



# Endogenous nitric oxide decreases hippocampal levels of serotonin and dopamine *in vivo*

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**1** Nitric oxide (NO) modulates the levels of various neurotransmitters in the CNS. Here we determined whether the specific nitric oxide synthase (NOS) inhibitor 7-nitroindazole (7-NI), the non-selective inhibitor of guanylate cyclase (GC) and NOS, methylene blue (MB), the NO-precursor L-arginine (L-Arg), and the selective soluble GC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) affect extracellular levels of serotonin (5-HT), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) in the rat ventral hippocampus by using microdialysis in freely moving animals.

**2** Local perfusion of 7-NI (1 mM) and MB (1 mM) significantly increased extracellular level of 5-HT, whereas DA was increased by 7-NI only. Systemic administration of 7-NI (50 mg kg<sup>-1</sup>) and MB (30 mg kg<sup>-1</sup>) increased the extracellular levels of 5-HT and DA. Extracellular levels of 5-HIAA was not influenced by local or systemic MB or 7-NI. In contrast, extracellular level of HVA was decreased by systemic MB and retrodialyzed MB, but was not influenced by 7-NI.

**3** Retrodialysis of L-Arg (2 mM) decreased the levels of 5-HT, DA, 5-HIAA and HVA in the hippocampus. Systemic administration of L-Arg (250 mg kg<sup>-1</sup>) decreased the level of 5-HT, but failed to influence DA, 5-HIAA and HVA.

**4** Local perfusion of ODQ (400 μM) did not affect 5-HT overflow in the hippocampus.

**5** We conclude that NOS inhibitors increased extracellular levels of 5-HT and DA in the rat ventral hippocampus after local or systemic administration, whereas the NO precursor L-Arg had the opposite effect. Thus, endogenous NO may exert a negative control over the levels of 5-HT and DA in the hippocampus. However, this effect is probably not mediated by cyclic GMP.

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**Abbreviations:** DA, dopamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; HVA, homovanillic acid; L-Arg, L-arginine; MB, methylene blue; 7-NI, 7-nitroindazole; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; s.e.mean, standard error of the mean

## Introduction

Nitric oxide (NO) is an unconventional transmitter molecule in the nervous system, which is synthesized from L-arginine (L-Arg) by nitric oxide synthase (NOS) (Garthwaite *et al.*, 1988). NO has been suggested to have multiple possible targets, among which the soluble guanylate cyclase (GC) is the most extensively characterized (Yun *et al.*, 1997).

Several *in vitro* studies have demonstrated that NO modulates the extracellular levels of various neurotransmitters in the central nervous system, e.g. serotonin (5-HT), dopamine (DA),  $\gamma$ -aminobutyric acid (GABA), and glutamate (Kaehler *et al.*, 1999; Strasser *et al.*, 1994; Lorrain & Hull, 1993; Segovia *et al.*, 1994). In addition, NO can inactivate the rate limiting enzyme in the synthesis of 5-HT, tryptophan hydroxylase (Kuhn & Arthur, 1996) and has been suggested to stimulate synaptic vesicle release from hippocampal synaptosomes (Meffert *et al.*, 1994). Furthermore, NO inhibits uptake of [<sup>3</sup>H]-DA by striatal synaptosomes (Lonart & Johnson, 1994) and transforms 5-HT into an inactive form (Fossier *et al.*, 1999; Pogun *et al.*, 1994). It is currently obscure which of the many possible targets of NO are important *in vivo*.

To date, there are no *in vivo* studies describing the role of NO in regulating hippocampal levels of 5-HT and DA.

However, local perfusion of *N*-methyl-D-aspartate (NMDA) into hippocampus, which is believed to increase the production of NO (Luo *et al.*, 1993), significantly decreased the extracellular levels of 5-HT and DA (Whitton *et al.*, 1994a,b; Tao & Auerbach, 1996). These findings indirectly indicate that NO may interact with the serotonergic and dopaminergic neurotransmission by suppressing the overflow of 5-HT and DA in the hippocampus.

Further characterization of NO's actions in the hippocampus could have several neurobiological implications, in light of the alleged role of NOergic and serotonergic mechanisms in anxiety and depression (Wiley *et al.*, 1995; Volke *et al.*, 1997; Faria *et al.*, 1997; Harkin *et al.*, 1999; Mongeau *et al.*, 1997).

The aim of present study was, using microdialysis in freely moving animals, to elucidate the possible role of NO in the regulation of extracellular levels of 5-HT and DA in ventral hippocampus after local and systemic application of drugs that affects the NO level.

## Methods

### Animals

All animal procedures were accepted by the Danish National Committee for Ethics in Animal Experimentation (j.no. 1998-

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561-44). Male Sprague-Dawley rats (MB Breeding Centre, Denmark) weighing 280–400 g were used. They were housed individually at  $20 \pm 2^\circ\text{C}$  in 12 h light/dark cycle (light on at 0700). Tap water and chow pellets were available *ad libitum*. The animals were kept for at least 2 weeks in the animal colony before entering experiments.

### Microdialysis and analytical technique

Under the fentanyl-fluanisone ( $0.0945$  and  $0.3$  mg kg<sup>-1</sup>, respectively) + midazolam ( $0.25$  mg kg<sup>-1</sup>) anaesthesia a guide cannula (CMA/12) was implanted just above the ventral hippocampus at AP -4; L -4.5; DV -5 relative to bregma (Paxinos & Watson, 1982) and was secured using three screws and dental acrylic. Immediately after the operation, the animal was injected with buprenorphin at  $0.03$  mg kg<sup>-1</sup> s.c. After 3–5 days, the rat was sedated with fentanyl-fluanisone + midazolam, and a concentric style (I-shaped) microdialysis probe (CMA/12 – 4 mm tip length) was inserted through the guide cannula and was secured with plastic glue. The animals were attached to a dual channel swivel (Instech technology, Plymouth, PA, U.S.A.), allowing unrestricted movement in the test cage.

The probes were perfused *in situ* overnight (14–16 h) with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 145, KCl 3, CaCl<sub>2</sub> 1.2, MgCl<sub>2</sub> 1. In order to study release of 5-HT,  $1$   $\mu\text{M}$  citalopram was added to the ACSF. During the experiment, ACSF was pumped at a rate of  $1.5$   $\mu\text{l min}^{-1}$  and samples were collected continuously for intervals of 20 min. Analysis of the samples was carried out within 1–12 h after collection. Before and after the experimental microdialysis session, recoveries of all probes were assessed by examining the amount of neurotransmitters from a known standard solution passing through the probe-membrane to the dialysate. All microdialysis disposables were obtained from CMA/Microdialysis AB (Stockholm, Sweden).

High pressure liquid chromatography (HPLC) with electrochemical detection was used for sample analysis (ESA Coulochem II with 5014B microdialysis coulometric analytic cell; ESA Inc. MA, U.S.A.). The mobile phase was composed of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 740  $\mu\text{M}$  1-octanesulphonic acid, 108  $\mu\text{M}$  Na-EDTA, 80 ml l<sup>-1</sup> acetonitrile and 100  $\mu\text{l l}^{-1}$  triethylamine with pH adjusted to 3.5 using H<sub>3</sub>PO<sub>4</sub>.

### Histology

At the end of the experiment the rat was anaesthetized with chloral hydrate ( $2.5$  mm kg<sup>-1</sup> i.p.) and cresyl-violete was perfused ( $1.0$   $\mu\text{l min}^{-1}$ ) through a dialysis probe for about 10 min to stain the surrounding tissue. Using this procedure only tissue intimately in contact with the probe was stained. After removal, the brain was fixed in a 4% formalin solution for at least 2 days. Subsequently, the brain was sliced on a microtome and the probe location was inspected under a microscope. Animals with misplaced probes were excluded from the study.

### Drugs

Drugs were administered directly *via* the probe, injected intraperitoneally (i.p.) or subcutaneously (s.c.). Doses and routes of systemic administration were chosen according to behavioural studies showing the drugs to have the intended effect (Volke *et al.*, 1997; 1998; Eroglu & Caglayan, 1997). All chemicals were of reagent grade or higher. L-Arg, 7-NI, triethylamine, 1-octanesulphonic acid, Tween-80 (Polyox-

ethylene sorbitan monooleate), and NaH<sub>2</sub>PO<sub>4</sub> were obtained from Sigma (St. Louis, MO, U.S.A.). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). Chloralhydrate, acetonitrile, dimethyl sulphoxide (DMSO) and Na-EDTA were obtained from Merck (Darmstadt, Germany). MB stock solution (10 mg methylthionine chloride per ml sterile water) was purchased from the local hospital pharmacy. Citalopram-HBr was kindly donated by H. Lundbeck A/S, Valby, Copenhagen.

ODQ was dissolved in DMSO and was made up to final volume by addition of ACSF. The final concentration of DMSO in the perfusion media was 0.3%. 7-NI was dissolved in a few drops of Tween-80, and was made up to final volume by addition of isotonic saline (injection studies) or directly dissolved in ACSF by a slight (50–60°C) heating procedure (perfusion studies). All other chemicals used for animal experimentation were dissolved in the ACSF or isotonic saline.

### Statistics

Microdialysis results are expressed as mean percentage change from the average of four sequential baseline measurements  $\pm$  standard error of the mean (s.e.mean). Statistical analysis (SPSS version 9.0) on dialysate 5-HT and DA-extracellular levels was carried out by comparing drug-treated and control animals for significant differences using analysis of variance with repeated measures over time. The data were corrected for multiple comparisons using the Bonferroni procedure (Grove & Andreasen, 1982). Differences were considered statistically significant when *P* was less than 0.05. The numbers of animals in each group are given in figure legends.

## Results

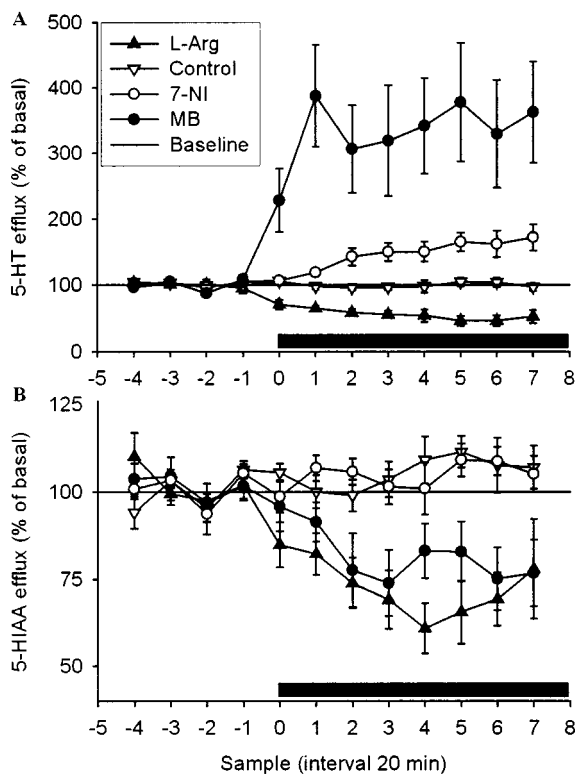
### *Hippocampal 5-HT release is increased by local perfusion of 7-NI or MB, and decreased by L-Arg (Figure 1)*

In the first series of experiments, we studied the effects of 7-NI (1 mM), MB (1 mM), and L-Arg (2 mM) on extracellular levels of 5-HT and 5-HIAA in the ventral hippocampus. Retrodialysis of MB caused an immediate and marked elevation of released 5-HT to a plateau ca. 350% of base level ( $P < 0.001$ ). Also 7-NI produced a significant increase in released 5-HT ( $P < 0.05$ ), albeit the time-course was slower and the maximal level reached was about 200% of baseline. No changes were observed in the extracellular 5-HIAA level after administration of 7-NI and MB.

In contrast, administration of L-Arg decreased 5-HT to 50% of baseline ( $P < 0.001$ ); the reduction occurred immediately and quickly reached a plateau, which was maintained throughout the experiment. The extracellular level of the metabolite 5-HIAA was significantly decreased by L-Arg; in a way similar to 5-HT ( $P < 0.001$ ).

### *Hippocampal 5-HT release is increased by systemic administration of 7-NI or MB, and decreased by L-Arg (Figure 2)*

7-NI ( $50$  mg kg<sup>-1</sup>, i.p.) and MB ( $30$  mg kg<sup>-1</sup>, s.c.) given systemically caused a significant increase in 5-HT release ( $P < 0.001$  and  $P < 0.01$ , respectively). The increase in 5-HT release occurred immediately after injection of 7-NI and peaked within 1 h at 350% of baseline, whereas MB caused



**Figure 1** Effect of local perfusion of L-Arg (2 mM), 7-NI (1 mM), MB (1 mM) or artificial CSF on 5-HT (A) ( $n=8$ ,  $n=12$ ,  $n=7$  and  $n=13$ , respectively) and 5-HIAA (B) ( $n=6$ ,  $n=7$ ,  $n=7$  and  $n=10$ , respectively) overflow in ventral hippocampus of rats. Drugs were infused into ventral hippocampus as indicated by bar. Results are expressed as per cent of basal efflux  $\pm$  s.e.mean.

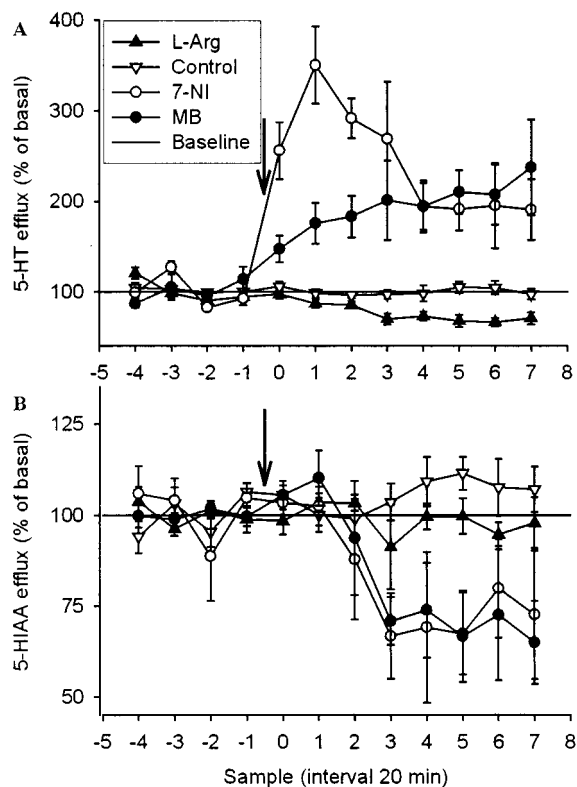
a slower but continuous increase in 5-HT release. Both 7-NI and MB failed to affect the extracellular level of 5-HIAA. In contrast, injection of L-Arg (250 mg kg<sup>-1</sup>, s.c.) significantly decreased the extracellular level of 5-HT ( $P<0.01$ ), but failed to influence 5-HIAA.

#### Hippocampal DA is increased by local perfusion of 7-NI or MB, and decreased by L-Arg (Figure 3)

Retrodialysis of the ventral hippocampus with 7-NI (1 mM) caused an immediate and marked elevation of the level of DA to about 4000% of baseline ( $P<0.001$ ). MB (1 mM) failed, however, to affect the extracellular DA. No changes were observed in extracellular HVA levels after administration of 7-NI, but a decrease was seen in the group retrodialyzed with MB ( $P<0.05$ ). L-Arg decreased the extracellular DA levels to 50% of baseline ( $P<0.05$ ). The reduction occurred immediately and it slowly progressed to a through after 3 h. In addition, the extracellular level of HVA was reduced by L-Arg ( $P<0.01$ ).

#### Hippocampal DA is increased by systemic administration of 7-NI or MB (Figure 4)

Systemic administration of 7-NI (50 mg kg<sup>-1</sup>, i.p.) and MB (30 mg kg<sup>-1</sup>, s.c.) caused a significant increase in the extracellular level of DA ( $P<0.01$  and  $P<0.05$ , respectively). The increase occurred immediately after injection of either drug and rapidly reached a plateau at 250% (MB) and 400% (7-NI) of baseline. MB caused a marked decrease in extracellular levels of HVA, which reached a plateau at 20% of basal ( $P<0.001$ ). The extracellular level of HVA after was



**Figure 2** Effect of systemic injection of 7-NI (50 mg kg<sup>-1</sup>, i.p.), MB (30 mg kg<sup>-1</sup>, s.c.), L-Arg (250 mg kg<sup>-1</sup>, s.c.) or vehicle on 5-HT (A) ( $n=5$ ,  $n=6$ ,  $n=7$  and  $n=13$ , respectively) and 5-HIAA (B) ( $n=4$ ,  $n=3$ ,  $n=9$  and  $n=13$ , respectively) overflow in ventral hippocampus of rats. Drugs were injected as indicated by the arrow. Results are expressed as per cent of basal efflux  $\pm$  s.e.mean.

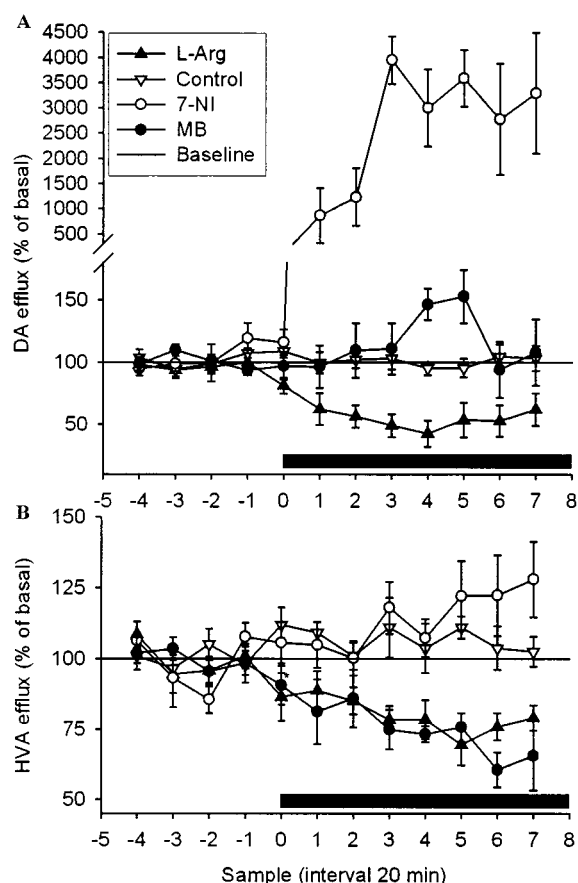
not influenced following the 7-NI injection. Systemic administration of L-Arg failed to influence DA and HVA.

#### Local perfusion of 1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ) fails to affect hippocampal 5-HT release (Figure 5)

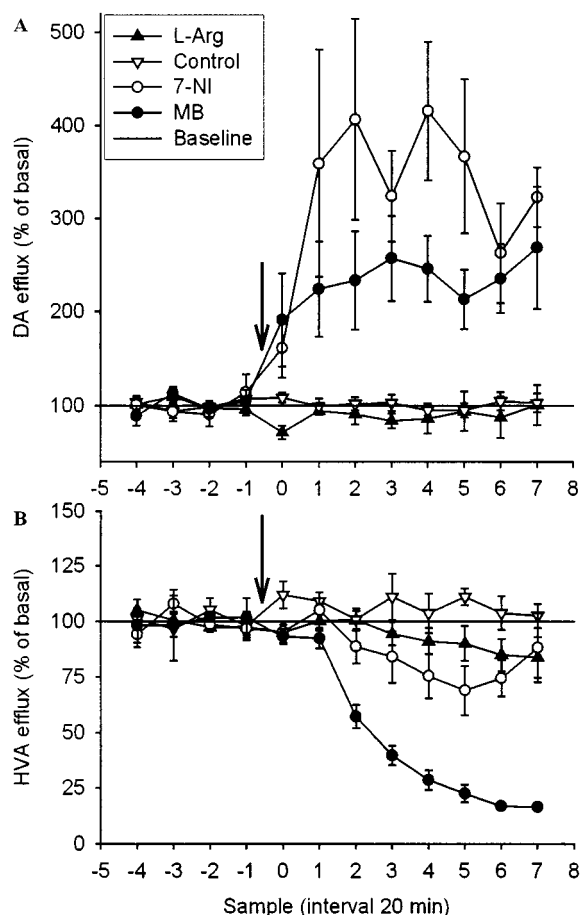
In order to clarify whether the possible effect of NO on the extracellular levels of 5-HT and DA is mediated by cyclic GMP, we examined the effect of addition of the selective NO-sensitive-sGC inhibitor ODQ to the perfusion media (containing 0.3% DMSO). In agreement with previous studies (Fedele *et al.*, 1996), DMSO alone at this concentration had no effect on 5-HT release (results not shown), so DMSO was not included in the perfusion media for the controls. Addition of ODQ to the perfusion media (400  $\mu$ M) did not influence the release of 5-HT compared with controls. However, due to the co-elution of ODQ with DA in our chromatographic system, we were not able to detect changes in DA levels.

## Discussion

The main finding of the present study is that both local and systemic administration of NOS inhibitors, 7-NI or MB, increased the efflux of 5-HT and DA from the ventral hippocampus in freely moving rats. These results agree with previous studies in which the NMDA receptor antagonists, MK-801 and D-AP5, increased extracellular levels of 5-HT and DA in the ventral hippocampus *in vivo* (Whitton *et al.*, 1992a,b; 1994a). Some studies have also shown similar effects of NOS inhibitors in other areas of the brain (Silva *et al.*, 1995;



**Figure 3** Effect of local perfusion of L-Arg (2 mM), 7-NI (1 mM), MB (1 mM) or artificial CSF on DA (A) ( $n=6$ ,  $n=7$ ,  $n=7$  and  $n=10$ , respectively) and HVA (B) ( $n=10$ ,  $n=7$ ,  $n=7$  and  $n=10$ , respectively) overflow in ventral hippocampus of rats. Drugs were infused into ventral hippocampus as indicated by bar. Results are expressed as per cent of basal efflux  $\pm$  s.e.mean.

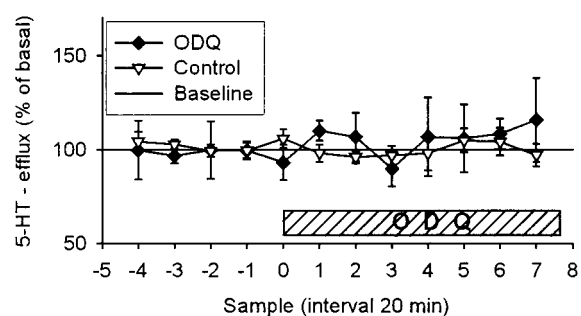


**Figure 4** Effect of systemic injection of 7-NI (50 mg kg<sup>-1</sup>, i.p.), MB (30 mg kg<sup>-1</sup>, s.c.), L-Arg (250 mg kg<sup>-1</sup>, s.c.) or vehicle on DA (A) ( $n=5$ ,  $n=5$ ,  $n=4$  and  $n=10$ , respectively) and HVA (B) ( $n=6$ ,  $n=4$ ,  $n=5$  and  $n=9$ , respectively) overflow in ventral hippocampus of rats. Drugs were injected as indicated by the arrow. Results are expressed as per cent of basal efflux  $\pm$  s.e.mean.

Desvignes *et al.*, 1999; Kaehler *et al.*, 1999). Thus, it appears that under basal conditions, endogenous NO plays an important role in negatively controlling extracellular levels of 5-HT and DA in the hippocampus. Retrodialysis of the ventral hippocampus with 7-NI induced a very large magnitude of increase in extracellular DA compared with the increase in 5-HT. However, as the perfusion media contained citalopram, the basal level of 5-HT was already elevated. Thus, it cannot be excluded that the true elevation of 5-HT produced by 7-NI was partially masked by citalopram.

In accordance with the effects of NOS inhibitors, perfusion with the endogenous NO-precursor, L-Arg, decreased the extracellular levels of 5-HT, DA, 5-HIAA and HVA. This probably represents the true effect of facilitated NO synthesis as L-Arg (1 mM) has been shown to increase NOS activity in the hippocampus (Vallebuona & Raiteri, 1994). Similar effects on the levels of 5-HT and DA have been obtained with local perfusion of NMDA into the ventral hippocampus (Whitton *et al.*, 1994a,b; Tao & Auerbach, 1996).

However, contradictory evidence also exists concerning the role of NO on the levels of the monoamines. For example, the NO donor hydroxylamine has been shown to increase DA release from striatal slices (Lonart *et al.*, 1993) which, however, may not depend on NO, since low L-Arg concentrations, had no effect on DA release itself (Silva *et al.*, 1995). Furthermore, L-Arg has been shown to induce DA release from the striatum and increase the extracellular levels of 5-HT and DA in the medial preoptic area *in vivo* (Strasser *et al.*, 1994; Lorrain &



**Figure 5** Effect of local perfusion of ODQ (400  $\mu$ M,  $n=5$ ) or artificial CSF ( $n=13$ ) on 5-HT overflow in ventral hippocampus of rats. ODQ was infused into ventral hippocampus as indicated by bar. Results are expressed as per cent of basal efflux  $\pm$  s.e.mean.

Hull, 1993). We believe that NO serves different roles in regulating transmitter levels in distinct brain areas and, at least in the ventral hippocampus, increased NO synthesis may result in suppression of 5-HT and DA overflow.

Two distinct isoforms of NOS, neuronal (nNOS) and endothelial NOS (eNOS), with different anatomical and intracellular localization, have been described in neurons (Dinerman *et al.*, 1994). It is likely that eNOS and nNOS serve different roles in the regulation of neurotransmitter release (Kano *et al.*, 1998). 7-NI has often been referred to as

being a selective inhibitor of neuronal NOS *in vivo*. This assumption may be incorrect, however, since it is based solely on the fact that 7-NI in some studies does not increase blood pressure of animals (Moore *et al.*, 1993). Moreover, direct evidence is missing as to whether 7-NI affects eNOS activity in neurons. By using knockout mice, Kano *et al.* (1998) have indirectly shown that 7-NI seems to inhibit both nNOS and eNOS in the cerebral cortex when administered locally. Thus, the contribution of different isoforms of NOS to the regulation of the levels of 5-HT and DA in the hippocampus remains to be established.

The GC inhibitor ODQ did not affect the 5-HT release. The dose of ODQ used has been shown to decrease the hippocampal cyclic GMP level by 50% (Fedele *et al.*, 1996). Thus, it seems likely that cyclic GMP does not have a major role in mediating the effects of NO on hippocampal transmitter levels under basal conditions. We cannot, however, exclude the possible role of cyclic GMP in case of increased concentration of NO. Further studies are warranted to clarify this matter.

Interestingly, not only local, but also systemic administration of 7-NI and MB increased the efflux of 5-HT and DA. There are several studies reporting potent anxiolytic and antidepressant-like effects of NOS inhibitors in various behavioural models (Wiley *et al.*, 1995; Volke *et al.*, 1997; Faria *et al.*, 1997; Harkin *et al.*, 1999). Furthermore, MB has been used as an experimental antidepressant in patients suffering from bipolar disorder (Narsapur & Naylor, 1983;

Naylor *et al.*, 1987). The present study raises the question whether the neurochemical basis of this action may be the change in 5-HT turnover similar to what is seen after established antidepressant therapy (Kreiss & Lucki, 1995). Proper answer to this question does, however, require further work. As conventional anxiolytic drugs are expected to suppress levels of 5-HT in the hippocampus (Nishikawa & Scatton, 1986) we believe that the mechanism underlying the anxiolytic-like activity of NOS inhibitors is not related to the change in the 5-HT efflux.

We conclude that NOS inhibitors increase the extracellular levels of 5-HT and DA in the rat ventral hippocampus after local or systemic administration whereas NO precursor L-Arg had the opposite effect. Thus, endogenous NO seems to exert a negative control over the levels of 5-HT and DA in the hippocampus. However, this effect is probably not mediated by the cyclic GMP.

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