EFFECTS OF FLUOXETINE ON ESTROUS CYCLE AND SEXUAL BEHAVIOR IN FEMALE RATS

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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COLLEGE OF ARTS AND SCIENCES

BY

JHIMLY SARKAR, M.S.

DENTON, TEXAS

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Date

To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Jhimly Sarkar entitled "Effects of fluoxetine on estrous cycle and sexual behavior in female rats." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

Lynda Uphouse

Lynda Uphouse, Ph.D., Major Professor

We have read this dissertation and recommend its acceptance:

Department Chair

Accepted: Jennifer Martin

Dean of Graduate School

DEDICATION

To my beloved parents, Mr. Adhir Kumar Sarkar and Mrs. Sikha Sarkar, who are the greatest inspiration and strength for my life and soul.

&

To Dr. Lynda Uphouse, my wonderful mentor, for being my friend, philosopher and guide. Everything I learned from her is a treasure for the rest of my life.

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My undeniable gratitude to my loving brother, Avijit, whose supports and care lead me through my way. Thanks for walking with me and carrying me when I was lost.

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ABSTRACT

JHIMLY SARKAR

EFFECTS OF FLUOXETINE ON ESTROUS CYCLE AND SEXUAL BEHAVIOR IN FEMALE RATS

DECEMBER 2007

Although highly effective for treatment of depression, antidepressants, such as fluoxetine (Prozac®), produce sexual dysfunction in a substantial number of patients. Mechanisms responsible for such sexual dysfunction were the focus of these studies. In a previous experiment, when female Fischer rats were treated daily with 10 mg/kg fluoxetine, both the estrous cycle and sexual behavior were disrupted. These results contrast with prior findings from which it has been suggested that fluoxetine does not disrupt estrous cyclicity. The current studies were designed to investigate explanations for the different outcomes and identify mechanisms for the cycle disruption. Fischer female rats received 15 days of 10 mg/kg fluoxetine with or without a 5 min daily exposure to a sexually active male. Male exposure delayed the onset of cycle disruption. Sprague-Dawley females were treated daily with 10 mg/kg fluoxetine to examine the generality of prior findings. Cycle disruption, though present, was less severe than in Fischer rats. A pair-fed group was added to the protocol to determine if reduced food intake contributed to the fluoxetine-induced sexual disruption in Fischer females. Pair-feeding mimicked the

effect of fluoxetine on the estrous cycle. In the next experiment, we questioned if hormonal priming could prevent the effect of fluoxetine on sexual behavior as measured by the lordosis reflex. In spite of hormonal priming, fluoxetine still reduced lordosis behavior. However, the magnitude of disruption was less than that seen in intact females. Finally we examined the effects of repeated fluoxetine treatment on serum luteinizing hormone (LH) both in ovariectomized and intact rats. Fluoxetine treatment failed to show any significant effect on serum LH. These experiments reinforce the possibility that a fluoxetine-induced disruption of the estrous cycle contributes to the drug-induced sexual dysfunction.

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LIST OF ABBREVIATIONS

Word	Abbreviation
1. 5-hydroxyindoleacetic acid	(5-HIAA)
2. Adrenocorticotrophic hormone	(ACTH)
3. Analysis of variance	(ANOVA)
4. Corticotrophin releasing hormone	(CRH)
5. Dopamine	(DA)
6. Enzyme-linked immunosorbent assay	(ELISA)
7. Follicle stimulating hormone	(FSH)
8. Gonadotropin releasing hormone	(GnRH)
9. Hypothalamic-pituitary adrenal	(HPA)
10. Hypothalamic-pituitary-gonadal	(HPG)
11. Lordosis to mount	(L/M)
12. Luteinizing hormone	(LH)
13. Monoamine oxidase inhibitors	(MAOIs)
14. Norepinephrine	(NE)
15. Obsessive compulsive disorder	(OCD)
16. Ovariectomized	(OVX)
17. Premenstrual dysphoric disorder	(PMDD)
18. Serotonin	(5-HT)
19. Selective serotonin reuptake inhibitors	(SSRIs)
20. Tricyclic antidepressants	(TCAs)

CHAPTER I

INTRODUCTION

Depression is one of the most common forms of mental illness, affecting 121 million people world wide [63]. Each year approximately 10% of the American population, or about 25 million American adults, suffer from some kind of depressive illness [16]. This disease not only affects the quality of life but it is also associated with a high risk of suicide [56,63]. Depression is usually treated with psychotherapy, antidepressant medications, or often a combination of both. Originally monoamine oxidase inhibitors (MAOIs), such as phenelzine and tranylcypromine sulfate, and tricyclic antidepressants (TCAs), such as imipramine, desipramine, amitriptyline, and clomipramine, were the main pharmaceutical agents for the treatment of depression [10,86]. MAOIs prevent the breakdown of neurotransmitters like serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE) and dopamine (DA), and thereby increase the amount of these neurotransmitters in the synaptic gap [10]. Tricyclic antidepressants work via inhibiting norepinephrine, serotonin or dopamine reuptake [86]. Although treatment of depressed patients with MAOIs and TCAs improve the symptoms of depression, they also lead to a wide range of side effects including severe hypertension, impaired cardiac contractibility, arrhythmias, sedation, weight gain, nervous system toxicity, cognitive impairment, and overdose-related death [22,51,57].

Before the discovery of selective serotonin reuptake inhibitors (SSRIs), antidepressant medication was mainly focused towards the NE system [12,13]. A role for 5-HT, however, had not been dismissed because of findings that the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were found to be lower in the brains of suicide victims [6,86]. Studies in blood platelets also indicated a reduction in the 5-HT level in platelets of depressed patients [34,37,73]. Moreover, tryptophan (5-HT precursor) treatment, which increased serotonin, was shown to reduce the symptoms of depression [16,86].

With the introduction of fluoxetine (Prozac®) in 1988, SSRIs started to gain much attention as antidepressant treatments. Since then, fluoxetine has become one of the most widely prescribed antidepressant drugs in the world [64,86]. In addition, fluoxetine is used for the treatment of a variety of disorders such as obsessive compulsive disorder (OCD), bulimia, anorexia nervosa, premenstrual dysphoric disorder (PMDD), panic disorder and obesity [71,86], many of which are more common in women than in men [22,71]. Like fluoxetine, other SSRIs including sertraline (Zoloft®), paroxetine (Paxil®), fluvoxamine (Luvox®), and citalopram (Celexa®) block the reuptake of serotonin into nerve terminals, thereby increasing the amount of 5-HT and serotonergic neurotransmission in the synaptic cleft [3,9,16,41,86]. Due to their documented effectiveness in the treatment of depression [41], SSRIs led to a new era in antidepressant medication.

Although highly effective for the treatment of depression, SSRIs produce sexual

side effects in a substantial number of patients [7,9,16,41,69]. Sexual side effects include impairment in all three phases (drive, arousal, and orgasm) of sexual behavior in both male and female patients [21]. It includes erectile difficulties and decreased or delayed ejaculation in male patients and orgasmic impairment, lowered desire, arousal and sexual dissatisfaction in both male and female patients [1,7,8,24,41,66]. In male patients, there is some relief from sexual dysfunction with phosphodiesterease inhibitors such as sildenafil citrate [4,26]; however, this treatment is less effective in females and as much as 32% of female patients suffer from some kind of sexual dysfunction during SSRI treatment [46,69,70]. Such adverse side effects often lead to discontinuation of treatment. Mood disorders, in particular depression, are twice as prevalent in women as in men [2]. The greater prevalence of mood disorders in female has implicated the presence of female gonadal hormones, estrogen and progesterone, in increasing the vulnerability towards the mood disorders. However, there are few well-controlled studies in which the issues and causes related to SSRI-induced sexual dysfunction in females have been carefully examined [23,28,29,43,60,81,83]. Therefore, the mechanisms responsible for SSRI-induced female sexual dysfunction are largely unknown.

An adequate amount of the female gonadal hormones, estrogen and progesterone, is required for normal reproductive functioning in both human and nonhuman females. The sequential releases of these gonadal hormones are controlled by the pituitary and the hypothalamus via the hypothalamic pituitary gonadal (HPG) axis. In human females, low blood levels of estrogen are directly correlated with multiple aspects of reduced

reproductive functioning such as vaginal dryness, pain and burning sensation during penetration, and a reduced sexual desire or libido (e.g. motivation for sexual behavior and satisfaction with sexual activities) [8,66]. Estrogen replacement therapy has been shown to improve these conditions in menopausal women [18,19,66]. Therefore, the disturbance of the hormonal balance or reproductive cyclicity might be expected to impact negatively on sexual behavior even in human females. In the case of non human mammals, such as female rats, sequential release of estrogen and progesterone initiates a cascade of events that lead to normal reproduction and sexual behavior [44]. These behaviors are abolished after ovariectomy (OVX) but can be restored with exogenous hormone treatment [40]. Estrogen, alone, can elicit sexual receptivity in female rats but the presence of progesterone is needed for the display of all components of female sexual behavior [39].

Serotonin has long been known to influence reproductive function in a variety of species including humans [65]. Serotonin is inhibitory to many components of reproductive functioning and a decrease in serotonergic activity facilitates sexual behavior in rodents [30,78-80]. In rats, serotonin reuptake inhibitors, such as clomipramine and fluvoxamine, are reported to decrease female gonadal hormones that are required for proper reproductive functioning [60]; and, in ovariectomized rats, 5-HT has a negative influence on the release of luteinizing hormone (LH) [84]. Fluoxetine increases extracellular serotonin, first by blocking the serotonin transporter and later via the desensitization of 5-HT_{1A} and 5-HT_{1B} inhibitory autoreceptors [53,76]. It is possible,

then, that antidepressant drugs may disrupt normal hormonal balance so that sexual dysfunction occurs. In support of this possibility is evidence that a disruption of vaginal cyclicity in female rats occur following treatment with a tricyclic antidepressant, imipramine [42].

Furthermore, serotonin has long been known to regulate food intake; and food restriction can lead to disruption of the reproductive cycle [5,17,27,28,31,85]. Since fluoxetine is anorectic, cycle disruption could result from such anorexia and consequent hormonal disruption. Finally, on the short term, SSRIs enhance activation of the hypothalamic-pituitary-adrenal axis (HPA) leading to release of corticotrophin releasing hormone (CRH) [36,47,59]; increased CRH can also disrupt normal reproductive cyclicity [72] by suppressing the pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus. Figure 1 shows the hormonal pathways of the HPA and HPG axes that are involved in the generation of proper reproductive signals and also how 5-HT can regulate these pathways.

GnRH is released into the hypothalamus-pituitary portal system and stimulates the release of pituitary hormones, follicle stimulating hormone (FSH) and LH, which then regulate ovarian function leading to sequential release of estrogen and progesterone. Initially, estrogen has a negative feedback effect of the release of LH, but estrogen also primes the organism for a future LH surge, which ultimately leads to proper ovulation. CRH release from the paraventricular nucleus of hypothalamus leads to an increase in

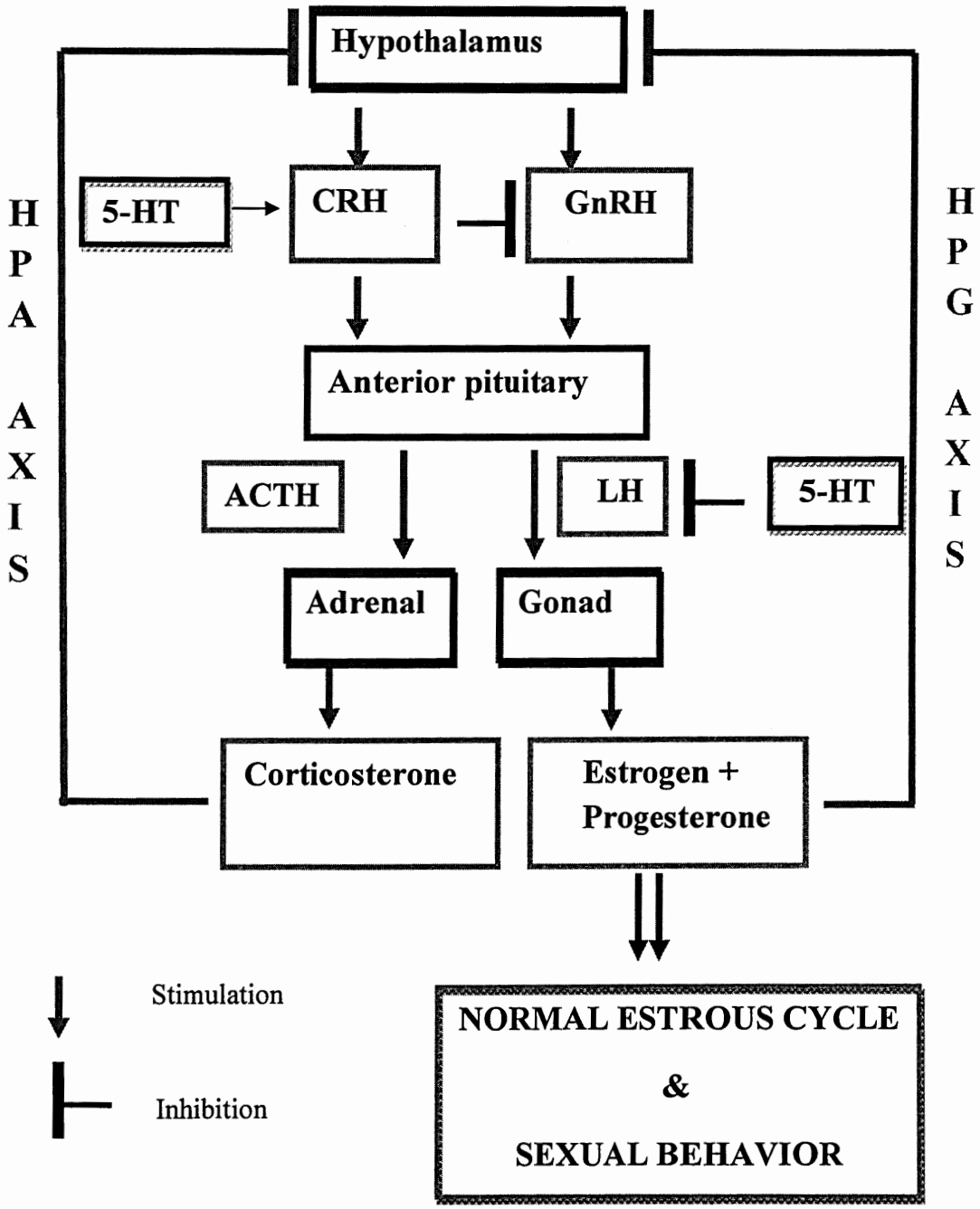


Figure 1: Hormonal pathways in regulating the reproductive cycle in female rats.

adrenocorticotrophic hormone (ACTH) release. Increased ACTH causes secretion of corticosterone from the adrenal gland. CRH, via neuronal pathways, exerts a negative effects on GnRH release and thereby leads to the disruption of the HPG axis [72]. Food restriction increases CRH and, therefore, suppresses the HPG axis. 5-HT is involved in the regulation of both GnRH and CRH pathways. At GnRH neurons, 5-HT decreases GnRH release and at the PVN, 5-HT increase CRH release. Therefore, fluoxetine by increasing 5-HT, increases CRH and leads to a decrease in LH. Food restriction also activates the HPA axis by stimulating the release of CRH and, therefore, suppresses the release of LH.

The female rat estrous cycle can be monitored by examination of sequential changes in the vaginal smear, the cell content of which is controlled by female gonadal hormones. With the stimulation of LH from the pituitary, the female gonad secretes estrogen; increased estrogen causes proliferation of the epithelium leading to the appearance of nucleated cells in the vaginal smear, and this is proestrus. After extended estrogen treatment, cornified cells appear in the vaginal smear; this is estrus. As estrogen levels decrease and progesterone is increased, leukocytes invade the epithelium. Leukocytes then start to engulf the proliferated cell layers inside the uterus, and this is diestrus. Rats are usually sexually receptive during the late proestrous and the estrous phase, when they have high estrogen in their blood. Rats are not receptive during the diestrous phase.

In previous studies, investigators have argued that repeated fluoxetine treatment does not disrupt the female rat's estrous cycle but does disrupt sexual behavior [28,43,83]. In contrast, a previous study in our lab showed a rapid disruption of vaginal estrus in Fischer rats after initiation of daily treatment with 10 mg/kg fluoxetine [81]. More than 50% of fluoxetine-treated females failed to demonstrate vaginal proestrus/estrus within the first 5 days of treatment and 100% showed an absence of proestrous/estrous smears during the next 5 days of treatment. Consistent with their diestrous-like vaginal smear and elongated cycles, fluoxetine-treated rats had significantly lower serum levels of progesterone than their vehicle-treated counterparts. This was a significant observation and, in contrast to earlier studies, led to the suggestion that the cycle disruption could be responsible for SSRI-induced female sexual dysfunction.

In the project by Matuszczyk et al. [43], sexual behavior and vaginal cyclicity of female Wister rats were examined during 3 weeks of treatment with 10 mg/kg fluoxetine. Fluoxetine reduced sexual behavior but had no disruptive effect on the female's vaginal cycle. Matuszczyk et al. [43] tested their female rats every day with male rats to measure sexual receptivity, whereas, in the study by Uphouse et al. [81], Fischer female rats were never exposed to the males. Heisler et al. [28] treated female Long–Evans rats with 5, 10 and 15 mg/kg of fluoxetine but never tested them for sexual receptivity. No disruption of vaginal cyclicity occurred during their treatment. Van de Kar and colleagues [83] used female Sprague–Dawley rats and treated them with 10 mg/kg fluoxetine for 15 days and

Table 1: Relevant literature regarding fluoxetine and reproduction.

Reference	Rat strain	Housing	Duration	Test	Major findings
Uphouse et al. [81] [a,1]	Fischer	Separate from male	25 days	Proestrous day only	Both cycle & behavior disrupted with a reduction in progesterone
Matuszczyk et al. [43][a,1]	Wister	With male	3 weeks	Every day	No cycle, but behavioral disruption
Heisler et al. [28] [a,2]	Long Evans	Not known	28 days	Not tested	No cycle disruption
Rehavi et al. [60] [a,1]	Not reported	Not known	3 weeks	Not tested	Decrease in female gonadal hormones (estrogen & progesterone)
Van de Kar et al. [83] [a 1]	SD	Not known	15 days	Not tested	No cycle disruption
Frye et al. [23] [b,3]	Long-Evans	Not known	15 days	After priming	Decrease in lordosis behavior

[a] Studies were done in intact rats model, [b] study done in OVX model

Dose- [1] 10 mg/kg, [2] 5, 10 & 15 mg/kg, [3] 10 & 20 mg/kg of fluoxetine

Fischer female rats were never exposed to the males. Heisler et al. [28] treated female Long–Evans rats with 5, 10 and 15 mg/kg of fluoxetine but never tested them for sexual receptivity. No disruption of vaginal cyclicity occurred during their treatment. Van de Kar and colleagues [83] used female Sprague–Dawley rats and treated them with 10 mg/kg fluoxetine for 15 days and also did not find any cycle disruption. Frye et al [23] used OVX Long-Evans rats and saw a decrease in lordosis behavior with 10 and 20 mg/kg fluoxetine in both acute and repeated conditions. The major findings of these prior studies are listed in Table 1.

Since our recent findings of an estrous cycle disturbance after fluoxetine contrasts with these previous reports, it was first necessary to identify any major procedural differences that might explain the different outcomes (Table 1). Of these, the two potentially most important differences were the way animals were housed and the strain of rats used. The presence of the male or even the olfactory cues from male's urine can influence the female's reproductive cycle in a variety of mammalian species. In particular, the presence of the male can induce estrus or synchronize estrous cycles possibly via increasing progesterone or LH [48,49,67]. While Matuszczyk et al. [43] tested their female rats on a daily basis, in the study by Uphouse et al. [81], Fischer female rats were never exposed to the males. It is possible, therefore, that the male's presence compensated for the effect of repeated fluoxetine injection on cycle disruption. The first experiment, described below, was designed to test this possibility.

The presence of the male, however, may not account for the absence of an effect of fluoxetine on estrous cyclicity in the studies by Van de Kar et al. [83] and Heisler et al. [28], where housing conditions were not described. Van de Kar and colleagues [83] used female Sprague–Dawley rats while female Fischer rats were used in our previous study. A number of phenotypic, neuroendocrine, and behavioral differences have already been well characterized between Fischer and other rat strains such as Sprague-Dawley. Fischer rats have significantly higher stress-induced levels of adrenocorticotropic hormone (ACTH) and corticosterone and show a significantly greater magnitude of stress-induced adrenal steroid receptor activation in the brain than Sprague-Dawley rats [20,54]. It is possible, therefore, that Fischer rats are uniquely susceptible to the SSRI-induced cycle block. If so, such a block should not occur in Sprague-Dawley females. This possibility was addressed in the second experiment described in this dissertation.

It was also important to identify potential mechanisms for the estrous cycle disruption in Fischer female rats. In the previous study by Uphouse et al. [81], Fischer rats lost a substantial amount of their body weight during fluoxetine treatment. Administration of 5-HT and its precursor leads to a reduction in food intake, eating rate and meal size [5,28]. In the human population, fat storage and female reproduction are highly correlated. A disruption of reproduction and menstrual cyclicity is seen in human females when negative energy balance is produced in the body due to anorexia or reduced food intake, potentially resulting from hormonal disturbances [32,85]. Menstrual cycles resume again when positive energy balance is restored [85]. Given the effects of food restriction on reproduction, it was also hypothesized that reduced food intake might be

responsible for the SSRI-induced sexual dysfunction in Fischer female rats. Treatment of intact female rats with 10 mg/kg fluvoxamine (SSRI) for 3 weeks was shown to decrease serum levels of the gonadal hormones, estrogen and progesterone [60]. Uphouse et al. [81] also found a reduction in blood serum progesterone in rats repeatedly treated with 10 mg/kg fluoxetine. Female rat sexual behavior is tightly linked to ovulation and the synchronous timing for both events is controlled by estrogen feedback to the HPG axis [44,74]. Normally cycling female rats show sexual receptivity only during the proestrous (ovulatory) portion of the reproductive cycle. Consequently, fluoxetine's ability to disrupt the estrous cycle may explain the SSRI's reported inhibitory effects on female rat sexual behavior. If so, exogenous treatment with estrogen and progesterone should attenuate effects of fluoxetine on sexual behavior.

Finally, experiments were designed to determine the effects of fluoxetine on LH. Successful ovulation depends on the interplay between gonadotropin releasing hormone (GnRH) and, consequently, LH pulse frequency generation and the production of estradiol [72]. A reduction in GnRH will slow follicular growth and reduce ovarian estradiol production. Several components of the HPA axis such as ACTH and CRH are known to reduce the GnRH pulse [72] and, as described earlier, SSRIs can enhance HPA axis activity. Thus, repeated fluoxetine may have disrupted reproductive cyclicity by reducing ovulatory signals.

The following experiments were designed to address each of the above questions. Specific aim A was designed to investigate the explanation for different outcomes reported by Uphouse et al. [81], Matuszczyk et al. [43], and Van de Kar et al. [83]. In

specific aim B, experiments were designed to investigate the mechanism of estrous cycle disruption in Fischer female rats. It was predicted that:

A.1. Daily exposure of female rats to male rats will reduce or prevent the estrous cycle disruption caused by repeated fluoxetine.

A.2. Sprague-Dawley rats will show less cycle disruption during repeated fluoxetine.

B.1. Pair-feeding (food restriction) will mimic the effects of fluoxetine on estrous cycle disruption and on sexual behavior.

B.2. Hormonal priming will prevent the effect of repeated fluoxetine and restore sexual behavior.

B.3. Rats treated with fluoxetine will have lower serum LH.

CHAPTER II

MATERIALS AND METHODS

A. Materials

Fluoxetine hydrochloride, estradiol benzoate, progesterone and sesame seed oil were purchased from Sigma/Aldrich Chemical Company (St. Louis, MO). Isoflurane (AErrane®) was purchased from Baxter Pharmaceutical Products Inc. (Deerfield, IL). Rat food (Harlan Teklad Rodent Diet) was purchased from Harlan Teklad (Madison, WI). Suture materials were purchased from Butler Company (Arlington, TX). Enzyme-linked immunosorbent assay (ELISA) kits for LH assay were purchased from Endocrine Technologies (Newark, CA). All other supplies came from Fischer Scientific (Houston, TX).

B. General Methods

Animal and housing conditions:

Adult female Fischer (F-344) and Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). After their arrival, rats were housed 2-3 per cage in polycarbonate cages (18.0/ 9.5/ 8.5 in.) with food and water available *ad libitum*. The animal facility rooms were maintained at 25°C and 55% humidity. Rats were housed in a 12 hr-12 hr light/dark cycle with lights off at 12 noon.

Vaginal smears:

After a two-week acclimation in the animal facility, rats were individually housed with *ad libitum* access to food and water, and their estrous cycles were monitored daily for at least two complete estrous cycles by observing their vaginal smear. Smearing was always done during the night cycle of the rats between 1PM to 4 PM; visibility was aided by red light. The female's vagina was flushed with distilled water containing methylene blue and the cell types present were determined by viewing under the light microscope. The relative proportions of nucleated cells, cornified cells, or leukocytes in the vaginal smear were used to determine the stage of the cycle (Figure 2). Smears with clusters of nucleated cells with some cornified epithelial cells but an absence of leukocytes were designated as a proestrous smear. Presence of predominantly cornified cells with some nucleated cells, but no leukocytes, were designated as estrous smear. Leukocytes present alone or with few nucleated cells and cornified cells, were designated as diestrous smears. Representative pictures of the various stages are shown in Figure 3.

Evaluation of sexual behavior:

Sexual behavior was measured on the day of proestrus for the control rats and on the predicted day of proestrus for the experimental rats. Testing of female sexual behavior took place between 1:00 and 4:00 PM. Except for the experiment with daily male rat exposure, females to be tested were moved from their home cage and were placed in a covered black cage to avoid light exposure; females then were moved to the testing room where the males were housed. In the testing room, visibility was aided by

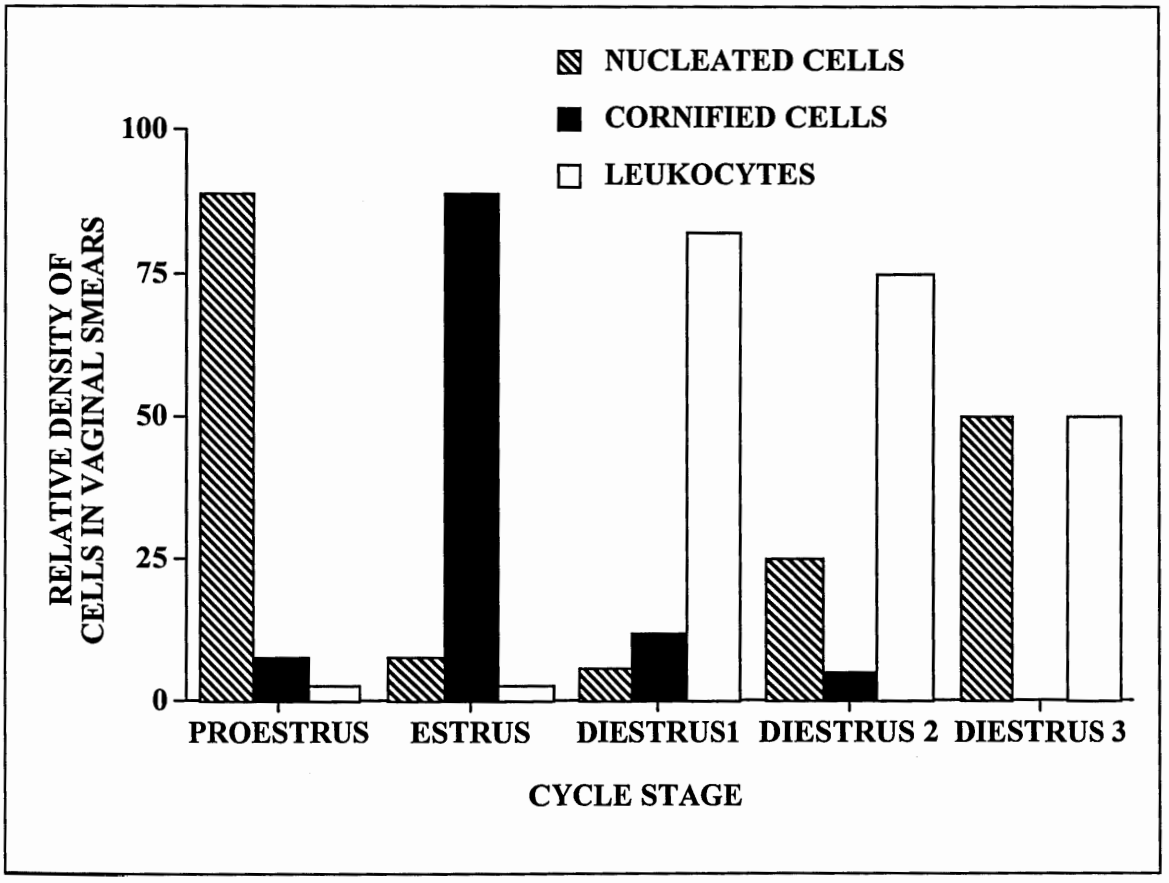


Figure 2: Distribution curve of different vaginal cell types for an ideal estrous cycle in female rats.

(Prepared by Dr. Lynda Uphouse)

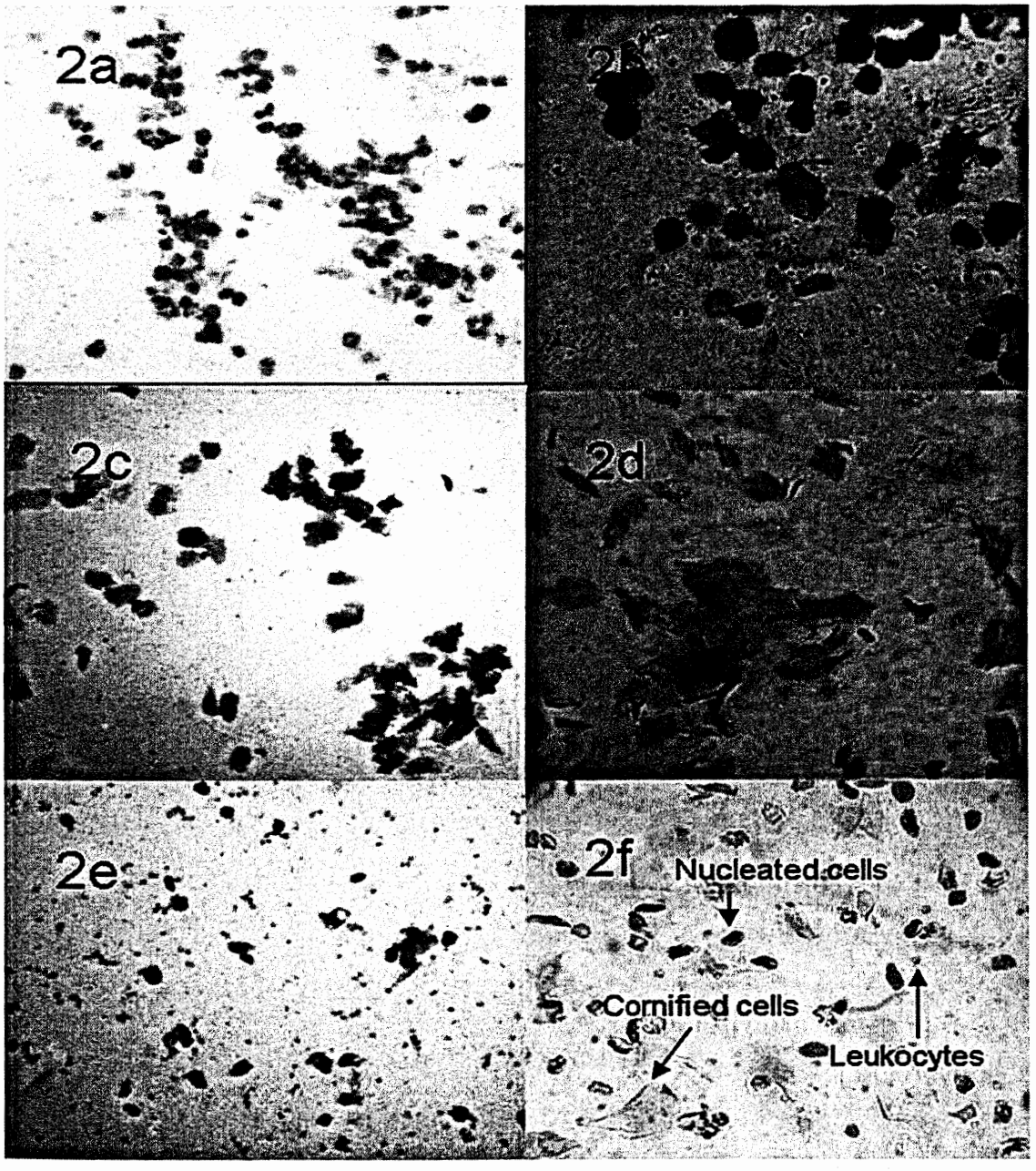


Figure 3: Vaginal cell types in different stages of the estrous cycle in female rats.

2a & 2b are proestrous smear showing a high proportion of nucleated cells. 2c & 2d are estrous smears with mostly cornified cells. 2e & 2f are mainly leukocytes with some nucleated and cornified cells indicative of a diestrous smear. 2a, 2c and 2e were originally taken in 100x magnification. 2b, 2d and 2f were originally taken in 200x magnification.

red lighting. For behavioral testing, females were placed into the home cage of a sexually active male until the male had achieved 10 mounts (for Experiment A.2, B.1 and B.2) or until the investigator scored the female as receptive or not receptive (Experiment A.1). When appropriate, sexual receptivity was quantified as the lordosis to mount (L/M) ratio [29], indicative of the frequency with which the female shows a lordosis response when mounted by the male. When multiple testing was used and extended mating might disrupt the female's cycle, the male was not allowed to complete mating. Instead, the presence or absence of a lordosis reflex in response to the male's mount was determined and females were rated as either receptive or nonreceptive. In the daily male exposure paradigm, a female was left in the male's cage for 5 min and judged as receptive or nonreceptive by the following criteria: presence of hop-dart (solicitation behavior), lordosis to touch, and lordosis to mount. In all cases, females were returned to their home cage at the completion of behavioral testing.

Preparation of fluoxetine for i.p. injections:

Fluoxetine was prepared for injection by dissolving 10 mg of fluoxetine hydrochloride in 1 ml of distilled H_20. The mixture was vortexed until fluoxetine was completely dissolved in water.

Data analysis:

Statistical tests, as described in the specific methods, were performed with SuperANOVA (v1.11) or StatView 4.5 from Abacus Concepts. The statistical reference was Zar [87].

C. Specific Methods

Experiment A.1: Effect of daily exposure to a male on repeated fluoxetine-induced cycle disruption in Fischer female rats.

After a two week acclimation to the laboratory, Fischer female rats were individually housed and their estrous cycles were monitored daily for 2 consecutive weeks. After the first week, body weight was monitored daily. Rats were enrolled in the study as they cycled into diestrus 1. Rats were injected (i.p.) daily for 10 days with fluoxetine (10 mg/ml in distilled water; at a dose of 10 mg/kg) or vehicle (distilled water, 1 ml/kg) and injections always began on diestrus 1. This led to a 4 to 5 day variation in the initiation of treatment. Thereafter, females were divided into 2 groups: fluoxetine and control group (10 mg/kg fluoxetine or vehicle, respectively). These two groups were exposed to a male rat every day. Male-exposed rats were moved to the male housing area after the presmearing and were kept there for the rest of the experiment. The male-exposed females were placed in the home cage of a sexually active male rat and left with the male for 5 minutes every day for 10 days. Thereafter, they were smeared, injected (i.p.) and returned to their home cage during the dark phase of the light-dark cycle. The female's behavior was scored on the following criteria: presence of hop-dart, lordosis to touch and lordosis to mount. A third group of rats, not exposed to males, were treated with 10 mg/kg fluoxetine as described by Uphouse et al. [81] and were used as a positive control for cycle disruption. This third group remained in the original housing area separate from the male housing.

Data analysis:

Body weight data were analyzed by repeated measure Analysis of Variance (ANOVA) with day of treatment as the repeated factor and type of treatment as the independent factor. Nonparametric data, such as the number of rats with normal cycles, were analyzed with Chi-Square tests.

Experiment A.2: Effects of repeated fluoxetine in Sprague-Dawley females.

Smearing of the rats prior to injection was done as described in experiment A.1. Females were then divided into two groups (i.e. fluoxetine-treated group and control group). Fluoxetine and control rats were provided with *ad libitum* access to food and water. Experimental procedures took place at the same time of the day, between 1:00 and 4:00 PM. Rats were smeared, and their body weight and food intake were also measured. Rats were then injected (i.p.) daily for the next 23-24 days with fluoxetine (in distilled water; 10 mg/kg) or vehicle (distilled water; 1ml/kg) and injections always began on the day following a fully cornified smear (e.g. on diestrus 1). Sexual behavior (as described in the general methods) was tested on the day of proestrus for the control rats or on the predicted day of proestrus for the experimental rats.

Data analysis:

Food intake and body weight data were analyzed as described in experiment A.1. Final lordosis response was compared by Student's t-test.

Experiment B. 1: The effect of pair-feeding on sexual behavior and estrous cycle disruption in female Fischer rats.

Smearing of the rats prior to injection was done as described in experiment A.1. Females were then matched by body weight and stage of the estrous cycle to produce matched triplets (i.e. fluoxetine-treated group, pair-fed group, and control). Fluoxetine and control rats were provided with *ad libitum* access to food and water. Pair-fed rats were matched to a fluoxetine rat so that their pretreatment estrous cycle was one day behind that of the fluoxetine rat. Pair-fed rats were given *ad libitum* access to water but their food allotment was restricted to that consumed by the fluoxetine pair-match one day earlier (e.g., at the same stage of the estrous cycle).

Data analysis:

Body weight and food intake data were analyzed by repeated measure ANOVA with day of treatment as the repeated factor and type of treatment as the independent factor. Post hoc comparisons between individual means, within days of treatment, were made with Tukey's test. Nonparametric data, such as the number of rats with normal cycles, were analyzed with Chi-Square tests.

Experiment B.2: Effects of repeated fluoxetine in hormonally primed OVX Fischer rats.

Surgical Procedures for OVX:

Female rats, approximately 60-90 days old, were anaesthetized with AErrane® and a single incision was made across the abdomen. The abdominal muscle was opened

and the ovarian tissue was ligated and removed. The muscle incision was sutured and the epidermal layer was stapled with a tissue stapler.

Hormonal treatment and drug administration:

Two weeks after OVX, the rats were injected (i.p.) daily with fluoxetine (10 mg/kg, 1 ml/kg) or vehicle (distilled water, 1 ml/kg) for the next 9 days. Body weight was measured each day immediately prior to injection. Estradiol benzoate (10 μg) and progesterone (500 μg) were dissolved in sesame seed oil and injections were given subcutaneously in a volume of 0.1 ml per rat. On the 8^{th} day of fluoxetine or vehicle treatment, rats received an injection of estradiol benzoate in the morning. Forty-eight hrs later (10^{th} day of the experiment), rats were injected with progesterone. Vaginal smears were taken 4 to 6 hrs after the progesterone injection to confirm vaginal responsiveness to estrogen. Then, female rats were placed into the home cages of sexually active males until the males had achieved 10 mounts. Receptivity was quantified as the L/M ratio [79]. After this first test (pretest), rats were injected i.p. with vehicle, 10 mg/kg fluoxetine or 20 mg/kg fluoxetine. Thirty min later, rats were tested again for sexual receptivity as described above.

Data analysis:

Sexual receptivity was analyzed by repeated measures ANOVA followed by post hoc comparison using Tukey's test for individual means.

Experiment B. 3. Effects of repeated fluoxetine on serum LH in Fischer rats.

Experiment B. 3. a :

In a prior experiment [81], 100% of the fluoxetine-treated rats had a blocked cycle after 10 days of repeated injection. Thus, it was hypothesized that a cycle block actually occurred prior to this time. Rats were injected daily with fluoxetine (10 mg/kg, 1ml/kg) or vehicle (distilled water, 1 ml/kg) for only 4 days until the first anticipated proestrous day. On the morning (between 10 A.M. and 12 noon) of their anticipated proestrous day, rats were smeared and injected with water or fluoxetine. Two hr later they were sacrificed by decapitation. Trunk blood was collected and centrifuged at 2500 x g for 5 minutes; centrifugation was repeated until a clear serum was obtained. Serum was stored at -80° C until used for determination of LH by ELISA.

LH ELISA:

The LH ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a polyclonal anti-LH antibody for solid phase (microtiter wells) immobilization and mouse anti-LH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. The mixture was incubated for 2 hrs; then the wells were washed with wash buffer 5 times to remove unbound labeled antibodies. TMB (3,3',5,5'-tetramethybenzidine), the color reagent solution, was then added and the solution was incubated for 20 min in the dark. The

addition of TMB solution resulted in the development of a blue color solution. With the addition of 2N HCl, the color reaction was stopped and a yellow solution was generated; the absorbency of that yellow solution was measured spectrophotometrically at 450 nm (blue range of light). The intensity of the color formed is proportional to the amount of enzyme present and is directly related to the amount of LH in the sample. A series of LH standards of known concentration were assayed in the same way to generate a standard curve (Figure 4). The concentration of LH in the test sample was measured based on the standard curve. Raw data were converted to ng LH/ml of serum and treatments were compared by Student's t-test.

Experiment B. 3. b:

Rats were OVX as described in experiment B.2. Two weeks later rats received either fluoxetine (10 mg/kg, 1ml/kg) or vehicle (distilled water, 1 ml/kg) injection for the next 5 days. On the morning of the 5^{th} day (between 8.00 to 10.00 AM), rats received their daily injection of fluoxetine, 2 hr before decapitation. Trunk blood was collected and processed for LH ELISA.

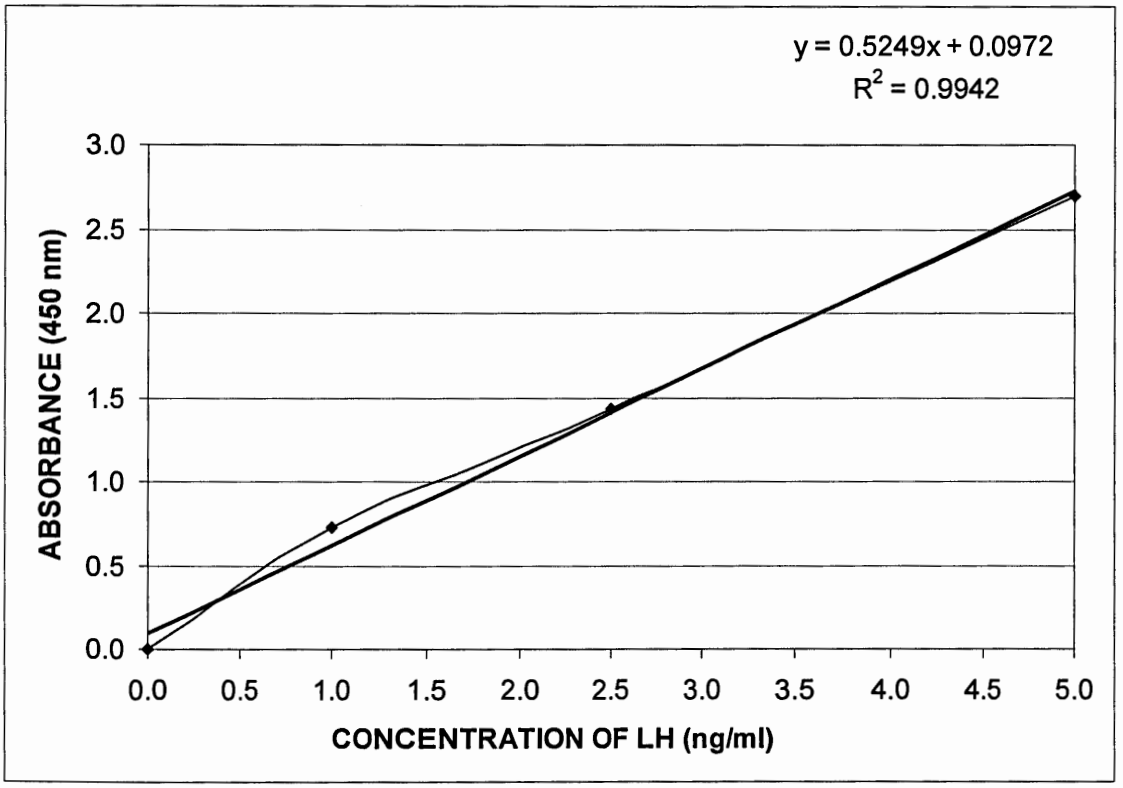


Figure 4: Standard curve for luteinizing hormone (LH) assay.

CHAPTER III

RESULTS

Experiment A. 1: Effect of exposure to a male on fluoxetine-induced estrous cycle disruption in Fischer female rats.

In a prior experiment [81], treatment with daily fluoxetine was reported to disrupt the estrous cycle of Fischer rats and to result in a decline in serum progesterone. Such a disruption was not reported in an earlier study in which females were exposed to a male rat every day [43]. The following experiment was designed to determine if exposure to a male would prevent fluoxetine's disruption of vaginal cyclicity.

It was hypothesized that the daily exposure of female Fischer rats to the male rats would reduce or prevent the estrous cycle disruption by repeated fluoxetine treatment but would have no impact on the reduction in body weight. There were three groups of rats. Two groups were exposed to a male rat every day and were housed in the same room with the male rats. These groups got either fluoxetine (10 mg/kg; 1ml/kg) or vehicle (distilled water, 1ml/kg). A third group (used as a positive control) of rats were treated with fluoxetine (10 mg/kg fluoxetine) but were not exposed to the males.

Both groups treated with fluoxetine lost weight, while the body weight of the vehicle-treated rats did not change (Figure 5). There was a significant effect of treatment $F_{2, 24} = 5.19$, $P \leq 0.002$), day of the experiment ($F_{10, 240} = 14.62$, $P \leq 0.0001$) and their

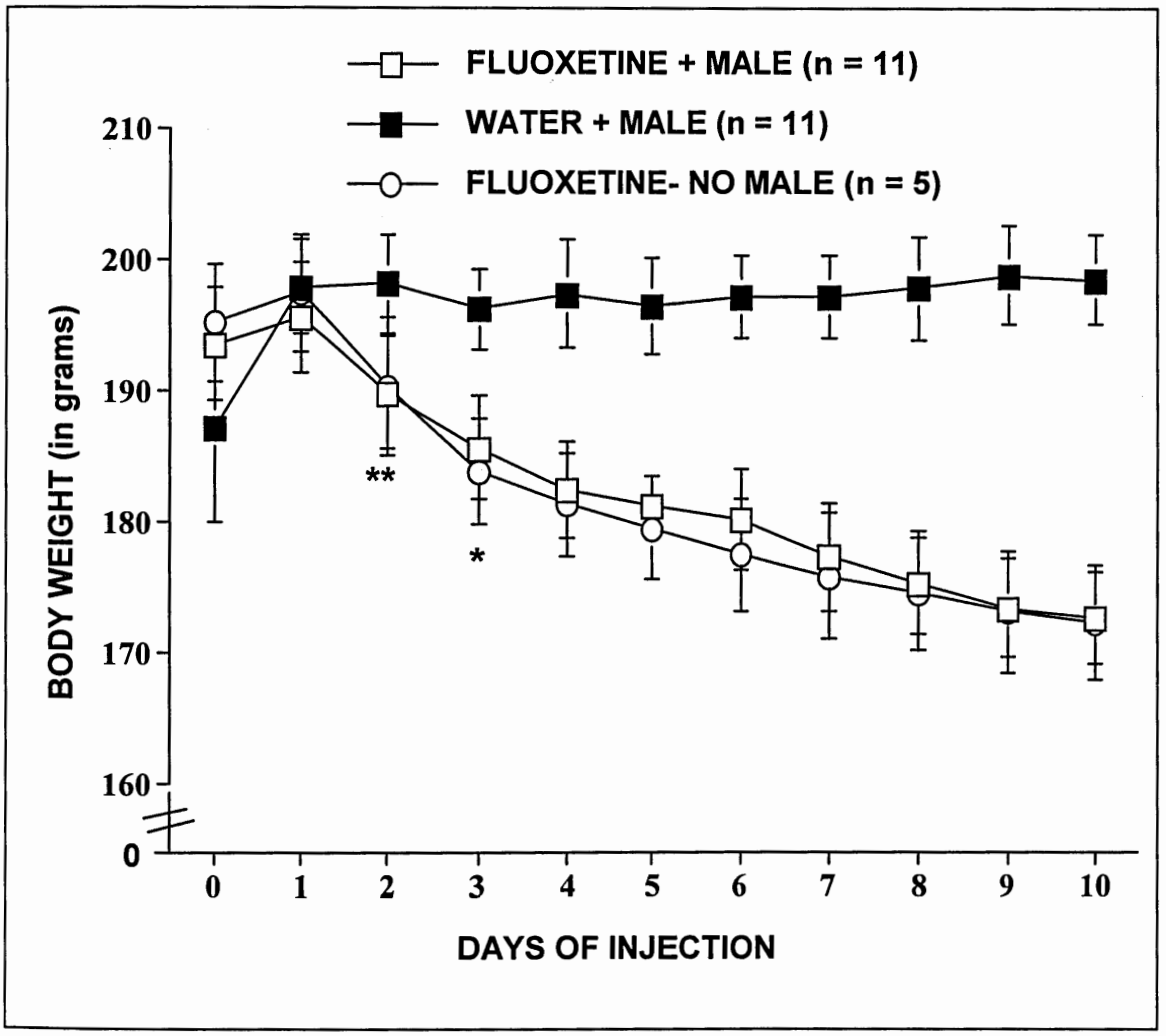


Figure 5: Body weight of female Fischer rats (with or without male exposure) during repeated fluoxetine or water treatment.

Data are for two male-exposed groups and also for one group, not exposed to males. Rats were given *ad libitum* access to food and water. Day 0 indicates body weight the day before the first injection; day 1 is the first day of injection. By day 3 (single asterisk), body weights of both groups of fluoxetine-treated rats were significantly less than their starting weight and then continued to be significantly lower for the rest of the experiment. By day 2 (double asterisk) and thereafter, body weights of both groups of fluoxetine-treated rats differed significantly from the water control group.

interaction ($F_{20, 240} = 8.17$, $P \leq 0.0001$). By day 3, body weights of both groups of fluoxetine-treated rats were significantly less than their starting weight ($q_{240, 10} \geq 4.47$, $P \leq 0.05$) and continued to be significantly lower for the rest of the experiment. By day 2, and for the remainder of the experiment, body weights of both groups of fluoxetine-treated rats significantly differed from the water control group ($q_{240,3} \geq 3.31$, $P \leq 0.05$).

Male exposure reduced the effect of fluoxetine treatment on the estrous cycle of the Fischer female rats (Figure 6). One hundred percent of the rats in the vehicle with male group cycled normally during the entire 10 days of the study. Consistent with prior studies [81], during the first 5 days after injection, only 40% of rats in the fluoxetine without male group showed a proestrous smear and none showed a proestrous smear during the second 5 day cycle. In contrast, 91% of rats in the fluoxetine with male exposure group showed a proestrous smear during the first 5 days and 54.5% showed a proestrous smear in the second 5 day cycle.

There was a significant treatment effect during the first (Chi square = 10.28, df = 2, $P \leq 0.006$) and second (Chi Square = 15.30, df = 2, $P \leq 0.0005$) cycles. There was also a significant effect of treatment on the number of days to show the first proestrous smear after initiation of injection ($F_{2,24} = 5.17$, $P \leq 0.02$). Fluoxetine and vehicle-treated rats with males had cycle lengths, respectively, of 5.00 ± 0.73 and 4.60 ± 0.36; fluoxetine-treated rats without males had an average cycle length of 8.80 ± 1.96 days. When only the rats in male-exposed groups were examined, there were no significant differences between the vehicle (100%) and the fluoxetine-treated rats (91%) during the first cycle

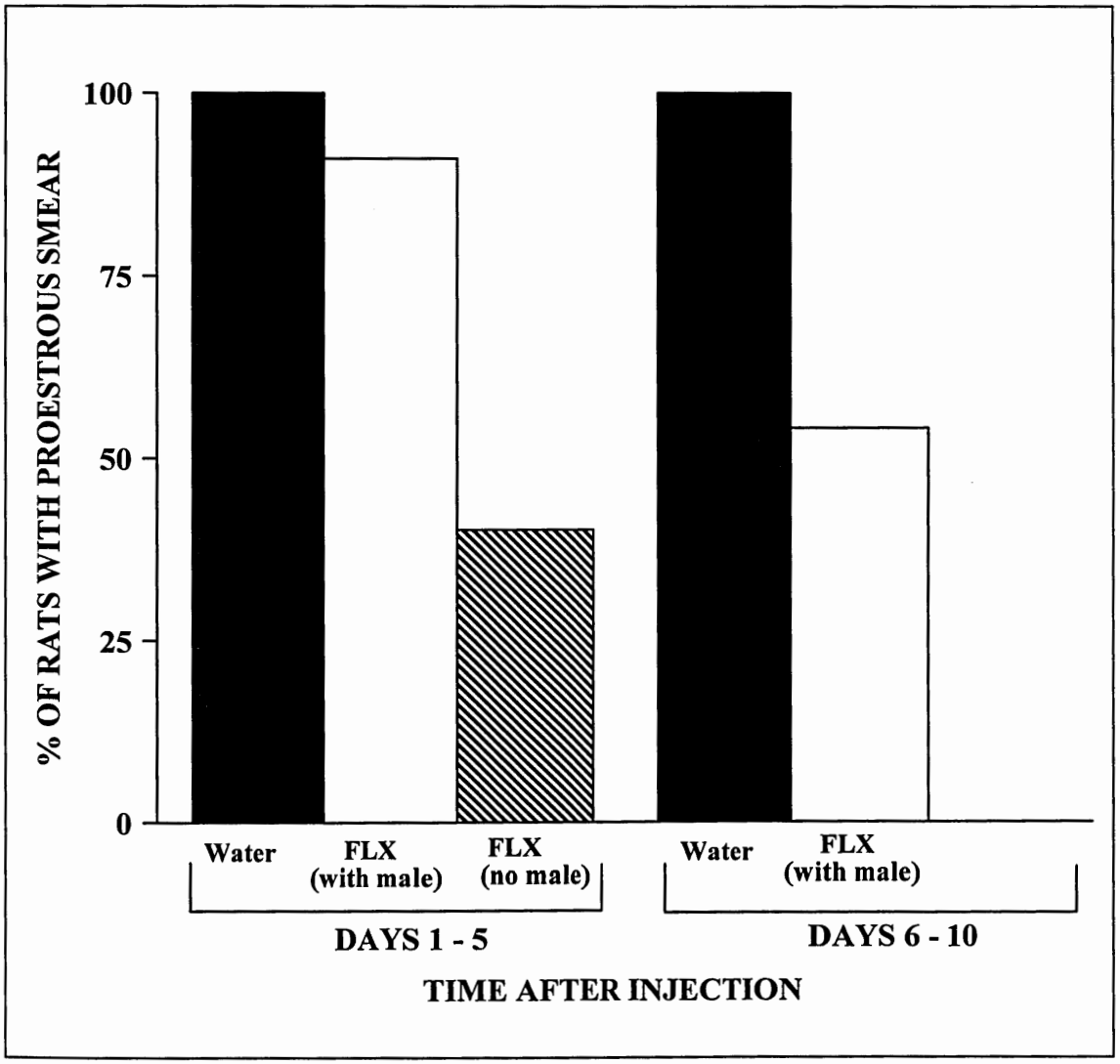


Figure 6: Effects of male exposure on the percentage of female Fischer rats showing proestrous smear after treatment with repeated fluoxetine or water.

Data are for two male-exposed fluoxetine-injected (10 mg/ kg) groups, and also for a fluoxetine-injected but not male-exposed group. Sample size is the same as in figure 4. Rats were given *ad libitum* access to food and water. Vaginal smears were monitored daily as described in the Methods. Plotted is the percentage of rats showing a proestrous smear in each treatment group. When no bars are present (e.g., fluoxetine no male group at cycle 2), there were no animals (0%) in that group that showed a proestrous smear. Data are for the first 5 days and the second 5 days after the beginning of the injections.

(Cycle 1, Chi Square = 1.04, df = 1, $P > 0.05$). However significantly fewer fluoxetine- treated rats (54.5%) cycled during the second 5 days of the experiment (Chi Square = 6.47, df = 1, $P \leq 0.01$). Thus, presence of the male reduced and/or delayed the disruptive effects of fluoxetine on the estrous cyclicity of female Fischer rats.

Experiment A.2: Effects of repeated fluoxetine in Sprague-Dawley females.

If a strain difference were responsible for the estrous cycle differences between the study by Uphouse et al. [81] and other previous studies [23, 28, 43, 83], then Sprague-Dawley rats should show minimal cycle disruption after repeated fluoxetine treatment. In contrast, body weight and food intake, consistently reported to decline after fluoxetine, should be reduced in Sprague-Dawley rats. This was evaluated in the following experiment.

Fluoxetine significantly reduced food intake (ANOVA for treatment, $F_{1,28} = 57.04$, $P \leq 0.0001$) in female Sprague-Dawley rats (Figure 7). This was most evident during the first ten days of treatment so there was a significant effect of day of treatment ($F_{23,644} = 10.29$, $P \leq 0.0001$) as well as a day by treatment interaction ($F_{23,644} = 6.45$, $P \leq 0.0001$). Food intake of fluoxetine-treated rats was significantly less than the vehicle control from day 1 through day 10 of treatment (all Tukey's $q_{644,2} \geq 2.77$, $P \leq 0.05$).

Fluoxetine treatment also significantly reduced the body weight of Sprague-Dawley rats (Figure 8). Vehicle-treated rats gained weight during the experiment while fluoxetine-treated rats lost weight. There was a significant effect of treatment ($F_{1,28} = 15.92$, $P \leq 0.0004$), day of the experiment ($F_{26,728} = 11.39$, $P \leq 0.0001$) and their

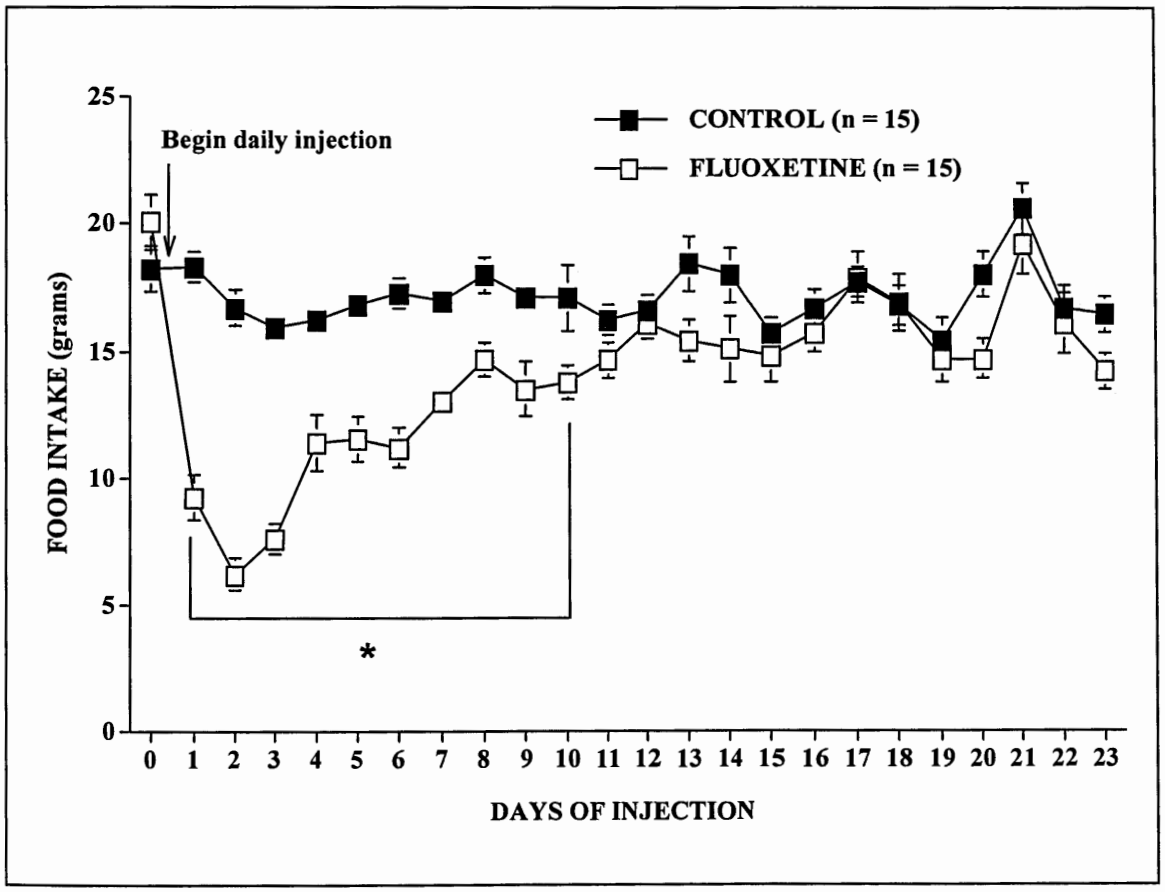


Figure 7: Food intake of female Sprague-Dawley rats during repeated fluoxetine treatment.

Intact, regularly cycling female rats were injected daily with 10 mg/kg fluoxetine (1 ml/kg) or an equivalent volume of distilled/deionized water. Day 0 indicates food intake for the day before the first injection; day 1 is the first day of injection. The injections continued daily for at least 23 consecutive days. Data are the mean ± S.E. food intake (in grams) for each indicated day. The single asterisk indicates days on which there was a significant decrease in food intake in fluoxetine-treated rats relative to the control rats.

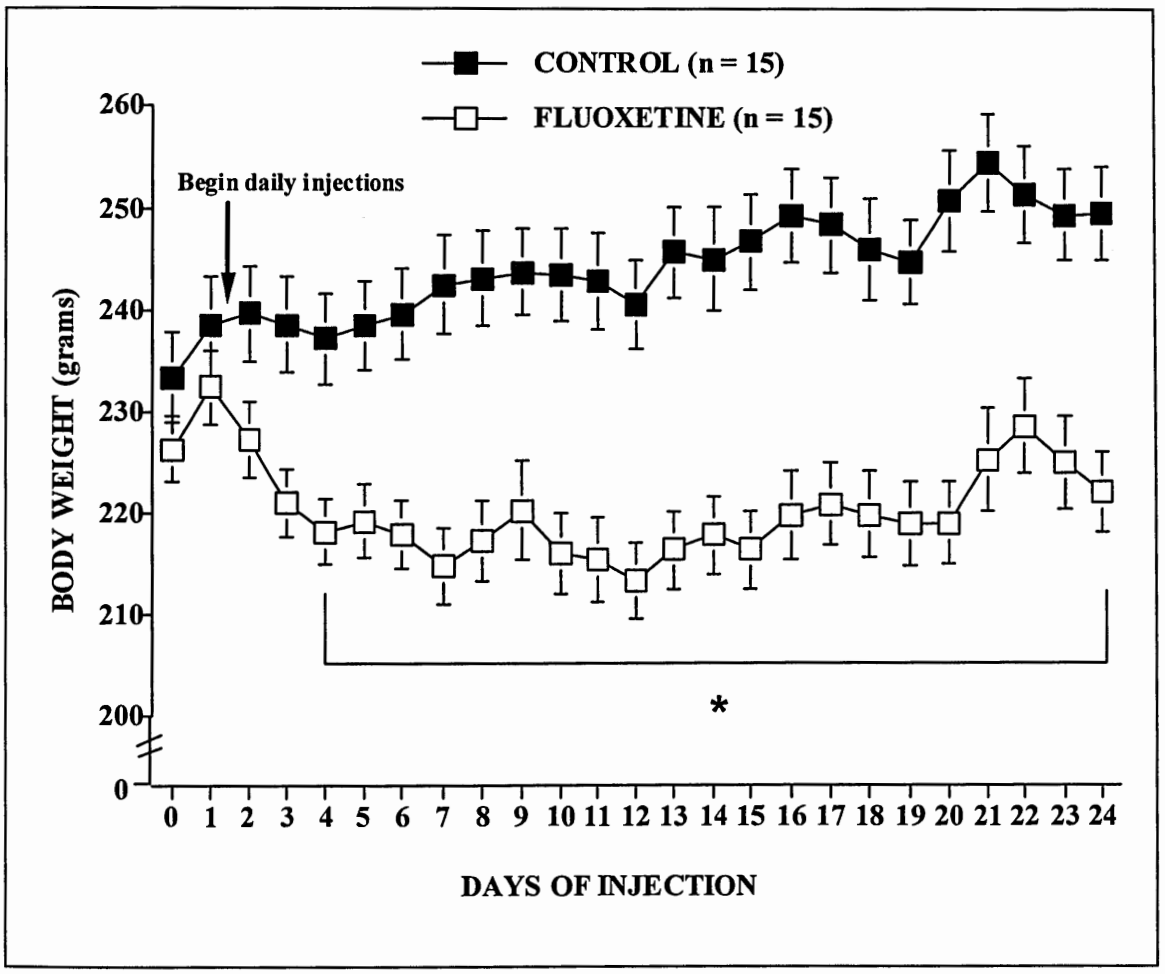


Figure 8: Body weight of female Sprague-Dawley rats during repeated fluoxetine treatment.

Intact, regularly cycling female rats were injected with 10 mg/kg fluoxetine (1 ml/kg) or an equivalent volume of distilled/deionized water. Day 0 indicates body weight the day before the first injection; day 1 is the first day of injection. The injections continued daily throughout the experiment. Data are the mean ± S.E. body weight (in grams) for each indicated day. The sample size from day 1- 23 is placed on the fig, for day 24, data are for 12 fluoxetine and 13 control rats. The single asterisk indicates days on which there was a significant decrease in body weight in the fluoxetine-treated rats relative to the control rats.

interaction ($F_{26,728} = 12.02$, $P \leq 0.0001$). With the exception of day 12 of treatment, the body weight of vehicle-treated rats was significantly greater than their starting weight (from day 8 and thereafter) (Tukey's $q_{728,27} \geq 2.77$, $P \leq 0.05$). Body weights of fluoxetine-treated rats were significantly less than their starting weight from day 4 through day 20 of treatment (Tukey's $q_{728,27} \geq 5.20$, $P \leq 0.05$). Thereafter, their body weights were not different from their starting weights. However, body weights of fluoxetine-treated rats remained significantly lower than that of their vehicle controls (all $P \leq 0.05$).

While less effective than in female Fischer rats, fluoxetine disrupted the estrous cycle of female Sprague-Dawley rats (Figure 9). Rats were injected with vehicle or fluoxetine (10 mg/kg, i.p.), beginning on diestrus 1, the day after a fully cornified vaginal smear, and continued for at least 23 days. Because injections were always initiated on the day after estrus, not every animal received the same number of injections. Prior to injection, Sprague-Dawley females showed both 4 and 5 day estrous cycles, which were equally distributed across the two treatment conditions (mean ± S.E. cycle length prior to treatment was 4.40 ± 0.10 and 4.40 ± 0.11 days for fluoxetine and vehicle treatments, respectively). In the control group, pre-injection cycle length was maintained after initiation of the daily injections [mean ± S.E. cycle length was 4.20 ± 0.07 days (median = 4.00, range = 4.00 to 5.00)]. In contrast to the vehicle-treated rats, 6 out of 15 of the fluoxetine-treated rats showed elongated cycles at least once during the experiment. Overall, the mean cycle length for fluoxetine-treated rats was 5.20 ± 0.46 (median = 4.30, range = 4.00 to 9.30) (see Table 2). Cycle lengths of fluoxetine and water treated

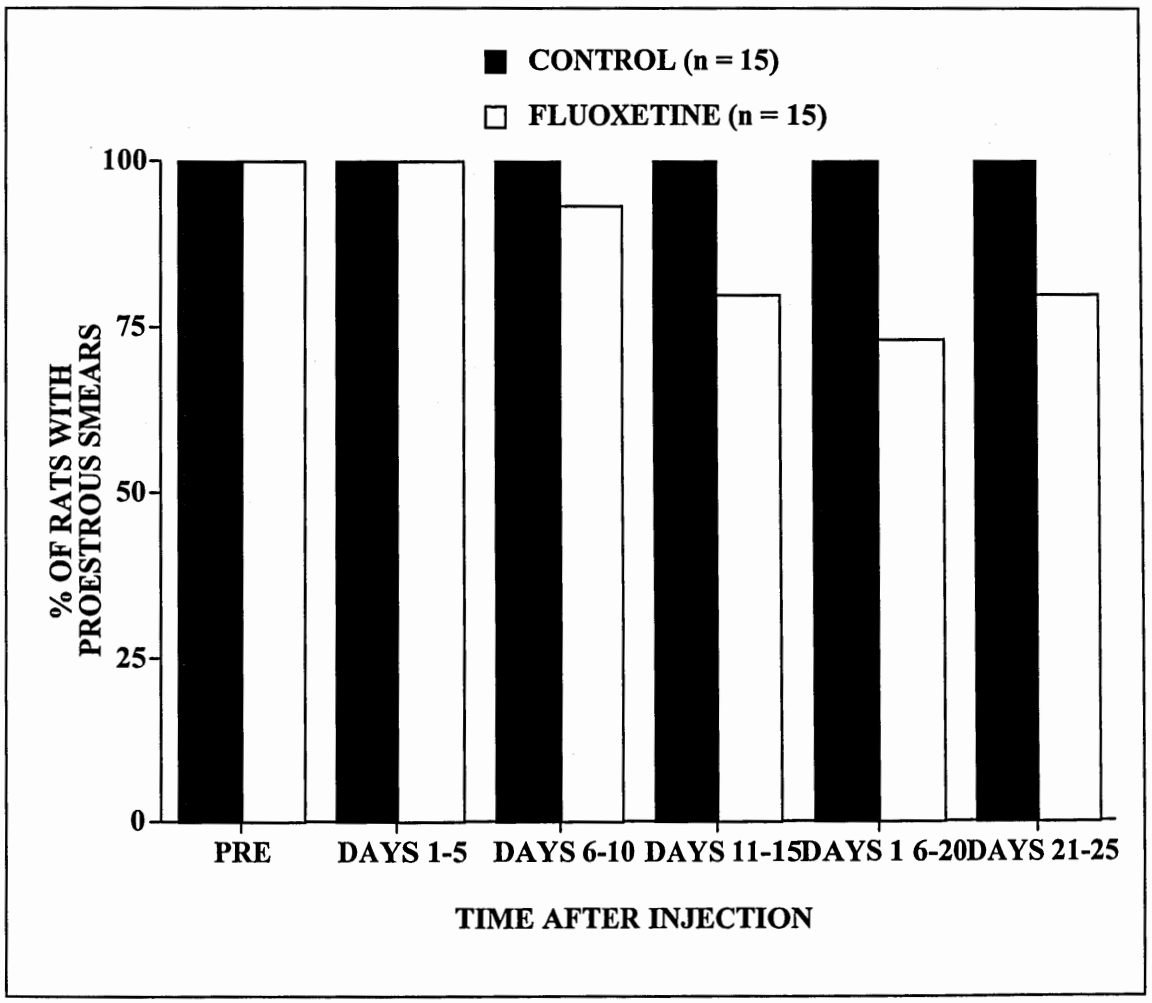


Figure 9: Percentage of female Sprague-Dawley rats showing a proestrous smear during repeated fluoxetine treatment.

Intact, regularly cycling Sprague-Dawley female rats were injected with 10 mg/kg fluoxetine (1 ml/kg) or an equivalent volume of distilled water beginning at diestrus 1 and continuing for 25 days. Data are the percentage of rats showing a proestrous smear in each of the treatment groups. Data were grouped into 5 day intervals for presentation. Sample size from day 1-day 23 is in the figure. From day 24 and thereafter, data are for 12 fluoxetine and 13 control rats. The majority of control animals continued to show regular cyclicity throughout the experiment. In contrast, 6 out of 15 fluoxetine-treated rats showed an elongated cycle at least once during the experiment.

Table 2: Cycle lengths of Sprague-Dawley female rats during repeated fluoxetine or vehicle treatment.

Repeated treatment	N	Mean cycle length ± S.E. (pre-treatment)	Mean cycle length ± S.E. (post-treatment)	Median cycle (range) (post-treatment)
Vehicle (water)	15	4.40 ± 0.11	4.20 ± 0.07	4.00 (4.00-5.00)
Fluoxetine	15	4.40 ± 0.10	5.20 ± 0.46	4.30 (4.00-9.30) *

Table 2: Data are the pre and post-treatment estrous cycle lengths for water and fluoxetine-treated rats. The vehicle-treated group maintained their pretreatment cycle length, whereas the cycle length was elongated in the fluoxetine-treated rats due to the presence of 6 rats that showed a disruption in their estrous cycle. The asterisk indicates significant difference between fluoxetine and vehicle-treated rats (Mann-Whitney U).

rats were significantly different (Mann-Whitney U = 161.5, df = 14 and 14, $P \leq .04$).

Fluoxetine treatment reduced sexual receptivity in some of the Sprague-Dawley rats (Figure 10). The low receptivity throughout the experiment resulted exclusively from the six rats that also showed disturbance of the estrous cycle; these six rats had an absence of sexual receptivity that occurred prior to an elongated vaginal cycle. With the exception of these six rats, the remaining fluoxetine-treated rats were sexually receptive during each proestrus. Similarly, when rats were tested on the last proestrous day prior to sacrifice, there was no significant difference between the groups (see Table 3). Thus, although fluoxetine treatment did disrupt the estrous cycle in some rats, the effects of fluoxetine on the estrous cycle of female Sprague-Dawley rats was not as robust as it was seen in the female Fischer rats [81].

Experiment B. 1: The effect of pair-feeding on sexual behavior and estrous cycle disruption in female Fischer rats.

The next experiment was designed to determine if the estrous cycle disruption after fluoxetine injection was due to a reduced food intake and consequent loss of body weight. If so, pair-feeding should mimic the effect of repeated fluoxetine on sexual behavior and estrous cycle disruption. Each rat from a group of control animals was matched for the pretreatment estrous cycle with a fluoxetine-treated rat and was pair-fed to its fluoxetine match. A secondary objective of the study was to test the hypothesis that the female's estrous cyclicity would be reinitiated after a more extended treatment with

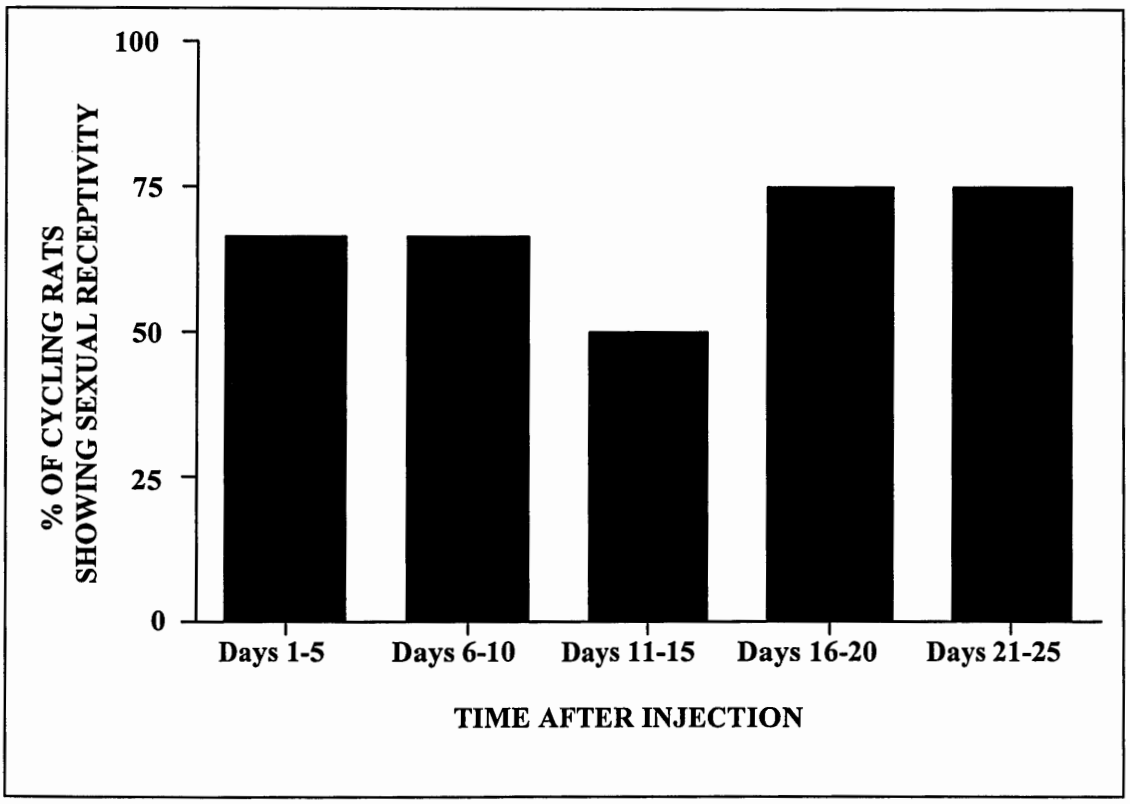


Figure 10: Percentage of female Sprague-Dawley rats showing sexual receptivity during repeated fluoxetine treatment.

Data are the percentage of fluoxetine-treated rats showing sexual receptivity each time they had a proestrous smear. Data were grouped into 5 day intervals for presentation. N's for the test intervals, respectively, were 12, 12, 10, 12, and 12, but do not necessarily include the same set of animals in each test interval.

Table 3: Lordosis behavior of Sprague-Dawley female rats during repeated fluoxetine or vehicle treatment.

Treatment	N	L/M Ratio ± S.E.
Vehicle	13	0.86 ± 0.08
Fluoxetine (10 mg/kg)	12	0.86 ± 0.06

Table 3: Data are the lordosis/mount ratios for rats tested on proestrus of the final week prior to completion of the experiment. Rats that failed to exhibit a proestrous smear during the last 5 days of the experiment were not tested. There was no significant difference in lordosis behavior between the two groups. Data are for 13 vehicle and 12 fluoxetine-treated rats.

fluoxetine. Repeated fluoxetine treatment reduced food intake in female Fischer rats (Figure 11). Food intake of pair-fed rats was identical to that of the fluoxetine-treated group. Therefore, food intake data were compared only between the fluoxetine-treated and control rats. Food intake for the first 16 days was included in the repeated measures ANOVA since every animal had received 16 days of injections. There was a significant effect of treatment ($F_{1,18} = 121.1$, $P \leq 0.0001$), day of treatment ($F_{16, 88} = 3.72$, $P \leq 0.0001$) and their interaction ($F_{16,288} = 6.83$, $P \leq 0.05$). Treatment of rats with fluoxetine reduced daily food intake within the first 24 hr of treatment and continued to do so throughout the 16 days of treatment (Tukey's, all $q_{288, 2} \geq 2.77$, $P \leq 0.05$). Since some animals received more than 16 days of injections, data after day 16 for these rats were compared by single factor ANOVA for each of the successive days. For these rats, food intake began to approach that of the control rats after day 16. With the exception of a significant difference in food intake on day 19, food intake across groups was not different after day 17.

Repeated fluoxetine treatment and pair-feeding significantly reduced the body weight of female Fischer rats (Figure 12). Unlike food intake, which was assessed 24 h after injection, body weight was assessed before sacrifice so that all animals were included in the sample for the first 17 days. Analysis of body weight data by repeated measures ANOVA was run for these first 17 days and independent ANOVA were used for the remaining days, as described above. For the first 17 days, there was a significant effect of treatment ($F_{2, 25} = 10.01$, $P \leq 0.0001$), day of treatment ($F_{17, 425} = 11.98$,

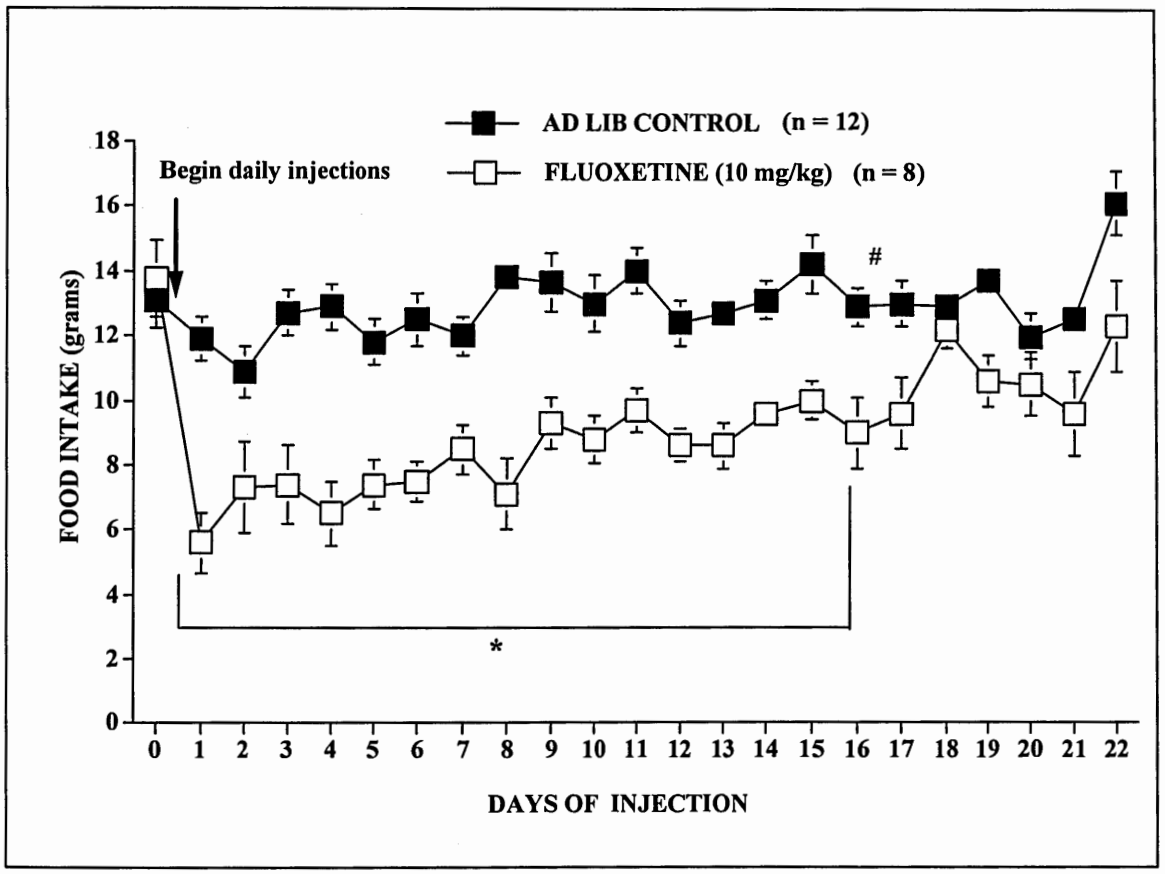


Figure 11. Food intake of female Fischer rats during repeated fluoxetine treatment.

Intact, regularly cycling female rats were injected with 10 mg/kg fluoxetine (1 ml/kg) or an equivalent volume of distilled/deionized water vehicle. Plotted are food intake data for fluoxetine-treated rats and for vehicle-treated rats with *ad libitum* access to food and water (AD LIB control). Day 0 indicates food intake for the day before the first injection; day 1 is food intake during the first day of injection. From day 0 through day 16, data are the mean ± SE food intake (in grams) for fluoxetine-treated and *ad libitum* control rats. Sample size until day 16 is in the figure. For days 17–22, data are from 8, 7, 7, 7, 7, and 7 fluoxetine-treated rats and 2, 12, 12, 8, 6, and 4 *ad libitum* control rats. The single asterisk indicates days on which there was a significant decrease in food intake relative to day 0. The # indicates the point at which the number of rats began to decrease.

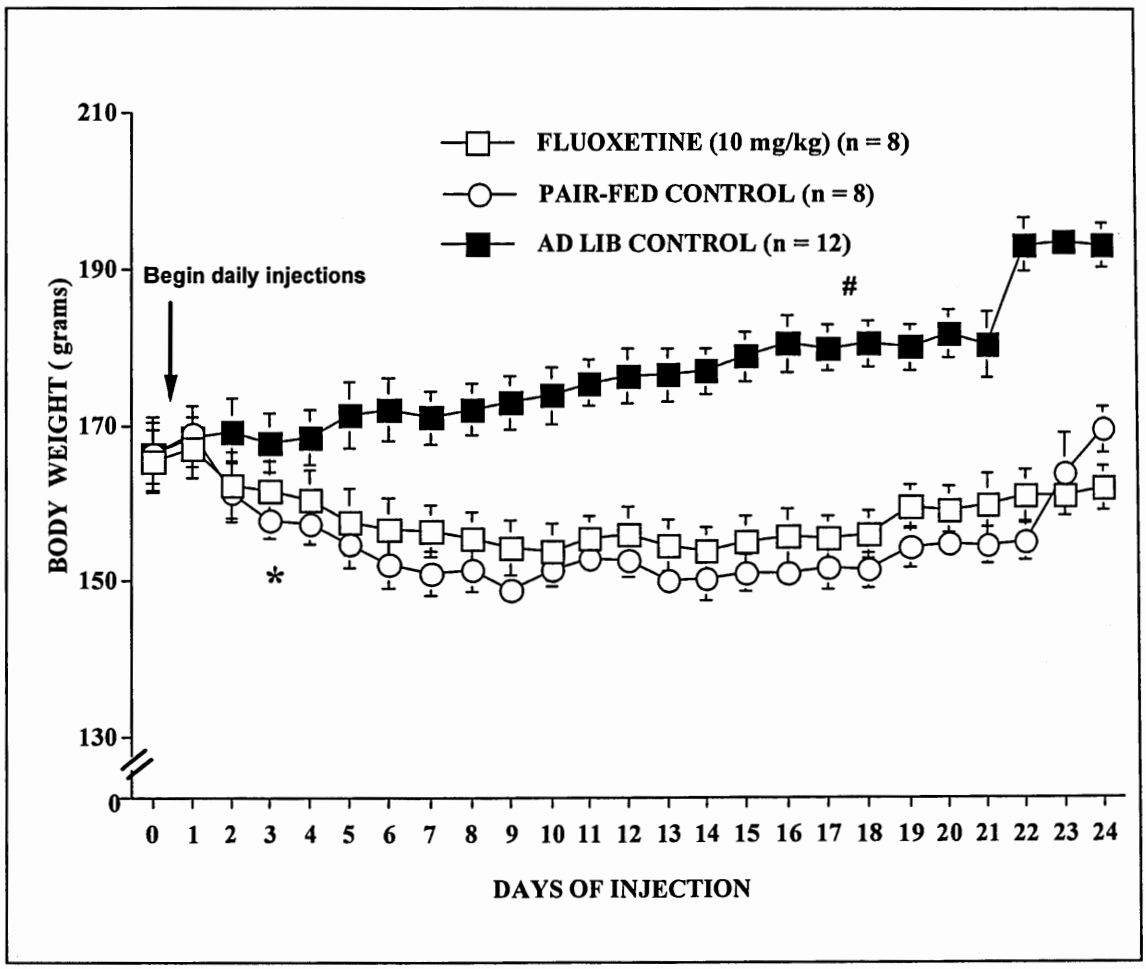


Figure 12. Body weights of female Fischer rats during repeated fluoxetine, pair-feeding or vehicle treatment.

Intact, regularly cycling female rats were injected with 10 mg/kg fluoxetine (1ml/kg) or an equivalent volume of distilled/deionized water vehicle. Vehicle-treated rats were either pair-fed with a fluoxetine-treated rat (Pair-Fed Control) or received *ad libitum* food and water (AD LIB Control). Day 0 indicates body weight the day before the first injection; day 1 is the first day of injection. From day 0 through day 16, data are the mean ± S.E. body weight (in grams) for fluoxetine-treated and *ad libitum* control rats. Sample size until day 16 is in the figure For days 17–24, data are from 8, 8, 7, 7, 7, 7, 7, and 5 fluoxetine-treated; 8, 7, 7, 7, 7, 6, and 3 pair-fed rats; and 12, 12, 12, 12, 8, 6, and 4 *ad libitum* controls. The single asterisk indicates the first day on which a significant decrease in body weight occurred relative to day 1. The # indicates the point at which the number of rats began to decrease.

$P \leq 0.0001$) and their interaction ($F_{34, 425} = 19.76$, $P \leq 0.0001$). Body weight of pair-fed and fluoxetine-treated rats was comparable (pair-fed rats had significantly smaller body weight only on days 9 and 13; $q_{425, 3} \geq 3.14$, $P \leq 0.05$).

Both repeated fluoxetine treatment and pair-feeding disrupted the estrous cycle in Fischer rats (Figure 13). Nine of the twelve control rats cycled regularly throughout the experiment while three control rats skipped a cycle at some point in the study. In contrast, both fluoxetine-treated and pair-fed rats showed a consistent disruption of the cycle. Consequently, there was a significant treatment effect on the frequency of disrupted cycles (Chi Square = 17.68, df = 2, $P \leq 0.0001$). However, with extended fluoxetine treatment, many of the fluoxetine-treated rats began to show signs of regaining estrous cyclicity. Six of the eight fluoxetine-treated rats showed some recovery from cycle disruption by the conclusion of the experiment so that there was a significant difference between the first 10 days and last 10 days of the experiment (Chi Square = 9.60, df = 1, $P \leq 0.002$). Four of these rats showed a proestrous smear by day 16 of injection and two more showed vaginal estrus by day 22. Moreover, when scored for the presence or absence of a lordosis reflex during the last 5 days of the experiment, five of these six fluoxetine-treated rats showed a lordosis response to the male rat's mount. In contrast to the fluoxetine-treated rats, only one pair-fed rat showed any evidence of reinitiation of vaginal cyclicity and this rat showed no response to the male. The recovery of estrous cyclicity (Figure 13) and sexual receptivity of the fluoxetine-treated rats and the restoration of food intake (Figure 11) in the fluoxetine-treated animals followed roughly the same time-course. Repeated fluoxetine treatment leads to a rapid

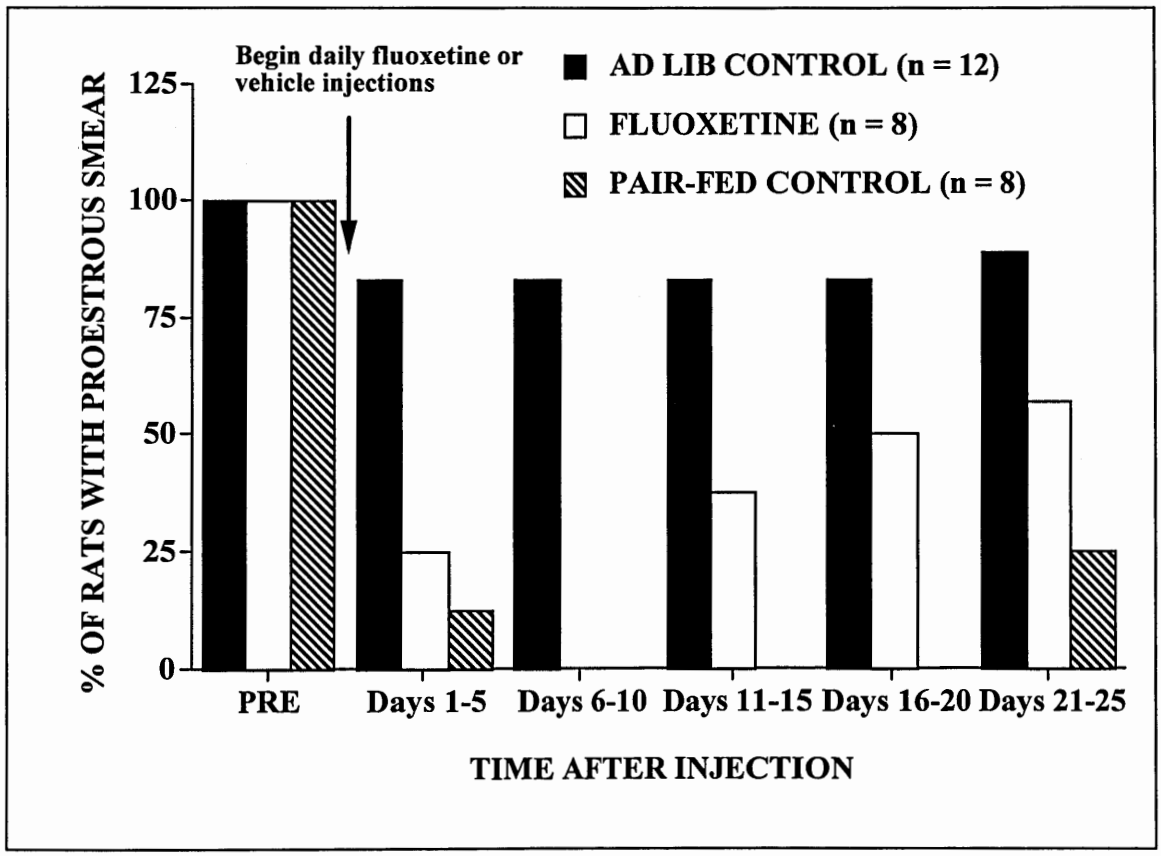


Figure 13: Percentage of female Fischer rats showing proestrous smear during repeated fluoxetine, pair-feeding or vehicle treatment.

Intact, regularly cycling female rats were injected with 10 mg/kg fluoxetine (1 ml/kg) or an equivalent volume of distilled/deionized water vehicle. Vehicle-treated rats were either pair-fed with a fluoxetine rat (Pair-Fed Control) or received *ad libitum* food and water (AD LIB control). Estrous cycles were monitored daily as described in the Methods. Plotted is the percentage of rats showing a proestrous smear in each treatment group. When no bars are present (e.g., days 6–10, 11–15, and 16–20), there were no animals (0%) in that group that showed a proestrous smear. Data are for the 5 days before treatment (PRE) and for blocks of 5 days thereafter. Throughout the experiment, the percentage of rats with nucleated or cornified cells was significantly greater in the *ad libitum* control than in either of the other two groups.

disruption of estrous cycle in female Fischer rats with the elimination of their sexual behavior. It also decreases food intake and body weight relative to the vehicle-treated rats. Food restriction mimicked the effect of fluoxetine on cycle disruption and sexual behavior. Thus, the anorectic properties of fluoxetine may be contributing, in part, toward the estrous cycle disruption.

Experiment B. 2: Effects of repeated and acute fluoxetine in hormonally primed OVX Fischer rats.

If a disturbance in the estrous cycle were responsible for fluoxetine-induced disruption of sexual behavior, then, in the absence of a cycle disruption, the normal sexual behavior should be restored. Hormonal priming in OVX rats should produce the appropriate conditions for sexual behavior. It was, therefore, hypothesized that such priming would reduce the effect of fluoxetine on sexual behavior. In addition, repeated fluoxetine has been reported to decrease the effects of a 5-HT receptor agonist [83] and the 5-HT_{1A} receptor mediates 5-HT's inhibitory effect on female rat sexual behavior [78,79]. Therefore, it was further hypothesized that repeated fluoxetine treatment would reduce any disruptive effect of acute fluoxetine on sexual behavior.

In the absence of the injection on the 10^{th} day, 9 days of repeated fluoxetine had no inhibitory effect on the lordosis behavior of OVXed and hormonally primed female Fischer rats (Table 4). OVX Fischer rats were injected daily for 9 days with 10 mg/kg fluoxetine or water vehicle (i.p., 0.1 ml/kg). On the 8th day of treatment, rats were injected with 10 μg estradiol benzoate followed 48 hr later with 500 μg progesterone

Table 4: Effects of repeated fluoxetine treatment on lordosis behavior in ovariectomized and hormone-primed female Fischer rats.

Repeated treatment	Mean L/M ratio ± S.E.	Median L/M ratio (Range)	N
Vehicle	1.0 ± 0	1.00 (1.00-1.00)	34
Fluoxetine (10 mg/kg)	0.92 ± 0.04	1.00 (0.80-1.00)	29

Table 4: Data are the lordosis/mount ratios for fluoxetine and vehicle-treated rats on the 10^{th} day of the experiment before their 10^{th} day injection. Ovariectomized rats were repeatedly treated for 9 consecutive days either with fluoxetine or vehicle; on the 8^{th} day, they were injected with estradiol benzoate (10 μg) and on the test day morning, they were injected with progesterone (500 μg). There was no significant difference in lordosis behavior between the two groups.

(s.c., 0.1 ml/rat). Lordosis behavior was monitored 4-6 h later, prior to injection on the 10th day. Two rats (both given repeated fluoxetine) could not be tested because the males refused to mount them. These two rats were excluded from the analysis. For the remaining rats, fluoxetine-treated rats were more variable with L/M ratios as low as 0.8, but both fluoxetine and water-treated rats had high levels of lordosis responsiveness. Thus, 24 hr after repeated fluoxetine treatment, lordosis behavior initiated by exogenous hormone priming was not reduced.

The effects of the acute treatment on the 10^{th} day injection with fluoxetine or water in rats repeatedly treated with fluoxetine or water on their sexual receptivity are shown in Figure 14. Data are for the same rats shown in Table 2 but after treatment on the 10th day. On the 10th day of the experiment, rats were tested for sexual receptivity and then injected with 0, 10 or 20 mg/kg fluoxetine. Thirty min later, sexual behavior was monitored for 10 mounts. There were significant effects of the type of repeated treatment (fluoxetine or water) ($F_{1,57} = 6.06$, $P \leq 0.02$), type of injection on the 10th day ($F_{2,57} = 43.47$, $P \leq 0.0001$), and their interaction ($F_{2,57} = 8.37$, $P \leq 0.0006$). In rats treated previously with water, both doses of fluoxetine injected on the 10th day significantly reduced lordosis behavior ($q_{6,60} = 5.21$ and 13.24, respectively). Similarly, in rats injected daily with 10 mg/kg fluoxetine, there was a decline in lordosis behavior after injection with fluoxetine. However, only for the higher dose of fluoxetine was the difference significantly different from the 10th day water injection ($q_{60,6} = 5.31$, $P \leq 0.05$). Repeated treatment with 10 mg/kg fluoxetine significantly reduced the effect of

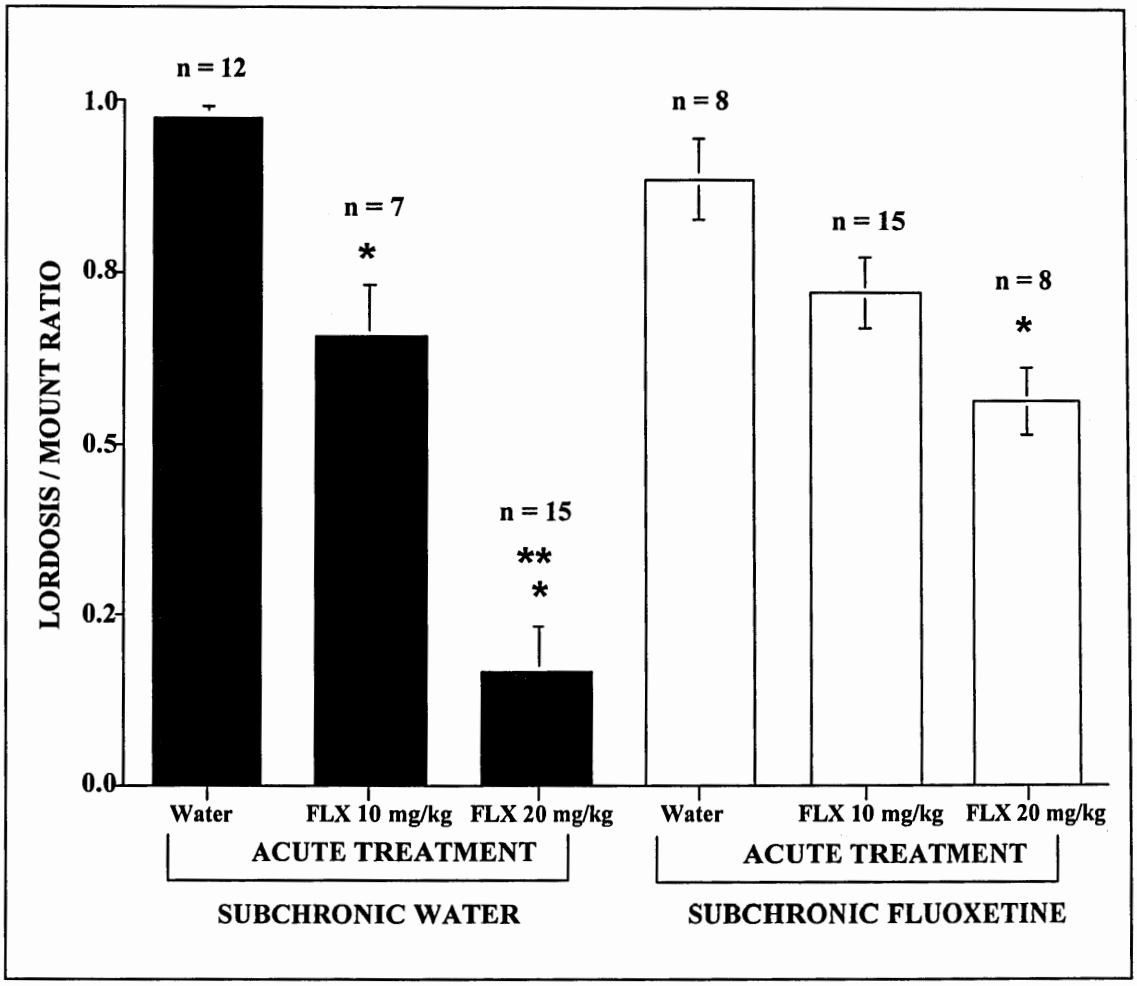


Figure 14: Effect of repeated treatment with fluoxetine or vehicle on the response to acute fluoxetine.

Data are the mean ± S.E. lordosis to mount ratios for rats previously treated with water or 10 mg/kg fluoxetine and injected on the 10th day with water, 10 mg/kg or 20 mg/kg fluoxetine. Single asterisks indicate a difference from the vehicle-treated rats. Double asterisks indicate a difference between repeated fluoxetine and repeated water treatment in the same acute treatment condition.

20 mg/kg fluoxetine on the 10th day of injection ($q_{60,6} = 6.47$, $P \leq 0.05$).

B. 3. Effect of fluoxetine injection on serum LH in female Fisher rats.

The final set of experiments were designed to test the hypothesis that repeated fluoxetine would reduce the LH surge. To test this hypothesis, on the day of diestrus 1 (day after a fully cornified smear), intact regularly cycling female rats were injected daily with fluoxetine (10 mg/kg) or vehicle (1 ml/kg). Four days later (at the time of the presumed preovulatory LH surge), females were sacrificed by decapitation and trunk blood was collected for ELISA measurement of serum LH.

In the control group, 5/8 of the females showed evidence of preovulatory uterine swelling (expected for females on the morning/afternoon of proestrous) but only 2 of the rats had nucleation/cornification of the vaginal smear. These two rats showed evidence of relatively high levels of LH in serum (e.g. 2.90 and 4.70 ng/ml, respectively). LH was undetectable in the remaining 6 rats. In the fluoxetine-treated group, 2/14 females had uterine swelling and both of these rats showed cornified vaginal smears. In addition, both of these rats had detectable LH (3.40 and 5.60 ng/ml, respectively) in blood serum. The remaining 12 rats showed diestrous smears, no uterine swelling, and undetectable levels of LH in blood serum.

Unfortunately, the failure of the control females to show high levels of LH in blood serum prevented us from drawing any conclusions from the experiment. In the absence of an indwelling cannula for repeated sampling of blood serum, it is very difficult to catch individual females at the precise timing of the preovulatory surge.

Therefore, we attempted an alternative approach to address the hypothesis.

OVX females were left undisturbed for 2 weeks. Two weeks after ovariectomy, when females are released from the negative feedback from ovarian estrogen and reportedly show relatively high levels of blood LH [35,84], rats were treated with fluoxetine. If fluoxetine blocks/reduces LH, then it might be anticipated that fluoxetine would also reduce the postovariectomy increase in LH. Females received 5 daily injections with fluoxetine (10mg/kg) or vehicle (distilled water 10 mg/kg). Rats were killed on the 5^{th} day, 2 hr after the last fluoxetine injection, and trunk blood was collected for the LH assay. There were no significant differences between fluoxetine and vehicle-treated females in serum LH (Figure 15, $t = 0.86$, $P > 0.05$).

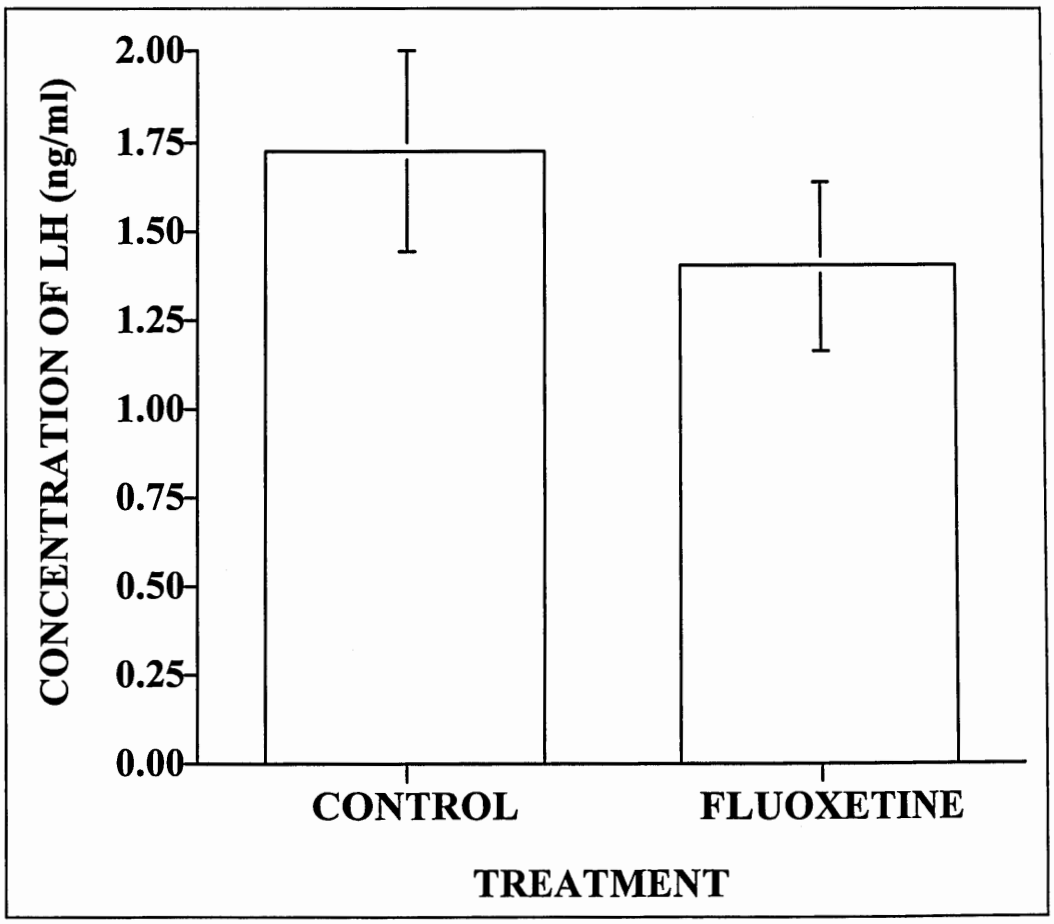


Figure 15. Effect of 5 days of fluoxetine on serum LH in OVX female Fischer rats.

Rats were OVX and 14 days later they received either a fluoxetine (10 mg/kg) or a vehicle (10 mg/kg distilled water) injection for 5 days. Data are for 6 fluoxetine and 6 vehicle-treated rats. Data are the mean ± S.E. serum LH (ng/ml).

CHAPTER IV

DISCUSSION

These studies were designed to evaluate potential explanations for the different conclusions by Uphouse et al. [81] and other investigators regarding the effect of fluoxetine on female rat estrous cyclicity. The second purpose was to identify possible mechanisms for the estrous cycle disruption in female Fischer rats after repeated fluoxetine treatment. Major findings of these studies were: 1) repeated fluoxetine disrupts the normal estrous cycle; 2) Fischer female rats are more vulnerable to this disruption than Sprague-Dawley rats; 3) fluoxetine's anorectic property may contribute toward the drug's disruption of estrous cyclicity and sexual behavior; and 4) fluoxetine's effect on female sexual behavior may involve a disturbance of the cyclic events normally leading to sexual receptivity.

Since the study by Matuszczyk et al. [43] was the only prior report in which the female's estrous cyclicity was a major focus of investigation, it was important to examine any procedural difference that could explain the absence of a disruption of estrous cyclicity in the study by Matuszczyk's et al. Because Matuszczyk et al. [43] tested female rats daily with male rats, it was possible that this daily testing had prevented the cycle disruption. The presence of the male or even the olfactory cues from male's urine can influence the female's reproductive cycle in a variety of mammalian species

[48,49,67]. In intact rats, this phenomena has been most thoroughly studied in aged or estrogenized females where the male's urine, via vomeronasal stimulation, leads to a cascade of events culminating in the release of GnRH and ultimately LH [50]. An increase in serum progesterone has been seen within 2 hr of exposure to the male's urine [50]. This is potentially important given the findings by Uphouse et al. [81] that serum progesterone was reduced in fluoxetine-treated female. The role of progesterone in facilitation of both sexual behavior and reproductivity is well documented [39,40]. In our experiment, exposure to a male rat reduced the effect of repeated fluoxetine on the estrous cycle disruption in comparison to the rats that were never exposed to male rat. Therefore, it is likely that Matuszczyk's daily testing procedure contributed to the absence of a fluoxetine-induced disturbance of the estrous cycle.

However, the use of a different rat strain may also have been a contributing factor. Although treatment with fluoxetine did disrupt the estrous cycle of Sprague-Dawley female rats, the degree of disruption was less than in the Fischer strain. These two genetically related strains (Fischer rats are inbred strains, maternally derived from outbred Sprague-Dawley strains) differ in a variety of traits. Important to the current study is their difference in HPA axis functioning [33]. Fischer rats have greater basal levels of corticosterone [62] and a heightened HPA response to stress [20,54]. In addition, Fischer rats are reported to have a hyperfunctional serotonergic system [25,62] and greater tissue levels of 5-HT in the hippocampus [14] and respond differently to serotonergic and antidepressant compounds [14,55,82]. Since fluoxetine leads to HPA axis activation and increases 5-HT, Fischer females, starting at a higher basal level, may

more easily attain a physiological state that is reproductively disruptive.

Another contributing factor to the strain difference may be the weight and adiposity difference between Fischer and Sprague-Dawley rats. Fischer rats have lower body weight and less adipose tissue (but greater adipose tissue relative to their body weight) than do Sprague–Dawley rats [68]. Fluoxetine's anorectic property is well recognized [5,17,28,31] and both Fischer and Sprague-Dawley rats responded to fluoxetine with a rapid decrease in their body weight. A disruption of reproduction and menstrual cyclicity is seen in human females when negative energy balance is produced in the body due to anorexia [32,85]. Menstrual cycles resume again when a positive energy balance is restored [85]. The mechanism for this is not conclusive [85] but probably includes activation of the HPA axis and, therefore, CRH release with consequent suppression of pulsatile release of GnRH and ultimately LH [11,61]. A reduction in the estrogen level is thought to contribute to this reproductive decline [15,38,85]. Since Sprague-Dawley rats began at a higher body weight then the Fischer rats, the percent decrease was less following fluoxetine. Consequently, Fischer rats may have been more vulnerable to anorexia induced disruption of the estrous cycle.

If increased vulnerability of the Fischer rats towards anorexia is one of the reasons for fluoxetine-induced estrous cycle disruption, then pair-feeding or food restriction should have the same effect on estrous cycle disruption as the repeated fluoxetine treatment. This hypothesis was tested by pair-feeding rats to the food intake of fluoxetine-treated females. Pair-feeding, like fluoxetine, led to a rapid disruption of both the estrous cycle and sexual behavior. With elongated treatment of fluoxetine, females

started to cycle again but the pair-fed match did not. Therefore, anorexia may disrupt the normal hormonal balance required for proper reproductive functioning and thereby explain the decline in sexual behavior.

If such a hormonal mechanism is responsible for fluoxetine's effect on female sexual behavior, then, exogenous hormonal priming should reduce the effect of fluoxetine on sexual behavior. OVX female rats fail to show sexual receptivity but priming with estrogen and progesterone restores the behavior [74,40]. In the current experiment, an acute treatment with fluoxetine reduced lordosis behavior of hormonally-primed OVX females with greater effects at a higher dose (20 mg/kg). Acute effects of fluoxetine on female rat sexual behavior are consistent with prior reports and with the inhibitory effects of 5-HT on lordosis behavior [15,23,45,77]. Since 5-HT's lordosis inhibiting effect resides, in part, from the neurotransmitter's activation of 5-HT_{1A} receptors [80], it is tempting to speculate that fluoxetine acutely reduces lordosis behavior via enhanced activation of 5-HT_{1A} receptors. Repeated treatment with fluoxetine leads to desensitization of 5-HT_{1A} receptors [58,83] that could explain the smaller effect of fluoxetine in females previously treated with 10 mg/kg fluoxetine.

Such a possibility may also explain the recovery of sexual behavior in intact female Fischer rats after 16-18 days of fluoxetine [81]. The role of 5-HT_{1A} receptors in SSRI-induced female sexual dysfunction has not yet been examined. However, Sukoff Rizzo et al. [75] reported that 5-HT_{1A} receptor antagonists reversed the effect of repeated fluoxetine treatment on non-contact penile erections in male rats.

Collectively these findings support the idea that fluoxetine-induced reduction of

sexual behavior and disruption of vaginal cyclicity may result from effects of the drug on the HPG axis function. If so, fluoxetine might be expected to decrease LH, and thereby, prevent follicular maturation and/or ovulation. However, fluoxetine failed to show any significant effect on serum LH. This is surprising since both acute and repeated fluoxetine have been reported to decrease serum LH levels [35,84] and may have resulted from our failure to sample animals at the appropriate time in the cycle. In the intact model, in the absence of an indwelling cannula for repeated sampling of serum, it is very difficult to catch individual females at the precise timing of the preovulatory LH surge. However, this does not explain the failure to see any change in serum LH in the OVX model. However, high LH levels in OVX rats (without hormonal priming) reflect a release of the HPG axis from estrogen's inhibitory feedback [35]. The preovulatory LH surge results from the positive feedback of estrogen and involves distinctly different mechanisms from this negative feedback [84]. Consequently, with the current data, we cannot rule out the possibility that intact females, treated with fluoxetine, fail to exhibit the preovulatory LH signal required for ovulation.

In summary, fluoxetine-induced disturbances in the HPG axis, leading to an estrous cycle block, may account for reported effects of the drug on female sexual dysfunction. The complete mechanism accounting for these effects of fluoxetine are not known. However, the following working model represents a starting point for future investigators (Figure 16). 5-HT, or reduced food intake can activate the HPA axis, which suppresses GnRH release and exerts an inhibitory effect on the HPG axis.

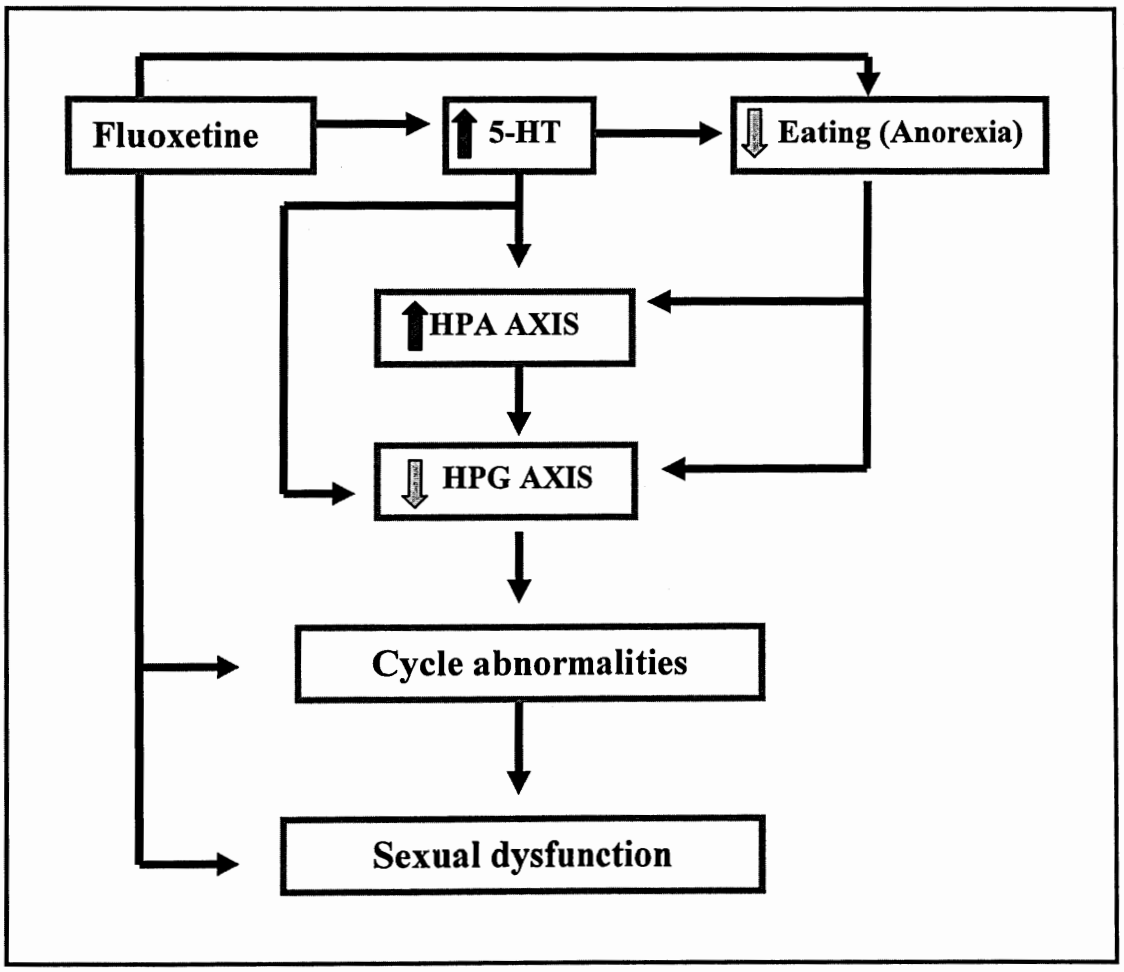
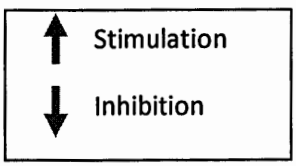


Figure 16: Proposed mechanistic model for fluoxetine-induced sexual dysfunction.



Inhibition of the HPG axis can impact negatively on reproductive functioning including estrous cyclicity and sexual behavior. Fluoxetine via increasing 5-HT can influence all of the above pathways and lead to sexual dysfunction.

In our experiments, we have pointed to the potential significance of both anorexia and gonadal hormones in fluoxetine's disruption of female rat estrous cyclicity. Future studies should be designed to assess the impact of these variables on the HPA axis with specific emphasis on the relative importance of the HPA axis as a contributor to the cycle disruption. It would also be interesting to examine effects of other SSRIs, like paroxetine or sertraline, that lead to weight gain instead of weight loss. If fluoxetine-induced anorexia leads to cycle disruption, then these SSRIs should have a lesser effect on the estrous cycle. Finally, if the cycle recovery following repeated fluoxetine treatment is due, in part, to desensitization of the $5\text{-}HT_{1A}$ receptor, then future experiments should be directed to test this hypothesis. Future experiments should also be directed toward examination of the molecular mechanisms whereby fluoxetine modifies HPG function.

Antidepressant induced sexual dysfunction occurs in a large number of male and female patients, but females are twice as affected by this as male patients [2]. Due to the presence of this undesirable side effect, patients often stop taking their medication; therefore, they fail to get effective treatment for their depression. In spite of the current awareness of the problem, few studies have actually accounted for the occurrence of SSRI-induced sexual dysfunction in females. These experiments are important given the assumption by other investigators that SSRI-induced sexual dysfunction occurs independent of the HPG axis. In human females, sexual desire and libido are also

controlled by gonadal hormones; therefore a disturbance of the HPG axis in human females could also lead to sexual dysfunction. Our findings have led to the generation of a neuroendocrine model of sexual dysfunction which could be of substantial value in the generation of therapeutic interventions for affected women.

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