Further Studies on the Mechanism of Serotonin-Dependent Anorexia in Rats

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Abstract. 4-(3-Indolyl-2-ethyl) piperidine (LM 5008), 2-(1-piperazinyl) quinoline (quipazine), and meta-chlorophenylpiperazine (mCPP) were studied for their ability to affect serotonergic mechanisms in vitro. Their relative potency in inhibiting serotonin (5-HT) uptake in vivo and reducing food intake in rats was also examined. mCPP was very potent in displacing 3H-5-HT bound to brain membranes (ICso, 6.2 x 10^-7 M), followed by quipazine, which showed an ICso of 3.8 x 10^-6 M. LM 5008 was the least effective with an ICso of 3.6 x 10^-5 M. mCPP and quipazine were less potent than d-fenfluramine in releasing 14C-5-HT from brain synaptosomes, while LM 5008 caused no significant effects at a concentration of 10^-5 M. Conversely, both in vitro and in vivo studies on 5-HT uptake showed that LM 5008 was the most potent compound in inhibiting 5-HT uptake and mCPP the least potent. Since a 50% reduction of food intake was not reached even with a dose of LM 5008 27-times higher than the EDso for inhibiting 5-HT uptake in vivo, it is suggested that even marked inhibition of 5-HT uptake at central synapses is not sufficient per se to trigger serotonin-dependent anorexia in the rat. Increased release and/or direct stimulation of post-synaptic receptors may be necessary to obtain this effect. This could be of interest for developing new agents which can cause anorexia by interacting with brain serotonin.

Key words: LM 5008 - Quipazine - Meta-chlorophenylpiperazine - d-Fenfluramine - Serotonin uptake - Serotonin release - Serotonin receptor - Anorexia

Various studies suggest that fenfluramine produces anorexia by inhibiting serotonin (5-HT) uptake and increasing its release from nerve terminals in the brain (Jespersen and Scheel-Kruger, 1973; Samanin et al., 1972; Garattini et al., 1975; Fuxe et al., 1975). Quipazine which, in addition to these effects (Hamon et al., 1973; Rodriguez et al., 1973), acts as an agonist at post-synaptic receptors (Rodriguez et al., 1973; Samanin et al., 1977a, b) also causes strong anorexia in rats (Samanin et al., 1977a) and this effect is prevented by pretreatment with methergoline (Samanin et al., 1977b), a 5-HT (5-hydroxytryptamine) antagonist (Mawson and Whittington, 1970). These studies have led to the suggestion that increased availability of 5-HT at post-synaptic receptors or direct receptor stimulation may produce anorectic effects in laboratory animals. Anorectic effects in rats have been described recently (Goudie et al., 1976; Blundell and Latham, 1978) with drugs, such as fluoxetine and Org 6482, which reportedly block 5-HT uptake specifically and potently at central synapses (Wong et al., 1975; Sugrue et al., 1976).

A striking exception, however, was noted in our laboratory with LM 5008, a new compound (LeFur and Uzan, 1977) which, at doses well above those reported to inhibit 5-HT uptake (LeFur et al., 1977), caused no reduction of food intake in rats. This raises the question whether blockade of 5-HT uptake is sufficient per se to trigger 5-HT-dependent anorexia. It has, however, been reported that the uptake of labeled 5-HT in striatal and hypothalamic synaptosomal preparations from animals given 10 mg/kg LM 5008 is not significantly different from that of control animals (LeFur and Uzan, 1977). Therefore, LM 5008 may not produce a substantial blockade of 5-HT uptake in specific brain areas particularly relevant for centrally 5-HT-mediated anorexia.

In an attempt to answer these questions and to obtain information as to which 5-HT mechanisms correlate closest with anorectic activity, we investigated how LM 5008 affects 5-HT mechanisms in vitro,
comparing it with quipazine and metachloro-phenylpiperazine (mCPP), a 5-HT agonist and uptake inhibitor with anorectic activity in rats (Garattini et al., 1976; Garattini, 1979). In vivo ED₅₀ of the three compounds for inhibiting brain 5-HT uptake and food intake were also measured. In one experiment, doses of quipazine and mCPP, which induce maximal anorexia, were compared with 10 mg/kg LM 5008, which induces no anorexia, for their ability to inhibit 5-HT uptake in various brain regions. In the same areas, the levels of the drugs under study were measured to permit more informed interpretation of the results.

Materials and Methods

Female CD₁ (Charles River, Italy) rats (200 ± 20 g) were used. The animals were housed at constant room temperature (21 ± 1°C) and relative humidity (50 %). They were allowed food and water ad libitum.

Uptake and Release by Brain Synaptosomes in vitro. Purified synaptosomes were obtained from whole brain tissue (Gray and Whittaker, 1962) and diluted with Krebs-Henseleit buffer to obtain a final protein concentration of 0.5–1 mg/ml. For uptake studies, 0.6 ml samples were incubated for 5 min at 30°C (or 0°C to assess passive diffusion) with 0.1 μM ¹⁴C-5-HT (58 μCi/mmol, Radiochemical Centre, Amersham) and drugs were added during a 5-min preincubation period. The reaction was stopped by cooling the tubes in ice and adding 0.5 ml ice-chilled Krebs-Henseleit buffer. Samples were then filtered through Millipore filters (0.65 μm pore size), washed with Krebs-Henseleit buffer, dissolved in Bray’s solution, and counted for radioactivity.

Release was studied using a superfusion system (Raiteri et al., 1974) to minimize interference from the reuptake mechanism. Portions of 1 ml synaptosome suspension, preincubated by incubation with ¹⁴C-5-HT (0.1 μM) for 15 min, were filtered and placed at the bottom of the superfusion chambers. Drugs were dissolved in Krebs-Henseleit solution and superfused from 0–20 min at a constant rate of 0.5 ml/min. The effluent and the filter were counted for radioactivity in a Packard Tri-Carb 2425 liquid scintillation spectrometer. The counting efficiency for ¹⁴C was checked for each sample using external standardization and averaged 85 % for unquenched samples. More detailed information about the methodology of the monoamine uptake and release study is given elsewhere (Mennini et al., 1978).

Binding of ³H-5-HT in vitro. Binding of ³H-5-HT was studied in rat brain cortical membrane preparations as described by Bennett and Snyder (1976). Proteins were adjusted to 1 mg/ml using a Bio Rad protein assay. The stock membrane preparation was maintained at −20°C until assay. After brief homogenization in a glass-Teflon homogenizer, 2 ml portions were preincubated at 37°C for 10 min, with or without 10 μM unlabeled 5-HT to determine nonspecific binding. Then, 7 nM ³H-5-HT (12.4 Ci/mmol, Radiochemical Centre, Amersham) and the test drug were added simultaneously and the mixture was incubated for 15 min. After addition of 5 ml 0.05 M TRIS HCl buffer (pH 7.4), the samples were rapidly vacuum filtered through Whatman GF/B filters. Each filter was then rinsed with 5 ml TRIS buffer and put in counting vials containing 10 ml Hydroluma (Supelchem). The counting efficiency for ³H was 50 %.

Food Intake. The animals were caged singly and trained to take their daily food (Altromin MT pellets for rats) from 9 a.m. – 1 p.m. for 10 days. On the day of the experiment, the animals were injected IP with various doses of quipazine maleate (Miles, Elkart, Indiana, U.S.A.), mCPP HCl (EGA-Chemie, W-Germany), LM 5008 HCl (Pharmuka, Gennelwiil, France) or an equal volume of saline. Immediately after injection the animals were placed in a cage containing a weighed amount of food, and 1 h later the remaining food was weighed to the nearest 0.1 g. The difference constituted the intake. For each drug, the dose inducing a 50% reduction of food intake (ED₅₀) was calculated using the following equation which represents the straight line obtained by the least squares method, fitting the percentage reduction of food intake against the dose: X = Y(50) - a/b, where Y(50) is the percent reduction of food intake, a is the intercept, and b the slope of the fitted straight line.

Effect of Drugs on 5-HT Depletion Induced by Fenfluramine in Whole Brain and Various Brain Areas in vivo. Various studies indicate that the ability of drugs to prevent the depletion of brain 5-HT induced by fenfluramine correlate fairly well with their potency in inhibiting 5-HT uptake (Ghezzi et al., 1973; Samanin et al., 1977b). The animals were given (IP) various doses of quipazine maleate, mCPP, or LM 5008 and 30 min later they received 15 mg/kg IP of fenfluramine HCl and 2 h later they were killed. The brains were quickly removed for fluorometric assay of 5-HT according to the method of Curzon and Green (1970). The ED₅₀ (dose inducing a 50% reversal of fenfluramine-induced depletion of 5-HT) was calculated as described for food intake studies. In one experiment, before fenfluramine was administered, the animals were given two maximal anorectic doses of mCPP and quipazine (2.5 and 5 mg/kg IP, respectively) and one dose of LM 5008 which caused no anorexia (10 mg/kg IP). In these animals the brains were dissected into the following samples by the procedure described by Glowinski and Iversen (1966): Hypothalamus; striatum; hippocampus; cortex (remaining telencephalon excluding striatum and hippocampus); and brainstem (excluding hypothalamus). These were assayed for 5-HT.

Determination of Drug Levels in vivo. The animals were injected IP with 2.5 mg/kg mCPP, 5 mg/kg quipazine, or 10 mg/kg LM 5008 and were killed 0.5, 1, and 2 h after drug administration. Brains were immediately removed and dissected as described above. The samples were stored at −20°C until assay. To 0.5–1 ml plasma, 2–1.5 ml 0.5 M phosphate buffer (pH 9.5) was added and the samples were extracted twice for 15 min with 5 ml benzene. After centrifugation the benzene extracts were combined and evaporated to dryness, 0.5 ml benzene, 50 μl 0.05 M trimethylamine, and 100 μl pentafluoropropionyl anhydride were added and the samples were heated (50°C) for 60 min. After the reaction, the samples were shaken with water and 5% aqueous ammonia solution, centrifuged, and 1 μl of the benzene phase was injected into a C. Erba Fractovap 2150 gas chromatograph with a 0.25 mm electron capture detector. The column was 2 m glass tube (4 mm inside diameter) packed with 3% OV17 on Gas Chrom P. The column temperature was 240°C for 1.0 min and quipazine and 200°C for mCPP assay. Under these experimental conditions, the minimum detectable amounts were 0.1 ng per injection; the linearity of the methods was 0.1–1 ng per injection. The recovery from biological samples was 85 ± 5 % for LM 5008, 82 ± 3 % for quipazine, and 91 ± 3 % for mCPP. Brains were homogenized (6 ml/g) in a cold mixture of acetone and 1 N formic acid (85:15, v/v). After centrifugation for 15 min at 4°C, the supernatant was mechanically shaken twice with heptane-chloroform (4:1). The organic phase was discarded and the aqueous phase used for extraction as described above.

Drugs. Drugs were as follows: 4-(3-Indolyloxy-2-ethyl) piperidine (LM 5008; Pharmuka, France); 2-(4-piperazinyl) quinoline maleate (quipazine; Miles, U.S.A.); and m-chlorophenylpiperazine (mCPP; Ega-Chemie, W. Germany).