Supplemental Material

Supplemental Fig. 1. Metabolic signals from the vessel wall during mGluR activation in low O_2. (A) NADH fluorescence changes from smooth muscle and endothelial cells during LDH inhibition with oxamate. Oxamate decreases NADH (-18.8 ± 2.1 %, n = 5, $P < 0.01$), suggesting these cells are more likely to take up lactate and convert it to pyruvate than the reverse reaction. (B) NADH changes from the vessel wall caused by the mGluR agonist t-ACPD. t-ACPD causes a biphasic NADH change with an initial dip (oxidation)(-7.2 ± 2.7%, $P < 0.05$) before increasing (reduction)(17.8 ± 4.6, $P < 0.05$, n = 6), a profile unlike the pure reduction response observed in astrocytes. (C) In the presence of LDH inhibition with oxamate—which blocks mGluR-mediated vasodilation—t-ACPD failed to significantly alter the NADH signal from the cells of the vessel wall (dip: -8.2 ± 4.5 %, $P > 0.05$; increase: 6.3 ± 5.3 %, $P > 0.05$, n = 6), suggesting astrocyte to vessel wall communication is compromised in oxamate. These data reveal a less prominent role for glycolysis and thus lactate production and release from the vessel wall compared to astrocytes.

Supplemental Fig. 2. Lowering the O_2 level produces inconsistent effects on vessel tone. (A and B) As vessel tone influences the polarity of diameter changes (*4*) we examined the effect of reducing O_2 on vessel diameter, but found inconsistent changes (7 dilations: 12.0 ± 1.2%; 4 constrictions: -12.2 ± 3.98; n = 14), suggesting O_2 induced tone cannot account for the disparate vessel responses we observed when mGluRs were activated in high and low O_2. Additionally, reducing O_2 did not induce anoxic depolarization or spreading depression, as we failed to observe cellular swelling of neuronal or glia somas (data not shown)(*29*).

Supplemental Fig. 3. Diagram depicting the proposed mechanism for how an increased glycolytic state promotes astrocyte-mediated vasodilations. Two separate but interacting pathways are shown. Decreased O_2 availability increases glycolysis and reduces oxidative metabolism. Glycolysis in asrtocytes leads to the production of NADH (excitation and emission photons indicate fluorescence) and pyruvate. Pyruvate can either enter oxidative metabolism (not shown) or be converted to lactate by lactate

dehydrogenase (LDH). Lactate is released in to the extracellular milieu by monocarboxylate transporters (MCT). Mounting evidence indicates that due to the isoforms of LDH and MCTs expressed in astrocytes, lactate production and release is favoured over lactate uptake and convesrsion to pyruvate, where the latter is thought to be the predominate process in neurons. A rise in endfoot Ca^{2+} caused by mGluR activation and subsequent Ca^{2+} release from internal stores or by photolysis of caged Ca^{2+} activates phospholipase A_2 (PLA_2) and may drive metabolic process through a number of different mechanisms including gliotransmission and transmitter uptake. PLA_2 leads to the generation of aracidonic acid (AA) which can be converted into vasoactive prostaglandin E_2 (PGE_2). PGE_2 efflux through prostaglandin transporter (PGT) relies on the counter transport of lactate from the extracellular space. Once liberated, PGE_2 is free to bind to G-protein coupled EP receptors on SMCs, eliciting relaxation via enhanced K^+ channel conductance.

Methods

Slice Preparation and Astrocyte Loading

Hippocampal and neocortical slices were prepared from juvenile (p 15-22), male, Sprague-Dawley rats. Care and use of animals was approved by the University of British Colombia Animal Care and Use Committee. Animals were anesthetized with Halothane (Sigma), decapitated and the brains rapidly removed into ice-cold high sucrose slicing solution containing (in mM): NaCl, 87; KCl, 2.5; NaHCO3, 25; CaCl2, 0.5; MgCl2, 7; NaH2PO4, 1.25; glucose, 25; sucrose, 75; saturated with 95% O_2 / 5% CO_2. Transverse hemisections, 400 μm thick, were cut on a vibrating slicer (Leica) and incubated at room temperature (22-24°C) in a chamber of ACSF containing (in mM): NaCl, 126; KCl, 2.5; NaHCO3, 26; CaCl2, 2.5; MgCl2, 1.5; NaH2PO4, 1.25; glucose, 10; saturated with 95% O_2 / 5% CO_2 for a minimum of 60 minutes prior to NADH imaging or further incubation for astrocyte dye loading. For the latter, slices were transferred to a 3 ml ACSF well containing the Ca^{2+} cage DMNPE-4-AM and/or the Ca^{2+} indicator Rhod-2-AM dissolved in DMSO (final DMSO concentration 0.2%) for 1.5 hours, which received continuous carbogen bubbling. Slices were then transferred to a large recovery chamber for at least 20 minutes before experimentation. Dye loading was restricted exclusively to astrocytes

as reported previously (*4*). To facilitate loading, male Sprague-Dawley rats (p15-18) were utilized. For experimentation a slice was transferred to a recording chamber and perfused with ACSF at a rate of 1–3 ml/min at room temperature. ACSF bubbled with 95% O_2, 5% CO_2, was defined as a high O_2 treatment and ACSF bubbled with 8-20% O_2, 5% CO_2, balanced N_2, was defined as a low O_2 treatment.

Imaging

A two-photon laser-scanning microscope (Zeiss LSM510-Axioskop-2 fitted with a 40X-W/0.80 numerical aperture objective lens) directly coupled to a tunable Mira Ti:sapphire laser (~100-fs pulses and 76MHz, pumped by a 5W Verdi laser; Coherent) provided excitation of Rhod2 or intrinsic NADH fluorescence. Images were acquired at depths between 50 and 100 μm into the brain slice in order to avoid damaged and unhealthy tissue at more superficial depths. For the t-ACPD experiments that did not utilize the Ca^{2+} cage, Rhod2 was excited at 835–840nm. Rhod2 epifluorescence was detected with an external photomultiplier tube (PMT) detector after passing through a 510 nm (40 nm band pass) and a 605 nm (55 nm band pass) emission filter. For the Ca^{2+} imaging and photolysis of caged Ca^{2+} within astrocyte compartments the Ti:sapphire laser was tuned to 730 nm, efficient for both uncaging and excitation of the Rhod-2 fluorophore which has a broad two-photon excitation cross section (*30*). The uncaging ROI was always within an astrocyte soma or endfoot (*31*). Laser intensities were the lowest possible for uncaging. Flash durations between 3 and 10 ms produced repeatable small Ca^{2+} transients. The laser intensity was carefully increased beyond this point until a larger more sustained Ca^{2+} signal, characteristic of internal release, occurred within the astrocyte, triggering a Ca^{2+} wave that propagated throughout the astrocyte network. Due to the highly nonlinear nature of two-photon microscopy (*31*), no uncaging occurred during the continuous excitation of Rhod2 required for imaging changes in astrocyte $[Ca^{2+}]_i$.

For NADH imaging the Ti:sapphire laser was tuned to 740 nm (*11*). NADH epifluorescence was detected with an external PMT detector after passing through a 450 nm (30 nm bandpass) emission filter. The laser power necessary for NADH excitation was typically two to three times that for standard flourophores, as others have reported

(*11*). To reduce photodamage a single NADH image was acquired every 30 s, which provided a stable NADH baseline line and adequate time resolution for measuring NADH changes and lumen diameter changes in response to the bath application of drugs.

Arterioles were identified with IR-DIC optics and chosen on the basis of their diameter, appearance of smooth muscle cells and health over an extended region. Although blood vessels in brain slices do not experience the same shear stresses as they do in vivo, repeatable dilations could be induced suggesting arterioles do maintain a degree of tone in brain slices. Vessel diameter changes were imaged by acquiring the transmitted laser light with an external PMT simultaneous with the fluorescence acquisition.

Lactate and PGE_2 Measurements

The assay used for measuring PGE_2 release was Specific Parameter PGE_2 ELISA kits (R&D systems) following protocols specified in supplier instructions. Extracellular lactate levels were measured using a Lactate Assay Kit (Biomedical Research Service Centre, SUNY Buffalo). This kit is based on the reduction of the tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan, which exhibits an absorption maximum at 492 nm. In both lactate and PGE_2 assays TTX (1 μM) and IBMX (100 μM) was added to dampen neuronal network activation and preserve cAMP levels respectively. In the latter, cAMP generation facilitates glycogen breakdown (*32*) which is thought to be an important step in astrocyte glycolysis (*19*).

Data Collection, Analysis and Statistics

Images were collected using 256 x 256 or 512 x 512 pixels and the scanning frame rate was 3.15 s or 7.86 s depending on the area scanned, and 8 line average was utilized. Image series were analyzed off-line, and luminal diameter measurements were made at multiple sites along the vessel. Quantification of lumen diameter, NADH and Ca^{2+} changes were performed with Zeiss LSM (version 2.8) software and ImageJ. Fluorescence signals were defined as F/F0 (%) = [(F1 - B1)/(F0 - B0)] x 100, where F1 and F0 are fluorescence in the astrocyte compartment at any given time point and at the beginning of the experiment respectively, and B1 and B0 are the corresponding

background fluorescence signals. For Rhod2, background values were taken from an adjacent area located at least 25 μm from the astrocyte soma. For NADH, background values were taken from the lumen of the vessel which was devoid of NADH fluorescence. This increased our signal to noise ratio by almost three fold, providing us with a measure of NADH changes greater than reported previously. Psuedo colour images were background subtracted, depict an absolute change in Rhod2 or NADH fluorescence and were generated by ImageJ using a 16-color Lut. Experimental values are presented as the mean ± SEM. A changes in the measured parameter is expressed as a percent change from baseline, where '-' indicates a decrease and no sign (positive) indicates an increase. Statistical analyses were performed using a two tailed Student's t test or ANOVA. $P < 0.05$ was accepted as statistically significant (* = $P < 0.05$, ** = $P < 0.01$).

Drugs

t-ACPD (100 μM) (purchased from Sigma-Aldrich, St. Louis) was applied for 5-10min. The longer drug application time was used for astrocytes and vessels imaged deeper in the slice (towards 100 μm). Lactate (1 mM) (Sigma) was applied for 5-10min and was pH corrected to 7.4. Sodium-Oxamate (2.5 mM) and Sodium-Iodoacetate (200 μM) (Sigma) were applied continuously and a minimum 15 min incubation period was used before t-ACPD was applied. Indomethacin (100 μM) (Sigma) was applied continuously and minimum 20 min incubation period was used before t-ACPD or lactate was applied. Tetrodotoxin (1 μM) (Alamone labs) and 1-Methyl-3-isobutylxanthine (IBMX) (100 μM) (Sigma) were always present during the release assays. Rhod2-AM (10 mM) (Molecular Probes, Eugene, Oregon) and the DMNPE-4-AM cage (10 μM) (synthesized by Graham Ellis-Davies) were added to the small incubation bath for 1.5 hours.