Supplementary methods

Detection of ROS by fluorescent indicators. We treated PC12 cells with 10 μM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydroflurescein diacetate, acetyl ester (CM-H_2DCFDA) (purchased from Molecular Probes), 5 μM diaminofluorescein-2 diacetate (DAF-2 DA) (purchased from Daiichi Pure Chemicals Co.), or 5 μM of 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoate (HPF) (Daiichi Pure Chemicals Co.) for 30 min to detect cellular H_2O_2, NO• or •OH, respectively. We took fluorescent images with a laser-scanning confocal microscope (Olympus FV300) using excitation and emission filters of 488 nm and 510 nm, respectively. HPF can be specifically oxidized by •OH, peroxynitrite ($ONOO^-$) and lipid peroxides, but neither H_2O_2, NO• nor O_2^-• (ref. 14). For the detection of cellular O_2^-•, we used 0.5 μM MitoSOX (purchased from Molecular Probes), and took images using excitation and emission filters of 543 nm and 565 nm, respectively. Fluorescent signals were quantified from 100 cells of each experiment using US National Institutes of Health Image software.

Staining of mitochondria. For staining of mitochondria, we co-stained with MitoTracker Green (MTGreen) (1 μM; Molecular Probes) and tetramethylrhodamine methyl ester (TMRM) (100 nM; Molecular Probes). Fluorescence from MTGreen is independent of the membrane potential, whereas that from TMRM is sensitive to the membrane potential. MTGreen and TMRM were detected using excitation at 488 and 543 nm, and emission filters of 510 and 565 nm, respectively.

Immunostaining. We purchased antibodies against HNE and 8-OH-G from Nikken Seil Co, and antibodies against TUJ-1 and GFAP from COVANCE and ThermoImmunon, respectively. We used BODIPY FL goat anti-mouse IgG (Molecular Probe) as a secondary antibody and visualized signals with a laser-scanning confocal microscope. Fluorescence signals in response to 8-OH-G and HNE were quantified with NIH Image software.

Intracellular Fenton reaction. We preincubated PC12 cells with 1 mM $CuSO_4$ for 30 min in medium containing 1% FCS, washed once with phosphate-buffered saline (PBS) containing $CaCl_2$ (0.1 g/l), $MgCl_2.6H_2O$ (0.1 g/l), glucose (1g/l) and sodium pyruvate

(0.036 g/l) (pH 7.2), and then exposed to the indicated concentration of ascorbate (vitamin C) for 1 h in phosphate-buffered saline as described above. As negative controls, $CuSO_4$ or ascorbate was omitted. Note that Cu^{+2} is reduced by ascorbate to Cu^{+}, which catalyzes the Fenton reaction to produce •OH from H_2O_2 that is being spontaneously produced in the cells.

Electron spin resonance measurement. We used *5*,*5*-dimethyl-1-pyrroline *N*-oxide (DMPO) as a free radical trapper, and detected electron spin resonance (ESR) signals with a KEYCOM ESR spectrometer type ESR-X01. As a standard of the reactant of •OH with DMPO, we produced •OH by the Fenton reaction in the mixture of 0.1 mM H_2O_2 and 1 mM $FeCl_2$ in the presence of 0.1 mM DMPO and subjected the whole solution to ESR measurement. For the measurements, we normalized the sensitivity of each experiment with the strength of the internal ESR signal derived from Mn^{2+}. To obtain a spectrum, ESR was scanned for 2 min, accumulated 10 times, and all signals were averaged.

For H_2 treatment, we prepared media containing 0.6 mM H_2 and 8.5 mg/l O_2, and filled a closed culture flask with 75% H_2, 20% O_2 and 5% CO_2 gases. We pretreated PC12 cells (2×10^6 cells in a 25 cm^2 flask) with 0.1 M DMPO and 2 mM $CuSO_4$ in DMEM containing 1% FCS for 30 min at 37 °C in the presence or absence of 0.6 mM H_2. After the removal of this medium, we exposed the cells to 0.2 mM ascorbate and 0.1 mM H_2O_2 in 0.3 ml of PBS in the presence or absence of 0.6 mM H_2 for 5 min at room temperature to produce •OH by the Fenton reaction, and scraped the cells into a flat cuvette for ESR measurement. In the other method, we preincubated PC12 cells (2×10^6 cells in a 25 cm^2 flask) in 0.3 ml of PBS containing 0.1 M DMPO and 30 μg/ml antimycin A for 7 min at room temperature in the presence or absence of 0.6 mM H_2, and then scraped the cells into a flat cuvette for ESR measurement. A differential spectrum was obtained by digitally subtracting one spectrum from the other to visualize the signals decreased by H_2 treatment.

Primary culture. We prepared primary cultures of neocortical neurons from 16-day rat embryos by the method described previously[19]. In brief, neocortical tissues were cleaned of meninges, minced, and treated with a protease cocktail (SUMILON). After mechanical dissociation by pipetting, we resuspended cells in nerve-cell culture medium

(SUMILON), and then plated onto poly-L-lysine-coated plates at a density of 5×10^4 cells / cm^2, changed to Neurobasal Medium (Invitrogen) with B-27 (Invitrogen) once every three days and then used neurons at day 11. One day before OGD, we changed the medium to Neurobasal Medium with B-27 minus AO (Invitrogen), and confirmed neuronal identity by immunostaining with antibodies to neuron marker TUJ-1, and astrocyte marker GFAP. We used preparations only containing over 90% neurons for experiments.

Oxygen-glucose deprivation. To initiate OGD, we replaced the culture medium with a glucose-deficient DMEM from which O_2 had been removed by bubbling in a mixed gas of either N_2 (95%):CO_2 (5%) or H_2 (95%):CO2 (5%) and maintained the culture for 60 min at 30 °C under an atmosphere of either N_2 (95%):CO_2 (5%) or H_2 (95%):CO_2 (5%). Treatment was terminated by exchanging the experimental medium with stocked culture medium and further incubation at 37 °C with air including 5% CO_2.

Reaction of H_2 in cell-free systems. We performed fluorescence spectroscopic studies with a Shimadzu RF-5300PC. For solution studies, we dissolved H_2 in water beyond the saturated level under 0.4 MPa of hydrogen pressure for 2 h and then used it under atmospheric pressure. We determined H_2 concentrations with a hydrogen electrode in each experiment.

To detect the reaction of H_2 with the oxidized form of cytochrome *c*, FAD, or NAD^+, we incubated solutions containing 10 μM cytochrome *c*, 1 mM FAD or 1mM NAD^+ with or without 0.8 mM H_2 in a closed cuvette at 23 °C for 30 min, and observed no reaction by absorbance at 415, 400 and 340 nm, respectively.

We monitored the reactivity of H_2 with various ROS by HPF, DAF-2, or nitroblue tetrazolium (NBT). We measured fluorescent signals of HPF and DAF-2 at 515 nm with excitation at 490 and 495 nm, respectively, and the reduction of NBT to NBT-diformazan by absorbance at 550 nm.

To detect the reaction of H_2 with •OH, we mixed hydrogen solution, phosphate buffer (10 mM at pH 7.4), ferrous perchlorate (0.1 mM), and HPF (0.4 μM). We initiated the Fenton reaction by adding H_2O_2 to 5 μM in a closed cuvette at 23 °C with gentle stirring and monitored fluorescence for 30 s.

To detect the reaction of H_2 with O_2^-•, we mixed solutions containing xanthine and

NBT (supplied by TREVIGEN) with or without 0.8 mM H_2 in a closed cuvette, initiated the reaction by adding xanthine oxidase at 23 °C and monitored for 5 min.

To detect the reaction of H_2 with H_2O_2, we incubated solutions including phosphate buffer (10 mM at pH 7.4) and H_2O_2 (10 μM) with or without H_2 (0.8 mM) in a closed glass tube at 23 °C for 30 min. We converted the remaining H_2O_2 to •OH by 0.2 μM horseradish peroxidase and then incubated with 10 μM HPF for 5 min.

To detect the reaction of H_2 with NO•, we incubated solutions containing phosphate buffer (10 mM at pH 7.4) and 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7, 0.1 μM, purchased from Dojin Chemicals Co.) with or without 0.8 mM H_2 in a closed cuvette at 23 °C for 30 min, and monitored the remaining NO• by incubation with 5 μM DAF-2 for 10 min.

To detect the reaction of H_2 with peroxynitrite ($ONOO^-$), we diluted a stock solution of 1 μM $ONOO^-$ in alkali 200-fold into 10 mM phosphate buffer with 0.4 μM HPF in the presence or absence of 0.8 mM H_2, and then examined HPF signals after 23 °C for 1 min.