

SEX DIFFERENCES IN THE EFFECTS OF VALIUM ON NEURONAL
ACTIVATION AND ELEVATED PLUS-MAZE BEHAVIOR IN THE RAT

By

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Abstract

Females are diagnosed with anxiety disorders more frequently than males; however, the majority of animal and preclinical drug research is done with only male subjects. This study aimed to replicate and extend previous research, which has found evidence for sex differences, by examining sex differences and the effects of chronic diazepam (DZ) treatment on behavior in the elevated plus-maze and neuronal activation as assessed by Fos expression in the medial prefrontal cortex (mPFC) and the medial nucleus of the amygdala (MeA). Behavioral results supported the anxiolytic properties of DZ; however, did not illustrate sex differences. Maze-evoked Fos results found evidence for laterality, as well a DZ influenced decrease in activation, in both regions. Sex was found to interact with laterality in the MeA. Increased Fos expression was seen in the MeA in females when compared to males. It is possible that the activation is due to the anxiety-inducing event more than the animal's response to that event since no significant sex differences were seen in the behavior in the elevated plus-maze. The current study supports and expands previous research regarding drug effects in the elevated plus-maze and maze-evoked Fos expression, however, does not support findings of sex differences in elevated plus-maze behavior.

Sex Differences in the Effects of Valium on Neuronal Activation and Elevated Plus-Maze Behavior in the Rat

The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) outlines the diagnosis criteria for anxiety disorders, including generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic disorder (PD), post-traumatic stress disorder (PTSD), and social phobia (or social anxiety disorder) (APA, 1995). Conventional treatment options include psychotherapy, pharmacotherapy, or a combination approach. Both antidepressants and anxiolytics have been found to be effective for some patients suffering from anxiety. One of the most commonly prescribed drugs is the benzodiazepine, diazepam (Valium).

Gender differences in the diagnosis of many of these disorders have been found and acknowledged (APA, 1995; Blanchard, Griebel, & Blanchard, 1995; Pigott, 1999). Specifically, PD with agoraphobia, GAD, and PTSD are diagnosed two to three times more frequently in women than men (Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004).

Investigations by Amenson and Lewinsohn (1981) and Ensel (1982) found gender discrepancies in depression and anxiety diagnostic rates remained after controlling for other possible confounds such as income, educational level, and different individual experiences with aversive events. Moreover, a recent fMRI study found that hormonal cycling in females has a strong influence on activation of hypothalamic-pituitary-adrenal (HPA) circuitry, and consequently, physiological responses to stress and anxiety, suggesting the cycle may be an underlying cause of gender differences in anxiety responses and disorders (Goldstein et al., 2005).

Despite the evidence suggesting clinical gender differences in the biological mechanisms and responses to stress and anxiety, the majority of drug screening and animal model research uses only male subjects (Blanchard, Griebel, & Blanchard, 1995; Charney & Nestler, 2004). In exploring sex differences using animal models, such as the elevated plus-maze, early studies found inconsistent results, thus signifying the necessity of further investigation to determine if sex differences can be seen in the models (Johnston, & File, 1991; Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993). More recent studies, which have built upon the preceding research and employed improved methodologies, have found strong evidence for sex differences, as well as elucidated a number of factors that appear to influence the emergence of differences in testing (Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993; Frye, Petralia, & Rhodes, 2000; Palanza, 2001; Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004; for review, see Charney, & Nestler, 2004).

A number of variables have been investigated and suggested to underlie these sex differences. Hormone levels and stages of the female rat estrous cycle have been found to influence a number of physiological and behavioral changes, including performance in models of anxiety, such as the elevated plus-maze, and response to drugs, such as diazepam (Frye, Petralia, & Rhodes, 2000). Specifically, rats in the proestrus phase were found to exhibit less anxiety-like behavior and have higher progesterone concentrations, when compared to rats in the estrous or diestrus phases, and males. Therefore, an increase in levels of progesterone has been implicated in enhancing anxiolytic behavior.

In addition to these physiological influences, a recent study by Cooke and Woolley (2005) examined the synaptic organization of the posterodorsal nucleus of the

medial amygdala in the rat, a structure involved in social behavior and the anxiety response, and found evidence of sexual dimorphism and lateralization, suggesting these structural differences may underlie the behavioral differences.

Preclinical research which has investigated the sex by drug interaction has shown inconsistent results of male and female responses to drug treatments; however, there is only a small amount of literature that has examined this interaction (Blanchard, Griebel, & Blanchard, 1995; Pollock, 1997). The anxiolytic effects of anti-anxiety drugs have been shown to be modulated by gonadal hormones, including progesterone and testosterone (Bitran, Hilvers, & Kellogg, 1993). However, Stock, Foradori, Ford, and Wilson (2000) investigated the effects of acute and chronic diazepam administration and gonadal hormone in male- and female-intact and gonadectomized rats and found no significant interactions. Given the inconsistency of findings, the interaction of drug and sex and hormones remains unclear.

This study aimed to investigate the influence of sex and chronic administration of the anxiolytic drug diazepam, on performance in the elevated plus-maze. The elevated plus-maze is commonly used and recognized as a reliable animal model of anxiety (Pellow, Chopin, File, & Briley, 1985; Hogg, 1996). It has also been used in many investigations examining sex differences, and results suggest that females may exhibit less anxiety-like behavior than males in the model (Imhof, Coelho, Schmitt, Morato, Carobrez, 1993; Frye, Petralia, & Rhodes, 2000; Stock, Fordadori, Ford, & Wilson, 2000; Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004). Furthermore, the influence of sex and drug exposure on brain activation was examined by measuring the expression of Fos as a marker of neuronal activation. This method of assessing brain activation is

particularly useful as it serves as an index of functional responses in behaving animals, and has been used in previous anxiety studies to examine structures proposed to be involved in anxiety, such as the medial nucleus of the amygdala (MeA) and the medial prefrontal cortex (mPFC) (Medeiros, Reis, & Mello, 2005; Kalisch, et al., 2004; Windle, Kershaw, Shanks, Wood, Lightman, & Ingram, 2004; Singewald, Salchner, & Sharp, 2003; Duncan, Knapp, & Breese, 1996).

It was hypothesized that sex differences would be present in the elevated plus-maze, supporting prior research, with females showing less anxious behavior than males. In regards to the drug effects, it was hypothesized that the drug-treated animals would show less anxious behavior when compared to the control animals. It was hypothesized that sex differences would be present within the drug-treated group and drug-treated females would display less anxiety-like behavior than drug-treated males. Finally, it was hypothesized that there would be evidence of sex differences in the expression of c-fos, with females showing less expression than males, in addition to evidence for an effect of drug status, with drug-treated animals displaying less expression than saline-treated animals.

Methods

Animals

Three timed pregnant female adult Sprague-Dawley rats (Charles River Laboratories; Durham, NC) arrived on ED14 and were individually housed and maintained on a 12:12 hour light-dark schedule, with lights on at 7:00am, with access to food and water *ad libitum*.

Pups were born on ED20. Litters were left undisturbed, with the exception of husbandry, until PD9, when the pups were sexed and tail-marked according to sex. After some litter redistribution, the final litter sizes were 10 (5 males; 5 females), 10 (6 males; 4 females), and 8 (4 males; 4 females). Litters were undisturbed, except husbandry and continued tail marking of pups, until weaning on PD23. Rats were then separated into same-sex pairs, and one group of three females, until PD59 when they were housed individually.

The adult Sprague-Dawley male ($n = 10$) and female ($n = 13$) rats (males 300-400g; females 200-250g) were maintained on a 12:12 hour light-dark schedule with lights on at 7 a.m., and had access to food and water *ad libitum*. Animals were divided into the following experimental groups: male controls ($n = 5$), male drug-treated ($n = 5$), female controls ($n = 6$), and female drug-treated ($n = 7$). One week prior to starting daily injections, animals were handled daily.

Drug administration

Animals were given daily intraperitoneal (i.p.) injections, according to their experimental group assignment, of either diazepam (DZ; 5 mg/kg), prepared in a vehicle solution of 40% propylene glycol, 10% alcohol, 5% sodium benzoate and benzoic acid, and 1.5% benzyl alcohol (Hospira; Lake Forest, IL), or saline, 0.9% at 1 ml/kg for control animals. Injections were administered between the hours of 8:00 and 11:00 a.m. for 20 or 21 consecutive days. Daily injection order of the four groups was counterbalanced.

Estrous cycle phasing of females

One week prior to behavioral testing, the estrous cycles of the females were determined and monitored. The rat estrous cycle lasts on average for 4-5 days and

consists of the following phases: proestrus, estrus, metestrus (diestrus I), and diestrus (diestrus II). The estrous cycle phase was determined for each female by examining vaginal epithelium collected by vaginal lavage, as described by Frye, Petralia, & Rhodes, (2000). In order to control for the effects of the different phases on behavioral testing, it was initially intended to test all females while in the diestrus phase (diestrus I and II); however, this was not accomplished in the current study.

Apparatus and test procedure

Elevated plus-maze testing occurred on two consecutive days (days 20 and 21 of injections), and the subjects were randomly assigned to a testing day. Subjects received a final injection of DZ or saline 1 hour prior to being tested. The wooden apparatus was constructed in-house and consisted of four arms – two open arms perpendicular to two closed arms (with 45 cm walls), each 30 cm long and 10 cm wide, and connected at the center by a 10 cm by 10 cm square. The closed arms and center square were painted black and the open arms were painted white. The entire apparatus was raised 50 cm off the ground.

Animals were placed in the center square of the maze, facing a closed arm, and videotaped for 5 minutes. Videotape recordings were later analyzed by a researcher blind to the experimental condition. As described by Stock, Fordadori, Ford, & Wilson (2000), behaviors of interest which were recorded by the observer included: number of entries and percentage of time spent in open arms (both believed to inversely represent anxiety levels), total number of entries (believed to be a measure of locomotor activity), and time spent in center (believed to be an inverse measure of activity). Entry into an arm was recorded when all four paws were observed to cross into the arm.

Perfusion and immunohistochemical index of Fos-LI

Animals were anesthetized with 100 mg/kg of pentobarbital approximately 2 hours after completing the behavioral task and transcardially perfused with cold saline followed by 4% paraformaldehyde (PFA). As shown in previous studies (Duncan, Knapp, & Breese, 1996), the 2 hour delay following the behavioral task is optimal for indexing Fos expression. Brains were removed and fixed in 4% PFA overnight at 4° C and then transferred to 30% sucrose in 0.1 M phosphate buffer for cryoprotection. Tissue was sliced using a freezing microtome (Model HM-430; Microm, Germany) at 40 µm, placed in 0.1 M phosphate buffer in 24-well plates, and stored at 4° C. The areas of interest which were assessed include: the medial prefrontal cortex (mPFC) and the medial nucleus of the amygdala (MeA), both components of the limbic system circuitry.

Sections were rinsed in Tris-buffered saline (TBS), washed in methanolic peroxide, and rinsed again in TBS. Sections were then washed in TBS + normal horse serum (TBS+), and incubated overnight at room temperature with the primary antibody (polyclonal rabbit anti-Fos – Calbiochem/EMD Biosciences; San Diego, CA) at a dilution of 1:10,000 in TBS+. Subsequently, sections were then rinsed in TBS and then incubated for 2 hours at room temperature with the secondary antibody (biotin-SP-conjugated affini-pure donkey anti-rabbit IgG – Jackson Immunoresearch; West Grove, PA.) at a dilution of 1:1,000 in TBS+. Sections were then washed in TBS, and incubated with the tertiary antibody (peroxidase-conjugated streptavidin – Jackson Immunoresearch; West Grove, PA) at a dilution of 1:1,600 in TBS and Triton X-100 for 1 hour at room temperature, and washed again in TBS. Sections were then developed using diaminobenzidine (DAB) reaction, followed by final washes in TBS.

Cell counting

Slices were mounted on charged, gelatin-covered slides, dried, dehydrated, and coverslipped. One section was quantified per structure for each animal. Using a photographic rat brain atlas as reference, sections containing the mPFC and MeA were selected. For the mPFC, a counting field of approximately 0.44 mm X 0.42 mm was used. For the MeA, a counting field of approximately 0.85 mm X 0.67 mm was used. Cells exhibiting Fos-LI were photographed through the 20x objective, viewed on the computer in a picture software program, zoomed in at 75%, and counted by the researcher, blind to experimental group assignment. Left and right structures for each were quantified separately. Due to the use of only one section per structure, and therefore no risk of double counting, all stained cells, ranging from light tan to black in color, were counted.

Statistics

Multiple two-way randomized ANOVAs were used with sex (male or female) and drug status (DZ or saline) as the two factors to compare number of open arm entries, total number of entries, time spent in center, and percentage of time spent in open arms in the elevated plus-maze. Three-way, mixed ANOVAs were used with hemisphere (left or right), sex (male or female), and drug status (DZ or saline) as the factors to compare number of cells stained for Fos-LI in the mPFC and the MeA. Significant interactions were further analyzed by post-hoc *t*-tests, with modified Bonferroni corrections, where appropriate.

Results

All animals' data were included in analysis, with the exception of one female in the saline-treated group, due to exclusion from behavioral testing, and therefore Fos assessment.

Estrous cycle phasing of females

Due to difficulties with the protocol and subsequent problems in accurately determining estrous cycle phases, subjects' phases were not used in determining assignment to a testing day; moreover, they were not used as an additional factor to analyze the data.

Elevated plus-maze

As seen in Figure 1, DZ-treated animals made more open arm entries than the saline-treated animals ($F_{1,18} = 5.128, p = 0.036$). No main effect of sex ($F_{1,18} = 0.821, p = 0.377$), nor a sex by drug status interaction ($F_{1,18} = 0.051, p = 0.823$) was found in open arm entries.

As shown in Figure 2, no main effect of sex ($F_{1,18} = 0.107, p = 0.747$), and no sex by drug status interaction ($F_{1,18} = 0.011, p = 0.919$) was found in the percentage of time spent on the open arms. A slight trend was observed with the DZ-treated animals spending a greater percentage of the time on the open arms than the saline-treated animals ($F_{1,18} = 3.162, p = 0.092$).

A main effect of DZ was also found in the total number of entries ($F_{1,18} = 6.077, p = 0.024$), as the DZ-treated animals made significantly more entries than the saline-treated animals (see Figure 3). No main effect of sex ($F_{1,18} = 2.825, p = 0.11$), nor a sex by drug status interaction ($F_{1,18} = 0.201, p = .659$) was found in total number of entries.

There was no main effects of drug status ($F_{1,18} = 0.004, p = 0.948$) nor sex ($F_{1,18} = 2.538, p = 0.129$), and no sex by drug status interaction ($F_{1,18} = 0.003, p = 0.958$), detected in the time spent in the center of the maze (see Figure 4).

Fos-Like Immunoreactivity

As shown in Figure 5, a main effect of hemisphere was found for the cell counts in the mPFC ($F_{1,18} = 6.526, p = 0.02$). The left mPFC showed a greater number of Fos-LI cells when compared to the right mPFC. A main effect for drug status was also found ($F_{1,18} = 28.049, p < 0.001$). Drug-treated animals showed lower cell counts in comparison to saline-treated animals. No main effect was found for sex ($F_{1,18} = 1.997, p = 0.175$). No significant interactions were found for sex by drug status ($F_{1,18} = 0.084, p = 0.775$), hemisphere by sex ($F_{1,18} = 2.194, p = 0.156$), hemisphere by drug status ($F_{1,18} = 1.244, p = 0.279$), nor hemisphere by sex by drug status ($F_{1,18} = 0.355, p = 0.559$).

As shown in Figure 6, a main effect of sex was found ($F_{1,18} = 7.304, p = 0.015$), with females expressing higher cell counts than males. A main effect was also found for drug status ($F_{1,18} = 22.850, p < 0.001$), as drug-treated animals showed lower numbers of stained cells than saline-treated animals. No main effect was seen for hemisphere ($F_{1,18} = 0.062, p = 0.807$).

A significant hemisphere by sex interaction was found for cell counts in the MeA ($F_{1,18} = 6.806, p = 0.018$). Post-hoc analyses did not find significant differences between the left and right hemispheres in males, nor females. Moreover, there was no significant difference found between the left hemisphere in males and females, nor the right hemisphere between males and females. No significant interactions were found for

hemisphere by drug status ($F_{1, 18} = 3.645, p = 0.72$), sex by drug status ($F_{1, 18} = 2.686, p = 0.119$), nor hemisphere by sex by drug status ($F_{1, 18} = 0.588, 0.453$).

Discussion

The results of this investigation support the hypothesis that chronic administration of the anxiolytic diazepam reduces anxiety-like behavior in the elevated plus-maze. The ethological conflict employed in this paradigm induces anxiety-like behaviors by opposing the desire to explore the novel maze, in the presence of open spaces and elevation. Drug-treated animals made more entries into the open arms of the maze than the saline-treated animals, suggesting a lowered anxiety level about exploring the open areas of the maze; therefore they may cross into the open arms more often and spend more time in the open arms. Moreover, although not significant, a trend was detected, with drug-treated animals tending to spend a greater percentage of time on the open arms than the saline-treated animals. This trend also supports the anxiolytic effect of treatment with diazepam. These findings support previous research which has found that diazepam reduces anxiety-like behavior in the elevated plus-maze.

Two measures of activity were used, however results were inconsistent. Drug-treated animals made significantly more arm entries than saline-treated animals, suggesting the drug was activity-enhancing. This does not support previous research. Diazepam has been shown to enhance activity levels at doses lower than 3 mg/kg, however, at doses higher than 3 mg/kg, such as 5 mg/kg as was used in this study, it has been shown to either not effect activity, or depress it to levels below saline-treated controls (Pellow, Chopin, File, & Briley, 1985; Stock, Fordadori, Ford, & Wilson, 2000; Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004). This has been shown with both

acute doses, as well as in chronic administrations – which differ among research designs, but have extended as far as 21 days of drug exposure, as was used in this study.

While the drug-treated animals showed higher levels of activity than the saline-treated animals, it is possible that the increased activity did not affect the assessment of anxiety-like behavior. Pellow, Chopin, File, and Briley (1985) found evidence that drugs which reduced anxiety-like behavior, such as acute diazepam, depressed activity levels to similar levels as did non-anxiolytics, such as haloperidol and imipramine. This suggests that reductions in anxiety-like behavior – increased open arm entries and time spent on open arms – cannot be explained by exploratory or locomotor behavior. While the effect on arm entry activity was in the opposite direction in this study, it is possible that the decrease in anxiety-like behavior seen in the drug-treated group is a reflection of decreased aversion to the open arms and not influenced by the increase in exploratory behavior.

This is supported by the lack of significant differences in the amount of time spent in the center. This measure has been proposed as a more sensitive measure of activity than total arm entries because it accounts for time spent not actively exploring the arms of the maze, and can be conceptually thought of as an inverse measure of activity (Fernandes, & File, 1996). Previous research findings by Stock, Fordadori, Ford, and Wilson (2000), in which this measure of activity was used in testing following a 21-day chronic administration of diazepam, also found no difference between drug and saline groups.

No sex differences were detected in anxiety-like behavior (number of open arm entries or percentage of time spent in open arms), which does not support the hypotheses;

however, this may be explained by a methodological weakness. Previous research has not only found evidence for sex differences using this model, but has also elucidated the influence of the phases of the females' estrous cycle on anxiety-like behavior in this maze (see Charney, & Nestler, 2000, for review; Frye, Petralia, & Rhodes, 2000). In the original design of this study, testing was to be done when females were in the diestrus phase, so as to control for the effects of phase; however, this was not the case at the time of testing.

As previously explained, the females' estrous cycles were to be observed starting one week prior to testing. The protocol, while successfully employed by other researchers, was new to the researcher, causing difficulties in accurately differentiating between phases. Due to scheduling restrictions, testing dates could not be adjusted to allow time to correct these problems; therefore, females were randomly assigned to a testing day, regardless of phase. As suggested by Frye, Petralia, and Rhodes (2000), negating to control for this variable in the design may have led to a wash-out effect in the females' behavioral data. Future investigations should take this into consideration in the design, and interpretations of results, whenever using males and females in anxiety-related research.

Using Fos-LI positive cells as an assessment of neuronal activation, the medial prefrontal cortex (mPFC) and the medial nucleus of the amygdala (MeA), both components of the limbic system, were examined after performance in the elevated plus-maze. Evidence of laterality was found in the mPFC, as a significantly greater number of Fos-LI positive cells were detected on the left than the right. At this point in time, no previous research has investigated laterality within this structure in the anxiety model

paradigm. In light of the evidence of laterality in this study, previous results in which the bilateral counts were averaged to achieve a single count for the structure, may have been skewed if there is in fact a difference between the hemispheres (Duncan, Knapp, & Breese, 1996; Singewald, Salchner, & Sharp, 2003; Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004; Medeiros, Reis, & Mello, 2005). It is possible that laterality in maze-evoked Fos expression in the mPFC could manifest functionally as influential in risk assessment and avoidance behavior seen in the maze, due to the role of the PFC in decision-making and judgment in humans. Future investigations should be done to further explore this finding of laterality in the mPFC, and if this is supported, the previous results and interpretations should be reconsidered.

There was also evidence for a significant effect of diazepam treatment, with drug-treated animals showing less expression of Fos-LI positive cells in the mPFC than the saline-treated animals. This supports the research hypothesis, suggesting lowered activation in this region by the maze due to treatment with an anxiolytic. Moreover, this finding extends the research of Medeiros, Reis, and Mellow (2005), who found similar results after an acute treatment of diazepam, of the same dose of 5 mg/kg as was used in this study, when using a restraint model of anxiety. As suggested in the aforementioned study, the prefrontal cortex is one of the areas where benzodiazepine receptors are found, thus it is possible that a functional effect of the anxiolytic involves reduced neuronal activation in this structure.

No sex differences were detected in anxiety-induced Fos expression in the mPFC, therefore there is no support for the hypothesized sex difference for this specific region. When considered in light of the behavioral findings of this investigation, it is possible

that not controlling for the estrous cycles of the females may be contributing to a similar masking effect of the possible differences. However, due to the slim amount of research investigating sex differences in Fos expression induced by this particular model of anxiety, more research is necessary to further examine this. Additionally, although no sex by drug interactions was hypothesized, nor found, this investigation extends previous research in that it employed a design which allowed for this possibility to be examined, and with additional methodological improvements, this may be more specifically studied.

The results for the medial nucleus of the amygdala give some support the research hypotheses for Fos expression, as well as suggest lateralization of activation in this region. Although not hypothesized, a significant hemisphere by sex interaction was found, however post hoc analyses did not reveal the source of significance. A recent investigation by Cooke and Woolley (2005) found evidence for structural lateralization in the MeA of the rat, and when combined with this finding, suggests that laterality should be examined further (for review of Cooke, & Woolley, 2005, see Appendix).

A possible explanation for not discovering the source of significance in this interaction may involve the lower-order, significant findings. A main effect of drug was found, with diazepam-treated animals expressing lower numbers of Fos-LI positive cells than saline controls. This main effect increased the variability within the male and female groups, and although did not interact significantly, this variability may be preventing the post hoc analyses between the sexes to reach significance. While the effects of acute DZ on Fos expression induced by a model of anxiety have been investigated in a limited number of studies, this is the first to examine the effects of chronic administration on

activation. The results suggest that chronic administration continues to yield anxiolytic effects on behavior and decreased neuronal activation in this region.

A main effect of sex was also found, with females expressing more Fos-LI positive cells than males, which refutes the research hypothesis. This suggests that although no behavioral differences were detected, there are differences in neuronal activation of the MeA between the sexes. Moreover, the results suggest that females have increased activation in the MeA when compared to males. It is difficult to speculate on the function of this difference because it is still unclear whether elevated plus-maze induced Fos expression is evoked by the stressful event itself and/or the animal's response to the event. This may also account for not finding significant sex differences in the maze, while finding significant sex differences in maze-evoked Fos expression. As aforementioned, given the lack of research using this model in examining sex differences in Fos expression, there are no previous findings to assess these results or conclusions against. This study extends the literature greatly, while also illustrating the necessity of further exploration of these variables.

In addition, it should also be noted that the model employed in this investigation, the elevated plus-maze, is only one of the many which have been used to model anxiety-like behavior in animals. It is possible that other models, which are designed based on a variety of other paradigms – such as conditioned fear or restraint stress – may have different results when applied to this design. While the elevated plus-maze is generally accepted as a valid model for both males and females, other models may not be (Pellow, Chopin, File, & Briley, 1985; Lister, 1990). Moreover, it is possible that other models are not effective at inducing Fos expression, and therefore cannot be used to replicate this

design and findings. And as suggested by Medeiros, Reis, and Mello (2005), the various models may also be different in their effectiveness and pattern of induction of Fos, which suggests that different models illustrate different facets of the anxiety response. With further investigations, these possibilities may be explored.

Given the constancy of differential anxiety disorder diagnosis rates for males and females, continued research on this topic is essential. This investigation examined a specific animal model for evidence of sex differences, and did not find significant sex differences. However, given the inconsistent findings when using this particular model to examine sex differences, it is possible that this model is not the most effective and reliable model of anxiety for comparing males and females.

This investigation also studied the effect of diazepam treatment on anxiety-like behavior. Unlike the majority of pharmacological manipulation research in which only an acute dose is used, a chronic administration was done, in an attempt to better model the clinical treatment regimen. In addition to observing behavioral effects, induction of Fos expression, a measure of neuronal activation, was also examined. This procedure allows for analyzing the functional neuronal response to the model of anxiety. It is through studies employing methods such as those included here that will hopefully lead to a better understanding of the gender differences and improved treatment options.

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Appendix

Clinical anxiety research

In response to a stressful or aversive stimulus, an adaptive fear response is initiated and later subsides when the danger is no longer present (Rosen, & Schulkin, 1998). However, in some individuals, this fear response is maladaptive, becoming exaggerated and dysfunctional. This pathological anxiety is commonly characterized by sufferers as involving intense states of negative affect and feelings of uncontrollability. Eysenck (1992) suggests that chronic hypervigilance biased to threatening stimuli underlies the outward manifestation of symptoms (as cited in Rosen, & Schulkin, 1998). Moreover, this chronic anxiety dysfunction may disrupt daily functioning, and commonly presents comorbid with major depression (Rosen, & Schulkin, 1998).

The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) defines anxiety disorders, distinguishing the various types by diagnostic criteria including generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic disorder (PD), post-traumatic stress disorder (PTSD), social phobia (or social anxiety disorder), and specific phobias (APA, 2000). In addition, DSM-IV-TR includes the prevalence rates and cultural and gender differences in diagnosis. Through community, epidemiological, and clinical studies, gender differences in diagnosis has been noted for a number of the disorders.

Panic Disorder (PD), with a lifetime prevalence rate of 1 – 2%, is diagnosed two to three times more frequently in women than in men. In Generalized Anxiety Disorder, with a lifetime prevalence of 5%, 55-60% of the diagnoses are female in clinical studies, but epidemiological studies suggest 2/3 of sufferers are female. Specific Phobias have

been found to have 7 – 11% lifetime prevalence, and across all subtypes, females are diagnosed twice as frequently as males. While no comment is made regarding gender differences in Post-Traumatic Stress Disorder (PTSD) diagnoses, which has a lifetime prevalence of 8%, an epidemiological study by Helzer, Robins, and McEvoy (1987) found that 70% of sufferers were female (as cited in Blanchard, Griebel, & Blanchard, 1995). Social Anxiety Disorder and Obsessive Compulsive Disorder (OCD) were found to be diagnosed equally as frequent in females and males in clinical studies, however, it was noted that community and epidemiological studies may find otherwise (APA, 2000). In light of these findings of gender differences in anxiety disorder diagnoses, a number of clinical studies have been conducted in order to investigate possible underlying factors.

Kendler, Walters, Neale, Kessler, Heath, and Eaves (1995) sought to understand the genetic and environmental risk factors for anxiety disorders in women. Using female monozygotic and dizygotic twins, the participants were interviewed about their anxiety and anxiety disorders, and the data were analyzed to determine the influence of genetics and both familial and individually unique environmental factors on the diagnosis of anxiety disorders. It was found that the genetic risk factors seen in women are neither completely nonspecific, nor completely specific for the disorders. Two factors appeared to hold the most influence: the liability to experience discrete and reoccurring episodes of dysphoria and a vulnerability to experience panic attacks. Familial environment was not found to be a significant influence on the diagnosis of an anxiety disorder, which refutes the public perception of these disorders. Finally, environmental factors which were unique to the individual (such as marital problems), appeared to influence diagnosis.

This investigation has a number of limitations, however. The researchers chose to focus only on females; therefore the sample did not include males and no direct comparison could be made regarding risk factors. This biased design suggests that the male diagnosis rates and risk factors are considered normal, and therefore the higher prevalence in females must be abnormal and likewise due to different risk factors. In addition, no genetic and environmental interactions were assessed, thus ignoring possible additional risks – such as genetic predisposition which only in accord with stressful life events result in an anxiety disorder diagnosis.

In addition to the assessment of risk factors, structural and physiological gender differences have also been investigated in anxiety. In a study by Goldstein et al. (2005), fMRI imaging was employed to examine gonadal and adrenal hormone modulation of the activation of the hypothalamic-pituitary-adrenal (HPA) axis in adult women. Twelve premenopausal women underwent two fMRI sessions and were shown neutral images and negative/aversive images during the scan; the order was counterbalanced across participants. The researchers used the women's menstrual cycles as a natural means of manipulating hormone levels; the group was counterbalanced, with half of the women participating in their first session 2-3 days after the onset of their menstrual cycle and their second session about 2 weeks after the onset, and the other half of the participants participating in their first session 2 weeks after onset and their second session 2-3 days after onset of the next cycle. Estrogen and progesterone levels are low during the early follicular phase (2-3 days after onset) and progesterone remains low, but estrogen levels increase during the late follicular phase (midcycle; about 2 weeks after onset). In addition, physiological arousal was measured by electrodermal activity (EDA).

The researchers found significantly greater magnitude of blood oxygenation level-dependent (BOLD) signal changes between neutral and negative image presentations during the early follicular phase when compared to the late follicular phase in a number of structures implicated in the fear response circuitry, including the central amygdale, hippocampus, and various cortical areas. This suggests that gonadal hormones influence anxiety arousal and responses in women, and is evidence of sexually dimorphic physiological mechanisms which may underlie the gender differences in anxiety disorders.

In addition to the gender differences seen in diagnosis and possibly disorder etiology discussed above, there is also evidence which suggests differences in pharmacological treatment. Current treatments that have been found to be effective in managing anxiety disorders include psychotherapy, pharmacotherapy, and combination therapy which combines the two approaches (Shekhar et al., 2001). Psychotherapy options include behavioral and cognitive-behavioral therapies, which have been found to be effective in the treatment of PD, Social Anxiety Disorder, OCD, specific phobias, and PTSD. Pharmacotherapy can include antidepressants and/or anxiolytics, and has been used effectively in managing symptoms in many of the disorders.

Antidepressants, categorized as tricyclics, monoamine oxidase inhibitors, or selective serotonin reuptake inhibitors, have been successful in some individuals suffering from PD, Social Anxiety Disorder, OCD, PTSD, and Generalized Anxiety Disorder. Anxiolytics have also been a successful treatment option for some individuals suffering from PD, Social Anxiety Disorder, and Generalized Anxiety Disorder. Benzodiazepines, such as diazepam, lorazepam, and alprazolam, have traditionally been

used. These drugs are thought to act as indirect agonists at GABA-A receptors, thus enhancing inhibitory neurotransmission by allosteric modulation. Diazepam is often considered the benchmark and is one of the most commonly used anxiolytics in clinical settings.

In addition to differential diagnostic rates in males and females, evidence has shown that there are gender differences in the metabolism of many psychotropic drugs, including anxiolytics (Pollock, 1997). Moreover, there have also been sex differences found in drug metabolism in animals (Stock, Ford, Biscardi, & Wilson, 1999). This illustrates the necessity of using males and females in preclinical testing.

Animal models of anxiety

In addition to the clinical research and case studies, researchers have also employed animal research to better understand the neuropathology of these anxiety disorders and to test the safety and efficacy of new drugs (Shekhar et al., 2001; Charney & Nestler, 2004). By using animal subjects, researchers are better able to control the study design and are also able to manipulate conditions and drugs that are not ethical to study directly in humans (Kalueff, & Tuohimaa, 2004). A number of behavioral models have been developed for the study of anxiety; current literature claims over 30 have validity (Charney, & Nestler, 2004).

These models may be developed using species-specific traits, conditioned or unconditioned stress responses, social conditions, prenatal or neonatal manipulations, or pharmacological manipulations (Shekhar et al., 2001; Charney, & Nestler, 2004; Kalueff, & Tuohimaa, 2004). With such variance in the underlying manipulations in the group of models, they have been found to have low convergent validity when tested in the same

subject (Palanza, 2001) and varying effectiveness as indices in anxiolytic screening or examining neurobiology (Shekhar et al., 2001; Kalueff, & Tuohimaa, 2004).

The elevated plus-maze is a commonly used and widely accepted model of anxiety which uses ethological conflict as a means of inducing anxiety (Charney, & Nestler, 2004; Hogg, S., 1996). Rats are known to be exploratory creatures, yet they also exhibit neophobia (fear of novel environments) and acrophobia (fear of heights) (Haug, & Whalen, (1999). By incorporating both height and open space, this model produces stress-evoked anxiety in the rat.

As described in Pellow, Chopin, File, and Briley (1985), the apparatus consists of four arms – two open arms perpendicular to two closed arms. Commonly used behavioral measures in this model include the number of entries into open arms and closed arms and time spent on open and closed arms. Those behavioral measures are then most commonly converted to percentage of entries into open arms and the percentage of time spent on open arms, both of which are believed to be inverse measures of anxiety level.

Additionally, the total number of arm entries are also calculated and used as a measure of overall level of activity/locomotion. Recently, Fernandes and File (1996) have suggested that time spent in center is a more sensitive measure of activity. This measure is an inverse assessment of time spent actively exploring the maze.

A number of studies have been conducted to validate the elevated plus-maze as a reliable model of anxiety (Pellow, Chopin, File, & Briley, 1985; Lister, R. G., 1990).

First proposed by Pellow, Chopin, File, & Briley (1985), the researchers used two strains of rats, and investigated the effects of initial placement in maze, light levels in the arms, repeated testing on behavior, as well as using physiological and pharmacological

validation methods. No significant differences in behavior were found between the two strains of rats in the maze. Initial placement in the maze, either facing an open or closed arm, did not have a significant effect on behavior. There was also no significant effect of equalizing light levels in both the closed and open arms. Results from three days of repeated testing did not differ significantly from novel testing results.

Physiological validation was done through measuring corticosterone concentrations; previous research has shown that aversive and stressful events increase plasma levels. Animals which had completed the maze had significantly higher concentrations than: animals which were sampled immediately after be removed from their home cage, rats confined to a closed arm for 20 minutes, and rats confined to an open arm for 20 minutes. Pharmacological validation was executed by testing a variety of drugs, including anxiogenics, anxiolytics, stimulants, and neuroleptics. Significant increases in open arm entries and percentage of time spent on open arms were only seen with clinically effective anxiolytics. Specifically, diazepam was tested in both acute and chronic (5 days) doses (1 or 2 mg/kg). Acute doses reduced total arm entries, but this effect was not detected when given chronically. Number of open arm entries was increased following acute doses, however, just missed significance. Chronic treatment significantly increased open arm entries. Both acute and chronic doses increased the percentage of time spent in open arms.

A variety of other procedural variables have also been tested for validation in the elevated plus-maze, including pretest manipulations, pharmacological manipulations, testing conditions, animal strain, and construction of the maze; the results are summarized in a review by Hogg (1996). Pretest manipulations have been found to have

an effect on behavior and drug effects, and therefore should be carefully considered in designing the experiment. Housing the subjects individually was found to reduce the animals' tendency to explore the open arms, and enhance the anxiolytic properties of diazepam. Pretest handling of subjects was found to influence drug effects; it was found to attenuate the anxiolytic effects of benzodiazepines. In addition, there is contradictory evidence regarding when behavioral effects of repeated testing emerge, as well as the quality (anxiogenic or anxiolytic) of the behavioral effects. These factors should be considered in experimental design, and considered when interpreting results.

These validation studies, however, used only male subjects. Therefore, while the elevated plus-maze is considered a reliable model of anxiety for males, the validity of the model for females is questionable due to the evidence of sex differences in maze behavior, and with male behavior being considered the standard. Fernandes, Gonzalez, Wilson, and File (1999) used factor analysis and found that the factors driving behavior in some animal models, such as the elevated plus-maze, may differ between males and females. They found for female rats, activity was the primary factor accounting for variance; while in males, anxiety was the primary factor accounting for variance. These findings suggest that factors controlling behavior in the maze are different between the sexes and results should be interpreted accordingly.

Although sex differences have been found in a number of nonreproductive behaviors, there remains a sex bias in animal anxiety research (Blanchard, Griebel, & Blanchard, 1995; Charney & Nestler, 2004). In a review by Blanchard, Griebel, and Blanchard (1995), it was found that an overwhelming majority, 90%, of preclinical psychopharmacology research on serotonergic drugs and anxiety has been conducted on

only male subjects. This is despite results that had been reported regarding the gender differences seen in clinical diagnosis and studies of anxiety (APA, 1995). A brief literature search on February 27, 2006 in the MEDLINE and PsycINFO databases using the keywords “anxiety,” “animal models,” and “rats” returned 272 results, only 25 of which reported using females in the research. While the majority of research involves only male subjects, the research that has been conducted using both males and females has found evidence of sex differences (Palanza, 2001).

One of the first investigations of sex differences in animal models of anxiety was done by Johnson and File (1991). The researchers used three models of anxiety – the social interaction test, the Vogel punished drinking test, and the elevated plus-maze. They found significant sex differences in the elevated plus-maze. Female rats made a significantly greater percentage of open arm entries than male rats. However, they found no significance in differences in the percent time spent on open arms, although they reported a trend of greater percentage of time spent on open arms by females when compared to males. The females also tended to make more total arm entries than the males, though this was not significant. This may indicate that females were overall more active than the males in this test.

As a foundational study in examining sex differences in anxiety through animal models, this investigation fueled future research by suggesting these differences can be seen in rats, as well as humans. However, a number of elements were not addressed in this design nor the discussion. These include, but are not limited to: subject age, and hormonal influences. Since this study, a number these variables and manipulations have been investigated.

Imhof, Coelho, Schmitt, Morato, and Carobrez (1993) again examined sex differences, but also looked at the influence of subject age on performance in the elevated plus-maze. Male and female Wistar rats were observed in the maze at ages 45, 60, 90, 120, or 150 days; each subject only performed in the maze once. Significant sex differences were seen at 45 days, as females spent a greater percentage of time in the open arms and overall made more entries into arms, when compared to males. No significant sex differences were seen at 60 days. However, significant sex differences were observed at 90 days, with females showing reduced aversion to the open arms as they spent a greater percentage of time in the open arms and a greater percentage of open arm entries, and showed a greater level of activity with more arm entries when compared to males. At 120 days though, the only sex difference detected was a greater level of activity in females, as indicated by an overall greater number of arm entries. Finally, at 150 days, no significant sex differences were observed. Overall, fear-like behavior increased as a function of age in both sexes.

The results of this investigation suggest that not only should subject sex be taken into consideration when designing and analyzing a study, but also the age of the subject. Consistent sex differences were only seen at 90 days, yet the pattern of behavior surrounding this time point suggests shifts in exploratory behavior may occur at different points in males and females. Males showed dramatic decreases in all three behavioral measures between 60 and 90 days, suggesting an increase in anxiety level. Females on the other hand showed this decrease in behavioral measures a little later, between 90 and 120 days. The researchers propose these results as evidence for sex differences in

anxiety-like behaviors in the elevated plus-maze, and also in support of using age-matching rather than weight-matching, especially in pharmacological studies.

Another variable which has been investigated in examining sex differences in animal models of anxiety is the estrous cycle in females. Frye, Petralia, and Rhodes (2000) used male and female Long-Evans rats in nine anxiety paradigms. Prior to behavioral testing, the females' estrous cycle was determined and monitored and subjects were then divided into groups based on the cycle phase they would be experiencing on testing day – proestrus, estrus, or diestrus. In the elevated plus-maze, proestrus females had significantly more open and closed arm entries than estrus females, diestrus females, and males. Moreover, the proestrus females spent a greater percentage of the time on an open arm than the other groups. These results illustrate that sex differences were only observed when the female rat was in proestrus and exhibiting reduced aversion to the closed arms and higher levels of overall activity, when compared to females rats in the other cycles and males. Up until this investigation, this distinction had not been made. These findings suggest that careful planning should be used when designing an anxiety study using females, and also that results which do not address this variable may have additional interpretations which were not originally considered.

Stock, Foradori, Ford, and Wilson (2000) also investigated the effects of hormones in the elevated plus-maze, in addition to also examining the influence of acute versus chronic administration of the anxiolytic diazepam. Male and female Long Evans rats were either sham-operated or gonadectomized at 60 – 70 days. One week following surgery, rats had silastic capsules either empty (control) or filled with diazepam (drug group) implanted subcutaneously. Acute treatment involved three days exposure, while

chronic treatment involved additional implants to ensure three weeks exposure. The researchers reported that the drug treated animals showed higher percentages of time spent on the open arms and greater number of open arm entries when compared to controls, however, no significant sex or hormone effects were seen between groups. These findings illustrate do not support earlier findings of sex differences in performance in the elevated plus-maze. Moreover, they do not support the previous findings in the Frye, Petralia, and Rhodes (2000) study which found significant hormone effects. However, in this investigation, hormone status did not include female estrous cycle phase differentiation, but rather the presence or absence of gonadal hormones. Finally, this study suggests that sex nor duration of administration of diazepam significantly affects behavior in the elevated plus-maze model of anxiety.

Fos expression as an assessment of neuronal activation

In addition to studying behavior, it is also helpful to investigate brain activation in trying to understand anxiety circuitry, drug effects, and sex differences. A common method employed to measure neuronal activation by an activating event, such as a behavioral task or administration of drug, is immunohistological assessment of Fos protein expression (Curran, & Morgan, 1995). Fos protein is the product of the protooncogene *c-fos*, an immediate early gene, which is expressed in response to hormone- and neurotransmitter-induced elevations in intracellular calcium and c-AMP (Sheng, & Greenberg, 1992). This is a particularly helpful approach to assessing brain activation because it serves as an index of functional responses in behaving animals and has been used in previous anxiety studies.

Duncan, Knapp, and Breese (1996) examined Fos expression in response to three animal models of anxiety: foot shock avoidance, elevated plus-maze, and an air-puff induced vocalization task. In this between-subjects design, each subject participated in only one of the models, and three control groups were also included: a group instrumentally conditioned to lever press for an appetitive reward, a group let undisturbed in their home cage, and a group that had been handled and placed in the novel environment of the air-puff chamber, however did not receive the air-puff. Two hours following the completion of the task, the rats were perfused and their brains extracted, as previous studies have shown that a two hour delay following the activating event or stimulus was optimal for immunohistological characterization of Fos.

All three of the anxiety models induced significantly more Fos expression in subjects than seen in the three control groups in a number of regions, including specific regions of the cortex, various nuclei of the amygdala, the accumbens shell and core, and nuclei of the hypothalamus. Specifically, after the elevated plus-maze, significantly more Fos was detected in limbic cortical regions, including the medial prefrontal cortex, than in controls. Significantly greater Fos expression was also seen in the various nuclei of the amygdala, when compared to the controls.

The inclusion of the instrumentally conditioned control group assessed whether a non-aversive behavioral task was an adequate activating event for Fos expression. It was found that this group was not statistically different than the other two control groups in Fos expression. Therefore, specificity in the activating event is crucial for Fos activation in these limbic and cortical areas; in this case, it was selective for an anxiety-related event.

Most of the common regions activated by the three models are associated with the limbic system, including the medial prefrontal cortex and the medial nucleus of the amygdala. This supports the hypothesis that these regions are involved in fear and anxiety circuitry. A number of previous Fos expression studies have also implicated these regions in functional circuitry involved in processing and responding to stressful situations. As suggested by the researchers, future studies employing anxiolytics may aid in differentiating anxiety circuitry from other stress-related activation. This study laid a foundation for additional research using pharmacological manipulations, and the combination of both pharmacological and behavioral manipulations, in order to further explore the influence of anxiety and anxiety drugs on neuronal activation, and anxiety circuitry.

Singewald, Salchner, and Sharp (2003) used a pharmacological manipulation to investigate the effects of anxiogenics on the induction of Fos expression. Four drugs which have all previously been shown to be anxiogenics, each by different mechanisms of action, in rats and humans, were used: FG-7142 (benzodiazepine inverse agonist), m-chlorophenyl piperazine (m-CPP, nonselective 5-HT_{2c} agonist), caffeine (adenosine receptor antagonist), and yohimbine (α_2 -adrenoreceptor antagonist). In a between-subjects design, which included a saline group, subjects were injected and then perfused and the brains extracted after two hours. The researchers did not include behavioral testing to verify the anxiogenic properties of the drugs, due to the resounding support from previous studies which used the same doses to induce anxiety, and in order to specify the activating event to be solely drug administration.

Cells expressing Fos were quantified in structures of interest, which included: forebrain regions, cortical regions, the hippocampus, nuclei of the amygdala, and the hypothalamus. Compared to the saline-injected control group, all of the drugs increased Fos expression significantly in the forebrain, including the medial prefrontal cortex, which supports the findings from Duncan, Knapp, and Breese (1996), which implicated this region in anxiety circuitry. Fos expression was also significantly increased in many cortical regions, and in the central, basolateral, and medial nuclei of the amygdala by all the drugs, when compared to saline controls, although m-CPP did not significantly increase expression in all of these areas. These findings lend further support to the involvement of these regions in an animal's neural response to anxiety.

This investigation, using pharmacological manipulations to study neuronal activation in response to anxiety, found similar results as is seen in behavioral models of anxiety, thus adding support to the involvement of the limbic structures, including the medial prefrontal cortex and the medial nucleus of the amygdala, in anxiety circuitry. A limitation of this study is that the exact involvement of these regions in anxiety cannot be differentiated. It is unclear whether the regions are involved in the activation of the anxiety response, or in the response to the aversive stimuli and state. Additional research is necessary in order to tease apart the relationship the activated regions have with these processes. The researchers suggested the use of anxiolytics in order to characterize the influence of the mediation of the anxiety response by such drugs on Fos expression.

Medeiros, Reis, and Mello (2005) examined the influence of three different types of drugs on stress-induced Fos expression. Dexamethasone, a synthetic glucocorticoid, was used, as it has previously been shown to reduce stress-induced Fos expression in the

paventricular nucleus of the hypothalamus. Diazepam, a benzodiazepine, was also used as it is commonly used in the treatment of clinical anxiety and has been shown to be an effective anxiolytic in many animal models of anxiety. Finally, imipramine, an antidepressant, was used because chronic antidepressant treatment is another form of pharmacotherapy used for clinical anxiety.

All groups received 20 days of injections: the control, dexamethasone, and diazepam groups received saline while the imipramine group received imipramine in order to more effectively model the clinical treatment course. On day 21, the control group received a final injection of saline, the dexamethasone and diazepam groups received their respective drug, and the imipramine group received a final injection of imipramine. All subjects were then restrained for 60 minutes in a plastic cylinder and were perfused, and the brains extracted, two hours later.

Cells positive for Fos were quantified in 40 brain regions/structures. The control group, which was restrained, however did not receive any drug manipulation, showed Fos expression in a number of brain regions, including the limbic structures, which supports previous research. Dexamethasone reduced Fos expression in the hippocampus and locus coeruleus, however, did not significantly reduce Fos expression in any other areas, when compared to saline controls. Chronic imipramine also reduced expression in the hippocampus and locus coeruleus, in addition to paventricular nucleus of the hypothalamus. Diazepam treatment resulted in significantly less Fos expression in the hippocampus, and a number of other areas.

Anxiety-induced Fos expression is a particularly helpful approach to assessing brain activation because it serves as a measure of functional neuronal responses in

behaving animals. This methodology should be used in future studies of sex differences in animal models of anxiety.

Figure 1

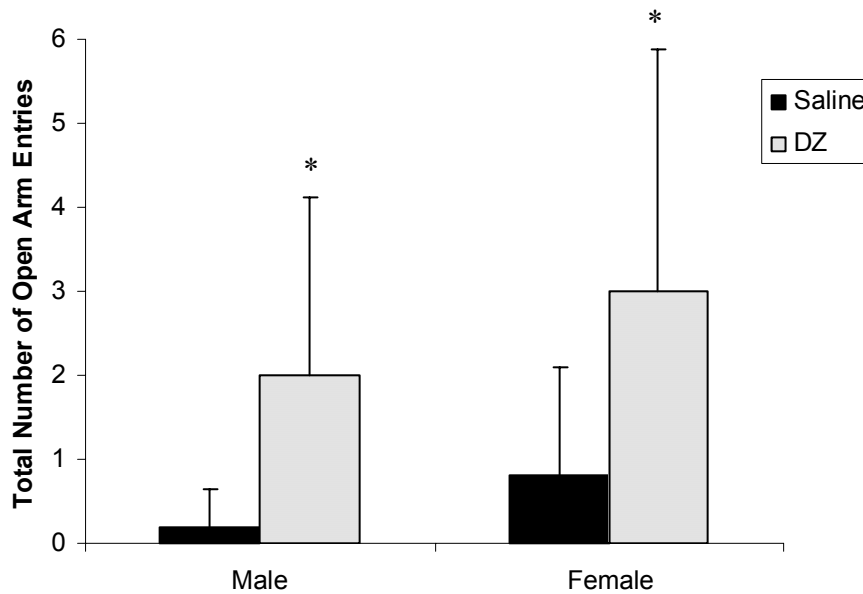
Number of Open Arm Entries

Figure 1. Mean number of open arm entries for the four experimental groups. No significant sex differences nor a sex by drug status interaction were seen. DZ-treated animals ($M = 2.58$, $SD = 2.539$) made significantly more open arm entries than saline-treated animals ($M = 0.50$, $SD = 0.972$).

* $p = 0.036$.

Figure 2

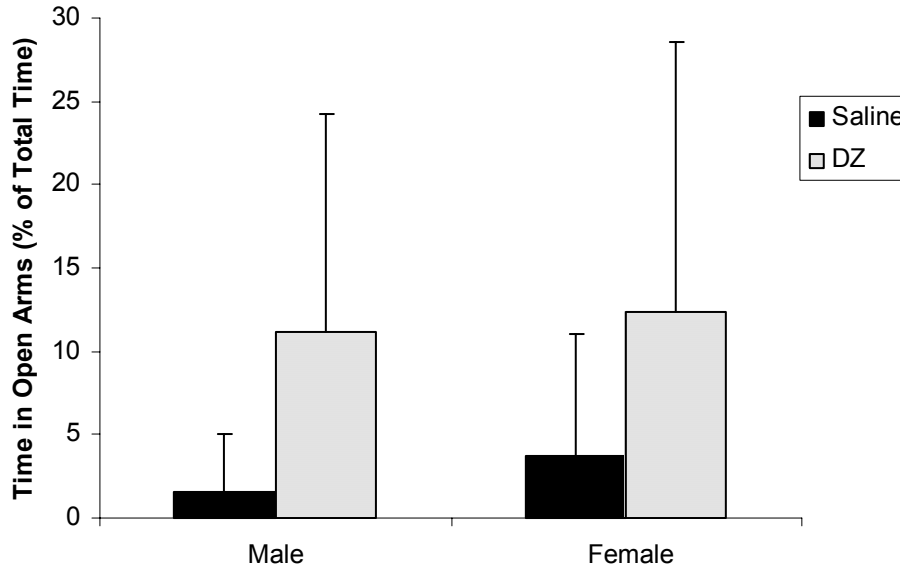
Percentage of Time Spent on the Open Arms

Figure 2. Mean amount of time spent on the open arms, shown as a percentage of the total time (5 minutes). No significant sex difference, nor sex by drug status interaction was found. A trend was observed, with DZ-treated animals ($M = 11.86$, $SD = 14.38$) spending greater percentage of the total time on the open arms than the saline-treated animals ($M = 2.67$, $SD = 5.51$), although these were not found to be significantly different ($p = 0.092$).

Figure 3

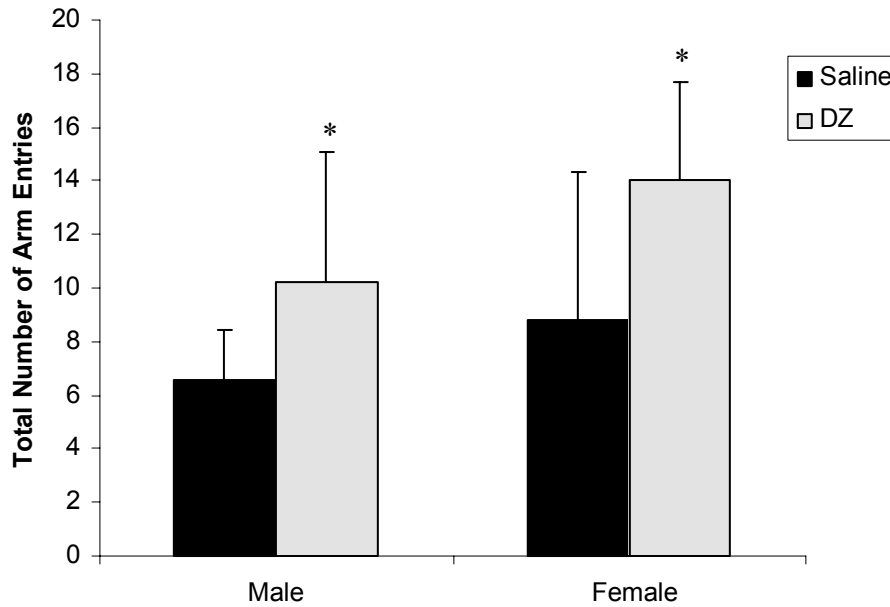
Number of Total Arm Entries

Figure 3. Mean number of total arm entries made by the four experimental groups. No main effect of sex nor a sex by drug status interaction was observed. A main effect of drug status was detected as DZ-treated animals made more total entries ($M = 12.42$, $SD = 4.44$) than the saline-treated animals ($M = 7.70$, $SD = 4.03$).

* $p = 0.024$

Figure 4

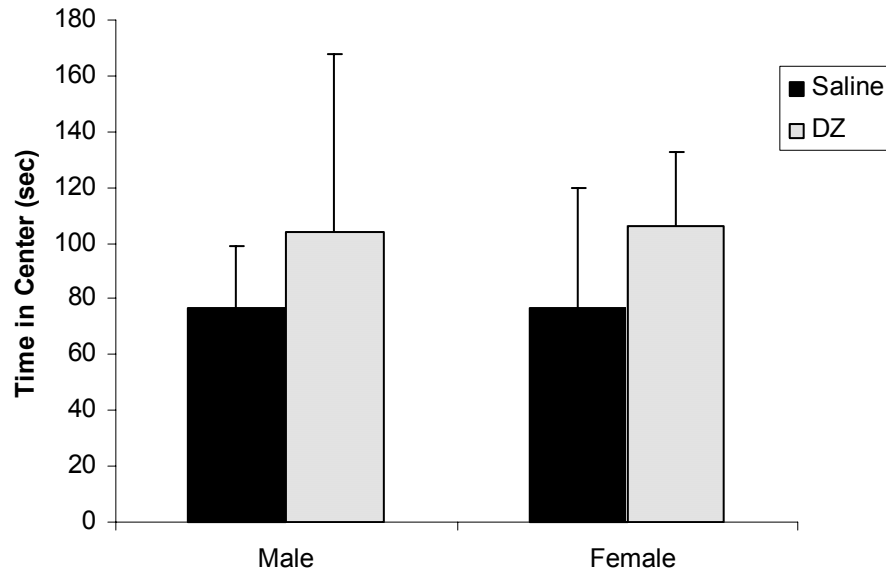
Time Spent in Center

Figure 4. Mean amount of time spent in the center by the four experimental groups. No significant sex or drug status differences were found, nor was a sex by drug status interaction found.

Figure 5
 Number of Cells Positive for Fos-LI in mPFC

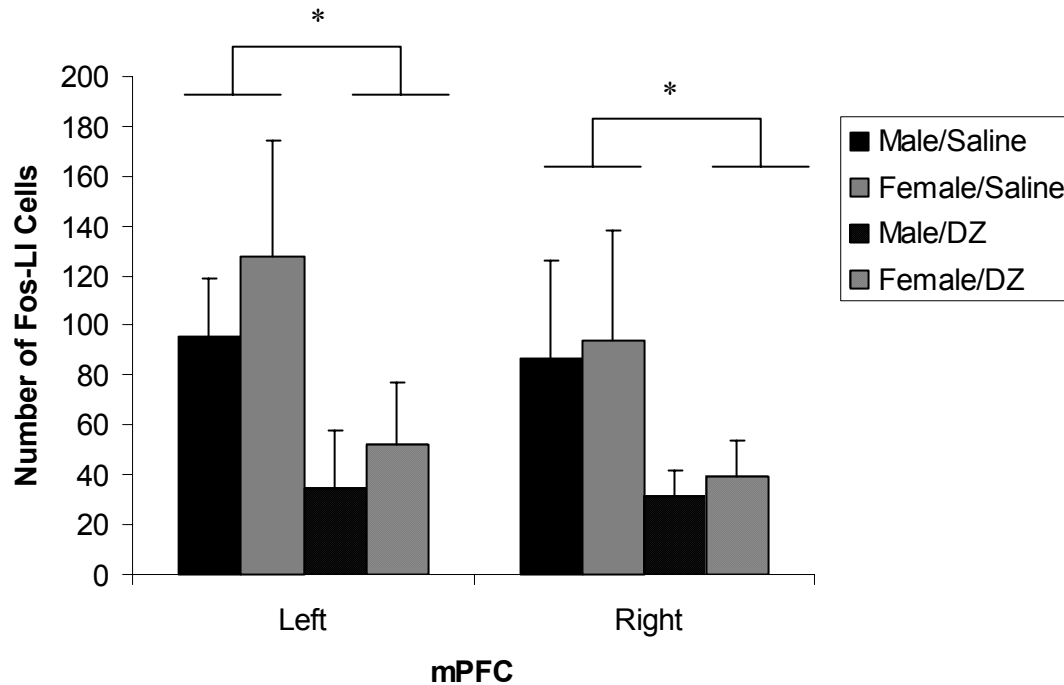


Figure 5. Mean number of cells positive for Fos-LI for left and right medial prefrontal cortex for each experimental group. Significant main effect of hemisphere was found, with a greater number counted in the left mPFC ($M = 75.23$, $SD = 46.125$) than in the right mPFC ($M = 60.59$, $SD = 39.110$), ($p = 0.020$). A main effect of drug status was also found, with fewer cells counted in drug-treated animals ($M = 40.417$, $SD = 16.729$) than in saline-treated animals ($M = 100.90$, $SD = 35.744$). Means were collapsed across hemisphere and sex. No main effect for sex was found. No significant interactions of sex by drug status, hemisphere by sex, hemisphere by drug status, nor hemisphere by sex by drug status were found.

* $p < 0.001$

Figure 6

Mean Number of Cells Positive for Fos-LI in MeA

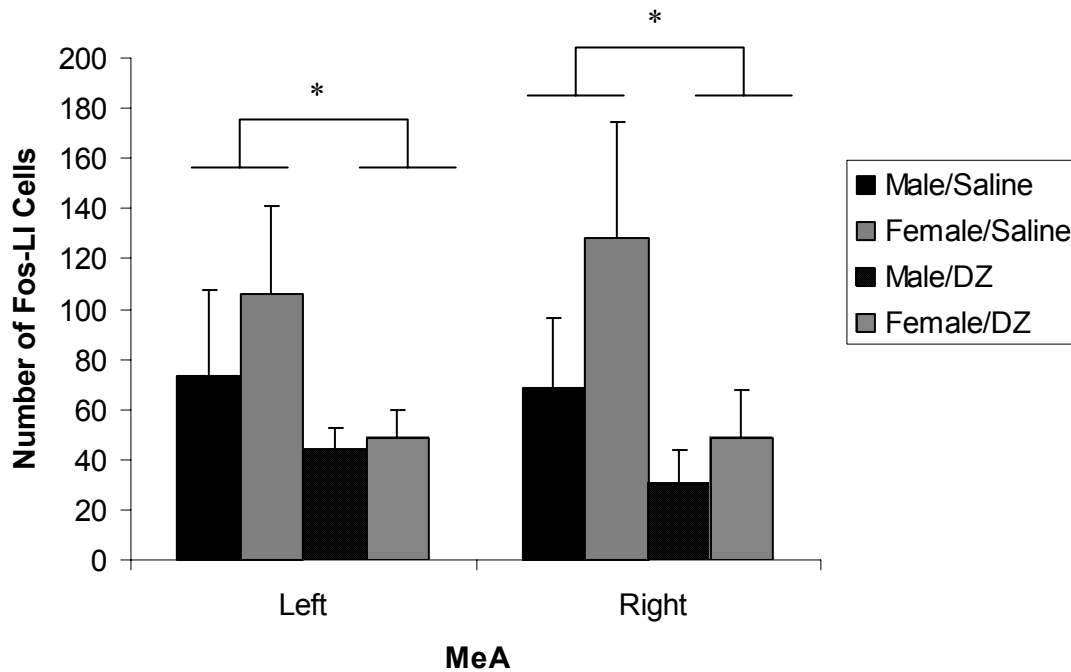


Figure 6. Mean number of cells stained for Fos-LI in the left and right medial nuclei of the amygdala. A significant hemisphere by sex interaction was found, however, post-hoc analyses did not elucidate the source of significance ($p = 0.018$). No significant interactions were found for hemisphere by drug status, sex by drug status, or hemisphere by sex by drug status. A main effect of sex was also found, with females showing higher cell counts ($M = 77.167$, $SD = 43.953$) than males ($M = 54.050$, $SD = 26.348$), ($p = 0.015$). Additionally, a main effect of drug status was also found, with lower numbers of stained cells in drug-treated animals ($M = 43.917$, $SD = 12.343$) in comparison to saline-treated animals ($M = 93.950$, $SD = 40.937$). There was no significant main effect of hemisphere.

* $p < 0.001$