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Functional interactions between 5-HT receptors and the serotonin transporter in pulmonary arteries

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5-HT, 5-hydroxytryptamine; SERT, serotonin transporter; LV, left ventricular; PAH, pulmonary arterial hypertension; RV, right ventricle; RVP, right ventricular pressure; S, septum; SAP, systemic arterial pressure; SD, Sprague-dawley.

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Abstract

Pulmonary arterial serotonin (5-HT) transporter (SERT)-, 5-HT receptor-expression and 5-HT-induced vasoconstriction can be increased in pulmonary hypertension. These variables were studied in normoxic and hypoxic fawn-hooded (FH) and Sprague-Dawley (SD) rats. Further, we compared the functional effects of SERT inhibitors and 5-HT receptor antagonists against 5-HT-induced vasoconstriction of pulmonary arteries. SERT and 5-HT_{1B} expression was greater in FH rat lungs than in SD rats, as was 5-HT-mediated vasoconstriction. The 5-HT_{2A} receptor antagonist ketanserin and the 5-HT_{1B} receptor antagonist SB224289 inhibited responses to 5-HT in all vessels. The combined 5-HT_{1B} receptor/SERT antagonist LY393558 was the most potent inhibitor of constriction in all vessels. SERT inhibitors, citalopram and fluoxetine, inhibited responses to 5-HT in SD vessels. However these inhibitors potentiated responses to 5-HT in FH vessels. After exposure of rats to two weeks of hypoxia, there was increased 5-HT mediated vasoconstriction and a profound decrease in SERT expression in both the FH and SD rat lung. Accordingly, citalopram had no effect on 5-HT-induced constriction in SD rat vessels and markedly less effect in FH rat vessels. Ketanserin, SB224289 and LY393558 inhibited responses to 5-HT in all hypoxic rat vessels. LY393558 was the most potent antagonist and there was synergy between the effects of fluoxetine and SB224289 when given simultaneously. The results suggest that, in FH rats, SERT inhibitors may increase pulmonary vasoconstriction but this can be inhibited by simultaneous 5-HT_{1B} receptor antagonism. There is synergy between the inhibitory effects of 5-HT_{1B} receptor antagonists and SERT inhibitors on 5-HT-induced pulmonary vasoconstriction.

Pulmonary arterial hypertension (PAH) is characterised by sustained elevation in pulmonary artery pressure. Familial PAH can be related to heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II receptor and/or polymorphisms in the gene encoding the serotonin (5-HT) transporter (SERT) (Lane et al., 2000; Eddahibi et al., 2001). Idiopathic PAH has no demonstrable cause and PAH can also occur secondary to many cardio-respiratory disorders. Regardless of the type of PAH however, the elevated pulmonary vascular resistance is associated with remodelling of muscular pulmonary arteries and arterioles which exhibit smooth muscle proliferation, medial hypertrophy and fibrosis (Fishman, 1998).

SERT mRNA is elevated in platelets from patients with PAH (Eddahibi et al., 2001). A polymorphism with long (L) and short (S) forms (Lesch et al., 1996) affects SERT function with the L allele inducing an increased rate of SERT gene transcription. The SERT polymorphism can also predict the severity of PAH in patients with chronic obstructive pulmonary disease (Eddahibi et al., 2003). Hence, it has been hypothesized that inhibitors of SERT may be useful in the treatment of PAH. One mechanism of action of SERT inhibitors treating clinical depression, however, is to cause an extracellular accumulation of 5-HT and increased 5-HT receptor activation (Slattery et al., 2004). The possibility that SERT inhibitors may similarly increase 5-HT activation in pulmonary arteries requires investigation. It is the 5-HT_{1B} and 5-HT_{2A} receptors that mediate contraction of human, rat and mouse pulmonary arteries (Keegan et al., 2001; MacLean et al., 1996a; MacLean et al., 1996b; Morecroft et al., 1999). Very recently, Liu *et al* (2004) have hypothesized that SERT and 5-HT_{1B} receptor activity interact and co-operate to effect pulmonary artery smooth muscle cell growth, suggesting that combined block would be the optimal therapeutic approach in PAH. Here we also examine if SERT and 5-HT_{1B} receptor activities interact or co-operate to affect contractile responses.

The Fawn-hooded (FH) rat is more sensitive to hypoxia-induced PAH than its Sprague-dawley (SD) controls. The FH rat has been studied as a model of human PAH as it has altered serotonergic function, an inherited storage defect to serotonin, increased circulating levels of 5-HT (Sato et al., 1992; Fujimori et al., 1998) and increased pulmonary vascular responsiveness to 5-HT (Ashmore et al., 1991). These are all factors observed in human PAH (MacLean et al., 2000). The FH rat also demonstrates increased lung endothelin-1 (Stelzner et al., 1992) and elevated circulating levels of endothelin-1 have also been reported in patients with PAH (Stewart et al., 1991). There is an increase in SERT activity in the brains of the FH rat (Hulihan-Giblin et al., 1993) although, surprisingly, expression of the SERT in the lung has not yet been studied.

As treatment of PAH with 5-HT_{2A} antagonists results in systemic hypotension (Domenighetti et al., 1997) this is unlikely to be a future therapeutic approach and hence we chose to examine the synergistic effects of the 5-HT_{1B} and SERT in this study. We examined the effects of LY393558 (1-[2-[4-(6-fluoro-1H-indol-3-yl)-3,6-dihydro-1(2H)-pyridinyl]ethyl]-3-isopropyl-6-(methylsulphonyl)-3,4-dihydro-1H-2,1,3-benzothiadiazine-2,2-dioxide), as this is both a SERT inhibitor (Ki ~1nM) and a 5-HT_{1B} receptor antagonist with a Ki at 5-HT_{1B} receptors of ~1nM (Pullar et al., 2001; Mitchell et al., 2001). We also studied the combination of fluoxetine and SB224289 (5, 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine]) *in vitro*. SB224289 is a selective 5-HT_{1B} receptor antagonist with a Ki of ~10nM (Roberts et al., 1997; Price et al., 1997). SB224289 has no reported affinity for SERT sites. To investigate the role of the 5-HT_{2A} receptor, we studied ketanserin which has a 10000-fold selectivity for the 5-HT_{2A} receptor over the 5-HT_{1B} receptor (Ki at 5-HT_{2A} receptors of ~ 1-10nM, Bard et al., 1997). We compared the effects of citalopram and fluoxetine to compare two SERT inhibitors with different pharmacological profiles. Citalopram is an extremely

selective SERT inhibitor with a Ki of ~1.8nM with no reported affinity for 5-HT receptors. Fluoxetine has a Ki at SERT sites of ~0.9-2nM but also has a relatively high affinity against the 5-HT_{2A} receptor (Ki: ~140 nM) (Owens et al., 1997).

In this study, we wished to test the following hypotheses: 1) SERT inhibitors may potentiate contractile responses to 5-HT. 2) In the presence of SERT inhibitors, contractile responses will be inhibited by co-administration of a 5-HT_{1B} receptor antagonist i.e. the actions of SERT and receptor inhibitors synergise to inhibit 5-HT-induced contractile responses. 3) the effects of SERT and 5-HT_{1B} receptor antagonism will be modified i) in the FH rat, which exhibits increased SERT expression, and ii) after hypoxic exposure, which inhibits SERT expression.

Methods

Isolated vessel study

All animal care and procedures were in accordance with institutional and international guidelines. FH rats (and control Sprague-Dawley (SD) rats) were studied (body weights were 250-350g). They were killed by sodium pentobarbitone (200mgkg^{-1}) and the lungs removed. Small pulmonary arteries of ~250μm i.d. were dissected and set up using wire myography as previously described (Keegan et al., 2001; MacLean et al., 1996b). Control vessels were set up at tensions equivalent to their mean *in vivo* right ventricular pressure (RVP, 15-20mmHg) whilst hypoxic rat vessels were set up at tensions equivalent to the elevated *in vivo* mean pressures observed after exposure to hypoxia (30-35mmHg). Following a 45 min equilibration period, the response to 50mMKCl was determined, the concentration which produced maximal contraction in these vessels. Cumulative response curves to 5-HT (Sigma, UK, 1nM-0.1mM) were constructed. One curve to 5-HT was constructed either in the absence or presence of inhibitor in each vessel. All inhibitors were allowed a 45 min equilibrium period prior to constructing the curves to 5-HT.

Exposure to hypoxia.

30-33 day old FH and SD rats were maintained in hypoxic conditions (equivalent to 10% O_2, 0.3% CO_2 balance N_2) in a hypobaric chamber for two weeks as described previously (Keegan et al., 2001; MacLean et al., 1996b).

Ex Vivo Assessment of PHT.

i) Measurement of right ventricular hypertrophy (RVH). RVH is assessed as RV weight divided by left ventricular plus septal weight. As the body weight of the age-matched SD and FH rats was significantly different, we corrected this ratio for body weight (g).

ii) Pulmonary artery remodelling. The percentage of remodelled vessels (<50μm diameter) was assessed by measuring vessels with a double elastic lamina and expressing this as a percentage of vessels examined.

In vivo measurements

Rats were pre-medicated with an intra-peritoneal injection of fentanyl **(**0.315mg.ml^{-1}) /fluanizone (10mg.ml^{-1}) [Hypnorm, Jansen], 0.9-1.1 ml.kg^{-1} and midazolam (0.5mg.kg^{-1}). The rats were placed on a thermostatically controlled pad and fitted with a rectal thermometer. Anaesthesia was maintained via a facemask with a mixture of nitrous oxide, oxygen (1:1 ratio) and 1% halothane. Systemic blood pressure was monitored through a 3F i.v. cannula (Portex Ltd, Hythe, UK) inserted into the ascending aorta via the right carotid artery. The RV was catheterised through the right external jugular vein and right atria using a 3F catheter. The catheter position within the RV was confirmed by the morphology of the pressure trace. Basal right ventricular pressure (RVP) and systemic blood pressures were made following a period of stabilization using an Elcomatic E751A pressure transducer connected to an MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbara, CA). Heart rate (bpm) was derived from the pressure traces. Results were analysed using the built in software package (Acq*Knowledge* 3.5).

Quantification of SERT expression by radioligand binding and TaqMan RT-PCR

We have established, using immunohistochemistry, that SERT expression is almost entirely selective to the pulmonary arteries with no significant expression being observed on other structures in the lung in FH or SD rats either before or after hypoxic exposure (see Figure 5, G for illustration). As it is the small pulmonary arteries that are of primary interest in PAH, and it is not possible to dissect these out due to their size and fragility, we chose to look whole lung in the knowledge that this reflected pulmonary artery expression.

Radioligand binding

Membrane Preparation

Lungs were washed and pulverised under liquid nitrogen and re-suspended in assay buffer:- 150mmol/L NaCl, 50mmol/L Tris-HCl, 1mmol/L EDTA, 10mmol/L $MgCl_2$, containing 500µg/ml soybean trypsin inhibitor, 10mmol/L benzamidine, 1µg/ml leupeptin, bacitracin, pepstatin A, antipain, and 10% glycerol, pH7.4. Following homogenisation, homogenate was filtered and centrifuged at 1200xg for 5mins at 4^{o}C. The supernatant fraction was centrifuged twice at 56,000xg for 30mins at 4^{o}C and resulting membrane pellet re-suspended in Tris-HCl buffer and homogenised. Protein estimation was by Pierce protein assay kit (Pierce Chemical Co, UK).

Saturation Binding Studies

Saturation studies were performed with membranes (25ug/ml) incubated in duplicate with ^{3}[H]-citalopram (83Ci/mmol) (Amersham, UK) (0.025-20nmol/L) in Tris-HCl assay buffer. For each assay, membranes were prepared from n=8-10 lungs. The reaction mixture was incubated in a final volume of 0.5mls for 60mins at 22^{o}C for the measurement of total binding. Non-specific binding was defined in the presence of 10µmol/L fluoxetine-hydrochloride (Tocris Cookson Ltd, UK). The reaction was terminated using a Brandel cell harvester and bound ^{3}[H]-citalopram separated from free by vacuum filtration over Whatman GF/C filters. Binding isotherms were analysed by a non-linear least square parametric curve fitting programme GRAPHPAD Prism, to derive a dissociation constant (K_D) and receptor number (Bmax).

Quantitative mRNA expression

After extraction of total RNA from whole lung using TRIZOL reagent (Life Technologies), real time fluorogenic reverse transcriptase-PCR was performed using Assays on Demand

gene expression probes for rat SERT, 5-HT_{2A}- and 5-HT_{1B}-receptor (Rn00564737, Rn00568473, Rn00573666 respectively; Applied Biosystems) according to the manufacturers instructions. Relative mRNA abundance was determined using the comparative delta CT method using 18S ribosomal RNA as internal control.

Immunohistochemistry

Immunohistochemistry was performed to identify the location of SERT and to confirm that the inhibition of SERT expression and binding after hypoxia could be observed at the protein level.

Paraffin sections (5µm thick) were mounted on poly-l-lysine slides. Slides were dewaxed in histoclear and sections rehydrated by immersion in ethanol (100%, 95% and 70%) then in distilled water. Antigen retrieval was carried out by microwaving in 10mM citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked using 3%H_2O_2 in methanol for 30 minutes. After 2 washes in PBS, the sections were preincubated in PBS supplemented with 0.5% BSA, 10% normal horse serum for 1 hour. Endogenous biotin was blocked using an Avadin/Biotin blocking kit (Vector Labs, UK) then incubated overnight with goat polyclonal anti-SERT Ab (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:50 in PBS containing 0.5% BSA, 15% normal horse serum. The sections were exposed for 1 hour to biotin-labelled anti-goat secondary Ab's (Vector Labs, UK) diluted 1:100 in PBS, then to streptavadin biotin horseradish peroxidase solution. Peroxidase staining was carried out using 3'3'-diaminobenzidine tetrahydrochloride dihydrate and hydrogen peroxide. Finally the sections were stained with hematoxylin.

Analysis

Unless otherwise stated, statistical comparisons were made by one-way ANOVA and differences ($P<0.05$) established using the Neuman-Kuels multiple comparisons test.

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Statistical analysis was always carried out between test groups and controls carried out simultaneously as shown in Figures and not against pooled data sets as shown in the Tables. Only when maximal responses were obtained were pEC_{50}, Bmax and apparent pK_B values calculated. Apparent pK_B values were calculated according to the following equation: pK_B=log (DR-1)-log[B]; where DR is the ratio of the mean EC_{50} value in the presence of antagonist to the mean EC_{50} value in the absence of antagonist for a particular agonist.

Results

Contractile responses to 5-HT in normoxic animals

5-HT induced a small contractile response in adult SD and FH rats and the responses were of a greater magnitude in the FH rats (Figure 1A, Table 1).

Effect of selective 5-HT antagonists.

In the SD rat and the FH rat vessels both ketanserin and SB224289 inhibited responses to 5-HT. In the SD rat, the value for the pK_B of SB224289 was 7.65 ± 0.20, consistent with an effect at contractile 5-HT_{1B} receptors (pK_B in FH rats was not calculated as no maximum response was achieved). The pK_B for ketanserin was 8.86 ± 0.20 and 8.20 ± 0.20 in SD and FH rat vessels respectively, consistent with an effect at contractile 5-HT_{2A} (Figure 1B and 1C, Table 1).

Receptor mRNA expression

In both the normoxic and hypoxic rats, there was a higher magnitude of 5-HT_{1B} receptor mRNA expression in lung tissue from the FH rats as compared with in SD. No difference in 5-HT_{2A} receptor expression was detected (Figure 1D and 1E). There was no significant increase in the expression of either receptor after exposure to hypoxia. The mRNA relative expression data for the 5-HT_{2A} receptor and shown in Figure 1D was as follows (n=4): Control SD: 3.55 ± 0.25; Control FH: 2.3 ± 0.44; Hypoxic SD: 2.75. ± 0.58; Hypoxic FH: 2.17 ± 0.3. The mRNA relative expression data for the 5-HT_{1B} receptor and shown in Figure 1E was as follows (n=4): Control SD: 2.5 ± 0.49; Control FH: 7.38 ± 1.06; Hypoxic SD: 4.9 ± 1.21; Hypoxic FH10.79 ± 1.83.

Effects of SERT inhibitors.

Figure 2A illustrates the effects of the SERT inhibitors on responses to 5-HT in SD control rats. This data are summarised, and statistically analysed, in Table 2. Whilst 0.1μM fluoxetine

had no effect, 1μM inhibited responses to 5-HT. Citalopram also inhibited responses to 5-HT. LY393558 had the most potent inhibitory effect at both 10 and 100nM.

As LY393558 inhibits both the transporter and 5-HT_{1B} receptor, the profound inhibitory effect of LY393558 suggested a synergy between these. To verify this we conducted separate experiments in SD rat vessels and examined the effects of fluoxetine alone and SB224289 alone and then examined the effects of the two antagonists when applied together at the same concentrations. The concentration of SB224289 selective for the 5-HT_{1B} receptor was chosen (200nM) and we selected a concentration of fluoxetine that had no inhibitory effect on its own (0.1μM). The results are shown in Figure 2B. It can be seen that the effects of the two combined are greater than the added effects of the two given separately, suggesting a synergistic interaction. The pEC_{50} values for the 5-HT control, SB224289 alone, fluoxetine alone and SB224289 plus fluoxetine were 5.56 ± 0.13, 4.74 ± 0.33, 5.72 ± 0.16 and 5.03 ± 0.5 respectively and the Emax values (expressed as % response of contraction to 50mM KCl) were 30 ± 4, 22 ± 5, 34 ± 9 and 10 ± 4 ($P<0.05$ vs 5-HT control). The response to KCl was not affected by hypoxia in either FH or SD rat vessels. Figure 2C illustrates the effects of the 5-HT transport inhibitors on responses to 5-HT in FH rats. This data are summarised, and statistically analysed, in Table 2. Unlike the inhibitory effect in the SD rat, citalopram actually increased the response to 5-HT in the FH rat vessels (Figure 2C). 0.1μM fluoxetine also increased the potency of 5-HT. LY393558 inhibited the responses to 5-HT in a dose-dependent fashion.

Haemodynamics and the effects of Chronic hypoxia

RV remodelling was elevated in the FH rat compared with the SD rat although there was no significant difference in the % of remodelled vessels or RV pressure (Table 3). When exposed to hypoxia, the FH rats developed a greater degree of RV hypertrophy when compared to the SD controls (Table 3). Hypoxia induced a greater degree of remodelling in the FH rats vs the

SD rats (Table 3). Mean systemic arterial pressure was the same in all groups of rats as was heart rate. Whilst RV pressure was elevated by ~2 fold in hypoxic FH and SD rats, the degree of elevation was the same in the SD and FH rat groups (Table 3).

Contractile responses to 5-HT in hypoxic animals

There was a 3 fold increase in the contractile response to 5-HT in the SD rats after exposure to hypoxia. The affinity for 5-HT was also increased (Figure 3A, Table 1). Both ketanserin and SB224289 significantly inhibited the response to 5-HT in the hypoxic vessels (Figure 3A, Table 1). The value for the pK_B of SB224289 was 8.16 ± 0.20 and the pK_B for ketanserin was 9.03 ± 0.26, consistent with an effect at 5-HT_{1B} and 5-HT_{2A} receptors respectively. In the FH rat there was an even greater (4 fold) increase in the maximum response to 5-HT after hypoxic exposure as well as an increase in affinity (Figure 3B, Table 1). Expression of 5-HT_{1B} receptor mRNA was higher in the hypoxic FH lung compared with the hypoxic SD lung (Figure 1E). Both ketanserin and SB224289 inhibited responses to 5-HT in these vessels. The pK_B of ketanserin was 8.2 ± 0.2. The pK_B of SB224289 was 8.16 ± 0.2.

Effects of SERT inhibitors

The effects of the 5-HT transport inhibitors on responses to 5-HT in hypoxic SD rat vessels are shown on Figure 3C and summarised in Table 2. Citalopram had no effect on responses to 5-HT. Fluoxetine inhibited responses at 1μM. LY393558 inhibited the responses to 5-HT in a dose-dependent fashion.

The effects of the 5-HT transport inhibitors on responses to 5-HT in hypoxic FH rat vessels is shown on Figure 3D and summarised in Table 2. Citalopram potentiated the potency of 5-HT but only at 1μM. Fluoxetine did not have an effect on responsiveness to 5-HT. LY393558 inhibited the maximum responses to 5-HT in a dose-dependent fashion.

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Quantification of SERT expression

SERT binding sites

Figure 4A illustrates that lung ^{3}[H]-citalopram binding was higher in the FH rat than in the SD rat. The affinity for ^{3}[H]-citalopram was not altered, the K_D for ^{3}[H]-citalopram binding was 0.77 ± 0.26 (SD) and 0.89 ± 0.17 (FH). After 2 weeks of hypoxia, however, specific binding was not detectable in either the FH or SD rat lungs.

SERT mRNA expression

Consistent with the immunohistochemistry and the binding studies, there was greater SERT mRNA expression in the FH rats compared to their controls and a marked inhibition after exposure to hypoxia (Figure 4B).

Localization of SERT and changes with hypoxia

SERT immunoreactivity was noted in the SD rat pulmonary arteries, especially at the medial/adventitial border (Figure 5A). Immunoreactive staining was more widespread in the pulmonary arteries of the FH rats, extending into the medial layer itself (Figure 5B). Staining was negligible in the vessels from the FH hypoxic rats (Figure 5C) and there was no visible SERT staining in the SD hypoxic vessels (Figure 5F). Figure 5G shows that SERT immunoreactivity was exclusive to the pulmonary arteries, with negligible immunoreactivity visible in other lung structures, including the airways. The example shown is from the SD rat but the FH rat exhibited the same distribution of SERT immunoreactivity.

Discussion

Previous studies have indicated that there is higher SERT ligand binding in various regions of the FH rat brain compared with control rat strains (Chen and Lawrence, 2000; Hulihan-Giblin et al, 1993). Here, we have shown that expression of SERT mRNA and ^{3}[H]-citalopram binding sites was higher in the FH rat lung compared with the SD rat lung controls. When sections of lung were examined, SERT immunoreactivity was seen to be concentrated in the pulmonary arteries, with negligible immunoreactivity observed in other lung structures. In SD rat pulmonary arteries, immunoreactive SERT was localised to the cells at the medial/adventitial border, which is a similar distribution to that we have previously described in mouse (MacLean et al, 2004). However, in FH rat pulmonary arteries, the immunoreactivity extended further into the medial layer. SERT immunoreactivity is also concentrated in the pulmonary arteries in man, and this is increased in patients with both primary and secondary PAH. In these patients, SERT immunoreactivity is distributed throughout the medial layer of pulmonary arteries (Eddahibi et al 2001, 2003). Hence, with respect to SERT expression, the blood vessels from the FH rat provide a good model in which to study the effects of SERT expression on pulmonary vascular contractile responses.

Compared with the SD rats, there was evidence of right ventricular (RV) hypertrophy. After a two-week exposure to hypoxia, the FH rats developed a greater degree of PAH as indicated by RV hypertrophy and increased pulmonary vascular remodelling. This is consistent with previous studies (Sato et al., 1992; Fujimori et al., 1998., Stelzner et al., 1992; Ashmore et al., 1991). We show here that the development of hypoxia-induced PAH in both the SD and FH rat was associated with a decrease in the expression of lung SERT as determined by Taqman RT-PCR, ligand binding and immunohistochemistry. In the hypoxic SD rat lungs, SERT expression was almost completely absent whilst expression of SERT mRNA and SERT immunoreactivity was evident in FH rat vessels but markedly reduced. We

have recently described a mouse that over-expresses the gene for human SERT and it also is similarly predisposed to hypoxia-induced PAH. There is also a decrease in SERT expression following hypoxic exposure in these mice (MacLean et al., 2004). The current study confirms that this was not a phenomenon only observed in mice and suggests that there is dissociation between SERT activity and PAH caused by hypoxic exposure. Mice over-expressing SERT developed spontaneous increases in RVP (MacLean et al., 2004). Whilst this was not the case for the FH rat, there was evidence of increased RV weight. Animal and clinical studies all suggest, therefore, that over-expression of the SERT cause a pre-disposition to PAH be it familial or secondary to hypoxic exposure. Consistent with this, SERT knock-out in mice protects against hypoxia-induced PAH (Eddahibi et al., 2000). Mouse and rat hypoxic models suggest, however, that persistence of SERT over-expression is not required for the progression of hypoxia-induced PAH (MacLean et al., 2004, this study).

5-HT causes pulmonary artery smooth muscle cell proliferation in a SERT-dependent fashion (Eddahibi et al., 2001, Fanburg and Lee, 2000). However, one mechanism of action of SERT inhibitors against clinical depression is an increase in extracellular accumulation of 5-HT (Slattery et al., 2004). If this occurred at pulmonary arteries then such elevations in 5-HT might cause increased pulmonary arterial vasoconstriction. Therefore, we wished to examine the effects of SERT inhibitors on contractile response to 5-HT in isolated pulmonary arteries.

All the SERT inhibitors reduced the contractile response to 5-HT in SD rat vessels. However, we show that in FH rat vessels, where the 5-HT_{1B} receptor is over-expressed, citalopram (0.1 and 1 μM) and fluoxetine (0.1μM) actually increased the potency of 5-HT. The effects of citalopram were, surprisingly, not concentration-dependent. This suggests either that citalopram is not as specific to the SERT as previously reported or that SERT activity affects vasoconstriction via more than one mechanism. There is more evidence for the latter, given that SERT activity can both affect the 5-HT available for receptor activation

(Slattery et al., 2004) and the amount of 5-HT passing into the cell may also affect 5-HT activation through superoxide production (Lee et al., 1999; Liu and Folz 2004). The higher concentration of fluoxetine (1μM) inhibited 5-HT contraction in the FH rat vessels. This is likely to be due to fluoxetine inhibiting the 5-HT_{2A} receptor at this concentration (Ki against 5-HT_{2A} receptor: ~0.14 μM, (Owens et al., 1997)) or inhibiting calcium sensitivity/uptake (Ungvari et al., 1999, 2000). As well as direct effects on 5-HT receptors, chronic treatment with SERT inhibitors may also result in up- or down-regulation of 5-HT receptors (Gray and Roth, 2001). For example, chronic fluoxetine can alter 5-HT_{2A} receptor signaling (Damjanoska et al., 2003), increase 5-HT_{1A} receptors (Hirano et al., 2002) and both up- and down-regulate the 5-HT_{2B} receptor (Kong et al., 2002). If SERT inhibitors are to be used for the treatment of PAH, these effects are worthy of consideration.

It required a combined block of both the SERT and the 5-HT_{1B} receptor with LY393558 to inhibit the contractile response to 5-HT in the FH rat vessels. In addition, we demonstrated a synergistic interaction between fluoxetine and SB224289 in SD rat vessels. Neither drug alone had an effect on the maximum response to 5-HT but together, they inhibited this by ~60%. The 5-HT_{1B} receptor is expressed in the pulmonary arteries of patients with PAH (Launey et al, 2002, Marcos et al., 2004). In these patients, expression is shown either to increase (Launey et al, 2002) or remain constant (Marcos et al., 2004). Regardless of whether on not 5-HT_{1B} receptor expression is increased, its co-operativity with the SERT in affecting both proliferation (Liu et al., 2004) and constriction (this study) indicates that maximum clinical effect may be gained by blocking both the 5-HT_{1B} receptor and SERT.

So why did citalopram and fluoxetine potentiate responses to 5-HT in FH rat vessels? Our working hypothesis is that, by inhibiting 5-HT reuptake, more 5-HT is accessible to stimulate 5-HT receptors. This would explain the requirement to block the receptors

simultaneously to confer inhibition of 5-HT-induced vasoconstriction as with LY393558 or higher concentrations of fluoxetine. Consistent with this, hypoxia both reduced SERT expression and increased 5-HT-induced vasoconstriction, mimicking the effects of citalopram and fluoxetine in the FH rat vessels. The vasoconstriction was increased most in the FH rats where there was enhanced 5-HT_{1B} receptor expression. The reduced SERT expression would mean that less 5-HT was removed from the 5-HT receptor sites.

We demonstrate that SERT expression is higher in lungs from the FH rat than the SD rat, yet contractile responses to 5-HT were greatest in the FH rat vessels. Hence, the higher SERT activity, which could remove 5-HT away from the receptors, must be over-ridden by receptor activation. Analysis of 5-HT_{1B} and 5-HT_{2A} expression confirmed that both receptors were present in the lungs of FH and SD rats. Accordingly, both ketanserin and SB224289 inhibited responses to 5-HT in FH and SD vessels. Lung 5-HT_{1B} receptor expression was, however, three-fold higher in the FH rat. It is possible that the increased 5-HT_{1B} receptor stimulation over-came any effects of higher SERT expression. In addition, we have previously shown that increased levels of ET-1 can increase 5-HT_1 receptor activation in rat pulmonary arteries (MacLean and Morecroft, 2000). There is increased lung ET-1 production in FH rats (Stelzner et al., 1992). Hence, this may also have increased 5-HT_{1B} receptor activation and contributed to the increased response to 5-HT observed in the FH rat vessels.

Consistent with our observation that hypoxia had no significant effect on either 5-HT_{1B}- or 5-HT_{2A}-receptor expression, both SB224289 and ketanserin inhibited responses to 5-HT in hypoxic SD and FH rats with similar pKB values to those observed in normoxic controls. In the absence of SERT sites in the hypoxic SD vessels, citalopram had no effect. Fluoxetine blocked the response to 5-HT, but again, only at the higher concentration which would inhibit the 5-HT_{2A} receptor. LY393558 was again the most potent antagonist. Citalopram only slightly potentiated 5-HT potency consistent with a reduction in SERT

expression. Again, LY393558 inhibited the maximum response to, and potency of 5-HT in a dose-dependent fashion.

In summary and conclusion, we have shown that SERT and 5-HT_{1B} expression is higher in FH rat lung than on SD rat lung. The contractile response of pulmonary arteries to 5-HT is increased in the FH rat, probably due to increased 5-HT_{1B}-mediated constriction. 5-HT mediated constriction is enhanced in hypoxic SD and FH vessels and this is associated with a decreased expression of SERT. The results also suggest that i) SERT inhibitors can potentiate contractile responses to 5-HT when the 5-HT_{1B} receptor is over-expressed and ii) this potentiation is due to inhibition of 5-HT uptake increasing the 5-HT available to activate the increased number of 5-HT receptors. An important additional finding is that combined 5-HT_{1B} and SERT inhibition synergize in blocking 5-HT-mediated constriction. The addition of 5-HT_{1B} antagonism prevents the increased 5-HT-mediated vasoconstriction induced by SERT inhibitors.

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Footnotes

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Figure legends

Figure 1.

Contractile responses to 5-HT in pulmonary resistance arteries from fawn-hooded (FH) and Sprague-Dawley (SD) rats. A. Responses in FH and SD vessels. B. Effect of the 5-HT_{2A} receptor antagonist ketanserin (10nM) and the 5-HT_{1B} receptor antagonist SB224289 (200nM) in SD rat vessels and C. Effect of ketanserin and SB224289 in FH rat vessels. All responses (A-C) are expressed as % of a response to 50mMKCl in each vessel and the number of animals is in parenthesis. D. Relative expression of lung 5-HT_{2A} receptor mRNA in lungs (n=4) from control and hypoxic SD and FH rats. E. Relative expression of lung 5-HT_{1B} receptor mRNA in lungs (n=4) from control and hypoxic SD and FH rats. Statistical analysis by one way ANOVA and Neuman-Kuels multiple comparisons test is shown for the maximum response in FH vs SD vessels. * $P<0.05$. All data are expressed as mean ± SE. Full statistical analysis is detailed in Table 1.

Figure 2.

The effect of SERT inhibitors on contractile responses to 5-HT in pulmonary resistance arteries. A. Effects in Sprague-Dawley (SD) rat pulmonary arteries. B. Effects of combining fluoxetine and the 5-HT_{1B} receptor antagonist SB224289 in SD vessels. C. A. Effects in Fawn-hooded rat pulmonary arteries. All responses (A-C) are expressed as % of a response to 50mMKCl in each vessel and the number of animals is in parenthesis. Statistical analysis is detailed in Table 2. All data are expressed as mean ± SE.

Figure 3

Effects of hypoxia on responses to 5-HT in rat pulmonary arteries. Effect of 2 weeks hypoxic exposure *in vivo* on responses to 5-HT (Hypoxic 5-HT) compared responses from normoxic rats (Normoxic 5-HT): effect of the 5-HT_{2A} receptor antagonist ketanserin (10nM) and the 5-HT_{1B} receptor antagonist SB224289 (200nM) in hypoxic Sprague-Dawley (SD) rat vessels (A) and hypoxic Fawn-hooded rat vessels (B). Effect of SERT inhibitors in hypoxic SD rat vessels (C) and hypoxic FH rat vessels (D). All data are expressed as mean ± SE. Statistical analysis is detailed in Tables 1 (A and B) and Table 2 (C and D).

Figure 4

SERT expression in the Fawn-hooded (FH) rat lung compared with Sprague-dawley (SD) rat lung and the effects of two weeks exposure to hypoxia. A. ^{3}[H]-citalopram binding in lungs (n=4) (binding was absent in lungs from hypoxic SD and FH rats). B. Relative expression of lung SERT mRNA in lungs (n=4). All data are expressed as mean ± SE.

Figure 5

Immunohistochemical localisation of the SERT in small pulmonary arteries from: A. Sprague Dawley control rat (SDC). B. Fawn-hooded control rat (FHC) C. Fawn-hooded rat after two weeks hypoxia (FHH) F. Sprague Dawley rat after two weeks hypoxia (SDH). D and E: SDC and FHC control sections incubated with secondary but not primary antibody. G: SERT immunoreactivity in SDC lung is shown to be concentrated in pulmonary arteries. Scale bars: 10μm (A-F), 25μm (G). *Key:* M, medial layer; A, adventitia; M/A, medial/adventitial border; EC, endothelial cell layer; PA, pulmonary artery; AW, airway.

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TABLE 1. *p*EC_{50} and E_{max} values for 5-HT-induced vasoconstriction in Fawn-hooded (FH) and Sprague-Dawley (SD) rat pulmonary resistance arteries: effect of the 5-HT receptor inhibitors ketanserin (5-HT_{2A}, 10nM) and SB224289 (5-HT_{1B}, 200nM).

	***p*EC_{50}**	**Emax**	**n**
SD rat			
Control	5.50 ± 0.19	20.9 ± 1.4	7
Control + ketanserin	4.75 ± 0.16**	22.5 ± 2.7	6
Control + SB224289	4.6 ± 0.16**	24.6 ± 3.0	6
Hypoxic	6.71± 0.08***	75.7± 6.4***	8
Hypoxic + ketanserin	5.64± 0.16‡‡‡	57.5± 7.8	6
Hypoxic + SB224289	5.43± 0.08‡‡‡	71.5± 8.5	6
FH rat			
Control	5.98 ± 0.15	31.8 ± 3.0†	15
Control + ketanserin	5.02 ± 0.16***	27.7 ± 7.0	6
Control + SB224289	nm	nm	6
Hypoxic	6.39 ± 0.10**	132 ± 10***	10

Hypoxic+ ketanserin	5.37 ± 0.10‡‡‡	144 ± 12	7
Hypoxic + SB224289	4.91 ± 0.15‡‡‡	115 ± 20	6

† P<0.05 vs SD control; **P<0.01 ***P<0.001 vs own control; ‡‡‡ P<0.001vs own hypoxic control group. All data are expressed as mean ± SE. n=number of animals. nm=no maximum response achieved.

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TABLE 2. pEC_{50} and E_{max} values for 5-HT-induced vasoconstriction in Fawn-hooded (FH) and Sprague-Dawley (SD) rat pulmonary resistance arteries: effect of SERT inhibitors.

	pEC_{50}	Emax	n
SD rat			
Control	5.67 ± 0.1	21.0 ± 1.0	10
Control + 0.1μM citalopram	6.15 ± 0.26	4.5 ± 0.6***	5
Control + 1μM citalopram	6.00 ± 0.17	11.3 ± 0.7***	6
Control + 0.1μM fluoxetine	5.90 ± 0.19	17.8 ± 3.0	6
Control + 1μM fluoxetine	5.36 ± 0.27	11.0 ± 1.5***	6
Control + 10nM LY393558	5.10 ± 0.05	7.2 ± 2.2***	6
Control + 100nM LY393558	5.00 ± 0.5	3.9 ± 2.2***	8
Hypoxic	6.71 ± 0.08***	75.7 ± 6.4***	8
Hypoxic + 0.1μM fluoxetine	6.55 ± 0.10	90.0 ± 8.3	6
Hypoxic + 1μM fluoxetine	5.73 ± 0.06‡‡‡	68.0 ± 4.9	6
Hypoxic + 0.1μM citalopram	6.69 ± 0.10	90.2 ± 5.2	6
Hypoxic + 1μM citalopram	6.48 ± 0.07	85.7 ± 7.3	6
Hypoxic + 10nM LY393558	5.76 ± 0.20‡‡‡	39.3 ± 8.9‡‡‡	7

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Hypoxic + 100nM LY393558	4.73 ± 0.14‡‡‡	21.2 ± 4.9‡‡‡	6
FH rat			
Control	5.47 ± 0.11	31.5 ± 0.11†	8
Control + 0.1μM citalopram	6.91 ± 0.27**	50.0 ± 9.0	7
Control + 1μM citalopram	6.30 ± 0.07*	26.6 ± 4.1	8
Control + 0.1μM fluoxetine	6.24 ± 0.13*	35.8 ± 1.8	6
Control + 1μM fluoxetine	5.71 ± 0.19	44.6 ± 13.8	6
Control + 10nM LY393558	nm	nm	6
Control + 100nM LY393558	nm	nm	6
Hypoxic	6.34 ± 0.08	126 ± 8.5	11
Hypoxic + 0.1μM fluoxetine	6.62 ± 0.15	125 ± 18	5
Hypoxic + 1μM fluoxetine	6.02 ± 0.10	90 ± 10	6
Hypoxic + 0.1μM citalopram	6.84 ± 0.14	123 ± 17	5
Hypoxic + 1μM citalopram	7.00 ± 0.14‡	124 ± 12	5
Hypoxic + 10nM LY393558	5.88 ± 0.18	87 ± 13‡	6
Hypoxic + 100nMLY393558	5.31 ± 0.30‡‡‡	64 ± 12‡‡	6

*P<0.05, **P<0.01, ***P<0.001 vs own control; † P<0.05 vs SD control; ‡ P<0.05 ‡‡ P<0.01, ‡‡‡P<0.001 vs own hypoxic control group. nm=no maximum response achieved. All data are expressed as mean ± SE. n=number of animals.

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TABLE 3. Body weight, vascular and right ventricular remodelling and haemodynamics in Sprague-Dawley (SD) and Fawn-hooded (FH) rats: effects of two weeks hypoxia.

	SD Normoxic	FH Normoxic	SD Hypoxic	FH Hypoxic	n
Body weight (g)	363 ± 11	255 ± 5†††	297 ± 8***	251 ± 4†††	7-9
% remodelled arteries	2.4 ± 2.4	6.8 ± 0.7	21.8 ± 1.2***	30.4±3.2***††	4-5
[RV/LV+S]/ body weight ($x10^{-4}$)	7.11 ± 0.54	10.72 ± 0.54†	15.98 ± 1.52***	21.94 ± 1.15***††	7-9
Heart Rate beats/min	379 ± 18	351 ± 12	426 ± 14	392 ± 12	7-9
Systolic RVP (mmHg)	26.2 ± 3.0	33.2 ± 2.2	57.8 ± 3.0***	62.4 ± 2.8***	7-9
Mean SAP (mmHg)	90.4 ± 9.5	79.3 ± 2.3	91.4 ± 5.1	72.4 ± 1.6	7-9

***P<0.001 vs own Normoxic control; †P<0.05, ††P<0.01, †††P<0.001 vs SD Normoxic/Hypoxic. All data are expressed as mean ± SE. n=number of animals. RV/LV+S, right ventricular/left ventricular plus septal weight (g); RVP, Right ventricular pressure; SAP, systemic arterial pressure.

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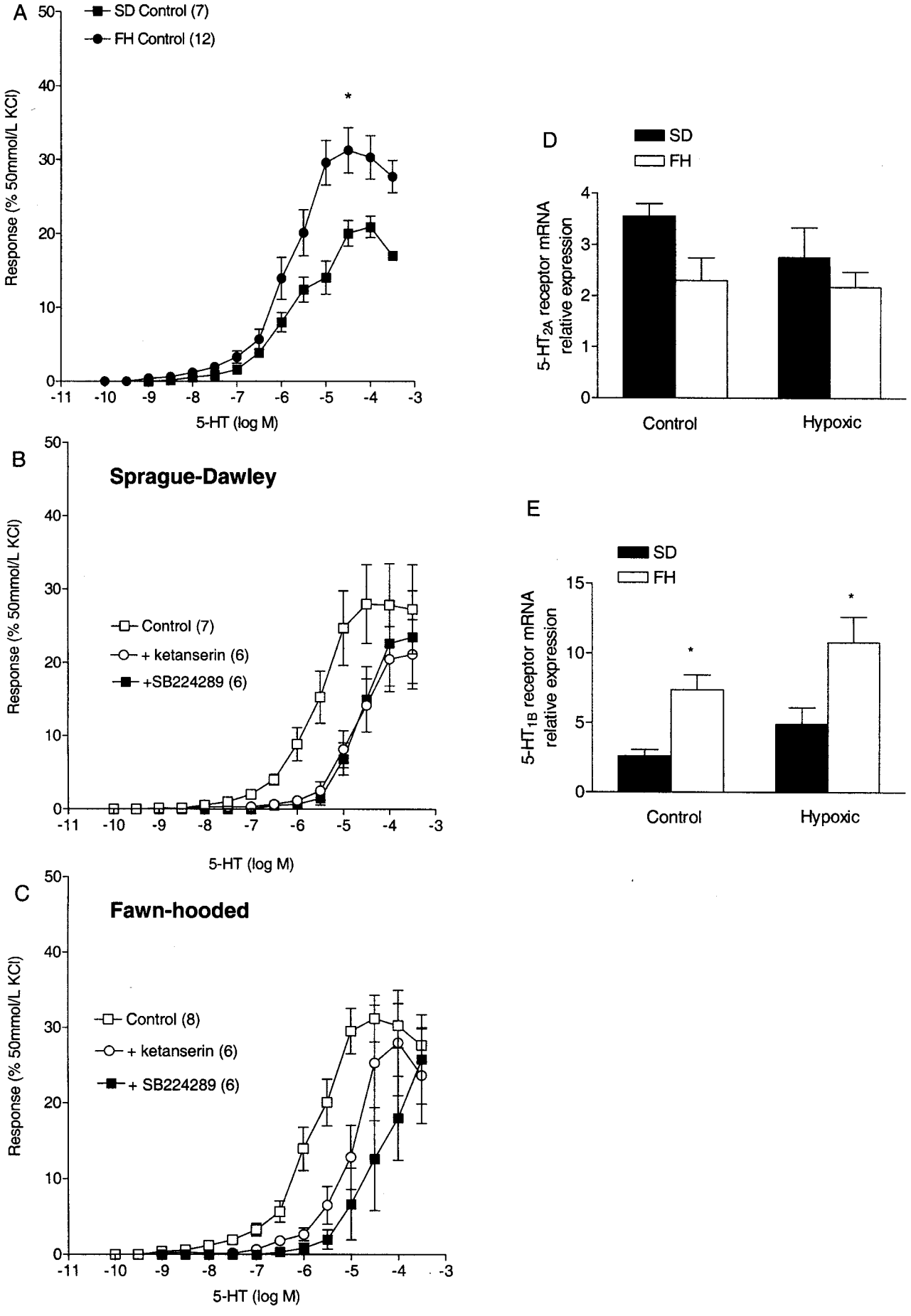

Fig 1

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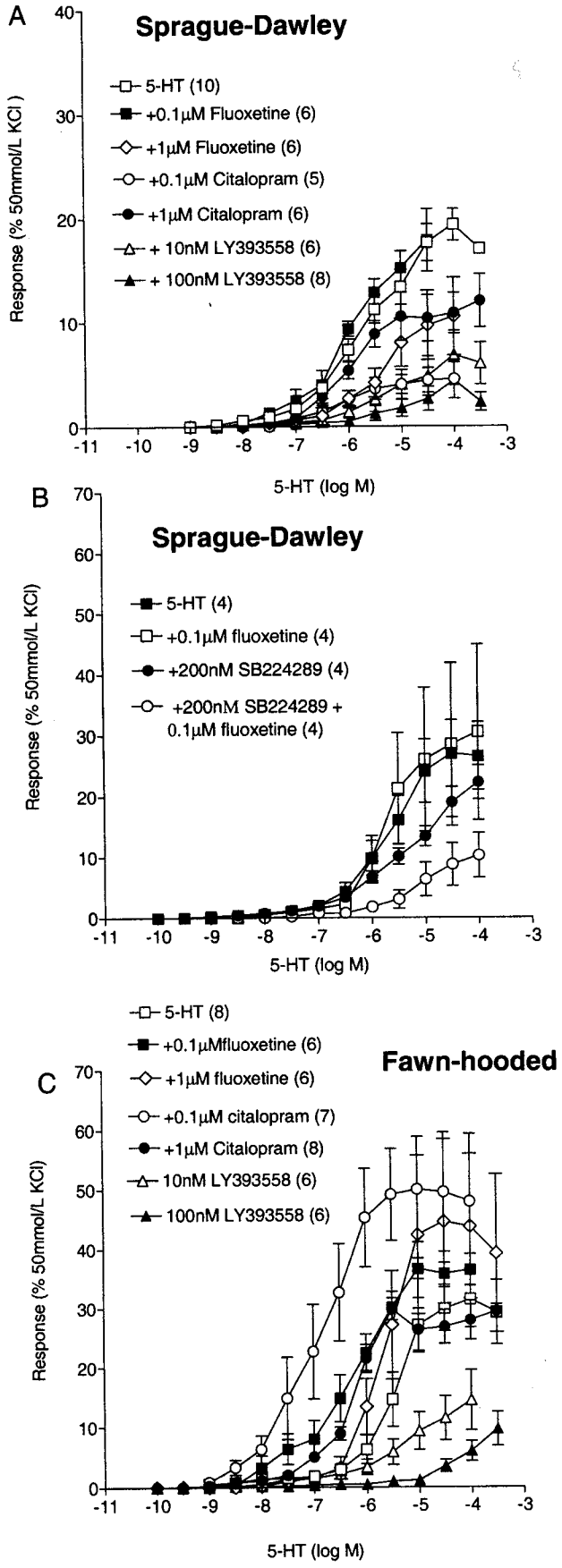

Fig 2

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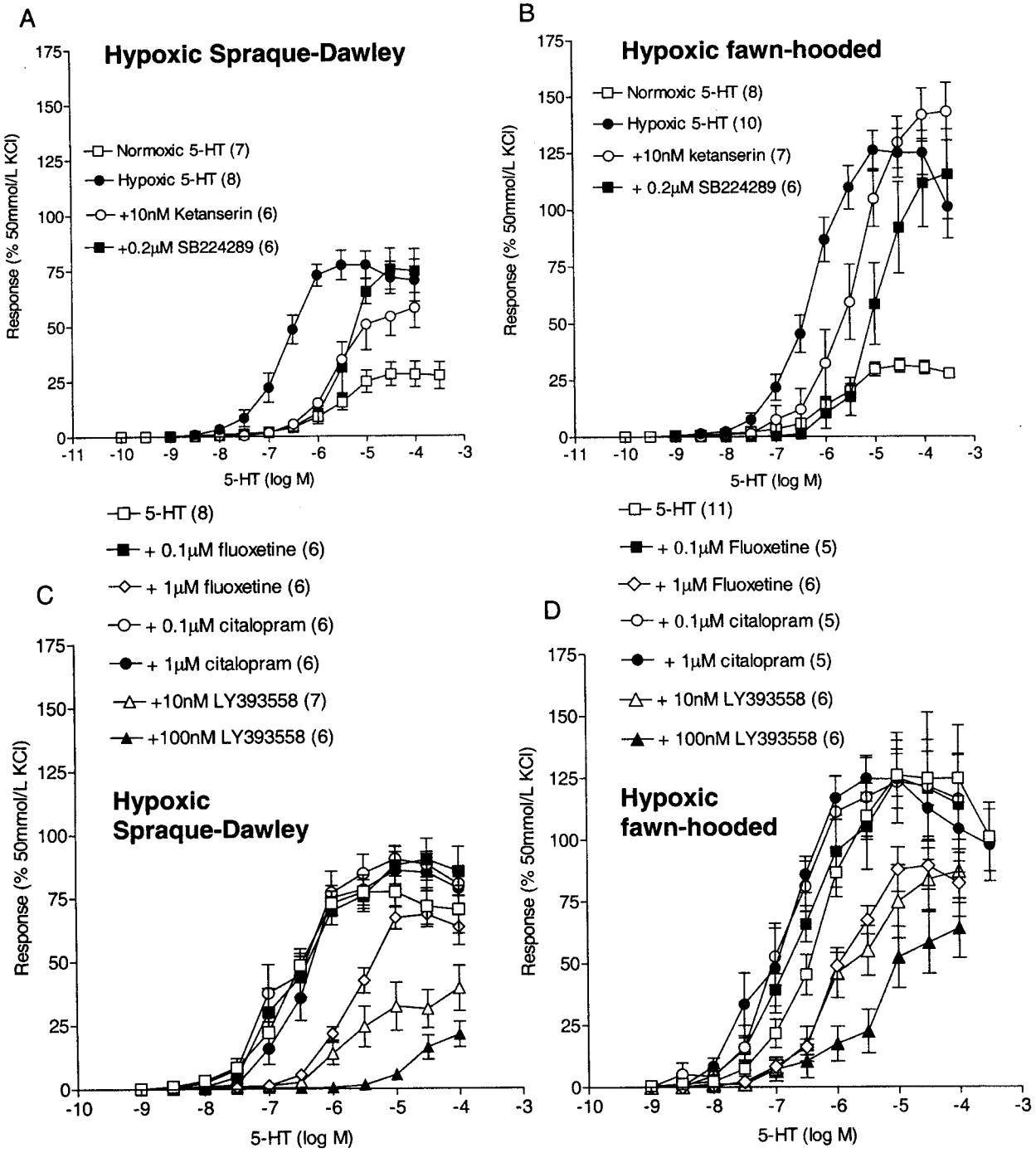

Fig 3

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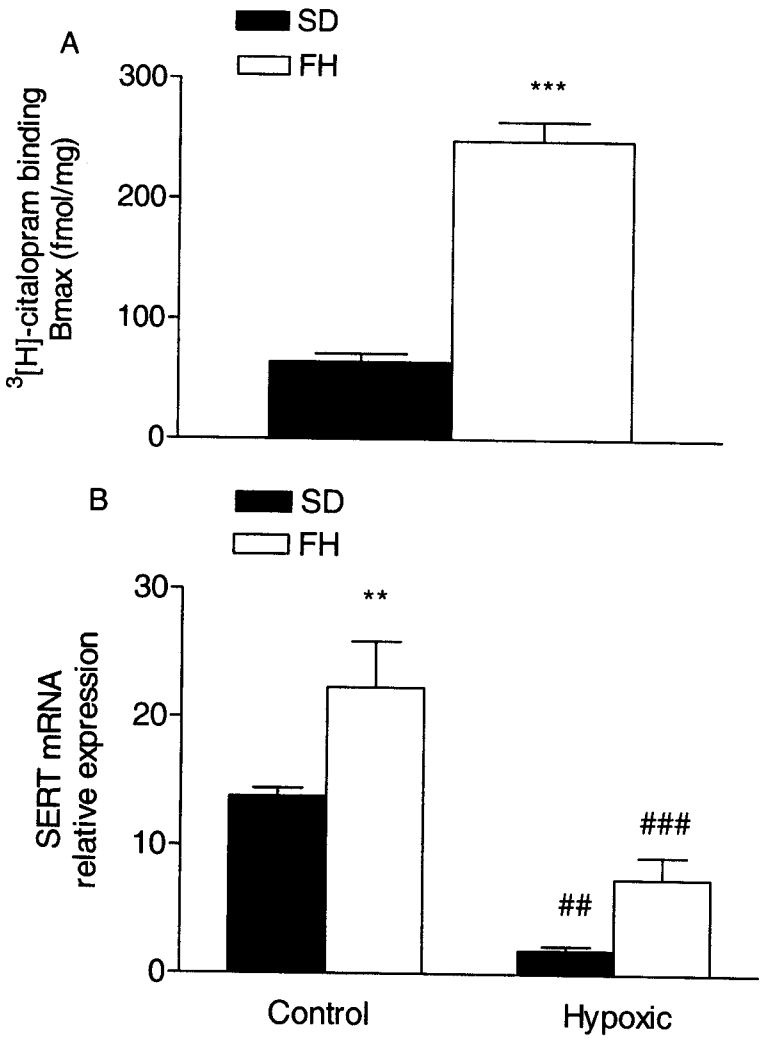

Fig 4

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Fig 5