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Sex matters: Females in proestrus show greater diazepam anxiolysis and BDNF- and parvalbumin-positive neurons than males

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Abstract

In humans and animal models, sex differences are reported for anxiety-like behavior and response to anxiogenic stimuli. In the current work, we studied anxiety-like behavior and response to the prototypical anti-anxiety drug, diazepam. We used 6th generation outbred lines of adult Long Evans rats with high and low anxiety-like behavior phenotypes to investigate the impact of proestrus on the baseline and diazepam-induced behavior. At three doses of diazepam (0, 0.1 and 1.0 mg/kg, i.p.), we measured anxiogenic responses on the elevated plus maze of adult male and female rats. We assessed parvalbumin and brain-derived neurotrophin protein levels in forebrain and limbic structures implicated in anxiety/stress using immunohistochemistry. At baseline, we saw significant differences between anxiety lines, with high anxiety lines displaying less time on the open arms of the elevated plus maze, and less open arm entries, regardless of sex. During proestrus, high anxiety females showed less anxiety-like behavior at 0.1 mg/kg while low anxiety females displayed less anxiety-like behavior at 0.1 and 1.0 doses, relative to males. Brain-derived neurotrophin protein was elevated in females in the medial prefrontal cortex and central amygdala

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest and also declare that the animal experiments comply with the current laws in the United States.

Author Contributions Statement

Authors Ravenelle and Donaldson designed the study; author Berman ran the statistics and wrote Data Analysis section; authors Asumadu and La wrote and piloted GUI with support from Dr. Yelleswarapu. All authors worked on edits and the final manuscript; authors Donaldson, La and Yelleswarapu wrote and edited the manuscript and finalized the graphics. Authors Mason, Ravenelle and Asumadu ran behavioral and post-mortem immunohistochemical studies.

Data Accessibility Statement

All data have been shared and archived in a public repository. They will be available through the EJN's data share (Figshare).

while parvalbumin-immunoreactive cells were greater in males in the medial prefrontal cortex. Parvalbumin-positive cells in high anxiety females were higher in CA2 and dentate gyrus relative to males from the same line. In sum, when tested in proestrus, females showed greater anxiolytic effects of diazepam relative to males, and this correlated with increases in neurotrophin and parvalbumin neuron density in corticolimbic structures.

Graphical abstract

Using outbred lines of animals phenotyped as showing high (HAn) and low anxiety (LAn)-like behavior, we confirmed HAn F6 offspring were less active on the open arms of the elevated plus maze (EPM) at baseline. HAn and LAn females were more active on the open arms of the EPM after diazepam treatment. Using a graphic user interphase, we counted a greater number of positively stained parvalbumin neurons (i.e., that stain a subset of GABA neurons) in CA2 and DG of the hippocampus.

Keywords

elevated plus maze (EPM); Long Evans rats; trait anxiety; stage of estrous

Introduction

Steroid hormones influence anxiety-like behavior (Fernandez-Guasti and Picazo 1992; Frye et al. 2000; Walf and Fryer 2007) and interact with the prototypical anti-anxiety medication, diazepam (Valium) in humans (Kelly et al. 2009) and in animals (Bitran et al. 1991; Díaz-Véliz et al. 2000; Olvera-Hernández and Fernández-Guasti 2011; Simpson et al. 2012). In Long Evans female rats in the proestrus stage of the estrous cycle (when estradiol and progesterone levels peak), increases in anxiety-like behavior are observed (Frye et al. 2000) as well as improvements in diazepam efficacy (Díaz-Véliz et al. 2000). Progesterone and diazepam modulate the γ -aminobutyric acid (GABA) system (Gomez et al. 2002; Smith 2002; Nin et al. 2011), which is implicated in anxiety disorders (Kalueff and Nutt 2007) and important in neural development (McCarthy et al., 2002; Möhler et al., 2004).

GABA plays a major inhibitory role in mammalian cortex, and in rodents, distinct calcium-binding proteins are localized to interneurons, nonpyramidal and pyramidal neurons, with

parvalbumin (PV) localized *almost* exclusively to GABA interneurons in mammalian cortex (layers II–V) and characterized as ‘basket- and chandelier’-like neurons that modulate pyramidal cells (Mikkonen et al. 1997; Pantazopoulos et al., 2006). Anti-anxiety drugs work, at least in part, by acting on PV-expressing GABA interneurons in the basolateral amygdala (Hale et al. 2010). Increases in PV-positive neurons have been demonstrated in the basolateral amygdala of rats reared in enriched environment, and these changes are associated with reduced anxiety-like behavior (Ravenelle et al. 2014; Urakawa et al. 2013).

Estrous changes also relate to fluctuations in brain-derived neurotrophin factor (BDNF) (Simpson et al. 2012), with BDNF expression or efflux peaking and falling with rising estradiol and declining progesterone, respectively (Bath et al. 2012; Sato et al. 2007). Early findings that increases in BDNF expression co-occur with distinct ages and stage of estrous cycle changes in estradiol, suggesting that the rise and fall of transcription factors such as BDNF may parallel changes in estradiol to contribute to hormonal regulation of behaviors (Toran-Allerand et al. 1999; Scharfman and MacLusky, 2006). Interestingly, fluctuations in BDNF are implicated in anxiety-like behavior in animals (Bath et al. 2012) and the pathogenesis and treatment of anxiety disorders in humans (Cohen et al. 2010). Further, when a single nucleotide polymorphism in the BDNF gene (Val66Met) was introduced into a mouse model (leading to lower BDNF), increased anxiety-like behavior was observed in the prepubertal and early adulthood stages, especially during estrus (Bath et al. 2012). Moreover, changes across the age axis in the levels of estradiol, are likely linked to cognitive and affective changes at adolescence and menopause (Scharfman and MacLusky 2006), as are age-related changes in BDNF levels (Croll et al. 1998; Mattson, Maudsley and Martin, 2004; Silhol et al., 2005).

The current study was designed to extend our previous findings showing greater diazepam effects on EPM in males displaying high anxiety-like behavior compared to females, and GABA neuron density positively correlating with low anxiety-like behavior. While we did not show similar diazepam effects on females in that work, we did not isolate the stage of estrous (Ravenelle et al. 2014). It is probable that given the variable stages of estrous for the females, there is greater variability in the female group. In addition, early work on inbred strains of high (HAB) and low (LAB) anxiety-like behavior Wistar rats, demonstrated males from the HAB line were more sensitive to the effects of 1.0 mg/kg diazepam (Liebsch et al., 1998; Bert et al., 2001). Given the preponderance of evidence reviewed suggesting estradiol and progesterone interact with EPM behavior and the anxiolytic effects of diazepam, possibly working via GABA and BDNF, we hypothesized that female rats synchronized to proestrus would show greater sensitivity to the anxiolytic effects of diazepam. We also expected that female rats would show these anxiety-like patterns that were correlated with immunoreactive patterns for parvalbumin (that stains a subset of GABA neurons) and BDNF.

Materials and methods

Animals and Housing

We used female ($n=61$) and male ($n=55$) Long-Evans rats weighing 250–350 g and 400–550 g, respectively. Animals were filial 6 (7th generation), derived from inter-generational

mating of extreme trait anxiety animals bred at the University of Massachusetts Boston vivarium. Original (F0) parental lines were obtained from Charles River Breeding Laboratories (Wilmington, MA) and phenotyped using the elevated plus maze, (i.e., lower quartile for percent time on open arms (OA) designated high anxiety (HAN) and upper quartile for %OA time, low anxiety (LAN)). HAN and LAN females (n=29–33 per group) were mated with unrelated same phenotyped males. Subjects were housed in pairs according to sex in polystyrene cages, with food and water available *ad libitum* and maintained on a 12h:12h light/dark cycle (lights on at 0700h). All protocols adhered to the applicable portions of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and received approval from the IACUC of the University of Massachusetts Boston.

Elevated Plus Maze

Anxiety screening and behavioral testing was accomplished using the elevated plus maze (EPM), a plus-shaped black Plexiglas apparatus (Med Associates, VT, USA) with a set of opposing open arms ($50 \times 6.5 \text{ cm}^2$) without walls and a set of opposing arms ($50 \times 6.5 \text{ cm}^2$) with high-enclosed walls ($50 \times 6.5 \times 15 \text{ cm}^3$) connected via a central platform ($6.5 \times 6.5 \text{ cm}^2$). On a given test day, same-sex animals were tested for a total of 5 min; each rat was placed at the central platform (elevated 70 cm above the floor), alternating placement with facing open and closed arms; percent open arm time and entries, as well as total number of entries were collected with the aid of automated Med Associates software. Animals were tested on EPM on four separate occasions, separated by a 3-week inter-trial interval (ITI) and novel maze placement. This ITI was adopted based on findings suggesting that this ITI is sufficient to remove the one-trial tolerance (Adamec and Shallow 2000; Ravenelle et al. 2014). Testing sessions consisted of a baseline test where no drug was administered (Baseline, $n=61$ females and $n=55$ males) followed by three counter-balanced sessions of diazepam (0.1 and 1 mg/kg) and vehicle administered 30 min during habituation to the testing area ($n=20$ females and $n=20$ males). We have previously found anxiety-like behavior on the elevated plus maze and novel object tests in these inter-generational lines with HAN animals showing fewer rears and less grid mobility in the center of the OFA, and making fewer approaches and contact with the novel object (unpublished findings).

Drugs and Injection Protocol

Diazepam (Sigma Chemical Co., St. Louis, MO, USA) and vehicle dosing occurred 30 min prior to the EPM; for dosing 0.1 and 1 mg/kg of diazepam (per ml; injection volume was calculated based on rats' weights) was suspended in 1% Tween (Sigma) 20/20 ml distilled water or equal volume vehicle injections of 1% Tween-20/dH₂O IP (VEH) using a within-subjects, counter-balanced design. The diazepam dose was chosen based on previously reported findings of the anxiolytic effects on EPM (Heldt et al. 2012; Ravenelle et al. 2014). On the day of testing, animals were transferred to the behavioral area and allowed to habituate for 15 min, weighed and then injected 30 min before placement on EPM.

Vaginal Cytology

We collected daily vaginal lavages for two weeks prior to testing in order to determine estrus cycling patterns, and only females showing 4-day cycles (n=20) were chosen; these females were monitored and used for all drug testing (adapted from Marcondes et al. 2002). After

placement into the study, vaginal swabs were taken to capture proestrus, and all EPM testing on females occurred during this stage, when estradiol and progesterone levels were high (Marcondes et al. 2001).

Perfusions and Immunohistochemistry (IHC)

At least seventy hours after the final diazepam and/or VEH treatment, animals (females were sacrificed during proestrus) were deeply anesthetized with Fatal Plus (Vortech Pharmaceuticals, Ltd., Dearborn, MI; 0.075–0.2 ml) and then transcardially perfused with isotonic saline followed by 4% paraformaldehyde. Brains were extracted and stored in cryoprotectant prior to frozen cryostat coronal sectioning (30 μ m). Later, we processed the free-floating sections for IHC by first rinsing in 0.05 M NaPBS for 1 h and then incubating in rabbit anti-BDNF (EMD Millipore, Billerica, USA), diluted 1:7,500 in NaPBS containing 0.4% Triton X-100 for 72 h at 4 $^{\circ}$ C. We then rinsed the sections and developed the protein stain using anti-rabbit secondary antiserum and avidin–biotin–peroxidase incubation (ABC Elite Kit, Vector Labs, Burlingame, CA, USA). Finally, the stain was revealed using 0.05% 3-diaminobenzidine hydrochloride (DAB) and 0.01% H_2O_2 . Next, the tissue was rinsed in RO H_2O before mounting on gelatin coated glass slides and covered with Permount and coverslips. We also ran IHC for parvalbumin (PV), a calcium-binding protein that selectively stains a subpopulation of GABA neurons. We followed the exact protocol as outlined above on separate coronal sections using a polyclonal antibody for PV (Millipore), diluted 1:6,000 in 0.05M NaPBS containing 0.4% Triton-X-100. We also ran immunohistochemical controls for each of the regions (i.e., hippocampal formation, VMH, CeA and mPFC), following each of the above-mentioned steps in the protocol except incubation in the primary antibody. For this step, tissue was incubated in the 0.05M NaPBS + 0.4% Triton-X-100 only.

Neuron Analysis

In order to quantify the BDNF and parvalbumin immunoreactive cells, we developed an image processing protocol using MATLAB programming of a computerized microscope setup connected to a digital camera for capturing images. Fixed brain sections were imaged using computerized Zeiss AxioObserver inverted microscope that is equipped with a CCD camera (AxioCam HRm3) connected to a desktop computer. To quantify the BDNF- and parvalbumin-positive cells, we developed a graphic user interface (GUI) using custom image processing MATLAB codes and ImageJ macros.

The BDNF images were obtained using a 40 $\times$ objective (EC Plan-Neofluar 40 $\times$ /0.75) while a 10 $\times$ objective (EC Plan-Neofluar 10 $\times$ /0.30) was used for parvalbumin-stained images. The stage of the microscope was moved in X, Y, and Z planes to obtain a Z-stack as well as tiled images, covering the entire area of the tissue sections. Experimenters used coded slides to avoid bias; each counted immunoreactive cells within regions of interest (medial prefrontal cortex, central amygdala, dentate gyrus, CA1, CA2, and CA3; Bregma range +2.2mm, –2.15 to –3.80 mm (Paxinos and Watson 2004)) within a 500 $\times$ 500 μ m reticle using every other serial section for a total of 6 sections per animal per slide (averaged), left and right sides for 6–10 animals per group. Each region of interest was then focus stacked and stitched in a batch process via ImageJ and fed into the MATLAB GUI that calculated parameters like reticle size, and reticle location for repeatability in addition to each stained structure's

indexed count, area, perimeter, circularity, eccentricity, relative stain intensity, and relative stain intensity's standard deviation, which all served as potential filter parameters.

Statistical Analyses

All graphing and statistical analyses were performed using GraphPad Prism (v. 6.0f) for Mac OS X and SPSS for Mac (v. 22.0). In order to verify normal distribution, the Kolmogorov-Smirnov test was carried out on all data. In the one instance where the data did not pass the normality test (0.1 mg/kg dose %OA time for females), an outlier test was run and the outlier was removed. Baseline activity on the EPM was analyzed using a two-way ANOVA (Between Factors: Sex and Trait) while diazepam effects were examined using a repeated measures three-way ANOVA (Between Factors: Sex and Trait; Within Factor: Drug; the levels of the Drug Factor were: VEH, 0.1 and 1.0 mg/kg). Where appropriate, *post hoc* tests were performed to make pairwise comparisons using the Holm-Sidak method with alpha adjustments. We also ran Pearson *r* correlations (two-tailed) to test the relation between the variables (behavioral data and immunohistochemical findings). Alpha was set at $p < 0.05$ for all analyses; exact *p* values are reported.

Results

Baseline Activity on EPM

At baseline, differences for Trait (main effect, $F_{1,112} = 21.96$, $P = 0.0002$) were observed with LAn animals having more %OA time relative to HAn (Fig. 1A); pairwise comparisons indicated LAn and HAn lines differed for proestrus females ($P = 0.00042$) and males ($P = 0.005$). The Trait $\times$ Sex interaction approached significance (interaction effect, $F_{1,112} = 3.753$, $P = 0.0552$). Analysis of baseline %OA entries also revealed differences for Trait (main effect, $F_{1,112} = 19.42$, $P = 0.0001$), all LAn animals had greater %OA entries relative to their HAn counterparts with LAn males significantly different from HAn males ($P = 0.0000764$) (Fig. 1B). When compared to males, proestrus females made many more Total Entries as indicated by a significant Sex effect (main effect, $F_{1,112} = 8.738$, $P = 0.0038$), and LAn tended to be more active than HAn animals, Trait effect approached significance (main effect, $F_{1,112} = 3.116$, $P = 0.0802$) (Fig. 1C).

Effects of diazepam on EPM

Following pretreatment with diazepam, we observed significant sex differences for %OA time, Sex effect (main effect, $F_{1,36} = 12.469$, $P = 0.000219$) and the Drug $\times$ Sex approached significance (interaction effect, $F_{1,36} = 4.028$, $P = 0.054$), with proestrus females showing greater anxiolytic effects of diazepam, particularly at the high dose (1.0 mg/kg). LAn animals showed greater increases in %OA time (main effect of Trait, $F_{1,36} = 8.373$, $P = 0.009$) as well as a Sex $\times$ Trait interaction (interaction effect, $F_{1,36} = 4.578$, $P = 0.037$) effect (Fig. 2A). There were also notable dose-dependent increases in %OA time as revealed by a significant effect of Drug (main effect, $F_{2,108} = 14.184$, $P = 0.000064$) and an interaction of Drug $\times$ Sex (interaction effect $F_{2,108} = 3.378$, $P = 0.00645$). Pairwise comparisons indicated %OA time for the 1.0 mg/kg dose differed from VEH ($P = 0.00315$) and the 0.1 mg/kg dose ($P = 0.000042$) (Fig. 2A).

Female rats in proestrus had a greater percentage of OA entries, Sex effect (main effect, $F_{1,36} = 15.349$, $P = 0.000383$) as did the LAn line, Trait effect (main effect $F_{1,36} = 6.027$, $P = 0.01905$), with the most notable differences for LAn females, Sex $\times$ Trait effect (interaction effect, $F_{1,36} = 4.687$, $P = 0.037107$). The high dose of diazepam induced the greatest anxiolytic effects, Drug effect (main effect $F_{2,36} = 10.514$, $P = 0.0041$), especially in LAn animals, Drug $\times$ Trait interaction (interaction effect, $F_{2,36} = 4.259$, $P = 0.00715$). Multiple comparisons indicated the high dose of diazepam differed from VEH and the low dose for LAn females in proestrus ($P = 0.000081$) and LAn males ($P = 0.0410$) (Fig. 2B). Evaluation of total entries indicated no diazepam related differences (Fig. 2C).

BDNF immunoreactivity (ir)

We used MATLAB programming to process the low magnification images of the stained tissue and the GUI to identify regions of interest and quantify the positively stained cells. There were significant increases in BDNF protein levels in mPFC for females in proestrus, Sex effect (main effect, $F_{1,37} = 17.041$, $P = 0.00001$) and LAn animals, Trait effect (main effect, $F_{1,37} = 11.719$, $P = 0.0021$) (Fig. 3A); no significant Sex $\times$ Trait effect was found (interaction, $F_{1,37} = 0.353$, $P = 0.043$). Analysis of BDNF protein levels in the CeA indicated HAn animals had a greater number of BDNF positive cells, Trait effect (main effect $F_{1,39} = 4.873$, $P = 0.046$) and that this was most pronounced for HAn females, Sex $\times$ Trait effect (main effect, $F_{1,39} = 8.21$, $P = 0.003$) (Fig. 3B) while evaluation of the VMH-ir did not reveal any significant differences (Fig. 3C). Figure 4A–D includes a photomicrograph of a coronal section at Bregma +2.70mm indicating the mPFC (square) and representative micrographs with the reticule placement for counts.

Parvalbumin immunoreactivity (ir)

Applying the GUI to quantification of stained cells in the mPFC, we observed significant increases in parvalbumin protein levels in mPFC for males, Sex effect ($F_{1,37} = 17.041$, $P = 0.0000557$) and LAn animals, Trait effect (main effect, $F_{1,37} = 11.719$, $P = 0.0023$) (Fig. 5A). We did not find a significant Sex $\times$ Trait effect (interaction, $F_{1,37} = 0.353$, $P = 0.2222$, *n.s.*). In the CeA, we noted a significant increase in PV-ir in HAn proestrus females, Sex effect (main effect $F_{1,37} = 6.530$, $P = 0.0129$), and in HAn lines (Trait effect, $F_{1,37} = 13.375$, $P = 0.0064$) with no significant interaction. In CA1, however, the Sex effect only approached significance ($F_{1,38} = 3.861$, $P = 0.057$) while in CA2, ANOVA revealed significant Sex (main effect, $F_{1,38} = 11.962$, $P = 0.00208$), trending significant Trait ($F_{1,38} = 3.800$, $P = 0.059$), and significant Sex $\times$ Trait interaction effects ($F_{1,38} = 20.581$, $P = 0.000014$). HAn proestrus females displayed the greatest number of PV immunoreactive cells (HAn vs. LAn females, $P = 0.0389$; pairwise comparison with Bonferroni adjustment) (Fig 5B). For CA3, we failed to observe a significant Sex or Trait, or Sex $\times$ Trait effects. In the granular layer of the hippocampus (dentate gyrus), we found a significant Sex effect ($F_{1,38} = 4.184$, $P = 0.0076$) but no other main or interaction effects, indicating no difference in PV-positive cells for any of the other conditions (Fig. 5C). Figure 6A–D depicts a line drawing of a coronal section of the rat brain, indicating the placement of the reticule in the drawing and the photomicrographs. All positively-stained cell counts were made with the use of the GUI to reduce experimenter bias and to increase reliability (Fig. 7 GUI).

Pearson *r* correlations

To determine associations between EPM outcomes and the immunohistochemical findings for BDNF and parvalbumin, Pearson *r* correlations were performed. Baseline %OA time and %OA entries showed a significant positive association ($r = 0.549$, $P = 0.000245$ (two-tailed)) thus, we report correlations with %OA time only. Baseline %OA time was significantly positively correlated with PV-ir in CA3 ($r = 0.441$, $P = 0.039775$), indicating the greater the activity on open arms, the greater the PV-ir. Percent Open Arm Time at each drug dose was positively correlated, VEH, 0.1 and 1.0 ($r = 0.407$, $P = 0.009122$; $r = 0.343$, $P = 0.030091$, respectively) and 0.1 and 1.0 mg/kg ($r = 0.719$, $P = 1.7412E-7$) (Table 1).

BDNF-ir in the CeA mPFC was significantly negatively correlated with diazepam-induced %OA Time ($r = -0.470$, $P = 0.023631$), demonstrating that lower BDNF protein levels were found in central amygdala when high %OA Time was recording. This suggests that neurotrophin levels in CeA may negatively impact anxiolytic responding. BDNF-ir in mPFC was significantly negatively correlated with PV-ir in mPFC ($r = -0.473$, $P = 0.022531$), indicating that when rats show more BDNF-ir stained cells in mPFC they show diminished PV-ir stains in the same regions (Table 1).

Discussion

Our findings demonstrate that female rats synchronized to the proestrus stage show a greater preference for the open arms on the EPM following diazepam treatment, compared to males. Female rats in proestrus and phenotyped as low anxiety made the most %OA entries and had the greatest %OA time. These findings support our hypothesis for synchronizing stage of estrous to minimize variance in the female group; as such, we were able to see sexually dimorphic effects of the anti-anxiety effects of diazepam, and for sex and trait differences in anxiety-like behavior on the EPM. After the termination of testing, we found sex-dependent differences in BDNF- and PV-ir in the mPFC, CeA, and hippocampal formation (CA2 and DG). We also found differences that were related to trait anxiety, notably greater BDNF-positive cells in mPFC for HAn animals. These findings also support our expectations for positive correlations between BDNF- and PV-ir and activity on the EPM. This relation is suggestive of a role for BDNF and PV protein levels in the variable responses to diazepam and the EPM test in females synchronized to proestrus.

Extreme trait anxiety rodent phenotypes are often used to study the biological and neuronal changes associated with anxiety disorders (Liebsch et al. 1998; Landgraf and Wigger 2002; Landgraf and Wigger 2003; Ravenelle et al. 2013). For example, animals displaying high anxiety-like behavior spend significantly less time on the open arms relative to those displaying low anxiety-like behavior (Landgraf and Wigger 2002; Landgraf and Wigger 2003; Millan 2003 for review; Ravenelle et al. 2013; Stead et al. 2006), as was reported in our current 7th generation of HAn / LAn lines. We extend those results with evidence for proestrus females displaying more percent OA time and entries (tested when estradiol and progesterone levels were rising) compared to males on baseline and VEH testing days (see also Weintraub et al. 2010). However, depending on the conditions during EPM testing (i.e., light intensity, administration of estradiol), increased open arm time can be found during other stages of the estrous cycle (Díaz-Véliz et al. 1997; Löfgren et al., 2006; Marcondes et

al. 2001; Mora et al. 1996) or decreased diazepam-anxiolysis following estradiol administration in ovariectomized rats (Nomikos and Spyraiki 1998). Moderate doses of progesterone (10 mg/kg) reduced anxiety-like behavior on the EPM (i.e., increased open arms) and while we did not confirm stage of estrous by measuring estradiol and / or progesterone levels, we used vaginal cytology to confirm proestrous. Additional work is needed where these levels are measured along with more exhaustive work including comparison female groups with hormone levels manipulated.

Our results also indicate that there were sex-dependent interactions with diazepam treatment, with both proestrus females and males showing greater anxiolytic effects following 1.0 mg/kg of diazepam, though the magnitude of the change in anxiety-like behavior was most pronounced in proestrus females. Conflicting results are found in the literature, with some studies illustrating an anxiolytic effect with acute treatment (1.0 mg/kg dose diazepam) and others failing to show any differences with chronic diazepam treatment or following a no-drug pre-maze exposure (Pellow et al. 1985; Cole and Rodgers 1995; Rodgers and Shepherd 1993; Rodgers et al. 1997; Violle et al. 2009). Additional differences might emerge based on test parameters; for instance, sexually mature female rats show less anxiety-like behavior than senescent, aged females (Byrnes and Bridges 2006). Simpson and colleagues (2012) reported sex-related differences in EPM activity, with females showing greater baseline movement in general, and on open arms in particular, using a randomized behavioral test schedule in an attempt to minimize distinct hormone effects on behavior, however, they did not monitor estrous cycle.

BDNF protein was elevated in proestrus females and in the HAn line in mPFC and CeA, while parvalbumin (that stains a subset of GABA interneurons and is activated during anxiety testing) immunoreactive cells were greatest in males in the same region. In proestrus females, PV-ir was significantly greater in CA2 and DG relative to males from the same line. While our results only point to a correlation between these changes and anxiety-like behavior, it is probable that BDNF and GABA proteins may contribute to the differential effects on anxiety-like behavior in proestrus females. For instance, earlier work demonstrates that the estradiol receptor alpha subtype (ER α) is co-localized with GABA in the hippocampus and implicated in development and adult plasticity (Rai and Jeswar 2012). Thus, it is not surprising that the response to diazepam is impacted by steroid hormone level given that metabolites for progesterone facilitate GABA efflux (Nin et al. 2011; Smith 2002). Moreover, Earnheart and colleagues (2007) demonstrate a link between diminutions in GABA_A receptor function and hippocampal neurogenesis and anxiety-like behavior.

The association between BDNF, changes in anxiety-like behavior and diazepam effects has also been investigated. A depression in transduction signaling critical for neuroplasticity (e.g., CAMKII, BDNF) is evident in mice that have been treated with diazepam (Huopaniemi et al. 2004). Pontén et al. (2011) reported changes in adult mice response to diazepam following neonatal exposure to propofol, and speculated these modifications were a result of increased neonatal BDNF levels in the prefrontal cortex and hippocampus, suggesting these changes might contribute to the later-life up-regulation of GABA_A, as reported by others (Lund et al. 2008). The present immunohistochemical findings showing greater BDNF protein levels in mPFC and increased PV-ir in dentate gyrus hippocampal

regions of female rats, support a potential association between BDNF, PV and the anxiolytic effects of diazepam.

Taken together, our data relate differences in BDNF protein levels and PV-positive GABA neurons to sex disparity in anxiety-like behavior and response to diazepam. Additional work is needed to provide direct evidence of this link between GABA density, BDNF protein, and estrogen and/or progesterone levels, and to examine whether similar findings regarding sex-diazepam interactions are observed in other behavioral tests, and relative to females at different stages of the estrous cycle.

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Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophin factor
CAMIIK	calmodulin-dependent protein kinase II
CA2	cornus ammonis
EPM	elevated plus maze
F0	filial zero (parental line)
GABA	gamma aminobutyric acid
HAn	high anxiety-like behavior
IACUC	Institutional Animal Care and Use Committee
IHC	immunohistochemistry
ir	immunoreactive
ITI	inter-trial interval
LAn	low anxiety-like behavior
mPFC	medial prefrontal cortex
NaPBS	sodium phosphate buffered saline
OA	open arm
PV	parvalbumin
RO	reverse osmosis

VEH vehicle (% Tween-20/dH₂O)

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Fig. 1.

A–C Females in proestrus and LAn animals display less anxiety-like behavior on EPM. (A) At baseline, %OA time on EPM differed between HAn and LAn lines with the latter showing less anxiety-like behavior (i.e., greater %OA time). (B) LAn animals also made more %OA entries relative to HAn. (C) Finally, females were more active in general with more total entries on the EPM. *** $p < 0.001$, HAn compared to LAn females; ## $p < 0.01$ and ### $p < 0.001$, HAn compared to LAn males. $N = 53$, ($n = 20$ – 33 animals per group).

Fig. 2.

A–C Diazepam elicits dose-dependent anxiolytic effects in LAn proestrus females. (A) %OA time was markedly increased in proestrus females from LAn lines following diazepam, and the drug induced anxiolytic behavior in a dose-dependent manner. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$, relative to VEH and 0.01 mg/kg dose, same trait. (B) Similar patterns were observed for %OA entries, greater anxiolytic behavior in proestrus females from LAn breeding lines, and in a dose-dependent manner. $*p < 0.05$, $***p < 0.0001$, relative to VEH and 0.01 mg/kg dose, same trait. (C) No significant differences between the sexes or anxiety lines were found for total entries. $N=40$, ($n=10$ animals per group).

Fig. 3.

A–C Increases in BDNF immunoreactivity in corticolimbic regions vary by sex. (A) Females in proestrus and HAn lines showed increased BDNF-positive cells in the mPFC, **** $p < 0.0001$, females relative to males; # $p < 0.05$, compared to LAn, same sex. (B) All females in proestrus displayed greater BDNF-ir in the central amygdala; * $p < 0.05$, females relative to males; # $p < 0.05$, compared to LAn, same sex. (C) No differences were noted for the ventromedial hypothalamus. N=6–10 animals per group (6 sections, left and right sides averaged).

Fig. 4.

A–D Sex differences in BDNF-immunoreactivity in the mPFC. Photomicrographs imaged using bright-field to show low magnification coronal section (Bregma +2.70 mm) indicating the placement of the reticule for counting, BDNF-stained cells in animals phenotyped as HAn / LAn. Cells were counted within 250 μm^2 region (square) in each brain section for representative (A) LAn proestrus female (B) HAn proestrus female (C) LAn male (D) HAn male; scale bar, 200 μm (for all images).

Fig. 5.

A–C Males and proestrus females from extreme trait anxiety lines show distinct parvalbumin protein levels. (A) PV-ir was elevated in males in the mPFC, $**p < 0.01$, males relative to females. (B) Significant increases in PV-positive cells were noted in LAn females in CA2, $\#p < 0.05$, relative to HAn females. (C) Female rats in proestrus showed PV-ir that was elevated in the dentate gyrus relative to males, $**p < 0.01$. $N = 17$ – 20 animals per group.

Fig. 6.

A–D Parvalbumin positive cells in mPFC are variable between the sexes. The figure shows a line drawing with a box insert identifying the location for counting in the mPFC (Bregma +3.20 mm) (above); photomicrographs from representative animals showing placement of reticule and PV-ir cells (below) (A) LAN proestrus female (B) HAN proestrus female (C) LAN male (D) HAN male; scale bar, 500 μ m (for all images).

Fig. 7.

A–D Screen shot of the MATLAB graphic user interface (GUI) used to quantify the immunoreactive cells. **Panel A** contains file read and save locations, reticle size and scale factor selection windows, and the image processing filter list. Here, the user selects an image to be processed from the list of raw images. In **Panel B**, the top image is a placeholder for where the selected image will be displayed. Here, the user can select the region of interest, which will be displayed in the bottom at higher magnification. Using the slide in **Panel C**, one can adjust filter parameters and refresh the calculations via the "Analyze" button on the top right. When the user is satisfied, data can be outputted via the "Save Data" button to a location defined along with a time stamp. The data output, **Panel D**, includes: an image list index, stained object index, sample name, ROI, handedness, total positively stained cell counts including those on the border, area (μm^2), aspect ratio, circularity, eccentricity, perimeter, mean stained structure intensity, standard deviation of stained structure intensity, cursor position reticle center, reticle size, the image's global maximum intensity, and the image's global minimum intensity.

Table 1

Correlation matrix of multiple variables measured for LAn and HAn animals

	Mean	SEM	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
(1) BL % OA Time	0.386	0.01	----								
(2) BDNF (mPFC)	40.37	1.77	-.091	----							
(3) BDNF (CeA)	22.56	1.44	-.215	.211	----						
(4) PV (mPFC)	5.19	0.23	.016	-.473*	-.276	----					
(5) PV (DG)	3.11	0.21	-.136	.495*	-.100	-.165	----				
(6) PV (CA3)	2.53	0.13	.441*	.112	-.208	-.331	.174	----			
(7) VEH	0.36	0.03	-.295	-.028	-.048	.201	.075	.028	----		
(8) 0.1 (mg/kg)	0.35	0.03	.126	.183	-.406*	.348	.312	-.348	.407**	----	
(9) 1.0 (mg/kg)	0.47	0.03	.096	.242	-.336	-.023	.323	-.237	.343*	.719	----

* $p < 0.05$,** $p < 0.01$,*** $p < 0.0001$

Abbreviations: BL % OA Time, baseline percent open arm time; BDNF, brain-derived neurotrophin factor; PV, parvalbumin; CeA, central amygdala; mPFC, medial prefrontal cortex; CA1, 2, 3 Cornu Ammonis (Ammon's horn); DG, dentate gyrus.