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Increase in serotonin$_2$ receptor density in rat cerebral cortex slices by stimulation of beta-adrenergic receptors

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Central noradrenergic and serotonergic pathways appear to modulate and balance each other. The interaction of norepinephrine and serotonin plays a role in the regulation of mood [1–3], blood pressure [4], pituitary hormone release [5], sleep patterns [6], and many other physiological and behavioral processes. Recent studies have found that the administration of β-adrenergic agonists to rats enhances behavioral responses to serotonergic stimulation [7, 8]. These serotonin-related behaviors, e.g. head twitching, resting tremor, forepaw treading, and hind-limb abduction, have been suggested to be mediated by central serotonin$_2$ (5-HT$_2$) receptors [9]. In the present study, we provide evidence for an interaction between β-adrenergic and serotonergic$_2$ receptor binding sites which may account for the serotonergic hyperactivity induced by β agonists. We report here that stimulation of β-adrenergic receptors by (-)isoproterenol in rat cerebral cortical brain slices increased the density of serotonin$_2$ receptor binding sites. Modulation of serotonin receptors by norepinephrine could represent an important component of central serotonergic and noradrenergic nervous system interaction.

Cerebral cortical slices (0.26 × 0.26 × 1–2 mm) were prepared from twelve to fifteen male Sprague–Dawley rats (150–250 g) as described previously [10] and immediately transferred to 250 ml of oxygen-saturated physiologic buffer [11] containing 0.1% (w/v) bovine serum albumin (Sigma Chemical Co.) at 37° and preincubated for 10–50 min, during which the buffer was continuously gassed with 95% O_2 and 5% CO_2. Preincubated slices were centrifuged at 200 *g* for 30 sec, the supernatant fraction was aspirated, and the slices were resuspended in 40 ml of fresh, gassed buffer at 37°. Slices were equally aliquoted into control and test groups, and each group was diluted to a total volume of 150 ml with 37° gassed buffer containing 0.5 mM sodium metabisulfite antioxidant (Sigma Chemical Co.) with or without 100 μM (−)isoproterenol (+)bitartrate (Sigma Chemical Co.). The incubation buffer was maintained at 37° in a shaking incubator and gassed (95% O_2/5% CO_2) throughout each experiment. Following the incubation, the slices were diluted 2-fold with ice-cold isotonic buffered saline and immediately homogenized using a Brinkmann Polytron. Membranes for binding assays were then prepared according to Bylund and Snyder [12], and protein concentrations were determined by the method of Lowry *et al.* [13].

Beta-adrenergic and 5-HT$_2$ receptor binding to cerebral cortical membranes (430–600 μg protein) were determined using [^{3}H]dihydroalprenolol ([^{3}H]DHA, Amersham, 50.0 Ci/mmole) and [^{3}H]spiperone (Amersham, 17.0 Ci/mmole), respectively, as described elsewhere [14, 15]. Nonspecific [^{3}H]DHA binding was determined in the presence of 5.0 μM *l*-alprenolol-*d*-tartrate (Sigma Chemical Co.), and nonspecific [^{3}H]spiperone binding was determined in the presence of 25 μM ketanserin tartrate (Janssen Pharmaceuticals).

Incubation of cerebral cortical slices with 100 μM (−)

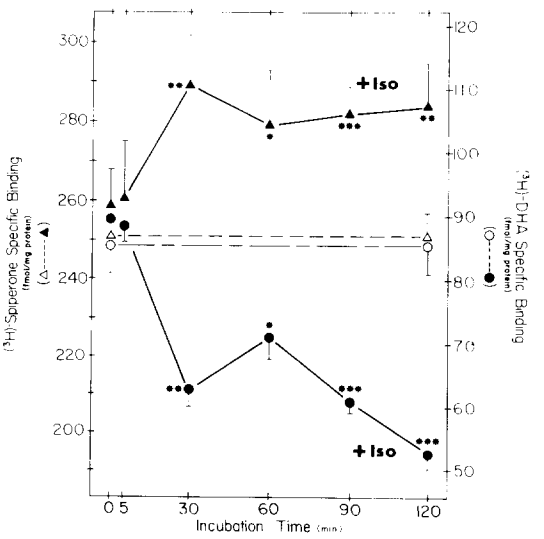

Fig. 1. Time course of isoproterenol-induced changes of β- and 5-HT$_2$-receptor binding sites in rat cerebral cortical membranes. Slices were incubated for various times at 37° with or without 100 μM (−)isoproterenol, after which membranes were prepared and assayed with a single concentration of ^{3}H-labeled ligand. Beta-adrenergic receptor binding was assayed with 1.45 nM [^{3}H]dihydroalprenolol ([^{3}H]DHA). Serotonin$_2$ receptor binding was assayed with 1.91 nM [^{3}H]spiperone. Assays were terminated by filtration through Whatman GF/B filters, and protein concentrations of 430–470 μg/ml and 430–470 μg/2 ml were used for β- and 5-HT$_2$-receptor assays respectively. Specific radioligand binding to control membranes did not vary significantly with incubation time, and the control values ± S.E.M. averaged over the entire incubation period were 85.4 ± 4.1 fmoles/mg protein for [^{3}H]DHA and 251 ± 5 fmoles/mg protein for [^{3}H]spiperone. Each point is the mean ± S.E.M. of four separate determinations for [^{3}H]DHA binding and six separate determinations for [^{3}H]spiperone binding, each determined in triplicate. The data shown are typical of results obtained in six experiments of similar design. Student's *t*-test was used to evaluate differences for significance. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$, when compared to controls.

isoproterenol reduced the specific binding of the β-receptor antagonist [^{3}H]DHA. We found a 26.5 ± 2.9% ($P < 0.01$; $N = 6$) reduction after 30 min (Fig. 1). The reduction in [^{3}H]DHA binding was maintained throughout a 120-min incubation. Beta-receptor binding in control slices was not changed significantly over this time course. In contrast, the specific 5-HT_2 receptor binding of [^{3}H]spiperone was *increased* in the slices incubated with 100 μM (−)isoproterenol. There was a 15.2 ± 5.1% ($P < 0.01$; $N = 6$) increase in 5-HT_2 receptor binding after 30 min of incubation (Fig. 1). The isoproterenol-induced increase in 5-HT_2 receptor binding was maintained for at least 120 min, and control binding did not change over this time course.

To determine if these changes in specific binding were due to changes in the density of receptors, i.e. B_{max}, or the apparent affinities of these receptors for their respective ligands, i.e. K_d, the specific binding of several different concentrations of tritiated ligand was determined and saturation analyses were performed as described elsewhere [14–17]. These studies indicated that the density of serotonergic$_2$ receptors was *increased* by 30.5 ± 7.3% ($P < 0.001$; $N = 6$) in the slices incubated with (−)isoproterenol (Fig. 2). In addition, the dissociation constant (K_d) for [^{3}H]spiperone was *increased* by 53.5 ± 19.8% ($P < 0.05$; $N = 6$) (Table 1). Incubation with isoproterenol decreased the B_{max} for [^{3}H]DHA by 19.6 ± 0.5% ($P < 0.01$; $N = 6$). Isoproterenol treatment also decreased the apparent affinity for [^{3}H]DHA, although this could have been due to residual isoproterenol in the membranes.

To investigate the pharmacological specificity of the isoproterenol-induced changes in 5-HT_2 and β-receptor binding, slices were coincubated with 100 μM (−)isoproterenol and 50 μM sotalol-HCl (Mead-Johnson), a specific β-receptor antagonist. The coincubation with sotalol prevented the isoproterenol-induced changes in β and 5-HT_2-receptor density (Table 1). This suggests that changes in both β and 5-HT_2 receptor B_{max} are mediated by β-receptor stimulation. The lowered binding affinity of [^{3}H]DHA in sotalol-treated tissue could be due to residual drug in the membranes. The reason for the change in affinity for [^{3}H] spiperone in the various groups is not clear.

To determine if β-receptor stimulation affects the binding of other receptor sites, we measured serotonin$_1$ (5-HT_1) and α_2-adrenergic receptor binding after incubating cerebral cortical slices with 100 μM (−)isoproterenol. Serotonin$_1$ receptor binding was determined using membranes pre-incubated in 10 μM pargyline-HCl (Sigma Chemical Co.) for 25 min at 37°. The membranes (740–800 μg protein) were incubated with 1.0 nM [^{3}H]serotonin (Amersham, 12.3 Ci/mmole) and 10 μM pargyline in 1 ml 50 mM Tris–HCl, pH 7.7, for 10 min at 37°. Total binding was determined in triplicate and nonspecific binding in duplicate in the presence of 20 μM serotonin-creatinine sulfate (Sigma Chemical Co.). Alpha$_2$-adrenergic receptor binding was determined by incubating membranes (450 μg protein) with 2.96 nM [^{3}H]clonidine (Amersham, 24.0 Ci/mmole) in 1 ml 50 mM Tris–HCl, pH 8.0, for 30 min at 25°. Total binding was determined in triplicate and nonspecific binding in duplicate in the presence of 5.0 μM clonidine-HCl (Boerhinger Ingelheim). Both serotonin$_1$- and alpha$_2$-receptor binding assays were terminated by rapid filtration through Whatman GF/B or GF/C filters with four washings with 5 ml of ice-cold 50 mM Tris–HCl, pH 8.0. Radioactivity was counted as described previously. Serotonin$_1$ receptor binding was not altered significantly after incubating slices for 60 or 120 min with isoproterenol (data not shown). In agreement with previous reports [18], alpha$_2$-receptor binding was increased 21.6 ± 2.3% ($P < 0.001$; $N = 6$) in slices incubated for 90 min with isoproterenol.

Although serotonin$_2$ sites have been shown previously to be separate from alpha$_2$-adrenergic sites, we have determined the apparent binding affinities of a number of serotonergic and adrenergic compounds at these sites and have clearly established a difference between these receptor sites. Binding of [^{3}H]spiperone or [^{3}H]clonidine to cerebral cortical membranes was determined as previously described in the presence of four or five concentrations of displacing agent ranging from 0.01 to 50.0 μM. The potency of each

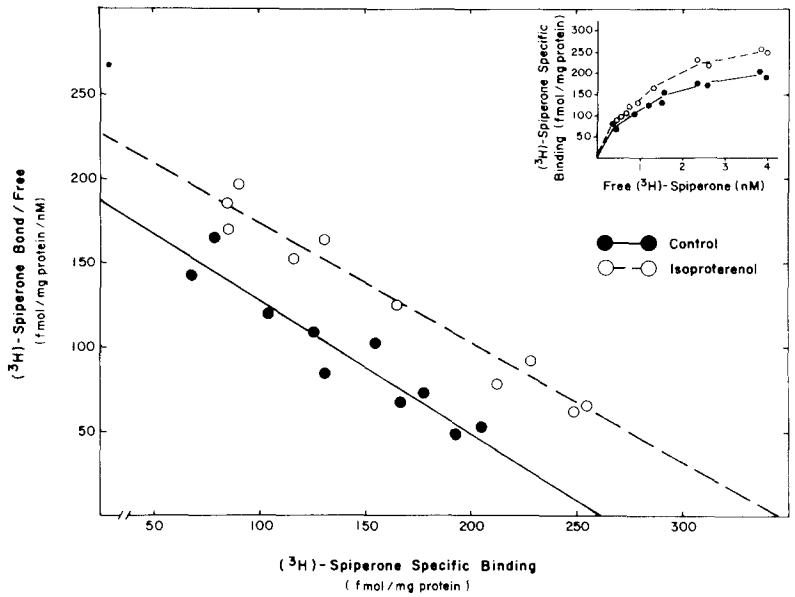

Fig. 2. Saturation analysis of specific 5-HT_2-receptor binding to rat cerebral cortical membranes. Slices were incubated for 60 min at 37° with or without 100 μM (−)isoproterenol. Membranes were prepared and saturation analyses were performed with ten concentrations of [^{3}H]spiperone ranging from 0.5 to 4.0 nM. Assays were terminated by filtration through Whatman GF/C filters, and a protein concentration of 300 μg/ml was used. Similar results were obtained in six other experiments of similar design. In each experiment, the B_{max} of [^{3}H]DHA binding in isoproterenol-incubated slices was decreased significantly from that of control slices.

Table 1. Effects of sotalol on isoproterenol-induced changes in cerebral cortical 5-HT_2 and β-receptor binding*

Treatment	[^{3}H]Spiperone		[^{3}H]Dihydroalprenolol	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	233 ± 6	0.86 ± 0.09	122 ± 6	2.16 ± 0.15
Isoproterenol	304 ± 17†	1.32 ± 0.17‡	98.1 ± 0.7§	4.88 ± 0.27†
Isoproterenol + sotalol	223 ± 13	0.48 ± 0.07‡	134 ± 10	6.59 ± 0.63†
Sotalol	244 ± 20	0.64 ± 0.06	134 ± 2	1.90 ± 0.12

* Cerebral cortical brain slices were incubated for 60 min in the presence of 100 μM (−)isoproterenol, 50 μM sotalol, or 100 μM (−)isoproterenol plus 50 μM sotalol. Control slices were incubated throughout the 60-min period with an equal volume of 0.5 mM sodium metabisulfite vehicle. Saturation analyses were performed on membrane homogenates of the slices using concentrations of [^{3}H]spiperone and [^{3}H]DHA ranging from 0.5 to 4.0 nM and 0.40 to 10.0 nM respectively. Values represent the mean ± S.E.M. of three or four separate Rosenthal determinations [14].

† $P < 0.001$, when compared to control.

‡ $P < 0.05$, when compared to control.

§ $P < 0.01$, when compared to control.

agent in inhibiting radioligand binding was taken as the inverse of the apparent inhibitory constant, K_i, where $K_i = IC_{50}/(1 + [^3\text{H-ligand}]/K_d)$. The IC_{50} (median inhibitory concentration) was calculated by probit analysis, and the K_d of [^{3}H]spiperone binding was determined by saturation analysis to be 0.86 nM. The K_d of [^{3}H]clonidine binding was 2.0 nM, as determined by U'Prichard *et al.* [19]. The potency series in inhibiting [^{3}H]spiperone binding was: spiperone (0.88 ± 0.22) ≥ ketanserin (1.20 ± 0.58) > MJ-13754-1 (3.04 ± 1.12) > rauwolszine (1,513 ± 312) > iprindole (2,155 ± 190) > yohimbine (2,578 ± 114) > clonidine (10,356 ± 3,399); the potency series in inhibiting [^{3}H]clonidine binding was: clonidine (0.179 ± 0.037) > rauwolszine (240 ± 19) ≥ yohimbine (278 ± 12) > MJ-13754-1 (4,915 ± 135) > iprindole (8,072 ± 983) > spiperone (17,396 ± 1,130) > ketanserin (>10^6). Apparent K_i values ± S.E.M. are in units of nM in parentheses. Both (−)isoproterenol and sotalol had extremely low affinities for the 5-HT_2 receptor with $K_i > 10^6$ nM in inhibiting specific [^{3}H]spiperone binding.

Our observation that 30 min of incubation with isoproterenol induced a down-regulation of β-adrenergic receptor binding is consistent with previous reports [10]. This down-regulation may be due to the induction of a high-affinity state of the β receptor to which the isoproterenol remains essentially irreversibly bound [10]. This decrease in β-receptor binding by isoproterenol has been shown in other studies to be reversed when membranes are incubated with GTP [10].

The increase in 5-HT_2 receptor density with β-adrenergic stimulation could be due to an alteration in presynaptic release or an interaction of these two receptor types on the same cell. Since dorsal and median raphe lesions and other alterations in serotonin stimulation do not alter 5-HT_2 receptor binding [20], an interaction of these two receptors on the same cell seems more likely. A direct interaction of isoproterenol and sotalol with the 5-HT_2 receptor is unlikely, since both have extremely low affinities for this site. Therefore, it appears that β-receptor stimulation modulates 5-HT_2 receptors. This modulation could occur through changes in membrane fluidity, cyclic AMP-dependent protein kinases, or other possible mechanisms.

Whatever the mechanism, an interaction between β-adrenergic and serotonin$_2$ receptors has several important implications. Recent studies indicating that administration of β-adrenergic agonists enhances serotonergic behavioral responses suggest that adrenergic agonists may act in part by enhancing the sensitivity to serotonin [7, 8]. Moreover, studies have indicated important interactions between central noradrenergic and serotonergic nervous systems in the actions of antidepressants. Electroconvulsive shock treatment (ECS) decreases β-adrenergic receptor binding [21] and the β-receptor stimulation of adenylate cyclase [22], whereas it increases 5-HT_2 receptor binding [23], and serotonin-induced behavioral responses [24]. It is possible that these enhanced behavioral responses by ECS are due to supersensitive 5-HT_2 receptors, which may in turn be due to enhanced stimulation of beta receptors by norepinephrine. Indeed, studies have shown that depletion of brain norepinephrine prevents the ECS-induced enhancement of serotonin-mediated behavior [25] and inhibition of catecholamine synthesis with α-methyl-*p*-tyrosine prevents the ECS-induced increase in 5-HT_2 receptor number in the cerebral cortex [26]. However, it is difficult to correlate changes in behavior to changes in receptor density, and recent studies have suggested that the serotonin syndrome may be mediated primarily by 5-HT_1 receptors [27].

In contrast to ECS, chronic tricyclic antidepressant treatment decreases both 5-HT_2 [15, 28] and beta-adrenergic receptors [14]. The role of serotonergic nerves in the down-regulation of 5-HT_2 receptors during chronic antidepressant treatment is ambiguous since depletion of brain serotonin does not prevent 5-HT_2 receptor down-regulation by amitriptyline [29]. In addition, prevention of the amitriptyline-induced down-regulation of beta-adrenergic receptors by combined amitriptyline–propranolol treatment does not block 5-HT_2 receptor down-regulation [30]. Thus, the down-regulation of 5-HT_2 receptors during chronic antidepressant treatment appears to be independent of beta-receptor stimulation and the presence of serotonin.

In summary, we have shown that incubation of cerebral cortical slices with 100 μM (−)isoproterenol decreased the density of β-adrenergic receptors and increased that of 5-HT_2 receptors. These changes appear to have been mediated by β-receptor stimulation since they were prevented by the selective β-receptor antagonist sotalol. In addition, isoproterenol increased α_2-adrenergic receptor binding, but had no significant effect on 5-HT_1 receptor binding in cerebral cortical slices.

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Properties of mitochondria treated with 1-chloro-2,4-dinitrobenzene*

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A role for GSH in mitochondria has so far not been established [1–3]. The substance may, however, be necessary for maintaining the sulphydryl groups of some mitochondrial enzymes [3, 4]. Such effects of GSH depend on its oxidizability to the disulphide form GSSG, and it should thus be effectively removed as a metabolically active substance by *S*-substitution. Specific blockage is theoretically feasible because GSH can react with various xenobiotics under catalysis by glutathione transferases [5]. CDNB is a favoured substrate for these enzymes which are now known also to be present in mitochondria [6]. This report describes the effect of CDNB on mitochondrial GSH and on the oxidative capacity of these depleted particles towards several substrates.

Materials and methods

[^{14}C]-pyruvate, [^{14}C]-NEM and [^{86}Rb]-rubidium chloride were purchased from Amersham International Ltd. (Amersham, U.K.) and dihydrolipoate from Sigma Ltd. (London, U.K.). The buffer used throughout was 0.125 M KCl containing 25 mM Tris HCl, pH 7.2 and 0.1 mM EDTA. Rat liver mitochondria were obtained as described previously [7] and used within 3 hr of preparation. Proteins were assayed by a biuret method [8], mitochondrial NPSH by the DTNB method [7, 9] and GSH by reaction with [^{14}C]-NEM followed by electrophoretic separation [1] then elution of the adduct with 1.5% acetic acid prior to counting. Pyruvate dehydrogenase was assayed by the release of [^{14}C]-CO_2 from 1-[^{14}C]-pyruvate [10]. The pyruvate dehydrogenase activity was maximized by preincubating mitochondria in buffer containing CCCP (0.5 μM) for 10 min. Lipoate dehydrogenase was assayed with 0.1 ml sonicated mitochondrial supsension added to 1 ml buffer containing lipoamide (100 nmoles), NADH (1 μmole) and CDNB (100 nmoles). Thiol formed was measured 10 min later with DTNB. Membrane potential ($\Delta\psi$) was determined with ^{86}Rb and valinomycin [11].

Results and discussion

When mitochondria are incubated with CDNB their concentration of GSH rapidly falls as shown by sedimentation and subsequent specific assay. The amount of GSH lost depends on the incubation time (since it slowly diffuses into the suspension medium [1]) and on the amount of CDNB

* Abbreviations used: BCNU, bischloronitrosourea; CCCP, carbonylcyanide-*m*-phenylhydrazone; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5-dithiobis (2-nitrobenzoic acid; GSH, glutathione; NEM, *N*-ethylmaleimide; NPSH, non-protein thiols.