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ORIGINAL ARTICLE

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Role of nitric oxide in the stress-induced release of serotonin in the locus coeruleus

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Abstract Serotonergic mechanisms within the locus coeruleus (LC) are thought to be important in various functions including the stress response. In this study we investigated a possible role of nitric oxide (NO) as an intermediary messenger in the regulation of the serotonin (5-HT) neurotransmission within the LC. Using the push-pull superfusion technique coupled with HPLC and electrochemical detection, the in vivo release of 5-HT was determined in time periods of 10 min in the LC of freely moving rats.

Superfusion with three different NO donors, SIN-1 (linsidomine), *S*-nitroso-*N*-penicillamine (SNAP) or 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANO) increased 5-HT release in the LC. Superfusion with the precursor of NO, L-arginine, for 1 h led to a sustained increase in 5-HT release. On the other hand, the NOS inhibitor *N*-methyl-L-arginine methyl ester (L-NAME) did not significantly change the release of 5-HT. Infusion of *N*-methyl-D-aspartate (NMDA) or kainic acid, as well as exposure of rats to noise stress or tail pinch increased the release of 5-HT in the LC. Superfusion with L-NAME prevented the increase in 5-HT outflow by all these procedures, while the inactive isomer D-NAME had no effect.

Taken together, the results of this study suggest that the release of 5-HT in the LC is facilitated by NO. Under resting conditions inhibition of NOS does not appear to substantially influence the release of 5-HT in the LC. However, there seems to be a facilitatory nitrergic influence on serotonergic responses evoked by excitatory amino acid receptor stimulation or various stress stimuli.

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Introduction

The interaction of the serotonergic system with noradrenergic neurons is thought to be important in stress-related disorders like panic disorder or depression (Asnis et al. 1992; Goddard et al. 1996). Several lines of evidence suggest a serotonergic modulation of neuronal activity in the locus coeruleus (LC), which is the major noradrenergic cell group in the brain: immunocytochemical (Pickel et al. 1977), autoradiographic (Leger and Descarries 1978) and release studies (Kaehler et al. 1999a) have confirmed that the LC receives a dense serotonergic innervation. Electrophysiological, biochemical and pharmacological studies have revealed a predominantly inhibitory role of 5-HT on the function of LC noradrenergic neurons (for review see Haddjeri et al. 1997). It has been demonstrated that neuronal release of 5-HT in the LC responds to stress (Singewald et al. 1997) and that this response is partly mediated indirectly via excitatory amino acids (EAA; Singewald et al. 1998).

It has been shown very recently that nitric oxide (NO) modulates EAA release in the LC (Singewald and Sinner 2000), suggesting a neuromodulator role of NO in the LC. Indeed, immunohistochemical studies have revealed the presence of nitric oxide synthase (NOS)-positive neurons and processes within and close to the LC (Xu et al. 1994). An extracellular NO signal was electrochemically detected in the LC area which is increased by locally applied *N*-methyl-D-aspartate (NMDA) and decreased by the locally or peripherally applied NOS inhibitor *N*-methyl-L-arginine methyl ester (L-NAME; Desvignes et al. 1997). However, it is not known whether NO interacts with the serotonergic transmission within the LC.

Therefore the push-pull superfusion technique (Philippu et al. 1996; Singewald and Philippu 1998) was applied to evaluate a possible role of NO in the modulation of basal and stress-induced release of 5-HT in the LC.

Materials and methods

Experimental procedures. Male Sprague-Dawley rats (230–260 g) were housed in a light-, temperature- and humidity-controlled environment. Protocols of experiments were approved by the Bundesmimisterium für Bildung, Wissenschaft und Kultur, Kommission für Tierversuchsangelegenheiten (GZ 68.210/15 Pr). Superfusion of the LC was carried out as previously described (Singewald et al. 1997). Briefly, the rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.). The head was fixed in a stereotaxic frame and a guide cannula with its stylet was sterotaxically inserted until the tip of the cannula was 2 mm above the right LC. The stereotaxic coordinates of the LC were (in mm): AP 0.8 posterior to interaural line, L 1.3, DV 2.8 above the interaural zero plane (Paxinos and Watson 1998). The guide cannula was fixed on the skull with stainless steel screw and dental cement. At least 2 days after surgery, the stylet of the guide cannula was replaced by a push-pull superfusion cannula with the following diameters (outer needle: o.d. 0.49 mm, i.d. 0.3 mm; inner needle: o.d. 0.2 mm, i.d. 0.1 mm). The push-pull cannula was 2 mm longer than the guide cannula thus reaching the LC. The conscious rats were placed in a large cage and the LC was superfused at a rate of 15 µl/min with artificial cerebrospinal fluid (aCSF), pH 7.2, of the following composition (mM): NaCl 140, KCl 3.0, $CaCl_2$ 1.25, $MgCl_2$ 1.0, Na_2HPO_4 1.2, NaH_2PO_4 0.3, glucose 3.0. Collection of the superfusate started after an equilibration period of at least 80 min so as to achieve stabilization of 5-HT release rate. The superfusate was continuously collected for time periods of 10 min in tubes kept at 0°C. Tubes used to collect the superfusate contained 4 µl of the following solution (mM, final concentrations): $Na_2S_2O_2$ 0.025, EDTA 1.5. The samples were stored at –80°C until biochemical analysis was carried out. At the end of the experiment the rat was killed with an overdose of sodium pentobarbital, the brain was removed and the localization of the cannula was histologically verified. Experiments with cannula localizations outside the LC were discarded. A microphotograph showing a typical localization of the push-pull cannula in the LC has been recently published (Singewald and Philippu 1998). Drugs used for superfusion of the LC were dissolved in aCSF. Solutions were prepared immediately before superfusion. For superfusion with decomposed NO donors, the solutions (200 µM) were kept at room temperature for 140 min before use. Noise stress (95 dB) and tail pinch (force: 3.5 N) were applied for 10 min as previously described (Singewald et al. 1995).

Biochemical analysis. 5-HT was determined by HPLC with electrochemical detection as previously described (Singewald et al. 1997). Briefly, the flow rate through an analytical column (Sepstick Microbore column 150×1 mm, 5 µm C18; BAS, West Lafayette, Ind., USA) was adjusted to 70 µl/min by a flow splitter, the pump flow rate was 1.5 ml/min. The analytical column was protected by a guard column (Sepstick Micropore 14×1 mm, 5 µm C8; BAS). The mobile phase consisted of 88% phosphate buffer (0.1 M, pH 3.5), 6% acetonitrile and 6% methanol. Fifty microliters of the superfusate were automatically injected by a refrigerated microsampler. Evaluations of 5-HT were carried out by comparing peak heights of samples with external standard solutions containing various concentrations of the compound to be determined.

Drugs. Drugs were of the highest grade available. *N*-methyl-D-aspartate (NMDA), kainic acid, L-arginine, 5-hydroxytryptamine (5-HT, serotonin), *N*-methyl-L-arginine methyl ester (L-NAME), *N*-methyl-D-arginine methyl ester (D-NAME) and 3-morpholinosydnonimine (SIN-1, linsidomine) were from Research Biochemical International (RBI, Natick, Mass., USA.); *S*-nitroso-*N*-penicillamine (SNAP) was obtained from Tocris Cookson (Langford, UK); 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propane amine (PAPANO) was from Cayman Chemical (Ann Arbor, Mich., USA).

Statistical analysis. Data are expressed as relative values. The mean release rates of 5-HT in the three samples preceding the administration of a drug or exposure to a sensory stimulus were taken as 1.0. Statistical analysis was carried out by Friedman's analysis of variance followed by Wilcoxon's signed rank test for paired data. Mann-Whitney test was used for analysis of differences between animals treated with L-NAME or D-NAME.

Results

Effect of NO donors on the basal release of 5-HT

The basal release rate of 5-HT in the LC was 1.45± 0.18 fmol/min (mean values ± SEM, *n*=19). During superfusion with the NO donors linsidomine, *S*-nitroso-*N*-penicillamine (SNAP) or 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANO) at 200 µM each, 5-HT release in the LC was increased by 100%–130%. After termination of superfusion with linsidomine and PAPANO, 5-HT release rates immediately dropped to basal levels, while the effect of SNAP persisted for another 10 min. Fifty µM of NO donors as well as the corresponding carrier compounds lacking ability of releasing NO were ineffective (Fig. 1).

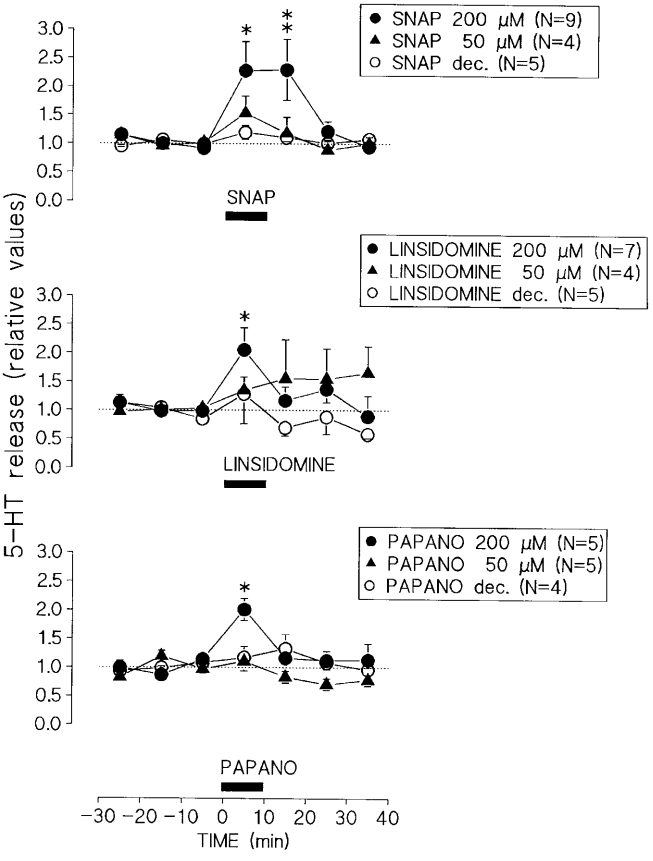

Fig. 1 Effects of NO donors on the basal release of 5-HT. The mean release rates in the three samples preceding drug superfusion were taken as 1.0. *Horizontal bars* denote the onset and duration of the superfusion with NO donors. Number of experiments *in parentheses* (*dec.* decomposed compound). Mean values ± SEM; **P*<0.05, ***P*<0.01

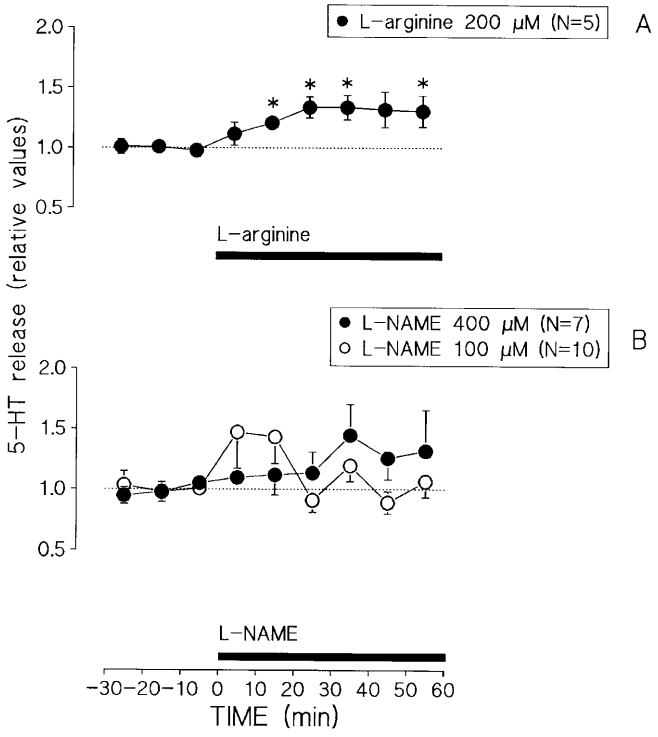

Fig. 2A, B Effects of L-arginine and L-NAME on the basal release of 5-HT. The mean release rates in the three samples preceding drug superfusion were taken as 1.0. *Horizontal bars* denote the onset and duration of the superfusion with **A** L-arginine or **B** L-NAME. Number of experiments *in parentheses*. Mean values ± SEM; **P*<0.05

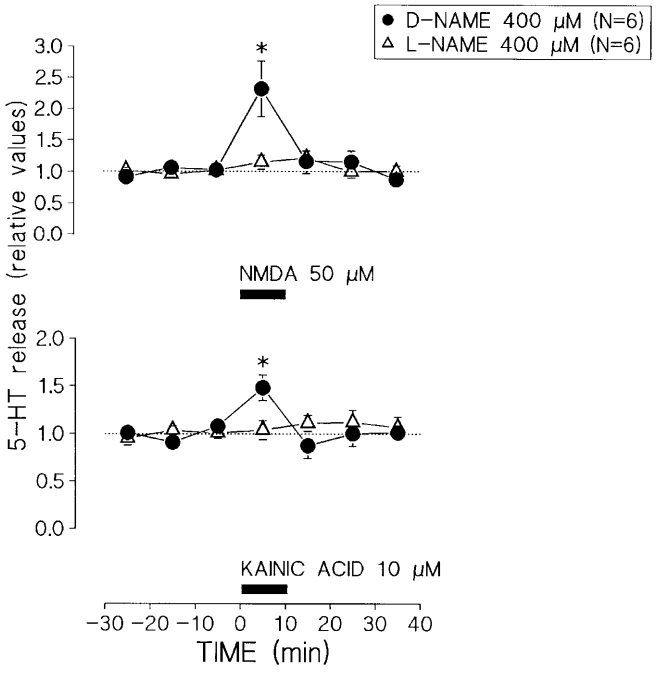

Fig. 3 Effect of L-NAME on the EAA receptor ligand-stimulated release of 5-HT. The mean release rates in the three samples preceding drug superfusion were taken as 1.0. *Horizontal bars* denote the onset and duration of the superfusion with NMDA or kainic acid. The superfusion with L-NAME/D-NAME was started 90 min before superfusion with NMDA or kainic acid and was continued to the end of the experiment. Number of experiments *in parentheses*. Mean values ± SEM; **P*<0.05

Effects of the NOS substrate L-arginine and the NOS inhibitor L-NAME on the basal release of 5-HT

Superfusion with the precursor of NO, L-arginine (200 μM), for 1 h led to a sustained increase in 5-HT release by 40% (Fig. 2A). On the other hand, the NOS inhibitor L-NAME (100 μM and 400 μM) did not influence the release of 5-HT (Fig. 2B).

Effects of L-NAME on the EAA receptor ligand-stimulated 5-HT release

Superfusion of the LC with 50 μM NMDA or 10 μM kainic acid for 10 min led to an increase in the release of 5-HT when D-NAME (400 μM) was present in the superfusion medium. D-NAME did not change the effects of EAA on 5-HT release in the LC (not shown), which were similar to those observed in the absence of D-NAME (Singewald et al. 1998). In the presence of 400 μM L-NAME the NMDA- and kainic acid-induced release of 5-HT was abolished (Fig. 3). D-NAME did not change the basal release of 5-HT (not shown).

Effects of L-NAME on stress-induced 5-HT release

Stress elicited by noise and tail pinch increased the release of 5-HT in the LC in the presence of D-NAME (400 μM; Fig. 4) to a similar extent than in the absence of D-NAME (Singewald et al. 1998). Superfusion of the LC with L-NAME (100 μM and 400 μM) reduced the tail pinch-induced release of 5-HT. The tail pinch- and noise-induced 5-HT release was abolished by 400 μM L-NAME (Fig. 4).

Discussion

In this study we found that NO exerts a stimulatory effect on serotonergic transmission in the LC of the freely moving rat. This is based on our findings that the NO precursor L-arginine as well as different NO donors elicited an increase in 5-HT extracellular levels.

So far the effect of NO on serotonergic neurotransmission (for review see Prast and Philippu 2001) has been investigated in only a few brain areas and led to varying results. In the striatum (Guevara-Guzman et al. 1994) and in the medial preoptic area (Lorrain and Hull 1993) the extracellular concentration of 5-HT was increased by applying locally either NO gas, NO donors or the NO precursor L-arginine. In the hippocampus endogenous NO decreases

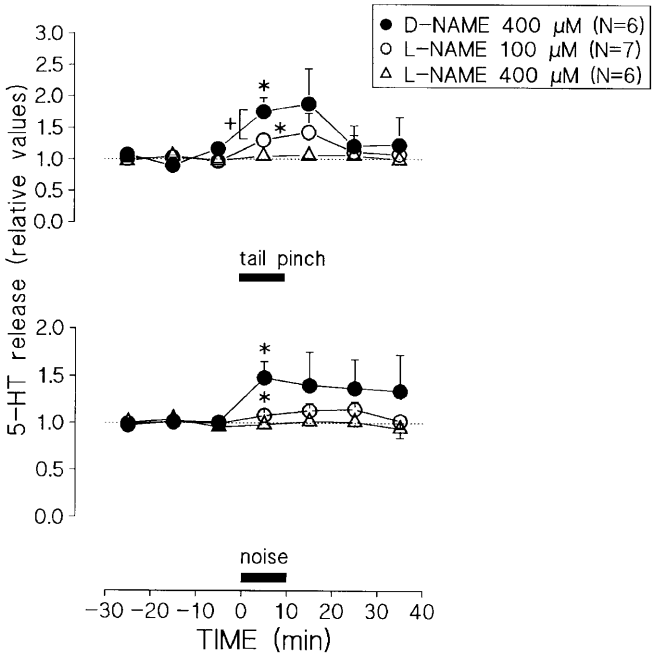

Fig. 4 Effects of L-NAME on the stress-induced release of 5-HT. The mean release rates in the three samples preceding noise and tail pinch were taken as 1.0. *Horizontal bars* denote the onset and duration of the stimulus. The superfusion with L-NAME/D-NAME was started 90 min before exposure to stimuli and was continued to the end of the experiment. Number of experiments *in parentheses*. Mean values ± SEM; **P*<0.05, $^{+}$*P*<0.05 D-NAME vs. L-NAME

the level of 5-HT (Wegener et al. 2000). Recently, it has been observed that NO exerts inverse concentration effects on the 5-HT release in the hypothalamus (Kaehler et al. 1999b) and the raphe nuclei (Smith and Whitton 2000): low concentrations of NO donors decreased the 5-HT release, while higher concentrations had the opposite effect.

In the present study we found that NO facilitates 5-HT release in the LC. It is tempting to speculate that NO directly stimulated the release of 5-HT. However, the processes underlying the NO-mediated increase in 5-HT release are not clear, because NO influences a large number of mechanisms and target systems. For example, in vitro studies on synaptosomes (Asano et al. 1997) have demonstrated that NO prevents 5-HT reuptake, which might lead to an increase in 5-HT extracellular levels. Furthermore, an indirect effect involving additional neurotransmitters in the LC (Singewald and Philippu 1998) may be suspected. Since there is a strong glutamatergic modulation of 5-HT release in the LC (Singewald et al. 1998) and NO has been shown to enhance glutamate release in this brain area (Singewald and Sinner 2000), we investigated whether the NO effect on 5-HT release involves a link via EAA.

LC superfusion with the NOS inhibitor L-NAME abolished the NMDA- and kainic acid-induced activation of serotonergic neurons, while the inactive isomer D-NAME had no effect. Thus it appears that the stimulatory effect of EAA receptor ligands on the release of 5-HT is dependent on the presence of endogenous NO. Thus NO is not only involved in excitatory mechanisms of EAA in the LC (Hall et al. 1998) but also facilitates the action of EAA on putatively inhibitory substrates such as serotonergic transmission within the LC. In support of such an idea heterogeneous effects of NO on the firing of LC neurons have been reported, involving mainly excitation (Pineda et al. 1996), no change, or inhibition (Xu et al. 1998).

Stressful and painful stimuli such as loud noise and tail pinch are known to evoke, in the LC, neuronal discharge and to enhance the release of 5-HT (for review see Singewald and Philippu 1998). The enhanced release of 5-HT by tail pinch and noise is attenuated by NMDA receptor antagonists, but not by AMPA/kainate receptor antagonists (Singewald et al. 1998). EAA receptor antagonists, even at high concentrations, attenuated but did not abolish the effects of the two stimuli on the 5-HT release, thus indicating that stress-induced 5-HT release is partially independent of the EAA input into the LC. On the other hand, the NOS inhibitor L-NAME eliminated the stress-induced release of 5-HT. This finding points to an important role of NO in mediating the action of EAA on the serotonergic transmission during stress. Furthermore, NO seems to be involved in the stimulatory effect of still undefined excitatory neurotransmitter(s) on stress-induced 5-HT release. Alternatively, NO might exert a direct stimulatory effect on 5-HT release during stress. It has been shown in other brain regions that foot shock (Ishizuka et al. 2000) or inescapable tail shock (Grahn et al. 2000) activates the nitrergic system and increases the extracellular level of NO metabolites. Similarly, in the LC stressful events, such as loud noise or tail pinch seem to activate formation of NO which influences the release of the inhibitory neurotransmitter 5-HT. This suggests that the nitrergic system is important for the co-activation of the serotonergic modulation and the excitatory input into the LC. It has been hypothesized that 5-HT release in the LC prevents overstimulation of LC neurons in response to stress stimuli (Singewald and Philippu 1998). The nitrergic influence on 5-HT-release might be of relevance to enhance such inhibitory functions.

Taken together, the results of this in vivo study suggest that the release of 5-HT in the LC is stimulated by NO. Endogenous NO appears to facilitate activation of serotonergic transmission evoked by NMDA, kainic acid or different stress stimuli.

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