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TDIQ (5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinoline) inhibits the consumption of "snacks" in mice

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Abstract

There is considerable evidence that α_2-adrenergic receptor activity exerts a pivotal role in initiation of feeding behavior. The appetite suppressant and monoamine release effects of TDIQ (5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinoline), a putative selective α_2-adrenergic compound, were compared to those of fenfluramine, a reference drug that produces an anorectic effect via presynaptic release and reuptake inhibition of serotonin. The drugs were administered to two groups of mice that had learned to consume either sweet milk or chocolate pellets (i.e. "snacks") during the low-activity/reduced-feeding "light" portion of their light/dark cycle. The selectivity of the drugs to suppress the consumption of snacks was determined by comparing doses of each drug that inhibited the animals' consumption of snacks to doses of each drug that have been shown, or were shown, to impact the motor (i.e. locomotor, rotarod, and inclined-screen side effect-like tests) or conditioned taste aversion (CTA) behavior of mice. An evaluation of TDIQ as a releaser of monoamines was determined in rodent brain synaptosomes. The administration of TDIQ or fenfluramine inhibited the consumption of the snacks, and a comparison of their ED_{50} doses indicated that TDIQ is about 3 times more potent than fenfluramine (1.3 mg/kg vs. 4.2 mg/kg, respectively) in the sweet milk test and almost equipotent to fenfluramine (19.4 mg/kg vs. 18.4 mg/kg, respectively) in the chocolate pellet assay. The selectivity of the appetite suppressant effect of TDIQ was differentiated from that of fenfluramine on the basis that TDIQ exhibited a wide separation between its dose–response effects that suppressed snack consumption and its minimal effects in tests that measured behavioral impairment. Moreover, TDIQ was distinguished from fenfluramine in that it displayed very low potencies as a presynaptic releaser of monoamines. Finally, TDIQ (0.3 mg/kg–30.0 mg/kg) did not induce a conditioned taste aversion. TDIQ may represent a novel chemical entity that exhibits a significantly favorable therapeutic-like (i.e. appetite suppressant) effect to side effect-like ratio.

Keywords: α_2-Noradrenergic; Anxiety; Binge eating; Eating disorders; Feeding behavior; Hypothalamus; Monoamines; Obesity; Paraventricular nucleus

1. Introduction

Current estimates have established a significant prevalence of overweight or obese individuals in developing and developed countries. In the United States, for example, 65% of adults are thought to have these conditions: 30% are considered to be slightly or moderately overweight and 35% are estimated to be obese (e.g., Abraham et al., 1983; Hedley et al., 2004). This is a major public health concern, since these people are at increased risk for morbidity and mortality associated with hypertension, cardiovascular disease, cancer (e.g., breast, uterine, prostate), and Type II diabetes mellitus (Pi-Sunyer, 1993). Current treatment options for overweight individuals include dietary advice, behavioral modification, exercise, surgical intervention, and pharmacologic therapy (e.g., Mertens and Van Gaal, 2000). Medications include agents that exert their appetite suppressant effect at peripheral and/or central sites of action. For example, orlistat (RO 18-0647) is a gastrointestinal lipase inhibitor that

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reduces (~30% decrease) an individual's absorption of fat (e.g., Sjostrom et al., 1998). Other medications are thought to reduce feeding through an enhancement of receptor activity, via presynaptic release and/or reuptake inhibition, of the central neurotransmitters norepinephrine, dopamine, and/or serotonin. These compounds include sibutramine, phendimetrazine, diethylpropion, phentermine, mazindol, phenmetrazine, and benzphetamine (e.g., Bray, 2000). Fenfluramine, an appetite suppressant with a long history of use, was withdrawn from the marketplace in 1997 following reports of cardiac valvulpathy (Connolly et al., 1997). The centrally acting drugs that remain on the market can produce various degrees of side effects such as nervousness, insomnia, hypertension, constipation, and/or abuse potential (e.g., Bray, 2000).

The regulation of feeding behavior and body weight is governed by interrelationships between an organism's central and peripheral physiological states and environmental conditions (e.g., Hill and Peters, 1998; West, 1996). The central control of dietary behavior probably involves the hypothalamus as a critical area in the brain. Many studies have emphasized the importance of neurotransmitter receptors at, or afferents passing through, this location and their role in the regulation of feeding behavior in response to the signals of hormonal agents (for review, see Kalra et al., 1999). For example, research investigations have gleaned intriguing insights about feeding behavior based on the discovery of hormones that inhibit eating such as leptin and its receptors in the medial hypothalamus, as well as the discovery of peptides that stimulate feeding such as ghrelin, agouti-related peptide (AgRP), and neuropeptide Y (NPY and NPY receptors) in the medial hypothalamus and, orexin A and melanin-concentrating hormone (MCH) in the lateral hypothalamus (e.g., Clark et al., 1984; Flier and Maratos-Flier, 2002; Häkansson et al., 1998; Qu et al., 1996; Tartaglia et al., 1995; Zhang et al., 1994). Interestingly, some of these latter substances may alter feeding behavior, at least in part, via interaction with receptors in the hypothalamus that respond to norepinephrine. The paraventricular nucleus (PVN) of the hypothalamus is one area that reportedly contains sites where norepinephrine is co-distributed with NPY and the infusion of either compound into this area increases the food intake of animals (e.g., Hastings et al., 1997; Matos et al., 1996). The latter effect may result from the stimulation of postsynaptic α_2-noradrenergic receptors and/or an antagonism of the inhibitory feeding effect of corticotropin-releasing factor (e.g., Morley and Levine, 1982). On the other hand, leptin, an adipocyte-secreted polypeptide hormone, may suppress appetite through a sequence of events that is thought to include antagonism of the activity of NPY and/or stimulation of hypothalamic α_2-noradrenergic autoreceptors (e.g., Brunetti et al., 1999; but see Commins et al., 1999). The latter action would inhibit the release of norepinephrine and, consequently, reduce postsynaptic α_2-noradrenergic receptor activity.

The above findings, as perhaps expected, have spurred investigations into the discovery of novel chemical entities that function as appetite suppressants. These research programs include the search for compounds that could exert a "leptin-like" effect or block the effect of NPY at central NPY receptors (e.g., Campfield et al., 1998; Chamorro et al., 2002; Norman et al., 2000). An approach that has not received as much attention involves the search for target molecules that would perturb, in a more direct fashion, the activity of α_2-noradrenergic receptors. This is perhaps not unexpected given the fact that classical α_2-noradrenergic drugs such as clonidine and yohimbine have produced inconsistent effects on feeding behavior (e.g., Berlin et al., 1986; Currie and Wilson, 1992; Kucio et al., 1991; Sax, 1991; Crow et al., 1998). However, it should be noted that these, and other, traditionally employed α_2-noradrenergic compounds also exhibit an interaction(s) at α_1-noradrenergic, imidazoline, 5-HT_{1A}, and/or 5-HT_{2B} receptors (e.g., Callado et al., 1996; Millan et al., 2000; Miralles et al., 1993; Newman-Tancredi et al., 1998; Wainscott et al., 1998). These latter sites may also exert a significant impact on an organism's feeding activities (e.g., Dourish, 1993) and, thus, obscure the role of α_2-noradrenergic receptors in feeding behavior.

Recent reports have described the pharmacological actions of TDIQ (5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinoline), a purported selective α_2-noradrenergic compound that produces, over a wide range of doses, anxiolytic-like activity in mice. In potential side effect-like tests, TDIQ exhibited minimal, if any, impairment in locomotor, rotarod, inclined screen, and heart rate/blood pressure (HR/BP) assays (Glennon et al., 2002; Malmusi et al., 1996; Young and Glennon, 2002; Young et al., 2006-this issue). From these data, it appears that TDIQ may exhibit a favorable therapeutic-like effect to side effect-like ratio. Furthermore, it has been suggested that TDIQ may produce its effect by reducing the activity of postsynaptic α_2-noradrenergic receptors (Young et al., 2006-this issue). If this same type of receptor is hyperactive in the PVN and exerts a prominent role in feeding behavior, then TDIQ may exhibit a suppressant effect on food ingestion.

In the present study, two groups of mice were provided the opportunity to consume either sweet milk or chocolate pellets (i.e. "snacks"), for a 30-min period per day, during the "light phase" of their light/dark circadian pattern. It is well documented that rodents are nocturnal animals that consume a considerable amount of their food rations during the dark phase of their light/dark cycle. During the light portion of the cycle, they display characteristics such as minimal general activity, lowered intake of food, and reduced norepinephrine activity (Armstrong, 1980; Martin and Myers, 1975). After the onset of the dark phase, however, the animals exhibit an increase in behavioral activities, including the initiation of feeding behavior (e.g., Armstrong, 1980). These latter actions are thought to be associated, to a significant degree, with increased norepinephrine activity and, in particular, an increased number of postsynaptic α_2-noradrenergic receptors (e.g., Goldman et al., 1985; Leibowitz and Brown, 1980; Leibowitz et al., 1985; Martin and Myers, 1975). In the current protocol, it was anticipated that each group of mice would learn to consume their snack, and perhaps concomitantly have increased postsynaptic α_2-noradrenergic receptor activity, during their "quiescent" state. It was then of interest to determine if TDIQ would inhibit the consumption of these snacks. The effects of TDIQ were compared to those of

fenfluramine, an appetite suppressant that produces its anorectic effect via presynaptic release and reuptake inhibition of serotonin (e.g., Rothman et al., 2001; Ziance et al., 1972). The selectivity of the appetite suppressant effects of the drugs in each group of mice was then evaluated by comparing doses of each drug that decreased consumption of the snacks to doses of each drug that have been shown, or are shown in the present study, to have an impact on the animals' motor behavior (Malmusi et al., 1996; Young et al., 2006-this issue; Ziance et al., 1972). The motor tests included locomotor, rotarod, and inclined-screen activities (e.g., Malick, 1987). In addition, a conditioned taste aversion (CTA) experiment was conducted to determine if doses of TDIQ that reduced snack intake might produce an inferred malaise state in mice (e.g., Bernstein et al., 1983; Garcia and Koelling, 1967). CTA is commonly used to assess whether a particular agent or treatment induces illness in animals; lithium chloride was used as a positive control in the present study. Finally, although TDIQ may reduce noradrenergic activity via an agonist effect at α_2-noradrenergic autoreceptors or an antagonist effect at postsynaptic α_2-noradrenergic receptors to influence the animals' consumption of snacks, it was also of interest to examine the possibility that it might produce an effect on feeding by the release of one or more monoamines from presynaptic receptor sites. Therefore, biochemical experiments were performed to evaluate the possibility that TDIQ might release dopamine, norepinephrine, or serotonin in rodent brain synaptosomes.

2. Materials and methods

2.1. Animals

Male ICR mice (Harlan Sprague Dawley Inc., Indianapolis, IN, USA), weighing 27–33 g at the time of testing, were used in the "snack" tests and the rotarod/inclined-screen procedure. The mice were housed individually in solid-bottomed plastic cages (38×22×15 cm) that contained wood shavings (Sani-Chips®, P.J. Murphy Forest Products, Montville, NJ, USA) for bedding material. They were housed in a temperature- and humidity-controlled room in a vivarium under a standard 12:12-h dark/light cycle (lights on at 0700). The mice had continuous, except during 30-min periods per day, access to chow pellets (Harlan/Teklad, LM-485, Mouse/Rat Nutritionally Complete Diet 7012, caloric density=3.2 kcal/g; Madison, WI, USA) and tap water. The experiments were conducted according to the standards set by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University and the NIH Guide for Care and Use of Laboratory Animals.

2.2. Snack test

2.2.1. Sweet milk preparations

Stock mixtures of sweet milk were prepared by combining one beaker (250 mL) of sugar (extra fine granulated 100% pure cane sugar, caloric density=4 kcal/g; Domino Foods, Inc.; Yonkers, NY, USA), two beakers (2×250 mL) of milk powder (Grade A and nonfat, caloric density=.4 kcal/g; Dairy America, Fresno, CA, USA), and 1500 mL of tap water. It was prepared fresh each day (at 0900) of the experiment and kept in a refrigerator until 1.5 h before use. The mice were presented the sweet milk in calibrated (50 mL capacity) Liquidiet Feeding Tubes (Product # 9019), attached to stainless steel Tube Holder (Product # 9263; Bio-Serv, Frenchtown, NJ, USA) clips and suspended in the animals' home cages.

2.2.2. Chocolate pellets

The pellets and the equipment for their delivery to the mice were obtained from Bio-Serv, Frenchtown, NJ, USA. The standardized pellets were "Supreme Chocolate Mini-Treats®" (Product # FO 5472-1). Each pellet was of round, spherical form and weighed 1 g; pellets were chosen at random to confirm their weight. The stated ingredients of each pellet were sucrose, corn starch, casein, dextrates, corn oil, vitamin mix, salt mix, choline, DL-methionine, L-cystine, flavoring agents and tableting binders. The stated nutritional content for a typical pellet was protein (19.8%), fiber (11.7%), moisture (10.0%), fat (6.1%), ash (4.2%), carbohydrate (52.0%), *encapsulated* vitamin C (1.08 g/kg) and vitamin D_3 (2080 IU/kg). The stated caloric density was 3.29 kcal/g. The mice were presented the pellets in Mouse Pellet Feeding Tubes (Product # 90045), attached to stainless steel Clip Holders (Product # 90055) and clipped over the top edge of the animals' home cage.

2.2.3. Snack test procedures

The sweet milk test was conducted in one group of mice (n=80) and the chocolate pellet test was conducted in another group of mice (n=112). All tests were conducted between 1300 and 1600 h. The experiments began with the presentation, for 30 min/day, of either the liquid feeding tubes (15 mL of sweet milk/tube) or pellet feeding tubes (3 pellets/tube) to the mice for an initial 8-day acclimation period. On days 1–3, a very high percentage (i.e. >80%) of the mice engaged, to various degrees, in covering the feeding devices with bedding material. This behavior gradually subsided and by the 8th day all of the animals had ceased the activity. For the next 10 days, the presentation, for 30 min/day, of either the sweet milk or chocolate pellets to the mice continued. The animals gradually learned to consume the snacks during this time and, between days 7 and 10, their consumption amounts became stable: the amount of sweet milk the mice consumed ranged from 1.2 mL to 3.4 mL (group mean=2.5 mL, S.E.M.=±0.09 mL) and the amount of chocolate pellets the mice ate ranged from 0.5 g to 2.0 g (group mean=1.26 g, S.E.M.=±0.12 g). Following the latter regimen, the mice in the sweet milk study were assigned to 10 groups (n=7–9/group) matched for baseline intake and the mice in the chocolate pellet study were assigned to 14 groups (n=7–9/group) matched for baseline consumption. On the next day, the mice in the sweet milk study were injected intraperitoneally (i.p.) with either saline vehicle, TDIQ (0.3, 1.0, 3.0, 10.0, or 17.0 mg/kg), or fenfluramine (1.0, 3.0, 10.0, or 30.0 mg/kg) 30 min before they were presented with their tubes of sweet milk. Similarly, the mice in the chocolate pellet investigation were injected i.p. with either saline vehicle, TDIQ (0.3, 1.0, 3.0, 10.0, 17.0, 30.0, or 40. mg/kg), or fenfluramine

(1.0, 3.0, 10.0, 17.0, 30.0, or 40.0 mg/kg) 30 min before they received their tubes of pellets. In each study, the mice were allowed to consume their snack for 30 min and, at the end of this period, the amount of snack each mouse consumed was recorded.

2.3. Rotarod and inclined-screen test

A separate group of mice ($n=80$) was used to conduct the rotarod test and the inclined-screen assay. The tests evaluated the ability of mice to maintain their balance on a rotating (4 rpm) cylinder and to navigate a 30° inclined screen (1/4-in. mesh screen), respectively. In brief, 90 min prior to drug or vehicle administration a group of mice was trained to remain on a rotarod (Economex® Rotarod; Columbus Instruments, Columbus, OH, USA) over four successive 1-min trials. Those mice that remained on the rod for at least two consecutive 60-s periods (i.e. 120 s) were retested 30 min before drug injection; mice that did not stay on the rotating rod for 120 s were not used in the experiment. Mice that were successful in the retesting session (i.e. one 60-s test) were assigned to a dose group ($n=10$/group) according to a table of random numbers (Winer, 1962) and then given an injection of either saline vehicle or fenfluramine (3.0, 10.0, 30.0, or 45.0 mg/kg) and returned to their home cage. After 30 min, the mice were tested again on the rotarod for ≤60 s. A notation was made if an animal did not fall, or fell, from the rotating rod. If a mouse fell from the rod, the time it spend on the rotarod was recorded (data noted but not presented or subjected to statistical evaluation). The percent of mice that fell (i.e. number of mice that fell ÷10) was recorded for the saline group and for each dose group of fenfluramine. Immediately following the rotarod test, the inclined-screen procedure was performed. In this test, mice were placed on the lower one-third (1/3) portion of the screen and those mice that climbed to the top of the screen within 60 s were assigned a score of passing and those that did not were given a score of failing. The percent of mice that were impaired (i.e. number of mice that failed to reach the top of the inclined plane ÷10) was recorded for the vehicle treatment and for each dose of fenfluramine. Further details of these procedures can be found in Young et al. (2006-this issue).

2.4. Conditioned taste aversion (CTA)

A separate group of mice ($n=56$) was used to complete the CTA study. Mice were acclimated to individual housing for 2 days in cages identical to their group-housing cages. On days 3 to 7, the mice were accustomed to restricted water access (20-h restriction) and then allowed to drink water from 2 tubes (see description of tubes in sweet milk test) for 20 min; 40 min later, the mice were provided water for 3 h and again restricted from water access for 20 h. This training protocol was repeated for 5 days (i.e. training days). On days 8 and 9 (conditioning days), the mice were presented with 0.005 M saccharin sodium salt (Sigma-Aldrich, St. Louis, MO) solution instead of water. Immediately thereafter they were injected (IP) with TDIQ (either .3, 1, 3, 10, or 30 mg/kg), 1.0 mL/kg of 0.9% saline vehicle (i.e. negative control), or 2% body weight of a 0.15-M LiCl solution (i.e. positive control agent for malaise-induction). Forty minutes later, they were presented with water for 3 h and then restricted from water access for 20 h. On day 10 (treatment break), the mice were provided with water as described in training days (see above). CTA was tested on days 11 and 12. In this procedure, the mice were presented with 2 tubes for drinking, one containing saccharin solution and the other containing water, and their consumption was recorded after 20 min by weighing the 2 tubes before and after testing. A preference score was determined from the ratio of saccharin solution ingested (in milliliters) to total fluid consumed (i.e. water and saccharin). The higher the preference score ratio, the more the mice preferred saccharin to water. The mean of total liquid consumption was 1.22 mL (S.E.M. = ±0.15 mL).

2.5. DA, NE, and 5-HT release assays

The procedures for the release studies have been reported previously (Rothman et al., 2001). Briefly, rat caudate (for DA release) or whole brain minus cerebellum and caudate (for NE and 5-HT release) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. Nomifensine (100 nM) and GBR 12935 (100 nM) were added to the sucrose solution for [^{3}H]5-HT release experiments to block any potential [^{3}H]5-HT reuptake into NE and DA nerve terminals. For the DA release assay, 100 nM desipramine and 100 nM citalopram were added to block [^{3}H]MPP$^+$ uptake into NE and 5-HT nerves. For the NE release assay, 50 nM GBR 12935 and 100 nM citalopram were added to block [^{3}H]MPP$^+$ uptake into DA and 5-HT nerves. After 12 strokes with a Potter-Elvehjem homogenizer, homogenates were centrifuged at 1000×g for 10 min at 0–4°, and the supernatants were retained on ice (synaptosomal preparation).

Synaptosomal preparations were incubated to steady state with 5 nM [^{3}H]MPP$^+$ (60 min) or 5 nM [^{3}H]5-HT (60 min) in Kreb-phosphate buffer [without bovine serum albumin (BSA)] (pH 7.4) that contained 154.4 mM NaCl, 2.9 mM KCl, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 5 mM glucose, 1 mg/mL ascorbic acid, and 50 μM pargyline plus 1 μM reserpine in a polypropylene beaker with stirring at 25 °C with the appropriate blockers. After incubation to steady state, 850 μl of synaptosomes preloaded with ^{3}H ligand was added to the 12×75 mm polystyrene test tubes that contained 150 μl of TDIQ in uptake buffer plus 1 mg/mL BSA. After 5 min ([^{3}H]5-HT) or 30 min (NE and DA assays), the release reaction was terminated by dilution with 4 mL of wash buffer (10 mM Tris–HCl, pH 7.4, containing 0.9% NaCl at 25 °C) followed by rapid vacuum filtration over GF/B filters (Whatman, Maidstone, UK) using a harvester (Brandel, Inc., Gaithersburg, MD, USA). The filters were rinsed twice with 4 mL of wash buffer using the harvester, and the retained tritium was counted by a liquid scintillation counter (Taurus: Titertek, Huntsville, AL, USA) at 40% efficiency after an overnight extraction in 3 mL Cytoscint (ICN Biomedicals Inc., Costa Mesa, CA).

2.6. Data presentation and statistics

In each snack test, each mouse served as its own control. The effect of drug treatment on mouse snack intake was determined by comparing the amount of snack consumed by the mice after the administration of either saline, TDIQ, or fenfluramine to the amount of snack they consumed during their baseline period (see test procedures). From these ratios, percent decreases in snack consumption were determined. The dose–response effect of each drug was analyzed by analysis of variance (statistically significant F value set at $p \leq 0.05$) and followed by Dunnett's post-hoc comparison test ($p \leq 0.05$) to determine statistical significance between control group versus each dose group. If a dose–response relationship occurred, then an effective dose 50% (ED_{50}) was calculated by the method of Finney (1952). The ED_{50} dose represents the calculated drug dose that would be expected to produce a 50% reduction in snack consumption by the mice. In the rotarod and inclined-screen tests, the control group was compared to each dose group by a z-test for the significance ($p \leq 0.05$; critical $z = \pm 1.96$) of difference between two proportions. If a dose–response relationship occurred, then an effective dose 50% (ED_{50}) was calculated and this value represents the expected dose of a compound where 50% of the mice would fall from the rotating rod or fail to reach the top of the inclined screen, respectively. In the CTA study, a preference score was determined from the ratio of saccharin solution ingested (milliliters) to total fluid consumed (milliliters of water and saccharin). The higher the preference ratio score, the more the mice preferred saccharin to water. The results were averaged per experimental group and analyzed using Dunnett's t-test ($p \leq 0.05$) for comparison of a control group (i.e. saline group) versus experimental groups (i.e. TDIQ groups and LiCl group). In the neurotransmitter release assays, EC_{50} values (nM, ±S.D.) were determined using the nonlinear least-squares curve-fitting program MLAB-PC (Civilized Software, Bethesda, MD, USA).

2.7. Drugs

TDIQ HCl (5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinoline hydrochloride) was synthesized in the Department of Medicinal Chemistry, Virginia Commonwealth University. Fenfluramine HCl was a gift from A.H. Robins Co., Richmond, VA, USA. Lithium chloride (LiCl) was purchased from Sigma-Aldrich (St. Louis, MO). Doses of each compound refer to the weight of the salt unless indicated otherwise. Each drug was dissolved in 0.9% saline, prepared fresh, and injected intraperitoneally (10 mL/kg injection volume) 30 min prior to testing.

3. Results

3.1. Snack tests

Mice learned, over a 10-day period, to consume their sweet milk and chocolate pellet "snacks". The administration of TDIQ or fenfluramine inhibited the consumption of these snacks in a dose-related manner (Fig. 1). Specifically, TDIQ suppressed the

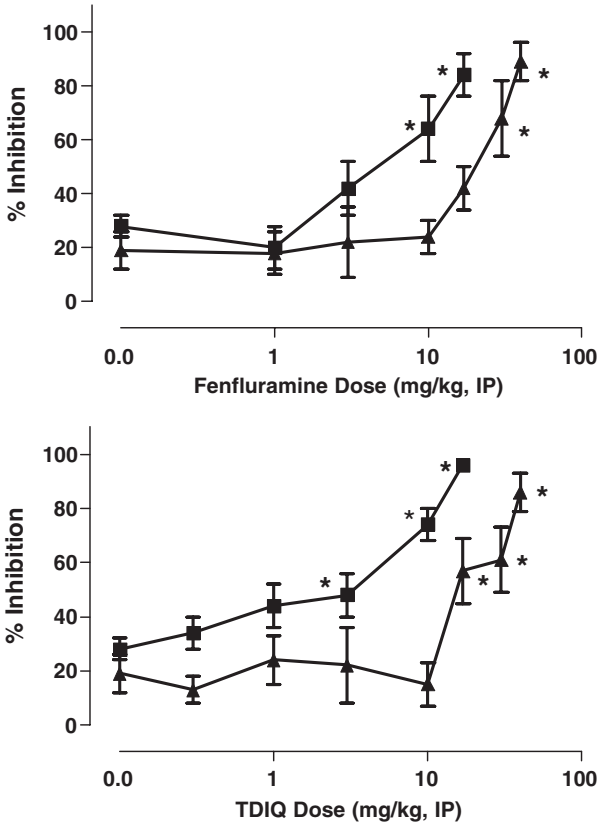

Fig. 1. Effects of fenfluramine (top) and TDIQ (bottom) in mice that learned to consume either sweet milk (■–■) or chocolate pellets (▲–▲) during the "light" portion of their light/dark cycle. Ordinate: Mean (±S.E.M.; $n = 7$–9 mice/dose) percent inhibition of each snack at each dose of fenfluramine or TDIQ. Abscissa: Doses of fenfluramine or TDIQ plotted on a logarithmic scale. An asterisk (*) denotes a dose that produced a statistically significant decrease in intake as compared with saline control ($p \leq 0.05$).

intake of sweet milk ($F(6, 49) = 16.43$, $p < 0.0001$) with an ED_{50} dose of 1.3 mg/kg (6.1 μmol/kg) that was approximately 15 times lower than its ED_{50} dose of 19.4 mg/kg (90.8 μmol/kg) that inhibited the consumption of chocolate pellets ($F(7, 62) = 7.74$, $p < 0.0001$; Fig. 1 and Table 4). Dunnett's post-hoc comparison test revealed that the response of the control group of mice was statistically different from the groups of animals that received 3 mg/kg, 10 mg/kg, or 17 mg/kg of TDIQ in the sweet milk procedure and 17 mg/kg, 30 mg/kg, or 40 mg/kg of TDIQ in the chocolate pellet assay. Fenfluramine also reduced the intake of sweet milk ($F(4, 35) = 21.33$, $p < 0.0001$) with an ED_{50} dose of 4.2 mg/kg (15.7 μmol/kg) that was more than four times lower than its ED_{50} dose of 18.4 mg/kg (68.7 μmol/kg) that decreased the consumption of chocolate pellets ($F(6, 51) = 7.98$, $p < 0.0001$; Fig. 1 and Table 4). Dunnett's post-hoc comparison test revealed that the response of the control group of mice was statistically different from the groups of animals that received 10 mg/kg or 30 mg/kg of fenfluramine in the sweet milk procedure and 30 mg/kg, or 40 mg/kg of fenfluramine in the chocolate pellet assay. Lastly, a comparison of the ED_{50} doses of the two compounds indicated that TDIQ is about 3 times more potent than fenfluramine (1.3 mg/kg vs. 4.2 mg/kg, respectively) in the sweet milk test and almost equipotent to

fenfluramine (19.4 mg/kg vs. 18.4 mg/kg, respectively) in the chocolate pellet assay.

3.2. Rotarod and inclined-screen tests

A total of 80 mice were tested (see Materials and methods) to meet the demand for 50 mice (i.e. 1 saline vehicle group and 4 dose groups of fenfluramine × 10 mice/group). Fenfluramine produced a dose-related (ED_{50} = 15.6 mg/kg) disruption of rotarod activity (Table 1). The *z*-tests revealed that the response of the control group (0.0) was statistically different ($p < 0.05$) from the response of the groups that received 10, 30, or 45 mg/kg of fenfluramine. In the inclined-screen assay, however, fenfluramine did not produce a complete dose–response function; 20% of mice were impaired at 45 mg/kg (the highest dose tested) of fenfluramine. The effect of TDIQ on rotarod and inclined screen activities has been reported previously (Young et al., 2006-this issue).

3.3. Conditioned taste aversion

TDIQ did not produce a conditioned taste aversion. Specifically, the administration of doses of TDIQ from 0.3 mg/kg to 30 mg/kg did not produce a significant change, as compared to the saline control group, in the saccharin preference ratio (Table 2). In contrast, the administration of LiCl resulted in a statistically significant decrease (i.e. CTA), as compared to saline control, in the ratio ($t = 15.28$, $df = 14$, $p < 0.0001$).

3.4. DA, NE, and 5-HT release tests

TDIQ was not a potent releaser of dopamine, norepinephrine, or serotonin in rat brain synaptosomes. As shown in Table 3, its EC_{50} values in these procedures ranged from ~1500 nM to ~6000 nM. The effects (EC_{50} values) of fenfluramine on monoamine release were published previously and are included in Table 3 for comparative purposes (Rothman et al., 2001).

Table 1
Results of fenfluramine and saline on rotarod and inclined-screen activities in mice

Drug	Dose (mg/kg) [a]	Rotarod activity % of mice disrupted [b]	Inclined-screen activity % of mice impaired [c]
Fenfluramine	3.0	0%	0
	10.0	40% *	0
	30.0	80% *	10
	45.0	90% *	20
	ED_{50} = 15.6 (9.2–26.7) mg/kg [d]		
Saline (10 mL/kg)		0%	0

[a] $n = 10$ mice/dose.
[b] Percent of mice that fell from rotarod.
[c] Percent of mice that failed to climb to the top of the screen.
[d] ED_{50} value followed by 95% confidence limits in parentheses.

Table 2
The effect of saline (negative control), TDIQ, and lithium chloride (positive control) on conditioned taste aversion in mice

Drug	Dose (mg/kg) [a]	Saccharin preference ratio [b]
Saline (10 mL/kg)		0.92 (0.02)
TDIQ	0.3	0.91 (0.03)
	1.0	0.88 (0.02)
	3.0	0.90 (0.01)
	10.0	0.87 (0.03)
	30.0	0.85 (0.05)
Lithium chloride (2% BW of a 0.15-M solution)		0.22 (0.04) *

[a] $n = 8$ mice/dose.
[b] Mean (±S.E.M.) ratio of (saccharin solution intake)÷(saccharin solution intake + water fluid intake).
* Dunnett's *t*-test; statistically different from saline control ($p < 0.0001$).

3.5. Summary of results

Table 4 summarizes the similarities and differences in dose–effect activities of TDIQ and fenfluramine in the snack, locomotor, rotarod, and inclined-screen tests in mice. Data were obtained from the present study or from data published previously in Ziance et al. (1972), Malmusi et al. (1996), or Young et al. (2006-this issue). All of these studies used the male ICR strain of mouse except the Ziance et al. (1972) study that used male Swiss–Webster mice to examine the effect of fenfluramine on locomotor activity. The present study also demonstrated that TDIQ does not produce a conditioned taste aversion (results not included in Table 4).

4. Discussion

The effects of TDIQ and fenfluramine were evaluated in two groups of mice that learned to drink sweet milk or eat chocolate pellets, for 30 min/day, during the "quiescent" portion of the animals' light/dark cycle. Both of these compounds inhibited the consumption of these snacks and a comparison of their ED_{50} doses revealed that TDIQ was 3 times more potent than fenfluramine in the sweet milk test and almost equipotent to fenfluramine in the chocolate pellet assay (Fig. 1 and Table 4). Curiously, each compound was more potent as an appetite suppressant in the sweet milk procedure than in the chocolate pellet test: ED_{50} doses of TDIQ were 1.3 mg/kg vs. 19.4 mg/kg, respectively, and ED_{50} doses of fenfluramine were 4.2 mg/kg vs. 18.4 mg/kg, respectively (Table 4). The reason(s) for this difference in potency is not clear but it can be speculated that it

Table 3
The effect of TDIQ and fenfluramine [a] on the release of norepinephrine (NE), serotonin (5-HT), and dopamine (DA) from rat brain synaptosomes

Drug	NE release EC_{50} (nM±S.D.) [b]	5-HT release EC_{50} (nM±S.D.)	DA release EC_{50} (nM±S.D.)
TDIQ	1485 (±180)	3990 (±550)	5745 (±345)
Fenfluramine [a]	739 (±57) [a]	79 (±12) [a]	>10,000 [a]

[a] Data reported previously (Rothman et al., 2001); included for comparative purposes.
[b] EC_{50} values (nM) followed by standard deviation (±S.D.) in parentheses.

Table 4
Comparison of the effects of TDIQ and fenfluramine in mice on the sweet milk, chocolate pellet, locomotor activity, rotarod, and inclined-screen tests

Drug	Sweet milk inhibition, ED_{50} (mg/kg)	Chocolate pellet inhibition ED_{50} (mg/kg)	Locomotor dose range/observed effect	Rotarod % effect at highest tested dose or ED_{50} (mg/kg)	Inclined-screen % effect at highest tested dose
TDIQ	1.3 (0.4–3.9)	19.4 (13.4–28.1)	1–30 mg/kg/No effect [a]	38% at 75 mg/kg [b]	0% at 75 mg/kg [b]
Fenfluramine	4.2 (1.9–9.5)	18.4 (12.0–28.4)	5–30 mg/kg/ Significant decreases [c]	15.6 (9.2–26.7)	20% at 45 mg/kg

Entries are either the calculated ED50 dose (mg/kg, 95% confidence limits), highest tested dose (with percent of effect), or dose range evaluated (with observed effect).
[a] Data previously reported (Malmusi et al., 1996).
[b] Data previously reported (Young et al., 2006-this issue).
[c] Data previously reported (Ziance et al., 1972).

might be related to the interaction of each drug to the different ingredients (e.g., palatability factor) in each snack (see Materials and methods) and/or in how (or at what rate of intake) the snacks were ingested (i.e. licking of the sweet milk vs. chewing the chocolate pellets). Further research will be needed, however, to clarify this issue. Whatever the reason, it is apparent that the mice in the sweet milk test, as compared to the mice in the chocolate pellet protocol, appeared to be more sensitive to the effect(s) of these drugs.

Historically, many appetite suppressants have caused serious side effects and, consequently, were withdrawn from the marketplace (for review, see Bray, 2000). Therefore, a prudent preclinical evaluation of an anorectic compound might (or should) emphasize how closely intertwined, or separable, an appetite suppressant effect is in relation to potential side effect-like activities in, for example, locomotor, rotarod, and inclined-screen tests (e.g., Malick, 1987). In the present study, the reference standard fenfluramine was administered to mice prior to the sweet milk test and it inhibited their intake with a dose–response effect that was separated narrowly from its dose–response effect that decreased locomotor activity or impaired rotarod performance (Ziance et al., 1972; Fig. 1, Tables 1 and 4). In the chocolate pellet test, fenfluramine produced a dose–response function that was not very separable from its dose–effect curve that reduced locomotor activity or disrupted rotarod coordination (Ziance et al., 1972; Fig. 1, Tables 1 and 4). Fenfluramine did, however, produce a wider separation of effect between its dose–response effects in the snack tests and the highest tested dose that produced maximal impairment in the inclined-screen procedure; only 20% of mice were disrupted at 45 mg/kg of fenfluramine (Fig. 1, Tables 1 and 4). Overall, the results obtained with fenfluramine demonstrate the occurrence of relatively narrow areas between the dose–response functions that inhibited the intake of snacks and its dose–response curves that produced disruptions in potential side effect-like tests (except the inclined-screen procedure). These results, however, are not inconsistent with pre-clinical and clinical observations of the effects of fenfluramine that indicate both an appetite suppressant effect and a major sedative side effect (e.g., Macko et al., 1972; Owen, 1975; Woodward, 1970).

TDIQ was evaluated as an appetite suppressant in mice and produced a relatively wide separation between doses that inhibited snack consumption and doses that produced little, or no, changes (i.e. impairment, increase, decrease) in locomotor, rotarod or inclined-screen tests (Malmusi et al., 1996; Young et al., 2006-this issue; Fig. 1 and Table 4). TDIQ produced dose-related inhibitions of sweet milk intake (ED_{50}=1.3 mg/kg) and chocolate pellet consumption (ED_{50}=19.4 mg/kg) that were lower than the highest dose tested that has been shown to produce some effect in the rotarod test: 38% of mice were impaired at 75 mg/kg of TDIQ (Young et al., 2006-this issue; Fig. 1 and Table 4). In other tests, TDIQ did not show marked impairment in locomotor or inclined-screen activities (Malmusi et al., 1996; Young et al., 2004-this issue; Table 4). Thus, the behavioral effects of TDIQ can be distinguished from the effects of fenfluramine on the basis that TDIQ appears to exhibit a wide separation between its dose–response effects that suppressed the consumption of snacks and its minimal effects in tests that measured behavioral impairment (Fig. 1 and Table 4). In addition, an experiment was performed to determine if TDIQ could induce inferred malaise in a CTA experiment (e.g., Bernstein et al., 1983; Garcia and Koelling, 1967). TDIQ doses of 0.3, 1.0, 3.0, 10, and 30 mg/kg did not alter significantly the saccharin preference ratio (Table 2), which indicates that TDIQ did not induce a CTA. In contrast, the administration of LiCl, which served as a positive control for the assay, produced a robust CTA as indicated by a statistically significant decrease in the ratio (Table 2). Taken together, the data above suggest that the appetite suppression effect of TDIQ is not due to malaise or induction of nonspecific general behavioral suppression. Finally, TDIQ can be differentiated from fenfluramine, and many of the currently marketed appetite suppressants, in that it exhibits very low potencies in the synaptosomal release assays (Table 3). Thus, the mechanism of action of TDIQ, unlike these other compounds, does not seem to include the release of a monoamine(s) from a presynaptic site(s).

Numerous studies have provided support for a role of endogenous norepinephrine in the control of anxiety and feeding behavior. In some types of anxiety disorders, for example, there may be an excessive degree of activity, and consequent dysfunction, of central α_2-noradrenergic receptors (e.g., Bremner et al., 1996; Redmond and Haung, 1979). The regulation of feeding behavior may involve the stimulation of postsynaptic α_2-noradrenergic receptors in the PVN, interruption of an organism's state of satiety, and subsequent initiation of eating (e.g., Goldman et al., 1985; Wellman, 2000). As such, the current results could be explained by speculating that the

initial placement of the feeding devices into the mouse cages produced changes in the animals' α_2-noradrenergic activity: efflux of norepinephrine, increased activity of postsynaptic α_2-noradrenergic receptors, and a behavioral response ("anxiogenic-like reaction"?) that resulted in the mice attempting to bury the devices with their bedding material (e.g., Njung'e and Handley, 1991; Young et al., 2006-this issue). However, the devices were presented on a daily basis and, eventually, the mice habituated (i.e. burying activity ceased) to their presence. During this process, it can be further speculated that the hyperactivity of postsynaptic α_2-noradrenergic receptors associated with burying behavior may have waned but not the increased activity of postsynaptic α_2-noradrenergic receptors in the PVN that is linked to feeding behavior. The latter activity might then have become increasingly salient until the animals' state of satiety was interrupted and the mice consumed the snacks. This feeding behavior was inhibited, however, in mice that were pretreated with doses of TDIQ. Moreover, the latter doses (and higher doses) do not, or are known not to, have a marked effect on motor behaviors or cardiovascular (i.e. heart rate and blood pressure) activities (Malmusi et al., 1996; Young et al., 2006-this issue; Table 4).

In view of the present results, it is tempting to speculate that mouse ingestive behavior and the effects of TDIQ may be of some relevance to the occurrence of certain (over)eating activities of humans. If excessive activity of postsynaptic α_2-noradrenergic receptors in the PVN is linked to hyperarousal or supersensitivity to external food stimuli, then TDIQ may be able to restore inhibitory synaptic control and, consequently, suppress eating behavior. For example, many overweight and obese people attempt to lose weight but find it difficult to limit their food intake and/or to maintain an adequate exercise program. It is not surprising, therefore, that these individuals oftentimes oscillate between weight loss and weight gain. A typical cycle may consist of weight loss, followed by behavioral symptoms that include excessive thoughts and/or hyperarousal reactions to external food stimuli and, eventually, increased food intake that results in a rapid regain of the lost weight. Such eating may, in fact, be unrelated to the person's energy requirements and be driven by food marketing and advertising campaigns that often promote predominately high fat and high sugar foods (e.g., Macht and Simons, 2000; Stunkard et al., 1996). Thus, these individuals may be less influenced by their internal physiological controls that strive to maintain energy balance and are more influenced by, or are hypersensitive to, external food stimuli that could be correlated to an excessive or inappropriate degree of activity of postsynaptic α_2-noradrenergic receptors in the PVN. The combination of increased activity of postsynaptic α_2-noradrenergic receptors and the presentation/availability of external food stimuli could be sufficient triggers to override internal controls that function to maintain energy balance. The latter events may indicate that α_2-noradrenergic regulation in the PVN is (or has become) different in some overweight/obese or "binge" eaters than in normal-weight individuals. An example of this condition could be an individual engaged in the practice of compulsive eating or "binge eating of comfort foods": the consumption of well marketed, palatable, available, and accessible foods that usually contain relatively high levels of sugar and fat (e.g., Dallman et al., 2003; Stunkard et al., 1996).

In summary, the present study explores further the involvement of an α_2-noradrenergic mechanism in the behavior(s) that governs food intake. It is speculated that an inappropriate or excessive degree of activity of postsynaptic α_2-noradrenergic receptors in the PVN may lead to an organism's over-consumption of food. Furthermore, the results obtained here warrant further research on the applicability of selective α_2-noradrenergic drugs as potential palliative compounds in the treatment of some types of overeating. TDIQ should be examined in other types of animal feeding (or fluid intake) paradigms. If positive results are obtained in such studies, then TDIQ might eventually be recommended as a potential therapeutic treatment in individuals who appear to be strongly influenced by external food stimuli. In such cases, TDIQ could be used to minimize the hyperactivity of postsynaptic α_2-noradrenergic receptors and lessen the effect that external food stimuli might have to trigger an individual's overeating. Of course, any appetite suppressant medication should be considered as just one component of a weight loss program. The most effective use of a compound such as TDIQ could come from it being coupled to patient counseling that centered on lifestyle alterations: changes in diet and increased exercise. Finally, it is recommended here that further research is needed to examine, more closely, the possible interrelationships between α_2-noradrenergic (hyper)activity, exposure to food advertising, feeding behaviors, sedentary lifestyle(s), and the prevalence of individuals who are overweight or obese.

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