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The endogenous neurotransmitter, serotonin, modifies neuronal nitric oxide synthase activities

MAUD BREARD[1], MARIE-AGNES SARI[2], YVES FRAPART[2], JEAN-LUC BOUCHER[2], CLAIRE DUCROCQ[1], & CATHERINE GRILLON[1]

[1]*Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Avenue de la terrasse, Gif sur Yvette 91190, France, and* [2]*Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, Université Paris V, Paris 75005, France*

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Abstract

Serotonin, an important neurotransmitter, is colocalized with neuronal nitric oxide synthase (nNOS), a homodimeric enzyme which catalyzes the production of nitric oxide ($NO^{\cdot}$) and/or oxygen species. As many interactions have been reported between the nitrergic and serotoninergic systems, we studied the effect of serotonin on nNOS activities. Our results reveal that nNOS is activated by serotonin as both NADPH consumption and oxyhemoglobin (OxyHb) oxidation were enhanced. The generation of L-citrulline from L-arginine (L-Arg) was not affected by serotonin in the range of 0–200 μM, suggesting an additional production of oxygen-derived species. But 5-hydroxytryptamine (5HT) induced the formation of both $O_2^{\cdot-}$ and H_2O_2 by nNOS, as evidenced by electron paramagnetic resonance (EPR) and by using specific spin traps. Overall, these results demonstrate that serotonin is able to activate nNOS, leading to the generation of reactive oxygen species (ROS) in addition to the $NO^{\cdot}$ production. Such a property must be considered *in vivo* as various nNOS-derived products mediate different signaling pathways.

Keywords: *Neuronal nitric oxide synthase, reactive oxygen species, superoxide, hydrogen peroxide, serotonin, neurotransmitter*

Abbreviations: *$NO^{\cdot}$, nitric oxide; nNOS, neuronal nitric-oxide synthase; BH4, 6R-tetrahydrobiopterin; CaM, calmodulin; 5HT, 5-hydroxytryptamine or serotonin; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide; BMPO, 5-tert-butyloxycarbonyl 5-methyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; SOD, superoxide dismutase; OxyHb, oxyhemoglobin; HPA, 4-hydroxyphenylacetic acid; NO_2HPA, 4-hydroxy-3-nitrophenylacetic acid*

Introduction

Serotonin (5-hydroxytryptamine, 5HT), one of the most important endogenous neuromediators, is involved in numerous physiological functions such as the regulation of sleep, mood, appetite and also in pathological situations concerning mental disorders. It is particularly well known for its major role in depression. In the dorsal raphe of the rat brain [1], in platelets [2] and in the gastrointestinal plexus of the guinea-pig [3], a proportion of serotoninergic neurons contain neuronal nitric-oxide synthase (nNOS).

nNOS is one of the three homodimeric NOS isoforms that catalyze the conversion of L-arginine (L-Arg) to nitric oxide ($NO^{\cdot}$) and L-citrulline by two sequential monoxygenase reactions [4]. This enzyme contains a C-terminal reductase domain which binds NADPH, FAD and FMN and an N-terminal oxygenase domain with binding sites for heme, 6R-tetrahydrobiopterin (BH4), and L-Arg. Between these

Correspondence: C. Grillon, Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Avenue de la terrasse, Gif sur Yvette 91190, France. Fax: 33 1 69 07 72 47. E-mail: catherine.grillon@icsn.cnrs-gif.fr

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two domains, an amino acid sequence binds calmodulin (CaM), an essential step in NOS activation [5]. In addition to producing $NO^{\cdot}$, nNOS is able to generate hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$). NO vs. superoxide production is mainly regulated by BH4. Formation of $O_2^{\cdot-}$ occurs in the absence of both BH4 and L-Arg. But, in the presence of BH4, both $NO^{\cdot}$ and $O_2^{\cdot-}$ are generated by nNOS depending on the concentration of L-Arg [6]. On the other hand, nNOS was also reported to produce H_2O_2 in the presence of BH4 and in the absence of, or with non-saturating levels of, L-Arg [7] and even in the presence of saturating levels of L-Arg [8]. Therefore, the regulation of nNOS activity, particularly the generation of various free radicals, is mainly under the control of the concentrations of L-Arg and BH4.

Numerous studies have demonstrated close relationships between the nitrergic and serotoninergic systems. $NO^{\cdot}$ was reported to act on 5HT turnover by inhibiting the activity of tryptophan hydroxylase, the main enzyme involved in 5HT biosynthesis [9,10]. On the other hand, 5HT is able to modulate $NO^{\cdot}$ levels. It induces the production of $NO^{\cdot}$ in endothelial cells [11], stomach and colon [12]. But a decrease in 5HT increases both nNOS expression and NOS activity in various parts of the brain [13]. In some cases, an interaction between 5HT and one of its receptors has been implicated in these effects [11,12].

In the present work, we investigated the possibility that 5HT may directly modulate nNOS activity. Experiments were carried out in various conditions with the aim of identifying the role of 5HT in the production of each nNOS-derived oxygen and nitrogen species. Our results show that 5HT enhanced purified recombinant rat nNOS activities. At low 5HT concentrations, NADPH consumption and oxyhemoglobin (OxyHb) oxidation were increased whereas $NO^{\cdot}$ production, evaluated by the formation of L-citrulline from L-Arg, remained unchanged. For greater 5HT concentrations, $NO^{\cdot}$ production decreased, suggesting an uncoupling of nNOS. nNOS-derived oxygen species were identified using spin trapping and electron paramagnetic resonance (EPR) spectroscopy for superoxide and ferric thiocyanate assay for hydrogen peroxide. This modulation of NOS activities by 5HT was specific to nNOS and we demonstrated here that 5HT has a different effect on eNOS and iNOS by decreasing citrulline formation.

Materials and methods

Materials

CaM and 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) were from Calbiochem (VWR International, Fontenay sous bois, France). BH4 was from Cayman Chemical Co. (Ann Arbor, MI, USA). L-[^{14}C]Arg was from Perkin Elmer Life Sciences (Courtaboeuf, France) and 5HT from Acros Organics (Noisy le Grand, France). L-Arg, L-citrulline, human hemoglobin, superoxide dismutase (SOD), catalase, NADPH, FMN, FAD, phosphodiesterase 3′, 5′-cyclic nucleotide activator (CaM)-agarose, hydrogen peroxide, dithiothreitol, diphenyleneiodonium chloride, 4-hydroxyphenylacetic acid (HPA) and 4-hydroxy-3-nitrophenylacetic acid (NO_2HPA) were from Sigma-Aldrich (Saint Quentin Fallavier, France). OxyHb was prepared as described previously [14]. The spin trap 5-*tert*-butyloxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was synthesized following the procedure described by Zhao et al. [15]. Recombinant full-length bovine eNOS, murine iNOS and the heme domain of rat brain nNOS ($nNOS_{OXy}$) were kindly provided by Dr D. J. Stuehr (Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA).

Expression and purification of nNOS

Recombinant full-length rat nNOS was isolated and purified from *Escherichia coli* (BL21) transformed with pCWNOS I plasmids containing rat brain NOS cDNA and pGroESL for expression of chaperone proteins. Culture of transformed *E. coli* and purification of full-length nNOS were performed as before [16], using affinity chromatography on immobilized CaM. The enzyme was purified in either the absence or presence of BH4 according to the needs of the experiments and stored aliquoted at -80°C. In order to remove L-Arg and imidazole introduced during the purification step, nNOS was filtered through Sephadex G25 equilibrated with 50 mM Hepes buffer pH 7.4 just before use. Heme content was determined optically from the [Fe^{II}-CO] − [Fe^{II}] difference spectrum using $\Delta\varepsilon_{444-470\,nm} = 76\,mM\,cm^{-1}$ [17] and the results were well correlated with those of protein quantification using the Bradford assay. nNOS specific activity was 800 ± 45 nmol citrulline min^{-1}-mg $protein^{-1}$ in 50 mM Hepes buffer, pH 7.4 and 300 ± 15 nmol citrulline min^{-1} mg $protein^{-1}$ in 0.1 M phosphate buffer, pH 7.4.

L-[^{14}C]citrulline formation assay

NOS activity was assessed by monitoring the formation of L-[^{14}C]citrulline from L-[^{14}C]Arg as described previously [18]. Briefly, purified nNOS (around 0.3 μg) was added to a mixture containing L-Arg (100 μM, including 2.3 kBq of L-[^{14}C]Arg), NADPH (500 μM), BH4 (4 μM), CaM (700 nM), $CaCl_2$ (1 mM), FMN (4 μM), FAD (4 μM), in the presence of increasing amounts of 5HT (0–500 μM) in 0.1 M phosphate buffer pH 7.4 in a total volume of 100 μl. The concentration of NOS was determined to obtain a maximal consumption of the substrate of

10% after 5-min incubation at 37°C. The reaction was stopped by adding 500 μl of 20 mM sodium acetate pH 5.5 containing 1 mM L-citrulline and 2 mM EDTA. The generated L-[^{14}C]citrulline was separated from L-[^{14}C]Arg by cation-exchange chromatography on a 1 ml Dowex-50W-X8 column, eluted with water and then quantified by liquid-scintillation counting with a Wallac 1414 Winspectral counter (Perkin Elmer, Courtaboeuf, France).

Oxyhemoglobin oxidation assay and NADPH consumption measurement

Other NOS activities were assessed in the same assay in 96-well microtiterplates by monitoring both OxyHb oxidation and NADPH consumption. The reaction was initiated by adding 0.3 μg of nNOS to microplate wells containing, unless otherwise stated, FAD (4 μM), FMN (4 μM), BH4 (4 μM), NADPH (500 μM), L-Arg (100 μM), CaM (700 nM), $CaCl_2$ (70 μM), OxyHb (60 μM) in phosphate buffer 0.1 M pH 7.4, in the presence of increasing amounts of 5HT (0–500 μM) in a total volume of 200 μl. Controls were performed by omitting nNOS or CaM in the reaction mixture. In some experiments, SOD and catalase (final concentration 400 and 2000 U/ml, respectively) were added to the reaction mixture. The reaction was performed at 37°C for 10 min and monitored at various wavelengths by a SpectraMax 190 96-well UV–visible spectrophotometer (Molecular Devices, Sunnyvale, CA) using SoftMaxPro software. For both assays, blank values were determined in parallel in the same microtiterplate in the absence of the enzyme but in the presence of all cofactors and then subtracted from the value obtained in the presence of nNOS. This calculation excludes both a potential autoxidation of NADPH or OxyHb during the assay and a reaction unrelated to the enzyme.

The conversion of OxyHb to methemoglobin by NO- and other reactive oxygen species (ROS) was evaluated by the disappearance of OxyHb, measured by the difference in absorption between 577 and 591 nm (isobestic point) according to Feelisch et al. [19]. As we ran the assay in microtiterplates, we established a standard curve for OxyHb in a 96-well plate in the same conditions of buffer and measurement as in the experiments and used it to quantify OxyHb disappearance.

NADPH oxidation was followed by monitoring the decrease in absorbance at 340 nm and quantitated, as for OxyHb, using a standard curve plotted using known NADPH concentrations.

H_2O_2 formation assay

The generation of H_2O_2 was evaluated by using the ferric thiocyanate assay [20] modified for microplate use. Furthermore, as the measured H_2O_2 can be the result of both the enzymatic production of nNOS and the self-dismutation of superoxide also produced by nNOS, the same experiments were performed according to Rosen et al. [7] in the presence of a spin trapping agent specific for $O_2^{\cdot-}$, DEPMPO, leading to the formation of DEPMPO-OOH adduct. As the self-dismutation rate of superoxide is around $3 \times 10^5\,M^{-1}\,s^{-1}$ at pH 7 [21], compared to the reaction rate of superoxide with DEPMPO which is close to $80\,M^{-1}\,s^{-1}$ [22], a large excess of DEPMPO was used (50 mM) and found to be sufficient to trap a maximum of superoxide (100 mM DEPMPO giving the same results, data not shown). We also checked that DEPMPO did not interfere with the ferric thiocyanate assay. Briefly, 2 μg of nNOS were added to microplate wells containing NADPH (500 μM), L-Arg (100 μM), CaM (700 nM), $CaCl_2$ (70 μM) in triethanolamine/HCl buffer 50 mM pH 7, in the absence or presence of 5HT (1 mM) in a total volume of 200 μl. The reaction was performed at 37°C for 10 min and stopped by addition of 100 μl of 1 M HCl. In some experiments, in order to trap $O_2^{\cdot-}$ produced by nNOS, DEPMPO (50 mM) was added before the enzyme. To 150 μl of each reaction mixture were added 20 μl of a ferrous ammonium sulfate aqueous solution (50 mM) and 30 μl of a potassium thiocyanate aqueous solution (1.8 M) and absorbance at 480 nm was monitored 10 min later. As for the other assays, blanks were determined in the absence of the enzyme and subtracted from the values measured in the presence of nNOS. To quantify H_2O_2 produced by nNOS, a standard curve was plotted in the same conditions as for the experiments with known concentrations of H_2O_2.

$O_2^{\cdot-}$ formation measured by spin trapping and electron paramagnetic resonance (EPR) spectroscopy

A typical incubation mixture contained 500 μM NADPH, 10 mM $CaCl_2$, 600 nM CaM, 10 μM BH4, 50 mM BMPO (final concentrations) and variable amounts (1–5 mM) of 5HT, in a total volume of 760 μl of 50 mM Hepes buffer containing 1 mM dithiothreitol. About 40 μl containing 10.5 μg of nNOS were mixed with the previous mixture and rapidly transferred into the Aqua-X sample cell (Bruker, Wissembourg) and data accumulation was started immediately. All experiments were carried out at 20°C in a Bruker EPR Elexsys 500 spectrometer operating at X-band frequency (9.82 GHz) and incorporating an shq 0011 cavity. The following instrument settings were used: field modulation frequency, 100 kHz; field modulation amplitude, 0.2 mT; time constant, 0.04 s; field sweep, 346–354 mT; microwave power, 6.3 mW; sampling time, 0.04 s; scan time, 20.8 s; and number of scans, 40.

In the presence of superoxide, BMPO leads to BMPO-OOH adduct which turns slowly into BMPO-OH. The two adducts have a very similar spectrum,

and so BMPO-OOH and BMPO-OH spectra were identified in incubations of BMPO performed in the presence of xanthine/xanthine oxidase (BMPO-OOH) or H_2O_2/$FeSO_4$ (BMPO-OH). The relative amounts of BMPO-OOH and BMPO-OH adducts observed in the incubation mixtures were estimated by simulations with Xsophe software (Bruker). The half-life of BMPO-OOH is reported to be around 9–23 min [23] and our results were calculated for the 1–6 min period of the reaction time. So, there is a very small contribution of BMPO-OH to the signals obtained. The amplitude of the second peak of the BMPO-OOH signal (max. 348.8 mT, $g = 2.0115$ and min. 349.2 mT, $g = 2.0098$) was measured and replotted as a function of time. The slopes of the curves, representing the formation rate of the adduct, obtained in the 1–6 min reaction times were compared under different conditions. Control experiments were performed in the absence of nNOS, or of NADPH, or in the presence of 100 μM diphenyliodonium chloride to check that the activity of nNOS was responsible for the generation of the observed superoxide, or in the presence of 100 U/ml SOD to check that the EPR signal is attributed to superoxide.

Peroxynitrite formation assay

Nitration of HPA by peroxynitrite was studied by HPLC analysis after reaction of nNOS in the presence of HPA. The reaction was performed in the same conditions as for the measurement of NADPH consumption and OxyHb oxidation except that OxyHb was omitted and HPA (1 mM) was added to the incubation mixture before the addition of the enzyme. Reaction mixtures were analyzed immediately at the end of the incubation after filtering using a cellulose membrane with 10,000 Da cut-off to remove proteins. HPLC analysis was performed on a C_{18} column equilibrated in 50 mM potassium phosphate, pH 3, containing 5% methanol at a flow rate of 1 ml/min. HPA and NO_2HPA were eluted by increasing the percentage of methanol from 5 to 45% in 10 min and then maintaining 45% methanol for 15 min. Compounds were monitored by UV detection at 280 and 365 nm. In these conditions, the retention times for HPA and NO_2HPA were, respectively 19 and 23.4 min.

The potential formation of 4-nitroserotonin from serotonin upon reaction with peroxynitrite was evaluated by the detection of 4-nitroserotonin using the same HPLC conditions, its retention time being 24.2 min.

UV/visible studies of the interactions of $nNOS_{oxy}$ with 5HT

Studies were carried out at room temperature in 1-cm path length cuvettes containing 0.8–1.2 μM $nNOS_{oxy}$ in 50 mM Hepes buffer, pH 7.4. 5HT was dissolved in buffer and increasing concentrations of 5HT were added to the sample cuvette whereas equivalents volumes of buffer were added to the reference cuvette. Differential spectra were recorded from 380 to 600 nm. Dissociation constant (K_s) was calculated from the plots of $1/\Delta A(\lambda_{432} - \lambda_{395})$ vs. 1/[5HT] using KaleidaGraph software.

Data analysis

All values were determined for several experiments as mentioned in the figure legends and means ± SEM were calculated. Statistical analysis was performed by means of Student's unpaired *t*-test.

Results

Effect of 5HT on nNOS activity

The effect of 5HT on nNOS activity was first studied by following the consumption of NADPH. The addition of increasing amounts of 5HT to nNOS induced a greater consumption of NADPH as shown in Figure 1(A). Only 5 μM 5HT was sufficient to increase nNOS activity significantly by 60%. In the range of 1–200 μM 5HT, NADPH consumption augmented 3.8-fold compared to the control. Above 200 μM 5HT, NADPH consumption remained between 3.8 and 4.9 times the control level without 5HT.

Taking into account that OxyHb reacts not only with $NO^{\cdot}$ but also with various oxygen species to give methemoglobin, we used this assay to examine a global activity of nNOS by evaluating the oxidation of OxyHb by all the products generated by nNOS (Figure 1(B)). Up to 200 μM 5HT, OxyHb oxidation increased with the concentration of 5HT and to 3.7 times the control. Over 200 μM 5HT, OxyHb oxidation remained between 3.7 and 4.7 times the control. These results are very well correlated with those obtained when following NADPH consumption.

For both assays, controls performed in the absence of CaM led to a very weak activity of nNOS (Figure 1(A) and (B)), whatever the serotonin concentration used, showing that nNOS is responsible for the increase in NADPH consumption and OxyHb oxidation in the presence of 5HT. Furthermore, the results obtained in the presence of an inhibitor of nNOS, diphenyleneiodonium, showing a lack of activity of nNOS in the presence of 5HT (Figure 1(A) and (B)) strengthen the fact that only nNOS is involved in the effects observed in the presence of 5HT, excluding an interference by reactions unrelated to NOS catalysis (for example, a weak contamination with another component able to react with 5HT or a metal-catalyzed oxygen reduction).

When SOD and catalase were added to the reaction mixture, the decrease in OxyHb was totally abolished

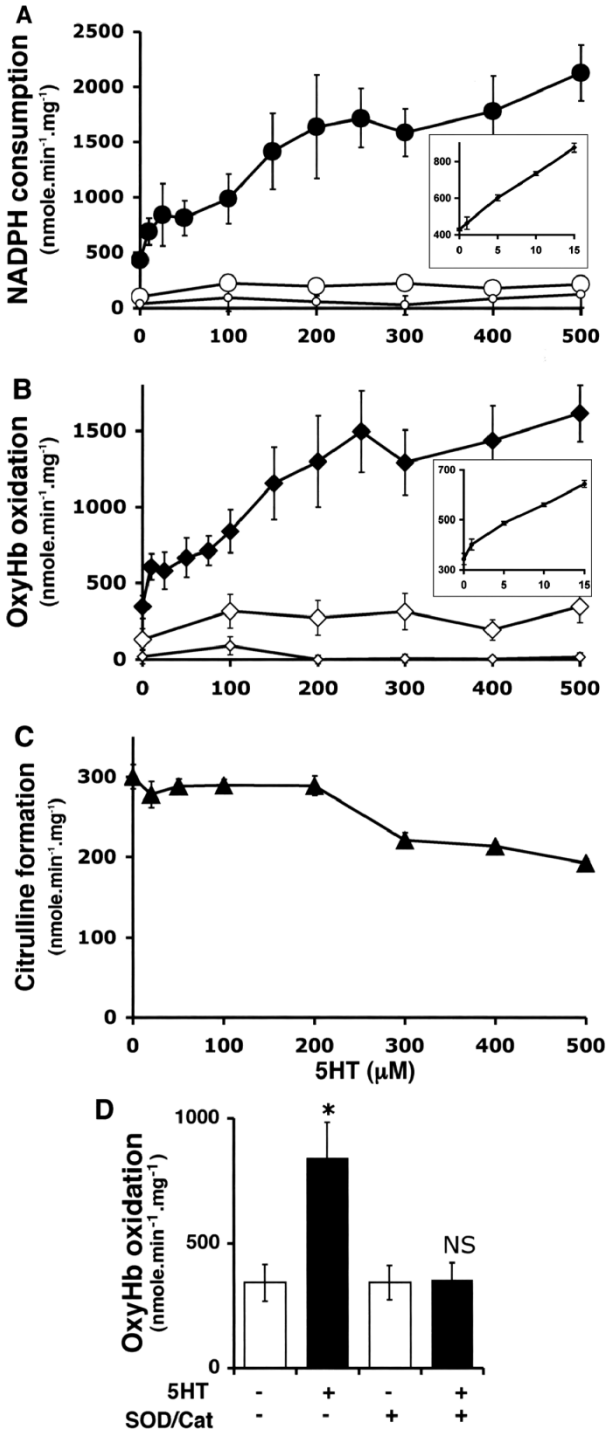

Figure 1. Effect of 5HT on nNOS activity assessed by NADPH consumption, OxyHb oxidation and L-[^{14}C]citrulline formation. Effect of 5HT (0–500 μM) on NADPH consumption (A) and on OxyHb oxidation (B) catalyzed by nNOS. For both measurements, the reaction was started by the addition of nNOS to the mixture of its cofactors (BH4, FAD, FMN, CaM, $CaCl_2$, NADPH), OxyHb (60 μM) and L-Arg (100 μM) (closed symbols). Control experiments were carried out in the absence of CaM (open symbols) or in the presence of 100 μM DPI (small open symbols). The reaction was performed at 37 °C for 10 min as described in Experimental Procedures. Results for low 5HT concentrations (0, 1, 5, 10, 15 μM) are shown in the insets. Data are expressed as mean values of NADPH consumption or OxyHb oxidation (nmol min^{-1} mg^{-1}) ± SEM from six determinations. Statistical significance: $p < 0.05$ for 5HT between 5 and 50 μM, $p < 0.01$ for 5HT ≥ 100 μM when compared to the control without 5HT; not significant for all 5HT concentrations in the absence of CaM when compared to control without 5HT and CaM; not significant for all 5HT concentrations in the presence of DPI when compared to the control without 5HT and with DPI. (C) Effect of 5HT on L-[^{14}C]-citrulline formation. The reaction was started by the addition of nNOS to the mixture of its cofactors (BH4, FAD, FMN, CaM, $CaCl_2$, NADPH) and L-[^{14}C]Arg (100 μM) as described in Experimental Procedures. L-[^{14}C]citrulline was separated from L-[^{14}C]Arg by ion-exchange chromatography. Results are expressed as mean values of citrulline formation (nmol min^{-1} mg^{-1}) ± SEM from six determinations. Statistical significance: $p < 0.01$ for 5HT ≥ 300 μM. (D) Effect of 5HT (100 μM) on OxyHb oxidation in the presence of SOD and catalase. The same experiment as in B was carried out in the presence of SOD (400 U/ml) and catalase (2000 U/ml) for 0 and 100 μM 5HT. Data are expressed as mean values of OxyHb oxidation (nmol min^{-1} mg^{-1}) ± SEM from six determinations. Statistical significance: ⋆, $p < 0.01$; NS, not significant.

(Figure 1(D)), showing that the increase in OxyHb oxidation is the consequence of the generation of ROS by nNOS in the presence of 5HT.

Finally, the effect of 5HT on nNOS activity was studied by following L-[^{14}C]citrulline formation from L-[^{14}C]Arg. As shown in Figure 1(C), 5HT (0–200 μM) was almost without effect on citrulline formation catalyzed by nNOS and by implication, to the same extent, on NO production. But, citrulline formation was reduced to between 73 and 64% of the control value for 5HT concentrations higher than 200 μM.

Taken together, these data show that for the low concentrations (10–200 μM) of 5HT, citrulline formation by nNOS was not affected, whereas NADPH consumption and OxyHb oxidation were strongly increased, suggesting an enhanced formation of ROS by nNOS.

Effect of 5HT on nNOS activity in the absence of L-Arg

To address the question of how 5HT might modify nNOS activity, we investigated the effect of 5HT in the absence of substrate. We chose a concentration of 100 μM 5HT to be in the middle of the concentration range where 5HT has only an effect on the generation of ROS. In the presence of BH4 and in the absence of L-Arg, conditions where nNOS was shown to produce mainly H_2O_2 [7], 5HT (100 μM) induced an increase in nNOS activities (Figure 2), where both NADPH consumption (Figure 2(A)) and OxyHb oxidation (Figure 2(B)) were increased 1.8- and 2.7-fold, respectively. These results are quite similar to the respective 2.6- and 2.4-fold increases obtained in the presence of L-Arg (Figure 2(A) and (B), first columns).

As BH4 is a cofactor of nNOS involved in electron transport, experiments were performed in its absence, using BH4-free purified nNOS. In the absence of both L-Arg and BH4, where nNOS was reported to generate mainly $O_2^{\cdot-}$ [7], both NADPH consumption

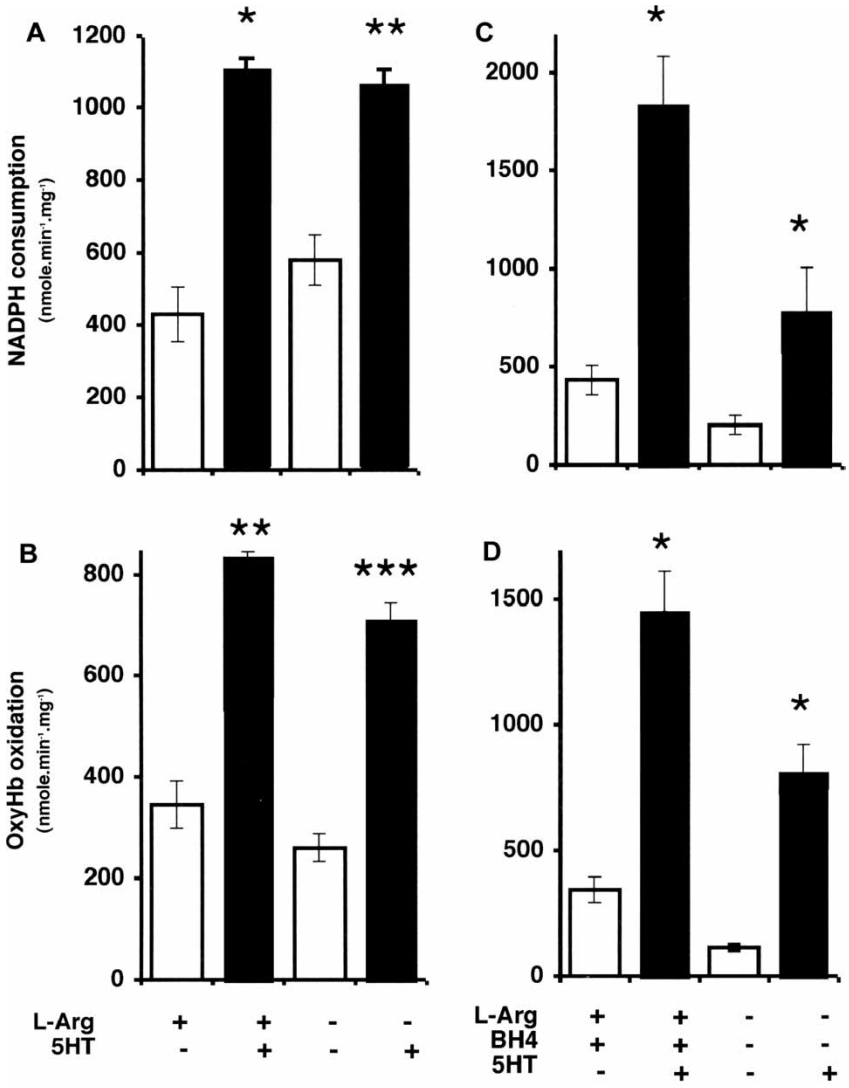

Figure 2. Effect of 5HT on nNOS activity in the presence or absence of L-Arg and BH4. The reaction was started by the addition of nNOS to the mixture of its cofactors (FAD, FMN, CaM, $CaCl_2$, NADPH) at concentrations described in Experimental Procedures, with OxyHb (60 μM), with or without L-Arg (100 μM) and/or BH4 (4μM). A, Effect of 5HT on NADPH consumption assessed by measuring absorbance at 340 nm. B, Effect of 5HT on OxyHb oxidation. The experiments without BH4 were carried out with BH4-free purified nNOS. Data are expressed as mean values of NADPH consumption or OxyHb oxidation (nmol min^{-1} mg^{-1}) ± SEM from one representative experiment out of three. Statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to the respective controls without 5HT.

and OxyHb oxidation are lower than in the presence of L-Arg and BH4 (Figure 2(C) and (D)). Addition of 100 μM 5HT led to 3.9- and 7.1-fold increases respectively in NADPH consumption and OxyHb oxidation.

Identification of nNOS-generated products in the presence of 5HT

NADPH consumption and OxyHb formation are usually used to evaluate the whole NOS activity but they are not precise enough to study the real effect of 5HT on nNOS and are sometimes artifact-prone. Therefore, we next investigated the nature of the ROS formed by nNOS in the presence of 5HT by using more specific methods. To obtain a sufficient amount of $O_2^{\cdot-}$ and H_2O_2 to be measured in these assays, we used greater amounts of nNOS and 5HT, but keeping the same 5HT/nNOS ratio as for the previous experiments, around 10,000–15,000.

First, to identify superoxide, we used spin trapping/EPR spectroscopy with an $O_2^{\cdot-}$ specific spin trap agent, BMPO [7], leading mainly to the formation of the adduct BMPO-OOH. As shown in Figure 3, in the presence of 100 μM L-Arg, very weak formation of BMPO-OOH by nNOS was observed, suggesting that, under these conditions, nNOS formed mainly $NO^{\cdot}$ and only very low amounts of superoxide. In the presence of 1 mM 5HT, a BMPO-OOH spectrum was observed and the formation rate of BMPO-OOH increased, showing the generation of superoxide by nNOS in the presence of 5HT, even in the presence of

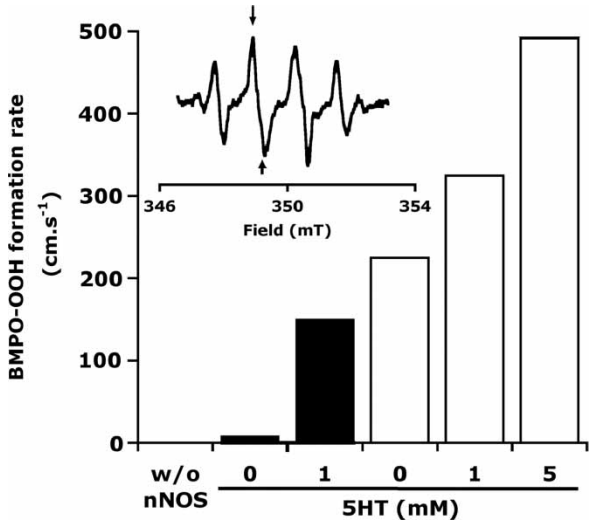

Figure 3. Effect of 5HT on $O_2^{\cdot-}$ generation by nNOS as assessed by spin trapping/EPR spectroscopy. The incubation mixtures consisted of BMPO (50 mM), nNOS (85 nM) and its cofactors (BH4, FAD, FMN, CaM, $CaCl_2$, NADPH) at concentrations mentioned in the Experimental Procedures. 5HT (0, 1 or 5 mM) was added to the mixture. Experiments were carried out in the presence (black columns) or absence (white columns) of 100 μM L-Arg. A negative control was performed without nNOS. The scans were all recorded under identical conditions, which are detailed in the Experimental Procedures. A typical EPR spectrum corresponding to BMPO-OOH is shown in the inset. Results are expressed as the rate of BMPO-OOH adduct formation given by the slope (in 10^{-6} cm s^{-1}) of the amplitude of the second peak (indicated by the arrows in the inset) measured during the first 6 min of the reaction.

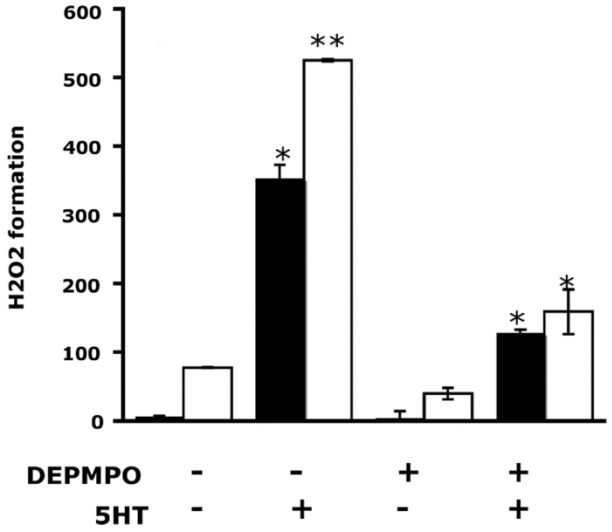

Figure 4. Effect of 5HT on H_2O_2 production by nNOS. The reaction was performed by adding nNOS (60–70 nM final concentration) to the mixture of its cofactors (BH4, CaM, $CaCl_2$, NADPH) without or with 1 mM 5HT and in the absence or presence of DEPMPO (50 mM) to trap the superoxide ions. Experiments were carried out in the presence (black columns) or absence (white columns) of 100 μM L-Arg. The reaction was stopped 10 min later, before adding the reactants to the ferrous thiocyanate assay. Results are expressed in nmol H_2O_2 generated, min^{-1} mg protein^{-1} (mean ± SEM of duplicates from one representative experiment out of two). Statistical significance: *, $p < 0.05$, **, $p < 0.01$ when compared to the respective controls without 5HT.

100 μM L-Arg. We checked that in the absence of nNOS, no formation of BMPO-OOH was observed (Figure 3), demonstrating that superoxide was produced from nNOS. In the absence of L-Arg, we found that nNOS produces $O_2^{\cdot-}$, as shown by the formation of the adduct BMPO-OOH as reported [24]. In the presence of 1 or 5 mM 5HT, the spectrum of BMPO-OOH was higher and the formation rate of the adduct was increased by factors of 1.4 and 2.2, respectively showing an increase in the rate of production of $O_2^{\cdot-}$ by nNOS in the presence of increasing concentrations of 5HT.

Then, in another set of experiments, we attempted to evaluate H_2O_2 produced by nNOS, using the ferric thiocyanate assay (Figure 4). At first, the experiment was performed in the presence of L-Arg. Without 5HT, H_2O_2 was undetectable, but in the presence of 1 mM 5HT, nNOS generated a measurable quantity of H_2O_2 estimated as 350 ± 20 nmol min^{-1} mg enzyme^{-1}. In parallel experiments, we used DEPMPO, an $O_2^{\cdot-}$ specific spin trapping agent to attempt to distinguish between H_2O_2 provided by nNOS and H_2O_2 resulting from $O_2^{\cdot-}$ dismutation. Under these conditions, the H_2O_2 measured in the presence of 5HT represented around 36% of the total measured H_2O_2, suggesting that nNOS generated more $O_2^{\cdot-}$ than H_2O_2.

Furthermore, in the absence of L-Arg, H_2O_2 production by nNOS was seen (Figure 4) as expected. The difference in H_2O_2 estimation in the absence and presence of DEPMPO was attributed to production of $O_2^{\cdot-}$ beside H_2O_2, confirming the previous results observed with EPR spectroscopy. As shown in Figure 3(B), the addition of 1 mM 5HT led to a great increase ($\times 6.7$) in the production of the total H_2O_2, observed without DEPMPO and enhanced 4-fold the real production of H_2O_2 by nNOS observed in the presence of the spin trap.

With the aim of revealing a potential formation of peroxynitrite, we added HPA to the reaction mixture and first checked that it did not interfere with the enzymatic activity in the absence or presence of 5HT by following NADPH consumption and OxyHb oxidation. After a 15- or 30-min incubation with the enzyme in the presence of 0.1 or 1 mM 5HT, we were unable to detect NO_2HPA by HPLC analysis. We were also unable to show the formation of 4-nitroserotonin, a product of the reaction between peroxynitrite and 5HT [25] (data not shown). If NO_2HPA and/or 4-nitroserotonin are formed, their concentrations should be below the detection limit of our HPLC method (around 0.2 μM for both NO_2HPA and 4-nitroserotonin).

Interactions of nNOS$_{oxy}$ with 5HT

The spectrum of the oxygenase domain of nNOS$_{oxy}$ in Hepes buffer displayed a broad absorption Soret band with maxima at 414 nm. This spectrum showed that nNOS$_{oxy}$ contained a mixture of the heme-Fe^{III} complex in the low and high spin states (LS and HS). The progressive addition of 5HT gave rise to a difference spectrum characterized by a minimum at 395 nm and a maximum at 432 nm (Figure 5). This effect of 5HT was in close analogy to the effect of imidazole and suggested that 5HT directly bound to the heme-Fe^{III} (type 2 interaction). This reaction was saturable (inset Figure 5) and the fit of the amplitude of the observed difference spectra $\Delta A(\lambda_{432} - \lambda_{395})$ vs. the 5HT concentration with a hyperbolic function led to a dissociation constant (K_s) of the nNOS$_{oxy}$–heme–Fe^{III}-5HT complex of 230 ± 45 μM.

Comparison of 5HT's effect on different NOS isoforms

Although we focused on the interaction of 5HT with nNOS, the effects of 5HT on other NOS isoforms were assessed with the aim of highlighting the characteristics of the 5HT effect. As shown in the Table I, NADPH consumption was enhanced by 5HT for the two constitutive isoforms but with a greater effect for nNOS (2.3-fold increase) than for eNOS (1.8-fold). OxyHb oxidation was also increased with a greater effect on nNOS (2.5-fold increase) than on eNOS (1.6-fold). Conversely, 5HT did not significantly affect NADPH consumption or OxyHb oxidation by iNOS. As previously shown, 100 μM 5HT did not affect citrulline formation by nNOS, but did inhibit eNOS and iNOS, with respectively 19 and 44% of citrulline production. Therefore, 5HT (100 μM) has a differential effect on the three NOS isoforms and shows a greater activation of nNOS.

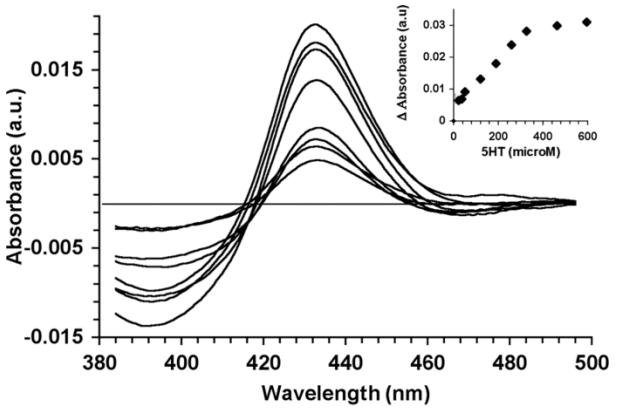

Figure 5. Representative difference spectra obtained upon addition of increasing concentrations of 5HT to nNOS$_{oxy}$. Stepwise additions of 5HT (22–596 μM final concentrations) to a solution of nNOS$_{oxy}$ (1 μM in Hepes buffer) was performed as described in Materials and Methods, and gave rise to a difference spectrum characterized by a minimum at 395 nm and a maximum at 432 nm. Inset, plot of the difference in absorbance $\Delta A(\lambda_{432} - \lambda_{395})$ vs. 5HT concentration.

Discussion

We demonstrate here that 5HT is able to modify nNOS activity. 5HT (5–200 μM) induced an increase in NADPH consumption by nNOS without modifying its production of citrulline and as a consequence, of NO$^{\cdot}$. Additionally, 5HT led to an increase in OxyHb oxidation by the products generated by nNOS. As OxyHb can react with NO$^{\cdot}$ ($3 \times 10^7\,M^{-1}s^{-1}$ [26]), $O_2^{\cdot-}$ ($4 \times 10^3\,M^{-1}s^{-1}$ [27]), $ONOO^-$ ($2 \times 10^4\,M^{-1}s^{-1}$ [28,29]) or H_2O_2 ([30] and our data showing that the H_2O_2 concentrations obtained in our experiments oxidized OxyHb (not shown)) to form methemoglobin, these results suggested that, in the presence of 5HT, nNOS generated ROS, in addition to the production of NO$^{\cdot}$ previously observed following citrulline formation. Superoxide and hydrogen peroxide were identified as oxygen species generated by nNOS in the presence of 5HT, both in the absence and presence of L-Arg. Although saturating concentrations of L-Arg (100 μM and more) were reported to inhibit superoxide generation by nNOS [6,31], this is not the case in the presence of 5HT.

As NO$^{\cdot}$ was reported to react very quickly with $O_2^{\cdot-}$ to form peroxynitrite with a formation rate around $10^{10}\,M^{-1}s^{-1}$ [32], nNOS, which simultaneously produces both species in the presence of 5HT, may also lead to the generation of peroxynitrite. But, we did not manage to show such a formation, maybe because of the assays based on the detection of nitration. In fact, it was shown that nitration of tyrosine *in vitro* by fluxes of NO$^{\cdot}$ and $O_2^{\cdot-}$ occurred only for a very small part (less than 0.1%) of the theoretical maximum [33]. Furthermore, the optimal conditions for peroxynitrite formation may not be achieved. In fact, peroxynitrite formation was shown to be dependent on the proximity of NO$^{\cdot}$ and $O_2^{\cdot-}$ and also on the relative rates of these two components [33]. On the other hand, our results confirm those of Weaver et al. suggesting that nNOS is not a good peroxynitrite synthase, unlike iNOS [34].

In our experiments, we were able to detect $O_2^{\cdot-}$ using spin trapping EPR experiments with BMPO despite the great difference in the reaction rates between superoxide and NO ($10^{10}\,M^{-1}s^{-1}$ [32]) and between superoxide and BMPO ($0.24\,M^{-1}s^{-1}$ [23]). This can be explained by the use of a large excess of BMPO (50 mM), or by a lack of peroxynitrite formation, or by excess superoxide formation compared to NO formation. In fact, Pignitter et al. [35] have recently shown that it is nearly impossible to trap superoxide with a spin trap when there is a concomitant generation of $O_2^{\cdot-}$ and NO$^{\cdot}$ because of the peroxynitrite formation. Therefore, in our studies,

Table I. Effect of 5HT on NOS isoform activities.

	NOS activity (% of control without 5HT)		
	nNOS	eNOS	iNOS
NADPH consumption	229 ± 52 (**)	178 ± 22 (**)	122 ± 9 (NS)
OxyHb oxidation	245 ± 41 (**)	163 ± 16 (**)	136 ± 10 (NS)
L-[^{14}C]citrulline formation	97 ± 2 (NS)	19 ± 3 (**)	44 ± 6 (**)

The activities of the different isoforms were measured as NADPH consumption, OxyHb oxidation to methemoglobin and production of L-[^{14}C]citrulline from L-[^{14}C]arginine, as described in Experimental Procedures. 5HT was tested at 100 μM. Results are the mean ± SEM for 6–18 determinations. (Statistical significance: **, $p < 0.01$; NS, not significant). The specific activities were around 800 ± 45, 120 ± 10 and 750 ± 20 nmol citrulline min^{-1} mg protein^{-1}, respectively for nNOS, eNOS and iNOS in 50 mM Hepes buffer, pH 7.4.

superoxide formation was likely underestimated. Conversely and for the same reasons, H_2O_2 produced by nNOS was likely overestimated.

The modification of nNOS activities by 5HT may be the result of a direct interaction between 5HT and nNOS. Such interaction was observed when 5HT was added to the oxygenase domain of nNOS ($nNOS_{oxy}$). This interaction is probably not involved in the observed effects on the enzyme activities, as they were observed for much lower concentrations (10–100 μM), but could be involved in the inhibition of L-citrulline formation (about-30%) and in the plateau reached for NADPH consumption and OxyHb oxidation, for concentrations of 5HT above 200 μM. This decrease in the enzyme activity could also be the consequence of the formation of reactive oxygen and nitrogen species by nNOS in the presence of 5HT. In fact, $NO^{\cdot}$, $ONOO^{-}$ and H_2O_2 have been shown to be feedback regulators of nNOS activity with IC_{50} of 0.8, 88 and >2 μM, respectively [36]. In our assays, the total production of H_2O_2 by nNOS in the presence of 5HT was around 4.6 μM/min, a higher value than the IC_{50} and the inhibition of citrulline formation by nNOS reached around 30%, which corresponds to the maximum inhibition that can be achieved by H_2O_2 [36].

5HT was shown to give different results with the other NOS isoforms. In contrast to nNOS, 5HT decreased citrulline formation by iNOS without significantly affecting NADPH consumption and OxyHb oxidation. On the other hand, data obtained with eNOS show an increase in NADPH consumption and in OxyHb oxidation, as was shown for nNOS but to a lesser extent. There was a strong parallel decrease in citrulline formation, suggesting that 5HT induced uncoupling of eNOS. This study demonstrated selectivity of the 5HT effect between the three NOS isoforms.

Therefore, 5HT, a well-known neuromediator, is the first endogenous molecule that is able to activate ROS production by nNOS without modifying $NO^{\cdot}$ formation over a wide range of concentrations. It has previously been reported that noradrenaline, another endogenous neuromediator, inhibits $NO^{\cdot}$ production by nNOS (assessed by citrulline formation) for similar concentrations (100 μM), but dopamine, a very similar catecholamine, was ineffective under the same conditions [37]. Melatonin, also, a 5HT metabolite, inhibits citrulline production by nNOS in the same concentration range, through an interaction with CaM binding [38]. Some endogenous derivatives from L-Arg were shown to modify nNOS activity. Agmatine strongly enhanced NADPH oxidation but decreased OxyHb oxidation and is known to induce H_2O_2 generation and cause the alteration of the prosthetic heme [39]. Very recently, N^{G}-monomethyl-L-Arg was reported to activate superoxide formation from nNOS in the presence of L-Arg or in the absence of both L-Arg and BH4 [31]. But this effect was correlated with a decrease in $NO^{\cdot}$ generation, suggesting an uncoupling of nNOS. However, this decrease in $NO^{\cdot}$ formation was assessed only for a concentration of 100 μM whereas N^{G}-monomethyl-L-Arg was shown to induce superoxide generation at 1 μM or less, depending on the presence of BH4 and/or L-Arg. So, the uncoupling of nNOS is not proven at the lower concentrations.

Several exogenous compounds have been shown to be activators of ROS generation by NOS. For example, paraquat, a pneumotoxicant, increases NADPH oxidation and $O_2^{\cdot-}$ generation. But, inversely, it is correlated with a decrease in $NO^{\cdot}$ production, suggesting an uncoupling of nNOS [40]. Adriamycin, a chemotherapeutic agent, has an analogous effect on endothelial NOS [41]. The effects of such redox-active drugs involve the shunt of electrons produced in the reductase domain of NOS, leading to an increase in $O_2^{\cdot-}$ production at the expense of $NO^{\cdot}$ production. Dinitrobenzene, a neurotoxicant, was reported to convert nNOS into a peroxynitrite synthase by increasing citrulline production and generating superoxide [42].

For 5HT, $NO^{\cdot}$ formation was not affected at least for the lowest concentrations. So the effects of 5HT are different from those of the previous effectors. Furthermore, 5HT is an electron donor, as demonstrated by Wrona and Dryhurst [43] and therefore cannot act like the previous molecules, which are electron acceptors. The mechanism of action of 5HT on nNOS remains to be determined. Although

redox-active compounds were shown to involve the reductase domain of nNOS, 5HT may have an effect on the oxygenase domain as demonstrated for high concentrations. Further investigations will be necessary to determine the site(s) of interaction of 5HT with nNOS and its consequences.

Our *in vitro* experiments were designed to examine whether 5HT can directly modulate nNOS activity. Our findings demonstrate that 5HT is able to modify the balance between ROS and $NO^{\cdot}$ production by nNOS. This suggests that the activity of nNOS may be regulated *in vivo*, not only by L-Arg and BH4 local concentrations as previously shown [44] but also by other endogenous molecules like 5HT. As the various nNOS products ($NO^{\cdot}$, $O_2^{\cdot -}$, H_2O_2, peroxynitrite) mediate different signaling pathways, the importance of 5HT in the regulation of nNOS activity must be considered in physiological and pathological situations. Therefore, the biological effects of serotonin remain complex and involve indirect interactions (via specific receptors for example) but also direct effects, as demonstrated in this study on NOS activity.

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