Principal Cell Spiking, Postsynaptic Excitation, and Oxygen Consumption in the Rat Cerebellar Cortex

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1Institute of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen N; 2Department of Clinical Neurophysiology, Glostrup Hospital, University of Copenhagen, Glostrup, Denmark; and 3Atomic Energy Commission, Institute of Biomedical Imaging, Molecular Imaging Research Center, Fontenay-aux-Roses, France

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Thomsen K, Piilgaard H, Gjedde A, Bonvento G, Lauritzen M. Principal cell spiking, postsynaptic excitation, and oxygen consumption in the rat cerebellar cortex. J Neurophysiol 102: 1503–1512, 2009. First published July 1, 2009; doi:10.1152/jn.00289.2009. 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 postsynaptic excitation? This question is related to the metabolic costs of different aspects of neurosignaling. The cerebellar cortex is well suited for addressing this problem because synaptic input to and spiking of the principal 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 postsynaptic excitation and PC spiking during evoked and ongoing 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 on postsynaptic excitation. In contrast, PC spiking was largely responsible for the increase in CMRO2, when ongoing neuronal activity was increased by γ-aminobutyric acid type A 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, whereas 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 be priori be assigned to specific neuronal activities.

INTRODUCTION

Theoretical calculations of the metabolic cost of neuronal signaling in human cerebral cortex have found that postsynaptic excitation is expensive, consuming up to sixfold more adenosine 5’-triphosphate (ATP) than the action potentials that evoke it (Attwell and Iadecola 2002; Lennie 2003). Both postsynaptic excitation and action potentials perturb the ionic balance of Na+, K+, and Ca2+ ions across the cell membrane (Amar 3rd 2000; Attwell and Laughlin 2001; Erecinska and Silver 1989; Lennie 2003), requiring energy and thus glucose and oxygen to restore it. Findings from primates (Rauch et al. 2008), felines (Viswanathan and Freeman 2007), and rats (Caesar et al. 2003; Lecoq et al. 2009; Offenhauser et al. 2005) all indicate that postsynaptic 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 as an indicator of neuronal oxidative metabolism (Wong-Riley 1989). The activity of cytochrome oxidase has been shown to vary between cell bodies and their processes, with the greatest activity being found postsynaptically in dendrites (Mjaatvedt and Wong-Riley 1988). 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 (Kageyama and Wong-Riley 1982). Because there is a close juxtaposition of energy supply and energy consumption at subcellular levels (Amar 3rd 2000), these studies indicate that large quantities of energy are consumed postsynaptically at excitatory synapses. Furthermore, cerebellar cortical oxygen consumption (CMRO2) responses to topically applied glutamate are proportional to the number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors present in the cortex (Sinha et al. 1999, 2004), consistent with the idea that postsynaptic excitation induces oxygen consumption via AMPA receptor activation.

The CMRO2 response to spiking has been more difficult to assess. Reducing cortical activity by increasing the dose of anesthesia affects spike rate and CMRO2 proportionately (Smith et al. 2002). However, the metabolic effects of spiking are difficult to distinguish from those of postsynaptic excitation due to massive recurrent circuitry in the neocortex (Thomson and Lamy 2007). In comparison, the organization of neuronal circuitry in the cerebellar cortex is largely feedforward, with spatial segregation of synaptic input to and spikes from its principal 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 (Llinás et al. 2004; see Cerebellar cortex in METHODS). Two other properties of the cerebellar cortex ensure feedforward neuronal signaling.

1) The lack of voltage-gated Na+-channels in the PC dendritic tree, preventing the backward propagation of action potentials into the dendrites (Llinás and Sugimori 1980).

2) The presence of inhibitory axon collaterals from PCs targeting neighboring PCs. Because PC axon collaterals are not excitatory, recurrent neuronal activation cannot occur (Orduz and Llinos 2007).

Purkinje cells are pacemakers, generating simple spikes endogenously via a resurgent Na+ current in the cell soma (Hausser and Clark 1997; Raman and Bean 1999). PCs also generate complex spikes in response to excitation by climbing
fibers (CFs; Llinás and Sugimori 1980), whereas excitatory input from PFs modifies spike timing (Lu et al. 2005; see Cerebellar cortex in METHODS). PC spiking can be functionally segregated from excitatory synaptic input by blocking ionotropic glutamate receptors, which abolishes postsynaptic excitation but leaves PC pacemaker spiking intact.

These characteristics—i.e., feedforward neuronal signaling and PC pacemaker spiking—allowed us to assess oxygen consumption in relation to different aspects of neurosignaling during evoked and ongoing neuronal activity. We found that the CMRO₂ response evoked by parallel fiber stimulation was due to postsynaptic excitation. In contrast, the increase in ongoing spiking induced by alleviating tonic inhibition with γ-aminobutyric acid type A (GABA_A) receptor blockers accounted for most of the concurrent increase in CMRO₂. Subsequent blockade of voltage-gated Na⁺ channels eliminated the spiking-dependent increase in CMRO₂, but left basal CMRO₂ intact. Our findings of a large component of the evoked CMRO₂ response dependent on postsynaptic excitation is in line with previously mentioned studies, confirming the importance of postsynaptic excitation for brain metabolism. Not all cortical energy is devoted to synaptic activity, however, because our study also found significant oxygen consumption due to PC pacemaker 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 were collected from 46 male Wistar rats (326 ± 32 g; Charles River Laboratories, Hamburg, Germany), 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 artificial cerebrospinal fluid [aCSF (in mM): 126 NaCl, 22 NaHCO₃, 1 Na₂HPO₄, 2.8 KCl, 0.88 MgCl₂, 1.45 CaCl₂, and 2.55 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 gases and for infusion of drugs. Probes and electrodes for measuring cerebellar blood flow (CBF), PC spiking, local field potentials (LFPs), and tissue Po₂ (tpO₂) were positioned along the same PF tract (i.e., on-beam). After this, anesthesia was switched to intravenous α-chloralose [1.2-O-(2,2,2-trichloroethyldiene)-α-D-glucopyranose] 2-hydroxypropyl-β-cyclodextrin (HBC) complex, dissolved in saline (0.5 g ml⁻¹; bolus: 1.6 ml kg⁻¹ administered intravenously [iv], continuous infusion: 1.1 ml kg⁻¹ h⁻¹ iv). Extra supplements of α-chloralose HBC complex (0.1 ml iv) were given on pilo-erection, increased blood pressure (>10%), or positive corneal reflex.

**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: postsynaptic excitation of PC dendrites in the ML layer, generation of PC spikes in the PC layer, and both propagation of PC spikes out of the cerebellar cortex and postsynaptic excitation of GrCs (whose axons become the PFs) in the GrC layer. Purkinje cells are pacemakers, generating simple (i.e., fast) spikes endogenously via a resurgent Na⁺ current in the cell soma (Hausser and Clark 1997; Raman and Bean 1999). They receive excitatory synaptic input from two sources: from PFs, modifying the timing of simple spike firing (Lu et al. 2005), and from climbing fibers (CFs), evoking complex spikes comprised of short bursts of high-frequency Na⁺ spikelets (Cavelier et al. 2002).

Because 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 pacemaker 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 pacemaker current. PC spiking frequency is regulated by tonic inhibition evoked by ML interneurons (Hausser and Clark 1997). The GABA_A receptor blocker bicuculline was used both to abolish the inhibitory component of PF stimulation and to alleviate interneuronal tonic inhibition, thereby pharmacologically increasing ongoing 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 postsynaptic excitation during evoked and ongoing neuronal activity. Evoked neuronal activity was achieved by stimulating the PFs, which are the nonmyelinated axons of granule cells. Ongoing PC firing and postsynaptic excitation were increased with the GABA_A receptor blocker bicuculline (0.2 mM) in 21 rats; in the remaining 9 rats, postsynaptic excitation was first inhibited with the ionotropic glutamate receptor blockers, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 1 mM) and dizocilpine maleate (MK801, 1 mM), before application of bicuculline. Of the rats receiving bicuculline first, 5 were subsequently given the voltage-gated Na⁺ channel blocker tetrodotoxin (TTX, 20 μM) to abolish both spiking and postsynaptic 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, 4 animals were given TTX alone to assess the effect of inhibiting voltage-gated Na⁺ channels on basal CMRO₂ because 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-cited drugs (≥30-min 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 three to four times to achieve a better signal-to-noise ratio of the tpO₂ signal. On 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 MΩ; tip diameter, ~2 μm) filled with 2 M NaCl and positioned in the Purkinje cell layer using a custom-built motorized micromanipulator. The preamplified (×10) signal was A/D-converted, amplified (spikes: ×2,000 and inverted; LFP: ×200), filtered (spikes: 300 to 6,000-Hz bandwidth; LFP: 0.1 to 2,400-Hz bandwidth), and digitally sampled using a Power 1401 interface and Spike2.5 software (both Cambridge Electronic Design [CED], Cambridge, UK). PC spikes were taken as those deflections of the high-frequency component exceeding 0.9 mV, which were identified as spikes by our spike-recognition software (Spike2, CED). The postsynaptic LFP was taken as the negative deflection of the low-frequency signal occurring after the stimulus artifact and the presynaptic LFP; the magnitude of the deflection was taken as LFP amplitude. LFP, used as a measure of postsynaptic excitation (Mathiesen et al. 2000), was calculated as LFP amplitude × stimulation frequency × duration of stimulation train. Spontaneous local field potentials (spLFPS) were obtained by low-pass filtering of the total electrical signal (0.1-6,000 Hz) 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

Tissue pO2 was measured continuously using a modified Clark-type polarographic oxygen electrode (OX-10, tip diameter: 3–10 μm, field of sensitivity: 2 × tip diameter; Unisense A/S, Aarhus, Denmark), which was previously described in detail (Offenhauser et al. 2005). 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 picoammeter (PA 2000, Unisense A/S). Signals were A/D converted and recorded at 1,000 Hz using a Power 1401 interface and Spike2.5 software (CED). 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; 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 wavelength was used to measure CBF changes in the ML layer and the red wavelength 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), sampled at 10 Hz, A/D converted, and digitally recorded using Spike2.5 software (CED).

Calculations and statistics

Oxygen consumption (CMRO2) was calculated from CBF and tPO2 measurements as described by Gjedde (2005a,b). The relationship between the three variables is

\[
\text{tPO}_2 = P_50 \left( \frac{2C_{\text{CBF}} \text{CMRO}_2}{C_{\text{CMRO}_2}} - 1 \right) - \frac{2\text{CMRO}_2}{3L}
\]

where \( P_{50} \) is the half-saturation tension of the oxygen–hemoglobin dissociation curve, \( h \) is the Hill coefficient of the same dissociation curve, \( C_a \) is the arterial oxygen concentration, and \( L \) is 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·100 g⁻¹·min⁻¹ and 219 μmol·100 g⁻¹·min⁻¹ (Zhu et al. 2002). The corresponding value of \( L \) was 5.45 mmol·100 g⁻¹·min⁻¹·mmHg⁻¹ for standard values of \( P_{50} \) (36 mmHg), \( h \) (2.7), and \( C_a \) (8 μmol l⁻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-min exposure to the drugs. CMRO2 responses to PF stimulation were calculated

FIG. 1. Cerebellar blood flow (CBF), tissue pO2 (tPO2), and change in cerebellar cortical oxygen consumption (ΔCMRO2) during parallel fiber (PF) stimulation. A: raw data showing PC spiking, tPO2, CBF, and arterial blood pressure (BP) during PF stimulation at increasing frequencies of 5, 10, and 15 Hz. CBF and tPO2 responses 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. B: averaged CBF and tPO2 responses to 15-Hz PF stimulation during control conditions in the 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; 30, 60, 90 s = 30-s poststimulation intervals in chronological order. ΔCMRO2 was calculated as stimulation – baseline values. C: averaged ΔCMRO2 to 5-, 10-, and 15-Hz PF stimulation in control conditions (cerebrospinal fluid [CSF]; n = 24) and in the presence of bicuculline (n = 11), CNQX + MK801 (n = 9) and tetrodotoxin (TTX; n = 4); Bicuculline had no effect on ΔCMRO2. CNQX + MK801 reduced ΔCMRO2 overall by 84.3 ± 2.7% compared with CSF values, whereas TTX entirely abolished ΔCMRO2. ns, nonsignificant; *P < 0.05 compared with CSF values. Data given as means ± SD. CNQX, 6-cyano-7-nitroquinoidaline-2,3-dione disodium; MK801, dizocilpine maleate.
Postsynaptic events are the main contributors to activity-dependent increases in CMRO₂ 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, whereas tpO₂ and CMRO₂ responses were measured in the PC layer. CMRO₂ responses were calculated from these two parameters. In control conditions, the evoked CBF, tpO₂, and CMRO₂ responses were frequency dependent (Fig. 1, A and B). Because parallel fiber stimulation results in both excitatory and inhibitory input to Purkinje cells (Llinás et al. 2004), we examined the role of both postsynaptic excitation and inhibition in these responses using CNQX + MK801 and bicuculline, respectively. Looking at inhibition first, we blocked GABAₐ receptors with bicuculline and found that CBF responses were reduced by 38.2 ± 5.6% (P = 0.0009, mean ± SE; n = 11, three-way ANOVA), but that neither tpO₂ nor CMRO₂ responses were affected (Fig. 1C). In comparison, inhibiting excitatory input by blocking ionotropic glutamate receptors with CNQX + MK801 substantially reduced all three responses (tpO₂ by 96.0 ± 7.0%, P = 0.0200; CBF by 82.9 ± 10.1%, P = 2.015 × 10⁻¹¹; and CMRO₂ by 20.6% (P = 0.0005). This result was confirmed in subsequent experiments using CNQX + MK801 given after bicuculline (titled MK801 panels, the negative deflection due to bicuculline was applied at the time point indicated in Fig. 1A, B, and C).

FIG. 2. Effect of drugs on local field potential (LFP). A: raw data showing LFPs in the presence of control conditions (artificial cerebrospinal fluid [aCSF]), bicuculline, CNQX + MK801 given after bicuculline (titled CNQX + MK801), all from one rat, and TTX + bicuculline (titled TTX) from another. LFP amplitude is the magnitude of the negative deflection occurring after the stimulus artifact, shown by the vertical distance between the 2 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 artifact and the LFP. Only the stimulus artifact is seen in the presence of TTX. B: linear relation between ∆CMRO₂ and ΣLFP for all treatments and all frequencies. ΣLFP was taken as a measure of postsynaptic excitation and was calculated as LFP amplitude × stimulation frequency × duration of stimulation train. The linear relation between ∆CMRO₂ and ΣLFP given by the dotted line with an intercept that approximates null confirms the dependence of ∆CMRO₂ on postsynaptic excitation. Note that there is no threshold of neuronal activity that must be crossed before increases in CMRO₂ are induced.

FIG. 3. The effect of blocking γ-aminobutyric acid type A (GABAₐ) receptors with bicuculline on neuronal activity and oxygen consumption measured in the Purkinje cell (PC) layer. A: raw data showing increased PC spiking and a concomitant decrease in tpO₂ due to bicuculline. Gray fields represent 5-min intervals during which control (aCSF) and bicuculline-influenced parameters were measured. Dotted line represents baseline tpO₂. B: raw data showing the increase in magnitude of the total electrical signal (TES; 0.1–6,000 Hz; sampling rate 25 kHz) during exposure to bicuculline. Bicuculline was applied at the time point indicated in A and measurements were made during the gray time intervals. TES was used to calculate the power of spontaneous local field potentials (spLFPs), taken as an indicator of spontaneous postsynaptic excitation. The rat shown here is different from the one in A, C: averaged data showing increases in spiking rate, spLFP, and CMRO₂ and a decrease in tpO₂ (n = 21). Spikes, spLFP, and CBF are given as means and 95% confidence intervals (CIs). TpO₂ and CMRO₂ are given as means ± SE. c, CSF; b, bicuculline. *P < 0.05, **P < 0.0005, ***P < 5 × 10⁻⁷.
84.3 ± 2.7%, \( P = 0.0227 \), mean ± SE; \( n = 9 \), three-way ANOVA). Inhibition of voltage-gated Na\(^{+}\) channels with TTX abolished the responses entirely (\( P = 1.27 \times 10^{-5} \), \( n = 10 \), three-way ANOVA; Fig. 2), confirming in vivo that inhibition of GABA\(_{A}\) receptors increases postsynaptic excitation (Mathiesen et al. 2000). This allowed us to gauge the effects of the applied drugs. Application of bicuculline increased \( \Sigma \) LFP by 88% compared with control conditions (\( P = 1.27 \times 10^{-5} \), \( n = 10 \), three-way ANOVA; Fig. 2), confirming us that the close relationship between CMRO\(_2\) and \( \Sigma \) LFP revealed a linear relation with an intercept approximating zero (\( y = 1.99x - 1.63 \), \( R^2 = 0.9231 \); Fig. 2). Because ionotropic glutamate receptor blockers do not affect presynaptic PF activity (Díez-Garcia et al. 2007; Sullivan et al. 2005), but do inhibit postsynaptic excitation, the close relationship between CMRO\(_2\) and \( \Sigma \) LFP and the effect of ionotropic glutamate receptor blockers on both these variables imply that postsynaptic excitation accounted for the whole CMRO\(_2\) response to PF stimulation.

**Bicuculline increases ongoing Purkinje cell spike rate, synaptic excitation, and CMRO\(_2\) in control conditions**

We then looked at ongoing 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 ongoing 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: 1) simple spiking due to PC pacemaker firing modified by interneuronal inhibition (see Cerebellar cortex in Methods) and 2) complex spiking evoked by climbing fiber input from the inferior olive nucleus. Because 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 ongoing 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; \( P = 1.64 \times 10^{-7} \), \( n = 21 \); 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. Bicuculline increases PC simple spike firing by reducing tonic GABAergic inhibition (Caesar et al. 2003; Thomsen et al. 2004). However, the increase in complex spikes may be due to a secondary effect of bicuculline on calcium-dependent K\(^{+}\) channels (Seutin and Johnson 1999).

Bicuculline also caused tpO\(_2\) to decrease from 30.2 ± 7.7 to 21.5 ± 8.2 mmHg (\( P = 7.25 \times 10^{-5} \), \( n = 21 \)), without affecting CBF, resulting in a rise in CMRO\(_2\) from 205.5 ± 37.0 to 235.0 ± 41.6 μmol O\(_2\)·100 g\(^{-1}\)·min\(^{-1}\) (\( P = 0.0019 \), \( n = 21 \)). Thus bicuculline increased both PC spiking rate and postsynaptic 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, postsynaptic excitation and spiking can be functionally segregated, given that simple spiking is endog-
enously generated by a resurgent Na⁺ pacemaker current in the cell soma (Hauser and Clark 1997; Raman and Bean 1999) and is thus not dependent on synaptic input. In seeking to evaluate the influence of spiking alone on CMRO₂ at the level of the PC soma, we used another group of rats in which postsynaptic excitation was inhibited using the ionotropic glutamate receptor blockers, CNQX + MK801, before applying bicuculline. CNQX + MK801 reduced spLF₉ by 79% from 0.0222 mV² (CI: 0.0099–0.0501 mV²) to 0.0046 mV² (CI: 0.0020–0.0103 mV²; P = 0.0034, n = 8; Fig. 4), confirming that ionotropic glutamate receptors were largely blocked. Synaptic input from climbing fibers was also blocked in that no complex spikes were observed in the presence of CNQX + MK801. None of the other parameters—i.e., PC spiking rate, tpO₂, CBF, or CMRO₂—was affected by CNQX + MK801.

The observation that basal CMRO₂ did not decline with the reduction in spLF₉ after application of CNQX + MK801 suggests that little spontaneous postsynaptic excitation was present in the PC layer during control conditions. This may be due to a combination of low granule cell activity (Chadderton et al. 2004) and increased GABAₐ tone due to anesthesia (Franks 2008).

During inhibition of postsynaptic excitation with CNQX + MK801, simple spikes reflected PC pacemaker activity (Hounsgaard and Yamamoto 1979). Applying bicuculline subsequent to CNQX + MK801 increased spike rate fourfold 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 spLF₉. At the same time, CBF rose by 11% and tpO₂ fell by 10%. These alterations were not significant in themselves, but did result in an increase in CMRO₂ to increase in both conditions was dependent on PC spiking and not on 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, 313.6 ± 28.2, 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 vs. 17.1 ± 4.6 mmHg, CMRO₂: 245.5 ± 18.9 vs. 248.4 ± 27.1 µmol O₂·100 g⁻¹·min⁻¹; mean ± SE; n = 6 + 6; Fig. 5A). This finding was supported by a [¹⁴C]-2-deoxyglucose ([¹⁴C]-2DG) study conducted in three sham-operated rats, where tissue glucose consumption during control conditions was shown to be 21.2 ± 2.8 µmol glucose·100 g⁻¹·min⁻¹ in the ML layer and 19.0 ± 1.8 µmol glucose·100 g⁻¹·min⁻¹ 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 those of either neighboring layer (tpO₂: 30.2 ± 2.0 mmHg; P = 0.0033 and CMRO₂: 205.8 ± 6.0 µmol O₂·100 g⁻¹·min⁻¹; 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, -8.6 ± 1.5, and -5.6 ± 2.8 mmHg, respectively; P = 0.3093; ΔCMRO₂ in ML, PC, and GrC layers: 29.2 ± 10.8, 29.5 ± 5.8, and 24.9 ± 10.8 µmol O₂·100 g⁻¹·min⁻¹, 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 (Brickley et al. 1996; Hausser and Clark 1997) 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 (Schaller and Caldwell 2003), thereby also preventing neurotransmission and postsynaptic excitation. We found that application of TTX abolished the CMRO₂ increment evoked by bicuculline without affecting basal CMRO₂ in the PC layer (CMRO₂ in control conditions: 205.0 ± 10.2 vs. CMRO₂ with TTX: 200.2 ± 10.2, P = 1.000; Fig. 6). This lack of effect on basal CMRO₂ was not expected in lieu of the hypothesis that almost all oxidative glucose metabolism in the cortex is devoted to neuronal signaling (Rothman et al. 1999).

Hypothetically, bicuculline could augment basal metabolism and the increase in basal CMRO₂ would then mask the true effect of TTX. Therefore TTX was applied alone to assess its effect on basal oxygen consumption. We measured tpO₂ in the GrC layer because little neuronal activity was present there during control conditions and any change in tpO₂ in the presence of TTX could thus be ascribed to alterations in basal oxygen consumption (Fig. 7). The little granule cell spiking and postsynaptic excitation present during control conditions were 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 × 10⁻⁴ mV² (CI: 2.6 × 10⁻⁴ to 11.9 × 10⁻⁴ mV²) in control conditions vs. 4.0 × 10⁻⁵ mV² (CI: 2.2 × 10⁻⁵ to 4.7 × 10⁻⁵ mV²) 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 tpO₂ nor CMRO₂ was 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 about 70% of cerebral metabolism remained after flattening of the electroencephalogram in anesthetized dogs (Astrup et al. 1981a).

**DISCUSSION**

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 postsynaptic excitation and to PC pacemaker firing separately. We also found that basal oxygen consumption in the anesthetized cerebellar cortex did not support neurosignaling because CMRO₂ remained unaffected after blocking neuronal activity with TTX.

**Postsynaptic excitation and CMRO₂ during parallel fiber stimulation**

We examined the evoked CMRO₂ response during PF stimulation and demonstrated that this response is entirely dependent on postsynaptic excitation of ionotropic glutamatergic receptors, in that a linear relation with an intercept of about 0 was found between CMRO₂ responses and Σ LFP, an index of postsynaptic excitation. This is in good agreement with previous studies showing that neuronal stimulation induces intracellular Ca²⁺ signaling, mitochondrial metabolism, and oxygen consumption via AMPA receptor activation (Caesar et al. 1996)
The feedforward organization of the neuronal circuitry in the cerebellar cortex, including spatial segregation of PC input and output, no recurrent PC excitation (Orduz and Llano 2007), and the inability of PC spikes to back-propagate into the dendrites (Llinás and Sugimori 1980), together with the pacemaker property of PCs (Hauser and Clark 1997; Raman and Bean 1999), form a good model for relating different aspects of neurosignaling to cortical oxygen consumption. In this setting, ongoing 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 threefold increase in firing rate. Complex spikes increased proportionately more than simple ones. Thus bicuculline changed the PC spiking pattern both qualitatively and quantitatively. Concurrent with the increase in spike rate, spLFP increased by 74% and CMRO_2 increased by 29.5 µmol O_2/100 g^-1·min^-1.

To evaluate the contribution of postsynaptic 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 postsynaptic excitation eliminated, Purkinje cells were seen to fire simple spikes due to the pacemaker current in the PC somata (Hounsgaard and Yamamoto 1979).

Applying bicuculline in the presence of CNQX + MK801 increased the spike rate by 25 Hz and CMRO_2 by 23.0 µmol O_2/100 g^-1·min^-1 without significantly altering spLFP. The increments in ongoing spike rate and CMRO_2 induced by bicuculline during inhibition of postsynaptic excitation were not significantly different from those induced during control conditions. Assuming the principle of summation, in which energy requirements of metabolic processes are additive (Wieser 1989), these findings imply that spontaneous postsynaptic excitation did not contribute significantly to CMRO_2 and that the increments in CMRO_2 induced by bicuculline in control conditions and during inhibition of postsynaptic excitation were attributable to the increase in ongoing PC spiking.

**No postsynaptic excitation during control conditions**

The observation that postsynaptic excitation played no role in the bicuculline-induced CMRO_2 increments led us to look at control conditions. Blocking glutamate ionotropic receptors with CNQX + MK801 affected neither spike rate nor basal CMRO_2, although it did reduce spLFP by 79%. We have previously shown that CNQX has no effect on basal CMR_glucone either (Caesar et al. 2008). Thus in contrast to the findings relating to PF stimulation, postsynaptic excitation is not a determinant of cortical metabolism during ongoing neuronal activity in the anesthetized cerebellum, regardless of whether postsynaptic 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 (Chadderton et al. 2004). This would result in little and disperse spontaneous postsynaptic excitation of the PCs, leaving PC pacemaker firing as the main determinant of incremental CMRO_2 during ongoing neuronal activity, as we have demonstrated.
Neurosignaling is not the major contributor to basal CMRO₂ in anesthetized rats

Using TTX, we examined our postulate that the lack of effect of ionotropic glutamate receptor blockade on basal CMRO₂ despite a clear-cut effect on the electrical signal can be explained by very low levels of spontaneous postsynaptic excitation due to anesthesia (Hentschke et al. 2005). Because TTX inhibits all Na⁺ channels in the cerebellum (Schaller and Caldwell 2003), it inhibits both neuronal signaling and PC pacemaker currents (Raman and Bean 1999). Accordingly, we found that TTX abolished both PF stimulation-evoked CMRO₂ responses and bicuculline-evoked increments in CMRO₂, without affecting basal oxygen consumption. This phenomenon has also been seen in the somatosensory cortex, where lamotrigine, an inhibitor of voltage-gated Na⁺ and Ca²⁺ channels, attenuated evoked CMRO₂ responses without affecting basal oxygen consumption (Kida et al. 2006). Earlier studies have shown that in the awake resting brain, 85% of energy consumption is associated with glutamate release and neurotransmission (Shulman et al. 2004), whereas in the anesthetized brain, 30% was due to synaptic activity (Astrup et al. 1981a). 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 anesthetized cerebellar cortex during control conditions (Maandag et al., 2007). 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 (Rolfe and Brown 1997). It is tempting to assign these cellular activities a major role in basal CMRO₂ (Astrup et al. 1981b; Erecinska and Silver 1989).

In conclusion, we have found that in the anesthetized rat, basal CMRO₂ greatly outweighed the CMRO₂ increments due to parallel fiber stimulation and PC pacemaker firing. In contrast to the awake condition in humans (Gusnard et al. 2001), baseline activities during anesthesia in rat cerebellar cortex did not embrace neuronal signaling. Instead, basal CMRO₂ may support activities that function to maintain cellular status quo. During parallel fiber stimulation, we found that the evoked CMRO₂ response was due to postsynaptic excitation and that during GABA_A receptor blockade with bicuculline, the increment in CMRO₂ was due to increased PC pacemaker firing. Our findings confirm the conclusions drawn from theoretical calculations of energy consumption during neurosignaling (Ames 3rd 2000; Attwell and Laughlin 2001; Lennie 2003) by demonstrating substantial oxygen consumption both by postsynaptic excitation at the level of the dendrites and by action potential generation at the level of the soma. Our findings imply that CMRO₂ responses cannot a priori be assigned to specific neuronal activities.

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