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Lactate is Oxidized Outside of the Mitochondrial Matrix in Rodent Brain

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1 **Lactate is Oxidized Outside of the Mitochondrial Matrix in Rodent Brain**
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Abstract

The nature and existence of mitochondrial lactate oxidation is debated in the literature. Obscuring the issue are disparate findings in isolated mitochondria, as well as relatively low rates of lactate oxidation observed in permeabilized muscle fibres. However, respiration with lactate has yet to be directly assessed in brain tissue with the mitochondrial reticulum intact. To determine whether lactate is oxidized in the matrix of brain mitochondria, oxygen consumption was measured in saponin-permeabilized mouse brain cortex samples, and rat prefrontal cortex and hippocampus (dorsal) subregions. While respiration in the presence of ADP and malate increased with the addition of lactate, respiration was maximized following the addition of exogenous NAD^+ , suggesting maximal lactate metabolism involves extra-matrix lactate dehydrogenase. This was further supported when NAD^+ -dependent lactate oxidation was significantly decreased with the addition of either low-concentration α -cyano-4-hydroxycinnamate or UK-5099, inhibitors of mitochondrial pyruvate transport. Mitochondrial respiration was comparable between glutamate, pyruvate, and NAD^+ -dependent lactate oxidation. Results from the current study demonstrate that permeabilized brain is a feasible model for assessing lactate oxidation, and support the interpretation that lactate oxidation occurs outside the mitochondrial matrix in rodent brain.

Short title: Mitochondrial lactate oxidation in permeabilized rodent brain.

Keywords: Lactate, Brain, Mitochondria, Respiration, Lactate dehydrogenase

47 **Introduction**

48 Lactate was once considered a dead-end waste product of glycolysis produced
49 primarily under anaerobic conditions. However, it is now appreciated that lactate is
50 continuously generated and consumed by oxidative cells (Gladden 2004). Lactate
51 shuttling between tissues serves to distribute oxidizable three-carbon precursors between
52 cells. In the cells of its origin, lactate production can help maintain the free NAD^+ pool
53 during high rates of glycolysis, and is the major cytosolic redox couple for
54 NAD^+/NADH . Although research examining lactate metabolism has been abundant, the
55 details of its uptake by mitochondria remain an apparent matter of controversy (Passarella
56 et al. 2015, Paventi et al. 2017).

57 In 1985, Brooks proposed a lactate shuttle hypothesis (Brooks 1985) (now
58 referred to as the cell-to-cell lactate shuttle), and later incorporated aspects which
59 suggested that lactate is oxidized by mitochondrial lactate dehydrogenase (LDH) (Brooks
60 et al. 1999a, 1999b). In mammalian skeletal muscle, the notion of direct mitochondrial
61 lactate oxidation has gathered support from some (Brooks et al. 1999b, Dubouchaud et al.
62 2000, Passarella et al. 2008, 2015), and discord from others (Rasmussen et al. 2002,
63 Sahlin et al. 2002, Ponsot et al. 2005, Gladden 2007, Yoshida et al. 2007). Evidence also
64 supports a modified version of the intracellular lactate shuttle (Hashimoto et al. 2006), in
65 which lactate is oxidized to pyruvate prior to its transport across the inner mitochondrial
66 membrane (Szczesna-Kaczmarek et al. 1984, Szczesna-Kaczmarek 1990, Elustondo et al.
67 2013, Jacobs et al. 2013).

68 Previous attempts to resolve the discrepancy in skeletal muscle have been
69 complicated by several factors. First, considering mitochondrial isolation requires

mechanical disruption of the mitochondrial reticulum and differential centrifugation to yield a relatively pure mitochondrial sample, results determined in isolated mitochondria are frequently under scrutiny due to the possibility of cytosolic contamination during the isolation process (Kras et al. 2016). Second, where several studies have reported negligible direct mitochondrial lactate oxidation (Rasmussen et al. 2002, Sahlin et al. 2002, Ponsot et al. 2005, Yoshida et al. 2007), work circumventing the need for isolated mitochondria using permeabilized skeletal muscle fibres demonstrated that lactate could be oxidized through the provision of exogenous NAD^+ (Elustondo et al. 2013, Jacobs et al. 2013). These results identified that NAD^+ is required to assess mitochondrial respiration supported by lactate, and since NAD^+ is not membrane permeable, suggest that the LDH enzyme is located outside of the mitochondrial matrix in skeletal muscle. These findings are expected consequences of a marked difference in NAD^+/NADH redox potential in the cytosol versus mitochondrial matrix (Williamson et al. 1967, Sahlin et al. 2002, Jones and Sies 2015).

Lactate readily crosses the blood-brain barrier via monocarboxylate transporters (MCTs) (Simpson et al. 2007), and is notably produced and oxidized in the brain (Boumezbeur et al. 2010, Dienel 2012, Schurr 2017). The astrocyte-neuron lactate shuttle hypothesis (Pellerin and Magistretti 2012, Schurr 2017) posits that lactate is exported by glial cells to be taken up and oxidized by neurons as an essential component of neural-glial metabolic cooperation. Isolated mitochondria from the brain offer a potential means of investigating the nature of mitochondrial metabolism in the CNS. However, isolating mitochondria from brain tissue tends to yield a heterogeneous mix of multicellular, if not multiregional mitochondrial populations (Kristian 2010). Isolating brain mitochondria is

further complicated by the extra care necessary to minimize cross-contamination of synaptic versus non-synaptic mitochondria (Kiss et al. 2016).

The recent development of the permeabilized brain preparation (Herbst and Holloway 2015) permits direct assessment of lactate oxidation in the brain without disruption of the mitochondrial reticulum. Therefore, the objective of the current study was to investigate and further clarify the nature of mitochondrial lactate oxidation using brain tissue. This was accomplished in both gross mouse cortex samples, as well as specific subregions of rat cortex (prefrontal) and hippocampus (dorsal). We report evidence that maximal mitochondrial lactate oxidation requires extramatrix LDH in rodent brain.

Methods

Animals and reagents

C57BL/6 mice ($n = 4$) were bred on-site at the University of Guelph, and were anesthetized with sodium pentobarbital before cervical dislocation as described previously (Herbst and Holloway 2016). Male Wistar rats ($n = 11$) were purchased from Charles River Laboratories (Montreal), and were anesthetized via an intraperitoneal injection of 250 mg/ml sodium pentobarbital (Vetoquinol, QC), prior to decapitation and dissection. All other chemicals were purchased from Sigma. All procedures in this study were approved by the University of Guelph Animal Care Committee and the St. Francis Xavier University Animal Care Committee, respectively, and conformed to the standards of the Canadian Council on Animal Care.

116 *Respirometry*

117 Permeabilization of mouse brain tissue for assessment of mitochondrial respiration was
118 performed as described previously (Herbst and Holloway 2015). Briefly, small (~2 mg)
119 samples were quickly dissected from mouse cortex, weighed, and placed into an
120 Oxygraph-2k (OROBOROS Instruments, Innsbruck, AT) containing a modified MiR05
121 respiration medium (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 10 mM
122 KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA). The method was developed to
123 ensure H₂O₂ could be determined, and therefore taurine was removed from the buffer, as
124 originally reported (Herbst and Holloway 2015); taurine was excluded in the current
125 study to be consistent with those experiments. MiR05 media was chosen following
126 preliminary experiments comparing it to another popular, but notably sucrose- and
127 lactobionate-free potassium salt buffer (*buffer z* (Perry et al. 2013)), due to differences in
128 maximal respiration (Supplementary figure S1). Samples were allowed approximately 5
129 minutes for residual substrates to deplete before saponin or substrate addition to avoid
130 drift during experiments.

131

132 Brain tissue was obtained from euthanized Wistar rats in a manner similar to the mice
133 (Herbst and Holloway 2015), except that brain subregions were dissected using a Brain
134 Matrix (Zivic Instruments, Pittsburgh). Specifically, prefrontal cortex (PFC) and dorsal
135 hippocampus (DH) subregions were dissected. PFC and DH were chosen, because they
136 continue to be areas of intense interest among broad fields of research. Prefrontal cortex
137 plays a major role in executive function, and important functions of the hippocampus
138 include short-to-long-term memory consolidation and spatial memory. Following

dissection, PFC and DH samples were quickly weighed, and placed into the oxygraph. Samples were allowed to incubate for 5 min prior to permeabilization with saponin (50 $\mu\text{g}\cdot\text{mL}^{-1}$). Respirometry was conducted at 37° C in 2 mL MiR05 with constant stirring at 750 rpm. Instrumental background O_2 consumption was corrected using equations determined under the same parameters used for experimental data collection.

Titration protocols

To ensure that saturating concentrations of NAD^+ and lactate were used in the subsequent respiratory protocols, titrations were performed on mouse cortical samples with NAD^+ (0.25, 0.5, 1, 1.5, 2.0, 2.5, 5 mM; in the presence of 100 mM lactate) and lactate (5, 10, 20, 40, 60, 80, 100 mM; in the presence of 2 mM NAD^+). To test the individual and summative effects of lactate and NAD^+ on mitochondrial respiration in the mouse cortex, 5 mM NAD^+ and 80 mM lactate were added separately to the respirometer, followed by their combined addition. Due to previous concerns that lactobionate contained within MiR05 respiration medium may have an inhibitory effect on lactate-mediated respiration (Passarella et al. 2015), we replaced MiR05 with potassium salt *buffer Z* in the presence of 5 mM ADP + 4 mM malate + 80 mM lactate + 5 mM NAD^+ , and compared the two media (Supplementary figure S1).

Maximal mitochondrial respiration in mouse cortex was stimulated in the presence of either 10 mM pyruvate, 10 mM glutamate, or 80 mM lactate + 5 mM NAD^+ . The monocarboxylate transporter inhibitor, α -cyano-4-hydroxycinnamate (CHC), was titrated in the presence of glutamate, pyruvate, or lactate + NAD^+ at 5 μM , 25 μM and 50 μM . CHC and UK-5099 (see subsequent paragraph) are recognized inhibitors of both

monocarboxylate transport in the plasma membrane, but more potently, mitochondrial pyruvate transport (Halestrap and Denton 1974, Halestrap 1975, Poole and Halestrap 1993, Gray et al. 2014). Saponin-permeabilization of the plasma membrane removes the role of plasma membrane monocarboxylate transporter inhibition in the current study. Due to slightly different maximal rates of respiration, inhibition experiments were expressed as a percentage of state III respiration for that substrate (control). In separate permeabilized mouse cortex samples, the LDH inhibitor oxamate (Oxm) was also titrated (10, 20 and 40 mM) in the presence of 5 mM ADP + 4 mM malate + 2 mM NAD^+ and either 80 mM lactate or 10 mM pyruvate ($n = 3-4$).

Experimental protocol

In rat PFC and DH, the respirometric protocol involved sequential addition of substrates and inhibitors as follows: 5 mM ADP, 4 mM malate, 40 mM lactate, 4 mM NAD^+ , 10 μM UK-5099, an inhibitor of mitochondrial pyruvate transport, 10 mM glutamate, and 1 mM aminooxyacetate, an inhibitor of the malate-aspartate shuttle aminotransferases. Malate concentration was chosen based upon separate experiments comparing 4 mM to each 2 mM and 0.5 mM malate (Supplementary figure S2). JO_2 was expressed as $\text{picomol O}_2 \times \text{s}^{-1} \times \text{mg}^{-1}$ tissue wet weight. ADP was added first to ensure further substrate-stimulated respiration was not dependent on the presence of ADP. Malate was subsequently added to ensure high rates of lactate (and NAD^+)-stimulated respiration. UK-5099 was added to determine if the rate of lactate-stimulated respiration would be reduced when mitochondrial pyruvate transport was inhibited. Glutamate was added at the end of the protocol to determine if off-target effects of pharmacological

experiments were present. Finally, aminooxyacetate was added as an inhibitor of aminotransferase to determine the potential impact of the malate-aspartate shuttle.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). One-way and two-way ANOVA were used to detect differences in substrate-linked and inhibitor-affected respiration in mouse samples. Two-way ANOVA with repeated measures was used to compare rat PFC to DH respiration. Bonferroni *post hoc* tests were used to identify specific differences among dependent variables. Bartlett's test did not support homogeneity of variances. Therefore, all data were square root – transformed prior to analysis. The alpha-level for statistical significance was set at $p = 0.05$. All data are reported as means $\pm$ standard deviation.

Results

Lactate oxidation in permeabilized mouse brain

Use of the permeabilized brain preparation provides the opportunity to analyze mitochondrial respiration in the brain without disrupting the mitochondrial reticulum. However, because lactate-stimulated respiration has not been previously assessed in the permeabilized rodent brain, we first established the appropriate concentrations of key substrates required for this preparation. Titration of NAD^+ (0-2.5 mM; apparent K_m for NAD^+ : 1.3 ± 0.5 mM) and lactate (0-100 mM; apparent K_m for lactate: 11.2 ± 3.5 mM) were performed in separate experiments to ensure that saturating concentrations of the respective substrates were added in subsequent protocols (Fig. 1). Further, where it has

208 been previously hypothesized that 60 mM lactobionate used in the respiration buffer may
209 inhibit LDH (Passarella et al. 2015), we replaced our buffer with the frequently employed
210 buffer Z (Perry et al. 2013) and observed no increase in respiration (Supplementary figure
211 S1), demonstrating that lactobionate does not have an inhibitory effect on lactate
212 metabolism.

213 214 *NAD-dependency on maximal lactate-supported respiration in mouse brain*

215 Previous work in skeletal muscle has shown that the provision of exogenous NAD^+ is
216 required to observe lactate respiration in permeabilized muscle fibers (Elustondo et al.
217 2013, Jacobs et al. 2013). Considering the mitochondrial inner membrane is impermeable
218 to NAD^+ and NADH (Di Lisa and Ziegler 2001), these previous findings suggest that
219 lactate is oxidized to pyruvate outside of the mitochondrial matrix before transportation
220 across the membrane. To determine whether these findings are corroborated in mouse
221 brain mitochondria, lactate- and NAD^+ -stimulated respiration were analyzed
222 independently, and in conjunction with one another. While no significant increase in
223 respiration was observed with either lactate or NAD^+ alone, their combined addition
224 resulted in an approximately 90% increase in respiration ($P < 0.001$; Fig. 2A). Therefore,
225 the requirement for the addition of NAD^+ not only allays concerns about residual NAD^+
226 contamination in the permeabilized brain preparation, but also supports that lactate was
227 oxidized outside of the mitochondrial matrix in these experiments. Moreover, lactate
228 respiration in the presence of NAD^+ was comparable to complex I-linked respiration
229 stimulated by either pyruvate or glutamate (Fig. 2B).

231 *Respiration supported by NAD-dependent lactate oxidation requires LDH and*
232 *mitochondrial monocarboxylate transport in permeabilized mouse brain*

233 To investigate whether inhibition of LDH affected NAD⁺-dependent lactate oxidation,
234 oxamate (inhibitor of LDH) was titrated (10, 20 and 40 mM) in the presence of 5 mM
235 ADP + 4 mM malate, 2 mM NAD⁺ and either 10 mM pyruvate or 80 mM lactate.
236 Whereas the effect of oxamate on pyruvate respiration was negligible, oxamate
237 significantly inhibited NAD⁺-dependent lactate respiration relative to control (Fig. 3A).
238 However, given reports that oxamate crosses the inner mitochondrial membrane
239 (Halestrap 1975), as well as the possibility that oxamate can affect pyruvate transport into
240 mitochondria (Martin-Requero et al. 1986), it was important to employ inhibitors of other
241 potential aspects of mitochondrial lactate metabolism. To determine whether lactate
242 either enters the mitochondrial matrix directly, or is first converted to pyruvate before
243 transport and oxidation, the mitochondrial pyruvate transport inhibitor α -cyano-4-
244 hydroxycinnamate (CHC; (Halestrap 1975)) was used in permeabilized mouse cortex
245 samples. In these experiments, titration of CHC produced a >50% inhibition of both
246 lactate- and pyruvate-stimulated respiration ($P < 0.001$). Together with our observations
247 of inhibition with oxamate, these experiments support that lactate is oxidized to pyruvate
248 prior to being transported across the inner mitochondrial membrane.

249 Because potential off-target effects of pharmacological experiments are always of
250 concern, glutamate respiration was also assessed in the presence of CHC to serve as a
251 positive control, as glutamate does not interact with mitochondrial monocarboxylate
252 transport and is also a complex I-linked substrate. Glutamate-stimulated respiration was

unaffected by CHC (Fig. 3B), suggesting that CHC is specific to monocarboxylate transport and does not negatively impact respiration.

Respiration supported by NAD-dependent lactate oxidation requires mitochondrial pyruvate transport in permeabilized subregions of rat brain

To further explore these findings, we utilized another inhibitor of mitochondrial pyruvate transport in permeabilized rat brain samples. In both rat PFC and DH, a more specific inhibitor of mitochondrial pyruvate transport, UK-5099 (Halestrap 1975, Gray et al. 2014) abolished ($P < 0.001$) NAD^+ -dependent, lactate-supported mitochondrial respiration (Fig. 4). This further emphasizes the necessity of pyruvate transport into the matrix to support lactate oxidation. As with CHC (Fig. 3B), glutamate-stimulated respiration was unaffected by UK-5099 (Fig. 4), supporting that UK-5099 is also specific to pyruvate transport, and does not negatively impact respiration by affecting the tricarboxylic acid cycle or electron transport chain in these samples. Glutamate respiration was significantly decreased however, following addition of the aminotransferase inhibitor aminooxyacetate (Fig. 4). This suggests that the malate-aspartate shuttle, which depends on aspartate aminotransferase, is required for glutamate/malate-dependent respiration. While the possibility of off-target effects with any inhibitor is acknowledged, these data suggest that the malate-aspartate shuttle, which depends on aspartate aminotransferase, is required.

276 Discussion

277 The present study sought to examine the functional location of mitochondrial
278 LDH in rodent brain. We observed an NAD^+ -dependency of lactate respiration in the
279 cortex and hippocampus, as well as ablated lactate oxidation upon inhibition of
280 mitochondrial pyruvate transport. Taken together, these findings corroborate previous
281 work in muscle suggesting that mitochondrial LDH is located outside of the
282 mitochondrial matrix. However, unlike skeletal muscle, we also observe modest lactate-
283 supported mitochondrial respiration prior to addition of exogenous NAD^+ . A similar
284 phenomenon is observed in liver, where NAD^+ remains bound to LDH localized to the
285 intermembrane space, even during acrylamide gel electrophoresis (Skilleter and Kun
286 1972). Thus, residual binding of NAD^+ to LDH outside of the matrix in our preparation is
287 plausible.

288 The nature of mitochondrial lactate metabolism has been a contentious topic. This
289 may be due, at least in part, to a heavy reliance on some indirect techniques (e.g.,
290 spectrophotometric monitoring of NADH change), and generalizations among species,
291 tissues and cell types. Recently, the mitochondrial metabolism of lactate in cultured
292 cancer cell lines was investigated more directly, using ^{13}C -labeled lactate and high-
293 resolution mass spectrometry (Chen et al. 2016). The results support the notion of a
294 mitochondrial L-lactate carrier (Taylor 2017) and matrix LDHB in lactate-supported
295 lipogenesis in proliferating, immortalized cells (Chen et al. 2016). Whether this
296 observation translates to mitochondria in mature, differentiated tissues like skeletal
297 muscle and brain has not, to our knowledge, been reported.

Questions about the purity of isolated mitochondria and a mismatch in pyruvate-lactate respiration in oxidative muscle fibers have also not been entirely explained. In the brain, however, analysis of respiration in permeabilized tissue demonstrates similar observable oxidation rates between both lactate and pyruvate, which suggests the brain may provide a better model of studying mitochondrial-associated LDH. While lactate is well-established to be produced and oxidized in non-oxidative and oxidative skeletal muscle fibers, respectively, evidence supports the shuttling of lactate in the brain between glia and neurons for similar purposes. As such, LDH isoforms associated with skeletal muscle (LDH-5) and the heart (LDH-1) have also been identified in brain tissue (Bittar et al. 1996, Hashimoto et al. 2008); it is of interest that these isoforms have been found to be expressed in a cell- and region-specific distribution, suggesting different regions of the brain may possess unique requirements for lactate oxidation. Moreover, within any given region, the location of mitochondrial LDH is of particular importance in understanding the cell-to-cell metabolic cooperation within the brain. The astrocyte-neuron lactate shuttle hypothesis supports that glucose is taken from the circulation and converted to lactate for oxidation in neurons (Bouzier-Sore et al. 2003, Pellerin and Magistretti 2012). An alternative hypothesis posits that the resulting lactate transport to neurons results only in the donation of electrons through the reduction of NAD^+ prior to the shuttling of pyruvate back to astrocytes for oxidation (reviewed in (Dienel 2012)). While the present study does not expand on these hypotheses, the commitment of lactate to the mitochondrial matrix would prevent the theoretical recycling of pyruvate, where evidence presented here and elsewhere (Cerdan et al. 2006) support that neither lactate nor pyruvate are directly or necessarily committed to mitochondrial oxidation.

321 The NAD^+ -dependent nature of maximal lactate respiration reported previously in
322 skeletal muscle (Elustondo et al. 2013, Jacobs et al. 2013), as well as here in the brain,
323 provide evidence that mitochondrial LDH is not located exclusively, if at all, in the
324 mitochondrial matrix of normal, differentiated brain tissue. Though previous studies have
325 demonstrated that the majority of cellular NAD^+ (>75%) is located inside the
326 mitochondria (Tischler et al. 1977, Di Lisa and Ziegler 2001), the cytosolic
327 NAD^+/NADH redox potential is reported to greatly exceed the mitochondrial
328 NAD^+/NADH potential ((Williamson et al. 1967, Jones and Sies 2015); see (Sahlin et al.
329 2002) for discussion)), suggesting that lactate oxidation is favored outside of the
330 mitochondrial matrix. In the cytosol, this is due to the equilibrium constant for the
331 associated redox couples (namely, lactate/pyruvate) combined with influential protein
332 binding sites for NADH (Jones and Sies 2015); changes in NADH are therefore primarily
333 mitochondrial (Sun et al. 2012, Blacker and Duchen 2016). Indeed, consistent with the
334 present study, lactate oxidation only occurs when NAD^+ is added. These results are
335 consistent with ideas about the nature of mitochondrial lactate oxidation in which LDH
336 acts external to the matrix (Gladden 2007, 2008, Hashimoto et al. 2008, Kane 2014,
337 Rogatzki et al. 2015).

338 Although little is known about the nature of NAD^+ synthesis and transport into
339 the mitochondria, current evidence supports that the concentration of NAD^+ within
340 different compartments is highly regulated (di Lisa 2001b). As such, where NAD^+
341 concentrations have been shown to respond to changes in ATP availability in the cytosol,
342 this is not true in the matrix (Vinogradov et al. 1972, Devin et al. 1997, Di Lisa and
343 Ziegler 2001). Therefore, extramatrix LDH may help couple adaptations in NAD^+

concentrations to changes in the availability of lactate as an energy substrate. This would be an important adaptive mechanism in the brain in particular, where substrate selectivity is regulated uniquely between cell types and brain regions. Though the relationship between cytosolic NAD^+ concentrations and lactate availability has yet to be investigated, the present data supports the NAD^+ -dependent nature of lactate oxidation in the cytosol of rodent brain.

The centrality of lactate to neuroenergetics (Schurr 2017), and to metabolic physiology in general (Hui et al. 2017), is undeniable. The experimental freedoms conferred by the permeabilized brain methodology permit evaluation of lactate oxidation in this tissue, and adds to the techniques with which mitochondrial function in brain can be explored to investigate pathologies, test various applications, and describe the phenotypic outcomes of transgenic animal models.

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Declarations of interest

Authors declare no conflicts of interest.

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Author contributions. All experiments involving mice were conducted in the laboratory of GPH at the University of Guelph. Experiments involving rats were conducted in the laboratories of DAK and KB at St. Francis Xavier University. EAFH and DAK conceived the study; EAFH, MAJG, KB, GPH and DAK contributed to the design of the study; EAFH, MAJG and DAK acquired, analyzed and interpreted the experimental data for the study; EAFH, MAJG, and DAK drafted the manuscript; EAFH, MAJG, KB, GPH and DAK revised the manuscript critically for intellectual content.

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Figure Captions

FIGURE 1. Titration of substrates in saponin-permeabilized mouse brain cortex. **A**, NAD^+ was titrated (0-2.5 mM) atop 100 mM lactate, 4 mM malate and 5 mM ADP. Apparent K_m for NAD^+ : 1.3 ± 0.5 mM. **B**, Lactate was titrated (0-100 mM) atop 2 mM NAD^+ , 4 mM malate and 5 mM ADP. Apparent K_m for lactate: 11.2 ± 3.5 mM. Results represent mean $\pm$ SD; $n = 4$.

FIGURE 2. Mitochondrial lactate oxidation in saponin-permeabilized mouse cortex requires extramatrix NAD^+ and is comparable to glutamate and pyruvate-supported JO_2 . **A**, 80 mM lactate (+Lactate); 2.5 mM NAD^+ (+ NAD^+) or both (+Lactate + NAD^+) were added to 5 mM ADP + 4 mM malate. Respiration with ADP + malate + lactate + NAD^+ was greater than any other condition ($^{***}P < 0.001$; $n = 3$). **B**, Mitochondrial JO_2 was comparable among 10 mM glutamate, 10 mM pyruvate and 80 mM lactate, added atop 5 mM ADP + 4 mM malate + 2.5 mM NAD^+ . Data are mean $\pm$ SD, $n = 4$.

FIGURE 3. Effects of inhibitors of lactate dehydrogenase and mitochondrial pyruvate transport on lactate-supported respiration in permeabilized mouse cortex. **A**, Oxamate (Oxm) was titrated (10, 20 and 40 mM) in the presence of 5 mM ADP + 4 mM malate + 2 mM NAD^+ and either 80 mM lactate or 10 mM pyruvate ($n = 3-4$). $^*P < 0.05$, $^{**}P < 0.01$, $^*P < 0.001$ lactate + NAD^+ versus control (no Oxm). $^{##}P < 0.001$ versus pyruvate + 20 mM Oxm condition; $^{###}P < 0.0001$ versus pyruvate + 40 mM Oxm condition. **B**, α -

cyano-4-hydroxycinnamic acid (CHC) was titrated (5, 25, and 50 μ M) in the presence of 5 mM ADP + 4 mM malate + 2 mM NAD^+ and either 80 mM lactate, 10 mM pyruvate or 10 mM glutamate ($n = 3-4$). *** $P < 0.001$ versus control for both lactate + NAD^+ and pyruvate. ### $P < 0.001$ glutamate versus each lactate + NAD^+ and pyruvate. Data are mean $\pm$ SD.

FIGURE 4. Mitochondrial respiration supported by exogenous NAD^+ -dependent lactate oxidation in permeabilized rat brain subregions relies on the pyruvate carrier. **A.** Representative oxygraphic trace of experiment: sequential additions of ADP (5 mM), malate (4 mM), lactate (40 mM), NAD^+ (4 mM), UK-5099 (10 μ M), glutamate (10 mM), and aminooxyacetate (1 mM) were made in saponin-permeabilized prefrontal cortex (PFC) and dorsal hippocampus (DH). Addition of lactate and NAD^+ increased respiration, while the subsequent addition of the mitochondrial pyruvate transport inhibitor UK-5099 decreased respiration. Glutamate respiration was unaffected by UK-5099, but inhibited by aminooxyacetate. Dashed line $P < 0.05$; dotted line $P < 0.0001$ between connecting points. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ PFC versus DH. # $P < 0.05$. Data are mean $\pm$ SD; $n = 10-11$.

A.

B.