Neural Activity Triggers Neuronal Oxidative Metabolism Followed by Astrocytic Glycolysis


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Materials and Methods

Brain slice preparation and electrophysiology. Natural litters of Sprague-Dawley (SD) rats were obtained from Charles River. Transgenic mice expressing GFP under the control of the GFAP promoter (SJ) were purchased from Jackson Labs (strain FVB/N-TgN(GFAPGFP)14Mes) and bred at the Laboratory Animal Services at Cornell Veterinary College. Genotyping was performed using standard PCR protocols provided by Jackson Labs. All preparations were performed in accordance with Cornell University animal use regulations (IACUC protocol 00-46-03). Transverse hippocampal slices (thickness 400 µm) were prepared using a vibratome (Campden Instruments) after decapitation and brain removal in ice-cold artificial cerebrospinal fluid (ACSF) at P22-28 (rats) or P21-24 (mice). At that age, rat brains have developed sufficiently to have reached oxidative capacities comparable to those of fully matured rats (S2). Slices were incubated for 1-3 hours in ACSF (NaCl 118 mM, KCl 3.0 mM, KH2PO4 1.0 mM, MgSO4 1.0 mM, glucose 20 mM, CaCl2 1.5 mM, NaHCO3 25 mM, osmolarity 290-300 mOsm/liter, pH 7.3) in a static bath chamber, saturated with 95% O2 and 5% CO2 at 34 °C. For imaging and electrophysiology, slices were transferred to a temperature-controlled perfusion system (Warner Instruments, heater-controller 324B, in-line heater SH-27B, bath chamber RC-27L) and suspended between two fine nylon grids with laminar flow (2.5 ml/min) above and below the slice at 34°C. For baseline recordings, the Schaffer collateral pathway was stimulated with 100 µs pulses at 0.2 Hz using a bipolar tungsten electrode (World Precision Instruments, microelectrode TST 33A10KT, stimulus isolator A365). Evoked local field potentials (LFP) were chosen as independent measure of neural activity (S3) and recorded from the CA1 stratum radiatum with borosilicate micropipettes with an outer diameter of 2 µm and a resistance of 1–2 MΩ. The stimulation current (typically 20–30 µA) was adjusted to evoke LFP with ~70% of the maximal amplitude. In some experiments, the AMPA/kainate receptor antagonist CNQX (25 µM) was added to the perfusate to block postsynaptic receptor activation without interfering with astrocytic glutamate uptake (S4). To induce focal neural activity, the Schaffer collateral pathway was stimulated with 100-µs pulses at 32 Hz for a brief period (5, 20, 60, or 240 s). After stimulation, LFP were monitored at 0.2 Hz. Hypoxia was induced in slices by perfusion with ACSF saturated with 95% N2 and 5% CO2 (S5).

Immunohistochemistry. Paraffin embedded rat brains from paraformaldehyde-perfused SD-rats (P24) containing transverse sections of the hippocampus were cut at 4 µm, deparaffinized, and rehydrated. Sections were microwaved in 0.01 M citrate buffer pH 6.0, for 10 min for antigen retrieval and blocked with 10% normal goat serum / 10%...
normal horse serum / 10% nonfat dry milk. They were incubated with primary antibodies in two combinations: (i) polyclonal rabbit anti-MAP2 (Chemicon AB5622, 1:50) with monoclonal mouse anti-GFAP (Chemicon MAB360, 1:50) and (ii) monoclonal mouse anti-cytochrome-oxidase subunit IV (Molecular Probes A21348, 1:10) with polyclonal rabbit anti-GFAP (DAKO, 1:1800). Biotinylated horse anti-mouse (adsorbed rat) secondary antibody (Vector Laboratories, Burlingame, CA) was followed by Texas Red Streptavidin DCS (Vector) to label monoclonal primary antibodies. Subsequently, slides were incubated with FITC conjugated goat anti-rabbit secondary antibody (Sigma) to label polyclonal antibodies. Slides were mounted with Vectashield DAPI (Vector) and imaged with epi-fluorescence microscopy.

**Multiphoton microscopy and spectroscopy.** The multiphoton microscopes consisted of modified Bio-Rad laser scanning instruments (MRC 600 upright and MRC 1024 inverted) with laboratory-built detection systems. Spectra Physics Ti:Sapphire lasers provided excitation of intrinsic NADH fluorescence at 740 nm with ~100-fs pulse-width and 80 MHz repetition rate. GFP was excited at 885-920 nm. Emission filters were chosen to separate intrinsic NADH fluorescence (range 410-490 nm) from GFP or Rhodamine123 fluorescence (510-650 nm). Images (512*512 pixels) were acquired within ~1 s. For multiphoton imaging and simultaneous electrophysiology in acute brain slices, dipping objectives (Olympus 20× NA 0.95 and Olympus LUMPlanFl 40× NA 0.8) were used. Laser power of ~30-40 mW (measured after the objective) was necessary to excite NADH fluorescence at a depth of ~70-100 µm, thereby bypassing the damaged outer layers and obtaining images from the viable core of the slices where stable baselines can be obtained (S6). Electrophysiology and imaging were synchronized using the pClamp 8.2 / Digidata1322A data acquisition system (Axon Instruments). Acute brain slices from GFAP-GFP mice (two-photon excitation with laser powers of 15-20 mW) and fixed tissues (epi-fluorescence excitation) were imaged on an inverted microscope with Zeiss C-Apochromat 10× NA 0.45 w.i., Olympus 20× NA 0.7 NA w.i., and Zeiss F-Fluar, 40× NA 1.3, o.i. objectives. Emission spectra of two-photon excited intrinsic tissue fluorescence were obtained by scanning small regions (30 µm²) with a dwell time of ~30 ms/µm² using an average excitation power of ~20 mW. The fluorescence was fiber-coupled to a spectrometer (Jobin Yvon, Edison, SPEX 270M) equipped with a liquid nitrogen cooled charged coupled device detector. To maximize fluorescence collection by an 550 µm multimode optical fiber with high transmission in the UV-visible range (OZ Optics), the stage was scanned over the stationary excitation beam using a motorized x-y stage (Merzhäuser IM 120*100). The combined spectral response of both the fiber and the grating were calibrated using a calibration lamp (Oriel Quartz Tungsten Halogen 63358). The wavelength calibration was performed using the spectral lines of Kr-Ar laser. 2P-excitation cross sections of NADH in aqueous solution were measured from 700 to 1000 nm by comparing to 10 µM fluorescein (S7).

**Statistical image analysis.** The linear correlation coefficient or Pearson correlation coefficient \((r)\) is a measure of the degree of linear correlation between two variables (S24). Given two arrays \(A\) and \(B\), the correlation coefficient is defined as:
This is a continuous variable that ranges between $-1$ for completely anti-correlated arrays, to $0$ for uncorrelated arrays, to $1$ for correlated arrays. In calculating the correlation between data sets throughout this work (e.g., for dip and overshoot arrays), the sum is over only those pixels that respond to stimulation, i.e. exhibit either a dip or an overshoot (or both). Pixels that fail to exhibit any response to stimulation are not included in the sum. The quantity $r^2$ is also known as the coefficient of determination and represents the fraction of the variation between the two arrays that can be explained by a linear relationship. Small values of $r^2$ tend to indicate either significant scatter in the data, or a nonlinear relationship between the two variables. Given an observed correlation coefficient, $r$, between two arrays (of $N$ pixels), we can calculate the probability that the magnitude of this correlation arose from uncorrelated arrays ($S8$).

$$P(r, N) = \frac{2}{\sqrt{\pi}} \frac{\Gamma \left( \frac{N-1}{2} \right)}{\Gamma \left( \frac{N-2}{2} \right)} \int_0^1 (1-x^2)^{(N-4)/2} \, dx$$

This provides a measure of significance on our calculated correlation or anti-correlation. The large array sizes typical of our data ($N \sim 10000$), give $P(r, N)$ values that become negligible very rapidly as a function of $r$. For example, even for a low correlation coefficient of $r = 0.1$ ($N = 10000$), we find that $P(r, N) \sim 10^{-22}$. This simply reflects the fact that large uncorrelated arrays yield $r = 0$ with a very narrow distribution in $r$. Hence, the correlation coefficients observed in our data analysis (e.g., $r = -0.84$ for dip and overshoot regions) prove an underlying correlation, because the probability that they arose from uncorrelated data is negligible (typically $P(r, N) < 10^{-100}$).

**Chemicals.** CNQX, β-NADH, and sodium cyanide were purchased from Sigma. Mitochondrial malate dehydrogenase was obtained from Roche.

**Supporting Online Material Text**

**A. Oxidative and glycolytic metabolic signatures upon focal neural activity**

Functional imaging techniques such as fMRI and PET utilize activity-dependent changes in neural energy metabolism and cerebral blood flow as an indirect measure of neuronal activity. Despite the widespread use of functional neuroimaging and decades of basic research, discordant views on the organization of brain energy metabolism and the coupling of neural activity and metabolism to blood flow have persisted ($S9–S11$). Due to space limitations, we could only cite a minor fraction of the most relevant literature in the main text. To provide in-depth information we have included more highly relevant publications reporting oxidative metabolism ($S12–S19, in addition to references 2, 3, 9, 23, 27, 30$) or glycolytic metabolism ($S20–S24, in addition to 1, 6, 19, 29, 30$) during focal neural activity in the supplementary references.
B. Advantages and limitations of brain tissue slices and the in vivo approach for the investigation of neurometabolic coupling

Brain tissue slices with fully preserved 3-dimensional neuron-glia interactions and sustained synaptic activity provide a powerful model for the investigation of neurometabolic coupling. Their geometry facilitates electrophysiological stimulations and recordings and pharmacological manipulations (no blood–brain barrier). Furthermore, brain slices circumvent the need for anesthesia that would accompany any presently feasible in vivo experiment. However, the preparation of slices subjects the brain tissue to a severe cold shock, a brief period of ischemia (30–60 s) and the outer layers to a mechanical trauma (S6). These factors substantially affect the initial baseline energy status and might interfere with the experimental outcomes.

The fact that brain slices are blood-free systems drastically facilitates the excitation and deconvolution of intrinsic optical signals, but it coincidently impedes the investigation of neurovascular coupling which should be reserved for the in vivo approach. However, in-vivo imaging experiments with the necessary resolution to resolve metabolic signatures in astrocytic and neuronal processes are not yet satisfactory because the complex and dynamic optical properties of the cerebral macro- and microcirculation on top of absorption, scattering and second-harmonic generation by connective and neural tissue interfere strongly with the exciting infrared light and the deconvolution of the relatively weak intrinsic fluorescence.
**Supporting figure S1**

![Intensity Histograms of Tissue Regions](image)

**Fig. S1.** Intensity histograms of tissue regions. The baseline intensities of the dip regions (mean ± SEM: 44.7 ± 0.1) are ~17% higher than those of the overshoot regions (38.30 ± 0.07) and ~7% higher than the average intensity of the entire tissue (41.87 ± 0.04). The high intensity of the dip regions, taken in conjunction with their punctate morphology confirms their mitochondrial origin. Even though the overshoot regions are clearly localized within the brightly fluorescent astrocytes (Fig. 4C), their baseline intrinsic fluorescence level is slightly lower (~9%) than the average intensity of the entire tissue. This can be explained by their cytoplasmic origin which excludes the fluorescence contribution of the bright and large astrocytic mitochondria (Fig. 2F).
References and Notes