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Estradiol increases consumption of a chocolate cake mix in female rats

Karen J. Boswell [a], Larry D. Reid [b,*], Christopher A. Caffalette [b], Karen T. Stitt [a], Lucas A. Klein [a], Aubrie M. Lacroix [b], Meta L. Reid [a]

[a] *Siena College, Loudonville, NY, USA*
[b] *Rensselaer Polytechnic Institute, Troy, NY, USA*

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Abstract

Female Sprague-Dawley rats were given an opportunity to eat chocolate cake mix (CCM) using a common brand of cake mix, while standard laboratory food was also available. They took large amounts of the CCM, often taking more than 20 g in 24 h. Some animals were given a single injection of 1 of 6 doses of estradiol valerate (ranging from 0.09 to 10.0 mg/kg) and others were given vehicle. Estradiol valerate provides for sustained release of estradiol. Those receiving estradiol ate more than those receiving vehicle at doses larger than 0.09 mg/kg. Further, with a dose of 10 mg/kg, greater intake among estradiol-treated females was apparent 2 months post-injection. Methodological issues of neophobia and conditioned avoidance were addressed in the study's design and may explain why increased intakes were observed here in contrast to the consensus that estradiol reduces food intake.

Keywords: Estradiol; Ingestion; Chocolate; Female rats; Estradiol valerate; Estrogen; Appetite; Obesity

1. Introduction

Based on data from adults in the USA (20+ years old) and defining obesity as a BMI ≥ 30, it is estimated that 8 million plus more women are obese than men. The incidence of obesity for women is 33.4% compared to 27.5% for men (Flegal et al., 2002). Women are exposed to higher levels of both endogenous and exogenous estrogenic compounds. Perhaps this might account for the different rates of obesity.

The hypothesis that higher rates of exposure to estrogenic compounds might account for the difference in obesity is *not* supported by reports from the animal laboratory. An early example is a study by Wade (1975) showing that estradiol reduced the consumption of food among female rats. Following Wade's experiment, there were many demonstrations supporting the hypothesis that estrogenic compounds lead to a reduction in food intake. A literature search, involving a computer-generated list of papers citing Wade's 1975 paper, yields a list of hundreds of reports. The consensus is that estrogenic compounds reduce food intake (Eckel, 2004; Horvath et al., 2004).

A different perspective emerges from our recent research. We were interested in following the observations (Reid, 1996; Reid and Hunter, 1984) that small doses of morphine (e.g., 1.0 mg/kg, not sufficiently large to induce analgesia) enhanced the intake of alcoholic beverages among rats, and doses of opioid antagonists reduced intakes. There is considerable evidence that the opioidergic system modulates intake of alcoholic beverages and intake of palatable ingesta (Reid, 1985; 1990, for reviews). Brawer and colleagues (e.g., Brawer et al., 1993; Desjardins et al., 1990, 1993) showed that large doses of estradiol valerate (EV) (2 mg a rat or about 10 mg/kg) selectively disrupted the arcuate nucleus of the hypothalamus and hence selectively reduced (in terms of the endogenous opioids) the β-endorphin content of brain. In the interest of determining which of the endogenous opioids is salient to intake of alcoholic beverages, we gave 2 mg of EV to female rats that were having daily opportunities to take alcoholic beverages. This dose of EV led, initially, to a transitory reduction in bodyweight and a marked reduction in intakes that was sustained for days. However, with

* Corresponding author.
E-mail address: reidl@rpi.edu (L.D. Reid).

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continued opportunity to take the beverage, the females' intakes increased until they were substantially larger than the control group's intakes (Reid et al., 2002). Once the increased intakes emerged, they were sustained for months (Reid et al., 2003b). The initial decrease in intakes was predictable based on the idea that decrements in opioidergic functioning (induced by the toxic effects of EV) would reduce appetite for alcohol much the same way opioid antagonists reduced intakes. The large increases in intakes manifest days after the injection of EV (Marinelli et al., 2003; Reid et al., 2002, 2003a,b) were not anticipated. A hormonal manipulation that induces a large increment in intake of alcoholic beverages, which is sustained for months without further intervention, might have relevance to alcoholism; consequently, further research was initiated.

Recently, we (Boswell et al., 2005) addressed the issue of whether the increased appetite for alcoholic beverage, seen after large doses of EV, is peculiar to alcoholic beverage or is a general increase in appetite that might affect intakes of many kinds of ingesta. Given the conclusion that there is considerable overlap in mechanisms that control ingestion in general and ingestion of alcohol (Reid, 1985; 1990), there were reasons to suspect that EV might enhance intakes of substances other than alcoholic beverages, contrary to the consensus that estradiol decreased intakes of ingesta (Eckel, 2004; Horvath et al., 2004).

Boswell et al. (2005) provided female rats with two different saccharin solutions, one (0.025%) that rats consume in large amounts and another (2.0%) that is taken in only small amounts (bittersweet to the human taste). EV-treated rats consumed more of the 0.025% solution and less of the 2.0% solution than vehicle-treated females. The effects on intake remained measurable for 3 months post-injection. It was also noted that the EV-treated rats did not show an enhanced appetite for laboratory food, as their rate of weight gain paralleled that of the vehicle-control animals once the acute effects of EV subsided. Large doses of EV can induce circumstances enhancing appetite for alcoholic beverages and some other ingesta (e.g., palatable saccharin solutions), but not all ingesta (e.g., bittersweet saccharin solution and ordinary laboratory food).

Procedural differences may provide an explanation for the discrepancy in our findings that estrogenic injections enhance intakes, compared with other published findings that they suppress intakes. After an injection of EV, female rats either lose weight or do not gain weight across a number of days. Vehicle-injected rats gain weight nearly daily (Flanagan-Cato et al., 2001; Reid et al., 2002). The acute effects of EV on weight may reflect a malaise caused by the initial effects of the drug. Supporting such a contention, de Beun et al. (1991) and Flanagan-Cato et al. (2001) have found that injections of estradiol induce taste and place aversions. The initial effects of the continuous estrogenic stimulation are probably related to a malaise, manifest as weight loss, resulting in reduced food intake. After the rats have adapted to continuous estrogenic stimulation, they gain weight at the rate of controls and an increase in appetite (relative to control group animals) for certain ingesta may emerge.

The following experiments extend the study of EV's effects to appetite for a complex palatable food. We chose chocolate cake mix batter (CCM), which had been used by Koch and Matthews (2001) and shown to be sensitive to drug effects. We took into account the possibility that the initial effects of EV might induce a conditional taste aversion and, therefore, did not pair the initial effects of EV with presentation of CCM.

2. General methods

There were four experiments. They shared a number of common features summarized in this part of the report.

2.1. Subjects

For each of the first three experiments, the subjects were 20 female Sprague-Dawley rats purchased from Taconic Farms (Germantown, NY, USA) with an average weight of nearly 200 g on arrival. The subjects of the fourth experiment were another 40 Sprague-Dawley females, purchased from the same supplier, when they weighed about 185 g. Upon arrival at the laboratory, they were housed individually. Throughout all procedures, standard laboratory food designed for rodents (PMI Nutritional International, Brentwood, MO) and water were always available. The windowless room housing their cages was maintained at about 22 °C, with 12 h/day of incandescent light beginning at 0800 h. The institutional review committees of Siena College and RPI, both of which adhere to the Guide for Care and Use of Laboratory Animals (National Academy of Sciences, 1996), approved the procedures of this study, as well as the general care of the animals.

2.2. Estradiol valerate (EV)

EV, from Sigma Aldrich, was administered intramuscularly, at various doses. The carrier of EV was sesame oil, from Sigma Aldrich. Injections were 0.1 ml/kg bodyweight. An injection of the same volume of oil served as a placebo in each experiment. EV was designed to provide sustained release of estradiol and a single injection provides estradiol for a period of 3 to 4 weeks (Dusterberg and Nishino, 1982).

2.3. Ingesta

Each experiment involved presentation of CCM. The mix, purchased from a local supermarket, was Duncan Hines Devil's Food brand consisting primarily of complex carbohydrates, sugar, and cocoa powder. The mix provides 4.19 kcal/g. A 517 g box of mix was combined with 320 ml of water, forming a thick batter that did not spoil (at least, across the weeks of these experiments) and was not subject to spillage.

CCM was placed in glass cups (11.2 cm high, 6.2 cm diameter at the top) which were held in place in the rats' cages by a pair of springs. When presented, it was available for 24 h/day except for a few minutes each day when the containers were weighed and refilled.

2.4. General procedures

After a few days (ranging between 2 and 4 days across experiments) to habituate to the living conditions of the laboratory, the procedures of the individual experiments began. Estradiol has been shown to induce taste and place aversions in rats (de Beun et al., 1991; Flanagan-Cato et al., 2001). To reduce the possibility of conditioned taste aversions, CCM was not presented after EV injections until the animals regained weight lost in response to the injection. Weight loss was greater when doses were larger, and the time needed to regain pre-injection weight varied across experiments, ranging from 6 to 11 days.

Rats were weighed daily at about noon. At about the same time, measures of amount of CCM eaten were made. A container with batter was weighed before being placed in a cage. Each day, containers were removed from cages, weighed, and additional batter added to replace what was consumed, and reweighed before being returned to the cages. To assess loss due to evaporation, four containers of CCM were treated similarly but only placed on the rack of cages. Amount lost due to evaporation varied slightly across days, very little among samples, and was about 1.5 g/day.

2.5. Data reduction and statistics

Bodyweights and differences in amount of food in containers across 24 h, corrected for loss due to evaporation (g eaten to the nearest 0.1 g) are the raw data of the experiments. Intakes of CCM were analyzed in terms of the g of CCM taken per kilogram of bodyweight (g/kg). However, given that the EV-treated rats tended to weigh less due to failure to gain weight in the days immediately following EV administration, it could be argued that any differences in intakes expressed in g/kg are a product of weight differences. Consequently, intakes are also reported in g.

The procedures of Experiments 3 and 4 involved presentation of CCM before injections of doses of EV or vehicle (see introduction of Experiment 4 for rationale). The g/kg of CCM eaten daily during these preliminary days of presentation are not presented, because they are not germane to determining EV's effects. Subsequent to some signs of neophobia, all subjects took substantial amounts of CCM. Groups were formed such that bodyweights as well as mean intakes in g/kg, were statistically equivalent. Then, groups were assigned at random to receive EV or vehicle.

The data from each experiment (CCM consumed over 24 h, reported in g/kg and in g) conform to an analysis of variance (ANOVA) for repeated measures with factors for doses of EV (including the vehicle-only dose) and days of presentation of CCM.

3. Experiment 1: large doses of EV and intake of CCM

The dose of EV used in the initial experiments assessing intake of alcoholic beverages was 2 mg per female or 10 mg of EV per kg of rat (mg/kg). Since the interest was in seeing if this same dose would affect ingestion of CCM, the dose used in this experiment was 10 mg/kg.

3.1. Method

About 2 weeks after arrival in the laboratory, two groups of rats ($n=10$ each) were formed and equated for weight. One group, randomly chosen, was injected with 10 mg/kg EV and carrier; the other received just carrier. On the day of injection, the mean weight of the rats receiving EV was 221.68 g (S.D. = 15.29) and the mean for the rats receiving vehicle was 219.50 (S.D. = 17.71), $t(18)=0.295$, $p=0.772$. CCM was introduced 10 days post-injection. For 4 weeks, the rats had access to CCM for 4 consecutive days followed by 3 days without batter. During the 5th week, after 4 days of CCM, half of the subjects were given access to CCM for an additional 4 days, while the other half had access to white cake mix for 4 days. The results of this differential exposure to different mixes are not presented, because the issue in question is addressed in Experiment 2. Following this, for the next 20 days, no cake mix was presented. Then, all rats were given access to CCM for 4 more days. The

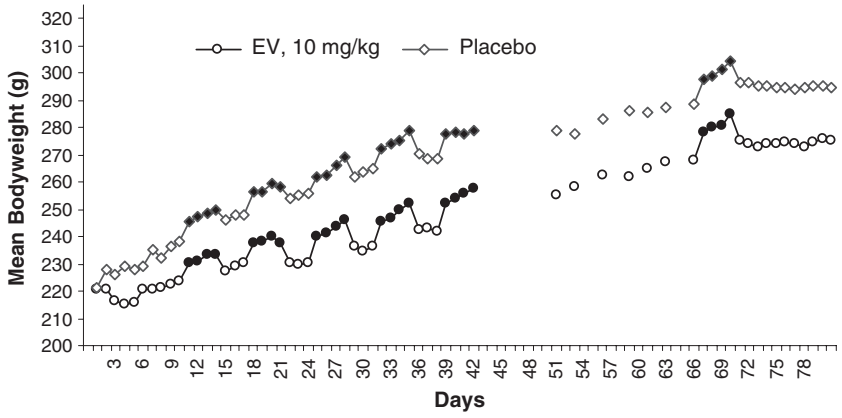

Fig. 1. The mean bodyweights of the two groups ($n=10$ each) are depicted across the days of the procedures. Weights were not taken on some days. The open data points are weights on days when no CCM was presented. Closed data points are weights when CCM was presented. Day 1 was the day of injections. Notice the reduction in weights of subjects of EV on days just after injections.

purpose of the final 4 days of access was to examine the longevity of the effects of a single injection of EV.

3.2. Results

The daily bodyweights of the two groups are summarized in Fig. 1 across the days following administration of EV. The loss of weight following an injection of EV, compared to the expected gain from the vehicle-injected females, is similar to that seen when we studied alcohol intake (Reid et al., 2002). Notice that after injections, the females receiving EV showed a slight loss in weight: for example, 4 days post-injection, the EV-treated rats had lost 6.23 g [$t(9)=3.179$, $p=0.011$] while the vehicle-treated rats had gained 9.44 g [$t(9)=5.98$, $p<0.001$]. At this point the two groups differed reliably in bodyweight, with the mean of the EV group=215.45 (S.D.=13.94) and the vehicle group mean=228.94 (S.D.=14.56), $t(18)=2.116$, $p=0.049$. The EV-treated animals did not begin to exceed their pre-injection weight until Day 9. Subsequently, they gained weight at a rate similar to that of females receiving vehicle, but remained somewhat smaller than controls. Subjects showed marked increments in bodyweight with the onset of taking CCM, and a statistically reliable decrease in bodyweight on the day immediately following removal of CCM. Weight loss with the end of presentations of CCM was unanticipated, and led to Experiment 2.

The amount of CCM eaten, expressed in terms of g/kg, for the two groups are summarized in Fig. 2. The first five series of 4 days' access were examined with a 2×20 mixed factorial ANOVA with factors of drug (EV, vehicle) and days of access to CCM. The ANOVA revealed a reliable effect for group, $F(1,18)=11.890$, $p=0.003$. The mean g/kg for the EV group was 106.62 (S.D.=11.01) and for the vehicle control group it was 85.73 (S.D.=15.68), with an effect size of $f=0.74$. There was also an effect for days [$F(19,342)=3.061$, $p<0.001$] but no reliable group×day interaction [$F(19,342)=0.539$, $p=0.944$]. In the absence of an interaction, the effect for days was not subjected to further analysis.

An ANOVA of intakes in g reveals that the EV-treated rats tended to consume more g than the vehicle controls, although the EV-treated animals were smaller. The mean g taken by the EV-treated animals was 25.92 (S.D.=2.66), compared with a mean of 22.65 (S.D.=5.01) for the vehicle controls [$F(1,18)=3.327$, $p=0.085$]. The effect size was $f=0.34$.

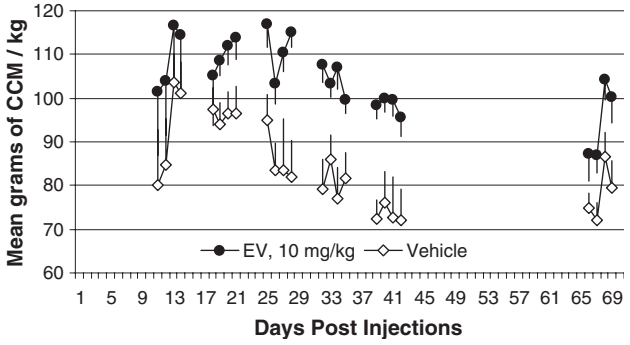

Fig. 2. The mean g of CCM per kg of bodyweight for each of two groups ($n=10$ for each group) are depicted. The bars are standard errors of the means.

Nearly 2 months post-injection, the rats were provided with a final 4 days of access to CCM. A 2×4 (drug×days) ANOVA was conducted to examine intakes. The EV-treated group consumed more g/kg CCM than the vehicle controls [$F(1,18)=7.770$, $p=0.012$]. The mean g/kg for the EV group was 94.60 (S.D.=14.01), and for the vehicle group the mean g/kg was 78.20 (S.D.=12.23); the effect size was $f=0.582$. There was a significant effect for days [$F(3,54)=10.262$, $p<0.001$], but in the absence of a significant drug×days interaction [$F(3,54)=0.587$, $p=0.626$] this effect was not examined further. An ANOVA of the last 4 days' CCM intake in g revealed $F(1,18)=2.426$, $p=0.137$ for the drug factor, with the EV group consuming a mean of 26.58 g (S.D.=4.35) compared with the vehicle group's mean of 23.54 (S.D.=4.38); the effect size for drug was $f=0.267$.

The EV-treated rats ate more g/kg CCM, on average, than their counterparts. Further, the enhanced eating persisted 60 days after the single injections of EV. The same dose of EV that enhances intakes of alcoholic beverages and palatable saccharin solutions also enhances intakes of CCM.

4. Experiment 2: changes in bodyweight following removal of access to chocolate and white cake mix

In Experiment 1, the weight loss associated with ending availability of CCM was unanticipated. We were curious to see if it would occur with a substance of similar complexity, but lacking cocoa. Consequently, in the next study, some female rats were given access to CCM while others were provided access to white cake mix (WCM).

In Experiment 1, reliable reductions in bodyweight occurred within 24 h after removal of rats' access to CCM. This phenomenon occurred on each of 10 occasions when CCM was withdrawn and measures were taken. CCM contains cocoa, a substance with known psychoactive components (e.g., the xanthine stimulants caffeine and theobromine). This experiment is a test of the idea that intake of a food containing cocoa powder induces changes in bodyweight regulation that are not seen with similar food without cocoa powder.

4.1. Method

Upon arrival at the laboratory, the females were housed individually. Standard laboratory food and water were always available. The CCM was the same used in Experiment 1. WCM was made by combining 302 g water with a standard box (517 g) of Duncan Hines White cake mix.

After 3 days habituating to their new housing and handling, the procedures began. The subjects were weighed daily. Based on initial weights, subjects were placed into two groups, such that mean weights were very similar [mean=207.09 g for CCM group, S.D.=3.79; mean=208.44 g for WCM group, S.D.=4.21, $t(16)=0.718$, $p=0.48$]. Groups were randomly assigned to get either CCM or WCM. Subjects then began a regimen of days of presentation of cake mix for 4 days

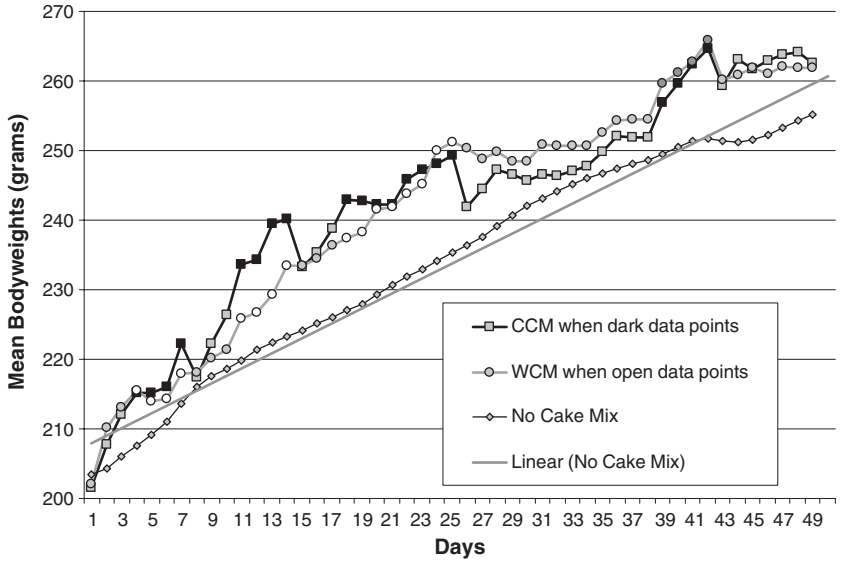

Fig. 3. The figure depicts mean body weights across the days of the experiment. The squares denote the means for the subjects who got CCM. The circles denote the means for the subjects who got WCM. The diamonds denote body weight gain for subjects who received no cake mix batter. When data points are gray, batter was not presented. The line without data points is a line of best fit for subjects getting no calorie-laden ingesta, just ordinary laboratory chow. For data points 39 to 42, CCM was presented to both groups.

followed by 3 days of no presentation. This procedure was followed for 2 weeks. For the third presentation, access to cake mix was extended to 8 consecutive days. Subsequent to these assessments, there were 13 days of no presentation of cake batter. Following this, all rats were given access to CCM for 4 consecutive days.

4.2. Results

Bodyweights are presented in Fig. 3. The data from Boswell et al. (2005), using 20 similar (vehicle-control) subjects given saccharin solutions to drink, in addition to the regular food and water, are presented to provide a context to judge the changes in weights that occur without presentation of cake mix. These comparison subjects lived in the same environment and were treated similarly to the subjects given CCM or WCM.

The rats, as expected, gained bodyweight across the days of the procedure. If no intervention is programmed, the trend line for the females not getting cake batter is the expected weight gain for this kind of subject, i.e., it represents the typical weight gain for female laboratory rats with standard food and water always available. With presentation of WCM and CCM, there were increases in bodyweights, relative to rats that received no batter.

The subjects took about the same amount of WCM and CCM (see Fig. 4). No reliable differences in intake were observed for any day of the procedure. Although we did not measure the amount of laboratory food taken by the females, it was noted that all subjects ate some of this food daily.

Despite taking similar amounts of batter, there were differences between the groups in bodyweights across the days when the different batters were presented and not presented, as can be seen in Fig. 3 and explored in more detail in Table 1. The percent body weight loss associated with the termination of CCM was 2.18%, 2.95%, and 3.29%, respectively, following the three episodes of access to the batter. With each termination of WCM, there was no or only slight weight loss. Subsequent to those three presentations, all rats were

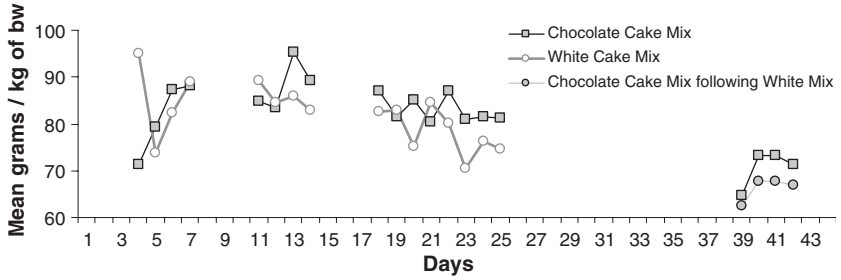

Fig. 4. Mean intake of batter for two groups in terms of g/kg of body weight. There was considerable overlap in distribution of intakes for the two groups and no statistically significant difference in intakes was tabulated for any day. The squares denote the means for the subjects who got CCM. The circles denote the means for the subjects who got WCM. Note for data points 39 to 42, CCM was presented to both groups.

Table 1
Weight change in response to removal of access to cake mix (weight in g)

Group	Last day		Day after		% change	t	p
	Mean	S.D.	Mean	S.D.			
Episode 1 (4-day access)							
CCM	222.23	3.66	217.34	4.17	−2.25	7.619	<0.001
WCM	217.98	9.41	218.07	7.94	0.04	0.121	0.906
Episode 2 (4-day access)							
CCM	240.10	9.63	233.22	9.58	−2.95	7.925	<0.001
WCM	233.45	8.10	233.49	8.06	0.02	0.041	0.920
Episode 3 (8-day access)							
CCM	249.27	9.80	241.75	9.27	−3.11	5.500	<0.001
WCM	251.29	11.34	250.36	11.71	−0.37	1.205	0.259
Episode 4 (4-day access: all received CCMB)							
CCM	264.60	13.71	259.23	12.91	−2.07	3.723	0.005
Previously WCMB	265.93	12.71	260.18	10.42	−2.21	6.162	<0.001

Weights of rats (n=10 per group) are in g; t-values are for correlated samples, df=9.

provided with access to CCM for 4 days. Upon termination, both groups: those initially receiving CCM as well as those initially receiving WCM, showed a similar amount of weight loss (Table 1, Episode 4).

5. Experiment 3: 1.5 mg/kg doses of EV and intake of CCM

The 10 mg/kg dose of EV provides supraphysiological doses of estradiol for a period of as many as 20 days and is apt to be toxic to the neurons of the arcuate nucleus of the hypothalamus (Desjardins et al., 1993). The next two experiments are explorations of considerably smaller doses that may not be toxic to the arcuate nucleus, but do provide continuous estradiol for a period of a number of days. This experiment assesses the dose of 1.5 mg/kg of EV, 11 to 19 days after it was administered.

5.1. Method

The 20 females of this experiment were the subjects of Experiment 2. The rats received no drug or treatment other than exposure to cake mix before the procedures of this experiment. Following the final 4-day presentation of CCM to all females, 10 days of no CCM elapsed before 10 rats were given 1.5 mg/kg EV and 10 were given vehicle. The EV and vehicle groups were comparable in bodyweight, prior history with cake mixes, and intake of CCM. The mean weight of the rats receiving EV was 262.11 (S.D.=11.36), and the mean weight of the vehicle control group was 263.96 (S.D.=8.78), $t(18)=0.399$, $p=0.695$. About 11 days post-injection, all rats were presented with CCM for 8 consecutive days.

5.2. Results

The EV-treated females, as those of Experiment 1, failed to gain weight across the first 5 to 6 days following injections whereas their counterparts gained weight across this period. The amounts eaten, in terms of g/kg, are summarized in Fig. 5. A 2×8 mixed factorial ANOVA with factors of group and days revealed a reliable effect for group [$F(1,18)=24.955$, $p<0.001$]. The mean g/kg eaten by the EV group was 98.69 (S.D.=11.28) compared with 74.76 (S.D.=10.11) for the vehicle-control group. The effect size was $f=1.09$. There was also a reliable effect for days [$F(7,126)=13.176$, $p<0.001$], but not for the interaction [$F(7,126)=1.587$, $p=0.145$]. In the absence of an interaction, the variation in intakes across days was not examined further.

Fig. 6 presents the mean g of CCM taken by the subjects across the 8 days of measurement. A 2×8 mixed factorial ANOVA showed a significant effect of group [$F(1,18)=13.346$, $p=0.002$]. The effect size was $f=0.79$. As was found with the analysis of intakes in g/kg, there was significant variation in g of intake across days [$F(7,126)=14.414$, $p<0.001$] but no group×day interaction [$F(7,126)=1.285$, $p=0.263$]. These data indicate that the EV-treated females were taking more g of batter although they were smaller.

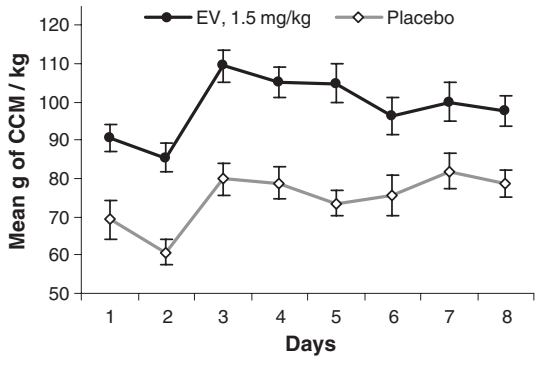

Fig. 5. The mean g of CCM eaten per kg of bodyweight on each day for each group is depicted. The bars are standard errors of the means.

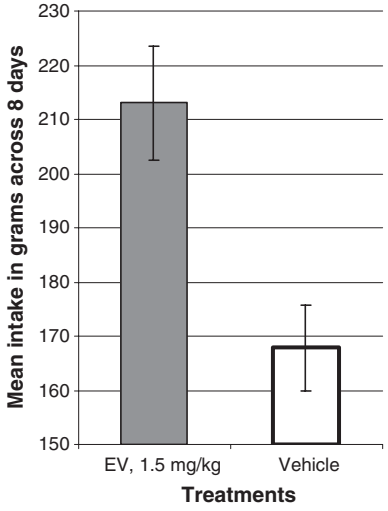

Fig. 6. The means of total grams of CCM eaten by the two groups of females, during the 8-day CCM was presented, are depicted. The bars are standard errors of the mean. There were 10 females in a group.

6. Experiment 4: dose–response assessment, EV and intake of CCM

The 1.5 mg/kg dose of EV is very large compared to the doses of estradiol that affect events of the estrous cycle. The question of whether considerably smaller doses might also enhance intakes is addressed by testing four doses of EV, repeatedly halving the 1.5 mg/kg dose used in Experiment 3: the doses assessed were 0.75, 0.38, 0.19 and 0.09 mg/kg of bodyweight, plus a vehicle control.

A methodological change was made here in comparison with Experiment 1, to reduce the possibility of neophobic responses to CCM affecting the assessment of EV's effects. In Experiment 1, one of the subjects receiving EV took no CCM for 6 days, but thereafter took it in amounts characteristic of her group. One subject receiving vehicle took no CCM for 2 days before taking it in characteristic amounts. This reluctance to take a novel food has been called neophobia. The data of both subjects showing neophobia were included in the results. If the neophobia of these two subjects happened to occur by chance within a group, it could have seriously confounded measures designed to assess appetite for an ingesta usually taken readily. Individual differences in degree of neophobia also increase the variability of intake within groups. In Experiment 3, neophobia was not a concern because all rats had considerable exposure to either white or chocolate mix, and all animals had at least 4 days' access to CCM prior to injection. To avoid the potential problem of neophobia in Experiment 4, CCM was presented for a period of days before injections, and injections given only after all subjects had demonstrated significant consumption of CCM.

6.1. Method

Forty subjects were assigned to one of five groups, arranging for nearly equal mean bodyweights across groups; groups were then randomly assigned to drug condition. By the end of an initial 8 days of exposure to CCM, all subjects were taking the amounts of CCM daily that we have come to expect without any experimental interventions. The groups' mean intakes a day before injections ranged from 98.3 to 106.9 g/kg, and an ANOVA indicated no reliable differences among them [$F(4,35)=0.391$, $p=0.814$]. The bodyweights among groups a day before injections were also nearly equivalent, ranging from 200.7 to 203.7 g [$F(4,35)=0.095$, $p=0.983$].

CCM was re-introduced 8 days after injections (the 1st measures are 9 days after injections), and presented for 8 consecutive days.

6.2. Results

The intakes in g/kg were examined with a 5×8 (drug×days) ANOVA. There was a significant difference among intakes of the groups [$F(4,35)=6.280$, $p=0.001$] (effect size $f=0.73$). There was also a significant effect of days [$F(7,245)=8.544$, $p<0.001$], showing a general tendency for intakes to increase and then decrease: the mean intakes were 96.0, 85.9, 101.0, 100.4, 103.5, 101.9, 94.0, and 91.6 g for days 1–8, respectively. The interaction was statistically significant [$F(28,245)=1.545$, $p=0.044$], but upon inspection, revealed no meaningful pattern.

Fig. 7 summarizes the g/kg consumed at each dose of EV. A post-hoc comparison of means (LSD, $\alpha=0.05$) indicated that the 0.09 mg/kg dose did not increase CCM intake compared with the vehicle-control. Intakes of the 0.19 mg/kg group were not reliably higher than the 0.09 mg/kg group. However, the doses of 0.19, 0.38, and 0.75 mg/kg were all associated with reliably higher intakes than the vehicle-control condition, and none of these doses differed from one another in terms of CCM intake.

An analysis of CCM intake in g led to conclusions nearly identical to the g/kg analysis regarding the dose–response relationship. There was an effect for drug, $F(4,35)=5.016$, $p=0.003$; for day, $F(7,245)=9.454$, $p<0.001$; and, for the interaction, $F(28,245)=1.415$, $p=0.087$. The overall mean g consumed each day ranged from a low of 20.0 g on Day 2 to a high of 24.8 on Day 5. The mean g intakes for each group were as follows: vehicle control=19.07 (S.D.=3.28), 0.09 mg/kg=21.42 (S.D.=3.67), 0.19 mg/kg=24.69 (S.D.=2.59), 0.38 mg/kg=25.42 (S.D.=2.71), and 0.75 mg/kg=24.88 (S.D.=4.66). Post-hoc comparisons of the group means (LSD, $\alpha=0.05$) showed the vehicle-control consumed less CCM than all other groups except the .09 mg/kg group. The 0.19, 0.38, and 0.75 mg/kg groups did not differ statistically from one another.

7. Discussion

A recently published, comprehensive review of the literature on regulation of energy homeostasis stated "Gonadal steroids, including estradiol and testosterone, have also been long known to reduce appetite, increase energy expenditure, and decrease adiposity" (Horvath et al., 2004, p. 236). In addition, it has been long known that ovariectomy leads to weight gains (see, Eckel, 2004, for another review). There is a nice symmetry to these long known generalizations (applications of estradiol reduce

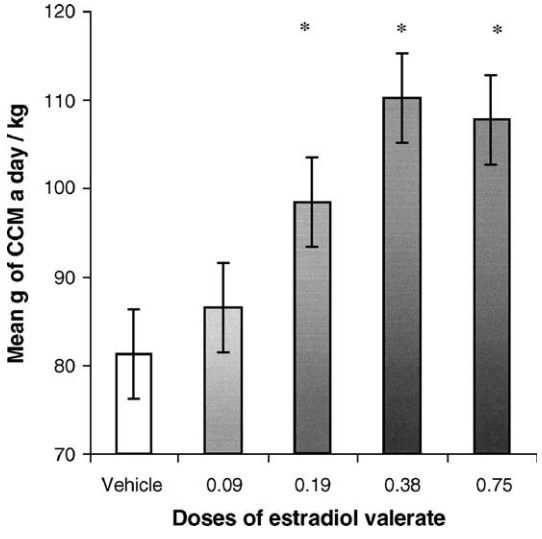

Fig. 7. Mean intake across the 8 days of opportunity to take CCM for each group ($n=8$), in terms of total g/kg for the 8 days, are depicted. The bars are standard errors of the means. *p-values<0.05 compared to vehicle controls.

appetite; ovariectomies enhance appetite). However, the generalization that estradiol reduces appetite is limited to certain methodological approaches. Procedures that minimize neophobia, and that disassociate a malaise accompanying the initial days of application of continuous estradiol from a presented ingesta, are salient to our observation that estradiol can increase intake of some substances. Yes, estrogenic applications can, and often do, reduce intakes and bodyweights initially, but those same applications can also increase intakes of some ingesta, including alcoholic beverages and CCM, subsequently. Estradiol does not induce a general increase in appetite. Bittersweet solutions, for example, are taken less during the same time that similar injections are increasing appetite for sweet solutions (Boswell et al., 2005). Estrogenic stimulation can enhance appetites for calorie-laden or otherwise palatable ingesta, and the enhancement can persist for weeks beyond a single injection of EV.

Measures of CCM-intake during Experiment 1 were made 4 days/week to reduce the experimenters' work on weekends. The rats, therefore, experienced abstinence from CCM every weekend. To our surprise, this resulted in rapid weight-loss with the end of presentation of CCM. With every end of daily intake of CCM that spanned 4 days or more, we observed about a 3% loss in bodyweights. There is no question about the statistical significance of the phenomenon (it was observed 23 times across the experiments with groups of $n=8$ or 10, with p-values <0.05 on each occasion leading to an infinitesimally small p-value for the combined data). Experiment 2 indicates that the weight loss is associated with the large intake in cocoa powder (chocolate) in the CCM. Tarka and colleagues have done extensive studies (e.g., Tarka et al., 1986; 1991) looking for signs of toxicity among laboratory animals fed cocoa powder and related substances. They found few signs of toxicity; however, their measurements did not include body-weight measures with the termination of days of ingestion. This does not mean, however, that cocoa powder (chocolate) is without potent effects. Modest amounts of chocolate intake can be lethal to dogs (Stidworthy et al., 1997).

We presume that the salient difference between the two mixes involves the cocoa powder, but cocoa is a chemically complex substance containing caffeine and theobromine. More study will be required to identify the component(s) responsible for the weight loss observed here. The salient mechanism might be how fast the two batters move through the gut, or it might be a disturbance in the hedonic regulatory processes of the brain. Morphine withdrawal, for example, apparently involves both of these kinds of mechanisms. Regardless of the mechanism involved, it is clear that the termination of presentation of CCM induces a disturbance in bodyweight regulation that is not seen with the termination of WCM. We have recently collected data indicating that removing access to a fat/sugar mixture (after several weeks of continuous access) does not lead to rapid weight loss; the weight changes are similar to those observed with WCM (Reid et al., 2005). Extensive intake of CCM induces changes that manifest themselves as a disturbance in homeostasis (allostasis, according to McEwen, 2004) when CCM presentation is terminated that is not observed with similar foods without cocoa powder.

The results of Experiment 4 indicate that estradiol's effect seems to asymptote at a dose between 0.19 and 0.38 mg/kg. The amounts of intake induced by these doses are similar to amounts induced by 1.5 and 10.0 mg/kg. This seemingly maximum effect might be due to a limit in the amount of CCM that these animals can ingest given the size of their gut. Possibly, with other ingesta, increments in intake may occur with increasing doses of EV. Alternatively, a dose in the range of 0.19 to 0.38 may be sufficient to saturate the estradiol receptors across the days of the experiment and hence further dosing may be superfluous. If there were measures extending beyond the days when we measured intakes, a dose–response relationship associated with duration of effect of doses greater than 0.38 mg/kg may be observed. As seen in Experiment 1, the effects of the 10 mg/kg dose were observed 2 months post-injection. Boswell et al. (2005) found that a 10 mg/kg dose enhanced intake of a saccharin solution for 3 months.

Experiment 4 did not determine the minimally effective dose of EV that will enhance eating. A t-test of the total g/kg over 8 days comparing the effects of 0.09 mg/kg to controls yields a $t(16)=1.88$, $p=0.09$, with an effect size of $f=0.594$. With respect to amount of circulating estradiol, there is a complicated relationship between duration of effect and peak effect that is difficult to untangle with EV. It will take a different kind of procedure to determine the minimally effective amount of estradiol that has to circulate for a critical period of time to establish an enhanced appetite for ingesta such as CCM. The possibility that the 0.09 g/kg dose (or a similar dose of estradiol provided in a different way) may have enhancing effects that do not exactly correspond to the time when we provided CCM leaves open the question of the minimally effective dose of estradiol (in terms of both peak and duration of effect) that is apt to enhance intakes.

A 2 mg/female dose of EV (about 10 mg/kg) leads to a disruption of the arcuate nucleus of the hypothalamus and produces effects similar to those of a lesion (Brawer et al., 1993). Lesions of the arcuate nucleus disrupt the usual circadian rhythm of ingestion (Reid et al., 1982) and this may be a factor in the enhanced ingestion. We do not know if doses as small as 0.19 mg/kg produce lesion-like effects. Smaller doses, such as 0.19 mg/kg, might involve a dampening of arcuate nucleus functioning, but not induce permanent changes; i.e., their effects may wane subsequent to the release of estradiol. The 10 mg/kg dose, however, probably induces a more permanent change (Brawer et al., 1993) and, therefore, induces an effect that far outlasts the release of estradiol. If that is the case, during a time of enhanced eating there may be no correlation to the amount of circulating estradiol. This is another potential reason that a relationship between estradiol and an enhanced appetite has not previously been observed.

If the doses used in Experiments 3 and 4 had not enhanced appetite for CCM, a reasonable conclusion would be that the effect seen with the large dose (i.e., 10 mg/kg) was related to that dose's probable toxic effect on the arcuate nucleus. However, the results indicate that much smaller doses can,

under some circumstances, enhance intakes of some kinds of ingesta. These results provide a new perspective and open the possibility that doses more commonly used as medicines might also enhance appetite. This possibility is supported by recent results of Juarez et al. (2005) showing that very small doses of estradiol, when given daily, can enhance intakes of alcoholic beverage.

An alternative is worth considering, since the smaller doses produced effects similar to the large dose used by Brawer et al. (1993). There is a possibility that the smaller doses of estradiol might also induce some toxicity with enduring effects. Continuous supraphysiological levels of estradiol, even if the levels are very low, might produce enduring changes in behavior such as eating, due to producing enduring changes in the structure of the neural control of appetite. These enduring changes in neural structure may not be manifest in usual tests for toxicity (e.g., indices of breast cancer) and, therefore, go unnoticed.

The smaller doses of EV, e.g., 0.38 mg/kg, clearly deliver supraphysiological doses of estradiol for a number of days, but the peak levels achieved shortly after injection wane gradually over days. We (Reid et al., 2003a) measured estradiol levels following 3.0 mg/kg of EV 4 and 12 days after the injection and found levels of 1416 and 278 pg/ml of serum, respectively. Extrapolating from that limited data, the levels of circulating estradiol 12 days after 0.38 mg/kg (a time when CCM intake was enhanced) is within the range of circulating estradiol during pregnancy.

We introduced this paper referring to the epidemic of obesity troubling citizens of the USA. We noted that women are more apt to be obese than men. We studied female rats getting a range of doses of EV, an estrogenic compound that has sustained effects. Do our results have any bearing on the incidence of obesity in human females?

Given the nature of our procedures, we did not provide the CCM across a sufficiently large number of days to observe the full development of obesity, if any, that might emerge with continuous opportunity to take CCM. We did observe an increase in appetite for a calorie-dense food that was sustained for days. Such a condition is likely to lead to obesity, if maintained over time. Some recently collected data in our laboratory suggests this is the case. We presented a mixture of fat and sucrose continuously for 2 months, and observed slightly higher daily intakes among the EV-treated females throughout this time. The EV group also showed reliably greater weight gain compared with controls (Reid et al., 2005).

Even our smallest dose of EV is large in comparison with a dose of estradiol that will reliably modify events associated with the estrous cycle in female rats. Further, and perhaps most importantly, the injection of EV provides a continuous dose of estradiol that is probably sufficient to saturate the estrogen receptors continuously for days (Dusterberg and Nishino, 1982). Are women exposed to such dosing? Hormone therapy both for problems associated with menopause and for birth control provides regimens of high doses of estrogenic drugs with sustained effects. Estrogenic compounds are among the most prescribed drugs in the USA. EV is a component of an injection touted by the World Health Organization as a once a month regimen to control births for use in developing countries, particularly those in Latin America (Bassol et al., 2000). (Incidentally, in a test, the women who used the injection, on average, gained 1.02 kg during a year with a few gaining more than 5 kg, there were no placebo controls.) In addition, a number of herbal "medicines" are estrogenic and are advertised as "treatments" for menopausal discomforts and aging. Popular diets, such as those based in certain soy products, have considerable potential to provide additional estrogenic stimulation. Presently, we do not know if women are exposed to sufficiently large doses of estrogens to enhance appetite for ingesta such as alcoholic beverages and foods containing cocoa. However, it is clear that many women are exposed to considerable amounts of exogenous estrogenic compounds, sometimes for many years. Gavaler et al. (2004) studied the estradiol levels in post-menopausal women receiving hormone replacement therapy. They found considerable variability in amounts of circulating estradiol. For over 10% of the women, the level of circulating estradiol was greater than six standard deviations above the mean level of untreated post-menopausal women. An effect of estradiol on appetite need not be large to be relevant. It is estimated that an additional, daily intake as small as 100 cal beyond the calories used, if sustained, is sufficient to establish obesity among women (Hill et al., 2003).

There are, of course, the obvious limitations of extrapolating data from simple laboratory experiments with rats to the complex world of food choices experienced by women. Our data are more directly relevant to the generalization that estradiol reduces intake of ingesta, since that generalization followed from conclusions derived from data collected with female rats. Clearly, our EV-treated rats did not have reduced appetites for CCM. Consequently, it is an overgeneralization to conclude that estradiol and similar estrogenic drugs are anorectic. The data of these experiments, showing that under some circumstances estrogenic stimulation can enhance appetite, sets the stage for a better understanding of estrogen's role in controlling ingestion.

While studying estradiol's effects on alcoholic intake in male rats, Juarez et al. (2005) observed that, in castrated male rats, treatment with estradiol, under some circumstances decreased intakes and under other circumstances increased intakes, findings similar to findings with EV in female rats (Reid et al., 2002).

Juarez et al. (2005), citing studies demonstrating that estrogen treatments modulate opioid receptors, theorized that estradiol's initial effects (reduced intakes) were due to an initial down regulation of opioid receptors (an effect similar to opioid antagonism), but subsequently, there was up regulation of opioid receptors (an effect similar to that of an opioid agonist). Such a sequence of events would account for estradiol's initial reduction in intake of alcoholic beverages, and other ingesta, as well as the subsequent enhanced intakes of alcoholic beverages and palatable food (such as CCM).

Theorizing that estradiol's effects on ingestion are related to its effects on opioidergic systems (particularly up and down regulation of opioid receptors) is not incompatible with the

more molar theorizing that the initial effects of estrogens set the circumstances for conditioned taste aversions, but that the conditions for that aversion wanes with the continuation of estradiol's presence. This theorizing is not incompatible with the more abstract idea that continuous high levels of estrogen, which is a condition of pregnancy, induces food avoidances during the initial period of pregnancy, but enhances appetite subsequently (particularly for carbohydrates) in preparation for the high demands for calories during pregnancy and lactation. The modulation of appetite by estradiol, via an opioidergic system, would have adaptive significance, protecting against potential toxins by sensitizing to bitter foods and enhancing the appetite for high calorie foods whose ingestion is not paired with nausea.

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