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Stimulus generalization by fenfluramine in a quipazine–ketanserin drug discrimination is not dependent on indirect serotonin release

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Abstract

The purpose of this study was to determine if animals trained to discriminate a serotonin$_{2A}$ (5-HT$_{2A}$) receptor agonist from a 5-HT$_{2A}$ receptor antagonist would also be sensitive to alterations in serotonin neurotransmission brought about by 5-HT reuptake inhibitors and releasers. Previous work from our laboratory has shown that the quipazine–ketanserin discrimination is mediated solely by the 5-HT$_{2A}$ receptor, thus providing a behavioral continuum of 5-HT$_{2A}$ receptor function. Rats were trained to discriminate quipazine (0.35 mg/kg) from ketanserin (1.0 mg/kg) on a variable interval-30 schedule of reinforcement. Following acquisition, substitution tests were conducted with the training drug, quipazine, and agents that have been shown to alter the synaptic levels of 5-HT, including fenfluramine, norfenfluramine, 5-methoxy-6-methyl-2-aminoindan (MMAI) and fluoxetine. All compounds substituted, except fluoxetine. Antagonist tests with mianserin and MDL 100,907 indicated that fenfluramine's and MMAI's substitution for quipazine was mediated by the 5-HT$_{2A}$ receptor. Animals were pretreated with PCPA to determine whether 5-HT release or direct agonism mediated the discriminative stimulus effects of fenfluramine and MMAI. PCPA blocked the substitution of MMAI but not of fenfluramine for quipazine. Analysis of ^{3}H-IP formation in cells showed that norfenfluramine dose-dependently stimulated phosphoinositide hydrolysis to levels similar to that of serotonin and quipazine. These results indicate that fenfluramine's substitution for quipazine in rats trained on a quipazine–ketanserin discrimination are due to direct agonism at the 5-HT$_{2A}$ receptor likely mediated by norfenfluramine, an active metabolite.

Keywords: Drug discrimination; Serotonin; Quipazine; Ketanserin; Fenfluramine; Norfenfluramine; MMAI

1. Introduction

Early drug discrimination studies suggested that generalization between direct and indirect 5-HT agonists is symmetrical. The indirect agonist, fenfluramine, which releases presynaptic stores and blocks reuptake of 5-HT, substitutes in rats trained to discriminate the direct agonist, quipazine (White et al., 1979), and vice versa, quipazine substitutes in fenfluramine-trained rats (White and Appel, 1981). We reasoned that by training with a highly specific cue, such as that generated by the reciprocal actions of an agonist and antagonist at a single, identified receptor, it might be possible to evaluate the behavioral consequences of altered neurotransmission at synapses where the receptor is localized. Previous work in our laboratory, characterizing the quipazine–ketanserin discrimination, revealed several features about this model which suggested that it would be ideally suited for the purposes of evaluating changes in 5-HT activity at 5-HT$_{2A}$ receptors (Smith et al., 1995). Advantages to using the quipazine–ketanserin discrimination include, first, the ability to study changes in neurotransmission at 5-HT$_{2A}$ receptors alone. Results from a series of converging studies including substitution, antagonism and ex vivo receptor autoradiography experiments suggested that this discrimination is mediated solely by 5-HT$_{2A}$ receptors. In substitution studies, DOI and MK 212 substituted fully for quipazine. The 5-HT$_2$ antagonists, MDL 100,907, mianserin, pizotifen and pirenperone, all substituted fully for ketanserin. In an antagonist study, MDL 100,907, a selective 5-HT$_{2A}$ antagonist, completely blocked quipazine discrimination. Results of ex vivo binding studies designed to estimate occupancy of 5-HT$_{2A}$ and 5-HT$_{2C}$

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receptors showed that the training dose of ketanserin blocked only 5-HT_{2A} receptors and not 5-HT_{2C} receptors. Together, these studies suggest that the quipazine–ketanserin discrimination is mediated by the 5-HT_{2A} receptor. A second advantage of using this model is that the behavior appears to reflect a continuum of 5-HT_{2A} receptor function ranging from full receptor activation (quipazine cue) through tonic activity (saline) to complete blockade (ketanserin cue) and is thus able to detect very subtle changes in 5-HT activation at the 5-HT_{2A} receptor. Dose–effect curves from earlier work (Smith et al., 1995) as well as the present paper support this conclusion. Third, we have previously shown this behavior to be sensitive to adaptive changes in the 5-HT_{2A} receptor system (Smith et al., 1995). Lastly, the agonist–antagonist paradigm has proven valuable in previous drug–drug discrimination studies that examined adaptive changes occurring after the administration of drugs acting at dopaminergic (Haenlein et al., 1985; Barrett et al., 1992) and benzodiazepine (Barrett and Smith, 1988) receptor sites.

The present studies were designed to determine if this paradigm also registers changes in neurotransmission at synapses where 5-HT_{2A} receptors are localized. As in previous research, we found that the indirect agonist, fenfluramine, substitutes for quipazine. This finding is consistent with the interpretation that rats trained to discriminate quipazine from ketanserin are sensitive to changes in synaptic 5-HT. The current manuscript describes a series of in vivo and in vitro studies which show that the fenfluramine substitution is not due to release of 5-HT, but rather reflects direct agonist action at 5-HT_{2A} receptors.

2. Methods

2.1. Subjects

The subjects were 72 Sprague–Dawley male rats (225–249 g) (Harlan Sprague–Dawley, Indianapolis, IN) individually housed and food-deprived to 85% of their free-feeding weights 1 week prior to the onset of the study. The rats were given enough food following training each day and on the weekends to maintain them at 85% of their expected free-feeding weights. Water was freely available at all times except during their 20-min training sessions. The animals were on a 12-h light–dark cycle with light onset at 6:00 a.m. All animal experimental procedures were approved by the Vanderbilt University Animal Care Committee.

2.2. Apparatus

Six experimental chambers (BRS/LVE Model No. RTC-024), each housed in a sound-attenuating chamber, were used for the experiments. The experimental chambers were equipped with a house light and two response levers and a pellet dispenser centered between the two levers. The start of the training session was signaled by the onset of the house light (7.5-W bulb). The light remained on until completion of the session. All pieces of equipment were controlled by MS-DOS-compatible computers using software written locally.

2.3. Training procedures

Rats were trained to lever-press for food reinforcement (45 mg Noyes Pellets) on a fixed ratio (FR) 1 schedule during daily 20-mm sessions. Following acquisition of the lever-press response, the reinforcement contingency was changed to a variable interval (VI) 10-s schedule of reinforcement and discrimination training initiated. The reinforcement schedule was incremented by 10 s each week until arriving at a final VI-30 schedule of reinforcement, which remained in effect for the duration of the study. At the end of 3 weeks of training, a 15-s time-out (TO) contingency was added to the training schedule. The TO contingency consisted of a 15-s period following incorrect responses during which reinforcement was not available. The purpose of the TO was to punish lever switching or probing as a strategy to identify the correct lever. The initial training doses were 0.5 mg/kg quipazine (QUIP) and 1.0 mg/kg ketanserin (KET). Following 5 weeks of training, the saline baseline was determined and it was found that the animals made 35% of their responses on the QUIP lever and 65% of their responses on the KET lever. The fact that the animals made more responses on the KET lever than the QUIP lever following saline implied that the QUIP cue was the more salient of the two training cues. In an effort to establish a saline baseline closer to 50%, the QUIP training dose was gradually lowered to 0.35 mg/kg and training resumed. Following an additional 28 days of training, the saline baseline was redetermined, and this time the rats made 45% of their responses on the QUIP lever and 55% of their responses on the KET lever. No further adjustments were made in the training doses.

The rats were given 20-min training sessions 5 days/week. Thirty minutes after being injected with either QUIP or KET, rats were placed in the experimental chamber and reinforced for responding on the correct lever. For half the rats, responding on the right lever was QUIP correct and responding on the left lever was KET correct; for the remaining animals, the conditions were reversed.

Throughout training, QUIP and KET were alternated every other day and training was given Monday–Friday. Discrimination learning was monitored twice weekly by calculating the percent correct responses (number of correct responses/total number of responses) that occurred during a 2.5-mm extinction session given at the beginning of training. These data provided a measure of discrimination learning unconfounded by reinforcement. During the remaining 17.5 min of the training session, the VI-30 schedule was in effect. Training continued until the percent

correct for the whole group averaged greater than 85% correct for both QUIP and KET. In the experiments described below, a subset of the original 72 rats trained on the QUIP–KET discrimination was used and most rats served as subjects in multiple experiments.

At the end of 18 months, the original group of rats had grown too old. A second group of rats ($n = 72$) was trained on the QUIP–KET discrimination using identical training procedures and experiments were continued.

2.4. Dose–response curve

Following acquisition of the discrimination, a dose–response curve was determined for both QUIP and KET during 5-min extinction test sessions. For this experiment, 63 rats were assigned to one of seven groups ($n = 9$), matched for choice behavior during the most recent saline test session. Independent groups of rats were injected with either QUIP (0.35, 0.175 and 0.0875 mg/kg), KET (1.0, 0.063 and 0.031 mg/kg) or saline and placed in the experimental chamber 30 min later. At the end of the 5-min test session, the animals were returned to their home cages. The dose–response curve presented is from the second group of animals trained on the QUIP–KET discrimination.

2.5. Substitution tests

In order to determine the sensitivity of the QUIP–KET discrimination to changes in neurotransmission, several compounds known to alter synaptic 5-HT levels were tested. The 5-HT uptake inhibitor, fluoxetine (10, 5.0, 2.5, 1.25 mg/kg), and the selective 5-HT-releasing agents, fenfluramine (3.0, 1.5, 0.75, 0.375 mg/kg) and 5-methoxy-6-methyl-2-aminoindan (MMAI) (1.25, 0.625 and 0.31 mg/kg) (Johnson et al., 1991), were tested to determine if they would substitute for QUIP. Norfenfluramine (1.2 mg/kg and1.0), an active metabolite of fenfluramine, was also tested for its ability to substitute. All experiments used a between-subjects design. For each drug tested, a saline group was included. All test compounds were administered subcutaneously. Rats were assigned to one of four or five independent groups ($n = 10–14$) for fluoxetine, fenfluramine and MMAI, and three groups ($n = 9$) for norfenfluramine. The rats received 1 week of retraining between all experiments. For each experiment, the groups were matched according to the percentage of responses on each lever during the most recent saline test.

To determine if fluoxetine in combination with a 5-HT_{1A} receptor antagonist would substitute for quipazine, 60 rats were assigned to one of four groups ($n = 15$). Two groups of rats were pretreated with 1 mg/kg WAY 100,635 and the other two groups were pretreated with saline. Thirty minutes later, one group of rats from each pretreatment condition was injected with 5 mg/kg fluoxetine and the other two groups were injected with saline. Thirty minutes following fluoxetine or saline administration, the animals were placed in the experimental chamber for a 5-min extinction test session. All the substitution curves were obtained from the first group of animals trained on the QUIP–KET discrimination, except norfenfluramine, which was determined in the second group.

2.6. Fenfluramine discrimination following PCPA pretreatment

If the substitution of fenfluramine for QUIP is mediated by fenfluramine's ability to release 5-HT, then depletion of 5-HT by *p*-chlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase, should block fenfluramine's substitution for QUIP. To test this possibility, 60 rats from the first group of QUIP–KET-trained animals, matched for choice behavior during the most recent saline test session, were assigned to one of four groups ($n = 15$). Two groups of rats were pretreated with 300 mg/kg PCPA daily for three consecutive days while the other two groups received saline injections during this same time period. No training was given during the 6 days of treatment. This experiment was conducted in the first group of QUIP–KET-trained animals. Seventy-two hours following the last injection, one group of animals from each pretreatment condition was injected with 1.5 mg/kg fenfluramine or saline and 30 min later placed in the experimental chamber and given a 5-min extinction test session. Training resumed 10 days following completion of the experiment.

2.7. Brain levels of 5-HT and 5-HIAA

In order to evaluate the effectiveness of the PCPA treatment, brain levels of 5-HT and 5-HIAA were determined in a subset of the first group of rats trained on the QUIP–KET discrimination. This group of rats was given treatment identical to that described above for the behavioral studies. Five rats were treated with 300 mg/kg PCPA for 3 days and five rats were injected with saline. Seventy-two hours later, the animals were sacrificed and brains harvested and analyzed by high-performance liquid chromatography.

Whole brain was homogenized in 0.1 M perchloric acid and centrifuged at $9000 \times g$ for 30 min. Fifty microliters of supernatant was then injected automatically (Waters 712 Autosample; Waters, Milford, MA) onto a reverse-phase column (5100 Chromosystems, Muchen, Germany) equilibrated with a mobile phase consisting of 7% methanol, 32 mM citric acid, 12.5 mM disodium phosphate, 0.5 mM octyl sodium sulphate and 0.05 mM disodium ethylenediamine tetraacetate pumped at 1.3 ml/min. 5-HT and 5-HIAA were monitored by electrochemical detection (Waters 464 pulsed electrochemical detector; Waters), operated at a potential of +800 mV vs. Ag/AgCl. Concentrations were determined by comparison with known standards. Data were analyzed using an automated acquisition system (millennium^{32}; Waters) and expressed as femtomoles per milligram of protein.

2.8. Antagonist studies

To determine if the discriminative stimulus effects of fenfluramine were mediated by the 5-HT_{2A} receptor, animals were tested for their ability to discriminate fenfluramine following pretreatment with the 5-$HT_{2A/C}$ receptor antagonist, mianserin, or with MDL 100,907, a selective 5-HT_{2A} receptor antagonist. Rats were assigned to one of six groups ($n=10$) and pretreated with saline or mianserin (0.2, 0.1, 0.05 and 0.025 mg/kg) 30 min before an injection of 1.5 mg/kg fenfluramine. Thirty minutes later, they were placed in the experimental chamber and given 5-min extinction test sessions. In a second group of rats, this procedure was replicated with MDL 100,907 or saline (0.2, 0.1, 0.05 and 0.025 mg/kg) followed by 2.5 mg/kg fenfluramine. Each experiment included a saline–saline control group.

Additional experiments were performed to determine whether pretreatment with fluoxetine would block the discriminative stimulus effects of fenfluramine. The rationale here was that fluoxetine, an uptake inhibitor, would prevent fenfluramine's entry into the presynaptic terminal and consequently prevent fenfluramine's release of 5-HT. Fluoxetine has been shown to block fenfluramine-induced release of 5-HT (Raiteri et al., 1995), and we predicted that it would block fenfluramine substitution. Rats were assigned to one of six groups ($n=10$) and pretreated with fluoxetine (10, 5, 2.5 and 1.25 mg/kg) or saline, 15 min prior to injection of 1.5 mg/kg fenfluramine. Thirty minutes later, the animals were given 5-min extinction test sessions.

Lastly, to determine if MMAI's substitution for QUIP was 5-HT_{2A} receptor-mediated, two groups of animals ($n=13$) were pretreated with either saline or 0.5 mg/kg MDL 100,907, 15 min prior to an injection of 1.25 mg/kg MMAI. Thirty minutes following MMAI administration, animals were given 5-min extinction tests. All antagonist studies were conducted in the first group of QUIP–KET-trained animals except the MDL 100,907–fenfluramine experiment, which was done in the second group of animals.

2.9. MMAI discrimination following PCPA pretreatment

PCPA pretreatment did not prevent fenfluramine from substituting for QUIP. As a positive control to verify PCPA's 5-HT-depleting effects, it was of interest to determine whether PCPA treatment would block the previously demonstrated substitution of MMAI, another select 5-HT-releasing agent, for QUIP. Sixty rats from the second group of QUIP–KET-trained animals, matched for their choice behavior during the most recent saline test session, were assigned to one of four groups ($n=10$). Two groups of rats were pretreated with 300 mg/kg PCPA daily for three consecutive days while the other two groups received saline injections during this same time period. No training was given during the treatment period. Seventy-two hours following the last PCPA or saline injection, one group of animals from each pretreatment condition was injected with 1.25 mg/kg MMAI or saline and 30 min later placed in the experimental chamber and given a 5-mm extinction test session. Training resumed 7 days following completion of the experiment.

2.10. Analysis of phosphoinositide hydrolysis in heterologous cell lines

NIH 3T3 fibroblast stably transfected with rat brain 5-HT_{2A} receptor cDNA (Julius, UCSF, San Francisco, CA, USA) were grown in 24 cell culture plates containing Dulbecco's modified Eagle's medium, 10% bovine calf serum and 200 mg/ml neomycin analogue G-41 8 sulfate. Cells were maintained in a humidified incubator in an atmosphere of 5% CO_2/95% air at 37 °C.

The accumulation of inositol monophosphate was assayed as described previously (Barker et al., 1994). Briefly, confluent cells were labeled for 20–24 hours (in the absence of serum) with 2 μCi/ml myo[^{3}H]inositol (20–25 Ci/mmol; NEN Life Science Products) in serum-free, inositol-free DMEM to label phospholipid pools. Labeling medium was aspirated and the cells washed three times with HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Cells were treated with drugs in the presence of lithium chloride (10 mM) and

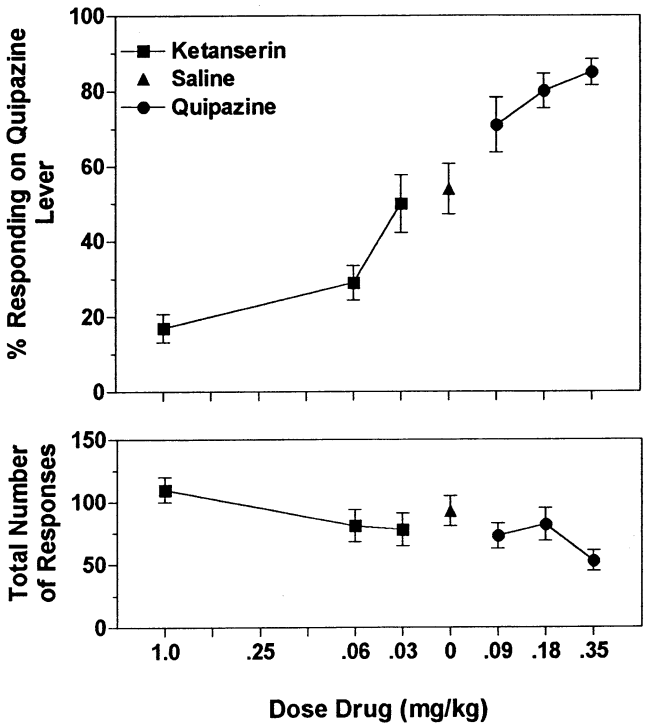

Fig. 1. Quipazine–ketanserin dose–response curve. The data shown are percent responding on the quipazine lever (upper panel) and mean number of responses during the 5-min test session (lower panel) as a function of varying doses of quipazine, ketanserin and saline during 5-min extinction test sessions. Ketanserin is plotted from highest to lowest dose, contrary to convention, in order to illustrate the continuum-like nature of the quipazine–ketanserin discrimination. Test doses are plotted as equal log units.

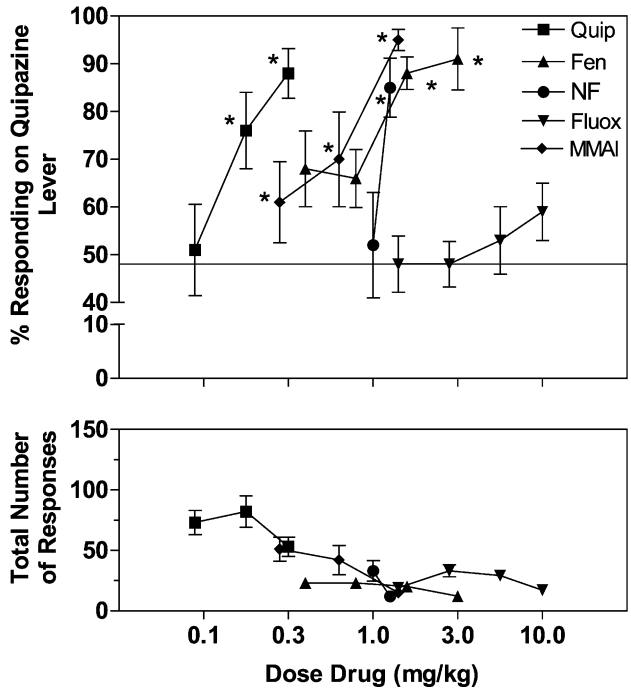

Fig. 2. Substitution tests with direct and indirect 5-HT_2 receptor agonists. The results of substitution tests with quipazine (included for comparison purposes), fenfluramine, norfenfluramine, fluoxetine and MMAI are shown in the upper panel. The total number of responses made during the 5-min extinction test is plotted in the lower panel. The data are expressed as percent responding on the quipazine lever (upper panel). Test doses are plotted as equal log units. The dashed line illustrates the mean saline control value. * Test doses of quipazine, MMAI, fenfluramine, norfenfluramine and fluoxetine that significantly differed from their respective saline controls.

pargyline (10 μM) and incubations were continued for 30 min. Reactions were terminated by aspiration of the medium and addition of methanol. [^{3}H]Inositol monophosphate ([^{3}H]IP) was extracted with chloroform/methanol and isolated by anion exchange chromatography. Radioactivity was quantitated by liquid scintillation counting.

2.11. Drugs

All drug doses were calculated as the salt. Quipazine, ketanserin, mianserin, fenfluramine, norfenfluramine, fluoxetine and WAY 100,635 were dissolved in saline. MDL 100,907 was dissolved in 2% tartaric acid and diluted with distilled water. MMAI was dissolved in distilled water. PCPA was prepared as a paste with Tween 80 and suspended in distilled water. Drugs were administered subcutaneously in a volume of 1 ml/kg, except PCPA, which was injected intraperitoneally and suspended in a volume of 2 ml/kg.

Quipazine maleate, ketanserin tartrate, and mianserin HCL were purchased from Research Biochemical (Natick, MA). Fenfluramine HCL, PCPA and serotonin were purchased from Sigma (St. Louis, MO). WAY 100,635 was purchased from Tocris Cookson (Ballwin, MO). Norfenfluramine was a gift from Lilly Research Laboratories, Indianapolis, IN, and MDL 100,907 from Marion Merrell Dow, Cincinnati, OH. MMAI was a generous gift from Dr. David Nichols (Purdue University, West Lafayette, IN).

3. Results

3.1. Discrimination training and dose–response function

Animals received a total of 48 training sessions prior to reaching the joint criteria of 85% correct for both training drugs and 50% responding on each lever following saline administration. A dose–response curve was determined for several doses of QUIP (0.35, 0.175 and 0.0875 mg/kg), KET (1.0, 0.06 and 0.03 mg/kg) and saline. As can be seen in Fig. 1, percent drug–lever responses for QUIP and KET were dose-dependent.

3.2. Substitution tests

The results of the substitution tests with fluoxetine, fenfluramine, MMAI and norfenfluramine are shown in

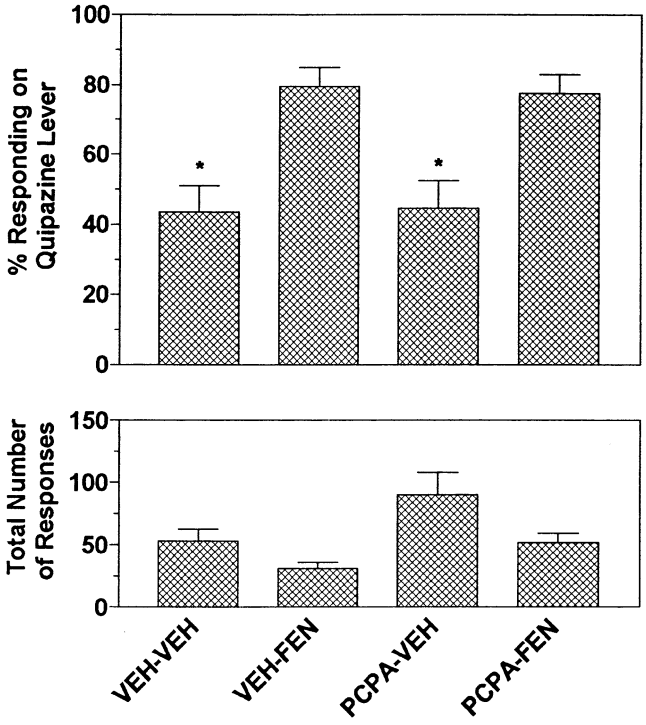

Fig. 3. Effects of PCPA pretreatment on fenfluramine's substitution for quipazine. Animals were pretreated with either 300 mg/kg PCPA or saline for 3 days. Seventy two hours after the final pretreatment injection, the rats were injected with 1.5 mg/kg fenfluramine and given 5-min extinction test sessions. Percent responding on the quipazine lever is shown in the upper panel and the total number of responses made during the 5-min test is shown in the lower panel. * Groups that differed significantly from VEH–FEN ($P < .05$, Newman–Keuls). Animals pretreated with PCPA or vehicle did not differ from one another.

Table 1
Serotonin and 5-HIAA brain levels following PCPA and saline treatment

	Saline	PCPA
5-HT	346 ± 41	48 ± 10 *
5-HIAA	420 ± 15	46 ± 10 *

* Groups that differ significantly from saline-treated animals ($P < .05$, t test).

Fig. 2. Fluoxetine did not substitute for QUIP at any of the doses tested. Later, a dose of 20 mg/kg fluoxetine was tested in the second group of QUIP–KET-trained rats and it also was not significantly different from saline (data not shown). Animals treated with 1.5 and 3.0 mg/kg fenfluramine made a significantly greater percentage of responses on the QUIP lever (88% and 90%, respectively) than saline-treated controls [$F(4,47) = 4.3$, $P < .0049$]. Similarly, all three test doses of MMAI produced significantly greater QUIP lever responding (61%, 70% and 95%) than saline-treated controls [$F(3,33) = 10.16$, $P < .0001$]. Lastly, animals injected with 1.2 mg/kg norfenfluramine made a significantly greater percentage of their responses on the QUIP lever than saline controls (87% vs. 43%, respectively) [$F(2,31) = 21.9$, $P < .0001$].

Also determined was whether fluoxetine (5 mg/kg) would substitute for QUIP when given in combination with WAY 100,635 (1.0 mg/kg), a 5-HT_{1A} antagonist. Under these conditions, fluoxetine still did not substitute for QUIP (SAL-FLU = 62 ± 6.3% and WAY-FLU = 67 ± 6.5%, $P > .05$) (data not shown).

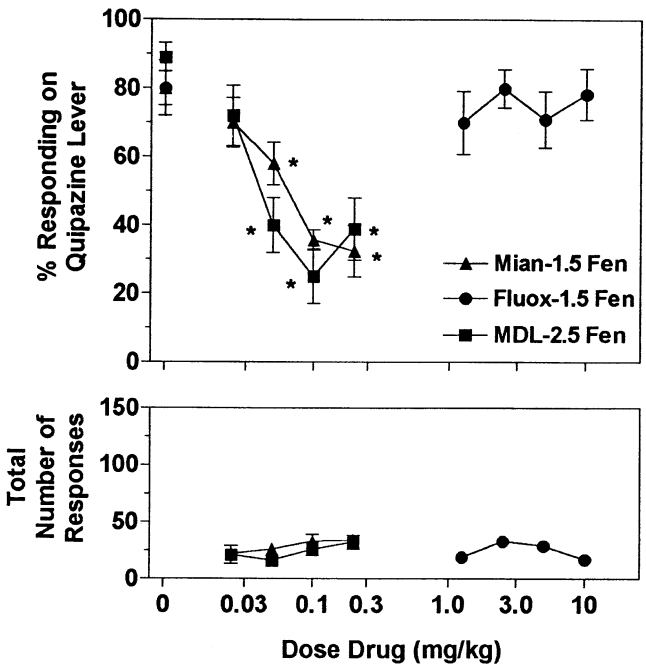

Fig. 4. Antagonism of stimulus effects of fenfluramine. Shown are the effects of pretreatment either with MDL 100,907, mianserin or fluoxetine on fenfluramine's substitution for quipazine (upper panel) and the total number of responses made during the 5-min extinction test session (lower panel). Test doses are plotted as equal log units. * Groups that were significantly different from saline ($P < .05$, Newman–Keuls).

3.3. Fenfluramine discrimination following PCPA pretreatment

To test the hypothesis that the substitution of fenfluramine for QUIP was mediated by 5-HT release, rats were pretreated with PCPA and then tested for their ability to discriminate fenfluramine (Fig. 3). Surprisingly, the substitution of fenfluramine for QUIP was not altered by PCPA pretreatment.

3.4. 5-HT and 5-HIAA levels following PCPA pretreatment

To confirm that pretreatment with PCPA was indeed depleting 5-HT levels, brain levels of 5-HT and 5-HIAA were determined in a subset of the rats trained on the QUIP–KET discrimination (Table 1). In animals pretreated with PCPA, brain levels of both 5-HT [$t = 7.9$, $df = 10$, $P < .0001$] and 5-HIAA [$t = 20.9$, $df = 10$, $P < .0001$] were significantly reduced relative to saline-pretreated controls. 5-HT and 5-HIAA levels in PCPA animals were 13.7% and 10.7% of saline-treated animals, respectively, indicating that the treatment procedure was effective.

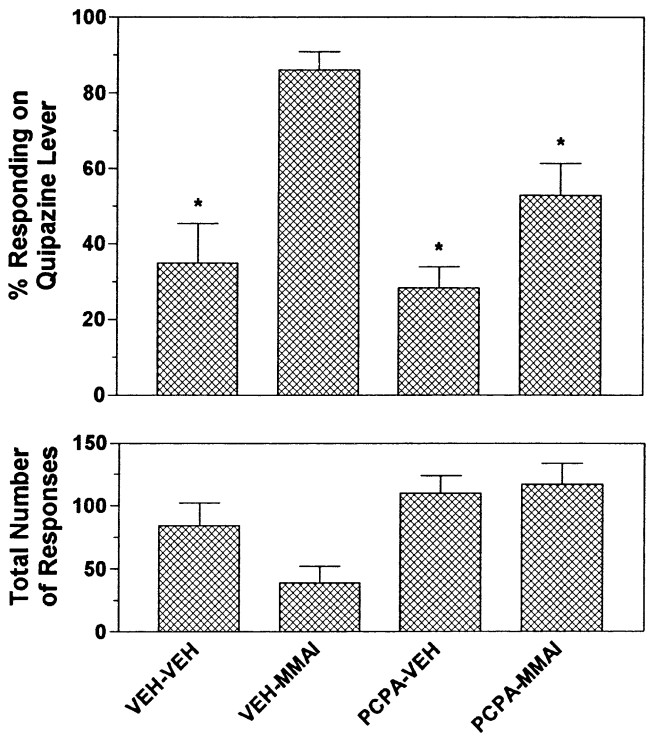

Fig. 5. Effects of PCPA pretreatment on MMAI's substitution for quipazine. Animals were pretreated with either 300 mg/kg PCPA or saline for 3 days. Seventy two hours after the final pretreatment injection, the rats were injected with 1.25 mg/kg MMAI and given 5-min extinction tests. Percent responding on the quipazine lever is shown in the upper panel and the total number of responses made during the 5-min test is shown in the lower panel. * Groups that differed significantly from VEH–MMAI-treated rats ($P < .05$, Newman–Keuls).

3.5. Antagonist studies

To determine the extent to which the 5-HT_{2A} receptor mediated the discriminative stimulus effects of fenfluramine, two antagonists, mianserin and MDL 100,907, were tested in combination with fenfluramine. Fig. 4 shows that both mianserin [$F(5,52)=10.7$, $P<.0001$] and MDL 100,907 [$F(5,48)$ 8.1, $P<.0001$] blocked fenfluramine's substitution for QUIP. To test the role that release of 5-HT plays in fenfluramine's substitution for QUIP, we determined if fluoxetine blocked fenfluramine substitution. Pretreatment with fluoxetine did not, however, block the discriminative stimulus effects of fenfluramine (Fig. 4) as evidenced by the fact that at no dose of fluoxetine did fenfluramine discrimination differ from saline-pretreated controls.

To determine if MMAI's substitution for quipazine was 5-HT_{2A} receptor-mediated, animals were tested on MMAI following pretreatment with either MDL 100,907 or saline. MDL 100,907 blocked the substitution of MMAI for quipazine ($t=2.78$, $df=21$, $P<.01$] with MDL 100,907 and saline-pretreated animals, making $57\pm11\%$ and $86\pm4\%$ of their responses on the quipazine lever, respectively (data not shown).

3.6. MMAI discrimination following PCPA pretreatment

The effects of serotonin depletion on MMAI discrimination are shown in Fig. 5. Animals pretreated with PCPA and tested on 1.25 mg/kg MMAI made a significantly lower percentage of their responses on the QUIP lever relative to saline-pretreated controls (53% vs. 86%, respectively) [$F(3,33)=8.8$, $P<.0002$]. These results suggest that release of 5-HT plays a major role in MMAI's substitution for QUIP.

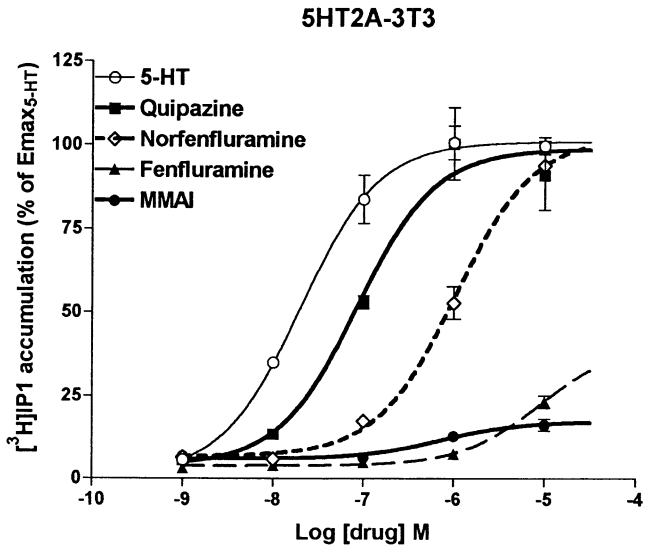

Fig. 6. Concentration response of 5-HT, norfenfluramine, quipazine, fenfluramine and MMAI stimulated PI hydrolysis in 3T3 cells transfected with 5-HT_{2A} receptor. Values are mean ± SEM normalized to the maximum responses of 5-HT from a single experiment performed in triplicate; the experiment was repeated three times with similar results. EC_{50} values were 20 nM for 5-HT, 85 nM for quipazine and 1 μm for norfenfluramine.

3.7. Characterization of direct agonist effects in vitro

In fibroblasts expressing the 5-HT_{2A} receptor QUIP, norfenfluramine dose-dependently stimulated the formation of [^{3}H]IP (Fig. 6). The maximal response of norfenfluramine was approximately the same as the maximal response of 5-HT and QUIP. MMAI and fenfluramine were less effective in stimulating [^{3}H]IP formation; at 10^{-5} M, [^{3}H]IP production was only 20–25% of the maximal 5-HT effect. The increase in the formation of [^{3}H]IP by 1 μm norfenfluramine was completely blocked by 1 μm ketanserin, a 5-HT_{2A} receptor antagonist.

4. Discussion

Previous work in our laboratory has shown that the QUIP–KET discrimination is uniquely sensitive to changes in 5-HT_{2A} receptor activity (Smith et al., 1995). In the present study, we examined the utility of this discrimination for detecting pharmacological alterations in synaptic levels of 5-HT. If effective, this behavior would presumably reflect neurotransmission only at synapses where 5-HT_{2A} receptors are present. Following training, dose–response curves were determined for QUIP and KET and found to be similar to those previously reported (Smith et al., 1995). In substitution tests, the 5-HT-releasing agents, MMAI and fenfluramine, substituted for quipazine. Norfenfluramine, an active metabolite of fenfluramine, also substituted for quipazine. In contrast, the 5-HT uptake inhibitor, fluoxetine, failed to substitute for quipazine. One possible explanation for the failure of fluoxetine to substitute for QUIP is that fluoxetine indirectly activates 5-HT_{1A} receptors in the raphe cell bodies, thereby inhibiting the release of 5-HT at terminal sites and hence preventing fluoxetine from substituting for QUIP (Romero et al., 1996). To test this possibility, animals were pretreated with WAY 100,635, a 5-HT_{1A} receptor antagonist, prior to fluoxetine. Fluoxetine did not, however, substitute for QUIP even in the presence of 5-HT_{1A} receptor blockade. Earlier substitution studies with fenfluramine and fluoxetine in rats trained to discriminate quipazine from saline reported similar substitution profiles (White et al., 1979). To our knowledge, MMAI has not previously been tested in rats trained to discriminate quipazine. An earlier study did, however, report partial substitution of LSD (75%), a 5-HT_{2A} receptor agonist, in animals trained to discriminate MMAI from saline, suggesting that there is some similarity in the stimulus properties of LSD and MMAI (Marona-Lewicka and Nichols, 1994). These investigators later reported that 5-HT reuptake inhibitors only partially substituted for MMAI (Marona-Lewicka and Nichols, 1998). These results, combined with our data document-

ing the failure of fluoxetine to substitute for quipazine, suggest that the discriminative stimulus effects of 5-HT uptake inhibitors differ from those of both direct agonists and 5-HT-releasing agents.

The substitution studies indicate that, in addition to direct 5-HT agonists, the 5-HT-releasing agents, fenfluramine and MMAI, will substitute for the discriminative stimulus effects of quipazine. Previous work has shown that fenfluramine's cue properties are blocked by nonselective 5-HT antagonists (McElroy and Feldman, 1984; White and Appel, 1981). The present finding — that the highly selective 5-HT_{2A} receptor antagonist, MDL 100,907 (Sorensen et al., 1993), completely blocked fenfluramine's substitution for quipazine — suggests that fenfluramine's cue in these animals is primarily mediated by the 5-HT_{2A} receptor. MDL 100,907 also blocked MMAI's substitution for quipazine. This is in contrast with earlier work in animals trained to discriminate MMAI from saline, which reported that the 5-HT antagonists, ketanserin and methiothepin, did not block MMAI's stimulus effects (Marona-Lewicka and Nichols, 1994). As with fenfluramine, the antagonist studies with MDL 100,907 suggest that MMAI's substitution for quipazine, in animals trained on a QUIP–KET discrimination, is mediated primarily by the 5-HT_{2A} receptor. The failure of fluoxetine to block fenfluramine's substitution for quipazine cast doubt on our assumption that release of 5-HT was mediating fenfluramine's substitution for QUIP and led to studies with PCPA.

Animals were pretreated with PCPA to evaluate whether fenfluramine's and MMAI's substitutions for quipazine were mediated by their actions as 5-HT-releasing agents. Brain levels of 5-HT following PCPA treatment were reduced to $<14\%$ of control animals showing that the treatment was successful in reducing serotonin availability. Surprisingly, PCPA had no effect on fenfluramine's substitution for quipazine, suggesting that its discriminative stimulus effects are not due to release of 5-HT. This conclusion is supported by our finding that pretreatment with fluoxetine does not block fenfluramine substitution. An earlier study reported a significant reduction in fenfluramine discrimination following PCPA pretreatment (White and Appel, 1981); however, this experiment was performed in rats trained to discriminate fenfluramine from saline. Since fenfluramine releases 5-HT, it is likely that the fenfluramine vs. saline training cue reflects activation of multiple receptors, whereas fenfluramine substitution in quipazine–ketanserin-trained animals is 5-HT_{2A} receptor-specific. The importance of the training conditions (drug, dose, etc.) to substitution studies has been well documented in the drug discrimination literature (Stolerman and D'Mello, 1981; Stolerman et al., 1984; White and Appel, 1982); the current results reinforce the evidence that substitution of one drug for another is largely determined by the stimulus conditions employed during training.

We next considered the possibility that the fenfluramine substitution is related to a direct agonist action at postsynaptic 5-HT receptors. Recent work (Fitzgerald et al., 2000) has documented that fenfluramine and its active metabolite, norfenfluramine, act as agonists at human 5-HT_{2A} receptors. In this manuscript, we show that these drugs are also agonists at rat 5-HT_{2A} receptors with norfenfluramine being more efficacious than the parent compound. Data by Mennini et al. (1991) suggest that brain concentrations of norfenfluramine would be sufficient to serve as discriminative stimuli 30 min postfenfluramine administration. Several investigators have previously suggested that norfenfluramine may play a role in fenfluramine's discriminative stimulus effects (Boja and Schechter, 1988; Goudie, 1977; McElroy and Feldman, 1984; Schechter, 1990). The present data in transfected cells provide a mechanism by which this may occur. The low intrinsic activity of MMAI as a direct agonist at 5-HT_{2A} receptors in vitro is consistent with in vivo results suggesting that its action is dependent on release of 5-HT (Marona-Lewicka and Nichols, 1994 and present results).

In conclusion, this series of experiments shows that release of 5-HT does not contribute significantly to fenfluramine's substitution for quipazine in rats trained to discriminate a continuum of 5-HT_{2A} receptor activation. Rather, the results are consistent with a significant role for direct agonist action of fenfluramine and especially its metabolite, norfenfluramine, at 5-HT_{2A} receptors. Importantly, the results provide convincing evidence that direct agonist activity at 5-HT_{2A} receptors is significant in the in vivo pharmacological actions of fenfluramine. In contrast, the 5-HT-releasing agent, MMAI, has no direct agonist action; its 5-HT_{2A} receptor-dependent substitution in QUIP–KET-trained animals suggests that this behavior may provide a sensitive index of neurotransmission at brain sites where 5-HT_{2A} receptors are localized.

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