Pharmacology Biochemistry and Behavior, Vol. 65, No. 3, pp. 369–373, 2000

0091-3057/00/$–see front matter

PII S0091-3057(99)00219-1

Supraadditive Effect of *d*-Fenfluramine Plus Phentermine on Extracellular Acetylcholine in the Nucleus Accumbens: Possible Mechanism for Inhibition of Excessive Feeding and Drug Abuse

PEDRO V. RADA*† AND BARTLEY G. HOEBEL*

**Princeton University, Department of Psychology, Princeton, NJ 08544; and*
†University of Los Andes, Laboratory of Behavioral Physiology, Merida, Venezuela

Received 5 February 1999; Revised 29 June 1999; Accepted 27 July 1999

RADA, P. V. AND B. G. HOEBEL. *Supraadditive effect of* d-*fenfluramine plus phentermine on extracellular acetylcholine in the nucleus accumbens: Possible mechanism for inhibition of excessive feeding and drug abuse.* PHARMACOL BIOCHEM BEHAV **65**(3) 369–373, 2000.—The combination of *d*-fenfluramine plus phentermine (*d*-FEN/PHEN) provides a tool for exploring neural mechanisms that control food intake and drug abuse. Prior research suggests that dopamine (DA) in the nucleus accumbens can reinforce appetitive behavior and acetylcholine (ACh) inhibits it. When rats were given *d*-fenfluramine (5 mg/kg, IP) DA increased to 169% ($p < 0.01$), and ACh decreased slightly. Phentermine (5 mg/kg, IP) increased extracellular DA to 469% of baseline and ACh increased slightly to 124% (both $p < 0.01$). The *d*-FEN/PHEN combination, however, increased both DA and ACh with a supraadditive effect on ACh to 172%. One interpretation is that dFEN/PHEN increases DA like a meal or drug of abuse, while also increasing ACh to stop further approach behavior. This leaves the animal "satiated," as defined by reduced intake of food or drugs. © 2000 Elsevier Science Inc.

Fenfluramine Phentermine Acetylcholine Microdialysis Accumbens Feeding Drug abuse Appetite Craving

DRUG abuse and overeating bear interesting relationships in terms of the underlying reward mechanisms (13,16). This raises the possibility that appetite suppressants could be used to reduce drug intake. Clinical studies found that *d,l*-fenfluramine plus phentermine (*d,l*-FEN/PHEN) was effective in weight control (31). This combination also showed signs of reducing alcohol or cocaine intake (10,28). However a serious side effect (cardiac valvular disease) was discovered, and fenfluramine was taken off the market in many countries. Since then, *d*-FEN/PHEN has been found to reduce food intake (26) and self-administration of cocaine in animals (6,27). In such studies the combination of two drugs was more effective than either drug alone. The present study investigates the neural mechanism for this additive effect.

Microdialysis studies suggest that *d,l*-FEN releases serotonin (5-HT) and blocks its reuptake (2,14), whereas PHEN preferentially releases and blocks reuptake of dopamine (29). A mixture of *d,l*-FEN/PHEN increases both extracellular 5-HT and DA in the nucleus accumbens (NAc) as one would predict (1,29). However, it was not clear how 5-HT and DA in the reward system would suppress drug intake without being a drug of abuse. Some additional mediator seemed likely.

Recent evidence suggests a possible role for accumbens cholinergic interneurons in behavior inhibition (13). For ex-

Requests for reprints should be addressed to B. G. Hoebel, Department of Psychology, Princeton University, Princeton, NJ 08544.

ample, acetylcholine (ACh) significantly increased in the posterior medial NAc during naloxone-induced withdrawal from chronic morphine (23,25), and ACh was released during the presentation of the conditioned stimulus in a conditioned taste aversion (21). Moreover, a conditioned taste aversion was induced by increasing extracellular ACh in the accumbens (30). In the course of a meal, DA is released in the appetitive phase and often remains high, while ACh is released during the satiation phase (18). This led us to test the hypothesis that *d*-FEN-PHEN might elevate extracellular levels of ACh and thereby provide a logical basis for the inhibition of ingestive behavior and drug self-administration.

In the present experiments, microdialysis of the posterior medial NAc (shell subregion) coupled to high-performance liquid chromatography was used to monitor DA and ACh following an acute systemic injection of *d*-FEN alone, PHEN alone or a combination of *d*-FEN/PHEN.

METHOD

Subjects, Surgery, and Microdialysis

Male Sprague–Dawley rats, weighing between 350–400 g, were individually housed with food and water ad lib. Animals were anesthetized with ketamine (50 mg/kg IP) and xylazine (10 mg/kg Ip) while bilateral guide shafts were stereotaxically implanted at coordinates: AP: +1.2 mm, L: 1.2 mm, and V: 4.0 mm with reference to bregma, midsaggital sinus, and the level skull surface, respectively. Rats recovered for at least a week before microdialysis probes were inserted and cemented in place extending an additional 5 mm to reach the posterior medial NAc (procedures approved by Institutional Animal Care and Use Committee).

Microdialysis probes were made of fused silica capillary tubing and 26-gauge stainless steel tubing, with a tip of cellulose membrane 2 mm long (8,9,20). Probes were cemented in place at least 16 h before the experiments, and were perfused with a Ringer's solution (142 mM NaCl; 3.9 mM KCl; 1.2 mM $CaCl_2$; 1.0 mM $MgCl_2$; 1.35 mM Na_2HPO_4; 0.3 mM NaH_2PO_4, pH: 7.35) at a flow rate of 0.5 μl/min overnight and at 1 μl/min during the experiments. A low concentration of neostigmine (0.3 μM) was added to the Ringer's to inhibit ACh degradation.

Acetylcholine and Dopamine Assays

ACh was measured by reverse-phase, high-performance liquid chromatography with electrochemical detection (HPLC-EC) using an ESA model 580 pump and mobile phase of 200 mM potassium phosphate (pH: 8.0) at a flow rate of 0.6 ml/min. Dialysate was injected into a 20-μl sample loop leading to a 10-cm C18 analytical column to separate ACh, which was then converted to betaine and hydrogen peroxide by an immobilized enzyme reactor (acetylcholinesterase and choline oxidase from Sigma Chemical Co. and columns from Varian Inc.). Detection was accomplished with an amperometric detector (Model 400 EG&G Princeton Applied Research Co.) that oxidized the hydrogen peroxide on a platinum electrode (BAS Inc.) set at 0.5 V with respect to a Ag-AgCl reference electrode (EG&G Princeton Applied Research Co.).

Dialysates were analyzed for DA and its metabolites DOPAC and HVA by reverse phase HPLC-EC (Coulochem model 5100 A, ESA Co.). DA was separated on a phase II (Brownlee Co.) column with a 3.2-mm bore and 3-μm C18 packing. The mobile phase was 60 mM sodium phosphate monobasic, 100 μM EDTA, 1 mM heptanosulfonic acid, and 4.5% v/v methanol (pH 3.6) at a flow rate of 1 ml/min.

Experimental Procedure

Samples were taken every 20 min until a stable baseline was reached (at least three baseline samples). Rats were then injected IP with either saline, *d*-FEN alone (5 mg/kg; Servier Inc.), PHEN alone (5 mg/kg; Sigma Chem. Co.), or a mixture of *d*-FEN/PHEN (5 mg/kg each), and then six more postinjection samples were taken. In each of these four treatment categories there were six rats for DA and six separate rats for ACh.

Statistics and Histology

Data were converted to percent of the mean of the three baseline samples and analyzed by two-way ANOVA for repeated measures comparing the drug injection to control saline injection followed by Tukey's test for multiple comparisons. To verify probe location in the NAc, rats received an overdose of sodium pentobarbital and were perfused with 0.9% saline solution followed by formalin. Brains were removed, frozen and sectioned at 40 microns from the anterior pole caudally until probe tracks were identified. Only rats with probes in the shell region of the posterior medial NAc were included in the analysis.

RESULTS

d-Fenfluramine Alone Increased Accumbens DA, Not ACh

d-Fenfluramine alone increased extracellular DA to 169 ± 31% of basal levels 40 min after the injection. This was statistically significant when compared to saline injection, $F(1, 8) = 3.08$, $p < 0.01$ (Fig. 1). DA metabolites DOPAC and HVA did not change significantly (Fig. 1). ACh was not altered significantly by the IP injection of *d*-FEN showing only a nonsignificant decrease to 89 ± 6% of baseline 80 min after the injection (Fig. 2).

Phentermine Alone Greatly Increased Accumbens DA, With a Small Effect on ACh

Phentermine produced a much larger increase in DA levels to 469 ± 158% 20 min after the injection, $F(1, 8) = 4.24$, $p < 0.01$ (Fig. 1). This increase in DA was correlated with a significant decrease in DOPAC, $F(1, 8) = 9.9$, $p < 0.01$ (Fig. 1), and decrease in HVA, $F(1, 8) = 2.62$, $p < 0.05$ (Fig. 1). Phentermine alone produced a small increase in extracellular ACh to 124 ± 7% of baseline. This was significant relative to the IP saline control injection, $F(1, 8) = 2.6$, $p < 0.05$ (Fig. 2).

Combined d-Fenfluramine and Phentermine Increased Both Extracellular DA and ACh with Supraadditive Effect on ACh

Injection of the *d*-FEN/PHEN mixture caused a large increase in extracellular DA release in the NAc to 671 ± 179%. This increase was significant when compared to the saline injection, $F(1, 8) = 6.29$, $p < 0.01$ (Fig. 1) or *d*-FEN alone, $F(1, 8) = 4.64$, $p < 0.01$, but not when compared to PHEN alone. Both metabolites (DOPAC and HVA) decreased significantly to 59 ± 10% and 68 ± 7%, respectively (Fig. 1). *d*-FEN/PHEN increased ACh levels in the NAc to 172 ± 18%. This effect was supraadditive and significant when compared to saline, $F(1, 8) = 4.62$, $p < 0.01$ (Fig. 2), to PHEN alone, $F(1, 8) = 3.26$, $p < 0.01$, or to *d*-FEN alone, $F(1, 8) = 3.161$, $p < 0.01$.

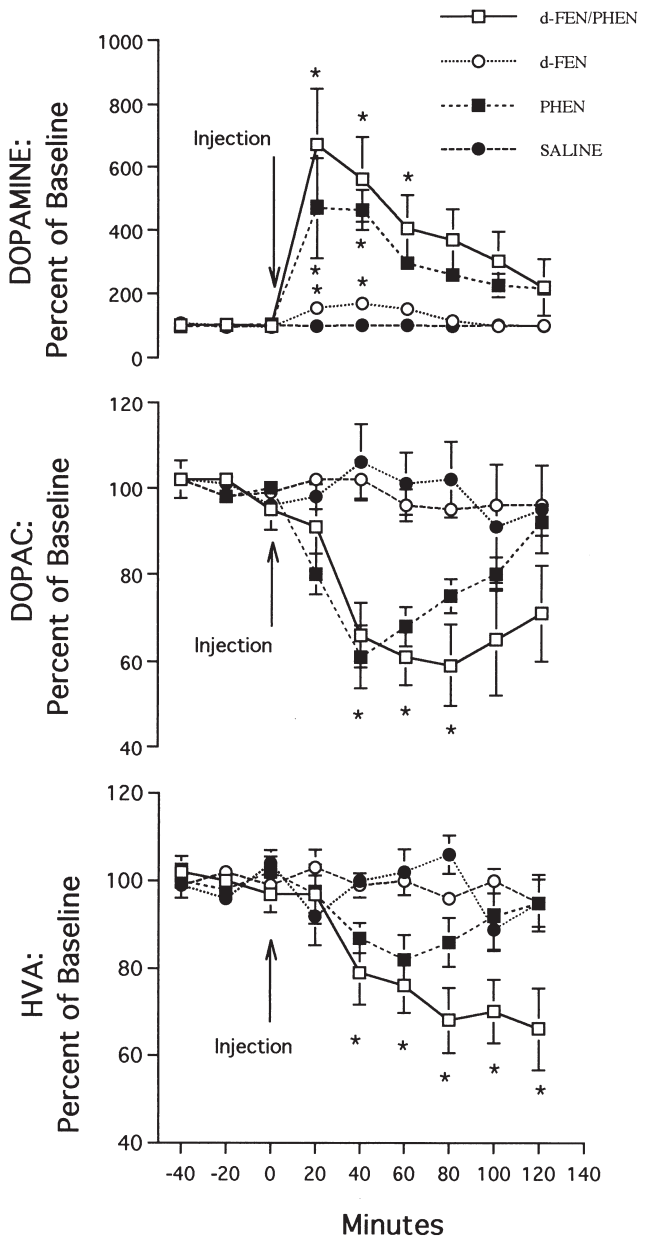

FIG. 1. Injection of *d*-FEN (open circles) and PHEN alone (closed squares) or together (open squares) (5 mg/kg of each, IP) changed extracellular levels of DA and its metabolites (DOPAC and HVA) in the NAc. A large effect came from PHEN, which increased DA immediately after the injection, and decreased DOPAC and HVA 40 min after the injection indicating DA reuptake block. *d*-FEN/PHEN increased DA to levels similar to those obtained by PHEN alone. Asterisks indicate $p < 0.05$ and error bars correspond to the standard error of the mean.

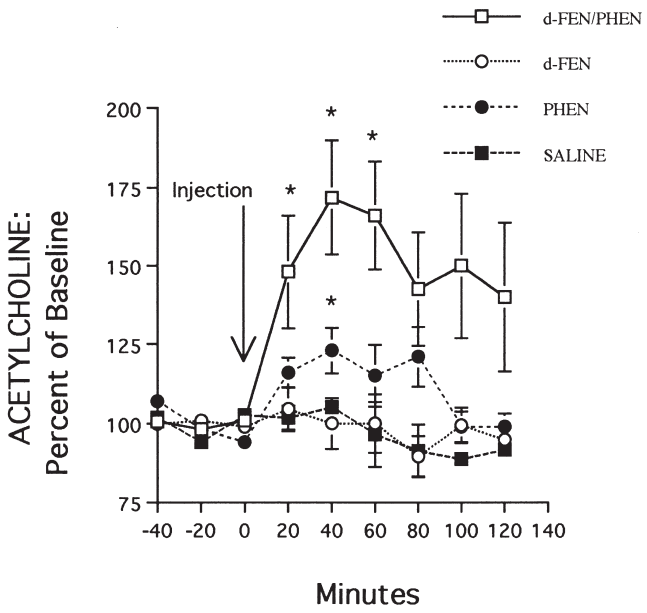

FIG. 2. Injection of *d*-FEN (5 mg/kg, IP) alone (open circles) did not change extracellular levels of ACh, while PHEN (5 mg/kg, IP) alone (closed circles) produced a mild increase in accumbens ACh. The combination of *d*-FEN and PHEN (open squares) induced a supraadditive increase in extracellular ACh reaching 172% of baseline.

DISCUSSION

The main finding is that *d*-FEN/PHEN not only increased extracellular DA like a drug of abuse (4,11), but it also increased ACh (72%). *d*-Fenfluramine alone nonsignificantly decreased ACh, and PHEN alone produced a mild significant increase. The observed ACh increase with the mixture cannot be explained simply as an additive effect; it was supraadditive for the specific dose used in this report.

The results in this report confirm earlier studies showing that PHEN increases extracellular DA levels in the NAc, and that a combination of PHEN and FEN also increases DA (1,29). It also confirms that PHEN or the mixture of *d*-FEN and PHEN blocks DA reuptake, as shown by the significant decrease in the intracellular metabolite DOPAC (Fig. 1). A new finding is that *d*-FEN mildly increased extracellular DA in the NAc. Others did not observe this, but they used lower doses of racemic *d,l*-FEN in which there is half as much *d*-FEN, and the *l*-FEN is a weak DA-receptor antagonist (5).

The mild increase in accumbens ACh observed after a systemic injection of PHEN alone was similar to that previously observed with amphetamine (3,15,17). It was suggested that this increase in ACh was due to amphetamine-induced release of DA that stimulated D_1 receptors on cholinergic neurons (15). Phentermine may have acted in this way; however, there is another possibility. PHEN may increase extracellular monoamines in hypothalamic satiety systems (16), which in turn, might activate a circuit that stimulates ACh release in the NAc.

The most interesting observation is the relatively large increase in accumbens ACh caused by the combination of two appetite suppresants. It is probably not a local accumbens effect, because in a prior study fluoxetine infused locally in the accumbens did not increase ACh, but instead decreased it. This was due to serotonergic inhibition of cholinergic interneurons at 5-HT_{1A} receptors (22). The supraadditive release of ACh in the accumbens observed after the injection of *d*-FEN/PHEN cannot be explained by stimulation of an excitatory receptor (D_1) and simultaneous local stimulation of an inhibitory receptor ($5HT_{1A}$). Instead, we suggest that some other system such as the hypothalamus is synergistically affected by

the *d*-FEN/PHEN combination, and this input stimulates ACh release in the NAc. Judging by the fact that some hypothalamic feeding factors such as norepinephrine and galanin lower accumbens ACh release (7,24), one can surmise that some hypothalamic satiety factors may raise ACh. Serotonin and DA are satiety agents in the hypothalamus (12,16), and thus could theoretically explain the increase in ACh seen in Fig. 2.

The observed supraadditive increase in extracellular ACh could be responsible for the behavioral inhibition caused by *d*-FEN/PHEN. ACh in the accumbens increases at the end of the meal, probably as a satiety signal (18). As confirmation, local neostigmine given to increase extracellular ACh during a meal stopped the meal (19). Furthermore, ACh increased during conditioned taste aversion (21) and a conditioned taste aversion was established by increasing extracellular ACh levels in the NAc (30). This suggests that an increase in cholinergic activity in the NAc may halt ingestive behavior (13).

A similar increase in extracellular ACh has been observed in the NAc during naloxone-induced withdrawal in morphine-dependent rats (23,25). Note, however, that in the conditioned-taste paradigm and morphine-withdrawal paradigm, extracellular DA is low while ACh is high, whereas natural satiety and *d*-FEN/PHEN treatment cause both DA and ACh to be elevated. Thus, *d*-FEN/PHEN seems to mimic satiety not withdrawal. The "satiety function" could help explain the inhibition of drug abuse reported with FEN/PHEN (6,10,28). Stated another way, FEN/PHEN may augment accumbens DA, as by a meal or drug of abuse, but also provide ACh to stop further behavior. Therefore, in the light of previous research, the results suggest that *d*-FEN/PHEN inhibits excessive food intake and drug abuse, at least in part, by increasing extracellular concentrations of ACh in the NAc.

ACKNOWLEDGEMENTS

This research was supported by USPHS Grant DA-10608.

REFERENCES

1. Balcioglu, A.; Wurtman, R. J.: Effects of fenfluramine and phentermine (fen-phen) on dopamine and serotonin in rat striatum: In vivo microdialysis study in conscious animals. Brain Res. 813:67–72; 1998.
2. Berger, U. V.; Gu, X. F.; Azmitia, E. C.: The substituted amphetamines 3,4-methylnedioxymethamphetamine, metamphetamine, *p*-chloroamphetamine and fenfluramine induce 5-hydroxytryptamine release via a common mechanism blocked by fluoxetine and cocaine. Eur. J. Pharmacol. 215:153–160; 1992.
3. Damsma, G., Tham, C.; Robertson, G. S.; Fibiger, H. C.: Dopamine D1 receptor stimulation increases acetylcholine release in the rat. Eur. J. Pharmacol. 186:335–338; 1990.
4. Di Chiara, G.; Imperato, A.: Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. USA 85:5274–5278; 1988.
5. Garattini, S.; Mennini, T.; Bendotti, C.; Invernizzi, R.; Samanin, R.: Neurochemical mechanism of action of drugs which modify feeding via the serotonergic system. Appetite Suppl. 7:15–38; 1986.
6. Glowa, J. R.; Rice, K. C.; Matecka, D.; Rothman, R. B.: Phentermine/fenfluramine decreases cocaine self-administration in rhesus monkeys. Neuroreport 8:1347–1351; 1997.
7. Hajnal, A.; Mark, G. P.; Rada, P. V.; Lenard, L.; Hoebel, B. G.: Norepinephrine microinjections in the hypothalamic paraventricular nucleus increase extracellular dopamine and decrease acetylcholine in the nucleus accumbens: Relevance to feeding reinforcement. J. Neurochem. 68:667–746; 1997.
8. Hernandez, L.; Paez, X.; Hamlin, C.: Neurotransmitter extraction by local intracerebral dialysis in anesthetized rats. Pharmacol. Biochem. Behav. 18:159–162; 1983.
9. Hernandez, L.; Stanley, B. G.; Hoebel, B. G.: A small, removable microdialysis probe. Life Sci. 39:2629–2637; 1986.
10. Hitzig, P.: Combined dopamine and serotonin agonists: A synergistic approach to alcoholism and other addictive behaviors. Maryland Med. J. 42:153–156; 1993.
11. Hoebel, B. G.; Hernandez, L.; Mark, G. P.; Pothos, E.: Microdialysis in the study of psychostimulants and the neural substrate for reinforcement: Focus on dopamine and serotonin. In: Frascella, J.; Brown, R., eds. Neurobiological approaches to brain–behavior interaction. NIDA Res. Monogr. 116:1–34; 1992.
12. Hoebel, B. G.; Leibowitz, S. F.: Brain monoamines in the modulation of self-stimulation feeding, and body weight. In: Weiner, H.; Hofer, M.A.; Stunkard, A. J., eds. Brain, behavior and bodily disease. New York: Raven Press; 59:103–142; 1981.
13. Hoebel, B. G.; Rada, P.; Mark, G. P.; Pothos, E.: Neural systems for reinforcement and inhibition of behavior: Relevance to eating, addiction and depression. In: Kahneman, D.; Diener, E.; Schwarz, N., eds. Well-being: The foundations of hedonic psychology. New York: Russell Sage Foundation; 1999:560–574.
14. Invernizzi, R.; Fracasso, C.; Caccia, S.; Garattini, S.; Samanin, R.: Effect of intracerebroventricular administration of *d*-fenfluramine and *d*-norfenfluramine, as a single injection or 2 hr infusion, on serotonin in brain: Relationship to concentrations of drug in brain. Neuropharmacology 30:119–123; 1991.
15. Keys, A. S.; Mark, G. P.: D-1 and D-2 dopamine receptor mediation of amphetamine-induced acetylcholine release in the nucleus accumbens. Neuroscience 86:521–531; 1998.
16. Leibowitz, S. F.; Hoebel, B. G.: Behavioral neuroscience of obesity. In: Bray, G. A.; Bouchard, C.; James, W. P. T., eds. Handbook of obesity. New York: Marcel Dekker; 1998:313–358.
17. Lindefors, N.; Hurd, Y.; Brene, S.; Persson, H.; Ungerstedt, U.: Amphetamine regulation of acetylcholine and gamma-aminobutyric acid in the nucleus accumbens. Neuroscience 48:439–448; 1992.
18. Mark, G. P.; Rada, P.; Pothos, E.; Hoebel, B. G.: Effects of feeding and drinking on acetylcholine release in the nucleus accumbens, striatum, and hippocampus of freely behaving rats. J. Neurochem. 58:2269–2274; 1992.
19. Mark, G. P.; Rada, P.; Pothos, E.; Hoebel, B. G.: Effects of feeding and drinking on acetylcholine release in the nucleus accumbens of freely moving rats. Soc. Neurosci. Abstr. 17:782; 1991.
20. Mark, G. P.; Schwartz, D. H.; Hernandez, L.; West, H. L.; Hoebel, B. G.: Application of microdialysis to the study of motivation and conditioning: Measurements of dopamine and serotonin in freely-behaving rats. In: Robinson, T. E.; Justice, J. B., eds. Microdialysis in the neurosciences. Amsterdam: Elsevier Science; 1991:369–385.
21. Mark, G. P.; Weinberg, J. B.; Rada, P. V.; Hoebel, B. G.: Extracellular acetylcholine is increased in the nucleus accumbens following the presentation of an aversively conditioned taste stimulus. Brain Res. 688:184–188; 1995.
22. Rada, P.; Mark, G.; Hoebel, B. G.: In vivo modulation of acetylcholine in the nucleus accumbens of freely moving rats: I. Inhibition by serotonin. Brain Res. 619:98–104; 1993.
23. Rada, P.; Pothos, E.; Mark, G. P.; Hoebel, B. G.: Microdialysis evidence that acetylcholine in the nucleus accumbens is involved in morphine withdrawal and its treatment with clonidine. Brain Res. 561:354–356; 1991.
24. Rada, P. V.; Mark, G. P.; Hoebel, B. G.: Galanin in the hypothalamus raises dopamine and lowers acetylcholine release in the nucleus accumbens: A possible mechanism for hypothalamic initiation of feeding behavior. Brain Res. 798:1–6; 1998.

25. Rada, P. V.; Mark, G. P.; Taylor, K. M.; Hoebel, B. G.: Morphine and naloxone, IP or locally, affect extracellular acetylcholine in the accumbens and prefrontal cortex. Pharmacol. Biochem. Behav. 53:809–816; 1996.
26. Roth, J. D.; Rowland, N. E.: Efficacy of administration of dexfenfluramine and phentermine alone and in combination, on ingestive behavior and body weight in rats. Psychopharmacology (Berlin) 173:99–106; 1998.
27. Rothman, R. B.; Elmer, G. I.; Shippenberg, T. S.; Rea, W.; Baumann, M. H.: Phentermine and fenfluramine. Preclinical studies in animal models of cocaine addiction. Ann. NY Acad. Sci. 844:59–74; 1998.
28. Rothman, R. B.; Gendron, T.; Hitzig, P.: Combined use of fenfluramine and phentermine in the treatment of cocaine addiction: A pilot case series. J. Subst. Abuse. 11:273–275; 1994.
29. Shoaib, M.; Baumann, M. H.; Rothman, R. B.; Goldberg, S. R.; Schindler, C. W.: Behavioural and neurochemical characteristics of phentermine and fenfluramine administered separately and as a mixture in rats. Psychopharmacology (Berlin) 131:296–306; 1997.
30. Taylor, K. M.; Davidson, K., Mark, G. P.; Rada, P.; Hoebel, B. G.: Conditioned taste aversion induced by increased acetylcholine in the nucleus accumbens. Soc. Neurosci. Abstr. 18:1066; 1992.
31. Weintraub, M.; Hasday, J. D.; Mushlin, A. I.; Lockwood, D. H.: A double-blind clinical trial in weight control: Use of fenfluramine and phentermine alone and in combination. Arch. Int. Med. 144:1143–1148; 1984.