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Nitric oxide from neuronal nitric oxide synthase sensitises neurons to hypoxia-induced death via competitive inhibition of cytochrome oxidase

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Abstract

Hypoxia/ischaemia is known to trigger neuronal death, but the role of neuronal nitric oxide synthase (nNOS) in this process is controversial. Nitric oxide (NO) inhibits cytochrome oxidase in competition with oxygen. We tested whether NO derived from nNOS synergises with hypoxia to induce neuronal death by inhibiting mitochondrial cytochrome oxidase. Sixteen hours of hypoxia (2% oxygen) plus deoxyglucose (an inhibitor of glycolysis) caused extensive, excitotoxic death of neurons in rat cerebellar granule cell cultures. Three different nNOS inhibitors (including the selective inhibitor *N*-4*S*-4-amino-5-2-aminoethyl-aminopentyl-*N*′-nitroguanidine) decreased this neuronal death by half, indicating a contribution of nNOS to hypoxic death. The selective nNOS inhibitor did not, however, block neuronal death induced either by added glutamate or by added azide (an uncompetitive inhibitor of cytochrome oxidase), indicating that nNOS does not act downstream of glutamate or cytochrome oxidase. Hypoxia plus deoxyglucose-induced glutamate release and neuronal depolarisation, and the nNOS inhibitor decreased this. Hypoxia inhibited cytochrome oxidase activity in the cultures, but a selective nNOS inhibitor prevented this inhibition, indicating NO from nNOS was inhibiting cytochrome oxidase in competition with oxygen. These data indicate that hypoxia synergises with NO from nNOS to induce neuronal death via cytochrome oxidase inhibition causing neuronal depolarisation. This mechanism might contribute to ischaemia/stroke-induced neuronal death *in vivo*.

Keywords: excitotoxicity, glutamate, ischaemia, mitochondria, neuronal nitric oxide synthase, stroke.

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Nitric oxide (NO) acts as a physiological neuromodulator and vasodilator in the brain, but also contributes to pathology (Duncan and Heales 2005; Guix *et al.* 2005; Moncada and Bolanos 2006). The main source of NO in the brain, in the absence of inflammation, is nNOS (NOS1). Within neurons, nNOS has been found in the post-synaptic density, attached to *N*-methyl-D-aspartate (NMDA)-type glutamate receptors via PDZ domains, and extracellular glutamate stimulates nNOS activity via activating NMDA receptors, causing an influx of calcium that stimulates nNOS via binding of calcium/calmodulin (Mungrue and Bredt 2004). nNOS has been implicated in excitotoxic death of neurons induced by excess extracellular glutamate acting on NMDA receptors, but the involvement of nNOS in this death is unclear – some authors find an involvement of nNOS and others do not (Keynes and Garthwaite 2004; Moncada and Bolanos 2006) and the involvement of nNOS in excitotoxicity has been suggested to be an artefact of cell culture systems (Keynes *et al.* 2004; Keynes and Garthwaite 2004). However, *in vivo* and *in vitro* inhibition of nNOS or gene-knockout of nNOS has been found to strongly protect brain neurons against ischaemia/reperfusion-induced death (Mei *et al.* 1996; Alonso *et al.* 2002; Moro *et al.* 2004; Scorziello *et al.* 2004; Moncada and Bolanos 2006). If nNOS is not involved in excitotoxicity it remains unclear how nNOS contributes to hypoxia/ischaemia-induced neuronal death.

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Abbreviations used: AAA-NG, *N*-4*S*-4-amino-5-2-aminoethyl-aminopentyl-*N*′-nitroguanidine; DETA, diethylenetriamine; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; iNOS, inducible nitric oxide synthase; INT, iodonitrotetrazolium chloride; LNMMA, NG-monomethyl-L-arginine; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NPLA, *N*-propyl-L-arginine; PI, propidium iodide; TMPD, tetramethyl-phenylenediamine.

The physiological target of NO is the ferrous haem of soluble guanylate cyclase (Moncada *et al.* 1991), but NO also binds to the ferrous haem of mitochondrial cytochrome oxidase (Brown and Cooper 1994; Cleeter *et al.* 1994; Moncada and Bolanos 2006). The latter binding causes acute and reversible inhibition of isolated mitochondrial cytochrome oxidase, respiration of isolated mitochondria, neuronal nerve terminals (synaptosomes) and neurons (Brown and Cooper 1994; Cleeter *et al.* 1994; Bal-Price and Brown 2001). nNOS has been found to bind to mitochondria (Haynes *et al.* 2004), and in particular to cytochrome oxidase, including within cerebellar granule neurons (Persichini *et al.* 2005). Activation of nNOS leads to inhibition of cytochrome oxidase in neurons (Almeida and Bolanos 2001). NO inhibition of cytochrome oxidase is in competition with oxygen, so that for example the apparent IC50 of NO for synaptosomal respiration was found to be 250 nmol/L at 150 μmol/L O_2, but at 30 μmol/L O_2 the apparent IC50 of NO for synaptosomal respiration was 60 nmol/L (Brown and Cooper 1994). This indicates that NO can dramatically increase the apparent K_M of respiration for oxygen, potentially sensitising neurons to hypoxia/ischaemia. Hypoxia normally kills neurons by the oxygen level decreasing below the K_M of cytochrome oxidase so that insufficient ATP is generated (if glycolysis is low), resulting in depolarisation of the plasma membrane, release of glutamate and activation of NMDA receptors (by the increased extracellular glutamate and decreased membrane potential) (Simon *et al.* 1984; Pohorecki *et al.* 1990). The K_M of cytochrome oxidase for oxygen is about 0.5 μmol/L in the absence of NO, but rises to 30 μmol/L in the presence of 60 nmol/L NO (Brown and Cooper 1994). Thus, the presence of NO may potentially sensitise neurons to hypoxic death and we have previously confirmed this in cultured cerebellar granule neurons exposed to NO from NO donors (Mander *et al.* 2005). In this work we tested whether the NO generated from nNOS in these neurons would be sufficient to sensitise them to hypoxic death. We find that it does, and this has implications for how nNOS contributes to hypoxic/ischaemic death of neurons *in vivo*.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), Versene, phosphate-buffered saline were purchased from Invitrogen (Carlsbad, CA, USA); *N*-propyl-L-arginine (NPLA), NG-monomethyl-L-arginine (LNMMA), 1400W and diethylenetriamine (DETA)-NONOate were obtained from Alexis (Lausen, Switzerland); all other chemicals were purchased from Sigma (St Louis, MO, USA).

Neuronal-glial and neuronal culture preparation

Mixed neuronal-glial cultures were prepared from 7-days post-natal Wistar rats. The cerebella were dissected, dissociated in Versene solution (1 : 5000), and plated at 0.25×10^6 cells/cm^2 in 24-well plates (in 500 μL of DMEM) coated with poly-L-lysine (15 μg/mL). Cultures were maintained in DMEM supplemented with heat-inactivated horse serum (5%) and fetal calf serum (5%), 2 mmol/L L-glutamine, 25 mmol/L KCl, 38 mmol/L glucose and 10 μg/mL gentamicin. For neuronal cultures, cytosine-D-arabinoside (10 μmol/L) that is toxic for proliferating (non-neuronal) cells was added 24 h after plating. Cytosine-D-arabinoside was not added to the mixed cultures. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cultures were used at 14–16 days *in vitro* (DIV) when nNOS is fully expressed (Samdani *et al.* 1997; Jurado *et al.* 2004).

Culture treatments

The cultures were treated with 10 mmol/L deoxyglucose to inhibit glycolysis, or deoxyglucose together with selective nNOS inhibitors either *N*-4*S*-4-amino-5-2-aminoethyl-aminopentyl-*N*′-nitroguanidine (AAA-NG) (1 μmol/L) or NPLA (100 μmol/L), or the non-specific NOS inhibitor LNMMA (5 mmol/L), or the selective inducible nitric oxide synthase (iNOS) inhibitor 1400W (25 μmol/L), or with the NMDA receptor antagonist 10 μmol/L MK801, or with the soluble guanylate cyclase inhibitor ODQ (10 μmol/L) and subjected to hypoxia. To induce hypoxia, mixed neuronal-glial or neuronal cultures were placed in a sealed, humidified and thermostated (37°C) container perfused with a gas mixture of 5% CO_2/2% O_2/93% N_2 for 16 h. For the normoxic treatment, cells were incubated in the same conditions but with a gas mixture of 5% CO_2/21% O_2/74% N_2. Some cultures were treated with 30 μmol/L NaN_3 (a cytochrome oxidase inhibitor) with or without deoxyglucose or AAA-NG, or with 200 μmol/L glutamate with or without AAA-NG for 16 h.

Assessment of cell viability

The viability of neuronal cells in the cultures was assessed by propidium iodide (PI, 2 μg/mL) and Hoechst 33342 (6 μg/mL) staining, using a fluorescence microscope (Axiovert S-100; Zeiss, Oberkochen, Germany) and filters for excitation at 365 nm and emission at 420 nm. The cell-impermeable nuclear dye propidium iodide stains the nuclei of cells that have lost plasma membrane integrity and are considered to be necrotic. Using the cell-permeable dye Hoechst 33342, the nuclear morphology of neurons was studied. Cells with relatively large nuclei with weak Hoechst-staining and lacking PI staining were considered to be viable, whereas cells showing nuclear shrinkage and intensive Hoechst staining, but still lacking PI staining were classified as chromatin condensed. Neuronal cells were distinguished from glial cells according to their nuclear morphology when stained with the Hoechst dye (neurons having round and slightly granulated nuclei, astrocyte having large and elongated nuclei, and microglia having round but evenly-stained nuclei) and according to cellular morphology. PI-positive or chromatin-condensed neuronal cells were counted in six microscopic fields in each well (two wells per treatment) and expressed as a percentage of the total number of neurons per field.

Nitrite measurement

Nitrite in cell-conditioned medium was measured by a fluorometric assay described in (Misko *et al.* 1993). Briefly, the samples were diluted 1 : 50 in 0.06 mol/L HCl containing 5 μg/mL 2,3-diaminonaphthalene that reacts with nitrite in acidic conditions to form

1-(*H*)-naphthotriazole, a fluorescent product, and incubated for 10 min at 20°C in the dark. Then, 0.3 mol/L NaOH was added to stop the reaction, and fluorescence intensity was measured in a spectrofluorophotometer (Shimadzu RF-1501; Kyoto, Japan) at λ_{ex} = 363 nm and λ_{em} = 426 nm. Nitrite concentration in the samples was calculated according to the calibration curve constructed using known concentrations of nitrite.

Glutamate levels

Extracellular glutamate was measured by adding glutamate dehydrogenase, diaphorase, NAD and iodonitrotetrazolium chloride (INT) to cell-conditioned medium and measuring the appearance of coloured formazan. The assay is based on the conversion of glutamate and NAD to oxoglutarate and NADH by glutamate dehydrogenase, and the subsequent conversion of NADH and colourless INT to NAD and red formazan by diaphorase. Cell culture media were collected immediately after treatments, supplemented with 0.04 mol/L KOH and 0.12% H_2O_2, and incubated at 70°C for 10 min to eliminate reducing substances. The samples were then allowed to cool to 20°C, and the pH was adjusted to 8.6 with H_2SO_4. These samples were diluted 1 : 4 in phosphate buffer (pH 8.6) with 0.3% Triton X-100, and then 1.5 mmol/L NAD, 0.1 U/mL diaphorase and 2.5 mmol/L INT were added. The absorbance at λ = 492 nm of these mixtures was measured using a spectrophotometer (Lambda 35; Perkin Elmer Instruments, Waltham, MA, USA) before and 5 min after adding 20 U/mL glutamate dehydrogenase. This increase in absorbance was proportional to the original amount of glutamate, and was calibrated using known concentrations of glutamate.

Tetramethyl-phenylenediamine oxidation measurements

Tetramethyl-phenylenediamine (TMPD) is a colourless electron donor for cytochrome oxidase. Activity of the oxidase results in one-electron oxidation of TMPD producing a highly coloured product (Wurster's blue). For the measurements of TMPD oxidation, the medium of mixed neuronal-glial cultures was decreased to 200 μL per well, and the cultures were incubated with 100 μmol/L of freshly-dissolved TMPD plus various other indicated treatments, under hypoxic or normoxic conditions for 30 min. After incubation, the samples were diluted five times in water (200 μL of medium from each well into 800 μL water in a 1 mL cuvette) and the absorbance at λ = 611 nm and at λ = 480 nm was measured using a spectrophotometer (Lambda 35; Perkin Elmer Instruments) and the absorbance difference $\Delta A = A_{611} - A_{480}$ was calculated (Wurster's blue has no absorbance at 480 nm and an absorbance peak at 611 nm, where phenol red has no significant absorbance). Medium taken from cells before the experiment and incubated without TMPD for 30 min in hypoxia or normoxia accordingly was used as a 'blank,' i.e. this absorbance was subtracted from the experimental measurements. Because TMPD autooxidises in the absence of cells, the cell-dependent TMPD oxidation results are plotted as the difference between values obtained from medium incubated with cells and medium incubated without cells under the same conditions.

Immunoassay of nNOS

Neuronal-glial cultures were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 30 min at 20°C. Cultures were washed twice with phosphate buffer and then incubated for 30 min in phosphate buffer containing 1% bovine serum albumin and 0.1% saponin. Primary antibody (monoclonal mouse anti-rat nNOS, 1 : 1000; Sigma) was added to cells in phosphate buffer containing 1% bovine serum albumin and 0.1% saponin for 1 h at 20°C. Cells were washed three times with phosphate buffer and then incubated with secondary antibody (Cy3 goat anti-mouse; Molecular Probes, Carlsbad, CA, USA) in phosphate buffer with 1% bovine serum albumin and 0.1% saponin. Cells were washed three times in phosphate buffer and then Hoechst 33342 was added in water for 5 min, to visualise nuclei of all cells. Cells were viewed using a fluorescence microscope with an excitation filter of 450–490 nm, and an emission filter of 515–565 nm for viewing nNOS distribution, and excitation 365 nm and emission 420 nm for viewing nuclei. Over 99% of the neurons in the cultures were nNOS-positive.

Detecting NOS by diaphorase activity

Neuronal-glial cultures were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 30 min at 20°C. Cultures were washed twice with phosphate buffer and then incubated for 30 min in phosphate buffer containing 1 mmol/L nitroblue tetrazolium, 1 mmol/L NADPH and 0.3% Triton X-100 at 37°C. NADPH was omitted in negative controls. The reaction was stopped when the signal was clearly distinguishable and background staining had not yet appeared (about 30 min). Cells were viewed using a phase contrast microscope. All the neurons investigated showed diaphorase activity.

Plasma membrane potential

Plasma membrane potential was measured using a bis-oxonol (supplied by Molecular Devices, Sunnyvale, CA, USA), which is a lipophilic anion that distributes across the plasma membrane according to the potential difference. The anion enters cells when they depolarise and binds to cytoplasmic proteins, which induces the bis-oxonol to fluorescence. A bottle of component A from the Fluorimetric Imaging Plate Reader membrane potential detection kit (Molecular Devices) was dissolved in 10 mL of buffer A (115 mmol/L NaCl, 1 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, 10 mmol/L HEPES, pH 7.4) and diluted 1 : 10 in Krebs-HEPES buffer (118 mmol/L NaCl, 25 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L Mg_2SO_4, 1 mmol/L $CaCl_2$, 38 mmol/L glucose, 6 mmol/L glutamine and 25 mmol/L HEPES, pH 7.4). DMEM was removed from neuronal-glial cell cultures, and cells were washed with the Krebs-HEPES buffer. Then the bis-oxonol solution in Krebs-HEPES was added to the cells (200 μL per well). Deoxyglucose (10 mmol/L) or deoxyglucose together with AAA-NG (1 μmol/L) was added to the wells (four wells per each treatment), and cells were incubated in hypoxia or normoxia for 1 h. Cells were viewed using a fluorescence microscope with filters for excitation 450–490 nm and emission 515–565 nm. Photos were taken of each well (2–3 randomly selected fields per well), with the same exposure time (0.3 s) for each picture. Fluorescence intensity of each image was calculated using ImageJ software (http://rsb.info.nih.gov/ij). The ability of the dye to respond to plasma membrane potential changes was tested by acute additions of 60 mmol/L KCl to normoxic untreated cultures to rapidly depolarise cells. KCl (60 mmol/L)

induced a rapid increase in the fluorescence from 6.54 ± 0.80 to 13.50 ± 0.90 indicating that the dye is able to rapidly sense a decrease in plasma membrane potential.

Statistical analysis

Each experiment was repeated three to eight times, and each repeat was done on cells from separate cultures. Data are expressed as mean ± SE. The data were analysed for significance using Student's *t*-test. In the figures, *p*-values are indicated as * (#, &) where $p < 0.05$, ** (##, &&) where $p < 0.01$ and *** (###, &&&) where $p < 0.001$.

Results

NO from nNOS contributes to hypoxia- plus deoxyglucose-induced neuronal death

We tested whether nNOS activity sensitises neurons to hypoxia in a mixed neuronal-glial culture. Mature cerebellar granule cell cultures of 14–16 DIV that are reported to express high levels of nNOS (Samdani *et al.* 1997; Jurado *et al.* 2004) were subjected to 16 h of relative hypoxia (2% O_2/5% CO_2 in nitrogen) with or without the glycolysis inhibitor 2-deoxyglucose (10 mmol/L). After the treatment, neuronal viability was assessed using PI and Hoechst 33342 staining to determine necrosis and chromatin condensation (an indication of apoptosis), respectively. Hypoxia alone did not cause any increase in necrotic (Fig. 1, upper chart) or chromatin-condensed (Fig. 1, lower chart) neurons. However, hypoxia together with deoxyglucose resulted in 40 ± 6% necrotic and 32 ± 7% chromatin-condensed neurons. Deoxyglucose plus 16 h of normoxia (21% O_2/5% CO_2 in nitrogen) had no effect on neuronal viability. Thus hypoxia or deoxyglucose alone cause no neuronal death, but when combined cause substantial neuronal death, presumably by energy depletion. No astrocytes or microglia showed any signs of chromatin condensation or necrosis in any of the conditions used indicating that cell death in these conditions was specific to neurons.

Two specific nNOS inhibitors AAA-NG (1 μmol/L) and NPLA (100 μmol/L), as well as a non-specific NOS inhibitor LNMMA (5 mmol/L) added in the culture medium at the start of the treatment partially prevented hypoxia plus deoxyglucose-induced neuronal necrosis (Fig. 1, upper chart): the percentage of necrotic neurons were 24 ± 5, 21 ± 8 and 19 ± 4, respectively. AAA-NG also reduced the percentage of chromatin-condensed neurons in hypoxia plus deoxyglucose treated cultures to 18 ± 5%. NPLA and LNMMA also showed a tendency to protect neurons against hypoxia plus deoxyglucose-induced chromatin condensation, but the effect was not statistically significant (Fig. 1, lower chart). An iNOS-specific inhibitor (1400 W), at a concentration (25 μmol/L) that we have previously shown to potently inhibit iNOS in these cultures (Mander *et al.* 2005), had no effect on hypoxia/deoxyglucose-induced

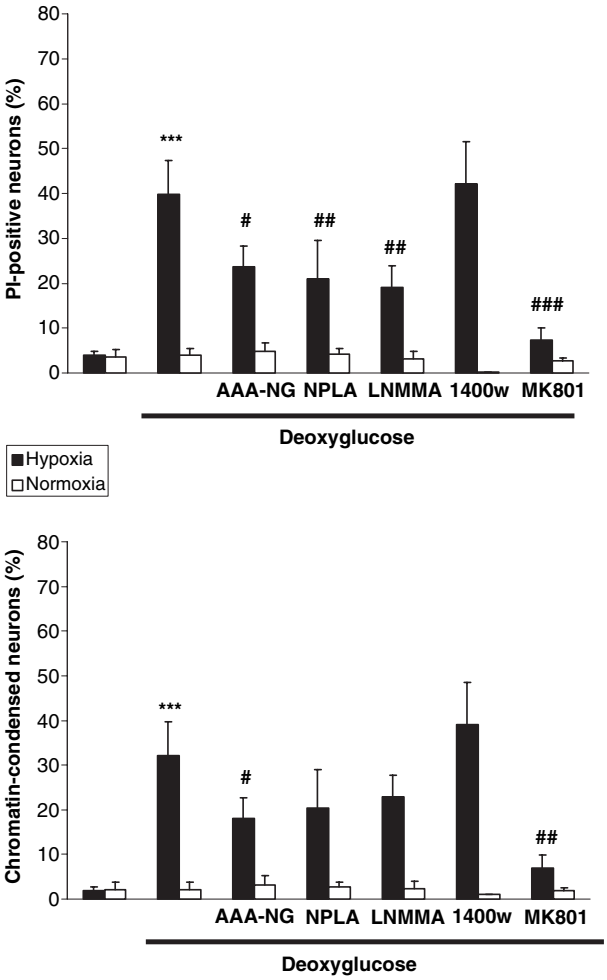

Fig. 1 The effect of nNOS inhibitors on hypoxia and deoxyglucose-induced neuronal damage in mixed neuronal-glial culture. Mixed neuronal glial cultures were treated with either 10 mmol/L deoxyglucose or deoxyglucose plus 1 μmol/L nNOS inhibitor AAA-NG or 100 μmol/L nNOS inhibitor NPLA or 5 mmol/L non-selective NOS inhibitor LNMMA or 25 μmol/L iNOS inhibitor 1400w or 10 μmol/L NMDA receptor antagonist MK801, and the cultures were subjected to hypoxic conditions (2% oxygen) for 16 h. Then cultures were stained with PI and Hoechst, to quantify necrotic (upper panel) and apoptotic (lower panel) neurons. Data are expressed as percentage of the total number of cells per microscope field. ***Significant difference compared to the control of the same treatment, $p < 0.001$; #, ## and ###significant difference compared to deoxyglucose-treated samples, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

neuronal death (Fig. 1). None of the NOS inhibitors had any effect on neuronal viability in normoxia, as well as in hypoxia without deoxyglucose (data not shown). These results indicate that nNOS (but not iNOS) contributes to the neuronal death induced by hypoxia/deoxyglucose in mixed neuronal/glial cultures.

In order to test whether the protective effect of nNOS inhibitors was on neurons directly, rather than glia, we tested

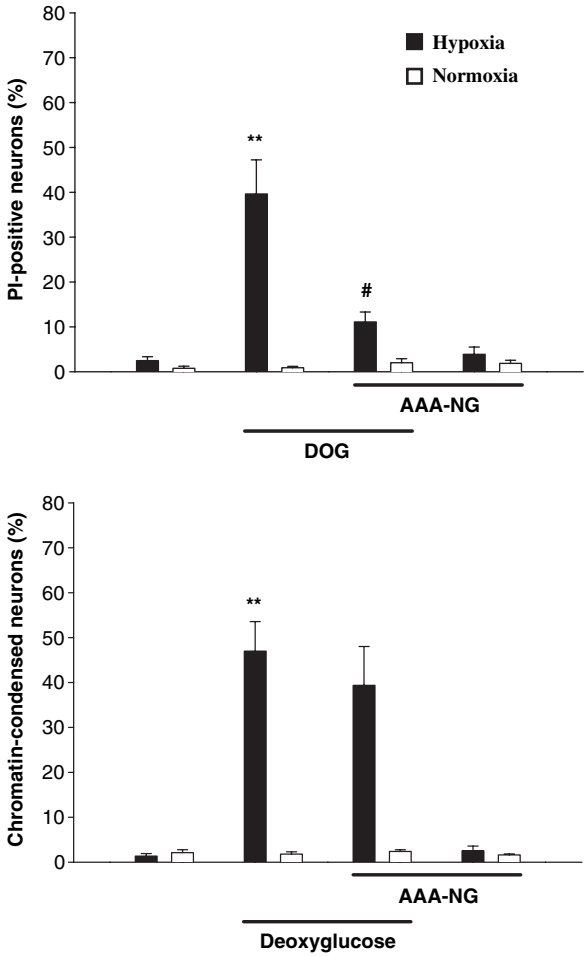

Fig. 2 The effect of nNOS inhibitor AAA-NG on hypoxia and deoxyglucose-induced neuronal toxicity in pure neuronal culture. Pure neuronal cultures were treated with either 10 mmol/L deoxyglucose or deoxyglucose with 1 μmol/L nNOS inhibitor AAA-NG and the cultures were subjected to hypoxic conditions (2% oxygen) for 16 h. Then cultures were stained with PI and Hoechst to quantify necrotic (upper chart) and chromatin-condensed (lower chart) neurons. Data are expressed as percentage of the total number of cells per microscopic field. **Significant difference compared to the hypoxic control, $p < 0.01$; #Significant difference compared to hypoxic deoxyglucose-treated samples, $p < 0.05$.

whether the most specific and potent nNOS inhibitor (AAA-NG) protected a pure culture of cerebellar granule neurons (without glia). As in the case of mixed neuronal-glial cultures, there was no change in neuronal viability after 16 h of hypoxia or deoxyglucose alone (about 2% dead, Fig. 2). However with hypoxia plus deoxyglucose the number of necrotic neurons increased to 40 ± 8% and chromatin condensed neurons to 47 ± 7%. AAA-NG rescued most of the neurons from hypoxia/deoxyglucose-induced necrosis, leaving only 11 ± 2% of them PI positive, however it had no significant effect in reducing the number of chromatin-condensed neurons.

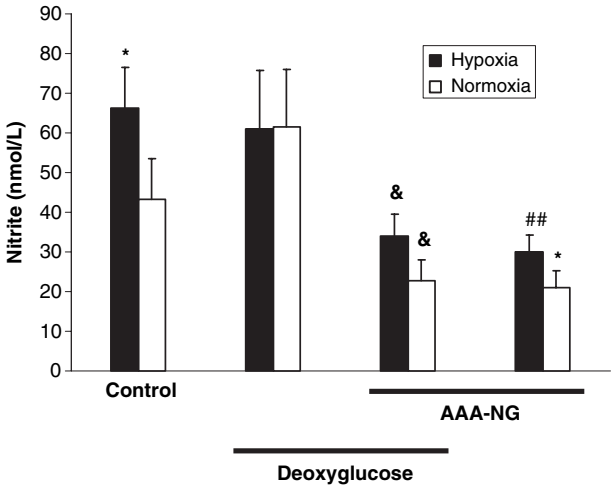

Fig. 3 The effect of nNOS inhibitor AAA-NG on nitrite level in neuronal-glial cultures. Mixed neuronal glial cultures were treated with 1 μmol/L AAA-NG with or without 10 mmol/L deoxyglucose for 16 h in hypoxia (2% oxygen) or in normoxia. The medium was collected from cells before and after the treatment, and nitrite measured. Data are presented as a difference in nitrite level before and after the treatment ('after treatment level' minus 'before treatment level'). *Significant difference compared to normoxic control, $p < 0.05$; ##Significant difference compared to hypoxic control, $p < 0.01$; &Significant difference compared to the samples treated with deoxyglucose only, $p < 0.05$.

To test the effect of the nNOS inhibitor AAA-NG on NO production, we measured nitrite levels in the mixed neuronal-glial culture medium before and after the above treatments (NO released from the cells is rapidly converted to nitrite). Figure 3 shows the increase in nitrite levels during the 16-h treatments, i.e. the NO production. In hypoxic cultures nitrite was produced more rapidly than in normoxia, although not in the additional presence of deoxyglucose (Fig. 3). AAA-NG significantly inhibited (but did not completely eliminate) nitrite production in all conditions. However, it should be noted that nitrite can be metabolised to nitrate and other products, so that rates of nitrite production may not be exactly the same as rates of NO production.

nNOS and guanylate cyclase participate in hypoxia plus deoxyglucose-induced glutamate release

Hypoxia/ischaemia cause neuronal death mediated by NMDA-type receptors (Pulsinelli and Duffy 1983; Yager *et al.* 1992). To test whether this is the case in our model, we tested the effect of MK801 (10 μmol/L), an NMDA receptor blocker, on hypoxia/deoxyglucose-induced neuronal toxicity. We found that MK801 almost completely prevented both necrosis and chromatin condensation of neurons in cultures exposed to hypoxia and deoxyglucose (Fig. 1). Thus the death was mediated by glutamate excitotoxicity via NMDA receptors.

Neuronal nitric oxide synthase might contribute to hypoxia/deoxyglucose-induced neuronal death either upstream

(via causing glutamate release or membrane depolarisation) or downstream of the NMDA receptor (via NMDA receptor activation of nNOS). To test whether nNOS activity could change the levels of released glutamate, we assessed glutamate in the medium collected from mixed neuronal-glial and pure neuronal cultures treated with hypoxia plus deoxyglucose with and without AAA-NG. In mixed cultures (Fig. 4, upper chart), hypoxia alone caused an increase in extracellular glutamate from 5 ± 1 to 13 ± 3 μmol/L, and hypoxia plus deoxyglucose caused a further increase to 27 ± 5 μmol/L. In pure neuronal cultures (Fig. 4, lower chart) there was no significant difference in medium glutamate levels between normoxic and hypoxic samples; however deoxyglucose plus hypoxia induced a large, significant glutamate release to the medium (to 31 ± 2 μmol/L). In mixed cultures, the nNOS inhibitor AAA-NG did not prevent hypoxia- and hypoxia plus deoxyglucose-induced glutamate release. However, in pure neuronal cultures AAA-NG did partly inhibit the hypoxia/deoxyglucose-induced glutamate release (to 17 ± 2 μmol/L), suggesting that nNOS has some role in hypoxia-induced glutamate release.

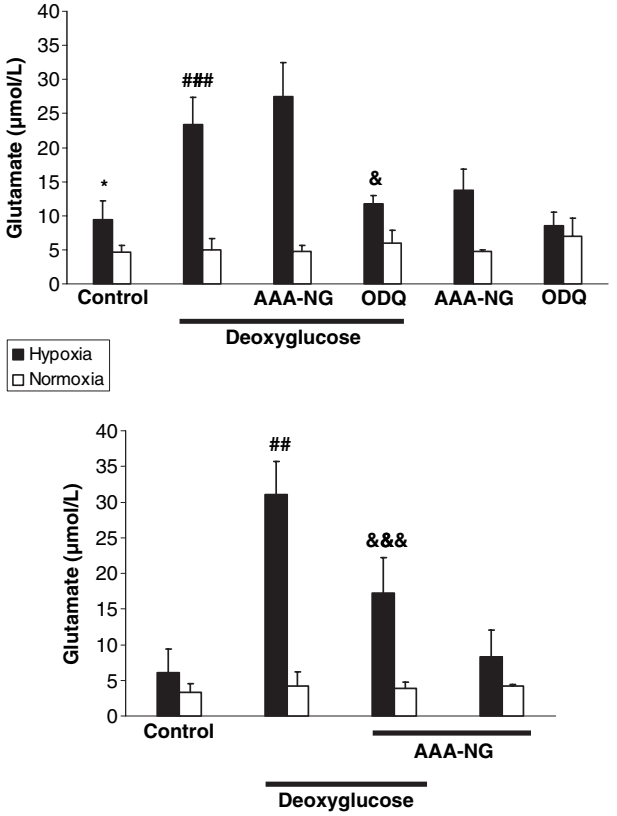

Fig. 4 The effect of nNOS inhibitor on glutamate level in mixed neuronal-glial and pure neuronal culture medium after hypoxia and deoxyglucose treatment. Mixed neuronal glial (upper chart) or pure neuronal (lower chart) cultures were treated with 1 μmol/L AAA-NG or 10 μmol/L ODQ (only mixed cultures) with or without 10 mmol/L deoxyglucose for 16 h in hypoxia (2% oxygen) or in normoxia. Extracellular glutamate was measured at the end of these treatments. *Significant difference compared to the normoxic control, $p < 0.05$; ## and ###Significant difference compared to the hypoxic control, $p < 0.01$ and $p < 0.001$, respectively; &&&Significant difference compared to hypoxia and deoxyglucose-treated sample, $p < 0.001$.

One of the well known targets of NO is soluble guanylate cyclase (Moncada *et al.* 1991). We tested whether this enzyme is involved in hypoxia and deoxyglucose-induced glutamate release by treating cells with a specific guanylate cyclase inhibitor ODQ (10 μmol/L) together with hypoxia and deoxyglucose and measuring glutamate in the medium. ODQ did reduce hypoxia plus deoxyglucose-induced glutamate accumulation in the medium, and had no effect on glutamate level on its own, either in hypoxia or in normoxia (Fig. 4 upper chart). However, ODQ treatment did not rescue neurons from hypoxia and deoxyglucose-induced death: the percentages of PI-positive neurons after 16 h of hypoxia were 26.5 ± 4.7 in deoxyglucose-treated and 24.2 ± 6.4 in deoxyglucose plus ODQ-treated cultures, and the percentages of chromatin-condensed neurons in these treatments were 48.0 ± 2.6 and 43.1 ± 3.5, respectively (five independent experiments). There was no effect of ODQ alone (in the absence of deoxyglucose) on neuronal viability in hypoxia or normoxia.

To test whether nNOS can mediate neuronal damage downstream of glutamate release, we incubated neuronal-glial cultures with high glutamate (200 μmol/L) with or without the nNOS inhibitor AAA-NG for 16 h. After glutamate treatment, 25 ± 5% of neurons in the cultures were PI-positive (Fig. 5, upper chart) and 16 ± 4% chromatin condensed (Fig. 5, lower chart). AAA-NG (1 μmol/L) was not able to prevent either type of glutamate-induced neuronal damage indicating that nNOS does not mediate glutamate toxicity in these cells and conditions. Thus nNOS must be acting upstream of the NMDA receptor, presumably either to promote glutamate release or membrane depolarisation, and these events may be downstream of cytochrome oxidase inhibition.

nNOS activity contributes to hypoxia-induced death via inhibition of mitochondrial cytochrome oxidase

Neuronal nitric oxide synthase might contribute to hypoxia/deoxyglucose-induced death either by NO/hypoxia synergy in inhibiting cytochrome oxidase, or alternatively by mediating death downstream of cytochrome oxidase inhibition. In order to test the latter possibility we examined whether the nNOS inhibitor AAA-NG would protect against death induced by an uncompetitive inhibitor of cytochrome oxidase, azide, which does not act in synergy with NO or oxygen (Petersen 1977). We incubated a neuronal-glial culture with 30 μmol/L NaN_3 plus deoxyglucose ± AAA-NG for 16 h. NaN_3 alone caused 13 ± 2% of neurons to become necrotic (Fig. 6, upper chart) with no significant change in the number of apoptotic cells (Fig. 6, lower chart). Deoxyglucose in the presence of NaN_3 increased the level of necrosis to 34 ± 5% and induced chromatin condensation in

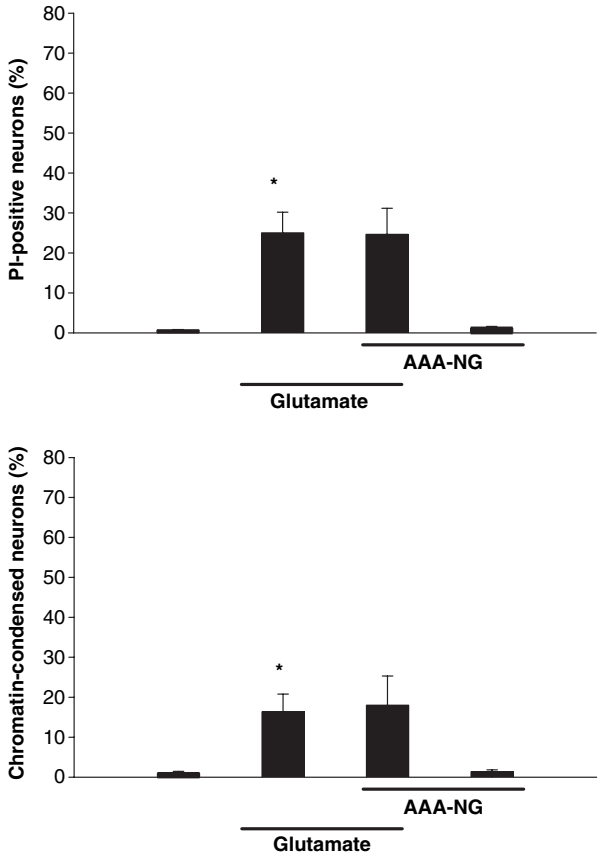

Fig. 5 The effect of nNOS inhibitor on glutamate-induced neuronal death. Mixed neuronal glial cultures were treated with 200 μmol/L glutamate with or without 10 mmol/L deoxyglucose or 1 μmol/L AAA-NG for 16 h in normoxia. Then cultures were stained with PI and Hoechst to quantify the percentage of neurons that were necrotic (upper panel) and chromatin condensed (lower panel). Data are expressed as percentage of the total number of cells per microscopic field. *Significant difference compared to control, $p < 0.05$.

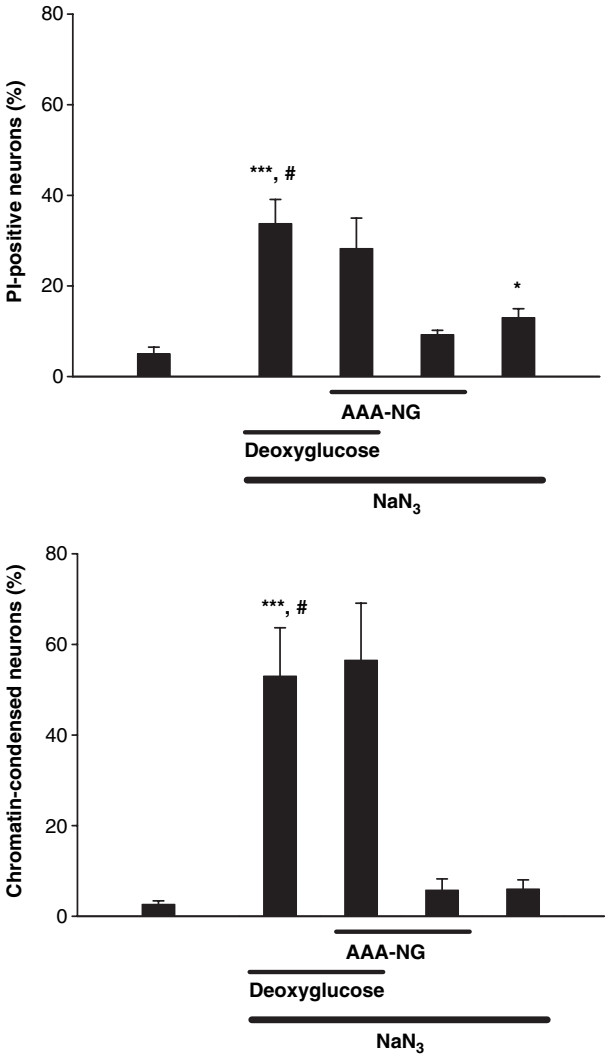

Fig. 6 nNOS inhibitor AAA-NG has no effect on NaN_3 plus deoxyglucose-induced neuronal death. Mixed neuronal glial cultures of 14–16 DIV were treated with 30 μmol/L NaN_3 and 10 mmol/L deoxyglucose plus or minus 1 μmol/L AAA-NG for 16 h in normoxia. Then cultures were stained with PI and Hoechst to quantify the percentage of neurons that were necrotic (upper panel) and chromatin condensed (lower panel). Data are presented as percentage of the total number of cells per microscopic field. *** and *Significant difference compared to the control, $p < 0.001$ and $p < 0.5$, respectively; ##Significant difference compared to samples treated with NaN_3 only.

53 ± 11% of neurons. AAA-NG had no protective effect on azide/deoxyglucose-induced death (Fig. 6), indicating that nNOS does not mediate death downstream of cytochrome oxidase inhibition, and is thus consistent with nNOS contributing to cell death at the level of cytochrome oxidase inhibition itself.

We directly tested whether nNOS and hypoxia were synergistically inhibiting cytochrome oxidase activity within the cells in neuronal-glial culture by measuring the oxidation rate of a cytochrome oxidase substrate TMPD. Hypoxia (2% O_2) markedly inhibited this measured cytochrome oxidase activity of the cells (relative to normoxia), and AAA-NG reversed this inhibition in the presence of hypoxia, but had no effect in normoxia (Fig. 7). Uncompetitive cytochrome oxidase inhibitor azide inhibited the oxidase activity both in hypoxia and normoxia (Fig. 7). Also cyanide (1 mmol/L) and the NO donor (1 mmol/L DETA-NONOate) inhibited oxidase activity in the cells, as expected (TMPD oxidation in untreated, cyanide-treated and DETA-NONOate-treated cultures in five separate experiments were 0.030 ± 0.008, 0.008 ± 0.004 and 0.007 ± 0.004 OD units, respectively). The above results indicate that the hypoxic conditions used inhibited cytochrome oxidase activity within the cells when nNOS was active but not when nNOS was inhibited by AAA-NG. Thus nNOS activity sensitises the cells to hypoxic-inhibition of cytochrome oxidase.

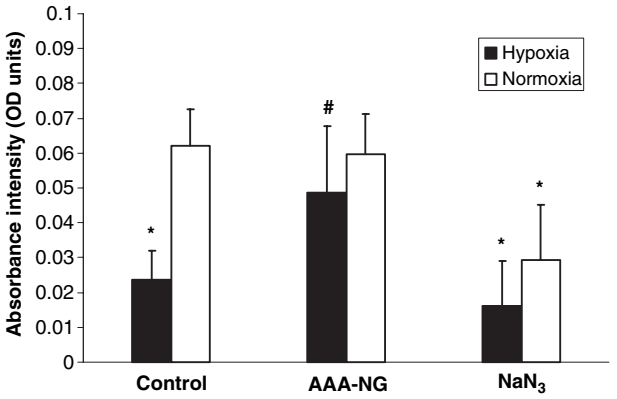

Fig. 7 The effect of nNOS inhibitor on cytochrome oxidase activity in hypoxia. Mixed neuronal glial culture medium was supplemented with freshly dissolved cytochrome oxidase substrate 100 μmol/L TMPD, and 1 μmol/L AAA-NG with or without 10 mmol/L deoxyglucose, or with 1 mmol/L NaN_3, and the cultures were incubated for 30 min in hypoxia (2% oxygen) or in normoxia. The level of Wurster's blue resulting from TMPD oxidation by cytochrome oxidase was assessed in a spectrophotometer. Because TMPD is also oxidised by the culture media, the data are plotted as the difference between absorbance values obtained from medium incubated with cells and medium incubated without cells under the same conditions. *Significant difference compared to the normoxic control, $p < 0.05$; #Significant difference compared to the hypoxic control, $p < 0.05$.

nNOS activity in hypoxia induces neuronal depolarisation

Inhibition of cytochrome oxidase may result in cell de-energisation and subsequent plasma membrane depolarisation. To test this, we incubated mixed neuronal-glial cultures with a bis-oxonol anionic, fluorescent dye that is excluded from hyperpolarised cells, but enters cells according to their depolarisation level and gives a fluorescence signal after binding to cytoplasmic proteins. One-hour treatment with hypoxia and 10 mmol/L deoxyglucose induced a significant increase in the membrane potential dye fluorescence in the cultures indicating that the cells were depolarised compared to the normoxic deoxyglucose-treated cells (Fig. 8). The cells showing depolarisation in hypoxia were mostly neurons, as revealed by viewing the same field in phase contrast (Fig. 8 photos). AAA-NG (1 μmol/L) applied together with hypoxia and deoxyglucose treatment reduced the fluorescence signal to the normoxic level, indicating the involvement of nNOS in hypoxia-induced neuronal depolarisation. To test whether NO could cause neuronal depolarisation the cultures were treated with 1 mmol/L of the NO donor DETA-NONOate in the presence of deoxyglucose in normoxia, and we found this caused a large decrease of plasma membrane potential (fluorescence increased from 8.6 ± 0.7 to 20.0 ± 4.4).

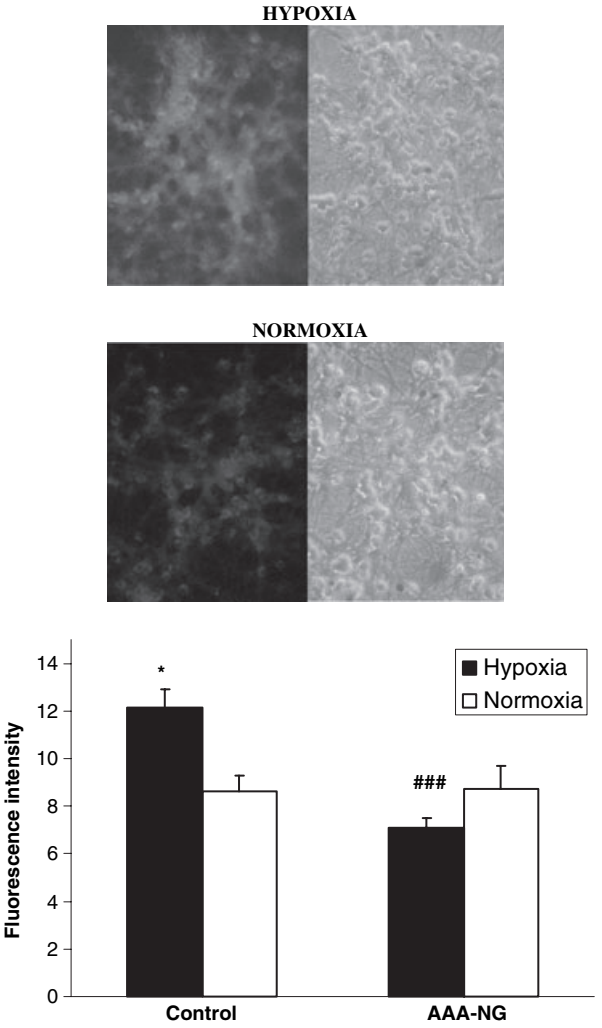

Fig. 8 The effect of nNOS inhibitor on plasma membrane potential. Mixed neuronal-glial cultures were incubated with a bis-oxonol dye together with 10 mmol/L deoxyglucose with or without 1 μmol/L AAA-NG for 1 h either in hypoxia or in normoxia, and cells were viewed by a fluorescence microscope and photographed. Representative fluorescence images (left) of deoxyglucose and hypoxia-treated (top) or normoxia-treated (bottom) samples are presented together with phase contrast images of the same field (right). The fluorescence intensity of images corresponding to the cell depolarisation level was calculated using ImageJ software and the data are presented in the graph below the photos (five independent experiments). *Significant difference compared to the normoxic control, $p < 0.05$; ###Significant difference compared to the hypoxic control, $p < 0.001$.

Discussion

N-4*S*-4-amino-5-2-aminoethyl-aminopentyl-*N*′-nitroguanidine (1 μmol/L), a potent, specific nNOS inhibitor, partially protected neurons against death induced by hypoxia/deoxyglucose in both a neuronal/glial co-culture and pure neuronal culture. Two other nNOS inhibitors had the same protective

effect, indicating that nNOS was indeed the effective target of these inhibitors. The nNOS inhibitor prevented hypoxic inhibition of cytochrome oxidase activity in the cells, indicating that NO from nNOS inhibited cytochrome oxidase in hypoxic conditions, or equivalently NO from nNOS raised the apparent K_M of cytochrome oxidase for oxygen. Another cytochrome oxidase inhibitor, azide, induced a similar level of neuronal death, over the same time course and conditions (plus deoxyglucose), indicating that cytochrome oxidase inhibition was sufficient to induce this death (if glycolysis was also inhibited). But the nNOS inhibitor did not protect against azide-induced neuronal death, indicating that NO from nNOS was not acting downstream of cytochrome oxidase, consistent with NO from nNOS acting at cytochrome oxidase itself.

An NMDA receptor antagonist, MK801, almost completely blocked hypoxia/deoxyglucose-induced neuronal death, indicating that the induced death is mediated by the NMDA-type glutamate receptor. However, the nNOS inhibitor had no effect on glutamate-induced neuronal death, indicating that NO/nNOS was acting upstream, rather than downstream of the NMDA receptor. Consistent with this we found that hypoxia/deoxyglucose induced a marked elevation in extracellular glutamate and the nNOS inhibitor lowered this elevation of glutamate in neuronal cultures. However, the decrease in glutamate was only partial in these cultures, and there was no effect of the nNOS inhibitor on the glutamate elevation in neuronal-glial cultures. This suggests that NO from nNOS may have an additional upstream effect on the NMDA receptor (other than elevating glutamate) such as depolarisation of the plasma membrane potential, resulting from cytochrome oxidase inhibition-induced energy depletion in the presence of deoxyglucose. We measured such a hypoxia plus deoxyglucose-induced neuronal depolarisation, which was nNOS dependent, and was replicated by adding an NO donor. An overall mechanism consistent with these results is presented in Fig. 9.

ODQ, a selective inhibitor of soluble guanylate cyclase, lowered the hypoxia-plus-deoxyglucose-induced glutamate release but had no effect on the neuronal death. This suggests that the NO-activated cyclase may amplify the glutamate release, but this action is not significantly involved in the subsequent death. Again this is consistent with cytochrome oxidase being the main target for the NO, followed by neuronal depolarisation as the main mechanism for neuronal death in response to hypoxia.

We have previously shown that NO inhibits neuronal respiration in competition with oxygen (Brown and Cooper 1994; Bal-Price and Brown 2001; Mander *et al.* 2005), and that NO from NO donors or glial iNOS is strongly synergistic with hypoxia in inducing neuronal death via cytochrome oxidase inhibition in these cerebellar granule neurons (Mander *et al.* 2005). However, it was previously unclear whether nNOS would generate sufficient NO to significantly

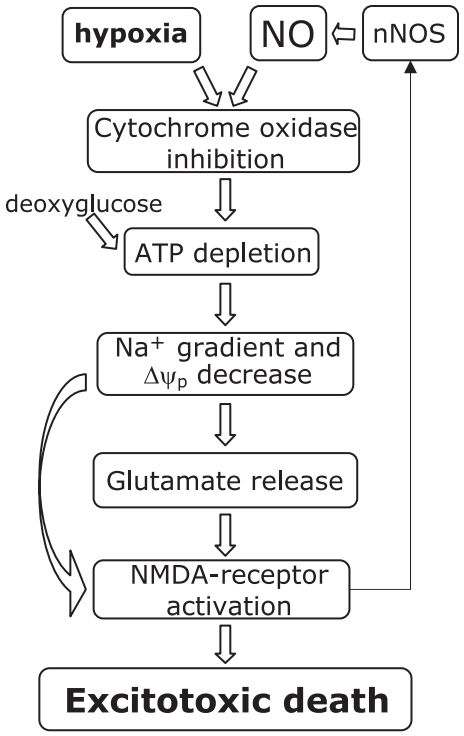

Fig. 9 A suggested model for the synergistic effect of nNOS and hypoxia on neurons.

inhibit mitochondrial respiration at low oxygen levels (Keynes *et al.* 2004; Keynes and Garthwaite 2004), and this is crucial to whether and how nNOS contributes to hypoxic/ischaemic damage in the brain. Almeida and Bolanos (2001) have provided evidence that glutamate stimulation of NMDA receptors results in a nNOS/NO-mediated reversible inhibition of mitochondrial ATP production in neurons, consistent with NO inhibition of cytochrome oxidase. Positron Emission Tomography studies in the intact brain are consistent with tonic NO inhibition of respiration in relatively hypoxic areas of the brain (Gjedde *et al.* 2005). nNOS has been found to bind directly to cytochrome oxidase in cerebellar granule neurons (Persichini *et al.* 2005), suggesting the possibility that nNOS provides NO directly to cytochrome oxidase. Gbadegesin *et al.* (1999) found that NO from NO donors enhanced glutamate-evoked NMDA receptor currents particularly under hypoxic conditions. They did not investigate the mechanism of this effect, but it is consistent with NO/hypoxic inhibition of cytochrome oxidase resulting in plasma membrane depolarisation that enhances NMDA receptor currents. Note, however, that NO can inhibit cytochrome oxidase by a variety of mechanism, some of which are not competitive with oxygen (Mason *et al.* 2006).

The presence of deoxyglucose, a well known inhibitor of glycolysis, was essential to see any neuronal death in hypoxic cultures, but had absolutely no effect on normoxic

cultures (Figs 1 and 2). This indicates that the insult generated by hypoxia is due to energy depletion, and that glycolysis can prevent this by supplying an alternative source of ATP. Hence *in vivo* ischaemia is much more damaging than hypoxia.

We have previously described the mild hypoxia/deoxyglucose culture model used here which induces neuronal death relatively slowly – little or no necrosis at 4 h, 27% necrosis at 12 h, 40% necrosis at 16 h (Mander *et al.* 2005). We used a mild level of hypoxia (2%), rather than anoxia, because we wanted to test whether NO from nNOS would modulate the sensitivity of cytochrome oxidase to oxygen, and this would be impossible in anoxia. We used 14–16 DIV culture, rather than a more usual 7–8 DIV, because nNOS expression is low at 7–8 DIV and high at 14–16 DIV (Samdani *et al.* 1997; Jurado *et al.* 2004).

Hypoxic conditions can arise in the brain during ischaemia, stroke, trauma, atherosclerosis or vascular dementia (Bouma and Muizelaar 1992; Sweeney *et al.* 1995; Desmond 1996). The brain is exceptionally sensitive to reduced oxygen and/or glucose supply, resulting in neuronal death (Juurlink and Sweeney 1997). Necrotic neurons are often at the core of the ischaemic infarction, with apoptotic neurons in the penumbra (Choi 1996). Our *in vitro* data presented here shows that neuronal and neuronal-glial cultures are able to survive mild hypoxia (2% oxygen) for 16 h, with little neuronal death. Two per cent oxygen is equivalent to about 20 μmol/L O_2, which is well above the K_M of respiration for oxygen in the absence of NO (about 0.5 μmol/L). However, in the presence of just 60 nmol/L NO the apparent K_M of neuronal respiration for oxygen increases to 30 μmol/L O_2 (Brown and Cooper 1994), so that cellular respiration would be inhibited at 20 μmol/L (2%) O_2 but not at 200 μmol/L (21%) O_2. Electrode measurements of NO in brain slices have shown that neuronal excitation increases the steady-state NO level by 8–80 nmol/L (Shibuki 1990; Shibuki and Okada 1991). Electrode measurements of oxygen levels in mammalian cortex *in vivo* range from 0 to 150 μmol/L O_2 but with a median of about 40 μmol/L O_2 (Feng *et al.* 1988). So our mild hypoxic conditions are only half the median *in vivo* level, and our 'normoxic' conditions (21%, 200 μmol/L O_2) are actually hyperoxic compared to *in vivo* conditions. Note, however, that oxygen gradients created by oxygen consumption of the cells will lower the local level of oxygen in the cells and mitochondria.

Our findings presented here show that NO from nNOS sensitises neurons to hypoxia/ischaemia-induced death via cytochrome oxidase inhibition. Reduced cerebral blood flow can lead to low oxygen (and glucose) levels, which in the presence of NO may cause inhibition of mitochondrial respiration and subsequent excitotoxic neuronal death. These findings may have relevance to how hypoxia/ischaemia damages the brain in pathologies such as stroke, vascular dementia and trauma.

Acknowledgement

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