Jung, Marianna E., Sexually Dimorphic Anxiety-Like Interoceptive Discriminative Stimuli. Doctor of Philosophy (Biomedical Sciences), December 1997, 150 pp, introduction, 2 chapters, discussion, bibliography, 109 titles.

This study compared gender differences in the anxiogenic stimuli induced by either a GABA-A antagonist, pentylenetetrazol (PTZ) or by a 5-HT1b/2 agonist, m-chlorophenylpiperazine (m-CPP) before and during ethanol withdrawal (EW). Rats were trained to discriminate either PTZ (16 mg/kg, IP) or m-CPP (1.2 mg/kg, IP) from saline in a two lever choice task for food reward. Male and female rats were gonadectomized or sham-operated, and ovariectomized (OVX) female rats were tested during replacement treatment with 17β-estradiol (2.5 mg, 21 day release, sc). The dose-response for the discrimination of the interoceptive stimulus (IDS) produced by PTZ (0 to 16 mg/kg) or m-CPP (0 to 1.2 mg/kg) was measured under all hormonal conditions. For m-CPP trained rats, latency to first lever-press response was also tested. Results: sham and estradiol-replaced female rats had higher ED50s for discrimination of the PTZ or m-CPP IDS than intact males or OVX rats. There is a dose-related impairment of operant responding after mCPP injection. Sham and estradiol replaced OVX rats showed an increased delay to the initiation of response after m-CPP injection as compared to sham or castrated male rats or OVX rats that showed no effect at the doses tested. Rats then received a chronic ethanol diet (6.5 %) for 10 days. At twelve hours of ethanol withdrawal,

they were tested for lever selection after saline injection. Fewer sham female and estradiol-replaced female rats responded on the drug lever during acute EW as compared to sham male, castrated or OVX rats. In general, the anxiogenic drug lever selection of OVX rats resembled that of male rats but was restored toward that of sham female rats by estradiol replacement. Castration did not alter the response of male rats to either PTZ or mCPP. Serum β-estradiol concentrations were determined by radioimmunoassay for sham, OVX, and estradiol-replaced female rats. The concentration was significantly higher in hormone-replaced female rats than in OVX. The estradiol concentration in sham female rats showed a cyclic pattern over 4 consecutive days, but this pattern did not correlate with any difference in IDS. Blood ethanol concentration (BEC) was determined using head space gas chromatography. BEC was higher in intact female rats than in intact male rats after ethanol injection (2 g/kg,ip), but did not differ during EW. Conclusions: females produce less anxiogenic IDS in response to either GABA inhibition or 5-HT1b/2 activation, but are more impaired by m-CPP in their ability to initiate operant responses than male rats. In addition, fewer intact females developed a spontaneous IDS during EW than males which is not the result of lower BEC. Estrogen appears to play a trophic role in altering responsiveness to anxiogenic stimuli.

SEXUALLY DIMORPHIC ANXIETY-LIKE INTEROCEPTIVE

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Marianna E. Jung, B.S., M.S.

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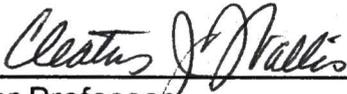
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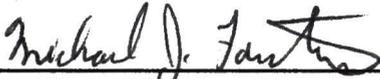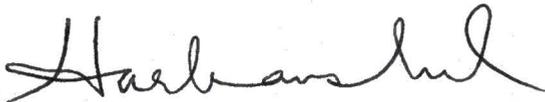
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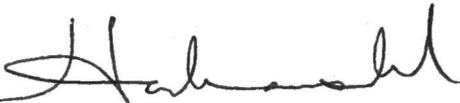
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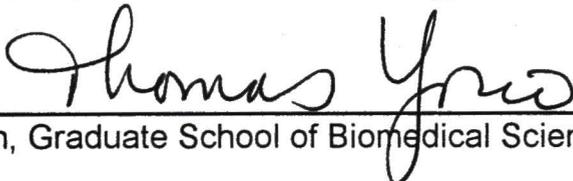
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Dean, Graduate School of Biomedical Sciences

Sexually Dimorphic Anxiety-Like Interoceptive Discriminative Stimuli

DISSERTATION

Presented to the Graduate Council of the

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy of Biomedical Sciences

By

Marianna Eunsun Jung, B.S., M.S.

December, 1997

ACKNOWLEDGMENT

TABLE OF CONTENTS

ABBREVIATION

ACTH: Adrenocorticotropic hormone

AEW: Acute ethanol withdrawal

BEC: Blood ethanol concentration

BZD: Benzodiazepine

CMC: Carboxymethylcellulose

CNS: Central nervous system

CSF: Cerebro spinal fluid

DOPA: Dihydroxyphenylalanine

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin

DZP: Diazepam

ED50:   Effective dose for 50 % response

EW: Ethanol withdrawal

FR 10:   Fixed-ratio 10 schedule

GABA: Gamma-aminobutyric acid

GAD: Glutamate decarboxylase

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: 5-hydroxytryptamine

HPA: Hypothalamic-pituitary-adrenal

IDS: Interoceptive (originated inside body) discriminative stimulus

IP: Intraperitoneal

m-CPP: m-chlorophenylpiperazine

mRNA: messenger-Ribonucleic-acid

OVX: Ovariectomy

PEW: Protracted ethanol withdrawal

PTZ: Pentylenetetrazol

SC: Subcutaneous

SEM: Standard error of mean

T: Testosterone

THP: Tetrahydroprogesterone

THDOC: Tetrahydrodioxy-corticosterone

LIST OF FIGURES

CHAPTER II.

LIST OF TABLES

INTRODUCTION

Statement of Problem

A number of preclinical and clinical studies indicate that men and women may experience mental health problems differently. Anxiety, a major psychiatric disorder, impairs the functional capacity of both men and women. However, males and females differ in physiologic and anatomic systems that may result in distinguishable behavioral outcomes of anxiety. Although abundant literature exists about gender differences, new interest has been generated in the potential role of gender factors in CNS disorders resulting from substance abuse. Ethanol is one of the most popular drugs of abuse in the United States, evoking social, economical, and health problems. This problematic aspect of ethanol abuse is clearly shown in the fact that alcoholism is a frequent psychiatric diagnosis in patients who commit suicide (Lejoyeux et al., 1994). Traditionally, alcoholism was considered a male disease because there were more male drinkers and more alcohol dependence in the male population than in the female population (Harford et al., 1992). Whereas, women were expected to be more moderate drinkers than men and less involved in work stress than men. Cultural or social norms influence the drinking habits of men and women. In modern society the roles of women are changing with concomitant changes in alcohol abuse. In addition, the incidence of psychiatric disorders or the frequency of seeking help for disorders has increased in the female population.

In my dissertation, I explored the role of gender factors in anxiety to determine the importance of gender in the treatment of these disorders, in particular, ethanol withdrawal (EW)-induced anxiety. Anxiety is a complex CNS disease with a variety of etiologies. In order to narrow the spectrum of potential mechanisms underlying a gender dependent susceptibility to anxiety, I studied the outcome on operant behavioral tasks that result from varying two major systems: 1) neurotransmitter systems, GABA and serotonin; and 2) the gonadal hormones. To do this, I utilized two distinguishable anxiogenic stimuli produced by an indirect GABA-A antagonist, pentylenetetrazol (PTZ) and a 5-HT1b/2 agonist, methyl-chlorophenylpiperazine (m-CPP) with gonadal or hormonal manipulation.

Specific aims for this project are:

1. To determine if male and female rats differ in the anxiogenic interoceptive (originated inside body) discriminative stimulus (IDS) induced by inhibition of GABA-A neurotransmission and if gonadal steroids are responsible for this difference.

2. To determine if male and female rats differ in the IDS and impaired behavioral initiation induced by activation of the 5-HT1b/2 receptor system and if gonadal steroids are responsible for this difference.

3. To determine if GABA-A receptor inhibition or 5-HT1b/2 receptor stimulation is involved in sexually dimorphic anxiogenic stimuli induced by EW and if gonadal steroids are responsible for this difference.

4. To determine if a difference in the blood ethanol concentrations between male and female rats accounts for a sexually dimorphic response to anxiogenic stimuli induced by EW

What Is Drug Discrimination?

Drug discrimination is a paradigm used to determine whether animals perceive an interoceptive stimulus or to determine if another stimulus shares properties with the trained stimulus. Typically, in this paradigm, rats are trained to perform an operant task to obtain food reinforcement. A correct response is to press one lever after injection of a drug (for example, an anxiogenic drug) and the other lever after a vehicle injection (Shearman and Lal, 1979). The first ten responses on the correct lever results in delivery of one food pellet (Fixed-Ratio 10 schedule). When acquisition of such response differentiation can be reliably established, the drug is said to produce an interoceptive (originated inside body) discriminative stimulus (IDS) that controls the differential responding in trained rats. Drug discrimination paradigms have been extensively used for identifying interoceptive stimuli, in particular those with anxiogenic properties. In this context, I conducted two separate sets of discrimination experiments to differentiate between components of the anxiety stimulus related to the GABA

and serotonin systems. PTZ is a prototypic drug that produces intense anxiety in humans (Rodin, 1958; Rodin and Calhoun, 1970) and experimental animals (Lal and Fielding, 1979; Shearman and Lal, 1979; Lal and Emmett-Oglesby, 1983). The indirect antagonist action of PTZ at GABA-A gated chloride ion channels is believed to be necessary for the anxiogenic action of the drug. m-CPP also produces anxiogenic stimuli in humans and animals. Recently, a m-CPP discrimination paradigm has been utilized to investigate neurobehavioral profiles of anxiety (Winter and Rabin, 1993; Callahan and Cunningham, 1994). Although both PTZ and m-CPP are anxiogenic drugs, it is important to notice similarities and dissimilarities between two drugs. Both drugs serve as a discriminative stimulus. However, only mCPP produces operant behavioral suppression (Kennett et al, 1986; Callahan and Cunningham, 1994). To my knowledge, my study is the first to characterize these properties of PTZ and m-CPP in a gender dependent manner.

Behavioral Evidence That Males and Females Differ in Response to Anxiogenic Stimuli

Although mechanisms behind sexually dimorphic anxiogenic responses are still unknown, males and females react in a different manner to certain anxiogenic stimuli. The gender difference for particular dimorphic responses is not predictable, because females may be more responsive to one stimuli than males but less responsive to another stimuli than males. Nonetheless, more

studies report that female rats are less anxious than male rats in response to a variety of anxiogenic stimuli. Steenbergen et al. (1989) employed an electric foot shock as an anxiogenic stimulus and compared an escape performance between male and female rats. They found that exposure to inescapable foot shocks resulted in a suppression of escape performance from one compartment to another compartment of an operant box. This behavioral disturbance was more severe in male rats than in female rats especially when shock duration was increased. Leret et al. (1994) measured animals' open arm activity in a elevated plus maze paradigm where a reduced open arm activity indicates anxiogenic behavior. They used exposure to an open field as a moderately stressful stimulus in rats. Exposure to an open field prior to testing in the elevated plus maze resulted in animals spending less time on the open arms of the elevated plus maze as compared to the pre-open field exposure test. The suppression of open arm activity was greater in male rats than in female rats, indicating that less anxiety was produced in female rats than male rats to that stimulus. Steenbergen et al. (1990) reported that males showed more suppression of open arm activity on the elevated plus maze than females after inescapable foot shock. Thus, these studies indicate that female rats show less anxiety-like response than male rats regardless of whether anxiety was expressed as increased initiation of behavior (escape) or decreased initiation of behavior (open arm entries).

Other examples of gender related differences in anxiogenic response also indicate that the direction of the gender difference is dependent on the type of behavior being expressed. Using anxiety produced by shock-stress, Heinsbroek et al. (1988) showed that shock-stress reduced locomotor activity to a greater extent in male rats than female rats. Likewise, forced swimming induced more immobility in male rats than female rats (Alonso et al., 1991). In contrast, Johnston and File (1991) demonstrated that female rats are more anxious (show a less frequent social interaction) than male rats in a social interaction test with same gender rats in an unfamiliar environment. When exposed to a nonpainful threat stimulus such as a cat or a cat odor, female rats show a greater frequency of defensive posture than male rats (Blanchard et al., 1991).

More women are prescribed anxiolytic drugs than men. Female anxiety patients are more vulnerable to the component of anxiety that involves retardation of the initiation of motor response whereas male patients are more sensitive to a hostility feature of anxiety (Katz et al., 1993). These results indicate the complex nature of anxiety which involves multiple features such as restlessness, nervousness, dysphoria, hostility, motoric retardation, impaired motivation, and aggressiveness (Katz et al., 1993). With these data in mind, it is reasonable to speculate that different neuronal and hormonal systems are involved in producing different behavioral outputs of anxiety between sexes. Given this, I believe that a gender-dependent susceptibility to aversive or anxiogenic stimuli depends upon the type of anxiogenic stimulus encountered. In this context,

employing different types of anxiogenic stimuli, PTZ, m-CPP, or EW may allow more accurate assessment of the mechanisms underlying gender dependent anxiogenic responses.

Gender Differences in the Brain

Anxiety is a CNS disorder, therefore it is essential to consider any known differences in brain between males and females. It is clear that there are morphological differences between the brains of males and females. However, the question is do such morphological differences give rise to the different anxiogenic responses between sexes. The sexually dimorphic regions of brain are bigger in males than in females. From a mechanistic point of view, the male hormone testosterone (T) appears to play a role in organization of brain structures during development and this effect may require local conversion of testosterone to estrogens. Work done by Gorski et al. (1978) demonstrated that the volume of the medial preoptic area of the hypothalamus is larger in male rats than in female rats in terms of brain weight. Subsequently, they were able to confirm that result by showing that both the volume and cell density of an intensely staining part of the preoptic area were greater in male rats than in female rats (Gorski et al., 1980). Later on, Swaab et al. (1992) reported that the volume of a putative homologue of this sexually dimorphic nucleus in the adult human hypothalamus was more than twice as large in men as in women and contained about twice as many cells. The same type of a gender difference was

observed in the rat cerebral cortex: the cortex was longer and wider in male rats than in female rats at 90 days of age (Reid et al., 1992). Roof and Havens (1992) reported that T contributes to morphologic gender difference in the hippocampus, an area of the limbic system. They found that the granule cell layer of the hippocampus was larger in male rats than in female rats and neonatal testosterone treatment of females resulted in a male-like hippocampus, indicating an organizational role of T in the developing brain.

The question arises, are these morphological differences related to the differences in behavioral responses to anxiety between males and females. Work done by Hines et al. (1992) provides a clue to that relationship. The volume of the medial nucleus of the amygdala is significantly greater in male rats than in female rats. This area is rich in T binding (Lisciotto and Morrell, 1994) and T metabolic enzymes such as aromatase (Beyer et al., 1993), indicating a role of T in this regions. The amygdala by way of the stria terminalis is involved in regulation of sexually dimorphic functions including aggression or sexual behavior. In particular, the role of the amygdala in aggression is shown in a study where an electrical brain stimulation of this area produces aggression or aversiveness in animals (Mecican and Delgado, 1953). Using direct stimulation of the amygdala, Adamec (1990) also showed increased anxiety-like behavior on the elevated plus maze. At the same time, a typically male feature of anxiety, aggression, is positively correlated with the T concentration in CSF (Higley, et

al., 1996). Another study suggests that aggressive behaviors are in part mediated by GABA-A antagonistic stimuli. For instance, electrical stimulation of the periaqueductal area produces aggressive behaviors such as jump or attacking in male rats. This phenomenon was potentiated by a GABA antagonist, PTZ, and attenuated by the GABA/BZD agonist, DZP, indicating GABA inhibition is in part responsible for the display of aggressive behaviors (Jung, 1994). Overall, T plays a role in organization of the developing brain including the areas responsible for aggression. Thus estrogen facilitation of the GABA-A system protect females from displaying the male typical anxiety response, aggression.

However, the importance of T appears to be limited to an early developmental period. In adult male rats, neither gonadectomy nor T replacement affects the sexual dimorphism in the medial preoptic area. On the other hand, the volume of this area in neonatally castrated adult male rats was significantly reduced as compared to that of the male rats castrated after the period of brain sexual differentiation (Gorski et al., 1978). Supporting this view, the maximal efficacy of the GABA-A stimulated chloride influx was not affected by 1 or 2 weeks of T treatment in adult male rats (Bitran et al., 1991). Castration of adult male rats also failed to alter the threshold for PTZ-induced seizures as compared to gonadally intact male rats (Kokka et al., 1992). Gonadectomy of adult male rats did not change the display of anxiety-like behavior on the

elevated plus maze (Zimmerberg and Farly, 1993; Astiningsih et al., 1996). In contrast, when male rats that had been castrated as newborns and were tested during adulthood for their anxiety-like behavior on the elevated plus maze, they showed significantly less anxiety-like behavior than sham-operated male rats (Lucion et al., 1996). In the same elevated plus maze model, gonadectomy did not alter the response of male rats whereas it enhanced the anxiogenic response of female rats (Zimmerberg and Farley, 1993). When one day-old chicks were treated with T and tested as adults, their attack scores were higher than a vehicle treated group. Since anxiety, in particular EW-induced anxiety is thought to occur predominantly in adulthood, I employed adult rats in the present study. *Given this, I hypothesized that sensitivity of castrated male rats to anxiogenic stimuli would not significantly differ from that of sham-operated male rats.*

Sexual dimorphism in the brain also exists at the neurochemical level. Ovtscharoff et al. (1992) examined sexual dimorphism of GABAergic neurons of the striatum during the prenatal period. They measured the densities of GABA-immunoreactive axons and cell bodies in the striata of male and female rats over a range of embryonic days. Throughout prenatal development, female striata had a higher density of GABA immunoreactive axons and cell bodies than male rats. The occurrence of such a gender difference at a critical period may launch a process of sexually dimorphic

response producing behavioral outcomes associated with anxiety. Since certain anxiogenic stimuli result from inhibition of GABAergic neurotransmission, a high GABAergic neuronal profile in female rats may persist until adulthood. This may contribute to their resistance to anxiogenic stimuli as illustrated earlier. Indeed, contribution of the GABAergic system to a sexually dimorphic response to anxiety has been suggested from several types of data.

GABAergic Involvement in Sexually Dimorphic Anxiogenic Response

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter whose presence is ubiquitous in the brain. There are two major types of GABA receptors; GABA-A and GABA-B, but primarily GABA-A receptors are involved in regulating anxiogenic stimuli. The GABA-A-gated chloride ion channel includes benzodiazepine (BZD) receptors that allosterically modulate chloride ion flow in response to GABA. The effect of GABA on GABA-A receptors is enhanced by BZD agonists and reduced by BZD inverse agonists (Hunkeler et al., 1981). In contrast, the GABA-B receptor is associated with calcium and is neither activated by GABA-A agonists (muscimol) nor blocked by GABA-A antagonists such as bicuculline and picrotoxin. GABA-A receptors have distinct binding sites for GABA agonist /antagonist, BZD, steroids, barbiturates, and the convulsant picrotoxin (Fahn, 1976).

Evidence is accumulating that activation of the brain GABA-A/BZD system produces more chloride ion flux in female rat brains than in male rat brains, at least in part due to an ovarian factor. Gonadally intact female rats have a higher GABA-stimulated chloride conductance than OVX rats (Bitran et al., 1991). Female rats at proestrus were more sensitive to the anxiolytic action of diazepam than OVX rats (Fernandez-Guasti and Picazo, 1990). In limbic areas such as the amygdala and the frontal cortex, female rats had a significantly greater number of BZD receptors than male rats (Farabollini et al., 1996). These results indicate that female rats should be less responsive to GABAergic antagonists than male rats. This is consistent with the higher PTZ seizure threshold observed in female rats as compared to male rats, (Kokka et al., 1992). Gonadectomy narrows this gender difference by decreasing the seizure threshold of female rats toward that of male rats. After acute handling or swim stress, intact female or OVX rats displayed a more pronounced increase in BZD receptors than intact or castrated male rats in cortex, hippocampus, and hypothalamus (Wilson and Biscardi, 1994). Similarly, acute swim stress increased GABA binding to membranes prepared from the forebrain in female mice but not in male mice (Akinci and Johnston, 1993). These changes were larger when measured in a relatively crude membrane preparation than in a well-washed membrane preparation. The authors speculated that the loss of endogenous modulators of GABA binding in the well-washed membrane preparation might account for the smaller gender difference. If the higher GABA-

A agonistic activity in females may be attributed to endogenous modulators of the GABAergic system, then recent studies indicating that there is steroidal modulation of GABA-A activity evoke the speculation that these modulators may be steroids of gonadal, adrenal and / or brain origin. The greater responsiveness of female rats to GABA-A agonists as compared to male rats demonstrates a phenomenon that is likely to occur in humans as well. Clinically, young women respond better to and require lower doses of BZD than young men (reviewed by Yonkers et al., 1992).

Steroidal Modulation of the GABAergic System

Studies have shown that estrogen influences the GABAergic system in the medial preoptic area of the hypothalamus or other steroid-sensitive brain regions. In those areas, estrogen target neurons are numerous and are thought to contain high concentrations of GABA and its synthesizing enzyme, glutamate decarboxylase (GAD) (Flugge et al., 1986). High concentrations of GABA and GAD are consistent with utilization of GABA as a neurotransmitter by a large number of neurons (Bach et al., 1992). A question arises about the functional relationship that exists between GABA and estrogen. Based on previous studies, estrogen appears to play an enhancing role in GABAergic activity. Perez et al. (1986) found that estradiol administration to OVX rats resulted in up-regulation of GABA receptors. In agreement, Maggi and Perez (1984) observed that estradiol treatment increased [3H]-muscimol (a GABA agonist) binding

activity in the striatum and this effect was blocked by the anti-estrogen tamoxifen. Schumacher et al. (1989) examined the effect of estrogen on [3H]-muscimol binding in the dorsal hippocampal formation in OVX and adrenalectomized female rats. Estradiol injection increased [3H]-muscimol binding in regions known to be rich in estrogen receptors. Canonaco et al. (1989) also reported that estrogen increased [3H]-muscimol binding in medial preoptic area in OVX female hamsters. These results indicate an enhancing role of estrogen in the GABA-A system.

Estrogen also modulates the GABA-A system at the level of GABA production and at the transporter. Estradiol treatment (2μg for 2 d) increased GAD mRNA levels in the medial basal hypothalamus in OVX rats (Unda et al. 1995). Following OVX, the level of GABA transporter mRNA decreased in the medial preoptic area of the hypothalamus (Herbison et al., 1995). In that study, estrogen treatment to OVX rats for 7 days reinstated the content of GABA transporter mRNA to a level similar to intact female rats. In addition, estrogen treatment for 7 days also increased the uptake of [3H]GABA in this brain region. These results clearly indicate that a female gonadal steroid, estrogen, directly or indirectly facilitates GABA-A agonistic activity. Higher GABA-A agonistic activity may more effectively counteract GABA-A antagonistic stimuli in female rats than in male or OVX rats. The hypotheses tested in the present study are: *Sham female rats are less sensitive to the GABA-A antagonistic IDS of PTZ, but more*

sensitive to a GABA/BZD agonist DZP blockade of the PTZ stimulus than male or OVX rats. Estradiol replacement to OVX rats reinstates a female-like sensitivity to PTZ or to DZP. Castration does not significantly alter the sensitivity of male rats to the PTZ discrimination stimulus.

Gender Differences in Serotonergic Systems in Response to Anxiety

In addition to GABA, serotonin (5-hydroxytryptamine, 5-HT) is also one of the major neurotransmitters implicated in modulation of anxiety. At least one clinically available anxiolytic is a serotonergic agent (buspiron, a 5-HT1a partial agonist). 5-HT containing neurons are known to be located in or near the raphe regions of the pons and the upper brain stem (Cooper et al., 1986). From these cell groups major projections ascend to limbic areas or the forebrain (reviewed by Iversen, 1984), emphasizing the role of serotonin in modulation of psycho-affective aspects. In general, inhibition of 5-HT neurotransmission is anxiolytic whereas its activation is anxiogenic. The non-selective 5-HT receptor antagonist, metergoline (Pigott et al., 1991) and a selective 5-HT2 antagonist, ritanserin (Colpaert et al., 1988) show anxiolytic activity in experimental animals. By comparison, a 5-HT1b/2 agonist m-CPP employed in the present study has anxiogenic activity in experimental animals and humans (Klein et al., 1991; Pigott et al., 1991; Germine et al., 1992).

m-CPP elicits a variety of pharmacological and physiological actions. Following systemic administration, m-CPP increases plasma corticosterone levels and body temperature (Wozniak et al., 1989), and decreases food intake (Fuller et al.,1981). Its anxiogenesis has been demonstrated in clinical and preclinical studies (Murphy et al., 1989; Zuardi, 1990). In humans, a single administration of m-CPP increases anxiety in normal subjects and in anxiety patients according to self-report using a behavioral rating scale (Klein et al., 1991; Pigott et al., 1991; Germine et al., 1992). In preclinical studies, animals injected with m-CPP showed reduced open arm activity in an elevated plus maze paradigm (Rezazadeh et al., 1993), decreased social interaction (Kennett et al., 1989), and impaired behavioral capacity. m-CPP administration also suppressed locomotion as measured by the number of cage crossings (Aulakh et al., 1987;1988) and this effect was confirmed by other laboratories (Kennett et al., 1986; Ulrichsen et al.,1992). In a two-lever choice task, m-CPP produced discriminative and rate suppressing effects in male rats (Winter and Rabin, 1993; Callahan and Cunningham, 1994). According to my preliminary observations, some animals injected with m-CPP failed to initiate or delayed initiation of a lever-press response. This was more prominent in female rats than in male rats. *These previous results and my preliminary observations led me to test two anxiogenic properties of m-CPP, impaired initiation of a lever-press response and the discrimination stimulus of m-CPP in male and female rats.*

Gender differences in the brain serotonergic system have long been investigated. Several lines of evidence indicate that the serotonergic system is more active in female rats than in male rats. Administration of the serotonin precursor tryptophan induces a characteristic serotonin behavioral syndrome including tremor, hindlimb abduction, forepaw treading, salivation, and occasional seizures (reviewed by Jacobs, 1976). This syndrome has been attributed to elevated brain levels of serotonin, since it is prevented by a serotonin antagonist methysergide (Jacobs, 1976). A gender difference in the serotonergic syndrome has been documented (Fischette et al., 1984): female rats exhibit the serotonin syndrome at a lower dose of a serotonin precursor, l-tryptophan than male rats. Biegon et al. (1979) examined the effect of pargyline (monoamine oxidase inhibitor) and tryptophan on the serotonin syndrome in male and female rats. Pargyline followed by a high dose of tryptophan (60 to 150 mg/kg) produced a similar serotonin behavioral syndrome. As observed by Fischette et al. (1984), female rats exhibited this syndrome at a lower dose of drugs than male rats, confirming their higher sensitivity to serotonergic agents than male rats. Other studies reported the same direction of a gender dependent sensitivity to serotonergic agents under stressful stimuli. Restraint stress increased binding of a 5-HT1A agonist, [3H] 8-OH-DPAT [8-hydroxy-2-(di-n-propylamino)tetralin] at 5-HT1A receptors in the rat dorsal hippocampus, and this binding level was higher in female rats than in male rats (Mendelson and McEwen, 1991). Inescapable electric footshock increased serotonergic activity

as indicated by increased metabolite concentrations in the frontal cortex, hypothalamus, amygdala, striatum, mesencephalon, and the medulla-pons area. (Heinsbroek et al., 1990). Shock-induced increments in serotonin metabolites were larger in female rats than in male rats. Thus, these data support a greater role for serotonin in anxiety-related behaviors of females than males.

The serotonerigic system of females appears to have a higher turnover rate for serotonin than that of males. The brain regional 5-HT and/or 5-hydroxyindoleacetic acid (5-HIAA) concentrations and the synthesis rates of 5-HT tended to be higher in the hippocampus of female rats than in male rats (Haleem et al., 1990). Although there were differences depending upon regions, the concentrations of 5-HT, 5-HIAA, tryptophan were generally higher in brainstem, hypothalamus/preoptic area, corpora striata, limbic forebrain, and cortex of female rats than male rats (Carlsson and Carlsson, 1988). These results indicate that females have the capacity for a greater serotonin response to stimuli than males.

A higher capacity of the serotonergic system in female rats than in male rats appears to be determined during an early developmental period. Concentration of 5-HT in the forebrain and midbrain of rats on postnatal day 10 to 14 were higher in female rats than in male rats (Giulian et al., 1973). In a study done by Giulian et al. (1973), OVX on day 1 significantly reduced brain 5-

HT concentrations compared to intact female rats. Castration on postnatal day 1 produced a trend toward elevation of 5-HT concentrations measured on postnatal day 12 as compared to intact male rats. T treatment of female rats on day 1 prevented the increase in 5-HT levels seen in control female rats on day 12 to 14. This response appears to end early in male brain, but continue in female brain. Injection of estrogen on day 1 after birth elevated brain 5-HT in male rats measured on day 12, but when estrogen was administered on postnatal day 11, no difference was obtained in male rats, but did enhance 5-HT concentrations in female rats on day 14. T did not alter 5-HT level in either gender at this period. These data suggest an enhancing role of estrogen versus an inhibitory role of testosterone in the serotonergic system, but only the effect of estrogen persists in adult female rats.

Based on the cited results, my initial hypotheses to test sexually dimorphic stimulus effects of m-CPP were: *female rats are more sensitive to the anxiogenic stimuli of m-CPP, impaired behavioral initiation or the discrimination stimulus than male or OVX rats. Estradiol replacement to OVX reinstates a female-like sensitivity to m-CPP. On the contrary, castration does not significantly alter the sensitivity of male rats to the m-CPP stimulus.*

Gender Difference in Anxiety-Like Stimuli Induced by EW

So far, I have described a sexually dimorphic GABAergic and serotonergic system associated with anxiogenic stimuli. The next question is how these systems are related to or altered by ethanol and ethanol withdrawal. There are gender differences in certain effects of ethanol. Female rats consume more ethanol than male rats, indicative of a higher reinforcing effect of ethanol in female rats than in male rats. Indeed, after ethanol treatment, female rats release a higher level of dopamine (a neurotransmitter responsible for a reinforcing effect) than male rats (Blanchard and Glick, 1995). Following administration of the same dose of ethanol, female rats show higher blood ethanol concentrations than male rats (Rivier, 1993). In our preliminary observations, female rats are less intoxicated than male rats during exposure to chronic ethanol diet. Similarly, women are less sensitive than men to the depressive effects of ethanol such as ataxia (Perkins et al., 1994). Whether or how these gender differences in the effects of ethanol could give rise to a gender specific development of ethanol dependence or EW-induced anxiety is the next question. My dissertation addresses this question in the following chapters.

A small hydrophilic compound, ethanol (C_2H_5OH) has no specific receptors and virtually affects all neuronal and hormonal systems. Among other actions of ethanol, a low dose of ethanol is anxiolytic. This effect of ethanol is thought to be mediated by potentiation of GABA-gated chloride channel

conductance (Allan et al., 1991). In general, termination of chronic drug treatment produces an opposite effect to their desired pharmacological effect. Thus, it is postulated that chronic ethanol treatment cessation functionally down-regulates the GABA-A receptor system, consequently resulting in a GABA-A antagonistic effect. This has been demonstrated in a behavioral study where the stimulus effect of EW substituted for the PTZ discrimination stimulus in male rats (Lal et al., 1987). The fact that the PTZ-like stimulus effect of EW is blocked by DZP (Lal and Emmett-Oglesby, 1983; Lal et al., 1987) and that BZDs are extensively used to treat EW further supports the GABA-A antagonistic nature of EW. *Because female rats have higher GABA-A agonistic activity than male rats as the result of estrogen actions, fewer sham and estradiol-replaced female rats are expected to develop the PTZ-like stimulus during EW than male or OVX rats.*

Ethanol has been recognized to alter the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier, 1993). A gender difference exists in this process with females reacting more robustly than males. Ethanol treatment resulted in a higher release of corticosterone in intact female rats than in intact male rats (Rivier, 1993). Following gonadectomy, the level of corticosterone in female rats decreased to that of male rats (Rivier, 1993), implying an enhancing role of an ovarian factor in the effect of ethanol on corticosterone release. The involvement of sex steroids in this difference is also inferred from the following experimental evidence. Plasma adrenocorticotrophic hormone (ACTH) response

to corticotropin-releasing hormone was significantly greater among women than among men (Gallucci et al., 1993). Plasma corticosterone levels were considerably higher in water deprived female rats than in male rats (Pericic and Pivac, 1995). Acute swim stress resulted in a higher corticosterone release in intact female rats than intact or castrated male rats (Wilson and Biscardi, 1994). Estrogen treatment of gonadectomized males further increased corticosterone secretion. These data indicate that estrogen enhances the HPA activity. As a result of the anxiolytic properties of corticosterone, a higher release of this steroid in female rats may more effectively counteract the anxiogenic stimuli of PTZ or the development of an endogenous PTZ-like stimulus during EW as compared to male rats. This further supports my hypothesis that fewer sham and estrogen-replaced female rats experience anxiety-like stimuli than male rats or OVX rats during EW in part due to an estrogen-related factor.

Using the m-CPP discrimination paradigm to determine if there is a gender dependent development of the anxiogenic cue during EW is a novel approach. Benefits of this approach are two fold: 1) It provides an alternate therapeutic strategy when GABA/BZD full agonists display dependence liability; 2) It allows a more accurate assessment of mechanisms underlying sexually dimorphic anxiety induced by EW. The behavioral changes observed after m-CPP treatment closely resemble or potentiate those during EW as tested in the elevated plus maze test (Rezazadeh et al., 1993). The final behavioral outcome

during EW may depend upon the magnitude of the gender dependent sensitivity of the GABAergic and the serotonergic system. In a clinical study, patients exhibiting anxiety during EW had decreased 5-HT metabolites (5-HIAA) in CSF as compared to a control group, indicating reduced central 5-HT activity during EW (Ballenger et al., 1979). Thus the hypotheses tested are: *fewer sham female rats develop an endogenous m-CPP-like stimulus during EW than male or OVX rats. Estradiol replacement to OVX reinstates a female-like response to EW. On the contrary, castration does not significantly alter the sensitivity of male rats to EW.* At the very least, this will characterize the role of sexual dimorphism in the relationship between the GABAergic and serotonergic systems associated with anxiety induced by EW.

CHAPTER 1

The experiments in this chapter were designed to test the following specific aims:

1) To determine if male and female rats differ in response to the anxiogenic interoceptive discriminative stimulus (IDS) induced by an indirect GABA-A antagonist pentylenetetrazol (PTZ) or to the blockade effect of a GABA/BZD agonist diazepam on IDS of PTZ;

2) To determine if male and female rats differ in development of an endogenous PTZ-like stimulus during ethanol withdrawal (EW);

3) To determine if gonadal factors are responsible for a sex difference in IDS of PTZ or an endogenous PTZ-like stimulus during EW;

4) To determine if a difference in blood ethanol concentration between male and female rats is responsible for a sex difference in the GABA/BZD system associated with anxiety.

Gender difference in the Pentylenetetrazol-Like Stimulus

Induced by Ethanol Withdrawal[1]

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Abstract

This study was designed to determine the role that estrogen plays in the potential gender difference of the anxiogenic stimuli produced by an indirect GABA-A antagonist, pentylenetetrazole (PTZ) or ethanol withdrawal (EW). Rats were trained to discriminate PTZ (16 mg/kg, IP) from saline in a two lever choice task for food reward. Male and female rats were gonadectomized or sham-operated, and ovariectomized (OVX) female rats were tested during replacement treatment with 17β-estradiol (2.5 mg, 21 day release, sc). The dose-response for the discrimination of the interoceptive stimulus (IDS) produced by PTZ (0 to 16 mg/kg) was measured under all hormonal conditions. Sham-operated females or β-estradiol-replaced OVX rats were less sensitive to the PTZ stimulus than OVX, sham or castrated male rats. Diazepam (DZP, 0 to 10 mg/kg, IP) injected prior to PTZ (16 mg/kg) blocked the PTZ stimulus at a lower dose in female and estrogen replaced OVX than in OVX rats and male rats (regardless of gonadal condition). During acute EW (12 hr after termination of chronic ethanol diet for 10 days), fewer sham female or β-estradiol-replaced OVX rats responded on the PTZ lever after saline injection than sham or castrated male rats. The ED_{50} for discrimination of the PTZ stimulus during protracted EW (36 hr) was lower than the ED_{50} prior to ethanol treatment in all groups. The percentage of OVX rats selecting the PTZ-lever fell between those of male

groups and other female groups in each behavioral test. β-Estradiol concentrations determined by a radioimmunoassay significantly increased in β-estradiol replaced OVX rats as compared to the pre-replacement value. However, in sham female rats, β-estradiol concentrations for consecutive days showed a four day cyclic pattern with a surge on day 2 which did not produce a change in their PTZ discrimination performance. In addition, after an IP injection of ethanol (2 g/kg), blood ethanol concentrations (BEC) were higher in gonadally intact female rats than in male rats. Therefore, these findings suggest that β-estradiol indirectly enhances GABAergic neurotransmission through mechanisms independent of the estrus cycle or changes in BEC, counteracting the GABA-A antagonistic stimuli of PTZ and reducing the development of a spontaneous PTZ-like stimulus during EW.

Introduction

Gender differences in behavioral responses to anxiogenic stimuli have been reported in numerous studies. However, results are somewhat controversial depending upon anxiety models employed. Female rats show a lower level of anxiogenic behavior than male rats in an elevated plus-maze test (Johnston and File, 1991) or in an inescapable footshock test (Heinsbroek et al., 1990). Female rats are less influenced by a shock stress-induced decrease in locomotor activity than male rats (Heinsbroek et al., 1988). Compared to male rats, female rats release more corticosterone after swim stress (Wilson and Biscardi, 1994). In contrast, female rats show a greater anxiogenic response than male rats in a social interaction test and a conflict test (Johnston and File, 1991). Since anxiety is a complex CNS disorder with numerous hormonal and neuronal factors involved, the sensitivity to anxiety may differ between males and females depending upon types of anxiety.

In this context, the present study focused on gender differences of a particular anxiogenic stimulus, one induced by an indirect GABA-A antagonist, PTZ or EW. Knowing that anxiety is one of the major withdrawal symptoms of ethanol abuse, avoidance of EW-induced anxiety is a critical factor in the maintenance of ethanol consumption in alcoholics. However, most studies have

focused on male subjects despite the fact that ethanol abuse and anxiogenic withdrawal symptoms may be common in both men and women. The primary goal of the present experiments is to characterize mechanisms underlying a potential gender difference in the PTZ discrimination stimulus or the PTZ-like stimulus induced by EW. To our knowledge, there has been no report that EW-induced anxiety was compared in male and female rats using the PTZ discrimination paradigm.

The stimulus effects of EW and PTZ share common aspects in that both stimuli are in part mediated through inhibition of GABA-A neurotransmission. This has been behaviorally demonstrated in a previous PTZ discrimination experiment using male rats (Lal et al., 1987). In that study, rats injected with saline selected a PTZ-lever over a saline-lever during EW, indicating that an endogenous GABA-A antagonistic stimulus develops during EW. This is an important property as far as a gender factor is concerned because certain features of the GABAergic system differ between males and females. In general, the GABA-A agonistic system appears to be more effective in females than in males. Several lines of evidence support this notion. Clinically, young women require lower doses of benzodiazepines (BZD) than young men (Yonkers et al., 1992). Preclinically, GABA-mediated chloride conductance in the GABA/BZD receptors is higher in intact female rats than in OVX rats (Bitran et al., 1991). At the steroidal level, a major female steroid, progesterone and its neuroactive
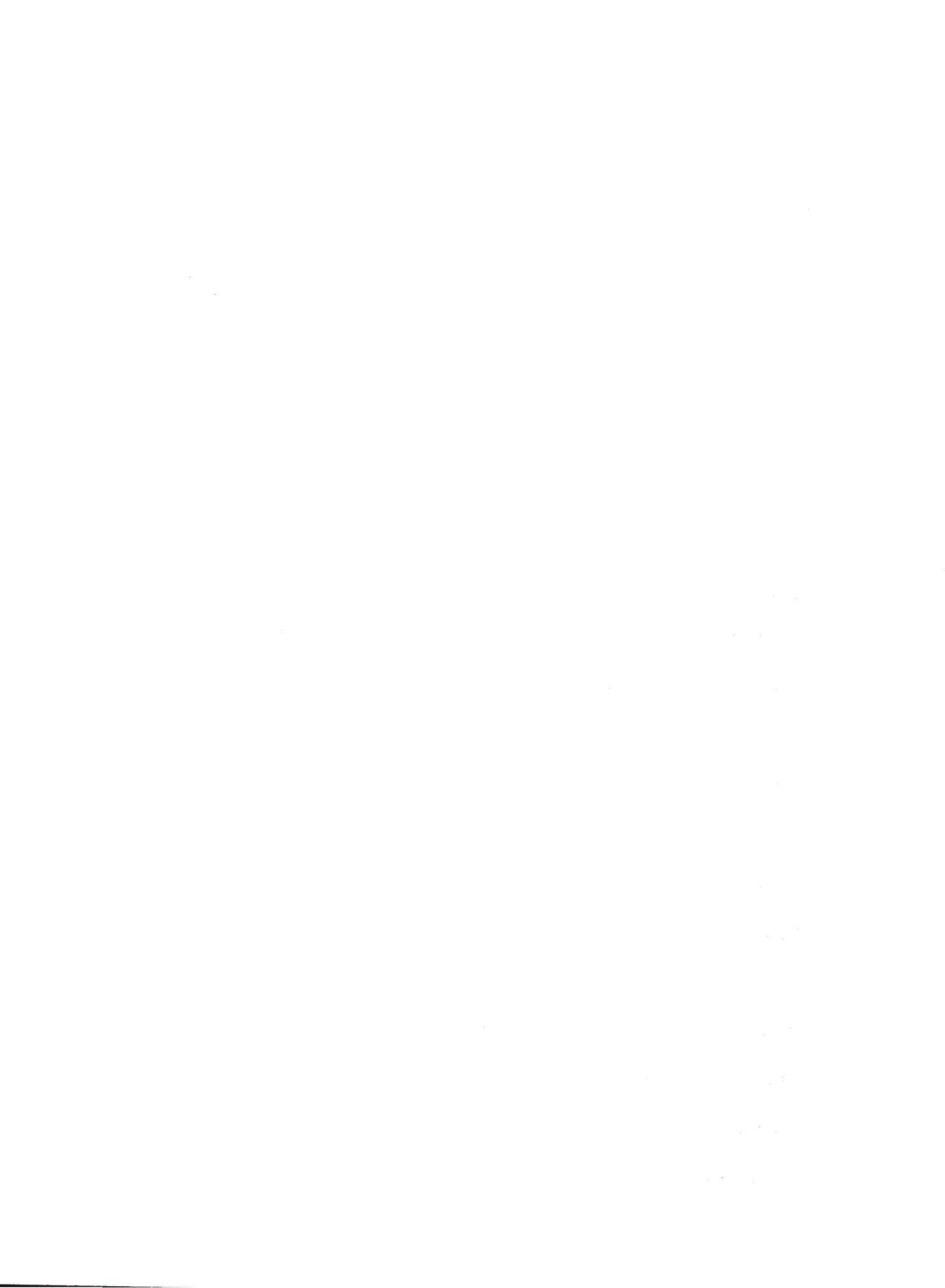
metabolites are thought to exert a GABA-A agonistic activity (Majewska, 1991).

These GABA-A agonistic steroids have been found in higher levels in the brain

and plasma of female rats as compared to male rats (Corpechot et al., 1993).

Behaviorally, the threshold for the PTZ-induced seizure is higher in female rats

than in male rats and this difference is abolished by ovariectomy (Kokka et al.,

1992). On the other hand, the suggestion has been made that a male hormone

exerts an organizational effect in aggression or anxiety-related behaviors during

the early developmental period rather than during adulthood. For instance, adult

male rats are not affected by the absence of gonadal hormones on the elevated

plus maze (Zimmerberg and Farly, 1993). Castration of adult male rats does not

alter the threshold for the PTZ-induced seizure (Kokka et al., 1992). Instead,

when day-old chicks were treated with testosterone oenanthate, the attack

scores increased from days 7 to 14 of birth as compared to a control group

(Astiningsih et al., 1996). Since alcoholism and its anxiogenic withdrawal

symptoms occur more frequently during adulthood, we dealt with adult male and

female rats. The hypotheses we tested are: That ovarian factors reduce the

sensitivity of female rats to the GABA-A antagonistic stimuli of PTZ or EW as

compared to male rats. In contrast, female rats are more sensitive than male

rats to a GABA-A/BZD agonist such as diazepam.

Although no previous data are available with regard to sexually dimorphic

anxiogenic stimuli of EW, a gender difference in certain effects of ethanol has

been long recognized. Female rats are less sensitive than male rats to depressive effects of ethanol such as sedation or ataxia (Unwin and Taberner, 1980). Prenatally, ethanol-exposed female rats make fewer performance errors during adulthood than a corresponding male group (Zimmerberg et al., 1989). Female rats consume more ethanol than male rats (Blanchard and Glick, 1995), indicative of a higher reinforcing effect of ethanol in female rats than in male rats. These differences do not give us direct information regarding a gender difference in EW. However, they imply the possibility that female rats are more resistant to a deteriorating effect of ethanol than male rats. This leads us to speculate that female rats are less influenced by EW-induced anxiety than male rats. In addition, it is of importance to examine whether a gender difference in metabolic rate of ethanol alters their anxiogenic response. In fact, given the same dose of ethanol, it has been reported that females rats have a higher blood ethanol concentration than male rats (Sutker et al., 1983). Thus, analysis of blood ethanol concentrations was reexamined in this study to exclude the possibility that a lower blood ethanol concentration in female rats during ethanol exposure results in fewer EW induced signs and symptoms as compared to male rats. In addition we measured the concentration of estradiol in intact rats and OVX rats before and after estradiol replacement in order to determine the relationship between estrogen concentration and anxiogenic response.

Methods

Animals

Adult male and female Long-Evans hooded rats (Charles River, Wilmington, MA) were housed individually with temperature (22-25^0 C) and humidity (55%) held constant. A 12 h light-dark cycle was maintained with lights on between 7 a.m. and 7 p.m. Animal body weights were maintained at 320-350 g for male rats and 290-310 g for female rats by limiting food (Purina rat chow) to 20 g/day for males and 16 g/day for females which included the food received during training. Food restriction was necessary to maintain the lever pressing response for food reward. Water was available ad libitum.

Discrimination training

Gonadally intact male and female rats were trained to press a lever for food reward under a fixed-ratio 1 (FR1) followed by a FR3 schedule. This procedure required animals to learn a lever-press response. After acquisition of a lever-press response, they were trained to discriminate between the PTZ and the saline stimulus under a FR10 schedule. Thus, they get food reward when they perform ten presses on one lever following PTZ (16 mg/kg, IP, 15 min) injection and the other lever following saline injection. Half of the male and female rats were trained with PTZ as the cue on the right lever, half were trained with PTZ on the left lever. Each training session was 10 minutes in length. An

equal number of saline and PTZ training sessions was given in an irregular order of presentation such that no condition occurred in more than three consecutive tests. Animals received at least 60 training trials prior to use in any behavioral experiment. Animals were selected for use in experiments when they achieved a 90% correct response rate for their last ten training sessions.

Discrimination testing

The percentage of animals selecting the PTZ lever was measured after treatment with increasing doses of PTZ (0, 4, 8, 16 mg/kg, IP). A cumulative dosing regime was applied in the following manner: fifteen (15) minutes after injection with saline, animals were tested for two (2) minutes to determine their lever selection. On completion of the lever selection, animals were immediately injected with PTZ 4 mg/kg and tested as above. The next dose injected was 4 mg/kg of PTZ which was the difference (4 mg/kg) between the second dose (8 mg/kg) and the first dose (4 mg/kg). In this manner, animals were given additional doses and tested until they received a cumulative dose of PTZ (16 mg/kg).

Drug test with DZP

The effect of a GABA-A/BZD agonist, diazepam (DZP) on the PTZ discrimination cue was tested. DZP (0, 0.625, 1.25, 2.5, 5.0, and 10 mg/kg, IP) was injected 15 min prior to PTZ injection (16 mg/kg) and 15 min later, rats were

placed into the chamber for their PTZ-lever selection. Each dose of DZP in combination with PTZ (16 mg/kg) was tested on a different day in a randomized order of DZP doses with a recovery period between tests. The test session ended with the delivery of a single food pellet on completion of FR10 or if neither lever was selected, after the session time had elapsed. A test duration was 2 min for all tests.

Chronic ethanol administration and ethanol withdrawal tests

Ethanol was administered in a nutritionally complete liquid diet containing 6.5% ethanol (w/v) as modified by Dodd and Shorey-Kutsche (1987). Animals received 100 ml of the ethanol diet each morning for 9 days. On the morning of the tenth day, 50 ml of the diet was given. Twelve hours later the diet tubes were removed and animals were fed with food pellets. In the morning of the first day after termination of the chronic diet, rats were injected with saline and were examined for their PTZ-lever selection.

Gonadectomy

This procedure was applied to male and female rats which had already acquired the PTZ discrimination task. Male (30) and female (30) rats were assigned into a sham-operated (N=15 for each gender) or a gonadectomized (N=15 for each gender) group. For an experiment 5, a separate group of PTZ-naïve female rats (15) were ovariectomized before the PTZ discrimination

training. For ovariectomy, under ether anesthesia, a small incision was made in the abdominal cavity directly above the ovaries. The ovaries of the OVX group were removed bilaterally whereas those of the sham group were left intact. The incisions were closed with stainless steel wound clips. For castration, a small incision was made in the scrotum. After removal of the testicles, two sutures were used to close the incision. Animals were returned to the colony room and were allowed a two week recovery prior to initiating behavioral anxiety tests.

β-Estradiol replacement

Our preliminary results indicate that castrated male rats do not significantly differ from sham-operated male rats in the PTZ discrimination stimulus. Thus, hormone replacement was conducted only for OVX female rats which had already acquired the PTZ discrimination task. Twelve ovariectomized female rats were subcutaneously implanted with β-estradiol pellets (2.5 mg for 21 days release). After a two day recovery period, they were subjected to behavioral tests.

Blood ethanol analysis

Subjects employed for this analysis were gonadally intact male (5) and female (5) rats. All rats were first anesthetized with a combination of ketamine (100 mg/kg) and chlorodiazepoxide (20 mg/kg) dissolved in saline. Thereafter a catheter was inserted into the right external jugular and the free end was fixed to

the skull. After a five day recovery period, they were injected with ethanol (2g/kg, 20 %, IP). Whole blood samples (100 μl) were taken from each rat through the jugular catheter at five different time points: 0 (prior to ethanol injection), 15, 30, 60, and 120 min after ethanol injection. After a sample was taken, the catheter was immediately flushed with an equal amount of saline. Ethanol concentration was analyzed by head space gas chromatography. An internal standard (t-butanol) was added to each blood sample (100 μl) in vials. The vials were then sealed with rubber stoppers and aluminum caps which were clamped with pliers. The samples were equilibrated at 80 degrees C and a sample of head-space gas was injected onto the gas chromatography column for determination of evaporated ethanol in the head space of the vials (Bonventre et al., 1982).

Blood β-estradiol analysis

Three groups of female rats were used for this assay: sham-operated (5), ovariectomized (5), and β-estradiol replaced ovariectomized (5) female rats. Whole blood samples (0.75 ml) were taken from each rat using the same method described in *'Blood ethanol analysis'*. For sham female rats, blood was collected for 5 consecutive days (day 1 to day 5) whereas OVX and β-estradiol replaced OVX rats, it was taken on day 1 and day 5 corresponding to sham female rats. Blood samples were immediately centrifuged and at least 250 μl of serum was

collected for assay. Each serum sample was kept frozen until assayed for β-estradiol concentration by radioimmunoassay. Serum samples were incubated with [^{125}I]estradiol in antibody coated tubes for 3 hours at room temperature. [^{125}I]Estradiol competes with estradiol in the sample for antibody sites. After incubation, separation of bound from free was achieved by decanting. Using a foam decanting rack, the contents of all tubes were aspirated and the radioactivity was counted using a gamma counter. The quantity of estradiol in the sample was determined by comparing the counts to a calibration curve.

Drugs

Pentylenetetrazol (PTZ) was purchased from Sigma Chemical Co (St Louis, MO). PTZ was freshly prepared and was dissolved in the saline solution (0.9 %). Diazepam (DZP, a gift from Hoffmann-La Roche, Nutley, NJ) was homogenized in 3 % carboxymethylcellulose (CMC) solution. β-estradiol (2.5 mg per pellet, 21 day release) was purchased from Innovative Research of America (Sarasota, FL).

Number of animals

The number of rats used for behavioral tests varied depending upon the number of rats which met the test criterion at the time of testing. In addition, during testing, animals which failed to emit a lever-press response (one FR10)

within a given test time (2 min) were not used in data analysis. The number of

animals in each experiment is indicated in the following table.

Table 1. Number of animals used in each experiment

Experiment	1	2	3	4	5	6	7
Sham-operated male	12	15	12	12			
Castrated male	12	13	12	12			
Sham-operated female	12	11	11	11			5
OVX 1	12	13	12	12	10		5
OVX + Estradiol	11	11	12	12			5
OVX 2					10		
Gonadally intact male						5	
Gonadally intact female						5	

OVX 1: Female rats that were ovariectomized after acquisition of the PTZ

discrimination task. OVX 2: Female rats that were ovariectomized before

acquisition of the PTZ discrimination task.

Experimental procedure

For all behavioral experiments with the exception of an experiment 5, five

experimental (gender) groups were tested: 1) sham-operated males, 2)
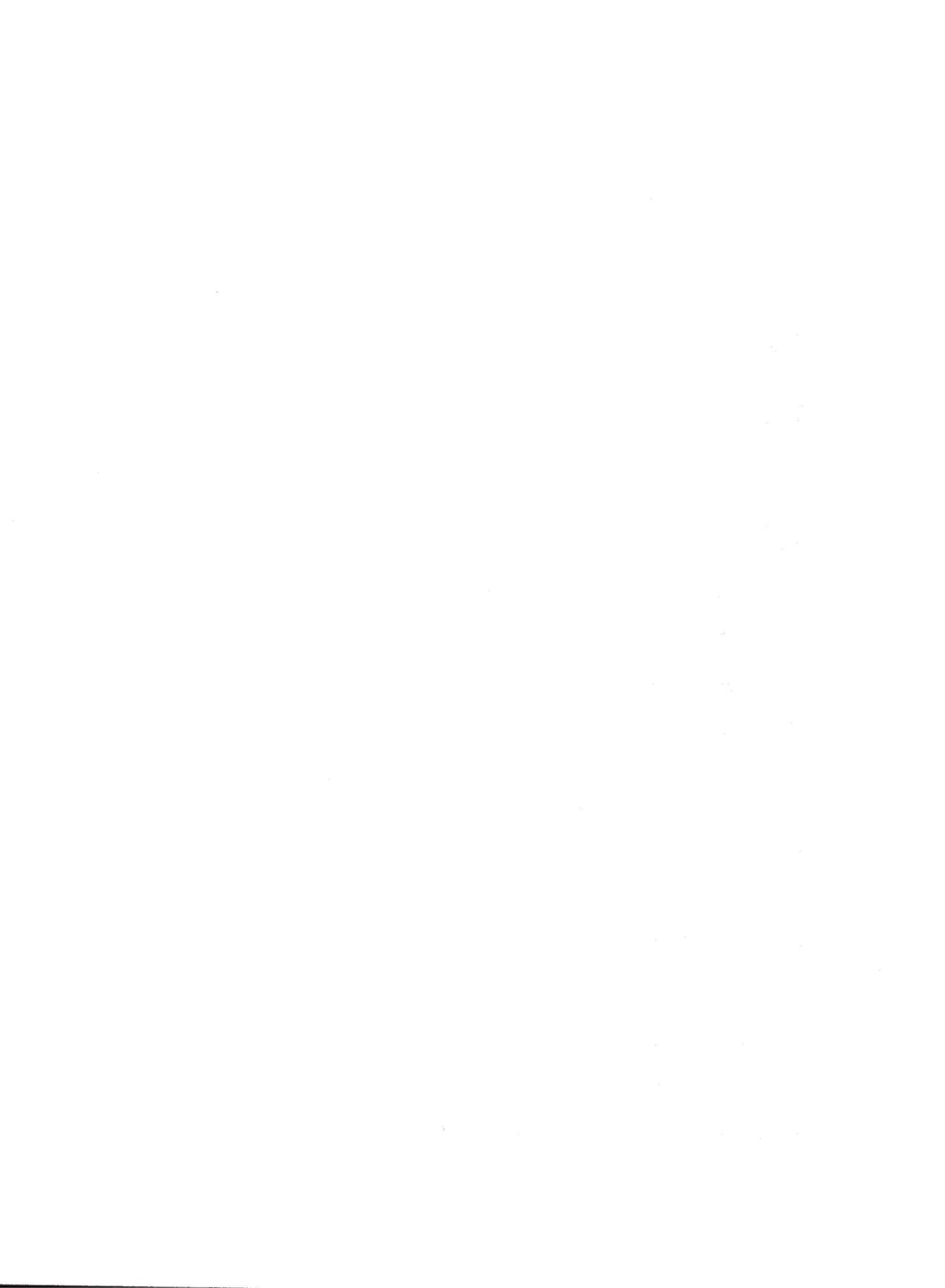
gonadectomized males, 3) sham-operated females, 4) OVX females, and 5) estradiol-replaced OVX females. For an experiment 5, a separate group of OVX female rats were used. Rats which met the criterion for the PTZ discrimination task by pressing the correct lever in 9 out of 10 consecutive training sessions were selected in behavioral tests (experiment 1 to 5).

Experiment 1 was designed to determine if the five experimental groups differ in their dose-response to a GABA-A antagonist, PTZ (0, 4, 8, and 16 mg/kg, IP) in the PTZ discrimination task. Animals were injected with saline and PTZ in a cumulative dosing method and tested for their PTZ-lever selection at each dose of PTZ.

Experiment 2 was designed to determine if the five experimental groups differ in their dose response to a GABA-A agonist, DZP (0, 0.625, 1.25, 2.5, 5.0, and 10 mg/kg, IP) to antagonize the PTZ discrimination stimulus (16 mg/kg, IP). Rats were injected with CMC (3 %, vehicle) or DZP 15 min prior to PTZ injection. 15 min later, they were tested for the PTZ-lever selection. In this manner, each dose of DZP was tested on a different day in a randomized order.

Experiment 3 was designed to determine if the five experimental groups differ in development of an endogenous PTZ-like stimulus during acute ethanol withdrawal (AEW, 12 h after termination of ethanol diet). Animals received

ethanol diet for 10 days. 12 hours after termination of ethanol diet, they were given a saline injection and tested for PTZ-lever selection.

Experiment 4 was designed to determine if the five experimental groups differ in their dose-response to the PTZ discrimination stimulus during protracted EW (36 h after termination of ethanol diet). Animals received ethanol diet for 10 days. 36 hours after removal of ethanol diet, they were given saline and PTZ 4, 8, and 16 mg/kg (cumulative doses). 15 min later, they were tested for PTZ-lever selection.

Experiment 5 was designed to compare the discriminative stimulus properties of PTZ in female rats trained with and without ovaries to determine possible differences in stimulus properties or intensity of the PTZ stimulus. PTZ-naïve female rats were ovariectomized and were trained for the PTZ discrimination as described in the methods section. After acquisition of the discrimination stimulus, rats were tested for their dose-response effect to PTZ. The dose-response curve was compared with that of female rats ovariectomized after acquisition of the PTZ discrimination task.

Experiment 6 was designed to determine if gonadally intact male and female rats differ in the blood ethanol concentration and clearance rate for blood ethanol before ethanol exposure and during EW. Gonadally intact male and female rats

were injected with 2 g/kg of ethanol and blood samples (100 μl) were collected at five time points: 0 (prior to ethanol injection), 15, 30, 60, and 120 min after ethanol injection. Blood samples were kept refrigerated until assayed. Animals then received chronic ethanol diet for 10 days. 12 hours after termination of the ethanol diet, rats were injected with 2 g/kg of ethanol. Blood samples (100 μl) were collected in the same manner as above. Ethanol concentration was analyzed by head space gas chromatography.

Experiment 7 was designed to determine if relationship exists between estrogen concentration and occurrence of the PTZ-induced discriminative stimulus in sham and OVX female rats. β-estradiol concentration was measured in sham female rats for 5 consecutive days (day 1 to 5). In OVX rats and β-estradiol replaced OVX rats, the concentration of β-estradiol was measured on corresponding days 1 and 5.

Data analysis

The data for selection of the PTZ lever were expressed as percentages (%) which were obtained as follows: ' % = 100 x [number of rats selecting the PTZ-lever / a total number of rats that completed the test] '. Each test session (a dose-response test, PTZ-lever selection tests during AEW or PEW) was repeated three times to obtain the mean and standard error of mean (SEM). For

the purposes of analysis, experimental groups defined by the variable gender included both gender, surgical condition, and hormonal treatment (5 groups). For dose-response tests (experiment 1 and 2), data were analyzed by two-way analysis of variance (gender X dose). For the data obtained during AEW (experiment 3), one way analysis of variance (gender) was used. For calculation of the ED50 for the PTZ discrimination dose-response effect, data were plotted as a logit PTZ lever selection versus a log dose of PTZ (experiment 4). A gender difference in ED50 was analyzed by two-way analysis of variance (gender X ethanol condition). Data that were continuous in nature (BEC and estradiol concentration) were directly subjected to either two-way (gender X time in experiment 6) or one-way (estradiol concentration over days in experiment 7) analyses of variance. The significance level was set as $P<0.05$. For determination of estradiol concentrations in sham female rats, data were collected for a 4 day cycle out of 5 days (experiment 7). A peak estradiol concentration was synchronized on day 2 because two of five rats had the peak on day 2.

Results

After 60 training sessions, animals acquired the PTZ discrimination task; they selected a PTZ lever after PTZ injection (16 mg/kg, IP) and a saline lever after saline injection.

Experiment 1: *Gender difference in the dose-response effect of the PTZ discrimination (Figure 1):* As the dose of PTZ increased, PTZ-lever selection also increased in all groups of rats with a dose and a gender group interaction [$F_{(12, 30)}=22.7$, $P < 0.001$]. Five experimental groups differed in the PTZ dose-response effect [$F_{(4,10)}=44.5$, $P<0.001$]. A repeated measure analysis by dose was conducted for a pair of experimental groups. The PTZ-lever selection (%) was lower in a sham female group than a sham male group [$F_{(1,4)}=108.9$, $P<0.001$] or in a OVX group [$F_{(1,4)}=25.9$, $P<0.007$]. The PTZ-lever selection (%) was also lower in an estradiol-replaced OVX group than a OVX group [$F_{(1,4)}=12.9$, $P<0.023$]. No significant differences were observed between two male groups (sham and castrated) and a sham female group and an estradiol-replaced OVX group.
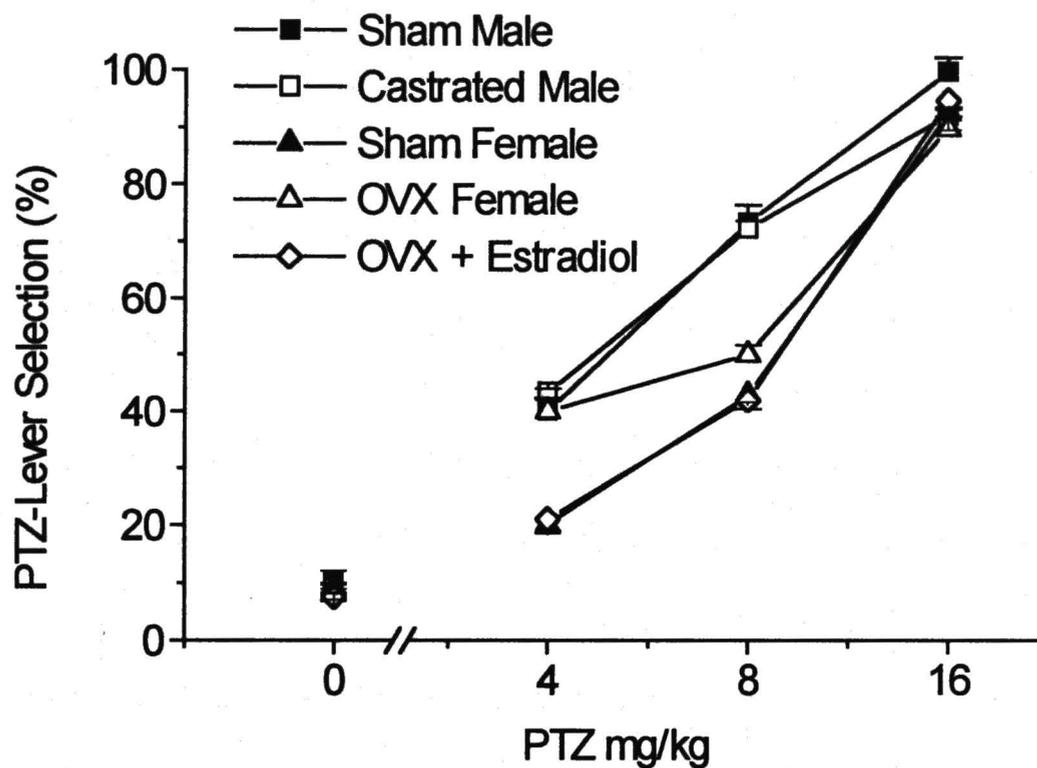
Figure. 1. Demonstration of a gender difference in the PTZ discrimination stimulus. Data points are mean values obtained from repeating each test three times and error bars are standard error of mean (SEM). $F(4,10) = 44.5$, $P<0.001$ by five experimental groups.

Experiment 2: *Gender difference in the effect of a GABA-A/BZD agonist,*

diazepam (DZP) on the PTZ discrimination stimulus (Figure 2): DZP (0, 0.625,

1.25, 2.5, 5.0, and 10 mg/kg, IP) injected prior to PTZ administration (16 mg/kg,

IP) blocked the PTZ discrimination stimulus. This effect of DZP was greater as

the dose of DZP increased [$F(4,52)=74.9$, $P<0.001$] and was different in five

experimental groups [$F(4,13)=28$, $P<0.001$] with a dose and a gender group

interaction [$F(4,16)=4.6$, $P<0.001$]. When a sham female group was compared

with a sham male or a castrated male group, the inhibitory effect of DZP on the

PTZ discrimination stimulus was more effective in a sham female group than in a

sham male [$F(1,6)=67.5$, $P=0.001$] or a castrated male [$F(1,50)=80.5$, $P=0.001$]

group. However, no significant difference was observed between a OVX group

and a sham female group or an estradiol replaced OVX group.
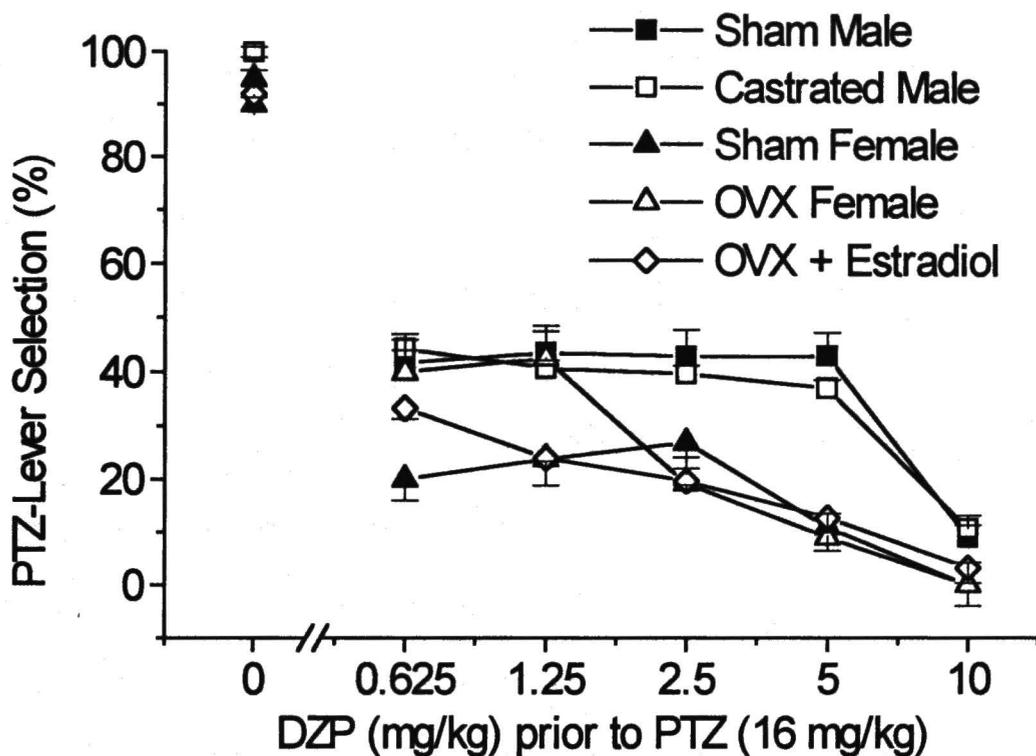
Figure 2. Demonstration of a gender difference in the effect of DZP on the PTZ discrimination stimulus. Data points are mean values obtained from repeating a test three times and error bars are SEM. $F(4,13)=28$, $P<0.001$ by five experimental groups.

Experiment 3: *Gender difference in the PTZ-like stimulus induced by acute ethanol withdrawal (AEW).* During AEW, animals injected with saline selected the PTZ-lever and the magnitude of this phenomenon differed in five experimental groups [$F(4,25)=6.6$, $P=0.001$]. When a sham female group was compared with a sham male group or a castrated male group, the PTZ-lever selection (%) was lower in a sham female group (23.8 ± 7 %) than a sham male group (50.1 ± 4 %) [$F(1,16)=22.7$, $P<0.001$] or a castrated male group (42.8 ± 1.8 %) [$F(1,10)=5$, $P=0.049$]. In addition, the PTZ-lever selection (%) was lower in an estradiol-replaced OVX group than a sham male group [$F(1,10)=13.5$, $P=0.004$]. Although statistically not significant, there was a trend that more OVX rats (33.5 ± 4.7 %) selected the PTZ-lever than sham female or estradiol-replaced OVX rats (23.8 ± 6.7 %). No significant differences were observed between two male groups (sham and castrated) and between a sham female and an estradiol-replaced OVX groups.
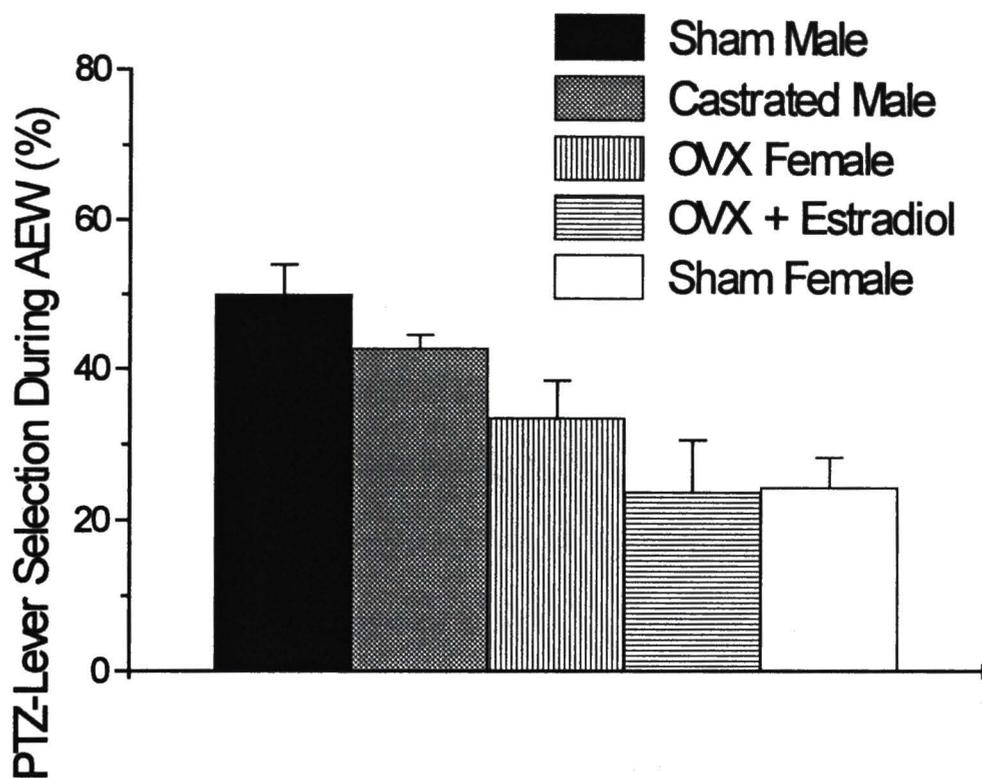
Figure 3. Demonstration of a gender difference in the PTZ-lever selection during

AEW. Data points are mean values obtained from repeating a test three times,

and error bars are SEM. $F_{(4, 25)}=6.6$, $P=0.001$ by five experimental groups.

Experiment 4: *Gender difference in the PTZ discrimination stimulus during protracted ethanol withdrawal (PEW).* Figure 4 represents the ED50s for the PTZ discrimination stimulus prior to chronic ethanol diet and during PEW. Before exposure to chronic ethanol diet, five experimental groups differed in their ED50s [$F(4,10)=27.8$, $P=0.006$]. A post hoc Tukey test indicated higher ED50 in a sham female group than that in a sham male group ($P=0.042$). When overall ED50s were compared before exposure to chronic ethanol diet and during PEW, ED50 during PEW were significantly lower than ED50 before chronic ethanol diet [$F(1,10)=123.9$, $P<0.001$], indicating that animals were sensitized to the PTZ stimulus during PEW. Five experimental groups differed in ED50s during PEW [$F(4,10)=3.9$, $P=0.037$]. Although a post hoc Tukey test indicated no difference in any pair of experimental groups, an overall gender difference during PEW appears to be contributed by a high ED50 (4 0 ± 0.25 mg/kg) in a sham female group versus a low ED50 (2.6 ± 0.3 mg/kg) in a sham male group. During PEW, ED50 (2.5 ± 0.4 mg/kg) in an OVX group was as low as that of a sham male (2.6 ± 0.3 mg/kg) or a castrated (2.7 ± 0.3 mg/kg) male group. This phenomenon was reversed by β-estradiol replacement, restoring the ED50 to the level of a sham female group.
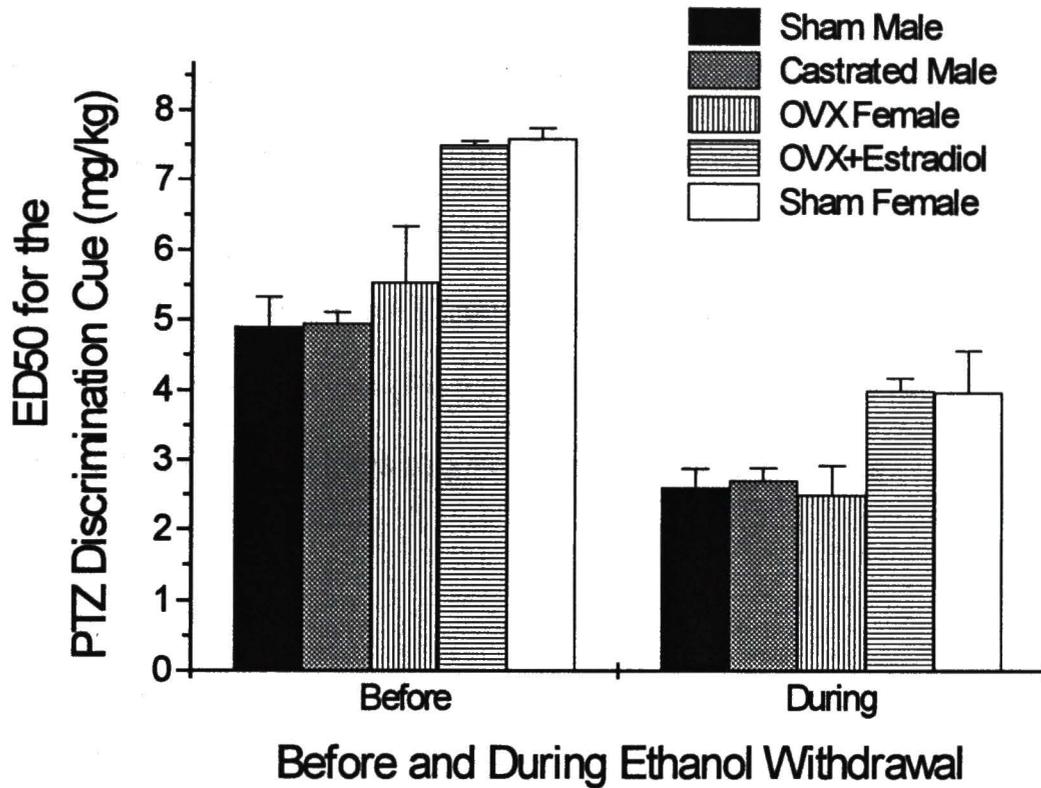
Figure 4. Demonstration of gender differences in ED50s for the PTZ

discrimination stimulus before chronic ethanol diet and during PEW. Data points

are mean values obtained from repeating a test three times, and error bars are

SEM. Left panel; ED50 before chronic ethanol diet. $F(4,10)=27.8$, $P=0.006$ by

five experimental groups. Right panel; ED50 during PEW. $F(4,10)=3.8$, $P=0.037$

by five experimental groups. $F(1, 10)=123.9$, $P<0.001$ by two ethanol conditions.
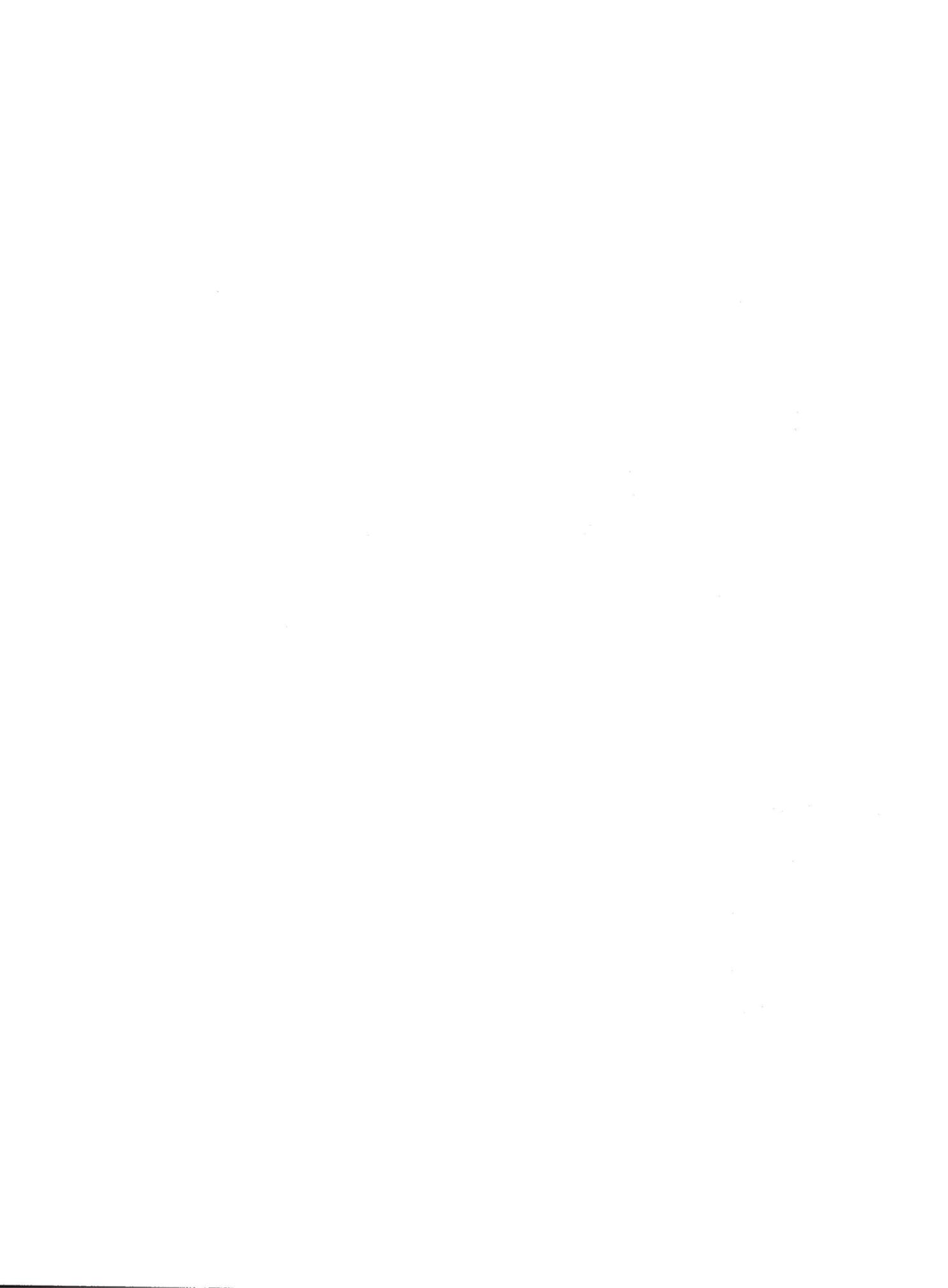
Experiment 5: *Comparison of the discriminative stimulus properties of PTZ in female rats trained with and without ovaries to determine possible differences in stimulus properties or intensity of the PTZ stimulus (Figure 5).*

As the dose of PTZ increases, the PTZ-lever selection increases in two groups of OVX rats [$F(3, 54)=27.1$, $P < 0.0001$]. Kruskal-Wallis one way analysis of variance indicates that the PTZ dose response does not differ between the two groups of OVX female rats ($P= 0.615$, df=1). In addition, the two groups did not differ in the number of trials required to reach training criteria.
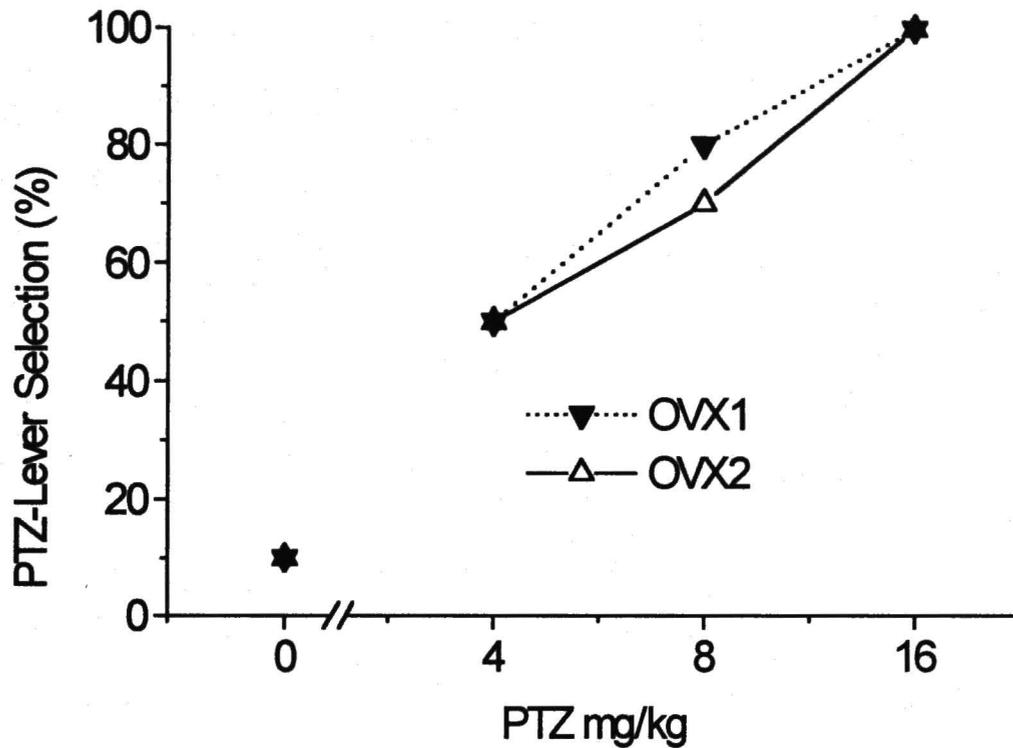
Figure 5. Demonstration of the dose-response effect of the PTZ discrimination stimulus in female rats trained with (OVX 2) and without ovaries (OVX 1). Data points are percentages (%) of rats which selected the PTZ-lever out of 10 rats in each group. P= 0.615, df=1.

Experiment 6: *Gender difference in blood ethanol concentrations* (Figure 6).
Before chronic ethanol diet, overall BEC was higher in a gonadally intact female
group than in a corresponding male group [$F(1,4)=103.2$, $P=0.001$]. As the time
after ethanol injection elapsed, BEC decreased [$F(3,12)=3.9$, $P=0.037$], but the
rate of clearance did not differ between male and female groups. During AEW,
there were no gender differences in BEC and the rate of clearance.
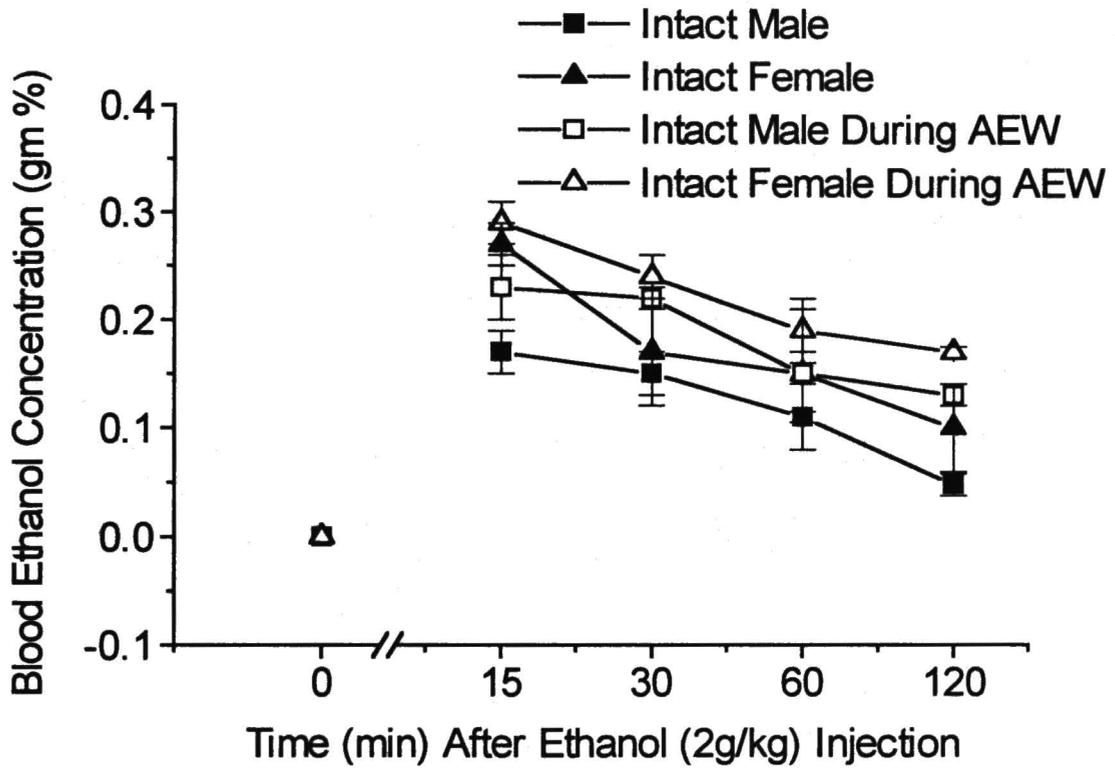
Figure 6. Demonstration of gender differences in BEC before and during AEW.

Data points are mean values, and error bars are SEM. $F(1,4)=103.2$, $P=0.001$

by two groups (male versus female) before EW.

Experiment 7: *Determination of β-estradiol concentrations and its influence on the PTZ discrimination stimulus (Figure 7).* Figure 7a shows that β-estradiol replacement in OVX rats significantly increased the serum β-estradiol concentration [$F(1,16)=300$, $P<0.001$] as compared to the pre-replacement value. Figure 7b illustrates that sham female rats undergo a 4 day cycle in serum estradiol concentrations which peak on day 2 as compared to day 1, 3, and 4 [$F(3,16)=13$, $P<0.001$]. The serum concentration on day 2 was also significantly higher than that in OVX rats [$F(1,13)=16.5$, $P=0.001$]. Despite the cyclic fluctuation in estradiol concentrations, performance of the PTZ discrimination task was stable as seen in Figure 7c.
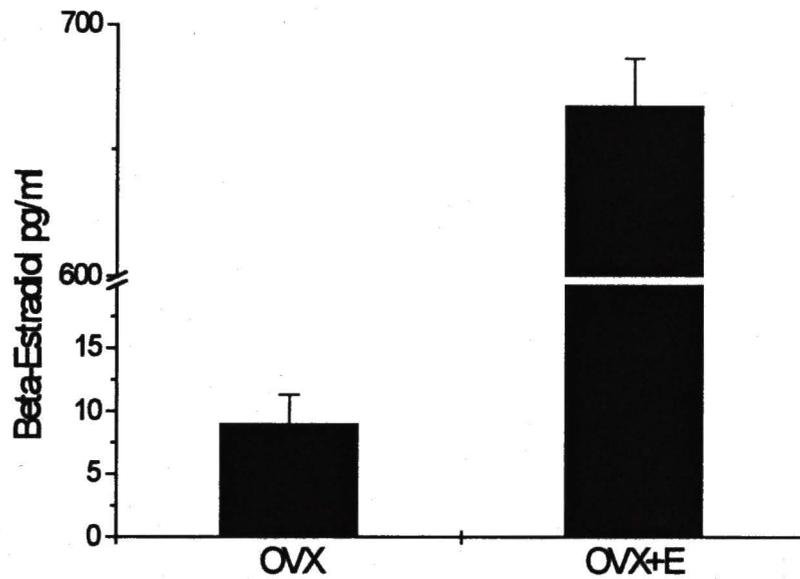
Figure 7a. Demonstration of β-estradiol concentrations in OVX rats before
(OVX) and after β-estradiol replacement (OVX + E). F(1,16)=300. P < 0.0001.
Data points are mean values, and error bars are SEM.
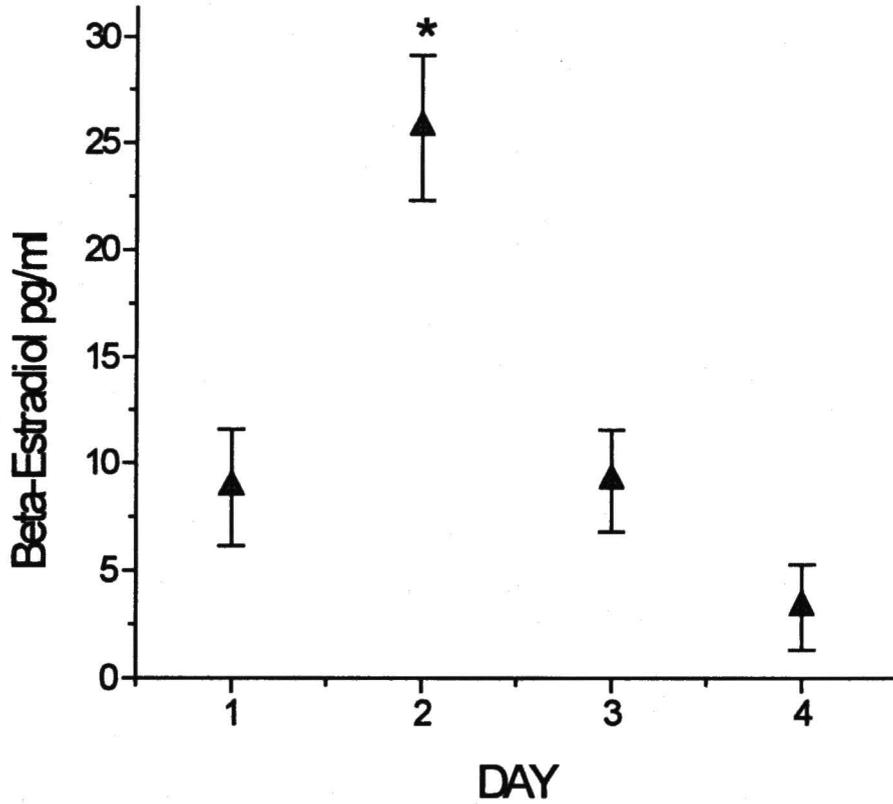
Figure 7b. Demonstration of β-estradiol concentrations in sham female rats for 4 consecutive days. Data points are mean values, and error bars are SEM. A symbol "*" represents a difference from the other three points. $F_{(2,37)}=47.6$, $P<0.001$.
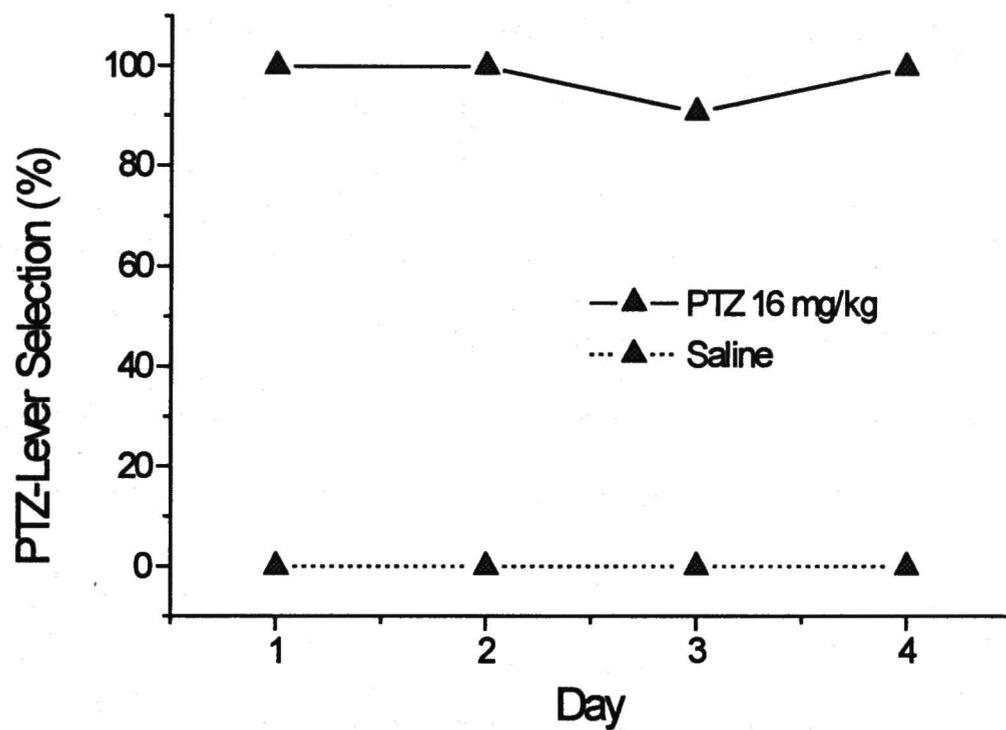
Figure 7c. Demonstration of the stable performance of the PTZ discrimination task for 4 consecutive days in sham female rats.

Discussion

The present findings provide experimental evidence that male and female rats differ in response to the anxiogenic stimuli induced by a GABA-A antagonist, PTZ or by an endogenous PTZ-like stimulus which develops during EW. In this study, we have shown a consistent phenomenon that fewer sham female rats respond to the PTZ-like anxiogenic stimulus than sham male rats. We have also demonstrated that estrogen is responsible, at least in part, for this gender difference by showing an effect of ovariectomy and estrogen replacement.

The gender difference observed in this study dose not appear to be due to different stimulus properties of PTZ induced by an ovarian steroid. This is supported by the finding of no difference in the PTZ-discrimination dose-response between two groups of OVX female rats: one was ovariectomized before training with PTZ and the other was ovariectomized after acquisition of the PTZ discrimination task. Since PTZ was as an independent variable, it is important to assure that animals were tested under the same stimulus condition. If PTZ provides different stimulus properties between groups, an accurate comparison can not be made from the behavioral outcomes which would be influenced by the different stimulus properties of PTZ.

OVX rats had a greater anxiogenic response than sham female rats and this phenomenon was reversed after estradiol replacement. These results indicate that a female steroid, estrogen enhances GABA-A agonistic activity, thus reducing the GABA-A antagonistic stimuli examined in this study. Similarly, ovariectomy abolished the gender difference in the threshold for the PTZ-induced seizure by decreasing the threshold of female rats toward that of male rats (Kokka, 1992). Adult female rats that received neonatal treatment with the estrogen antagonist, tamoxifen or prepubertal ovariectomy showed a greater anxiogenic response than control females. Thus, they spent a shorter time on the open arms of the elevated plus maze than control females that received vehicle during the neonatal period (Zimmerberg and Farly, 1993). The inhibitory activity of estrogen on anxiogenic stimuli appears to result from enhancement of GABAergic neurotransmission. At the receptor level, GABA-stimulated chloride conductance in GABA-A/BZD receptors is more effective in intact female rats than in OVX rats (Bitran et al., 1991). The DZP data in the present study also provide behavioral evidence that the GABA-A agonistic system in female rats is more active than in male rats. At a given dose, DZP blocks the PTZ discrimination stimulus more effectively in sham female rats than in sham or castrated male rats. Indeed, in a clinical study, young women have been reported to require lower doses of BZDs than young men (Yonkers et al., 1992).

The role of estrogen in enhancing the GABAergic system is indirectly demonstrated by work from Akinci and Johnston (1993). They found that acute swim stress in cold water increased GABA binding in membrane samples prepared from the forebrain of female mice, but not of male mice. The increment of GABA binding was larger when measured in a crude membrane preparation than in a well-washed membrane preparation in female mice. The authors (Akinci and Johnston, 1993) interpreted that result as the loss of endogenous modulators of GABA binding in the well-washed membrane preparation in female mice. It is plausible to speculate that the endogenous modulator of GABA binding could be estrogen. As Fink et al. (1996) referred to estrogen as "Nature's psychoprotectant", estrogen may provide a protective mechanism to counteract or suppress GABA-A antagonistic anxiogenic stimuli. Since EW produces an endogenous PTZ-like stimulus, the protective mechanism of estrogen may also occur to protect against the GABA-A antagonistic component of EW.

Within our data, there is a general trend that ovariectomy does not completely abolish the sexually dimorphic anxiogenic response between male and female rats. In addition, sham female rats undergo a cyclic variation in estrogen levels, but their performance in the PTZ discrimination stimulus is not cyclic in nature. In other studies there was no direct effect of ovarian factors or the estrus cycle on anxiogenic responses. In the elevated plusmaze, neither

OVX nor estrus cycle modified anxiogenic behavior in rats (Nomikos and Spyraki, 1988). OVX did not influence ethanol-induced motor incoordination or spontaneous open field activity in mice (Beaker et al., 1985). Forced swimming induced immobility in male rats to a greater extent than in female rats (Alonso et al., 1991). The immobility level was similar in the different stages of the estrous cycle of female rats. These data are consistent with the general hypothesis that periodic or continuous estrogen exposure at a threshold concentration of estrogen is necessary to maintain greater GABA-A agonistic activity in females than males, but that acute increases in estrogen activity do not directly facilitate GABA-A function.

With regard to male hormones, their role in anxiety-related behavior is less defined than that of female hormones. In general male hormones appear to influence anxiety-associated behaviors during the developmental period rather than during adulthood. The results of our experiments are consistent with those of other groups that have determined that castration of adult male rats produces no significant change in anxiogenic response.

Tischkau and Ramirez (1993) demonstrated that ovariectomy reduced the progesterone receptors in the hypothalamus of female rats more than 80%. Estrogen replacement (10 µg / rat for 4 days) to OVX rats restored the progesterone receptors to a level equivalent to that of intact females. At the
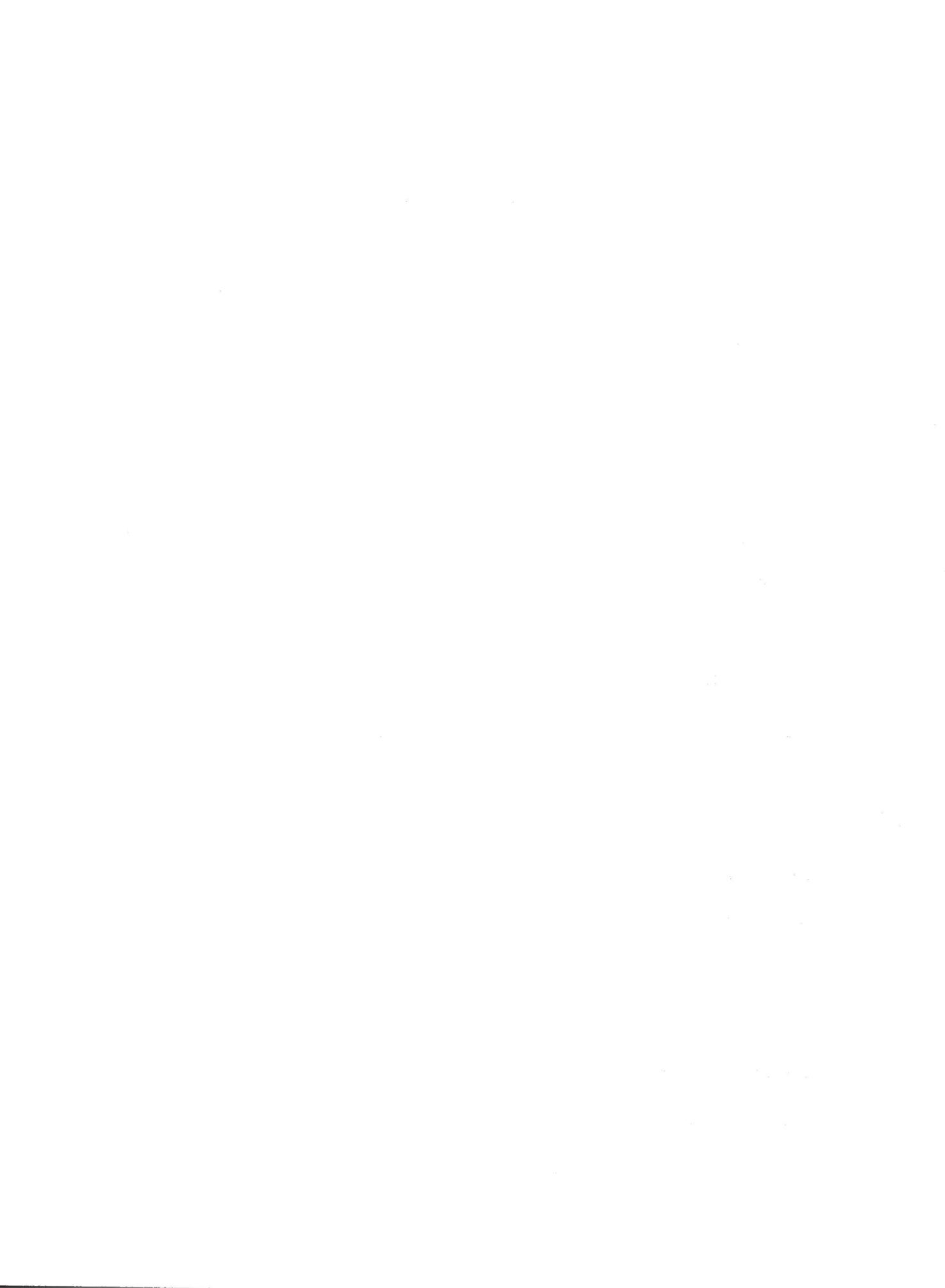
genomic level, progesterone mRNA expression in venous vessels of female rats decreased after ovariectomy (Knauthe et al., 1996). This effect was reversed by chronic treatment with estradiol (1 mg/rat/day sc). The fact that a chronic treatment with estradiol was required for inducing progesterone mRNA further supports the idea of an indirect anxiolytic activity of estrogen. At the very least, these results lead us to speculate that estrogen could also contribute to higher GABA-A agonistic activity by inducing a higher level of progesterone receptors or increasing the expression of GABA-A subunits that bind progesterone metabolites.

An unique feature of progesterone different from estrogen is that progesterone is metabolized to GABA-A agonistic steroids such as tetrahydroprogesterone (THP) or tetrahydrodioxy-corticosterone (THDOC) (Majewska, 1991). These steroids are found in the brain of adreno/gonadectomized rats, which indicates their brain-origin. More importantly, progesterone itself and its metabolites, THP and THDOC occur higher levels in the brain of female rats than in male rats (Corpechot et al., 1993). Enzymes which convert progesterone to THP are detected at higher levels in the brains of female rats than in male rats. GABA-A agonistic properties of these neuroactive steroids have been demonstrated at the behavioral level as having anxiolytic activity. In a two-chambered mouse exploration test (Crawey et al., 1986), THDOC increased the number of

transitions (indice of anxiolytic activities) made by mice between two compartments and enhanced the tendency of mice to explore a novel environment. In a conflict test, THDOC increased the number of punished licking responses under electric shock (Crawey et al., 1986). Acute swim stress increased the levels of THP or THDOC in the cerebral cortex, hypothalamus, and plasma of the rats (Crawey et al., 1986). It has been suggested that the increased levels of these steroids play a role in attenuating stressful stimuli by acting at the GABA-A receptors. These reports emphasize a higher capacity for GABA-A agonistic activity in female rats than in male rats.

With regard to BEC, given the same dose of ethanol, female rats have a higher BEC than male rats (Sutker et al., 1983; present data). These data exclude the possibility that the lower intensity of EW-induced anxiogenic stimuli in sham female rats is due to a lower BEC during the development of dependence than in male rats. Our data also indicate that males and females do not differ significantly in the clearance rate for ethanol either before or after exposure to ethanol diet. In agreement with our findings, no gender difference was found in the activity of alcohol dehydrogenase and acetaldehyde dehydrogenase in the gastric mucosa of male and female rats (Maly et al., 1992). These data indicate that a difference in the metabolic rate of ethanol is not responsible for the difference in anxiogenic response between male and female rats.

Similarly, the metabolic rate of PTZ does not appear to be a contributing factor to the gender difference observed in this study. PTZ distributes rapidly from the systemic circulation to the CNS, including sites of action (reviewed by Ramzan and Levy, 1985). Esplin and Woodbury (1956) demonstrated that the biological activity of PTZ declined with the half-life of about 120 min in male rats. By comparison, Ramzan and Levy (1985) reported that the half-life of female rats was 116 ± 25 min. Thus, the half-life of PTZ does not appear to significantly differ between male and female rats. A lack of gender difference in the half-life of PTZ would argue against the possibility that female rats are less responsive to the stimulus effect of PTZ due to their lower blood concentration of PTZ or higher PTZ clearance rate as compared to male rats.

This notion is further supported by our observation that there was no male-female difference during the acquisition phase of the PTZ discrimination task. In a study done by Kokka et al. (1992), PTZ seizure threshold increased in male rats pretreated with ethanol, but not in corresponding female rats. Suggestion can be made that male rats are less sensitive to the PTZ-induced seizure than female rats under a certain condition. Finn and Gee (1994) measured the PTZ threshold dose for onset of myoclonic twitch, clonus or tonic hindlimb extention in male, diestrus female, and estrus female rats. There was no significant gender- and estrus cycle-related differences in the PTZ threshold dose for onset of all three convulsion measures. Administration of an

anticonvulsant, $3\alpha,5\alpha$-progesterone, prior to PTZ injection increased the PTZ threshold dose for the three convulsion measures. There was a gender- and estrus cycle-related difference in the $3\alpha,5\alpha$-progesterone-induced increase in the PTZ threshold dose for myoclonic twitch onset, but not for onset of clonus or tonic hindlimb extention: the PTZ threshold dose was higher in diestrus female rats than in estrus or in male rats. This gender difference is not identical to what we have seen in the present study. If a pharmacokinetic difference between males and females determines a gender-dependent sensitivity to PTZ, the divergent gender differences in PTZ actions would not have occurred. Perhaps, multiple mechanisms may mediate the pharmacological action of PTZ. Additional to its action at the GABA/BZD receptors, PTZ reduces transient inward currents of Na^+ and Ca^{++} and increases the inactivation of K^+ currents in response to a depolarizing pulse (Klee et al., 1973). Depending upon the mechanisms involved, one gender may be more sensitive to PTZ than the other. Moreover, female rats are less responsive to PTZ whether the PTZ stimulus is exogenously administered or endogenously developed during AEW as compared to male rats (present data). Above cited studies and the present findings support the hypothesis that the pharmacodynamic rather than pharmacokinetic factors are involved in gender-dependent anxiogenic sensitivity.

In conclusion, the present study provides evidence that the sexually dimorphic anxiogenic response to PTZ or EW is in part due to estrogen-mediated factors directly or indirectly acting on the GABAergic system. Our findings extend the use of the PTZ discrimination paradigm to determine gender differences in the anxiogenic stimuli associated with EW. Hence, this study may provide a step toward development of improved preclinical or clinical strategies for the gender specific treatment of anxiety disorders, in particular during EW or for ethanol abuse-related problems.

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CHAPTER 2

The rationale for simultaneous employment of the m-CPP discrimination paradigm and the PTZ model is several fold. Like the GABAergic system, the serotonergic system plays a significant role in regulation of anxiety. Anxiety is a multivalent phenomenon involving numerous neuronal factors. Thus, behavioral outcome in each model may reflect some but not all of these factors. For instance, there is a striking dissimilarity between anxiogenesis of PTZ and m-CPP in behavioral manifestations: m-CPP causes impaired behavioral initiation which is not an action of PTZ. Moreover, employing the m-CPP paradigm will allow the study of the interaction between the GABA and serotonin systems associated with anxiety. This may prove beneficial by encouraging research or therapeutic strategies for the treatment of anxiety or EW-induced anxiety which are free of the adverse consequences of GABA/BZD therapies such as dependence liability or unwanted sedative side effects in both men and women.

The experiments in this chapter were designed to test the following specific aims:

1) To determine if male and female rats differ in response to the anxiogenic interoceptive discriminative stimulus (IDS) or impaired behavioral initiation induced by a 5-HT1b/2 agonist, m-CPP;

2) To determine if male and female rats differ in development of an endogenous the m-CPP-like stimulus during EW;

3) To determine if gonadal factors are responsible for a gender difference in IDS, impaired behavioral initiation induced by m-CPP, or an endogenous m-CPP-like cue during EW;

4) To determine if a difference in blood ethanol concentration between male and female rats is responsible for a gender difference in the stimulus effects of m-CPP.

Gender Difference in Anxiogenic Stimuli Induced by

m-Chlorophenylpiperazine or Ethanol Withdrawal

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Abstract

This study compared gender differences in the anxiogenic stimuli induced by a 5-HT1b/2 agonist, m-chlorophenylpiperazine (m-CPP) or by ethanol withdrawal (EW). Subjects employed in behavioral tests were gonadectomized or sham-operated male and female rats and 17β-estradiol (2.5 mg, 21 day release, sc) replaced ovariectomized (OVX) female rats. Rats were trained to discriminate m-CPP (1.2 mg/kg, IP) from saline in a two lever choice task for food reward. Latency to first lever-press response and the dose-response for the discrimination of the interoceptive stimulus (IDS) produced by m-CPP (0 to 1.2 mg/kg) were measured under all hormonal conditions. Results: Sham and estradiol replaced OVX rats showed an increased delay to the initiation of response after m-CPP injection and had higher ED50 for discrimination of the m-CPP IDS as compared to sham male, castrated or OVX rats. Rats then received a chronic ethanol diet (6.5 %) for 10 days. At twelve hours of EW, they were tested for lever selection after saline injection. Fewer sham female and estradiol-replaced female rats responded on the drug lever during acute EW as compared to sham male, castrated or OVX rats. The anxiogenic drug lever selection of OVX rats resembled that of male rats but was restored toward that of sham female rats by estradiol replacement. Castration did not alter the response of male rats to mCPP. Conclusions: females are more impaired in their ability to initiate operant responses, but produce less anxiogenic IDS in

response to 5-HT1b/2 activation than male rats. In addition, fewer sham females developed a spontaneous IDS during EW than male or OVX rats. Estrogen appears to play a trophic role in altering responsiveness to anxiogenic stimuli. (Supported by NIAAA #AA06890 and #AA09567).

Introduction

As the number of female alcoholics increases in the United States, a gender difference in ethanol-related problems has gained increased attention in alcohol research. However, despite the recognized importance of the gender factor as a research or a treatment variable of alcoholism, little information is available establishing a gender difference in ethanol withdrawal (EW)-induced anxiety. This topic is important because anxiety is the major driving force for maintaining ethanol consumption in both male and female alcoholics. The primary goal of the present study is to provide preclinical strategies to prevent EW-induced anxiety in males and females by characterizing a potential gender difference in serotonergic or other sexually dimorphic factors associated with anxiety.

A 5-HT1b/2 partial agonist, m-chlorophenylpiperazine (m-CPP) is a metabolite of the antidepressant drug, trazodone (Li et al, 1990). In general, antidepressant drugs are anxiogenic in their initial effects, followed by antidepressant actions after chronic treatment. Studies employing human subjects report that a single administration of m-CPP elicits a significant increase on a behavioral rating scale of anxiety in normal subjects (Klein et al., 1991) as well as in patients suffering from an anxiety disorder (Pigott et al., 1991; Germine et al., 1992). The implication that serotonin is involved in the

action of ethanol has been made in several studies. In a clinical study, m-CPP intensified craving in abstinent alcoholics (Naranjo and Bremner, 1994). m-CPP also increased a plasma concentration of cortisol (Lawlor et al., 1989) and this effect was impeded during the abstinence phase of alcoholics (Krystal et al., 1996). In the hippocampus of alcoholics, the maximal number of ^{3}H-paroxetine (a selective 5-HT reuptake inhibitor) binding sites decreased as compared to that of controls (Chen et al., 1991). The authors (Chen et al., 1991) interpreted these results as a reduction of serotonergic nerve terminal density resulting from ethanol abuse related neuronal damage. These studies indicate a serotonergic dysfunction associated with ethanol abuse. In an animal study using the elevated plus maze paradigm, m-CPP potentiated the EW-induced anxiogenic response in male rats (Rezazadeh et al., 1993). So far, no study has reported a gender difference in anxiogenic stimuli induced by m-CPP or an endogenous m-CPP-like anxiogenic stimulus during EW.

Anxiety is a complex phenomenon involving multiple features such as restlessness, nervousness, dysphoria, hostility, motoric retardation, impaired motivation, and aggressiveness (Katz et al., 1993). Assuming that different neuronal and hormonal systems are involved in different anxiogenic outputs, it is not surprising that anxiety is expressed differently between sexes. Indeed, it has been reported that women are more vulnerable to the motor retardation component of anxiety whereas men are more sensitive to a hostility feature of

anxiety (Katz et al., 1993). In the light of this fact, m-CPP serves as a good pharmacological tool to study anxiety between males and females because m-CPP produces two distinguishable anxiogenic properties: behavioral suppression (Lawlor et al, 1989; Ulrichsen et al., 1992) and a discriminative cue (Winter and Rabin, 1993; Callahan and Cunningham, 1994; Wallis and Lal, 1997). m-CPP inhibits the exploratory and ambulatory activities of rats in an open field paradigm (Klodzinska et al., 1989) where a higher activity level in the open field indicates less anxiety. m-CPP also serves as a discriminative stimulus (Winter and Rabin, 1993; Callahan and Cunningham, 1994) as indicated by the animals ability to learn a discrimination between the m-CPP stimulus and a vehicle stimulus in an operant procedure. In this context, the present study utilized two measures of the anxiogenesis of m-CPP, impaired behavioral initiation and the discriminative stimulus of m-CPP in an ethanol-naive state and during EW in male and female rats.

Studies have reported gender differences in the serotonergic system. Female rats exhibit the serotonin syndrome (i.e., head shaking or hindlimb abduction) at a lower dose of the serotonin precursor, l-tryptophan, than male rats (Fischette et al., 1984). Female rats have higher brain concentrations of 5-HT and its metabolites, 5-hydroxyindoleacetic acid (5-HIAA) than male rats. These results indicate that the serotonergic system is more effectively expressed

in females than in male that led us to examine male-female differences in the behavioral response to the serotonergic agonist mCPP.

The hypotheses to be tested in the present study were : 1) female rats are more sensitive to the anxiogenic properties of an exogenously applied serotonergic drug, m-CPP, whereas; 2) fewer female rats develop an EW-induced interoceptive anxiogenic stimulus than male rats. The influence of estrogen on these anxiogenic responses was tested by means of gonadectomy of male and female rats and estrogen replacement in OVX female rats.

Method

Animals

Adult male and female Long-Evans hooded rats (Charles River, Wilmington, MA) were housed individually with temperature (22-25^0 C) and humidity (55%) held constant. A 12 h light-dark cycle was maintained with lights on between 7 a.m. and 7 p.m. Animal body weights were maintained at 320-350 g for male rats and 290-310 g for female rats by limiting food (Purina rat chow) to 20 g/day for male rats and 16 g/day for female rats which included the food received during training. Food restriction was necessary to maintain the lever pressing response for food reward. Water was available ad libitum.

Preliminary training

This procedure required animals to learn a lever-press response for food reward. Animals were trained under a fixed-ratio 1 (FR1) schedule where they have to emit one press on any of two levers to get one food pellet. The number of a lever-press response for one food pellet was gradually increased to 10 (FR10). After acquisition of a FR10 schedule, they were subjected to the following preselection phase.

Preselection of subjects

Since m-CPP produces behavioral suppression, this procedure eliminated rats unable to emit a lever-press response after m-CPP injection. For this preselection phase, three groups of rats were employed: gonadally intact male (20) and female rats (20), and OVX female rats (22). They were injected with either saline or m-CPP (1.2 mg/kg, IP) in a randomized order. 15 min later, rats were placed into the operant boxes and tested for at least ten presses on any lever for food reward. Initially, a session time was set up to 2 hours for the first 20 sessions. Rats which were able to perform a FR10 schedule within 20 sessions were selected and the percentage of these rats out of total rats was calculated in each group. Session times were then decreased to 20 min for the subsequent discrimination training.

Discrimination training

Preselected rats were trained to discriminate between m-CPP and saline under a FR10 schedule. Thus, they get food reward when they emit ten presses on one of two levers following m-CPP (1.2 mg/kg, IP, 15 min) administration and the other lever following saline injection. Half of the male and female rats were trained with m-CPP as the cue on the right lever whereas the other half were trained with m-CPP on the left lever. Each training session was 20 minutes in length. An equal number of saline and m-CPP training sessions was given in an irregular order of presentation such that no condition occurred in more than three consecutive tests. Animals received at least 60 training trials prior to use in any experiment. Typically, when stimulus control has been well established, animals rarely respond on the incorrect lever. Animals were selected for use in experiments when they achieved a 90% correct response rate for their last ten training sessions.

Discrimination testing

The m-CPP discrimination threshold was measured by a cumulative dosing regime. Fifteen (15) minutes after injection with saline, animals were tested for their lever selection. On completion of this test, animals were immediately injected with the lowest dose of m-CPP (0.25 mg/kg) and tested as above. The next dose injected was the difference (0.25 mg/kg) between the second dose (0.5 mg/kg) and the first dose (0.25 mg/kg). In this manner, animals were given additional doses and tested until they received the

cumulative m-CPP dose of 1.2 mg/kg. The data were expressed as the percentage of animals selecting the m-CPP lever at each dose for their discrimination ability.

Chronic ethanol administration

Ethanol was administered in a nutritionally complete liquid diet containing 6.5% ethanol (w/v) as modified by Dodd and Shorey-Kutsche (1987). Animals received a 100 ml aliquot of ethanol diet each morning for 9 days. On the morning of the tenth day, a 50 ml aliquot of diet was given so that all animals receive the same dose of ethanol before testing. Twelve hours later the diet tubes were removed and animals were fed with food pellets. On the following morning [acute ethanol withdrawal (AEW), 12 hours after termination of ethanol diet)], they were injected with saline. Fifteen min later, their lever selection was tested as described above. On the second day after termination of chronic ethanol diet [protracted ethanol withdrawal (PEW), 36 hours after termination of chronic ethanol diet], they were injected with saline and m-CPP (0.25, 0.5, and 1.0 mg/kg IP) in a cumulative dosing method and tested for their lever selection.

Gonadectomy

Under isoflurane anesthesia, a small incision was made in the abdominal cavity directly above the ovaries. The ovaries of the OVX group were removed bilaterally. The incisions were closed with stainless steel wound clips. For castration, testicular areas were cleaned, and a small incision was made in the

scrotum. After removal of the testicles, two sutures were used to close the incision. After a few resting hours, animals were returned to the colony room. Animals were allowed a two week recovery period prior to initiating behavioral tests.

β-Estradiol replacement

Our preliminary results indicate that castrated male rats do not significantly differ from sham male rats in the m-CPP discrimination stimulus. Thus, hormone replacement was conducted only to OVX female rats which had already acquired the m-CPP discrimination task. Ovariectomized female rats were subcutaneously implanted with β-estradiol pellets (2.5 mg for 21 days release). After a two day recovery period, they were used for behavioral tests.

Drug

M-CPP was purchased from Sigma Chemical Co (St Louis, MO) and was freshly dissolved in the saline solution (0.9 %). β-estradiol (2.5 mg per pellet, 21 day release) was purchased from Innovative Research of America (Sarasota, FL).

Number of animals

The number of rats used for behavioral tests (experiment 3 to 6) varied depending upon the number of rats which met a test criterion at the time of

testing with the exception of experiments 1 and 2. Experiments 1 and 2 did not

test the discriminative ability of animals. In addition, animals which failed to emit

a lever-press response (one FR10) within a given test time (10 min) were not

used in data analysis. The number of animals in each experiment was indicated

in the following table.

Table 2. Number of animals used in each experiment

Experiment	1	2	3	4	5	6
Sham-operated male		11	12	14	14	
Castrated male		8	10	10	10	
Sham-operated female		9	9	11	11	11
OVX	22	10	10	10	10	
OVX + Estradiol		12	12	11	11	
Gonadally intact male	20					
Gonadally intact female	20					

Experimental procedure

For all behavioral experiments with the exception of experiment 1, five

experimental groups were tested: 1) sham-operated males, 2) gonadectomized

males, 3) sham-operated females, 4) OVX females, and 5) estradiol-replaced

OVX females. For an experiment 1, three experimental groups were tested: 1) gonadally intact male, 2) gonadally intact female, and 3) OVX rats.

Experiment 1 was designed to determine if male and female rats differ in behavioral impairment induced by m-CPP. Gonadally intact male and female rats and OVX rats were injected with m-CPP (1.2 mg/kg, IP) or saline 15 min prior to placing into the boxes. They were then tested for any lever press under a FR10 schedule. The initial session length for this experiment was two hours for both saline and m-CPP sessions. After 20 sessions, rats able to emit at least 10 lever-presses on either a drug or a saline lever were preselected. The percentage (%) of preselected rats out of total rats in each group was calculated.

Experiment 2 was designed to determine if the five experimental groups differ in initiation latency for a lever-press response under the m-CPP stimulus and if an ovarian steroid, β-estradiol contributes to this difference. Rats were injected with saline and m-CPP (0.25, 0.5, 1.0, and 1.2 mg/kg, IP) in a cumulative dosing method. 15 min later, they were tested for the initiation latency at each dose of m-CPP. Initiation latency is defined as the time (min) between a session-start and completion of 10 lever-presses for food reward.

Experiment 3 was designed to determine if the five experimental groups differ in the m-CPP discrimination and if an ovarian steroid, β-estradiol contributes to this

difference. Rats which met the criterion for the m-CPP discrimination stimulus were tested for a dose-response effect of m-CPP. Thus, they were injected with saline and m-CPP (0.25, 0.5, 1.0, and 1.2 mg/kg, IP) in a cumulative dosing method. 15 min later, they were placed into the box for their lever-selection at each dose of m-CPP. ED50 for a dose-response curve was calculated in each experimental group.

Experiment 4 was designed to determine if the five experimental groups differ in development of an endogenous m-CPP-like stimulus during acute ethanol withdrawal and if an ovarian steroid, β-estradiol contributes to this difference. Animals received chronic ethanol diet for 10 days. 12 hours after termination of ethanol diet, they were given saline injection and tested for m-CPP-lever selection. The percentage of rats which selected a drug-lever over a saline-lever out of total rats tested was calculated in each experimental group.

Experiment 5 was designed to determine if five experimental groups differ in the m-CPP discrimination stimulus during PEW. Animals received chronic ethanol diet for 10 days. 36 hours after termination of ethanol diet, animals were injected with saline and m-CPP (0.25, 0.5, 1.0, and 1.2 mg/kg, IP) in a cumulative dosing method and tested for m-CPP-lever selection at each dose of m-CPP. ED50 for a dose-response curve was calculated and compared with ED50 before chronic ethanol diet (in Experiment 3).

Experiment 6 was designed to determine if relationship exists between estrogen concentrations and occurrence of the m-CPP discriminative stimulus in sham and OVX female rats. β-Estradiol concentrations in sham female rats (day 1 to day 5) were cited from the data in Chapter 1 (Figure 7b). The m-CPP discriminative stimulus was measured in sham female rats after injection of either m-CPP (1.2 mg/kg) or saline. It was determine if the m-CPP discriminative stimulus shows a cyclic pattern as did estradiol concentrations in sham female rats.

Data analysis

For the purposes of analysis, experimental groups defined by the variable gender included both gender, surgical condition, and hormonal treatment (5 groups). The data for selection of the m-CPP lever were expressed as percentages (%) which were obtained as follows: ' % = 100 x [number of rats selecting the mCPP-lever / a total number of rats that completed the test] '. The test in experiment 1 was conducted only once and the data were analyzed by non-parametric statistics, Kruskal-Wallis. Data that were continuous in nature (initiation latency in experiment 2) were subjected directly to two-way analyses of variance (gender X dose). Dose-response tests (experiment 3), the mCPP-lever selection tests during AEW (experiment 4) or PEW (experiment 5) were repeated three times to obtain the means and standard error of means (SEM). Two-way analysis of variance (gender X dose) was used for data analysis in

experiments 2 and 3. One-way analysis of variance (gender) was used to analyze data obtained from experiment 4. For calculation of the ED50 for the m-CPP discrimination stimulus, data were plotted as a logit m-CPP lever selection versus a log dose of m-CPP (experiment 5). The gender difference in ED50 was analyzed by two-way analysis of variance (gender X ethanol condition in experiment 5). The significance level was set as $P<0.05$.

Results

After 60 training sessions, animals acquired the m-CPP discrimination task; they selected a m-CPP lever after m-CPP injection (1.2 mg/kg, IP) and a saline lever after saline injection.

Experiment 1: Gender difference in impaired operant response initiation after m-CPP injection. After m-CPP treatment (1.2 mg/kg), fewer sham female rats (40 %) were able to emit lever-press responses on either a saline or a m-CPP lever than intact male rats (80 %) or OVX rats (59 %) [Kruskal-Wallis test statistic = 6.55, P=0.038, df=2].
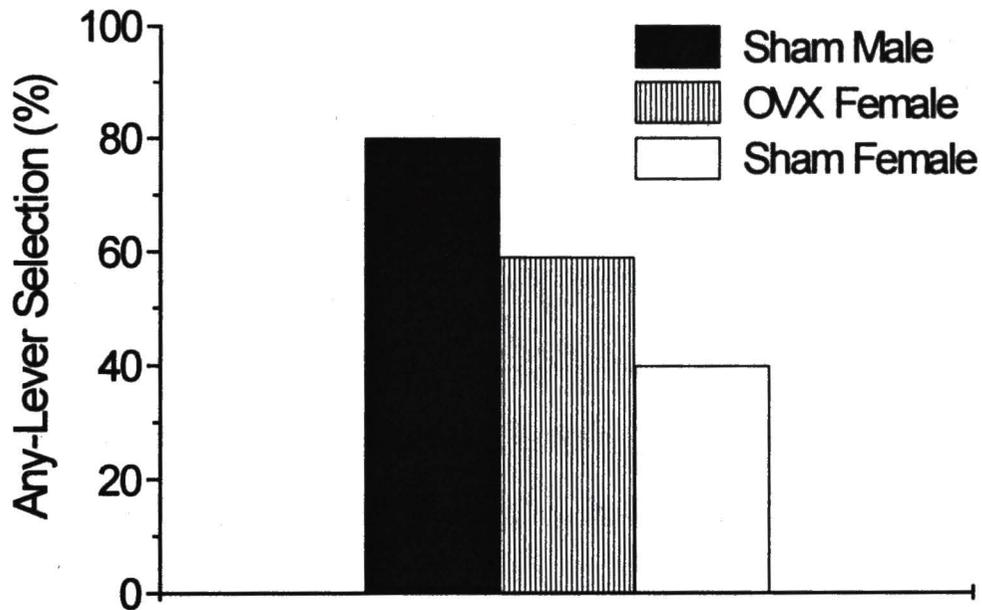
Figure 1. Demonstration of a gender difference in impaired operant response initiation under the m-CPP stimulus effect (1.2 mg/kg). Y axis indicates the percentages of rats which initiated any lever press after m-CPP injection. Kruskal-wallis test statistic =6.56. df=2, P= 0.038 by three experimental groups.

Experiment 2: Gender difference in initiation latency of any lever-press response after m-CPP injection (Figure 2): Administration of m-CPP (0.25, 0.5, 1.0 mg/kg, IP) resulted in significantly delayed initiation latency of any lever-press response. This effect was different in five experimental groups [$F(4,46)=22.5$, $P=0.009$] and greater as the dose of m-CPP increased [$F(3, 138)=5.2, P=0.002$]. When a pair of groups was compared, initiation latency of a sham female group was higher than that of a sham male group [$F(1,18)=8.02, P=0.01$] or an OVX female group [$F(1,17)=5.1, P=0.038$]. Latency of a β-estradiol replaced OVX group was higher than that of an OVX group [$F(1,20)=5.3, P=0.033$]. No significant difference was found between sham male and castrated male groups or sham female and estradiol-replaced female groups.
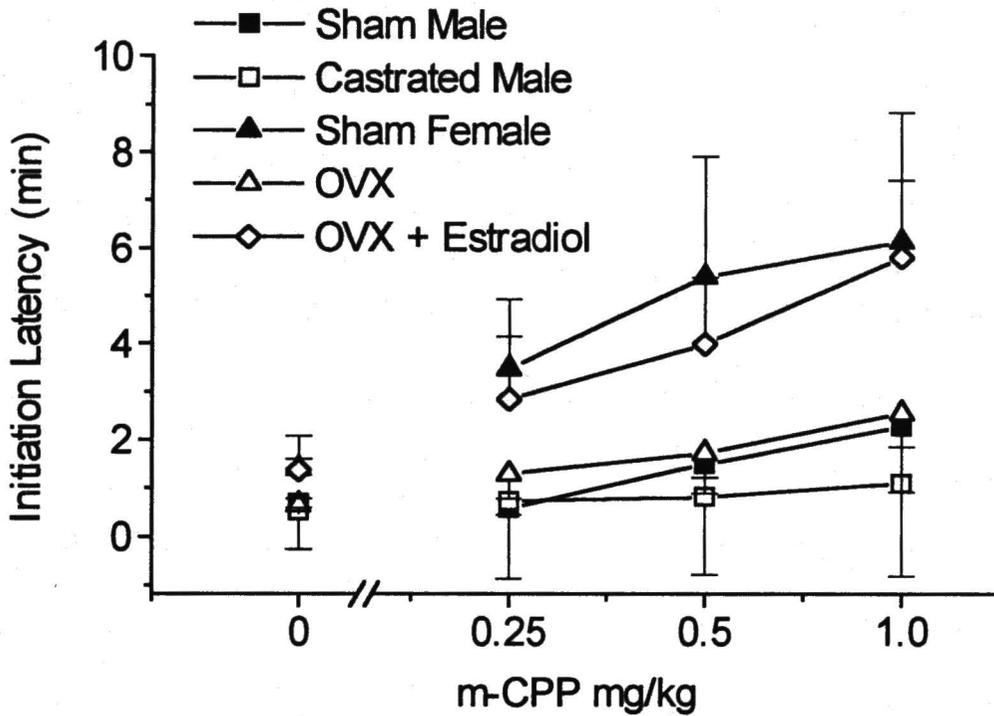
Figure 2. Demonstration of a gender difference in initiation latency of a lever-press response after m-CPP injection. Data points are mean values, and error bars are SEM. $F_{(4,46)}=22.5$, $P=0.009$ by five experimental groups. $F_{(3,138)}=5.2$, $P=0.002$ by dose.

Experiment 3: Gender difference in a dose-response effect of the m-CPP discrimination stimulus (Figure 3). As the dose of m-CPP increased, m-CPP lever selection increased in all groups of rats [F(3, 28)=254, P< 0.001]. Five experimental groups differed in the m-CPP discrimination stimulus [F(4,14)=5.2, P=0.009]. When a pair of groups was compared, the mCPP-lever selection (%) was lower in a sham female group than a sham male group [F(1,8)=5.3, P=0.05] or OVX group [F(1,6)=9, P=0.024]. In addition, the mCPP-lever selection (%) was lower in a β-estradiol replaced OVX group than an OVX group [F(1,4)=32.6, P=0.005]. No significant difference was found between male groups (sham and castrated) or a sham female and an estradiol-replaced female groups.
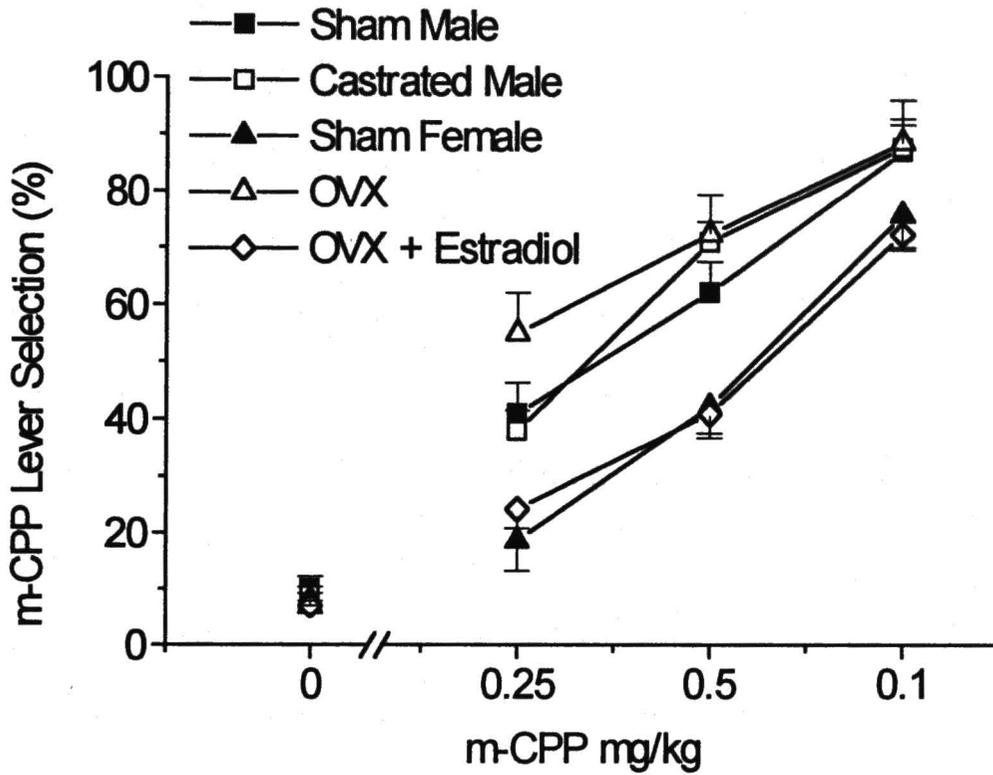
Figure 3. Demonstration of a gender difference in the m-CPP discrimination stimulus. Data points are mean values obtained from repeating a test three times and error bars are SEM. F(4,14)=5.2, P=0.009 by five experimental groups. F(3, 28)=254, P< 0.001 by dose.

Experiment 4: *Gender difference in an endogenous m-CPP-like stimulus induced by AEW (Figure 4).* Twelve hours after termination of chronic ethanol diet, animals injected with saline selected a m-CPP-lever over a saline-lever. Five experimental groups differed in the m-CPP lever selection during AEW [$F(4,28)=8.4$, $P<0.001$]. When a pair of experimental groups were compared, the m-CPP lever selection (%) was lower in a sham female group than a sham male group [$F(1,4)=16.6$, $P=0.015$] or an OVX group [$F(1,16)=16.2$, $P=0.001$]. In addition, the m-CPP lever selection (%) was also lower in a β-estradiol replaced OVX than an OVX group [$F(1,4)=8.7$, $P=0.042$]. No significant difference in the m-CPP lever selection was found between male groups (sham and castrated) or sham female and estradiol-replaced female groups.
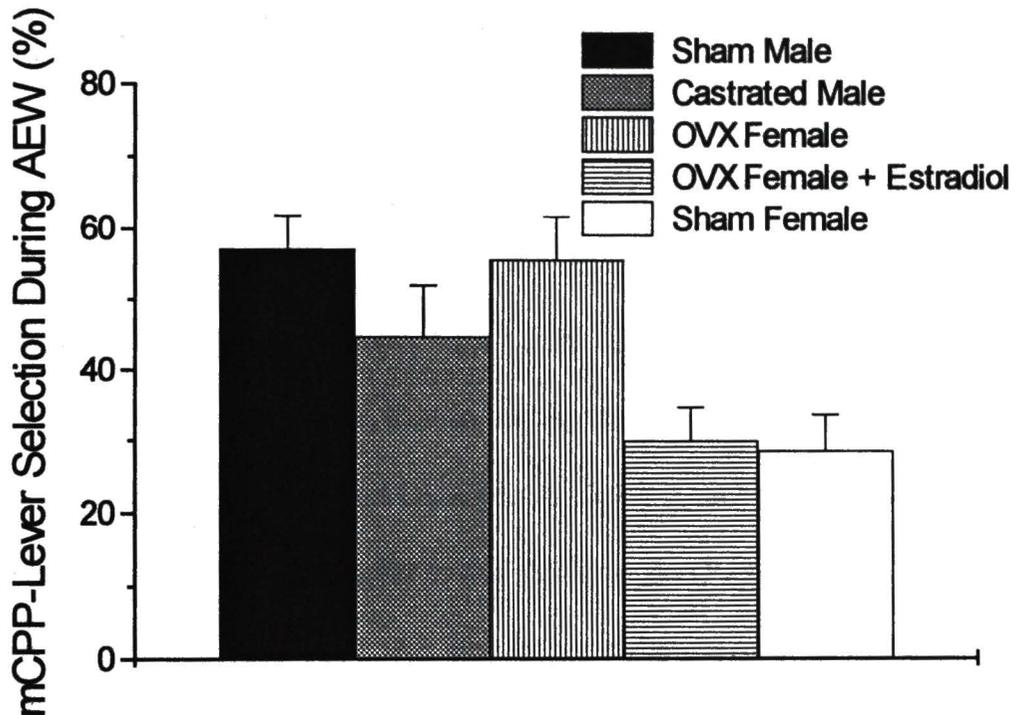
Figure 4. Demonstration of a gender difference in an endogenous m-CPP-like stimulus occurring during AEW. Data points are mean values obtained from repeating a test three times and error bars are SEM. $F(4,28)=8.4$, $P<0.001$ by five experimental groups.

Experiment 5: Gender difference in the ED50 for the m-CPP discrimination stimulus during PEW (Figure 5). Figure 5 illustrates that ED50 for the m-CPP discrimination stimulus during PEW were decreased as compared to those prior to chronic ethanol diet [$F(1, 10)=22.6$, $P=0.001$]. ED50s in five experimental groups differed during PEW [$F(4, 10)=8.1$, $P=0.004$]. When a pair of experimental groups was compared for ED50 during PEW, the ED50 in a sham female group was higher than that in a sham male group [$F(1,4)=8.5$, $P=0.04$]. ED50 in a β-estradiol replaced OVX group (0.2 ± 0.002 mg/kg) was also significantly higher as compared to the ED50 in an OVX group (0.075 ± 0.002 mg/kg) [$F(1,4)=5.3$, $P=0.007$]. No significant differences in ED50 were found between male groups (sham and castrated) or sham female and estradiol-replaced OVX groups during PEW.
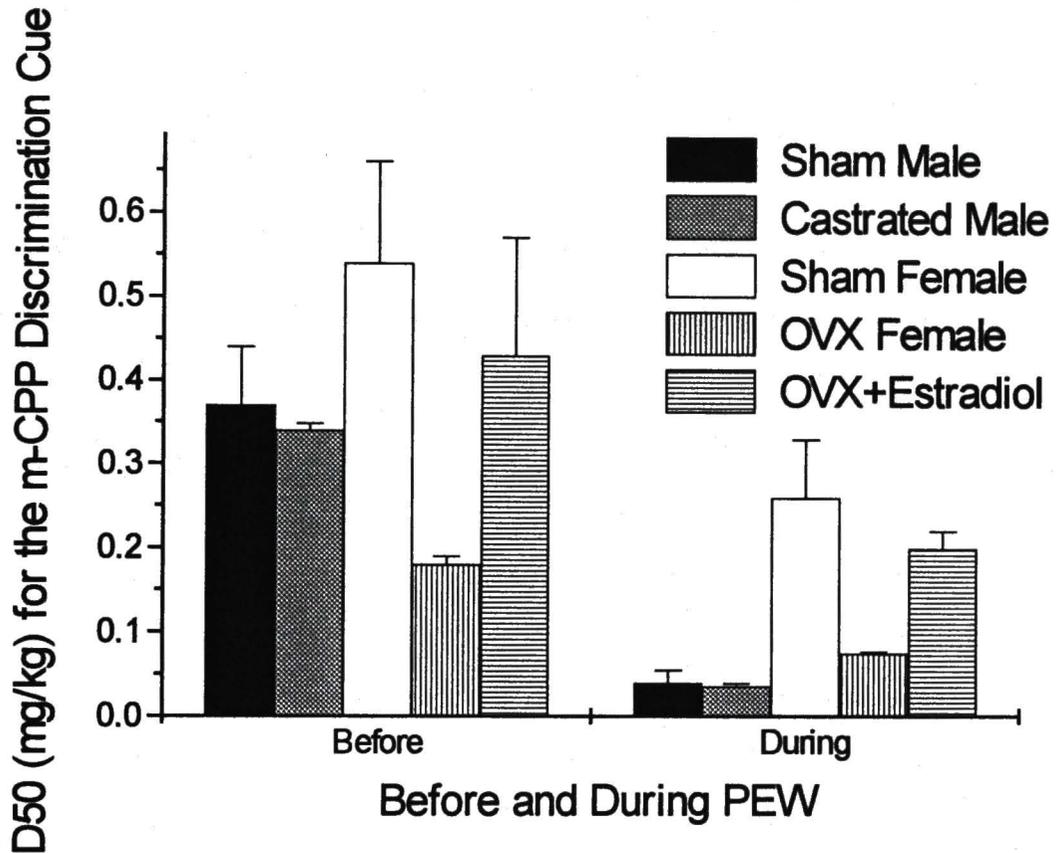
Figure 5. Demonstration of gender differences in ED50 for the m-CPP discrimination stimulus before and during EW. Data points are mean values obtained from repeating a test three times and error bars are SEM. Left panel; ED50 before chronic ethanol diet. Right panel; ED50 during PEW. $F_{(4, 10)}=8.1$, $P=0.004$ by five experimental groups. $F_{(1, 10)}=22.6$, $P=0.001$ by ethanol conditions (before and during EW).

Experiment 6: *The influence of β-estradiol concentrations on the m-CPP*

discrimination stimulus. Figure 7b in 'Chapter 1' illustrates that sham female rats

undergo a 4 day cycle in serum β-estradiol concentrations which peak on day 2

as compared to day 1, 3, and 4 [$F(3,16)=13$, $P<0.001$]. The serum concentration

on day 2 in sham female rats was also significantly higher than that in OVX rats

[$F(1,13)=16.5$, $P=0.001$]. Despite the cyclic fluctuation in estradiol

concentrations, performance of the m-CPP discrimination task was stable as

seen in Figure 6.
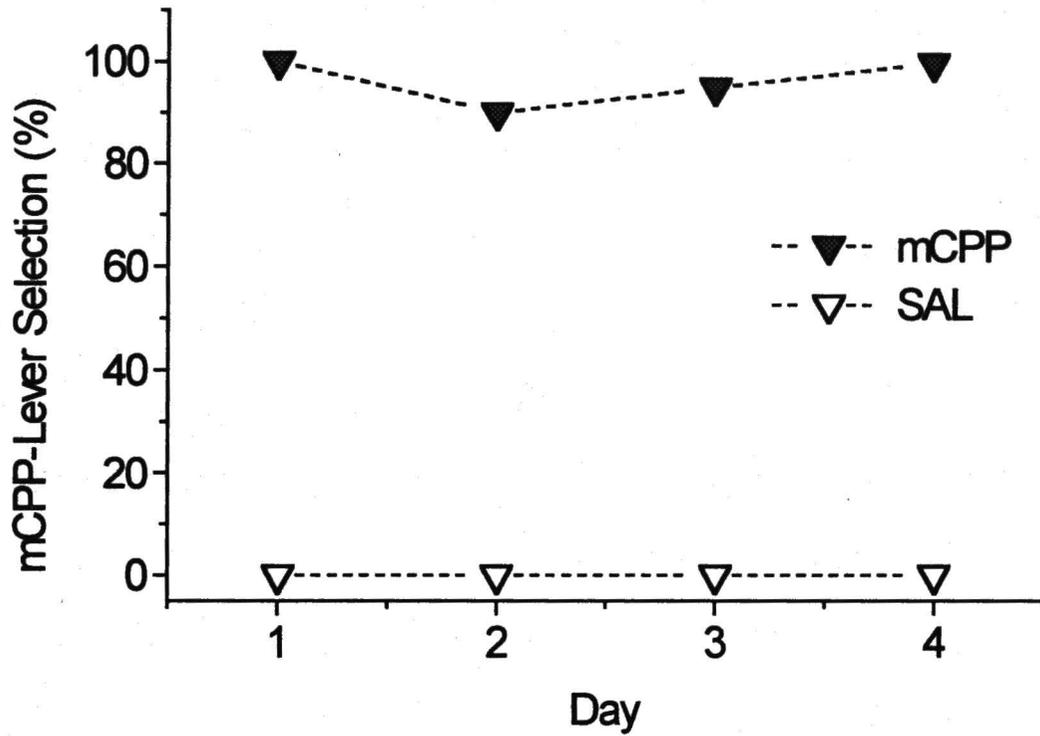
Figure 6. Demonstration of the stable performance of the m-CPP discrimination task for 4 consecutive days in sham female rats.

Discussion

The present data demonstrate that male and female rats differ in response to the two types of anxiogenesis produced by m-CPP, impaired behavioral initiation and the discriminative stimulus. Female rats are more susceptible to impaired behavioral initiation than males. In addition, fewer female than male rats select the mCPP lever at a given dose of drug. An ovarian steroid, β-estradiol, may be responsible for at least part of the male-female difference in anxiogenic response. Removal of ovarian estrogen by OVX produces a behavioral pattern similar to that of male rats and β-estradiol replacement reinstates the anxiogenic response of OVX rats toward that of sham-operated female rats. These findings suggest that the gender difference in m-CPP-mediated anxiety varies depending upon the types of anxiety.

Behavioral and pharmacological effects of m-CPP associated with anxiogenic stimuli have been examined in previous studies. In an elevated plus maze paradigm, administration of m-CPP reduced the open arm activity of male rats and EW potentiated this effect (Rezazadeh et al., 1993). In a two-lever choice task, m-CPP produced discriminative (Wallis and Lal, 1997; Winter and Rabin, 1993; Callahan and Cunningham, 1994) and rate suppressing effects in male rats (Winter and Rabin, 1993; Callahan and Cunningham, 1994). However, the present study is the first to examine a gender difference in the

anxiogenic properties of m-CPP using a discrimination paradigm, before and during EW. The impaired behavioral initiation observed in the present study is similar to the reported suppression of locomotor activity induced by acute m-CPP administration to adult male rats (Ulrichsen et al., 1992). In contrast, Haleem (1993) found no gender difference in locomotor activity of rats injected with m-CPP, as measured by means of cage crossing scores. It is an important question whether the interrupted initiation of an operant response (present result) is merely due to motoric disruption that resembles the suppressed locomotion seen in previous studies or to attention deficits. The impaired initiation of response is not due to the lack of operant learning. This is confirmed by our behavioral observations that: 1) Before exposition to the m-CPP stimulus, rats acquired a lever-press response for food reward; and 2) Rats injected with m-CPP sporadically exhibited normal movement inside the operant box. Since m-CPP causes no significant changes in cognitive performance (Silverstone et al., 1994), the impaired initiation is unlikely to result from an attention deficit. Instead, these behavioral disruptions may reflect one feature of anxiogenic phenomena induced by m-CPP. In support of this view, anxiety patients frequently report retarded initiation of activities. Interestingly enough, more female patients report such a symptom of anxiety than male patients (Katz et al., 1993). This is consistent with our data that under the m-CPP stimulus, more sham female or β-estradiol replaced OVX rats failed or delayed to begin a lever-press response than sham and castrated male rats or

OVX rats (Figure 1 and 2). More female rats injected with a given dose of l-tryptophan (a serotonin precursor) exhibit the serotonergic behavioral syndrome (i.e., limb abduction, forepaw treading, head weaving, or tremor) than male rats (Dickinson and Curzon, 1986: Fischette et al., 1984). The concentration of 5-HT and its metabolite, 5-hydroxytryptamine-indoleaceticacid (5-HIAA) are higher in whole brain of female rats than in male rats (Kato, 1960; Carlsson and Carlsson, 1988a). This may result from a higher concentration of 5-HT synthesizing enzymes, tryptophan hydroxylase (Carlsson and Carlsson, 1988b) and a subsequent higher production of 5-HT (Rosecrans, 1970; Vaccari et al., 1977). At a behavioral level, inescapable foot-shock increased the concentration of 5-HIAA in the frontal cortex, hypothalamus, amygdala, striatum, mesencephalon, and the medulla-pons area to a greater extent in female rats than in male rats (Heinsbroek et al., 1990). The data presented in the current study is consistent with higher endogenous serotonin concentrations in female than male rats.

Another possible mechanism underlying a gender difference in anxiogenic properties is inferred from the interaction between stress and the hypothalamic-pituitary-adrenal (HPA) system. m-CPP activates the HPA axis and subsequently results in the release of corticosterone to a greater extent in female rats as compared to male rats (Haleem, 1993). That study establishes that female rats respond more robustly than male rats to the stimulus effect of m-CPP at the level of the HPA axis. Thus, it is plausible to speculate that a higher

activation of the HPA axis induced by m-CPP may also contribute to the gender difference observed in our study.

In contrast to the gender difference in impaired initiation, sham female or β-estradiol replaced OVX rats exhibited a lower sensitivity to the m-CPP discrimination stimulus than male rats or OVX rats (Figure 3 and 4). Based on previous studies, we expected to observe a higher sensitivity of female rats than male rats to the m-CPP discrimination stimulus. One explanation for our finding could be that female rats particularly sensitive to the operant inhibition effects of mCPP were eliminated during training for this experiment due to their failure to initiate the operant response. Consequently, the pre-selected female group may consist of rats that are insensitive to the m-CPP stimulus. These results indicate that two components of anxiety; impaired behavioral initiation and the discrimination cue may not be identical in their underlying mechanism.

m-CPP acts at different subtypes of 5-HT receptors such as 5-HT1b, 5-HT2a, and 5-HT2c. 5-HT2c agonist, MK212, 5-HT1b agonist, RU24969, and 5-HT1b/2c agonist, TFMPP, all suppressed the rate of a lever-press response (Callahan and Cunningham, 1994). A 5-HT antagonist with high affinity for 5-HT2c receptors prevented hypo-locomotion induced by m-CPP in rats. On the other hand, a 5-HT2c agonist, MK212 showed the highest substitution for the m-CPP discrimination cue as compared to other 5-HT receptor subtype ligands

(Callahan and Cunningham, 1994). Previous studies are consistent with the involvement of a wide range of serotonergic receptor subtypes in impaired behavioral initiation and the m-CPP discriminative stimulus, however, 5HT2 receptor agonists appear to show the greatest substitution for the m-CPP stimulus.

Similar gender dependent differences were found in the m-CPP discrimination stimulus before and during EW. Therefore, there may be a common mechanism for the stimulus effects of m-CPP and EW. Inhibition of the GABA-A system exerts the male-typical features of aggression. This is shown in a study where electrical brain stimulation of the periaqueductal gray produces aggressive behavior. This behavior is potentiated by a GABA-A antagonist pentylenetetrazol (PTZ), but attenuated by a GABA/BZD agonist diazepam (Jung, 1994). During EW, the anxiety-like interoceptive stimulus shares properties with both mCPP (Rezazadeh et al., 1993) and PTZ (Lal et al., 1988). While increased serotonergic activity may not be directly responsible for the aggressive nature of anxiety in males during ethanol withdrawal, it may contribute to the difference in aggression between males and females. A potential mechanism for a serotonergic role in the gender difference of aggression is supported by reports that: 1) m-CPP mediates dynorphin release from a peripheral organ (Majeed et al., 1987); 2) Dynorphin produces aversive or anxiety-like properties (Pfeiffer et al., 1986); and 3) Estrogen may decrease

the release of dynorphin (Wagner et al., 1994). Estrogen blockade of m-CPP stimulated dynorphin release may contribute to the low sensitivity of sham female or β-estradiol replaced OVX rats to the discrimination stimulus of m-CPP as compared to males.

Previous studies (Dickinson and Curzon, 1985) indicate that the intensity of the 5-HT behavioral syndrome is not significantly related to the stage of the estrus cycle (Fischette et al., 1984). Our previous data (Figure 6b in Chapter 1) show that sham female rats undergo a 4 day cyclic variation in estrogen concentration with a surge on day 2 whereas their operant performance for the m-CPP discrimination did not show a cyclic pattern (Figure 6). Nevertheless, we have shown that the anxiogenic response of β-estradiol-replaced OVX rats resembles that of sham female rats. Presumably, estrogen may modulate the anxiogenic stimuli of m-CPP by acting as a trophic factor not by acutely modulating serotonergic response.

The role of male hormones in anxiety-related behavior is less well defined than that of female hormones. Male hormones appear to influence anxiety-associated behaviors during the developmental period rather than during adulthood. Castration of the adult male rats failed to alter the threshold for the PTZ-induced seizure (Kokka et al., 1992). Anxiogenic behavior of adult male rats was not affected by castration on the elevated plus maze (Zimmerberg and

Farly, 1993). However, when newborn castrated male rats were tested during adulthood on the elevated plus maze, they showed a significantly higher open arm activity than sham-operated male rats, indicating that they are less anxious (Lucion et al., 1996). When one day old chicks were treated with testosterone oenanthate, their attack scores were higher than vehicle treated control chicks in adulthood. In contrast, there was no difference in the open arm activity on the elevated plus maze before and after gonadectomy in adult male rats (Astiningsih et al., 1996). In the same elevated plus maze model, gonadectomy did not alter the response of male rats whereas it enhanced the anxiogenic response of female rats (Zimmerberg and Farley, 1993). Thus, our results showing no significant difference in anxiogenic response of male rats before and after gonadectomy further support the notion that male gonadal hormones exert less influence on anxiogenic stimuli during adulthood.

Given the same dose of ethanol, female rats have a higher BEC than male rats (Sutker et al., 1983; refer Figure 5 in Chapter 1). These data exclude the possibility that the lower intensity of EW-induced anxiogenic stimuli in sham female rats is due to a lower BEC in female rats during the development of dependence than in male rats. Our previous data also indicate that males and females did not differ significantly in the clearance rate for ethanol either before or after exposure to ethanol diet. These data indicate that a difference in the

metabolic rate of ethanol is not responsible for the difference in anxiogenic response between male and female rats.

In summary, estrogen appears to play a protective role in females, reducing the discriminative properties of serotonergic stimuli whether they result from an exogenously administered drug or ethanol withdrawal. In contrast, impairment of behavioral initiation is facilitated by estrogen, clearly indicating that the underlying mechanism for producing the discriminative properties of the serotonergic stimuli may differ in nature from that producing the behavioral impairment.

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DISCUSSION

The existence of sex differences in the expression of anxiety has long been recognized, but the present studies provide the following new data: 1) The interoceptive discriminative stimuli (IDS) of PTZ and m-CPP are sexually dimorphic: fewer female rats perceive the IDS of PTZ or m-CPP than male rats. 2) Unlike PTZ, m-CPP impairs behavioral initiation in a sex dependent manner: more female rats are impaired in behavioral initiation than male rats. 3) Gonadectomy or hormone replacement alters anxiogenic response in a manner such that the response of OVX rats resembles that of male rats and estrogen replacement reinstates a female-like response in OVX rats. In contrast, castration does not alter either the stimulus effect of PTZ or that of m-CPP. 4) EW produces a sexually dimorphic endogenous PTZ and/or m-CPP like stimulus: fewer sham-operated female and estrogen-replaced OVX rats develop a PTZ or a m-CPP like stimulus than sham-operated male, castrated, or OVX rats during AEW. 5) Estrogen modulates the anxiogenic stimulus of PTZ or m-CPP through a mechanism independent of the stage of the estrus cycle or BEC.

The present results support the idea that the GABAergic and serotonergic systems are involved in a sexually dimorphic anxiogenic response through

estrogen-mediated factors. This indicates that the central nervous system neurotransmitters, GABA and serotonin, interact with hormones of gonadal origin (i.e., estrogen) in regulation of anxiety. Brain-body communication is also seen in the activity of the HPA axis in response to stressful stimuli. Upon stress, the HPA axis is activated and corticosterone is released. Corticosterone acts acutely as an anxiolytic drug. Acute treatment of corticosterone increases open arm activity on the elevated plus maze (Andreatini and Leite, 1994). A gender difference is observed in response of the HPA axis to anxiety such that the release of corticosterone is greater in female rats than in male rats (Pericic and Pivac, 1996). Thus, one can not exclude the possibility that PTZ or m-CPP stimulates the HPA axis which subsequently releases corticosterone to a higher extent in female rats than in male rats. In fact, Haleem et al. (1993) demonstrated that m-CPP administration increased plasma corticosterone and this effect of m-CPP was higher in female rats than in male rats. If more corticosterone is released in female rats than in male rats following administration of PTZ or m-CPP, it may more effectively counteract the drug induced anxiogenic stimuli in female rats as compared to male rats. Ethanol is also known to activate the HPA axis. Both ethanol administration and EW increase plasma corticosterone concentration (Bano et al., 1996). Since the HPA axis responds to stressful stimuli more actively in female rats, their corticosterone release induced by EW could be higher compared to male rats. Applying this logic, an anxiolytic property of corticosterone may contribute to

lower development of an endogenous PTZ or m-CPP like stimulus in sham-operated and estrogen-replaced female rats than male or OVX rats during EW (present data).

Fewer female Rats respond to the GABA-A Antagonistic Stimuli Than Male Rats in Part Due to Estrogen-Mediated Factors

The present results show that fewer sham and estradiol-replaced female rats respond on the PTZ-lever than sham male, castrated, and OVX rats at a given dose of PTZ. These findings are consistent with previous studies. Behaviorally, female rats develop PTZ-induced seizure at a higher dose of PTZ than male rats, and ovariectomy abolishes this difference (Kokka et al., 1992). At the neurotransmitter level, female rats have a significantly higher number of GABA/BZD receptors in limbic areas as compared to male rats (Farabollini et al., 1996). In addition, gonadally intact female rats have a higher GABA-stimulated chloride conductance than OVX rats (Bitran et al., 1991). Thus, present findings strengthen the notion that fewer female rats experience GABA-A antagonistic anxiogenic stimuli in part due to higher GABA-A agonistic activity in female rats as compared to male rats.

Gender Difference in The Stimulus Effect of m-CPP Varies Depending Upon the Anxiogenic Nature of m-CPP

The present results obtained from the m-CPP paradigm demonstrate that activation of the 5-HT1b/2 receptor system serves as an effective discriminative stimulus in female as well as male rats (Winter and Rabin, 1993; Callahan and Cunningham, 1994; Wallis and Lal, 1997). Unlike the stimulus effect of PTZ, the anxiogenic nature of m-CPP is also expressed as impaired behavioral initiation. After m-CPP injection, some animals fail to initiate or delay initiation of a lever-press response. This effect predominantly occurs in sham and hormone-replaced female rats (present results). In contrast, fewer sham and hormone-replaced female rats respond on the m-CPP lever than male rats or OVX rats at a given dose of m-CPP. This indicates that a gender difference in anxiogenic response depends on the type of anxiogenic stimulus applied and estrogen appears to play a role in producing this difference:

Previous studies report that the production of serotonin is higher in female rats than in male rats as measured by the concentration of 5-HT, its precursor (tryptophan), and its metabolites (5-HIAA) (Giulian et al., 1973; Haleem et al., 1990). In addition, female rats display the serotonin syndrome at a lower dose of serotonin precursor, l-tryptophan than male rats (Fischette et al., 1984; Biegon et al, 1979). Restraint stress increases the binding of [3H] 8-OH-DPAT (5-HT1A agonist) at 5-HT1A receptors in the dorsal hippocampus to a greater extent in female rats than in male rats (Mendelson and McEwen, 1991). Increasing brain serotonin levels by administration of the precursor l-tryptophan

results in suppression of operant response (Iversen, 1984). Based on these previous reports, I expected that more female rats would respond on the m-CPP lever or would be impaired in initiation of a lever-press response than male rats at a given dose of m-CPP. This hypothesis turned out to be true for the effect of mCPP on behavioral initiation, but not for its IDS. Katz et al. (1993) reported that more female anxiety patients are impaired in initiation of activity, while more male patients display a hostility feature of anxiety. Castration does not alter either IDS or impaired initiation as compared to sham male rats. The present results and those of previous authors are consistent with a higher serotonin turnover in females than males, resulting in the impaired initiation of activity.

Gender Difference in EW-Induced Anxiogenic Stimuli

A serotonergic component of EW-induced anxiety is shown in a study where administration of a 5H2 antagonist, mianserin prevented the EW induced anxiogenic behavior in the elevated plus maze test (Lal et al., 1993). In the same model, m-CPP given during EW potentiated anxiogenic behavior as compared to saline-injected male rats (Rezazadeh et al., 1993). These findings indicate that the serotonergic receptor system is involved in the stimulus effect of EW. Many investigators have demonstrated that EW has GABA-A antagonistic features. Our results confirm that there are both GABA-A antagonistic and serotonergic agonistic properties of EW in both males and females. Rats injected with saline select the PTZ or mCPP lever over a saline lever during EW

(present data), indicating that EW produces an endogenous PTZ or m-CPP-like stimulus. However, a gender difference was observed in the occurrence of an endogenous m-CPP or PTZ-like stimulus during EW. Gonadectomy and hormone replacement studies clearly indicate a role of estrogen in maintaining the gender difference in the development of an endogenous anxiogenic IDS during EW.

Steroidal Modulation of Anxiety-Like Stimuli: Estrogen Plays a Protective Role Against Anxiogenic Stimuli

The results of our studies indicate that estrogen plays a protective role against the anxiogenic stimuli produced by either PTZ or m-CPP. The next question is whether the role played by estrogen is direct or indirect. There was no relationship between estradiol concentrations over 4 consecutive days of the estrus cycle and the occurrence of an IDS in response to PTZ or m-CPP injections in sham-operated female rats. Similarly, there was no change in forced swimming-induced immobility at different stages of the estrous cycle (Alonso et al., 1991). At the neuronal level, the estrus cycle did not affect GABAergic neuronal activity as measured by the rate of GABA accumulation in steroid sensitive brain areas such as the medial preoptic area of the hypothalamus (Grattan et al., 1996). In addition, Loscher et al. (1992) reported no changes in the concentration of GABA, glutamate, aspartate, and glycine during any stage of the estrus cycle of female rats. These results support the

idea that estrogen modulates anxiety through a mechanism independent of the estrus cycle.

Sex steroids exert a large part of their action through interaction with gene expression resulting in regulation of a protein synthesis (Chan and O'Malley, 1976). Knowing that GABA and serotonin receptors are membrane bound proteins, it is conceivable that estrogen is involved in the synthesis or degradation of the GABA and serotonin receptors. Estrogen may act as a transcriptional factor for the synthesis of such receptor proteins or other secondary factors involved. Indeed, estrogen has been reported to induce P receptors in the hypothalamus (Brown et al., 1987). In addition, P receptor mRNA expression in venous vessels of female rats decreased after ovariectomy (Knauthe et al., 1996). This effect was reversed by chronic treatment with estradiol. THP is a metabolite of a female gonadal hormone progesterone (P) and THDOC is structurally related to an adrenal hormone, corticosterone (Majewska, 1986). P and its metabolite THP are potent anxiolytics acting as GABA-A agonists and may serve as secondary factors initiated by estrogen for anxiolytic activity.

Other actions of estrogen may involve other receptor systems. Activation of the kappa opioid receptor system produces an aversive stimulus that may be antagonized by estrogen (Wagner et al., 1994). Kappa opioids reduce the firing

rate of dopaminergic neurons, an effect that is reduced by estrogen. In addition the author suggested that estrogen may decrease the release of an endogenous kappa agonist, dynorphin. Because the release of dynorphin would be less in females, they may show less anxiogenic response to a given stimulus. m-CPP mediates dynorphin release from duodenum (Majeed et al., 1987). If this effect of m-CPP also occurs in the brain, m-CPP induced dynorphin release may exacerbate anxiety or the aversiveness of EW. In fact, it has been documented that following m-CPP administration, a craving for alcohol increased in abstinent alcoholics (Naranjo and Bremner, 1994). This increased craving may partially be attributed to exacerbated anxiety or aversiveness of EW induced by m-CPP. With this scenario, the anxiogenic IDS of m-CPP could represent the aversive nature of anxiety which in part results from activation of dynorphin release. A given dose of ethanol, increases the release of dopamine in the nucleus accumbens of female rats to a greater extent than of male rats (Blanchard and Glick, 1995). If the mesolimbic dopaminergic system is more active in female rats than in male rats, in part due to an estrogen-mediated mechanism, it may more effectively counteract the aversive component of anxiogenic stimuli in females as compared to male or OVX rats.

Another indirect action of estrogen to modulate anxiety may occur through the HPA axis. For instance, animals exposed to ether stress showed an increase in plasma ACTH and estradiol concentrations (Lesniewska et al.,

1990). This response was markedly higher in female rats than in male rats. OVX female rats responded to ether stress as intact male rats did. After estradiol replacement to OVX females, the pattern of plasma ACTH and estradiol concentrations was similar to that of intact female rats. On the other hand, after castration and testosterone replacement plasma ACTH and estradiol responses to ether stress were similar to those observed in intact male rats. These results suggest that estrogen induces activation of the HPA axis whereas testosterone has little effect on its' activity. In addition, stressful stimuli result in an increase of the GABA/BZD receptors and circulating corticosterone concentrations (Wilson and Biscardi, 1994). These phenomena were more prominent in female rats than in male rats. Moreover, there was a positive correlation between the concentration of circulating corticosterone and the GABA/ BZD receptors in female rats, but not in male rats (Wilson and Biscardi, 1994). Presumably, estrogen activates the HPA axis increasing basal levels of corticosterone, which could act directly as an anxiolytic or indirectly through the GABA/BZD system.

Ethanol does not have its own specific receptors and affects numerous organs and tissues. The GABAergic system may be more affected by ethanol than the serotonergic system. Indeed, a role of the GABAergic system has been demonstrated for the actions of neurosteroids, defined as steroids originated from brain (Baulieu et al., 1987). There are two types of neurosteroids, GABA-A agonistic and GABA-A antagonistic (reviewed by Majewska, 1991). GABA-A

agonistic neurosteroids are tetrahydroprogesterone (THP) and tetrahydro-deoxycortico-sterone (THDOC). GABA-A antagonistic steroids are pregnenolone sulfate and dehydroepiandrosterone sulfate. Female rats produce more GABA-A agonistic neurosteroids than male rats (Corpechot et al., 1993). In addition, enzymes such as 5-alpha-reductase and 3-alpha-hydroxysteroid oxidoreductase, which metabolize P to THP are found at higher levels in female rats than in male rats (Mellon, 1994). All four steroids P, THP, THDOC, and corticosterone are anxiolytic and these activities are more effectively displayed in female rats than in male rats (Gallucci et al., 1993), perhaps during EW as well. There is little data about how the serotonergic system responds to EW in a different manner between males and females. Bano et al. (1996) reported that brain tryptophan concentration and 5-HT synthesis and turnover were decreased in ethanol-withdrawn rats. A clinical study also reported that anxiety patients during EW showed a decreased 5-HT metabolite (5-hydroxyindoleacetic acid, 5-HIAA) in CSF (cerebrospinal fluid) compared to a control group (Ballenger et al., 1979), indicating a reduced central 5-HT activity during EW. If both the serotonergic and GABAergic system are reduced during EW, the net impact may be less in females than males because the basal level of these systems is greater in females than males. This may explain why fewer female rats develop a spontaneous m-CPP or PTZ-like stimulus during EW.

Sexually Dimorphic Anxiogenic Response is Not Attributed to Pharmacokinetic Factors

The present results from BEC show that BEC is higher in intact female rats than in intact male rats. These results are consistent with previous reports. Given the same dose of ethanol, female rats have a higher BEC than male rats (Sutker et al., 1983; present data). When doses of ethanol were equated for body weight, women reached higher peak BEC than men (Goist et al., 1985; Tabakoff et al., 1983). However, when the BEC was corrected for differences in body water content between men and women, no gender difference in ethanol metabolism could be found (Tabakoff et al., 1983). As the time after ethanol injection elapses, BEC decreased in all groups tested, but no gender and time interaction was found. In accordance of these findings, no gender difference was found in the activity of alcohol dehydrogenase and acetaldehyde dehydrogenase in the gastric mucosa of male and female rats (Maly et al., 1992). These results exclude the possibility that reduced BEC during ethanol exposure was responsible for the less frequent development of anxiety during EW in female rats as compared to male rats.

Similarly, the metabolic rate of m-CPP or PTZ does not appear to be a contributing factor to the gender difference observed in this study. Under the same stimulus effect of m-CPP, female rats are more vulnerable to impaired behavioral initiation whereas they are less responsive to the IDS of m-CPP than

male rats (present data). By analogy, female rats are less responsive to the IDS of PTZ than male rats (present data), but no gender- or estrus-cycle related difference is seen in PTZ-induced myoclonic twitch, clonus or tonic hindlimb extention (Finn and Gee,1994). When an anticonvulsant, 3α,5α-progesterone, was administered prior to PTZ injection, the PTZ threshold dose was higher in diestrus female rats than in estrus or in male rats for myoclonic twitch onset, but not for onset of clonus or tonic hindlimb extention. These divergent gender dependent sensitivity to PTZ would not be seen, if the PTZ clearance rate is higher in female rats than in male rats. In addition, even in the absence of the exogenous PTZ stimulus, fewer female rats develop the PTZ-like stimulus than male rats during AEW (present data). Furthermore, the half-life of PTZ does not appear to differ between male (Esplin and Woodbury, 1956) and female rats (Ramzan and Levy,1985). Therefore, it is plausible to speculate that different anxiogenic response to m-CPP or PTZ in male and female rats is mediated by mechanisms other than pharmacokinetic factors.

CONCLUSIONS

Using PTZ and m-CPP discrimination paradigms in addition to gonadal or hormonal manipulations, the present results demonstrate the following.

1) Male and female rats differ in their behavioral expression of anxiety under the same stimulus property condition.

2) Females are more susceptible to disruption of operant behavior by serotonergic agonists than males.

3) Females are more resistant to GABA antagonistic stimuli than males.

4) Fewer females develop spontaneous anxiety-like IDS during EW than males.

5) Overall, the gender differences in the expression of anxiety appear to be dependent upon the presence of basal estradiol levels in females.

6) The role of estrogen appears to be trophic rather than stimulatory, because there was no significant relationship of the estrus cycle to a change in the expression of anxiogenic stimuli.

7) Male hormones do not appear to play a significant role in the gender differences in the expression of anxiety in adult rats.

8) The gender differences in the development of a spontaneous anxiety-like IDS during EW are not secondary to pharmacokinetic differences in ethanol metabolism between males and females, therefore they are most likely to be pharmacodynamic in nature.

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