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Ovarian hormones modulate 'compulsive' lever-pressing in female rats

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ABSTRACT

Life events related to the female hormonal cycle may trigger the onset of obsessive–compulsive disorder (OCD) or exacerbate symptoms in women already suffering from it. These observations suggest a possible role for ovarian hormones in the course of this disorder. Yet, the mechanisms that may subserve the modulatory effect of ovarian hormones are currently unknown. The aim of the present study was therefore to test the role of ovarian hormones in the signal attenuation rat model of OCD. Experiment 1 compared the behavior of pre-pubertal and adult male and female rats in the model, and found no age and sex differences in compulsive responding. Experiment 2 found that compulsive responding fluctuates along the estrous cycle, being highest during late diestrous and lowest during estrous. Acute administration of estradiol to pre-pubertal female rats was found to attenuate compulsive behavior (Experiment 3), and withdrawal from chronic administration of estradiol was shown to increase this behavior (Experiment 4). These findings extend the use of the signal attenuation model of OCD to female rats, and by demonstrating that the model is sensitive to the levels of ovarian hormones, provide the basis for using the model to study the role of ovarian hormones in OCD. In addition, the present findings support the hypothesis that the increased risk of onset and exacerbation of OCD in women post-partum may be a result of the decrease in the level of estradiol, which was elevated during pregnancy.

Introduction

Obsessive–compulsive disorder (OCD) is a psychiatric disorder with a life time prevalence of 1–3% (Sasson et al., 1997), characterized by recurrent, intrusive and unwanted thoughts (obsessions) and/or repetitive ritualistic behaviors (compulsions) (American Psychiatric Association, 1994). Several lines of evidence suggest that ovarian hormones play a modulatory role in OCD (Uguz et al., 2007). The age of onset of OCD in women is later than in men, and has a bi-modal distribution with the first peak occurring between 13 and 16 years of age and the second peak between 22 and 32 years, i.e. around puberty and child-bearing years, respectively (Brandes et al., 2004). In addition, premenstruum, pregnancy and post-partum are associated with an increased risk of onset and exacerbation of OCD (Abramowitz et al., 2003; Labad et al., 2005; Maina et al., 1999; Vulink et al., 2006). Yet, the mechanisms that may subserve the modulatory effect of ovarian hormones are currently unknown. Uncovering these mechanisms may be advanced by the use of appropriate animal models of OCD, which closely mimic its behavioral and neural manifestations.

Up to date there have been only a few studies that assessed the role of ovarian hormones in compulsive behavior in animals (Agrati et al., 2005; Fernandez-Guasti et al., 2006; Ulloa et al., 2004), and these studies have been conducted using the 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide) model of OCD, developed by Yadin et al. (1991). In this model, an acute injection of the 5-HT1A agonist 8-OH-DPAT leads to a decrease in spontaneous alternation in a T-maze, and this decreased alternation is hypothesized to model perseveration and indecision in OCD (Yadin et al. 1991). Although the predictive validity of this pharmacological model is fair, its suitability for studying the neurobiology of OCD has been questioned (Joel, 2006a).

The aim of the present study was therefore to test the role of ovarian hormones in the signal attenuation rat model of OCD, which has been shown to involve neural systems implicated in OCD (for review see Joel, 2006b). This model was developed on the basis of the theoretical proposition that compulsive behaviors result from a deficit in the feedback associated with the performance of normal goal-directed responses (e.g., Baxter, 1999; Gray, 1982; Malloy, 1987; Pitman, 1987, 1991; Reed, 1977; Szechtman and Woody, 2004, for review see Otto, 1992). In the model, attenuation of a signal indicating that a lever-press response was effective in producing food, leads, in a subsequent extinction test, to excessive lever-pressing that is not accompanied by an attempt to collect a reward. This behavior, which we have named 'compulsive' lever-pressing because it may be analogous to the excessive and unreasonable behavior seen in OCD, is abolished by the selective serotonin reuptake inhibitors fluoxetine, paroxetine and fluvoxamine, but not by the anxiolytic drug, diazepam, the antipsychotic, haloperidol, or the tricyclic antidepressant, desipramine (Joel and Avisar, 2001; Joel and Doljansky, 2003; Joel et al.,

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2004), in accordance with the differential efficacy of these drugs in alleviating obsessions and compulsions in OCD patients (e.g., Dolberg et al., 1996; Piccinelli et al., 1995; Zohar et al., 1992). In addition, manipulations of the rat orbital cortex affect compulsive lever-pressing (Joel et al., 2005a,b; Joel and Klavir, 2006), in line with the fact that functional imaging findings in OCD patients consistently implicate the orbitofrontal cortex in this disorder (for a recent review see Friedlander and Desrocher, 2006). Finally, dopaminergic and serotonergic manipulations have been shown to affect compulsive lever-pressing (Flaisher-Grinberg et al., 2008; Joel et al., 2001, 2004; Joel and Doljansky, 2003), in line with clinical evidence implicating these systems in OCD (for review see Goodman et al., 1990; McDougle et al., 1993; Stein, 2002).

Experiment 1 assessed sex and age differences in the behavioral response to signal attenuation. Specifically, compulsive lever-pressing of pre-pubertal (7 weeks) and mature (4 months) female and male rats was assessed. Experiment 2 assessed compulsive lever-pressing in female rats at the different stages of the estrous cycle. Because this experiment revealed a close association between compulsive lever-pressing and estradiol levels in female rats as reported in the literature (Haim et al., 2003; Shaikh, 1971; Spornitz et al., 1999), Experiment 3 tested the effects of an acute administration of estradiol on this behavior. Finally, since Experiment 3 revealed that estradiol had an anti-compulsive effect in the model, Experiment 4 tested whether withdrawal from repeated administration of estradiol, as occurs post-partum, will result in an increase in compulsive lever-pressing. It should be noted that in Experiments 3 and 4, rather than using ovariectomized rats as has been done in previous studies (Fernandez-Guasti et al., 2006), we chose to use pre-pubertal female rats, in which the levels of ovarian hormones are quite stable (Noda et al., 2002).

Experiment 1: The effects of age and sex in the signal attenuation model

In order to test the effects of age and sex in the signal attenuation model, compulsive behavior of male and female rats, aged 7 weeks and 4 months (at the Test day) was assessed. The above ages were chosen since before 60 days of age rats are defined as being at a developmental stage of adolescent, while rats at the age of 60 days and above are defined as being at the developmental stage of adulthood (Tirelli et al., 2003). Moreover, the age of sexual maturity and of first ovulation in female rats is typically above 40 days (Kohn and Clifford, 2002; Ricu et al., 2008).

Methods

Subjects

Male and female Sprague–Dawley rats (Harlan, Jerusalem, Israel), 5 weeks or 4 months old at the beginning of the experiment, were housed 4–5 to a cage under a reversed 12-h light–dark cycle (lights on 1900–0700). Rats were maintained on a 22-h food restriction schedule (see below), with water freely available. They were weighed twice a week to ensure that their body weight was not reduced to below 90% of the weights of free-feeding rats, based on growth curves (Harlan, http://www.harlan.com/models/spraguedawley.asp). All experimental protocols were carried out according to the guidelines of the Institutional Animal Care and Use Committee of Tel Aviv University.

Apparatus and behavioral procedure

Behavioral testing was conducted in eight operant chambers (Campden Instruments, Loughborough, UK), housed in sound-attenuated boxes and equipped with a 3 W house light, a Sonalert module (Model SC 628) that could produce a 80 dB 2.8 kHz tone, and two retractable levers on either side of a food magazine (fitted with a 3 W magazine light), into which 45 mg Noyes precision food pellets (Noyes, Sandown Chemical Limited, Hampton, England) could be delivered. Access to the food magazine was through a hinged panel, the opening of which activated a micro-switch. Equipment programming and data recording were computer controlled.

Prior to the beginning of the experiment, rats were handled for about 2 min daily for 5 days. A 22-h food restriction schedule began simultaneously with handling and continued throughout behavioral testing. Food was provided in the home cage at least half an hour after the end of the session. On the last 3 days, 20–30 food pellets used as reinforcement for operant training were introduced after handling into the home cages on a tray. The tray was removed from the cage after each rat was observed to consume at least 2 pellets.

The post-training signal attenuation (PTSA) procedure included 4 stages:

Stage 1: magazine training. On Days 1–3, rats were trained to collect food pellets from the food magazine in the operant chamber, with the levers retracted. On each trial, a single food pellet was dropped into the food magazine, simultaneous with the onset of a compound stimulus consisting of the magazine light and the tone. The compound stimulus was turned off after the rat's head entered the food magazine or after 15 s had elapsed, and a 30-s intertrial interval began (for more details see Fig. 1). On each day, each rat was trained until it completed 30 trials in which it inserted its head into the food magazine during stimulus presentation (collected trials), or until a total of 40 trials was reached. The number of collected trials and the total number of trials were recorded.

Stage 2: lever-press training. On Day 4, rats received a session of pre-training using a free-operant schedule. The houselight was on and one lever was present in the operant box throughout the entire session. Responding on this lever (reinforced lever, RL) resulted in the delivery of a single food pellet into the magazine, accompanied by the presentation of the compound stimulus (magazine light and tone). The stimulus was turned off after the rat's head entered the food magazine or after 15 s from the rat's first lever-press had elapsed. The lever designated as RL was counterbalanced over subjects and remained the same for each rat over the entire experimental procedure. Each rat was trained until it completed 30 trials, that is, pressed the lever and inserted its head into the food magazine during stimulus presentation. Rats that failed to attain 30 completed trials within 30 min, were returned to the test chamber at the end of the day for an additional session. On Days 5–7, rats were trained to lever-press in a discrete-trial procedure. On each trial, both levers were inserted into the chamber. Responding on the RL resulted in the delivery of a single food pellet into the magazine, accompanied by the presentation of the compound stimulus. The levers were retracted and the compound stimulus was turned off, after the rat's head entered the food magazine or after 15 s (Day 5, 10 s Days 6 and 7) from the rat's first lever-press had elapsed. Further lever-presses on the RL as well as responding on the other lever (nonreinforced lever, NRL) had no programmed consequences (for more details see Fig. 1). Each trial was followed by a 30-s intertrial interval. Each rat was trained until it completed 40 trials, that is, pressed the lever and inserted its head into the food magazine during stimulus presentation, or for a total of 60 trials.

In order to assess acquisition of the lever-press response, the number of trials on which the rat did not press the RL (unpressed trials) and the number of trials on which the rat pressed the RL without inserting its head into the food magazine (uncompleted trials) were recorded in addition to the number of completed trials. In order to assess rats' tendency for excessive lever-pressing, the number of lever-presses on the NRL, and the number of lever-presses on the RL after the

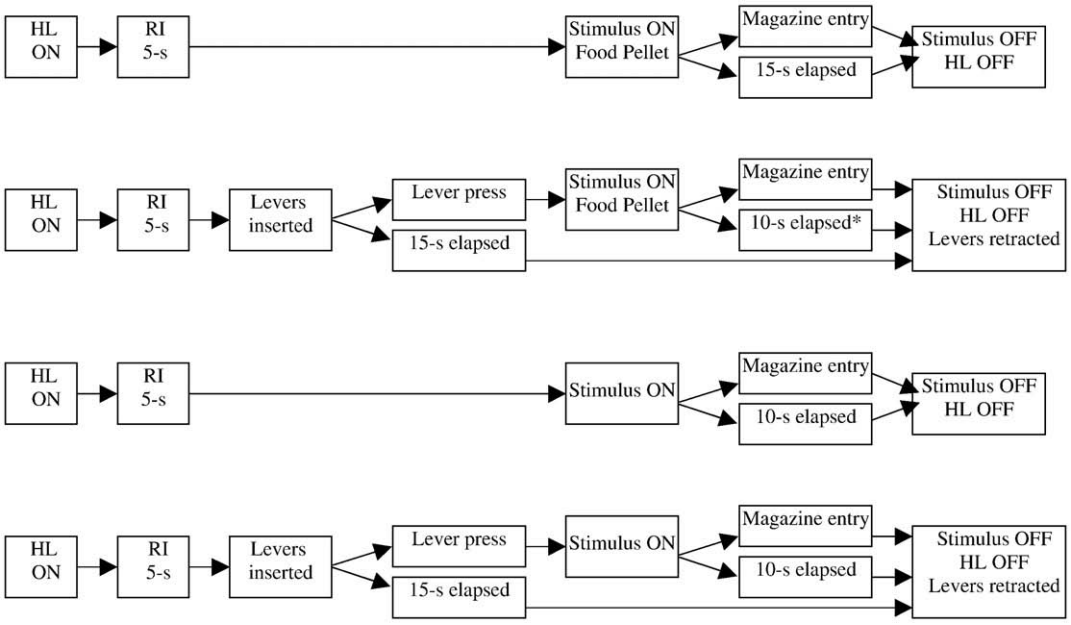

Fig. 1. A schematic diagram of the organization of a trial in each of the different training stages of the post-training signal attenuation procedure. HL, houselight; RI, random interval; * on the first day of lever-press training (Day 5) this time limit was 15 s.

first response (extra lever-presses, ELP) were recorded. The latter measure was further subdivided into ELP in uncompleted trials (that is, ELP not followed by insertion of the head into the food magazine while the stimulus is on; ELP-U), and ELP in completed trials (ELP-C).

Stage 3: signal attenuation. On Days 8–10, with the levers retracted, rats were exposed to the presentation of the compound stimulus as on Days 1–3, but no food was delivered to the food magazine (for more details see Fig. 1). Rats received 30 such trials on each day, and the number of collected trials was recorded. Rats that had more than 12 collected trials on Day 10 were returned to the test chamber at the end of the day for an additional session.

Stage 4: test. On the following day, rats were trained as in the lever-press training stage, except that no food was delivered to the food magazine, that is, pressing the lever resulted in the presentation of the compound stimulus only (for more details see Fig. 1). The session lasted 60 trials. The behavioral measures recorded included the same measures recorded in the lever-press training stage. *Compulsive lever-pressing is operationally defined as the number of ELP-U in the test stage of the post-training signal attenuation procedure.*

Statistical analysis

Rats' performance on the test stage was analyzed using ANOVA with main factors of Age (pre-pubertal/mature) and Sex (male/female). Significant interactions were followed by post hoc least significant difference (LSD) comparisons. For all comparisons, significance was assumed at $p<0.05$ (two-tailed). Performance on the magazine training, lever-press training and signal attenuation stages was also analyzed, to ensure that differences in performance at the test stage were not a result of an earlier difference. Performance on the magazine training stage was analyzed using mixed ANOVA with main factors of Age and Sex and a repeated measurements factor of Days performed on the number of uncollected trials (i.e., trials on which the rat did not enter the magazine during stimulus presentation) on the three sessions of the magazine training stage. Performance on the lever-press training stage was analyzed using mixed ANOVA with main factors of Age and Sex and a repeated measurements factor of Days performed on the number of unpresssed trials and on the number of ELP-C on the three sessions of lever-press training (the variability of the other variables was too low to enable statistical analysis, as all rats achieved 40 completed trials and most rats had no uncompleted trials). Performance on the signal attenuation stage was analyzed using a mixed ANOVA with main factors of Age and Sex and a repeated measurements factor of Days performed on the number of collected trials (i.e., trials on which the rat's head entered the magazine during stimulus presentation) on the three sessions of the signal attenuation stage.

Results

Of the 44 rats that underwent PTSA, the data of one rat were lost due to computer failure at the Test and two rats were excluded from the analysis because their performance on one of the behavioral measures in the test was more than 4 standard deviations higher than their group mean. Thus, the final analysis included 10, 10, 12 and 9 rats in the pre-pubertal females, pre-pubertal males, mature females and mature males groups, respectively.

On the first day of *magazine training*, mature female rats had more uncollected trials compared to the other groups, but this difference was not present on the next 2 days of magazine training (Fig. 2A, an Age×Sex×Days ANOVA revealed a significant main effect of Age, $F(1,37)=5.9$, $p<0.05$, and a significant Age×Sex×Days interaction, $F(2,74)=3.78$, $p<0.05$, only). Post hoc LSD confirmed that the mature female group was significantly different from the other three groups on the first training day (all p's<0.05) only (all p's>0.2).

On the first day of *lever-press training*, mature female and male rats performed a higher number of unpressed trials compared to pre-pubertal male and female rats (Fig. 2B, an ANOVA revealed a significant main effect of Age, $F(1,37)=10.55$, $p<0.005$, and a significant Age×Days interaction, $F(2,74)=8.43$, $p<0.005$, only). Post hoc LSD comparisons comparing mature rats to pre-pubertal rats on each of the 3 days confirmed that this difference was significant on the first day of training only ($p<0.0005$). Mature rats also performed a higher number of ELP-C compared to pre-pubertal rats, and this difference persisted throughout training (Fig. 2C, an ANOVA revealed a significant main effect of Age, $F(1,37)=4.97$, $p<0.05$, only).

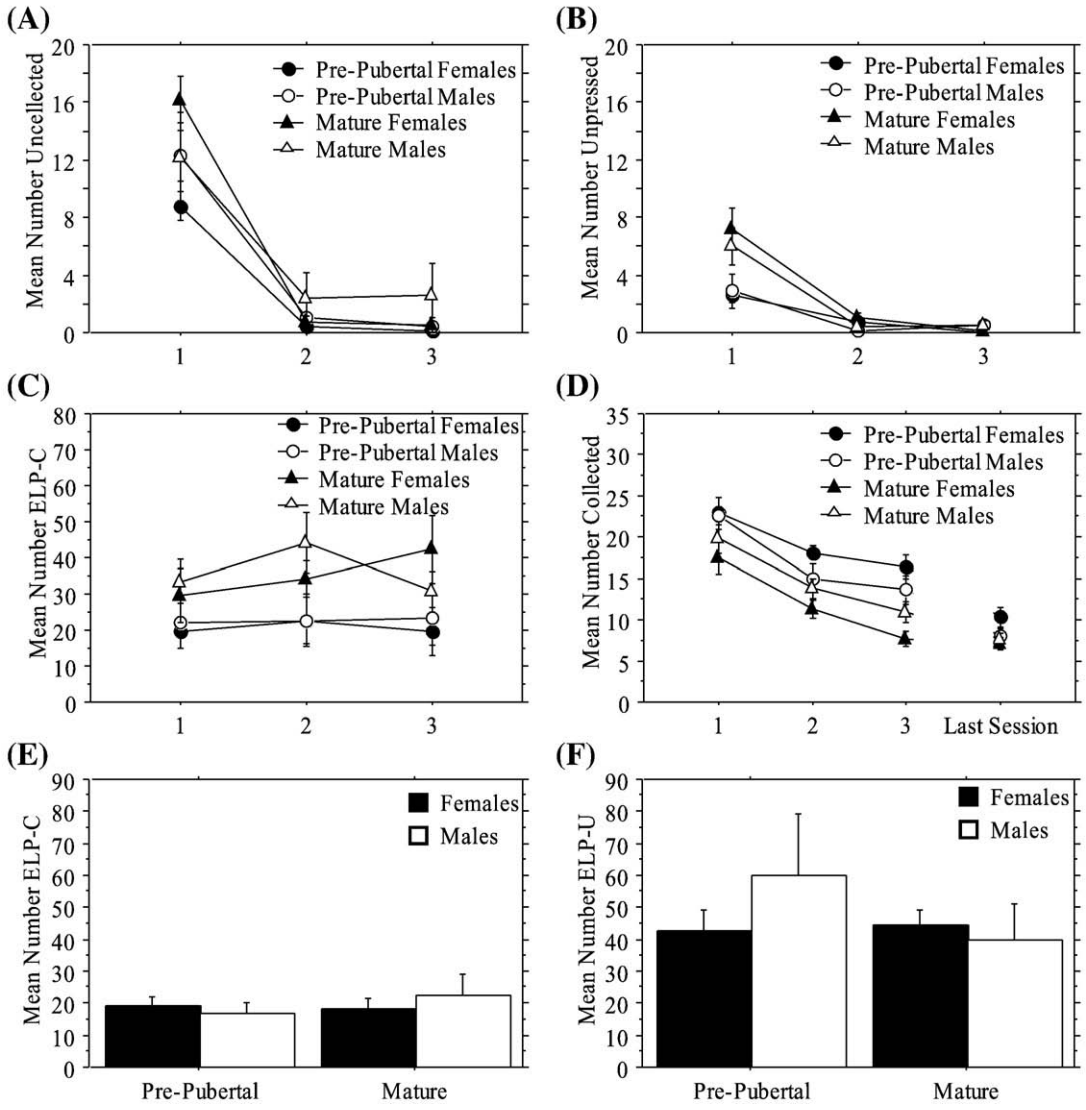

Fig. 2. Mean and standard error of the number of (A) uncollected trials on the three sessions of the *magazine training stage* [a significant main effect of Age, $F(1,37)=5.9$, $p<0.05$, and a significant Age×Sex×Days interaction, $F(2,74)=3.78$, $p<0.05$], (B) unpressed trials [a significant main effect of Age, $F(1,37)=10.55$, $p<0.005$, and a significant Age×Days interaction, $F(2,74)=8.43$, $p<0.005$], and (C) extra lever presses that were followed by magazine entry (ELP-C) on the three sessions of the *lever-press training stage* [a significant main effect of Age, $F(1,37)=4.97$, $p<0.05$], (D) collected trials on the first three sessions and on the final (3rd or 4th) session of the *signal attenuation stage* [a significant main effect of Age, $F(1,37)=17.93$, $p<0.0005$, and a significant Age×Sex interaction, $F(1,37)=4.83$, $p<0.05$], (E) extra lever presses that were followed by magazine entry (ELP-C) and (F) that were not followed by magazine entry (ELP-U) on the *test* day of the PTSA procedure (no significant difference was found) (Experiment 1).

There were also age- and sex-related differences in the *signal attenuation stage*. Specifically, mature rats extinguished magazine approach faster compared to pre-pubertal rats, and this difference was particularly evident within females, as mature females were the fastest and pre-pubertal females the slowest to extinguish (Fig. 2D, an ANOVA revealed a significant main effect of Age, $F(1,37)=17.93$, $p<0.0005$, and a significant Age×Sex interaction, $F(1,37)=4.83$, $p<0.05$). As detailed above, rats that performed more than 12 collected trials on the third session of signal attenuation were given another session. Analysis of the number of collected trials on the last (i.e., third or fourth) session of signal attenuation revealed no significant differences between the four groups, although the pre-pubertal female group tended to exhibit a higher number of collected trials compared to the other three groups which performed similarly.

Figs. 2E and F present the mean number of extra lever-presses that were followed by magazine entry (ELP-C) and that were not followed by magazine entry (ELP-U), respectively, in female and male, pre-pubertal and mature rats during the *test*. There were no significant differences between the four groups in the number of ELP-C and of ELP-U (all F's<1).

Experiment 2: Assessing the relation between stages of the estrous cycle and compulsive lever-pressing

Methods

Subjects

As in Experiment 1, except that female rats, 3–4 months old, were used. Vaginal smears were used to determine the stage of the estrous cycle on the Test day for each rat.

Apparatus and behavioral procedure

Apparatus and behavioral procedure were identical to the ones used in Experiment 1 with the exception that in the first replication of Exp. 2, a 1-month pause was introduced at the end of the lever-press training stage because most rats did not show a regular estrous cycle (i.e., two estrous cycles, 4–5 days long each). Training was resumed at age 4 months, with additional two sessions of lever-press training. The remaining procedure was identical to the procedure used in Experiment 1. The second replication started with 4-month-old rats. Data were therefore first analyzed using ANOVAs with Replication as an

additional main factor. Because the effect of Replication, as well as its interaction with estrous stage, were not significant, data from the two replications were combined.

Vaginal smears/vaginal cytology

The estrous cycle of Sprague–Dawley female rats lasts 4–5 days and consists of four stages: proestrous, estrous, metestrous (or diestrous 1) and diestrous (or diestrous 2 and 3). Vaginal smears were taken each day at the same time, starting from Day 3 of handling throughout the entire experiment, and estrous cycle stages were determined based upon the following smear characteristics: estrous (E) primarily consisted of anucleated cornified cells; metestrous (diestrous 1, D1) consisted of the same proportion of leukocytes, cornified, and nucleated epithelial cells; diestrous 2 (D2) and diestrous 3 (D3) primarily consisted of leukocytes, and proestrous (P) consisted of a predominance of nucleated epithelial cells (Jenkins et al., 2001; Marcondes et al., 2002). Rats that cycled normally during training, but for at least 4 days prior to the Test as well as on the day of the Test remained in diestrous, were characterized as irregular cyclers (D) (Hubscher et al., 2005).

Statistical analysis

Rats' performance on the test stage of the PTSA procedure was analyzed using ANOVA with a main factor of Stage (P/E/D1/D2/D3/D) performed on the number of ELP-C and ELP-U. Performance on the lever-press training and signal attenuation stages was also analyzed, to ensure that differences in performance at the test stage were not a result of an earlier difference.

Results

Of the 59 female rats that underwent PTSA, 4 rats had unclear vaginal smears, one rat fell ill during training, and 3 rats were excluded from the analysis because their performance on one of the behavioral measures in the test was more than 4 standard deviations higher than their group mean. Thus, the final analysis included 12, 4, 11, 6, 10 and 8 rats in the proestrous, estrous, diestrous 1, diestrous 2, diestrous 3 and irregular cycler groups, respectively.

No differences were found between the six groups at the lever-press training and signal attenuation stages (data not shown, all p's>0.45). Figs. 3A and B present the mean number of ELP-C and ELP-U, respectively, on the test stage. There were no differences between the six groups in the number of ELP-C, $F(5,45)=0.825$, $p>0.53$. However, the number of ELP-U varied during the estrous cycle, being highest at late diestrous and proestrous and lowest at estrous, $F(5,45)=2.493$, $p<0.05$ (see Fig. 3 for the results of post hoc comparisons).

Experiment 3: The effects of acute administration of estradiol benzoate on compulsive lever-pressing

Methods

Subjects

As in Experiment 1 except that 4-week-old female rats were used.

Apparatus and behavioral procedure

Apparatus and behavioral procedure were identical to the ones used in Experiments 1 and 2.

Drug

17β-Estradiol 3-benzoate (Sigma Aldrich, Israel) was dissolved in corn oil to a dose of 0.05 mg/kg and administered s.c. in a volume of 1 ml/kg, one or 24 h prior to the Test. No-drug controls received an equivalent volume of corn oil either 1 or 24 h prior to the Test. The dose of estradiol was chosen on the basis of behavioral studies performed in other laboratories using acute (Van den Buuse and Eikelis, 2001) and chronic (Galea et al., 2001) administration regimens.

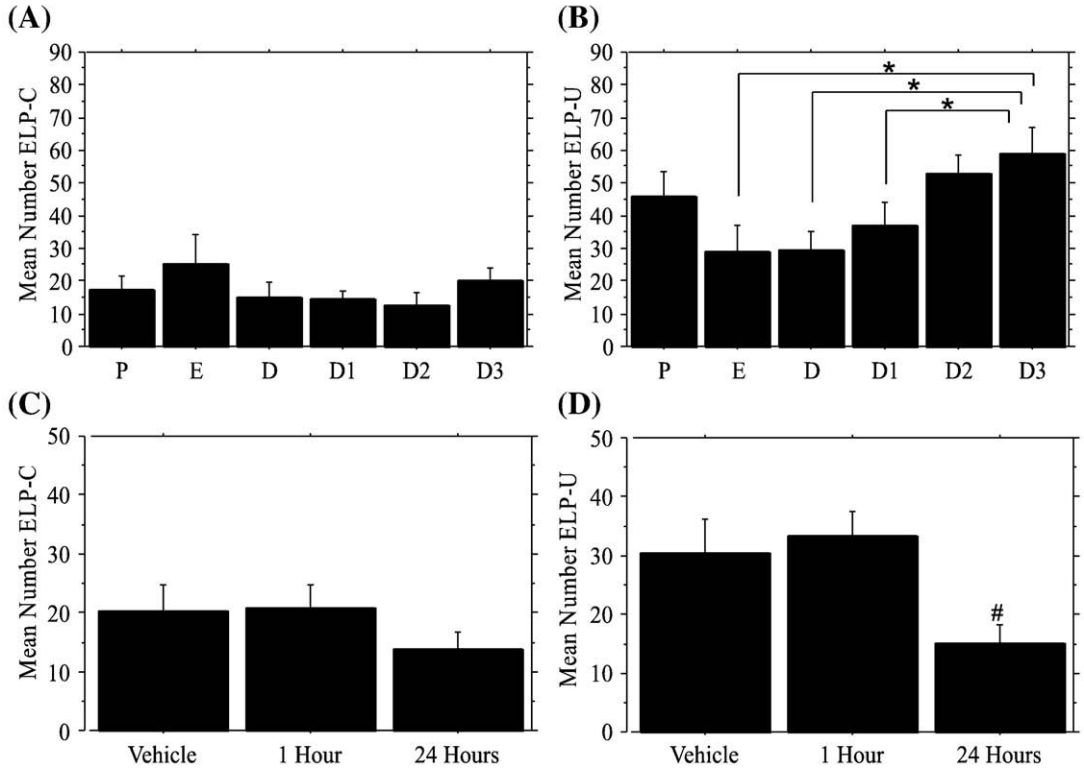

Fig. 3. Mean and standard error of the number of extra lever presses that (A, C) were followed by magazine entry (ELP-C) and (B, D) were not followed by magazine entry (ELP-U) of (A, B) female rats along the estrous cycle on the test day of the PTSA procedure (Experiment 2), and (C, D) female rats treated with vehicle or with 0.05 mg/kg of estradiol 1 or 24 h before the test day of the PTSA procedure (Experiment 3). *Significantly different from group D3 ($p<0.05$). #Significantly different from the vehicle group ($p<0.05$).

Statistical analysis

There were no differences between the two vehicle groups (1 h/24 h) and therefore their data were combined. Rats' performance in the test stage of the PTSA was analyzed using ANOVA with a main factor of Treatment (estradiol-1 h/estradiol-24 h/vehicle) on the number of ELP-C and ELP-U.

Results

Forty three female rats were randomly allocated to the 4 drug conditions. One rat from the estradiol-24 h group was excluded from the analysis because of an injection failure, and 3 rats, one from the estradiol-24 h group and 2 from the vehicle-24 h group were excluded from the analysis because their performance on at least one of the behavioral measures in the test was more than 4 standard deviations higher than their group mean. Thus, the final analysis included 13, 11, 9 and 6 rats in the estradiol-1 h, estradiol-24 h, vehicle-1 h and vehicle-24 h groups, respectively.

No differences were found between the three groups at the lever-press training and signal attenuation stages (data not shown, all p's>0.2). Figs. 3C and D present the mean number of ELP-C and ELP-U, respectively, on the test. There were no differences between the three groups in the number of ELP-C, $F(2,36)=0.852$, $p=0.435$. However, administration of estradiol 24 h prior to the test significantly decreased the number of ELP-U, whereas administration of estradiol 1 h prior to the test had no effect (an ANOVA revealed a significant effect of Treatment, $F(2,36)=3.922$, $p<0.05$, see figure for the results of post hoc analysis).

Experiment 4: The effects of withdrawal from chronic administration of estradiol benzoate on compulsive lever-pressing

The finding in Exp. 3 that acute administration of estradiol decreased ELP-U raises the possibility that withdrawal from repeated estradiol administration will have an opposite effect, namely to increase compulsive lever-pressing. This possibility is of great interest given that one plausible hypothesis for the observed association between post-partum and the onset or exacerbation of OCD symptoms in women is that the sudden decline in estrogen levels following delivery causes these symptoms to appear or worsen (Hill et al., 2007). This "withdrawal hypothesis" has been suggested in the past in relation to other psychiatric disorders linked to the female hormone cycle such as post-partum depression, premenstrual dysphoric disorder and others (Sherwin, 2005). Experiment 4 therefore attempted to mimic the withdrawal from estradiol in the post-partum period of the female rat by chronically administering estradiol for 7 days ("3rd trimester of pregnancy") and then assessing compulsive behavior on the third day of withdrawal from estradiol ("post-partum"). Rats' compulsive behavior was compared to compulsive behavior of rats treated with vehicle or with estradiol for the entire period. The hormone-withdrawal regimen we have used here has been used successfully in the past to asses "post-partum depression" in ovariectomized female rats (Galea et al., 2001; Stoffel and Craft, 2004). Because in the present study this treatment regimen was applied in young, non-ovulating female rats, we assessed its efficacy in mimicking the post-partum period by also testing the rats in the Forced Swim Test (FST), an animal model of depression (Cryan et al., 2005; Porsolt et al., 1977).

Methods

Subjects

As in Experiment 1 except that 4-week-old female rats were used. The experiment was run in two replications. Data were therefore first analyzed using ANOVAs with Replication as an additional main factor. Because the effect of Replication, as well as its interaction with Treatment, were not significant, data from the two replications were combined.

PTSA

Apparatus and behavioral procedure were identical to the ones used in Experiments 1–3. However, in order to minimize any possible effects of estradiol administration on the acquisition of lever-press responding, hormone administration began following 2 days of lever-press training and continued through 4 days in which the behavioral procedure was stopped. Then lever-press training was resumed for an additional 2 days and training continued as in Experiments 1–3. The test was conducted on the third day of withdrawal from estradiol (Fig. 4A).

The forced swim test (FST)

Apparatus — a vertical cylindrical glass container (50 cm tall×20 cm diameter), filled to a depth of 14.5–16 cm (adjusted to each rat individually) with tap water at 24±0.5 °C. This depth was sufficient to ensure that animals could not touch the bottom of the container with their hind legs. *Behavioral procedure* — was adapted from the procedure described by Galea et al. (2001) and Pliakas et al. (2001). Testing was conducted in two sessions, with Session 1 starting approx. 4 h following the Test stage of the PTSA procedure, and session 2 following 24 h later. On both sessions, rats were placed in the water for 10 min. Although the traditional exposure time on the 1st session is 15 min, a pilot test we conducted revealed that at the age of 7 weeks, female Sprague–Dawley rats had difficulties after 10 min in the water. Following each swimming session, rats were removed from the water, partially dried with paper towels, and placed in a warmed enclosure for 30 min until completely dried. The water was changed and the cylinder was thoroughly washed and wiped with a damp cloth between subjects in order to remove the presence of any potential alarm substances (Abel, 1991). Both sessions were videotaped from the side of the cylinders and were later scored by an observer who was blind to the rat's condition. The behaviors scored in the FST were: (1) struggling — quick movements of the forelimbs such that the front paws break the surface of the water; (2) swimming — movement of forelimbs or hind limbs in a paddling fashion; and (3) immobility — floating in the water, doing only those movements necessary to keep the head above the water. Increased immobility in the second session is considered to model "depressive-like" behavior in the FST (Borsini and Meli, 1988; Dalvi and Lucki, 1999; Porsolt et al., 1977). The data presented below were collected during the second session (10 min).

Drug

17β-Estradiol 3-benzoate (Sigma Aldrich, Israel) was dissolved in corn oil to a dose of 0.2 mg/kg and administered s.c. in a volume of 1 ml/kg daily. Group estradiol-vehicle (EV) received estradiol for 7 days followed by 4 days of vehicle (an equivalent volume of corn oil). Group estradiol–estradiol (EE) received estradiol for 11 days. No-drug controls (Group vehicle–vehicle, VV) received vehicle for 11 days. The dose of estradiol was chosen on the basis of behavioral studies performed in other laboratories using chronic administration regimens (Galea et al., 2001; Stoffel and Craft, 2004).

Vaginal smears/vaginal cytology

As in Exp. 2. Smears were collected at the end of the day (around 1600 h) over a 5 day period starting from the Test day of the PTSA. Only rats that did not show signs of estrous or proestrous on the day of the Test in the PTSA were included in the statistical analysis.

Statistical analysis

The number of ELP-C and ELP-U on the test stage of the PTSA was analyzed using ANOVAs with a main factor of Treatment (estradiol–vehicle/estradiol–estradiol/vehicle–vehicle). Performance on the last 2 days of the lever-press training stage and on the 3 days of signal

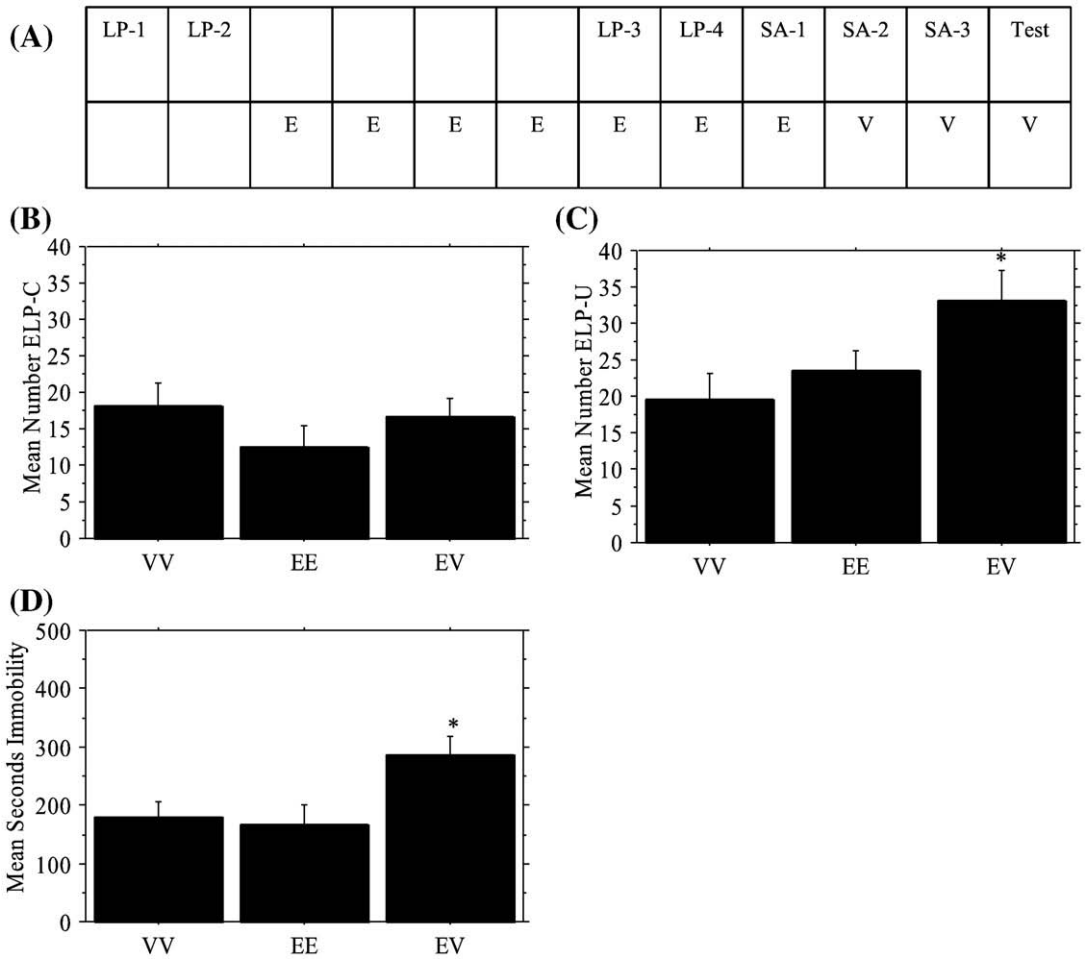

Fig. 4. (A) The timing of estradiol (E) and vehicle (V) injections relative to the days of the PTSA procedure in the estradiol-vehicle group. (B, C) Mean and standard error of the number of extra lever presses that (B) were followed by magazine entry (ELP-C) and (C) were not followed by magazine entry (ELP-U) of female rats treated chronically with vehicle (VV), 0.2 mg/kg of estradiol (EE), or 0.2 mg/kg of estradiol followed by vehicle (EV) on the test day of the PTSA procedure. (D) Mean and standard error of the number of seconds female rats of the three groups spent immobile on the second day of the FST (Experiment 4). *Significantly different from the vehicle (VV) group ($p<0.05$).

attenuation was also analyzed, to ensure that differences in performance at the test stage were not a result of an earlier difference. Rats' performance on the test session of the FST was analyzed using ANOVA with a main factor of Treatment (estradiol–vehicle/estradiol–estradiol/vehicle–vehicle) performed on the mean time rats spent immobile. A significant main effect was followed by post hoc LSD comparisons. For all comparisons, significance was assumed at $p<0.05$ (two-tailed).

Results

Seventy nine female rats were randomly allocated to the 3 drug conditions. Data of 4 rats were lost due to computer failure at the Test. Twenty nine rats (9 from group VV, 12 from group EE and 8 from group EV) were excluded because they showed signs of estrous or proestrous on the day of the Test in the PTSA, and 2 rats (1 from group EE and 1 from group VV) were excluded from the analysis because their performance on at least one of the behavioral measures in the test was more than 4 standard deviations higher than their group mean. Thus, the final analysis included 18, 11 and 15 rats in the estradiol–vehicle, estradiol and vehicle groups, respectively.

PTSA

No differences were found between the three groups on the lever press training and signal attenuation stages (data not shown, all p's>0.1). Figs. 4B and C present the mean number of ELP-C and ELP-U, respectively, on the test. There were no differences between the three groups in the number of ELP-C, $F(2,41)=0.90$, $p=0.414$. However, rats tested following withdrawal from chronic administration of estradiol performed a higher number of ELP-U compared with the other two groups, $F(2,41)=3.892$, $p<0.05$.

FST

Thirty six rats (10 — Group VV; 10 — Group EE; 16 — Group EV) were tested in the FST. Rats that were tested following withdrawal from estradiol administration (Group EV) showed increased immobility in the FST compared with the other two groups (Fig. 4D, $F(2,33)=4.382$, $p<0.05$). These results are compatible with results of previous studies which used similar hormone regimens in order to mimic the postpartum period in rats (Galea et al., 2001; Stoffel and Craft, 2004).

Discussion

The present study is the first to assess female rats in the signal attenuation rat model of OCD. In this model, attenuation of an external signal of reward leads, in a subsequent extinction test, to excessive lever-presses that are *not* followed by magazine entry (ELP-U). This behavior has been termed 'compulsive' lever-pressing (for review see Joel, 2006b). The main findings of the present study are that (1) a similar behavioral response to signal attenuation is evident in male and female Sprague–Dawley rats, both at pre-puberty and at adulthood (Experiments 1); (2) compulsive lever-pressing in female

rats fluctuates during the estrous cycle, being lowest at estrous and rising gradually through diestrous (Experiment 2); (3) acute administration of estradiol to pre-pubertal female rats decreases compulsive lever-pressing; and (4) withdrawal from repeated administration of estradiol results in an increase in compulsive lever-pressing in pre-pubertal female rats.

Sex and age differences in the PTSA procedure

There were several age and sex differences during the early stages of training in the PTSA procedure. Specifically, mature females were slower to acquire and faster to extinguish magazine approach compared to the other groups, although all groups reached a similar level of performance by the end of the magazine training and signal attenuation stages, respectively. In addition, during lever-press training, mature male and female rats were slower to acquire lever-pressing compared to pre-pubertal rats, as reflected in their higher number of unpressed trials on the first day of training. Finally, mature rats exhibited a higher number of ELP-C throughout lever-press training compared to pre-pubertal rats. These latter findings are in line with a previous report that mature rats are impaired at the initial acquisition of lever-pressing (Port et al., 1996). Although there are no studies of sex and age differences in the acquisition and extinction of magazine approach, there is a previous report of sex and age differences in Pavlovian conditioning. Specifically, Balda et al. (2006) found that adult female mice displayed a stronger cocaine conditioned place preference compared to adult male mice, and both groups showed stronger place preference compared to male and female adolescent mice, which performed similarly.

The important finding of Experiment 1 is that in spite of the age and sex differences at the early stages of training, pre-pubertal and mature male and female rats performed similarly on the test stage of the PTSA procedure. Specifically, the groups did not differ in the number of excessive lever-presses that were followed by magazine entry (ELP-C) and that were not followed by magazine entry (ELP-U). As mentioned above, previous experiments performed using the signal attenuation model of OCD assessed the effects of the procedure in mature male rats only (Flaisher-Grinberg et al., 2008; Joel and Avisar 2001; Joel and Doljansky, 2003; Joel and Klavir, 2006; Joel et al., 2001, 2004, 2005a,b, for review, see Joel, 2006b). The fact that in the present study, signal attenuation was found to induce similar behavioral effects in both pre-pubertal and mature male and female rats is in line with the phenomenology of the disease in human patients, as the gender ratio has been found to be similar in adolescents and adults (Geller, 2006; for a brief review see Turner, 2006). This is in contrast with the observation that in children, there is greater vulnerability and male predominance of 3:2 (Flament et al., 1988; Geller et al., 1998, 2006; Turner, 2006).

Compulsive lever-pressing on the different stages of the estrous cycle

Mature female rats exhibited different levels of compulsive lever pressing depending on the stage of the estrous cycle they were in during the test stage of the PTSA procedure. Specifically, the number of ELP-U was found to be highest during diestrous (D2, D3), lowest during estrous (E), and intermediate during proestrous (P) and metestrous (D1). Rats with an irregular cycle which remained in diestrous for at least 4 days prior to the test (D) also exhibited low levels of compulsive lever-pressing. In contrast, there were no significant differences between the number of ELP-C during the different stages of the estrous cycle. The demonstration of cyclic changes in ELP-U but not in ELP-C suggests that compulsive lever-pressing (rather than excessive lever-pressing in general) is modulated by ovarian hormones. Importantly, there were no differences between the groups on the earlier stages of the PTSA procedure, suggesting that the different level of compulsivity on the test was not a result of earlier differences.

Changes in compulsive responding during the estrous cycle have previously been reported using the 8-OH-DPAT model of OCD, and were very similar to those observed in the present study. Specifically, the efficacy of 8-OH-DPAT to induce perseveration (the measure of compulsive responding in this model) was highest at late diestrous and proestrous and lowest at estrous (Agrati et al., 2005; Fernandez-Guasti et al., 2006).

Inspection of plasma levels of ovarian steroids during the estrous cycle in several rat strains, including Sprague–Dawley (Haim et al., 2003; Shaikh, 1971; Spornitz et al., 1999), reveals a striking similarity between the level of estradiol, but not progesterone, and of compulsive behaviors, both in the signal attenuation model (present study) and in the 8-OH-DPAT model (Agrati et al., 2005; Fernandez-Guasti et al., 2006). This correlation suggests that estradiol levels may be modulating compulsive behaviors.

Effects of acute estradiol administration

Acute administration of estradiol 24 h before the test to pre-pubertal female rats significantly decreased the number of ELP-U, but not of ELP-C, suggesting that estradiol exerts an anti-compulsive effect. The finding that estradiol does not exert an anti-compulsive effect when administered 1 h before the test suggests that the anti-compulsive effect of estradiol is mediated by estradiol's genomic (classic) mechanism. Specifically, estradiol, as well as other steroid hormones, is known to have two kinds of mechanism of action, classic (or genomic) and non-traditional (or non-genomic). These mechanisms differ in their cellular sites of action and the rapidity of their effects, with the non-traditional mechanism acting within seconds or minutes, and the classic mechanism acting within hours (for reviews, see Brann et al., 1995; McEwen and Alves, 1999). Through its genomic action, estradiol can interact with several neural systems implicated in the pathophysiology of OCD (for reviews see Stein, 2000; Friedlander and Desrocher, 2006) including, the serotonergic system (for a brief review see Benmansour et al., 2008), the dopaminergic system (Karakaya et al., 2007; Quinlan et al., 2008), and the orbitofrontal cortex (Dreher et al., 2007).

Another support for an anti-compulsive effect of estradiol is a recent study, which used aromatase knockout (estrogen-deficient) mice. It was found that these mice exhibit increased barbering (plucking of hair and whiskers), grooming and wheel running compared to wild type mice (Hill et al., 2007). Interestingly, this increase in compulsive behaviors was observed only in male mice (Hill et al., 2007). In contrast, a study assessing the effects of acute administration of estradiol to ovariectomized rats in the 8-OH-DPAT model, failed to find a clear anti-compulsive effect of estradiol, although such an effect was exerted by an acute administration of estradiol and progesterone (Fernandez-Guasti et al., 2006). The discrepancy between the effects of estradiol on compulsive behavior in the two models may stem from the fact that the present study used intact rats while Fernandez-Guasti et al. (2006) used ovariectomized rats. This is because there are previous reports that the effects of estradiol in intact rats are observed in ovariectomized rats only with hormone replacement therapy (Viau and Meaney, 2004).

Withdrawal from repeated estradiol administration

Pre-pubertal female rats that underwent withdrawal from repeated administration of estradiol exhibited a higher number of compulsive lever-presses compared with rats continuously treated with either vehicle or estradiol. The effect of estradiol-withdrawal was specific to compulsive lever-pressing, as the three groups did not differ in the number of ELP-C, nor were there differences between the three groups at the earlier stages of training. In addition to increased compulsivity, “withdrawal” rats spent a longer time immobile in the FST. This latter finding is in line with previous reports of increased

immobility following withdrawal from repeated estradiol administration in ovariectomized rats, and suggests that pre-pubertal rats may also be used in a hormonal model of post-partum. The finding of increased compulsivity following withdrawal from repeated estradiol administration is in line with the hypothesis that "withdrawal" from estradiol may account for the worsening of symptoms post-partum in OCD (Hill et al., 2007) as well as in other psychiatric disorders (e.g., post-partum depression, Sherwin, 2005).

Summary and conclusions

The present study extends the signal attenuation model to female rats. In addition, it suggests that this model is appropriate for studying the role of ovarian hormones in compulsive behaviors, by demonstrating that compulsive lever-pressing fluctuates during the estrous cycle and is affected by manipulations of estradiol. The present study reveals that acute administration of estradiol exerts an anti-compulsive effect and that withdrawal from repeated estradiol administration has an opposite effect, namely to increase compulsive responding.

When attempting to interpret these results it is important to note that the effects of estradiol manipulations were demonstrated in pre-pubertal rats whereas fluctuations in compulsivity during the estrous cycle as well as evidence from humans on the possible modulation of OCD symptoms by ovarian hormones were obtained in sexually mature subjects. As there are well-documented neural and behavioral differences between pre-pubertal and sexually mature females (for review see Spear, 2000), further studies are needed to test whether the present results would generalize to the sexually mature female population.

Although conclusions from the present set of experiments should be drawn with caution, the finding that withdrawal from repeated estradiol administration exerts a pro-compulsive effect supports the hypothesis that the increased risk of onset and exacerbation of OCD in women post-partum is partly a result of the decrease in estradiol levels, which were elevated during pregnancy. The present findings on the effects of acute and repeated estradiol administration may also account for the pattern of change in compulsive lever-pressing during the estrous cycle. Specifically, compulsive lever-pressing was found to be lowest on estrous and to gradually rise during diestrous reaching its pick on D3-proestrous. Circulating levels of estradiol start to rise during late D2 and remain high until the beginning of proestrous, when they rapidly decline and reach their lowest level at late proestrous (Haim et al., 2003; Shaikh, 1971; Spornitz et al., 1999). Exp. 3 revealed that estradiol exerts an anti-compulsive effect 24 h, but not 1 h, following its administration. Exp. 4 revealed that repeated administration of estradiol does not affect compulsive lever-pressing (the estradiol–estradiol group), and that withdrawal from 7 days of estradiol administration exerts a pro-compulsive effect. Because during the estrous cycle estradiol levels are high for around 36 h only, it is possible that estradiol withdrawal does not exert a pro-compulsive effect, but rather, the protective effects of estradiol are seen, albeit only following the decrease in estradiol levels (that is, at estrous). This protective effect gradually dissipates throughout diestrous. We are not aware of reports of changes in OC symptoms around ovulation in OCD patients, but according to our data we may predict that OC symptoms should be low at that time, as the hormonal changes before estrous/ovulation are similar in rats and humans. OC symptoms were reported, however, to increase several days before menstruation (Labad et al., 2005; Vulink et al., 2006). This exacerbation of symptoms may be accounted for by the pro-compulsive effect of withdrawal from estradiol, because estradiol levels start rising 3 days post-ovulation, remain high for around 7 days and then rapidly decline before menstruation (MacGregor et al., 2006).

Although the present study focused on the ability of estradiol to modulate compulsive lever-pressing, progesterone may also modulate this behavior. Indeed, in the 8-OH-DPAT model, co-administration of estradiol and progesterone exerted an anti-compulsive effect (Fernandez-Guasti et al., 2006), and in the object burying model, acute administration of progesterone decreased burying behavior (Schneider and Popik, 2007), whereas withdrawal from repeated administration of progesterone increased it (Gallo and Smith, 1993). Further studies are needed to reveal the role of progesterone as well as of estradiol–progesterone interactions in compulsive behaviors.

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