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Abstract

The relative contributions of glycolysis and oxidative phosphorylation to neuronal presynaptic energy demands are unclear. In rat hippocampal neurons, ATP production by either glycolysis or oxidative phosphorylation alone sustained basal evoked synaptic transmission for up to 20 min. However, combined inhibition of both ATP sources abolished evoked transmission. Neither action potential propagation failure nor depressed Ca^{2+}-influx explained loss of evoked synaptic transmission. Rather, inhibition of ATP synthesis caused massive spontaneous vesicle exocytosis, followed by arrested endocytosis, accounting for the disappearance of evoked postsynaptic currents (PSCs). In contrast to its weak effects on basal transmission, inhibition of oxidative phosphorylation alone depressed recovery from vesicle depletion. Local astrocytic lactate shuttling was not required. Instead, either ambient monocarboxylates or neuronal glycolysis was sufficient to supply requisite substrate. In summary basal transmission can be sustained by glycolysis, but strong presynaptic demands are met preferentially by oxidative phosphorylation, which can be maintained by bulk but not local monocarboxylates or by neuronal glycolysis.

Significance Statement

Neuronal energy levels are critical for proper central nervous system function, but the relative roles for the two main sources of ATP production, glycolysis and oxidative phosphorylation, in fueling presynaptic function in unclear. Either glycolysis or oxidative phosphorylation can fuel low-frequency synaptic function, and inhibiting both underlies loss of synaptic transmission via massive vesicle release and subsequent failure to endocytose lost vesicles. Oxidative phosphorylation, fueled by either glycolysis or endogenously released monocarboxylates, can fuel more metabolically demanding tasks such as vesicle recovery after depletion. Our work demonstrates the flexible nature of fueling presynaptic function to maintain synaptic function.

Introduction

Neurons are energetically demanding cells, requiring a staggering amount of ATP to maintain ion gradients during signaling, and to power presynaptic vesicle cycling and filling. Although postsynaptic function is especially demanding (Attwell and Laughlin, 2001; Harris et al., 2012), the energetic cost of presynaptic function has recently been highlighted (Rangaraju et al., 2014; Pathak et al., 2015; Jang et al., 2016; Lujan et al., 2016). With >20,000 presynaptic ATP molecules consumed per glutamate vesicle (Attwell and Laughlin, 2001; Harris et al., 2012) not including ATP used for the restoration of presynaptic sodium and potassium gradients, the estimated 1×10^6 molecules of ATP at the presynaptic terminal would be rapidly exhausted without re-supply (Rangaraju et al., 2014).

Glucose is the predominant source of ATP in the central nervous system. After crossing the blood brain barrier, glucose is utilized by neurons and astrocytes to produce ATP through glycolysis and oxidative phosphorylation. Glycolysis quickly converts glucose into two molecules of pyruvate and a net total of two ATP. This provides cells with a rapid source of ATP for a short period of time, particularly in the absence of oxygen. Mitochondrial oxidative phosphorylation produces ATP at a slower rate but has a higher yield (32 ATP versus 2 produced with glycolysis; Pfeiffer et al., 2001). Substrates other than metabolically derived pyruvate can also feed the TCA cycle and oxidative phosphorylation. These include astrocyte-derived lactate (Schurr et al., 1988; Izumi et al., 1997; Gallagher et al., 2009; Ivanov et al., 2011; Wyss et al., 2011), ketone bodies (Edmond et al., 1987; Auestad et al., 1991; Izumi et al., 1998; McKenna, 2012; Takahashi et al., 2014), or glutamate (Hertz et al., 1988; Sonnewald et al., 1996; McKenna, 2007, 2012).

The relative importance of glycolysis and oxidative phosphorylation in fueling presynaptic function remains unclear. Glycolysis may be particularly vital for vesicle

endocytosis (Rangaraju et al., 2014), localization of synaptic vesicle machinery to synaptic terminals (Jang et al., 2016), and glutamate accumulation in synaptic vesicles (Ikemoto et al., 2003). Recent evidence also suggests that glycolytic failure quickly alters the presynaptic action potential waveform to depress transmission at the calyx of Held synapse (Lujan et al., 2016). However, other studies have demonstrated a prime effect of oxidative phosphorylation in powering synaptic transmission and information processing (Schurr et al., 1988; Izumi et al., 1997; Ivanov et al., 2011; Wyss et al., 2011; Hall et al., 2012).

Some of the confusion may arise because few of the above studies directly queried the relative roles of glycolysis and oxidative phosphorylation on presynaptic function under controlled conditions that reduce secondary explanations for transmission deficits. In a reduced, controlled environment, we find that although continuous ATP production is indeed important to sustain transmitter release, ablation of evoked transmission requires tandem inhibition of glycolysis and oxidative phosphorylation. We tracked the loss of transmission to massive vesicle exocytosis, which in the absence of ATP synthesis, failed to elicit commensurate vesicle endocytosis and recovery. We also found a privileged role for oxidative phosphorylation in fueling recovery of synaptic transmission after rapid depolarization-induced vesicle depletion even when glycolysis was inhibited, suggesting the availability of alternative substrates for oxidative phosphorylation. Neurons appear to power oxidative phosphorylation with either the glycolytic breakdown of glucose or the utilization of extracellular monocarboxylates, presumably from surrounding astrocytes. However, inhibiting either fuel source alone did not affect recovery. We conclude that evoked transmission exhibits flexibility in ATP sourcing that yields resilience of presynaptic function to changes in metabolic conditions.

Materials and Methods

Hippocampal Cell Culture

Cell cultures were produced and maintained as previously reported (Mennerick and Zorumski, 1995; Moulder et al., 2007) Briefly, hippocampal (neuron) and cortical (astrocyte) tissue of postnatal day 1-4 Sprague-Dawley rat pups of both sexes (85% female) were harvested using protocols approved by the Washington University Animal Studies Committee. The tissue was digested by 1 mg/ml papain, and mechanically dispersed. First, astrocytes were plated on collagen microcultures in Eagle's medium (Life Technologies) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 17 mM D-glucose, 400 µM glutamine, 500 U/ml penicillin, and 50 µg/ml streptomycin and maintained at 37°C in a humidified incubator (5% CO_2/95% air) before neuronal plating. Neurons were plated at a low density (~100 cells/mm^{-2}). +/-Astrocyte microcultures were prepared as previously described (Sobieski et al., 2015). Briefly, 25-mm glass coverslips were stamped with a polydimethylsiloxane microstamp coated with 0.5 mg/ml collagen to create 150-200 µm diameter microdots and backfilled with a non-permissive substrate poly-L-lysine grafted polyethylene glycol (PLL(20 kDa)-g[3.5]-PEG(2 kDa); Surface Solutions, Dübendorf, Switzerland) at a concentration of 10 µg/ml in PBS for 1 hour and then washed with 1x PBS. Unless otherwise stated, experiments were performed between 9-14 days *in vitro*.

Electrophysiology

Whole-cell electrophysiological recordings were performed at room temperature on the stage of an Eclipse TE2000-S inverted microscope. Data were collected using with a Multiclamp 700B amplifier and Digidata 1550 data acquisition board (Molecular Devices) using pClamp 10 software. During experiments in which both GABAergic and glutamatergic autaptic PSCs were studied, the intracellular pipette solution consisted of (in mM): 140 potassium chloride, 4 NaCl, 10 4-(2-hydroxyethyl)-1-

144 piperazineethanesulfonic acid (HEPES), 5 EGTA, and 0.5 $CaCl_2$. The pH was adjusted
145 to 7.25 with KOH. Experiments designed to examine excitatory postsynaptic currents
146 alone employed an internal solution containing 140 mM potassium gluconate instead of
147 potassium chloride to isolate glutamate-mediated currents. No exogenous ATP or
148 MgATP was added via the whole-cell pipette solution. Previous work on autaptic
149 hippocampal neurons has demonstrated that at least 10 min of recording time is required
150 for ~10% of the pipette contents to reach presynaptic terminals (Rosenmund and
151 Stevens, 1996). Therefore, whole-cell recordings obtained shortly after break-in should
152 rapidly establish postsynaptic ion gradients but effectively isolate presynaptic events
153 compromised by preceding metabolic manipulations.
154 Extracellular solution during voltage-clamp recordings typically consisted of (in
155 mM): 138 NaCl, 4 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, and 0.01 D-2-amino-5-
156 phosphonovalerate (D-APV, Tocris), pH 7.25, adjusted with NaOH. Miniature PSCs and
157 experiments evoking vesicle release directly from synaptic terminals were recorded in
158 saline containing 1 μM TTX. For current clamp recordings, saline contained 2,3-Dioxo-
159 6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 1 μM) and
160 bicuculine (50 μM). All PSCs were autaptic, recorded from single-neuron microcultures,
161 except for mPSC analysis during metabolic inhibition in Figure 3, where recordings were
162 performed on cells in multi-neuron microcultures. This was done to ensure that the
163 majority of presynaptic events arose from cells whose metabolic state was not
164 influenced by the whole-cell recording pipette over the time periods required to monitor
165 mPSCs.
166 Whole-cell recording pipettes were pulled from borosilicate glass capillary tubes
167 (World Precision Instruments) and had final open-tip resistances 2-6 MΩ. Unless
168 otherwise stated, neurons in voltage-clamp mode were held at -70 mV. Evoked autaptic
169 PSCs were elicited with a 1.5 ms depolarizing pulse to 0 mV. Data were sampled at 20

kHz, filtered at 10 kHz for PSC recordings. Access resistance was compensated to 90% for evoked autaptic PSC recordings. Miniature PSCs were sampled at 5 kHz and filtered at 1 kHz; access resistance was not compensated. For recording action potentials in current clamp mode, neurons were biased with small, tonic current injection when necessary to hold the membrane potential near -65 mV. Depolarizing current pulses in increments of 5-10 pA were applied for 1200 ms until a single action potential was elicited. When solution exchange or drug delivery was performed, solutions were dispensed to the target neuron by a gravity-driven local perfusion system from a common tip with an exchange time of ~100 ms.

Endocytosis measurement

Fixable FM1-43 (FM1-43FX) dye labeling of vesicle cycling was performed as previously detailed (Moulder et al., 2010). High density cell cultures were subjected various metabolic conditions in the presence of 0.5 – 1 μM TTX and 1 μM NBQX, 25 μM D-APV for 20 min prior to probing for vesicle cycling with FM1-43FX. The probe was conducted by challenging cells for 2 min with 5 μM FM1-43FX (Thermo Fisher Scientific) in 45 mM KCl to induce vesicle cycling. In the case of labeling in 0 Ca^{2+} solution, 100 μM EGTA was included to chelate residual Ca^{2+} during the probe. In other experiments FM1-43FX was included during the 20 min metabolic challenge. In all cases, non-internalized FM1-43FX fluorescence was removed with a brief application of saline supplemented with 500 μM Advasep-7 (CyDex). Cultures were washed 5 times with saline then fixed for immunostaining with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 10 min at room temperature. Cultures were then washed with PBS followed by a 10 minute incubation in 4% NGS and 0.04% Triton in PBS. Primary antibody, anti-synapsin I (1:1000) was applied for 3 h at RT and washed twice in PBS followed by 30 minute incubation in secondary antibody Alexa Fluor 633 conjugate

196 (Thermo Fisher Scientific), and glass coverslipping with Fluoromount G (Southern
197 Biotechnology Associates).
198 FM1-43FX and anti-synapsin I images were captured on an inverted Eclipse
199 TE2000-S microscope using a 60X objective with a 1.4 numerical aperture and a C1
200 scanning confocal laser (488, and 633 nm) with EZ-C1 software (Nikon). Z-stack images
201 were acquired with alternating laser lines while all gain and acquisition settings were
202 held constant within a given experiment. Two-dimensional projected images were
203 created and analyzed using Image J software and regions of interest to measure FM1-
204 43FX uptake/intensity were selected blindly using synapsin I-labeled puncta. FM1-43FX
205 fluorescence intensity was background subtracted, and puncta with intensity values
206 below background were assigned a value of 0.
207
208 **Data Analysis**
209 Data was analyzed and plotted using MetaMorph 7, Clampfit 10 (Molecular
210 Devices), MiniAnalysis 6 (Synaptosoft), Excel 2011 (Microsoft), Prism 6 (GraphPad), and
211 ImageJ. Unless otherwise stated, data in figures and text are given as mean ± SEM.
212 Student's two-tailed unpaired *t* test was used to compare 2 groups unless otherwise
213 noted. If more than 2 parameters were compared between 2 groups, a Bonferroni
214 correction was applied. A One-Way ANOVA was used if comparing more than two
215 experimental conditions and a Two-way ANOVA was implemented when comparing the
216 effects of at least two parameters over time. To test differences in variance between
217 groups, an F-test was conducted. Non-parametric data were analyzed using a Kruskal-
218 Wallis one-way ANOVA followed by Dunn's multiple comparisons test. Significance was
219 defined as a corrected p-value < 0.05. The reported n refers to the number of neurons
220 in each group within a particular experiment, except in imaging experiments where it

refers to the number of pre-synaptic terminals. In all cases at least 3 independent cultures were surveyed, each contributing equally to final N values.

Materials

D-APV, NBQX, TTX, were obtained from Tocris Biosciences. All materials without identified suppliers above were obtained from Sigma-Aldrich. 2-Deoxy-D-glucose (2DG, 10 mM), oligomycin (oligo; 1 μM), and alpha-cyano-4-hydroxycinnamic acid (4-CIN, 100 μM) are key pharmacological reagents used herein. 2DG and oligo are commonly used at these concentrations to inhibit glycolysis and oxidative phosphorylation, respectively (5-20 minutes; Schurr et al., 1999; Rangaraju et al., 2014; Pathak et al., 2015; Lujan et al., 2016). At concentrations below 250 μM, 4-CIN effectively blocks lactate transport into neurons with very few off-target effects (Izumi et al., 1997; Erlichman et al., 2008; Choi et al., 2012; Tang et al., 2014), although at higher concentrations may have off-target effects (Chih et al., 2001). In our studies, an effect of 4-CIN was observed only when 4-CIN was combined with another metabolic manipulation (2DG). This strongly suggests that 4-CIN at the concentration employed acted through the intended mechanism.

Results

Acute inhibition of both glycolysis and oxidative phosphorylation is required to interfere with evoked synaptic transmission

We initially tested the effect of acute inhibition of glycolysis and/or oxidative phosphorylation on action potential-evoked synaptic transmission. Incubations in metabolic poisons (2DG to inhibit glycolysis, oligo to inhibit oxidative phosphorylation, or the combination) were performed 15 minutes prior to establishing whole-cell, patch-clamp recordings from autaptic hippocampal neurons (Figure 1A). The whole-cell

recording configuration allowed direct control of postsynaptic ion gradients, so presynaptic variables were effectively isolated by metabolic manipulations (see Methods). Evoked postsynaptic currents (PSCs) were recorded immediately after break-in and were typically measured for ~5 minutes at a 0.04 Hz stimulation frequency. Inhibiting either glycolysis with 2DG (Figure 1B), or oxidative phosphorylation with an ATP synthase inhibitor, oligo (Figure 1C), had no significant effect on the ability to evoke PSCs in hippocampal neurons (Figure 1E). We observed no difference in effects on glutamate- (N = 10, 7 cells, 2DG and oligo respectively) and GABA-mediated (N = 6, 10 cells, respectively) PSCs, so results were pooled. Average evoked PSC amplitude was variable but did not differ among control (6.0 ± 0.7 nA, N = 19), oligo (4.3 ± 1.3 nA, N = 20), and 2DG conditions (6.8 ± 3.6 nA, N = 17). However, combined oligo and 2DG (2DG+oligo) treatment significantly suppressed PSC amplitude (0.15 ± 0.02 nA, N = 16), in most cases completely suppressing evoked PSCs (Figure 1D, E). PSCs were not altered further during whole-cell recordings (5 min duration) in metabolic inhibitors; there was no time-dependent decrement in PSCs with continued treatment with 2DG or oligo alone (Figure 1F), and PSCs did not emerge during recording in the continued combined presence of 2DG+oligo.

Interestingly, inhibition of oxidative phosphorylation alone or in the presence of 2DG was initially associated with suppressed axosomatic voltage-gated sodium currents (Figure 1G,H), which drive presynaptic action potentials (Bekkers and Stevens, 1991; Prakriya and Mennerick, 2000). This is in contrast to recent results at the calyx of Held, where axonal action potentials were particularly sensitive to glycolytic inhibition (Lujan et al., 2016). In our experiments the sodium currents in both oligo treatment groups grew over time and were not significantly different from the glucose control group by 5 min recording time, despite the lack of ATP in the pipette solution (Figure 1H). We posit that the growth of sodium currents likely reflects the re-establishment of K^+ and Na^+ gradients

during cell dialysis with the patch pipette solution. We conclude that oxidative phosphorylation is most important for maintaining these ion gradients and that glycolysis alone is not sufficient.

To further characterize the effect of oligo on excitability, we examined somatic action potentials in current-clamp recordings, using just-suprathreshold current injection titrated for each cell. In this independent sample of neurons, both maximum rise rate and peak amplitude of action potentials were depressed following 15 min of oligo incubation compared with sibling control cells (Figure 1I). Despite initial suppression of sodium currents in both oligo-treated groups (Figure 1H) and the effects of oligo on action potential waveform (Figure 1I), transmission failure occurred only in neurons where both glycolysis and oxidative phosphorylation were inhibited (Figure 1E). PSCs in this condition did not recover despite the recovery of sodium current (Figure 1H). These observations indirectly suggest that the disruption of sodium current alone is unlikely to account for the depressed transmission when both glycolysis and oxidative phosphorylation are inhibited.

Inhibition of glycolysis and oxidative phosphorylation abolishes evoked vesicle release.

To test more directly whether action potential propagation deficits solely underlie suppressed evoked PSCs, we challenged 2DG+oligo-treated cells with 100 mM K^+ (substituted for Na^+) in the presence of tetrodotoxin (TTX; 0.5-1 μM) immediately following the 15 minute incubation in saline with metabolic inhibitors or glucose control. This evoked vesicle exocytosis through directly depolariziation of terminals in the absence of action potential firing (Prakriya and Mennerick, 2000). Direct depolarization of unclamped presynaptic terminals elicited antagonist-sensitive PSCs in control neurons (Figure 2A) but not in 2DG+oligo-treated cells (Figure 2B). Metabolically

poisoned neurons lost both the antagonist-sensitive peak (synchronous) release and sustained release (Figure 2C,D).

Both action potentials and hyperkalemia require Ca^{2+} influx for exocytosis. Depression of presynaptic Ca^{2+} influx following ATP loss could explain PSC loss. To by-pass Ca^{2+} influx we utilized hypertonic sucrose, a Ca^{2+} independent secretagogue (Rosenmund and Stevens, 1996). Sucrose (0.5 M) elicited antagonist-sensitive PSCs from control neurons (Figure 2E, F, black trace) but not from 2DG+oligo-treated cells (Figure 2E,F, blue trace). The loss of evoked release when action potentials and Ca^{2+} influx are circumvented strongly suggests that neither action potential propagation nor Ca^{2+} influx is the primary dysfunction underlying the loss of evoked PSCs after combined inhibition glycolysis and oxidative phosphorylation. This outcome suggests that releasable synaptic vesicles may be unavailable following metabolic inhibition.

Metabolic poisoning fosters massive spontaneous vesicle release

Although K^+- and sucrose-evoked vesicle release was completely abolished, we were surprised that spontaneous miniature PSCs (mPSC) were still present in 2DG+oligo-treated cells (Figure 3B,C) and were comparable to mPSCs in controls (Figure 3A). We found no significant difference in the mean frequency (Figure 3D), amplitude (Figure 3E), 10-90% rise time (Figure 3F), or decay time (Figure 3G) between control and 2DG+oligo-treated excitatory mPSCs (mEPSCs) and inhibitory mPSCs (mIPSCs). These data suggest the presence of synaptic vesicles in the terminals, at least initially, despite the inability to evoke release. On closer inspection of mPSCs, we realized that metabolically compromised cells exhibited significantly greater variance in mPSC frequency than control cells (Figure 3D; $F = 6.052$, $p < 0.0002$). We reasoned that loss of ATP production may initiate vesicle release, and the large variance in release frequency may correspond to different stages of metabolic poisoning in different

neurons.

We hypothesized that loss of presynaptic ATP induces massive spontaneous vesicle exocytosis, which may contribute to the loss of evoked vesicle release. Thus, neurons may experience increased mPSC frequency throughout incubation in the metabolic poisons as presynaptic ATP levels are diminished; after 15 min incubation in 2DG+oligo neurons with a high mPSC frequency are still exocytosing, while cells with low mPSC frequency have exhausted the ability to exocytose. Any exocytosis would be independent of action potentials, as TTX was present during incubation of 2DG+oligo.

We directly tested whether loss of ATP affects vesicle fusion by recording neurons throughout the 15 min incubation period in 2DG+oligo (Figure 3H-M) instead of following the incubation period (Figure 3A-G). The protocol allowed us to observe changes in mPSC frequency and evoked transmission within individual cells during metabolic inhibition. We first evoked release in the presence of TTX with K^+ application as in Figure 2A-D (Figure 3H,I black traces) and then measured mPSC frequency in the presence of either glucose control or metabolic inhibitors over the course of 15 min. Following 15 min of glucose (control) or 2DG+oligo treatment, we re-assessed evoked PSCs. PSCs were mildly depressed in control cells, but were abolished in the 2DG+oligo treatment (Figure 3H-J). In the same cells, mPSC frequency was initially high following the initial K^+ challenge, reflecting residual presynaptic Ca^{2+} (Figure 3K, L). In control neurons, mPSC frequency immediately subsided, but in 2DG+oligo cells, the mPSC frequency remained elevated before decreasing to near-control levels by 15 min (Figure 3K-M).

In a separate experiment mPSC frequency was quantified over the 15 min incubation in either 2DG+oligo or glucose controls without the accompanying K^+-evoked PSC challenge. We once again observed a significant increase in mPSC frequency in the presence of 2DG+oligo compared to glucose controls (Figure 3N-P). Thus, the large

initial mPSC frequency elicited by K^+ in Figure 3H-M was not necessary to initiate the sustained elevation of mPSC frequency in metabolically poisoned cells. These observations suggest that tandem inhibition of glycolysis and oxidative phosphorylation produces massive synaptic exocytosis. Although some degree of spontaneous release still persists following 15 min of poisoning, evoked release is nearly abolished.

Endocytic failure after metabolic poisoning

Because vesicles are recycled, mass exocytosis alone would not account for the loss of evoked vesicle release following incubation in 2DG+oligo. Recent work has shown that ATP levels also influence vesicle endocytosis, with glycolysis playing a particularly important role (Rangaraju et al., 2014; Pathak et al., 2015; Jang et al., 2016). To test whether endocytosis was arrested in metabolically poisoned neurons, we measured vesicle cycling with FM1-43FX to label vesicle internalization at presynaptic terminals, identified with synapsin I (Figure 4A). Cultures were incubated for 20 minutes in the indicated conditions. Following incubation, vesicle cycling was probed by adding FM1-43FX and 45 mM K^+ for 2 min. Neither 2DG (Figure 4C,G burgundy bar) nor oligo (Figure 4,D,G teal bar) altered vesicle cycling, but co-administration of 2DG+oligo significantly reduced vesicle cycling during the probe period (Figure 4E,G blue bar).

To confirm that diminished cycling resulted from metabolic poisoning and not from prolonged vesicle exocytosis, we simulated the extended exocytosis observed in 2DG+oligo-treated cells using 45 mM K^+ (substituted for Na^+) present for 20 min with TTX but without any metabolic inhibition. Exposure to 45 mM K^+ produced a similar steady-state mPSC frequency compared with 2DG+oligo treatment (25.74 ± 11.72 Hz, n = 5 K^+-challenged cells), but vesicle cycling during a subsequent 2 min probe in FM1-43FX was not significantly affected (Figure 4B,G orange bar). Finally, we measured FM1-43FX labeling in a control group lacking Ca^{2+} in the probe condition to diminish

vesicle cycling during FM1-43FX loading. Cycling was significantly reduced (Figure 4F,G gray bar), comparable to when neurons were metabolically poisoned. This control condition also confirms the synaptic nature of labeling.

Experiments in Figure 4 confirm that vesicle cycling, rather than vesicle filling or other potential explanations, is associated with the loss of evoked transmission during combined 2DG+oligo treatment. However, the experiments do not specifically determine whether arrested endocytosis contributes. To test this, we incubated cells in FM1-43FX throughout the entire 20 min period of poisoning, during which massive exocytosis occurs (Figure 3M). As a control, we again depolarized cells in the presence of TTX to produce a comparably stimulated rate of exocytosis, but without metabolic inhibition. The net FM1-43FX labeling during the 20 min incubation in 2DG+oligo (Figure 5A-B, blue bar) was no different than control (Figure 5A-B, black bar), while K^+ stimulation produced the FM1-43FX labeling expected of ongoing vesicle cycling (Figure 5A-B, orange bar). The lack of staining in the unstimulated control (Figure 5A, left panel; Figure 5B, black bar) presumably reflects the low rate of mPSCs per synapse in the 15 min incubation. Overall, the results demonstrate that the massive exocytosis during metabolic inhibition is not followed by compensatory endocytosis. Thus, arrested endocytosis likely explains the loss of evoked vesicle release. The results demonstrate an important role for continuous ATP production, flexibly driven by either glycolysis or oxidative phosphorylation, in synaptic vesicle cycling.

Oxidative phosphorylation powers recovery following intense presynaptic transmission.

Although either glycolysis or oxidative phosphorylation proved capable of sustaining basal transmission (Figure 1), we wondered if a more energetically demanding stimulus would reveal a privileged role for presynaptic glycolysis or oxidative

403 phosphorylation. We examined the recovery of excitatory PSCs (EPSCs) after a brief,
404 strong depolarization designed to empty all recycling vesicles (Sara et al., 2002; Chung
405 et al., 2010). Glutamate synaptic signaling and uptake may be more energetically
406 demanding than GABA signaling (Waldvogel et al., 2000; Chatton et al., 2003), so we
407 focused on EPSCs. As schematized in Figure 6A, we measured baseline evoked
408 EPSCs, applied 90mM K^+ for 30s to deplete the vesicle pool, and then measured the
409 recovery of EPSCs with action potential stimulation every 25 s for ~2 min.
410 2DG incubation did not significantly affect EPSC recovery after vesicle depletion
411 compared to glucose-incubated controls (Figure 6B,D). However, oligo alone
412 significantly diminished recovery (Figure 6C,E). Slower recovery did not result from
413 action potential deficits because sodium action currents remained at pre-challenge levels
414 for both oligo treated cells and control cells (Figure 6G). These data suggest that
415 glycolysis alone cannot adequately sustain presynaptic recovery following a high-
416 demand stimulus. Rather, oxidative phosphorylation, perhaps because of its larger ATP
417 yield, has a privileged role in fueling recovery of presynaptic function after vesicle
418 depletion. Further, the lack of a 2DG effect implies that on-demand glycolysis is not
419 necessary to supply pyruvate for oxidative phosphorylation during the challenge. This
420 suggests that neurons power presynaptic function with reserve or alternative substrates
421 for oxidative phosphorylation when glycolysis is inhibited.
422
423 **Endogenous monocarboxylate powers oxidative phosphorylation when glycolysis**
424 **is inhibited**
425 Attractive candidates for alternative metabolic substrates during glycolytic
426 inhibition include monocarboxylates, such as lactate (Schurr et al., 1988; Izumi et al.,
427 1997; Ivanov et al., 2011; Wyss et al., 2011). Extracellular recording solutions do not
428 contain exogenous monocarboxylates, so based on prevailing views lactate would be

shuttled on demand from astrocytes to neurons during increased glutamate signaling (e.g., following vesicle depletion) to supply the TCA cycle with pyruvate (Pellerin and Magistretti, 1994, 2012). Microcultures provide a unique opportunity to test local shuttling, and we tested the local shuttle hypothesis by culturing neurons in the absence of contact with an astrocyte bed (Sobieski et al., 2015). We previously showed that neurons without astrocyte contact exhibit altered synchrony of evoked glutamate release (-astrocyte; Figure 7B) compared to astrocyte-supported controls (+astrocyte; Figure 7A), but many other facets of evoked transmission are intact (Sobieski et al., 2015). Recovery of EPSCs following the vesicle depletion challenge was slightly depressed in -astrocyte neurons compared with +astrocyte neurons when recorded in control medium (Figure 7C), but this effect was not statistically significant. Furthermore, incubation/recording in saline with lactate (1.5 mM) in place of glucose did not increase EPSC recovery after vesicle depletion (Figure 7D). Therefore, any deficit in the recovery of -astrocyte EPSCs cannot be accounted for by the loss of astrocyte-derived lactate.

Although these observations exclude a strong role for local, on-demand monocarboxylate shuttling as envisioned by the astrocyte-neuron lactate shuttle hypothesis (Pellerin and Magistretti, 1994, 2012), it is possible that bulk lactate levels derived from the summed contributions of surrounding astrocytes participates in fueling recovery from vesicle depletion. We tested the importance of global lactate efflux by pharmacologically inhibiting monocarboxylate transport with the monocarboxylate transport inhibitor 4-CIN, 100 μM for 15 min. 4-CIN had no effect on EPSC recovery after total vesicle depletion (Figure 8A,C). These results suggest that either neuronal glycolysis or perhaps the breakdown of alternative fuel reserves can support synaptic transmission when lactate shuttling alone is inhibited.

Reducing either neuronal glycolysis (Figure 6D) or monocarboxylate transport (Figure 8C) to diminish supply of substrates to the TCA cycle yielded little effect on

455 EPSC recovery after vesicle depletion. These data suggest two sources of fuel for
456 oxidative phosphorylation, either of which can support EPSC recovery following
457 depletion. We next examined the combined effects of inhibition of monocarboxylate
458 transport and glycolysis. Co-administration of 2DG and 4-CIN significantly reduced
459 EPSC recovery after vesicle depletion (Figure 8B,D). Thus, although inhibition of neither
460 monocarboxylate transport (Figure 8A,C) nor neuronal glycolysis alone (Figure 6B,D)
461 altered recovery, combined inhibition of both partially arrested EPSC recovery,
462 equivalent to the effect of oligo (Figure 6C,E). These data suggest that oxidative
463 phosphorylation continuously supplies ATP necessary for synaptic vesicle recovery after
464 depletion, and substrate for oxidative phosphorylation can originate from either
465 extracellular monocarboxylates or from neuronal glycolysis.
466

467 **Discussion**

468 In this study, we investigated the sources of ATP synthesis that drive basal
469 presynaptic function and recovery of function following high energetic demand. Our work
470 shows that ongoing ATP production, either through glycolysis or oxidative
471 phosphorylation, sustains evoked synaptic transmission for up to 20 min. Only the
472 combined inhibition of glycolysis and oxidative phosphorylation abolished PSCs evoked
473 at low frequency. The loss of evoked transmission likely results from massive
474 spontaneous vesicle release and subsequent failure to endocytose vesicles. Our data
475 also suggest that oxidative phosphorylation has a privileged role in powering the rapid
476 recovery of EPSCs after vesicle depletion. Neuronal presynaptic oxidative
477 phosphorylation appears to be flexible in use of energetic substrates, as either neuronal
478 glycolysis or astrocyte-derived monocarboxylates sufficed to fuel recovery. Overall, our
479 work elucidates the sources of presynaptic ATP during basal and high-demand activity.

Our work adds mechanistic details to previous studies that have highlighted the energetic demand of neurons in brain slices. Excitatory postsynaptic potentials in hippocampal slices are very sensitive to glucose deprivation (Schurr et al., 1988; Izumi et al., 1994, 1997). However, the roles of presynaptic and postsynaptic factors and of glycolysis versus oxidative phosphorylation have been unclear. In slices, energetic depression leads to accumulation of neurotransmitters and other secondary changes. It can be difficult to know whether changes to transmission are a direct consequence of changes to ATP levels or to these secondary consequences. Further, the trauma of tissue preparation can cause important metabolic changes that affect results (Takano et al., 2014). Although cultures have their own limitations, they allow experimental control not possible *in situ*, including our isolation of local versus global astrocyte contributions.

Several of our observations appear to conflict with other recent studies. Figure 1H suggests that ion gradients are rapidly compromised by inhibition of ATP produced by oxidative phosphorylation. Other recent work found that the Na^+/K^+ ATPase is a relatively light burden on presynaptic ATP levels (Rangaraju et al., 2014). One reconciliation could be that ATP demands are compartmentalized, with more ATPase burden near the soma, where our whole-cell recordings of sodium currents and action potentials were made. However, another complication is that at the calyx of Held synapse, presynaptic axonal action potential waveform is quite sensitive to glycolytic inhibition due to collapse of ion gradients (Lujan et al., 2016). There are two surprising things about these results compared with ours. First, we found that inhibition of oxidative phosphorylation alone, rather than glycolysis alone, produced changes to sodium current and action potential waveform. Second, because hippocampal glutamate release is quite sensitive to changes in action potential waveform (Prakriya and Mennerick, 2000; Meeks and Mennerick, 2004; Sobieski et al., 2015; Lujan et al., 2016), if the somatic action potential waveform changes we observed following oligo treatment alone were

propagated to terminals, we would have expected strong alteration of transmitter release. Instead, we found that the changes to excitability produced by oligo alone were not associated with deficits in evoked PSCs.

Our results also differ from others with regard to the role of glycolysis on endocytosis. Previous work suggests that endocytosis relies heavily on glycolysis (Rangaraju et al., 2014; Pathak et al., 2015). In contrast, we observed that vesicle endocytosis, measured by FM1-43FX fluorescence (Figure 4C, G burgundy bar), and PSCs recovered well following sole inhibition of glycolysis (Figure 6D). The reasons for the discrepancies are unclear, but could relate to use of different methods to measure presynaptic activity.

Our observations uncovered massive vesicle exocytosis during combined glycolytic and oxidative phosphorylation inhibition. Similar effects have been observed following oxygen-glucose deprivation (Allen et al., 2004; Fujimoto et al., 2004) or pharmacological manipulation of glycolysis and oxidative phosphorylation (Lujan et al., 2016). Endocytic failure following the mass vesicle exocytosis appears sufficient to explain the loss of evoked PSCs (Figure 5); however, we cannot exclude other contributing mechanisms, such as ATP-dependent vesicle filling or vesicle priming, to the loss of evoked PSCs. The precise cause of the massive exocytosis remains unclear. Collapse of ion gradients and consequent depolarization-dependent calcium influx or mitochondrial efflux could participate (Lujan et al., 2016). Other Ca^{2+}-independent possibilities include electrostatic effects of ATP on synaptogatmin-1 function and ATP dependent SNARE complex disassembly (Park et al., 2012).

The persistence of spontaneous mPSCs following metabolic inhibition could suggest differential sensitivity of vesicle pools underlying spontaneous and evoked neurotransmission (reviewed in Crawford and Kavalali, 2015). It is tempting to speculate that the different molecular identities of the two pools afford them different energetic

sensitivity, or that ATP depletion shifts the evoked pool of vesicles into a spontaneous-only mode of release. Alternatively, the residual mPSCs during the period of abolished evoked transmission could simply reflect a few remaining functional terminals that were not readily detected in our measures of evoked release. In the future it will be important to explore the differential neuroenergetics of spontaneous and evoked release in more detail.

An important conclusion from our work is that oxidative phosphorylation plays a more prominent role than glycolysis in fueling recovery of transmission following brief, intense metabolic challenge. Vesicle endocytosis, as probed by FM1-43FX uptake (Figure 4D, G teal bar), was unperturbed by pre-treatment with oligo. This suggests that the depression of recovery of evoked EPSCs following depolarizing challenge (Figure 6C, E) likely results from a mechanism other than endocytic arrest, such as a deficit in replenishment of vesicles from the reserve pool or vesicle filling.

The importance of oxidative phosphorylation for EPSC recovery after a strong presynaptic stimulus could implicate the astrocyte-neuron lactate shuttle (Pellerin and Magistretti, 1994; Takahashi et al., 1995; Bittner et al., 2010). However, neither pharmacological inhibition of monocarboxylate transport (Figure 8A,C) nor removal of local astrocyte support (Figure 7A-C) altered EPSC recovery. Thus, local, on-demand lactate shuttling from astrocytes in response to increased activity does not account for presynaptic recovery. In fact, EPSC recovery after vesicle depletion was only reduced by obstructing both glycolysis and monocarboxylate transport to neurons (Figure 8B,D), effectively cutting off two main fuel supplies for oxidative phosphorylation. These findings suggest that oxidative phosphorylation fueling presynaptic recovery can be effectively sourced by either neuronal glycolysis or by ambient, global monocarboxylates.

Interestingly, basal synaptic transmission was not ablated with co-incubation of 2DG and 4-CIN as it was with 2DG and oligo (Figure 1D,E). The inability of combined

2DG/4-CIN to depress basal evoked synaptic transmission most likely results from incomplete inhibition of monocarboxylate transport by 4-CIN. Alternatively, additional substrates for oxidative phosphorylation could maintain basal function. One alternative is glutamate, which can be converted to alpha-ketoglutarate, an important TCA intermediate and potential source of pyruvate (McKenna, 2007). Ketone bodies represent another alternative fuel and direct source for acetyl-Co-A (Izumi et al., 1998). Typically ketone bodies are derived peripherally, with questionable relevance to dissociated cultures, but they also may be produced by astrocytes (Takahashi et al., 2014). In future work, it would be intriguing to query to roles of these fuel sources in fueling synaptic transmission and presynaptic function.

The relative importance of oxidative phosphorylation over glycolysis is also interesting because of the presynaptic compartmentalization of glycolysis and oxidative phosphorylation within the neuron. Early and more recent work have found that glycolytic machinery in the presynaptic cytosol maintains K^+ homeostasis by fueling Na^+/K^+-ATPase activity (Knull, 1978; Lipton and Whittingham, 1982; Lujan et al., 2016). Mitochondria, on the other hand, reside in only 25-40% of presynaptic terminals (Chavan et al., 2015; Pathak et al., 2015). Despite the sporadic localization of mitochondria to presynaptic terminals, our work as well as others demonstrate that oxidative phosphorylation is important for presynaptic function (Verstreken et al., 2005; Kang et al., 2008; Hall et al., 2012; Sun et al., 2013; Pathak et al., 2015). Thus, sparse mitochondria apparently produce sufficient ATP to supply neighboring boutons (Pathak et al., 2015).

In conclusion, synaptic transmission is surprisingly resilient to changes in metabolic substrate availability. Either glycolysis or oxidative phosphorylation suffices to fuel basal synaptic transmission, but loss of both leads to a massive release of synaptic vesicles, diminished vesicle endocytosis, and evoked PSC loss. Further metabolic

584 challenges, such as strong depolarization, reveal a preferential role of oxidative
585 phosphorylation in producing the ATP required for presynaptic recovery. In turn oxidative
586 phosphorylation is fueled by either neuronal glycolysis or by transport of ambient
587 monocarboxylates, likely from astrocytes. Impeding both fuel supplies weakens EPSC
588 recovery. Taken together, our data showcase the flexibility of neurons to changes in
589 metabolic substrate availability in order to maintain presynaptic function.
590

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743

744 **Figure Legends.**
745

746 **Figure 1.** Acute inhibition of glycolysis and oxidative phosphorylation impairs evoked
747 vesicle release. **A-D**. Representative evoked autaptic EPSCs elicited in each of the
748 indicated conditions, after 15 min pre-incubation in the conditions prior to establishing
749 the whole-cell recording. '2DG' designates substitution of 2DG (10 mM) for glucose.
750 Oligo was applied at 1 μM. The dashed boxes indicate presynaptic stimulation currents,
751 including an inward sodium current highlighted in panel G. **E.** Neurons exhibiting PSCs
752 or no detectable PSC, from the four conditions, color-coded according to the text labels
753 in A-D (n = 16-20 neurons per condition). 2DG+oligo exhibited significantly more
754 neurons lacking evoked PSCs than control glucose (chi-square, $X^2 = 30.35$, $p < 0.0001$).
755 **F.** Summary of evoked PSCs obtained following 5 min stimulation (0.04 Hz), normalized
756 to initial currents obtained on membrane break-in. There was no significant difference in
757 PSC peak size of glucose, 2DG, or oligo alone after 5 min of recording compared to
758 immediate break-in ($p > 0.05$ for all conditions; two-way repeated measures ANOVA with
759 Bonferroni corrections performed on raw data). No PSCs were evident at time 0 or 5 min
760 after break-in in the 2DG+oligo condition, hence the lack of bar. **G.** Representative
761 sodium currents measured immediately on break-in in the four experimental conditions
762 taken from the time period indicated by the dashed boxes in A-D. Colors correspond to
763 the text labels in A-D. **H.** Summary of sodium current obtained immediately after break-in
764 and after 5 min of stimulation, normalized to 0 min glucose sodium current. At 0 min, the
765 oligo and 2DG+oligo conditions were significantly smaller than glucose controls ($p <$
766 0.01, 0.0001 for oligo and 2DG+oligo, respectively; two-way repeated measures ANOVA
767 with Bonferroni corrections performed on raw data) At 5 min there was no significant
768 difference between glucose and the three remaining conditions ($p > 0.05$ for all
769 conditions, two-way repeated measures ANOVA with Bonferroni corrections performed

on raw data). Data are represented as mean ± SEM. **p ≤ 0.01. ***p ≤ 0.001. n.s, non-significant. **I.** Action potential waveform changes resulting from oligo-only treatment. Current-clamp recordings were established after incubations as above, and a family of depolarizing currents (5-10 pA increment, 1200 ms) was injected to elicit a just-suprathreshold action potential. Peak and maximum rate of rise were measured in 27 control neurons and 25 oligo-treated neurons. The inset shows an exemplar action potential from one cell in each condition superimposed.

Figure 2. Presynaptic vesicle release is also impaired when action potentials and calcium influx are by-passed. **A,B.** Representative autaptic currents evoked by 100 mM K^+ for 3 s, as indicated by the horizontal bar. Red traces indicate the same treatment, but in the presence of a cocktail of postsynaptic receptor blockers (1 μM NBQX, 50 μM bicuculline), revealing non-synaptic currents elicited by K^+. **C,D.** Summary of antagonist-sensitive peak PSC amplitude and total charge transfer for the two conditions (n=12,12; p < 0.001, 0.01, respectively, unpaired, two-tailed Student's *t*-test with Bonferroni corrections) measured from the antagonist-subtracted currents. **E.** Representative traces of hypertonic sucrose (0.5 M) used to elicit exocytosis of releasable vesicles from neurons in the glucose control (black trace) and 2DG+oligo-treated (blue trace) neurons. **F.** Summary of sucrose-elicited charge transfer from glucose-treated (n=5 neurons) and 2DG+oligo-treated (n=5 neurons) cells (p < 0.05, unpaired, two-tailed Student's t test). Individual data points represent single neurons, bars represent mean ± SEM. *p ≤ 0.05. **p ≤ 0.01. ***p ≤ 0.001.

Figure 3. Metabolically compromised neurons exhibit high variance in mPSC frequency and display gradual, massive exocytosis. **A-C.** Representative traces of the indicated experimental conditions, drug concentrations as in Figures 1 and 2. Examples B and C

represent different cells in the same condition with different spontaneous mPSC release frequency. **D.** Summary of pooled mPSC frequency in the two conditions, revealing the higher variance in mPSC frequency in 2DG+oligo (n=21, 22 neurons in glucose and 2DG+oligo conditions, respectively; p=0.0002, *F*-test). The difference between conditions was not significantly different ($p > 0.05$, unpaired, two-tailed Student's t-test with Bonferroni corrections). **E-G.** Summary of mPSC parameters, separated by mEPSCs (n=9, 7 neurons in glucose and 2DG+oligo conditions, respectively) and mIPSCs (n=8, 8 neurons in glucose and 2DG+oligo conditions, respectively). No significant differences were found on any of the parameters between glucose and 2DG+oligo ($p > 0.05$, unpaired, two-tailed Student's t-test with Bonferroni corrections). **H, I.** Representative traces documenting the effect of control (glucose) or 2DG+oligo application on evoked and spontaneous release during recordings from a neuron on a multi-neuron island. During recordings in TTX, K^+ was applied as in Figure 2 to elicit transmitter release from surrounding terminals. Shown are antagonist-subtracted currents at baseline (black) and following 15 min of control or 2DG+oligo application. **J.** Summary of the change in total antagonist-subtracted (receptor-generated) charge following 15 min incubation. Some depression in control K^+-evoked PSCs (n = 7) was noted. However, 2DG+oligo (n = 6) caused a complete loss of evoked PSC. A repeated measures ANOVA revealed a significant interaction between treatment condition and time (*$p<0.01$). **K, L.** mPSC recordings from the neurons shown in H and I. The traces document mPSCs at three 30-s epochs following the baseline K^+ application, as labeled. Initially (time =0), mPSC frequency is high in both cells, a residual effect of K^+ application. **M.** Summary of mPSC frequency change from 30-s epochs during incubation in 2DG+oligo. Same 13 cells represented in panel J. 2DG+oligo elicited a significantly more sustained mPSC frequency than control (*$p < 0.005$ main effect of treatment and interaction between time and treatment). **N-P.** The increased mPSC

822 frequency increase was not the result of interaction with K^+ stimulation. **N,O.** Sample
823 records from control and poisoned neurons, without preceding K^+ stimulation. **P.**
824 Summary data from multiple cells at early and late time points. A two-way, repeated-
825 measures ANOVA revealed a main effect of poison and a drug by time interaction
826 ($p<0.05$). Asterisks indicate Bonferroni corrected post-hoc testing, ****$p \leq 0.0001$. n.s,
827 not significant. Individual data points represent neurons, and bars represent mean ±
828 SEM.
829
830
831 **Figure 4.** Synaptic vesicle cycling is impaired following combined inhibition of glycolysis
832 and oxidative phosphorylation. **A.** Representative images of presynaptic terminals
833 immunopositive for synapsin I (left, magenta) after induced vesicle cycling in the
834 presence of FM1-43FX (center, green), and merged (right, white) showing the
835 internalization of FM1-43FX during vesicle endocytosis in the presence of glucose. **B–F.**
836 Example merged images after 20 min pre-incubation in solution containing 45 mM KCl
837 (B), 2DG (C), oligo (D), 2DG+oligo (E), or 0 mM Ca^{2+} (F). **G**. Summary of cycling (A; n
838 =150 presynaptic terminals for each condition from three replications). Scale bar, 10 μm.
839 *$p < 0.05$ for all the comparisons between each of the 45 mM KCl, 2DG, and oligo
840 conditions and either the 2DG+oligo or 0 mM Ca^{2+} condition (45 mM KCl vs. 2DG +
841 oligo, $p = 0.0127$; 2DG vs. 2DG+oligo, $p < 0.0001$; oligo vs. 2DG + oligo, $p = 0.0026$; 45
842 mM KCl vs. 0 mM Ca^{2+}, $p < 0.0001$; 2DG vs. 0 mM Ca^{2+}, $p < 0.0001$; oligo vs. 0 mM
843 Ca^{2+}, $p < 0.001$). The 2DG+oligo condition was not significantly different from the 0 mM
844 Ca^{2+} condition (NS, $p = 0.0746$, Kruskal-Wallis one-way ANOVA followed by Dunn's
845 multiple comparisons test). Data are represented as mean ± SEM. *$p \leq 0.05$. n.s, non-
846 significant.
847

848 **Figure 5.** Endocytosis is inhibited during metabolic arrest despite continued exocytosis.
849 FM1-43FX incubation occurred during 20 min glucose (control), sustained
850 depolarization, or 2DG+oligo. **A.** Representative images show merged fluorescence of
851 synapsin labeling (magenta) and FM1-43 uptake (green). Scale bar 10 μm. **B.** Summary
852 of 150 terminals from 3 independent experiments reveals that while continued cycling is
853 evident with sustained depolarization, FM1-43 uptake is lost during metabolic arrest,
854 despite strong, prolonged exocytosis evident in Figure 3 (***$p < 0.001$)
855
856 **Figure 6.** Oxidative phosphorylation plays a privileged role in recovery from intense
857 presynaptic activity. **A.** Schematic and representative data showing autaptic EPSCs
858 evoked by action potentials in normal glucose solution before (left) and after (right)
859 vesicle depletion with strong, sustained depolarization with 90 mM K^+ for 30 sec.
860 Stimulus action currents in this figure and subsequent figures are blanked for clarity. **B,**
861 **C.** Representative recovery traces from 2DG (B) and oligo alone (C) treatments,
862 showing poor recovery from vesicle depletion in the oligo alone condition. **D, E.**
863 Summary of recovery of action potential-evoked EPSCs following the depletion protocol
864 for 2DG compared to glucose control (D, $P > 0.05$, two-way ANOVA), and glucose saline
865 in the presence (+oligo) or absence (-oligo) of 1 μM oligomycin (E, $p < 0.05$ two-way
866 ANOVA). **F, G**. Summary of axosomatic sodium currents following the depletion protocol
867 for 2DG compared to glucose control (F, $p > 0.05$, two-way ANOVA), and glucose saline
868 in the presence or absence of oligomycin (G, $p > 0.05$, two-way ANOVA). Data are
869 represented as mean ± SEM. *$p \leq 0.05$. n.s, non-significant.
870
871 **Figure 7.** Local, on-demand monocarboxylate shuttling does not support the oxidative
872 phosphorylation required for recovery. **A, B.** Recovery of evoked EPSCs after 30 sec of
873 90 mM K^+ from microcultures containing (+astrocyte) or missing (-astrocyte) a local

874 astrocyte bed. Note that both +astrocyte and -astrocyte microcultures were obtained
875 from the same plates. **C.** Summary of recovery following vesicle depletion in +astrocyte
876 (black) and –astrocyte (red) microcultures ($p > 0.05$, two-way ANOVA). **D.** Summary of
877 recovery of sibling -astrocyte EPSCs incubated in control medium (light red) or in lactate
878 (dark red, 1.5 mM, 15 min; $p > 0.05$, two-way ANOVA). Data are represented as mean ±
879 SEM. n.s, non-significant.
880
881 **Figure 8.** Oxidative phosphorylation fueling presynaptic recovery is flexibly supplied by
882 neuronal glycolysis or by monocarboxylate transport. **A, B.** Representative traces of
883 EPSC recovery following monocarboxylate transport inhibition alone (A; 4-CIN, 100 μM)
884 or combined inhibition of glycolysis and monocarboxylate transport (B; 2DG+4-CIN) for
885 15 min. **C, D.** Summary of recovery after 30 sec of 90 mM K^+ following incubation in 4-
886 CIN alone (C, $p > 0.05$, two-way ANOVA) or combined 2DG+4-CIN (D, $p < 0.05$, two-
887 way ANOVA). Data are represented as mean ± SEM. *$p \leq 0.05$. n.s, non-significant