ONLINE METHODS

Animals. Experiments were carried out in accordance with protocols approved by the San Francisco Veterans Affairs Medical Center Animal Studies subcommittee. Swiss Webster mice were obtained from Simonsen. Wild-type and p47phox−/− mice on the C57/Bl6 background were obtained from Jackson Laboratories. The p47phox−/− mice were maintained as homozygotes by using fresh breeder stock, fully backcrossed to wild-type C57/Bl6 mice after eight generations. Sod2-overexpressing mice (Sod2+) were generated previously and subsequently outbred to the C57/Bl6 background and maintained as heterozygotes. Cultures were prepared from Sod2− × wild-type matings, which yield Sod2−/− and wild-type littermate embryos. Cell culture reagents were obtained from Mediatech and all other reagents were obtained from Sigma-Aldrich, except where noted. Data analyses for all studies were performed by observers blinded to the experimental conditions.

Neuron cultures. Cultures were prepared from the cortices of embryonic day 16 mice and plated in 24-well culture plates or poly-d-lysine–coated glass coverslips. After 1 d in culture, 10 μM cytosine arabinoside was added for 24 h to prevent glial proliferation. The neurons were subsequently maintained with serum-free NeuroBasal medium (Gibco) containing 5 mM glucose and used after 9–10 d in vitro. These cultures contained >95% neurons and no detectable microglia. Experiments were initiated by changing the culture medium with a balanced salt solution (BSS) containing 1.2 mM CaCl2, 0.8 mM MgSO4, 5.3 mM KCl, 0.4 mM KH2PO4, 137 mM NaCl, 0.3 mM NaHPO4, 5 mM glucose and 10 mM 1,4-piperazinediethanesulfonate buffer (pH 7.2). Drugs were added from concentrated stocks in BSS. Where used, drugs were added to the culture medium 10 min before the addition of NMDA or H2O2.

Immunostaining. Formaldehyde-fixed cultures were immunostained as described previously using rabbit antibody to 4HNE (Alpha Diagnostics International, 1:500 dilution), mouse antibody to microtubule-associated protein 2 (MAP2, Chemicon International, 1:500 dilution) and rabbit antibody to p47phox (Millipore, 1:500 dilution). Antibody binding was visualized with Alexa Fluor 488–conjugated antibody to mouse IgG or Texas Red 594–conjugated antibody to rabbit IgG (Invitrogen). Images were obtained using confocal microscopy using step-wise 1-μm z stack sampling. For quantification of 4HNE immunostaining, neurons were designated as 4HNE-positive if fluorescence was 50% greater than background fluorescence. Neurons were analyzed in three randomly chosen optical fields from each of four wells per experiment, with >100 neurons per well for a total of 400–500 neurons per experiment.

p47phox membrane translocation. We used MAP2 immunostaining to identify the neuronal plasma membrane. The area of p47phox immunoreactivity overlapping with MAP2 immunoreactivity was analyzed in confocal images through each neuron and expressed as a ratio to the area of p47phox immunoreactivity that was not overlapping with MAP2. These ratios were calculated for all cells in each treatment group and compared by the Kruskall-Wallis test with the Dunn post hoc test for multiple group comparisons.

PKC peptide inhibitors. TAT-conjugated peptide inhibitors were prepared with the amino acid sequences described previously and generously provided by D. Mochly-Rosen (Stanford University). Stock solutions in DMSO were diluted >1:10,000 into BSS for use in cell cultures or into sterile water for use in mice.

Superoxide detection. We added 5 μM dHEth (Invitrogen) to cultures 10–20 min before the addition of NMDA or H2O2 and maintained it throughout the duration of the experiment. The cultures were photographed at the specified time points with a fluorescence microscope (Axiovert 40 CFL, Zeiss) using 510–550-nm excitation and >580-nm emission. Neurons were designated as ethidium-positive if the mean perikarya fluorescence was greater than 50% above background fluorescence22. Neurons were analyzed in three randomly chosen optical fields from each of four wells per experiment, with >100 neurons per well for a total of 400–500 neurons per experiment. For real-time imaging, images were acquired with 510 ± 15-nm excitation at 2-min intervals (40-ms exposure). The change in raw fluorescence for each neuron in the field of view was then normalized to baseline fluorescence before stimulus. Values were averaged from 8–12 neurons per experiment, with the n being the number of experiments from independent culture preparations.

For in vivo experiments, dHEth was prepared as a 1 mg ml−1 solution in 1% DMSO (vol/vol) and administered at 1 mg per kg of body weight by intraperitoneal injection. Adult male C57/Bl6 wild-type or C57/Bl6 p47phox−/− mice were anesthetized with 2% isoflurane and placed in a stereotaxic frame. The right hippocampus (anterior-posterior 2.5 mm, medial-lateral 2 mm and dorsal-ventral 2 mm from Bregma and the cortical surface) was injected with 6 nmol of NMDA in 1 μl of saline vehicle over 5 min, beginning 30 min after the dHEth injections. Anesthesia was discontinued immediately after the NMDA injections. Mice receiving NMDA also received 1 mg per kg CNQX, and some mice also received 15 mg per kg apocynin or 0.2 mg per kg TAT-conjugated peptide PKC inhibitors. CNQX, apocynin and the peptide PKC inhibitors were given by intraperitoneal injection 20–30 min before the NMDA injections. All drugs were prepared in sterile saline and controls received equal volumes of saline vehicle.

Mice were killed and perfusion-fixed with 4% formaldehyde (wt/vol) 30 min after the NMDA injections. Cryostat sections were prepared and photographed with a confocal fluorescent microscope with excitation at 510–550 nm and emission >580 nm to detect oxidized ethidium species. We analyzed five sections from each brain, which were taken at 40-μm intervals to span the hippocampus. The fluorescence of ten representative CA1 hippocampal neuron cell bodies was measured and normalized to background fluorescence in the stratum radiatum. The normalized values for the 50 neurons were averaged to generate a single value for each brain.

Intracellular calcium imaging. Neurons were loaded for 30 min with 4 nM Fura-2 a.m. (Molecular Probes) and washed once with BSS before imaging. Images were acquired at 1-min intervals with 30-ms exposures, using excitation at both 340 and 380 nm and emission at >510 nm. The mean fluorescence measured in each neuron in the field of view for both 340- and 380-nm excitation. Raw fluorescence was then normalized to baseline levels before stimulus and the ratio of 340/380-nm fluorescence was calculated from these normalized values.

Cell death. Death of cultured neurons was quantified by trypan blue staining 24 h after NMDA exposures. Neuron death in mouse hippocampus was evaluated 3 d after NMDA injections by the Fluoro-Jade B method (Histo-Chem). We collected five coronal sections from each animal, which were spaced 40 μm apart and spanned the hippocampus. The total number of Fluoro-Jade B–positive neurons was counted in the hippocampal CA1 cell field of both hemispheres. Data from each animal were expressed as the mean number of degenerating neurons per hippocampal section.

Statistical analyses. Except where noted, data are expressed as mean ± s.e.m. and were assessed using one-way ANOVA followed by Tukey’s test for multiple comparisons between groups.