Principle cell spiking, post-synaptic excitation and oxygen consumption in the rat cerebellar cortex by

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Running head: Spiking, synaptic excitation and oxygen

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Abstract

One contention within the field of neuroimaging concerns the character of the depicted activity - does it represent neuronal action potential generation, i.e., spiking, or post-synaptic excitation? This question is related to the metabolic costs of different aspects of neurosignaling. The cerebellar cortex is well-suited for addressing this problem, as synaptic input to and spiking of the principle cell, the Purkinje cell (PC), are spatially segregated. Also, PCs are pacemakers, able to generate spikes endogenously. We examined the contributions to cerebellar cortical oxygen consumption (CMRO₂) of post-synaptic excitation and PC spiking during evoked and on-going neuronal activity in the rat. By inhibiting excitatory synaptic input using ionotropic glutamate receptor blockers, we found that the increase in CMRO₂ evoked by parallel fiber (PF) stimulation depended entirely upon post-synaptic excitation. In contrast, PC spiking was largely responsible for the increase in CMRO₂ when on-going neuronal activity was increased by GABAₐ receptor blockade. In this case, CMRO₂ increased equally during PC spiking with excitatory synaptic activity as during PC pacemaker spiking without excitatory synaptic input. Subsequent inhibition of action potential propagation and neurotransmission by blocking voltage-gated Na⁺-channels eliminated the increases in CMRO₂ due to PF stimulation and increased PC spiking, but left a large fraction of CMRO₂, i.e., basal CMRO₂, intact. In conclusion, while basal CMRO₂ in anesthetized animals did not seem to be related to neurosignaling, increases in CMRO₂ could be induced by all aspects of neurosignaling. Our findings imply that CMRO₂ responses cannot a priori be assigned to specific neuronal activities.
Keywords
bicuculline, GABA_A receptors, electrophysiology, local field potentials, CNQX

Introduction
Theoretical calculations of the metabolic cost of neuronal signaling in human cerebral cortex have found that post-synaptic excitation is expensive, consuming up to six-fold more ATP than the action potentials that evoke it (4; 26). Both post-synaptic excitation and action potentials perturb the ionic balance of Na^+, K^+ and Ca^{2+} ions across the cell membrane (1; 5; 14; 26) requiring energy and thus glucose and oxygen to restore it.

Findings from primates(36), felines (50) and rats (8; 25; 33) all indicate that post-synaptic excitation is a major consumer of cortical oxygen. Evidence supporting this comes from cytochrome oxidase histochemical studies. Cytochrome oxidase is the terminal enzyme in the mitochondrial electron transport chain and, in brain slices, is used an indicator of neuronal oxidative metabolism (52). The activity of cytochrome oxidase has been shown to vary between cell bodies and their processes with the greatest activity being found post-synaptically in dendrites (32). Differentiation of cytochrome oxidase activity is found even among dendrites of the same cell, dendritic segments receiving the greatest excitatory input having the greatest cytochrome oxidase activity (23). As there is a close juxtaposition of energy supply and energy consumption at sub-cellular levels (1), these studies indicate that large quantities of energy are consumed post-synaptically at excitatory synapses. Furthermore, CMRO_2 responses to topically applied glutamate are proportional to the number of AMPA receptors present in the cortex (44; 45), consistent with the idea that post-synaptic excitation induces oxygen consumption via AMPA receptor activation.
The CMRO\textsubscript{2} response to spiking has been more difficult to assess. Reducing cortical activity by increasing the dose of anesthesia affects spike rate and CMRO\textsubscript{2} proportionately (46). However, the metabolic effects of spiking are difficult to distinguish from those of post-synaptic excitation due to massive recurrent circuitry in the neocortex (49). In comparison, the organization of neuronal circuitry in the cerebellar cortex is largely feed-forward with spatial segregation of synaptic input to and spikes from its principle cell, the Purkinje cell, with excitatory synaptic input to PC dendrites in the molecular (ML) layer, and PC spike generation and propagation in the PC and granule cell (GrC) layers, respectively ((28); see The cerebellar cortex in the Methods section).

Two other properties of the cerebellar cortex ensure feed-forward neuronal signaling:

1. The lack of voltage-gated Na\textsuperscript{+}-channels in the PC dendritic tree, preventing the backwards propagation of action potentials into the dendrites (27), and

2. The presence of inhibitory axon collaterals from PCs targeting neighboring PCs. As PC axon collaterals are not excitatory, recurrent neuronal activation cannot occur (34).

Purkinje cells are pace-makers, generating simple spikes endogenously via a resurgent Na\textsuperscript{+} current in the cell soma (19; 35). PCs also generate complex spikes in response to excitation by climbing fibers (CFs; (27)), while excitatory input from PFs modifies spike timing ((29); see The cerebellar cortex in the Methods section). PC spiking can be functionally segregated from excitatory synaptic input by blocking ionotropic glutamate receptors, which abolishes post-synaptic excitation but leaves PC pace-maker spiking intact.
These characteristics, i.e., feed-forward neuronal signaling and PC pace-maker spiking, allowed us to assess oxygen consumption in relation to different aspects of neurosignaling during evoked and on-going neuronal activity. We found that the CMRO$_2$ response evoked by parallel fiber stimulation was due to post-synaptic excitation. In contrast, the increase in on-going spiking induced by allievating tonic inhibition with GABA$_A$ receptor blockers accounted for most of the concurrent increase in CMRO$_2$. Subsequent blockade of voltage-gated Na$^+$ channels eliminated the spiking-dependent increase in CMRO$_2$, but left basal CMRO$_2$ intact. Our findings of a large component of the evoked CMRO$_2$ response dependent upon post-synaptic excitation is in line with previously mentioned studies, confirming the importance of post-synaptic excitation for brain metabolism. Not all cortical energy is devoted to synaptic activity, however, as our study also found significant oxygen consumption due to PC pace-maker firing in the cell soma and a large component not related to neurosignaling at all.

**Methods**

**Ethical information**

The protocol for the present study was approved by the Danish National Ethics Committee according to the guidelines set forth in the European Council’s Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

**Animals**
Data was collected from 46 male Wistar rats (Charles River, Germany; 326 ± 32 g), kept in plastic-bottomed cages with wood shavings, given free access to tap water and Altromin 1314 rat chow, and acclimatized to a 12:12-h light:dark cycle. Under isoflurane anesthesia (5% induction, 2% surgery), an open cranial window and surrounding agar well were placed over the cerebellar vermis. After the underlying dura was removed, the cortex was continuously superfused with aCSF (126 mM NaCl, 22 mM NaHCO₃, 1 mM Na₂HPO₄, 2.8 mM KCl, 0.88 mM MgCl₂, 1.45 mM CaCl₂ and 2.55 mM glucose). The animals were tracheotomized and artificially ventilated with oxygen-enriched air ensuring PₐO₂ > 100 mmHg and PₐCO₂ = 36.8 ± 2.7 mmHg. Two catheters were placed in the left femoral artery and vein for measuring arterial blood pressure and blood gasses and for infusion of drugs. Probes and electrodes for measuring cerebellar blood flow (CBF), PC spiking, local field potentials (LFPs) and tissue pO₂ were positioned along the same PF tract, i.e., on-beam. After this, anaesthesia was switched to intravenous α-chloralose (1,2–0-[2,2,2-trichloro-ethylidene]-[alpha]-d-gluco-furanose) HBC complex, dissolved in saline (0.5 g ml⁻¹; bolus: 1.6 ml kg⁻¹ iv, continuous infusion: 1.1 ml kg⁻¹ h⁻¹ iv). Extra supplements of α-chloralose HBC complex (0.1 ml iv) were given upon pilo-erection, increased blood pressure (> 10%) or positive corneal reflex.

The cerebellar cortex

The cerebellar cortex has three layers, each containing different cell types as well as different PC subcellular components, i.e., the superficial ML layer containing inhibitory interneurons and PC dendrites, the middle PC layer containing PC somata, and the profound GrC layer containing granule cells and PC axons. The function of each layer is
clearly delimited – post-synaptic excitation of PC dendrites in the ML layer, generation of PC spikes in the PC layer, and propagation of PC spikes out of the cerebellar cortex as well as post-synaptic excitation of GrCs (whose axons become the PFs) in the GrC layer. Purkinje cells are pace-makers, generating simple, i.e., fast, spikes endogenously via a resurgent Na⁺ current in the cell soma (19; 35). They receive excitatory synaptic input from two sources: from PFs, modifying the timing of simple spike firing (29), and from climbing fibers (CFs), evoking complex spikes comprised of short bursts of high-frequency Na⁺ spikelets (9). As PF input to PCs occurs directly via PF:PC synapses and indirectly via interneurons, PF stimulation is biphasic with an initial excitatory followed by an inhibitory component. The neurotransmitter released at PF:PC and CF:PC synapses is glutamate, which we took advantage of to functionally segregate PC pace-maker spiking from synaptic excitation. In the presence of ionotropic glutamate receptor blockers, there was no synaptic excitation from either PFs or CFs, and PC spiking was entirely due to somatic pace-maker current. PC spiking frequency is regulated by tonic inhibition evoked by ML interneurons (19). The GABA_A receptor blocker, bicuculline, was both used to abolish the inhibitory component of PF stimulation and to alleviate interneuronal tonic inhibition, thereby pharmacologically increasing on-going PC spiking frequency.

Protocol
The animals were assigned to one of three groups. In one group \((n = 30)\), tpO₂ and CBF were measured in the PC layer, defined as the cortical depth at which both simple and complex PC spiking were observed and where the amplitude of the simple spikes was
maximal (313.6 ± 28.2 μm). In the remaining two groups, tpO2 and CBF were measured either in the ML layer (146.0 ± 10.9 μm; n = 6) or the GrC layer (476.7 ± 30.1 μm; n = 10), which lie directly above, respectively below, the PC layer.

The aim of the present study was to evaluate the role of post-synaptic excitation during evoked and on-going neuronal activity. Evoked neuronal activity was achieved by stimulating the PFs, which are the non-myelinated axons of granule cells. On-going PC firing and post-synaptic excitation were increased with the GABA_A receptor blocker, bicuculline (0.2 mM), in 21 rats; in the remaining 9 rats, post-synaptic excitation was first inhibited with the ionotrophic glutamate receptor blockers, CNQX (1 mM; 6-cyano-7-nitroquinoxaline-2,3-dione disodium) and MK801 (1 mM; dizocilpine maleate), before application of bicuculline. Of the rats receiving bicuculline first, five were subsequently given the voltage-gated Na⁺ channel blocker, TTX (tetrodotoxin; 20 μM) to abolish both spiking and post-synaptic excitation. In the remaining two groups, the response to bicuculline was assessed in either the ML or the GrC layers to compare the responses in these layers to that of the PC layer. In the GrC group, four animals were given TTX alone to assess the effect of inhibiting voltage-gated Na⁺ channels on basal CMRO₂, as little neuronal activity was present in this layer. All drugs were dissolved in aCSF and applied topically to the cortex. Where two or more drugs were applied to the same animal, each new drug was added to the previous one(s) in the superfusate. Note that the ionotropic glutamate receptor blockers, CNQX and MK801, were always applied together. In the PC group in control conditions and during steady-state in the presence of the above drugs (≥ 30 minutes exposure), PFs were stimulated at 5, 10 and 15 Hz (square wave, 0.2 ms, 1.5
mA, 30 s, intertrain interval 120 s), which was repeated 3 - 4 times to achieve a better signal-to-noise ratio of the tpO₂ signal. Upon completion of the protocols, the animals were killed by an intravenous injection of air or by an overdose of pentobarbital.

**Electrophysiology**

Action potentials and LFPs were recorded using a single-barreled glass microelectrode (impedance 2 – 3 MOhm; tip diameter ~ 2 μm) filled with 2 M NaCl and positioned in the Purkinje cell layer using a custom-built motorized micromanipulator. The pre-amplified (x10) signal was A/D-converted, amplified (spikes: x2000 and inverted; LFP: x200), filtered (spikes: 300–6000 Hz bandwidth; LFP: 0.1–2400 Hz bandwidth), and digitally sampled using a Power 1401 interface and Spike 2 software (both Cambridge Electronic Design (CED), Cambridge, UK). PC spikes were taken as those deflections of the high-frequency component (300 – 6000 Hz) exceeding 0.9 mV, which were identified as spikes by our spike-recognition software (Spike 2, Cambridge Electronic Design, Cambridge, UK). The post-synaptic LFP was taken as the negative deflection of the signal occurring after the stimulus artifact and the pre-synaptic LFP, and the magnitude of the deflection was taken as LFP amplitude. ∑LFP, used as a measure of post-synaptic excitation (31), was calculated as LFP amplitude x stimulation frequency x duration of stimulation train. Spontaneous local field potentials (spLFPs) were obtained by low-pass filtering of the signal at 300 Hz and calculation of the power content. Digital sampling rates were 25 kHz for spikes and spLFPs and 5 kHz for evoked LFPs.

**Tissue pO₂**
TpO₂ was measured continuously using a modified Clark-type polarographic oxygen electrode (OX-10, tip diameter: 3 – 10 μm, field of sensitivity: 2 x tip diameter; Unisense A/S, Aarhus, Denmark), which has been described in detail previously (33). The oxygen electrode was placed within 0.25 mm of the microelectrode; both were inserted to exactly the same cortical depth. The oxygen electrode was connected to a high impedance picoamperometer (PA 2000, Unisense A/S). Signals were A/D converted and recorded at 1000 Hz using a Power 1401 interface and Spike 2.5 software (CED, Cambridge, UK). The oxygen electrode was calibrated in air-saturated and oxygen-free saline (0.9% at room temperature) before and after each experiment.

Cerebellar blood flow

Cerebellar blood flow (CBF) was measured continuously using laser Doppler flowmetry (LDF). The LDF probe (type: 415-260; wave lengths: green, 543 nm and red, 780 nm; fiber separation: 140 μm for both wave lengths; Perimed, Järfälla, Sweden) measured CBF changes down to depths of 250 μm (green) and 500 μm (red) and was positioned as close as possible to the oxygen electrode. The green wave length was used to measure CBF changes in the ML layer, the red wave length to measure CBF changes in the PC and GrC layers. The LDF signal was smoothed with a time constant of 0.2 s (PeriFlux 4001 Master; PeriMed, Järfälla, Sweden), sampled at 10 Hz, A/D converted and digitally recorded using Spike 2.5 software (CED, Cambridge, UK).

Calculations and statistics
Oxygen consumption (CMRO2) was calculated from CBF and tpO2 measurements as described by Gjedde (16; 17). The relationship between the three variables is

\[ tpO_2 = P_{50}^h \left[ \frac{2 \cdot C_a \cdot CBF}{CMRO_2} - 1 \right] - \frac{2 \cdot CMRO_2}{3 \cdot L} \]

where \( P_{50} \) is the half-saturation tension of the oxygen-hemoglobin dissociation curve, \( h \) the Hill coefficient of the same dissociation curve, \( C_a \) the arterial oxygen concentration, and \( L \) the effective diffusion coefficient of oxygen in brain tissue. The value of \( L \) was determined from baseline values of rats in similar conditions of anesthesia in which CBF and CMRO2 were reported in the literature to be 53 ml (100g\(^{-1}\)) min\(^{-1}\) and 219 μmol (100g\(^{-1}\)) min\(^{-1}\) (53). The corresponding value of \( L \) was 5.45 μmol (100g\(^{-1}\)) min\(^{-1}\) mmHg\(^{-1}\) for standard values of \( P_{50} \) (36 mmHg), \( h \) (2.7) and \( C_a \) (8 μmol ml\(^{-1}\)). CMRO2 levels were calculated from CBF and tpO2 averages obtained during 300 s intervals immediately preceding application of drugs and during steady-state after ≥30 minutes’ exposure to the drugs. CMRO2 responses to PF stimulation were calculated from CBF and tpO2 averages obtained during each 30 s stimulation period and the immediately preceding 30 s baseline.

Statistical analyses of on-going neuronal activity data were performed using either Student’s paired t-tests, or 1- or 2-way ANOVAs followed by Student’s paired t-tests, corrected by Bonferroni’s factor. Statistical analyses of evoked neuronal activity (i.e., PF stimulation) data were performed using 3-way ANOVAs. P < 0.05 was considered statistically significant. PC firing rates and ΣLFPs are given as means and 95% confidence intervals; all other data is given as means ± SEM.
Results

Postsynaptic events are the main contributors to activity-dependent increases in CMRO$_2$

during parallel fiber stimulation

To evaluate cortical energy consumption in vivo during activation, PFs in the rat cerebellum were stimulated at frequencies of 5, 10 and 15 Hz, while tpO$_2$ and CBF responses were measured in the PC layer. CMRO$_2$ responses were calculated from these two parameters. In control conditions, the evoked CBF, tpO$_2$ and CMRO$_2$ responses were frequency-dependent (Fig. 1a, b). As parallel fiber stimulation results in both excitatory and inhibitory input to Purkinje cells (28), we examined the role of both post-synaptic excitation and inhibition in these responses using CNQX+MK801 and bicuculline, respectively. Looking at inhibition first, we blocked GABA$_A$ receptors with bicuculline and found that CBF responses were reduced by $38.2 \pm 5.6\%$ ($p = 0.0009$, mean ± SE; $n = 11$, 3-way ANOVA), but that neither tpO$_2$ nor CMRO$_2$ responses were affected (Fig. 1c).

In comparison, inhibiting excitatory input by blocking ionotropic glutamate receptors with CNQX+MK801 reduced all three responses greatly (tpO$_2$ by $96.0 \pm 7.0\%$, $p = 0.0200$; CBF by $82.9 \pm 10.1\%$, $p = 2.015 \times 10^{-11}$; and CMRO$_2$ by $84.3 \pm 2.7\%$, $p = 0.0227$, mean ± SE; $n = 9$, 3-way ANOVA). Inhibition of voltage-gated Na$^+$ channels with TTX abolished the responses entirely (tpO$_2$: $p = 0.0423$, CBF: $p = 0.0003$ and CMRO$_2$: $p = 0.0241$; $n = 4$, 3-way ANOVA).

We wished to compare the CMRO$_2$ responses with the corresponding levels of post-synaptic excitation elicited by PF stimulation at 5, 10 and 15 Hz. To this end, evoked local
field potentials (LFPs) were used to calculate $\Sigma$LFP, an index of post-synaptic activity (31). This allowed us to gauge the effects of the applied drugs. Application of bicuculline increased $\Sigma$LFP by 88% compared to control conditions ($p = 1.27 \times 10^{-5}$, $n = 10$, 3-way ANOVA; Fig. 2), confirming in vivo that inhibition of GABA$_A$ receptors increases post-synaptic excitation (40). Conversely, CNQX+MK801 reduced $\Sigma$LFP by 90% ($p = 3.897 \times 10^{-14}$, $n = 7$, 3-way ANOVA), showing that post-synaptic excitation was largely blocked. Application of TTX abolished the LFP signal ($p = 2.128 \times 10^{-5}$, $n = 4$, 3-way ANOVA), demonstrating that action potential propagation and resulting neurotransmission during PF stimulation was entirely inhibited. Thus, during PF stimulation, CNQX+MK801 inhibited CMRO$_2$ responses by 84.3% and $\Sigma$LFP by 90%. Plotting CMRO$_2$ responses against $\Sigma$LFP revealed a linear relation with an intercept approximating zero ($y = 1.99x - 1.63$, $R^2 = 0.9231$; Fig. 2). As ionotropic glutamate receptor blockers do not affect pre-synaptic PF activity (12; 47) but do inhibit post-synaptic excitation, the close relationship between CMRO$_2$ and $\Sigma$LFP and the effect of ionotropic glutamate receptor blockers on both these variables imply that post-synaptic excitation accounted for the whole CMRO$_2$ response to PF stimulation.

Bicuculline increases on-going Purkinje cell spike rate, synaptic excitation and CMRO$_2$ in control conditions

We then looked at on-going neuronal activity in control conditions and in conditions of inhibited synaptic excitation with CNQX+MK801. In both conditions, neuronal activity was increased using the GABA$_A$ receptor blocker, bicuculline. Two measures of on-going neuronal activity were assessed, PC spiking and the spontaneous local field potential
(spLFP), which was taken as a measure of spontaneous synaptic activity. Two classes of PC spiking were observed, simple spiking due to PC pace-maker firing modified by interneuronal inhibition (see *The cerebellar cortex* in the Methods section), and complex spiking evoked by climbing fiber input from the inferior olive nucleus. As our software program could not distinguish between the two classes of spikes, shifts from one to the other were noted by personal observation.

In the PC layer under control conditions, application of bicuculline caused on-going PC spiking to increase by 150% from 12.8 Hz (CI: 10.5 – 15.5 Hz) to 33.1 Hz (CI: 27.3 – 40.2 Hz; \( p = 1.647 \times 10^{-7}, n = 21 \); Fig. 3) and spLFP to increase by 74% from 0.0258 mV\(^2\) (CI: 0.0192 – 0.0346 mV\(^2\)) to 0.0449 mV\(^2\) (CI: 0.0334 – 0.0604 mV\(^2\); \( p = 0.0117, n = 21 \)). The firing rate of complex spikes appeared to increase more than that of simple ones. While bicuculline increases PC simple spike firing by reducing tonic GABAergic inhibition (8; 48), the increase in complex spikes may be due to a secondary effect of bicuculline on calcium-dependent K\(^+\) channels (42). Bicuculline also caused \( t_pO_2 \) to decrease from 30.2 ± 7.7 mmHg to 21.5 ± 8.2 mmHg (\( p = 7.254 \times 10^{-5}, n = 21 \)), while CBF remained constant, resulting in a rise in CMRO\(_2\) from 205.5 ± 37.0 µmol O\(_2\)/100g/min to 235.0 ± 41.6 µmol O\(_2\)/100g/min (\( p = 0.0019, n = 21 \)). Thus, bicuculline increased both PC spiking rate and post-synaptic excitation, increased CMRO\(_2\) and augmented complex spiking.

During blockade of synaptic excitation, PF pacemaker activity is responsible for the increase in CMRO\(_2\).
In Purkinje cells, post-synaptic excitation and spiking can be functionally segregated, as simple spiking is endogenously generated by a resurgent Na$^+$ pace-maker current in the cell soma (19; 35) and is therefore not dependent upon synaptic input. Wishing to evaluate the influence of spiking alone on CMRO$_2$ at the level of the PC soma, we used another group of rats in which post-synaptic excitation was inhibited using the ionotropic glutamate receptor blockers, CNQX+MK801, before applying bicuculline. CNQX+MK801 reduced spLFP by 79% from 0.0222 mV$^2$ (CI: 0.0099 – 0.0501 mV$^2$) to 0.0046 mV$^2$ (CI: 0.0020 – 0.0103 mV$^2$; p = 0.0434, n = 8; Fig. 4), confirming that ionotropic glutamate receptors were largely blocked. Synaptic input from climbing fibers was also blocked, as no complex spikes were observed in the presence of CNQX+MK801. None of the other parameters, i.e., PC spiking rate, tpO$_2$, CBF or CMRO$_2$ were affected by CNQX+MK801. The observation that basal CMRO$_2$ did not decline with the reduction in spLFP after application of CNQX+MK801 suggests that little spontaneous post-synaptic excitation was present in the PC layer during control conditions. This may be due to a combination of low granule cell excitability (10) and increased GABA$_A$ tone due to anesthesia (15).

During inhibition of post-synaptic excitation with CNQX+MK801, simple spikes reflected PC pace-maker activity (21). Applying bicuculline subsequent to CNQX+MK801 increased spike rate 4-fold from 8.3 Hz (CI: 5.9 – 11.6 Hz) to 33.2 Hz (CI: 23.7 – 46.6 Hz, p = 0.0002, n = 9, Fig. 4) without affecting spLFP. At the same time, CBF rose by 11% and tpO$_2$ fell by 10%. These alterations were not significant in themselves, but did result in an increase in CMRO$_2$ from 216.2 ± 24.7 µmol O$_2$/100g/min
to 239.2 ± 24.7 µmol O₂/100g/min (p = 0.0265, n = 9). In the presence of
CNQX+MK801, bicuculline did not alter the type of spike which remained simple,
indicating that the increase in PC spiking was due to increased pacemaker activity. Thus,
bicuculline given under these conditions increased PC pacemaker activity and CMRO₂
with no increase in spLFP.

Summarizing the above, we found that the bicuculline-induced increases in PC spike rate
and CMRO₂ were equivalent in control conditions and during inhibition of post-synaptic
excitation regardless of the magnitude of spLFP. These findings suggest that the increase
in CMRO₂ in both conditions was dependent upon PC spiking and not upon spontaneous
synaptic excitation. This conclusion is supported by the finding of very little spontaneous
synaptic excitation under control conditions in the PC layer.

Laminar analysis of the metabolic response to bicuculline

The possibility that the bicuculline-evoked increase in CMRO₂ in the PC layer was only a
reflection of a greater increase in one of the neighboring layers was also examined. To
this end, tpO₂ was measured in three groups of rats at 146.0 ± 10.9 µm, 313.6 ± 28.2 µm
or 476.7 ± 30.1 µm below the surface of the cortex corresponding to the ML, PC or GrC
layers, respectively. Basal tpO₂ and CMRO₂ were similar in the ML and GrC layers
(tpO₂: 18.7 ± 4.4 mmHg vs 17.1 ± 4.6 mmHg, CMRO₂: 245.5 ± 18.9 µmol O₂/100 g/min
vs 248.4 ± 27.1 µmol O₂/100 g/min; mean ± SE; n = 6 + 6; Fig. 5a). This finding was
supported by a [¹⁴C]-2DG study conducted in 3 sham-operated rats, where tissue glucose
consumption during control conditions was shown to be 21.2 ± 2.8 µmol

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363 consumption during control conditions was shown to be 21.2 ± 2.8 µmol
364 glucose/100g/min in the ML layer and 19.0 ± 1.8 µmol glucose/100g/min in the GrC
layer (p = 0.3103, n = 3; Fig. 5b). The PC layer, which was not distinguishable using the
[14C]-2DG method, displayed a significantly higher basal tpO2 and a significantly lower
basal CMRO2 than either neighboring layer (tpO2: 30.2 ± 2.0 mmHg, p = 0.0033 and
CMRO2: 205.8 ± 6.0 μmol O2/100 g/min, p = 0.0012, n = 21, one-way ANOVA; Fig. 5a).
These data imply that the PC layer was metabolically more quiescent than its neighboring
layers during control conditions.
Application of bicuculline caused tpO2 to decrease and CMRO2 to increase in all layers
with no effect on CBF. Moreover, the bicuculline-evoked increases in tpO2 and CMRO2
did not differ significantly between layers (ΔtpO2 in ML, PC and GrC layers: -4.2 ± 2.8
mmHg, -8.6 ± 1.5 mmHg and -5.6 ± 2.8 mmHg, respectively; p = 0.3093; ΔCMRO2 in
ML, PC and GrC layers: 29.2 ± 10.8 μmol O2/100 g/min, 29.5 ± 5.8 μmol O2/100 g/min
and 24.9 ± 10.8 μmol O2/100 g/min, respectively; p = 0.9284; both analyses performed as
one-way ANOVAs, means ± SE; n = 6 + 21 + 6). Thus, bicuculline increased oxygen
consumption to a similar extent in all three layers, confirming in vivo that all layers of the
cerebellar cortex are under the influence of tonic GABAergic inhibition (6; 19), and that
the increase in CMRO2 observed in the PC layer is not due to a tpO2 sink in a
neighboring layer.

TTX does not affect basal CMRO2

TTX inhibits action potential generation and propagation by blocking voltage-gated Na+
channels (41), thereby also preventing neurotransmission and post-synaptic excitation.
We found that application of TTX abolished the CMRO2 increment evoked by
bicuculline without affecting basal CMRO$_2$ in the PC layer (CMRO$_2$ in control conditions: 205.0 ± 10.2 vs. CMRO$_2$ with TTX: 200.2 ± 10.2, p=1.000; Fig. 6). This lack of effect on basal CMRO$_2$ was not expected in lieu of the hypothesis that almost all oxidative glucose metabolism in the cortex is devoted to neuronal signaling (39). Hypothetically, bicuculline could augment basal metabolism and the increase in basal CMRO$_2$ would then mask the true effect of TTX. Therefore, TTX was applied alone to assess its effect on basal oxygen consumption. We measured tpO$_2$ in the GrC layer as little neuronal activity was present there during control conditions and any change in tpO$_2$ in the presence of TTX could thus be ascribed to alterations in basal oxygen consumption (Fig. 7). The little granule cell spiking and post-synaptic excitation present during control conditions was abolished by TTX (spikes: 3.5 Hz (CI: 2.0 – 4.3 Hz) in control conditions vs 0.5 Hz (CI: 0.3 – 1.3 Hz) with TTX, p = 0.0367; spLFP: 3.4 x 10$^{-4}$ mV$^2$ (CI: 2.6 x 10$^{-4}$ – 11.9 x 10$^{-4}$ mV$^2$) in control conditions vs 4.0 x 10$^{-5}$ mV$^2$ (CI: 2.2 x 10$^{-5}$ – 4.7 x 10$^{-5}$ mV$^2$) with TTX, p = 0.0295; both $n = 4$; Fig. 7). At the same time, TTX abolished spiking in the PC layer (18.3 Hz (CI: 6.8 – 46.6 Hz) in control conditions vs 0.1 Hz (CI: 0 – 0.2 Hz) with TTX; p = 0.0023; $n = 4$). In spite of the effect of TTX on neuronal activity, neither tpO$_2$ nor CMRO$_2$ were affected. This finding suggests that most cortical oxygen consumption in the cerebellum during anesthesia is not due to neuronal signaling, which is in good agreement with an earlier study showing that ~70% of cerebral metabolism remained after flattening of the EEG in anesthetized dogs (2).
The present study measured the energy expenditure of different aspects of neuronal activity in rat cerebellar cortex during stimulation and ongoing neuronal activity. We found that different subcellular elements were active during different stimulation paradigms, allowing us to quantitate energy consumption due to post-synaptic excitation and to PC pace-maker firing separately. We also found that basal oxygen consumption in the anesthesized cerebellar cortex did not support neurosignaling, as CMRO$_2$ remained unaffected after blocking neuronal activity with TTX.

**Post-synaptic excitation and CMRO$_2$ during parallel fiber stimulation**

We examined the evoked CMRO$_2$ response during PF stimulation and demonstrated that this response is entirely dependent upon post-synaptic excitation of ionotropic glutamatergic receptors, as a linear relation with an intercept of ~0 was found between CMRO$_2$ responses and ΣLFP, an index of post-synaptic excitation. This is in good agreement with previous studies showing that parallel fiber stimulation induces intracellular Ca$^{2+}$ signaling, mitochondrial metabolism and oxygen consumption via AMPA receptor activation (7; 11; 25; 33; 37; 44; 45).

PF stimulation also evokes action potential propagation through the parallel fibers, neurotransmitter release at axon terminals and re-uptake by Bergmann glial processes, as well as some retrograde stimulation of granule cells (13; 22). All these activities are energy-consuming (1; 5; 26), are not inhibited by blocking ionotopic glutamate receptors, but nonetheless did not contribute to the evoked CMRO$_2$ responses. We speculate that the cortical surface stimulation employed in this study resulted in an activated beam of PFs confined to the upper regions of the ML layer. Due to the small
sampling volume of the oxygen electrode and its placement in the PC layer, any oxygen consumption due to activated PFs, axon terminals and Bergmann glial processes in the upper ML layer would not be “seen”, explaining the apparent lack of oxygen consumption by these pre- and peri-synaptic activities in the present study.

**Oxygen consumption during PC pace-maker firing**

The feed-forward organization of the neuronal circuitry in the cerebellar cortex, including spatial segregation of PC input and output, no recurrent PC excitation (34), and the inability of PC spikes to back-propagate into the dendrites (27), together with the pace-maker property of PCs (19; 35) form a good model for relating different aspects of neurosignaling to cortical oxygen consumption. In this setting, on-going PC spiking was modified pharmacologically using bicuculline, a GABA$_A$ receptor blocker. In the presence of bicuculline, PC spiking rose by 20 Hz, representing an almost three-fold increase in firing rate. Complex spikes increased proportionately more than simple ones. Thus, bicuculline changed the PC spiking pattern both quantitatively and qualitatively. Concurrent with the increase in spike rate, spLFP increased by 74% and CMRO$_2$ increased by 29.5 µmol O$_2$/100g/min.

To evaluate the contribution of post-synaptic excitation to these effects of bicuculline, the ionotropic glutamate receptors were blocked with CNQX+MK801 before applying bicuculline. CNQX+MK801 abolished complex spikes and reduced evoked LFPs by 92%, indicating that synaptic inputs from both climbing and parallel fibers were effectively inhibited. With post-synaptic excitation eliminated, Purkinje cells were seen to fire simple spikes due to the pace-maker current in the PC somata (21).
Applying bicuculline in the presence of CNQX+MK801 increased spike rate by 25 Hz and CMRO₂ by 23.0 μmol O₂/100g/min without significantly altering spLFP. The increments in spike rate and CMRO₂ induced by bicuculline during inhibition of post-synaptic excitation were not significantly different from those induced during control conditions. Assuming the principle of summation where energy requirements of metabolic processes are additive (51), these findings imply that spontaneous post-synaptic excitation did not contribute significantly to CMRO₂ and that the increments in CMRO₂ induced by bicuculline in control conditions and during inhibition of post-synaptic excitation were attributable to the increase in PC spiking.

*No post-synaptic excitation during control conditions*

The observation that post-synaptic excitation played no role in the bicuculline-induced CMRO₂ increments lead us to look at control conditions. Blocking glutamate ionotropic receptors with CNQX+MK801 affected neither spike rate nor basal CMRO₂, although it did reduce spLFP by 79%. We have previously shown that CNQX has no effect on basal CMRglucose either (7). Thus, in contrast to the findings relating to PF stimulation, post-synaptic excitation is not a determinant of cortical metabolism during on-going neuronal activity in the anesthesized cerebellum, regardless of whether post-synaptic excitation is augmented (as in the presence of bicuculline) or inhibited (as in the presence of CNQX+MK801). We hypothesize that during anesthesia, spontaneous action potentials running through the PFs are few and asynchronous, in agreement with the low spiking rate of their progenitors, the granule cells (10). This would result in little and disperse spontaneous post-synaptic excitation of the PCs, leaving PC pacemaker firing as the main
Neurosignaling is not the major contributor to basal CMRO$_2$ in anesthetized rats

Using TTX, we examined our postulate that the lack of effect of ionotropic glutamate receptor blockade on basal CMRO$_2$ despite a clear-cut effect on the electrical signal can be explained by very low levels of spontaneous post-synaptic excitation due to anesthesia (20). As TTX inhibits all Na$^+$ channels in the cerebellum (41), it inhibits both neuronal signaling and PC pace-maker currents (35). Accordingly, we found that TTX abolished both PF stimulation-evoked CMRO$_2$ responses and bicuculline-evoked increments in CMRO$_2$, without affecting basal oxygen consumption. This phenomenon has also been seen in the somatosensory cortex, where lamotrigine, an inhibitor of voltage-gated Na$^+$ and Ca$^+$ channels, attenuated evoked CMRO$_2$ responses without affecting basal oxygen consumption (24). Earlier studies have shown that in the awake resting brain, 85% of energy consumption is associated with glutamate release and neurotransmission (43), while in the anesthetized brain, 30% was due to synaptic activity (2). In this context, our finding that TTX had no effect on basal oxygen consumption was unexpected, but does support our hypothesis that very little neuronal signaling occurs in the anesthesized cerebellar cortex during control conditions (30). Basal cellular metabolism, i.e., standard metabolic rate, is to a large degree determined by mitochondrial proton leak, maintenance of membrane potential, and lipid/protein synthesis (38). It is tempting to assign these cellular activities a major role in basal CMRO$_2$ (3; 14).
In conclusion, we have found that in the anesthetized rat, basal CMRO$_2$ greatly outweighed the CMRO$_2$ increments due to parallel fiber stimulation and PC pace-maker firing. In contrast to the awake condition in humans (18), baseline activities during anesthesia in rat cerebellar cortex did not embrace neuronal signaling. Instead, basal CMRO$_2$ may support activities which function to maintain cellular status quo. During parallel fiber stimulation, we found that the evoked CMRO$_2$ response was due to post-synaptic excitation and that during GABA$_A$ receptor blockade with bicuculline, the increment in CMRO$_2$ was due to increased PC pace-maker firing. Our findings confirm the conclusions drawn from theoretical calculations of energy consumption during neurosignaling (1; 5; 26) by demonstrating substantial oxygen consumption both by post-synaptic excitation at the level of the dendrites and by action potential generation at the level of the soma. Our findings imply that CMRO$_2$ responses cannot *a priori* be assigned to specific neuronal activities.

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Reference List


**Figure legends**

Fig. 1. CBF, tpO2 and ΔCMRO2 during PF stimulation.

Panel a: Raw data showing PC spiking, tpO2, CBF, and arterial BP during PF stimulation at increasing frequencies of 5, 10, and 15 Hz. ΔCBF and ΔtpO2 are frequency-dependent.

Duration of PF stimulation is indicated by the short black lines beneath the CBF trace.

The stimulation frequencies were 5, 10 and 15 Hz from left to right.

Panel b: Averaged CBF and tpO2 responses to 15 Hz PF stimulation during control conditions in left panel, and the corresponding calculated CMRO2 response in right panel (n = 24). Base = the immediately preceding 30 s baseline before stimulation; stim = 30 s stimulation; 30s, 60 s, 90 s = 30 s post-stimulation intervals in chronological order.

ΔCMRO2 was calculated as stimulation – baseline values.

Panel c: Averaged ΔCMRO2 to 5, 10 and 15 Hz PF stimulation in control conditions (CSF; n=24) and in the presence of bicuculline (n=11), CNQX+MK801 (n=9) and TTX (n=4). Bicuculline had no effect on ΔCMRO2. CNQX+MK801 reduced ΔCMRO2 overall by 84.3 ± 2.7% compared to CSF values, while TTX entirely abolished ΔCMRO2. ns, nonsignificant; *, p < 0.05 compared to CSF values. Data given as means ± sd.

Fig. 2: Effect of drugs on LFP.
Panel a: Raw data showing LFPs in the presence of control conditions (aCSF), bicuculline, CNQX+MK801 given after bicuculline (entitled CNQX+MK801), all from one rat, and TTX+bicuculline (entitled TTX) from another. LFP amplitude is the magnitude of the negative deflection occurring after the stimulus artefact, shown by the vertical distance between the two arrowheads. In the presence of CNQX+MK801, the negative deflection becomes positive. In the bicuculline and CNQX+MK801 panels, the presynaptic action potential (depicted by the tilted arrow in panel CNQX+MK801) is seen intercalated between the stimulus artefact and the LFP. Only the stimulus artefact is seen in the presence of TTX.

Panel b: Linear relation between $\Delta$CMRO$_2$ and $\Sigma$LFP for all treatments and all frequencies. $\Sigma$LFP was taken as a measure of post-synaptic excitation and was calculated as LFP amplitude x stimulation frequency x duration of stimulation train. The linear relation between $\Delta$CMRO$_2$ and $\Sigma$LFP given by the dotted line with an intercept that approximates null confirms the dependency of $\Delta$CMRO$_2$ on post-synaptic excitation. Note that there is no threshold of neuronal activity which must be crossed before increases in CMRO$_2$ are induced.

Fig. 3. The effect of blocking GABAA receptors with bicuculline on neuronal activity and oxygen consumption measured in the PC layer.

Panel a: Raw data showing increased PC spiking and a concomitant decrease in tpO$_2$ due to bicuculline. Gray fields represent 5 minute intervals during which control (aCSF) and bicuculline-influenced parameters were measured. Dotted line represents baseline tpO$_2$. 
Panel b: Raw data showing the increase in magnitude of the total electrical signal (TES; 0.1-6000Hz, sampling rate 25 kHz) during exposure to bicuculline. Bicuculline was applied at the timepoint indicated in Panel a and measurements were made during the gray time intervals. TES was used to calculate the power of spontaneous local field potentials (spLFP), taken as an indicator of spontaneous post-synaptic excitation. The rat shown here is different from the one in Panel a.

Panel c: Averaged data showing increases in spike rate, spLFP, and CMRO₂, and a decrease in tpO₂ (n=21). Spikes, spLFP and CBF are given as means and 95% CI (confidence intervals). TpO₂ and CMRO₂ are given as means ± SD. c=CSF, b=bicuculline. * p<0.05, ** p<0.0005, *** p<5 x 10⁻⁷.

Fig. 4. The effects of blocking ionotropic glutamate receptors with CNQX+MK801, followed by blocking GABAA receptors with bicuculline.

Panel a: Raw data from one animal showing decreased amplitude of the total electrical signal (TES, 0.1–6000 Hz) and, in this case, a slight increase in tpO₂ during blockade of synaptic excitation with CNQX+MK801. Subsequently, blockade of GABAA receptors with bicuculline increased PC spike rate and produced a slight decrease in tpO₂. Gray fields represent 5 minute intervals during which control (CSF), CNQX+MK801-influenced and bicuculline+CNQX+MK801-influenced parameters were measured.

Panel b: Averaged data showing increased spike rate and CMRO₂ and decreased spLFP. The variations in CBF and tpO₂ were not significant in themselves but did result in significantly increased CMRO₂ after application of bicuculline. Spikes, spLFP and CBF are given as means and 95% CI (confidence intervals). CMRO₂ and tpO₂ are given as
Fig. 5. Laminar representation of tpO₂, CMRO₂ and CMR₆Glu in cerebellar cortex during control conditions.

Panel a: [14C]-2DG study showing glucose consumption during control conditions in the cerebellar cortex. To the left, a Nissl stained frontal slice of the cerebellar cortex with the inset showing the ML layer (light gray) and GrC layer (dark gray) surrounding a core of white matter (white) is shown. To the right, an autoradiographic image of an adjacent slice is shown. Autoradiographic signal intensities were color-coded according to the quantitative CMR₆Glu scale (μmol glucose/100 g/min) to the right. Note that the color-code of white matter is blue, while that of both the ML and GrC layers is in shades of green.

No difference in glucose consumption was found between the ML and GrC layers. Due to the resolution of the 2DG method, the PC layer could not be distinguished. *n=3.

Panel b: Averaged data showing tpO₂ and CMRO₂ in the ML, PC and GrC layers. tpO₂ and CMRO₂ were similar in ML and GrC layers. In the interjacent PC layer, tpO₂ was greater and CMRO₂ was less than corresponding values in the neighboring layers. *n=6+10+6. Data is given as means ± SD. **, p<0.005, 2-way ANOVA.

Fig. 6. The effect of blocking voltage-gated Na⁺ channels with TTX after neuronal activity and oxygen consumption have been increased with bicuculline.
Panel a: Raw data from the PC layer showing first an increase in spiking and total electrical signal (TES, from which spLFP is calculated) due to bicuculline followed by a decrease in both parameters after the addition of TTX. Gray fields represent 5 minute intervals during which control (aCSF), bicuculline-influenced and TTX+bicuculline (TTX)-influenced parameters were measured.

Panel b: Calculated CMRO₂ of the same rat as in Panel a showing an increase in oxygen consumption during exposure to bicuculline followed by a decrease during exposure to TTX. Note that while TTX abolished the CMRO₂ increment due to bicuculline, it did not affect basal CMRO₂, which is given by the black dotted line. The gray fields and the time scales are the same in both Panels a and b.

Panel c: Averaged data showing decreases in spike rate, spLFP, and CMRO₂ due to TTX \( (n=5) \). Spikes and spLFP are given as means and 95% CI (confidence intervals). CMRO₂ is given as means ± SD. c=CSF, b=bicuculline, t=ttx+bicuculline. * p<0.05, ** p<0.01.

Fig. 7. The effect of TTX on neuronal activity and metabolic parameters.
more superficial layers, which is exemplified by the abolished spiking in the PC layer.

c=CSF, t=TTX. n=4. * p<0.05, ** p<0.005.
a. PC spikes [Hz], tpO₂ [mmHg], CBF [au], aBP [mmHg]

b. ΔCBF [au], ΔtpO₂ [mmHg], ΔCMRO₂ [mmol O₂/100g/min]

c. ΔCMRO₂ [mmol O₂/100g/min] vs. PF stimulation frequency (5 Hz, 10 Hz, 15 Hz)

- CSF
- bicuculline
- CNQX+MK801
- TTX

* ns
b.

aCSF bicuculline CNQX+ MK801 TTX

$Y = 1.9929x - 1.627$

$R^2 = 0.9231$
a.
Pc spiking [Hz]

b.
TES [mV]

c.
Spikes [Hz]       spLFP [mV]

CMRO₂ [µmol O₂/100g/min]
a. PC firing [Hz]

b. Spikes [Hz]
b.

- Nissl stain
- CMR$_{glu}$

**tpO$_2$ [mmHg]**

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<th></th>
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**CMRO$_2$**

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a.  

b.  

c.  

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**CMRO2 [µmol O2/100 g/min]**

**TES [mV]**

**PC spikes [Hz]**

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a. 

PC firing [Hz]
TET [mV]
tpO₂ [mmHg]
CBF [au]
BP [mmHg]

b. 

PC spikes [Hz]
GrC spLFP [10⁻³ x mV²]
CBF [au]
GrC tpO₂ [mmHg]
CMRO₂ [µmol O₂/100g/min]