Neuroprotective and neurotoxic properties of the ‘inert’ gas, xenon

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Background. Antagonists of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors have been shown not only to have neuroprotective effects but also to exhibit neurotoxic properties. In this study, we used c-Fos, a protein product of an immediate early gene, as a marker of neuronal injury to compare the neuroprotective effects of xenon and the neurotoxic properties of xenon, nitrous oxide, and ketamine, three anaesthetics with NMDA receptor antagonist properties.

Methods. We used an in vivo rat model of brain injury in which N-methyl-DL-aspartic acid (NMA) is injected subcutaneously (s.c.) and c-Fos expression in the arcuate nucleus is used as a measure of injury. To examine the neurotoxic potential of each of the three anaesthetics with NMDA receptor antagonist properties, c-Fos expression in the posterior cingulate and retrosplenial (PC/RS) cortices was measured.

Results. Xenon dose-dependently suppressed NMA-induced c-Fos expression in the arcuate nucleus with an IC50 of 47 (2)% atm. At the highest concentration tested (75% atm) NMA-induced neuronal injury was decreased by as much as that observed with the prototypical NMDA antagonist MK801 (0.5 mg kg ±1 s.c.). Both nitrous oxide and ketamine dose-dependently increased c-Fos expression in PC/RS cortices; in contrast, xenon produced no significant effect. If the dopamine receptor antagonist haloperidol was given before either nitrous oxide or ketamine, their neurotoxic effects were eliminated.

Conclusions. Uniquely amongst anaesthetics with known NMDA receptor antagonist action, xenon exhibits neuroprotective properties without co-existing neurotoxicity. The reason why ketamine and nitrous oxide, but not xenon, produce neurotoxicity may involve their actions on dopaminergic pathways.

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Although glutamate is probably the most important excitatory neurotransmitter in the mammalian brain and is essential for normal brain function, the presence of excessive amounts of glutamate can lead to cell death. The term excitotoxicity, first coined by Olney,1 denotes the process whereby activation of glutamate receptors, especially those of the N-methyl-D-aspartate (NMDA) subtype, leads to excess calcium entry into cells which in turn triggers a

1 Declaration of interest. Professor Maze and Professor Franks are Board members of an Imperial College spin-out company (Potexeon Ltd) that is interested in developing clinical applications for medical gases, including xenon. Both Professor Franks and Professor Maze are paid consultants in this activity. In addition, Air Products have funded, and continue to fund, work in the authors’ laboratories that bears on the actions of xenon as an anaesthetic and neuroprotectant and Air Products has a financial stake in Potexeon Ltd. However, none of the work described in this manuscript was funded by either company.
biochemical cascade resulting in neuronal death. Neurotoxicity induced by over-activation of NMDA receptors is thought to sustain ongoing neuronal injury and death in acute processes such as stroke and head trauma, as well as in chronic neurodegenerative disorders. Consistent with this, a large body of evidence has shown that NMDA receptor antagonists can be neuroprotective in both in vitro and in vivo models of neuronal injury and can prevent deterioration of cognitive function after cardiopulmonary bypass.2

Xenon, a noble gas with anaesthetic properties, has recently been found to be an NMDA receptor antagonist,8 another gaseous anaesthetic, nitrous oxide can also inhibit the NMDA receptor.10 Consequently, these two gases might be considered good candidates as neuroprotectants because they can be easily administered and rapidly enter the brain. However, a major deterrent to the use of NMDA antagonists as neuroprotective agents is the profound psychotomimetic behavioural changes which such drugs can produce.11 Histological data in studies conducted with the NMDA receptor antagonists ketamine, phencyclidine (PCP), and dizocilpine maleate (MK801) revealed pyramidal neuronal damage in the region of the posterior cingulate and retrosplenial (PC/RS) cortices,12,13 which may underlie these behavioural changes; similar pathological changes have been reported with nitrous oxide.14 Whether or not NMDA antagonism alone is sufficient to produce this neurotoxicity, however, is not known and it is possible that perturbation of other neurotransmitter systems is involved. For example, NMDA receptor antagonists including ketamine and nitrous oxide, can activate dopamine receptors or increase dopamine release both in vivo and in vitro.15–17 Moreover, major antipsychotics with dopamine D2 receptor antagonist properties can prevent ketamine’s psychotomimetic side-effects.18 In addition, a recent in vitro study suggests that xenon can decrease dopamine release.19

The aims of the present study were to determine: (i) whether xenon has a neuroprotective effect in an in vivo model of neuronal excitotoxicity; (ii) whether xenon exhibits typical NMDA receptor antagonist neurotoxicity in the PC/RS cortices; and (iii) whether dopamine contributes to the neurotoxicity exhibited by anaesthetics with NMDA receptor antagonist properties. We used an immediate early gene (c-fos)-encoded protein (c-Fos), in order to assess neuronal injury as reported previously.20–24

Methods
This study conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986.

Effect of xenon on NMA-induced c-Fos expression in the arcuate nucleus
Nine groups (n=3–4 in each group) of female Sprague–Dawley rats (240–260 g) were treated randomly as follows: group 1 received NMA, 100 mg kg⁻¹ body wt subcutaneously (s.c.), and was exposed to 25% oxygen and 75% nitrogen; groups 2–5 were exposed to either 20, 40, 60, or 75% xenon plus 25% oxygen (with the remainder being nitrogen, where necessary) for 15 min before injection of NMA, 100 mg kg⁻¹ s.c.; group 6 was exposed to 25% oxygen and 75% nitrogen, and received saline, 8 ml kg⁻¹ s.c.; group 7 received 75% xenon and 25% oxygen for 15 min before injection of saline 8 ml kg⁻¹ s.c.; group 8 was exposed to 25% oxygen and 75% nitrogen; and received MK801, 0.5 mg kg⁻¹ s.c.; group 9 was exposed to 25% oxygen and 75% nitrogen; and received MK801 0.5 mg kg⁻¹ s.c., followed by NMA, 100 mg kg⁻¹ s.c. 15 min later. Doses of NMA and MK801 were selected from a previous study.14 Female animals were used because this gender has been shown to be more sensitive to excitoxins, particularly for NMDA antagonists that are rapidly metabolized,25 for agents that are less susceptible to metabolism, such as nitrous oxide, neurotoxicity is the same for both sexes.25 Rats were randomly assigned between groups without reference to the day in their oestrous cycle.

Neurotoxicity of xenon, nitrous oxide, ketamine, and MK801
Eleven groups (n=3 in each group) of animals were treated as follows: group 1 was exposed to 25% oxygen and 75% nitrogen; groups 2–4 were exposed to 40, 60, or 75% xenon, plus 25% oxygen (with the remainder being nitrogen, where necessary) for 90 min; groups 5–7 were exposed to 40, 50, or 60% nitrous oxide, plus 25% oxygen (with the remainder being nitrogen) for 90 min; groups 8–10 received ketamine, 25, 50, or 100 mg kg⁻¹ s.c., respectively, and were exposed to 25% oxygen and 75% nitrogen; group 11 received MK801, 0.5 mg kg⁻¹ s.c. and was exposed to 25% oxygen and 75% nitrogen. Doses of ketamine were selected on the basis of a previous study,12 and were below those necessary to induce a loss of righting reflex.

Effect of dopamine D2 receptor antagonist haloperidol on nitrous oxide- and ketamine-induced neurotoxicity
Five groups (n=3 in each group) of animals were treated randomly as follows: group 1 received saline, 2 ml kg⁻¹ s.c. and were exposed to 75% nitrogen and 25% oxygen; group 2 received saline before exposure to 75% nitrous oxide and 25% oxygen for 90 min; group 3 received haloperidol 2.5 mg kg⁻¹ s.c., 30 min before exposure to 75% nitrous oxide and 25% oxygen for 90 min; group 4 received saline, 2 ml kg⁻¹ s.c., before ketamine 50 mg kg⁻¹ s.c.; group 5 received haloperidol 2.5 mg kg⁻¹ s.c., 30 min before ketamine 50 mg kg⁻¹ s.c.
Gas exposure

During exposure to 20, 40, 60, and 75% xenon or 40, 60, and 75% nitrous oxide as mentioned above, the gas mixture was introduced into a chamber (International Market Supply, Cheshire, UK) using calibrated flow meters. After a flush at a flow rate of 4 litres min⁻¹ for 3 min, the flow rate was reduced to 40 ml min⁻¹ for maintenance. The humidity in the chamber was maintained between 40 and 60% using silica gel (Merck, Leicestershire, UK) and the carbon dioxide level was maintained below 0.6% with soda lime.

Perfusion, brain harvesting, and tissue processing

For excitotoxicity experiments, rats were killed 3 h after NMA administration. For neurotoxicity experiments, rats were killed 90 min after exposure. Animals were deeply anaesthetized with sodium pentobarbital 100 mg kg⁻¹ i.p., perfused transcardially with 100 ml heparinized saline followed by 4% paraformaldehyde 500 ml in 0.1 M phosphate buffer. The whole brain was removed and further fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) overnight. The appropriate area of brain was sliced and embedded in paraffin.

Fig 1 Representative coronal sections showing half of the arcuate nucleus of the hypothalamus stained for c-Fos positive immunoreactivity. (A) Three hours after subcutaneous injection of NMA (100 mg kg⁻¹) the c-Fos positive neurons are easily visualized by dense black staining. (B) For animals injected with NMA but also exposed to 75% xenon there was a marked reduction in the number of c-Fos positive neurons. The scale bar represents 100 µm and the third ventricle is marked V3.

Immunohistochemistry

Sections were incubated for 30 min in 0.3% H₂O₂ in methanol in order to quench endogenous peroxidase and thereafter washed three times in PBS. Following this, the sections were incubated for 1 h in a ‘blocking solution’ consisting of 3% donkey serum and 0.3% Triton X in PBS (PBT) and subsequently incubated overnight at 4°C in 1:6000 goat anti-c-Fos antibody (sc-52-G, Santa Cruz Biotechnology, Santa Cruz, CA) in PBT with 1% donkey serum. The sections were then rinsed three times with PBT and incubated with 1:200 donkey anti-goat IgG (Vector Laboratories, Burlingame, CA) in PBT with 1% donkey serum for 1 h. The sections were washed again with PBT and incubated with avidin–biotin–peroxidase complex (Vector Laboratories) in PBT for 1 h. The sections were rinsed three times with PBS and stained with 3,3′-diaminobenzidine (DAB) with nickel ammonium sulphate to which hydrogen peroxide was added (DAB kit, Vector Laboratories) to achieve immunohistochemical visualization. When the staining was complete, the sections were rinsed in PBS followed by distilled water and mounted on glass slides, dehydrated with 100% ethanol, cleared with 100% xylene, and covered with cover slips. Unless otherwise mentioned, all reactions were performed at room temperature.

Quantitative counting of c-Fos neurons

The sections were photographed using a digital camera (model C2020Z, Olympus Optical, Southhall Middlesex, UK) attached to a microscope (Olympus model BX50). Under identical conditions, photographs were taken from each of the three sections per animal and counted for c-Fos positive neurons (dense black nuclear staining; see Fig. 1) by an author who was blinded to the treatment. The sum of c-Fos positive neurons for the three representative sections was the aggregate score of each animal for either the arcuate nucleus or PC/RS cortices and results are reported as mean.
The statistical analysis was performed by one-way analysis of variance, followed by Newman–Keuls test. A $P$ value $<0.05$ was regarded as statistically significant.

Results

**NMA-induced excitotoxicity in the arcuate nucleus of the hypothalamus, the effects of xenon and MK801**

Subcutaneously injected NMA (100 mg kg$^{-1}$) produced a large number of c-Fos positive neurones in the arcuate nucleus of the hypothalamus (1188 (197), mean (SEM)) that was greatly in excess of the number (37 (8)) observed in control animals injected with saline. Figure 1A is a representative section showing c-Fos positive neurones in the arcuate nucleus following NMA treatment. Xenon exposure produced a marked and concentration-dependent reduction in the number of c-Fos positive neurones (Figs 1B and 2) with an IC$_{50}$ of 47 (2)% atm. At the highest concentration tested (75% atm) the number of c-Fos positive neurones did not differ significantly from the number observed in saline-injected animals ($P>0.05$). In addition, the number of c-Fos positive neurones from animals exposed to xenon alone at the highest concentration investigated (75% atm) did not differ significantly ($P>0.05$) from animals exposed to nitrogen (Fig. 2, inset). As a positive control we tested the ability of the prototypical NMDA antagonist MK801 to block the induction of c-Fos positive neurones in the hypothalamus. MK801 0.5 mg kg$^{-1}$ (Fig. 2, inset) injected s.c. reduced the number of NMA-induced c-Fos positive neurones to a value (89 (23)) that was comparable with that found in the presence of 75% xenon (Fig. 2).

**Neurotoxicity in the posterior cingulate and retrosplenial cortices following administration of xenon, nitrous oxide, ketamine, and MK801**

Using c-Fos expression in layers III/IV of the posterior cingulate and retrosplenial cortices as a marker for neurotoxicity, we investigated the extent to which the NMDA antagonists xenon, nitrous oxide, ketamine, and MK801 exhibited neurotoxicity alone. Figure 3 illustrates representative sections showing the posterior cingulate and retrosplenial cortices following various treatments. In control animals (Figs 3A and 4) the number of c-Fos positive neurones was only 109 (29) and this did not change significantly ($P>0.05$) in the presence of xenon up to 75% atm (Figs 3B and 4). This was in marked contrast to the effects of nitrous oxide (Figs 3C and 4) and ketamine (Figs 3D and 4), both of which increased the number of c-Fos positive neurones in a dose-dependent manner. As a positive control, the effects of MK801 at the dose (0.5 mg kg$^{-1}$) that we had observed gave maximum neuroprotection to the neurotoxic effects of NMA were investigated (Fig. 2, inset). This dose of MK801 produced a large and highly significant increase in the number of c-Fos positive neurones in the posterior cingulate and retrosplenial cortices.

**Effect of haloperidol on nitrous oxide- and ketamine-induced neurotoxicity**

The extent to which the neurotoxicity produced by nitrous oxide and ketamine could be blocked by the dopamine D2 receptor antagonist haloperidol was investigated. Pretreatment with haloperidol greatly decreased the number of c-Fos positive neurones induced by either nitrous oxide or ketamine alone (Fig. 5), and for both agents haloperidol pretreatment reduced the induction of c-Fos positive neurones to a level comparable with that found in control animals (Fig. 5).

Discussion

**c-Fos as a rapid and sensitive indicator of neuroneal injury**

The $c$-$f$os gene, a member of a class of immediate early genes, is readily induced by a variety of physiological and pathological factors. For example, noxious stimulation including peripheral sensory stimulation, seizures, and that caused by pharmacological agents including glutamate receptor agonists and antagonists, can cause an increase of $c$-$f$os-encoded protein in the spinal cord or higher brain centres.$^{20-23}$ Thus, while c-Fos protein is a neuroneal
marker for neuroneal stress induced by non-lethal stimulation, under certain conditions, including exposure to excessive amounts of NMDA receptor agonists or antagonists, c-Fos protein can be used as a marker for neuroneal injury before cell death or apoptosis. Furthermore, c-fos gene induction and its protein expression precedes heat-shock protein expression (a known cell stress indicator which is not induced by normal physiological stimuli) or histopathological changes. Therefore, the protein encoded by the c-fos gene can be used as a rapid and sensitive marker of neuroneal injury when neurones are under lethal stress and, although there may not be a direct causal link, c-Fos expression has been used as an indicator to measure both neuroprotection by, as well as neurotoxicity of, NMDA antagonists.

Neuroprotection by xenon

One of the aims of the present study was to use c-Fos as a marker for neuroneal injury to examine whether or not xenon could act as a neuroprotectant. We used an in vivo paradigm that involves the induction of a selective type of neuroneal injury in the arcuate nucleus of the hypothalamus following s.c. injection of a glutamate receptor agonist, NMA. Excessive activation of the NMDA subtype of the glutamate receptor produces excitotoxicity, which is implicated in both acute (e.g. stroke, seizures, hypoxia, trauma) and chronic (e.g. Huntington’s disease) neuroneal injury. Moreover, some NMDA receptor antagonists have shown remarkable efficacy in experimental models of neuroneal injury although this promise has not been translated into clinical utility. One of the possible reasons for the failure to translate this neuroprotective potential to clinical use may be the difficulty that most NMDA receptor antagonists have in penetrating the blood–brain barrier and reaching the effect site. Xenon, however, is a small apolar atom that rapidly reaches equilibrium with the brain when present in the inspired gas. Our results show that, at concentrations that are easily attainable at normal pressures, xenon is an effective neuroprotectant (Figs 1 and 2). Indeed, at the
highest concentration we tested (75% atm), which is close to the concentration required for surgery, the neuroprotection achieved was comparable with that obtained using the potent NMDA receptor antagonist MK801 (Fig. 2, inset).

**Neurotoxicity of NMDA antagonists**

Although NMDA receptor antagonists have neuroprotective properties, they can also cause psychotomimetic effects in humans and abnormal locomotor activities in rodents. A neuroneal correlate (although not necessarily the cause) of the ability to produce these behavioural effects is the damage which NMDA antagonists produce in PC/RS cortices which can be assessed by c-Fos expression. The data in Figures 3 and 4 show that, unlike nitrous oxide, ketamine and MK801, xenon had no effect on c-Fos expression in PC/ RS cortices, and that concentrations of xenon that gave

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**Fig 4** Concentration or dose–response effects of xenon, nitrous oxide, and ketamine on the number of c-Fos positive neurons in the posterior cingulate and retrosplenial cortices. Results are means (SEM). *P<0.05; **P<0.01 relative to control. The data for MK801 (0.5 mg kg⁻¹), a prototypical NMDA receptor antagonist are presented as a positive control.

**Fig 5** Haloperidol, a dopamine D2 receptor antagonist, blocks nitrous oxide and ketamine-induced neurotoxicity. Data show the number of c-Fos positive neurones in the posterior cingulate and retrosplenial cortices following exposure to 75% nitrous oxide or s.c. injection of ketamine (50 mg kg⁻¹) compared with control. Results are means (SEM). **P<0.01 relative to control.
maximum neuroprotection (75% atm) showed no intrinsic neurotoxicity. This corroborates recent work on the neurotoxicity of nitrous oxide and ketamine and a study that showed nitrous oxide enhanced the neurotoxicity induced by ketamine while this was inhibited by xenon. The observation that xenon is able to counteract the neurotoxic effects of ketamine, for which there is strong evidence of NMDA receptor involvement, suggests that xenon, in addition to its ability to inhibit NMDA receptors, is likely to have additional targets.

It has been suggested that the behavioural changes produced by ketamine, phencyclidine, and MK801 in healthy individuals are similar to those seen in schizophrenia, which in turn has been linked to a dysfunctional dopamine system. Ketamine is known to induce cortical release of dopamine and D2 dopamine receptor antagonists ameliorate ketamine-induced prefrontal cortical impairment in rats. Like ketamine, nitrous oxide also causes dopamine metabolic changes in the cortex of rats which is seen as an increase of 3,4-dihydroxyphenylalanine, the major metabolite of dopamine in the rat brain; we recently confirmed this finding in a tissue culture preparation. In contrast, xenon does not increase dopamine release in PC12 cells. It is noteworthy that, of the neurotransmitters in cortical afferent neurones, only dopamine is distributed to frontal and cingulate areas. All of the above argue for a possible role of dopamine in the neurotoxic effects of NMDA receptor antagonists. The data presented here (Fig. 5), showing that the dopamine D2 receptor antagonist haloperidol blocks the toxicity produced by both nitrous oxide and ketamine, provides supporting evidence for this view.

Possible clinical implications

Xenon, when used as a general anaesthetic, has virtually no side-effects and rapidly distributes into the CNS when breathed. Its ability to act as a neuroprotectant together with its apparent lack of neurotoxicity sets xenon apart from other anaesthetics, such as nitrous oxide and ketamine, with known NMDA antagonist properties. Thus, xenon might be considered as the anesthetic of choice when neuronal injury can be anticipated, for example in cardiac surgery requiring coronary artery bypass.

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