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Involvement of Endogenous Opioid Peptides in Fenfluramine Anorexia

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MAJEED, N. H., W. LASOŃ, B. PRZEWŁOCKA AND R. PRZEWŁOCKI. *Involvement of endogenous opioid peptides in fenfluramine anorexia.* PHARMACOL BIOCHEM BEHAV **25**(5) 967–972, 1986.—The effect of chronic fenfluramine (20 mg/kg, once daily) injections on the brain and peripheral immunoreactive (ir) dynorphin (DYN), alpha-neoendorphin (ANEO) and beta-endorphin (BE) was studied in rats. Fenfluramine injected repeatedly for 5 and 9 days induced anorexia. In the same animals there were no significant changes in the ir-DYN and ir-ANEO contents in the brain and pituitary. However, the ir-DYN and ir-ANEO contents in the gastrointestinal tract (duodenum) were markedly decreased after 5 and 9 days of fenfluramine injection. In contrast to ir-DYN and ir-ANEO, there was an increase in the hypothalamic and a decrease in the anterior lobe of pituitary ir-BE content. There was no significant change in the neurointermediate (NI) lobe of the pituitary. The results of our study suggest that part of the fenfluramine anorexia may be mediated by the peripheral prodynorphin and central beta-endorphin systems.

Fenfluramine Opioid peptides Anorexia Hypothalamus Pituitary Gastrointestinal tract

REPEATED administration of fenfluramine leads to progressive changes in its effectiveness, in reducing food intake, both in animals and man [11, 24, 26]. Tolerance has been reported to develop towards the anorectic effect of fenfluramine. The mechanism by which fenfluramine induces anorexia is not well known, but many studies showed that the ability of the drug to release 5-HT is probably the most important neurochemical event related to this behavioural effect of fenfluramine [4, 8, 10, 35]. However, the mechanisms by which tolerance towards the fenfluramine anorexia develops still remain unclear.

An elevation in the ir-BE and ir-Met-enkephalin levels in the hypothalamus was reported in chronically fenfluramine-treated rats [6, 7, 16]. However, there are no data concerning the involvement of dynorphin/alpha-neoendorphin, the two endogenous opioid peptides which are derived from the same prodynorphin precursor [19] and are distributed in a parallel manner in the central and peripheral nervous systems in rats [29]. We found previously that acute fenfluramine injections in rats induced a dose-dependent increase in the brain beta-endorphin and a decrease in the gut (duodenum) dynorphin/alpha-neoendorphin levels [27]. A vast body of evidence suggests that opiates and endogenous opioid peptides may play an important role in the regulation of appetite [15, 28, 30, 32]; moreover, since opiates clearly modify the serotonergic mechanisms [31], a question arises whether endogenous opioid peptides may participate in the fenfluramine-induced anorexia.

METHOD

Male Wistar rats weighing 200–220 g were housed under a 12:12 light:dark cycle with the dark at 6 p.m. and kept on a feeding schedule which allowed free access to the usual dry-pellet diet for 4 hours a day (from 10 a.m. to 2 p.m.). The body weight and food intake were measured daily, and the animals were then divided into six groups, each consisting of at least 10 animals. Three groups of rats were treated with 0.9% NaCl for 5, 9 and 14 days, and the other three groups were injected intraperitoneally (IP) with fenfluramine (Les Laboratories Servier France), 20 mg/kg, for 5, 9 and 14 days. After the last injection of either saline or fenfluramine, the food intake (in grams) was measured for 2 hours and the same animals were killed by decapitation for biochemical assays. The hypothalamus, pituitary and duodenum were rapidly removed, weighed and then incubated in 0.1 N HCl at 95°C for 10 min. After homogenization the homogenates were centrifuged at 10.000 g for 15 min and the supernatants were adjusted to pH 7.5. After further centrifugation the aliquots of the supernatants were appropriately diluted and assayed for immunoreactive beta-endorphin, dynorphin and alpha-neoendorphin.

Iodination Procedure

The peptides were iodinated with Na ^{125}I using the chloramine-T method. Five μg of the synthetic peptide were diluted in 50 μl of 0.5 M phosphate buffer, pH 7.4, and were

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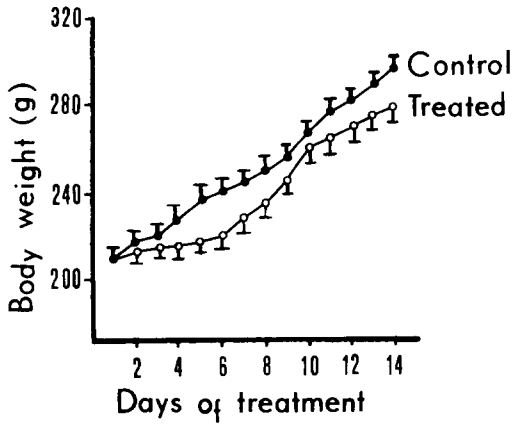

FIG. 1. Effect of chronic fenfluramine (20 mg/kg) injections on the increase in body weight in the rat. Significant differences between the control and fenfluramine-treated rats are evident on day 4 onwards.

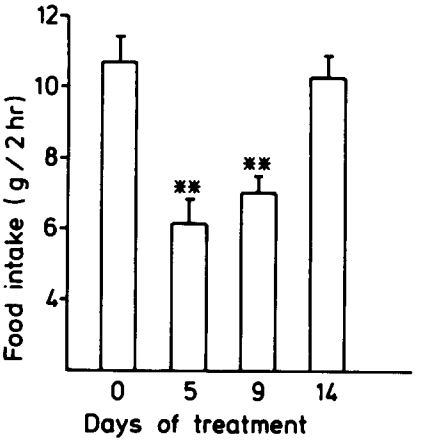

FIG. 2. Effect of repeated fenfluramine (20 mg/kg) injections on the food intake (expressed as grams per a 2-hour period) in the rat. Values represent means±SEM of at least 10 animals per group. $^{**}p<0.01$, Duncan test.

TABLE 1

THE EFFECT OF CHRONIC FENFLURAMINE (20 mg/kg) INJECTIONS ON THE IMMUNOREACTIVE DYNORPHIN AND ALPHA-NEOENDORPHIN CONTENTS IN THE HYPOTHALAMUS, NEUROINTERMEDIATE (NI) AND ANTERIOR LOBES OF THE PITUITARY IN THE RAT

Treatment (days)	Hypothalamus (pmoles/g)	NI-pituitary (pmoles/g)	Anterior pituitary (pmoles/g)
		ir-dynorphin	
0	34.6 ± 2.5	1332.1 ± 114.5	97.6 ± 9.3
5	35.9 ± 1.8	1201.9 ± 139.0	83.0 ± 5.5
9	32.0 ± 4.3	1467.0 ± 100.0	89.8 ± 9.8
14	36.3 ± 3.0	1450.0 ± 108.0	91.7 ± 6.3
		ir-alpha-neoendorphin	
0	91.2 ± 4.4	1967.0 ± 138.0	112.7 ± 5.3
5	96.3 ± 7.6	1796.0 ± 126.0	97.8 ± 6.2
9	92.9 ± 4.1	2121.0 ± 107.0	109.5 ± 8.1
14	95.7 ± 3.5	2052.0 ± 117.0	106.8 ± 5.8

Values represent means ± SEM of at least 8 animals per group.

put into a conical vial which contained one millicurie of Na ^{125}I. Then 10 μl of 0.2% chloramine-T solution in 0.05 M phosphate buffer, pH 7.4, were poured into the vial. The mixture was gently shaken for 30 sec and the reaction was stopped with 10 μl of a 0.2% sodium metabisulfite solution.

The labeled peptide was purified by gel filtration on a column (0.9×20 cm) of Bio Gel P4, 200–400 mesh. The peptide was eluted with acetic acid containing 0.1% bovine serum albumin to prevent peptide adsorption. The range of a specific activity of the iodinated peptide was 300–600 Ci/mmole.

Generation of Antibodies

Antibodies against alpha-neoendorphin ("Agathe"), dynorphin ("Goldy") and beta-endorphin ("DA") were developed by dissolving 4.5 mg of peptide and 25.0 mg of thyroglobulin in 4 ml of distilled water, and cooled at 0°C. Two and a half mg of carbodiimide, dissolved in 0.5 ml of water, were added to the above mixture and stirred overnight at 0°C. After an exhaustive dialysis against 0.9% NaCl the conjugated peptide was lyophilized. The conjugated peptide was emulsified with Freund's complete adjuvant and injected intradermally at multiple sites on the back of two male New Zealand white rabbits. After an initial dose of 1.5 mg the rabbits were booster-injected with 0.5 mg immunogen each at 3-week intervals. Fourteen days after the last injection the animals were test bled, and the antiserum of the animal which proved to have the highest titer and sensitivity was used for a radioimmunoassay determination of the peptide.

Radioimmunoassay (RIA) Procedures

The dynorphin RIA was performed with the antiserum

TABLE 2

THE EFFECT OF CHRONIC FENFLURAMINE (20 mg/kg) INJECTIONS ON THE IMMUNOREACTIVE BETA-ENDORPHIN CONTENT IN THE HYPOTHALAMUS, NEUROINTERMEDIATE (NI) AND ANTERIOR LOBES OF THE PITUITARY IN THE RAT

Treatment (days)	Hypothalamus (pmoles/g)	NI-pituitary (nmoles/g)	Anterior pituitary (nmoles/g)
0	19.4 ± 1.8	385.7 ± 50.0	21.2 ± 1.45
5	26.7 ± 2.0*	391.1 ± 62.0	16.1 ± 0.4*
9	18.4 ± 1.4	365.5 ± 58.0	19.6 ± 1.9
14	19.6 ± 1.6	371.8 ± 49.5	20.8 ± 1.7

Values represent means ± SEM of at least 8 animals per group.
*$p<0.05$, Duncan test.

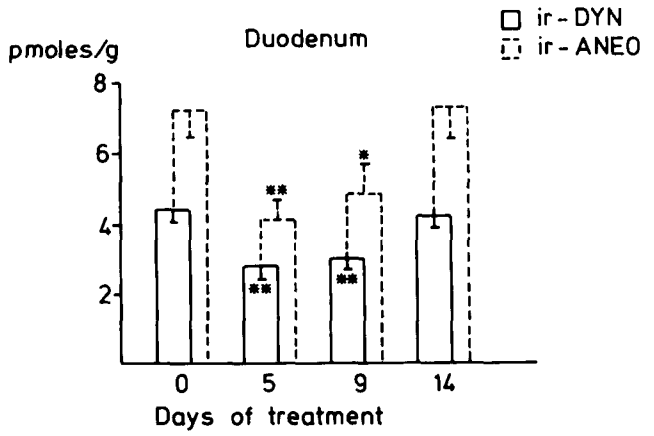

FIG. 3. Effect of repeated fenfluramine (20 mg/kg) injections on the ir-DYN and ir-ANEO contents in the rat duodenum. Values represent means±SEM of at least 8 animals per group. *$p<0.05$, **$p<0.025$, Duncan test.

directed against the synthetic porcine dynorphin A_{1-13}, according to the procedure described previously [18]. Dynorphin A exhibited the same cross-reactivity as dynorphin A_{1-13}. The antiserum exhibited a negligible avidity for dynorphin A_{1-8}, alpha-neoendorphin, β-neoendorphin, Leu-enkephalin and beta-endorphin.

The alpha-neoendorphin RIA was performed with the antiserum directed against synthetic alpha-neoendorphin, as described previously [29]. The antiserum recognized alpha-neoendorphin and β-neoendorphin with a high avidity, but did not cross-react with dynorphin A, Met-enkephalin or Leu-enkephalin and beta-endorphin.

The beta-endorphin RIA was performed as described previously [17]. The antiserum recognized beta-endorphin but did not cross-react with Leu-enkephalin, dynorphin A or alpha-neoendorphin. Assays were carried out in Eppendorf tubes (1.5 ml); the incubation mixture consisted of 50 μl of the neutralized tissue extract (pH 7.4) or standard solution, 100 μl of the antiserum against alpha-neoendorphin (final dilution 1:15000), dynorphin (final dilution 1:50000) and beta-endorphin (final dilution 1:100000), 50 μl of the ^{125}I-labeled ligand, all of them diluted to a final volume of 0.5 ml of "buffer D." The mixture was equilibrated at 4°C for 20 hours for alpha-neoendorphin and dynorphin, and for 48 hours for beta-endorphin; afterwards antibody-bound peptides were separated from the unbound peptides by adding 500 μl of a mixture containing 1% charcoal and 0.5% bovine serum albumin in the RIA buffer. After vortexing for 5 sec the suspension was allowed to stand for 10 min and was centrifuged for 3 min at 3000 g. The radioactivity was measured in the supernatant.

The Gel filtration chromatography was performed with a sephadex G-50 superfine column (0.9×50 cm), eluted with 2 N of acetic acid containing 0.1% bovine serum albumin, at a flow rate of 4.8 ml/hr at 4°C. Fractions of 700 μl were collected, lypophilized, redissolved in 350 μl of RIA-buffer, and aliquots were assayed for ir-dynorphin. The recovery of the synthetic peptide from the column was 90%.

Statistical Analysis

The data were analyzed by the ANOVA and the significance is indicated by the Duncan test.

RESULTS

Since control groups treated with saline for 5, 9 and 14 days showed no significant differences, they were pooled as one control for the statistical analysis.

Figure 1 shows the body weights of rats after daily injections of saline or fenfluramine, 20 mg/kg, for 14 days. The body weights of rats receiving fenfluramine increased at the rate slower than those of the control animals. Figure 2 shows the effect of chronic fenfluramine, 20 mg/kg, injected for 5, 9 and 14 days, on the food intake (g/2 hr). There was a significant reduction in the food intake following fenfluramine injection for 5 and 9 days, $F(3,46)=12.5$, $p<0.01$; however, tolerance towards the anorectic effect of fenfluramine was observed 14 days after the drug injection, as the fenfluramine-treated rats consumed the same amount of food as the saline-treated ones.

In the same animals the levels of ir-DYN, ir-ANEO and ir-BE were differentially affected by chronic treatment. There were no significant changes in the brain and pituitary ir-DYN and ir-ANEO contents after 5, 9 and 14 days of fenfluramine treatment (Table 1). However, a time-dependent decrease in the duodenal content of ir-DYN and ir-ANEO, $F(3,47)=2.59$ and $F(3,41)=3.67$, $p<0.05$, respectively, was observed (Fig. 3). There was a significant increase in the hypothalamic and a decrease in the anterior lobe of the pituitary ir-BE content after 5 days of fenfluramine treatment, $F(3,50)=2.46$ and $F(3,50)=1.57$,

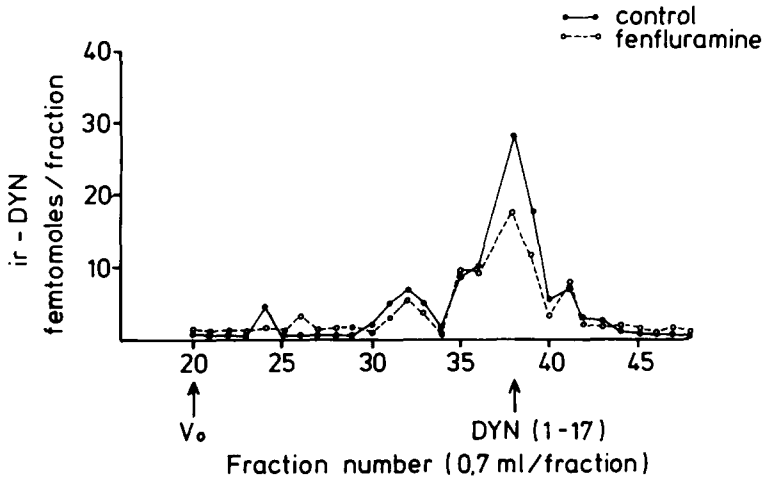

FIG. 4. Sephadex G-50 superfine column chromatography of a duodenal extract from the control and chronically fenfluramine-treated (20 mg/kg IP, for 5 days) rats. The dynorphin immunoreactivity in the assay was calculated in femtomoles per fraction.

$p<0.05$, respectively, while no significant change in the NI-pituitary was found (Table 2). In the animals tolerant towards the anorectic effect of fenfluramine the levels of those peptides were also normal in the brain, pituitary and gut.

The immunoreactive dynorphin measured by the RIA in the duodenum of saline- and fenfluramine-treated rats was further characterized using a sephadex G-50 superfine column. Fractions of the eluates were radioimmunoassayed with antisera directed towards dynorphin. There were several small immunoreactive peaks, but the dominant one had its elution volume similar to that of the synthetic dynorphin (1–17) (Fig. 4). In the duodenum of fenfluramine-treated rats immunoreactive peaks were smaller than those in saline-treated rats.

DISCUSSION

The present study indicates that anorexia induced by a chronic fenfluramine treatment is correlated with a decrease in the peripheral dynorphin/alpha-neoendorphin and, to a lesser extent, with an increase in central beta-endorphin levels.

We observed previously that an acute fenfluramine treatment induced a dose-dependent decrease in the duodenal ir-DYN content [27]. Since we found that there was a time-dependent decrease in the duodenal content of ir-DYN and ir-ANEO after a chronic fenfluramine treatment, it might be speculated that the decreased level of these peptides in the gastrointestinal tract indicates their enhanced release from the gut. Recently we found that fenfluramine enhanced the release of ir-DYN from the duodenum in vitro (unpublished data). It is well known that dynorphin is present in the periphery [37], and is the most potent opioid peptide found there in comparison with other opioids [14]. As opioid and serotonin interactions were reported to occur in the periphery [3, 13, 34], we assumed that at least part of the fenfluramine anorexia might be mediated by dynorphin and related peptides. This is most probably due to the stimulation of gastrointestinal opiate receptors [1], and hence results in delaying the gastric emptying [5, 9, 36] and inhibiting the upper gastrointestinal peristalsis [22,23]. These changes in the gastrointestinal physiology may mediate some satiety signal that link up the gut with the brain through the vagus and sympathetic nerves. Moreover, the effect of opioid peptides on some gut peptides, such as gastrin and CCK, which were involved in the peripheral control of the food intake should not be excluded. Since opioid peptides were reported to affect the release of the gut gastrin and CCK [20,21], fenfluramine enhanced the release of the duodenal dynorphin/alpha-neoendorphin which, in turn, could influence the release of CCK which is recognized peripheral affecting appetite [12].

In contrast to ir-DYN and ir-ANEO, there was a significant increase and a decrease in the ir-BE content in the hypothalamus and anterior pituitary, respectively, after 5 days of fenfluramine treatment. In accordance with our data, some other authors reported an elevation in the hypothalamic content of ir-BE and also ir-Met-enkephalin in rats treated chronically with fenfluramine [6, 7, 16]. Since we observed an increase in the hypothalamic and a decrease in the anterior pituitary ir-BE after both acute [27] and chronic (this study) fenfluramine treatment, it may be speculated that the increased and decreased hypothalamic and anterior pituitary ir-BE concentrations, respectively, may result from inhibition of the brain and stimulation of the anterior pituitary BE release.

A vast body of evidence indicates that serotonin plays an inhibitory role in the food intake [2, 25, 33]. Since endogenous opioid peptides enhance feeding when injected centrally [15,28], we assumed that part of the fenfluramine anorexia may be due to inhibition of the brain and stimulation of the pituitary BE release. If the release of an excessive amount of ir-BE from the anterior pituitary is involved in the chronic fenfluramine anorexia, a logical target of this effect is stimulation of opiate receptors in the gastrointestinal tract [1]. It is noteworthy that an intracerebroventricular injection of a methionine enkephalin analogue reverses the suppressive effect of the serotonin agonist quipazine on the tail pinch-induced feeding [33]. This indicates that endogenous opiates play some role in maintaining the feeding drive, and that serotonergic-opiate interactions on the satiety processes

should be considered. Tolerance towards the anorectic effect of fenfluramine was developed after 14 days of drug injection, as the fenfluramine-treated rats consumed the same amount of food in comparison with the saline-treated ones. In the same animals the levels of ir-DYN, ir-ANEO and ir-BE were back to normal, which testifies that these peptides may play some role in the chronic fenfluramine anorexia.

In conclusion, the present and our previous finding show that endogenous opioid peptides in the brain and periphery are differentially and independently modulated by acute and chronic fenfluramine treatment. Moreover, the part of anorexia induced by fenfluramine treatment may be mediated by activation of the peripheral (gut) prodynorphin and, to a lesser extent, by inhibition of the brain and stimulation of the pituitary beta-endorphin systems.

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