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5,7-Dihydroxytryptamine-induced lesions of serotonergic neurons and desipramine-induced down-regulation of cortical beta adrenoceptors: a re-evaluation

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Studies on mechanisms of desipramine (DMI) induced desensitization of the norepinephrine (NE) receptor coupled adenylate cyclase system in brain have shown that the endogenous agonist NE plays a pivotal role for both the development of subsensitivity and the down-regulation of beta adrenoceptors [1–4]. Moreover, an intact serotonergic neuronal input is co-required for the down-regulation of central beta adrenoceptors by tricyclic antidepressants [5–7], monoamine oxidase (MAO) inhibitors, and electroconvulsive treatment [8]. In animals with a reduced synaptic availability of serotonin (5-HT), cortical beta adrenoceptors display a marked decrease in agonist affinity as determined in competition-binding studies [9, 10], with no significant changes occurring in the K_d values of antagonist binding. Since the previously reported IC_{50} values for isoproterenol were derived from rather shallow displacement curves [9] and the density of beta adrenoceptors was determined by Scatchard analysis of antagonist binding, information on high and low agonist affinity of beta adrenoceptors is desirable but not available. Consequently, we have re-evaluated the effect of chronic treatment with DMI and of specific lesions of serotonergic neurons with 5,7-dihydroxytryptamine (5,7-DHT) on beta adrenoceptors in rat cortex by using non-linear regression analysis of competition-binding curves.

Materials and methods

Male Sprague–Dawley rats (275–300 g) from Harlan Industries (Indianapolis, IN) were used in all experiments. The animals were maintained under standard laboratory conditions with a 12-hr light/dark cycle and with free access to water and standard pelleted rat chow. Serotonergic neurons were lesioned by intraventricular injection of 5,7-DHT as the creatinine sulfate salt (150 μg in 20 μl of saline with 0.1% ascorbate) after pretreatment (45 min) with DMI (25 mg/kg, i.p.) to prevent the destruction of noradrenergic neurons. Sham-operated animals (controls) received DMI followed by an intraventricular injection of a corresponding volume of vehicle. Ten days following lesioning, the rats were injected daily with DMI (15 mg/kg, i.p.) for 1 week. Twenty-four hours after the last injection, the rats were decapitated, the brain was rapidly removed and the cortex was frozen at −80° until assayed. The levels of 5-HT and NE were determined by high performance liquid chromatography-electron capture as previously described [4, 11]. Only data from tissue samples showing selectivity of the lesions (5-HT < 10% of control values) were included in the results. The density of beta adrenoceptors was determined according to Bylund and Snyder [12], using a 50 mM Tris buffer with 10 mM $MgCl_2$. Competition-binding curves for inhibition of specific [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding by (−)-isoproterenol were constructed using a concentration of 3 nM [^{3}H]DHA and isoproterenol over a range of 0.001 to 100 μM. Specific [^{3}H]DHA binding represents total binding minus non-specific binding assayed in the presence of 10 μM *d,l*-propranolol. To determine high and low agonist affinity states of beta adrenoceptors, the individual competition-binding curves were subjected to non-linear regression analysis using the program LIGAND of Munson and Rodbard [13]. Each curve was analyzed using a one-site and a two-site model. A two-site model was selected as it yielded a significant reduction in the residual variance using the F-test criterion ($P < 0.001$). Non-specific binding was "floated", i.e. picked by the computer based on the competition curve being analyzed. Proteins were analyzed according to Lowry *et al.* [14]. The significance of the data was evaluated by the two-tailed Student's *t*-test.

Drugs and chemicals. [^{3}H]DHA (sp. act. 45–60 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). (−)-Isoproterenol HCl, 5,7-DHT and *d,l*-propranolol HCl were purchased from the Sigma Chemical Company (St. Louis, MO). Desipramine HCl was donated by the Dow Chemical Corp. (Cincinnati, OH).

Results

Effect of desipramine on the concentration of beta adrenoceptors with high and low agonist affinity conformation in the cortex. In cortical tissue of normal animals, non-linear regression analysis of competition binding curves revealed that approximately 75% of beta adrenoceptors were in the high affinity conformation for the agonist isoproterenol with the remaining 25% being in the low affinity conformation. For isoproterenol, the ratio of K_L to K_H was about 400–800. Chronic DMI treatment caused the well known decrease in the number of beta adrenoceptors [R_T], but this reduction was entirely confined to beta adrenoceptors in the high affinity conformation [R_H] with no significant changes occurring in the fraction of receptors showing low affinity [R_L] (Table 1). DMI did not alter the affinities of either receptor population.

Consequences of 5,7-dihydroxytryptamine-induced lesions on the down-regulation of beta adrenoceptors by desipramine. Lesioning of 5-HT neurons with 5,7-DHT significantly increased the number of cortical beta adrenoceptors but this increase was restricted to receptors in the low affinity conformation (Table 2). In agreement with results of previous studies using Scatchard analysis of binding data [5–8], DMI failed to reduce the density of beta adrenoceptors [R_T] in cortex of lesioned animals to a level observed in sham-operated animals (125 ± 9 vs 78 ± 4 fmol/mg protein in lesioned vs non-lesioned animals respectively). However, the drug did decrease the number of beta adrenoceptors in the high affinity conformation [R_H] to the same degree (Table 2)as it did in tissue from sham-operated animals (Table 1). DMI did not alter the marked increase of beta adrenoceptors in the low affinity conformation. Although there were fewer beta adrenoceptors in the high agonist affinity conformation, the remaining receptors had the same affinity for isoproterenol in their high and low affinity conformations as they did in the 5,7-DHT-saline group (Table 2).

Discussion

In confirmation of studies by Hancock and Marsh [15] who used [^{125}I]cyanopindolol as the radioligand, we found that the reduction in the density of cortical beta adrenoceptors following chronic treatment with DMI was predominantly confined to the beta adrenoceptor population displaying high affinity for the agonist isoproterenol [R_H]. This adrenoceptor population represents about 75% of total beta adrenoceptors in the cortex. Previous studies using Scatchard analysis of saturation isotherms have shown that selective lesions of serotonergic neurons with the neurotoxin 5,7-DHT prevent the down-regulation of beta adrenoceptors by DMI (see Introduction). Two-site non-

Table 1. Effect of desipramine on cortical beta adrenoceptors: Non-linear regression analysis of competition binding curves

Treatment	Dissociation constants (nM)		Beta adrenoceptor concentration (fmol/mg protein)		
	K_H	K_L	R_T	R_H	R_L
Sham-Saline	45 ± 5 (6)	39,800 ± 8,200 (6)	115 ± 5 (6)	86 ± 4 (6) (75%)	29 ± 2 (6) (25%)
Sham-DMI	51 ± 4 (5)	44,800 ± 10,600 (5)	78 ± 4* (5)	57 ± 4* (5) (73%)	21 ± 2 (5) (27%)

Rats were given desipramine (DMI) (15 mg/kg, i.p., per day) for 1 week. The animals were killed 24 hr after the last injection. Competition binding curves were constructed using cortical membrane preparations, as described in Materials and Methods, and were analyzed by non-linear regression analysis using the program LIGAND (two-site model). R_T designates the total number of receptors, while R_H and R_L define beta adrenoceptor populations with high and low agonist affinity conformations respectively. K_H and K_L designate the corresponding dissociation constants. Values are means ± SEM; the numbers in parentheses indicate the number of animals.

*P < 0.001.

Table 2. Consequences of 5,7-DHT-induced lesions on cortical beta adrenoceptors and their down-regulation by desipramine: Non-linear regression analysis of competition binding curves

Treatment	Dissociation constants (nM)		Beta adrenoceptor concentration (fmol/mg protein)		
	K_H	K_L	R_T	R_H	R_L
5,7-DHT–Saline	61 ± 12 (7)	21,600 ± 2,300 (7)	170 ± 11* (7)	90 ± 9 (7) (53%)	80 ± 3* (7) (47%)
5,7-DHT–DMI	51 ± 7 (6)	21,900 ± 4,800 (6)	125 ± 9† (6)	51 ± 6‡ (6) (41%)	74 ± 5§ (6) (59%)

Serotonergic neurons were lesioned by intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) as described in Materials and Methods. Ten days after lesioning, the animals were given desipramine (DMI), (15 mg/kg, i.p., per day) for 1 week. The animals were killed 24 hr after the last injection. Competition binding curves were constructed using cortical membrane preparations, as described in Materials and Methods, and were analyzed by non-linear regression analysis using the program LIGAND (two-site model). For definitions of abbreviations see legend to Table 1. Values are means ± SEM; the numbers in parentheses indicate the number of animals.

* P < 0.001 (5,7-DHT vs sham-saline in Table 1).
† P < 0.01 (5,7-DHT–DMI vs 5,7-DHT–Saline).
‡ P < 0.005 (5,7-DHT–DMI vs 5,7-DHT–Saline).
§ P < 0.001 (5,7-DHT–DMI vs Sham-DMI in Table 1).

linear regression analysis of agonist competition-binding curves obtained with cortical tissue from animals with selective lesions of 5-HT neurons revealed that the high agonist affinity fraction of beta adrenoceptors was, in fact, reduced by DMI to the same level as in cortical membrane preparations of normal animals. Since it is the high affinity state of the beta adrenoceptor formed in the presence of the agonist that determines stimulation of adenylate cyclase [16], the present results can satisfactorily explain data demonstrating that the responsiveness of the cyclic AMP generating system to isoproterenol and NE is reduced in lesioned animals to approximately the same extent as in control animals [4, 6]. Our results may also explain why the increased number of beta adrenoceptors reported following 5,7-DHT lesions as determined by Scatchard analysis of antagonists binding [6, 17] is not accompanied by an increased cyclic AMP response to isoproterenol and/or NE [4, 6]. Though guanine nucleotides cause a significant interconversion of R_H to R_L in membrane preparations from peripheral tissue [18], the rightward shift of competition binding curves by GTP is only marginal or absent in cortical membrane preparations ([15, 19, 20] and unpublished results from this laboratory). It remains thus to be elucidated whether this lack of GTP sensitivity and the observed selective change in R_H or R_L indicate two different receptor species rather than different affinity states of the same receptor.

In conclusion, the estimation in the present studies of beta adrenoceptors in the high and low agonist affinity conformation by non-linear regression analysis of competition-binding curves has provided new evidence for a functional linkage between serotonergic and noradrenergic neuronal systems in brain. The molecular basis of the substantial and exclusive loss by DMI of beta adrenoceptors in the high agonist affinity conformation and of the highly significant changes in the ratio of beta adrenoceptors in the high to low agonist affinity conformation following impairment of serotonergic input remains to be elucidated.

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Departments of Pharmacology and Psychiatry
Vanderbilt University School of Medicine
Nashville, TN 37232, U.S.A.

D. HAL MANIER
DAVID D. GILLESPIE
FRIDOLIN SULSER*

* Author to whom all correspondence should be addressed.

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Influence of intracellular folates on methotrexate metabolism and cytotoxicity

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The antifolate methotrexate (MTX) is given clinically in high doses to overcome tumor resistance. This therapy is administered in association with 5-formyltetrahydrofolate (folinic acid; leucovorin; ^5N-CHO-H_4PteGlu) to prevent antifolate toxicity [1]. Increased intracellular folate levels are thus potentially achieved not only in normal but also in tumor cells. This could possibly affect the sensitivity of the latter to subsequent MTX therapy since folate repletion of tumor cells inhibits MTX polyglutamate (MTXPG) formation [2, 3]. In the present report, we have examined the effects of varying the tumor cell intracellular folate pool size on MTX cytotoxicity and have confirmed that tumor cell sensitivity to MTX decreases as folate pools rise because of impaired MTXPG formation.

Methods and results

Chemicals. [3′,5′,7-^{3}H]MTX (sp. act. 20 Ci/mmol) and [3′,5′,7,9-^{3}H]folic acid (sp. act. 18 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). (6*S*)-[3′,5′,7,9-^{3}H]Folinic acid and unlabeled (6*S*)-folinic acid were synthesized by the method of Moran and Colman [4]. Unlabeled MTX was obtained from the National Cancer Institute (Bethesda, MD). All other chemicals were of reagent grade and purchased from the Fisher Scientific Co. (Pittsburgh, PA) or the Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Gibco Laboratories (Chagrin Falls, OH) and treated with dextran-coated charcoal (cFBS) at room temperature until less than 1% of the [^{3}H]thymidine added before the procedure remained.

Propagation of cells in culture. MCF-7 cells, a line of human breast cancer cells in continuous monolayer culture [5], were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Flow Laboratories) supplemented with 10% FBS, penicillin (200 μg/ml) and streptomycin (200 μg/ml) under 5% CO_2 at 37°. Prior to all experiments, MCF-7 cells were folate depleted by transferring them to folate-free DMEM (Flow Laboratories) containing 10% charcoal-treated FBS (cFBS) for 10 days until cell growth stopped. The cells were then refed with the same medium containing either added 10 μM folic acid or 10 μM (6*S*)-folinic acid, and experiments were performed approximately 5 days later when both refed cell types were at the same logarithmic cell growth rate. In all experiments, the folate-containing medium was removed, and the cells were washed and transferred to folate-free DMEM to remove exchangeable folate 1 hr prior to MTX exposure.

MTX cytotoxicity. We first examined the consequences of two different folate exposures on MTX cytotoxicity. Results are illustrated in Fig. 1. Folic acid refed cells were significantly more sensitive to MTX than folinic acid refed cells with an IC_{50} of 0.04 μM in the folic acid cells compared to 0.25 μM for the folinic acid refed cells.

Intracellular folates. We next examined the consequences of the two different folate exposures on total intracellular folate pools in MCF-7 cells. Total intracellular folates and intracellular monoglutamated folates were assayed after logarithmic growth had resumed following folate depletion and refeeding with either 10 μM [^{3}H]folic acid (final sp. act. 2.25 Ci/mmol) or 10 μM [^{3}H]-(6*S*)-folinic acid (final sp. act. 0.11 Ci/mmol). The methods used were described recently by Allegra *et al.* [6]. The following results represent the mean ± SD of three experiments: total folate pools after 10 μM (6*S*)-folic acid exposure were over 10-fold lower (7.22 ± 0.74 nmol/g) than after 10 μM folinic acid exposure (89.2 ± 20.1 nmol/g). The distribution of intracellular folates was then determined by HPLC (N = three experiments): 5-CH_3H_4PteGlu pools represented a slightly higher percentage of intracellular folates in folic acid compared to folinic acid refed cells (53.7 ± 2.88% vs 45.4 ± 0.74%), whereas the combined H_4PteGlu-10-CHO-H_4PteGlu pools made up a greater portion of intracellular folates in folinic acid refed cells (46.0 ± 0.57 vs 33.5 ± 1.90%). Intracellular folinic acid represented 8.59 ± 1.13% of intracellular folates in folinate-refed cells and 2.03 ± 0.65% in folate-refed cells. Intracellular folic acid was only identified in cells exposed to folic acid (10.8 ± 1.43% of intracellular folates).

MTX uptake. To determine the mechanisms underlying the decrease in MTX cytotoxicity observed in the folinic acid refed cells, we examined if higher intracellular folate