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$5HT_2$ RECEPTOR CHANGES IN RAT CORTEX AND PLATELETS FOLLOWING CHRONIC RITANSERIN AND CLORGYLINE ADMINISTRATION

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Abstract—Chronic administration of clorgyline or ritanserin to adult rats for 28 days followed by a 3 day drug-free period results in a significant decrease in $5HT_2$ receptor number (B_{max}) in rat frontal cortex from 315.23 ± 10.72 fmol/mg protein to 249.63 ± 13.99 fmol/mg protein and 222.55 ± 17.17 fmol/mg protein, respectively. On rat blood platelets, ritanserin significantly increases recept number from 26.18 ± 3.83 fmol/mg protein to 50.94 ± 7.96 fmol bound/mg protein, whereas clorgyline has no significant effect (21.32 ± 4.78 fmol/mg protein). Following both drug regimens, the affinity (K_d) of the respective ligands for the receptor is not significantly different from controls: the mean K_d value of the three groups for [^{3}H]ketanserin is 1.57 ± 0.05 nM in cortex and 0.83 ± 0.25 nM for [^{125}I]iodolysergic acid diethylamide (LSD) on platelets. Clorgyline increases serotonin (5HT) and noradrenaline (NA) levels in cerebellum, and decreases 5-hydroxyindole acetic acid (5HIAA) and homovanillic acid (HVA): ritanserin does not change the levels of the amines or their metabolites. The data shows that platelet and brain changes are not comparable after ritanserin administration. The receptor binding data demonstrates that curve fitting to two data points provides information which is comparable to and as statistically robust as that obtained from eight point saturation curves. Thus, if pilot studies show that the data follows a rectangular hyperbola, two point assays (optimal at 0.1 K_d and 3 K_d) can be used to obtain estimates of B_{max} and K_d.

Three 5HT receptor subtypes have been identified: $5HT_1$, $5HT_2$, and $5HT_3$ (Ref. 1 for review). $5HT_1$ receptors have a high affinity (nM) for 5HT and can be labelled with [^{3}H]5HT [2], whereas $5HT_2$ receptors have a low affinity (μM) for serotonin (5HT), were initially labelled with [^{3}H]spiperone [3] and stimulate inositol phospholipid (PI) hydrolysis [4]. More selective ligands have now been developed including [^{3}H]ketanserin [5] and [^{125}I]iodo-lysergic acid diethylamide (LSD) [6] thus allowing further receptor characterization. $5HT_3$ receptors are labelled by [^{3}H]quipazine [7] and were identified in the periphery [8, 9] but are also found in the CNS [10, 11].

$5HT_2$ receptors are most concentrated in the frontal cortex, but are present in the striatum and mesolimbic areas [5] and on blood platelets [12]. They mediate contraction of vascular smooth muscle and certain 5HT related behaviours e.g., the head twitch response [13].

Chronic administration of some antidepressants decrease $5HT_2$ receptor density [14]; these decreases appear to be functionally linked since chronic imipramine or iprindole treatments also decrease cortical 5HT-induced PI hydrolysis [15]. Chronic administration of $5HT_2$ antagonists, such as ketanserin or ritanserin, also decrease rat cortical $5HT_2$ receptor number but with no apparent change in the K_d, implying that this decrease is probably not due to direct competition between drug and radioligand [16]. The decrease in $5HT_2$ receptor density following chronic treatment with some $5HT_2$ antagonists is a paradox since antagonists are generally expected to increase receptor number. On the other hand, acute administration of $5HT_2$ antagonists (e.g. mianserin and ritanserin) causes both K_d and B_{max} to decrease suggesting that, in this case there is a direct drug effect on the receptor [17].

We have examined $5HT_2$ receptors in frontal cortex and platelets, of adult male rats, after chronic treatment with ritanserin, a selective and long-lasting $5HT_2$ antagonist [16] and clorgyline, a specific monoamine oxidase type A inhibitor (MAOI) [18], to compare the effects of an antagonist with a drug which increases synaptic 5HT (effectively acting like an agonist) and to determine if $5HT_2$ receptor changes in frontal cortex parallel changes on blood platelets.

Binding data was initially analysed using direct linear plots of data from eight concentrations of radioligand. Dunn *et al.* [19], however, reported that estimates of B_{max} and K_d can be made by fitting a curve to data from only two concentrations of radioligand. Their procedure requires the curve to be a rectangular hyperbola and their data suggests that the two optimal concentrations of radioligand are 1 × K_d and 4 × K_d. We also assessed our data using this two point procedure.

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MATERIALS AND METHODS

Animals. Adult male Wistar rats were randomly assigned to three groups of 12 animals and were maintained on a 12:12 hour light:dark cycle with free access to food and fluid. They received ritanserin (10 mg/kg/day) or clorgyline (2 mg/kg/day) in drinking water for 28 days. These drug schedules have been used in other studies [16, 18, 20]. Drugs were dissolved in dilute tartaric acid (50 mM) at pH 5 (pH of domestic water). Fluid intake was monitored daily to ensure adequate drug was being administered; animals were weighed weekly and dose schedules readjusted. After 28 days, there was a 3 day drug free period after which the animals were anaesthetized by inhalation of Enflurane (Abbott) and approximately 10 ml of blood per animal was collected from the exposed inferior vena cava into a syringe containing 2 ml of anticoagulant (1% EDTA and 3.2% citric acid trisodium salt). The anaesthetized animals were then decapitated, their brains rapidly removed, and the frontal cortices and cerebella dissected on ice and stored at −70° prior to analysis.

Platelet preparation. Blood samples were centrifuged (600 *g* × 5 min, 5°), platelet rich plasma (PRP) was removed and the blood volume restored using phosphate buffered saline plus glucose (NaCl 139 mM, Na_2HPO_4 8.6 mM, NaH_2PO_4 1.4 mM, glucose 11 mM, EDTA 5 mM, pH 7.2). This process was repeated five times to maximize the platelet yield. The total PRP from individual samples was centrifuged (600 *g* × 10 min) to remove red cell contamination and then incubated at 37° for 10 min to metabolize any endogenous free 5HT. An aliquot of PRP was taken, platelets were counted using a Coulter counter and the remainder was pelleted by centrifugation (20,000 *g* × 15 min) and stored at −70°.

[^{3}H]*Ketanserin binding to rat frontal cortex.* Cortical $5HT_2$ receptors were measured using a modified published method [5]. Cortices were homogenised (10% w/v) in Tris–HCl buffer (50 mM, pH 7.7) using a polytron (setting 7, 10 sec). The homogenate was centrifuged (37,000 *g* × 15 min, 4°) and the supernatant discarded. The pellet was resuspended in cold buffer and re-centrifuged. The resultant membrane pellet was resuspended in buffer (10 mg of original tissue/ml) and incubated with eight concentrations of [^{3}H]ketanserin (0.06–4 nM); non-specific binding was determined using methysergide (1 μM). Two hundred and fifty microlitres of membrane suspension was used per tube and the final assay volume was 0.5 ml. Samples in triplicate, were incubated for 15 min at 37° in a shaking water bath. The incubation was terminated by rapid filtration under vacuum using a Brandel Cell Harvester and Whatman GF/C filters. Filters were washed with 3 × 4 ml of ice-cold Tris buffer (pH 7.7) and placed in vials containing 6 ml of scintillation fluid (Optiphase "Hisafe" II). Bound radioligand was measured in a scintillation counter on the following day.

[^{125}I]*Iodo-LSD binding to rat platelets.* $5HT_2$ receptors on platelets were measured according to the method of Engel *et al.* [6] as modified by Brazell *et al.* [21]. Since the density of $5HT_2$ receptors on platelets is approximately 8% of that in the cortex, the more sensitive $5HT_2$ radioligand, [^{125}I]iodo-LSD was used in the platelet assays [6]. Using a polytron (setting 7, 10 sec), platelet membrane pellets were resuspended in assay buffer (Tris 50 mM, ascorbic acid 7 mM, EDTA 3 mM, NaCl 125 mM, KCl 5 mM, pH 7.4) at a final tissue concentration of approximately 133 × 10^8 platelets per ml. The platelets were incubated with seven concentrations of [^{125}I]iodo-LSD (0.06–4 nM) and ketanserin tartrate (1 μM) was used to assess non-specific binding. One hundred and fifty microlitres of platelet membrane suspension was used per tube and the total incubation volume was 250 μl. Duplicate samples were incubated for 30 min at 37° in a shaking water bath by which time binding equilibrium with the ligand was reached (Brazell, unpublished data). Samples were filtered under vacuum using a Brandel Cell Harvester and Whatman GF/C filters presoaked in a solution of 0.5% Triton and 0.3% polyethylenemine. Filters were washed with 3 × 4 ml of ice-cold buffer and bound radioligand was measured in a gamma counter.

Protein concentration was determined using the method of Lowry *et al.* [22] as modified by Peterson [23] with bovine serum albumin as the protein standard. Direct linear plot analysis was used to obtain values of B_{max} and K_d [24].

Two point estimations of B_{max} *and* K_d. B_{max} and K_d values obtained from direct linear plot eight point saturation curves were compared with those obtained by fitting curves to two of the points. The procedure involved the use of a non-linear regression programme (Enzfitter). Three pairs of radioligand concentrations ($1 \times K_d$ and $4 \times K_d$), ($0.5 \times K_d$ and $2 \times K_d$) and ($0.1 \times K_d$ and $3 \times K_d$) were examined.

Estimation of 5HT, 5HIAA, NA and HVA in cerebellar samples. Cerebellar homogenates, in 1 ml of 0.1 M perchloric acid, were centrifuged (13,000 *g* × 10 min, 4°), and the supernatant stored at −70° until analysed using high performance liquid chromatography (HPLC) with electrochemical detection (ECD). Separation and detection was performed at 10° using an ACS 351 pump (HPLC Technology) and an on-line degasser (ERC3510, Erma an Inc.) coupled to a Chromspher C18 cartridge column (length, 10 cm; internal diameter, 3 mm; particle size, 5 μm). The cartridge was protected by a guard column and a saturation pre-column (Chrompack UK Ltd). ECD was carried out with an LC-2A detector (BAS Inc.); the working electrode was maintained at +0.75 V with respect to an Ag/AgCl reference electrode. The mobile phase consisted of a 0.1 M citrate-phosphate buffer containing 1.5 mM octane sulphonic acid, 12% methanol and 1 mM EDTA, at pH 2.65; the flow-rate was 0.6 ml/min. Peaks were displayed, integrated and stored using a Shimadzu C-R3A recorder coupled to an FDD-1A disk drive (Dyson Instruments Ltd).

Chemicals and drugs. [^{125}I]Iodo-LSD (sp. act.: 2200 Ci/mmol) and [^{3}H]ketanserin hydrochloride (sp. act.: 61 Ci/mmol) were obtained from New England Nuclear (Hertfordshire, U.K.); ketanserin tartrate was obtained from Janssen Pharmaceutica (Beerse, Belgium). All other drugs were gifts from the following sources: ritanserin from Janssen Pharmaceutica, clorgyline from May and Baker Phar-

maceuticals (Essex, U.K.) and methysergide from Sandoz Pharmaceuticals (East Hanover, NJ).

RESULTS

Effect of the chronic drug regimens on rat weight

Initially, the control group weighed 372.92 ± 7.85 g, the clorgyline group, 400 ± 9.37 g and the ritanserin group, 391.67 ± 7.37 g. An analysis of variance (ANOVA) showed no significant difference between the groups. At the end of the experiment (completed by all the animals), controls weighed 430 ± 8.43 g, the clorgyline group, 413 ± 7.67 g and the ritanserin group, 437.71 ± 6.48 g; ANOVA showed no significant difference between the groups. The control and ritanserin groups gained weight throughout the 32 days. The overall gain in the control group was 55 ± 8.94 g (13% increase) and in the ritanserin group, the gain was 45.21 ± 6.39 g (10% increase). The clorgyline group, however, showed a weight loss of 17.5 ± 7.06 g (4% decrease) after the first week, but in the last week of treatment the clorgyline group gained weight at a rate similar to that of the other two groups, and at 32 days there was an overall gain of 13.33 ± 6.61 g (3% increase).

$5HT_2$ receptor changes in cortex and platelets after drug regimens

The B_{max} value for cortical $5HT_2$ receptors was 315 ± 10.72 fmol/mg protein (Table 1). In the clorgyline group, B_{max} was significantly decreased (21%, $P < 0.05$) to 249.63 ± 13.99 fmol/mg protein. There was also a significant decrease (29%, $P < 0.001$) in B_{max} in the ritanserin group to 222.55 ± 17.17 fmol/mg protein. K_d values were not significantly changed by the treatments, the mean K_d value for [^{3}H]ketanserin was 1.57 ± 0.05 nM.

The B_{max} value for $5HT_2$ receptors on platelets was 26.18 ± 3.83 fmol/mg protein. Chronic clorgyline treatment decreased receptor number to 21.32 ± 4.78 fmol/mg protein (i.e. by 19%); however, this difference did not reach statistical significance ($P < 0.4$). The ritanserin regimen significantly increased receptor number to 50.94 ± 7.96 fmol/mg protein (i.e. by 95% $P < 0.05$). K_d values did not differ from the controls, the mean K_d value for [^{125}I]iodo-LSD was 0.83 ± 0.25 nM. As the platelet membranes were not extensively washed, white cell contamination might have caused a big variation in the protein content/number of platelets. However, when B_{max} values were expressed in terms of platelet number, there was still a significant ($P<0.01$) increase in receptor number following the ritanserin regimen and a non significant decrease in receptor number in the clorgyline group.

Comparison of B_{max} and K_d values using curve fitting to various sets of two data points

All the curve fitting procedures provided B_{max} values within approximately 5% of each other and within 10% of the B_{max} value obtained using the

Table 1. Effect of chronic clorgyline and ritanserin administration on $5HT_2$ receptor number in rat cortex and rat blood platelets

	Cortex		Platelets	
	B_{max} (fmol bound/mg protein)	K_d (nM)	B_{max} (fmol bound/mg protein)	K_d (nM)
Control	315.23 ± 10.72	1.51 ± 0.20	26.18 ± 3.83	0.80 ± 0.18
Clorgyline	249.63 ± 13.99*	1.54 ± 0.23	21.32 ± 4.78	0.41 ± 0.08
Ritanserin	222.55 ± 17.17†	1.66 ± 0.16	50.94 ± 7.96*	1.28 ± 0.41

Each value represents the mean ± SE of six (platelet) and seven (cortex) separate experiments which, in the case of the cortex were carried out in triplicate and in the case of the platelets were carried out in duplicate. For each cortical sample the assay was repeated; due to radioligand expense, this was not possible for the platelet samples. Unpaired two-tailed *t*-tests were used for comparisons of the drug treated group with the controls, * $P < 0.05$, † $P < 0.001$.

Table 2. A comparison of B_{max} values for $5HT_2$ receptors in rat cortex obtained by fitting curves to various sets of two data points

	B_{max} (fmol of bound ligand per mg protein)		
	Control	Clorgyline	Ritanserin
Eight point curve (Enzfitter)	332.09 ± 16.33	253.98 ± 16.86*	255.16 ± 16.55*
Two point curve ($1 \times K_d$ and $4 \times K_d$)	350.03 ± 26.79	260.55 ± 21.11*	262.41 ± 19.41*
Two point curve ($0.5 \times K_d$ and $2 \times K_d$)	343.43 ± 33.01	231.70 ± 15.00*	262.25 ± 23.46*
Two point curve ($0.1 \times K_d$ and $3 \times K_d$)	340.16 ± 21.27	247.74 ± 23.21*	240.82 ± 19.84*
Direct linear plot method	315.23 ± 10.72	249.63 ± 13.99*	222.55 ± 17.17†

Values represent the mean B_{max} value ± SE of seven separate experiments, which were performed in triplicate. For each example, the assay was repeated. Unpaired two-tailed *t*-tests were carried out for comparisons with the control animals, * $P < 0.05$, † $P < 0.001$.

Table 3. A comparison of K_d values for $5HT_2$ receptors in rat cortex obtained by fitting curves to various sets of two data points

	K_d values (nM)		
	Control	Clorgyline	Ritanserin
Eight point curve (Enzfitter)	1.90 ± 0.35	1.68 ± 0.32	2.46 ± 0.37
Two point curve ($1 \times K_d$ and $4 \times K_d$)	2.40 ± 0.85	2.04 ± 0.54	2.86 ± 0.65
Two point curve ($0.5 \times K_d$ and $2 \times K_d$)	1.54 ± 0.27	1.40 ± 0.21	2.20 ± 0.54
Two point curve ($0.1 \times K_d$ and $3 \times K_d$)	1.73 ± 0.24	1.28 ± 0.26	1.89 ± 0.29
Direct linear plot method	1.57 ± 0.22	1.73 ± 0.22	1.66 ± 0.16

Each value represents the mean K_d value ± SE of seven separate experiments which were carried out in triplicate. For each sample, the assays were repeated. Unpaired two-tailed t-tests were used for comparisons of the drug treated animals with the controls.

direct linear plot method (Table 2). The significant findings regarding B_{max} using the eight point direct linear plot method i.e. that chronic clorgyline or ritanserin administration reduces cortical $5HT_2$ receptor numbers, were confirmed when a measure of B_{max} was obtained using a curve fitting procedure on eight data points and also on the various combinations of two data points.

The K_d values for [^{3}H]ketanserin obtained using the curve fitting procedure confirmed the findings obtained with the direct linear plot method i.e. that there were no significant differences between the groups (Table 3). However, when the various methods of data analysis were examined together, the overall K_d for the clorgyline group was 10% lower than the control K_d whereas the K_d for the ritanserin group was approximately 20% higher.

Measurements of amines and their metabolites in the cerebellum after chronic drug regimens

As determined by HPLC, there was an 85% increase in 5HT and a 22% decrease in 5HIAA in the cerebellum of the clorgyline treated animals (Fig. 1). There was also a 62% increase in NA and a 61% decrease in HVA. In the ritanserin treated animals, there were no significant changes in 5HT, 5HIAA, NA or HVA levels relative to control levels.

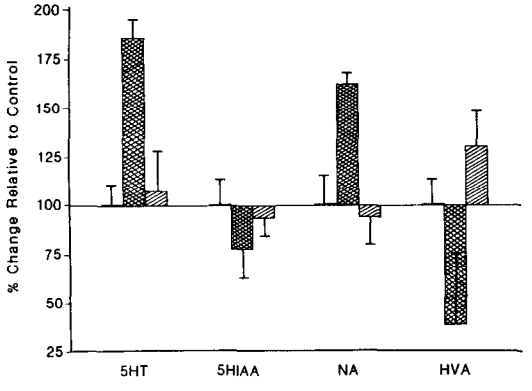

Fig. 1. Changes in amines and their metabolites in the cerebellum after chronic clorgyline and ritanserin administration. Each value represents the mean ± SE of 12 separate values expressed in terms of per cent change with respect to the control. (□) control, (▩) clorgyline (⌐) ritanserin.

DISCUSSION

Our data on weight changes in response to the drug regimen suggests that neither vehicle nor ritanserin are toxic or have appetite suppressing effects, since the animals in both groups gained weight during the study. The initial, small decreases in weight (<5%) observed during the first week of the clorgyline regimen are unlikely to be due to toxicity as this drug has been used clinically in doses of 0.5 mg/kg [25], and the weight gain in the latter part of the study was comparable to that in the other groups. Durcan *et al.* [20] reported that chronic antidepressant drug treatment, e.g. desipramine and clorgyline, temporarily suppress food and fluid intake in rats with the maximum effect occurring in the first week. This suppression may result from increased levels of catecholamines present at the postsynaptic dopamine or β-adrenergic receptor in the perifornical region of the hypothalamus [26] or, alternatively, to changes in the serotonergic system [27]. Clorgyline, by elevating brain NA and 5HT levels [18], may initially have an appetite suppressing effect which is later reversed as a result of receptor adaptation.

The interpretation of the receptor binding data was dependent on analysis of eight point saturation curves. This was compared with that obtained using two data points. It was found that in terms of accuracy, it is acceptable to employ two point binding assays. Data analysis involving two points will, theoretically, have an error twice that obtained with the eight point analysis as the SE is inversely proportional to the square root of the sample size. The errors in the two point analyses of K_d and B_{max} are in fact larger than those obtained with the eight point analysis but are smaller than predicted. The error in the B_{max} values was always less than 10% whereas the error in the K_d values was substantially higher and ranged from 12 to 27%. Our data suggests that if two point assays are employed, then ligand concentrations of $0.1 \times K_d$ and $3 \times K_d$ provide the "best" estimate of B_{max} and K_d. Some eight point

data should be examined to establish that it can be fitted to a rectangular hyperbola and to establish a rough estimate of K_d. The technique is particularly useful when limited amounts of tissue e.g. specific brain nuclei or large numbers of samples e.g., from clinical trials, require analysis. The procedure also saves time and is more economical.

Analysis of the receptor binding data shows that on platelets, clorgyline causes a 19% decrease in $5HT_2$ receptor number and although this decrease is not significant, it follows the expectation that a drug which chronically elevates 5HT will cause a decrease in $5HT_2$ receptor number. In the cortex, clorgyline significantly decreases $5HT_2$ receptors by 21%. In both tissues, the K_d remains unchanged. Ritanserin, however, increases platelet $5HT_2$ receptor number, which is predictable but somewhat unexpected in light of our cortical findings. This study in brain confirms the findings of Leysen *et al.* [16] that chronic antagonist administration decreases cortical $5HT_2$ receptor number. As *in vivo* drug induced changes in receptor number are generally considered to be long term events involving changes in receptor synthesis or turnover [28] and since platelets are anucleate, these changes may occur when the platelets are at the megakaryocyte stage. The data shows that platelet receptor changes do not always reflect receptor changes in the brain. This could be due to the fact that neurones receive inputs from many systems and also have a greater synthetic capacity than platelets. Alternatively, the apparent difference in receptor regulation between platelets and cortex could be due to differences in selectivity of the ligands used as it has recently been reported that ketanserin binds to sites involved in amine release in addition to $5HT_2$ receptors [29].

There are several possible explanations for the antagonist induced down regulation of $5HT_2$ receptors in cortex. Such "apparent" down-regulation may be due to residual "free" ritanserin competitively inhibiting [^{3}H]ketanserin binding and hence reducing B_{max}. Since the K_d value for [^{3}H]ketanserin binding remains unchanged, this seems unlikely and also, the preparative washing of the membranes will ensure removal of most free drug. Alternatively, as ritanserin binds tightly to the receptor site and is difficult to remove [16], the reduction in B_{max} may be due to irreversible receptor blockade rather than to down-regulation since in this case K_d would be unchanged. However, brain levels of ritanserin in chronically treated animals (14 mg/kg/day for 25 days), followed by a 3 day drug-free period have been reported to be below 1 ng/g tissue [16]. Also [^{3}H]ketanserin would be predicted to have a competitive effect as it is a cogener of ritanserin. Finally, although it has been shown that K_d values increase and B_{max} values decrease within 2 hr of acute drug administration, over a period of 48 hr, i.e. less than our "wash out" period, both values return to control levels as the drug dissociates from the receptor [16].

Since clorgyline increases 5HT levels, it suggests that the receptor changes are 5HT mediated, although MAOIs also affect other amines. However, we found no change in 5HT or 5HIAA in the ritanserin treated animals suggesting that ritanserin does not mediate its effect via 5HT. It is possible that changes in cerebellar amine levels might not reflect cortical changes, but as clorgyline increases both cerebellar and cortical 5HT and NA and decrease 5HIAA [18] it is likely that ritanserin induced changes in cerebellar amine levels will be similar to cortical changes. Amitriptyline induced down regulation of $5HT_2$ receptors also seems to be independent of 5HT levels as down regulation is unaffected by neuroterminal destruction [30]. Hence amitriptyline and ritanserin may have direct effects on the receptor or on its effector system, a conclusion which is supported by a report that ritanserin elevates diacyglycerol (DAG) in membranes by inhibiting diacylglycerol kinase [31]. By acting on protein kinase C and stimulating phosphorylation of various proteins, DAG could promote receptor down regulation either directly or via a change in some other receptor population. Alternatively, if 5HT is not necessary for the induction of $5HT_2$ receptor down regulation, 5HT may not be the endogenous ligand for the receptor. This seems unlikely on the basis of our clorgyline data. However, it has been reported, by one group [32] (but is as yet unconfirmed) that a peptide from bovine brain (2–10 kD) acts on the $5HT_2$ receptor and that $5HT_2$ antagonists may mimic this peptide and hence cause down regulation. This peptide has also been proposed to decrease the responsiveness of the $5HT_1$ recognition sites [32] and this allows the possibility that changes could be related to receptor "cross talk".

In conclusion, the platelet data, showing that ritanserin causes $5HT_2$ receptor supersensitivity, indicates that antagonist induced down-regulation of $5HT_2$ receptors is not a universal response and suggests that the complexity of the cortex compared with platelets either in terms of interacting neuronal systems or in terms of an interaction between the various receptor populations present, is in some way responsible for these findings. There is already some evidence for an interaction between $5HT_1$ and $5HT_2$ receptors [33] and their second messenger systems [34] and, although a model to explain the down regulation of $5HT_2$ receptors in terms of receptor "cross talk" is presently unclear, future research could proceed in this direction.

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