Energetic substrate availability regulates synchronous activity in an excitatory neural network

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Abstract

Neural networks are required to meet significant metabolic demands associated with performing sophisticated computational tasks in the brain. The necessity for efficient transmission of information imposes stringent constraints on the metabolic pathways that can be used for energy generation at the synapse, and thus low availability of energetic substrates can reduce the efficacy of synaptic function. Here we study the effects of energetic substrate availability on global neural network behavior and find that glucose alone can sustain excitatory neurotransmission required to generate high-frequency synchronous bursting that emerges in culture. In contrast, obligatory oxidative energetic substrates such as lactate and pyruvate are unable to substitute for glucose, indicating that processes involving glucose metabolism form the primary energy-generating pathways supporting coordinated network activity. Our experimental results are discussed in the context of the role that metabolism plays in supporting the performance of individual synapses, including the relative contributions from postsynaptic responses, astrocytes, and presynaptic vesicle cycling. We propose a simple computational model for our excitatory cultures that accurately captures the inability of metabolically compromised synapses to sustain synchronous bursting when extracellular glucose is depleted.

Introduction

Accurately processing, storing, and retrieving information comes at a considerable metabolic cost to the central nervous system (1). It is currently thought that the human brain is responsible for 20% of all energy consumed by the body, whilst comprising only 2% of the total body weight (2). The amount of energy expended on different components of excitatory

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53signaling in the brain has been estimated (3)(4), and mechanisms mediating synaptic
54transmission (including glutamate accumulation in vesicles) are predicted to monopolize 41%
55of all ATP turnover in the cortex (5). Theoretical considerations suggest that cortical
56networks therefore maximize the ratio of information transmitted to energy consumed (6).
57This finding could explain why the mean firing rate of neurons measured *in vivo* is much
58lower than that expected to maximize the brain's total coding capacity, i.e. where neurons fire
59at approximately half their maximum rates, a behavior that is only very rarely observed in
60practice (3)(7). Mathematical models of energy-efficient neurotransmission led to the
61surprising conclusion that synaptic vesicle release probability is low and synaptic failures
62should occur often (8)(5). However, even with these adaptations for energetic efficiency the
63metabolic demands of neural networks remain a large proportion of the body's total energy
64budget.
65
66To meet this energetic demand, the cortex has evolved an extensive neurovascular coupling
67that can increase blood flow to regions of high activity. Astrocytes and other glial cells in
68contact with blood vessels are important regulators of brain energy supply and play a key role
69providing neurons with a readily accessible fuel source (9)(10). The nature of this neuron-
70astrocyte relationship remains controversial however, with conflicting theories concerning the
71primary substrate of resting *versus* active neural metabolism as well as the relative fluxes
72through metabolic pathways in the two cell types (11)(12)(13). Hemodynamic signals based
73on blood-oxygen-level-dependent functional magnetic resonance-based imaging (BOLD
74fMRI) show that oxygen uptake during neural activity is disproportionally small compared to
75that required for complete oxidation of glucose (i.e. $6O_2$ per glucose consumed), suggesting
76that glycolysis is the major metabolic pathway of active cortex (14). This observation led to a
77proposal that neural activity induces aerobic glycolysis in astrocytes, which then produce

78lactate to serve as the main fuel source for neurons (15)(16). Whilst, as an energetic substrate,
79lactate can support some aspects of synaptic function (17)(18), the astrocyte-neuron lactate
80shuttle hypothesis challenges the long-standing consensus that glucose is the principal fuel
81source of neuronal metabolism (12). Other recent studies continue to support the idea that
82significant amounts of glucose feed directly into neuronal glycolysis however (e.g. (19)(20)
83(21)), and advancements in fluorescent imaging demonstrate that activity stimulates
84glycolysis, but not lactate uptake (22).

85

86Presynaptic nerve terminals are unusual in the sense that many lack mitochondria but are able
87to satisfy the sizable ATP-consumption requirements of synaptic vesicle recycling (23)(24)
88(25). ATP production must be able to increase rapidly in order to meet acute changes in
89presynaptic demands, and so it is perhaps not surprising that recent work has highlighted the
90importance of locally-derived glycolytic ATP generation and glucose transport in the
91synaptic vesicle cycle (26)(27). Neuronal activity has been associated with localization of key
92glycolytic enzymes to the presynaptic terminal (28), and several of these proteins are
93enriched in synaptic vesicles (29)(30) where they are found to be essential for synaptic
94vesicle re-acidification and glutamate uptake (31)(32). Vesicle recycling is a highly dynamic
95and energetically-demanding process (33)(34) for which ATP supply *via* glucose oxidation
96alone is presumably too slow (35)(36). Consequently, metabolic stress induced at individual
97excitatory presynapses by substrate depletion has been shown to reduce the number of
98functional release sites and depress rates of synaptic vesicle recovery (37)(38). It is not yet
99understood, however, whether these changes in central carbon metabolism directly affect
100global network behavior. In this study we set out to determine the consequences of energetic
101substrate depletion on a network model of human cortical neurons.

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103Our approach was to develop a combined experimental-computational model sufficiently
104detailed to be relevant to the problem at hand yet sufficiently simple to provide an intuitive
105picture of how metabolism governs important aspects of cortical network behavior. For these
106experiments we used induced cortical glutamatergic neurons (iNs) derived from human
107embryonic stem cells (hESCs) by overexpression of neurogenin 2 (NGN2) (39) from a
108genetically safe harbor in order to maximize induction efficiency and improve network
109homogeneity (40). We cultured human iNs together with rat astrocytes on multi-electrode
110arrays (MEAs); extensive characterization of their electrophysiology has been performed
111previously (39)(41) and suggests individual iNs constitute excitatory cortical layer 2/3
112neurons equipped with AMPA receptors. For computational conceptualization of our
113experimental results we extended a simplified version of the spiking neural network model
114used by Guerrier *et al.* (2015) to describe the emergence of synchronous bursting driven by
115synaptic dynamics (42). We found a reduced version of this model captured the same effects
116and incorporated metabolic regulation of synaptic vesicle recovery in order to interpret
117experimental data derived from cultured excitatory networks.

118

119**Results**

120

121***Emergence of synchronized bursts during excitatory network development***

122We cultured human iNs together with rat astrocytes on MEAs from day 3 of induction
123onwards. From day 12 we recorded 10min of electrical activity at three regularly-spaced
124intervals (10:00 AM GMT every Monday, Wednesday, and Friday) each week for a total of
125six weeks (Materials and Methods). To ensure the observed network behavior was
126representative we repeated this experiment on three separate occasions, each time using a

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127different hESC clone for induction and a new preparation of astrocytes for co-culture. The
128emergence of spontaneous bursting after 3-4 weeks from induction was a consistent feature
129of developing networks (Fig. 1). Bursts were easily identified upon visual inspection of
130recorded data and consisted of a characteristic, high-frequency spike train (Fig. 1A). Timings
131of bursts were synchronized across all participating electrodes whilst spontaneous action
132potentials occurring within and between bursts were not. A custom-built synchronous burst
133detection algorithm (SI Appendix) was used to analyze bursts from raw electrode data and
134showed that synchronous bursting spread and then stabilized across the network over the
135course of development. Network maturation was accompanied by a gradual increase in
136synchronous burst frequency (Fig. 1B), but precise frequencies varied significantly between
137cultures. Although we do not present an extensive analysis here, we also experienced that,
138during later stages of network maturation, synchronous bursts underwent different degrees of
139higher level organization, including the appearance of compound bursting (43) and burst
140compactification (44).

141

142**Figure 1. Synchronous bursting characteristics. A)** Synchronized bursts consisting of
143trains of action potentials are clearly visible in raw electrode data. Representative data from
144two neighboring electrodes showing temporal correlation of bursts occurring in control
145conditions (i). Synchronized bursts are obliterated (although spontaneous action potentials
146persist) by the presence of the AMPA/kainite receptor antagonist CNQX (ii), but this effect is
147immediately reversible following a wash-off (iii). Total inhibition of electrical activity upon
148treatment with TTX (iv). For illustrative purposes, upper panels displaying fast time scale are
149smoothed using a 2ms Gaussian window. **B)** Numbers of synchronous bursts per minute
150(SBPM) gradually increases and stabilizes over the course of network development (data
151from three independent 6-week-long experiments each using distinct hESC clones and

152astrocyte preparations). Two-way ANOVA reports time (days from induction) as the major

153source of variation, $P_{time} < 0.0001$; N=3. **C)** When mature, the same cultures were subject to

154CNQX treatment, revealing the dependence of synchronous bursting on excitatory

155glutamatergic signaling. Zero synchronous bursts were observed in the presence of CNQX.

156

157Several groups have similarly described the emergence of network-wide synchronous bursts

158in cultures of disassociated primary rat neurons (45)(46)(47) and human iNs (48). The

159frequency of synchronous bursting reported by Frega et al. (4.1 ± 0.1 burst/min) (48) lies

160within the SBPM range that we observe in mature cultures, suggesting that the two

161phenomena are closely related. The similar characteristics of synchronous bursting are

162perhaps expected given that iNs were used in those experiments also, but one cannot rule out

163possible differences caused by viral targeting or contributions from excitatory/inhibitory

164neuronal contamination in astrocyte preparations (49). To confirm that synchronous bursts

165are dependent on excitatory glutamatergic signaling we subjected each culture to treatment

166with 40μM cyanquixaline (CNQX) following the developmental time course. CNQX is a

167specific, competitive inhibitor of excitatory AMPA/kainite receptors (50). 10min incubation

168of cultures in the presence of this drug resulted in total inhibition of synchronous bursts

169without affecting spontaneous firing of action potentials (Fig. 1A, 1C and S1) (see also

170Supplementary Dataset 1). The effect was immediately reversible following a single wash

171with fresh media. Subsequent administration of 1μM tetrodotoxin (TTX), a potent voltage-

172gated sodium channel blocker, silenced the network entirely (Fig. 1A and S1) (see also

173Supplementary Dataset 1). Conversely, administration of 40μM bicuculline, a competitive

174antagonist of the primary inhibitory GABA receptor, had no detectible effect on synchronous

175bursts (not shown).

176

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177***Kinetics of vesicle re-acidification determine synchronous bursting frequency***

178Having confirmed that neuronal communication *via* release of the excitatory neurotransmitter
179glutamate is responsible for coordinating synchronous bursting, we were interested to know
180what aspects of neurotransmission determine synchronous bursting frequency. To evaluate
181the role of the synaptic vesicle cycle we sought to inhibit a process known to be important for
182vesicle maintenance and recovery following exocytosis. Various pathways and vesicle pools
183are suggested to participate in synaptic vesicle recycling (33)(34), and the currently accepted
184knowledge is concisely summarized by KEGG pathway entry hsa04721. Common to all
185pathways is vesicle re-acidification by the vacuolar-type ATPase (v-ATPase) required to
186generate the electrochemical proton gradient that is an essential prerequisite for the uptake of
187glutamate into synaptic vesicles (51)(52).

188

189We evaluated the response of cultures to acute pharmacological inhibition of the v-ATPase
190by rounds of 10min incubation in media treated with drug or vehicle, transfer to the MEA
191recording device for a 200sec equilibration, and a 10min recording of network activity
192(Materials and Methods). We found that 50nM concanamycin A (CMA), a highly specific
193inhibitor of the v-ATPase (53), significantly reduced the number of synchronous bursts per
194minute (SBPM) (Fig. 2, vehicle: 4.76 ± 1.76; CMA: 0.30 ± 0.15) suggesting that vesicular
195re-acidification is a key determinant of the frequency of network-wide synchronous bursts.
196Conversely, treatment with 80μM dynasore (54), an inhibitor of all the major dynamin
197isoforms required for vesicle endocytosis, had no significant effect after the first or second
19810min incubation (Fig. 2B, vehicle: 3.90 ± 1.20; dynasore 10min: 4.33 ± 1.11; dynasore
19920min: 4.00 ± 1.50).

200

201 **Figure 2. Inhibiting the vesicle recycling and maintenance pathways. A)** Incubating
202 cultures in the presence of 50nM CMA for 10min significantly reduces the frequency of
203 synchronous bursting compared to 10min incubation in the presence of vehicle alone. CMA
204 *versus* vehicle P = 0.032; N=3. **B)** 80µM dynasore had no detectible effect on SBPM after the
205 first, or second, 10min incubation. Dynasore 10min *versus* vehicle P = 0.4, dynasore 20min
206 *versus* vehicle P = 0.48; N=3.

207

208 ***Glucose depletion reduces synchronous burst rate***

209 To assess the consequences of energetic substrate restriction on synchronous bursting we
210 developed a timing-based protocol of substrate depletion and repletion (Fig. 3A and Materials
211 and Methods). On the basis of recent work, 20-30min in the absence of extracellular glucose
212 is sufficient to impair presynaptic transmission (55)(37)(38), which is far shorter than the 16h
213 time window during which the survival of cultured neurons remains uncompromised (56). As
214 with previous pharmacological experiments, we repeated multiple rounds of 10min
215 incubation, 200sec equilibration, followed by a 10min recording after which transfer of a
216 culture into fresh media was always performed regardless of whether or not it contained an
217 alternative mix of substrates (Fig. 3A and Materials and Methods). This was to rule out any
218 confounding effects of mechanical perturbation or media acidification/oxygenation. We
219 found that synchronous burst frequency (as measured by SBPM) decreased following the first
220 10min incubation in the absence of 25mM glucose and 0.22mM pyruvate and was
221 significantly reduced after the second 10min incubation, with far fewer synchronous bursts
222 occurring at regularly-spaced intervals across the subsequent 10min recording (Fig. 3B and
223 S2, glucose and pyruvate: 6.23 ± 2.00; no substrate 10min: 4.60 ± 1.01; no substrate 20min:
224 0.30 ± 0.12) (see also Supplementary Dataset 2). This phenomenon was significantly
225 reversed after the reintroduction of 25mM glucose alone (Fig. 3B and S2, glucose alone: 3.95

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226± 1.20) (see also Supplementary Dataset 2), indicating that a high SBPM can be sustained in
227absence of the oxidative substrate pyruvate.
228
229**Figure 3. Glucose depletion decreases SBPM. A)** Cartoon schematic of substrate depletion-
230repletion experimental protocol described in Materials and Methods. Each wash-incubate-
231equilibrate-record epoch was performed using fresh media regardless of substrate
232composition. **B)** Synchronous burst frequency decreases following 10min incubation in the
233absence of extracellular glucose (25mM) and pyruvate (0.22mM), and significantly further
234still following a second 10min incubation. Replenishment of glucose alone is sufficient to
235restore a significantly higher SBPM. No substrate 20min *versus* glucose and pyruvate P =
2360.013; no substrate 20min *versus* no substrate 10min P = 0.003; glucose alone *versus* no
237substrate 20min P = 0.012; N=4. **C)** Only the metabolically active D-isoform of glucose (not
238L-glucose) can sustain a significantly higher SBPM in physiologically-relevant conditions
239containing 5mM D- or L-glucose, 5mM DL-lactate (racemic mixture) and 0.22mM pyruvate.
240L-glucose 20min *versus* D-glucose control P < 0.001; L-glucose 20min *versus* L-glucose
24110min P = 0.027; D-glucose replenishment *versus* L-glucose 20min P = 0.014; N=4.
242
243In order to confirm our observations are not related to the artificially high levels of glucose
244(25mM) conventionally used in cell culture media, a consequence of osmotic stress possibly
245experienced upon exchange of the growth media, or complete absence of an alternative
246oxidative substrate altogether, we repeated the substrate depletion-repletion protocol under
247more physiologically-relevant conditions (57). We retained 0.22mM pyruvate and
248supplemented with 5mM DL-lactate (racemic mixture) at all time points, reducing the total
249concentration of extracellular glucose to 5mM. To control for the possible effect of osmotic
250stress during periods of glucose depletion we replaced D-glucose with its non-metabolically-

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251active isoform L-glucose. In accordance with previous experiments, we found that
252substituting 5mM D-glucose with 5mM L-glucose led to a slight decrease in synchronous
253burst frequency after 10min incubation followed by a significant drop in SBPM during the
254second 10min recording (Fig. 3C and S3, D-glucose: 7.02 ± 1.02; L-glucose 10min: 2.65 ±
2550.60; L-glucose 20min: 1.0 ± 0.35) (see also Supplementary Dataset 3). This effect was
256significantly reversed upon replacement of L-glucose with D-glucose (Fig. 3C and S3, D-
257glucose replenishment: 5.48 ± 1.51) (see also Supplementary Dataset 3), confirming that the
258metabolically active form of glucose alone can sustain a high synchronous bursting
259frequency. The continued presence of substrates that can only be used to generate ATP by
260oxidative phosphorylation implies that the glycolytic substrate glucose is required to sustain a
261high SBPM. Specificity for glucose was further supported by substituting 5mM glucose for
2625mM galactose, a glycolytic substrate whose transport and pre-processing means it passes
263through glycolysis more slowly, but yields the same ATP molar equivalent to glucose during
264oxidative phosphorylation (Chapter 16.1.11. in (58)), which also failed to rescue the higher
265synchronous bursting frequency (Fig. S4) (see also Supplementary Dataset 4).
266
267Finally, to conceptualize how these features lead to the emergence of synchronous bursting
268initiated by spontaneous action potential firing we have built upon the results of Guerrier *et*
269*al*. (42) to describe our excitatory network with a model based on random, sparse synaptic
270connections equipped with short-time synaptic plasticity (STSP), three synaptic vesicle pools,
271and a mechanism linking vesicle maintenance and recovery rates to energetic substrate
272availability. In our implementation of this computational model we explored the possibility of
273simplifying STSP dynamics further and found that the assumption of just a single vesicle
274pool (Fig. 4A) remains sufficient to recapitulate the synchronous bursting phenotype we

275observed experimentally during glucose depletion (Fig. 4B, Material and Methods and SI
276Appendix).
277
278**Figure 4. Computational model of synchronous bursting. A)** Cartoon schematic showing
279that presynaptic vesicle recycling involves several steps suggested to depend on ATP
280supplied by glycolysis, including endocytosis and vesicle re-acidification by the v-ATPase. In
281our computational model we encompassed contributions from all pathways in a single term
282describing the rate of synaptic vesicle recovery and maintenance. **B)** Simulations reveal that
283in high levels of extracellular glucose (left), synchronized bursting persists at a higher
284frequency than when extracellular glucose is low (right), as modelled by reducing the rate of
285vesicle recovery and maintenance. Upper panels display raster plots of spike timings from all
286400 neurons in the simulated network, middle panels the total spike count across the network
287as a function of time, and lower panels the corresponding fluctuations in membrane potential
288of a representative neuron.
289

290Discussion

291
292In this work we used cultured networks of excitatory human iNs and rat astrocytes as an
293experimental model system to study the influence of energetic substrate availability on global
294network behavior. Glucose depletion dramatically reduced the prevalence of network-wide
295synchronous bursts mediated by excitatory neurotransmission, an effect that could not be
296rescued by a purely respiratory substrate such as lactate or pyruvate. Our results build upon
297earlier work focused on the effects of energetic substrate availability on neuronal function,
298which revealed that glucose is essential for synaptic transmission even though intracellular
299ATP levels remain normal in the presence of oxidative fuel sources (59)(60)(61)(62).

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300

301Using CNQX to inhibit synaptic transmission we found that network-wide synchronous 302bursts are an emergent property of excitatory synaptic communication rather than 303synchronization of intrinsically bursting neurons. In a simple computational model, we 304demonstrated that this type of synchronized bursting is sensitive the rate of synaptic vesicle 305recycling and maintenance, which is in turn sensitive to energetic substrate availability. Our 306model revealed that a single vesicle pool was sufficient to recapitulate our experimental 307observations, which is particularly important given the lack of conclusive experimental 308evidence for the involvement of different pathways and vesicle pools during synaptic vesicle 309recycling (33)(34), about which we prefer to remain agnostic. An exception however, is our 310acceptance that synaptic vesicle re-acidification is a central component of all vesicle 311recycling and maintenance pathways, which explains the decrease in SBPM we observed 312following pharmacological inhibition of the v-ATPase. Our study therefore suggests that 313vesicle re-acidification plays an important role as a determinant of synchronous bursting 314frequency.

315

316Recent lines of evidence indicate that fully-functional presynaptic transmission is dependent 317on activity-induced glycolysis (31)(32)(26)(28) and this has led to a proposal that a rapid 318supply of ATP is required to power the synaptic vesicle cycle at nerve terminals (36). This 319claim is further supported by studies demonstrating a decrease in vesicle recovery rates and 320impaired vesicle maintenance within individual synapses in the absence of extracellular 321glucose (19)(37)(38), but we note other factors such as the inability of the presynaptic action 322potential to trigger vesicle release cannot be fully excluded (55). Our experimental results are 323consistent with the idea that efficacy of the synaptic vesicle cycle may diminish upon a drop 324in extracellular glucose concentration, and as a proof-of-concept we have accommodated this

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325in our computational description of excitatory cultures. Following the approach of Lucas *et*
326*al*. (38), we modified the time constant for vesicle recovery in the model to account for the
327fact that under low glucose conditions, when energy charge (ATP:ADP/AMP ratio) is more
328sensitive to increased ATP consumption rates, the proportion of functional vesicles available
329for release is reduced (31)(32)(26)(28)(27). As observed experimentally, when vesicle
330recovery and maintenance is compromised in response to a decrease in extracellular glucose
331concentration, there is a corresponding decrease in synchronous burst frequency (Fig. 4B). A
332possible explanation for why a reduction in glucose availability might impair the synaptic
333vesicle cycle is that v-ATPase activity is more reliant on glycolytically-derived ATP (31)
334(32). However, other stages of vesicle recycling are likewise energetically demanding
335processes that depend on both oxidative (63) and non-oxidative supply of ATP (26)(28).
336Therefore, although it has been argued that availability of vesicles does not become rate-
337limiting during ATP depletion (64)(65), and despite the fact that in our hands the inhibitor
338dynasore had no effect on synchronous bursting frequency, the impairment of endocytosis
339upon glucose restriction, particularly involving rapid "kiss-and-run" (34) or dynamin
340independent (66) mechanisms, cannot be completely ruled out.

341

342Dependence on glucose might be relevant for presynaptic function beyond the synaptic
343vesicle cycle (67) because many nerve terminals are thought to lack mitochondria (23)(24)
344suggesting that a considerable proportion of presynaptic ATP supply may be glycolytic in
345origin. Our experiments do not exclude the possibility that postsynaptic function also
346becomes compromised by the removal of glucose since reduced excitatory postsynaptic
347potential propagation also leads to a decrease in SBPM in our computational model.
348Postsynaptic compartments of neurons have considerable energy requirements associated
349with reversal of ion fluxes and membrane potential maintenance, but ATP for these processes

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350is thought to be supplied almost exclusively *via* oxidative metabolism (3)(4)(5). The inability
351of pyruvate or lactate to sustain a high SBPM in our experiments suggests that the observed
352dependence on glucose is not limited to its role as a substrate for oxidative metabolism
353however, because these substrates have been shown to support many aspects of neuronal
354function (17)(18)(38) that can be left to depend exclusively on ATP supplied by oxidative
355phosphorylation. Galactose was also unable to sustain a high SBPM, implying that
356glycolytically-derived ATP is likely an important energy contributor to synchronous bursting.
357Alternatively, it has been hypothesized that astrocytically-derived lactate can be used as a
358substrate for oxidative metabolism by neurons (15)(16), and thus in principle could become
359rate-limiting for neuronal activity during glucose depletion, in a fashion that simply cannot be
360rescued by the presence of lactate and pyruvate in the extracellular media. As well as
361providing vital support for synaptic function, the presence of astrocytes in co-cultures is
362known to critically shape the metabolic profile of both neurons and astrocytic metabolic
363gene-expression profiles (68) that may in turn affect the glycolytic capacity of both cell types
364*in vitro*. How well these expression patterns correspond to those of intact brain is currently
365not completely clear however (67), and the finding that culture microenvironments
366potentially alter preferences in bioenergetic pathway use (69) should be taken into
367consideration when using experimental results to infer the relative contributions of various
368metabolic pathways *in vivo*. In addition, metabolic reprogramming from aerobic glycolysis to
369oxidative phosphorylation has been shown to occur during neuronal differentiation (70)
370meaning dependence on glucose as a non-oxidative fuel source may depreciate after further
371maturation.

372

373It is also important to highlight the effects that glucose depletion can exert on neuronal
374activity through cellular signaling. Most likely this would occur indirectly *via* the AMP-

375activated protein kinase pathway that regulates the activity of proteins involved in fuel supply
376and ATP turnover in response to changes in energetic demands (71). In the brain there is no
377good evidence for a direct glucose-sensing mechanism such as that thought to exist in
378pancreatic β-cells (72), but it is understood that many neuronal cell types express ATP-
379sensitive potassium channels that provide an additional level of coupling between
380intracellular energy status and membrane excitability (73)(74). This finding has been
381suggested to underpin the effect that ketogenic diets can have to reduce risk propensity to
382epileptic seizures (75). As such, treatment with inhibitors of glycolysis including 2-deoxy-D-
383glucose has recently been suggested as a route towards effective seizure management (76).
384The rationale for such treatments is based on the observation that inhibition of glycolysis
385suppresses network excitability and epileptiform bursting both *in vivo* and in hippocampal
386slices (77)(78)(79), which complements the results we present here showing that glucose
387depletion decreases synchronous bursting frequency in cultured networks and that this can
388potentially be attributed to glycolytic cessation.

389

390In summary, our results show how network-wide synchronous activity emerging from
391excitatory coupling and synaptic vesicle dynamics is regulated by energetic substrate
392availability in a simplified cultured network model of neurons and astrocytes. The failure to
393sustain a high synchronous bursting frequency in the absence of any metabolic fuel source
394can be explained by the fact that synaptic transmission is a highly energetically-demanding
395process that requires ATP for vesicle maintenance and recovery at the presynapse in addition
396to reversal and restoration of ion fluxes and membrane potential at the postsynapse. Sources
397of ATP for these processes may involve contributions from neuronal or astrocytic glycolysis,
398which would necessitate the particular dependence of synchronous bursting frequency on
399glucose. However, it is very likely that glucose oxidation also contributes toward the supply

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PloS one, Vol. 14, No. 8, 2019.

400of ATP required to sustain synaptic activity. Thus, our combined experimental-computational 401approach paves the way for establishing an effective and pragmatic model for (dys)regulation 402of metabolism in the (un)healthy human brain. By making experimental data and 403computational code available to the wider community we hope to contribute to the further 404advancement of knowledge on this important subject.

405

406Materials and Methods

407

408*Cell culture*

409Human embryonic stem cells H9 [Wisconsin International Stem Cell (WISC) Bank, WiCell 410Research Institute, WA09 cells] were cultured according to WiCell stem cell protocols in 6-411well plates on Matrigel (Corning, hESC-Qualified) in StemFlex (Gibco). Use of stem cell line 412H9 was approved by the Steering Committee of the UK Stem Cell Bank and for the Use of 413Stem Cell Lines (ref: SCSC18-05).

414Primary mixed glial cultures were derived from P0-P2 neonatal Spraque Dawley rats and 415were generated along the previous guidelines (80), with minor modifications (81). The pups 416were euthanized following Schedule 1 rules and regulations from the Home Office Animal 417Procedures Committee UK (APC). Mixed glia cells were maintained for 10 days in culture 418after which flasks were shaken for 1h at 260rpm on an orbital shaker to remove the loosely 419attached microglia, and then overnight at 260rpm to dislodge oligodendrocyte precursors. 420Astrocyte cultures were then maintained in glial culture medium (Dulbecco’s modified 421eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine and 4221% pen/strep) and passaged at a ratio of 1:3, every 10-14 days. Cells were passaged at least 423once before co-culturing with iNs and were only used between passages 2 and 5.

424

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PloS one, Vol. 14, No. 8, 2019.

425*Induction of NGN2- hESCs and culturing iNs on MEAs*

426Gene targeting and generation of dual GSH-targeted NGN2 OPTi-OX hESCs was performed 427as described previously (40). The day prior to initiation of the reprogramming process (day 4280), NGN2-hESC colonies grown to 70-80% confluency were disassociated using Accutase 429(Sigma-Aldrich) and isolated NGN2- hESCs were seeded in 6-well plates at a density of 43025,000cells/cm^2 on Matrigel (Corning, hESC-Qualified) in StemFlex (Gibco) supplemented 431with RevitaCell (Gibco). Inducible overexpression of NGN2 began on day 1 by transferring 432cells into D0-2 induction medium (DMEM/F12, 1% Pen/Strep, 1x non-essential amino acid 433solution (NEAA, Gibco), 1% (v/v) N-2 supplement (Gibco), 1x Glutamax (Gibco), and 434doxycycline (dox) at 4μg/ml). On day 3, cells were dissociated using Accutase and re-435suspended at a density of 4000cells/μl in >D2 medium (Neurobasal-A Medium 436(ThermoFisher), 1% pen/strep, 1x Glutamax, 1x B27 supplement (Gibco), 10ng/ml brain-437derived neurotrophic factor (BDNF), 10ng/ml human recombinant neurotrophin-3 (NT-3), 438and dox at 4μg/ml) supplemented with RevitaCell. Rat astrocytes were dissociated using 4390.05% trypsin-ethylenediaminetetraacetic acid (EDTA) and re-suspended at a density of 4404000cells/μl in >D2 medium supplemented with RevitaCell. Astrocytes were then mixed at a 441ratio of 1:1 with dox-treated NGN2- hESCs in >D2 to make a final seeding density of 4422000cells/μl for each cell type.

443

444Cells were seeded onto a selection of 8x8 (60 electrodes in total, excluding corners) electrode 445MEAs (60MEA200/30iR-Ti or 60ThinMEA100/10iR-ITO, Multichannel Systems) covered 446by Teflon-sealed lids (Multichannel Systems) and pre-coated overnight at 4˚C with 500μg/ml 447poly-D-lysine (PDL) in ultrapure water. For seeding, MEAs were first incubated for 1h at 44837˚C, 5% CO_2 with a 20μl drop of laminin solution (20μg/ml laminin in DMEM) covering 449the electrode region. The laminin drop was aspirated immediately prior to seeding and

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PloS one, Vol. 14, No. 8, 2019.

450replaced with a 15μl drop of re-suspended cell mixture (total density of 4000cells/μl). MEAs
451were incubated with 15μl drops of cell suspension for 1h at 37˚C, 5% CO_2 to allow cells to
452adhere before being topped up to 1ml with >D2 medium (without RevitaCell). Cultures were
453maintained throughout lifespan at 37˚C, 5% CO_2 with 500μl media replenished every second
454day. 2μM cytosine β-D-arabinofuranoside was added to cultures on day 5 to inhibit astrocyte
455proliferation and kill undifferentiated NGN2- hESCs and dox was excluded from >D2 media
456from day 8 onward.

457

458***Recording procedures***

459For recordings, MEAs incubated at 37˚C, 5% CO_2 were transferred to the MEA recording
460device (MEA2100-2x60-System, Multichannel Systems). All recordings were performed in
461atmospheric conditions with stage and custom-built heated lid held at 37˚C. Developmental
462time course recordings took place every second or third day, 10min after a 37˚C, 5% CO_2
463incubation following half-media change. MEAs were allowed to equilibrate for 200sec on the
464MEA recording device and recording sessions lasted 10min with local field potentials from
465all electrodes sampled at 25kHz.

466

467A strict regime of media exchange, incubation, equilibration, and recording was enforced for
468all pharmacological and substrate depletion-repletion experiments. For drug treatments, MEA
469cultures were first washed (2x total media exchange) in >D2 media with vehicle (all drugs
470were diluted in DMSO or ddH_2O as required for treatment and corresponding concentration
471of the dissolving agent used as vehicle controls) to control for the effects of mechanical
472perturbation, and subsequently incubated at 37˚C, 5% CO_2 for precisely 10min. MEAs were
473then transferred to the recording device (pre-heated to 37˚C) and equilibrated for 200sec prior
474to a 10min recording sampling at 25kHz. This procedure was repeated, immediately

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PloS one, Vol. 14, No. 8, 2019.

475following each recording, under the multiple test conditions (inclusion of pharmacological
476compound in fresh >D2 media) necessary for each experiment. Cultures were exchanged into
477fresh >D2 media on termination of the final recording and returned to incubation at 37˚C, 5%
478CO_2. The same protocol was employed for substrate depletion-repletion experiments using
479>D2 media based on Neurobasal-A medium lacking glucose and sodium pyruvate
480(ThermoFisher). In this case media supplemented with the appropriate combinations of 25 or
4815mM D- or L-glucose, 0.22mM sodium pyruvate, 5mM sodium DL-lactate (racemic
482mixture), and 5mM D-galactose served as test conditions.

483

484Recorded data were processed and analyzed using the MC Rack/MC Tools software
485(Multichannel Systems) and a custom-built synchronous burst detection algorithm described
486in the SI Appendix. The algorithm calculates the number of synchronous bursts per minute
487(SBPM) for each recording and was used to extract this value from all biological replicates
488and conditions for further statistical analysis. Two-way analysis of variance (ANOVA) was
489performed in GraphPad Prism to assess the influence of biological conditions (time, energy
490substrate composition, or drug) and biological replicates (N = number of cultures) on SBPM;
491resulting p-values for conditions and number of replicates for each experiment are displayed
492in the figure legends. Student's t-tests were used to compare mean SBPMs from pairs of
493biological conditions relevant to experimental interpretation; corresponding p-values are
494displayed in figure legends and annotated in figures with * signifying $P < 0.05$ and **
495signifying $P < 0.01$. Error bars are standard error of the mean (SEM) and SBPM values
496reported in main text are mean ± SEM. Representational experimental data (Supplementary
497Datasets 1- 4) have been deposited in Figshare (see Data Deposition for link and DOIs).

498

499***Computational modelling***

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PloS one, Vol. 14, No. 8, 2019.

500We simulated a modified version of the excitatory neural network model described by
501Guerrier *et al*. (42) consisting of 20x20 (400) connected neurons organized on a square
502lattice. Membrane potential of each neuron was modelled using the simplified Hodgkin-
503Huxley model and neurons were connected randomly according to a probability distribution
504that decays as a function of distance between pairs on neurons (see SI Appendix and (42)).
505We believe this type of random synaptic connectivity accurately reflects that which emerges
506in our experimental cultures over the course of development. In the original work (42), the
507fraction of available *free*, *docked*, and *recovering* synaptic vesicles are simulated,
508corresponding to the proposed existence of multiple vesicle pools and recovery pathways (33)
509(34). Here we simulated only the fraction of docked vesicles, assuming that recovery is
510described by a single rate constant encompassing these mechanisms and have shown that this
511is sufficient to recapitulate the emergence of synchronous bursting across the simulated
512network (SI Appendix). To model the effect of glucose depletion on synaptic vesicle
513recovery, we allow for modulation of this rate by energy substrate availability, assuming that
514when the levels of extracellular glucose are low the overall rate of recovery decreases as a
515function of recent presynaptic energy consumption. Although we take inspiration from a
516similar approach used in (38), we do not intend to model the exact functional form of this
517glucose dependence and instead sought only to capture the desired properties using a
518simplified model of presynaptic metabolism (SI Appendix).

519

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521

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PloS one, Vol. 14, No. 8, 2019.

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526

527

Supporting Information

528

529The file Supporting_Information.pdf accompanying this manuscript includes:
530SI Appendix
531Figures S1- S4
532Table S1
533

534

References

5351. Ames 3rd A. CNS energy metabolism as related to function. Brain Res Brain Res Rev.
536 2000;34:42–68.
5372. Mink JW, Blumenschine RJ, Adams DB. Ratio of central nervous system to body
538 metabolism in vertebrates: its constancy and functional basis. Am J Physiol.
539 1981;241(3):R203-12.
5403. Attwell D, Laughlin SB. An energy budget for signaling in the grey matter of the
541 brain. J Cereb Blood Flow Metab. 2001;21(10):1133–45.
5424. Lennie P. The cost of cortical computation. Curr Biol. 2003;13(6):493–7.
5435. Harris JJ, Jolivet R, Attwell D. Synaptic energy use and supply. Neuron.
544 2012;75(5):762–77.
5456. Levy WB, Baxter RA. Energy efficient neural codes. Neural Comput. 1996;8(3):531–
546 43.
5477. Perge JA, Koch K, Miller R, Sterling P, Balasubramanian V. How the optic nerve
548 allocates space, energy capacity, and information. J Neurosci. 2009;29(24):7917–28.
5498. Levy WB, Baxter RA. Energy-efficient neuronal computation via quantal synaptic

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D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

550 failures. J Neurosci. 2002;22(11):4746–55.

5519. Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial

552 and neuronal control of brain blood flow. Nature. 2010;468(7321):232–43.

55310. Nortley R, Attwell D. Control of brain energy supply by astrocytes. Curr Opin

554 Neurobiol. 2017;47:80–5.

55511. Chih CP, Lipton P, Roberts Jr. EL. Do active cerebral neurons really use lactate rather

556 than glucose? Trends Neurosci. 2001;24(10):573–8.

55712. Chih CP, Roberts Jr. EL. Energy substrates for neurons during neural activity: a

558 critical review of the astrocyte-neuron lactate shuttle hypothesis. J Cereb Blood Flow

559 Metab. 2003;23(11):1263–81.

56013. Barros LF, Weber B. CrossTalk proposal: an important astrocyte-to-neuron lactate

561 shuttle couples neuronal activity to glucose utilisation in the brain. J Physiol.

562 2018;596(3):347–50.

56314. Fox PT, Raichle ME, Mintun MA, Dence C. Nonoxidative glucose consumption

564 during focal physiologic neural activity. Science. 1988;241(4864):462–4.

56515. Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic

566 glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl

567 Acad Sci U S A. 1994;91(22):10625–9.

56816. Magistretti PJ, Allaman I. Lactate in the brain: from metabolic end-product to

569 signalling molecule. Nat Rev Neurosci. 2018;19(4):235–49.

57017. Schurr A, West CA, Rigor BM. Lactate-supported synaptic function in the rat

571 hippocampal slice preparation. Science. 1988;240(4857):1326–8.

57218. Izumi Y, Benz AM, Katsuki H, Zorumski CF. Endogenous monocarboxylates sustain

573 hippocampal synaptic function and morphological integrity during energy deprivation.

574 J Neurosci. 1997;17(24):9448–57.

sinc(*i*) Research Institute for Signals, Systems and Computational Intelligence (fich.unl.edu.ar/sinc)
D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

57519. Bak LK, Schousboe A, Sonnewald U, Waagepetersen HS. Glucose is necessary to
576 maintain neurotransmitter homeostasis during synaptic activity in cultured
577 glutamatergic neurons. J Cereb Blood Flow Metab. 2006;26(10):1285–97.
57820. Patel AB, Lai JC, Chowdhury GM, Hyder F, Rothman DL, Shulman RG, et al. Direct
579 evidence for activity-dependent glucose phosphorylation in neurons with implications
580 for the astrocyte-to-neuron lactate shuttle. Proc Natl Acad Sci U S A.
581 2014;111(14):5385–90.
58221. Lundgaard I, Li B, Xie L, Kang H, Sanggaard S, Haswell JD, et al. Direct neuronal
583 glucose uptake heralds activity-dependent increases in cerebral metabolism. Nat
584 Commun. 2015;6:6807.
58522. Diaz-Garcia CM, Mongeon R, Lahmann C, Koveal D, Zucker H, Yellen G. Neuronal
586 Stimulation Triggers Neuronal Glycolysis and Not Lactate Uptake. Cell Metab.
587 2017;26(2):361–374 e4.
58823. Shepherd GM, Harris KM. Three-dimensional structure and composition of CA3--
589 >CA1 axons in rat hippocampal slices: implications for presynaptic connectivity and
590 compartmentalization. J Neurosci. 1998;18(20):8300–10.
59124. Chavan V, Willis J, Walker SK, Clark HR, Liu X, Fox MA, et al. Central presynaptic
592 terminals are enriched in ATP but the majority lack mitochondria. PLoS One.
593 2015;10(4):e0125185.
59425. Devine MJ, Kittler JT. Mitochondria at the neuronal presynapse in health and disease.
595 Nat Rev Neurosci. 2018;19(2):63–80.
59626. Rangaraju V, Calloway N, Ryan TA. Activity-driven local ATP synthesis is required
597 for synaptic function. Cell. 2014;156(4):825–35.
59827. Ashrafi G, Wu Z, Farrell RJ, Ryan TA. GLUT4 Mobilization Supports Energetic
599 Demands of Active Synapses. Neuron. 2017;93(3):606–615 e3.

sinc(*i*) Research Institute for Signals, Systems and Computational Intelligence (fich.unl.edu.ar/sinc)
D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

60028. Jang S, Nelson JC, Bend EG, Rodriguez-Laureano L, Tueros FG, Cartagenova L, et al.
601 Glycolytic Enzymes Localize to Synapses under Energy Stress to Support Synaptic
602 Function. Neuron. 2016;90(2):278–91.

60329. Knull HR. Compartmentation of glycolytic enzymes in nerve endings as determined by
604 glutaraldehyde fixation. J Biol Chem. 1980;255(13):6439–44.

60530. Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, et al. Molecular
606 anatomy of a trafficking organelle. Cell. 2006;127(4):831–46.

60731. Ikemoto A, Bole DG, Ueda T. Glycolysis and glutamate accumulation into synaptic
608 vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate
609 kinase. J Biol Chem. 2003;278(8):5929–40.

61032. Ishida A, Noda Y, Ueda T. Synaptic vesicle-bound pyruvate kinase can support
611 vesicular glutamate uptake. Neurochem Res. 2009;34(5):807–18.

61233. Sudhof TC. The synaptic vesicle cycle. Annu Rev Neurosci. 2004;27:509–47.

61334. Alabi AA, Tsien RW. Perspectives on kiss-and-run: role in exocytosis, endocytosis,
614 and neurotransmission. Annu Rev Physiol. 2013;75:393–422.

61535. Pfeiffer T, Schuster S, Bonhoeffer S. Cooperation and competition in the evolution of
616 ATP-producing pathways. Science. 2001;292(5516):504–7.

61736. Ashrafi G, Ryan TA. Glucose metabolism in nerve terminals. Curr Opin Neurobiol.
618 2017;45:156–61.

61937. Sobieski C, Fitzpatrick MJ, Mennerick SJ. Differential Presynaptic ATP Supply for
620 Basal and High-Demand Transmission. J Neurosci. 2017;37(7):1888–99.

62138. Lucas SJ, Michel CB, Marra V, Smalley JL, Hennig MH, Graham BP, et al. Glucose
622 and lactate as metabolic constraints on presynaptic transmission at an excitatory
623 synapse. J Physiol. 2018;596(9):1699–721.

62439. Zhang Y, Pak C, Han Y, Ahlenius H, Zhang Z, Chanda S, et al. Rapid single-step

sinc(*i*) Research Institute for Signals, Systems and Computational Intelligence (fich.unl.edu.ar/sinc)
D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

625 induction of functional neurons from human pluripotent stem cells. Neuron.
626 2013;78(5):785–98.
62740. Pawlowski M, Ortmann D, Bertero A, Tavares JM, Pedersen RA, Vallier L, et al.
628 Inducible and Deterministic Forward Programming of Human Pluripotent Stem Cells
629 into Neurons, Skeletal Myocytes, and Oligodendrocytes. Stem Cell Reports.
630 2017;8(4):803–12.
63141. Lam RS, Topfer FM, Wood PG, Busskamp V, Bamberg E. Functional Maturation of
632 Human Stem Cell-Derived Neurons in Long-Term Cultures. PLoS One.
633 2017;12(1):e0169506.
63442. Guerrier C, Hayes JA, Fortin G, Holcman D. Robust network oscillations during
635 mammalian respiratory rhythm generation driven by synaptic dynamics. Proc Natl
636 Acad Sci U S A. 2015;112(31):9728–33.
63743. Bertram R, Satin L, Zhang M, Smolen P, Sherman A. Calcium and glycolysis mediate
638 multiple bursting modes in pancreatic islets. Biophys J. 2004;87(5):3074–87.
63944. Paavilainen T, Pelkonen A, Makinen ME, Peltola M, Huhtala H, Fayuk D, et al. Effect
640 of prolonged differentiation on functional maturation of human pluripotent stem cell-
641 derived neuronal cultures. Stem Cell Res. 2018;27:151–61.
64245. Wagenaar DA, Pine J, Potter SM. An extremely rich repertoire of bursting patterns
643 during the development of cortical cultures. BMC Neurosci. 2006;7:11.
64446. Eytan D, Marom S. Dynamics and effective topology underlying synchronization in
645 networks of cortical neurons. J Neurosci. 2006;26(33):8465–76.
64647. Pasquale V, Massobrio P, Bologna LL, Chiappalone M, Martinoia S. Self-organization
647 and neuronal avalanches in networks of dissociated cortical neurons. Neuroscience.
648 2008;153(4):1354–69.
64948. Frega M, van Gestel SH, Linda K, van der Raadt J, Keller J, Van Rhijn JR, et al. Rapid

sinc(*i*) Research Institute for Signals, Systems and Computational Intelligence (fich.unl.edu.ar/sinc)
D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

650 Neuronal Differentiation of Induced Pluripotent Stem Cells for Measuring Network
651 Activity on Micro-electrode Arrays. J Vis Exp. 2017;(119).
652 49. Hildebrand B, Olenik C, Meyer DK. Neurons are generated in confluent astroglial
653 cultures of rat neonatal neocortex. Neuroscience. 1997;78(4):957–66.
654 50. Blake JF, Brown MW, Collingridge GL. CNQX blocks acidic amino acid induced
655 depolarizations and synaptic components mediated by non-NMDA receptors in rat
656 hippocampal slices. Neurosci Lett. 1988;89(2):182–6.
657 51. Maycox PR, Deckwerth T, Hell JW, Jahn R. Glutamate uptake by brain synaptic
658 vesicles. Energy dependence of transport and functional reconstitution in
659 proteoliposomes. J Biol Chem. 1988;263(30):15423–8.
660 52. Naito S, Ueda T. Characterization of glutamate uptake into synaptic vesicles. J
661 Neurochem. 1985;44(1):99–109.
662 53. Huss M, Ingenhorst G, Konig S, Gassel M, Drose S, Zeeck A, et al. Concanamycin A,
663 the specific inhibitor of V-ATPases, binds to the V(o) subunit c. J Biol Chem.
664 2002;277(43):40544–8.
665 54. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a
666 cell-permeable inhibitor of dynamin. Dev Cell. 2006;10(6):839–50.
667 55. Lujan B, Kushmerick C, Banerjee TD, Dagda RK, Renden R. Glycolysis selectively
668 shapes the presynaptic action potential waveform. J Neurophysiol. 2016;116(6):2523–
669 40.
670 56. Sobieski C, Warikoo N, Shu HJ, Mennerick S. Ambient but not local lactate underlies
671 neuronal tolerance to prolonged glucose deprivation. PLoS One. 2018;13(4):e0195520.
672 57. Zilberter Y, Zilberter T, Bregestovski P. Neuronal activity in vitro and the in vivo
673 reality: the role of energy homeostasis. Trends Pharmacol Sci. 2010;31(9):394–401.
674 58. Stryer L, Berg JM, Tymoczko JL. Biochemistry (5th ed.). New York: W.H. Freeman;

sinc(*i*) Research Institute for Signals, Systems and Computational Intelligence (fich.unl.edu.ar/sinc)
D. S. Tourigny, M. K. A. Karim, R. Echeveste, M. R. N. Kotter & J. S. O Neill; "Energetic substrate availability regulates synchronous activity in an excitatory neural network"
PloS one, Vol. 14, No. 8, 2019.

675 2002.

676 59. Cox DW, Bachelard HS. Attenuation of evoked field potentials from dentate granule
677 cells by low glucose, pyruvate + malate, and sodium fluoride. Brain Res.
678 1982;239(2):527–34.

679 60. Cox DW, Morris PG, Feeney J, Bachelard HS. 31P-n.m.r. studies on cerebral energy
680 metabolism under conditions of hypoglycaemia and hypoxia in vitro. Biochem J.
681 1983;212(2):365–70.

682 61. Fleck MW, Henze DA, Barrionuevo G, Palmer AM. Aspartate and glutamate mediate
683 excitatory synaptic transmission in area CA1 of the hippocampus. J Neurosci.
684 1993;13(9):3944–55.

685 62. Kanatani T, Mizuno K, Okada Y. Effects of glycolytic metabolites on preservation of
686 high energy phosphate level and synaptic transmission in the granule cells of guinea
687 pig hippocampal slices. Experientia. 1995;51(3):213–6.

688 63. Pathak D, Shields LY, Mendelsohn BA, Haddad D, Lin W, Gerencser AA, et al. The
689 role of mitochondrially derived ATP in synaptic vesicle recycling. J Biol Chem.
690 2015;290(37):22325–36.

691 64. Heidelberger R, Matthews G, Sterling P. Roles of ATP in Depletion and
692 Replenishment of the Releasable Pool of Synaptic Vesicles. J Neurophysiol.
693 2002;88(1):98–106.

694 65. Neher E. What is rate-limiting during sustained synaptic activity: Vesicle supply or the
695 availability of release sites. Front Synaptic Neurosci. 2010;2:1–6.

696 66. Xu J, McNeil B, Wu W, Nees D, Bai L, Wu LG. GTP-independent rapid and slow
697 endocytosis at a central synapse. Nat Neurosci. 2008;11(1):45–53.

698 67. Yellen G. Fueling thought: Management of glycolysis and oxidative phosphorylation
699 in neuronal metabolism. J Cell Biol. 2018;217(7):2235–46.

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700 68. Mamczur P, Borsuk B, Paszko J, Sas Z, Mozrzymas J, Wiśniewski JR, et al.
701 Astrocyte-neuron crosstalk regulates the expression and subcellular localization of
702 carbohydrate metabolism enzymes. Glia. 2015;63(2):328–40.
703 69. Sünwoldt J, Bosche B, Meisel A, Mergenthaler P. Neuronal Culture
704 Microenvironments Determine Preferences in Bioenergetic Pathway Use. Front Mol
705 Neurosci. 2017;10:1–11.
706 70. Zheng X, Boyer L, Jin M, Mertens J, Kim Y, Ma L, et al. Metabolic reprogramming
707 during neuronal differentiation from aerobic glycolysis to neuronal oxidative
708 phosphorylation. Elife. 2016;5:1–25.
709 71. Hardie DG, Ross FA, Hawley SA. AMPK: A nutrient and energy sensor that maintains
710 energy homeostasis. Nat Rev Mol Cell Biol. 2012;13(4):251–62.
711 72. Kojima I, Medina J, Nakagawa Y. Role of the glucose-sensing receptor in insulin
712 secretion. Diabetes, Obes Metab. 2017;19:54–62.
713 73. Haller M, Mironov SL, Karschin A, Richter DW. Dynamic activation of K ATP
714 channels in rhythmically active neurons. J Physiol. 2001;537(1):69–81.
715 74. Tanner GR, Lutas A, Martinez-Francois JR, Yellen G. Single KATP Channel Opening
716 in Response to Action Potential Firing in Mouse Dentate Granule Neurons. J Neurosci.
717 2011;31(23):8689–96.
718 75. Lutas A, Yellen G. The ketogenic diet: Metabolic influences on brain excitability and
719 epilepsy. Trends Neurosci. 2013;36(1):32–40.
720 76. Shao L-R, Rho JM, Stafstrom CE. Glycolytic inhibition: A novel approach toward
721 controlling neuronal excitability and seizures. Epilepsia Open. 2018;1–7.
722 77. Stafstrom CE, Ockuly JC, Murphree L, Valley MT, Roopra A, Sutula TP.
723 Anticonvulsant and antiepileptic actions of 2-deoxy-D-glucose in epilepsy models.
724 Ann Neurol. 2009;65(4):435–48.

725 78. Shao L-R, Stafstrom CE. Glycolytic inhibition by 2-deoxy-d-glucose abolishes both
726 neuronal and network bursts in an in vitro seizure model. J Neurophysiol.
727 2017;118(1):103–13.

728 79. Shao L-R, Wang G, Stafstrom CE. The Glycolytic Metabolite, Fructose-1,6-
729 bisphosphate, Blocks Epileptiform Bursts by Attenuating Voltage-Activated Calcium
730 Currents in Hippocampal Slices. Front Cell Neurosci. 2018;12:168.

731 80. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell
732 cultures from rat cerebral tissue. J Cell Biol. 1980;85(3):890–902.

733 81. Syed YA, Baer AS, Lubec G, Hoeger H, Widhalm G, Kotter MR. Inhibition of
734 oligodendrocyte precursor cell differentiation by myelin-associated proteins.
735 Neurosurg Focus. 2008;24(3–4):E5.

736

737

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