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Evidence for a control of plasma serotonin levels by 5-HT_{2B} receptors in mice

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Running title. 5-HT_{2B} receptor and plasma serotonin

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Abstract

A correlation between high plasma serotonin levels and total pulmonary resistance was reported in more than 80% of pulmonary hypertensive patients. When submitted to chronic hypoxia (10% O_2 for more than 3 weeks), wildtype mice develop lung vascular remodeling and pulmonary hypertension. We previously reported that, by contrast, the development of these hypoxia-dependent alterations is totally abolished in mice with permanent (genetic) or transient (pharmacologic) inactivation of the serotonin 5-HT_{2B} receptor. In the present study, we asked whether 5-HT_{2B} receptors could be involved in the control of plasma serotonin levels. Further investigating the chronic-hypoxic-mouse model of pulmonary hypertension, we first show that in wildtype mice, plasma serotonin levels and 5-HT_{2B} receptors expression were significantly increased after chronic exposure to hypoxia. This increase appeared before significant changes in remodeling factors could be detected and persisted when the pathology was established. Conversely, in mice with either genetically or pharmacologically inactive 5-HT_{2B} receptors, plasma serotonin levels were not modified by chronic hypoxia. We then confirmed that 5-HT_{2B} receptors can control plasma serotonin levels by providing *in vivo* evidence that an acute agonist stimulation of 5-HT_{2B} receptor triggers a transient increase in plasma serotonin that is serotonin transporter dependent and blocked by 5-HT_{2B} receptor selective antagonist or genetic abalation. Our data support the notion that a 5-HT_{2B} receptor-dependent regulation of serotonin uptake is implicated in the control of plasma serotonin levels.

Introduction

Primary pulmonary hypertension is a rare but fatal condition characterized by an elevation in pulmonary arterial pressure that is associated with pulmonary vasculature remodeling (Loscalzo, 2001). Several studies have suggested a role for serotonin (5-hydroxytryptamine, 5-HT) in pulmonary hypertension (for review see Egermayer et al., 1999). A correlation between high plasma serotonin levels and total pulmonary resistance, first observed in a patient with a defect in platelet serotonin storage capacity (Hervé et al., 1990), was confirmed in more than 80% of pulmonary hypertensive patients (Hosoda, 1994; Hervé et al., 1995; Kéreveur et al., 2000). In rats, roles for serotonin in the initiation and progression of monocrotaline induced pulmonary hypertension have been suggested since plasma serotonin levels are raised, and pulmonary arteries show hyper-reactivity to serotonin. In these experiments, treatments with 5-HT_2R antagonists inhibited the development of pulmonary hypertension along with a decrease in the plasma serotonin concentrations and the number of proliferative cells, substantiating the pivotal role of plasma serotonin in the development of monocrotaline- or interleukin-6-induced pulmonary hypertension (Miyata et al., 2001).

The main peripheral sources of serotonin are as a neurotransmitter and local hormone in the gastrointestinal tract enterochromaffin cells and pulmonary neuroepithelial bodies. Blood serotonin, which is concentrated in the platelet-dense granules (>99%), is almost absent from plasma (nanomolar range) (Da Prada and Picotti, 1979). Defective platelet uptake (Awabdy et al., 2003) or release from activated platelets could generate increased plasma serotonin as in platelet storage diseases (Hervé et al., 1990). Furthermore, lungs have been reported to remove efficiently “free” serotonin from plasma (Gillis and Pitt, 1982). High levels of plasma serotonin

could, thus, result from an impaired endothelial metabolism that would decrease 5-hydroxyindole-acetic acid (5-HIAA) levels and/or serotonin uptake. As well as being a pulmonary vasoconstrictor, plasma serotonin may exert a co-mitogenic influence on pulmonary vascular smooth muscle cells (Liu and Fanburg, 2005) and contribute to both hypoxia-induced acute vasoconstriction and chronic vascular remodeling trough various serotonin receptors (5-HTRs) and the serotonin transporter (SERT).

When exposed to few weeks of hypoxia (10% O_2), wildtype mice develop pulmonary hypertension and lung vascular remodeling recapitulating at least in part the human pathology. Smooth muscle and endothelial cells from human and rodent pulmonary arteries express mRNAs for 5-HT_{1B}, 5-HT_{2A}, 5-HT_7 and 5-HT_{2B}Rs and SERT (Ullmer et al., 1995). Mice deficient for 5-HT_{1B}Rs or for SERT are still responsive to chronic hypoxia though the increase in pulmonary artery blood pressure and pulmonary vascular remodeling are reduced when compared to control mice. Using the chronic-hypoxic-mouse model of pulmonary hypertension, we previously reported that the hypoxia-dependent increases in pulmonary blood pressure and lung remodeling are totally abolished in mice with genetically or pharmacologically inactive 5-HT_{2B}Rs (Launay et al., 2002). An interference of the 5-$HT_{2B}^{-/-}$ mice congenital cardiomyopathy to the lung hypoxic response has been ruled out since: (i) The cardiac phenotype of 5-$HT_{2B}^{-/-}$ mice is characterized by left ventricular dysfunctions, but no apparent right ventricular alterations, (ii) the basal pulmonary pressure in 5-$HT_{2B}^{-/-}$ mice is indistinguishable from $^{+/+}$ mice and (iii) in $^{+/+}$ mice exposed to chronic hypoxia in presence of RS-127445, one of the most selective 5-HT_{2B}R antagonist, all tested lung parameters are not significantly different from untreated values, clearly demonstrating that the activation of 5-HT_{2B}Rs is required for hypoxia-induced pathology independently of any prior cardiac phenotype. In both humans and mice, pulmonary

hypertension is associated with a substantial increase in 5-HT_{2B}R, 5-HT_{1B}R, but not 5-HT_{2A}R expression in pulmonary arteries (Launay et al., 2002). Recently, a heterozygous mutation causing premature truncation of the 5-HT_{2B}R protein was found in one patient with fenfluramine-associated pulmonary hypertension (Blanpain et al., 2003) that clearly increases proliferative index of this mutant receptor (Deraet et al., 2005). However, the link between 5-HT_{2B}Rs and pulmonary hypertension pathophysiology remains to be discovered.

Since a correlation between plasma serotonin levels and total pulmonary resistance was consistently observed in pulmonary hypertensive patients, we asked whether 5-HTRs could participate in the control of plasma serotonin levels. In the present study, we are reporting that 5-HT_{2B}Rs are involved in a carrier-dependent control of plasma serotonin levels in mice.

Materials and Methods

Reagents. LY-266097, 1-(2-chloro-3,4-dimethoxybenzyl)-6-methyl-1,2,3,4-tetrahydro-9*H*pyrido[3,4-b]indole hydrochloride, and RS-127445, 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine, were kindly provided by Lilly and Roche companies. BW-723C86, 1-[5-(2-thienylmethoxy)-1H-3-indolyl]propan-2-amine HCl and all other chemicals were reagent grade, purchased from usual commercial sources. The radioactive compound [^{3}H]LY-266097 (925 GBq/mmol) was provided by Dr J. Würch (Roche, Basel), whereas [^{3}H]serotonin binoxalate (745 GBq/mmol) and [^{3}H]$NaBH_4$ (2.28 TBq/mmol) were purchased from DuPont NEN.

Hypoxia. The 5-HT_{2B}R mutant mice ($^{-/-}$) were generated in the 129PAS pure genetic background that was used as controls ($^{+/+}$) (Nebigil et al., 2000). Mice 6 weeks of age (20-25 g) were randomly divided into groups of 50% male 50% female mice, maintained either in room air (21% O_2) or in normobaric chambers (21–23°C) with hypoxic air (10% O_2, 90% N_2, two or five weeks (Launay et al., 2002). Normoxic mice were kept in the similar room with the same 12/12 light-dark cycle. Classically, groups of 10 mice were treated with vehicle, a highly selective 5-HT_{2B}R (full neutral) antagonist RS-127445 at 1 mg/kg/day or dexfenfluramine at the "therapeutic" dose of 2.5 mg/kg/day delivered by mini-osmotic pumps (Alzet) from the beginning of the hypoxic treatments. All animal care and procedures were in accordance with institutional guidelines and European regulations.

Cardiovascular evaluations. Mice were anesthetized with intraperitoneal (i.p.) injection of ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg). Right ventricular systolic pressure (RVSP) was measured by insertion into the right ventricle of a 26-gauge needle connected to a

pressure transducer. After a blood sample was collected by cardiac puncture for measurements, the pulmonary artery was cannulated through an incision in the right ventricle and perfused with Earle's balanced salt solution (37°C, 20 cm H_2O pressure).

Lung parameters. Lung vascular bed around pulmonary arteries was collected and prepared for culture (Launay et al., 2002). The culture was maintained in DMEM supplemented with serotonin-depleted serum (10%) for 24-h, washed with HBSS and grown for 24-h in serum-free medium {DMEM/F-12 (1:1) with 5 µg/ml insulin, 5 µg/ml transferrin, 30 nmol/L selenium, 20 nmol/L progesterone, and 100 µmol/L putrescine} before serotonin uptake was performed.

To measure serotonin uptake, after incubation for 1 min with 25, 50, and 100 nmol/L [^{3}H]serotonin binoxalate, cells were lysed by addition of 0.1N NaOH and radioactivity was counted using liquid scintillation spectrometry. Specific serotonin uptake was assessed as the difference between total uptake and uptake in the presence of 1 µmol/L paroxetine. Due to a lack of materials, only three concentrations of [^{3}H]serotonin were used, leading only to an estimate of the initial rates (1 min uptake) of specific serotonin uptake *vs.* serotonin concentration. After a non-linear regression analysis according to a hyperbolic model (EZ-FIT program), we determined the Michaelis-Menten constant (Km) and initial rate (Vi) of serotonin uptake into lung vascular cells.

Dosage of the 5-HT_{2B}R expression was performed by binding experiments using a selective tritiated radioligand (LY-266097). Briefly, cell membranes were prepared by four cycles of homogenization (Brinkman P10 disrupter) and centrifugation (48,000 x g for 15 min). The assay was established to achieve steady state conditions and to optimize specific binding (Kellermann et al., 1996). 50 µg of membrane proteins were incubated with 1 nM [^{3}H]LY-

266097 at 4°C for 60 min. Nonspecific binding was determined using 1 µM RS-127445. Assays were terminated by vacuum filtration through glass fibre filters (GF/B), which had been pretreated with 0.1% polyethyleneimine. Total and bound radioactivity was determined by liquid scintillation counting. Greater than 80% specific binding was achieved in these assays.

For elastolytic activities, conditioned media (380 µL) were incubated at 37°C with 200 µg [^{3}H]elastin produced by radiolabeling of purified insoluble elastin from bovine nuchal ligament (Elastin Products Co) with [^{3}H]$NaBH_4$ as previously described (Takahashi et al., 1973). After 24 hours, the radioactivity in 250 µL of the supernatant was determined by liquid scintillation spectrometry as a measure of elastolysis. To control for non-enzymatic degradation, radiolabeled elastin was incubated with medium from tissue-free cultures. Elastase activity was related to a standard curve generated with human leukocyte elastase (0.075 to 5.0 ng) (Elastin Products Co).

Blood parameters. Plasma 3,4-dihydroxyphenylglycol (DHPG) and 5-hydroxyindole-acetic acid (5-HIAA) levels were measured by HPLC and serotonin in both plasma and whole blood by radioenzymology (Berlin et al., 1995; Kéreveur et al., 2000).

Acute injections. Acute intraperitoneal (i.p.) injections of the preferential 5-HT_{2B}R agonist BW 723C86 (10 mg/kg), selective 5-HT_{2B}R antagonist RS-127445 (1mg/kg), dexfenfluramine (10 mg/kg) or nordexfenfluramine (10 mg/kg) were performed on 5-$HT_{2B}R^{+/+}$ and $^{-/-}$ mice before blood was collected by cardiac puncture for measurements over a 30 minutes time period. Pre-treatment with paroxetine was performed by i.p. injection twice a day of the selective SERT blocker paroxetine (1mg/kg) over two days, reducing platelet serotonin uptake by more than 90%.

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Statistics. The reported data represent the mean of individual values±SEM (n=number of individuals at the end of treatments as indicated in the text). Comparisons were performed using the non-parametric Kruskal-Wallis test. Significance was set at $P < 0.0001$. This level of significance also applies to correlation between individual values, assessed by Kendall rank coefficients. For the group comparisons, statistical comparisons were made by one way ANOVA. Difference between groups was established using the Bonferonni-Dunn multiple comparison tests ($P < 0.0001$).

Results

The hypoxia-dependent increase in plasma serotonin levels is 5-HT_{2B}R-controlled.

We previously reported that 5 weeks of hypoxia increased RVSP, media wall thickness, pulmonary elastase activity, expression of 5-HT_{2B}Rs, and thymidine incorporation in lungs of wild-type but not of 5-$HT_{2B}R^{-/-}$ or 5-HT_{2B}R-antagonist-, RS-127445, treated $^{+/+}$ mice (Launay et al., 2002). A progressive increase in RVSP was detected in $^{+/+}$ mice treated from two to five weeks under hypoxia (10% O_2) (Fig. 1A). We tested serotonin levels in blood samples of the hypoxia-treated mice after acute, two weeks or five weeks of hypoxia. Strikingly, as the expression of 5-HT_{2B}Rs in lungs (Fig. 1B), plasma serotonin levels (Fig. 1C) were significantly increased in $^{+/+}$ mice after exposure to two weeks of chronic hypoxia, before significant changes in remodeling factors could be evidenced (e.g. elastase activity: Fig. 1D) but not in hypoxic 5-$HT_{2B}R^{-/-}$ mice. No change in whole blood serotonin could be evidenced.

This increase in plasma serotonin levels indeed persisted after 5 weeks of hypoxia when the pulmonary hypertensive pathology was fully established (Fig. 2A-C) but not in hypoxic 5-$HT_{2B}R^{-/-}$ or 5-HT_{2B}R-antagonist-, RS-127445, treated $^{+/+}$ mice. Furthermore, the increase in plasma serotonin levels was potentiated in the presence of dexfenfluramine in $^{+/+}$ mice but not in hypoxic 5-$HT_{2B}R^{-/-}$ or RS-127445-treated $^{+/+}$ mice (Fig. 2C). No significant reduction of whole blood serotonin could be observed in hypoxic $^{-/-}$ and $^{+/+}$ mice. By contrast and as expected, in hypoxic and dexfenfluramine exposed $^{-/-}$ and $^{+/+}$ mice, a significant reduction of whole blood serotonin was observed, thus independently of 5-HT_{2B}Rs (Fig. 2B). Following the increase in plasma serotonin levels, the serotonin main catabolite 5-HIAA was also increased in chronic hypoxia dexfenfluramine treated $^{+/+}$ mice (Fig. 2D). Although not reaching statistical significance ($P > 0.05$), a trend toward a decreased serotonin degradation as expressed by an increased ratio

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between plasma levels of serotonin and 5-HIAA was observed in both hypoxic 5-$HT_{2B}R^{-/-}$ and $^{+/+}$ mice. These first observations suggest a role of 5-HT_{2B}Rs in plasma serotonin regulation that precedes other pulmonary hypertension parameters.

The hypoxia-dependent increase in plasma serotonin levels is correlated with RVSP and 5-HT_{2B}R-expression.

When comparing the plasma serotonin levels and the others parameters affected by hypoxia, we observed significant ($P < 0.0001$) correlations between all the individual plasma serotonin values and RVSP values (Fig. 3A), lung 5-HT_{2B}R expression site numbers (Fig. 3B), or plasma 5-HIAA values (Fig. 3C). Moreover, the ratios serotonin/5-HIAA were also significantly correlated with plasma DHPG values, confirming that these ratios reflect monoamine oxidase A (MAOA) activity (Fig. 3E). However, the correlation was not significant between individual plasma and whole blood serotonin levels (Fig. 3D). These results further substantiate the evidence that 5-HT_{2B}Rs are required for the regulation of plasma serotonin levels, independently of platelets, which store most of the whole blood serotonin.

5-HT_{2B}Rs affect lung serotonin uptake activity.

We determined the parameters of serotonin uptake activity in mouse lung vascular cultures after different treatments. Significant variations in the lung uptake activity were detected, which were entirely depending on changes in estimated SERT initial rate of uptake (Vi). Chronic hypoxia decreased the lung serotonin uptake activity in $^{+/+}$ mice (Table 1) but not its Km (reflecting SERT affinity for serotonin). As expected, chronic hypoxia in the presence of dexfenfluramine nearly completely blocked the lung serotonin uptake activity in both $^{+/+}$ and 5-$HT_{2B}R^{-/-}$ mice (Table 1).

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A significant increase (27%) in basal normoxic serotonin uptake Vi was observed in 5-$HT_{2B}R^{-/-}$ compared to $^{+/+}$ mice, with no effect of hypoxia (Table 1). No change in expression levels of SERT could be detected in any genotype or treatment (189±5 fmoles/mg of protein) as assessed by paroxetine binding experiments (not illustrated). These results show that 5-HT_{2B}Rs are required to regulate hypoxic and basal normoxic lung serotonin uptake activity.

Direct *in vivo* activation of 5-HT_{2B}Rs results in an increase of plasma serotonin levels.

To test if normoxic mice do respond to activation of 5-HT_{2B}Rs, we acutely injected 5-HT_{2B}R agonists and assessed the serotonin plasma levels. Over a 30 minutes time period, plasma levels were extremely stable after vehicle injection in mice. A significant increase in plasma serotonin levels over this control value was observed at 10 minutes after i.p. injection of the 5-HT_{2B}R preferential agonist BW-723C86 (10 mg/kg) (76±12% over basal, n=6) in $^{+/+}$ mice (figure 4A). By contrast, in 5-$HT_{2B}R^{-/-}$ mice (figure 4B) or in 5-HT_{2B}R-antagonist, RS-127445- (1 mg/kg, n=4) treated $^{+/+}$ mice (figure 4C), this increase could not be detected. Interestingly, acute i.p. injection of nordexfenfluramine (10 mg/kg, n=6) fully mimicked the BW-723C86-dependent increase in plasma serotonin levels in timing (peak at 10 minutes) and magnitude (82±17% over basal) in $^{+/+}$ mice, with again no effect on 5-HT_{2B}R inactive mice. However, acute i.p. dexfenfluramine injection (10 mg/kg, n=6) produced a faster (5 minutes) and sharper increase in plasma serotonin, which was observed in both $^{+/+}$ and 5-HT_{2B}R inactive mice. The dexfenfluramine effect was clearly distinct from that of nordexfenfluramine, since the nordexfenfluramine as the BW-723C86 effects on peripheral serotonin release required 5-HT_{2B}Rs. No variation in whole blood serotonin could be detected in this time window in either

genotype after similar injections (not illustrated). Activation of 5-HT_{2B}Rs can thus increase plasma serotonin levels.

The *in vivo* 5-HT_{2B}R-dependent increase in plasma serotonin levels is SERT dependent.

We tested the effect of SERT inhibition using a pretreatment by the highly selective SERT blocker paroxetine or $SERT^{-/-}$ on dexfenfluramine, BW-723C86, or nordexfenfluramine *in vivo* acute i.p. injection. Strikingly, no change in plasma serotonin levels could be detected after acute injection of nordexfenfluramine or BW-723C86 when the mice were pretreated by paroxetine (1mg/kg, n=6) twice a day for two days, either in $^{+/+}$, 5-$HT_{2B}R^{-/-}$, and $SERT^{-/-}$ mice (not illustrated). In contrast, a lower but significant increase in plasma serotonin levels over the control value was still observed at 5 minutes after dexfenfluramine injection in $^{+/+}$ mice (62±4% of plasma level without paroxetine) (figure 5A) and in 5-$HT_{2B}R^{-/-}$ mice after paroxetine treatment (64±8% of plasma level without paroxetine) (figure 5B). No variation in total blood serotonin could be detected in this time window in either genotype after similar injections (not illustrated). The BW-723C86- and nordexfenfluramine-dependent increases in plasma serotonin levels appear entirely 5-HT_{2B}R- and SERT-dependent, whereas that of dexfenfluramine appears 5-HT_{2B}R-independent and only partially SERT-dependent.

Discussion

We are presenting here the first evidence that 5-HT_{2B}Rs are required to control the plasma serotonin levels in mice. In chronic $^{+/+}$ hypoxic mice, the plasma serotonin levels parallel lung expression of 5-HT_{2B}R, and are significantly increased before any significant vascular remodeling could be evidenced, and potentiated by dexfenfluramine. In mice with either permanent (5-HT_{2B}R homozygous mutation) or transient (5-HT_{2B}R selective antagonist) inactivation of 5-HT_{2B}Rs, plasma serotonin levels are not modified by chronic hypoxia. We also observed that the values for plasma serotonin levels and 5-HT_{2B}Rs expression are significantly correlated. In addition, we show that acute injection of BW-723C86, a preferential 5-HT_{2B}R agonist, triggers a rapid increase in plasma serotonin levels, which is entirely SERT-and 5-HT_{2B}R-dependent as shown by genetic and pharmacologic ablation of either protein and mimicked by nordexfenfluramine but not dexfenfluramine. These data demonstrate the participation of both 5-HT_{2B}R and SERT in the control of plasma serotonin levels *in vivo*.

An interference of the 5-$HT_{2B}^{-/-}$ mice congenital defects has been ruled out since treatment of $^{+/+}$ mice by RS-127445, the most selective 5-HT_{2B}R antagonist, reproduces the results obtained with 5-$HT_{2B}^{-/-}$ mice. The serotonergic anorectic agent and amphetamine derivative, dexfenfluramine, increases the risk of developing pulmonary hypertension (Rich et al., 2000). *In vitro*, dexfenfluramine and its main metabolite nordexfenfluramine have been shown to inhibit serotonin reuptake and to stimulate its release from brain synaptosomes (Mennini et al., 1981). Moreover, both compounds bind to 5-HT_2Rs with appreciable affinity. In fact, dexfenfluramine binds weakly to rodent and human 5-$HT_{2A,B,C}$Rs but nordexfenfluramine behaves as a high affinity agonist for 5-HT_{2B} and 5-HT_{2C}Rs and more moderately for 5-HT_{2A}Rs (Porter et al., 1999; Fitzgerald et al., 2000; Rothman and Baumann, 2002; Setola et al., 2003 and

supplementary table). In mice, chronic exposure to dexfenfluramine 2.5 mg/kg/day leads to complete conversion into nordexfenfluramine with a final plasma concentration of about 500 nmol/L (Caccia et al., 1985; Launay et al., 2002), fully stimulating 5-HT_{2B}Rs but not 5-HT_{2A}Rs (Supplementary table), 5-HT_{2C}Rs being below detection level in the periphery. Chronic effects of dexfenfluramine therefore appeared nordexfenfluramine-mediated.

The mechanisms leading to hypoxia-induced plasma serotonin elevation might be a consequence of platelet defects or of a slower serotonin uptake by platelets due to a kinetic change in SERT activity (Awabdy et al., 2003). We did not observed any significant variation in whole blood serotonin content (>99% of which is represented by platelets) in hypoxic mice (Fig. 2B) thus eliminating major changes in serotonin synthesis, platelet number or their serotonin uptake capacity. Furthermore, the absence of correlation between individual values of plasma and blood serotonin (Fig. 3D) indicates that mechanisms controlling serotonin platelet uptake and plasma levels are not coordinately regulated by hypoxia. Moreover, mature platelets express 5-HT_{2A}Rs but not 5-HT_{2B}Rs, and ketanserin, a selective 5-HT_{2A}R antagonist, does not significantly change pulmonary artery pressure in hypoxic mice (Marcos et al., 2003). Recently, platelets serotonin uptake inhibition by both dexfenfluramine and nordexfenfluramine (IC50, dexfenfluramine approximately 3 μmol/L; nordexfenfluramine approximately 10 μmol/L) has been reported, but no serotonin efflux occurs at these concentrations (Johnson et al., 2003). Together with our chronic *in vivo* results, these findings indicate that regulation of platelets serotonin content and that of plasma serotonin are under different molecular controls in hypoxic mice.

A change in metabolism might also explain the increase in plasma serotonin levels in hypoxic pulmonary hypertension. Lung endothelial cells control serotonin clearance by

catabolism into 5-HIAA by MAOA and lung is known as the main site of serotonin removal from plasma (Gillis and Pitt, 1982). Low oxygen levels are expected to directly decrease MAO activity and thus serotonin metabolism. The non-significant reduction in serotonin degradation observed in hypoxic 5-$HT_{2B}^{-/-}$ or $^{+/+}$ mice (Fig. 3C-E) indicates that 5-HT_{2B}Rs have no major contribution to the MAOA activity under hypoxia and the increase in plasma serotonin cannot be explained solely by changes in MAOA activity. In conclusion, a generalized deficit of the serotonin metabolism could not explain the increase in plasma serotonin encountered during hypoxic pulmonary hypertension with no changes in whole blood serotonin.

Since the lung endothelium is the major site of carrier-mediated peripheral serotonin fast removal (Gillis and Pitt, 1982), reduced SERT uptake activity in lungs might also raise plasma serotonin levels. Recent evidence obtained by positron emission tomography scan of SERT ligands in healthy volunteers confirmed that lung is the main fast high-uptake organs in the body (Suhara et al., 1998; Takano et al., 2002). Our result showing a reduced rate of lung serotonin uptake upon chronic hypoxia, which is not observed when the 5-HT_{2B}R are inactivated (Table 1), agrees with previous reports in lung of hypoxic rats and mice (Jeffery et al., 2000). In perfused rat lung, SERT can mediate an efflux of serotonin (James and Bryan-Lluka, 1997) and the serotonin efflux caused by SERT substrates (such as amphetamine derivatives) is carrier-mediated (Hilber et al., 2005). Together, the reduced lung serotonin uptake and the increased plasma serotonin levels in hypoxic mice suggest that hypoxia-dependent 5-HT_{2B}R activation can trigger the increase in plasma serotonin, probably via a negative regulation of SERT activity. Whether this regulation is restricted to lungs remains to be determined.

Another site known to express 5-HT_{2B}Rs is the gut, which is the main site of peripheral serotonin synthesis by enterochromaffin cells. The recent observation that, in perfused rat ileum,

dexfenfluramine (EC50 around 100 μmol/L) or nordexfenfluramine (EC50 around 10 μmol/L) can increase serotonin levels in the venous effluent strongly support their ability to increase its release from the small intestine (Rezaie-Majd et al., 2004). Given the plasma concentrations of dexfenfluramine and nordexfenfluramine in sub-micromolar range (Caccia et al., 1985; Launay et al., 2002) and the nanomolar affinity of nordexfenfluramine for 5-HT_2Rs (Supplementary table), a direct action at 5-HT_2Rs to the regulation of serotonin release from intestine seems unlikely but cannot be totally excluded.

In a different experimental *in vivo* setup, our data show that acute injection of the 5-HT_{2B}R preferential agonist BW-723C86, as nordexfenfluramine (another preferential 5-$HT_{2B/2C}$R agonist), significantly increases plasma serotonin levels with identical kinetic and 5-HT_{2B}R-dependency, whereas dexfenfluramine acts with a faster kinetic and generates a serotonin increase that is independent of 5-HT_{2B}Rs (Fig. 4). Evidence that the mechanism by which nordexfenfluramine induces serotonin efflux is different from that underlying dexfenfluramine-induced release had been previously documented: work in synaptosomes showed that nordexfenfluramine acts, at least in part, on the release of a serotonin cytoplasmic pool (Mennini et al., 1981), whereas dexfenfluramine can induce a Ca^{2+}-dependent serotonin release from a vesicular pool (Gobbi et al., 1998); independent work using microdialysis showed also that dexfenfluramine, but not nordexfenfluramine, uses calcium to increase extracellular serotonin (Puig de Parada et al., 1995).

Although evaluated in the periphery, our work substantiates a dual mode of action for these compounds in modulating plasma serotonin levels. Our observations that acute injection of dexfenfluramine triggers serotonin releasing effects that are rapid, mainly paroxetine-insensitive and independent of 5-HT_{2B}Rs, support a rapid mechanism of serotonin release by

dexfenfluramine. The modulation by BW-723C86 or nordexfenfluramine of serotonin plasma levels is blocked by pharmacological (paroxetine at 1mg/kg, Fig. 5) or genetical (mutant mice, not illustrated) SERT inhibition. In humans, similar paroxetine treatment leads to plasma concentration less than 500 nmol/L (Lindsay De Vane, 1999). Thus assuming a similar bioavailibity in mice, it is unlikely that this concentration could affect 5-HT_{2A}, 5-HT_{2C}, or 5-HT_{2B}Rs (Supplementary table). The elimination of both BW-723C86- and nordexfenfluramine-induced plasma serotonin increases in 5-HT_{2B}R $^{-/-}$ mice, in 5-HT_{2B}R-antagonist treated $^{+/+}$ mice, by selective SERT blocker and in SERT $^{-/-}$ mice, strongly supports the hypothesis that serotonin release is controlled by 5-HT_{2B}Rs via a regulation of SERT uptake activity. Reports showing that both dexfenfluramine and nordexfenfluramine at μmol/L concentrations generated no efflux of serotonin from platelets (Johnson et al., 2003) and that in ileum both compounds increased serotonin levels but at concentrations over 10 μmol/L (Rezaie-Majd et al., 2004) support the implication of other organs such as the lungs in this serotonin release.

A 5-HT_{2B}R-dependent SERT phosphorylation (Launay et al., 1998) in vascular endothelium could be an essential component of the 5-HT_{2B}R-dependent regulation of plasma serotonin independently of platelets. But the molecular pathway leading to the control of SERT activity by 5-HT_{2B}Rs remains to be molecularly detailed. Our data support the hypothesis that the 5-HT_{2B}R is an essential partner in signaling pathways regulating the plasma serotonin levels via a control of SERT activity. The previous observations that, in response to hypoxia, increases in RVSP and in lung vascular remodeling are reduced in mice deficient for SERT, together with our finding that paroxetine blocks 5-HT_{2B}R-efflux effects, may explain the paradoxical reports showing that some SERT inhibitors have putative beneficial effects in pulmonary hypertension (Marcos et al., 2003) whereas injection of serotonin potentiates the development of pulmonary

hypertension in rats exposed to chronic hypoxia (Eddahibi et al., 1997). Nevertheless, our work demonstrates that 5-HT_{2B}R can control plasma serotonin levels in mice and further suggests that such a control may participate to other types of pulmonary hypertension in other organisms.

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FOOTNOTES

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Legends for Figures

Figure 1- Kinetic of hypoxia-dependent increase- In $^{+/+}$ mice exposed to hypoxia from 0 (AH, n=10), 2 (H2W, n=10) and 5 (H5W, n=14) weeks, RVSP is progressively increasing (A). Lung 5-HT_{2B}R expression (B) and plasma serotonin (C) are significantly higher after 2 weeks of hypoxia than in vehicle-treated normoxic $^{+/+}$mice (VE, n=19), but not elastase activity (D). Any statistical difference from normoxic untreated control values is indicated by an asterisk ($P < 0.0001$).

Figure 2- Hypoxia-dependent increase in plasma serotonin levels is 5-HT_{2B}R-controlled- In $^{+/+}$ mice exposed to hypoxia for 5 weeks (H5W, n=14), RVSP (A) and plasma serotonin (C) are significantly higher than in vehicle-treated normoxic $^{+/+}$mice (VE, n=19). Simultaneous exposure of $^{+/+}$ mice to hypoxia in the presence of the specific 5-HT_{2B}R antagonist RS-127445 (RS5W, n=9) prevents both increases. The presence of dexfenfluramine (DF5W, n=16) with hypoxia increases significantly RVSP (A), plasma serotonin (C) and plasma 5-HIAA (D), but decreases significantly the whole blood serotonin (B) as compared to normoxic and hypoxic values. By contrast, none of the 5-$HT_{2B}^{-/-}$ mice exposed to hypoxia for 5 weeks (n=7) exhibits any significant change in RVSP, plasma 5-HIAA or plasma serotonin *vs.* vehicle-treated normoxic $^{-/-}$ mice (n=9), neither in the presence of RS-127445 (n=9) nor dexfenfluramine (n=8). Exposure of 5-$HT_{2B}^{-/-}$ mice to dexfenfluramine with hypoxia decreases significantly the whole blood serotonin and basal normoxic value is above control. Any statistical difference from normoxic untreated control values is indicated by an asterisk, and from chronic hypoxia values by a cross ($P < 0.0001$).

Figure 3- Correlations between individual values of plasma serotonin levels with other parameters involved in pulmonary hypertension- Highly significant correlations ($P < 0.0001$,

star) are observed between all wildtype individual values at 5 weeks for plasma serotonin levels and RVSP (A) (n=58), the number of 5-HT_{2B}R specific binding sites (B) (n=54), plasma 5-HIAA levels (C) (n=58). However, the correlation was not significant between individual plasma serotonin levels and whole blood serotonin contents (D) (n=58). Furthermore, the serotonin/5-HIAA plasma ratios were also significantly correlated with plasma DHPG values confirming they reflect MAOA activity (E) (n=22). {r=rank of correlation, normoxia (open circles), hypoxia (gray circles), hypoxia with dexfenfluramine (open squares), hypoxia with RS-127445 (black squares)}.

Figure 4- Injection of 5-HT_{2B}R agonists increase plasma serotonin levels- Over a 30 minutes time period, measures of plasma serotonin levels are extremely stable after vehicle injection in control (+/+ VE), RS-127445- (1 mg/kg, n=4) treated mice (+/+ VERS) and mutant (-/- VE) mice. A significant increase ($P < 0.0001$, star, n=6) in plasma serotonin levels over control values is observed at 10 minutes after BW-723C86 (10 mg/kg) (+/+ BW) and nordexfenfluramine (10 mg/kg) (+/+ NDF) i.p. injection in $^{+/+}$ mice (A), which is not detected in 5-$HT_{2B}R^{-/-}$ mice (B) or in RS-127445 treated $^{+/+}$ mice (C). However, acute dexfenfluramine i.p. injection (10 mg/kg) produced a significant increase ($P < 0.0001$, star, n=6) in plasma serotonin in $^{+/+}$ (+/+ DF), RS-127445 treated $^{+/+}$ mice (+/+ DFRS) and 5-$HT_{2B}R^{-/-}$ (-/- DF) mice at 5 minutes.

Figure 5- Paroxetine blocks 5-HT_{2B}R agonists-dependent increase in plasma serotonin levels- A significant increase (star, $P<0.0001$, n=6) in plasma serotonin levels over control values (VEP) is observed at 5 minutes after acute dexfenfluramine i.p. injection in paroxetine pre-treated mice in both $^{+/+}$ (+/+ DFP) (A) and 5-$HT_{2B}R^{-/-}$ (-/- DFP) (B) mice. However, i.p. injection of BW-723C86 (+/+ BWP) or nordexfenfluramine (+/+ NDFP) in paroxetine pretreated mice did not modified plasma serotonin levels in either $^{+/+}$, or 5-$HT_{2B}R^{-/-}$ mice.

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Table 1

Parameters of lung serotonin uptake

	Km	Vi
	μmol/L	pmol/min/mg prot
VE	4.53±0.58	10.86±0.47
H5W	4.89±0.48	8.18±1.27 *
DFH5W	4.54±0.61	0.58±0.08*†
RSH5W	4.48±0.64	10.30±0.98
VEKO	4.61±0.69	13.80±0.95*†
H5WKO	4.64±0.91	13.87±1.50*†
DFH5WKO	4.71±0.47	1.41±0.12*†
RSH5WKO	4.43±0.49	13.90±0.93*†

Parameters of serotonin uptake were evaluated in lung explants from normoxic $^{+/+}$mice (VE), hypoxic $^{+/+}$mice (H5W), hypoxic $^{+/+}$ mice in the presence of dexfenfluramine (DFH5W), hypoxic $^{+/+}$ mice in the presence of 5-HT_{2B}R-specific antagonist RS-127445 (RSH5W), normoxic $^{-/-}$mice (VEKO), hypoxic $^{-/-}$mice (H5WKO), hypoxic $^{-/-}$mice in the presence of dexfenfluramine (DFH5WKO) or hypoxic $^{-/-}$mice in the presence of RS-127445 (RSH5WKO). Any statistical difference from normoxic untreated control values is indicated by an asterisk, and from chronic hypoxia values by a cross ($P < 0.0001$).

Figure 1

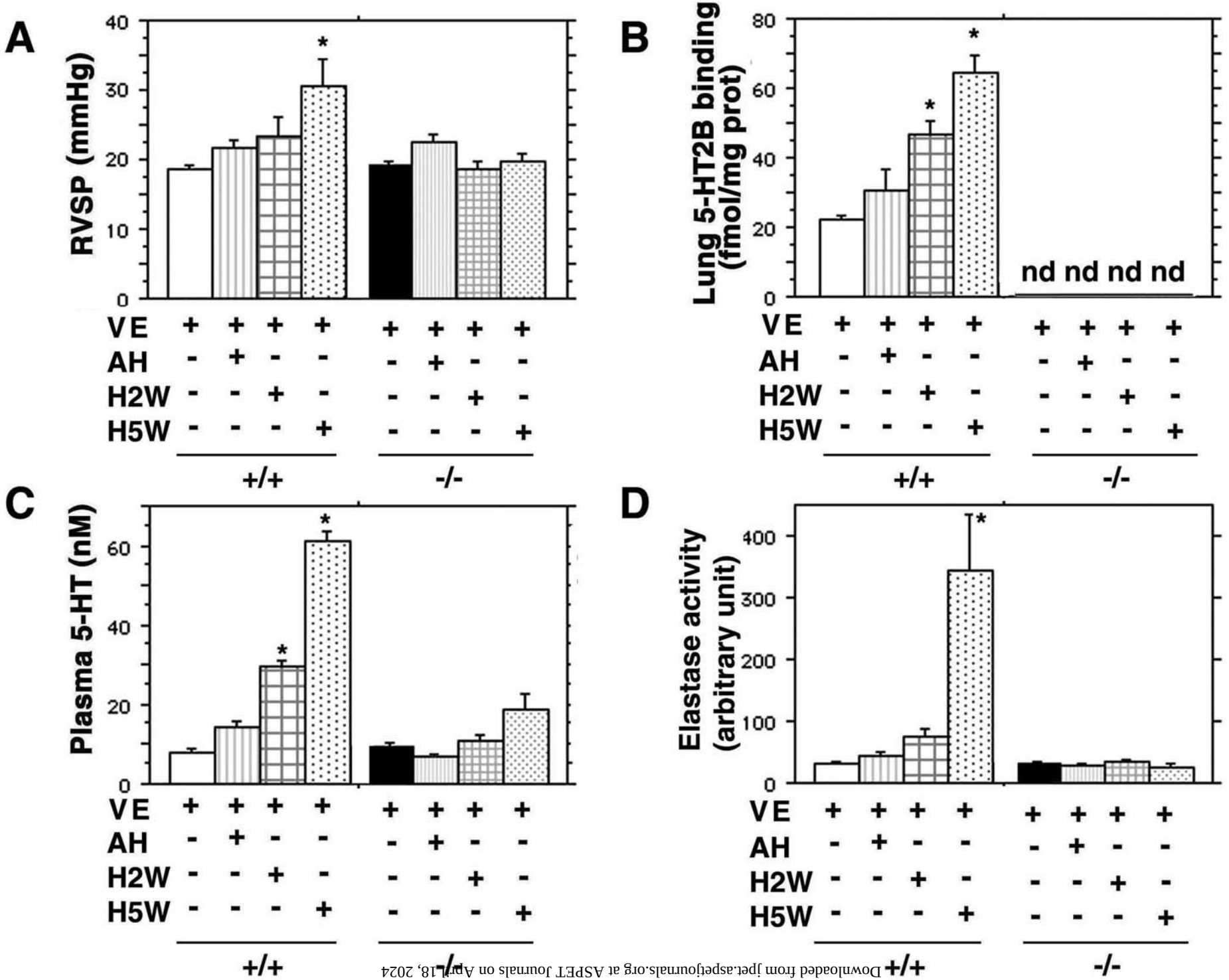

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Figure 2

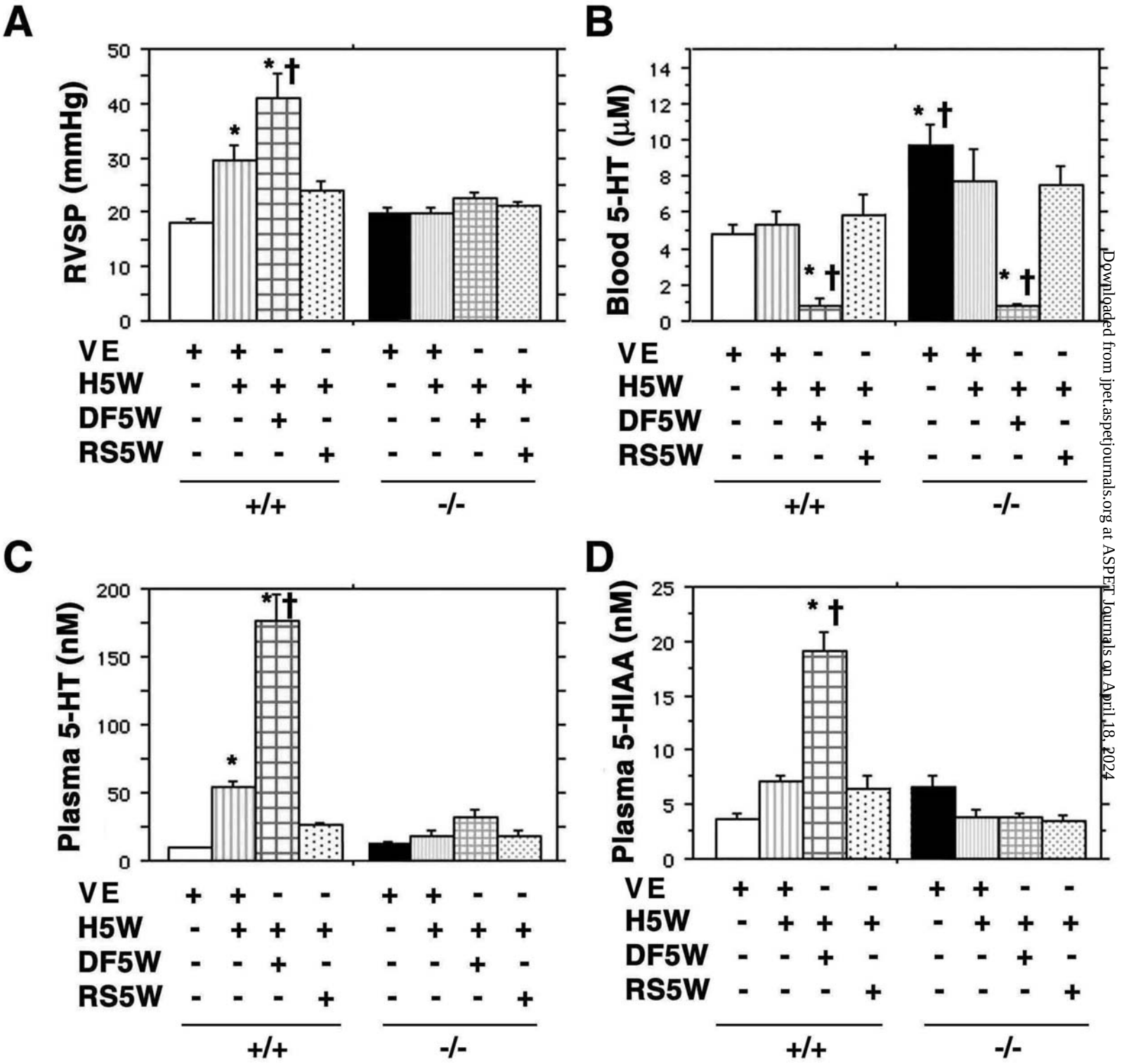

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Figure 3

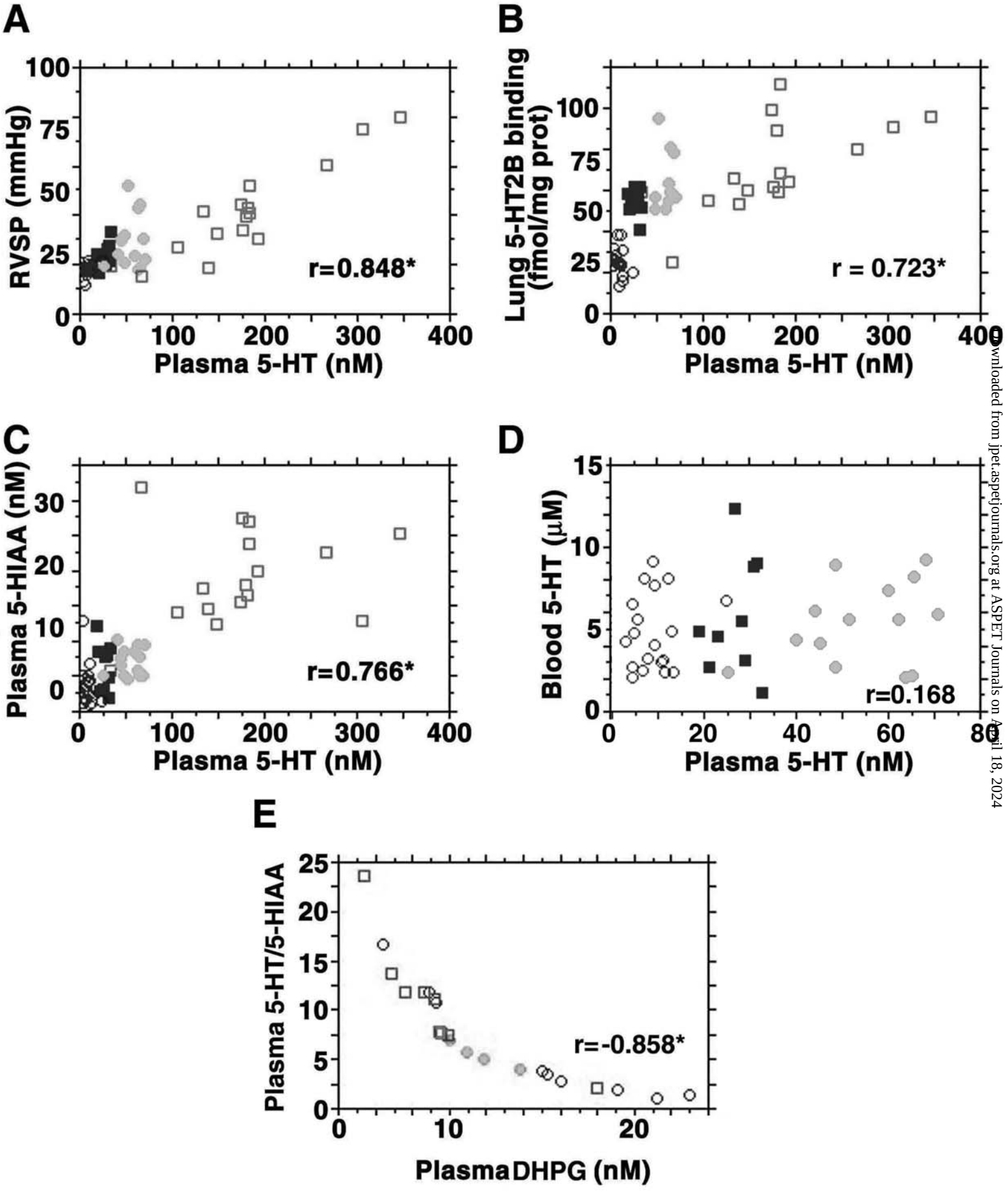

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Figure 4

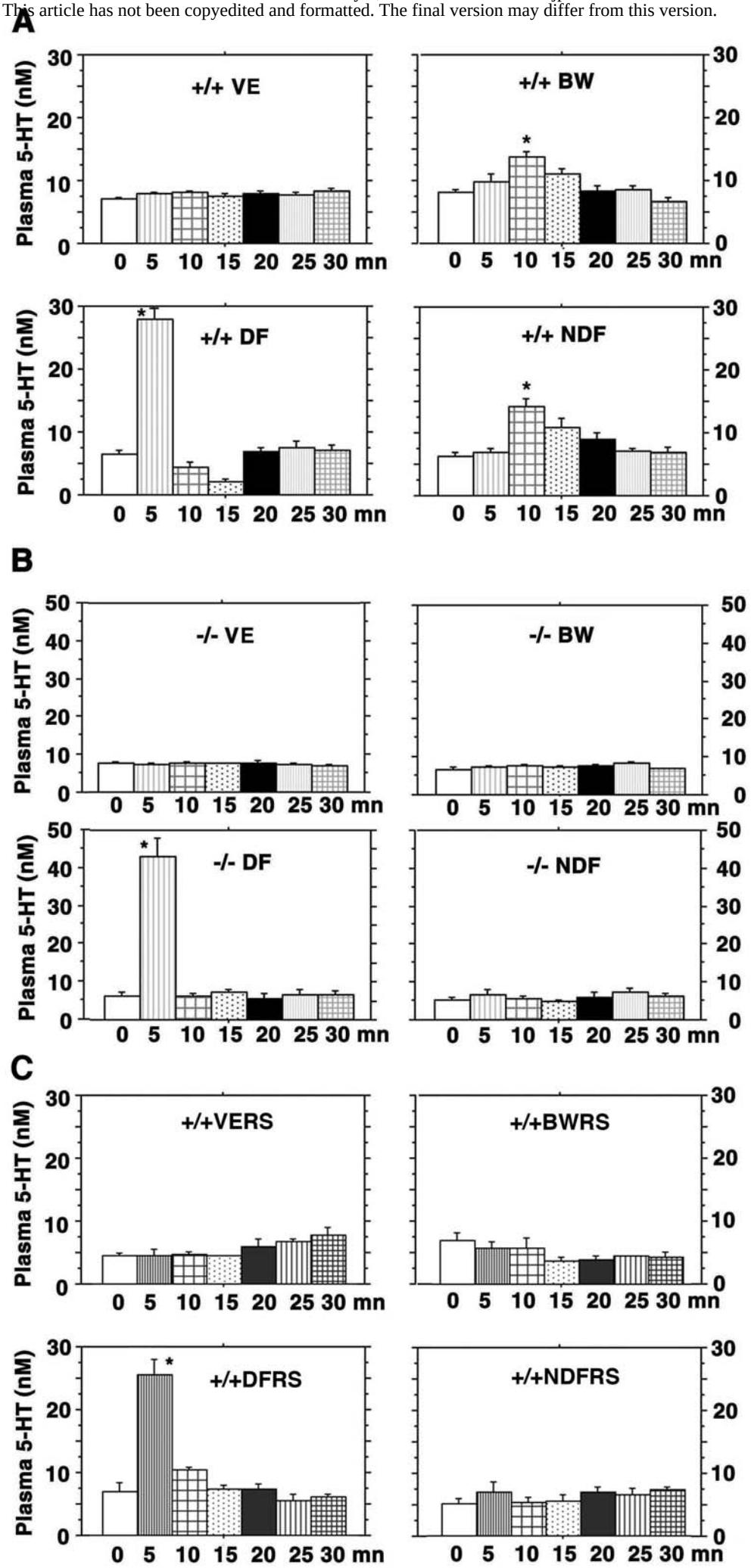

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Figure 5