Free Radical Research, March 2009; 43(3): 206–213

informa
healthcare

Serotonin binds to purified neuronal nitric oxide synthase: A possible explanation for ROS production induced by 5HT in the presence of nNOS

MAUD BRÉARD, & CATHERINE GRILLON

Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Avenue de la terrasse, Gif sur Yvette 91190, France

(Received 9 October 2008; revised 3 December 2008)

Abstract
Serotonin (5HT) was shown to induce in vitro the production of ROS in the presence of neuronal nitric oxide synthase (nNOS) in addition to the basal $NO^{\bullet}$ formation. With the aim of understanding this mechanism, this study investigated the potential binding of 5HT to nNOS. By using [^{3}H]5HT, it is reported here that 5HT binds to nNOS, but only when the enzyme is active and in a superoxide-dependent manner. This binding is prevented by DPI but not by L-NAME. The formation of 5HT-nNOS complex was shown to be very well correlated with the production of ROS by 5HT in the presence of nNOS. A mechanism involving nNOS only in its initial step is proposed to explain both the formation of 5HT-nNOS complex and the production of ROS observed in the presence of nNOS and 5HT.

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

Abbreviations: *$NO^{\bullet}$, nitric oxide; nNOS, neuronal nitric-oxide synthase; BH4, 6R-tetrahydrobiopterin; CaM, calmodulin; 5HT, 5-hydroxytryptamine or serotonin; SOD, superoxide dismutase; OxyHb, oxyhaemoglobin; L-NNA, L-N^G-nitroarginine; L-NAME, L-N^G-nitroarginine methyl ester; DPI, diphenyleneiodonium chloride; ROS, reactive oxygen species*

Introduction

Both serotonin (5HT) and nitric oxide ($NO^{\bullet}$) are important neuromediators responsible, respectively, for the vasoconstriction and the vasodilatation of blood vessels. Numerous studies have demonstrated close relationships between the nitrergic and serotoninergic systems [1]. Both molecules interfere with the other one. $NO^{\bullet}$ acts on 5HT turnover by inhibiting tryptophan hydroxylase activity, the main enzyme involved in 5HT biosynthesis [2]. On the other hand, 5HT modulates $NO^{\bullet}$ levels in endothelial cells, stomach and colon [3] and a decrease in 5HT increases both nNOS expression and NOS activity in various parts of the brain [4]. Very recently, a physical interaction between nNOS and the serotonin transporter was demonstrated, leading to reciprocal modulation of their activity [5].

Based on these results, we wondered about a possible direct effect of 5HT on nNOS activity and we demonstrated, in a recent *in vitro* study, that 5HT induced $O_2^{-\bullet}$ and H_2O_2 production in the presence of nNOS without affecting the transformation of L-arginine to L-citrulline and $NO^{\bullet}$ [6]. In the present work, we investigated possible direct binding of 5HT to nNOS and we compared the conditions of 5HT-nNOS complex formation with those leading to the production of ROS. These results led us to propose a mechanism explaining both the binding of 5HT to nNOS and the production of reactive oxygen species (ROS) in the simultaneous presence of nNOS and 5HT.

Correspondence: Catherine Grillon, Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, Rue Charles Sadron, 45071 Orléans Cedex 2, France. Tel: (33) 2 38 25 78 04. Fax: (33) 2 38 25 55 61. Email: grillon@cnrs-orleans.fr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802676662

Materials and methods

Materials

Calmodulin (CaM) was from Calbiochem (VWR International, Fontenay sous Bois, France), BH4, L-N^G-nitroarginine (L-NNA and N^G-nitro-L-arginine methyl ester (L-NAME) were from Cayman Chemical Co. (Ann Arbor, MI) and 5HT hydrochloride was from Acros Organics (Noisy le Grand, France). L-Arg, human haemoglobin, superoxide dismutase (SOD), catalase, bovine serum albumin (BSA), NADPH, FMN, FAD, diphenyleneiodonium chloride (DPI), ferricytochrome C, potassium ferricyanide, sodium ascorbate and cysteine were from Sigma-Aldrich (Saint Quentin Fallavier, France). Oxyhaemoglobin (OxyHb) was prepared as described previously [7].

nNOS preparation

Recombinant full-length bovine nNOS was purified as described previously [6] and then submitted to an ADP-Sepharose affinity chromatography. The nNOS purity was 94–96% and the specific activity was 800 ± 45 nmol citrulline/min × mg protein in 50 mM Hepes buffer, pH 7.4. SDS-PAGE analysis also attested that nNOS preparation was not contaminated by nNOS fragments like its reductase domain. In some experiments, purified recombinant full-length rat nNOS from Cayman Chemicals was used. Its specific activity was 513 nmol $NO^{\bullet}$/min × mg protein in 50 mM Hepes buffer, pH 7.4. Exactly the same results were obtained on 5HT binding using nNOS from both origins (data not shown).

Binding of 5HT to nNOS

The reaction was initiated at 37°C by adding 1.5–3 μg of nNOS to microtubes containing the cofactors (FAD (4 μM), FMN (4 μM), BH4 (4 μM), NADPH (500 μM), CaM (700 nM), $CaCl_2$ (70 μM)) and L-Arg (100 μM) in 0.1 M phosphate buffer pH 7.4 in a total volume of 200 μl, unless otherwise stated, in the presence of 1 μM [^{3}H]5HT creatinine sulphate monohydrate (1.11 TBq/mmol, Perkin Elmer). In some experiments, increasing concentrations of unlabelled 5HT, BSA (100 nM), L-NAME (100 μM), DPI (100 μM), SOD (400 U/ml), catalase (2000 U/ml), ascorbate (1 mM) or cysteine (1 mM) were added to the reaction mixture. The reaction was stopped by addition of Laemmli buffer containing 5% mercaptoethanol and heating at 95°C for 5 min. After SDS-PAGE of the samples, the gel was incubated in isopropanol/acetic acid/water (5:2:13) for 1 h and then in Amplify (GE Healthcare) for 30 min. The dried gel was exposed to a film (HyperfilmTM, GE Healthcare) for ~ 30–40 days at −80°C before development. The intensity of the [^{3}H]5HT-nNOS band depends on this exposure time.

Oxyhaemoglobin oxidation assay and NADPH consumption measurement

The reaction was initiated by adding 0.3 μg of nNOS to microplate wells containing the cofactors, L-Arg (100 μM), OxyHb (60 μM), 5HT (100 μM) in 50 mM Hepes buffer pH 7.4 in a total volume of 200 μl. In some experiments, DPI, L-NNA or L-NAME (100 μM) were used. The reaction was monitored for 10 min at 37°C by a SpectraMax 384 96-well UV-visible spectrophotometer (Molecular Devices) using SoftMaxPro software. Blank values were determined in the absence of the enzyme but in the presence of all cofactors and subtracted from the value obtained with nNOS. This calculation excludes both potential auto-oxidation of NADPH or OxyHb during the assay and a reaction unrelated to the enzyme. The conversion of OxyHb to methemoglobin by $NO^{\bullet}$ and/or other reactive oxygen species was evaluated by the disappearance of OxyHb, measured by the difference in absorption between 577–591 nm according to Feelisch and Kubitzek [8]. We established a standard curve for OxyHb in a 96-well plate in the same conditions 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.

Cytochrome C and ferricyanide reduction assay

Cytochrome C and ferricyanide reduction by nNOS were assessed in 96-well microtiter plates. Wells contained FAD (4 μM), FMN (4 μM, excepted for ferricyanide assay), CaM (700 nM), $CaCl_2$ (70 μM), 5HT (100 μM) in 50 mM Hepes buffer pH 7.4. Ferricytochrome C (40 μM) or potassium ferricyanide (1 mM) and nNOS (0.5 μg) were added just before the beginning of the reaction, which was initiated by adding NADPH (500 μM final concentration) to obtain a final volume of 200 μl. The reaction was performed at 37°C (27°C for ferricyanide) for 10 min and monitored at 550 nm for cytochrome C assay and 420 nm for ferricyanide assay. Blank values were determined as previously. Standard curves of ferrocytochrome C and ferricyanide were established in the same conditions and used to quantify the appearance of ferrocytochrome C or the disappearance of ferricyanide.

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

We have previously shown that 5HT affects nNOS activity by inducing ROS generation in addition to $NO^{\bullet}$ production [6]. We investigated here a potential direct binding of 5HT to nNOS.

Binding of 5HT to nNOS

After 5-min incubation of recombinant rat nNOS (100 nM) with [^{3}H]5HT (1 μM), followed by denaturing SDS-PAGE analysis of the samples, the autoradiography showed a band corresponding to the molecular weight of nNOS monomer (155 kDa) which was attributed to the complex [^{3}H]5HT-nNOS (Figure 1A, control nNOS). The resistance of the 5HT-nNOS complex to heating at 95°C for 5 min in the presence of 2-mercaptoethanol suggests strongly a covalent linkage between 5HT and nNOS. This band was lacking at the initial time of the reaction (T_0) and the label remained for increasing times up to 20 min. In some experiments, we observed a slower kinetic of 5HT binding depending likely on the batch of nNOS used (control, Figure 1B compared to Figure 1A). Control experiments performed with increasing concentrations of non-radioactive 5HT added to the reaction (Figure 1A) or with BSA (100 nM), as irrelevant protein in addition to nNOS (Figure 1B, top) showed, respectively, that [^{3}H]5HT (and not a hypothetical radiolysis derivative) does bind to nNOS and that [^{3}H]5HT does not bind to BSA. On the contrary, an important binding was shown on catalase when it was added to the reaction mixture (Figure 1B, bottom). And, as for nNOS, the binding of [^{3}H]5HT to catalase appears after a 5-min incubation. Taken together, these results demonstrate very rapid binding of 5HT to

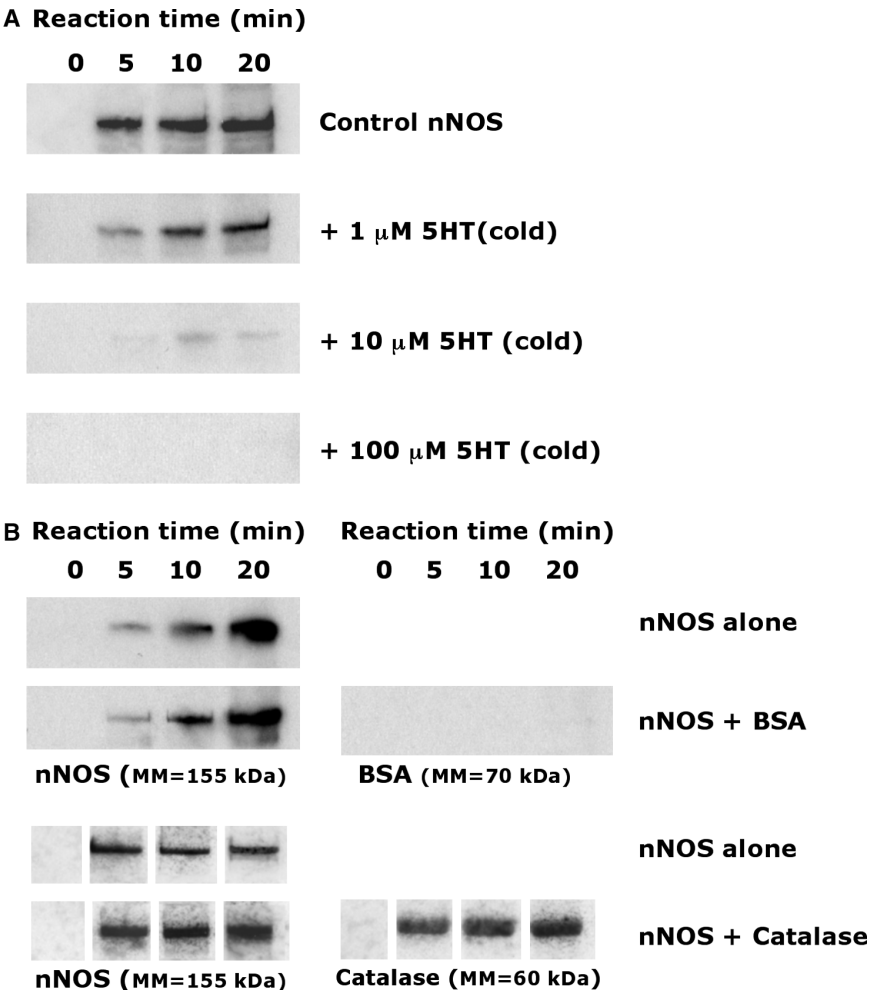

Figure 1. Binding of [^{3}H]5HT to nNOS. (A) [^{3}H]5HT (1 μM) was incubated with recombinant rat nNOS (1.5–3 μg) with or without unlabelled 5HT. (B) [^{3}H]5HT (1 μM) was incubated with nNOS, with nNOS and BSA or with nNOS and catalase. SDS-PAGE from reaction mixtures removed at various times and autoradiography were performed. Bands corresponding to [^{3}H]5HT linked to nNOS monomer (MM = 155 kDa) or BSA (MM = 70 kDa) or catalase monomer (MM = 60 kDA) were visualized according to the reaction time and the conditions of the reaction.

nNOS (and also to catalase when present) during the first 5 min of the reaction.

Conditions required for 5HT binding to nNOS

In the absence of nNOS cofactors, the [^{3}H]5HT-nNOS band did not appear after 5-min reaction (Figure 2A) and was only very weak for longer times (15–20 min), showing that the [^{3}H]5HT binds to nNOS only when the enzyme was active. In the presence of L-NAME, an inhibitor of oxygenase domain belonging to the L-Arg analogue family, the binding of 5HT to nNOS was very similar to that of the control, whereas with DPI, an inhibitor of flavoenzymes which acts at the reductase domain of NOS, there was a very weak binding with respect to the control nNOS lanes (Figure 2A). To address the question of a potential involvement of reactive oxygen species produced in 5HT-nNOS complex formation, we used SOD, catalase or superoxide scavengers. The addition of SOD (400 U/ml), ascorbic acid (1 mM) or cysteine (1 mM) to the reaction mixture totally prevented 5HT binding even after a 20-min reaction (Figure 2B), suggesting the involvement of superoxide in this reaction. Catalase (2000 U/ml) alone did not affect 5HT binding, excluding the involvement of hydrogen peroxide, but catalase and SOD did (Figure 2B). Independently of catalase activity, the absence of 5HT binding to nNOS in the presence of SOD alone, which scavenges only superoxide and furthermore generates hydrogen peroxide, strenghtens the fact that hydrogen peroxide is not involved in this mechanism. From the precedent experiment performed in the presence of L-NAME, we can conclude that $NO^{\bullet}$ is not involved in 5HT binding to nNOS. These results were also confirmed by experiments carried out in the absence of L-Arg (data not shown).

Therefore, the binding of 5HT to nNOS is dependent on enzyme activity and among ROS produced by nNOS, only superoxide is involved in the binding of 5HT to nNOS and not hydrogen peroxide or $NO^{\bullet}$.

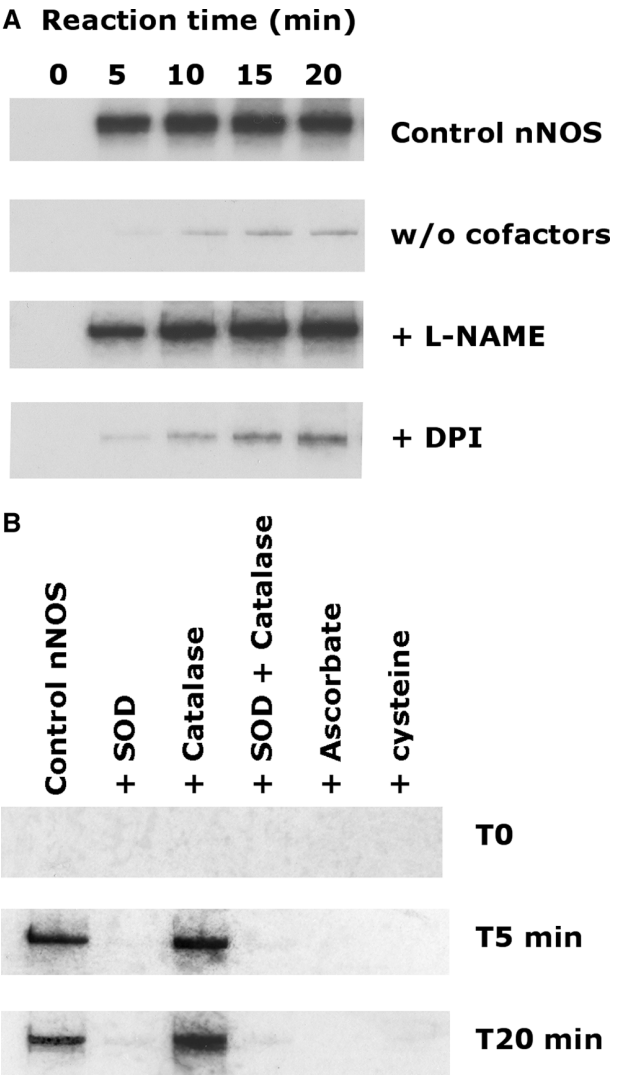

Figure 2. Binding of [^{3}H]5HT to nNOS according to nNOS activity and superoxide production. (A) [^{3}H]5HT (1 μM) was incubated with recombinant rat nNOS (1.5–3 μg) and L-Arg, in the presence or absence of cofactors or in the presence of L-NAME or DPI (100 μM). (B) [^{3}H]5HT (1 μM) was incubated with nNOS (1.5–3 μM) and SOD (400 U/ml), catalase (2000 U/ml), ascorbate or cysteine (1 mM). Bands corresponding to [^{3}H]5HT linked to nNOS monomer (MM = 155 kDa) were visualized according to the reaction time and the conditions of the reaction.

Relation between 5HT binding to nNOS and ROS production in the presence of nNOS and 5HT

In order to compare the role of 5HT both on the binding to nNOS and on the induction of ROS production in the presence of nNOS, complex formation and nNOS activity were both evaluated in various conditions. Taking into account that OxyHb reacts not only with $NO^{\bullet}$ [9] but also with various oxygen species ($O_2^{-\bullet}$ [10], $ONOO^-$ [11] and H_2O_2 [12]) to give methemoglobin, we used this assay to examine a global activity of nNOS. As shown in Figure 3, L-NAME (100 μM) and L-NNA (100 μM), inhibitors of the nNOS oxygenase domain activity, failed to inhibit either NADPH consumption (Figure 3A) or OxyHb oxidation (Figure 3B) in the presence of 100 μM 5HT, whereas they strongly inhibited these activities in the absence of 5HT. Conversely, DPI (100 μM) strongly and similarly inhibited the consumption of NADPH (Figure 3A) and the oxidation of OxyHb (Figure 3B), both in the absence and presence of 100 μM 5HT.

To investigate a potential involvement of the reductase domain in the effect of 5HT, the activity of the reductase domain of nNOS was assessed by the reduction of cytochrome C or ferricyanide. In these conditions, the presence of 5HT (100 μM) did not affect the reduction of cytochrome C (Figure 4A) or ferricyanide (Figure 4B) significantly, excluding an

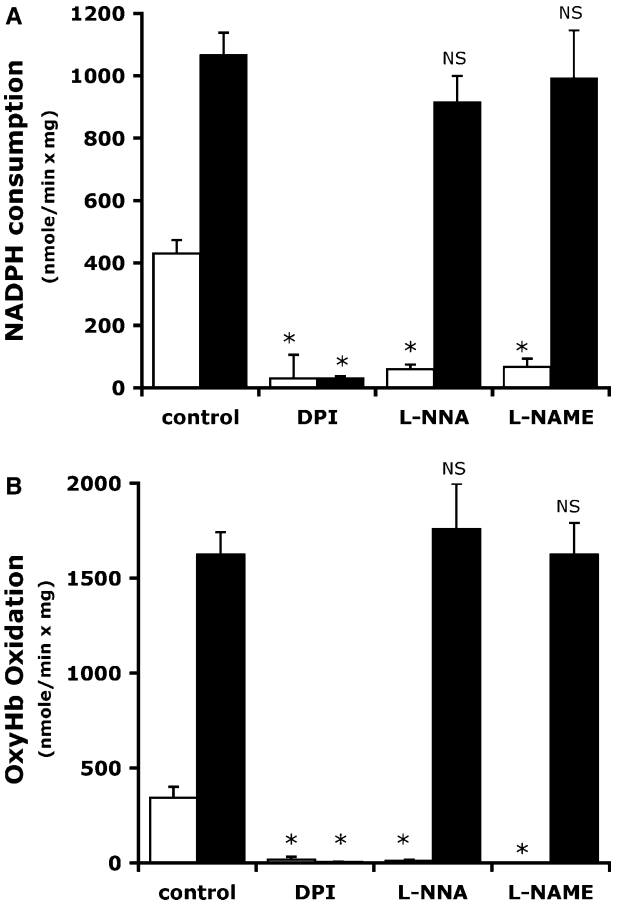

Figure 3. Effects of NOS inhibitors on NADPH consumption (A) and on OxyHb oxidation (B). Recombinant rat nNOS (0.3 μg) was added to the mixture of its cofactors and L-Arg in the presence (filled bars) or absence (open bars) of 5HT (100 μM) and with or without DPI, L-NNA or L-NAME (100 μM). The reaction was performed at 37°C for 10 min. Data are expressed as mean values of NADPH consumption or OxyHb oxidation (nmole/min × mg) ± SEM from two to eight determinations. Statistical significance (Student's unpaired *t*-test): ⋆ $p < 0.001$; NS, not significant when compared with the respective control with or without 5HT.

effect of 5HT on electron flux in the reductase domain of nNOS.

Discussion

In a previous study, we demonstrated that 5HT induced the production of ROS (H_2O_2 and $O_2^{-\bullet}$) in the presence of nNOS without affecting the production of $NO^{\bullet}$ [6]. According to the literature, such results were difficult to explain if we consider that all products come from nNOS activity. In fact, production of superoxide by NOS, following incubation with various molecules such as adriamycin [13], paraquat [14] and very recently superoxide itself [15], is linked to uncoupling of the enzyme, leading to a decrease in $NO^{\bullet}$ production. In this paper, we studied a different possibility. We demonstrate, first, that 5HT is able to bind nNOS, depending on its activity, and particularly on its production of superoxide. Second, the binding of 5HT to nNOS is inhibited by inhibitors of the total nNOS activity, but not by inhibitors of only oxygenase domain. Third, the electron flux in the reductase domain is not affected by the presence of 5HT. And, finally, 5HT binding to nNOS is very well correlated with the 5HT-induced production of ROS in the presence of nNOS. So, the binding of 5HT to nNOS may be an explanation of the ROS production in the presence of 5HT and nNOS which we described previously [6].

An irreversible and SOD-dependent binding of 5HT to rat liver microsomal proteins was previously shown by Uemura et al. [16] in the presence of NADPH and O_2. Furthermore, Perez-Reyes and Mason [17] demonstrated the formation of a radical (a semiquinone imine) upon incubation of 5HT with $O_2^{-\bullet}$. All these results lead us to propose a model explaining both effects of 5HT on nNOS: (Figure 5) 5HT oxidation by nNOS-produced superoxide leads to H_2O_2 formation and $5HT^{+\bullet}$ radical, which can bind to nNOS or which can regenerate by consuming NADPH leading to $NADP^+$ and superoxide.

So, the observed increase in NADPH consumption is due to the addition of NADPH used by nNOS and NADPH used to regenerate 5HT from $5HT^{+\bullet}$. In the same way, the production of $O_2^{-\bullet}$ and H_2O_2 results from this reaction and not from nNOS activity. Thus, 5HT does not modify nNOS activity but induces an independent ROS production. This could explain why we observed previously that $NO^{\bullet}$ production by nNOS is not affected by 5HT. This is in agreement with the fact that electron flux in the reductase domain was not affected by 5HT.

This mechanism involves an initial production of superoxide to initiate the cycle. The possibility that the initial $O_2^{-\bullet}$ come from another source than nNOS (flavins for example) was excluded: first because at T_0, 5HT did not bind to catalase (Figure 1B, bottom) although it was incubated with flavins and all cofactors before the addition of nNOS; second because ROS production observed by oxyhaemoglobin oxidation is calculated by subtracting the blank consisting of all cofactors including flavins and 5HT without nNOS; third, because we demonstrated previously that calmodulin removing inhibits the effect of 5HT on ROS production [6]; and finally because DPI inhibits both 5HT binding on nNOS and ROS production. Therefore, the presence of nNOS is required to produce the initial $O_2^{-\bullet}$.

As nNOS was shown to produce a small amount of superoxide in basal conditions even in the presence of BH4 and L-Arg [6,18], this would be the initiation of the reaction. In the same way, and to strengthen these results, we reported previously that, in the absence of L-Arg (when nNOS produces mainly superoxide), 5HT increased ROS production [6]. Thus, superoxide production by nNOS may be the initiator of the chain reaction leading to ROS

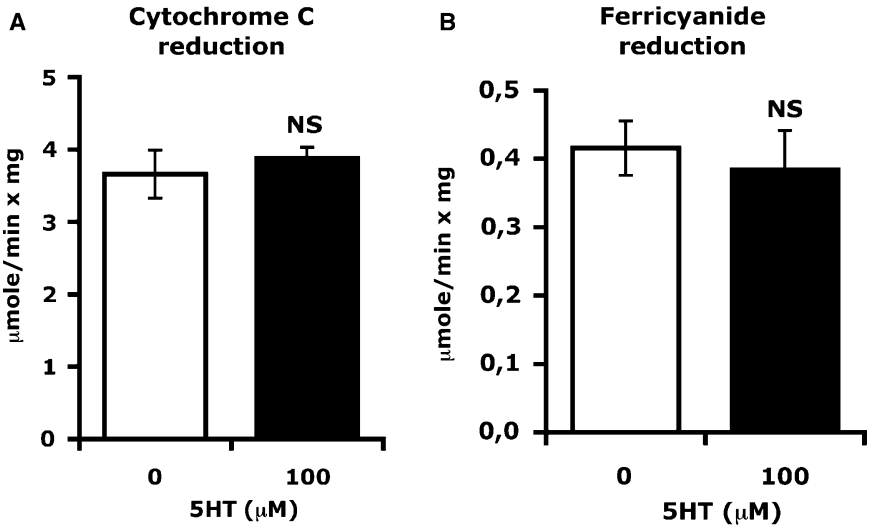

Figure 4. Effects of 5HT on reduction of cytochrome C and ferricyanide by nNOS. NADPH (500 μM) was added to the reaction mixture containing recombinant bovine nNOS (0.5 μg) with or without 5HT (1 μM) and, according to the experiment, cytochrome C (40 μM) (A) or ferricyanide (1 mM) (B). The reaction was performed at 37°C or 27°C, respectively, for 10 min. Data are expressed as mean values of reduced-ferricytochrome C or ferricyanide (μmole/min × mg) ± SEM from eight to nine determinations. Statistical significance: NS, not significant.

production and to 5HT binding to nNOS. The final production of $O_2^{-\cdot}$ would support the reaction, leading to an amplification of ROS production.

Whether the initial superoxide comes from the oxygenase or the reductase domain of nNOS remains unclear. According to the literature, nNOS is able to produce superoxide [19] (mainly by its oxygenase domain [20]) but ~ 10% of its production appears to depend on enzyme catalysed electron transport [20]. On the other hand, we demonstrated here that, contrary to DPI which is known to totally inhibit nNOS activity, i.e. both $NO^{\cdot}$ [21] and $O_2^{-\cdot}$ [19] production, L-NAME does not inhibit 5HT binding to nNOS and ROS production. However, L-NAME (100 μM) was reported to maintain 30% of $O_2^{-\cdot}$ formation by nNOS [19] and that may be enough to initiate the formation of the 5HT radical.

The notion of the formation of a radical reacting with nNOS to form a covalent complex was strengthened by our results showing very strong binding between 5HT and nNOS. The possible involvement of a 5HT semiquinone-imine radical was based on the work of Perez-Reyes and Mason [17]. By using EPR experiments they have shown the formation of the 5HT semiquinone imine (imino-indole ring form), suggesting that this radical form is the most stable and this radical was supposed to be involved in the stimulation of NADPH oxidation and oxygen consumption by 5HT. In our case, we observed, in addition, the binding of 5HT radical to nNOS.

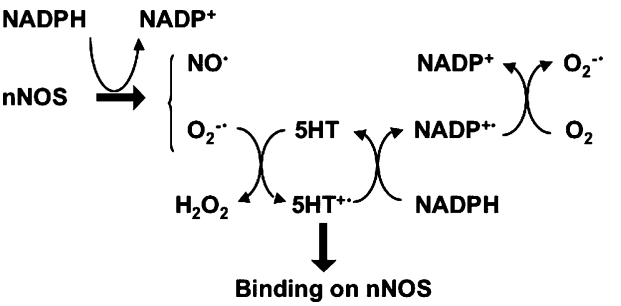

Figure 5. A proposed model that illustrates the effect of 5HT: Binding of 5HT to nNOS and production of $O_2^{-\cdot}$ and H_2O_2; adapted from Perez-Reyes and Mason [17].

Such a radical would be able to react with thiol functions of proteins. The fact that BSA (used here as an irrelevant protein) contains only one thiol could explain the lack of binding of 5HT to BSA, but 5HT binds to bovine catalase which contains four cysteines. In the case of bovine or rat nNOS, 24 cysteine residues are present. As nNOS still produce $NO^{\cdot}$, it is likely that Cys415, involved in the interaction with the heme, and the two cysteines necessary to maintain the zinc thiolate and consequently the stability of the dimer are not modified. Logically, 5HT binding should occur on Cys present in the close area where superoxide is produced if 5HT can be there. However, as explained below, this nNOS domain remains to be determined. In this way, the identification of cysteine(s) bound to 5HT would also help to clarify the nNOS domain involved in superoxide production. On the other hand, the fact that 5HT binds also to catalase in the presence of nNOS suggests that either superoxide and/or 5HT radical diffuse from the site of production to the medium. So, as shown for catalase, 5HT binding can occur everywhere if Cys are available.

In our *in vitro* model, nNOS is the only possible target for 5HT binding but it cannot be excluded that, *in vivo*, 5HT binds to other proximal proteins.

In the brain, 5HT is mostly concentrated in vesicules and a low level is in the cytoplasma, in possible contact to nNOS. Besides such a compartimentalization, a physical interaction between nNOS and the serotonin transporter (SERT) has been reported, suggesting proximity between nNOS and 5HT inside cells [5]. A 5HT content ~ 0.4 μM has been reported in cultured human neurons from hippocampus and cortex [22], which is not far from the concentrations we used in this work. Furthermore, the possibility that such a reaction takes place *in vivo* is strengthened by the fact that endogenous 5HT levels have been reported to be increased in pathological conditions (lesions or ischemia [23], primary pulmonary hypertension [24]).

Serotonin was recently shown to be transamidated to fibrinogen [25] or to small GTPases by transglutaminases [26]. In this case, an enzyme is responsible for the binding and the serotonylation is considered as a signal transduction pathway leading to the release of platelet α-granules. In the same way, we have shown here that 5HT is able to bind purified nNOS upon $O_2^{-\bullet}$ production by nNOS and to induce ROS formation in the presence of NADPH and O_2. Therefore, in addition to its usual roles mediated by various specific receptors, serotonin seems to play new roles because of its ability to bind other molecules and particularly proteins.

In conclusion, we demonstrated that 5HT is able to bind to nNOS and we propose a mechanism, fitting with all our results, involving the 5HT radical formation upon superoxide production by nNOS, resulting in 5HT binding to nNOS, NADPH consumption and ROS production. The present *in vitro* study suggests that, *in vivo*, in the presence of nNOS, 5HT may increase the production of ROS. And, finally, our results could be widened to a more general situation: in the presence of any source of superoxide and in the presence of NADPH and O_2, 5HT may result in deleterious effects *in vivo* due to amplification of ROS production.

Acknowledgements

We wish to thank Claire Ducrocq for very useful discussions concerning this work and Professor J. Y. Lallemand for providing a fellowship for M.B.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Kadowaki M, Kuramoto H, Kuwahara A. Morphological relationship between serotoninergic neurons and nitrergic neurons for electrolytes secretion in the submucous plexus of the guinea pig distal colon. Brain Res 1999;831:288–291.

[2] Kuhn D, Arthur R. Inactivation of brain tryptophan hydroxylase by nitric oxide. J Neurochem 1996;67:1072–1077.

[3] Stoner M, Scherr A, Lee J, Wolfe L, Kellum J. Nitric oxide is a neurotransmitter in the chloride secretory response to serotonin in rat colon. Surgery 2000;128:240–245.

[4] Ramos AJ, Tagliaferro P, Lopez-Costa JJ, Lopez EM, Pecci Saavedra J, Brusco A. Neuronal and inducible nitric oxide synthase immunoreactivity following serotonin depletion. Brain Res 2002;958:112–121.

[5] Chanrion B, Mannoury la Cour C, Bertaso F, Lerner-Natoli M, Freissmuth M, Millan MJ, Bockaert J, Marin P. Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity. Proc Natl Acad Sci USA 2007;104:8119–8124.

[6] Breard M, Sari MA, Frapart Y, Boucher JL, Ducrocq C, Grillon C. The endogenous neurotransmitter, serotonin, modifies neuronal nitric oxide synthase activities. Free Radic Res 2007;41:413–423.

[7] Murphy ME, Noack E. Nitric oxide assay using hemoglobin method. Methods Enzymol 1994;233:240–250.

[8] Feelisch M, Kubitzek D. The oxyhemoglobin assay. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. Chichester, England: John Wiley & Sons Ltd; 1996. p 455–477.

[9] Kelm M, Dahmann R, Wink D, Feelisch M. The nitric oxide/superoxide assay. Insights into the biological chemistry of the NO/O-2. Interaction. J Biol Chem 1997;272:9922–9932.

[10] Sutton HC, Roberts PB, Winterbourn CC. The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin. Biochem J 1976;155:503–510.

[11] Romero N, Radi R, Linares E, Augusto O, Detweiler CD, Mason RP, Denicola A. Reaction of human hemoglobin with peroxynitrite. Isomerization to nitrate and secondary formation of protein radicals. J Biol Chem 2003;278:44049–44057.

[12] Weiss SJ. Neutrophil-mediated methemoglobin formation in the erythrocyte. The role of superoxide and hydrogen peroxide. J Biol Chem 1982;257:2947–2953.

[13] Vasquez-Vivar J, Martasek P, Hogg N, Masters BS, Pritchard KA Jr, Kalyanaraman B. Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. Biochemistry 1997;36:11293–11297.

[14] Margolis AS, Porasuphatana S, Rosen GM. Role of paraquat in the uncoupling of nitric oxide synthase. Biochim Biophys Acta 2000;1524:253–257.

[15] Sun J, Druhan LJ, Zweier JL. Dose dependent effects of reactive oxygen and nitrogen species on the function of neuronal nitric oxide synthase. Arch Biochem Biophys 2008;471:126–133.

[16] Uemura T, Matsushita H, Ozawa M, Fiori A, Chiesara E. Irreversible binding of 5-hydroxytryptamine and 5-hydroxytryptophan metabolites to rat liver microsomal protein. FEBS Lett 1979;101:59–62.

[17] Perez-Reyes E, Mason RP. Characterization of the structure and reactions of free radicals from serotonin and related indoles. J Biol Chem 1981;256:2427–2432.

[18] Cardounel AJ, Xia Y, Zweier JL. Endogenous methylarginines modulate superoxide as well as nitric oxide generation from neuronal nitric-oxide synthase: differences in the effects of monomethyl- and dimethylarginines in the presence and absence of tetrahydrobiopterin. J Biol Chem 2005;280:7540–7549.

[19] Pou S, Keaton L, Surichamorn W, Rosen GM. Mechanism of superoxide generation by neuronal nitric-oxide synthase. J Biol Chem 1999;274:9573–9580.

[20] Yoneyama H, Yamamoto A, Kosaka H. Neuronal nitric oxide synthase generates superoxide from the oxygenase domain. Biochem J 2001;360:247–253.

[21] Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R, Nathan CF. Inhibition of macrophage and endothelial

cell nitric oxide synthase by diphenyleneiodonium and its analogs. Faseb J 1991;5:98–103.
[22] Fatima Shad K, Arshad Saeed S. The metabolism of serotonin in neuronal cells in culture and platelets. Exp Brain Res 2007;183:411–416.
[23] Westergaard E. The effect of serotonin on the blood-brain barrier to proteins. J Neural Transmission 1978:(supp), 14: 9–15.
[24] Kereveur A, Callebert J, Humbert M, Herve P, Simonneau G, Launay JM, Drouet L. High plasma serotonin levels in primary pulmonary hypertension. Effect of long-term epoprostenol (prostacyclin) therapy. Arterioscler Thromb Vasc Biol 2000;20:2233–2239.
[25] Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. Nature 2002;415:175–179.
[26] Walther DJ, Bader M A. unique central tryptophan hydroxylase isoform. Biochem Pharmacol 2003;66:1673–1680.

This paper was first published online on iFirst on 4 February 2009.