In Situ Respiration and Bioenergetic Status of Mitochondria in Primary Cerebellar Granule Neuronal Cultures Exposed Continuously to Glutamate

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Mitochondria play a central role in neuronal death during pathological exposure to glutamate (excitotoxicity). To investigate the detailed bioenergetics of the in situ mitochondria, a method is described to monitor continuously the respiration of primary cerebellar granule neuron cultures while simultaneously imaging cytoplasmic Ca\(^{2+}\) and mitochondrial membrane potential. Coverslip-attached cells were perfused in an imaging chamber with upstream and downstream flow-through oxygen electrodes. The bioenergetic consequences of chronic glutamate exposure were investigated, including ATP supply and demand, proton leak, and mitochondrial respiratory capacity during chronic glutamate exposure. In 25 mM K\(^+\) medium supplemented with 10% dialyzed serum, cells utilized 54% of their respiratory capacity in the absence of receptor activation (37% for ATP generation, 12% to drive the mitochondrial proton leak, and the residual 5% was nonmitochondrial). Glutamate initially increased mitochondrial respiration from 51 to 68% of capacity, followed by a slow decline. It was estimated that 85% of this increased respiration was because of increased ATP demand, whereas 15% was attributable to a transient mitochondrial proton leak. N-Methyl-d-aspartate receptor activation was only responsible for 62% of the increased respiration. When adjusted for cell death over 3 h of glutamate exposure, respiration of the viable cells remained near basal and protonophore stimulated respiration to the same extent as control cells. Pyruvate-supplemented media protected cells from glutamate excitotoxicity, although this was associated with mitochondrial dysfunction. We conclude that excitotoxicity under these conditions is not because of an ATP deficit or uncoupling. Furthermore, mitochondria maintain the same respiratory capacity as in control cells.

Although glutamate is the dominant excitatory neurotransmitter in the central nervous system, excessive glutamate exposure can cause neuronal cell death by a process known as glutamate excitotoxicity (1, 2). This phenomenon amplifies brain damage following stroke, traumatic brain injury, and epilepsy where massive glutamate release from neurons in the affected region causes pathological activation of N-methyl-D-aspartate (NMDA)-selective glutamate receptors and contributes to neuronal cell death in the surrounding tissue (3). In primary neuronal culture models, necrosis is not instantaneous but rather occurs after a latent period followed by an uncontrolled rise in cytoplasmic free Ca\(^{2+}\), ([(Ca\(^{2+}\)]\(_{c}\)), known as delayed Ca\(^{2+}\) deregulation (DCD) (4, 5).

A central role for mitochondria in cerebellar granule neuron (CGN) excitotoxicity is firmly established, based on fluorescent monitoring of changes in mitochondrial membrane potential (\(\Delta\psi_m\)) (6–8), mitochondrial Ca\(^{2+}\) accumulation (9, 10) and morphology (11), and on the protection afforded by mitochondrial depolarization prior to glutamate under conditions where glycolysis is adequate (12–14). However, there is very limited information on in situ mitochondrial respiration (15–18), even though the classic oxygen electrode chamber has proven to be the single most important apparatus for monitoring the function of isolated mitochondria. This is primarily because of the requirement for large numbers of cells in suspension when using conventional oxygen electrodes.

The approach taken in the present study was to develop a “cell respirometer” that retains the cells on their coverslips, reduces the dead volume as far as possible, and continuously superfuses media over the cells, detecting the difference in the upstream and downstream oxygen concentrations with sensitive flow-through micro oxygen electrodes. Because the assembly is mounted on a digital imaging microscope, a representative sample of the entire cell field can be continuously imaged to monitor cell morphology and survival together with fluorescent signals from one or more indicators, such as FURA-PE3 for \([\text{Ca}^{2+}]_c\), and TMRM\(^+\) for mitochondrial and plasma membrane potentials. In experiments to monitor mitochondrial function during potentially excitotoxic glutamate exposure, it is important to distinguish between a change in population respiration because of stochastic cell death and one that reflects a uniform decline in single cell respiration. By imaging a representative field, cell viability can be continuously assessed and used to correct the response.

The present study tested hypotheses that chronic glutamate exposure increases ATP demand, that oxidative phosphorylation becomes rate-limiting, that mitochondrial uncoupling is an
early event (7, 19), and that mitochondrial respiratory capacity is impaired (15).

**EXPERIMENTAL PROCEDURES**

Reagents—TMRM was from Molecular Probes (Eugene, OR). Fura-PE3-AM was from Tef Labs (Austin, TX). 10 kDa of dialyzed fetal bovine serum and all other reagents were from Sigma.

Cerebellar Granule Neuron Preparation and Indicator Loading—CNGs were prepared from 5- to 7-day-old rat Wistar pups essentially as described previously (20). 3 × 10^6 cells were allowed to settle for 30–40 min onto 22 × 40-mm coverslips previously coated with 33 μg/ml poly-d-lysine and then maintained at 37 °C in a 95/5% air/CO₂ incubator. Cells were used after 7–10 days in culture. Prior to assembling the perfusion chamber, cells in culture media were incubated at 37 °C with 3 mM TMRM plus 1 μM tetraphenylboron for 2–2.5 h and with 2 μM FURA-PE3-AM for 50–80 min. Under these conditions, TMRM fluorescence was in nonquench mode (21), meaning that a decrease in either plasma membrane potential (Δψ_m) or Δψ_o, is reflected in a decrease in whole-cell fluorescence. Fura-PE3-AM was used to monitor [Ca^{2+}]. Because it was retained sufficiently by the granule neurons to provide reliable 340/580 nm signal over 2–5 h, whereas FURA2 AM was essentially lost after 3 h.

**Respiration Measurements Using the Cell Respirimeter**—The respirometer is shown schematically in Fig. 1. A closed RC-30 imaging chamber (Warner Instruments, Hamden, CT) was assembled submerged in standard buffer without serum (to avoid cell drying or bubble trapping) with a lower 22 × 40-mm coverslip with attached cells, a 250-μm gasket (yielding a chamber volume of 130 μL), and a blank upper coverslip. The chamber was mounted on an Olympus IX 70 inverted fluorescence microscope equipped with a 40× oil-immersion objective. Miniature polarographic Clark-type oxygen electrodes constructed for perfusion systems with 1/16 inch fittings (Microelectrodes, Inc., Bedford, NH) monitored upstream and eluant oxygen tension. A peristaltic pump placed distal to the downstream oxygen electrode was used to set the system flow rate, which was typically 30–60 μl/min to provide adequate oxygen depletion for detection by the downstream electrode. The standard perfusion medium used for respiration measurements consisted of (in mM) 105 NaCl, 25 KCl, 20 TES, 15 glucose, 1.3 MgCl₂, 1.3 CaCl₂, 1.2 Na₂SO₄, 0.4 KH₂PO₄, 0.2 NaHCO₃, 10% dialyzed fetal bovine serum, 3 mM TMRM, and 1 μM tetraphenylboron, pH 7.3, at 37 °C. Variations of the standard buffer are specified under “Results.” The upstream electrode controlled for changes in oxygen tension in the inflow and was used to check the downstream electrode base line at the beginning and end of the perfusion, via a tubing shunt that allowed buffer flow to bypass the chamber (Fig. 1).

A dual-pen chart recorder was wired to each OM-4 oxygen meter (which delivered the polarizing voltage to the electrodes (Microelectrodes, Inc., Bedford, NH)) to provide a continuous trace of respiration (which delivered the polarizing voltage to the electrodes (Microelectrodes, Inc., Bedford, NH)) to provide a continuous trace of respiration using a 250-μm gasket, resulting in an ~130-μL chamber volume. The inlet and outlet ports of the chamber were connected to upstream and downstream flow-through polarographic oxygen electrodes (Microelectrodes Inc.) resting on the microscope stage. Each oxygen meter delivered a polarizing voltage to each electrode and had outputs that were connected to a dual-pen chart recorder for monitoring the signals over time. A tubing manifold was used to connect different buffers to the system. Cells were perfused with a peristaltic pump at 30–60 μl/min in the standard buffer (or supplemented according to each experiment) for a 30-min equilibration period and then a 30-min baseline period to establish baseline-respiration, FURA-PE3, and TMRM. During the 30-min equilibration period, buffer flow to the chamber was redirected to the shunt tubing for 3–5 min to check that both oxygen electrodes gave the same air-saturated buffer readings. The shunt was also opened at the end of each experiment to assess downstream electrode drift over time. Cells were imaged with a 40× oil-immersion objective, and a single field-of-view was kept throughout each experiment.

Responses to temperature variations. To avoid this, a custom-made acrylic incubator was constructed to surround and maintain the entire microscope stage, objective and incubation reservoirs at 37 °C, eliminating buffer cooling and temperature artifacts.

**Statistics**—Analyses were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Tests used were two-way ANOVA, one-way ANOVA with Tukey’s post test, and unpaired Student’s t test. The level of statistical significance was taken as p < 0.05.

**RESULTS**

Assessing Neuronal Bioenergetics Using Flow-through Oxygen Electrodes—Fig. 2 illustrates some of the considerable

![Diagram of experimental setup](http://www.jbc.org)
advantages of the respirometer over traditional closed-chamber, stirred-suspension oxygen electrodes to monitor coverslip-attached cell respiration. First, the cells remain attached to their coverslips; second, the perfusion mode permits long term experiments and medium exchange; and third, simultaneous monitoring of cell viability and function can be performed.

During steady-state perfusion, the respiratory rate is the product of the flow rate multiplied by the difference between the upstream and downstream oxygen tensions. Fig. 2 illustrates basic bioenergetic information obtained from a respirometer trace (Fig. 2A), and the associated images obtained by using the potentiometric probe TMRM+ and the Ca2+ probe FURA-PHE3 (Fig. 2B). The following respiratory rates were determined: basal (\(V_{basal}\)), maximal uncontrolled respiration in the presence of 2 \(\mu\)M FCCP (\(V_{FCCP}\)), oligomycin (0.2 \(\mu\)g/ml)-insensitive (\(V_{oligo}\)), and residual nonmitochondrial (\(V_{rot/myx}\)) respiration in the presence of 2 \(\mu\)M myxothiazol plus 1 \(\mu\)M rotenone (myx/rotx). Flow was stopped to prime upstream tubing with each compound, resulting in a transient oxygen depletion. The shunt was opened to assess electrode drift. The shunt was closed, resulting in the downstream electrode signal going off-scale momentarily because of transient depletion of oxygen in the chamber while the shunt was open; the chamber did not, however, go anoxic, as the oxygen meter provided an independent means of monitoring the absolute oxygen tension. Numbers below the downstream oxygen electrode trace represent the mean ± S.E. (\(n = 4\) independent preparations) of the respiration in nmol of O/min × 106 cells at each steady state. The perfusion rate was 44 \(\mu\)l/min. Basal respiration was monitored for 30 min, followed by perfusion with 2 \(\mu\)M FCCP for 10 min to assess maximal respiration. Cells were given 50 min of recovery in standard buffer and then sequentially perfused for 15 min with 0.2 \(\mu\)g/ml oligomycin and 20 min with 2 \(\mu\)M myxothiazol plus 1 \(\mu\)M rotenone (myx/rotx). Flow was stopped to prime upstream tubing with each compound, resulting in a transient oxygen depletion. The shunt was opened to assess electrode drift. 

Fig. 2 shows the effects of these additions on FCCP-PHE3 and TMRM+ fluorescence of a representative single CGN soma. Brief FCCP perfusion decreased TMRM+ fluorescence by ~50% because of mitochondrial release of TMRM+ and consequent re-equilibration across the plasma membrane (6), and modestly increased the FCCP-PHE3 340/380 nm ratio. Cytoplasmic Ca2+ levels rapidly recovered during FCCP washout, whereas TMRM+ fluorescence took 50 min to return close to pre-FCCP values. The kinetics of TMRM+ recovery are primarily because of slow re-equilibration of probe across the plasma membrane, which has a low surface-to-volume ratio compared with the mitochondrial inner membrane (6, 21).

Under the present conditions (elevated KCl in the presence of serum) ATP synthase inhibition by oligomycin perfusion resulted in rapid Ca2+ deregulation. Thus glycolysis alone is
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Glutamate Stimulates Granule Neuron Respiration—Switching from the standard perfusate to one supplemented with 250 μM glutamate and 25 μM glycine (subsequently referred to as glutamate) rapidly and robustly increased respiration by 32 ± 4% (IV_{glutamate} − V_{basal}, V_{basal}) well below the maximal respiratory capacity observed with FCCP (Fig. 4, A versus B). Specifically, a peak glutamate response utilized 40–50% of the reserve CGN ATP synthesis capacity. This increase was followed by a slow decline in respiration over the remaining perfusion period (Fig. 4B, solid line). Two possible explanations for the slow decline are as follows: a gradual reduction in mitochondrial activity within all CGNs or a complete loss of mitochondrial function in some neurons but not in others. Transmission, TMRM fluorescence, and FURA-PE3 images containing ~80–120 CGNs indicated that some cells maintained relatively normal morphology, low FURA-PE3 340/380 nm ratios, and some TMRM+ fluorescence, whereas other cells showed gross morphological changes, high FURA-PE3 340/380 nm ratios (followed by inevitable loss of FURA-PE3 after a further 20–40 min), and no TMRM+ signals (Fig. 4, C–E). Because of these two distinct cell populations, the slow respiration decline seems better explained by the complete loss of mitochondrial function (i.e. no substrate oxidation) in the latter cells rather than a uniform reduction in mitochondrial activity in all cells. It is notable that the synchronous Ca2+-deregulation of cells in the presence of oligomycin (Fig. 3) is associated with respiratory failure with no intervening uncontrolled respiration.

FIG. 3. Effect of oligomycin and FCCP on basal respiration. Cells were equilibrated for 30 min in standard buffer (A and C) or buffer supplemented with 10 mM pyruvate (B and D). The basal state was recorded over a 15-min period. This was followed by perfusion with 0.2 μg/ml oligomycin (oligo) followed by 2 μM FCCP to assess uncontrolled respiration. A and B, respiration was normalized to the basal rate (A, 0.96; B, 1.11 nmol of O/min × 10^6 cells) and expressed per total (solid lines) or viable (dashed lines) cell. C and D, time course of TMRM fluorescence (dashed lines; FU, fluorescence units) and FURA-PE3 340/380 nm ratio (solid lines) changes in a representative cell soma from each experiment. Fractions represent the proportion of cells examined that failed to maintain [Ca2+]i homeostasis after oligomycin. Data are from a single CGN preparation cultured 7 days in vitro.

FIG. 4. Effects of 3 h of continuous glutamate/glycine perfusion on CGN respiration, TMRM+ fluorescence, and FURA-PE3 340/380 nm ratio. 3 × 10^6 cells were equilibrated for 30 min in standard buffer. Respiration for each preparation was normalized to the 30–60-min basal period. A, initial respiratory capacity assessed by perfusion with 2 μM FCCP (n = 6). Error bar represents ± 1 S.D. of peak uncontrolled respiration. B, 180-min perfusion with standard buffer containing 250 μM glutamate plus 25 μM glycine (glu), followed by standard buffer with 2 μM FCCP to assess final respiratory capacity (n = 4). Basal respiration was 1.23 ± 0.29 nmol of O/min × 10^6 cells (mean ± S.E.). Solid line, normalized respiration per 10^6 total cells; dashed line, normalized respiration per 10^6 viable cells. Bars show S.E.s. C–E, representative cell soma TMRM+ fluorescence (dashed lines; FU, fluorescence units) and FURA-PE3 340/380 nm ratio (solid lines) of CGNs divided into three groups as follows: those that survived 3 h of continuous glutamate (C), those that Ca2+-deregulated immediately (D), and those that recovered from initial Ca2+ load imposed by glutamate but deregulated after a variable lag period (E). Twenty cells from each experiment were randomly selected for quantitative fluorescence analysis. Fractions are the number of cells in each group.

Ca2+ homeostasis after oligomycin. Thus oligomycin addition in the absence of exogenous pyruvate causes an ATP depletion that results in impaired glycolysis and subsequent mitochondrial respiratory inhibition because of a failure of substrate supply. This can be prevented by exogenous pyruvate even though the substrate cannot contribute to cellular ATP generation in the presence of oligomycin.
Consequently, respiration was corrected for viable cell number with each image taken (Fig. 4B, dashed line). In the corrected trace, viable CGNs maintain respiration at or slightly above basal for the entire 3-h glutamate exposure. Because experimental variation in viability was large, it is uncertain if the apparent rise in viable cell respiration during the final 60 min of glutamate reflects a true increase in viable cell metabolism above basal. Additionally, the maximal respiratory capacity of the viable cells with FCCP was within the range of that determined for cells not exposed to glutamate (Fig. 4A). Thus surviving cells do not undergo a loss of respiratory capacity, and contradictory reports in the literature (15) are most probably because of cell death.

Viability was significantly lower after 3 h of continuous glutamate exposure compared with 4 h of control perfusions (Table 1). After correcting for viability, each replicate showed a reserve ATP synthesis capacity after 3 h of continuous glutamate, although this was variable (100 ± 60%) and was not significantly different from that determined in control perfusions after 1 (84 ± 10%; Fig 4A) or 4 h (76 ± 15%; Table 1).

The Initial Increased Respiration in the Presence of Glutamate Is a Combination of Increased ATP Turnover and Mitochondrial Proton Leak—To determine whether glutamate-stimulated respiration was because of increased ATP demand or increased H⁺ leak, 10-min glutamate-stimulated CGNs were treated for 15 min with 0.2 μg/ml oligomycin plus glutamate (Fig. 5). Brief oligomycin perfusion was used to minimize subsequent cell death (see Fig. 3). The oligomycin-insensitive respiration was significantly greater in CGNs acutely treated with glutamate compared with time-matched controls (50% of basal for glutamate treated versus 40% of basal for controls). Nonmitochondrial respiration was not significantly different between these experiments, indicating that the increased oligomycin-insensitive rate was because of a greater mitochondrial H⁺ leak or utilization of the proton current for some process other than ATP synthesis.

In one experiment (not shown), CGNs were treated with glutamate for 10 min as in Fig. 5B and then perfused for 15 min with oligomycin in the absence of glutamate. The oligomycin-insensitive rate (43%, with 11% nonmitochondrial) was close to that determined for control perfusions (Fig. 5A; range 36–42%), suggesting that the increased H⁺ leak may rapidly reverse as glutamate is removed.

To determine whether the increased H⁺ leak persists during longer glutamate exposure, CGNs were perfused for 140 min with glutamate followed by 15 min with oligomycin plus glutamate (Fig. 5C). After correction for nonviable cells, oligomycin-insensitive respiration returned to control levels and was significantly lower than after 25 min of total glutamate exposure (Fig. 5, C versus B). However, variation after additionally correcting for nonmitochondrial respiration meant that the H⁺ leak after 155 min of glutamate exposure was not significantly different from either the control or 25-min glutamate leak rates (one-way ANOVA, control, 33.7 ± 1.8%; 25-min glutamate exposure, 44.5 ± 0.6%; 155-min glutamate exposure, 35.7 ± 3.7%).

The NMDA Receptor Is the Primary Contributor to Glutamate-stimulated Respiration—Although glutamate activates multiple receptors, it is the NMDA receptor that is responsible for excitotoxicity under the present conditions (23). To assess the NMDA receptor contribution to the metabolic demand imposed by glutamate, cells were sequentially exposed to glutamate and NMDA (Fig. 6A) or NMDA prior to glutamate (Fig. 6B). The NMDA and glutamate responses were independent of the order of addition (Fig. 6, A versus B). Respiration stimulated by NMDA was 62% of that stimulated by glutamate. Consistent with this finding, glutamate-stimulated respiration was reduced by 67% in the presence of the selective NMDA receptor antagonist MK801 (Fig. 6B). 5 μM MK801 completely blocked the NMDA response, confirming that this concentration was saturating under the conditions used (Fig. 6A). The responses elicited via the NMDA and non-NMDA components were virtually additive compared with the total glutamate response (Fig. 6B).

Pyruvate Increases Initial Maximal Respiration and Protects against Glutamate Excitotoxicity—Supplementing the standard medium with 10 mM pyruvate did not affect basal respiration (Fig. 7B versus Fig. 4B) but significantly increased the maximal FCCP rate from 84 ± 9% (n = 6; Fig. 4A) to 127 ± 16% (n = 3; Fig. 7A) above basal (student’s t test, p = 0.02), indicating that substrate supply to mitochondria via glycolysis is limiting in the fully uncontrolled state, as in the absence of mitochondrial ATP synthesis (Fig. 3).

In the presence of pyruvate, the initial respiratory increase to glutamate was not significantly different compared with experiments without pyruvate (n = 4; 44 ± 8% versus 32 ± 4%, with versus without pyruvate; p = 0.22, Student’s t test). Although cell

### Table I

**CGN viabilities with each treatment as a function of time**

| Time | Control | +Glutamate | Glutamate + MK801 | Glutamate + pyruvate | FCCP + glutamate |
|------|---------|------------|-------------------|----------------------|------------------|
| min  |         |            |                   |                      |                  |
| 60   | 100 ± 0 | 99.5 ± 0.5 | 100 ± 0           | 100 ± 0              | 100 ± 0          |
| 80   | 100 ± 0 | 97.1 ± 1.3 | 94.1 ± 1.4        | 94.3 ± 2.9           | 92.9 ± 0.5       |
| 100  | 99.7 ± 0.3^a| 95.1 ± 1.3^ab | 93.5 ± 1.4^ab | 92.8 ± 2.7^ab | 88.6 ± 1.1^b |
| 120  | 99.2 ± 0.5^a| 94.3 ± 1.3^ab | 93.1 ± 1.3^ab | 92.1 ± 3.1^ab | 86.2 ± 1.3^b |
| 140  | 98.5 ± 0.7 | 90.8 ± 1.9^ab | 92.9 ± 1.2^b  | 91.7 ± 3.2^ab | 85.3 ± 2.2^b |
| 160  | 97.9 ± 1.0^a| 82.1 ± 3.0^b  | 90.5 ± 0.6^b  | 90.9 ± 3.0^b  | 82.9 ± 3.6^b |
| 180  | 97.1 ± 1.2^a| 73.4 ± 6.0^b  | 86.7 ± 1.7^ab | 90.5 ± 2.9^b  | 79.6 ± 4.1^ab |
| 200  | 95.2 ± 2.3^a| 67.6 ± 7.6^b  | 82.4 ± 3.2^ab | 89.1 ± 2.9^b  | 74.3 ± 4.5^ab |
| 220  | 93.6 ± 3.4^a| 62.2 ± 8.5^b  | 76.4 ± 4.7^ab | 87.0 ± 3.5^b  | 69.1 ± 7.8^ab |
| 240  | 91.4 ± 4.1^a| 54.7 ± 10.1^b | 71.6 ± 6.0^ab | 82.9 ± 4.4^ab | 57.7 ± 10.8^ab |

2-Way ANOVA: p values

- Time: <0.0001
- Treatment: 0.003
- Time × treatment: <0.0001
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Fig. 5. Oligomycin-insensitive respiration of CGNs and effects of acute and chronic glutamate. A, following 70 min of standard buffer, buffer with 0.2 μM oligomycin was perfused followed at 85 min by 2 μM myxothiazol plus 1 μM rotenone (myxo/rot). B, at 60 min cells were perfused with 250 μM glutamate plus 25 μM glycine (glu) for 10 min, followed by oligomycin plus glutamate/glycine at 70 min and myxothiazol plus rotenone at 85 min. C, at 60 min cells were perfused with glutamate/glycine, followed at 200 min by oligomycin plus glutamate/glycine and at 215 min by rotenone plus myxothiazol. Respiration data are normalized to the average basal rate for each preparation (n = 4 for each condition; values in nmol of O/min × 10^6 cells; A, 0.94 ± 0.15; B, 0.90 ± 0.12; C, 1.39 ± 0.21). Cells were used at 7–10 days in vitro. Rates next to each steady-state are expressed as percent of basal viable cell respiration; means sharing common superscripts are not significantly different, as assessed by 1-way ANOVA. D–F, representative cell soma TMRM^+ fluorescence (dashed line; FU, fluorescence units) and FURA-PE3 340/380 nm ratio over the time course of each experiment. Arrows above traces correspond to additions specified in A–C.

Fig. 6. Contribution of the NMDA receptor to the glutamate-induced respiratory response. CGNs were equilibrated for 45–60 min in standard buffer before recording. Cells were perfused with 250 μM glutamate plus 25 μM glycine (glu), 300 μM NMDA plus 25 μM glycine (NMDA), 5 μM MK801, NMDA plus MK801, or glutamate/glycine plus MK901 as shown. Numbers on each trace represent percent increase in respiration above the basal 100% period. Data are from a single granule neuron preparation cultured either 7 (A) or 8 (B) days in vitro. Basal respiration averaged 1.08 (A) and 1.19 (B) nmol of O/min × 10^6 cells. Cell viability, assessed as described in Fig. 4 and the text, was used to correct the raw traces for dead cells.

viability was significantly improved by pyruvate (Table I), viable cell respiration declined steadily with time after glutamate and responded weakly to FCCP (Fig. 7B), in contrast to glutamate controls that had robust FCCP responses.

Although the initial respiratory response to glutamate was not enhanced, cells in 10 mM pyruvate-supplemented buffer exhibited greater initial \([\text{Ca}^{2+}]\), responses to glutamate, with mean peak FURA-PE3 340/380 nm increasing by 0.64 ± 0.03 (Fig. 7, C–E; n = 80 cells). Similarly, TMRM^+ dropped to a greater extent after glutamate (71 ± 1%; n = 80 cells) than when using the standard buffer without pyruvate, indicating a greater plasma and/or mitochondrial depolarization.

Whereas pyruvate relieves substrate limitation, and hence increases uncontrolled respiration in the presence of oligomycin (Fig. 3B) or in the basal state (cf. Fig. 7A and Fig. 4A), the decrease in uncontrolled respiration following 3 h of glutamate exposure in the presence of pyruvate (Fig. 7B) is an apparent contradiction. More cells survive glutamate exposure in the presence of pyruvate; however, sustained \([\text{Ca}^{2+}]\), levels are higher (cf. Fig. 7C and Fig. 4C), suggesting that the respiratory capacity of viable cells may be compromised by \(\text{Ca}^{2+}\).

Submaximal FCCP Facilitates Cell Death during Initial Glutamate Exposure—In the presence of 10% serum, 1 μM FCCP is largely bound to albumin and is not sufficient to fully relieve CGN respiratory control (data not shown). Perfusion of cells with 1 μM FCCP starting 15 min prior to glutamate exposure increased respiration 41% above basal (Fig 8B). This was associated with a significant 22 ± 2% TMRM^+ reduction but no significant FURA-PE3 340/380 change (basal, 1.59 ± 0.02, n = 40; 1 μM FCCP, 1.62 ± 0.02, n = 40 cells; Student’s t test). Subsequent glutamate perfusion in the continued presence of 1 μM FCCP resulted in a significantly lower respiratory increase (8%, n = 2) compared with experiments in the absence of pronophore (32 ± 4%; n = 4). Additionally, in the presence of FCCP, the average peak FURA-PE3 340/380 increase following glutamate was significantly greater than without FCCP (0.50 ± 0.05, n = 40 cells versus 0.32 ± 0.02, n = 80 cells; p < 0.0001 by Student’s t test). Viable cell respiration was sustained at or very close to the initial 1 μM FCCP-stimulated rate throughout the experiment. During the 1st h of glutamate/FCGP exposure, the presence of 1 μM FCCP was the only condition that resulted in a significantly lower viability when compared with controls (Table I).

Excess FCCP Leads to Rapid, Uniform Changes in Cell Morphology following Glutamate Exposure without Complete Loss of Respiration—Pretreating cells with 5 μM FCCP increased respiration to the same extent as 2 μM FCCP, but with much slower kinetics (Fig. 9A). Whole-cell TMRM^+ fluorescence was...
reduced by 90% (Fig. 9B). Subsequent glutamate addition resulted in immediate loss of Ca²⁺ homeostasis in all cells examined and a rapid decline in respiration (Fig. 9, A and B). After 90 min of exposure, ~98% of cells showed changes in morphology (Fig. 9, C and D). Using the current criteria for dead cells (i.e. loss of TMRM⁺ and Ca²⁺ homeostasis and changes in morphology), 98% were considered dead. At this time, respiration was 19% of peak rate, indicating that at least 19% of cells considered dead were still consuming oxygen. Although this does not change the overall conclusions from the preceding experiments, it does limit the precision with which it is possible to use these criteria to correct for cell death.

Viable Cell FURA-PE3 Ratios Show an Inverse Correlation with Respiratory Capacity—Because the cells are perfused under conditions of partial plasma membrane depolarization (25 mM K⁺), [Ca²⁺]ᵢ is elevated because of Ca²⁺ channel activation (24). In the absence of NMDA receptor activation (control or glutamate plus MK801) [Ca²⁺]ᵢ decreased significantly during the course of the experiment (Fig. 10). At the end of the experiment, [Ca²⁺]ᵢ in glutamate-exposed cells was not significantly higher than before glutamate addition, although with glutamate plus pyruvate [Ca²⁺]ᵢ was significantly enhanced. There was a significant (p = 0.02, r² = 0.314) negative correlation between the FCCP respiratory response and the average viable cell FURA-PE3 340/380 nm ratio at the end of each experiment, indicating that chronically elevated [Ca²⁺]ᵢ may lead to loss of cell substrate oxidation capacity (Fig. 11). This is consistent with the reduced respiratory capacity observed after synchronous Ca²⁺ deregulation with oligomycin (Fig. 3).

After 4 h of control perfusions, viable cell TMRM⁺ was not significantly different from the basal 30–60-min period (Fig. 10B). Perfusions with glutamate and glutamate plus pyruvate resulted in significantly lower viable cell TMRM⁺ compared with the basal 30–60-min period prior to glutamate addition, consistent with NMDA receptor-mediated plasma membrane depolarization, while 0.5 µM MK801 attenuated this effect.

**DISCUSSION**

Bioenergetic parameters measure the extents of displacement from equilibrium (e.g. Gibbs free energy, protonotive force, membrane potential, redox state, and ATP/ADP ratios), the rates of energy flux (e.g. metabolic rates, ATP turnover, proton flux, and respiration), or combinations of these parameters (e.g. proton conductance that is determined as proton leak per nV of proton electrochemical gradient). Although there is considerable literature detailing the rates of glucose or pyruvate utilization and lactate or CO₂ production in CGN cultures (reviewed in Refs. 25–27), technical problems have limited the investigation of the primary mitochondrial flux parameter, namely the rate of electron transport or proton cycling as monitored by respiration. The classic oxygen electrode chamber has proven to be the single most important apparatus for monitoring the function of isolated mitochondria, because careful experimental design allows determination of ATP turnover, proton leaks, maximal electron transport activity, and other extensive parameters (for review see Ref. 28). However, a conventional cylindrical chamber is entirely unsuitable for monitoring the respiration of a neuronal monolayer attached to a substrate. This study shows that the respiration of a neuronal monolayer can reliably be measured over extended periods using flow-through polarographic oxygen electrodes.
rate slightly underestimates true oxygen utilized under these conditions because not all the 3 × 10⁶ cells initially used attach to the coverslip, and some plated cells do not end up in the perfusion area once the chamber is assembled.

Most values in the existing literature are expressed per mg of cell protein. Hertz and Peng (26) report a value of 44 nmol of O/min × mg of cell protein for CGNs in 55 mM K⁺. Based on a value of 76 μg of protein/10⁶ cells (29), this translates to 3.3 nmol of O/min × 10⁶ cells. Slightly higher values (62 nmol of O/min × mg of cell protein, or 4.7 nmol of O/min × 10⁶ cells) were reported by Atlante et al. (15) for CGNs in 18 mM K⁺, whereas Almeida et al. (30) measured a basal rate of 6.5 nmol of O/min × mg of cell protein for cortical neuronal cells at 30 °C. In studies expressed per 10⁶ cells, Bal-Price et al. (17, 31) reported value of 3.9–4 nmol of O/min × 10⁶ CGNs in 25 mM K⁺. However, with the exception of the present study, it must be remembered that all cited investigations required scraping or trypsinization of the cells to remove them from their substrates, almost certainly resulting in some cell damage. Significantly, we are aware of no studies that report the extent of protonophore-relieved respiratory control seen in such preparations.

In the basal state, in situ CGN mitochondria operate at about 50% of maximal respiration (Fig. 2A). Oligomycin reduced respiration by 61%, suggesting that ATP turnover accounts for this percentage of basal cell respiration. However, this is an underestimate because oligomycin hyperpolarizes mitochondria, enhancing the activity of the highly potential dependent constitutive inner membrane proton leak (22). Rolfe et al. (32) showed that oligomycin reduced hepatocyte respiration by ~64%; however, when corrected for membrane potential changes in H⁺ leak, ATP turnover was calculated to account for 69% of basal cell respiration. If CGN H⁺ leak is similarly dependent on Δψm, then the reported oligomycin-insensitive respiration is about 20% higher than it would be in the absence of inhibitor (Fig. 12). By using this correction, a better estimate is that 68% of CGN respiration supports ATP turnover, 21% supports H⁺ leak, and the remaining 11% (in the presence of rotenone plus myxothiazol) supports nonmitochondrial reactions.
Pyruvate supplementation did not significantly affect basal respiration, indicating that in this state glycolysis provides adequate substrate to the mitochondrion. However, during uncontrolled respiration glycolytic substrate supply to the mitochondria limits respiratory chain activity because pyruvate significantly enhanced the FCCP effect. Moreover, under the present incubation conditions, which prevent induction of apoptosis, these CGN preparations had insufficient glycolytic activity to maintain prolonged basal function in the presence of oligomycin (Fig. 3). This is in contrast to previous work with CGNs maintained in low K⁺ media (12, 33). Most important, pyruvate decreases cell death (Fig. 3) in the presence of oligomycin even though it cannot contribute to ATP production, suggesting that mitochondrial polarization is protective, perhaps by maintaining NADPH and glutathione reduction.

Acute Effects of Glutamate—A key finding from this study is that under the present conditions glutamate exposure acutely stimulates CGN respiration submaximally. From first principles it would be predicted that NMDA receptor activation would lead to an increase in CGN mitochondrial respiration on three counts as follows: utilization of the proton gradient to drive Ca²⁺ uptake into the matrix, increased plasma membrane Ca²⁺-ATPase activity to expel Ca²⁺ entering the cell, and increased Na⁺/K⁺-ATPase activity in response to increased Na⁺ entry. In fact, a 32% increase in basal respiration was observed immediately following glutamate infusion (Fig. 4B). This increase utilized about 40–50% of the reserve ATP-generating capacity of CGN mitochondria. Pyruvate supplementation did not further increase the response to glutamate (Fig. 7B). Thus, glycolysis was capable of supplying necessary substrate to sustain the higher respiratory chain activity, and oxidative phosphorylation was capable of supplying sufficient ATP to meet the energy demand imposed by glutamate.

Atlante et al. (15) exposed CGNs to glutamate/glycine in Mg²⁺-free low K⁺ medium for 30 min, washed, and suspended the neurons by scraping and determined respiration in the presence of 10 mM glucose. They reported a 20% decrease in respiration relative to control cells; however, because cell viability was not monitored, it is unclear whether this represented a change in respiration per viable cell. Surprisingly, they observed a total inhibition of respiration by ouabain both in
control and glutamate-exposed neurons, suggesting that their assay was insufficiently sensitive to detect the respiration under conditions of low ATP demand. In their study, classic respiratory control was performed on homogenates from parallel cell preparations, and a 50% decrease in state 3 respiration was observed with succinate as substrate with little change in state 4 rate (15). This was incorrectly interpreted as indicating rapid "uncoupling" of oxidative phosphorylation, i.e. an increased H⁺ leak, which would have manifested as an increased state 4 respiration. The decreased state 3 rate could reflect either an inhibited respiratory chain or a decreased yield of mitochondria because of cell death.

In a related study, Almeida et al. (34) exposed cortical neurons cultured in 78-cm² dishes to glutamate for 15 min, re-stored the culture medium, and monitored respiration after 24 h by trypsinizing the cells, washing, storing on ice, and assaying in a conventional Clark-type oxygen electrode chamber. They reported a modest decrease in respiration, but the ability of the preparation to respond to succinate indicated extensive damage resulting from the manipulations.

The glutamate-induced initial increase in respiration can be analyzed both to determine the proportion of the response because of NMDA- and non-NMDA-mediated effects and also to distinguish between increased ATP demand and increased utilization of the H⁺ current via H⁺ leaks (classic uncoupling) or to drive matrix Ca²⁺ accumulation. Comparison of the effects of glutamate and the selective agonist NMDA (Fig. 6) shows that 62% of the increase in respiration is because of NMDA receptor activation. Clearly ion influx through this receptor stimulates ATP turnover but not to such a degree that demand exceeds supply. Thus, the protective effect of MK801 (Table I; Fig. 10) is likely a consequence of limiting one or more Ca²⁺-dependent processes rather than reducing ATP demand. The non-NMDA-driven respiration is additive to the NMDA effect and could be because of the metabolic effects of the metabotropic glutamate receptor, AMPA/kainate receptor activation, or glutamate uptake into neurons or contaminating astrocytes causing an increased ATP demand by the Na⁺/K⁺-ATPase. However, no respiratory inhibition was observed on addition of the glutamate transport inhibitor DL-threo-β-benzoyloxyaspartic acid (100 μM, data not shown).

The analysis to distinguish ATP turnover from uncoupled respiration was performed. The second analysis was performed by acute addition of oligomycin to inhibit ATP synthase (Fig. 5). Again, it should be noted that under these conditions these CGN preparations do not possess sufficient glycolytic activity to maintain Ca²⁺ homeostasis in the absence of mitochondrial ATP synthesis, and rapid cell death ensues. The slight 10% increase in oligomycin-insensitive respiration observed 25 min following glutamate addition (Fig. 5) does not persist, confirming the absence of a significant population of rapidly respiring "uncoupled" mitochondria in the glutamate-exposed cells. The transient increase could (from first principles) be due to the utilization of the H⁺ current to drive matrix Ca²⁺ accumulation during the initial period of glutamate exposure. However, in this case a similar increase would be predicted following oligomycin alone, which causes cytoplasmic Ca²⁺ to rise to a similar extent as that in glutamate-treated cells (Fig. 5).

The CGN preparations used in Fig. 4B and Fig. 5, B and C, responded similarly to glutamate, with a peak respiration occurring 6 min after glutamate and reaching the same extent (30 ± 5% in Fig. 5B, 39 ± 7% in Fig. 5C, and 32 ± 4% in Fig. 4B). Oligomycin blocked oxidative phosphorylation after a short delay (Figs. 3 and 5). During this time, glutamate-stimulated respiration declined to 19–21% above basal or about 0.18 nmol of O/min x 10⁶ viable cells (Fig. 5C and Fig. 4B), suggesting that 50% of the glutamate-induced increase is attributable to a mitochondrial proton leak. However, because oligomycin hyperpolarizes mitochondria and glutamate slightly depolarizes mitochondria (6), it is necessary to correct the leak for changes in ΔΨm. A more realistic estimate is that H⁺ leak accounts for about 15% of the increase with glutamate, whereas the remaining 85% would be attributable to ATP turnover (Fig. 12). Because this uncoupling rapidly reverses upon glutamate washout, it does not seem to reflect a small minority of cells entering a fully uncoupled state but rather may be a consequence of activating a regulated H⁺ conductance (e.g. an uncoupling protein).

Chronic Glutamate Exposure—An important finding of this study is that mitochondria in surviving CGNs do not show diminished respiratory capacity after 3 h of continuous glutamate exposure. Previous attempts to monitor the effects of glutamate exposure on neuronal respiration (e.g., Ref. 35) have not allowed for stochastic cell death, and so it has been unclear whether reported chronic decreases in respiration were because of cell death or a uniform respiratory inhibition. If the assumption is made that Ca²⁺ deregulated cells having altered morphology and no TMRM⁺ fluorescence do not respire, then the 45% remaining viable cells after 3 h glutamate show no decrease in respiration, or even a statistically insignificant increase (Fig. 4B). These cells retain protonophore-releaseable respiratory control. Moreover, viable cell respiration with 1 μM FCCP is maintained at the pre-glutamate level throughout 3 h glutamate exposure (Fig. 5B), again suggesting no loss of mitochondrial function.

The analysis of cell respiration makes the assumption that Ca²⁺ deregulated, morphologically altered cells lacking TMRM⁺ fluorescence do not consume oxygen. This was tested by using oligomycin to cause a synchronous Ca²⁺ deregulation in the whole-cell population that was monitored in the respirometer (Fig. 3). No prominent increase in oligomycin-insensitive respiration was seen despite an initial FURA 340/380 nm ratio increase from about 1.4 to 3. Furthermore, extended incubation with oligomycin resulted in further respiratory inhibition, reduction in TMRM⁺ signal, and altered cell morphology. From this perspective, the assumption that glutamate-treated cells that undergo these changes do notrespire seems valid. However, when 98% of cells were considered dead (i.e. nonrespiring) following treatment with excess FCCP plus glutamate, respiration declined by 81% rather than the expected 98% (Fig. 9). This indicates that (a) some cells considered "dead" do consume oxygen, and (b) the viable cell respiration traces overestimate the true rate at which these cells consume oxygen. Although this uncertainty does not affect our conclusions that cells exposed to glutamate retain normal mitochondrial function until cell death, it is clear that more experiments are required to determine the variable(s) that more accurately differentiate an oxygen-consuming cell from one that is truly dead.

Because rat brain mitochondria possess a high Ca²⁺ uniporter activity sufficient to utilize the full capacity of the respiratory chain when extramitochondrial [Ca²⁺] is elevated (36), the lack of respiratory increase associated with the increased [Ca²⁺], following oligomycin was initially surprising (Fig. 3), suggesting that elevated [Ca²⁺] was not sufficient to activate the uniporter significantly, that the uniporter was inhibited, or that mitochondrial respiration was severely limited because of lack of substrate. Glycolytic failure did occur with oligomycin because uncontrolled respiration with pyruvate supplementation was much greater than with glucose alone (Fig. 3). Despite this, the failure to observe a prominent increase in respiration after oligomycin-induced Ca²⁺ deregulation can-
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not be attributed to substrate supply problems because by-passing glycolysis with exogenous pyruvate did not stimulate state 4 respiration.

The resting $[\text{Ca}^{2+}]_c$ of granule neurons in 25 mM KCl is about 100 nM (37), well below the threshold of about 500 nM for initiating mitochondrial $\text{Ca}^{2+}$ accumulation (36). Even with 70 mM KCl to fully activate L-type $\text{Ca}^{2+}$ channels, steady-state $[\text{Ca}^{2+}]_c$, only reaches about 200 nM (37). Therefore, the basal rate of $\text{Ca}^{2+}$ entry in 25 mM KCl is quite low relative to influx necessary to achieve mitochondrial loading. In Fig. 3, C and D, it is seen that oligomycin caused FURA 340/380 nm to plateau at about 3.0; this is more readily seen in Fig. 3D with pyruvate present. The FURA plateau at 3 likely represents the critical concentration of about 500 nM at which the mitochondria began accumulating $\text{Ca}^{2+}$ at a slow rate that matched the continued rate of $\text{Ca}^{2+}$ entry across the plasma membrane (36). Subsequent FCCP addition further increased this ratio to $>$4 in Fig. 3D. Because FCCP depolarizes mitochondria, thus inhibiting $\text{Ca}^{2+}$ accumulation, the further FURA increase is consistent with the plateau at 3 representing the point at which mitochondria began accumulating $\text{Ca}^{2+}$. The plateau was not sustained in the cell depicted in Fig. 3C, suggesting failure of mitochondrial $\text{Ca}^{2+}$ accumulation. This is consistent with the rapid loss of TMRM$^+$ in this cell. Careful inspection of the respiration traces in Fig. 3, A and B, provides an indication as to the amount of oxygen utilized for $\text{Ca}^{2+}$ uptake. Oligomycin addition initially reduced respiration to 33 and 34% of basal rate, respectively. This was followed by a very small increase (about 4% in both cases) to a final constant state 4 rate. In both cases the increase amounted to 0.04 nmol of O/min $\times 10^6$ cells. We suggest that this small increase represents the utilization of the proton motive force to drive $\text{Ca}^{2+}$ accumulation under these conditions. This is an exceedingly small rate of $\text{Ca}^{2+}$ uptake, consistent with the idea that $\text{Ca}^{2+}$ entry through L-type $\text{Ca}^{2+}$ channels is relatively low under the current conditions. Thus, $[\text{Ca}^{2+}]_c$ did not increase to such an extent to prominently activate the $\text{Ca}^{2+}$ uniporter.

Taken together, the data indicate that glutamate-induced DCD is associated with a loss of respiration rather than pronounced uncoupling. Does $\text{Ca}^{2+}$ deregulation precede or follow loss of respiration? This remains an unresolved issue because these alternatives cannot adequately be resolved in these experiments, particularly given the synchronous changes in TMRM$^+$ and FURA-PE3 signals as cells begin to lose $\text{Ca}^{2+}$ homeostasis. Although there is no indication of a slow decline in mitochondrial function of viable cells after glutamate, it may be that mitochondrial respiration rapidly shuts down, resulting in $\text{Ca}^{2+}$ deregulation. This possibility cannot be distinguished from the alternative that $\text{Ca}^{2+}$ deregulation results in loss of respiration.

Chronic Glutamate Exposure with Pyruvate—Pyruvate supplementation significantly attenuates glutamate excitotoxicity (Table I). This protection cannot be attributed to enhanced mitochondrial substrate supply because glutamate did not maximally stimulate respiration and pyruvate did not further enhance glutamate-stimulated rate, in contrast to its effects on uncontrolled respiration. Surprisingly, cells that remained viable after 3 h of continuous glutamate exposure exhibited a pronounced reduction in maximal respiratory capacity (Fig. 7B), indicating an impairment downstream of pyruvate supply to the mitochondria. Four observations indicate that elevated cytoplasmic $\text{Ca}^{2+}$ levels may be involved. First, there was a significant negative correlation between $[\text{Ca}^{2+}]_c$, and mitochondrial responsiveness to FCCP (Fig. 11). Second, $\text{Ca}^{2+}$ transients immediately following glutamate addition were significantly greater with pyruvate present (Fig. 7). Third, cytoplasmic $\text{Ca}^{2+}$ levels after 3 h of glutamate exposure were significantly higher than before glutamate addition (Fig. 10). Fourth, oligomycin-induced $\text{Ca}^{2+}$ deregulation resulted in impaired uncontrolled respiration (cf. Fig. 3B and Fig. 7A). Clearly, pyruvate protection from glutamate excitotoxicity cannot be ascribed to improved mitochondrial function, as indicated by respiratory capacity. This is not surprising given that chronic glutamate exposure imposed very little energy demand above the basal state (Fig. 4). Reduction in the reserve ATP-generating capacity is therefore not an event associated with DCD. Previous studies (18, 38–42) have shown that pyruvate-supplemented media protect neurons from glutamate- or NMDA-mediated excitotoxicity. However, it is unclear how pyruvate paradoxically increases $\text{Ca}^{2+}$ influx and protects against excitotoxicity.

Conclusions—The electrode has proven over the years to be the most versatile apparatus for investigating the function of isolated mitochondria and cells that exist in suspension. The lack of a similar device for cultured neurons and other cells attached to a substrate has restricted investigations of in situ mitochondrial function in such preparations. By combining continuous flow oxygen electrodes with a digital fluorescence imager, it is possible to obtain quantitative information on mitochondrial and cellular energy supply and demand while simultaneously viewing a representative field to monitor cell viability together with parameters such as membrane potentials and $[\text{Ca}^{2+}]_c$. The hypotheses that glutamate stimulates CGN respiration maximally, that the increase is entirely the result of increased ATP demand, and that excitotoxicity is associated with a loss of reserve ATP-generating capacity are not valid for the present experimental conditions, although the data support the hypothesis that the NMDA receptor is a significant contributor to the overall energy demand imposed by glutamate. It has not escaped our attention that studies to investigate neuronal bioenergetic changes associated with apoptosis and other stresses are equally feasible with this technique.

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