Published in final edited form as:

Horm Behav. 2009 September ; 56(3): 322–331. doi:10.1016/j.yhbeh.2009.06.009.

Tamoxifen produces conditioned taste avoidance in male rats: An analysis of microstructural licking patterns and taste reactivity

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Abstract

Estrogen receptor activation has been shown to reduce body weight and produce a conditioned reduction in food intake in male rats that is putatively mediated by estradiol's suggested aversive effects. Evidence has shown that the selective estrogen receptor modulator tamoxifen used in the prevention and treatment of breast cancer may also produce a change in food intake and body weight, which are known to impact cancer development and survival. The purpose of the present study was to examine whether tamoxifen produces a conditioned reduction in intake similar to estradiol by producing a conditioned aversion. A one bottle lickometer test was used to examine conditioned changes in sucrose drinking, while the taste reactivity test was used to measure rejections reactions, which serve to index aversion in rats. A backward conditioning procedure that consisted of 3 conditioning days and one vehicle test day was used to examine for conditioned changes in 0.3 M sucrose intake and taste reactivity. Our results show that tamoxifen produced a conditioned reduction in sucrose drinking in a one bottle fluid intake test that was similar to the effects produced by estradiol (positive control); however, no active rejections reactions were produced by either tamoxifen (1 and 10 mg/kg) or estradiol. The present results suggest that tamoxifen, at the doses used in the present study, acts as an estrogen receptor agonist to regulate food intake and that the conditioned reduction in intake produced by tamoxifen and estradiol reflects conditioned taste avoidance rather than conditioned taste aversion.

Keywords

selective estrogen receptor modulators; lickometer; body weight; sucrose; taste reactivity

Introduction

Selective estrogen receptor modulators (SERMs) are a unique class of drug that act as both estrogen receptor agonists and antagonists depending on the target tissue (Jordan, 2006). For example, the SERM tamoxifen has been shown to block the growth factor effects of estrogens on breast cancer cells (Forrest, 1971) while mimicking the effects of estrogens in the uterus (Gielen et al., 2008; Pole et al., 2005). Tamoxifen's anti-estrogenic effect in breast tissue makes

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it a useful therapeutic tool in both the prevention and treatment of breast cancer. However, due to the mixed agonist/antagonist effects, tamoxifen can produce a variety of effects throughout the body that involve both blocking and mimicking the effects of estrogens (see Fitzpatrick (1999) for a review). For example, tamoxifen use has been associated with hot flashes, sweating, insomnia, anxiety and sexual dysfunction (Mourits et al., 2001). Even though tamoxifen is an effective pharmacological treatment option for male and female breast cancer patients, tamoxifen's effects outside of breast tissue are important as they may impact the clinical use of tamoxifen. For example, estrogen receptor activation has been shown to regulate food intake and body weight (Geary, 2004), which are two factors that can impact cancer development and survival (Camoriano et al., 1990; Eliassen et al., 2006; Enger et al., 2004; van den Brandt et al., 2000). It is, therefore, of interest to examine the effects of tamoxifen on food intake and body weight.

Results of a variety of animal studies have shown that estrogens act in an unconditioned manner to reduce both the amount of food consumed, as well as body weight, in both male and female rats and mice (Asarian and Geary, 2006; Geary et al., 2001). The anorexic effects of estrogens have been suggested to be mediated by both peripheral and central actions (Butera and Beikirch, 1989; Gandelman, 1983). Some of the brain areas implicated in mediating the anorexic effects of estrogens include portions of the hypothalamus such as the paraventricular hypothalamus (Butera and Beikirch, 1989), ventromedial hypothalamus (Nunez et al., 1980), medial preoptic area (Dagnault and Richard, 1997) and arcuate nucleus (Clegg et al., 2007).

There have been several clinical investigations that have reported limited or mixed results on the effects of tamoxifen on body weight. Some previous studies have reported that tamoxifen may increase weight gain, while others have reported no effect in female breast cancer patients (Demark-Wahnefried et al., 2001; Kumar et al., 1997; Rohatgi et al., 2002). To date, no reports on the effects of tamoxifen on body weight in male patients have been made available. Animal studies that have examined both food intake and body weight have also yielded conflicting evidence. Tamoxifen has been shown to reduce food intake and body weight in ovariectomized (OVX)-female rats (Wade and Heller, 1993; Wallen et al., 2001). Tamoxifen was, however, reported to antagonize estradiol's suppressive effects on body weight in OVX-Syrian hamsters (Wade and Powers, 1993). In orchidectomized-male rats tamoxifen has been shown to inhibit body weight gain compared to controls (Fitts et al., 2004). Changes in food intake following tamoxifen administration have not been reported in males, to date.

In addition to estrogens' unconditioned regulation of food intake, the results of previous studies have also shown that estrogens produce conditioned changes in ingestion in both males and females as indexed by a conditioned taste avoidance. When given access to a sucrose solution that has been previously paired with estradiol administration, rats, mice, Mongolian gerbils, and humans will avoid consuming the estradiol-paired tastant during subsequent presentations (De Beun et al., 1991; Flanagan-Cato et al., 2001; Ganesan and Simpkins, 1990; Ganesan and Simpkins, 1991; Ganesan, 1994; Miele et al., 1988; Ossenkopp et al., 1996; Young et al., 1989). When examining the conditioned effects of tamoxifen on food intake in OVX-female rats, Fudge et al. (2009) reported that tamoxifen produced a conditioned reduction in sucrose drinking that was similar to the reduction in intake produced by estradiol. These findings suggest that tamoxifen acts as an estrogen receptor agonist to produce conditioned changes in ingestion in OVX-female rats. To date, there has been only one report on the conditioned effects of tamoxifen on food intake in males, however, no significant effects were found. Lopez et al., (2006) demonstrated that when paired with a novel saccharin solution, tamoxifen does not produce conditioned taste avoidance in male rats.

It has been suggested that the conditioned reduction in food intake produced by estradiol may be due to aversive effects, possibly nausea, and has thus been historically referred to as

conditioned taste aversion (Bernstein et al., 1986; De Beun et al., 1991; Gustavson et al., 1989). In humans, increased levels of estradiol have been reported to produce aversive effects such as nausea and vomiting (Goodman and Gilman, 1975; Young et al., 1989). Additional evidence for the aversive effects of estradiol has come from studies that examined the role of the area postrema (AP) in mediating estradiol-induced conditioned reductions in intake. AP is a brain stem structure that is suggested to serve as a toxin-sensitive region that mediates nausea (Borison and Wang, 1953; Ossenkopp and Eckel, 1995). Bernstein et al. (1986) reported that AP lesions abolish estradiol-induced (0.9 mg/ml minipump infusion) conditioned reductions in food intake in male rats, thus further supporting the hypothesis that estradiol-induced illness mediates the observed conditioned reductions in ingestion. Other evidence that estradiol produces an aversive effect, at least in male rats, comes from place learning studies that have shown that estradiol produces conditioned place avoidance in males (De Beun et al., 1991).

The purpose of the present study was to examine and compare the effects of tamoxifen to the effects of estradiol on sucrose drinking patterns, gustatory conditioning (measured as conditioned taste avoidance) and body weight gain. As well, the present study examined whether aversive effects are a mediating factor in the effects of tamoxifen and estradiol on food intake in male rats. A lickometer apparatus was used to quantify the volume of fluid intake, as well as to examine several ingestive behaviors related to licking patterns, to measure conditioned taste avoidance and putative underlying mechanisms. Changes in anxiety related behaviors and locomotor activity, which could potentially influence intake measures in rats, were also examined. The taste reactivity test was used to index active oral rejection reactions in order to determine whether any observed conditioned reductions in intake, produced by tamoxifen and/or estradiol in the lickometer apparatus, are true taste aversions (conditioned rejection responses) or only conditioned taste avoidances (see Parker 2003).

Materials and Methods

Animals

Naive male Long Evans rats (Charles River, St Constant, Quebec), weighing between 230-310 g at the beginning of the experiment, were pair housed in polypropylene cages (45 × 22 × 20 cm). Rats had continuous access to food (Prolab) and water throughout the entire experiment, unless otherwise stated. The colony room was kept on a 12:12h-light/dark cycle (lights on at 0700h) at a room temperature of 21 ± 1°C. All experimental procedures occurred during the light phase (0900 – 1630h) and were approved by the institutional Animal Care Committee and carried out according to the guidelines set out by the Canadian Council on Animal Care.

Drugs and Groups

Tamoxifen citrate (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 10% ethanol/90% saline and intraperitoneally (IP) injected (Young et al., 2001) at a dose of 1 and 10 mg/kg and a volume of 2 ml/kg. The doses of tamoxifen were chosen on the basis of the results of prior studies that examined food intake, body weight and anxiety (Gray et al., 1993; Walf and Frye, 2005). It is important to note that the 1 mg/kg dose of tamoxifen used in the present study is calculated to be just slightly below the equivalent dose that is prescribed to patients (20 mg) based on surface area, mg/m². Human equivalent doses were calculated using a standard conversion formula provided by the FDA (www.fda.gov/cder/cancer/animalframe.htm). Vehicle control injections (10% ethanol/90% saline, vehicle) were administered at a volume of 2 ml/kg, IP. The positive control 17β-Estradiol (estradiol, Sigma-Aldrich) was dissolved in 10% ethanol/90% saline and injected subcutaneously (SC) at a dose of 50 µg/kg and a volume of 1 ml/kg. The 50 µg/kg dose of estradiol was chosen based on previous evidence that demonstrated that this dose effectively produces conditioned taste avoidance in male rats (De Beun et al., 1991), as well as previous studies that suggested that pharmacological doses of

estrogens are needed to produce conditioned taste avoidance (Ganesan and Simpkins, 1990; Ossenkopp et al., 1996; Roesch, 2006). Even though different routes of administration were used to inject tamoxifen (IP) and estradiol (SC), differential pharmacokinetics are unlikely as both compounds are lipid soluble. It has previously been shown that the release of fat soluble compounds does not differ if administered IP or SC (Goodman and Gilman, 1975).

Experiment 1: Sucrose Intake and Microstructure of Lick Patterns

Lickometer Test Chamber—The lickometer test chamber consisted of a clear Plexiglas box (31 × 41 × 31 cm) with a graduated glass cylinder and metal spout mounted on the front of the chamber. The end of the spout was mounted 8 cm above the chamber floor. The lickometer allowed for a microstructural analysis of drinking behavior by collecting the frequency and temporal correlates of licks on the spout (Davis and Smith, 1992). To record drinking measures, a non-invasive current (~60 nA) was passed through the drinking spout. When the animal's tongue contacted the spout, the circuit was completed and recorded by the Contact 108 lick analysis system (Dilog Instruments, Tallahassee, FL). The volume of sucrose solution consumed was quantified by reading fluid levels in the graduated glass cylinder. Custom made programs (Baird et al., 1999; Kaplan et al., 2001) were used to generate ingestive behavior variables from the frequency and temporal features of the licks. The microstructural variables of drinking that were analyzed are the number of licks (total number of tongue contacts with the spout), the number of bursts (a series of licks, minimum of 3, with an interlick interval upper limit of 1 sec (Baird et al. 1999)) and the size of burst (number of licks per burst). It has been suggested that the number of bursts of drinking reflects the postingestive consequences of food consumption (see Spector et al. 1988 for review), while the size of burst reflects the hedonic properties/palatability of the tastant (see Davis and Smith 1992; Spector et al. 1988; Spector et al. 1998).

Automated Activity Test Chamber—Locomotor activity data (Ossenkopp and Kavaliers, 1996) were collected using eight VersaMax Animal Activity Monitors (AccuScan Instruments, model VMA16TT/W, Columbus, OH). Each test chamber consisted of a clear Plexiglas chamber (40 × 40 × 30 cm) with grey coverings on two sides to prevent visual contact between animals in adjacent boxes. A Plexiglas lid with several air holes was placed on each of the test chambers during testing. Locomotor activity was monitored via two sets of infrared photobeams, with 16 sensors per bank of photobeams, located along the sides of the test chamber. One set of photobeams was located 2.5 cm above the floor with sensors every 2.54 cm along the perimeter to monitor horizontal activity. Another set of photobeams was located 8 cm above the floor to monitor vertical activity. Data were collected and analyzed with a Versamax Analyzer (AccuScan Model CDA-8, Columbus, OH). The multiple locomotor variables (Ossenkopp and Mazmanian, 1985) quantified were: total distance (total distance traveled in cm), number of horizontal movements (frequency of separate horizontal movements lasting more than 1 s with a minimum 1 s rest period between incremental counts), horizontal movement time (total time spent ambulating in the open field), number of vertical movements (frequency of rears with a minimum 1 s time period between rear counts) and vertical time (time in seconds spent in a rearing posture activating the upper bank of sensors).

A margin area, which consisted of a 3 infrared beam wide area (out of a possible 16 beams with 2.54 cm spacing) next to the walls of the open field, was spatially defined and used to quantify thigmotaxis. The ratio of time spent in the defined margin (amount of time in seconds the animal spent near the walls divided by the amount of time in seconds spent in the entire open field apparatus) was used as a measure of thigmotaxis.

Water Deprivation Schedule—Twenty hours prior to the start of the experiment, animals were water deprived and placed on a water deprivation schedule for the remainder of the

experiment. The experiment consisted of eight consecutive water baseline training days, to stabilize fluid intake, and four experimental days that occurred 72 h apart. During baseline training, animals had access to distilled water for 30 min in the lickometer apparatus. On experimental days, animals had access to 0.3 M sucrose for 30 min in the lickometer. On non-experimental days, water was returned to the home cage for 30 min in lieu of drinking in the lickometer. At the end of each day (1600h) throughout the experiment, including both experimental and non-experimental days, water was made available in the home cage for 30 min.

Experimental Procedure—Twenty-four hours after the last water baseline day, a conditioning procedure began that consisted of three experimental days (Conditioning Days 1-3). Animals (n=8 per drug group) were injected with either tamoxifen (1 and 10 mg), estradiol (50 µg/kg) or vehicle 20 min before being placed in the lickometer test chamber with access to 0.3 M sucrose for 30 min. On experimental day 4 (Test Day), only vehicle injections were administered to probe for possible conditioned drug effects. This experimental procedure has previously been reported to effectively produce a conditioned reduction in intake by both tamoxifen and estradiol in OVX-female rats (refer to Fudge et al., 2009). Immediately following the drinking session in the lickometer on Conditioning Days 1-3 and the Test Day, animals were tested in the automated Versamax open field apparatus for 30 min to quantify locomotor activity. It is important to note that on Conditioning Day 1 the open field test apparatus was a novel environment, which has previously been shown to induce an anxiety-associated thigmotaxis response in rats (Ossenkopp et al., 1994; Treit and Fundytus, 1988). Body weight was recorded on all experimental days prior to injections.

Statistical Analyses—A one-way Analysis of Variance (ANOVA) was used to examine for possible group differences in baseline measures on the last day of baseline training. The ANOVA contained a between subjects factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle). A mixed-factor ANOVA was used to analyze lickometer, open field and thigmotaxis data that were collected on Conditioning Days 1-3. For each behavioral test, the ANOVA contained a between subject factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle) and a within subject factor of Day (three levels for lickometer, open field and thigmotaxis measures: Conditioning Days 1-3). Post hoc comparisons were carried out using Tukey's HSD. To examine for possible conditioned effects, Test Day data were analyzed separately with a one-way ANOVA containing a between subject factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle). For body weight, a mixed-factor ANOVA, which consisted of a between subject factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle) and a within subject factor of Day (four levels: Conditioning Days 1-3 and Test Day), was used to analyze changes in cumulative body weight gain across experimental days. Body weight gain was calculated by subtracting the body weight measures taken on Day 0 (Conditioning Day 1 was chosen as Day 0 as body weight was measured prior to any experimental manipulations) from Days 3, 6 and 9 (which correspond to Conditioning Days 2 and 3 and the Test Day, respectively). Statistical significance was determined using Greenhouse-Geisser corrected degrees of freedom and an α level of 0.05.

Experiment 2: Taste Reactivity Test

Intraoral Cannulation—Naïve male rats were implanted with an intraoral cannula according to the procedures described by Fudge et al. (2006). Following 24 h of food deprivation, rats were anesthetized with separate IP injections of 100 mg/kg ketamine and 20 mg/kg xylazine solutions in distilled water. A 15-gauge stainless steel needle was inserted into the dorsal mid-neck of the rat and fed subcutaneously under the ear and along the jaw until just ahead of the mandible. The needle was inserted inside the oral cavity until it protruded through the mouth from behind the first maxillary molar. Intramedic polyethylene tubing (PE90) was inserted into

the needle, which was then withdrawn leaving the tubing in its path. A 20-gauge intramedic luer stub adapter was attached to the tubing at the back of the neck. To secure the tubing within the mouth, an O-ring was placed onto the tubing. The tubing was then heat flared with a small soldering iron to secure the O-ring. After surgery, the rats were individually housed in hanging wire cages. Forty-eight hours after surgery, the cannulae were flushed with distilled water on three consecutive days so that they remained clear of food debris during recovery.

Taste Reactivity Test Chambers—The taste reactivity test chambers were made of a Plexiglas box (29 × 29 × 25 cm) sitting on a clear glass plate with a mirror mounted at a 45° angle below the glass plate. While in the chamber, each animal's cannula was attached to an infusion pump (model 341-A; Sage Instruments, Cambridge, MA) via a 1 m length of polyethylene tubing (PE 90). The infusion pump produced a constant infusion rate (0.78 ml/min) of the tastant into the oral cavity through the cannula. A digital video camera (SONY DCR-DVD201, London, On, Canada) located approximately 1 m from the mirror was used to videotape the ventral view of the animal's upper body and record the orofacial and somatic responses elicited during infusion. The camera was connected to an IBM-type computer that was used to store the video files.

Testing Procedure—On three separate adaptation days, the water-replete animals were placed in the taste reactivity chamber and given intraoral infusions of distilled water for 5 min to habituate the rats to the experimental conditions. Twenty-four hours later, experimental manipulations commenced on three experimental days that were each separated by 72 hours. The first two experimental days were conditioning days (Conditioning Day 1 and 2). As the taste reactivity test is a time consuming procedure and changes in drinking were observed early during the conditioning phase in Experiment 1, only 2 conditioning days were used in Experiment 2. On these days (Conditioning Days 1 and 2), all animals received an injection of either tamoxifen (1 mg/kg, n = 9, and 10 mg/kg, n = 7), estradiol (50 µg/kg, n = 7) or vehicle (n = 8) 20 min prior to the start of intraoral sucrose infusions in the taste reactivity chamber. On the last experimental day, Test Day, all animals received vehicle injections to examine for potential drug conditioning effects. The sucrose infusion schedule consisted of seven 1-min infusions of 0.3 M sucrose with a 10-min time delay between each infusion. Body weight was recorded on experimental days prior to injections.

Behavioral Quantification—Videotapes were scored in slow motion (1/5 speed) to identify individual behavioral responses. The behaviors were scored based on categories described by Grill and Norgren (1978) and elaborated on by Ossenkopp and Eckel (1995) and Parker (1995). All behaviors were scored on a frequency basis. The taste reactivity test is a measure of consummatory behaviors and is an index of palatability and aversion in rats. The ingestive consummatory responses measured were rhythmic mouth movements (MM, rhythmic movement of the lower mandible), paw licking (PL, licking one and/or both forelimbs and hindlimbs) and tongue protrusions (TP, both medial and lateral tongue protrusions were scored separately). The active rejection reactions scored were gapes (a large amplitude opening of the mandible with retractions of the corners of the mouth), chin rubs (mouth or chin in direct contact with the floor or wall of the chamber and body projected forward) and head shakes (rapid twisting of the head from side to side that may or may not include the expulsion of fluids from the mouth due to the rapid movement of the head).

Statistical Analyses—Total ingestive behaviours and total rejection reactions were calculated for each experimental day by summing the frequency of all ingestive behaviors and all rejection reactions, respectively, on each experimental day. However, the ANOVAs revealed no significant group differences for either ingestive behaviors or rejection reactions ($F_s < 1.941$, $p_s > 0.05$). It is possible that the non-significant results may have been due to the

different frequencies with which some of the behaviors were produced (i.e.: mouth movements are produced at a high frequency compared to tongue protrusions and paw licks). A separate analysis of each individual ingestive behavior and rejection reaction was also conducted. The sum of each ingestive behaviour (tongue protrusions, mouth movements and paw licking) and each active rejection reaction (gapes, chin rubs and head shakes) on Conditioning Days 1-2 and the Test Day was calculated by adding the number of each behavior in the 7 infusions to produce a total daily frequency score. A mixed-factor ANOVA with a between subject factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle) and a within subject of Day (Conditioning Days 1 and 2) was used to examine the effects of tamoxifen and estradiol on the individual taste reactivity behaviours measured during the conditioning phase. For the Test Day, a one-way ANOVA with a between subject factor of Drug (four levels: tamoxifen (1 and 10 mg/kg), estradiol and vehicle) was used. Body weight gain was calculated by subtracting the body weight measures taken on Day 0 (Conditioning Day 1 was chosen as Day 0 as body weight was measured prior to any experimental manipulations) from Days 3 and 6 (which correspond to Conditioning Day 2 and the Test Day, respectively). Statistical significance was based on Greenhouse–Geisser corrected degrees of freedom and an α level of 0.05.

Results

Experiment 1: Volume and Lickometer Measures

Fluid Intake—There were no significant differences in the volume of fluid consumed among any of the groups on the last baseline day before conditioning began ($F(3,28) = 0.675$, $p > 0.05$, Figure 1). Statistical analysis of fluid intake volume on Conditioning Days 1-3 revealed a significant Day $\times$ Drug interaction ($F(6,56) = 13.861$, $p < 0.001$) and a main effect of Drug ($F(3,28) = 7.513$, $p < 0.05$) for tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals (refer to Figure 1). On Conditioning Days 2 and 3, tamoxifen (1 and 10 mg/kg) and estradiol significantly reduced sucrose intake compared to vehicle controls (Tukey's HSD, $ps < 0.05$). Even though estradiol reduced sucrose drinking, estradiol-treated males drank significantly more sucrose on Conditioning Day 2 than tamoxifen (10 mg/kg)-treated males (Tukey's HSD, $p < 0.05$); however, no such difference was observed on Conditioning Day 3. On the Test Day, there was a significant main effect of Drug ($F(3,28) = 12.723$, $p < 0.001$) for tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals. These results suggest that tamoxifen (1 and 10 mg/kg) and estradiol produced conditioned taste avoidance on the Test Day as sucrose intake was significantly reduced in animals treated with tamoxifen (1 and 10 mg/kg) and estradiol compared to vehicle controls (Tukey's HSD, $ps < 0.05$).

Number of Licks—There were no significant group differences in the number of licks on the spout during baseline ($F(3,28) = 0.17$, $p > 0.05$). The ANOVA revealed a significant Day $\times$ Drug interaction ($F(6,56) = 16.283$, $p < 0.001$) and a main effect of Drug ($F(3,28) = 9.869$, $p < 0.001$, see Figure 2) on Conditioning Days 1-3. Post hoc analyses indicated that on Conditioning Day 1, estradiol-treated rats licked the spout significantly more than vehicle controls (Tukey's HSD, $p < 0.05$). On Conditioning Day 2, tamoxifen (1 and 10 mg/kg) and estradiol significantly reduced the number of licks on the spout compared to controls (Tukey's HSD, $ps < 0.05$); however, estradiol-treated males licked the spout significantly more than tamoxifen (1 and 10 mg/kg)-treated males (Tukey's HSD, $ps < 0.05$). On Conditioning Day 3, similar results were observed as tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals licked the spout significantly less compared to controls (Tukey's HSD, $ps < 0.05$). On the Test Day, the ANOVA revealed a significant main effect of Drug ($F(3,28) = 14.318$, $p < 0.001$). Tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals licked the spout significantly less often than the vehicle control animals (Tukey's HSD, $ps < 0.05$). These reductions in number of licks are consistent with the data reported in the fluid intake results.

Number of Bursts—No significant group differences were observed for the baseline data ($F(3,28) = 0.062, p > 0.05$). As well, there were no significant differences in the number of bursts produced in either tamoxifen- or estradiol-treated animals during conditioned taste avoidance acquisition on Conditioning Days 1-3 ($F_s < 2.911, p_s > 0.05$). On the Test Day, a main effect of Drug ($F(2,28) = 3.269, p < 0.05$) was revealed such that tamoxifen (1 mg/kg)-treated animals produced significantly fewer bursts than did the vehicle controls (Tukey's HSD, $p < 0.05$, see Figure 3).

Size of Bursts—There were no significant group differences during baseline ($F(3,28) = 0.271, p > 0.05$). On Conditioning Days 1-3, a mixed-factor ANOVA indicated that there was a significant Day $\times$ Drug interaction ($F(6,56) = 3.294, p < 0.05$; refer to Figure 4). Post hoc analysis showed that on Conditioning Day 2, tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals had a significantly smaller size of bursts than vehicle controls (Tukey's HSD, $p_s < 0.05$). On the Test Day, a main effect of Drug failed to reach significance ($F(3,28) = 2.787, p = 0.059$).

Locomotor Activity and Thigmotaxis—The ANOVA revealed a significant Day $\times$ Drug interaction ($F(6,56) = 2.552, p < 0.05$) for the time spent ambulating in the open field when males were treated with tamoxifen and estradiol. However, post-hoc analysis (separate one way ANOVA analysis for each conditioning day) revealed no significant differences among groups in the time spent ambulating on any of the conditioning days (Tukey's HSD, $p_s > 0.05$, see Figure 5 B). A significant main effect of Drug ($F(3,28) = 3.424, p < 0.05$) during the conditioning phase was found for the number of vertical movements (rears) produced in tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals. Post hoc analyses indicated that animals treated with 10 mg/kg of tamoxifen reared significantly less than animals treated with 1 mg/kg of tamoxifen (Tukey's HSD, $p < 0.05$, see Figure 5 A). No other significant differences were found ($F_s < 2.124, p_s > 0.05$).

No significant effects on thigmotaxis were observed for the tamoxifen- or estradiol-treated groups ($F_s < 0.957, p_s > 0.05$). This suggests that the changes in sucrose intake produced by either tamoxifen or estradiol were likely not the result of sedative effects on locomotor activity or changes in anxiety (as measured by thigmotaxis).

Body Weight—The ANOVA revealed a significant main effect of Drug ($F(3,28) = 13.842, p < 0.001$) for tamoxifen and estradiol on the cumulative body weight gained in treated male rats on Days 3, 6 and 9. Post hoc analyses indicated that tamoxifen (1 and 10 mg/kg)- and estradiol-treated animals had a significantly lower body weight gain than vehicle controls (Tukey's HSD, $p_s < 0.05$). Animals treated with 10 mg/kg of tamoxifen produced a greater reduction in the cumulative amount of body weight gained compared to animals treated with estradiol (Tukey's HSD, $p < 0.05$, refer to Figure 6).

Experiment 2: Taste Reactivity Measures

Ingestive Behaviors—On Conditioning Day 1 and 2, the mixed-factor ANOVA revealed a significant Day $\times$ Drug interaction ($F(3,27) = 10.254, p < 0.001$) and a main effect of Drug for the total number of tongue protrusions produced ($F(3,27) = 5.802, p < 0.05$) during the sucrose infusions. Post hoc analysis revealed that males treated with 10 mg/kg of tamoxifen produced significantly more tongue protrusions than males treated with 1 mg/kg of tamoxifen, estradiol or vehicle controls on Conditioning Day 1 (Tukey's HSD, $p_s < 0.05$, refer to Figure 7A). On Conditioning Day 2, tamoxifen (1 and 10 mg/kg) and estradiol significantly reduced the total number of tongue protrusions produced during the sucrose infusions compared to vehicle controls (Tukey's HSD, $p_s < 0.05$, refer to Figure 7 A). For paw licks during the conditioning phase, the mixed-factor ANOVA revealed a main effect of Drug ($F(3,27) = 4.755$,

$p < 0.05$) such that tamoxifen (1 and 10 mg/kg) and estradiol significantly reduced the number of paw licks compared to vehicle controls (Tukey's HSD, $ps < 0.05$, refer to Figure 7 A).

On the Test Day, similar results to those which were observed on Conditioning Day 2 were found as the ANOVA revealed a significant main effect of Drug for the total number of tongue protrusions ($F(3,27) = 8.951$, $p < 0.05$) and paw licks ($F(3, 27) = 7.698$, 0.05). Post hoc analysis indicated that tamoxifen (1 mg/kg) and estradiol significantly reduced the total number of tongue protrusions, while tamoxifen (1 and 10 mg/kg) and estradiol reduced the number of paw licks, produced during the 7 sucrose infusions on Test Day (Tukey's HSD, $ps < 0.05$). Refer to Figure 7 A and B. No other significant differences were observed on Conditioning Days 1 and 2 or the Test Day ($F_s < 2.318$, $ps > 0.05$).

Active Rejection Reactions—Few to no active rejection reactions were observed on the experimental days. Statistical analysis of this limited data revealed that no significant group differences in the number of gapes, chin rubs or head shakes were observed on Conditioning Days 1 and 2 or the Test Day ($F_s < 2.640$, $ps > 0.05$).

Body Weight—The ANOVA revealed a significant Day $\times$ Drug interaction ($F(3,27) = 13.216$, $p < 0.001$) and a main effect of Drug ($F(3,27) = 29.853$, $p < 0.001$) for tamoxifen and estradiol on the cumulative body weight gained in treated male rats on Days 3 and 6 in Experiment 2. Post hoc analyses indicated that tamoxifen (1 and 10 mg/kg) treated animals had a significantly lower body weight gain than estradiol and vehicle controls on Days 3 and 6 (Tukey's HSD, $ps < 0.05$, refer to Figure 8).

Discussion

The results of Experiment 1 suggest that tamoxifen, like estradiol, produced conditioned taste avoidance in male rats as a significant reduction in sucrose consumption was observed during the conditioning phase and, more importantly, on the Test Day in both tamoxifen- and estradiol-treated animals. These results are consistent with a previous study from our laboratory that demonstrated with the same methodology that tamoxifen produces conditioned taste avoidance in a similar manner as estradiol in OVX-female rats (Fudge et al., 2009). The combined results suggest that tamoxifen acts as an estrogen receptor agonist to produce conditioned taste avoidance in both male and female rats. However, an examination of the pattern of drinking in Experiment 1 revealed no consistent changes in either the number or size of bursts of drinking on Conditioning Days 1-3 or on the Test Day. On the Test Day, only the 1 mg/kg dose of tamoxifen significantly altered the number of bursts of drinking. A change in the size of bursts was produced by tamoxifen and estradiol, but this occurred on Conditioning Day 2 only. It is, therefore, unclear what mechanisms are involved in mediating the effects of tamoxifen and estradiol on the palatability or postingestive consequences as revealed by changes in the size and number of bursts of drinking, respectively (Davis and Smith 1992; Spector et al. 1988; for review see Spector et al. 1998). This is likely due to variability in licking measures and the limited data available for analysis, as drinking levels robustly decreased over conditioning days.

Although the results from Experiment 1 suggest that tamoxifen produces conditioned taste avoidance in male rats, conflicting evidence has previously been reported. Lopez et al. (2006) reported that tamoxifen administration, when paired with the ingestion of saccharin, did not produce conditioned taste avoidance in male rats. One possible explanation for these divergent results in males may be the different methodologies used. Differences in dose, route of administration, tastant, and conditioning paradigm are evident. It has previously been demonstrated that pharmacological, but not physiological, doses of estradiol produce conditioned taste avoidance in rats (De Beun et al., 1991; Ganesan and Simpkins, 1990;

Ossenkopp et al., 1996; Roesch, 2006). The doses and routes of administration of tamoxifen (0.5 mg/kg, SC and 10 µg per rat, ICV) used by Lopez et al. (2006), when compared to the doses and route of administration used in the present study (1 and 10 mg/kg, IP), may not have been sufficient to produce conditioned taste avoidance in male rats. Additionally, Lopez et al. (2006) used only one saccharin-tamoxifen pairing with forward conditioning to examine conditioned taste avoidance, while the present study used three tamoxifen-sucrose pairings (Conditioning Days 1-3) with backward conditioning.

When examining the unconditioned effects of tamoxifen on food intake, previous studies have reported that tamoxifen mimics estrogen's unconditioned regulatory effects on food intake in OVX-female rats. In these previous studies, tamoxifen, like estradiol, reversed the increase in food intake produced by ovariectomy (Wade and Heller, 1993). These previous results suggest that tamoxifen acts as an estrogen receptor agonist to reduce food intake in OVX-female rats in an unconditioned manner. The results of Experiment 1 are consistent with these previous findings as a conditioned taste avoidance was observed in both estradiol- and tamoxifen-treated male rats, and they indicate that tamoxifen acts as an agonist in mediating food intake in a conditioned manner. Together the present findings along with previous findings in OVX-female rats suggest that tamoxifen mimics both the conditioned and unconditioned effects of estradiol on food intake (Wade and Heller, 1993). It is important to note, however, that the unconditioned regulatory effects of tamoxifen on food intake have not been previously reported for male rats. It is possible that tamoxifen may regulate food intake in an unconditioned sexually dimorphic manner. However, no sex differences in the unconditioned effects of estrogens on food intake have been reported to date.

It is also possible that the changes in intake in Experiment 1 may reflect unconditioned mechanisms and not solely a conditioned change in intake. This concern is especially valid given that intake measures were taken every 72 h and it is well known that the unconditioned effects of estradiol are observed for 24 h or more after estradiol administration and can last for days (see Geary, 2004 for review). However, it also has been reported that after 5 consecutive days of estradiol injections (0.1-1 mg per rat) in OVX-female rats the unconditioned effects of estradiol on ingestion are no longer observed 3 days after the cessation of treatment (Schemmel et al., 1982). According to these results, it is highly unlikely that the present reductions in intake are solely the result of the unconditioned effects of estradiol as all experimental days were conducted 3 days apart. Moreover, Ganesan and Simpkins (1990) have suggested that the conditioned effects of estradiol on ingestion are separate from, but act synergistically with, the unconditioned effects. The results of their study showed that estradiol produced a greater suppression in the ingestion of a novel versus a familiar food item. Additional evidence from this research group has also reported that there is a distinct difference in the unconditioned versus the conditioned effects of estrogens on food intake. It was shown that estradiol administration increased food intake (unconditioned effect) in female Mongolian gerbils, while still producing a conditioned taste avoidance (conditioned effect) (Ganesan, 1994). This evidence of separate, synergistic processes for the conditioned and unconditioned effects of estrogens on food intake suggests that the intake results in the present study likely reflect both the conditioned and unconditioned effects of estradiol and tamoxifen.

The taste reactivity results in Experiment 2 of the present study further indicate that tamoxifen, similar to estradiol, produced a conditioned reduction in sucrose drinking as a conditioned reduction in ingestive behaviors was observed. However, very few active rejection reactions were observed, which suggests that estradiol and tamoxifen do not produce aversive effects to induce a conditioned reduction in sucrose intake. Rather, a shift in taste palatability, as evidenced by the reduction in ingestive behaviours, is produced. Thus, the reduction in intake observed in Experiment 1 likely reflects conditioned taste avoidance and not conditioned taste aversion. The reduction in sucrose palatability in Experiment 2 is consistent with previous

reports that estradiol treatment reduces sucrose intake by altering taste perception. Atchley et al. (2005) reported that estradiol treatment significantly decreases the positive-feedback signals elicited by brief access to dilute sucrose solutions, thus indicating a reduction in sucrose palatability. Further evidence that estradiol treatment reduces palatability was reported by Curtis et al. (2004) who reported a sex difference in sucrose taste perception (see also Clarke and Ossenkopp, 1998). OVX-female rats were reported to lick dilute sucrose solutions less than males indicating that OVX-female rats are less sensitive to low concentrations of sucrose. The results of these drinking studies suggest that estradiol reduces sucrose taste perception, and consequently sucrose palatability, which is further supported by the present taste reactivity test results demonstrating a significant decrease in ingestive responses to an infusion of an estradiol-paired sucrose solution.

The taste reactivity results in Experiment 2 are also consistent with the results reported by Flanagan-Cato et al. (2001) that estradiol (10 µg per rat or approximately 33 µg/kg assuming an average weight of 300 g) does not elicit aversion to produce a conditioned reduction in intake in OVX-female rats. However, Ossenkopp et al. (1996) reported that estradiol produced conditioned active rejection reactions in male rats (100 µg/kg). One possible explanation for the differential effects of estradiol on active rejection reactions in male rats reported here and by Ossenkopp et al. (1996) is a difference in dose. The dose used by Ossenkopp et al. (1996) (100 µg/kg) is significantly higher than the dose in the present study (50 µg/kg). It is possible that a dose of 100 µg/kg of estradiol may produce a toxic effect in male rats that results in an aversive (nausea) response to the estradiol-paired tastant.

Even though the results of Experiment 2 do not indicate that estradiol produced a conditioned reduction in intake in Experiment 1 by inducing aversive toxic effects, additional support for the production of aversive effects by estradiol in male rats has been reported. The results of a place learning study reported that estradiol (50 µg/kg) produced aversive effects in males thus causing place avoidance (De Beun et al., 1991). However, several other studies have shown that estradiol at a lower dose (10 µg) and at a higher dose (1 mg) produces place preferences in OVX-female rats (Frye and Rhodes, 2006; Walf et al., 2007). It is, therefore, unclear whether estradiol produces aversive effects in place learning or if there is a sex difference in this type of conditioning.

Additional evidence for aversive effects has also been reported. Estradiol-induced (0.9 mg/ml, minipump infusion) conditioned reductions in intake in male rats have been shown to be mediated by AP, a brain stem structure that is suggested to serve as a toxin-sensitive region that mediates nausea (Bernstein et al., 1986; Borison and Wang, 1953; Eckel and Ossenkopp, 1996; Ossenkopp and Eckel, 1995). However, it is reasonable to assume that the dose of estradiol that was infused (0.9 mg/ml) is a relatively high dose and therefore likely produced a toxic effect that involved AP signaling to reduce food intake (Bernstein et al, 1986). Additionally, care needs to be taken when interpreting the results of AP lesion studies that examine the aversive effects of estrogens without a direct measure of rejection responses in the taste reactivity test. There is extensive evidence that AP plays a significant role in mediating food intake above and beyond mediating toxicity effects. AP has been shown to be involved in the reduced feeding effects of many different peptides that regulate food intake, such as CCK. This peptide has been shown to produce reduced sucrose intake (satiety) without inducing aversive responses in the taste reactivity test (Eckel and Ossenkopp, 1994), as has the orexigenic hormone ghrelin (Gill and Lutz, 2006; Van der Kooy, 1984, for review of the role of AP in feeding refer to Price et al., 2008). Lesions of AP have been shown to inhibit hyperphagia produced by ghrelin, thus suggesting that AP lesions have a more general effect on food intake beyond simply mediating nausea/malaise (Gill and Lutz, 2006). Therefore, as Bernstein et al. (1986) did not measure taste reactivity responses but only food intake, it is perhaps premature to conclude that this evidence is suggestive of aversive toxic effects induced

by estradiol in conditioning a reduction in intake. The present results suggest that, at this dose of estradiol (50 µg/kg), an aversion interpretation of the estradiol-induced conditioned reductions in intake is not warranted, as the conditioned reduction in intake is produced without production of any active rejection reactions to indicate a true aversion.

When examining the changes in cumulative body weight gain, water-deprived tamoxifen- and estradiol-treated male rats in Experiment 1 showed a significant reduction in body weight gain across experimental days compared to their vehicle controls. These results are consistent with previous reports on the anorexic effects of tamoxifen in OVX-female rats and orchidectomized male rats (Fitts et al., 2004; Wade and Heller, 1993; Wallen et al., 2001). It is important to note that intact males were used in the present study, thus suggesting that tamoxifen's regulation of body weight does not seem to be affected by gonadal status. The results of Experiment 1 also suggest that it is possible that tamoxifen may regulate body weight in a dose dependent manner as the high dose of tamoxifen (10 mg/kg) produced the greatest reduction in the amount of body weight gained compared to estradiol. This dose response stands in contrast to the sucrose intake measures in Experiment 1 that did not reveal any effect of dose. However, it is possible that a floor effect, caused by the robust reduction in sucrose drinking, prevented any observable dose response. Overall, the results of Experiment 1 suggest that tamoxifen likely acts as an estrogen receptor agonist in terms of regulating body weight gain.

In Experiment 2, tamoxifen (1 and 10 mg/kg) also significantly reduced the amount of body weight gained compared to vehicle controls. Estradiol, however, did not alter the amount of body weight gained in water-replete male rats. This suggests that there may have been an interaction between the animal's water deprivation state and estradiol's effect on body weight. The effects of tamoxifen, however, did not show any such interaction. It is unclear whether tamoxifen regulates body weight in a manner different from estradiol. At first glance, these results seem to also indicate that tamoxifen may regulate food intake through an alternate, non-estrogenic mechanism. However, it is important to note that there is evidence from yoked-feeding studies that demonstrates that estrogen receptor activation regulates body weight independently from food intake (Mueller and Hsiao, 1980; Roy and Wade, 1977). Therefore, the observed changes in body weight in the present results do not necessarily reflect any consequence of, or relationship with, the observed change in sucrose drinking behaviors produced by either estradiol or tamoxifen. It is possible that the difference in the observed body weight gain effects of estradiol and tamoxifen in Experiment 2 may reflect differences in the affinity of these compounds for the different subtypes of estrogen receptors involved in regulating body weight. Future studies are clearly needed to further examine the role of estrogen receptors in mediating body weight independent of feeding.

Estrogen receptor modulation has many regulatory effects that could also account for the conditioned reduction in sucrose consumption produced by tamoxifen and estradiol in Experiment 1. For example, it is possible that tamoxifen and estradiol altered locomotor activity in such a manner as to interfere with drinking. There is, however, little evidence to show that estradiol administration alters general locomotor activity in male rats. In the present study, male rats treated with 10 mg/kg tamoxifen displayed fewer rearing responses than males treated with 1 mg/kg on Conditioning Day 2 and 3. This reduction in number of rears is unlikely to account for the reduction in sucrose intake on Conditioning Day 2 or 3, especially since no significant differences from vehicle were observed on those days. Additionally, there were no differences in the number of vertical movements (rears) between tamoxifen doses on the Test Day, when sucrose drinking was also significantly reduced. Therefore, the observed conditioned taste avoidance produced by tamoxifen (1 and 10 mg/kg) cannot be explained by alterations in general locomotor activity. Estrogen receptors have also been shown to regulate anxiety behaviors (Walf et al., 2007; Walf and Frye, 2007). The present results indicate that neither tamoxifen nor estradiol significantly altered thigmotaxis in a novel open field on

Conditioning Day 1 when compared to vehicle controls. Thus, it is unlikely that either tamoxifen or estradiol altered sucrose intake or produced conditioned taste avoidance by enhancing locomotor-related anxiety.

Anticipated Significance

The anticipated significance of the present study is the indication that the conditioned, along with the unconditioned, effects of SERMs on food intake are an additional concern in the clinical use of these drugs (Kumar et al., 1997). The present results indicate that tamoxifen may have the potential to produce conditioned taste avoidance in patients undergoing preventative and adjuvant treatment. This is of particular concern when patients, including male patients, undergo dietary changes to enhance their health. The introduction of a new diet along with tamoxifen use may result in a decrease in compliance to the new healthy diet plan due to conditioned taste avoidance. It is, therefore, important to characterize and understand both the unconditioned and conditioned effects of SERMs on food intake and body weight regulation. At this time, however, no clinical reports of tamoxifen-induced conditioned taste avoidance have been published. Future studies that investigate the conditioned effects of tamoxifen use on feeding behavior in patients are needed.

Acknowledgments

We would like to thank the following persons for volunteering their time in the lab: Alex Di Cicco, Andrew Lockey and Mona Madady. Funding for this study was provided by NSERC Discovery Grants R2195A01 and RO557A01 to Klaus-Peter Ossenkopp and Martin Kavaliers, respectively, and NIH Grant DC-07389 to John-Paul Baird. Melissa Fudge was supported by an NSERC Canada Graduate Scholarship.

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Figure 1.

Group mean volume intake (ml) $\pm$ SEM across all experimental days. TAM = tamoxifen, estradiol = 17β -estradiol and vehicle = 10% ethanol/90% saline. Baseline represents last water baseline day. * = $p < 0.05$ versus all drug groups. # = $p < 0.05$ versus tamoxifen (10 mg/kg).

Figure 2.

Group mean number of licks $\pm$ SEM across all experimental day. TAM = tamoxifen, estradiol = 17β -estradiol and vehicle = 10% ethanol/90% saline. Baseline represents last water baseline day. * = $p < 0.05$ versus all drug groups. ** = $p < 0.05$ versus vehicle. # = $p < 0.05$ versus tamoxifen (1 and 10 mg/kg).

Figure 3.

Group mean number of bursts $\pm$ SEM across all experimental days. TAM = tamoxifen, estradiol = 17β -estradiol, vehicle = 10% ethanol/90% saline. Baseline represents last water baseline day. ** = $p < 0.05$ versus vehicle.

Figure 4. Group mean size of bursts $\pm$ SEM across all experimental days. TAM = tamoxifen, E2 = 17β -estradiol and vehicle = 10% ethanol/90% saline. Baseline represents last water baseline day. * = $p < 0.05$ versus all drug groups.

Figure 5.

Group mean number of vertical movements (rears, A) and mean time spent ambulating (B) +/- SEM across all experimental days. TAM = tamoxifen, estradiol = 17 β -estradiol and vehicle = 10% ethanol/90% saline. # = $p < 0.05$ versus tamoxifen (1 mg/kg).

Figure 6.

Group mean change in body weight from baseline (grams) $\pm$ SEM across all experimental days. The change in body weight (in grams) was calculated by subtracting the body weight (grams) on Conditioning Day 1 from the body weight (grams) of the animals on each experimental day. TAM = tamoxifen, E2 = 17 β -estradiol and vehicle = 10% ethanol/90% saline. * = $p < 0.05$ versus all drug groups. # = $p < 0.05$ versus estradiol.

Figure 7 A and B.

Group mean tongue protrusions (A) and paw licks (B) $\pm$ SEM on Conditioning Day 1 and 2 and the Test Day. TAM = tamoxifen, E2 = 17β -estradiol and vehicle = 10% ethanol/90% saline. * = $p < 0.05$ versus all drug groups. ** = $p < 0.05$ versus vehicle. # = $p < 0.05$ versus all other groups.

Figure 8.

Group mean change in body weight from baseline (grams) $\pm$ SEM across all experimental days. The change in body weight (in grams) was calculated by subtracting the body weight (grams) on Conditioning Day 1 from the body weight (grams) of the animals on each experimental day. TAM = tamoxifen, E2 = 17 β -estradiol and vehicle = 10% ethanol/90% saline. # = $p < 0.05$ versus tamoxifen (1 and 10 mg/kg).