondrial ultrastructure and protein levels. Related to Figure 4.



(A) *Quantitative structural analysis of mitochondria from astrocyte endfeet in male 16p11.2$^{df/+}$ (n = 7) and WT (n = 7) littermates.*
(B) *Western blot quantification of relative levels of proteins associated with mitochondria fusion or fission (OPA-1, DRP1, MFN1&2) in the cerebral cortex of male 16p11.2$^{df/+}$ and WT littermates (n = 5-8).*
(C) *Western blot quantification of relative PGC-1 α protein levels in primary brain endothelial cells from male 16p11.2$^{df/+}$ (n = 7) and WT (n = 5) littermates.*
(D) *Example of immunoblot for N-terminal (NT)-PGC-1 α protein in primary brain endothelial cells from male 16p11.2df/+ (n = 5) and WT (n = 5) littermates (tubulin as loading control).*
All data in A-C are mean with individual values. n = number of animals per group.

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Chapter VIII: Discussion

Neurometabolic research is shifting and progressively evolving from its glucose- and neuron-centric view of cerebral metabolism to include important contributions from non-neuronal cells such as astrocytes and alternative fuel substrates such as lactate. It is now widely accepted that the brain can produce and meet its energy requirements, at least in part, with alternative substrates (e.g. lactate, fatty acids, ketone bodies) (Aubert et al., 2005; Daniel et al., 1971; Falkowska et al., 2015; Jensen et al., 2020; Magistretti & Allaman, 2018; Nehlig, 2004; Panov et al., 2014, 2022; Riske et al., 2017; Romano et al., 2017). However, there remains much controversy as to the conditions under which these alternative metabolic fuels are used, or even preferred, and their specific cellular (e.g. neuron vs astrocyte) or system (e.g. central vs peripheral) origin.

The extent to which there is metabolic coupling between the two main cortical compartments (neurons and astrocytes) and the direction of exchanges are contested. However, there is strong evidence of collaboration and that the key benefits resulting from this collaboration, such as tolerance to glucose deprivation (Sobieski et al., 2018) and exercise-induced benefits to executive functions (Hayek et al., 2019), depends on extracellular cortical lactate. Extracellular cortical lactate concentrations can be manipulated through signaling processes that increase glycolysis or glycogenolysis, or peripheral increases in lactate (e.g. exercise or artificially by infusion) (Alberini et al., 2018; Boumezbeur et al., 2010b; Dienel, 2012; Falkowska et al., 2015; Huang et al., 2021; Matsui et al., 2017, 2017; Quistorff et al., 2008; Schurr et al., 1999; Siebenmann et al., 2021; van Hall et al., 2009; Xue et al., 2022).

Despite often being studied as an isolated organ, the brain is dependent on the rest of the body for energy. Brain function and metabolism are influenced by acute changes in metabolite blood concentrations (Cai et al., 2022; Dienel, 2019a; Jensen et al., 2020; Li et al., 2022; Rebelos

et al., 2021; Rehni & Dave, 2018; van Hall et al., 2009; Xue et al., 2022), hormone release (Ahima & Antwi, 2008; Głombik et al., 2020; Roh et al., 2016; Roh & Kim, 2016), hepatic function (Bjerring et al., 2018; O'Hare & Zsombok, 2016; Palafox-Sánchez et al., 2021; Pocai, Obici, et al., 2005) and the composition of the gut microbiome (Selkrig et al., 2014; Soto et al., 2018; Wachsmuth et al., 2022). Therefore, the overarching objective of this thesis was to investigate the systemic (peripheral) contributions to extracellular brain metabolite pools. Our experiments focused on systematically altering metabolite availability and access to investigate the impacts of these altered states on extracellular glucose and lactate both at rest and following activity-induced challenges.

As its initial objective, this thesis investigated the influence of blood-borne metabolites on extracellular brain glucose and lactate. We investigated how increasing blood levels of monosaccharides (glucose, fructose, galactose), ketone bodies (β-hydroxybutyrate) and other monocarboxylates (lactate, pyruvate), through intraperitoneal administration, differentially impacted cortical extracellular metabolite (glucose and lactate) fluctuations in CD1 mice (see **Chapter II**; Béland-Millar et al., 2017) and in a mouse model of ASD (16p11.2 deletion; see **Chapter VII**; Béland-Millar et al., 2023). As a follow-up, we investigated the acute contribution of BBB transport and peripheral lactate on cortical extracellular metabolite fluctuations by pharmacologically inhibiting the ubiquitous hexose (glucose) and monocarboxylate (lactate) transporters and systemic variations in metabolite availability (see **Chapter VI**). Finally, we investigated the impact of chronic systemic changes in metabolite availability on key metabolic enzymes, both peripherally and centrally, in a two-way model in which activity levels (exercise vs sedentary) and diets (normal chow vs. high-fat diet) were manipulated (see **Chapter III**; Béland-Millar et al., 2020).

As a secondary objective, this thesis examined the effect of metabolite availability on activity-dependent fluctuations of cortical glucose and lactate. First, we investigated the impact of peripheral metabolite administration on cortical extracellular metabolite fluctuations of the primary visual cortex during visual stimulation (see **Chapter V**; Béland-Millar & Messier, 2018). Unfortunately, these findings were not robustly replicable in the primary motor cortex using a voluntary wheel-running paradigm. High levels of inter and intra-subject variability suggested the need to study the relative impact of different motor behavior tasks of varied difficulty on extracellular fluctuations of glucose and lactate (see **Chapter IV**; Béland-Millar & Messier, 2022). **Chapter VI** provides insight into the relative contribution of blood-borne metabolites on motor-induced extracellular metabolite fluctuations through pharmacological inhibition of glucose and lactate BBB transporters. **Chapter VII** (Béland-Millar et al., 2023) also investigated motor-induced extracellular metabolite fluctuations in a mouse model of autism spectrum disorder (ASD).

Methodological strengths and limitations

Biosensors

The use of electrochemical biosensors is ubiquitous throughout this thesis. In short, this technique involves the insertion of microelectrodes into brain tissue. The electrode is coated with an enzyme that reacts with the molecule of interest, and the resulting chemical reaction creates an electrical current that is measured by the electrode. The relative strength of this electrical current provides an indication of the metabolite concentration through time. The strengths and limitations of this particular methodological approach have been extensively detailed, both within the chapters of this thesis (e.g. **Chapter II**, see Discussion – Methodological considerations, Béland-Millar et al., 2017; **Chapter IV**, see Discussion - Technical Limitations

of the Biosensors, Béland-Millar & Messier, 2022) and in other published work (Ganesana et al., 2019; Kotanen et al., 2018). Like other techniques, such as microdialysis, electrochemical biosensors enable the (semi-)quantitative measurement of molecules in the extracellular space of the brain. For the work herein, biosensors will measure the fluctuations of metabolically relevant glucose and lactate metabolites. However, in contrast to comparable approaches, the electrochemical biosensors enable faster temporal sampling (1Hz or 1 measurement per second) and greater ecologically validity in the behaviors assessed. Faster and highly sensitive sampling rate is critical to studies of metabolism where compartmental metabolite exchanges and changes in the rate of metabolic pathways occur on the order of seconds. Though the mice are tethered, once the electrodes are implanted, they can move about freely in their cage, able to explore, run, drink and eat. This allows the measurement of metabolite fluctuations in response to biologically relevant behaviors, limiting the artificial implications of electrical stimulations or artificially imposed testing conditions.

The use of electrochemical biosensors does come with their own limitations: a) relative measure of metabolite presence, b) limited measurements of directional flux and c) biofouling. Any interpretation of results obtained using these electrodes should take into considerations these caveats. Though the pre- and post-calibrations measures, one can estimate metabolite concentrations based on the strength of the electrical current (nA) measured by the electrode. However, due to manufacturing variations, biofouling (see below) as well as insertion-variations, nA measurements of comparable metabolite concentrations can vary. Thus, these electrodes are much more suited to relative measures of metabolite fluctuations. It is also important to consider that measurements of extracellular metabolite fluctuations, as enabled by these electrodes and other techniques, do not in themselves inform on the directional flux of said metabolites but

rather estimate net-sums of extracellular metabolite fluctuations. The changing extracellular metabolite concentrations are the result of many factors which include, but are not limited to, BBB transfer of metabolites in and out of the brain, as well as metabolite influx and efflux of all major cellular compartments, including neurons, astrocytes and other glial cells. Information on directional flux is key to questions brain metabolism in the context of the ANLS and NALS. Technological limitations make this information hard to access, though there have been some recent developments that may prove useful and allow breakthroughs in this ongoing debate (see section entitled *Future directions*). Finally, biofouling is the accumulation of biological material or cells on the electrode surface which can inhibit enzymatic reactions thereby reducing the electrode sensitivity. In preliminary experiments, we have demonstrated that within a single mouse, relative electrodes readings are comparable for at least 3 days of testing, suggesting that biofouling likely can become a significant concern but only beyond three days of continuous recording.

Thus, given the nature of the biosensors and the readings they provide, data are best analysed as a relative measure of fluctuations, presented as percent change from baseline. Though this facilitates comparison within and across subjects (for the reasons stated above), it does impose upon the reader an important consideration with regards to differences in absolute metabolite concentration when reviewing the resulting graphs. According to our own readings, which are in line with results obtained by others (Hanna et al., 2020; Horn & Klein, 2010; Samala et al., 2011), extracellular brain values of glucose and lactate averaged 1.4 ± 0.59 and 0.9 ± 0.56 mmol/L, respectively. Other studies have found higher extracellular glucose and lower extracellular lactate values than those observed here (Diggs-Andrews et al., 2010; Park et al., 2021; Skwarzynska et al., 2022; Thinnes et al., 2021) – however methodological and regional

differences may play an important part in this variability (Kleinridders et al., 2018; Lee et al., 2020). Differences in glucose and lactate concentrations are even more pronounced in the blood, where we obtained average readings averaging between 8 to 10 and 1.7 to 3.1 mmol/L, respectively. Given the variable baseline concentrations, a relative increase identified in percentages could be misleading with regards to absolute concentrations. For example, a graph depicting 400% increase in blood lactate would represent blood lactate concentrations of approximately 10 mmol/L, a value comparable to baseline blood glucose concentrations. This could give readers the impression that blood lactate levels greatly exceed that of glucose and be interpreted as lactate having a greater relative contribution. Therefore, it is important to consideration the interpretation of relative fluctuations within the context of how these could translate to absolutely values.

Metabolite and inhibitor injections

Metabolites. Although biosensors allow monitoring of extracellular metabolite fluctuations within the brain of behaving mice, artificial means were used to impact metabolite concentrations and transport. The glucose concentrations administered via intraperitoneal injection were representative of normal rodent intake during a meal (Messier & White, 1984). For ease of comparison, the same concentration was used for all metabolites (2g/kg). It is important to consider that the same concentration does not imply the same metabolic impact. For example, the breakdown of single glucose molecule yields two lactate molecules.

Secondly, speed and volume of metabolite transport from the intraperitoneal space towards systemic circulation is not identical to the kinetics of oral administration and may have differential impacts. When compared to oral administration, intraperitoneal glucose results in elevated blood glucose concentrations for both male and female mice. Stable isotope-labelled

glucose tolerance tests revealed that intraperitoneal glucose administration (in comparison to oral routes of administrations) results in heightened absorption rates, as demonstrated by increased exogenous (administered) glucose entering circulation, and does not supress endogenous (hepatic) glucose production (Small et al., 2022).

Pharmacological inhibition of transporters. Although studies have yet to compare the kinetic absorptions rates of oral vs. intraperitoneal administration of the drugs used in this thesis, intraperitoneal administration has been determined to be an adequate administration route for drug delivery (Al Shoyaib et al., 2019), and both transport inhibitors used, AZD3965 and WZB117, have been demonstrated to be effective when administered intraperitoneally (Liu et al., 2012; Silva et al., 2021).

AZD3965 and WZB117 primarily impact MCT1 and GLUT1 transporters, respectively (Liu et al., 2012; Silva et al., 2021). These transporters are the predominant lactate and glucose transporters found within the cerebral vasculature (BBB) but are also ubiquitously expressed throughout the body (Halestrap & Wilson, 2012; Mueckler & Thorens, 2013). As such, there is a risk that any observation may be, at least partially, due to the effect of these inhibitors on the various peripheral organs dependent on these transporters for their normal function. Another important consideration was the use of DMSO which has multiple potential side effects, including toxicity, increasing BBB permeability, and modulating metabolism (see **Chapter VI** section entitled *Discussion – Technical limitations and considerations* for additional details). DMSO was nonetheless used because of the absence of alternative dilution strategies. These methodological limitations severely restrict our ability to make specific conclusions. Nonetheless they allowed us to make a number of interesting observations that provide some insights into the role of the BBB for the use of glucose and lactate under various conditions. For example,

Chapter VI clearly demonstrates the speed with which endothelial transporter expression is altered by pharmacological blockade.

Metabolic heterogeneity

Finally, there are limitations to the generalization of any regional metabolic observations. Fundamentally, the brain is heterogenous (Aiello et al., 2015; Borowsky & Collins, 1989; Palombit et al., 2022; Wong et al., 2006). This includes varying levels of vascular (and therefore oxygen) penetration, relative distribution of different cell (e.g. neurons, astrocytes, oligodendrocytes, microglia) and sub-cell types (e.g. reactive astrocytes), cellular and regional proclivities for different metabolic pathways (e.g. OXPHOS, PPP, glycolysis, fatty acid oxidation, glycogenolysis) as well as varying energetic needs.

Metabolic phenotypes are commonly observed across different cell types and brain regions. Astrocytes (Bélanger et al., 2011; Takahashi, 2021; Xiong et al., 2022) and endothelial cells (Eelen et al., 2015, 2018; Leung & Shi, 2022), as well as structures such as the cortex and amygdala (Kleinridders et al., 2018), are primarily glycolytic. In contrast, neurons are considered to be primarily oxidative (Ivanov et al., 2014; Magistretti & Allaman, 2015) and brain regions such as the thalamus preferentially consume glucose through the pentose phosphate pathway (PPP) (Kleinridders et al., 2018).

The passage of time also impacts metabolic heterogeneity. From a macroscopic perspective, the passage of time (aging) increases metabolic heterogeneity within the brain (Lee et al., 2004). On the smaller circadian timescale, in mice, hippocampal cells favor glycolytic energy processing early-on but as the day progresses, hippocampal cells increase the portion of glucose processed through OXPHOS (Brancati et al., 2021), perhaps as a consequence of decreasing glycogen availability (Bellesi et al., 2018).

Contribution of the blood-brain barrier and blood-borne metabolites to neuroenergetics

Blood-borne metabolites maintain extracellular metabolite pools.

Due to the brain's limited capacity for energy storage (i.e. glycogen), it is metabolically reliant on the continuous peripheral processing (e.g., hepatic glucose production) and delivery of metabolites (e.g., through GLUT1 and MCT1 transporters on the BBB) (Obel et al., 2012). Consequently, the maintenance and replenishment of extracellular cerebral metabolite pools that feed cortical activity are also highly dependent on metabolite availability and transport across the BBB. Physical activity and diet cause acute changes in peripheral metabolite availability that subsequently alter glucose uptake and induce changes in extracellular metabolite pools (Hwang et al., 2017; LaManna et al., 2009; Lobley et al., 2014; McNay et al., 2000).

The endothelial bi-directional transporters, MCT1 and GLUT1, and concentration gradient ensure blood-borne metabolite transport across the BBB and into the CNS. Herein we have confirmed the importance of BBB transport to maintaining extracellular metabolite pools as well as demonstrated the capacity of alternative metabolic fuels to sustain extracellular metabolite pools, which is consistent with the capacity of these alternative fuels to sustain neuronal signaling (Bouzier-Sore et al., 2002; Silva et al., 2022; Smith et al., 2003; Wyss et al., 2011).

Brain activity, or signalling, induces important transient and regional fluctuations in energetic needs (see **Chapter I** section entitled *The Brain: An Energetically Demanding Organ*). The brain is reliant on the dynamic nature of the peripheral vasculature and BBB transport to deliver the necessary metabolic substrates required meet these local heightened needs. Neuronal activation induces a plethora of compensatory mechanisms to increase metabolite availability and delivery. These mechanisms include increased cerebral blood flow (CBF) (Ances, 2004;

Attwell et al., 2010; Chaigneau et al., 2007), hepatic changes in metabolite production (Lam, 2007; Nonogaki & Iguchi, 1997; Pocai, Obici, et al., 2005) and enhanced cerebral glycogenolysis (DiNuzzo et al., 2012; Magistretti et al., 1986; Nadeau et al., 2018; Swanson et al., 1992).

As a result of these changes and the heightened regional metabolic consumption, activation is transiently and regionally coupled with an increase in extracellular cortical lactate and decrease in extracellular glucose (see **Chapter I** section entitled *Introduction - Metabolite fluctuations*). We demonstrated the importance of the BBB, and therefore blood-born metabolites, (see **Chapter VI**), the differential impact of peripherally administered alternative fuels (see **Chapter V**; Béland-Millar & Messier, 2018), and the relevance of the novelty and intensity of a given task (see **Chapter IV** and **Chapter V**; Béland-Millar & Messier, 2022; Béland-Millar & Messier, 2018) to creating and sustaining this pattern of extracellular metabolite fluctuations. These findings suggest that, to varying degrees depending on the task and fuel in question, blood-borne alternative fuels underlie to the frequently observed activity-induced extracellular fluctuations. In turn, this implies that the activity-induced increases in extracellular lactate are not exclusively endogenously produced, either by neurons or astrocytes, which has important repercussions on our understanding of the ANLS vs NALS debate (see **Chapter I** section entitled *Astrocyte-to-Neuron Lactate Shuttle*).

Adaptability

As demonstrated by the work herein and others (Dienel, 2012; Pan et al., 2000; Siebenmann et al., 2021; Svart et al., 2018; van Hall et al., 2009; Brocchi et al., 2022; Prins, 2008), the brain can adapt quickly adapt to metabolic challenges. When a task is repeated, the cerebral energy consumed to execute it decreases, likely through the development of efficient neuronal pathways that decrease the energetic demand of the task (Zhan et al., 2018; Sayala et

al., 2005). Similarly, the BBB quickly adapts to its transport of blood-borne metabolites when inhibited (see **Chapter VI**) by rapidly modulated the cortical endothelial MCT1 and GLUT1 expression. The immediate increase in endothelial MCT1 during the most restrictive challenge (inhibition of both GLUT1 and MCT1) is a novel and striking evidence of the speed at which the BBB can adapt its transporter expression to accommodate the energetic needs of the brain as well as a compelling visualization of the importance of monocarboxylate entry as a rapid-response mechanism.

The sections above discuss the rapid extracellular metabolite pools fluctuations and the brain adaptation to acute changes in environmental metabolite availability. Both exercise and diet have been shown to impact cerebral metabolism, both acutely and chronically. The impacts are broad and include modulating transporter expression (Béland-Millar et al., 2020; Forero-Quintero et al., 2017; Leino et al., 1999; Matsui et al., 2019; Puchowicz et al., 2007; Shcherbakova et al., 2022; Takimoto & Hamada, 2014; Xue et al., 2022), expression of key regulatory enzymes for various metabolic pathways (Béland-Millar et al., 2020; Dienel, 2019a; Düking et al., 2022; Lutas & Yellen, 2013; Sorochynska et al., 2019; Takimoto & Hamada, 2014; Xue et al., 2022), neurotransmitter production (Bhattacharya et al., 2023; Briguglio et al., 2018; Guo et al., 2021; Heijnen et al., 2016; Lin & Kuo, 2013; Meeusen & De Meirleir, 1995; Sarbadhikari & Saha, 2006; Wurtman, 1983), mitochondrial fitness (Bernardo et al., 2016; Burtscher et al., 2021; Gusdon et al., 2017; Kaliszewska et al., 2021; Li et al., 2019; Marques Neto et al., 2020; O'Reilly et al., 2023; Pathak & Baar, 2023; Radak et al., 2007; Silvestri & Giacco, 2022; Steiner et al., 2011; Zhao et al., 2021) and executive functions (Audiffren & André, 2019; Francis & Stevenson, 2013; Freeman et al., 2014; Gomez-Pinilla & Hillman, 2013; Hillman et al., 2008; Kaliszewska et al., 2021; Klimova et al., 2020; Kramer et al., 2006;

Mandolesi et al., 2018; Otaegui-Arrazola et al., 2014; Proctor et al., 2017; Raichlen & Alexander, 2017; Spencer et al., 2017). However, adaptation in response to long-term changes in metabolite availability (diet) *and* physical activity (exercise) in rested rats are not as well understood. Thus, we investigated changes in key metabolites, metabolic enzymes and transporters following a 5 week change in diet (normal chow vs. high-fat diet) and physical activity (sedentary vs. treadmill) (see **Chapter III**; Béland-Millar et al., 2020). Our work found that, in muscle, changes in diet and exercise primarily altered the expression of key metabolic enzymes that feed OXPHOS. The brain, however, exhibited restricted changes. The cortex of rats fed a high-fat diet (regardless of their exercise condition) had lower glycogen concentrations and elevated MCT1 expression. This is consistent with another study that revealed increased hippocampal monocarboxylate transport following a hyperpalatable diet and exercise paradigm (Brochier, 2013). Interestingly, many of exercise-derived metabolic benefits can be reproduced by peripherally injected lactate (Hashimoto et al., 2021; Morland et al., 2017; Nalbandian & Takeda, 2016). These effects could be due to the lactate-dependent increase of BDNF expression through a SIRT1-dependent pathway (Hayek et al., 2019). BDNF has also been shown to underlie many of the beneficial effects of exercise on vasculature, mitochondria, metabolism, executive functions, and apoptosis protection (Marosi & Mattson, 2014). In addition, the activation of the hydroxycarboxylic acid receptor 1 (HCAR1), a lactate receptor, induces cerebral angiogenesis (Morland et al., 2017).

Taken together, we can extrapolate the importance of blood-borne lactate, and its transport, to cerebral bioenergetics and hypothesize that it may be partially responsible for the shift towards monocarboxylate brain metabolism and elevated MCT1 expression in times of energetic challenges or changes. Future investigations into cerebral metabolism should take into

consideration the plastic contribution the BBB and its transport of peripheral metabolites, specifically alternative fuels such as lactate.

Disease states

There is a long list of disease states associated with altered cerebral metabolism (Bernardo et al., 2016; Blass, 2001; Burtscher et al., 2021; Butterfield et al., 2022; Ernst et al., 1994; Felmlee et al., 2020; Koepsell, 2020; Lombard, 1998; Nguyen et al., 2021; Profaci et al., 2020; Watts et al., 2018), including Autism Spectrum Disorders (ASD) (Anil Kumar et al., 2017; Cho et al., 2017; Keeratitanont et al., 2022) and Alzheimer's Disease (Ardanaz et al., 2022). Alzheimer's disease has been found to have important metabolic dysregulations that precede clinical symptoms (Swerdlow, 2020), including a reduction in GLUT1 (Winkler et al., 2015; Kyrtata et al., 2021) as well as a reduction in brain lactate and MCTs (Lu et al., 2015; Zhang et al., 2018; Tang et al., 2019) (though Hong and colleagues found an increase in hippocampal MCT3 in 2 and 3 month sold APP/PS1 mice (Hong et al.,2020)). Glucose transport deficiencies, either GLUT1 or GLUT3, recapitulate many symptoms often observed in brain pathologies (Winkler et al., 2015; Zhao et al., 2010). Reductions in the expression or plasticity (translational or post-translation) of transporters responsible for the influx of blood-borne metabolites is a common feature in brain pathologies and has important functional implications (Koepsell, 2020).

Here we demonstrated an altered metabolic profile of an ASD mouse model (see **Chapter VII**; Béland-Millar et al., 2023). We hypothesized that these results are due to this mouse model possessing a common ASD phenotype: a hypermetabolic brain (Cho et al., 2017; Sharma et al., 2018). The ASD brain is characterized by inefficient neuronal networks, partly due to inefficient pruning (Faust et al., 2021; Frith, 2003; Hill & Frith, 2003; Pagani et al., 2021), thus creating a metabolic sink and saturating glucose transport to meet the elevated energetic

needs of inefficient signaling. Saturated transport combined with a rigid infrastructure unable to upregulate glucose or monocarboxylate transport may underlie some of the physiological and behavioral abnormalities observed in this ASD mouse model or other pathologies.

Astrocyte dysfunctions are also being discovered as common hallmarks of brain pathologies (Siracusa et al., 2019; Phatnani & Maniatis, 2015), including ASD (El-Ansary, 2010; Zeidán-Chuliá et al., 2014) and Alzheimer's diease (Siracusa et al., 2019; Phatnani & Maniatis, 2015). Interestingly, ketogenic diets have been shown to improve ASD (Castro et al., 2015; Li et al., 2021) and Alzheimer symptoms (Tabaie et al., 2021), as well as impact astrocyte morphology (Gzielo et al., 2019; Koppel et al., 2021; Düking et al., 2022). Combined with our observations of the main dysregulated metabolic machinery being endothelial mitochondria biogenesis and recapitulation of the observations with an *endothelial specific* haplodeficiency mouse model (see **Chapter VII**; Béland-Millar et al., 2023), there is compelling evidence suggesting that the origins of these metabolic deficiencies may be non-neuronal. Endothelial and astrocytic functions are intimately intertwined with both astrocytes (Heithoff et al., 2021; Liu et al., 2018; MacVicar & Newman, 2015) and glycolysis (Leung & Shi, 2022) regulation cerebral vasculature and therefore metabolite availability.

Glucose sparing and preferential upregulation of monocarboxylate pathways

The notion of glucose sparing is not novel. Changes in diet and physical activity, for example, elevate blood lactate and β-hydroxybutyrate which subsequently spares cerebral glucose use (Leino et al., 2001; Kemppainen et al., 2005; Hwang et al., 2017; LaManna et al., 2009; Lobley et al., 2014; McNay et al., 2000; Cox et al., 2016; Dienel, 2012; Tabernero et al., 1996; Zilberter & Zilberter, 2020). However, previous studies neglected to systematically assess the ability of alternative fuels (e.g. lactate and β-hydroxybutyrate) to modulate extracellular

metabolite pools. We demonstrated a common pattern of elevated blood lactate and extracellular cortical glucose, which led us to hypothesize that the blood-borne lactate was sparing glucose use within the brain, resulting in its accumulation (see **Chapter II**; Béland-Millar et al., 2017). Through the peripheral pharmacological inhibition of the glucose (GLUT1) and lactate (MCT1) transporters found on the BBB, we confirmed that this observation is at least partially driven by peripheral lactate availability and that inhibition of monocarboxylate transport reduced extracellular glucose pools (see **Chapter VI**).

The seemingly preferential upregulation of monocarboxylate transport or use in acute (see **Chapter VI**) or chronic (see **Chapter III**) metabolic challenges emphasizes the important contribution of blood-borne alternative metabolites to sustaining extracellular metabolic pools and consequently fueling (Jensen et al., 2020; Silva et al, 2022; Magistretti & Allaman, 2018) or modulating (Bozzo et al., 2013; Vaccari-Cardoso et al., 2023; de Castro Abrantes et al., 2019) brain activity. This is consistent with evidence of cerebrovascular control of lactate transport through MCT1 playing a role in hippocampal neurogenesis, cognition, and lactate homeostasis (Wang et al., 2019) as well as preferential increase of MCT1 expression in endothelial capillaries of rat having undergone a 4–6-week ketogenic diet (Leino et al., 2001). Though the exact function and benefit of this preferential MCT1 upregulation remains to be elucidated, it is consistent with the notion that lactate may be a preferential metabolic fuel for the brain (Bouzier-Sore et al., 2003), as well as in agreement with increased expression of astrocytic lactate transporter (MCT2) during motor or pharmacologically induced bioenergetic challenges (Matsui et al., 2019). The exact (or relative) speed at which translation or membrane insertion of all of these transporters can be upregulated is mostlyunknown, but evidence, including our own (see **Chapter VI**), suggests that these transporters can be readily adaptable and upregulated quickly

in response to environmental challenges (Felmlee et al., 2020; Kumagai et al., 1995; Leão et al., 2020). For example, cell surface expression of MCT2 was increased in cell cultures as quickly as 1 minute following application of various agents reproducing synaptic and metabolic changes (Pierre et al., 2009). Approximately 10% of GLUT1 and MCT1 proteins are cytoplasmic (Duelli & Kuschinsky, 2001; Leino et al., 1999). This internal transporter storage likely underlies their dynamic ability to rapidly upregulate these transporters.

Overall, the objective of this thesis was to investigate the influence of blood-borne metabolites on extracellular brain glucose and lactate, as well as assess the effects of metabolite availability on activity-dependent fluctuations of extracellular cortical glucose and lactate. Throughout the thesis, we observed a universal pattern of uncoupling between blood and extracellular brain metabolite (increased blood lactate and increased brain glucose), characterized the importance of blood-borne metabolites (specifically lactate) to maintaining extracellular metabolite pools and demonstrated the cohesive adaptation of the brain to modulate its monocarboxylate uptake in response to chronic changes in metabolite availability. Taken together, these results are indicative of the important role of alternative fuels (e.g. lactate or ketone bodies) in meeting the bio-energetic requirements of the brain through a concerted effort with the rest of the body to provide a converging downstream metabolite substrate (e.g. lactate) that could be easily imported across the BBB. This would allow the brain to optimize its transport and metabolic pathways while still being able to benefit from the ingestion of a variety of fuels. Though we propose a favored metabolic pathway to adapt in changing environmental conditions, this of course does not negate evidence of the brains ability to metabolize other fuels. Beyond its metabolic properties, tightly regulated and preferential upregulation of monocarboxylate pathways may ensure that lactate can continue to meet its important signaling

functions in times of metabolic challenges to modulate neuronal signals (excitability), plasticity and memory (Magistretti & Allaman, 2018).

Though most of the evidence and observations provided in this work support the hypothesis provided above, there are nonetheless results which are not as congruent. Namely, limiting cortical access to peripheral glucose during motor behavior depleted extracellular glucose pools despite elevated extracellular lactate (see **Figure 3** in **Chapter VI**). If extracellular lactate was an efficient metabolic replacement, one would assume that it would not be elevated following diminished extracellular glucose. In conditions of limited glucose availability such as hypoglycemia, neurons increase their oxidation of glucose through the TCA cycle (Jiang et al., 2009). This, in itself, would *not* result in a lactate efflux as glucose and its downstream products would be consumed within the neuron. However, increased extracellular lactate may represent an astrocytic lactate gradient to support neuronal needs (Herzog et al., 2013). This is consistent with observations of astrocyte-to-neuron lactate gradients, abolished lactate gradients in the presence of MCT1 or MCT2 inhibitors and enhanced neuronal uptake of glucose during low glucose availability (Mächler et al., 2016; Shah et al., 2019). Finally, this elevated lactate could represent a vascular response, increasing delivery of lactate in response to limited glucose availability (Abi-Saab et al., 2002; Dienel, 2012).

Implications

Extracellular metabolite pools are the means through which non-neuronal cells (e.g., endothelial cells and glial cells) bolster and modulate neuronal signalling (Bonvento & Bolaños, 2021; Briquet et al., 2022; Hu & Wilson, 2002; Magistretti & Allaman, 2018; Turner & Adamson, 2011). The work presented in this thesis explores the fundamental shifts in extracellular metabolite pools in response to varying metabolite availability and activity induced-

metabolic needs. The discovered patterns of metabolic adaptation and extracellular fluctuation support findings of the contribution of non-neuronal cells to neuronal metabolism and the use of alternative fuels (e.g., lactate) to support the metabolic needs of neuronal signalling. What's more, the glucose-sparing effect, and the converging metabolic adaptions to improve monocarboxylate uptake, further support research suggesting that lactate may be the preferential metabolic fuel when present in large enough quantities (Bouzier-Sore et al., 2003; Dienel, 2012). This naturally raises the question: why did our brains switch from a monocarboxylate dominant metabolism to a carbohydrate dominant metabolism (Hawkins et al., 1971; Leino et al., 1999)? Research into this question may center around the impacts of lactic acidosis which decreases tissue pH and alters cerebral and mitochondrial function (Hillered et al., 1984; Rehncrona, 1985), thereby promoting a switch to carbohydrate metabolism to avoid such impacts. Or perhaps, a simple switch to a carbohydrate rich diet is sufficient to induce a carbohydrate-centric cerebral metabolic profile.

Importantly, these findings stress the acute contribution of peripheral metabolite availability on the transient fluctuations of extracellular metabolites, both during activation and at rest. This highlights the cooperative nature of the peripheral and central nervous system in maintaining cerebral metabolic homeostasis. As we have demonstrated, this homeostatic maintenance of extracellular metabolite pools can be altered in disease states. Altered metabolism is present in a plethora of neurological diseases (Cleland et al., 2021; Duarte et al., 2013). There is evidence to suggest that these metabolic disturbances are caused, at least in part, by altered function in non-neuronal cells such as astrocytes and endothelial cells (Grammas, 2011; Lee et al., 2022; Li et al., 2019; Molofsky et al., 2012; Peteri et al., 2019; Ricci et al., 2009; Siracusa et al., 2019; Yuan et al., 2023). Interestingly, the cognitive impact of some of

some of these disease states can be reduced when the brain is directly provided with additional metabolic substrates (Cisternas et al., 2019; Cunnane, 2022; Cunnane et al., 2016, 2020; Duran-Aniotz & Hetz, 2016; Morrill & Gibas, 2019; Suzuki et al., 2011; van Gemert et al., 2022), suggesting that the cerebral metabolic machinery in non-neuronal cells responsible for maintaining extracellular metabolite pools play a key role in mediating the cognitive symptoms of these disorders.

Finally, support for the metabolic cooperation between neurons and non-neuronal cells, specifically astrocytes, provided by this body of work and the studies cited herein have significant implications for mathematical modeling studies and imaging techniques (Buxton, 2010; Jolivet et al., 2010; Magistretti & Pellerin, 1996). Specific knowledge regarding the transport and metabolism of metabolites across the various cerebral compartments (endothelial cells, astrocytes, neurons) is critical in the development of accurate of mathematical modeling studies. Therefore, any future mathematical modeling studies should take into consideration the extracellular fluctuations, and their modulating variables identified in this work to inform their models and ensure that they replicate *in vivo* findings. Imaging techniques such a BOLD fMRI and PET scans are interpreted with the underlying assumptions that changes in oxygen and tracer metabolism, respectively, are representative of neuronal metabolism, either at rest or following stimulation (Drew, 2019; Magistretti & Pellerin, 1996). These assumptions may need to be re-evaluated in light of the contribution of oxygen-independent metabolic pathways (e.g., glycolysis and PPP) and non-neuronal cells to local and global cerebral metabolism. Cerebral metabolic deficits in neuronal disorders observed with these techniques (Duran-Aniotz & Hetz, 2016; Frith, 2003; Keeratitanont et al., 2022) need to consider that their measurements may be influenced by

or are mainly the result of non-neuronal metabolism or deficiencies in non-neuronal metabolic machinery.

Summary

This body of work highlights the brain's adaptability to varying metabolite availability as well as hints to collaborative and synergistic links between systems (central and peripheral) and cellular compartments (endothelial, astrocytes and neurons) that play key roles in maintaining and modulating extracellular metabolite pool levels. When systemic availability of various metabolites is increased, there appears to be converging metabolic pathways that promote peripheral lactate production (likely through the liver) which in turn increases cortical extracellular glucose, likely through glucose-sparing mechanisms (see **Chapter II**; Béland-Millar et al., 2017). Further support for this hypothesis can be found when observing the effects of inefficient metabolites (galactose; see **Chapter II**; Béland-Millar et al., 2017), pharmacological inhibition of the endothelial transporters GLUT1 and MCT1 (see **Chapter VI**) and chronic changes in diet and exercise (see **Chapter III**, Béland-Millar et al., 2020). The additive impacts of pharmacological transporter inhibition on cortical extracellular metabolite fluctuations and endothelial transporter expression further nuances the contribution of peripheral metabolites on cortical extracellular pools in response to the shifting needs and availability (see **Chapter VI**). Favoring cortical monocarboxylate transport also appears to occur when alternative metabolites are chronically increased (see **Chapter III**; Béland-Millar et al., 2020).

Following activation, extracellular cortical glucose and lactate pools are uncoupled, with extracellular lactate rising and glucose decreasing (Béland-Millar & Messier, 2018, 2022; Hu & Wilson, 1997b; Pellerin & Magistretti, 1994). Varying the availability of metabolites can influence these typical activation-induced extracellular metabolite fluctuations. The enhanced

capacity of systemic lactate injections to attenuate these trends over fructose suggests that it may more efficiently replace glucose as a metabolic fuel, especially when energy needs are estimated to be high in visual stimulation paradigms (see **Chapter V**; Béland-Millar & Messier, 2018). Motor tasks in the utilised tethered system effectively replicated the expected activity-induced extracellular cortical fluctuations, including their dependence on intensity and novelty of the task (see **Chapter IV**; Béland-Millar & Messier, 2022). This allowed us to develop a usable model for voluntary and imposed motor behavior in this tethered experimental setup.

Pharmacological transporter inhibition illustrated the importance of peripheral monocarboxylate transport in maintaining motor-induced extracellular metabolite fluctuations (see **Chapter VI**). Notable, these results suggest that both endogenous (created in the brain) and exogenous (created in the body) lactate contribute to brain energetics. Finally, an ASD mouse-model exhibited attenuated motor-induced extracellular cortical lactate, enhanced cerebral glucose uptake and stable cerebral oxygen levels (see **Chapter VII**; Béland-Millar et al., 2023). Together this suggests elevated shuttling of glucose through non-oxidative pathways, which likely includes the PPP for enhanced antioxidant capacity.

Future directions

The study of brain metabolism is particularly complex due to the interactions of various signalling and metabolic pathways within and across cells. For example, any effect observed following the altered cerebral lactate transport may be the result of inefficient lactate clearing impacting local pH, impaired access to this alternative fuel source, its role in BBB maintenance and metabolic homeostatic signalling or neuronal excitability. To definitively answer questions highlighted throughout this work would require a leap in technology that would enable the tracing of substrates across compartments and throughout their various metabolic steps without

hinderance to normal biological processes. Researchers are working towards this goal by using metabolic isotope tracing studies to identify where metabolites travel to within and across cells (Dong et al., 2022; Wang et al., 2022).

A key-component in the astrocyte-neuron collaboration appears to be the neuronal MCT2 transporter. MCT2 expression in astrocytes is correlated with GFAP expression (Hanu et al., 2000), which is a measure of astrogliosis (reactive astrocytes) (Brahmachari et al., 2006; Eng & Ghirnikar, 1994). Many studies have found that astrocytes and altered metabolism play a key role in various disorders (Chen et al., 2022; Mulica et al., 2021; Beard et al., 2022; Zulfiqar et al., 2019), including a study demonstrating that diminished astrocytic LDHA expression and lactate homeostasis are associated to depressive-like behaviors (Yao et al., 2023). Together, this paints a picture of aberrant metabolic cooperation through altered monocarboxylate production and transport that subsequently impacts key executive functions. Further understanding the impacts of aberrant metabolic pathways may bring us closer to understanding the distinctive and overlapping roles of glucose and lactate, as well as glycolysis and OXPHOS metabolism, in various cellular compartments.

Glutamate and potassium may have differential and non-additive impacts on astrocytic metabolism (Rimmele et al., 2018), which may be regulated in part by the potassium-induced inhibition of glutamate transport (Rimmele et al., 2017). Astrocytes play an important role in memory formation (Kol et al., 2020; Zhou et al., 2021; Kol & Goshen, 2021) and control of neuronal excitability (Verhog et al., 2020; Bellot-Saez et al., 2017; Wang et al., 2021). The latter likely being modulated through HCAR1 (lactate receptor) mechanisms (Skwarzynska et al., 2022; de Castro Abrantes et al., 2019). Interestingly, MCT2 is co-localized HCAR1 and its expression is enriched in correlation with ATP-sensitive potassium transporters (KATP) (Medel

et al., 2022). Therefore, future work may want to shift its focus to potassium-induced releases in lactate or work towards disentangling if the lactate-to-neuron shuttle, and consequently lactate contributions to neuronal metabolism or extracellular metabolite pools, is impacted by the specific substrates downstream of signalling (e.g., glutamate vs potassium).

Altogether, MCT2, lactate-responsive mechanisms (HCAR1) and neuronal uptake of extracellular lactate pools appears central to normal executive functions and susceptible to dysfunction in various disease states. Given the ubiquitous observations of aberrant metabolic profiles in disease states and the contribution of lactate signalling and transport to executive functions altered by said disease states, further probing into aberrant metabolism, transport, utilisation, and signalling focusing on these elements (e.g., lactate, HCAR1, MCT2) may prove promising. Recent work has demonstrated that MCT2 overexpression recovered cognitive functions in a rat stroke model (Yu et al., 2021) and that HCAR1 regulates neurogenesis in an ischemia mouse model (Kennedy et al., 2022). Though there is evidence to suggest that in some disorders, such as Alzheimer's disease, altered metabolism precedes clinical symptoms (Hammond & Lin, 2022; Salvadó, 2022), there is still much work to be done to determine if these metabolic profiles are symptoms or causal factors.

In addition, this author would encourage future studies to continue expanding our understanding of the collaborative nature of metabolism at the metabolite (glucose and lactate), cellular (neurons and astrocytes), and whole body (blood and brain) level. From decreasing glucose uptake and use, to being a key signalling molecule to induce exercise-like benefits, blood-borne lactate production is a key player in shaping cerebral metabolism. Continued investigations into the ANSL vs NALS debate should not neglect to measure, control and manipulate these variables. This extends particularly to investigations into the impact of diets on

neuronal functions and disease prognosis that are impacted by uptake and utilization of circulating metabolites.

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