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Effect of Mefenorex on 5-HT Release: Studies In Vitro on Rat Hypothalamic Slices and In Vivo by Microdialysis

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OROSCO, M., C. MORET, M. BRILEY AND S. NICOLAIDIS. *Effect of mefenorex on 5-HT release: Studies in vitro on rat hypothalamic slices and in vivo by microdialysis.* PHARMACOL BIOCHEM BEHAV **50**(4) 485-490, 1995. – Mefenorex, used for 20 years as an anorexic drug, has not been studied so far with regard to its central mechanism of action, although its chemical structure suggests a serotonergic mechanism. In the present study, the effect of mefenorex on serotonin (5-HT) release was investigated both in vitro, on rat hypothalamic slices and in vivo, using microdialysis in the paraventricular (PVN)-ventromedian (VMH) hypothalamic area while mefenorex was applied locally by means of counterdialysis. In vitro, mefenorex increased the spontaneous release of ^{3}H 5-HT from hypothalamic slices but not the electrically evoked release. This suggests a 5-HT releasing action of mefenorex not mediated through the terminal autoreceptor. The in vivo study confirmed the enhanced release and provided additional information. The delayed and modest increase of the 5-HT intracellular metabolite 5-HIAA may be indicative of an inhibition of reuptake. The dopaminergic system was also, but more modestly, activated by mefenorex. The increase in 5-HT release together with the inhibition of its reuptake may represent the main mechanism of action of mefenorex, and the secondary activation of the dopaminergic system may contribute in its anorexigenic effect at the level of the PVN-VMH area.

Mefenorex Anorexigenic drug Serotonin Hypothalamus

MEFENOREX was described as an appetite regulator more than 20 years ago (22) and since then, is being used as an anorexigenic drug. According to its chemical structure, it was first classified as an amphetamine-related compound (12). However, the behavioral profile of mefenorex, especially its lack of potency to substitute for amphetamine in discriminative responses, suggested marked differences with amphetamine (6,7). At the time of the first behavioral studies, monoaminergic mechanisms were not systematically investigated, and if they were, relevant techniques were not always used (21). Today it appears necessary to better understand the monoaminergic mechanism of this therapeutically used anorexigenic agent.

Although little is known about the action of mefenorex on monoaminergic activity, its structural analogy to other anorexigenic agents suggests a serotonergic mechanism of action. Anorexigenic agents have been shown to facilitate 5-HT release while blocking its reuptake by a nontetrodotoxin sensitive mechanism as it was shown in in vivo (2,5) or in vitro using rat brain slices (8), rat brain synaptosomes (14), or rat platelets (9). Serotonin neurotransmission itself is modulated in part by terminal autoreceptors (10,15) that can be blocked by the autoreceptor antagonist methiothepin. As a result, in slice preparations, methiothepin increases the release of 5-hydroxytryptamine (5-HT) both in vitro (16) and in vivo (4).

In the present investigation, the effect of mefenorex was assessed first on hypothalamic slices. This approach allows showing whether mefenorex may affect both the basal outflow of 5-HT and the terminal autoreceptor. The investigations on brain slices have been completed using an in vivo preparation assessed by the microdialysis technique. Freely moving rats had the microdialysis probes aimed at the feeding-related nuclei, paraventricular (PVN), and ventromedian (VMH), and the monoaminergic function of these regions was assessed

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both in basal conditions and following the application of mefenorex by means of the counterdialysis technique.

METHOD

Male rats (Iffa Credo), weighing 200–250 g, housed under a 12 D : 12 L cycle were used in both in vitro and in vivo experiments.

In Vitro Study

Animals were sacrificed between 1000 and 1100 h, and brains immediately removed and dissected. Slices (0.4 mm thick) from hypothalamus were incubated for 30 min at 37°C in Krebs solution containing 0.1 μmol/l ^{3}H-5-HT creatinine sulphate (Amersham, France; specific activity 10–20 Ci/mmol) and equilibrated with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution was the following (millimolar concentrations): NaCl (118.0), KCl (4.7), $CaCl_2$ (1.3), $MgCl_2$ (1.2), NaH_2PO_4 (1.0), $NaHCO_3$ (25.0), glucose (11.1), disodium ethylenediamine tetraacetic acid (EDTA) (0.004), and ascorbic acid (0.11). At the end of the incubation period, single slices were transferred to glass chambers and superfused with Krebs solution, which was continuously equilibrated with O_2 (95%) plus CO_2 (5%). The temperature of the superfusate was 37°C, and the rate of superfusion was 0.5 ml/min. At 2 h 20 min (S1) and 3 h 04 min (S2) after the onset of superfusion, slices were stimulated for 2 min by an electrical field generated in the chamber between two platinum electrodes (3 Hz, rectangular pulses of 20 mA current strength and 2 ms duration). Collection of samples of the superfusate Krebs solution began 8 min before the first period of electrical stimulation. The samples were collected at 4-min intervals. At the end of the experiment, the slices were solubilized with 0.5 ml Soluene 100 (Packard) and the radioactivity in the superfusate samples and the slices was determined by liquid scintillation spectrometry (Packard Tricarb 2500 TR). The first stimulation period (S1) was used as a control and mefenorex was added to the superfusion medium 20 min before the second stimulation period (S2) and remained present throughout the rest of the experiment. The amount of tritium released per 4-min sample was expressed as a fraction of the total tritium content of slices at the onset of the respective collection period. The overflow of tritium, induced by electrical stimulation, was calculated as the total increase of radioactivity above the resting outflow obtained in the sample immediately preceding the onset of stimulation. The spontaneous outflow of radioactivity obtained during the 4 min preceding the first period of stimulation and the corresponding fraction of radioactivity released before the second period were Sp1 and Sp2, respectively. To quantify the changes of stimulated tritium overflow induced by mefenorex, the ratio S2/S1 was calculated indicating the ratio of fractional release between the second and the first period of electrical stimulation. In the same manner, the ratio Sp2/Sp1 concerning basal release was calculated. The amount of [^{3}H]5-HT in each slice of hypothalamus at the end of the experiment was also determined.

In the control experiments, the schedule was exactly the same except for mefenorex being replaced by its vehicle.

Statistical evaluations were performed by using the analysis of variance followed by Bonferroni's test for comparisons with control groups.

In Vivo Study

Animals were housed individually in cylindrical Plexiglas cages designed to allow microdialysis and stressless administrations in unrestrained animals. The temperature of the room was maintained at 24 ± 1°C, and the lights were turned on from 0600 to 1800 h. Food and water were available ad lib except during the experimental period.

Rats were anesthetized with pentobarbital (Sanofi, 50 mg/kg) after pretreatment by a muscle relaxant, xylasine (Rompun®, Bayer). The animal was placed in a stereotaxic frame (Kopf Instruments). A guide cannula (Carnegie) for the microdialysis probe was aimed at the space between the paraventricular nucleus (PVN) and the ventromedian hypothalamus (VMH). The coordinates of the guide tip were [according to the atlas of Paxinos and Watson (19)] − 1.9 mm anterior, 0.5 mm lateral to and 7 mm ventral to bregma. The dialysis probe protruded 2 mm beyond the guide tube, reaching a point 9 mm ventral to bregma. The guide cannula and a securing device (screw) were fixed to the skull with stainless steel screws and dental cement. At least 1 week was allowed for postoperative recovery before the experiments began. During this period, the animals were accustomed to the experimental conditions in their home cages. They were permanently connected to the dialysis system of catheters to be used in the experiment, protected by a metal sheath and kept out of the animals' reach by means of a counterbalanced beam. This arrangement allowed normal movements. Soon after the operation, the ani-

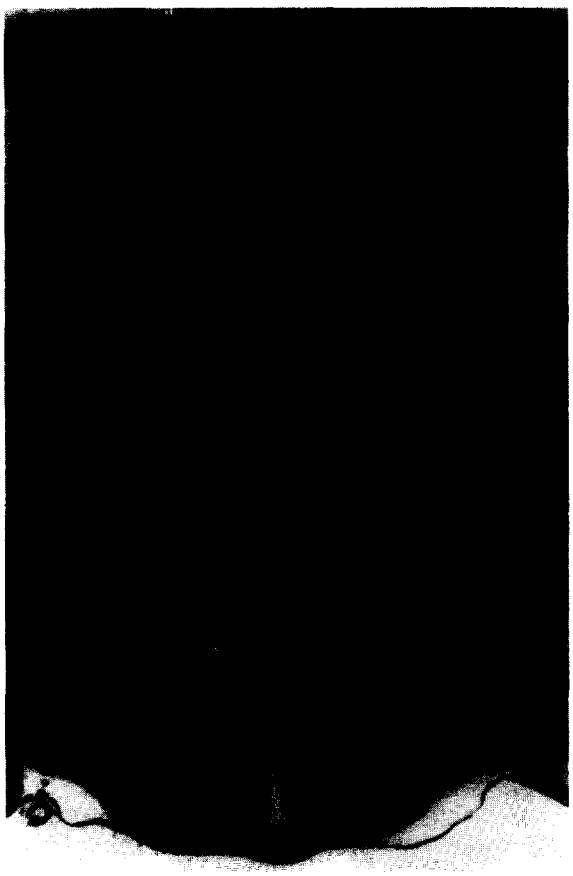
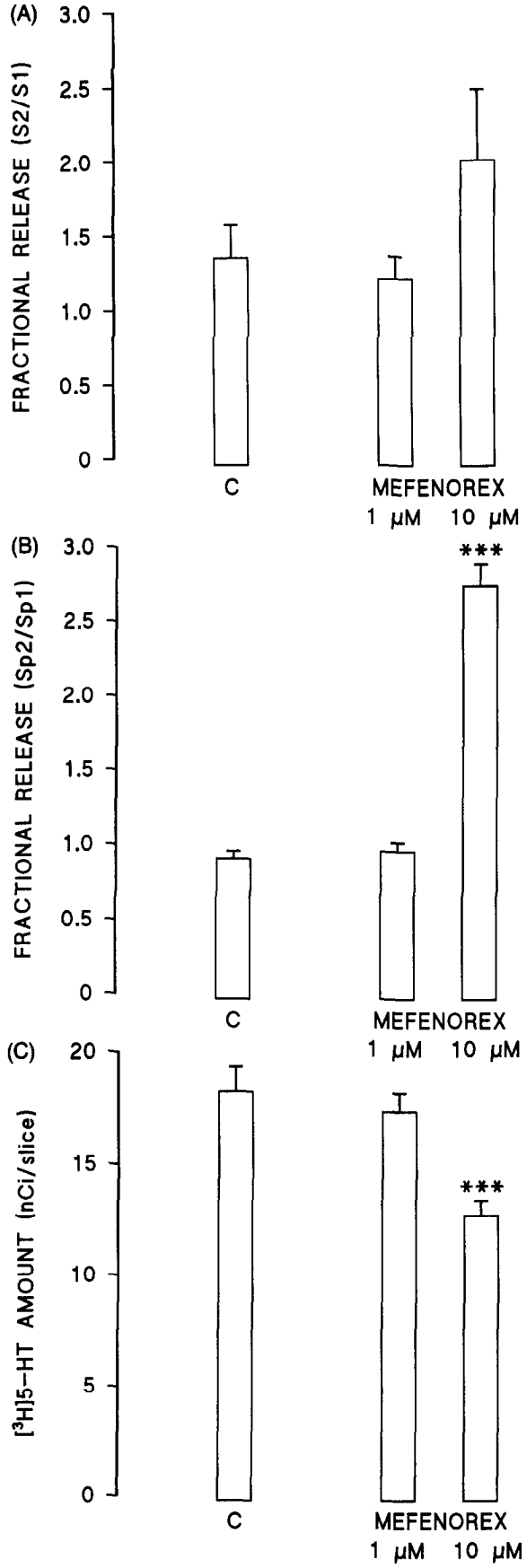


FIG. 1. Coronal section at the level of anterior hypothalamus depicting the location of the microdialysis probe between the paraventricular nucleus (PVN) and the ventromedian hypothalamus (VMH).



mals showed normal sleep (shown in parallel experiments), feeding, and body weight gain (17). All experiments were performed in animals' own home cages.

The microdialysis probes (Carnegie) were 2 mm long (dialysis section), with a diameter of 0.5 mm and a 20,000 molecular weight cutoff. According to our in vitro calibration test, the recovery was around 10% for both monoamines and their metabolites.

The perfusion was performed with a Ringer type solution containing 147 mM Na^+, 2.3 mM Ca^{++}, 4 mM K^+, and 155.6 mM Cl^-. A system of catheters connected to a two-way swivel placed on the beam allowed perfusion of fluid into the probe and collection of the perfusates. The flow rate (2 μl/min) allowed the collection of 30 μl samples every 15 min into microvials contained in a refrigerated microcollector (Carnegie) controlled by a computer.

Because the experiment was conducted in the home cage where the tubes were permanently connected to the rat, the only change on the day of experiment was the insertion at 0900 h of the microdialysis probe through the guide. The first samples were taken 3 h after insertion of the probe. After collecting four baseline dialysis samples, the perfusion fluid was switched to one of the same composition containing 4 mM mefenorex, so that 80 μmol were locally perfused over a 10-min period. Food was removed during the experiment in order not to interfere with the effect of spontaneous feeding (18).

The dialysates were analyzed by means of reverse-phase liquid chromatography with electrochemical detection (Albedo 100®, Europhor) at a potential of 750 mV. The chromatographic system consisted of a 20 μl sample loop leading to a 10 cm column (Europhor) with a 3.2 mm internal diameter and 3 μ C-18 packing. The mobile phase consisted of an acetate buffer containing 100 μM EDTA, 1 mM octanesulfonic acid, and 6% v/v acetonitrile at pH 3.1. The compounds analyzed were noradrenaline (NA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), and 5-hydroxyindolacetic acid (5-HIAA).

At the end of each experiment, the animals were given a lethal dose of pentobarbital (250 mg/kg) and cardiac perfusion of saline and then 10% formol was performed. The brain was removed, further hardened in 10% formol, and then serial coronal sections were cut (60 μm). Following glycerin staining, the sections were observed at low magnification by an observer unaware of the data obtained during the microdialysis experiments to check for the position of the dialysis probe (Fig. 1). The animals whose brain did not show a correct position of the guide and the probe were discarded by the independent observer.

FIG. 2. Effect of mefenorex on [^{3}H]5-HT release elicited by electrical stimulation (A), spontaneous outflow of [^{3}H]5-HT (B), and content of [^{3}H]5-HT in slices of rat hypothalamus. (A) Ordinate: fraction of the total tissue [^{3}H]5-HT released by a 2-min period of electrical stimulation (3 Hz, 20 mA, 2 ms) expressed as the ratio (S2/S1) obtained between the second period of stimulation carried out in the presence of the drug (S2) and the first period (S1). (B) Ordinate: ratio between the spontaneous outflow of [^{3}H]5-HT obtained during the 4 min preceding the second stimulation (Sp2) and the corresponding fraction of [^{3}H]5-HT released before the first period (Sp1). (C) Ordinate: [^{3}H]5-HT accumulation in slices expressed in Ci/slice determined at the end of the experiment. Mefenorex in the concentrations indicated was added to the medium 20 min before S2. Shown are mean values ± SEM of 9–10 experiments per group. ***$p < 0.001$ when compared to the corresponding control value (C).

As is usual in experiments of this type, the percentage of variation relative to the mean of the four baseline samples was calculated for each animal for NA, DA, DOPAC, 5-HT, and 5-HIAA. The results were then expressed as means of percentage variations ± SEM, and the statistical significance was calculated by a paired Student's *t*-test, each animal being its own control.

RESULTS

In Vitro Study

In control experiments, the overflow of tritium induced by the first period of electrical stimulation (S1) and by the second one (S2) represented 2.13 ± 0.19% (n = 10) and 2.89 ± 0.58% (n = 10) of the total tissue radioactivity, respectively. They did not differ significantly. The basal release (Sp1 and Sp2) taken as the fraction collected just prior to the onset of each stimulation was 1.63 ± 0.06 (n = 10) and 1.48 ± 0.12 (n = 10) of the total tissue radioactivity, respectively. They did not differ significantly.

Mefenorex, added before the second period of electrical stimulation, had the tendency to increase the release of [^{3}H]5-HT evoked electrically (Fig. 2A) at 10 μM; however, this effect did not reach significance. At this concentration, mefenorex increased the spontaneous outflow of [^{3}H]5-HT significantly in comparison with controls (Fig. 2B).

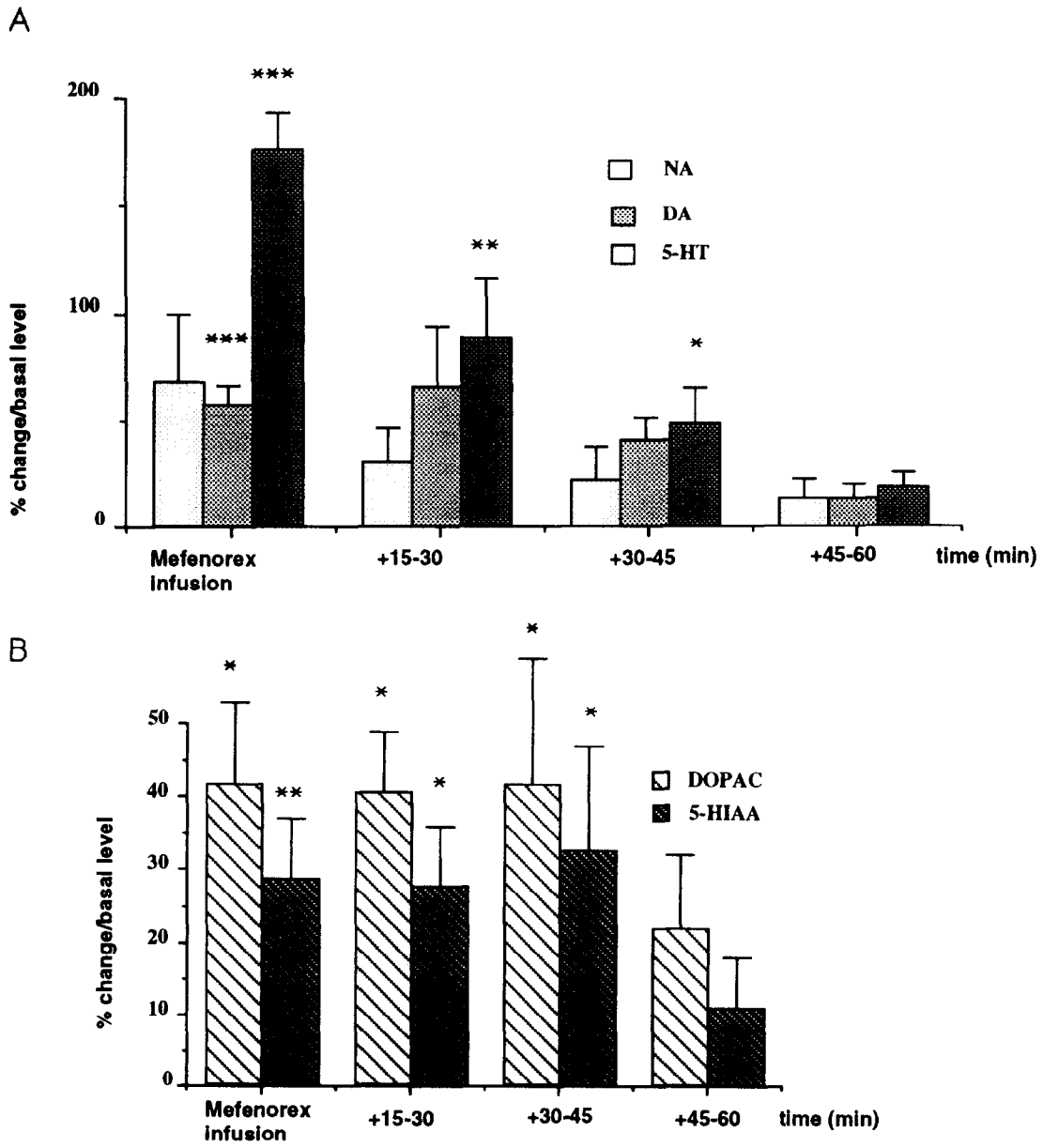


FIG. 3. Changes in the monoamines, NA, DA, and 5-HT (A) and in the metabolites, DOPAC and 5-HIAA (B) in 15 min dialysates samples, during and after Mefenorex infusion (20 μg) through the perfusion fluid. Results are expressed as percent variations of the mean baseline samples preceding the infusion ± SEM, n = 8. *p < 0.05, **p < 0.01, ***p < 0.001.

The [^{3}H]5-HT tissue content at the end of the experiment was significantly decreased in hypothalamic slices superfused with 10 μM mefenorex (Fig. 2C) with respect to control tissue.

In Vivo Study

During the sample corresponding to mefenorex infusion, 5-HT abruptly increased (+178%, $p < 0.001$), remained high during the following 15-min period (+90%, $p < 0.01$), started to decrease during the 30–45 min period, and returned to the preinfusion level 45 min later (Fig. 3A). Its metabolite 5-HIAA showed a lesser increase (+29%, $p < 0.01$) that started from the infusion sample and lasted until the 45–60-min period, i.e., a little longer than the 5-HT changes (Fig. 3B).

NA also increased during mefenorex infusion (+68%), but to a lesser extent than 5-HT before regularly decreasing towards basal levels 60 min later. Despite the magnitude of this increase, it did not reach statistical significance, due to individual variability (Fig. 3A).

Mefenorex significantly increased DA (+58%, $p < 0.001$ during the infusion sample). This increase lasted 45 min before returning to basal levels (Fig. 3A). The DA metabolite, DOPAC, showed the same profile of variation as did the corresponding 5-HT metabolite, 5-HIAA, in terms of duration with a somewhat higher magnitude (+42%) (Fig. 3B).

DISCUSSION

Both the in vitro and in vivo experiments agree in showing that mefenorex induces the release of 5-HT like other anorexigenic agents do.

The 5-HT releasing capacity of mefenorex is supported by several observations. In the in vitro preparation, mefenorex does not change the evoked overflow of [^{3}H]5-HT but it does increase its basal release by 200% at 10 μM. Contrary to methiothepin, a nonselective 5-HT$_1$ receptor antagonist, which, at 10 μM, enhances both the release of [^{3}H]5-HT induced by electrical stimulation and the basal one (16), the action of mefenorex on 5-HT release appears not to be mediated through the terminal autoreceptor. The reduced amount of [^{3}H]5-HT remaining in the tissue at the end of the experiment is consistent with increased spontaneous outflow of [^{3}H]5-HT in the presence of mefenorex.

The in vivo experiment also shows a clearcut increase in 5-HT in the VMH-PVN region, an area that promotes satiety, in response to the local infusion of mefenorex applied by means of counterdialysis. This technique has previously been used in similar investigations on other 5-HT-releasing anorexigenic drugs (3,20).

The in vivo procedure provides additional information on the effects of mefenorex because the same samples allow us to measure the 5-HT metabolite, 5-HIAA, and also the catecholamines together with their metabolite DOPAC. It was shown that, in microdialysis conditions, a delayed and smaller increase in 5-HIAA is a characteristic feature of an enhanced 5-HT release (11,18).

In the PVN-VMH region, mefenorex appears to also activate the dopaminergic activity, although to a lesser degree. Dopaminergic activation in the hypothalamus was shown to promote satiety (13). However, in contrast to the effect of amphetamine, mefenorex does not exert a significant releasing effect on NA. In addition, it must be noticed that the ratio DOPAC over DA is higher than the ratio 5-HIAA over 5-HT. Because both metabolites are produced intracellularly, the ratio of each metabolite over its respective monoamine is indicative of the rate of its reuptake. This may suggest that mefenorex brings about more reuptake inhibition for 5-HT than for DA; this second mechanism may add some specificity to the serotonergic mechanism of mefenorex and account for its inhibitory effect on feeding. However, this reasoning is based mainly on experiments on brain homogenates that do not necessarily apply to findings from microdialysis studies (1).

In conclusion, the present data indicate that the anorexigenic effect of mefenorex may result from its 5-HT-releasing and perhaps 5-HT reuptake inhibiting action at the level of the hypothalamic satiety promoting centers. The modest dopaminergic activation induced by mefenorex may also contribute in promoting an additional and perhaps synergistic mechanism of satiety at the level of the VMH-PVN area.

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