

ACCELERATED COMMUNICATION

3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy") Induces Fenfluramine-Like Proliferative Actions on Human Cardiac Valvular Interstitial Cells in Vitro

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ABSTRACT

Recent findings have implicated the 5-hydroxytryptamine 2B (5-HT_{2B}) serotonin receptor in mediating the heart valve fibroplasia [valvular heart disease (VHD)] and primary pulmonary hypertension observed in patients taking the now-banned appetite suppressant fenfluramine (Pondimin, Redux). Via large-scale, random screening of a portion of the receptorome, we have discovered that the amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") and its *N*-demethylated metabolite 3,4-methylenedioxyamphetamine (MDA) each preferentially bind to and activate human recombinant 5-HT_{2B} receptors. We also demonstrate that MDMA and MDA, like fenfluramine and its *N*-deethylated metabolite norfenfluramine, elicit prolonged mitogenic responses in human valvular interstitial cells via activation of

5-HT_{2B} receptors. We also report that pergolide and dihydroergotamine, two drugs recently demonstrated to induce VHD in humans, potentially activate 5-HT_{2B} receptors, thus validating this assay system for its ability to predict medications that might induce VHD. Our discovery that MDMA and a major metabolite, MDA, induce prolonged mitogenic responses in vitro similar to those induced by fenfluramine and norfenfluramine in vivo (i.e., valvular interstitial cell fibroplasia) predict that long-term MDMA use could lead to the development of fenfluramine-like VHD. Because of the widespread abuse of MDMA, these findings have major public health implications. These findings also underscore the necessity of screening current and future drugs at h5-HT_{2B} receptors for agonist actions before their use in humans.

In September of 1997, the highly effective appetite suppressant fenfluramine (Pondimin), a component of the drug combination "Fen-Phen", and the optically pure (+)-isomer dexfenfluramine (Redux) were voluntarily removed from the marketplace at the urging of the United States Food and Drug Administration because of their association with heart valve fibroplasia and dysfunction, a

condition known as valvular heart disease (VHD). Since then, several independent echocardiographic studies of patients who received long-term fenfluramine therapy revealed an increased prevalence of valvular heart disease (Connolly et al., 1997; Jick et al., 1998; Weissman et al., 1998; Weissman, 2001). Histopathological examination of resected valves has revealed proliferative foci containing interstitial cells and increased levels of extracellular matrix (Steffee et al., 1999). Identical pathology has been seen in resected valves harvested from persons undergoing

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ABBREVIATIONS: VHD, valvular heart disease; 5-HT, 5-hydroxytryptamine; h, human; PPH, primary pulmonary hypertension; MDMA, 3,4-methylenedioxymethamphetamine; HEK, human embryonic kidney; VIC, interstitial valvular cells; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; DA, dopamine; NE, norepinephrine; GBR12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine; IP, inositol phosphate; RTI-229, 3β-(4-iodophenyl)tropane-2β-pyrrolidine carboxamide; MDA, 3,4-methylenedioxyamphetamine; SB206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole.

long-term administration of certain ergot derivatives (e.g., ergotamine and methysergide) and from those suffering from carcinoid syndrome (Steffee et al., 1999).

Recently, we proposed that drugs (and/or their metabolites) associated with VHD should preferentially bind with high affinity to a single, proximal molecular target (receptor, channel, or transporter), whereas similar medications (e.g., fluoxetine, phentermine) not associated with VHD would not (Rothman et al., 2000). Via screening of VHD-associated and non-VHD-associated drugs at a limited number of recombinant receptors, transporters, and ion channels, we discovered that VHD-associated drugs shared high affinity for only the human 5-HT_{2B} (h5-HT_{2B}) receptor (Rothman et al., 2000). In functional assays, we demonstrated that VHD-associated drugs were all h5-HT_{2B} receptor agonists (Rothman et al., 2000). Fitzgerald et al. (2000) also reported that fenfluramine and a major metabolite, norfenfluramine, were agonists at recombinant h5-HT_{2B} receptors and independently suggested that the 5-HT_{2B} receptor was responsible for fenfluramine-induced VHD. Launay et al. (2002) subsequently discovered that activation of the 5-HT_{2B} receptor is also responsible for fenfluramine-induced primary pulmonary hypertension (PPH).

Recently, we pioneered the use of large-scale screening of psychoactive drugs at a huge panel of recombinant receptors (i.e., "receptorome") to identify the κ -opioid receptor as the site of action of the novel hallucinogen Salvinorin A (Roth et al., 2002; Sheffler and Roth, 2003). We now report the results of a receptorome screen of the club drug 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") that we performed to identify novel molecular targets responsible for the actions of MDMA in humans. These studies reveal that MDMA, like fenfluramine and other drugs known to be associated with heart valve fibroplasia, preferentially binds to and activates h5-HT_{2B} receptors. Additionally, we demonstrate that MDMA induces prolonged mitogenic responses in primary cultures of human cardiac interstitial valve cells. Because drugs that activate h5-HT_{2B} receptors induce VHD and PPH in humans, these findings have major public health implications.

Materials and Methods

HEK293 and COS-7 Cell Culture and Transfection. Stably and transiently transfected cells were maintained as detailed previously (Roth et al., 2002). For transfections with the h5-HT_{2B} receptor, HEK293 or COS-7 cells were seeded in 100-mm dishes and transfected using 6 μ g of plasmid and 36 μ l of Fugene (Roche, Indianapolis, IN) according to the manufacturer's protocol as described previously (Rothman et al., 2000).

Radioligand Binding Assays and Phosphatidylinositol Hydrolysis Assays. Radioligand binding assays were performed as described previously using the resources of the National Institute of Mental Health Psychoactive Drug Screening Program (Rothman et al., 2000). Phosphatidylinositol hydrolysis assays were performed using HEK293 cells transiently expressing human 5-HT_{2B} receptors as reported previously (Rothman et al., 2000).

VIC Isolation and Culture. Human heart valves were obtained from donor hearts deemed unsuitable for transplantation, or from hearts that were removed from transplant recipients at the Cleveland Clinic Foundation. All Cleveland Clinic patients who have tissue surgically removed have authorized its subsequent use for research purposes (protocols approved by the CCF IRB 2378). To remove the cells from the tissue, the specimens were placed into

sterile containers, immersed in a solution of collagenase-II (2 mg/ml; Worthington Biochemicals, Freehold, NJ) in serum-free medium, then agitated in an incubated shaker (140 rpm, 20 min, 37°C). After return to the sterile flow hood, all surfaces were rubbed with a sterile cotton swab to remove the endothelial cells. The valve specimens were then finely minced and then digested with collagenase-III (1 mg/ml) in an incubated shaker (4 h, 140 rpm, 37°C). Each resulting cell suspension was filtered (70 μ m) to remove debris, and the cell pellet was resuspended in DMEM/Ham's F12 medium (1:1, containing low glucose with HEPES) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). The culture was incubated in a humidified atmosphere of 95% air/5% CO₂ at 37°C with changes of medium every 48 h.

[³H]Thymidine Deoxyribose Incorporation Assay. Subconfluent VIC seeded in 24-well clusters were incubated overnight in serum-free DMEM (Invitrogen). Cells were then treated over the course of 3 days with various concentrations of test agents. Twelve hours before the end of the treatment period, cells were pulsed with 2 to 5 μ Ci/ml [³H]thymidine deoxyribose (PerkinElmer Life Sciences, Boston, MA). After treatment, the medium was removed and the cells were washed thoroughly with ice-cold phosphate-buffered saline, pH 7.4. Ice-cold 10% trichloroacetic acid was then added and the cells were incubated for 30 min at 4°C. The cells were again washed thoroughly with ice-cold phosphate-buffered saline, pH 7.4, and then lysed in 0.5 N NaOH. After neutralization with glacial acetic acid, samples were assayed for [³H]thymidine deoxyribose incorporation by liquid scintillation counting. Values are reported as the mean \pm S.E.M. of triplicate determinations and are representative of three independent experiments.

Immunoblot Analysis of Erk 1/2^{MAPK} Phosphorylation. VIC seeded in 24-well clusters were incubated overnight first in DMEM containing 5% dialyzed fetal bovine serum and then in serum-free DMEM. Cells were treated over the course of 15 min with 10 μ M fenfluramine, norfenfluramine, MDMA, or MDA. After treatment, the medium in each well was replaced with 200 μ l of 1 \times Laemmli sample buffer and collected. Samples were resolved on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were probed for phospho-Erk 1/2^{MAPK} immunoreactivity using a 1:1000 dilution of polyclonal primary antibody (Cell Signaling Inc., Beverly, MA) and a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. Immunoreactivity was revealed using LumiLight horseradish peroxidase substrate (Roche) and imaged on a Kodak Digital Science Image Station 440CF (Eastman Kodak, Rochester, NY). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD). Samples were similarly analyzed for total Erk 1/2^{MAPK} immunoreactivity, and the resulting values were used to correct phospho-Erk 1/2^{MAPK} measurements for slight differences in sample protein content. Values are reported as the mean \pm S.E.M. of duplicate determinations and are representative of three independent experiments.

[³H]DA, [³H]NE, and [³H]5-HT Release Assays. Following published procedures (Rothman et al., 2001), rat caudate (for [³H]DA release) or whole brain minus cerebellum and caudate (for [³H]NE and [³H]5-HT release) was homogenized in ice-cold 10% sucrose containing 1 μ M reserpine. Nomifensine (100 nM) and GBR12935 (100 nM) were also added to the sucrose solution for [³H]5-HT release experiments to block any potential [³H]5-HT reuptake into NE and DA nerve terminals. After 12 strokes with a Potter-Elvehjem homogenizer, homogenates were centrifuged at 1000g for 10 min at 0 to 4°C and the supernatants were retained on ice (synaptosomal preparation). Each rat brain (approximately 1200 mg) produced enough tissue for 250 test tubes for the [³H]DA and [³H]5-HT release assays and for 125 test tubes for the [³H]NE release assay.

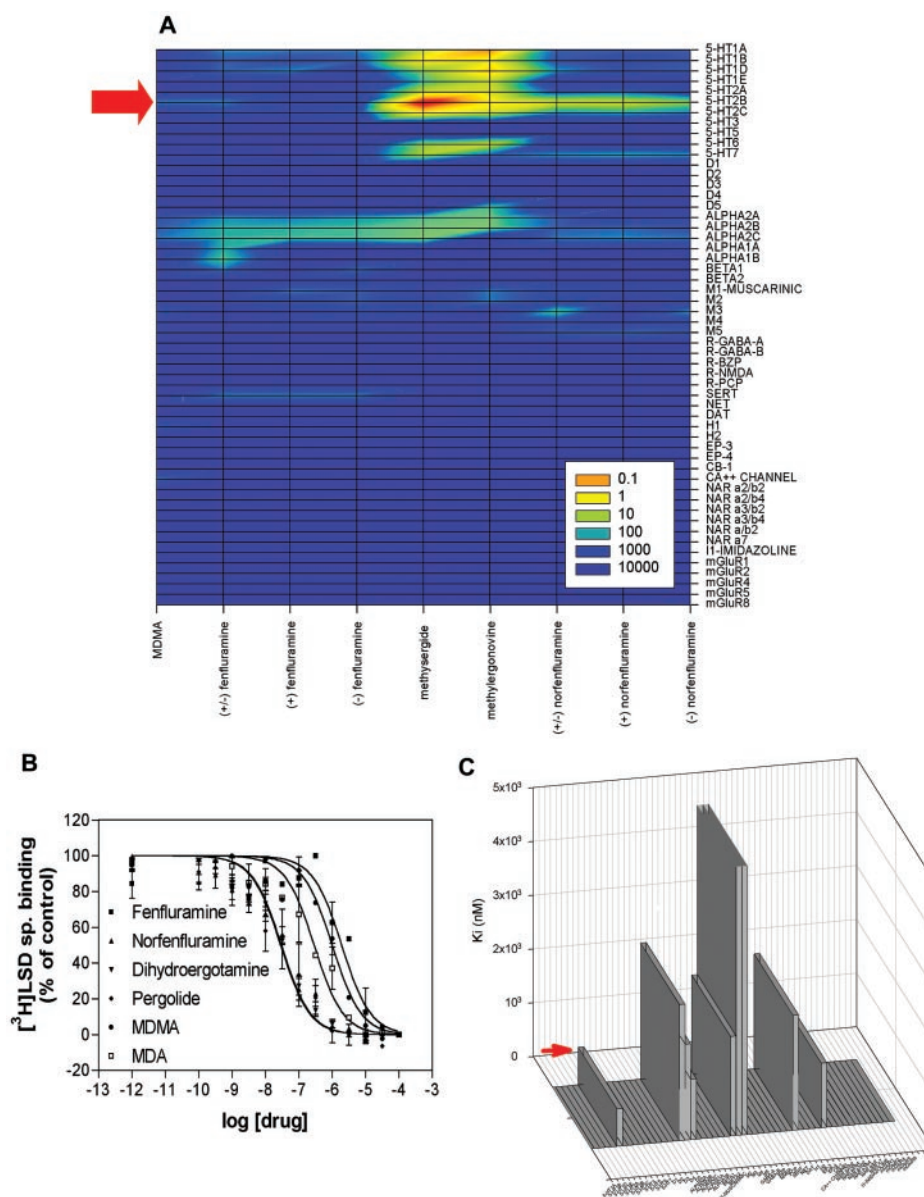


Fig. 1. Large-scale screening of the receptorome reveals that MDMA preferentially interacts with the human 5-HT_{2B} serotonin receptor. Top, K_i values for various drugs screened at a large number of mainly human recombinant receptors, ion channels, or transporters using the resources of the National Institute of Mental Health Psychoactive Drug Screening Program. For these studies, test compounds were initially screened at 10 μ M. When greater than 50% inhibition of radioligand specific binding was obtained, K_i values were determined in quadruplicate. A three-dimensional mesh plot of the data was made in which the K_i values were color-coded. The red arrow indicates that MDMA has preferentially high affinity for h5-HT_{2B} receptors. Bottom left, representative isotherms showing radioligand displacement from h5-HT_{2B} receptors expressed in COS-7 cells, the nonlinear regression of which was used to determine IC₅₀ values. K_i values were calculated using the Cheng-Prusoff approximation. Bottom right, K_i values for MDMA in bar chart format; the arrow shows the K_i value for the h5-HT_{2B} receptor; K_i values >10,000 nM are set to zero for clarity. Red arrow, K_i value for MDMA.

Synaptosomal preparations were incubated to steady state with 5 nM [³H]DA (30 min), 7 nM [³H]NE (60 min), or 5 nM [³H]5-HT (60 min) in uptake buffer without bovine serum albumin, plus 1 μ M reserpine, in a polypropylene beaker with stirring at 25°C. Nomifensine (100 nM) and GBR12935 (100 nM) were added to the buffer for [³H]5-HT release experiments, whereas RTI-229 (5 nM) was added to the buffer for [³H]NE release experiments. After incubation to steady state, 850 μ l of synaptosomes preloaded with [³H]neurotransmitter were added to 12 \times 75-mm polystyrene test tubes that contained 150 μ l of test drug in uptake buffer. After 5 min ([³H]DA and [³H]5-HT) or 30 min ([³H]NE), the release reaction was terminated by dilution with 4 ml of wash buffer (10 mM Tris-HCl, pH 7.4, containing 0.9% NaCl at 25°C) followed by rapid vacuum filtration over Whatman GF/B filters using a Brandel Harvester (Brandel Inc.,

Gaithersburg, MD). The filters were rinsed twice with 4 ml of wash buffer using the Brandel Harvester, and the retained tritium was counted by a Taurus liquid scintillation counter at 40% efficiency after an overnight extraction in 3 ml of Cytoscient (ICN Biomedicals Inc., Costa Mesa, CA).

Results

Screening the Receptorome Reveals the h5-HT_{2B} Receptor As a Primary Molecular Target for MDMA and MDA. To discover novel molecular targets for the effects of psychoactive compounds (see Roth et al., 2002, for a recent example), we screened the club drugs MDMA and MDA at a

large number of recombinant (mostly human) neurotransmitter and hormone receptors, ion channels, and transporters. To our surprise, MDMA exhibited preferentially high affinity for the h5-HT_{2B} receptor (Fig. 1A), a receptor previously implicated in fenfluramine-induced VHD (Fitzgerald et al., 2000; Rothman et al., 2000) and PPH (Launay et al., 2002). As shown in Fig. 1A and Table 1, other valvulopathic drugs are also characterized by preferentially high affinities for h5-HT_{2B} receptors (Fig. 1A; Table 1). We also discovered that two additional commonly prescribed medications bind to and activate 5-HT_{2B} receptors: 1) pergolide, a drug used in treating Parkinson's disease that was recently associated with VHD of the fenfluramine-type (Pritchett et al., 2002) and 2) dihydroergotamine, a drug used in treating migraine headaches, which was reported several years ago to induce VHD (Creutzig, 1992). As is shown in Table 1, both pergolide and dihydroergotamine have high affinities for h5-HT_{2B} receptors.

We subsequently examined the abilities of MDMA and its *N*-demethylated metabolite (MDA) to activate human, recombinant 5-HT_{2B} receptors. These studies identified MDA as a more potent and efficacious agonist than MDMA (Fig. 2A; Tables 1 and 2). In this regard, we reported previously that the *N*-dealkylated metabolites of drugs known to induce either VHD or PPH (e.g., norfenfluramine and methylephedrine) are also more potent and efficacious 5-HT_{2B} receptor agonists than their respective parent compounds (Table 2; Rothman et al., 2000). Importantly, the EC₅₀ values for activating phosphoinositide hydrolysis at h5-HT_{2B} receptors for MDMA (2000 nM) and MDA (190 nM) are nearly identical to the plasma concentrations found in humans after a single recreational dose (150 mg) of MDMA in humans. For instance, after a single 150-mg dose of MDMA, de la Torre et al. (2000) reported a C_{max} for MDMA of 2000 nM and a C_{max} for MDA of 150 nM. Table 2 also demonstrates that both pergolide and dihydroergotamine, drugs recently demonstrated to induce VHD of the fenfluramine-type in humans (Pritchett et al., 2002), are also potent h5-HT_{2B} agonists.

Because the (+)-stereoisomer of fenfluramine, dexfenfluramine, also used as an anorexigenic (Redux), was associated with VHD and PPH, we evaluated optically pure preparations of MDMA and MDA for potency and efficacy at human 5-HT_{2B} receptors. We detected no significant difference in efficacy between the *R*- and *S*-stereoisomers of either MDMA or MDA; with respect to potency, the *S*-stereoisomer of MDMA was slightly more potent than the *R*-stereoisomer, whereas the *R*- and *S*-stereoisomers of MDA exhibited no

statistically significant difference in potency (Fig. 2B and Table 2).

MDMA and MDA are widely appreciated to release the biogenic amine neurotransmitters from nerve terminals via a carrier-mediated exchange mechanism see (Baumann et al., 2000; Rothman and Baumann, 2002). We thus determined the EC₅₀ values of the compounds under consideration for releasing [³H]5-HT, [³H]NE, and [³H]DA from rat brain synaptosomes. Norfenfluramine is more potent than fenfluramine at releasing [³H]NE and [³H]DA. MDMA is most potent at releasing [³H]5-HT, but still potently releases [³H]NE and [³H]DA (Table 2). MDA differs from MDMA in that its most potent action is in releasing [³H]NE (Table 2). There is a pronounced enantioselectivity in the actions of MDMA and MDA as indicated by the more potent effects of (*S*)-MDA and (*S*)-MDMA compared with (*R*)-MDA and (*R*)-MDMA, respectively (Table 2). Perhaps the key feature to emerge from this analysis is that the potency of (*R*)-MDMA, MDA and its stereoisomers, in the biogenic amine release assays is similar to their potency at 5-HT_{2B} receptors, indicating that MDMA will activate 5-HT_{2B} receptors at typical pharmacological doses.

Valvulopathic Drugs Induce Prolonged Mitogenic Responses in Human Heart Valve Interstitial Cells.

Because much of the evidence implicating 5-HT_{2B} receptor activation in drug-induced VHD is inferential, we set out to directly test the mitogenic activity of valvulopathic drugs using primary cultures of human heart valve interstitial cells (hVICs). In preliminary studies, we established that hVICs express functional 5-HT_{2B} receptors coupled to phosphoinositide hydrolysis (data not shown). We next evaluated the abilities of selected VHD-associated drugs to elicit mitogenic responses from hVICs. For these studies, we incubated serum-starved hVICs for 48 h with fenfluramine, norfenfluramine, MDMA, MDA, SB206553 (a 5-HT_{2B/2C} antagonist), or 5-HT and measured [³H]thymidine incorporation into newly synthesized DNA.

The VHD-associated drugs fenfluramine and norfenfluramine each induced statistically significant mitogenic responses in hVICs (Fig. 3A). MDMA, MDA, and 5-HT, but not

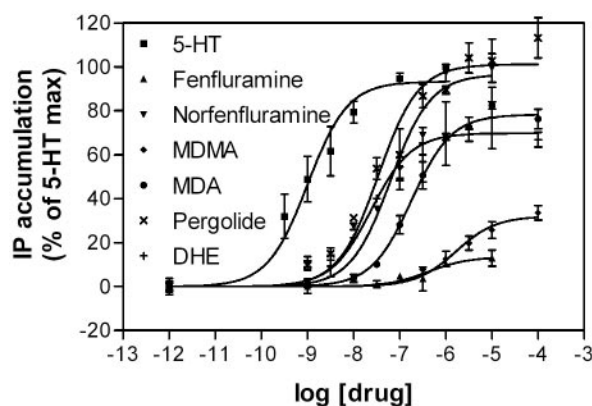


Fig. 2. MDMA and MDA potently activate h5-HT_{2B}-serotonin receptors in vitro. Concentration-dependent stimulation of phosphatidylinositol hydrolysis via activation of h5-HT_{2B} receptors expressed in HEK293 cells by fenfluramine, norfenfluramine, (*R,S*)-MDMA, (*R*)-MDMA, (*S*)-MDMA, (*R,S*)-MDA, (*R*)-MDA, or (*S*)-MDA was assayed as described under *Materials and Methods*. Data represent mean \pm S.E.M. for $n = 3$ separate experiments of percentage stimulation of [³H]IP accumulation relative to the full agonist 5-HT.

TABLE 1

MDMA, MDA, and other valvulopathic drugs bind to human recombinant 5-HT_{2B} receptors

Data represent mean \pm S.E.M. for at least three separate determinations in which K_i values were measured by radioligand displacement assays (see *Materials and Methods*). Fenfluramine is known to induce VHD in humans.

Drug	K_i (pK _i)
	nM
Fenfluramine	4134 (5.9 \pm 0.2)
Norfenfluramine	15 (7.83 \pm 0.06)
Dihydroergotamine	15.2 (7.82 \pm 0.06)
Pergolide	14 (7.8 \pm 0.1)
(<i>R,S</i>)-MDMA	500 (6.30 \pm 0.08)
(<i>R,S</i>)-MDA	100 (6.8 \pm 0.1)

compared with vehicle-treated cells, suggesting that 5-HT_{2B} receptors regulate basal mitogenesis in hVICs. In fact, we have observed that basal Erk 1/2^{MAPK} phosphorylation, which is quite high in serum-starved VICs compared with serum-starved HEK cells (data not shown), hinders the detection of a statistically significant mitogenic response to drug treatment.

Discussion

The major finding of the present study is that MDMA and MDA, in a manner identical to drugs demonstrated to induce VHD and PPH in humans, bind to and activate human recombinant 5-HT_{2B} receptors and induce mitogenesis in human heart valve interstitial cells in vitro. Importantly, MDMA and MDA activate h5-HT_{2B} receptors within the same concentration ranges at which they 1) occur in plasma

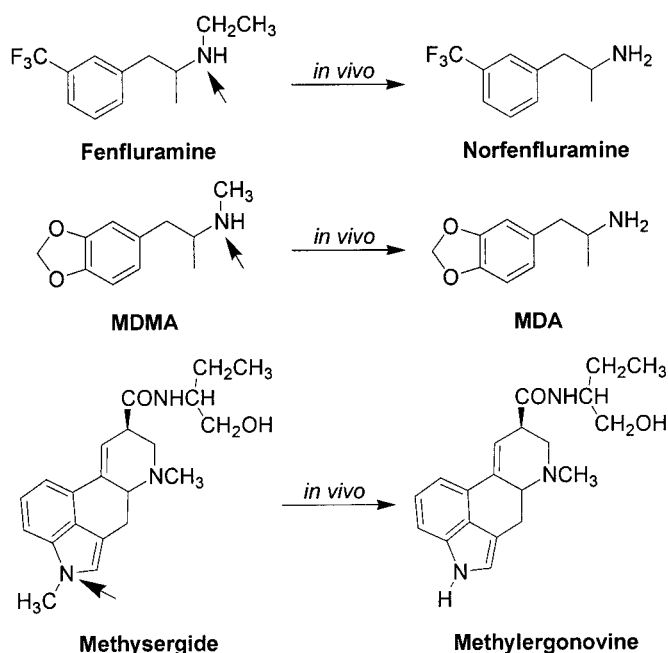
MDMA and MDA, similar to other valvulopathic drugs, activates h5-HT_{2B} serotonin receptors in vitro

Data represent mean \pm S.E.M. For estimates of efficacy (relative to 5-HT) and potency (EC_{50}) for activation of PI hydrolysis ($n = 3$ separate experiments) or estimates of potency (EC_{50}) for in vitro neurotransmitter release, assays used rat brain synaptosomes preloaded with [3 H]5-HT, [3 H]DA, or [3 H]NE (see *Materials and Methods* for details). Data for methysergide and methylergonovine are from ref. 10. The chemical structures are shown below the table, and the arrows indicate the dealkylated nitrogen. Drugs in bold are those known to induce VHD in humans.

Drug	pEC ₅₀ for 5-HT _{2B} -Mediated PI Hydrolysis	Relative Efficacy for 5-HT _{2B} -Mediated PI Hydrolysis	Release EC ₅₀		
			5-HT	DA	NE
	<i>nM</i>			<i>nM</i>	
5-HT	1 (9.0 ± 0.1)	1.00 ± 0.06	44 ± 3	>10,000	>10,000
Fenfluramine	400 (6.4 ± 0.2)	0.13 ± 0.02	108 ± 4	>10,000	740 ± 30
Norfenfluramine	60 (7.2 ± 0.1)	0.96 ± 0.03	104 ± 3	1900 ± 200	170 ± 10
Dihydroergotamine	30 (7.52 ± 0.09)	0.73 ± 0.02	N.D.	N.D.	N.D.
Pergolide	53 (7.27 ± 0.09)	1.12 ± 0.04	N.D.	N.D.	N.D.
(<i>R,S</i>)-MDMA	2000 (5.8 ± 0.1)	0.32 ± 0.02	72 ± 3	278 ± 7	110 ± 10
(<i>R</i>)-MDMA	900 (6.0 ± 0.2)*	0.27 ± 0.02	340 ± 20	3700 ± 100	560 ± 40
(<i>S</i>)-MDMA	6000 (5.2 ± 0.2)*†	0.38 ± 0.03	74 ± 3	142 ± 4	136 ± 9
(<i>R,S</i>)-MDA	190 (6.73 ± 0.05)	0.80 ± 0.02	160 ± 7	190 ± 6	108 ± 7
(<i>R</i>)-MDA	150 (6.83 ± 0.05)	0.76 ± 0.02	310 ± 10	900 ± 30	290 ± 10
(<i>S</i>)-MDA	100 (6.9 ± 0.1)	0.81 ± 0.04	100 ± 4	98 ± 4	50 ± 5
Methysergide	150 (6.8 ± 0.1)	0.18 ± 0.02	N.D.	N.D.	N.D.
Methylergonovine	0.8(9.2 ± 0.1)	0.40 ± 0.02	N.D.	N.D.	N.D.

* Significantly different ($P < 0.05$) from the other enantiomer by two-tailed t test.

† Significantly different from racemate ($P < 0.05$) by two-tailed t test.



after a single recreational dose and 2) release biogenic amines, an activity widely accepted to be a major pharmacological effect of these agents. We also report that two commonly prescribed medications reported to induce VHD in humans, pergolide and dihydroergotamine (Creutzig, 1992; Pritchett et al., 2002), also activate h5-HT_{2B} receptors in vitro. Previous studies suggested that VHD-associated drugs cause heart valve dysfunction via activation of heart valve

interstitial cell 5-HT_{2B} receptors. Our current report brings to five the number of medications known to activate 5-HT_{2B} receptors (e.g., fenfluramine, ergotamine, dihydroergotamine, pergolide, and methysergide), each of which induces VHD of the fenfluramine-type in humans. With the exception of fenfluramine, all of the drugs currently reported to produce VHD in humans are ergot derivatives. Because of the widespread use of ergot derivatives for treating diseases such as migraine headaches and Parkinson's disease, these findings are likely to have negative implications for drug discovery efforts that use ergolines or ergopeptines as lead candidates. Our finding that amphetamine derivatives (e.g., fenfluramine, MDMA, MDA, and norfenfluramine) also activate h5-HT_{2B} receptors demonstrates that drugs of other classes also need to be screened for potential valvulopathogenic actions. In this regard, we are in the process of a large-scale screen of various drugs currently approved for use in humans to identify potential valvulopathogenic drugs by virtue of their ability to bind to and activate recombinant h5-HT_{2B} receptors (V. S. Setola, S. J. Hufeisen, K. J. Grande-Allen, I. Vesely, R. A. Glennon, B. Blough, R. B. Rothman, B. L. Roth, manuscript in preparation).

Because there is no suitable animal model for predicting the valvulopathogenic actions of drugs, we evaluated the mitogenic effect of various drugs on hVICs, a novel in vitro model system. Because hVICs are the cells affected in drug-induced VHD, hVICs represent the most physiologically and pharmacologically relevant model system for VHD prediction. We report here that several drugs known to induce VHD in humans, as well as MDMA and MDA, elicit prolonged mitogenic responses in hVICs. Our results strongly suggest, therefore, that MDMA and MDA are valvulopathogenic; retrospective echocardiographic studies in human MDMA users are currently in progress to test this notion.

These studies also showed that h5-HT_{2B} receptor activation plays a critical role in the transduction of a mitogenic signal by VHD-associated drugs, strongly supporting the hypothesis that h5-HT_{2B} receptor agonists are likely to cause VHD. In this regard, we demonstrated that mitogenesis was abrogated by coincubation with a 5-HT_{2B/2C}-selective antagonist (SB206553; see <http://kidd.bioc.cwru.edu/pdsp.php> for comprehensive pharmacological profile of SB206553). Because human cardiac valves express large quantities of 5-HT_{2B} receptors and do not express appreciable amounts of 5-HT_{2C} receptors (Fitzgerald et al., 2000), it is likely that the inhibition by SB206553 is principally caused by 5-HT_{2B} receptor blockade. It is conceivable that the residual stimulation of [³H]thymidine incorporation not blocked by SB206553 might be caused by activation of mitogenic 5-HT_{2A} receptors, because human heart valves express 5-HT_{2A} mRNA [although sheep VICs apparently predominantly express 5-HT_{2A} receptors (Xu et al., 2002)], and the drugs studied herein are low-affinity, low-efficacy 5-HT_{2A} agonists (Nash et al., 1994; Rothman et al., 2000; Roy et al., 2000). Arguing against such a role for 5-HT_{2A} receptors in the mitogenic response of hVICs is the observation that the genetic ablation of 5-HT_{2B} receptors, but not of 5-HT_{2A} receptors (J. Gingrich, personal communication), interferes with myocardioblast proliferation during embryonic development, suggesting that the activation of mitogenic pathways by 5-HT_{2A} receptors in heart valves is not essential for cardiac development (Nebigil et al., 2000a,b). Taken together, these results imply that

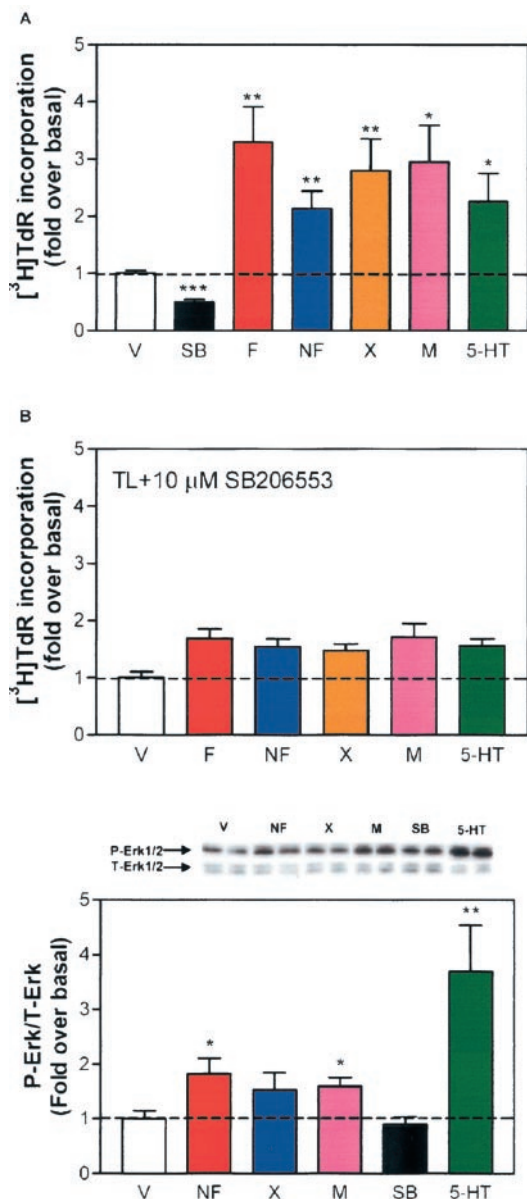


Fig. 3. MDMA and MDA induce mitogenesis in human heart valve interstitial cells in vitro. A, stimulation of [³H]thymidine deoxyribose incorporation in VICs treated for 48 h with either vehicle (V), 5-HT, the 5-HT_{2B/2C} receptor antagonist SB206553 (SB), fenfluramine (F), norfenfluramine (NF), MDMA (X), or MDA (M) reveals a prolonged mitogenic response that is blocked by pretreatment with SB (B). All drugs used at 10 μM except SB206553, which was used at 1 μM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001, significant difference from vehicle-treated cells by two-tailed *t* test. C, immunoblot analysis of Erk 1/2 phosphorylation in VICs treated for 10 min with norfenfluramine, MDMA, MDA, SB206553, or 5-HT reveals a short-term mitogenic response (i.e., increase in percent of total cellular Erk 1/2 phosphorylated; see *Materials and Methods* for details) after exposure to 5-HT_{2B} receptor agonists. *, *P* < 0.05; **, *P* < 0.01, significant difference from vehicle-treated cells by two-tailed *t* test.

activation of mitogenic pathways by 5-HT_{2A} receptors is inessential for cardiac development and that the 5-HT_{2B} receptor is most likely responsible for the mitogenic responses induced by valvulopathogenic drugs. Other findings implicating the h5-HT_{2B} receptor as the proximal molecular target responsible for fenfluramine-like VHD are the observations that h5-HT_{2B} receptors 1) are enriched in human heart valves; 2) are essential for normal cardiac development; and 3) induce, upon activation, prolonged mitogenic responses in heterologous expression systems (Fitzgerald et al., 2000; Nebigil et al., 2000b).

Our discovery that pergolide and dihydroergotamine, two drugs reported to induce VHD in humans (Creutzig, 1992; Pritchett et al., 2002), also activate h5-HT_{2B} receptors in vitro validates the use of recombinant h5-HT_{2B} receptors to screen for valvulopathogenic potential. Of equal importance, recent data have implicated the 5-HT_{2B} receptor in the pathogenesis of primary pulmonary hypertension, a severe and frequently fatal illness (Launay et al., 2002). Importantly, in this regard, fenfluramine use increases the risk of developing primary pulmonary hypertension (Abenhaim et al., 1996). Thus, these data further highlight the necessity of screening current and potential pharmacotherapies for agonist potencies and efficacies at human 5-HT_{2B} receptors and validate the use of 5-HT_{2B} receptor-expressing cell lines as models to do so. The data presented herein are thus of major public health importance because they suggest that MDMA abuse, which is at an all-time high, puts an expanding population at increased risk for developing VHD and primary pulmonary hypertension.

References

- Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, Higenbottam T, Oakley C, Wouters E, Aubier M, et al. (1996) Appetite-suppressant drugs and the risk of primary pulmonary hypertension. International Primary Pulmonary Hypertension Study Group. *N Engl J Med* **335**:609–616.
- Baumann MH, Ayestas MA, Dersch CM, Partilla JS, and Rothman RB (2000) Serotonin transporters, serotonin release and the mechanism of fenfluramine neurotoxicity. *Ann NY Acad Sci* **914**:172–186.
- Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, and Schaff HV (1997) Valvular heart disease associated with fenfluramine-phentermine. *N Engl J Med* **337**:581–588.
- Creutzig A (1992) Ergotamine-induced heart valve fibrosis and coronary microangiopathy? *Dtsch Med Wochenschr* **117**:1736.
- de la Torre R, Farre M, Ortuno J, Mas M, Brenneisen R, Roset PN, Segura J and Cami J (2000) Non-linear pharmacokinetics of MDMA ('ecstasy') in humans. *Br J Clin Pharmacol* **49**:104–109.
- Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine PA, Sun JH, Link JR, Abbaszade I, Hollis JM, et al. (2000) Possible role of valvular serotonin 5-HT_{2B} receptors in the cardiopathy associated with fenfluramine. *Mol Pharmacol* **57**:75–81.
- Jick H, Vasilakis C, Weinrauch LA, Meier CR, Jick SS, and Derby LE (1998) A population-based study of appetite-suppressant drugs and the risk of cardiac-valve regurgitation. *N Engl J Med* **339**:719–724.
- Launay JM, Herve P, Peoc'h K, Tournois C, Callebert J, Nebigil CG, Etienne N, Drouet L, Humbert M, Simonneau G, et al. (2002) Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nat Med* **8**:1129–1135.
- Nash JF, Roth BL, Brodtkin JD, Nichols DE, and Gudelsky GA (1994) Effect of the R(–) and S(+) isomers of MDA and MDMA on phosphatidyl inositol turnover in cultured cells expressing 5-HT_{2A} or 5-HT_{2C} receptors. *Neurosci Lett* **177**:111–115.
- Nebigil CG, Choi DS, Dierich A, Hickel P, Le Meur M, Messaddeq N, Launay JM, and Maroteaux L (2000a) Serotonin 2B receptor is required for heart development. *Proc Natl Acad Sci USA* **97**:9508–9513.
- Nebigil CG, Launay JM, Hickel P, Tournois C, and Maroteaux L (2000b) 5-Hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. *Proc Natl Acad Sci USA* **97**:2591–2596.
- Pritchett AM, Morrison JF, Edwards WD, Schaff HV, Connolly HM, and Espinosa RE (2002) Valvular heart disease in patients taking pergolide. *Mayo Clin Proc* **77**:1280–1286.
- Roth BL, Baner K, Westkaemper R, Siebert D, Rice KC, Steinberg S, Ernsberger P, and Rothman RB (2002) Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist. *Proc Natl Acad Sci USA* **99**:11934–11939.
- Rothman RB and Baumann MH (2002) Therapeutic and adverse actions of serotonin transporter substrates. *Pharmacol Ther* **95**:73–88.
- Rothman RB, Baumann MH, Dersch CM, Romero DV, Rice KC, Carroll FI, and Partilla JS (2001) Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. *Synapse* **39**:32–41.
- Rothman RB, Baumann MH, Savage JE, Rauser L, McBride A, Hufeisen SJ, and Roth BL (2000) Evidence for possible involvement of 5-HT_{2B} receptors in the cardiac valvulopathy associated with fenfluramine and other serotonergic medications. *Circulation* **102**:2836–2841.
- Roy A, Brand NJ, and Yacoub MH (2000) Expression of 5-hydroxytryptamine receptor subtype messenger RNA in interstitial cells from human heart valves. *J Heart Valve Dis* **9**:256–261.
- Sheffler DJ and Roth BL (2003) Salvinorin A: the 'magic mint' hallucinogen finds a molecular target in the kappa opioid receptor. *Trends Pharmacol Sci* **24**:107–109.
- Steffee CH, Singh HK, and Chitwood WR (1999) Histologic changes in three explanted native cardiac valves following use of fenfluramines. *Cardiovasc Pathol* **8**:245–253.
- Weissman NJ (2001) Appetite suppressants and valvular heart disease. *Am J Med Sci* **321**:285–291.
- Weissman NJ, Tighe JF Jr, Gottdiener JS, and Gwynne JT (1998) An assessment of heart-valve abnormalities in obese patients taking dexfenfluramine, sustained-release dexfenfluramine, or placebo. Sustained-Release Dexfenfluramine Study Group. *N Engl J Med* **339**:725–732.
- Xu J, Jian B, Chu R, Lu Z, Li Q, Dunlop J, Rosenzweig-Lipson S, McGonigle P, Levy RJ, and Liang B (2002) Serotonin mechanisms in heart valve disease II: the 5-HT₂ receptor and its signaling pathway in aortic valve interstitial cells. *Am J Pathol* **161**:2209–2218.

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