NOx− concentrations in the rat hippocampus and striatum have no direct relationship to anaesthesia induced by ketamine†

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Using microdialysis, we have examined the effects of ketamine on concentrations of total nitric oxide oxidation products (NOx−) in the rat hippocampus and striatum in vivo to investigate the relationship between anaesthesia and NOx− production in the brain. Ketamine 25, 50 and 100 mg kg−1 i.p. increased NOx− concentrations to mean 125 (σd 13)%, 165 (11)% and 193 (13)% of basal, respectively, in the hippocampus and to 122 (12)%, 147 (7)% and 177 (14)% of basal in the striatum. Local perfusion with ketamine 50 and 100 μmol litre−1 into the hippocampus or striatum increased NOx− concentrations to 212 (32)% and 291 (17)% of basal, respectively, in the hippocampus and to 148 (20)% and 201 (18)% of basal in the striatum. Ketamine 50 and 100 mg kg−1 i.p. caused dose-dependent prolongation of loss of the righting reflex (LRR) and 100 mg kg−1 i.p. also caused loss of the corneal reflex (LCR). Local perfusion of ketamine did not provoke LRR or LCR. Inhibition of NOS by L-NAME 100 mg kg−1 i.p. decreased hippocampal NOx− concentrations to 58 (7)% of basal and did not provoke LRR or LCR. Although the effect of ketamine-induced increases in hippocampal NOx− concentrations was significantly depressed by L-NAME, LRR was not affected. These data imply that NOx− concentrations in the hippocampus or striatum have no direct relationship to the anaesthetic efficacy of ketamine, although this requires further investigation.

Keywords: measurement techniques, microdialysis; pharmacology, nitric oxide; anaesthetics i.v., ketamine, brain, hippocampus; brain, striatum; rat

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Nitric oxide is a highly reactive and unstable free radical species and has been demonstrated to be a mediator of diverse physiological functions, including signal transmission, transduction and defence mechanisms in the central and peripheral nervous system. Several studies have suggested a role for nitric oxide in mediating central nociceptive pathways and possible involvement in the mechanism of wakefulness and anaesthesia.

Nitric oxide exerts many of its effects by increasing intracellular concentrations of cyclic guanosine monophosphate (cGMP) in target cells via activation of soluble guanylate cyclase. Stimulation of excitatory amino acid (EAA) receptors increases nitric oxide production. Suppression of excitatory transmission stimulated by excitatory amino acids or augmentation of inhibitory transmission via gamma-aminobutyric acid (GABA) receptors results in a sedating effect. Some anaesthetics may suppress excitatory transmission at any step in the glutamate receptor–nitric oxide–cGMP pathway. In fact, there is increasing evidence that blocking the glutamate receptor–nitric oxide–cGMP pathway may be associated with the development of anaesthesia. For example, 2-amino-6-(trifluoromethoxy)benzothiazole (riluzole), both a pre- and postsynaptic inhibitor of glutamate, exerts an anaesthetic action. Non-competitive antagonists at NMDA receptors, such as MK-801, SKF10047, phencyclidine and dextromethorphan, can potentiate anaesthesia.

Ketamine is also known as a non-competitive antagonist of NMDA receptors. Because ketamine blocks excitation of NMDA receptors, it may influence nitric oxide production in the brain. Several reports have demonstrated that ketamine affects the glutamate receptor–nitric oxide–cGMP pathway in vitro, however, this has not been confirmed in living animals by direct measurement of nitric oxide or nitric oxide oxidation products.

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In this study, using in vivo brain microdialysis coupled with online analysis of total nitric oxide oxidation products (NOx−), we have investigated the effects of ketamine, with or without S(+)-N3-[imino(nitroamino)methyl]-L-ornithine methyl ester hydrochloride (l-NAME), an NOS inhibitor, on NOx− concentrations in the rat hippocampus and striatum to determine the relationship between NOx− concentration and anaesthesia induced by ketamine.

Materials and methods

The study was approved by the Animal Welfare Committee of Yokohama City University School of Medicine. The microdialysis technique in freely moving rats was used. Adult male Sprague–Dawley rats (7–8 weeks old, weighing 250–350 g) were anaesthetized with pentobarbital 50 mg kg−1 i.p. and then placed in a stereotaxic apparatus (Model SR-6, Narisige Scient Instrument LAB, Japan). A guide cannula (CMA 10, Carnegie Medicine, Sweden) for penetration of a microdialysis probe was stereotaxically implanted into the right hippocampus or right striatum according to the atlas of Paxinos and Watson,12 as described previously.13 The probe was fixed in place with cranioplast cement. A stainless steel dummy probe was inserted into the guide cannula until a dialysis probe was inserted.

The rats were kept in a 12-h light–dark cycle in a regulated environment (23 ± 2°C, 50% humidity, free access to water and food) for at least 48 h after the surgical procedure. At the beginning of the experiment, a microdialysis probe (3 mm long dialysis membrane, 0.24 mm od, molecular weight cut-off 20 000 Da; CMA11, Carnegie Medicine, Sweden) was inserted through the implanted guide cannula into the hippocampus or striatum. The probe was perfused with artificial cerebrospinal fluid (NaCl 147.0 mmol litre−1, KCl 4.0 mmol litre−1, CaCl2 3.0 mmol litre−1, pH 7.4) at a constant flow rate of 2 μl min−1 with a microdialysis pump (Model CMA 102, Carnegie Medicine, Sweden).

The rats were kept in a plastic cage where they could move freely and had access to food and water during the dialysis experiment. After 2 h of dialysis equilibration, the dialysate was collected every 20 min in an automated sample injector connected to an automated nitric oxide detector–HPLC–spectrophotometer system (ENO-20, Eicom, Japan). Briefly, in this system, NO2− and NO3−, the stable oxidation products of nitric oxide in the dialysate, were separated using a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm, Eicom, Japan). NO2− was reduced to NO− in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). NO− was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The absorbance of the colour of the dye product at 540 nm was measured by a flow-through spectrophotometer. The quantity of NO2− and NO3− was calculated and recorded on a personal computer using specialized software (PowerChrom, ADInstruments Pty Ltd, Australia). NOx− was the sum of NO2− and NO3−. This assay system has been demonstrated as feasible for online assessment of nitric oxide production in living animals by measuring NOx−.14,15

At the end of the experiments, rats were killed by an overdose of diethyl ether and the correct position of the probe was verified by histological examination of the fibre tract.

Ketamine was purchased from Sankyo Co., Tokyo, Japan. l-NAME was purchased from Research Biochemicals International, Natick, MA, USA. All test agents were administered when three consecutive stable dialysis samples (basal level) had been collected after an equilibration period of 2 h of brain perfusion.

Anaesthesia was defined as loss of the righting reflex (LRR)16 and loss of the corneal reflex (LCR).17 LCR was defined as a deeper level of anaesthesia than LRR. These methods have been demonstrated to be related to the depth of anaesthesia in the rat.18

All data are expressed as mean (SD) values of NOx− in extracellular fluid (ECF) in the rat hippocampus or striatum. Statistical analysis was performed using StatView for Windows (Abacus Concepts, Inc., CA, USA). The Kruskal–Wallis test was used to analyse differences in NOx− concentrations between different experimental groups. If the overall difference in NOx− concentrations between different experimental groups was significant, differences in mean NOx− concentrations at individual times between control and each drug-treated group were analysed using the Mann–Whitney U test. Percentage change in NOx− was calculated by comparing NOx− concentrations after treatment with basal levels in each group, obtained by averaging NOx− concentrations in the three consecutive dialysate samples collected before treatment. Behavioural effects were analysed using the Mann–Whitney U test. Differences were considered significant at P<0.05.

Results

In vitro recovery of NO2− and NO3− was 30.9 (1.8)% and 35.1 (3.4)% (n=4), respectively, determined by perfusing the microdialysis probe directly in a mixture of NaNO2 10−6 mol litre−1 and NaNO3 10−6 mol litre−1 standard solution at 35°C, at a flow rate of 2 μl min−1. Accordingly, basal NOx− in the dialysate was 0.15 (0.04) μmol litre−1 (n=58) and 0.21 (0.05) μmol litre−1 (n=28) in the rat hippocampus and striatum, respectively. Therefore, given similar in vivo recovery, basal NOx− concentrations in ECF were 0.42 (0.12) μmol litre−1 and 0.59 (0.14) μmol litre−1 in the rat hippocampus and striatum, respectively.

Figure 1 shows the effects of ketamine on NOx− concentrations in the ECF of the hippocampus or striatum. Ketamine 25, 50 and 100 mg kg−1 i.p. induced increases in NOx− concentrations both in the hippocampus (Fig. 1A) and striatum (Fig. 1B) in a dose-dependent manner. Mean maximum NOx− concentrations after ketamine 25, 50 and
Ketamine and NOx− concentrations in the CNS in rats

Fig 1 Effects of ketamine i.p. (A, B) or local perfusion (C, D) on NOx− concentrations in ECF in the rat hippocampus (A, C) or striatum (B, D). Ketamine was administered after three consecutive stable dialysis samples (basal) had been collected. A: Ketamine 25, 50 or 100 mg kg−1 was administered i.p. C, D: Ketamine 50 or 100 μmol litre−1 was perfused locally into the hippocampus (C) or striatum (D). Data are mean (SD)

Table 1 Effects of ketamine with or without l-NAME, on loss of the righting reflex (LRR) (mean (SD)) and loss of the corneal reflex (LCR). l-NAME i.p. was administered alone or with ketamine 50 mg kg−1 i.p., 60 min later. *P<0.05 vs ketamine 50 mg kg−1 (Mann-Whitney U test).

Drugs | Duration of LRR (min) | LCR
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Control (n=7) | | Absent
Ketamine 25 mg kg−1 i.p. (n=11) | Absent | Absent
Ketamine 50 mg kg−1 i.p. (n=12) | 16.3 (11.1) | Absent
Ketamine 100 mg kg−1 i.p. (n=12) | 45.7 (19.4)* | Present
l-NAME 100 mg kg−1 i.p. (n=6) | Absent | Absent
Ketamine 50 mg kg−1 i.p. + l-NAME 100 mg kg−1 i.p. (n=6) | 16.9 (10.5) | Absent

100 mg kg−1 i.p. were 0.57 (0.07) μmol litre−1 (n=6, P>0.05 vs control), 0.67 (0.07) μmol litre−1 (n=6, P<0.05 vs control) and 0.87 (0.11) μmol litre−1 (n=7, P<0.05 vs control) (125 (13)% 165 (11)% and 193 (13)% of basal, respectively) in the hippocampus, and 0.71 (0.09) μmol litre−1 (n=5, P>0.05 vs control), 0.90 (0.06) μmol litre−1 (n=5, P<0.05 vs control) and 1.13 (0.16) μmol litre−1 (n=5, P<0.05 vs control) (122 (12)% 147 (7)% and 177 (14)% of basal, respectively) in the striatum. Local perfusion of ketamine 50 and 100 μmol litre−1 into the hippocampus or striatum through the microdialysis probe provoked dose-dependent increases in NOx− concentrations in both the hippocampus (Fig. 1C) and striatum (Fig. 1D). Mean maximum NOx− concentrations were 0.91 (0.29) μmol litre−1 (n=6, P<0.05 vs control) and 1.31 (0.22) μmol litre−1 (n=6, P<0.05 vs control) (212 (32)% and 291 (17)% of basal, respectively) in the hippocampus, and 0.92 (0.18) μmol litre−1 (n=4, P<0.05 vs control) and 1.14 (0.21) μmol litre−1 (n=4, P<0.05 vs control) (148 (20)% and 201 (18)% of basal, respectively) in the striatum.

Ketamine 25 mg kg−1 i.p. did not provoke LRR or LCR. Ketamine 50 and 100 mg kg−1 i.p. provoked dose-dependent prolongation of LRR but only the 100 mg kg−1 dose resulted in LCR (Table 1). Local perfusion of ketamine 50 and 100 μmol litre−1 into the hippocampus or striatum via the microdialysis probes did not provoke LRR or LCR.

l-NAME 100 mg kg−1 i.p., an NOS inhibitor, caused a decrease in NOx− concentrations in the hippocampus. The mean minimum NOx− concentration was 0.24 (0.02) μmol litre−1 (n=6, P<0.05 vs control) (58 (7)% of basal) and NOx− concentrations remained decreased for 180 min. l-NAME 100 mg kg−1 i.p. did not provoke LRR or LCR. When NOx− concentrations were reduced by pretreatment...
Discussion

We have demonstrated that ketamine increased NOx- concentrations in the hippocampus and striatum in both anaesthetized and non-anaesthetized rats in vivo and inhibition of the activity of NOS attenuated this effect of ketamine.

The mechanism of general anaesthesia remains largely unknown. Many theories consider that general anaesthesia is primarily the result of altered synaptic transmission. Depression of excitatory transmission and enhancement of inhibitory transmission are related to general anaesthesia. As nitric oxide plays an important role in excitatory synaptic transmission, more and more attention has been paid to the effects of nitric oxide on anaesthesia. In the central nervous system (CNS), glutamate receptor–nitric oxide–cGMP pathways have been demonstrated in the cerebellum, hypothalamus, midbrain, striatum and hippocampus. Nitric oxide may be an important neurotransmitter in these regions. Activation of NMDA receptors promotes an increase in intracellular calcium which causes activation of NOS, which then increases nitric oxide production. The glutamate receptor–nitric oxide–cGMP pathway in the hippocampus is believed to play an important role in several physiological (i.e. learning and memory) and pathophysiological (i.e. epilepsy and neurodegenerative diseases) conditions.

Ketamine acts as an antagonist of NMDA-mediated transmission by blocking the phencyclidine site at the NMDA receptor complex. Therefore, we expected that ketamine may decrease nitric oxide production by blocking NMDA receptors. In fact, some studies showed that ketamine inhibits nitric oxide production and NOS activity in vitro although there are no in vivo data to support this. As Ohta and colleagues demonstrated that NOx- concentrations in the dialysate were related to nitric oxide production in vivo using in vivo brain microdialysis, in our study we measured NOx- concentrations in anaesthetized and freely moving rat brains. Our results showed that NOx- concentrations dose-dependently increased in rats anaesthetized with ketamine. To examine if these phenomena were related to the effect of ketamine-induced general anaesthesia, we also tested the effects of ketamine on NOx- concentrations in non-anaesthetized rats by local perfusion of ketamine 50 or 100 μmol litre⁻¹ into the hippocampus or striatum. Although these concentrations are clinically relevant serum concentrations after anaesthesia with ketamine in animals, local perfusion at these doses into the hippocampus or striatum did not provoke LRR or LCR. Also, under these conditions, ketamine caused dose-dependent increases in NOx- concentrations in the hippocampus and striatum. These results demonstrate that ketamine induced dose-dependent increases in NOx- concentrations in the rat hippocampus and striatum in both anaesthetized and non-anaesthetized animals.
Our results are different from previous in vitro studies using mouse-activated macrophage-like cells or lipopolysaccharide-treated rat alveolar macrophages. One of the reasons for the discrepancy is that the concentration of ketamine used in their studies was much higher than the serum concentration of ketamine used for anaesthetic doses. Reported serum concentrations of ketamine for anaesthesia in animals are less than 80 μmol litre⁻¹. However, the concentration of ketamine used in the study of Shimuoka and colleagues was up to 600 μmol litre⁻¹, which was much higher than that used for local perfusion into the brain in our study. Although Galley and Webster demonstrated that ketamine decreased NOS activity at concentrations as low as 0.01 μmol litre⁻¹, they measured NOS activity in vitro using a method based on the quantitative conversion of oxyhaemoglobin to methaemoglobin while we quantitatively assayed NOx⁻ by microdialysis. This may be a reason for the discrepancy.

Another explanation is that they studied a different isoform of NOS (iNOS), expressed in endothelium, vascular smooth muscle and activated neutrophils and macrophages, while most of the NOS isoforms in the brain are cNOS and nNOS. In an in vivo study in rat cerebellar slices, ketamine did not alter basal cGMP concentrations when no exogenous stimulant was given, but suppressed exogenous l-glutamate- and NMDA-stimulated cGMP formation. This study implied that ketamine may not directly affect NOS or guanylate cyclase activities in the brain.

Differences in in vivo and in vitro experiments may also be an important reason for the discrepancy. In animals, the hippocampus and striatum receive neuronal inputs from other regions of the brain. Tonic inhibitory and phasic excitatory regulation of the activity of glutamate receptors has been demonstrated in the hippocampus and striatum in vivo. The activity of the glutamate receptor–nitric oxide–cGMP pathway in the hippocampus and striatum may be affected by neuronal inputs from other regions of the brain. In an in vitro preparation, these neuronal inputs are removed. Thus basal activity and release of most neuronal systems may be profoundly affected by removal of inputs from other regions of the brain.

The reason why ketamine, an NMDA receptor antagonist, increases NOx⁻ concentrations in the rat hippocampus and striatum is not known. A possible mechanism may be that ketamine increases NOx⁻ concentrations via an increase in acetylcholine (ACh) release in the brain. In our previous study, ketamine produced a dose-dependent increase in ACh from the hippocampus and striatum in vivo. In the study of Giovanni and colleagues, intracerebroventricular administration of the NMDA antagonists, 3-((RS)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), 2-amino-7-phosphonoheptanoic acid (D-AP7) or MK-801 increased ACh outflow from the rat hippocampus. ACh was the first neurotransmitter shown to indirectly affect the tone of blood vessels by causing release of a substance termed endothelium-dependent relaxing factor, which has been demonstrated to be nitric oxide. A recent study showed that ACh and nicotine may evoke nitric oxide release in the rat hippocampal slice. Therefore, in our study, the effect of ketamine-evoked increases in NOx⁻ concentrations in the hippocampus and striatum may have been caused partly by ketamine-induced increase in ACh in this region.

Another explanation is that ketamine may affect the outflow of endogenous EAA. In the study of Liu and Moghaddam, local application of an NMDA receptor antagonist, 2-amino-5-phosphonovaleric acid (APV), dose-dependently increased basal outflow of endogenous glutamate and aspartate in the hippocampus and striatum of freely moving rats, possibly because of a decrease in presynaptic ongoing inhibitory processes that control basal outflow of EAA. Similarly, in our study, ketamine may have decreased these presynaptic ongoing inhibitory processes, resulting in an increase in the outflow of glutamate or aspartate, or both, which in turn activated other subtypes of glutamate receptors not blocked by ketamine. As stimulation of non-NMDA receptors and metabotropic glutamate (mGlu) receptors increased nitric oxide production in the rat hippocampus in vivo, the increased endogenous glutamate and/or aspartate may activate non-NMDA receptors and/or mGlu receptors to increase NOx⁻. This remains to be elucidated.

Moreover, Smirnova, Stinnakre and Malled demonstrated the existence of an inhibitory presynaptic mechanism that controls the release of glutamate. Local perfusion of the NMDA receptor antagonist, CPP, into the hippocampus resulted in a decrease in GABA outflow from the rat hippocampus. Thus ketamine may decrease presynaptic GABA outflow, which in turn increases the release of endogenous glutamate and/or reduces inhibition of non-NMDA and mGlu receptors, resulting in an increase in NOx⁻ release from the hippocampus and striatum.

As nitric oxide is synthesized by NOS, inhibition of NOS activity by an NOS inhibitor would be expected to decrease nitric oxide production. In our study, l-NAME, an NOS inhibitor, significantly decreased NOx⁻ concentrations. This is in agreement with a previous study and proves that NOx⁻ concentrations in dialysate are related to nitric oxide production in the hippocampus and striatum in vivo. The fact that l-NAME significantly decreased the magnitude of ketamine-enhanced NOx⁻ concentrations and pretreatment with l-NAME significantly depressed the stimulating effect of ketamine on NOx⁻ production implies that by whichever mechanism, inhibition of NOS activity attenuates the stimulating effect of ketamine on NOx⁻ production in vivo.

The effect of nitric oxide on anaesthesia is still controversial. Johns, Moscicki and DiFazio showed that inhibition of NOS activity reduced the minimum alveolar anaesthetic concentration of halothane in the rat. But Adachi and colleagues reported an opposite effect. Also, it is not proved that inhibition of NOS activity reduces the anaes-
thetic requirements for i.v. anaesthetics. In our study, ketamine produced a dose-dependent increase in NOx-concentrations in the hippocampus. If NOx- concentration in the hippocampus are related to the anaesthetic efficacy of ketamine, decreased NOx- concentrations in the hippocampus by pretreatment of rats with l-NAME should decrease the duration of LRR induced by ketamine. However, our results did not support this speculation. The duration of LRR provoked by ketamine 50 mg kg⁻¹ i.p. was not decreased by pretreatment with l-NAME 100 mg kg⁻¹ i.p. This dose of l-NAME has been demonstrated to be sufficient to inhibit more than 90% of NOS activity in the rat hippocampus in vivo and decreased NOx- concentrations to 58% of basal in our study. Although these data did not allow us to draw firm conclusions on the relationship between NOx- concentrations in the hippocampus and the anaesthetic efficacy of ketamine, our data imply that NOx- concentrations in the hippocampus are not related to the anaesthetic efficacy of ketamine.

In summary, ketamine produced an increase in NOx- concentrations in the hippocampus and striatum in both anaesthetized and non-anaesthetized rats in vivo. Inhibition of NOS activity by l-NAME attenuated this effect of ketamine without diminishing the anaesthetic effects of ketamine. These results imply that NOx- concentrations in the hippocampus or striatum are not related directly to the anaesthetic efficacy of ketamine, although further investigation is required.

References

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