Activity-dependent Increases in Local Oxygen Consumption Correlate with Postsynaptic Currents in the Mouse Cerebellum *In Vivo*

Claus Mathiesen,¹ Kirsten Caesar,¹ Kirsten Thomsen,¹ Tycho M. Hoogland,² Brent M. Witgen,¹ Alexey Brazhe,³ and Martin Lauritzen^{1,4}

¹Department of Neuroscience and Pharmacology, and Center for Healthy Aging, University of Copenhagen, DK-2200 Copenhagen N, Denmark,

²Netherlands Institute for Neuroscience, NL-1000 GC Amsterdam, The Netherlands, ³Biological Faculty, Moscow State University, Moscow 119991, Russia,

and ⁴Department of Clinical Neurophysiology, Glostrup Hospital, DK-2600 Glostrup, Denmark

Evoked neural activity correlates strongly with rises in cerebral metabolic rate of oxygen (CMRO₂) and cerebral blood flow (CBF). Activity-dependent rises in CMRO₂ fluctuate with ATP turnover due to ion pumping. *In vitro* studies suggest that increases in cytosolic Ca²⁺ stimulate oxidative metabolism via mitochondrial signaling, but whether this also occurs in the intact brain is unknown. Here we applied a pharmacological approach to dissect the effects of ionic currents and cytosolic Ca²⁺ rises of neuronal origin on activity-dependent rises in CMRO₂. We used two-photon microscopy and current source density analysis to study real-time Ca²⁺ dynamics and transmembrane ionic currents in relation to CMRO₂ in the mouse cerebellar cortex *in vivo*. We report a direct correlation between CMRO₂ and summed (i.e., the sum of excitatory, negative currents during the whole stimulation period) field EPSCs (Σ fEPSCs) in Purkinje cells (PCs) in response to stimulation of the climbing fiber (CF) pathway. Blocking stimulus-evoked rises in cytosolic Ca²⁺ in PCs with the P/Q-type channel blocker ω -agatoxin-IVA (ω -AGA), or the GABA_A receptor agonist muscimol, did not lead to a time-locked reduction in CMRO₂, and excitatory synaptic or action potential currents. During stimulation, neither ω -AGA or (μ -oxo)-bis-(*trans*-formatotetramine-ruthenium) (Ru360), a mitochondrial Ca²⁺ uniporter inhibitor, affected the ratio of CMRO₂ to fEPSCs or evoked local field potentials. However, baseline CBF and CMRO₂ decreased gradually with Ru360. Our data suggest that *in vivo* activity-dependent rises in CMRO₂ are correlated with synaptic currents and postsynaptic spiking in PCs. Our study did not reveal a unique role of neuronal cytosolic Ca²⁺ signals in controlling CMRO₂ increases during CF stimulation.

Introduction

Coupling of neuronal activity, energy metabolism, and blood flow forms the basis of noninvasive functional imaging used to map brain function in humans (Raichle and Mintun, 2006). Glucose oxidation provides nearly all the energy required by neurons to support brain activity in the resting state (Clarke and Sokoloff, 1994) as well as during activation (Lin et al., 2010). It is now recognized that neurotransmitter signaling plays a key role in regulating cerebral blood flow (CBF), and both neurons and astrocytes mediate this control (Lauritzen, 2005; Attwell et al., 2010). In comparison, less is known regarding the local *in vivo* control of the cerebral metabolic rate of O₂ (CMRO₂), as most knowledge of mitochondrial function was obtained from isolated

mitochondria, dissociated cell cultures, or brain slices (Kann and Kovács, 2007). CMRO₂ is controlled by the ADP/ATP ratio, as well as by changes in the mitochondrial Ca²⁺ concentration that stimulate the activity of tricarboxylic acid (TCA) cycle dehydrogenases and central enzymes in the respiratory chain (Gunter et al., 2004). In addition, respiration is controlled by the Ca²⁺-dependent mitochondrial aspartate-glutamate transporter, which increases mitochondrial nicotinamide adenine dinucleotide (NADH) levels (Satrústegui et al., 2007). Indeed, recent studies suggest that glutamate-dependent oxidative phosphorylation in mitochondria is exclusively triggered by extramitochondrial Ca²⁺ in physiological concentration ranges (Gellerich et al., 2010). Furthermore, rapid rises in oxygen consumption mediated by increased cytosolic Ca²⁺ concentrations have been demonstrated in cultured Purkinje cells (PCs) (Hayakawa et al., 2005). These observations support the notion that cytosolic Ca²⁺ signaling may function as a rapid feedforward mechanism to control CMRO₂ and thereby control cytosolic ATP levels in nervous system tissue (Gunter et al., 2004); however, the influence of cytosolic Ca²⁺ on CMRO₂ has not been investigated in the intact brain. We studied this relationship *in vivo* with preserved perfusion (i.e., glucose and oxygen supplies), testing the hypothesis that activity-driven increases in cytosolic Ca²⁺ in PCs are rate

Received Sept. 5, 2011; revised Oct. 20, 2011; accepted Oct. 20, 2011.

Author contributions: C.M., K.C., K.T., A.B., and M.L. designed research; C.M., K.C., K.T., T.M.H., B.M.W., and A.B. performed research; C.M., K.T., T.M.H., and A.B. analyzed data; C.M., K.T., T.M.H., A.B., and M.L. wrote the paper.

This study was supported by the Lundbeck Foundation via the Lundbeck Foundation Center for Neurovascular Signaling, the NOVO-Nordisk Foundation, the Danish Medical Research Council, the NORDEA foundation for Center for Healthy Aging, and Foundation Leducq. We thank Micael Lönstrup for excellent surgical assistance and Peter Rasmussen for providing the initial CSD Matlab script.

Correspondence should be addressed to Dr. Claus Mathiesen, Institute of Neuroscience and Pharmacology, Panum Institute 18.5, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark. E-mail: mathiesenator@gmail.com.

DOI:10.1523/JNEUROSCI.4526-11.2011

Copyright © 2011 the authors 0270-6474/11/3118327-11\$15.00/0

Figure 1. Experimental setup and functional anatomy of mouse cerebellar PCs. **A**, Dorsal view of the mouse brain, indicating the location of the cranial window over the cerebellar vermis lobule VI (red box). a, Anterior, p, posterior. **B**, Side view of the mouse brain, indicating placement of 16-channel (16-ch.) probe, LDF probe, tpO₂ electrode and bipolar stimulation (stim.) electrode. **C**, Counterclockwise order: sagittal, transverse, and top views of PCs in the cerebellum of *Pcp2-EGFP* mice expressing cell-specific green fluorescent. **D**, Top, The sagittal orientation of PC dendrites. Time series of CF-evoked Ca²⁺ responses (top view, with numbers indicating time in seconds from stimulation onset and yellow bar indicating the 10 Hz, 15 s stimulus).

limiting for the corresponding CMRO₂ responses. Our data suggest that cytosolic Ca²⁺ per se does not directly modulate CMRO₂ responses; instead, our results suggest that *in vivo* CMRO₂ responses correlate significantly with postsynaptic currents.

Materials and Methods

Animal handling

All procedures involving animals were approved by the Danish National Ethics Committee according to the guidelines set forth in the European Council Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Forty four male white Naval Medical Research Institute mice [6–8 weeks old, Crl:NMRI(Han)] were examined using pharmacological interventions designed to inhibit Ca²⁺ entry into either cells or mitochondria in the vermis region, lobule VI of the cerebellar cortex. CBF, tissue partial pressure of oxygen (tpO₂) and excitatory synaptic currents as indicated by either current source density (CSD) analysis or extracellular local field potentials (LFPs) in response to climbing fiber (CF) stimulation were measured in 32 mice, while cytosolic Ca²⁺ responses to the same stimulation paradigm were examined in 12 mice using two-photon microscopy. To compare stimulus-evoked Ca²⁺ responses to the anatomy of cerebellar PCs shown in Figure 1C, we also examined three transgenic *Pcp2* mice (9–11 weeks old) expressing the green fluorescent Purkinje cell protein-2 (*Pcp2-EGFP*; Jackson Laboratory).

Mice were anesthetized by intraperitoneal injections of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) (Sigma-Aldrich), and were given supplemental doses of ketamine every 20 min. Body temperature was maintained at 37°C using a temperature controller and heating blanket (model TC-1000 Temperature Controller, CWE). The trachea was cannulated for mechanical ventilation with a small-animal ventilator (SAAR-830, CWE). A catheter (TYGON S54HL 0.010 × 0.030 mm, VWR International) was inserted into the left femoral artery for continuous monitoring of blood pressure. A blood sample taken at the beginning of each experiment was used to adjust respiration to obtain physiological blood gas values [pO₂, 95–110 mmHg; partial pressure of CO₂, 35–40 mmHg; pH, 7.35–7.45]. A custom-made metal plate allowing cranial access was fixed to the skull with cyanoacrylate gel (Loctite

Adhesives). A craniotomy (~4 mm diameter) was made above lobule VI of the medial vermis region of the cerebellum, and the dura was removed. The craniotomy was filled with 1% agarose (type III-A, low electroendosmosis; Sigma-Aldrich) and was moistened with artificial CSF (aCSF) (in mM as follows: NaCl 120, KCl 2.8, NaHCO₃ 22, CaCl₂ 1.45, Na₂HPO₄ 1, MgCl₂ 0.876, and glucose 2.55; pH 7.4) at 37°C, aerated with 95% air/5% CO₂. For imaging experiments using the two-photon microscope, part of the craniotomy was covered with a glass coverslip, which permitted pharmacological interventions and electrical recordings. At the end of experiments, mice were killed by intravenous injection of air followed by decapitation.

Climbing fiber stimulation

A coated, bipolar stainless-steel electrode (0.25 mm contact separation; SNEX 200, RMI Corporation) was stereotactically lowered into the inferior olive as described previously (Caesar et al., 2003). PCs were identified by their ability to fire simple spikes (SSs) and complex spikes (CSs) spontaneously, with the production of a complex spike 5–8 ms after electrical stimulation of the inferior olive. Stimulation of CFs also resulted in an LFP with a classical laminar profile and potential reversal (Mathiesen et al., 1998). Positioning was optimized by means of the maximal response of LFP in the cerebellar vermis region to continuous low-frequency stimulation (0.5 Hz). Pulses of 0.2 ms constant current with an intensity of 0.15 mA (ISO-flex, A.M.P.I.) were used at 2, 5, and 10 Hz for 15 s. The test stimulation during pharmacological interventions was 10 Hz for 15 s. The stimulation was controlled by a Sequencer file running within the Spike2 software (version 7.02, Cambridge Electronic Design).

Electrophysiological recordings

PC spike activity and LFPs were recorded with single-barreled glass microelectrodes (borosilicate glass; tip diameter 2–3 μm, outer diameter 1.5 mm; inner diameter 0.86 mm; Sutter Instrument) containing aCSF at a depth of 50–100 μm below the brain surface. An Ag/AgCl ground electrode was placed near the cranial window submerged in aCSF. These analog signals were first amplified using a differential amplifier (gain ×10, bandwidth 0.1–10,000 Hz; model DP-311, Warner Instruments) followed by additional amplification using CyberAmp 380 (Molecular Devices; gain ×100, bandwidth 0.1–10,000 Hz).

In electrophysiological experiments, laminar LFP profiles from which CSD maps were calculated (see CSD calculation, below) were recorded using a vertical 16-channel Michigan probe with 50 μm between electrodes (A1x16–5 mm–50–413, NeuroNexus Technologies) and a 16-channel amplifier (gain $\times 1000$, bandwidth 1–10,000 Hz; PGA16, Multichannel Systems). All electrical analog signals were digitally sampled at least five times the low-pass filter frequency using a power1401mk II interface (Cambridge Electronic Design) connected to a personal computer running Spike2 software.

Stimulation artifacts were removed from PC spike traces with a Spike2 script (artrem.s2s, Cambridge Electronic Design). PC spike activity was quantified by calculating the root mean square (rms) of the spike trace, while spike interval histogram (INTH) analysis was applied to illustrate changes in PC spiking rhythm. The poststimulus refractory period is the period from the end of stimulation to the recovery of spiking to pre-stimulus levels and reflects the activity of the Ca^{2+} -gated K^+ current (gK_{Ca}) (Llinas, 1981; Womack et al., 2009).

CSD calculation

CSD is related to the second-order spatial derivative of the LFP under the assumptions of constant extracellular electrical conductivity, homogeneous cortical in-plane activity and equal distance between recorded potentials (Nicholson and Freeman, 1975; Nakagawa and Matsumoto, 1998). CSD was calculated from averaged laminar LFP profiles using custom-written Matlab scripts (The MathWorks), which calculated the difference of the difference between LFP amplitudes for every running set of three neighboring channels. The CSD maps obtained in this manner identified the location and amplitude of the negative and positive currents. At a cortical depth of 50 μm , the amplitude of the negative current is proportional to the magnitude of the field (i.e., extracellular) EPSC (fEPSC) and reflects Na^+ influx into PCs via AMPA receptor channels, while the delayed positive current represents K^+ efflux out of PCs via P/Q-type gK_{Ca} (Llinas, 1981; Womack et al., 2009). The reverse current pattern is observed at a depth of 100 μm due to the anatomical and electrophysiological properties of the PCs (Llinas, 1981). We calculated the summed fEPSCs (ΣfEPSC) as fEPSC amplitude $\times$ stimulation frequency $\times$ duration of stimulation train, which represents the transmembrane Na^+ flux into PCs during the stimulation period (Mathiesen et al., 1998).

Cerebellar cortical blood flow measurement

CBF was recorded continuously using a laser-Doppler flowmetry (LDF) probe at a fixed position 0.3 mm above the pial surface in a region devoid of large vessels (wavelength 780 nm; 250 μm fiber separation allowing CBF measurement to a depth of 1 mm; Perimed) (Fabricius et al., 1997). The probe was placed close to the microelectrodes recording electrophysiological variables and oxygen (Fig. 1B). The LDF signal was smoothed with a time constant of 0.2 s (PeriFlux 4001 Master, Perimed), sampled at 10 Hz, analog-to-digital (A/D) converted, and digitally recorded and smoothed (time constant 1 s) using the Spike2 software. The LDF method does not measure CBF in absolute terms, but is valid in determining relative changes in CBF during moderate flow increases (Fabricius and Lauritzen, 1996). Evoked rises of CBF are expressed as a percentage of baseline. No significant changes in CBF baseline were observed during the experiments.

Local tpO_2 measurements

Local tpO_2 was recorded with a modified Clark-type polarographic oxygen microelectrode (OX-10, Unisense A/S). The small tip size (3–5 μm) assured reliable tpO_2 measurements, and its built-in guard cathode removed all oxygen from the electrolyte reservoir, enabling the measurement of tpO_2 over time under different treatment conditions with excellent long-term stability (signal drift 0–0.5%/h). The field of sensitivity is a sphere of $2\times$ tip diameter. The oxygen electrodes were calibrated in air-saturated and oxygen-free saline (0.9% at 37°C) before and after each experiment, with reproducible oxygen measurements. The tip of the O_2 microelectrode was positioned in the center of the sampling volume of the laser-Doppler probe (Fabricius et al., 1997) at a depth of 50–75 μm , where maximal fEPSCs were found. The oxygen electrode was connected to a high-impedance picoampere meter (PA 2000,

Table 1. Control levels of dendritic Ca^{2+} coverage, spike activity, CSD, CBF, tpO_2 , and CMRO_2 responses to CF stimulation (15 s at 10 Hz)

	Control levels
Ca^{2+} ($N = 12$)	
Peak (%)	13.6 ± 2.3
Coverage (%)	20.5 ± 3.9
Spikes ($N = 7$)	
Spontaneous (rms)	0.43 ± 0.09
Evoked (rms)	0.33 ± 0.09
Postrefractory (s)	16.0 ± 4.9
CSD ($N = 9$)	
Negative current (mV^2)	-0.51 ± 0.06
Positive current (mV^2)	0.206 ± 0.076
CBF ($N = 16$)	
Amplitude (% of baseline)	28.6 ± 2.9
tpO_2 ($N = 16$)	
Amplitude (mmHg)	-4.9 ± 0.9
Baseline (mmHg)	28.4 ± 1.7
CMRO_2 ($N = 16$)	
Amplitude (% of baseline)	19.9 ± 2.1

Unisense A/S) sensing the currents of the oxygen electrode. Signals were A/D converted and recorded at 10 Hz (Power 1401 data acquisition interface and Spike 2 software). Noise, including heartbeat and mechanical ventilation artifacts, were minimized using smoothing (time constant 1 s; Spike 2). Current recordings were transformed to millimeters of mercury using the calibrations with saturated and oxygen-free standard solutions.

Calculation of CMRO_2

CMRO_2 was calculated off-line from simultaneously obtained recordings of tpO_2 and CBF. Baseline values of tpO_2 and CBF were taken as the mean of a 20 s period obtained before the onset of stimulation. These values were then combined with reported values for CBF and CMRO_2 previously obtained (Zhu et al., 2002) in rats (53 ml (100 g) $^{-1}$ min $^{-1}$ and 219 μmol (100 g) $^{-1}$ min $^{-1}$, respectively) to calculate the corresponding effective diffusion coefficient of oxygen in brain tissue (L). The pertinent relationship is as follows:

$$\text{tpO}_2 = P_{50} h \sqrt{\frac{2C_a \text{CBF}}{\text{CMRO}_2}} - 1 - \frac{2\text{CMRO}_2}{3L}, \quad (1)$$

where P_{50} is the half-saturation tension of the oxygen–hemoglobin dissociation curve, h is the Hill coefficient of the same dissociation curve, and C_a is the arterial oxygen concentration (Gjedde et al., 2005). Standard values used for these calculations were as follows: $P_{50} = 36.0$ mmHg; $h = 2.7$; and $C_a = 8 \mu\text{mol ml}^{-1}$; these values correspond to an L value of 11.3 μmol (100 g) $^{-1}$ min $^{-1}$ mmHg $^{-1}$.

Two-photon imaging

Twelve mice were examined with a commercial two-photon microscope (SP5 multiphoton/confocal Laser Scanning Microscope, Leica), a MaiTai HP Ti:Sapphire laser (Millennia Pro, Spectra Physics), and a 20×1.0 numerical aperture water-immersion objective (Leica). The excitation wavelength was set to 820 nm. The frame size was typically 128×128 pixels (185 ms/frame), or 256×256 pixels (344 ms/frame), while a size of 512×512 pixels (661 ms/frame) was used to obtain an overview of dendrite activation.

Bolus loading of synthetic Ca^{2+} indicator

Oregon Green BAPTA-1/AM (10 mM OGB-1/AM, Invitrogen) in dimethylsulfoxide plus 20% Pluronic F-127 (BASF Global) was diluted in aCSF to yield a final dye concentration of 0.8 mM. The dye was then bolus loaded through a micropipette at two to four sites at depths of 50–100 μm below the surface of the cerebellar cortex (4–6 psi, 1–5 s; Pneumatic Pump, World Precision Instruments).

Image analysis

Stimulation of the inferior olive resulted in robust activation of CFs, which evoked Ca^{2+} increases in PC dendrites in the field of view. Two methods were applied to extract the Ca^{2+} elevations: spatial independent component analysis (ICA) and a correlation-based method.

Spatial independent component analysis. Spatial ICA using the fast ICA algorithm developed by Aapo Hyvärinen (www.cis.hut.fi/projects/ica/fastica/) was applied to 128×128 -pixel frames. Evoked responses were attributable to a single spatial component, with activated dendrites appearing as tubular structures with high pixel intensity and matching the sagittal orientation of PC dendrites in Pcp2-EGFP mice (Fig. 1D). To assess drug effects on evoked calcium responses, we used the parameter coverage, which represents the fraction of pixels within a component that had intensity values above a set threshold in response to stimulation relative to the total number of pixels. This intensity threshold was set as the whole-frame mean intensity plus 5 SDs. Coverage was a more reliable measure of direct drug effects than fluorescence intensity changes, since the remaining responding areas after drug applications often showed similar intensity changes to control conditions, while coverage changed significantly.

Correlation-based method. A correlation-based method was applied to 256×256 -pixel frames. For each pixel, time tracks after mean centering and normalization to the SDs were cross-correlated with an “on-off” signal of the following form:

$$0.5(1 + (10(t - t_{\text{start}})) \cdot \tanh(-10(t - t_{\text{stop}}))) \quad (2)$$

where t_{start} and t_{stop} define the stimulation period. This transformation results in a matrix of correlation coefficients measuring the similarity of the time course to a step-like signal that increases over the period of stimulation. These maps were binarized by 95% significance levels, and only pixels with at least five neighbors within a radius of three pixels were preserved. Ninety-five percent significance levels were derived from Monte Carlo simulations in which the same procedure was applied to 10^6 surrogate random time courses with values sampled from the Poisson distribution for each stimulation period. The time courses, spatially averaged from activated areas, were fitted with smoothing cubic splines (using the SciPy library function *splrep* with smoothing parameter $s = 2$) over the stimulation periods. The maxima of these smooth spline representations were taken as the response amplitudes. This data processing was performed with custom-written scripts for open-source SciPy (scipy.org), NumPy (numpy.org), and Matplotlib (matplotlib.sf.net) Python libraries. The relative increases in fluorescence ($\Delta F/F$) were calculated by averaging all dendrite-associated pixels within each frame and dividing the fractional change of fluorescence over time by the prestimulus baseline value of the same pixels.

Figure 2. CF-evoked calcium, electrophysiological, and metabolic responses in the mouse cerebellum *in vivo*. **A**, Depth profile of CF-evoked LFPs recorded at 50 μm intervals (left) and calculated CSD map (right) with characteristic negative currents (hot colors, −) and positive currents (cold colors, +). At a depth of 50 μm, the negative current represents the fEPSC and the delayed positive current represents the postsynaptic g_{KCa} . **B**, PC spike activity before stimulation included both spontaneous simple and complex spikes (CS, SS, top) as shown in the spike INTH (B1, bottom). CS rate increased and SS rate decreased in response to CF stimulation (B2, bottom) followed by a poststimulus SS refractory period (B3, top and bottom). The PC spike activity returned to baseline levels ~30 s after the end of stimulation (B4, top and bottom). **C–F**, CF stimulation (yellow-shaded region, 10 Hz, 15 s) also evoked Ca^{2+} rises in identified PC dendrites ($\Delta F/F$) (**C**) that returned to normal almost immediately after the end of stimulation, as well as longer lasting changes in CBF (**D**), tpO_2 (**E**), and CMRO_2 (**F**). Calibration, **C**, **D**, **F**, 10% changes; **E**, 5 mmHg.

Figure 3. CF stimulation induces frequency-dependent responses in Ca^{2+} coverage, fEPSCs, CBF, tpO_2 , and CMRO_2 . **A**, Ca^{2+} coverage (see Materials and Methods) during CF stimulation at 2, 5, and 10 Hz. Responding dendrites are indicated by red pixels superimposed on an averaged projection image obtained from the raw data. The number of pixels with a statistically significant Ca^{2+} signal (the coverage) increased with increasing stimulation frequency. **B**, Boxplot of Ca^{2+} coverage as a function of stimulation frequency ($N = 6$). **C**, CSD map of evoked postsynaptic activity in PC in response to stimulation with negative currents (hot colors, $-$) and positive currents (cold colors, $+$). At a depth of $50 \mu\text{m}$, the negative current represents the fEPSC and the delayed positive current represents the postsynaptic $\text{gK}_{\text{Ca}^{2+}}$. **D**, Boxplot of the sum of negative currents ($\Sigma \text{fEPSC} = \text{fEPSC amplitude} \times \text{stimulation frequency} \times \text{duration of stimulation train}$) for each stimulation period ($N = 6$). **E–G**, CF stimulation also evoked frequency-dependent changes in CBF (**E**), tpO_2 (**F**), and CMRO_2 (**G**). The yellow bar indicates stimulus duration of 15 s. **H, I**, Boxplots show peak increases of CBF (**H**) and CMRO_2 (**I**) ($N = 6$). Peak increases of CBF (red circle), CMRO_2 (green triangle), and Ca^{2+} coverage (orange star) were plotted as a function of the sum of negative currents (ΣfEPSC) in **J**. Whiskers indicate SEM.

Drug application

All drugs were dissolved in aCSF. Muscimol (5-aminoethyl-3-hydroxyisoxazole; Sigma-Aldrich), a classical potent agonist of GABA_A receptors, was used to induce tonic inhibition; the inhibition was relieved by application of bicuculline ($[\text{R}-(\text{R}^*, \text{S}^*)]-5-(6,8\text{-dihydro-8-oxofuro}[3,4\text{-e}]-1,3\text{-benzodioxol-6-yl})-5,6,7,8\text{-tetrahydro-6,6-dimethyl-1,3-dioxolo}[4,5\text{-g}]\text{isoquinolinium Cl}^-$; Sigma-Aldrich), a potent and specific GABA_A antagonist, or gabazine (6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide; SR 95531, Sigma-Aldrich), a competitive GABA_A antagonist. These drugs were applied by placing drops of the dissolved compound (2 mM) on the agarose covering the vermis region. In separate experiments, we typically applied $40 \mu\text{M}$ ω -agatoxin-IVA (ω -AGA; A6719, Sigma-Aldrich), a potent and selective P/Q-type channel blocker, to block postsynaptic Ca^{2+} entry in PCs (McDonough et al., 2002) or $0.036\text{--}0.36 \text{ mM}$ Ru360 $[(\mu)[(\text{HCO}_2)(\text{NH}_3)_4\text{Ru}]\text{OCl}_3$; catalog #557440, Calbiochem], a selective and potent mitochondrial uniporter blocker, to block Ca^{2+} influx into mitochondria (Ying et al., 1991).

Statistical analysis

The baseline and slope of the evoked tpO_2 were estimated using linear correlation, and tpO_2 amplitude was calculated as the difference between the stimulus-evoked negative tpO_2 response and the immediately preceding baseline (OriginPro 8.1). CBF and CMRO_2 responses were normalized to the immediately preceding 20 s baseline, and the maximal response amplitude was estimated using nonlinear curve fitting (Extreme; OriginPro 8.1). Original, non-normalized data were analyzed statistically using correlation analyses to compare Ca^{2+} transients obtained using two-photon imaging with electrophysiological signals obtained in other experiments using the same experimental protocol. Drug effects of muscimol and ω -AGA were analyzed using a paired t test. As different concentrations of Ru360 were given to separate groups of mice, Ru360 data were analyzed using a mixed-effects model (R, R Development Core Team, R Foundation for Statistical Computing) followed by a paired t test to test for significance at specific time points. Significance level was

set at $\alpha = 0.05$ (two tailed). Data are presented as mean values $\pm$ SEM in the scatterplots and tables.

Results

Frequency dependence of neuronal Ca^{2+} , postsynaptic currents, CBF, and CMRO_2 during evoked climbing fiber activity

We measured a number of parameters to describe the relationship among transmembrane synaptic currents, cytosolic Ca^{2+} , and CMRO_2 . Evoked calcium responses in the molecular layer of the cerebellar cortex were imaged using two-photon microscopy. In parallel, we recorded CBF, tpO_2 , CSD, LDF, and single-unit activity from PC (Fig. 1A–C) (Offenhauser et al., 2005). All data are summarized in Table 1.

Figure 1C illustrates the sagittal orientation of PC dendrites in a Pcp2-eGFP transgenic mouse line. Stimulation of the CFs resulted in fluorescence increases in parasagittal bands indicating activated PC dendrites (Fig. 1D). The bands were usually several dendrites thick, in line with previous studies using pH-sensitive dyes (Chen et al., 1996; Hanson et al., 2000). The cytosolic Ca^{2+} increases in PC dendrites decayed rapidly after stimulation (Fig. 2C). In contrast, the CBF, tpO_2 , and calculated CMRO_2 time courses were more prolonged (Fig. 2D–F).

CF stimulation also gave rise to PC EPSPs (Fig. 2A, left), which were transformed into a CSD ratio map (Fig. 2A, right). The CSD maps showed a negative current corresponding to the averaged fEPSC with a maximum 5–8 ms after stimulation, reflecting Na^+ entry via PC AMPA receptors. A delayed maximum positive current was recorded at the same depth 10–20 ms after stimulation, reflecting an outward $\text{gK}_{\text{Ca}^{2+}}$ gated by voltage-sensitive P/Q-type Ca^{2+} channels (Llinas, 1981; Womack et al.,

Table 2. Changes from control levels of Ca^{2+} rises, spike activity, current source density, CBF, and CMRO_2

	N	Changes from control level (%)
Ca^{2+}		
Peak		
Muscimol 3'–60'	8	$-100 \pm 0^{***}$
Bicuculline/gabazine	8	$-4.4 \pm 7.2\$\$$
ω -AGA 30'–50'	4	-19.9 ± 34.8
ω -AGA 60'–90'	4	$-62.2 \pm 13.1^*$
Coverage		
Muscimol 3'–60'	8	$-100 \pm 0^{***}$
Bicuculline/gabazine	8	$-1.5 \pm 15.0\$\$$
ω -AGA 30'–50'	4	$-66.8 \pm 23.8^*$
ω -AGA 60'–90'	4	$-73.2 \pm 11.5^{**}$
Spikes		
Spontaneous		
Muscimol	8	$-88.6 \pm 10.2^*$
Bicuculline/gabazine	7	$2.5 \pm 7.6\%$
ω -AGA 30'–50'	7	-11.8 ± 7.7
ω -AGA 60'–90'	7	$-49.9 \pm 11.4^*$
Evoked		
Muscimol	8	-20.1 ± 10.8
Bicuculline/gabazine	7	16.7 ± 57.3
ω -AGA 30'–50'	7	-10.0 ± 6.8
ω -AGA 60'–90'	7	-6.7 ± 17.4
Postrefractory		
Muscimol	8	ND
Bicuculline/gabazine	7	ND
ω -AGA 30'–50'	7	$-89.0 \pm 11.0^*$
ω -AGA 60'–90'	7	$-89.9 \pm 8.2^*$
CSD		
Negative current		
Muscimol 10'–20'	6	8.3 ± 10.8
Muscimol 40'–60'	9	$28.2 \pm 11.5^*$
Bicuculline/gabazine	8	1.5 ± 18.2
ω -AGA 30'–50'	2	-19.9 ± 8.7
ω -AGA 60'–90'	2	-26.2 ± 11.0
Positive current		
Muscimol 10'–20'	6	$-54.0 \pm 14.3^*$
Muscimol 40'–60'	9	$-77.4 \pm 8.8^*$
Bicuculline/gabazine	8	$-25.1 \pm 16.2\$$
ω -AGA 30'–50'	2	-29.1 ± 9.1
ω -AGA 60'–90'	2	-46.3 ± 2.9
CBF		
Amplitude		
Muscimol 10'–20'	6	-9.2 ± 14.5
Muscimol 40'–60'	8	$-37.1 \pm 6.1^*$
Bicuculline/gabazine	8	12.8 ± 9.5
ω -AGA 30'–50'	7	$-39.7 \pm 17.9^*$
ω -AGA 60'–90'	7	$-37.2 \pm 10.6^*$
Ru360 30'	3	$42.0 \pm 31.8^*$
Ru360 60'	3	21.0 ± 30.7
CMRO_2		
Amplitude		
Muscimol 10'–20'	6	33.2 ± 29.1
Muscimol 40'–60'	8	$-47.6 \pm 6.8^*$
Bicuculline/gabazine	8	17.3 ± 14.9
ω -AGA 30'–50'	7	25.1 ± 20.1
ω -AGA 60'–90'	7	$-38.3 \pm 14.0^*$
Ru360 30'	3	10.5 ± 6.1
Ru360 60'	3	-14.9 ± 6.1
$\Delta\text{CMRO}_2/\text{LFP}$		
Ru360 30'	3	0.7 ± 4.0
Ru360 60'	3	-2.8 ± 3.4

Negative values indicate inhibition, while positive values indicate increases. Using the paired *t* test, statistically significant differences from control levels at $p < 0.05$, 0.01, or 0.001 are indicated by *, **, and ***, respectively; significant differences between the muscimol group and the bicuculline/gabazine group are indicated by \$, \$\$, and \$\$\$, respectively. Ru360 data represent the average of three concentrations. Changes in current source density are evaluated using negative currents representing the fEPSC and positive currents representing the Ca^{2+} -gated K^+ current.

2009). The CSD maps also revealed a second positive current at a depth of 100 μm with the same timing as the fEPSC (i.e., 5–8 ms after stimulation), which most likely corresponded to another outward K^+ current (Frey et al., 2009). PC single-unit recordings showed random firing of both SSs and CSs, as shown in the spike INTH (Fig. 2B1). CF stimulation increased CS and reduced SS firing (Fig. 2B2); this was followed by a poststimulus refractory period of SS firing (Fig. 2B) with longer spike intervals (Fig. 2B3). In normal physiological conditions, the refractory period lasted ~ 30 s, after which PC spike activity returned to baseline levels (Fig. 2B4, top).

CFs were stimulated at frequencies of 2, 5, and 10 Hz, evoking increases in synaptic activity, postsynaptic complex spiking, dendritic Ca^{2+} , CBF, and CMRO_2 (Fig. 3). Under control conditions, the evoked Ca^{2+} , ΣfEPSC , CBF, and CMRO_2 responses increased in a frequency-dependent manner. An increased number of pixels with a significant Ca^{2+} signal was observed as a function of the stimulus frequency (Fig. 3A,B), indicating that higher rates of CF stimulation recruited more cells. In parallel sets of experiments, we demonstrated that postsynaptic activity as measured by the ΣfEPSC responses correlated linearly with the stimulation frequency (Fig. 3C,D), and that in the same mice, the increases in ΣfEPSC were accompanied by proportional increments in CBF and CMRO_2 (Fig. 3F,I). Combined results from two-photon microscopy and electrophysiological experiments are shown in Figure 3F. A linear correlation was found between normalized ΣfEPSC responses and CMRO_2 ($r^2 = 0.999$, $p = 0.015$) (Fig. 3J). The observations that Ca^{2+} and CMRO_2 correlated linearly to the stimulus frequency and that the correlation analysis revealed a clear linear relationship between these two parameters and ΣfEPSC (Fig. 3J) validates comparison of the imaging and electrophysiological data across different experiments.

The effect of GABA_A receptor blockade on stimulus-evoked Ca^{2+} , CBF, and CMRO_2

To explore the coupling among cytosolic Ca^{2+} , CBF, and CMRO_2 transients, we modulated the level of tonic synaptic inhibition with muscimol (Krogsgaard-Larsen and Johnston, 1978), a potent GABA_A agonist, that suppresses PC simple spike output and, notably, suppresses PC dendrite Ca^{2+} responses during CF stimulation (Schreurs et al., 1992; Callaway et al., 1995). We evaluated pharmacological effects on Ca^{2+} responses by quantifying the total area covered by activated cells in the field of view (Table 2). Muscimol abolished the evoked cytosolic Ca^{2+} responses in PC dendrites within 3 min of application (Fig. 4A,F), and Ca^{2+} responses remained undetectable throughout the period of muscimol exposure. The effects of muscimol were reversed by application of the GABA_A receptor antagonists bicuculline or gabazine (Fig. 4A, right; Table 2). While the magnitude of the fEPSC was not affected during muscimol application (Fig. 4B,C; Table 2), the delayed positive current representing the P/Q Ca^{2+} channel-mediated $\text{gK}_{\text{Ca}^{2+}}$ (McDonough et al., 2002) was reduced (Fig. 4C, bottom). This synaptically activated $\text{gK}_{\text{Ca}^{2+}}$ returned to normal after application of either bicuculline or gabazine (Table 2; Fig. 4C). Spontaneous PC SS firing decreased within 3–10 min of muscimol application (Fig. 5A, top), while spontaneous CS firing was still present (Fig. 5A, middle). Evoked PC CS firing also remained constant throughout muscimol treatment (Fig. 5A, bottom). Since spontaneous CS firing remained unaffected over the course of the experiment, it is unlikely that muscimol diffused to the cerebellar nuclei, which, via inhibitory projections to the inferior olive, could alter CS firing patterns (Stam et al., 2010). In summary, the delayed $\text{gK}_{\text{Ca}^{2+}}$ current and spontaneous SS

Figure 4. Muscimol decreases dendritic Ca^{2+} responses in PCs before attenuation of CBF and CMRO_2 responses. CFs were stimulated at 10 Hz for 15 s, indicated by the yellow bars in **D** and **E**. **A**, Ca^{2+} coverage is indicated by blue pixels before, and at 3–20 and 40–60 min after muscimol application. (Note that no dendrite Ca^{2+} coverage is seen at these last two time points.) After reversal with the GABA_A receptor antagonists bicuculline or gabazine, Ca^{2+} coverage is indicated by red pixels. The inhibition of Ca^{2+} responses by muscimol was reversed by GABA_A receptor antagonists, indicating the specificity of the muscimol effect on GABA_A receptors. In comparison, muscimol had no effect on the fEPSCs (hot colors, –, at depth of 50 μm) in **B**, but did decrease the positive currents (cold colors, +, at depth of 50 μm) due to silencing of the g_{KCa} . Data are summarized for all animals in the boxplots of negative current (fEPSC, top) and positive current (bottom) in **C**; the latter was significantly reduced by muscimol at both time periods. Bic, Bicuculline; Gab, gabazine. **D, E**, The corresponding rises in CBF (**D**) and CMRO_2 (**E**) remained unchanged at 3–20 min (second panel) compared with control conditions (first panel), while at 40–60 min (third panel) both variables decreased. The mean CBF and CMRO_2 are represented as solid lines, while dotted lines represent $\pm$ SEM values, and control CBF and CMRO_2 responses are shown as superimposed gray traces. Complete reversal was observed after topical application of bicuculline or gabazine (Gabaz) (fourth panel). $n = 8$. **F**, Typical example of time-dependent effects of muscimol on the peak increases of CBF (red cross), CMRO_2 (green triangle), fEPSC (red circle), and positive current (blue circle) and Ca^{2+} coverage (orange star), relative to control levels during the first 20 min of muscimol application illustrates the rapid decrease in dendritic Ca^{2+} responses and g_{KCa} compared with the other variables. Statistical information appears in Table 2.

activity decreased significantly after muscimol application, while fEPSC and CS firing were unaffected.

Although activity-evoked rises of CBF and CMRO_2 were unaffected up to 20 min during muscimol exposure (Figs. 4D,E; Table 2; $n = 6$), they did decrease at a later time point (Fig. 4D,E; Table 2; $n = 8$). Baseline CMRO_2 and CBF levels were unaffected by GABA_A receptor antagonists. Importantly, evoked Ca^{2+} signals declined much faster than either CBF or CMRO_2 responses beginning at ~ 3 min postapplication. Ca^{2+} responses were reduced in conjunction with g_{KCa} (delayed positive current). A difference of the decline in the Ca^{2+} responses relative to the Ca^{2+} -gated K^+ current could be explained by the larger sampling volume of the CSD recording (Fig. 4F). The significant difference in timing between the effects of muscimol on Ca^{2+} transients and CMRO_2 responses suggests only a weak correlation between cytosolic Ca^{2+} and CMRO_2 .

The effect of P/Q-type voltage-gated Ca^{2+} channel block on stimulus-evoked Ca^{2+} , CBF, and CMRO_2

To further explore the importance of Ca^{2+} , from a predominantly neuronal origin, in mediating stimulus-evoked rises in CBF and CMRO_2 , we blocked P/Q-type Ca^{2+} channels with ω -AGA (McDonough et al., 2002). ω -AGA induced a gradual decline in the number of PC dendrites exhibiting a Ca^{2+} response (44% of control at 30–50 min), with nearly complete absence of Ca^{2+} signals after 60–90 min (Fig. 6A,E; Table 2). The fEPSCs (excitatory negative currents) were unaffected after 30–50 min, but reduced after 60–90 min (Fig. 6B,E; Table 2). Thus, ω -AGA had an immediate (30 min) effect on PC cytosolic Ca^{2+} responses (McDonough et al., 2002) and a delayed (60 min) effect

on neurotransmitter release from CF terminals (Doroshenko et al., 1997). ω -AGA also decreased the evoked CBF responses after 30–50 min with no further reduction at 60–90 min. This finding suggests that part of the blood flow response could be dependent upon the measured Ca^{2+} response (Fig. 6C,E; Table 2).

Importantly, CMRO_2 responses were unchanged at 30–50 min, but were reduced after 60–90 min of ω -AGA exposure (Figs. 6D,E; Table 2). Thus, the time course of the decrease in CMRO_2 responses followed the decrease in fEPSCs, but not that of the PC cytosolic Ca^{2+} responses (Fig. 6E; Table 2), which is consistent with the notion that neurometabolic coupling in cerebral and cerebellar cortex primarily reflects synaptic rather than spiking activity (Viswanathan and Freeman, 2007; Lecoq et al., 2009; Thomsen et al., 2009). CMRO_2 responses also decreased together with decreases in spontaneous and evoked PC spiking (Fig. 5B). The poststimulus refractory period in SS firing was shortened after 30 min of ω -AGA application, most likely because of reduced Ca^{2+} entry in the PC dendrites (Table 2). Figure 6E shows the reductions in CMRO_2 , fEPSC, and Ca^{2+} coverage induced by ω -AGA with time; a linear correlation between CMRO_2 and fEPSC amplitude was observed ($r^2 = 0.976$, $p < 0.0001$) (Fig. 6F), but none was detected between CMRO_2 and Ca^{2+} coverage. Our finding that there was a dissociation between CMRO_2 and cytosolic Ca^{2+} responses questions the hypothesis that evoked rises in CMRO_2 require neuronal Ca^{2+} signaling.

The effect of blocking the mitochondrial uniporter on stimulus-evoked CBF and CMRO_2

Mitochondria lie in close juxtaposition to Ca^{2+} channels of cellular organelles, including those of the plasma membrane (Rizzuto and

Pozzan, 2006). Microdomains with high Ca^{2+} concentrations form at the mouths of these channels; besides contributing to global Ca^{2+} responses, these microdomains may directly supply mitochondria with Ca^{2+} (Pivovarova et al., 1999). Since our results showed that evoked CMRO_2 does not rely on cytosolic Ca^{2+} , we hypothesized that increases in mitochondrial Ca^{2+} occurring concurrently but independently of global cytosolic Ca^{2+} responses could. Therefore, to test the importance of mitochondrial Ca^{2+} for activation-induced oxygen consumption, the mitochondrial uniporter was blocked. Ru360, a cell-permeable oxygen-bridged dinuclear ruthenium red amine complex, specifically blocks Ca^{2+} uptake into mitochondria in intact cells (Matlib et al., 1998). We found that topical application of this drug caused baseline CMRO_2 and CBF to decrease throughout the first 60 min of exposure to Ru360 with no further reduction after this ($-7.2 \pm 4.1\%$ and $-16.0 \pm 3.8\%$, respectively; $p < 0.01$ in both cases). In contrast, a biphasic effect of Ru360 was observed with an initial increase (up to 30 min) followed by a decrease on stimulus-evoked CMRO_2 and CBF as well as on evoked field LFPs ($p < 0.05$). Thus, the ratio of CMRO_2 to corresponding LFPs remained constant, implying that work-dependent respiration is unaffected when blocking mitochondrial uniporters (Table 2). The constant CMRO_2/LFP ratio in the face of decreasing LFP suggests that Ru360 exerted its effects on synaptic mechanisms. Similarly, the ratio between CMRO_2 transients and fEPSCs was constant in the presence of ω -AGA (Fig. 6F). We surmise that activation-evoked increases in CMRO_2 are more closely linked to active transport associated with synaptic and action currents rather than to neuronal Ca^{2+} signals.

Discussion

Our study investigated the relationships among CF-evoked neuronal Ca^{2+} increases, CMRO_2 , and CBF in the intact mouse cerebellum. We demonstrate that (1) evoked PC Ca^{2+} signals, CMRO_2 , and CBF scale linearly with stimulus frequency (Fig. 3F,I); (2) that tonic activation of GABA_A receptors abolishes evoked Ca^{2+} signals long before abolishing CBF and CMRO_2 transients (Fig. 4F); and (3) that blockade of P/Q-type Ca^{2+} channels with ω -AGA reduces cytosolic Ca^{2+} and CBF but not CMRO_2 responses (Fig. 6E). We conclude that increased cytosolic Ca^{2+} in neurons is not required to control activity-driven CMRO_2 , an idea consistent with CMRO_2 being mainly regulated by ion pumps (Erecińska and Silver, 1989). This is supported by our finding of a conserved ratio between evoked field potentials and CMRO_2 transients in the face of changing mitochondrial oxygen consumption induced by Ru360.

Figure 5. Spontaneous and evoked PC spike activity. **A**, Spontaneous and evoked PC spike activity (evaluated as the rms of the recording taken from 0.3 to 5 kHz) under control conditions (top trace) and in the presence of muscimol (middle trace). The spontaneous PC spike rate decreased after application of muscimol; in comparison, the complex spike amplitude increased, whereas the overall rms value remained constant during stimulation. The bottom traces indicate the complex spike signature evoked by CF stimulation under control conditions (trace 1) and in the presence of muscimol (trace 2). Trace 3 represents a spontaneously occurring complex spike during muscimol application. Both evoked and spontaneous complex spike amplitudes were increased after muscimol application. **B**, Spontaneous and evoked PC spike activity (rms) under control conditions (top trace) and in the presence of ω -AGA (bottom trace). Horizontal red arrows indicate the duration of the poststimulus refractory period, which is the time period from the end of stimulation to the recovery of spike activity back to prestimulus levels. The poststimulus refractory period reflects g_{KCa} activity mediated by P/Q Ca^{2+} channel activity, which was nearly abolished by ω -AGA. The yellow bars in all panels indicate the duration of CF stimulation at 10 Hz and 15 s.

Control of brain energy supply and consumption by activity

Increases in neuronal activity primes cellular respiration to supply ATP to the ATPases, which restore ionic gradients across the nerve cell membrane. This accounts for up to 95% of ATP turnover (Erecińska and Silver, 1989). ATP breakdown transiently increases the level of ADP and the feedback of ADP and inorganic phosphate to the mitochondria. This strategy is the default mechanism by which activity controls cellular respiration and ATP supply (Gunter et al., 2004). In addition, Ca^{2+} signaling can increase oxidative phosphorylation in two ways (Gunter et al., 2004; Pardo et al., 2006; Satrustegui et al., 2007; Gunter and Sheu, 2009). Mitochondrial Ca^{2+} influx via the Ca^{2+} uniporter is followed by activation of dehydrogenases localized to the mitochondrial matrix, resulting in increased TCA cycle activity and ATP production (Satrustegui et al., 2007). Alternatively, small cytosolic Ca^{2+} signals can induce ATP production through a mechanism independent of mitochondrial Ca^{2+} elevation. Such a mechanism requires Ca^{2+} -dependent mitochondrial carriers (e.g., the malate–aspartate shuttle), which transfers NADH

Figure 6. ω -AGA reduces evoked Ca^{2+} and CBF responses independently of preserved fEPSC and CMRO₂ responses. CFs were stimulated at 10 Hz for 15 s, as indicated by the yellow bar in **C** and **D**. **A**, Ca^{2+} coverage (red pixels) before and after application of ω -AGA reveals reduced dendritic Ca^{2+} coverage at 30–50 and 60–90 min with ω -AGA. **B**, CSD analysis with negative currents (hot colors, $-$) and positive currents (cold colors, $+$). At a depth of 50 μm , the negative current represents the fEPSC and the delayed positive current represents the postsynaptic gK_{Ca} . The fEPSCs remained constant during the first 50 min of ω -AGA exposure, while at 60–90 min the fEPSCs were reduced. In comparison, gK_{Ca} was reduced from 30 min onward (**B**, second and third panels). In **C** and **D**, traces are represented as mean value $\pm$ SEM (dashed lines). **C**, ω -AGA induced decreased CBF responses at 30–50 and 60–90 min after exposure, suggesting a Ca^{2+} -dependent component of CBF control. Gray traces in middle and right panels indicate the control response. **D**, CMRO₂ was unaffected at 30–50 min, but was reduced 60–90 min after ω -AGA exposure. The reduction in CMRO₂ coincided with the reduction in fEPSCs and occurred later than the reduction in dendritic Ca^{2+} responses. **E**, Time course of reduction in CMRO₂ responses (green triangles), Ca^{2+} coverage (orange stars), and fEPSC (red circles) relative to control levels during the 90 min following application of ω -AGA ($N = 2$). Ca^{2+} responses started to decrease before the fEPSC and CMRO₂ responses, which decreased in parallel ($N = 4$). **F**, Linear correlation between fEPSC and CMRO₂ responses ($r^2 = 0.976$, $p < 0.0001$; see Table 2).

equivalents into the mitochondria (Pardo et al., 2006; Satrustegui et al., 2007). Thus, neurons could match or possibly anticipate the ATP consumption of ion pumps by using cytosolic Ca^{2+} elevations to increase mitochondrial respiration (Gunter et al., 2004). Recently, a study of isolated brain mitochondria suggested that brain ATP production and thus CMRO₂ are controlled by a feedforward mechanism exclusively involving non-mitochondrial Ca^{2+} (Gellerich et al., 2010). Furthermore, in isolated PCs *in vitro*, increased cytosolic Ca^{2+} evoked by high extracellular K^+ induced both mitochondrial depolarization due to mitochondrial Ca^{2+} entry via the uniporter and an immediate burst in O_2 consumption (Hayakawa et al., 2005). In the cerebellum, the mitochondrial marker cytochrome oxidase is localized to PCs, while Bergmann glia exhibit considerably lower oxidative capacities (Kasischke, 2008). It is therefore a reasonable assumption that the CMRO₂ responses we measured primarily reflect respiration in neurons.

Mechanisms of activity-driven CMRO₂ responses

Application of the GABA_A receptor agonist muscimol abolished or reduced spontaneous PC SS activity almost instantaneously and abolished PC dendritic Ca^{2+} responses within a few minutes (Figs. 4A, 5A). At the same time, baseline levels of CBF and CMRO₂ were unchanged (Fig. 4D,E) (Caesar et al., 2008b; Thomsen et al., 2009). CF-evoked fEPSC and complex spiking responses remained constant (Figs. 4B, C, 5A), indicating that the ion fluxes, and hence the workload, on the Na^+/K^+ ATPase was unchanged. Only after 40 min of drug exposure did CBF and CMRO₂ responses begin to decline (Fig. 4D,E). Gabazine, a specific antagonist of GABA_A receptors, reversed the effects of muscimol on cytosolic Ca^{2+} , CBF, and CMRO₂ responses (Fig.

4A,D,E), suggesting that the muscimol effect required specific interaction with GABA_A receptors. Previous studies have indicated that the stimulation-evoked rises in CBF can be reduced by 80–90% without concomitant reductions in the evoked electrophysiological signal (Offenhauser et al., 2005; Caesar et al., 2008b; Leithner et al., 2010). CF stimulation evokes large rises in lactate that are coupled to rises in synaptic activity and spike rate (Caesar et al., 2008a). Thus, in the cerebellum preservation of normal function and restoration of ionic gradients is powered by both glycolysis and respiration (Reinert et al., 2011). Likewise, stimulation of parallel fiber beams increases respiration and aerobic glycolysis (Thomsen et al., 2009). Therefore, under normal conditions there is a dynamic balance between respiration and glycolysis, and when CMRO₂ is reduced, glycolysis is assumed to be increased acutely. Therefore, the linear correlation for EPSC versus CMRO₂ applies for unperturbed and “drug-naïve” systems, while becoming uncoupled when the level of tonic synaptic inhibition increases (Caesar et al., 2008b).

Our current data, obtained in mice, displayed a slower time course of the muscimol effect than our previous observations in rats, which exhibited a faster onset of the depression of CBF and CMRO₂ responses following muscimol exposure (Caesar et al., 2008b). Thus, the cytosolic Ca^{2+} and CMRO₂ responses were more obviously dissociated in time in mice. We examined this dissociation using a specific blocker of P/Q-type Ca^{2+} channels, ω -AGA (McDonough et al., 2002). ω -AGA did not influence basal CBF, CMRO₂, or CS firing, but did lead to a decline in the evoked Ca^{2+} rises in PC dendrites (Fig. 6A,E). The onset of this decline was delayed compared with that observed during muscimol application (Fig. 4F), which may be explained by a slower diffusion rate of ω -AGA. Thus, 30 min after application, Ca^{2+}

and CBF responses were moderately affected (Fig. 6C), while CMRO₂ responses remained constant (Fig. 6D). The physiological effect of ω -AGA was additionally verified by the observation that the drug reduced the poststimulation pause in spontaneous SS activity (Fig. 5B; Table 2). ω -AGA led to a gradual reduction in the fEPSC amplitude that closely matched the concomitant reduction in CMRO₂ responses (Fig. 6E,F) due to blocking presynaptic Ca²⁺ channels on CF terminals (Doroshenko et al., 1997). These experiments demonstrated that cytosolic Ca²⁺ transients did not modulate CMRO₂ responses to CF stimulation. Notably, the reduction in CMRO₂ seen after prolonged ω -AGA exposure was not due to a direct effect of Ca²⁺ on cellular respiration, but due to a reduced workload (i.e., reduced ion flux over the plasma membrane, as indicated by decreased fEPSC amplitude). The close proximity of mitochondria to Ca²⁺ channels in the plasma membrane and endoplasmic reticulum (Rizzuto and Pozzan, 2006) raises the possibility of mitochondrial Ca²⁺ transients regulating cellular respiration independently of transient increases in cytosolic Ca²⁺. Therefore, we blocked mitochondrial Ca²⁺ entry with Ru360, which resulted in a gradual time-dependent decrease in basal CMRO₂. By contrast, stimulation-evoked CMRO₂ responses, showed an initial enhancement followed by a reduction in the presence of Ru360. Importantly, LFP amplitude followed a similar time course as CMRO₂, resulting in a conserved ratio between CMRO₂ and LFP during the entire exposure to Ru360. This finding suggests that the different CMRO₂ responses in the presence of Ru360 are due to variations in workload as was found with ω -AGA. Together with our results demonstrating dissociations between Ca²⁺ and CMRO₂ during muscimol and ω -AGA application, these data support the notion that cytosolic Ca²⁺ does not modulate activity-evoked rises in CMRO₂ in the intact cerebellum. Furthermore, blocking Ca²⁺ channels in neurons and mitochondria with ω -AGA and Ru360 did not affect the amount of O₂ consumed per unit work. Our study does not rule out the possibility of presynaptic Ca²⁺ transients affecting CMRO₂ via decreased neurotransmitter release, as suggested by the reduction in fEPSCs and LFPs or the possibility of postsynaptic Ca²⁺ transients in PC dendrites contributing to downstream mechanisms (e.g., dilation of local blood vessels, as suggested by the coordinated reduction of CBF and Ca²⁺ responses seen with ω -AGA). We did find that preserved function of the mitochondrial Ca²⁺ uniporter was necessary for maintaining basal, ongoing CMRO₂ and CBF levels.

In conclusion, we performed Ca²⁺ imaging of PC dendrites in the cerebellar cortex of anesthetized mice to evaluate the contribution of cytosolic Ca²⁺ transients to the control of activity-dependent increases in CMRO₂. Our observations suggest that cytosolic Ca²⁺ increases are not rate limiting for activity-dependent increases in CMRO₂. Here we report that CMRO₂ is strongly related to the level of synaptic and action potential currents. We suggest that activity-driven rises in CMRO₂ are triggered by a feedback mechanism involving changes in the ATP/ADP ratio. One explanation for the observed lack of influence of Ca²⁺ on *in vivo* CMRO₂ may be that the exchange rate between the mitochondrial and cytosolic metabolite pools is fast relative to the neuronal TCA cycle flux (de Graaf et al., 2004). Therefore, in healthy mouse brain tissue, there may be limited requirement for either increased uptake of NADH by mitochondria or an enhanced production of TCA intermediates via Ca²⁺-dependent mechanisms (Satrústegui et al., 2007). One limitation of our study is that, while the majority of the Ca²⁺ signal derives from PCs, we cannot exclude smaller contributions from Bergman glia to the Ca²⁺ signal and even to the increase in CMRO₂. Also, our

study is restricted to one particular structure and neuronal circuit, and we cannot generalize across brain regions or species. Further studies are needed to extend the findings from the CF–PC circuit in the mouse cerebellum to other circuits.

References

- Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA (2010) Glial and neuronal control of brain blood flow. *Nature* 468:232–243.
- Caesar K, Thomsen K, Lauritzen M (2003) Dissociation of spikes, synaptic activity, and activity-dependent increments in rat cerebellar blood flow by tonic synaptic inhibition. *Proc Natl Acad Sci U S A* 100:16000–16005.
- Caesar K, Hashemi P, Douhou A, Bonvento G, Boutelle MG, Walls AB, Lauritzen M (2008a) Glutamate receptor-dependent increments in lactate, glucose and oxygen metabolism evoked in rat cerebellum *in vivo*. *J Physiol* 586:1337–1349.
- Caesar K, Offenhauser N, Lauritzen M (2008b) Gamma-aminobutyric acid modulates local brain oxygen consumption and blood flow in rat cerebellar cortex. *J Cereb Blood Flow Metab* 28:906–915.
- Callaway JC, Lasser-Ross N, Ross WN (1995) IPSPs strongly inhibit climbing fiber-activated [Ca²⁺]_i increases in the dendrites of cerebellar Purkinje neurons. *J Neurosci* 15:2777–2787.
- Chen G, Hanson CL, Ebner TJ (1996) Functional parasagittal compartments in the rat cerebellar cortex: an *in vivo* optical imaging study using neutral red. *J Neurophysiol* 76:4169–4174.
- Clarke DD, Sokoloff L (1994) Circulation and energy metabolism of the brain. In: *Basic neurochemistry: molecular, cellular, and medical* (Siegel GJ, Agranoff BW, Albers RW, Molinoff PB, eds), pp 645–680. New York: Raven.
- de Graaf RA, Mason GF, Patel AB, Rothman DL, Behar KL (2004) Regional glucose metabolism and glutamatergic neurotransmission in rat brain *in vivo*. *Proc Natl Acad Sci U S A* 101:12700–12705.
- Doroshenko PA, Wopmann A, Miljanich G, Augustine GJ (1997) Pharmacologically distinct presynaptic calcium channels in cerebellar excitatory and inhibitory synapses. *Neuropharmacology* 36:865–872.
- Erecińska M, Silver IA (1989) ATP and brain function. *J Cereb Blood Flow Metab* 9:2–19.
- Fabricsius M, Lauritzen M (1996) Laser-Doppler evaluation of rat brain microcirculation: comparison with the [14C]-iodoantipyrine method suggests discordance during cerebral blood flow increases. *J Cereb Blood Flow Metab* 16:156–161.
- Fabricsius M, Akgören N, Dirnagl U, Lauritzen M (1997) Laminar analysis of cerebral blood flow in cortex of rats by laser-Doppler flowmetry: a pilot study. *J Cereb Blood Flow Metab* 17:1326–1336.
- Frey U, Egert U, Heer F, Hafizovic S, Hierlemann A (2009) Microelectronic system for high-resolution mapping of extracellular electric fields applied to brain slices. *Biosens Bioelectron* 24:2191–2198.
- Gellerich FN, Gizatullina Z, Trumbeckaite S, Nguyen HP, Pallas T, Arandarcikaite O, Vielhaber S, Seppet E, Striggow F (2010) The regulation of OXPHOS by extramitochondrial calcium. *Biochim Biophys Acta* 1797:1018–1027.
- Gjedde A (2005) Blood-brain transfer and metabolism of oxygen. In: *Blood-brain barriers: from ontogeny to artificial interfaces*, (Dermietzel R, Spray DC, Nedergaard M, eds), pp 523–550. Hoboken: John Wiley and Sons.
- Gunter TE, Sheu SS (2009) Characteristics and possible functions of mitochondrial Ca(2+) transport mechanisms. *Biochim Biophys Acta* 1787:1291–1308.
- Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD (2004) Calcium and mitochondria. *FEBS Lett* 567:96–102.
- Hanson CL, Chen G, Ebner TJ (2000) Role of climbing fibers in determining the spatial patterns of activation in the cerebellar cortex to peripheral stimulation: an optical imaging study. *Neuroscience* 96:317–331.
- Hayakawa Y, Nemoto T, Iino M, Kasai H (2005) Rapid Ca²⁺-dependent increase in oxygen consumption by mitochondria in single mammalian central neurons. *Cell Calcium* 37:359–370.
- Kann O, Kovács R (2007) Mitochondria and neuronal activity. *Am J Physiol Cell Physiol* 292:C641–C657.
- Kasischke KA (2008) A new pathway for lactate production in the CNS. *J Physiol* 586:1207–1208.
- Krogsgaard-Larsen P, Johnston GA (1978) Structure-activity studies on the inhibition of GABA binding to rat brain membranes by muscimol and related compounds. *J Neurochem* 30:1377–1382.

- Lauritzen M (2005) Opinion: reading vascular changes in brain imaging: is dendritic calcium the key? *Nat Rev Neurosci* 6:77–85.
- Lecoq J, Tiret P, Najac M, Shepherd GM, Greer CA, Charkpak S (2009) Odor-evoked oxygen consumption by action potential and synaptic transmission in the olfactory bulb. *J Neurosci* 29:1424–1433.
- Leithner C, Royle G, Offenhauser N, Fuchtemeier M, Kohl-Bareis M, Villringer A, Dirnagl U, Lindauer U (2010) Pharmacological uncoupling of activation induced increases in CBF and CMRO₂. *J Cereb Blood Flow Metab* 30:311–322.
- Lin AL, Fox PT, Hardies J, Duong TQ, Gao JH (2010) Nonlinear coupling between cerebral blood flow, oxygen consumption, and ATP production in human visual cortex. *Proc Natl Acad Sci U S A* 107:8446–8451.
- Llinas RR (1981) Electrophysiology of cerebellar networks. In: *Handbook of physiology, section I, the nervous system, Vol II, motor control, Part 2*, American Physiological Society (Brooks VB, ed), pp 831–877. Baltimore, MD: Williams and Wilkins.
- Mathiesen C, Caesar K, Akgören N, Lauritzen M (1998) Modification of activity-dependent increases of cerebral blood flow by excitatory synaptic activity and spikes in rat cerebellar cortex. *Journal of Physiology* 512:555–566.
- Matlib MA, Zhou Z, Knight S, Ahmed S, Choi KM, Krause-Bauer J, Phillips R, Altschuld R, Katsube Y, Sperelakis N, Bers DM (1998) Oxygen-bridged dinuclear ruthenium amine complex specifically inhibits Ca²⁺ uptake into mitochondria in vitro and in situ in single cardiac myocytes. *J Biol Chem* 273:10223–10231.
- McDonough SI, Boland LM, Mintz IM, Bean BP (2002) Interactions among toxins that inhibit N-type and P-type calcium channels. *J Gen Physiol* 119:313–328.
- Nakagawa H, Matsumoto N (1998) ON and OFF channels of the frog optic tectum revealed by current source density analysis. *J Neurophysiol* 80:1886–1899.
- Nicholson C, Freeman JA (1975) Theory of current source-density analysis and determination of conductivity tensor for anuran cerebellum. *J Neurophysiol* 38:356–368.
- Offenhauser N, Thomsen K, Caesar K, Lauritzen M (2005) Activity-induced tissue oxygenation changes in rat cerebellar cortex: interplay of postsynaptic activation and blood flow. *J Physiol* 565:279–294.
- Pardo B, Contreras L, Serrano A, Ramos M, Kobayashi K, Iijima M, Saheki T, Satrustegui J (2006) Essential role of aralar in the transduction of small Ca²⁺ signals to neuronal mitochondria. *J Biol Chem* 281:1039–1047.
- Pivovarov NB, Hongpaisan J, Andrews SB, Friel DD (1999) Depolarization-induced mitochondrial Ca accumulation in sympathetic neurons: spatial and temporal characteristics. *J Neurosci* 19:6372–6384.
- Raichle ME, Mintun MA (2006) Brain work and brain imaging. *Annu Rev Neurosci* 29:449–476.
- Reinert KC, Gao W, Chen G, Wang X, Peng YP, Ebner TJ (2011) Cellular and metabolic origins of flavoprotein autofluorescence in the cerebellar cortex in vivo. *Cerebellum* 10:585–599.
- Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca²⁺: molecular determinants and functional consequences. *Physiol Rev* 86:369–408.
- Satrústegui J, Pardo B, Del Arco A (2007) Mitochondrial transporters as novel targets for intracellular calcium signaling. *Physiol Rev* 87:29–67.
- Schreurs BG, Sanchez-Andres JV, Alkon DL (1992) GABA-induced responses in Purkinje cell dendrites of the rabbit cerebellar slice. *Brain Res* 597:99–107.
- Stam AH, de Vries B, Janssens AC, Vanmolkot KR, Aulchenko YS, Henneman P, Oostra BA, Frants RR, van den Maagdenberg AM, Ferrari MD, van Duijn CM, Terwindt GM (2010) Shared genetic factors in migraine and depression Evidence from a genetic isolate Evidence from a genetic isolate. *Neurology* 74:288–294.
- Thomsen K, Piilgaard H, Gjedde A, Bonvento G, Lauritzen M (2009) Principal cell spiking, postsynaptic excitation, and oxygen consumption in the rat cerebellar cortex. *J Neurophysiol* 102:1503–1512.
- Viswanathan A, Freeman RD (2007) Neurometabolic coupling in cerebral cortex reflects synaptic more than spiking activity. *Nat Neurosci* 10:1308–1312.
- Womack MD, Hoang C, Khodakhah K (2009) Large conductance calcium-activated potassium channels affect both spontaneous firing and intracellular calcium concentration in cerebellar Purkinje neurons. *Neuroscience* 162:989–1000.
- Ying WL, Emerson J, Clarke MJ, Sanadi DR (1991) Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex. *Biochemistry* 30:4949–4952.
- Zhu XH, Zhang Y, Tian RX, Lei H, Zhang N, Zhang X, Merkle H, Ugurbil K, Chen W (2002) Development of O-17 NMR approach for fast imaging of cerebral metabolic rate of oxygen in rat brain at high field. *Proc Natl Acad Sci U S A* 99:13194–13199.