Differential nitric oxide synthase activity, cofactor availability and cGMP accumulation in the central nervous system during anaesthesia

H. F. Galley1*, A. E. Le Cras1, S. D. Logan2 and N. R. Webster1

1Academic Unit of Anaesthesia and Intensive Care, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK. 2Department of Biomedical Sciences, University of Aberdeen, UK

*Corresponding author

We investigated the effects of anaesthesia on dynamic nitric oxide production, concentrations of tetrahydrobiopterin and the accumulation of cyclic GMP (cGMP) in the rat central nervous system (CNS). Rats were assigned to anaesthesia with halothane, isoflurane, pentobarbital, diazepam, ketamine or xenon (n=6 per group). After 30 min, [14C]-arginine (i.v.) was given and, after a further 60 min of anaesthesia, rats were killed and exposed immediately to focused microwave radiation. After removal of the brain and spinal cord, nitric oxide production from radiolabelled arginine (and hence nitric oxide synthase activity during anaesthesia) was measured as [14C]-L-citrulline by scintillation counting. cGMP was determined by enzyme immunoassay and tetrahydrobiopterin by fluorescence HPLC, in brain regions and the spinal cord. Nitric oxide synthase activity was similar in all brain regions but was lower in the spinal cord, and was unaffected by anaesthesia. cGMP was similar in all areas of the CNS and was significantly decreased in rats anaesthetized with halothane. Isoflurane produced similar effects. In contrast, ketamine and xenon anaesthesia increased cGMP in the spinal cord, brainstem and hippocampus. Diazepam and pentobarbital had no effect. Tetrahydrobiopterin concentrations were similar in all areas of the CNS and were increased in the cortex and hippocampus after anaesthesia. We have shown profound differential effects of anaesthesia on the nitric oxide pathway in the rat CNS.


Keywords: brain, cerebral cortex; brain, hippocampus; pharmacology, nitric oxide; rat

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Nitric oxide is synthesized from L-arginine by the action of nitric oxide synthase (NOS), and type I or neuronal NOS is the predominant isoform of the enzyme within the central nervous system (CNS).1 All NOS enzymes have a requirement for tetrahydrobiopterin for catalytic activity, and NOS activity is governed by tetrahydrobiopterin availability.2 The primary way in which nitric oxide mediates cellular and intercellular communication is through the activation of soluble guanylyl cyclase, resulting in cGMP formation and the regulation of neuronal ion channels. Nitric oxide is a component of the excitatory and inhibitory neurotransmitter pathways involved in anaesthesia,3 and anaesthetics have been shown to alter aspects of the nitric oxide pathway, including cGMP accumulation, metabolites of nitric oxide and NOS activity,3–6 although some data are conflicting.

It has generally been assumed that changes in cGMP entirely represent nitric oxide-dependent changes, and levels of cGMP have been used previously as a measure of nitric oxide production, despite the potential for nitric oxide-independent effects on cGMP. In addition, there are no data on the effect of anaesthesia on rate-limiting cofactors for NOS and no in vivo studies of NOS activity in the CNS during anaesthesia. We therefore investigated the effects of anaesthesia, using a range of agents, on NOS activity and concentrations of tetrahydrobiopterin and cGMP in the rat CNS.

Materials and methods

These investigations were performed in accordance with UK Home Office regulations and all animals remained anaesthetized to a noxious stimulus throughout.

Female rats (230–270 g, six per group) were used for all experiments and were maintained on normal rat chow. The
use of a single sex minimizes any differences due to gender, and females do not fight when caged together. Rats were assigned randomly to anaesthesia with halothane 1.5% in oxygen; isoflurane 2% in oxygen; pentobarbital 50 mg kg\(^{-1}\) as a bolus followed by 50 mg kg\(^{-1}\) h\(^{-1}\); diazepam 10 mg kg\(^{-1}\) as a bolus followed by 5 mg kg\(^{-1}\) h\(^{-1}\); ketamine 50 mg kg\(^{-1}\) as a bolus followed by 5 mg kg\(^{-1}\) h\(^{-1}\); or xenon 80% in oxygen. Anaesthesia was induced with either 1.5% halothane (halothane group only) or 2% isoflurane (other groups). After surgical tracheostomy, rats were ventilated to maintain normocapnia (\(\text{PCO}_2\) was maintained between 4 and 5 kPa).\(^7\) Saline (5 ml kg\(^{-1}\) h\(^{-1}\)) was infused continuously via the femoral vein. After surgical preparation, anaesthesia was continued with the allocated agent for 30 min. [\(^{14}\text{C}\)]L-arginine (Amersham Life Sciences, Amersham, UK) was then administered (15 \(\mu\text{Ci kg}^{-1} = 10 \text{mg kg}^{-1}\) L-arginine in saline i.v.). Anaesthesia was continued for a further 60 min. Animals were killed by cervical dislocation and subjected instantaneously to focused microwave radiation to stop all CNS enzyme activity and maintain cGMP levels, as described previously.\(^8\) \(^9\) One group of rats (\(n=6\)) were completely untreated (untreated control group) and another group (\(n=6\)) received [\(^{14}\text{C}\)]L-arginine via a tail vein without anaesthesia and were killed 60 min later (treated control group).

The brain was removed and placed in 1 M dithioerythritol (Sigma, Poole, Dorset, UK) to prevent autooxidation of pterins. The brain was dissected rapidly into the medulla, hippocampus, cerebellum and cerebral cortex. Laminectomy was performed simultaneously and the cervical and thoracic spinal cord were removed. Samples were frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\).

Nitric oxide production from an i.v. dose of [\(^{14}\text{C}\)]arginine (and hence NOS activity during anaesthesia) was measured as labelled citrulline by scintillation counting, as described previously for the measurement of \textit{in vitro} NOS activity.\(^10\) Arginine, including residual labelled arginine, was removed from tissue homogenates before scintillation counting, using a cation exchange column; retention of radiolabelled arginine was consistently \(>95\%\).

cGMP was measured using an enzyme immunoassay kit (R&D Systems Europe, Oxford, UK) with prior acetylation of samples and standards to increase assay sensitivity (typically \(>0.37 \text{ pmol ml}^{-1}\)), as recommended by the manufacturer. Intra- and inter-assay precision was 6.2\% (\(n=8\)) and 6.6\% (\(n=8\)) respectively.

Tetrahydrobiopterin concentrations were measured by HPLC after selective oxidation with iodine, using a modification of the method described by Fukushima and Nixon.\(^11\) Under acid conditions, all pterins are converted to fluorescent biopterin, whilst under alkali conditions tetrahydrobiopterin is converted to non-fluorescent material, with only biopterin detected. Tetrahydrobiopterin can therefore be measured as the difference between biopterin concentrations under acid and alkali conditions, using HPLC with fluorescent detection.

Prepared samples were injected into a C18 Techsil column (10 \(\mu\text{m}\) particle size; HPLC Technology, Macclesfield, Cheshire, UK) using a Gynkotech HPLC system (HPLC Technology). The mobile phase was 10% (v/v) methanol in water and samples were calibrated against biopterin (Sigma), which had also undergone acid–alkali oxidation. Biopterin was detected fluorometrically at excitation and emission wavelengths of 350 and 440 nm respectively. The accuracy of the technique was 78.3 (77.0–79.8)\% in recovery experiments (\(n=30\)), using 30 ng ml\(^{-1}\) biopterin in the presence of 1 mg ml\(^{-1}\) bovine serum albumin to mimic tissue protein content. The precision of the assay was 1.8\% within runs and 8.1\% between runs (\(n=10\)).

Results from all assays were expressed in terms of tissue homogenate protein concentration, determined using the Bradford reagent (Sigma).

\textbf{Statistical analysis}

Results are expressed as median and range. Statistical analysis, performed using Microsoft Excel with the Astute statistical add-in, was determined by Kruskal–Wallis analysis of variance with Mann–Whitney \textit{U} post \textit{hoc} testing as appropriate. \(P<0.05\) was considered significant.

\textbf{Results}

The adequacy of microwave exposure in halting enzyme activity and maintaining cGMP levels was confirmed in preliminary experiments using untreated animals. cGMP concentration, with and without 5 s microwave exposure respectively, was 2.1 (1.7–2.4) and 0.07 (0–0.08) pmol mg\(^{-1}\) protein in cerebellum and 2.6 (2.1–3.2) and 0.08 (0–0.08) pmol mg\(^{-1}\) protein in spinal cord (\(n=3\)).
Table 1 shows [14C]citrulline accumulation in treated controls (i.e. animals were not anaesthetized but received [14C]arginine). Accumulation in the spinal cord was significantly lower than in other CNS regions (P=0.0135). The effect of i.v. [14C]arginine on cGMP and tetrahydrobiopterin concentrations is shown in Fig. 1. In all brain regions the concentrations of both cGMP (Fig. 1A) and tetrahydrobiopterin (Fig. 1B) after [14C]arginine were unaltered compared with untreated controls (i.e. animals were not anaesthetized and did not receive [14C]arginine). However, the cGMP concentration was higher in the cerebellum than other brain regions or spinal cord (P<0.05) (Fig. 1A).

Effect of anaesthesia

Spinal cord

In the spinal cord, tetrahydrobiopterin concentration was similar in all anaesthetized animals, but was higher than in non-anaesthetized animals (P<0.05) (Fig. 2A). Spinal cord [14C]citrulline accumulation was unaltered by anaesthesia (Fig. 2B). CGMP concentrations in the spinal cord were significantly different after anaesthesia (P<0.0001). Post hoc testing revealed that anaesthesia with xenon or ketamine increased cGMP concentration (P<0.05) (Fig. 2C) and halothane and isoflurane anaesthesia decreased cGMP concentration in the spinal cord (P<0.01 and P<0.05 respectively) (Fig. 2C).

Brain

Tetrahydrobiopterin concentrations remained unchanged compared with control rats in the cortex and hippocampus, irrespective of anaesthesia (Fig. 3). However, tetrahydrobiopterin concentrations were altered in the brainstem and cerebellum (P=0.0192 and P=0.0012 respectively) (Fig. 3). On post hoc testing, tetrahydrobiopterin was increased after xenon anaesthesia (P<0.05 in brainstem and P<0.01 in cerebellum) (Fig. 3). No other anaesthetics had any effect in these brain regions.

Release of [14C]citrulline (and hence NOS activity during anaesthesia) was not affected by any anaesthetic in any brain region (Fig. 4).

Cyclic GMP concentrations were altered in the brainstem (P=0.0001), cerebellum (P=0.005), cortex (P<0.0001) and hippocampus (P=0.0003) after anaesthesia. As in the spinal cord, xenon or ketamine anaesthesia resulted in significantly increased cGMP concentrations in the brainstem (both P<0.05), cortex and hippocampus (xenon P<0.05, ketamine
Halothane anaesthesia resulted in decreased cGMP concentrations in all brain regions (\(P<0.05\) in brainstem and cerebellum, \(P<0.01\) in cortex and hippocampus). Isoflurane decreased cGMP concentrations in the cerebellum and cortex (both \(P<0.05\)) (Fig. 5).

**Discussion**

We found that halothane and isoflurane decreased cGMP concentrations in the spinal cord and most brain regions. In contrast, ketamine and xenon anaesthesia increased cGMP concentrations in the spinal cord and most brain regions. We also observed that diazepam and pentobarbital had no significant effect on cGMP levels in any brain region.

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\(\text{Fig 3}\) Effect of anaesthesia on tetrahydrobiopterin in rat brain after an i.v. dose of [\(1^\text{4}\text{C}\)]L-arginine. Box and whisker plots show median, 25th and 75th centiles and range. Shaded boxes=anaesthetized animals; empty boxes=unanaesthetized, treated controls (\(n=6\) per group). NS (non-significant) and \(P\) values refer to Kruskal–Wallis analysis of variance. C=control; X=xenon; H=halothane; I=isoflurane; K=ketamine; D=diazepam; P=pentobarbital. *Significantly different from unanaesthetized rats (\(P<0.05\), Mann–Whitney \(U\)-test); **significantly different from unanaesthetized rats (\(P<0.01\), Mann–Whitney \(U\)-test).

\(\text{Fig 4}\) Effect of anaesthesia on [\(1^\text{4}\text{C}\)]L-citrulline in the rat brain after an i.v. dose of [\(1^\text{4}\text{C}\)]L-arginine. Box and whisker plots show median, 25th and 75th centiles and range. Shaded boxes=anaesthetized animals; empty boxes=unanaesthetized, treated controls (\(n=6\) per group). NS (non-significant) refers to Kruskal–Wallis analysis of variance. C=control; X=xenon; H=halothane; I=isoflurane; K=ketamine; D=diazepam; P=pentobarbital.
concentrations in the brain and spinal cord. However, NOS activity, measured as $[^{14}\text{C}]\text{L}-\text{citrulline}$ after an i.v. dose of $[^{14}\text{C}]\text{L}-\text{arginine}$, was unaffected by any of the anaesthetic agents tested. Tetrahydrobiopterin concentration was increased in anaesthetized animals, independently of the anaesthetic used.

A technique similar to that described here was used by Northington et al. to measure in vivo nitric oxide release in the brain, although labelled arginine was directly infused into sheep cerebellum using a microdialysis probe. These authors found increased nitric oxide release in response to $N$-methyl-$d$-aspartate (NMDA) and decreased nitric oxide after an NOS inhibitor was administered. In the present study, we administered $[^{14}\text{C}]\text{arginine}$ i.v. as a bolus dose to quantify nitric oxide release, and hence NOS activity, across a defined time window. We have demonstrated the accumulation of $[^{14}\text{C}]\text{citrulline}$ in the brain and spinal cord after an i.v. dose of labelled l-arginine, indicating that $[^{14}\text{C}]\text{l}-\text{arginine}$ was able to reach the CNS. Administration of labelled arginine had no effect on concentrations of either cGMP or tetrahydrobiopterin, as no difference was seen between treated and untreated control rats, and all subsequent comparisons were made using anaesthetized animals that had received $[^{14}\text{C}]\text{arginine}$. Labelled citrulline concentration was lowest in the spinal cord, which may reflect either differences in the delivery of i.v. arginine to the spinal cord compared with the brain, or genuinely lower nitric oxide production in the spinal cord than in the brain. A previous study using the in vitro conversion of $[^{14}\text{C}]\text{arginine}$ to $[^{14}\text{C}]\text{citrulline}$ also reported lower NOS activity in the spinal cord than in the brain, as in the present study.

The anaesthetic agents used in the present study were chosen for their differing chemical structures and disparate suggested modes of action. Xenon is an inert gas that inhibits NMDA receptor channels. Halothane is a halogenated hydrocarbon that is approximately 25–30% metabolized. Isoflurane is a fluorinated ether and is less than 5% metabolized. Ketamine is an NMDA receptor antagonist, diazepam acts at the benzodiazepine site on GABA$_A$ receptors, and pentobarbital is a short-acting barbiturate that is thought to act at GABA$_A$ receptors. We found that citrulline from $[^{14}\text{C}]\text{arginine}$, and hence NOS activity during the time window of the study, was not affected by any of the anaesthetics studied. Previous in vitro studies have produced conflicting results. A direct inhibitory effect of halothane and isoflurane on NOS activity in the rat brain has been described in vitro. However, other studies found no effect of halothane, isoflurane or enflurane on rat brain NOS. Ketamine and pentobarbital had no effect on rat brain NOS activity in one study but inhibited activity in another study. Different techniques, which included citrulline accumulation and the conversion of oxy- to met-haemoglobin, were used in these in vitro studies. Both are accepted, validated techniques for the indirect quantification of NOS activity. The present study used the conversion of exogenous $[^{14}\text{C}]\text{l}-\text{arginine}$ to $[^{14}\text{C}]\text{l}-\text{citrulline}$ in a physiological in vivo situation, as used in a previous study in sheep. An improved version of the in vitro citrulline assay also confirmed no direct effect of halothane on the activity or kinetics of NOS isolated from the rat CNS.

Of course, nitric oxide production from brain homogenates...
in an artificial in vitro situation may not reflect effects in vivo.

In our in vivo study we found that both halothane and isoflurane anaesthesia markedly decreased cGMP concentrations throughout the rat CNS. There have been conflicting results from previous studies of the effects of anaesthetics on cGMP, and the majority of these studies have been in vitro. It has been shown that hypercapnia and hypoxia have profound effects on nitric oxide and cGMP, which illustrates the need to maintain normal arterial blood gas values. In the present study normocapnia was maintained, so the changes we observed in cGMP were not the result of ventilation-induced hypercapnia. Potential non-specific post-mortem changes in cGMP were eliminated using microwave irradiation, as described previously.

The decreased cGMP concentration reported previously in anaesthetized, treated animals has been attributed to altered motor function as a result of anaesthesia/sedation, as paralysis for 15 min also decreased cGMP, and increased locomotor activity induced by tremors and seizures or forced running increased cGMP. However, we found that cGMP concentration was decreased by halothane and isoflurane, was unaltered by pentobarbital and diazepam, and was increased by ketamine and xenon. Interestingly, in the present study, we noted that, although nociceptive responses were abolished during xenon and ketamine anaesthesia, some degree of spontaneous motor function was retained.

In rat cerebellar slices in vitro, ketamine was found to depress NMDA or glutamate-stimulated cGMP but not non-receptor-mediated cGMP accumulation. We found increased cGMP concentrations in response to the NMDA receptor antagonists ketamine and xenon. It might be expected that these agents would cause decreased cGMP accumulation by blocking NMDA-receptor mediated NOS activation. However, a recent study using microwaved probes in the brains of intact rats reported that products of nitric oxide are increased during administration of ketamine, but cGMP was not measured. There are no previous studies of the effects of xenon on cGMP.

Alterations in cGMP can occur through mechanisms other than altered nitric oxide release. Soluble guanylate cyclase, the enzyme that catalyses the conversion of GTP to cGMP, is activated about 400-fold by nitric oxide, about 20-fold by hydroxyl ions and about 4-fold by carbon monoxide. However, it is the regulation of cGMP concentration via phosphodiesterases, which catalyse the breakdown of cGMP to 5’-GMP, that is likely to be particularly important. In the present study, microwave radiation will not only inactivate guanylate cyclase, thus terminating further cGMP production, but will also inactivate phosphodiesterases and hence terminate further cGMP breakdown. The effect of anaesthetic agents on phosphodiesterases remains unknown. The altered cell membrane function that has been reported in response to some anaesthetics could also lead to the intracellular accumulation of cGMP. In the present study, this will manifest as an increase in cGMP in brain homogenates, but in in vitro studies the effect would appear as decreased release of cGMP into the incubation medium.

We found that tetrahydrobiopterin concentrations increased in the spinal cord in all anaesthetized rats and in some brain areas after anaesthesia with xenon. These changes were not associated with NOS activity measured as [14C]citrulline accumulation, suggesting that NOS does not occur in the same distribution as sites of tetrahydrobiopterin synthesis. The significance of these findings is unclear. Tetrahydrobiopterin is also a cofactor for tyrosine and tryptophan hydrolases, and in situ hybridization has shown that GTP-cyclohydrolase, the rate-limiting enzyme for tetrahydrobiopterin synthesis, is co-localized with these enzymes and not with NOS, suggesting that nitric oxide-producing cells may obtain their tetrahydrobiopterin from that released into the circulation.

There are no previous data on the effects of anaesthetics, either in vivo or in vitro, on biopterins. However, it has been shown that prolonged hypoxia increases cerebellar nitric oxide formation, through up-regulation of GTP cyclohydrolase I expression and hence tetrahydrobiopterin levels, again emphasizing the importance of maintaining and within normal limits. Alteration of cell membrane function or transport systems for secreted products by anaesthetics may also lead to the accumulation of cell products such that concentrations appear increased.

In summary, we have shown that anaesthesia affects cGMP production in the rat brain and spinal cord. The discrepancy between the changes in cGMP in the absence of altered NOS activity, measured as [14C]citrulline production or tetrahydrobiopterin concentrations, suggests that these effects may act via nitric oxide-independent cGMP regulation, possibly through effects on the metabolism of cGMP.

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