

# Superoxide ( $\cdot\text{O}_2^-$ ) Production in CA1 Neurons of Rat Hippocampal Slices Exposed to Graded Levels of Oxygen

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Submitted 21 September 2006; accepted in final form 31 May 2007

**D'Agostino DP, Putnam RW, Dean JB.** Superoxide ( $\cdot\text{O}_2^-$ ) production in CA1 neurons of rat hippocampal slices exposed to graded levels of oxygen. *J Neurophysiol* 98: 1030–1041, 2007. First published June 6, 2007; doi:10.1152/jn.01003.2006. Neuronal signaling, plasticity, and pathologies in CA1 hippocampal neurons are all intimately related to the redox environment and, thus tissue oxygenation. This study tests the hypothesis that hyperoxic superfusate (95%  $\text{O}_2$ ) causes a time-dependent increase in superoxide anion ( $\cdot\text{O}_2^-$ ) production in CA1 neurons in slices, which will decrease as oxygen concentration is decreased. Hippocampal slices (400  $\mu\text{m}$ ) from weaned rats were incubated with the fluorescent probe dihydroethidium (DHE), which detects intracellular  $\cdot\text{O}_2^-$  production. Slices were loaded for 30 min using 10  $\mu\text{M}$  DHE and maintained using one-sided superfusion or continuously loaded using 2.5  $\mu\text{M}$  DHE and maintained using two-sided superfusion (36°C). Continuous loading of DHE and two-sided superfusion gave the highest temporal resolution measurements of  $\cdot\text{O}_2^-$  production, which was estimated by the increase in fluorescence intensity units (FIUs) per minute (FIU/min  $\pm$  SE) over 4 h. Superoxide production (2.5  $\mu\text{M}$  DHE, 2-sided superfusion) was greatest in 95%  $\text{O}_2$  ( $6.6 \pm 0.4$  FIU/min) and decreased significantly during co-exposure with antioxidants (100  $\mu\text{M}$  melatonin, 25  $\mu\text{M}$  MnTMPyP) and lower levels of  $\text{O}_2$  (60, 40, and 20%  $\text{O}_2$  at  $5.3 \pm 0.3$ ,  $3.3 \pm 0.1$ , and  $1.6 \pm 0.2$  FIU/min, respectively). CA1 cell death after 4 h (ethidium homodimer-1 staining) was greatest in 95%  $\text{O}_2$  and lowest in 40 and 20%  $\text{O}_2$ . CA1 neurons generated evoked action potentials in 20%  $\text{O}_2$  for >4 h, indicating viability at lower levels of oxygenation. We conclude that  $\cdot\text{O}_2^-$  production and cell death in CA1 neurons increases in response to increasing oxygen concentration product (=  $\text{PO}_2 \times \text{time}$ ). Additionally, lower levels of oxygen (20–40%) and antioxidants should be considered to minimize superoxide-induced oxidative stress in brain slices.

## INTRODUCTION

Superoxide anion ( $\cdot\text{O}_2^-$ ), generated from the mitochondria, is the most abundant form of reactive oxygen species (ROS) in neurons (Emerit et al. 2004; Nicholls 2004; Sugawara and Chan 2003). Its levels are tightly regulated under normal conditions because it can, paradoxically, have both physiological and pathophysiological effects on cellular function (Droge 2002; McCord 1995). For example, in the CA1 hippocampus,  $\cdot\text{O}_2^-$  and other ROS act as signaling molecules in processes such as long-term potentiation (LTP) (Gahtan et al. 1998; Klann and Thiels 1999; Klann et al. 1998; Knapp and Klann 2002) and oxidative preconditioning induced by cerebral ischemia (Furuichi et al. 2005; Ravati et al. 2000) and breathing

hyperbaric oxygen (Nie et al. 2006). Excess production of  $\cdot\text{O}_2^-$ , however, can occur as a result of mitochondrial dysfunction with deleterious consequences for neurons (Emerit et al. 2004). In fact, there is a selective vulnerability of the CA1 pyramidal neurons to  $\cdot\text{O}_2^-$ -induced oxidative stress, which is thought to be critical in producing neuronal damage (Weinstock and Shoham 2004; Wilde et al. 1997). For example,  $\cdot\text{O}_2^-$  is reduced sequentially to form hydrogen peroxide and hydroxyl radicals. Excess  $\cdot\text{O}_2^-$  also reacts with nitric oxide to form peroxynitrite. All of these  $\cdot\text{O}_2^-$ -derived reactive species are highly toxic to neurons in excess concentrations (Emerit et al. 2004; Moncada and Bolanos 2006).

In the present study, we examined the effect of sustained oxygen exposure on  $\cdot\text{O}_2^-$  production in CA1 hippocampal neurons in tissue slices. The  $\text{PO}_2$  in the intact CNS ranges from <10 to 35 Torr for a rat breathing normobaric air (reviewed in Dean et al. 2003). Therefore tissue  $\text{PO}_2$ 's exceeding 35 Torr in the CNS are, presumably, hyperoxic. Work from our lab has demonstrated that the tissue  $\text{PO}_2$  profiles measured in 300- to 400- $\mu\text{m}$ -thick brain slices (rat) equilibrated with  $\geq 20\%$   $\text{O}_2$  are also hyperoxic from the surface to the core (Dean et al. 2005; Mulkey et al. 2001). Surprisingly, nearly all experiments using the in vitro brain slice are performed with solutions equilibrated with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ , which produces a tissue  $\text{PO}_2$  of  $\sim 440$  to  $\sim 300$  Torr (from surface to core, 150  $\mu\text{m}$  deep), which is the equivalent of a rat breathing >2.0 atmospheres absolute (ATA)  $\text{O}_2$  (Dean et al. 2003). These supraphysiological  $\text{PO}_2$ 's likely perturb redox homeostasis, which is intimately linked to cellular excitability (Droge 2002). In fact, recent evidence from our lab shows that normobaric and hyperbaric hyperoxia increases the excitability of CA1 neurons in hippocampal slices and activates neural plasticity (Dean et al. 2004; Garcia et al. 2004).

In this study, we hypothesized that 95%  $\text{O}_2$  causes a significant time-dependent increase in  $\cdot\text{O}_2^-$  levels in the CA1 region in hippocampal slices relative to lower levels of oxygen (60, 40, and 20%  $\text{O}_2$ ). The results of this study demonstrate that 95%  $\text{O}_2$  significantly increases the rate of  $\cdot\text{O}_2^-$  production and caused the greatest cell death over 4 h relative to lower levels of oxygen. These findings suggest that lower levels of control oxygen (e.g., 20 or 40%), supplemental antioxidants or both procedures should be considered for use with brain slices.

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## METHODS

*Solutions and materials*

Artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.3 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 1.24 KH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl, and 10 glucose, equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>, was used for tissue slicing (2–4°C), incubation (22–24°C), and the experimental control condition (35–36°C). Additional levels of oxygen tested included ACSF equilibrated with 60, 40, or 20% O<sub>2</sub> in 5% CO<sub>2</sub> and balance N<sub>2</sub> (35–36°C). Dihydroethidium (DHE) was purchased from Invitrogen (San Diego, CA) as stabilized 5 mM stock in DMSO and 20 μl aliquots were made and stored at –80°C. DHE was added to the ACSF at a final concentration of 10 μM for the preincubation protocol or at 2.5 μM for the continuous loading protocol. DHE solution was prepared fresh immediately before each experiment under dark conditions. Carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone (FCCP) was purchased from Sigma (St. Louis, MO) and prepared as a 5 mM stock (in ethanol) and used at a final concentration of 1 μM. Manganese(III)tetrakis(1-methyl-pyridyl)porphyrin pentachloride (MnTMPyP) was purchased from Calbiochem (La Jolla, CA) and used at a final concentration of 25 μM. Melatonin was purchased from Sigma and used at a final concentration of 100 μM.

*Preparation of hippocampal slices*

Animal procedures were done in agreement with the Wright State University and University of South Florida Laboratory Animal Care and Use Committees guidelines. Wright State University and University of South Florida are accredited by AAALAC and covered, respectively, by National Institutes of Health Assurance Nos. A360-01 and A4100-01.<sup>1</sup> Sagittal cortico-hippocampal slices (400 μm) were freshly prepared from Sprague Dawley rats (≥ P21 days, >100 g). Bilateral, sagittal tissue blocks, each containing one lobe of the hippocampus, were cut in ice-cold ACSF (4–6°C, equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>) with a vibratome (Pelco 101, series 1000). Preincubation of hippocampal slices with DHE (10 μM) began 30 min after slicing and was done in the dark with control ACSF (95% O<sub>2</sub>-5% CO<sub>2</sub>) at 37°C for 30 min. Next, slices preincubated with DHE were washed for 10 min in ACSF (95% O<sub>2</sub>) before being transferred to a thermoregulated (35°C) tissue slice bath (model RC-27L, Warner Instruments) with ~1.0 ml volume mounted on the stage of a Nikon TE2000 inverted epifluorescence microscope. In experiments using one-sided superfusion, the brain slice was immobilized between the bottom of the tissue bath, made from a cover glass, and a removable top grid of fine nylon mesh.

A second method for measuring ·O<sub>2</sub><sup>-</sup> production with DHE was to use two-sided superfusion of hippocampal slices while continuously loading a lower concentration of DHE (2.5 μM) via the ACSF (35–36°C). In these experiments, the brain slice was immobilized between a bottom stationary nylon mesh in the bath and a removable grid of nylon fibers. This second method allowed for direct exposure of oxygenated solution (containing DHE) to the neurons imaged at the bottom of the slice with the ×10 objective on the inverted microscope. As reported in the following text, this method was superior to the method of preloading and one-sided superfusion for monitoring oxygen-induced ·O<sub>2</sub><sup>-</sup> production. Thus the majority of the study was conducted using this latter method; i.e., Figs. 3–7. All solutions were equilibrated with a specific level of O<sub>2</sub> for ≥30 min at 35–36°C before attempting to image slices. Solutions containing DHE in the superfusate were replaced every 30 min with fresh media to minimize excessive oxidation of the fluorogenic probe.

<sup>1</sup>Experiments (Figs. 1–5) and initial analyses were conducted at Wright State University, Dayton, OH. The final analyses, manuscript preparation and additional experiments requested after peer review (Figs. 6 and 7) were conducted at the University of South Florida, Tampa, FL, using the same research equipment.

*Generating and scavenging superoxide*

Superoxide anion production was stimulated with FCCP (1 μM), which has been shown previously to stimulate mitochondrial ·O<sub>2</sub><sup>-</sup> production in vitro (Bindokas et al. 1996; Scanlon and Reynolds 1998; Sengpiel et al. 1998). MnTMPyP is a cell-permeable superoxide dismutase (SOD) and catalase (CAT) mimetic and has been used at a concentration of 25 μM to quench ·O<sub>2</sub><sup>-</sup> production (Dantas et al. 2002; Wang et al. 2004). Light-inactivated MnTMPyP (25 μM, 12 h visible light) was used to control for possible nonspecific effects of MnTMPyP on fluorescence intensity. Melatonin was used at a concentration of 100 μM as a broad spectrum antioxidant, which has been shown to be particularly effective at scavenging ·O<sub>2</sub><sup>-</sup> (Uchida et al. 2004).

*Visualization and measurement of superoxide in hippocampal slices*

Relative rate of ·O<sub>2</sub><sup>-</sup> production was measured by monitoring an increase in ethidium fluorescence over time generated by the oxidation of DHE by ·O<sub>2</sub><sup>-</sup> (Bindokas et al. 1996; Nakamura et al. 2001; Zhao et al. 2003). DHE is oxidized to the fluorescent product ethidium by intracellular ·O<sub>2</sub><sup>-</sup>, which intercalates with the cell's DNA and is thought to further enhance its fluorescent properties. Cellular fluorescence was imaged using a Nikon extra long working distance (ELWD, 16 mm) Plan Fluor ×10 infinity-corrected objective (0.30 NA) with Hoffman modulation contrast optics.<sup>2</sup> The oxidized product of DHE (ethidium) was visualized by alternatively exciting dye-loaded cells at 525 nm and then capturing their fluorescence emission at 590 nm. Baseline measurements of FIU were initiated within 15 min of transferring the slice to the tissue bath chamber. Measurements of FIU of individual neurons were taken every 1–5 min thereafter with minimum exposure times (200 ± 50 ms) to limit phototoxicity and photobleaching. Observations lasted 30 min to 4 h, depending on the experimental protocol. Images were captured using a digital camera (CoolSnap HQ, Roper Scientific, Tucson, AZ) and processed using MetaFluor software (Universal Imaging, Sunnyvale, CA).

*Data analysis*

During each DHE experiment, several cells within the CA1 pyramidal region were selected to monitor real-time production of ·O<sub>2</sub><sup>-</sup> and to gauge the outcome of the experiment. Subsequently, experiments were analyzed off-line using MetaFluor to measure changes in FIU of individual neurons, which are reported here. Several criteria were used to select cells for off-line analysis. In preincubated slices (10 μM DHE × 30 min) only cells with an initial baseline of 400–600 FIU were analyzed. A baseline FI of <300 units presumably reflected poor retention of DHE in cells, whereas a baseline FI >600 units appeared to indicate early oxidative stress, possibly from cells damaged near the surface of the slice; these cells were more apt to lyse and disappear before the experiment ended. Cells that disappeared (lysed) within the first 60 min of measurement were excluded from analysis. Average FIU for each cell was calculated as the change in fluorescence from baseline, ΔFI = (FI – FI<sub>b</sub>), where FI<sub>b</sub> is the baseline fluorescence defined by FI at the start of the experimental recordings. Superoxide production was reported as a change in absolute FIU at a given time point, or as a slope of the fluorescence intensity over time (ΔFIU/min), which reflected the rate of ·O<sub>2</sub><sup>-</sup> production. Each cell served as its own control. Fluorescence values were measured using MetaFluor software and were analyzed and plotted using Excel, SigmaPlot and KyPlot software packages. Statistical comparisons of

<sup>2</sup>Hoffman modulation contrast (HMC) optics were not required for these experiments however. The slit aperture in the HMC modified objective, which would reduce the amount of transmitted light, was not a problem when working with DHE because neutral density filters were still required to avert photobleaching during repeated DHE excitation.

absolute FIU and slopes of  $\cdot\text{O}_2^-$  production ( $\Delta\text{FIU}/\text{min}$ ) between graded  $\text{O}_2$  concentrations were made using an unpaired Student's *t*-test and  $P < 0.05$  was considered significant. One-way ANOVAs were performed on the slope of the FIU/time, and multiple comparison tests were performed using a Tukey-Kramer's test for pairwise comparisons ( $P < 0.05$ ). All FIU values are reported as the means  $\pm$  SE. All summary data were collected from at least three and  $\leq 12$  independent experiments in different slices.

### Visualization and measurement of cell death in CA1 cell layer

Relative levels of CA1 cellular viability in hippocampal slices exposed to graded levels of oxygen (0–4 h) were assessed using ethidium homodimer-1 (EH-1, 12 mM stock in DMSO) at a final concentration of 6  $\mu\text{M}$ . EH-1 fluorescence increases with increased numbers of dead cells and has been used to qualitatively estimate relative tissue viability in hippocampal slices (Bickler and Hansen 1998; Pinheiro et al. 2006). After recovery from sectioning (60 min in 95%  $\text{O}_2$ ), brain slices were exposed to 95, 60, 40, or 20%  $\text{O}_2$  in 5%  $\text{CO}_2$  and balance  $\text{N}_2$  for  $\leq 4$  h and then preincubated with EH-1 (20 min at 37°C). After incubation with EH-1, the slices were washed for 15 min and transferred to the imaging chamber. Images were captured using the same excitation/emission filters and ELWD ( $\times 10$ ) objective used with DHE. Five individual brains (4 slices/brain) were used for each level of oxygen ( $n = 20$  slices/level of  $\text{O}_2$ ) with slices sampled at times 0 and 4 h. EH-1 fluorescence was quantified by measuring the average EH-1 fluorescence within an  $800 \times 80 \mu\text{m}$  area positioned over the CA1 pyramidal region. Summary data are presented as the group average FIU  $\pm$  SE, and significance was evaluated for *time 0* versus 4 h at each level of  $\text{O}_2$  using a Student's unpaired *t*-test.

### Intracellular recordings of CA1 neurons during and after exposure to 20% $\text{O}_2$

Intracellular recordings were made in CA1 pyramidal neurons using sharp-tipped, high-resistance (50–110 M $\Omega$ ) microelectrodes filled with 3 M potassium acetate in bridge-balance mode using an Axoclamp 2A amplifier (Molecular Devices). After recovery from slicing in 95%  $\text{O}_2$  (1 h, 22°C) and incubation in 20%  $\text{O}_2$  (4 h, 22°C), slices were transferred to a recording chamber and superfused continuously with ACSF equilibrated with 20%  $\text{O}_2$  (36°C). Action potentials of CA1 neurons were evoked using positive current injection (0.2–1.0 nA; 250 ms). Experimental recordings were maintained up to an additional 5 h in 20% (36°C) after the initial 4-h incubation (22°C). Data were recorded and analyzed off-line using AxoScope 9 (Molecular Devices). In some experiments, oxygen-dependent excitability of CA1 neurons was assessed by briefly (10 to 20 min) switching from ACSF equilibrated with 20%  $\text{O}_2$ –95%  $\text{O}_2$ .

## RESULTS

### Pharmacological manipulation of CA1 hippocampal superoxide production in slices exposed to one-sided superfusion after 30-min DHE preloading

Data in Fig. 1 were obtained from slices preincubated with 10  $\mu\text{M}$  DHE for 30 min, washed, and then exposed to ACSF equilibrated with 95%  $\text{O}_2$  for an additional 45 min during imaging. The DHE-loaded cells at the bottom of the slice were visualized and single-cell traces of 10 CA1 neurons in the same slice show a rapid time-dependent increase in FIU, which reflects increased  $\cdot\text{O}_2^-$  production during the 45-min exposure to 95%  $\text{O}_2$ . Note the considerable variability in the rate of  $\cdot\text{O}_2^-$  production between CA1 neurons (Fig. 1A). The absolute

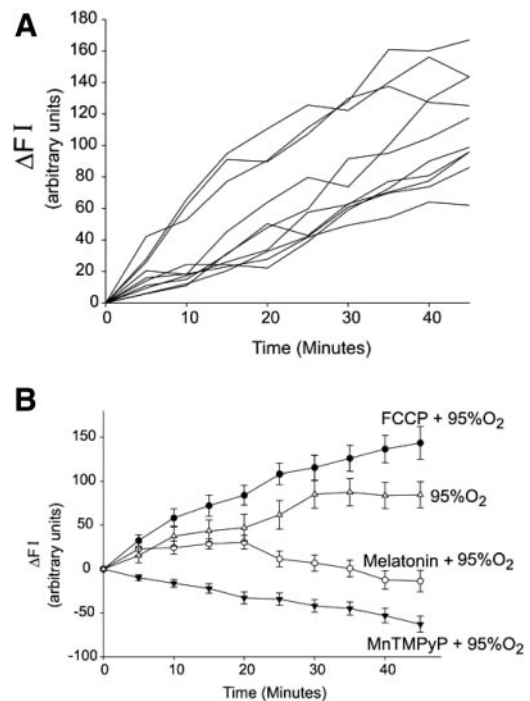


FIG. 1. Effects of agents that increase (FCCP) and decrease (melatonin, MnTMPyP) the rate of  $\cdot\text{O}_2^-$  production in 95%  $\text{O}_2$  in CA1 neurons; 1-sided superfusion. *A*: single-cell measurements of  $\cdot\text{O}_2^-$  production in 10 CA1 neurons from a single slice exposed to 95%  $\text{O}_2$  for 45 min. Experiments were performed using slices preloaded with 10  $\mu\text{M}$  dihydroethidium (DHE) for 30 min, washed, and then submerged in artificial cerebrospinal fluid (ACSF) while resting directly on the glass bottom of the tissue slice bath. *B*: uncoupling mitochondrial respiration with carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP,  $n = 80$ ; 1  $\mu\text{M}$ ) had no significant effect on the rate of  $\cdot\text{O}_2^-$  production relative to 95%  $\text{O}_2$  alone. Conversely, exposure to the superoxide dismutase mimetic MnTMPyP ( $n = 60$ ; 25  $\mu\text{M}$ ) and the broad spectrum antioxidant melatonin ( $n = 60$ ; 100  $\mu\text{M}$ ) significantly decreased the rate of  $\cdot\text{O}_2^-$  production in 95%  $\text{O}_2$  ( $P > 0.001$ ). Refer to the text for further discussion of how a negative slope for fluorescence intensity unit (FIU)/min is interpreted.

increase in FIU from  $t = 0$  to 45 min ranged from 65 to 165 FIU. In actuality, this period represents the final 45 min during 90 min of exposure to 95%  $\text{O}_2$  if the time from slicing (0–45 min) plus the duration of the experimental run (46–90 min) are considered.

Figure 1*B* shows the summary data from six to eight hippocampal slices per condition during application of FCCP, MnTMPyP, or melatonin for 45 min while superfused with ACSF equilibrated with 95%  $\text{O}_2$ . Superoxide production in 95%  $\text{O}_2$  alone is also shown for comparison. The mitochondrial uncoupler, FCCP, was used as a positive control ( $n = 80$  cells) to establish that DHE is sufficiently sensitive to detect increases in  $\cdot\text{O}_2^-$  and to determine if the mitochondria were a source for  $\cdot\text{O}_2^-$  production in our experiments. Adding FCCP to the superfusate during 95%  $\text{O}_2$  resulted in a peak value of  $143 \pm 19$  FIU and at a rate of  $3.0 \pm 0.8$  FIU/min during the first 45-min period. Superoxide production with FCCP was not significantly higher, however, than that with 95%  $\text{O}_2$  alone, which caused a peak increase of only  $85 \pm 15$  FIU at a rate of  $2.8 \pm 0.6$  FIU/min over 45 min. Exposure to FCCP for  $>60$  min caused a decrease or plateau in the slope of FI, the significance of which is discussed in the following text.

To determine the relative specificity of DHE for  $\cdot\text{O}_2^-$  in the CA1 hippocampus, control experiments were performed in



95% O<sub>2</sub> with the  $\cdot\text{O}_2^-$  scavenger and SOD and CAT mimetic, MnTMPyP ( $n = 60$  cells). Slices were exposed to MnTMPyP prior to (10 min) and during  $\cdot\text{O}_2^-$  measurements. MnTMPyP significantly reduced  $\cdot\text{O}_2^-$  production relative to 95% O<sub>2</sub> alone ( $P < 0.001$ ). Notice that the slope of FIU/min was negative ( $-1.3 \pm 0.2$  FIU/min) with the  $\cdot\text{O}_2^-$  scavenger, indicating that little or no  $\cdot\text{O}_2^-$  was being produced. This was expected considering that previous reports indicate that MnTMPyP is a highly specific  $\cdot\text{O}_2^-$  scavenger (Klann 1998; Wang et al. 2004). Additionally, the broad spectrum antioxidant melatonin ( $n = 60$  cells) significantly decreased the rate of  $\cdot\text{O}_2^-$  production ( $-1.3 \pm 0.2$  FIU/min) during exposure to 95% O<sub>2</sub> after a modest increase ( $30 \pm 8$  FIU) during the first 20 min of the experiment ( $P < 0.001$ ).

#### Superoxide production during graded O<sub>2</sub> exposure using 30-min DHE preloading and one-sided superfusion

Figure 2, A–D, shows a comparison of fluorescence images of the CA1 region of individual hippocampal slices exposed to graded levels of oxygen over 0, 1, and 4 h (from left to right). These  $\cdot\text{O}_2^-$  measurements were made in hippocampal slices using one-sided superfusion and exposure (from top to bottom)

to 95, 60, 40, and 20% O<sub>2</sub> after 30 min of preincubation with 10  $\mu\text{M}$  DHE. Notice the apparent increase in FIU of each consecutive image at 95% O<sub>2</sub>, which is considerably greater than that observed with lower levels of oxygen (Fig. 2, A–D). Summary data show that peak measurements of  $\cdot\text{O}_2^-$  production during 4 h exposure to 95, 60, 40, and 20% O<sub>2</sub> were  $193 \pm 27$  ( $n = 80$ ),  $61 \pm 10$  ( $n = 60$ ),  $51 \pm 15$  ( $n = 60$ ), and  $12 \pm 6$  FIUs ( $n = 60$ ), respectively (Fig. 2E). Notice that the slope of FIU/min in 95% O<sub>2</sub> was greatest during the first 45 min of  $\cdot\text{O}_2^-$  measurement, which was followed by a plateau in FI after 60 min. At levels of O<sub>2</sub> < 95%, the slope of  $\cdot\text{O}_2^-$  production became negative (60 and 40%) after  $\sim 60$  min or less (20%; Fig. 2E).

To better assess the oxygen-dependent effect on  $\cdot\text{O}_2^-$  production using preloaded slices and one-sided superfusion we analyzed the slopes of  $\cdot\text{O}_2^-$  production within the initial 45 min of exposure to 95, 60, 40, and 20% O<sub>2</sub>. The rate of  $\cdot\text{O}_2^-$  production with 95% O<sub>2</sub> was significantly different ( $P < 0.001$ ) than 60, 40, and 20% O<sub>2</sub>. However, the rates of  $\cdot\text{O}_2^-$  production with 60, 40, and 20% O<sub>2</sub> were not significantly different from each other ( $P > 0.05$ ); refer to Fig. 5B.

We attributed the plateau and negative slopes in FIU/min after 45 min were due to: leakage of DHE from the intracellular

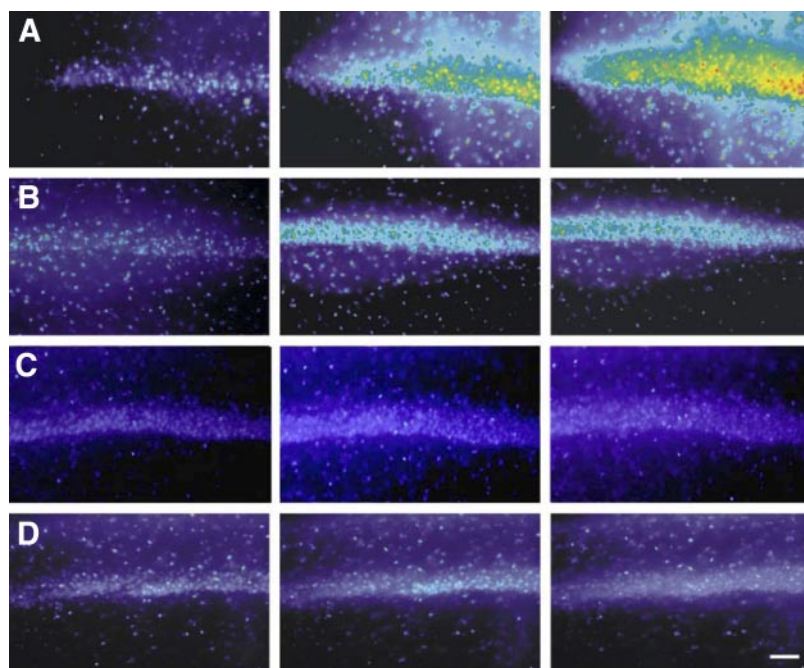
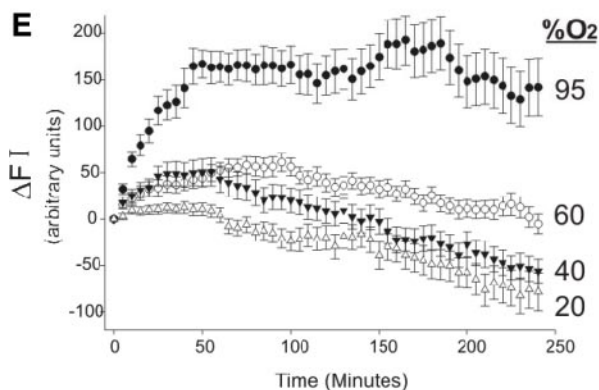


FIG. 2. Superoxide radical imaging of the CA1 hippocampus; 1-sided superfusion. Slices were preincubated with 10  $\mu\text{M}$  DHE for 30 min, washed, and then submerged in ACSF while resting directly on the glass bottom of the tissue slice bath. A: representative pseudocolored images of the CA1 hippocampal region ( $\times 10$  objective, bottom surface of slice) demonstrating  $\cdot\text{O}_2^-$  production in 95% O<sub>2</sub>, 60% O<sub>2</sub> (B), 40% O<sub>2</sub> (C), and 20% O<sub>2</sub> (D) at times 0, 1, and 4 h (left to right); scale bar = 200  $\mu\text{m}$ . E: summary data showing the effects of graded oxygen exposure for 4 h on  $\cdot\text{O}_2^-$  production; 95% O<sub>2</sub> ( $n = 80$  cells), 60% O<sub>2</sub> ( $n = 60$ ), 40% O<sub>2</sub> ( $n = 60$ ), and 20% O<sub>2</sub> ( $n = 60$ ) with 6–8 slices/O<sub>2</sub> level. Time 0 includes 30 min of recovery following slicing plus 30 min of DHE loading. Thus the total time the slices were exposed to 95% O<sub>2</sub> was 5 h. Refer to the text for further details regarding how changes in the slope of DHE fluorescence/min were interpreted.



compartment, a gradual decrease in DHE concentration resulting from oxidation, and/or decreased availability of unbound nucleic acids for ethidium to bind to. Regarding the first explanation, DHE passively diffuses into cells and its retention in cells has not been well characterized, so it is conceivable that probe leakage could occur that exceeds the rate of ethidium oxidation. The second explanation is that the concentration of the DHE decreases within the intracellular compartment as it is irreversibly oxidized by  $\cdot\text{O}_2^-$ . The third explanation is that the plateau in fluorescence may result from ethidium intercalating with all the available nucleic acids, and thus preventing further binding necessary for increased fluorescence (Morgan et al. 1979). This could conceivably result in the ethidium diffusing back into the extracellular space, possibly contributing to the observed increase in background fluorescence observed with time during one-sided superfusion.

*Pharmacological manipulation of CA1 hippocampal superoxide production in slices exposed to two-sided superfusion and continuous DHE loading*

We hypothesized that the time-dependent plateau in FIU/min in 95%  $\text{O}_2$  shown in Fig. 2E resulted from the finite availability of DHE in preloaded slices exposed to one-sided superfusion. Accordingly, we tried an alternative method in which a lower concentration of DHE was continuously supplied to the slice throughout the duration of the experiment. Additionally, the slice was elevated on a nylon mesh grid to permit direct exposure of both slice surfaces to oxygenated ACSF.

Superoxide production in 10 individual cells is shown in Fig. 3A as a time-dependent increase in FI over 4 h during exposure to 95%  $\text{O}_2$ . Notice that the increase in  $\cdot\text{O}_2^-$  production over time does not plateau and that the response is essentially linear (Fig. 3B, 95%  $\text{O}_2$  trace). Also notice that the rate of  $\cdot\text{O}_2^-$  production and absolute change in peak fluorescence significantly exceeds that measured using one-sided superfusion and DHE preloading (cf. Fig. 2E, 95%  $\text{O}_2$  trace); see following text, Fig. 5. Figure 3B shows the summary data from hippocampal slices following the application of FCCP, MnTMPyP, and melatonin for 1 h while superfused with ACSF equilibrated with 95%  $\text{O}_2$ . Superoxide production in 95%  $\text{O}_2$  alone was estimated at  $5.7 \pm 0.7$  FIU/min ( $n = 60$  cells) with a peak fluorescence after 1 h of  $688 \pm 29$  FIU. Adding FCCP further increased  $\cdot\text{O}_2^-$  production ( $P < 0.001$ ) in 95%  $\text{O}_2$  to  $992 \pm 24$  FIU over 1 h and resulted in a greater rate of superoxide production at  $8.3 \pm 0.8$  FIU/min ( $n = 80$  cells). These observations support the role of the mitochondria as a source of intracellular  $\cdot\text{O}_2^-$  production as reported by others (Bindokas et al. 1996). Exposure to FCCP for longer periods of time ( $>120$  min) resulted in cell death (lysing) and a gradual plateau in FI (not shown).

In studies using antioxidants, the slices were briefly pretreated with MnTMPyP and melatonin (10 min) prior to and during subsequent  $\cdot\text{O}_2^-$  measurements. During exposure to 95%  $\text{O}_2$ , application of MnTMPyP caused only a very modest increase of  $98 \pm 3$  FIU over 1 h at the rate of  $0.8 \pm 0.1$  FIU/min ( $n = 60$  cells), which was significantly decreased ( $P < 0.001$ ) relative to 95%  $\text{O}_2$  alone. Light inactivation (12 h) of MnTMPyP (25  $\mu\text{M}$ ) rendered the antioxidant less effective as a SOD and CAT mimetic:  $\cdot\text{O}_2^-$  production was significantly

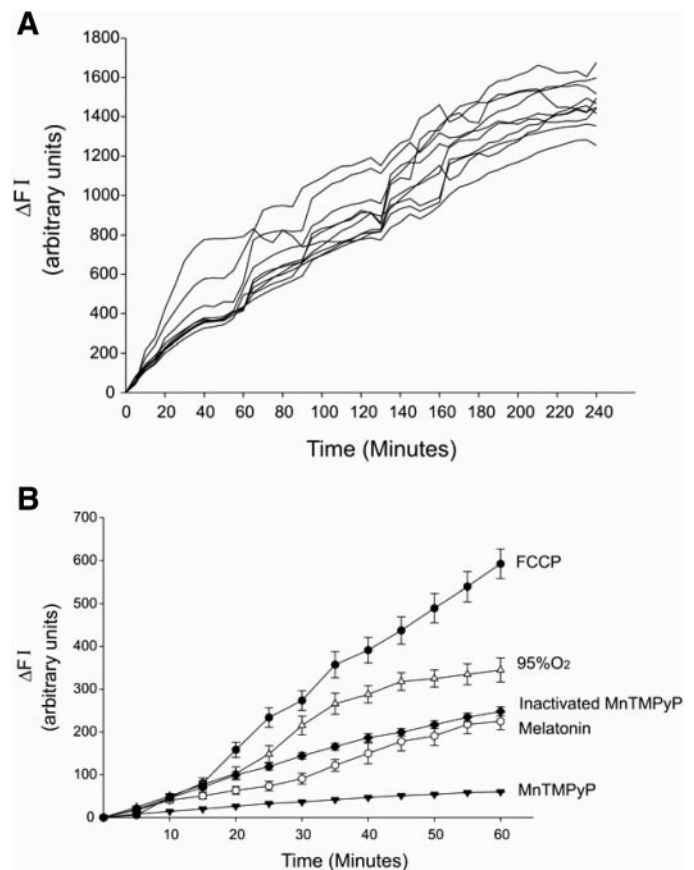


FIG. 3. Effects of agents that increase (FCCP) and decrease (melatonin, MnTMPyP)  $\cdot\text{O}_2^-$  production in 95%  $\text{O}_2$  in CA1 neurons; continuous loading of DHE and 2-sided superfusion. A: single-cell monitoring of  $\cdot\text{O}_2^-$  production in 10 CA1 neurons exposed to 95%  $\text{O}_2$  for 4 h. Notice that  $\cdot\text{O}_2^-$  measurements can be maintained for  $\leq 4$  h without a plateau or negative slope in FIU/min; cf. Figs. 1B and 2E. B: uncoupling mitochondrial respiration with FCCP ( $n = 80$ ; 1  $\mu\text{M}$ ) significantly increased  $\cdot\text{O}_2^-$  production ( $P > 0.001$ ) relative to 95%  $\text{O}_2$  alone ( $n = 60$ ). superoxide dismutase (SOD) mimetic MnTMPyP ( $n = 60$ ; 25  $\mu\text{M}$ ) and melatonin ( $n = 60$ ; 100  $\mu\text{M}$ ) significantly reduced  $\cdot\text{O}_2^-$  production during exposure to 95%  $\text{O}_2$  ( $P > 0.001$ ). Light-inactivated MnTMPyP ( $n = 50$ ; 25  $\mu\text{M}$ ) reduced  $\cdot\text{O}_2^-$  production during exposure to 95%  $\text{O}_2$  but was significantly higher than activated MnTMPyP ( $P > 0.001$ ).

lower in CA1 neurons ( $n = 50$ ) during exposure to 95%  $\text{O}_2$  ( $P > 0.001$ ), but it was only  $\sim 30\%$  of the inhibition of  $\cdot\text{O}_2^-$  production that occurred using normally active MnTMPyP. Likewise, melatonin (100  $\mu\text{M}$ ) caused a similar inhibition of  $\cdot\text{O}_2^-$  production in 95%  $\text{O}_2$  as light inactivated MnTMPyP and significantly decreased  $\cdot\text{O}_2^-$  production ( $P < 0.001$ ) causing an increase of  $344 \pm 27$  FIU over 1 h at the rate of  $2.9 \pm 0.1$  FIU/min ( $n = 60$  cells).

*Superoxide production during graded  $\text{O}_2$  exposure using continuous DHE loading and two-sided superfusion*

Figure 4 shows a comparison of fluorescence images of the CA1 region of individual hippocampal slices exposed to 95, 60, 40, and 20%  $\text{O}_2$  over 0, 1, and 4 h (from left to right). Note the dramatic increase in fluorescence relative to the initial baseline and that the fluorescence is proportional to the level of oxygen over the entire 4 h of study (Fig. 4, A–D). In these studies, exposure to 95, 60, 40, and 20%  $\text{O}_2$  produced an increase in peak fluorescence of  $1,265 \pm 65$ ,  $813 \pm 45$ ,  $535 \pm$



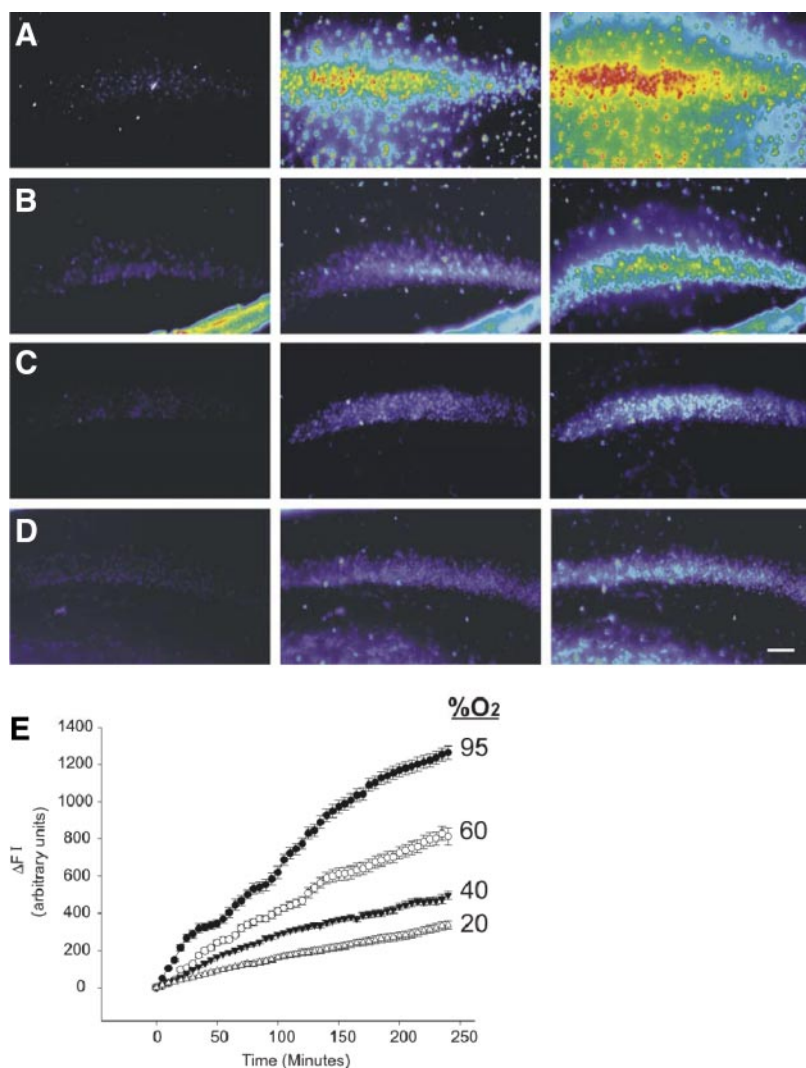


FIG. 4. Superoxide radical imaging of the CA1 hippocampus; 2-sided superfusion. Slices were continuously exposed to 2.5  $\mu\text{M}$  DHE while submerged in ACSF and supported between 2 nylon grids so that oxygenated ACSF flowed across both surfaces of the slice. Measurements of tissue PO<sub>2</sub> profiles in 400- $\mu\text{m}$ -thick hippocampal slices reveal that this configuration produces a symmetrical V-shaped PO<sub>2</sub> profile in the slice with the minimum value at  $\sim 200$   $\mu\text{m}$  deep; i.e., the core of the slice (Dean et al. 2005). Representative pseudocolored images of the CA1 hippocampal region ( $\times 10$  objective, bottom surface of slice) demonstrating  $\cdot\text{O}_2^-$  production in 95% O<sub>2</sub> (A), 60% O<sub>2</sub> (B), 40% O<sub>2</sub> (C), and 20% O<sub>2</sub> (D) at times (from left to right) 0, 1, and 4 h; scale bar = 200  $\mu\text{m}$ . E: summary data showing the effects of graded oxygen exposure for 4 h on  $\cdot\text{O}_2^-$  production; 95% O<sub>2</sub> ( $n = 60$  cells), 60% O<sub>2</sub> ( $n = 60$ ), 40% O<sub>2</sub> ( $n = 60$ ), and 20% O<sub>2</sub> ( $n = 60$ ) with 6 slices/O<sub>2</sub> level. Time 0 includes 60 min of incubation in 95% O<sub>2</sub> after slicing, without DHE. Thus the total time the slices were exposed to 95% O<sub>2</sub> was 5 h. Refer to the text for further details.

23, and  $337 \pm 22$  FIU at the rate of  $6.6 \pm 0.4$ ,  $5.3 \pm 0.3$ ,  $3.3 \pm 0.1$ , and  $1.6 \pm 0.2$  FIU/min over 4 h, respectively (Fig. 4E). The rates of  $\cdot\text{O}_2^-$  production were significantly different between 95, 60, 40, and 20% O<sub>2</sub> ( $P < 0.001$ ). In addition, this method resulted in no plateau in FI over the 4-h study period (cf. Fig. 2E).

#### Comparison of one-sided versus two-sided superfusion and method of DHE loading

Comparison of summary data for hippocampal slices shows the rate of  $\cdot\text{O}_2^-$  production during exposure to FCCP, MnTMPyP, and melatonin in 95% O<sub>2</sub> with two- and one-sided superfusion (Fig. 5A). Notice that with two-sided superfusion the rate of  $\cdot\text{O}_2^-$  production was significantly higher during exposure to FCCP ( $P < 0.001$ ) and significantly lower with MnTMPyP and melatonin ( $P < 0.001$ ). Conversely  $\cdot\text{O}_2^-$  production with FCCP using one-sided superfusion was not significantly different from 95% O<sub>2</sub> alone ( $P > 0.05$ ). However, experiments using one-sided superfusion showed that both MnTMPyP and melatonin significantly reduced  $\cdot\text{O}_2^-$  production in 95% O<sub>2</sub> relative to 95% O<sub>2</sub> alone ( $P < 0.001$ ).

The rate of  $\cdot\text{O}_2^-$  production during the first 45 min was directly proportional to oxygen level in the ACSF using the two-sided superfusion, and the rate of  $\cdot\text{O}_2^-$  production was significantly different between all levels of oxygen ( $P < 0.001$ ). Using the one-sided superfusion we observed a significant difference in the rate of  $\cdot\text{O}_2^-$  production during the first 45 min in 95% O<sub>2</sub> relative to lower levels of oxygen. However, no oxygen-dependent effect on  $\cdot\text{O}_2^-$  production was detected at 20, 40, and 60% using one-sided superfusion (Fig. 5B). A comparison of absolute  $\cdot\text{O}_2^-$  production in response to 95, 60, 40, and 20% O<sub>2</sub> using one- and two-sided superfusion is shown in Fig. 5C. Data from experiments with two-sided superfusion (2-SP, ■,  $n = 260$  cells) and one-sided superfusion (1-SP, ▨,  $n = 260$  cells) are grouped into four columns representing (left to right) 95, 60, 40, and 20% O<sub>2</sub> over 30, 60, 120, and 240 min ( $x$  axis). Notice the dramatic time-dependent increase in FIU that occurs with two-sided superfusion and continuous loading relative to slices that were visualized with one-sided superfusion and DHE preloading.

#### Oxygen dose and cell death during two-sided superfusion

EH-1 staining was used to determine if greater CA1 cell death occurred at lower levels of oxygen due to hypoxia and/or

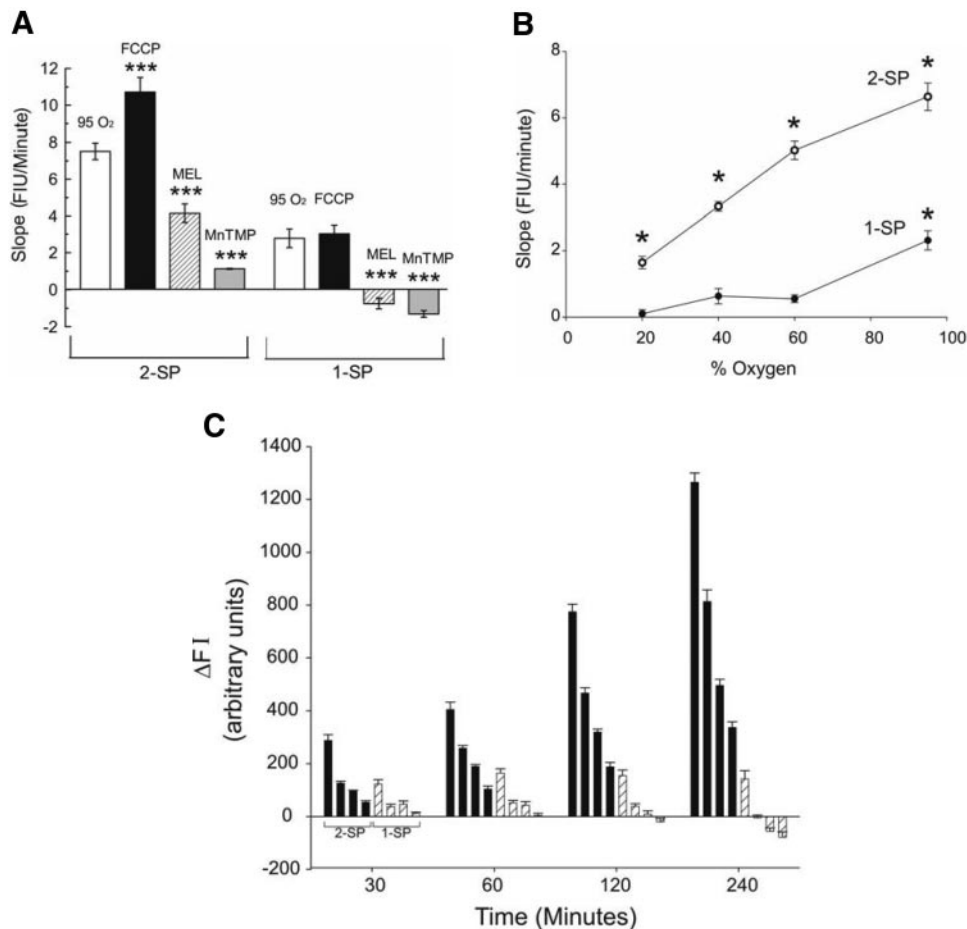


FIG. 5. Comparison of summary data using the 2 techniques of DHE loading and slice maintenance. *A*: exposure to FCCP, MnTMPyP, and melatonin in 95% O<sub>2</sub> with 2-sided superfusion (2-SP) and 1-sided superfusion (1-SP). FCCP caused a significant increase in ·O<sub>2</sub><sup>-</sup> production ( $P < 0.001$ ) during 2-SP but not 1-SP. Melatonin (MEL) and MnTMPyP (MnTMP) significantly reduced ·O<sub>2</sub><sup>-</sup> production relative to 95% O<sub>2</sub> alone using both 1- and 2-SP ( $P < 0.001$ ). *B*: comparison of the rates of ·O<sub>2</sub><sup>-</sup> production (FIU/min) during the initial 45 min of exposure to 95, 60, 40, and 20% O<sub>2</sub> using 1- and 2-SP. The rates of ·O<sub>2</sub><sup>-</sup> production were all significantly different (\*) for each level of oxygen with 2-SP ( $P < 0.001$ ). Using 1-SP, 95% O<sub>2</sub> was significantly different from 60, 40, and 20% O<sub>2</sub> ( $P < 0.001$ ), but 60, 40, and 20% O<sub>2</sub> were not different from each other ( $P > 0.05$ ). Values are means  $\pm$  SE. *C*: comparison of CA1 hippocampal ·O<sub>2</sub><sup>-</sup> production in response to 4-h exposure to graded levels of O<sub>2</sub> at times 30, 60, 120, and 240 min using 1- or 2-SP. Summary data show ·O<sub>2</sub><sup>-</sup> production in response to 95, 60, 40, and 20% O<sub>2</sub> (from left to right) with 2-sided superfusion and continuous loading (2-SP, ■,  $n = 260$ ) and 1-sided superfusion after 30 min of DHE preloading (1-SP, ▨,  $n = 260$ ). The magnitude of the time-dependent increase in FIU was much greater with 2-SP. Each column shows the mean change in FIU for each level of oxygen.

higher levels of oxygen due to hyperoxia. Figure 6, *A–D*, shows EH-1 staining in hippocampal slices before (*left*) and after 4-h exposure (*right*) to (*top to bottom*) 95, 60, 40, and 20% O<sub>2</sub> at 36°C. As shown in panel E, the highest level of cell death was observed after 4-h exposure to 95% O<sub>2</sub> ( $t = 0$  vs.  $t = 4$  h, Student's unpaired  $t$ -test,  $P < 0.001$ ). Sixty-percent O<sub>2</sub> caused a modest but significant increase in cell death ( $P < 0.05$ ), and no time-dependent increase in cell death was observed using 40 and 20% O<sub>2</sub>. This pattern of hyperoxia-induced cell death is similar to that which others have reported using the organotypic hippocampal slice preparation (Graulich et al. 2002; Pomper et al. 2001) and suggests that hyperoxia (60 and 95% O<sub>2</sub>) and increased ·O<sub>2</sub><sup>-</sup> may increase oxidative stress-induced cell death.

#### Cellular viability in 20% O<sub>2</sub>: electrophysiological recordings of evoked action potentials

Intracellular recordings from a total of 12 neurons in six hippocampal slices were used to determine if CA1 neurons remain functional after prolonged exposure to 20% O<sub>2</sub>. We postulated that if action potentials could be measured in CA1 neurons during and after 4-h exposure to the lowest level of O<sub>2</sub> (20%), then this would confirm that CA1 neurons were indeed viable. It also would be further evidence that the decrease in ·O<sub>2</sub><sup>-</sup> production in 20% O<sub>2</sub> was not due to increased CA1 cell death at the lowest level of oxygenation.

A representative electrophysiological experiment in 20% O<sub>2</sub> shown in Fig. 7, *A–C*, demonstrates evoked action potentials in a CA1 neuron in response to a 0.4-nA positive current pulse (250 ms). This particular recording was made at 36°C after incubation in 20% O<sub>2</sub> for 4 h (22°C). A brief exposure to 95% O<sub>2</sub> (10 min) increased evoked action potential frequency (Fig. 7*B*). Switching back to 20% O<sub>2</sub> reversed the oxygen-dependent increase in excitability (Fig. 7*C*). Figure 7, *D–F*, shows evoked action potentials from three individual neurons maintained in 20% O<sub>2</sub> for 7, 8, and 9 h, respectively (36°C). These data demonstrate that neurons are viable after extended incubation in 20% O<sub>2</sub>. These findings are consistent with previous studies from our lab (Garcia et al. 2005) that have demonstrated that lowering oxygen decreases neuronal excitability but does not abolish it.

#### DISCUSSION

This study provides a comprehensive description of superoxide (·O<sub>2</sub><sup>-</sup>) production in cells of the CA1 hippocampal slice exposed to sustained, graded oxygenation. There were five important findings. First, two methods for loading DHE and maintaining the slices (preincubation with 10 μM DHE followed by 1-sided superfusion and continuous loading with 2.5 μM DHE and 2-sided superfusion) gave quantitatively different results for estimating oxygen-dependent ·O<sub>2</sub><sup>-</sup> production in the brain slice. The latter scenario—2-sided superfusion and

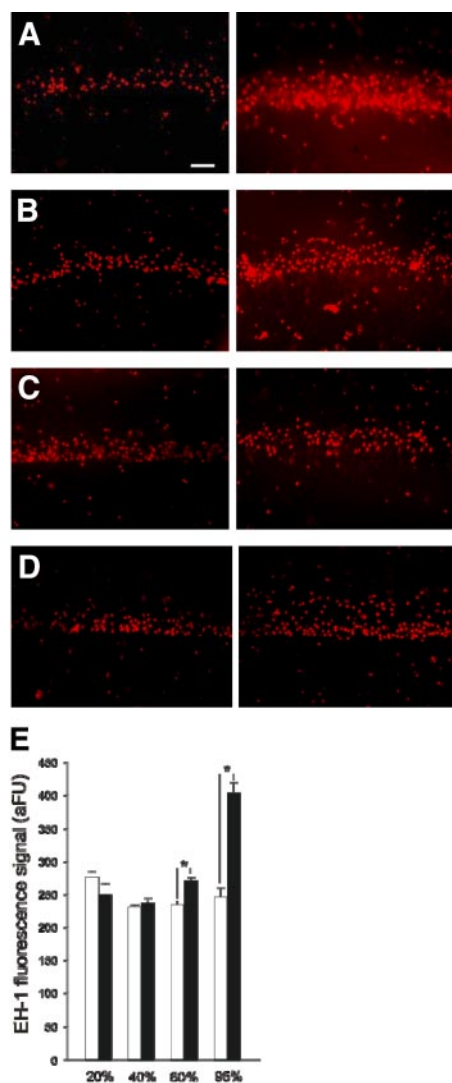


FIG. 6. Cell death in CA1 hippocampal neurons exposed to graded levels of oxygen. Representative images of CA1 hippocampal slice neurons before (left) and after (right) 4-h exposure to 95% O<sub>2</sub> (A), 60% O<sub>2</sub> (B), 40% O<sub>2</sub> (C), and 20% O<sub>2</sub> (D). Note the highest labeling of EH-1-labeled neurons followed 4-h exposure to 95% O<sub>2</sub>; scale bar = 200  $\mu\text{m}$ . E: summary data show significant cell death occurred after 4 h with 95% O<sub>2</sub> ( $P < 0.001$ ) and 60% O<sub>2</sub> ( $P < 0.05$ ) but not with 40 and 20% O<sub>2</sub>. ET-1 fluorescence is presented as group averaged FIU  $\pm$  SE measured within an  $80 \times 800 \mu\text{m}$  region of CA1 neurons in individual slices. (\*:  $P < 0.05$  for average CA1 fluorescence for  $t = 0$ ,  $\square$  vs.  $t = 4$  h,  $\blacksquare$ ).

continuous DHE loading—gave the best temporal resolution of  $\cdot\text{O}_2^-$  production. Second, we observed that under control conditions used for maintaining slices (95% O<sub>2</sub>,  $\sim 36^\circ\text{C}$ ),  $\cdot\text{O}_2^-$  production in the CA1 hippocampus is significantly increased during 4 h relative to lower levels of oxygen that are not hypoxic, at least as defined by the absolute value for tissue PO<sub>2</sub>. Third,  $\cdot\text{O}_2^-$  production during hyperoxia (95% O<sub>2</sub>) is minimized by adding the antioxidants melatonin or MnTMPyP to the ACSF. Fourth, cell death is likewise greatest after 4 h of exposure to 95% O<sub>2</sub>. No significant time-dependent increase in CA1 cell death occurred in hippocampal slices maintained at  $\sim 36^\circ\text{C}$  in ACSF equilibrated with 20 and 40% O<sub>2</sub>. Finally, CA1 neurons remain viable—that is, capable of generating action potentials—during and after prolonged exposure ( $\geq 4$  h) to 20% O<sub>2</sub> at  $\sim 36^\circ\text{C}$ .

#### Measurement of oxygen-dependent hippocampal superoxide production

Oxygen-dependent  $\cdot\text{O}_2^-$  production in the CA1 hippocampus was characterized using two methods of  $\cdot\text{O}_2^-$  measurement. For one-sided superfusion, the brain slice was supported against the glass coverslip at the bottom of the tissue slice chamber. CA1 cells were visualized from the bottom after 30 min of loading with 10  $\mu\text{M}$  DHE. A drawback to this method was that the surface of the slice being imaged was not directly exposed to the oxygenated ACSF. Additionally, the rate of increase in DHE fluorescence reached a plateau before 60 min (95% O<sub>2</sub>). At lower levels of O<sub>2</sub>, DHE fluorescence decreased over time, and no differences were noted in the rate of  $\cdot\text{O}_2^-$  production as a function of O<sub>2</sub> level (20–60% O<sub>2</sub>). A decrease in the plateau of DHE fluorescence likely occurred from irreversible oxidation and/or cellular leakage of the probe, and thus  $\cdot\text{O}_2^-$  production may have been underestimated using this method.

These limitations were addressed by using two-sided superfusion and continuous loading with a lower concentration of DHE (2.5  $\mu\text{M}$ ). By raising the slice up off the floor of the tissue bath on a nylon mesh, the cells being imaged on the bottom surface of the slice were brought into direct contact with a constant flow of oxygenated ACSF. Using a  $\times 10$  objective enabled suitable resolution and working distance to image neurons in the CA1 region. Using two-sided superfusion, we

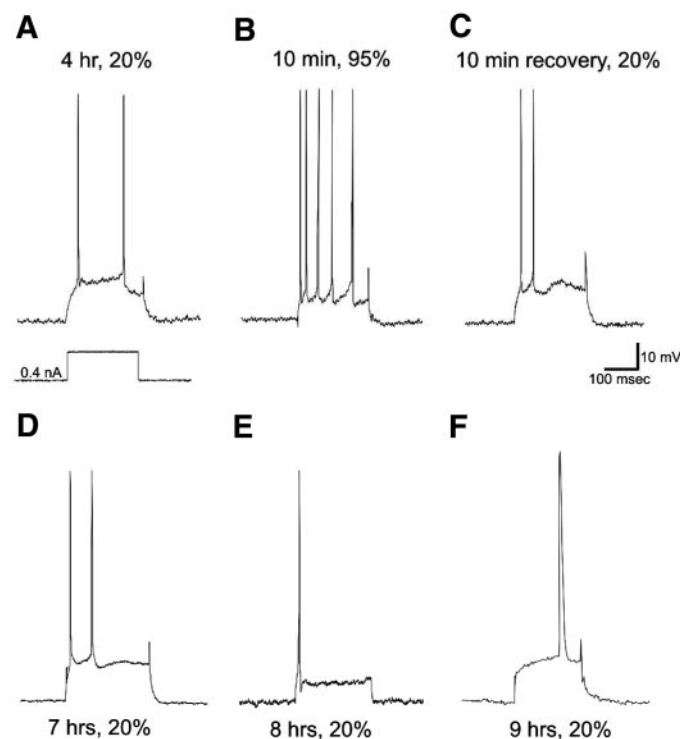


FIG. 7. Hippocampal CA1 neurons remain viable after prolonged exposure to 20% O<sub>2</sub>. A: representative electrophysiological experiment demonstrated evoked action potentials after 4-h exposure to 20% O<sub>2</sub>. B: exposure to 95% O<sub>2</sub> for 10 min in the same cell caused an increase in evoked action potential frequency, and this increased excitability reversed when returning back to 20% O<sub>2</sub> (C). Evoked action potentials were recorded from 3 individual neurons maintained in 20% O<sub>2</sub> for 7 h (D), 8 h (E), and 9 h (F). All recordings were made using sharp electrodes (50–110 M $\Omega$ ) in current clamp mode. Evoked action potentials were in response to 0.4 nA. Membrane potential ranged from  $-55$  to  $-70$  mV in all cells recorded.



determined that  $\cdot\text{O}_2^-$  production in CA1 cells was proportional to the " $\text{O}_2$  concentration product," which is defined as  $\text{PO}_2 \times$  duration of exposure to  $\text{O}_2$  (Fig. 4E). This method improved diffusion of oxygen to the slice and enabled detection of a higher resolution fluorescence signal that neither plateaued nor decreased over time. The maximum change in FIU with 95%  $\text{O}_2$  after 4 h was approximately seven times greater using two-sided superfusion and continuous loading. Furthermore, because DHE is potentially toxic to cells at high concentrations ( $>50 \mu\text{M}$ ) (Zhao et al. 2003), using a lower concentration (2.5  $\mu\text{M}$ ) applied immediately before visualizing cells likely minimized toxicity. We also attempted to use one-sided perfusion with continuous DHE loading (2.5  $\mu\text{M}$ , data not shown), but the images quickly became blurry, and it was difficult to visualize individual neurons within the CA1 region. This likely occurred because the oxidized DHE became trapped between the bottom surface of the slice and cover glass during one-sided superfusion. It's important to note that the  $\text{PO}_2$  profiles of slices maintained in one- and two-sided superfusion are considerably different (Dean et al. 2005).

#### *Cell death and cellular viability as a function of $\text{O}_2$ tension*

Using EH-1 staining and electrophysiology, we show that the lower rate of  $\cdot\text{O}_2^-$  production with 60, 40, and 20%  $\text{O}_2$  as compared with 95%  $\text{O}_2$  was not due to increased cell death from hypoxia (Fig. 6). To the contrary, the highest level of EH-1 staining and thus cell death was observed after 4-h incubation in 95%  $\text{O}_2$ . These data suggest that CA1 neurons in hippocampal slices are vulnerable to oxidative stress from prolonged exposure ( $\leq 4$  h) to hyperoxic control conditions (Fessel et al. 2002; Rice 1999). This finding supports the earlier findings of Pomper et al. (2001), who showed that CA1 cell death in organotypic slice cultures, prepared from younger rats, was greatest in 95%  $\text{O}_2$  and less in 20%  $\text{O}_2$ . Likewise, cell death was greatest in hippocampal and cerebral cortical neuronal cultures incubated in 95 and 21%  $\text{O}_2$  and less in 9%  $\text{O}_2$  (Brewer and Cotman 1989; Kaplan et al. 1986).

Our electrophysiological measurements from individual CA1 neurons in slices maintained in 20%  $\text{O}_2$  provided further evidence that CA1 neurons remained viable during prolonged exposure to the lowest level of  $\text{O}_2$  tested; that is, they continue to generate action potentials that overshoot 0 mV. Excitability, however, was lower during exposure to 20%  $\text{O}_2$  and increased during brief exposure to 95%  $\text{O}_2$ . Similar observations have been made previously in our lab when comparing CA1 neuronal excitability of hippocampal slices maintained in 95, 60, and 40%  $\text{O}_2$  (Garcia et al. 2005). It is our opinion (Dean et al. 2003) and others (Bingmann and Kolde 1979; Bingmann et al. 1982; Fessel et al. 2002; Pomper et al. 2001; Shibata and Blatteis 1991) that neuronal activity that is traditionally characterized as "normal" in 95%  $\text{O}_2$  has in fact been altered by an underlying level of oxidative stress due to hyperoxia. We propose that the brain slice preparation has increased excitability from the general stimulatory effects of hyperoxia (Dean et al. 2003; Garcia et al. 2005; this study, Fig. 7). It is likely that the lower level of excitability observed using lower levels of  $\text{O}_2$  reflect the reduced excitability of the deafferented in vitro brain slice preparation rather than a hypoxic condition. Further studies are needed to determine if slice procedures and recovery from slicing should be done in lower oxygen. The findings of the

present study, however, indicate that this possibility should be considered.

The relationship between hyperoxia and decreased cell viability needs to be assessed in a rigorous study in which the neuroprotective effects of various antioxidants and  $\text{O}_2$  levels are established using a combination of electrophysiology, real-time measurements of ROS and measurements of cell death. This is especially important because the levels of certain endogenous antioxidants are reduced during incubation and superfusion of brain slices with ACSF (Rice 1999; Rice et al. 1994).

#### *Source of superoxide production*

Increasing the concentration of molecular  $\text{O}_2$  provides a source for the generation of ROS (Kato et al. 1997; Li et al. 2004). The general consensus is that the mitochondria appear to be the primary source of  $\cdot\text{O}_2^-$  production in neurons (Emerit et al. 2004). In fact, several investigators have reported that  $\cdot\text{O}_2^-$  production in hippocampal neurons is generated from the mitochondria under physiological and pathological conditions (Bindokas et al. 1996; Li et al. 2004; Murakami et al. 1998). Our data support this by demonstrating that uncoupling mitochondrial respiration with FCCP increases the production of  $\cdot\text{O}_2^-$  in hippocampal neurons as previously reported (Bindokas et al. 1996). An increase in ethidium fluorescence indicates probe oxidation by  $\cdot\text{O}_2^-$ ; however, increased ethidium fluorescence does not rule out further reactions that  $\cdot\text{O}_2^-$  may undergo to produce additional ROS and reactive nitrogen species: hydrogen peroxide, hydroxyl radical, peroxyxynitrite and nitrogen dioxide (Bindokas et al. 1996). Regardless, for purposes of this study, DHE enables us to estimate oxygen-dependent  $\cdot\text{O}_2^-$  production.

#### *Considerations with fluorescence measurement of superoxide*

Several methods of ROS detection exist, but the most accurate and sensitive method for detecting intracellular ROS in cells and tissues involves fluorescence-based techniques (Budd et al. 1997; Castilho et al. 1999; Gomes et al. 2005; Zuo and Clanton 2002). The fluorogenic probe most commonly used for intracellular  $\cdot\text{O}_2^-$  detection is DHE, and it has been extensively used to measure intracellular  $\cdot\text{O}_2^-$  in the hippocampus (Bindokas et al. 1996; Kawase et al. 1999; Kovacs et al. 2001; Muranyi and Li 2006; Peterson et al. 2002). Intracellular oxidation of DHE yields the two-electron oxidized product, ethidium, which binds to DNA and enhances the fluorescence signal ( $\lambda_{\text{ex}} = 480 \text{ nm}$ ;  $\lambda_{\text{em}} = 586 \text{ nm}$ ). The ethidium fluorescence resulting from the oxidation of DHE is inhibited by endogenous and exogenous  $\cdot\text{O}_2^-$  scavengers, including superoxide dismutase and melatonin (Bindokas et al. 1996; Budd et al. 1997; Castilho et al. 1999; Uchida et al. 2004). However, like all fluorogenic probes, there are a number of caveats associated with using DHE, including the probe's specificity. To our knowledge, no fluorogenic probe is exclusively oxidized by a specific ROS, but DHE is a fairly specific probe for detecting intracellular  $\cdot\text{O}_2^-$  (Bindokas et al. 1996), and its use has been reviewed and critiqued (Budd et al. 1997; Zuo and Clanton 2002). To test the specificity of DHE for  $\cdot\text{O}_2^-$  production, we confirmed that  $\cdot\text{O}_2^-$  production was inhibited completely by a  $\cdot\text{O}_2^-$  scavenger MnTMPyP. Light-inactivated

MnTMPyP, by contrast, was only 2/3 as effective at inhibiting ·O<sub>2</sub><sup>-</sup> production. The remaining one-third of ·O<sub>2</sub><sup>-</sup> scavenging activity exhibited by light-inactivated MnTMPyP could indicate an insufficient amount of light was used or that DHE is reacting with another form of ROS family. We also were able to demonstrate a further increase in ·O<sub>2</sub><sup>-</sup> production during 95% O<sub>2</sub> with FCCP, which was consistent with other studies (Bindokas et al. 1996; Prehn 1998).

With regard to our fluorescence measurements, DHE is permanently oxidized by ·O<sub>2</sub><sup>-</sup>, and the signal produced is irreversible after oxidation, which occurs in all ROS-sensitive probes. In theory, the signal should never decrease, only increase during ·O<sub>2</sub><sup>-</sup> production or plateau when no ·O<sub>2</sub><sup>-</sup> is generated. Therefore the decrease in ethidium fluorescence that we observed is presumably indicative of probe leakage and/or photobleaching and resulted in a negative slope of FIU/min. Last, DHE should only be used as a qualitative measure of ·O<sub>2</sub><sup>-</sup> production because the yield of ethidium produced per ·O<sub>2</sub><sup>-</sup> radical is not stoichiometric (Benov et al. 1998). Therefore our data were reported as an estimate of oxygen-induced ·O<sub>2</sub><sup>-</sup> production.

#### Implications of hyperoxia-induced superoxide formation

Recent experiments in our lab using the hippocampal slice preparation have shown that normobaric hyperoxia (95% O<sub>2</sub>) and hyperbaric oxygen (HBO) increase synaptic activity and firing rate in CA1 neurons, resulting in increased excitability (Dean et al. 2003; Garcia et al. 2004). For example, HBO often induces one or more secondary population spikes in CA1 neurons, referred to as mild epileptiform activity. At higher levels of hyperoxia, these excitatory effects of HBO on synaptic activity are often only partially reversible, resulting in oxygen-induced potentiation of the synaptic response. Increased excitability of hippocampal neurons during HBO is attenuated by ·O<sub>2</sub><sup>-</sup> scavenger MnTMPyP (Garcia et al. 2003). This is consistent with studies from Klann et al. (1998), which show MnTMPyP inhibited the induction of hippocampal LTP, and that ·O<sub>2</sub><sup>-</sup> is an important signal transduction molecule in its induction (Kishida et al. 2005; Knapp and Klann 2002). Therefore evidence supports the role for ·O<sub>2</sub><sup>-</sup> in mediating hyperoxia-induced hyperexcitability in hippocampal slices.

The pathological consequences of increased ROS production are well known (Lynn et al. 2005; Serrano and Klann 2004). In fact, the hippocampus is particularly vulnerable to hyperoxia-induced oxidative stress (Fukui et al. 2001; Onodera et al. 2003; Serrano and Klann 2004; Shimabuku et al. 2005), and this can be prevented by an overexpression of superoxide dismutase in transgenic rats (Chan et al. 1998). In addition, supplemental antioxidants (e.g., melatonin) decrease lipid peroxidation, increase glutathione peroxidase activity, and prevent cognitive deficits in the rat hippocampus under conditions of oxidative stress (Baydas et al. 2005; Gonenc et al. 2005; Maharaj et al. 2005). Our results show that melatonin (100 μM) significantly reduces hippocampal ·O<sub>2</sub><sup>-</sup> production during hyperoxia, suggesting that supplemental melatonin may reduce excess oxidative stress that likely occurs in hippocampal slices over the time spent in 95% O<sub>2</sub>. Melatonin, when used at a concentration of 10 to 100 μM, significantly protects

hippocampal slices from ischemic damage due to excess ·O<sub>2</sub><sup>-</sup> production (Uchida et al. 2004).

In conclusion, using DHE we have demonstrated that ·O<sub>2</sub><sup>-</sup> production in the CA1 hippocampus is tightly coupled to tissue oxygen concentration ranging from 20 to 95% and that 95% O<sub>2</sub> causes the greatest level of ·O<sub>2</sub><sup>-</sup> production over the duration of a 4-h experiment. Superoxide production in 95% O<sub>2</sub>, however, is significantly reduced using the antioxidant melatonin or MnTMPyP. Considering that neurons are sensitive to their redox environment, it is likely that 95% O<sub>2</sub> perturbs normal redox signaling, especially because endogenous antioxidants are significantly reduced in this preparation (Brahma et al. 2000; Rice 1999; Rice et al. 2002). Thus 95% O<sub>2</sub> may lead to hyperexcitability (Garcia et al. 2005) and hyperoxic preconditioning (Dong et al. 2002; Freiberger et al. 2006), which may confound certain types of experiments in neurons. These data indicate that hippocampal ·O<sub>2</sub><sup>-</sup> production is proportional to oxygen concentration, and thus investigators seeking to minimize oxidative stress in the brain slice preparation should consider using a lower level of control oxygen, supplemental antioxidants, or both.

#### GRANTS

This work was supported by Office of Naval Research Grants N000140410172 to J. B. Dean and N000140610105 to D. P. D'Agostino and ONR-DURIP Equipment Grant N000140210643 to J. B. Dean.

#### REFERENCES

- Baydas G, Reiter RJ, Akbulut M, Tuzcu M, Tamer S. Melatonin inhibits neural apoptosis induced by homocysteine in hippocampus of rats via inhibition of cytochrome c translocation and caspase-3 activation and by regulating pro- and anti-apoptotic protein levels. *Neuroscience* 135: 879–886, 2005.
- Benov L, Stejneger L, Fridovich I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Rad Biol Med* 25: 826–831, 1998.
- Bickler PE, Hansen BM. Hypoxia-tolerant neonatal CA1 neurons: relationship of survival to evoked glutamate release and glutamate receptor-mediated calcium changes in hippocampal slices. *Brain Res Dev Brain Res* 106: 57–69, 1998.
- Bindokas VP, Jordan J, Lee CC, Miller RJ. Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *J Neurosci* 16: 1324–1336, 1996.
- Bingmann D, Kolde G. Burst activity elicited in hippocampal slices by a rise of PO<sub>2</sub>. *Pfluegers Arch* 382: R38, 1979.
- Bingmann D, Kolde G, Speckmann EJ. Effects of elevated pO<sub>2</sub> values in the superfusate on the neural activity in hippocampal slices. In: *Physiology and Pharmacology of Epileptogenic Phenomenon*, edited by Klee MR, Lux HD, Speckmann EJ. New York: Raven, 1982, p. 97–104.
- Brahma B, Forman RE, Stewart EE, Nicholson C, Rice ME. Ascorbate inhibits edema in brain slices. *J Neurochem* 74: 1263–1270, 2000.
- Brewer GJ, Cotman CW. Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. *Brain Res* 494: 65–74, 1989.
- Budd SL, Castilho RF, Nicholls DG. Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett* 415: 21–24, 1997.
- Castilho RF, Ward MW, Nicholls DG. Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurochem* 72: 1394–1401, 1999.
- Chan PH, Kawase M, Murakami K, Chen SF, Li Y, Calagui B, Reola L, Carlson E, Epstein CJ. Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. *J Neurosci* 18: 8292–8299, 1998.
- Dantas APV, Tostes RCA, Fortes ZB, Costa SG, Nigro D, Carvalho MHC. In vivo evidence for antioxidant potential of estrogen in microvessels of female spontaneously hypertensive rats. *Hypertension* 39: 405–411, 2002.

- Dean JB, Mulkey DK, Garcia III AJ, Putnam RW, Henderson III RA. Neuronal sensitivity to hyperoxia, hypercapnia and inert gases at hyperbaric pressures. *J Appl Physiol* 95: 883–909, 2003.
- Dean JB, Mulkey DK, Henderson III RA, Potter SJ, Putnam RW. Hyperoxia, reactive oxygen species, and hyperventilation: oxygen sensitivity of brain stem neurons. *J Appl Physiol* 96: 784–791, 2004.
- Dean JB, Potter SJ, Garcia III, AJ, Henderson III RA, Putnam RW. Oxygen tension profiles differ in rat brain slices superfused on one side versus two sides. *FASEB J* 19: A645, 2005.
- Dong H, Xiong L, Zhu Z, Chen S, Hou L, Sakabe T. Preconditioning with hyperbaric oxygen and hyperoxia induces tolerance against spinal cord ischemia in rabbits. *Anesthesiology* 96: 907–912, 2002.
- Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95, 2002.
- Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. *Biomed Pharmacother* 58: 39–46, 2004.
- Fessel J, Arbeson WC, Winder D, Roberts LJ. Standard brain slice protocols result in neuronal oxidative injury in hippocampal brain slices. *Free Rad Biol Med* 33: S433, 2002.
- Freiberger JJ, Suliman HB, Sheng H, McAdoo J, Piantadosi CA, Warner DS. A comparison of hyperbaric oxygen versus hypoxic cerebral preconditioning in neonatal rats. *Brain Res* 1075: 213–222, 2006.
- Fukui K, Onodera K, Shinkai T, Suzuki S, Urano S. Impairment of learning and memory in rats caused by oxidative stress and aging, and changes in antioxidative defense systems. *Ann NY Acad Sci* 928: 168–175, 2001.
- Furuichi T, Liu W, Shi H, Miyake M, Liu KJ. Generation of hydrogen peroxide during brief oxygen-glucose deprivation induces preconditioning neuronal protection in primary cultured neurons. *J Neurosci Res* 79: 816–824, 2005.
- Gahtan E, Auerbach JM, Segal M. Reversible impairment of long-term potentiation in transgenic Cu/Zn-SOD mice. *Eur J Neurosci* 10: 538–544, 1998.
- Garcia III AJ, Henderson III RA, Putnam RW, Dean JB. Oxidative stress induced by hyperbaric oxygen increases the synaptic response of CA1 neurons in the rat hippocampus. *FASEB J* 17: A71, 2003.
- Garcia III, AJ, Henderson III, RA, Putnam RW, Dean JB. Hyperoxia increases neuronal excitability by affecting membrane properties and excitatory input within the CA1 hippocampus. *FASEB J* 18: A1058, 2004.
- Garcia III AJ, Henderson III RA, Putnam RW, Pearson JC, Dean JB. Neuronal excitability at 0.95 atmosphere absolute (ATA<sub>0.95</sub>) is elevated relative to a lesser degree of oxygenation in the rat CA1 hippocampus. *Soc Neurosci Abstr* 729.18, 2005.
- Gomes A, Fernandes E, Lima JLFC. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods* 65: 45–80, 2005.
- Gonenc S, Uysal N, Acikgoz O, Kayatekin BM, Sonmez A, Kiray M, Aksu I, Gulecer B, Topcu A, Semin I. Effects of melatonin on oxidative stress and spatial memory impairment induced by acute ethanol treatment in rats. *Physiol Res Acad Scie Bohemoslovaca* 54: 341–348, 2005.
- Graulich J, Hoffmann U, Maier RF, Ruscher K, Pomper JK, Ko HK, Gabriel S, Obladen M, Heinemann U. Acute neuronal injury after hypoxia is influenced by the reoxygenation mode in juvenile hippocampal slice cultures. *Brain Res Dev Brain Res* 137: 35–42, 2002.
- Kaplan FS, Brighton CT, Boytim MJ, Selzer ME, Lee V, Spindler K, Silberberg D, Black J. Enhanced survival of rat neonatal cerebral cortical neurons at subatmospheric oxygen tensions in vitro. *Brain Res* 384: 199–203, 1986.
- Katoh S, Mitsui Y, Kitani K, Suzuki T. Hyperoxia induces the differentiated neuronal phenotype of PC12 cells by producing reactive oxygen species. *Biochem Biophys Res Commun* 241: 347–351, 1997.
- Kawase M, Murakami K, Fujimura M, Morita-Fujimura Y, Gasche Y, Kondo T, Scott RW, Chan PH. Exacerbation of delayed cell injury after transient global ischemia in mutant mice with CuZn superoxide dismutase deficiency. *Stroke* 30: 1962–1968, 1999.
- Kishida KT, Pao M, Holland SM, Klann E. NADPH oxidase is required for NMDA receptor-dependent activation of ERK in hippocampal area CA1. *J Neurochem* 94: 299–306, 2005.
- Klann E. Cell-permeable scavengers of superoxide prevent long-term potentiation in hippocampal area CA1. *J Neurophysiol* 80: 452–457, 1998.
- Klann E, Roberson E, Knapp L, Sweatt J. A role for superoxide in protein kinase C activation and induction of long-term potentiation. *J Biol Chem* 273: 4516–4522, 1998.
- Klann E, Thiels E. Modulation of protein kinases and protein phosphatases by reactive oxygen species: implications for hippocampal synaptic plasticity. *Prog Neuropsychopharmacol Biol Psychiatry* 23: 359–376, 1999.
- Knapp LT, Klann E. Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C. *J Neurosci* 22: 674–683, 2002.
- Kovacs R, Schuchmann S, Gabriel S, Kardos J, Heinemann U. Ca<sup>2+</sup> signalling and changes of mitochondrial function during low-Mg<sup>2+</sup>-induced epileptiform activity in organotypic hippocampal slice cultures. *Eur J Neurosci* 13: 1311–1319, 2001.
- Li J, Gao X, Qian M, Eaton JW. Mitochondrial metabolism underlies hyperoxic cell damage. *Free Rad Biol Med* 36: 1460–1470, 2004.
- Lynn S, Huang EJ, Elchuri S, Naemuddin M, Nishinaka Y, Yodoi J, Ferriero DM, Epstein CJ, Huang T-T. Selective neuronal vulnerability and inadequate stress response in superoxide dismutase mutant mice. *Free Rad Biol Med* 38: 817–828, 2005.
- Maharaj DS, Maharaj H, Antunes EM, Maree DM, Nyokong T, Glass BD, Daya S. 6-Hydroxymelatonin protects against quinolinic-acid-induced oxidative neurotoxicity in the rat hippocampus. *J Pharmacy Pharmacol* 57: 877–881, 2005.
- McCord JM. Superoxide radical: controversies, contradictions, and paradoxes. *Proc Soc Exp Biol Med* 209: 112–117, 1995.
- Moncada S, Bolanos JP. Nitric oxide, cell bioenergetics and neurodegeneration. *J Neurochem* 97: 1676–1689, 2006.
- Morgan AR, Evans DH, Lee JS, Pulleyblank DE. Review: ethidium fluorescence assay. II. Enzymatic studies and DNA-protein interactions. *Nucl Acids Res* 7: 571–594, 1979.
- Mulkey DK, Henderson III RA, Olson JE, Putnam RW, Dean JB. Oxygen measurement in brainstem slices exposed to normobaric hyperoxia and hyperbaric oxygen. *J Appl Physiol* 90: 1887–1899, 2001.
- Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF, Chan PH. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J Neurosci* 18: 205–213, 1998.
- Muranyi M, Li P-A. Hyperglycemia increases superoxide production in the CA1 pyramidal neurons after global cerebral ischemia. *Neurosci Lett* 393: 119–121, 2006.
- Nakamura K, Bindokas VP, Kowlessur D, Elas M, Milstien S, Marks JD, Halpern HJ, Kang UJ. Tetrahydrobiopterin scavenges superoxide in dopaminergic neurons. *J Biol Chem* 276: 34402–34407, 2001.
- Nicholls DG. Mitochondrial dysfunction and glutamate excitotoxicity studied in primary neuronal cultures. *Curr Mol Med* 4: 149–177, 2004.
- Nie H, Xiong L, Lao N, Chen S, Xu N, Zhu Z. Hyperbaric oxygen preconditioning induces tolerance against spinal cord ischemia by upregulation of antioxidant enzymes in rabbits. *J Cereb Blood Flow Metab* 26: 666–674, 2006.
- Onodera K, Omoi N-O, Fukui K, Hayasaka T, Shinkai T, Suzuki S, Abe K, Urano S. Oxidative damage of rat cerebral cortex and hippocampus, and changes in antioxidative defense systems caused by hyperoxia. *Free Rad Res* 37: 367–372, 2003.
- Peterson SL, Morrow D, Liu S, Liu KJ. Hydroethidine detection of superoxide production during the lithium-pilocarpine model of status epilepticus. *Epilepsy Res* 49: 226–238, 2002.
- Pinheiro AC, Gomez RS, Massensini AR, Cordeiro MN, Richardson M, Romano-Silva MA, Prado MA, De Marco L, Gomez MV. Neuroprotective effect on brain injury by neurotoxins from the spider *Phoneutria nigriventer*. *Neurochem Int* 49: 543–547, 2006.
- Pomper JK, Graulich J, Kovacs R, Hoffmann U, Gabriel S, Heinemann U. High oxygen tension leads to acute cell death in organotypic hippocampal slice cultures. *Brain Res Dev Brain Res* 126: 109–116, 2001.
- Prehn JH. Mitochondrial transmembrane potential and free radical production in excitotoxic neurodegeneration. *Naunyn-Schmiedeberg's Arch Pharmacol* 357: 316–322, 1998.
- Ravati A, Ahlemeyer B, Becker A, Krieglstein J. Preconditioning-induced neuroprotection is mediated by reactive oxygen species. *Brain Res* 866: 23–32, 2000.
- Rice ME. Use of ascorbate in the preparation and maintenance of brain slices. *Methods Enzymol* 18: 144–149, 1999.
- Rice ME, Forman RE, Chen BT, Avshalomov MV, Cragg SJ, Drew KL. Brain antioxidant regulation in mammals and anoxia-tolerant reptiles: balanced for neuroprotection and neuromodulation. *Comp Biochem Physiol Toxicol Pharmacol* 133: 515–525, 2002.



- Rice ME, Perez-Pinzon MA, Lee EJK.** Ascorbic acid, but not glutathione, is taken up by brain slices and preserves cell morphology. *J Neurophysiol* 71: 1591–1596, 1994.
- Scanlon JM, Reynolds IJ.** Effects of oxidants and glutamate receptor activation on mitochondrial membrane potential in rat forebrain neurons. *J Neurochem* 71: 2392–2400, 1998.
- Sengpiel B, Preis E, Kriegelstein J, Prehn JH.** NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria. *Eur J Neurosci* 10: 1903–1910, 1998.
- Serrano F, Klann E.** Reactive oxygen species and synaptic plasticity in the aging hippocampus. *Ageing Res Rev* 3: 431–443, 2004.
- Shibata M, Blatteis CM.** High perfusate PO<sub>2</sub> impairs thermosensitivity of hypothalamic thermosensitive neurons in slice preparations. *Brain Res Bull* 26: 467–471, 1991.
- Shimabuku R, Ota A, Pereyra S, Veliz B, Paz E, Nakachi G, More M, Oliveros M.** Hyperoxia with 100% oxygen following hypoxia-ischemia increases brain damage in newborn rats. *Biol Neonate* 88: 168–171, 2005.
- Sugawara T, Chan PH.** Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxidants Redox Signal* 5: 597–607, 2003.
- Uchida K, Samejima M, Okabe A, Fukuda A.** Neuroprotective effects of melatonin against anoxia/aglycemia stress, as assessed by synaptic potentials and superoxide production in rat hippocampal slices. *J Pineal Res* 37: 215–222, 2004.
- Wang T, Liu B, Qin L, Wilson B, Hong JS.** Protective effect of the SOD/catalase mimetic MnTMPyP on inflammation-mediated dopaminergic neurodegeneration in mesencephalic neuronal-glia cultures. *J Neuroimmunol* 147: 68–72, 2004.
- Weinstock M, Shoham S.** Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. *J Neural Transmission* 111: 347–366, 2004.
- Wilde GJ, Pringle AK, Wright P, Iannotti F.** Differential vulnerability of the CA1 and CA3 subfields of the hippocampus to superoxide and hydroxyl radicals in vitro. *J Neurochem* 69: 883–886, 1997.
- Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B.** Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Rad Biol Med* 34: 1359–1368, 2003.
- Zuo L, Clanton TL.** Detection of reactive oxygen species and nitrogen species in tissues using redox-sensitive fluorescent probes. *Methods Enzymol* 352: 307–325, 2002.