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Sex differences in diazepam effects and parvalbumin-positive GABA neurons in trait anxiety Long Evans rats

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Abstract

In clinical populations, prevalence rates for a number of anxiety disorders differ between males and females and gonadal hormones are thought to contribute to these differences. While these hormones have been shown to modulate the anxiolytic effects of the benzodiazepine agonist diazepam in some models, findings are inconsistent. Here, we tested for sex differences in response to anxiogenic stimuli following a 30-min diazepam (1.0 mg/kg) pre-treatment in male and female rats showing high (HAn) and low (LAn) anxiety-like behavior on the elevated plus maze. Acute diazepam administration resulted in decreased anxiety-like behavior only in HAn males as demonstrated by a significant increase in percent open arm time in the elevated plus maze (EPM). Immunohistochemical analysis for parvalbumin (PV; a calcium-binding protein that selectively stains GABAergic neurons) in central amygdala (CeA), caudate putamen (CPu) and the hippocampus indicated the number of GABAergic interneurons in these areas differed across sex and anxiety trait. In the CPu, females had significantly more PV-immunoreactive (IR) cells than males, and LAn females had greater PV-IR neurons than HAn females. In the CeA, males displayed an increased number of PV-IR neurons compared to females, with no differences found between LAn and HAn. Further, trait differences were evident in the CA2 region of the hippocampus, regardless of sex. Taken together, these data suggest that gonadal hormones and trait anxiety may influence the sensitivity to the anti-anxiety effects of diazepam and these differences may be due in part to the distribution of GABA-containing interneurons.

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Keywords

Anxiety; GABA; Diazepam; Elevated Plus Maze; Striatum; Amygdala

1. Introduction

It is estimated that 15% of the world's population is suffering from an anxiety disorder (Narrow et al., 2002) with lifetime prevalence rates at 19% for males and 31% for females (Bremner 2004). Pre-clinical models in which animals are selectively bred for enhanced anxiety reactions are a helpful tool used in neurobiological studies of anxiety-like traits (Landgraf and Wigger, 2002; Landgraf and Wigger, 2003). Although alterations in γ -aminobutyric acid (GABA) transmission have been implicated, the exact mechanisms underlying anxiety disorders remain elusive (Heidt et al., 2012).

Classic benzodiazepines such as diazepam are commonly used to treat anxiety disorders. Diazepam facilitates action at GABA_A receptors and these receptors are found throughout limbic brain regions, including the caudate putamen (CPu), the central nucleus of the amygdala (CeA) and the CA1 and CA2 regions of the hippocampus (Bristow and Martin, 1988; Heidt et al., 2012). Previous studies have illustrated that benzodiazepines also modulate neuroendocrine function (De Souza, 1990; Giordano et al., 2003), however, the exact effects of diazepam on the HPA axis are not yet fully understood. Selectively bred male rats classified by high or low anxiety-like behavior show differential anxiolytic reactions to diazepam (1 mg/kg). While high anxiety-like behavior animals exhibit a diazepam-induced decrease in anxious behavior as indicated by a 20-fold increase in percent open arm time on the elevated plus maze (EPM), the diazepam-induced decrease in anxious behavior in the low anxiety-like behavior animals is 2.5 fold (Liebsch et al., 1998). Although both anxiety lines exhibit elevated corticosterone (CORT) and adrenocorticotrophic hormone (ACTH) levels post EPM exposure, this increase is more pronounced in high anxiety-like behavior animals (Liebsch et al., 1998).

Sex differences have been observed in anxiety disorders with females exhibiting up to three times the prevalence rate for general anxiety disorder (Yonkers and Ellison, 1996). Animal models have also illustrated sex differences in anxiety on several measures including the EPM, open field and social interaction tests (Gonzalez et al., 1998) suggesting a possible influence of hormones on anxiety states. Both estrogen receptor (ER) forms alpha and beta have been implicated in GABAergic cell regulation and amygdala GABA_A cell densities are associated with general activity on the EPM (Yilmazer-Hanke et al., 2003). Female ERalpha knock-out (KO) mice exhibit increases in spontaneous activity while ERbeta KO mice spend less time on the open arms of the EPM indicating loss of ERbeta subtype increases anxiety-like behavior (Krezel, et al., 2001; Ogawa et al., 2003). Circulating gonadal hormones are also believed to influence sensitivity to benzodiazepines in humans (Sundström et al., 1997) although in rats, previous work has found that intact females and males both show an increase in open arm time and entries following acute and chronic diazepam administration (Singwald 2007). Estrogen has been found to rapidly alter dopaminergic neurotransmission through membrane-bound receptors on GABAergic neurons in the CPu (for review, see

Becker 1999), an area that has been implicated in anxiety states in both clinical and animal models (Hasler et al., 2007; Favilla et al., 2008).

Still other brain regions have been shown to contribute to the behavioral expression of anxiety. Immediate early gene neural mapping of anxiogenic stimuli has revealed that in anxiety paradigms fear and stress areas often overlap (Singewald 2007) and acute diazepam administration increases c-fos-immunoreactive (IR) cells in the hypothalamic paraventricular nucleus (PVN), CeA and the supraoptic nucleus (SON) (Rodgers and Dalvi, 1997). High anxiety behavior animals show a blunted c-fos IR response in the medial prefrontal cortex (mPFC) after diazepam administration compared to their low anxiety behavior counterparts (Herman et al., 2005). The amygdala, an important part of the limbic system and regulatory region for HPA axis activation, shows differential c-fos-IR in trait anxiety animals with enhanced reactivity in high anxiety lines (Anand and Shekhar, 2003; Blanchard et al., 2005; Frank et al, 2006; Kalueff and Nutt, 2007). The hippocampus has also been implicated in the regulation of the HPA axis as chronic stress induces dendritic atrophy and decreased glucocorticoid receptor expression in this region (Ulrich-Lai and Herman, 2009). Moreover, local GABA neuron modulation has been described in many of the brain regions that show dysregulation in animals with trait anxiety, such as hippocampus (Klausberger et al., 2003) amygdala (Marowsky et al., 2005) and the CPu (Tepper and Bolam, 2004).

In the present study, we set out to determine how sex and trait anxiety might interact to influence the anxiolytic action of diazepam, and whether differences in the number of GABA interneurons in the CeA, CPu or hippocampus might correlate with baseline and/or drug responses. Females and males from the fourth filial generation were of selectively outbred high (HAn) and low (LAn) anxiety Long Evans rats tested in the EPM after both vehicle and diazepam administration and we measured the distribution of GABA neurons within regions implicated in the regulation of anxiety.

2. Materials and Methods

2.1 Experimental animals

Sixteen female (250–350g) and sixteen male (500–700g) adult Long Evans rats were obtained from pre-existing extreme trait anxiety lines bred at the University of Massachusetts Boston. The trait anxiety lines were phenotyped on the elevated plus maze, we chose animals that were in the lower quartile for percent time on open arms (OA) as high anxiety (HAn) and those in the upper quartile for most %OA time as low anxiety (LAn). HAn and LAn females (n=16 per group) were mated with unrelated same phenotyped males. For the current study, all animals were procured from filial four of the trait anxiety lines and were housed in standard Plexiglas (48 × 25 × 22 cm) laboratory cages 2–4 rats per cage according to trait anxiety and sex resulting in four groups (n=8 per group): HAn/female, HAn/male, LAn/female and LAn/male. Animals were group housed according to weight guidelines outlined in the Guide for the Care and Use of Laboratory Animals and all protocols were approved by the IACUC of the University of Massachusetts Boston.

2.2 Elevated plus maze

The EPM is a plus-shaped maze constructed of black Plexiglas (Med Associates, VT) with two opposing open arms ($50 \times 6.5 \text{ cm}^2$) without walls and two 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 ($6.5 \times 6.5 \text{ cm}^2$). The maze was set at an elevation of 70 cm above the floor. Animals were placed at the central platform and allowed to explore the maze for 5 min. Percent arm entries and percent open arm time were dependent measures of anxiety-like behavior and the total number of closed arm entries was used as an indicator of locomotor activity. All data were recorded via computer using automated Med Associates software. Animals were tested on EPM three separate times with at least three weeks between each testing session and in a novel room. This inter-trial interval (ITI) and novel maze placement was chosen based findings suggesting that this ITI is sufficient to remove the one-trial tolerance (Ademac and Shallow, 2000). Testing sessions consisted of a baseline test where no drug was administered (Baseline) and two sessions where either diazepam (1 mg/kg) or vehicle (VEH) was administered 30 min prior to EPM testing. Testing began at adulthood (PND 100) and continued at PND 145 and PND 166 for remaining EPM + VEH/Drug sessions.

2.3 Drug Treatment

Prior to placement in the EPM (30 min) animals were administered either diazepam (1 mg/kg/ml, IP) suspended in 1% Tween 20/20 ml distilled water or equal volume vehicle injections of 1% Tween-20/dH₂O IP (VEH) using a within-subjects design. The diazepam dose was chosen based on previously reported findings of the anxiolytic effects on EPM (Heidt et al., 2012). Animals were randomly assigned to drug condition in a counter-balanced manner to avoid order bias. On each test day, animals were transferred to the testing area and allowed to habituate for 15 min and then weighed before injection.

2.4 Transcardial Perfusion

At the termination of the behavioral testing, all animals were administered a lethal dose of Fatal-Plus (approximately 0.075-0.2 ml i.p.). Animals were transcardially-perfused with 0.9% isotonic saline followed by 4% paraformaldehyde to fix the brain tissue. Brains were extracted, blocked and stored in a 20% sucrose-4% paraformaldehyde and then a 10% sucrose-4% paraformaldehyde solution prior to frozen sectioning. Serial 30 μm sections were taken of the striatum, hippocampus and amygdala and stored in 4% paraformaldehyde briefly prior to immunohistochemistry.

2.5 Immunohistochemistry

Sections (30 μm) were rinsed in 0.05M NaPBS and immersed for 1 h at RT in 1.5% Goat Serum-NaPBS. Next, slices were incubated overnight at RT in the primary anti-parvalbumin rabbit pAB in 0.4% TritonX-NaPBS (1:6000; EMD Millipore, Billerica MA). Sections were then washed in 10% Goat Serum-NaPBS followed by an incubation for 1 h at RT in biotin-goat anti-rabbit in 0.4% TritonX-NaPBS (1:600; Vector Labs, Burlingame CA) and then washed in NaPBS. The sections were then incubated in an avidin-biotin solution (45:45:10,000; Vector Labs, Burlingame CA) for 1 h at RT and later flushed in NaPBS. Sections were later developed in diaminobenzidine-peroxidase solution (DAB kit; Vector

Labs, Burlingame CA) to visualize PV-IR cells. Finally, tissue was rinsed in RO H₂O before mounting on gelatin coated glass slides and made permanent. Sections were imaged at 20× magnification using a SPOT Flex Monochrome microscopy camera and SPOT 5.0 software (Diagnostic Instruments, Sterling Heights MI). PV-IR cells were counted within a 250² μm region in the area of interest using ImageJ (NIH) software. The three experimenters who made the counts were blind to the experimental condition of the representative tissue and showed high inter-rater reliability (Pearson $R = 0.98$).

2.6 Statistical analysis

We analyzed baseline differences for each outcome measure using a two-way ANOVA (sex and trait (low and high)). Comparisons for the drug condition were carried out using a three-way ANOVA with trait and sex as between subject factors and drug (VEH and diazepam) as the within-subjects factor across each of the dependent measures (%OA time, %OA entries and Total Entries) from the EPM tests. Separate, two-way ANOVAs were used to determine differences in the number of PV-IR cells for each brain region with sex and trait as between-subjects factors. Where appropriate, we used Bonferroni post-hoc tests for pair-wise comparisons. We also ran a Pearson r correlation matrix to determine the relation between the immunohistochemical data (parvalbumin immunoreactivity) and the behavioral data (%OA Time and %OA entries). Data were analyzed and graphed (group means $\pm$ SEM) using GraphPad Prism for Mac OS X (v. 5). 3.

Results

3.1 Baseline activity on the EPM (%OA time and %OA entries)

We measured anxiety-like behavior (%OA time, %OA entries) and general activation (total activity) on the EPM three weeks before the start of drug trials to establish that the offspring (filial 4) of selectively bred HAn and LAn offspring exhibited the expected phenotypes and to provide a baseline for subsequent testing. As expected, HAn animals exhibited higher anxiety-like behavior on the %OA time measure (decreased %OA time) compared to LAn animals as indicated by a significant main effect of Trait [$F(1,28)=35.22, p<0.0001$]. In addition, the analysis indicated a significant main effect of Sex [$F(1,28)=10.54, p<0.01$], with males showing an overall decrease in anxiety like behavior (increased %OA time) compared to females. Post-hoc comparisons revealed within the LAn trait group, males exhibited less anxiety-like behavior as indicated by an increase in the percent of time spent on OA compared to LAn females ($p<0.05$) (Fig. 1A). Similarly, HAn rats also made fewer %OA entries (increased anxiety like behavior) compared to LAn rats, as indicated by a significant Trait effect [$F(1,28)=9.080, p<0.01$] (Fig. 1B). No significant effects were found for total entries (data not shown). These findings confirm that offspring of rats selectively bred for high anxiety exhibit increased anxiety-like behaviors on the EPM compared to those selectively bred for low anxiety.

3.2 Effects of diazepam on %OA time in the EPM

In order to assess whether sex and trait anxiety interact to influence the anxiolytic effects of diazepam, the response to anxiogenic stimuli (EPM) in HAn and LAn rats was assessed following either diazepam (1 mg/kg) or vehicle. Analysis with a three-way ANOVA

revealed significant main effects for Trait [$F(1,64)=6.312, p<0.05$], Sex [$F(1,64)=4.629, p<0.05$] and Drug [$F(1,64)=4.922, p<0.05$]. In addition to a significant Sex $\times$ Trait [$F(1,64)=5.512, p<0.05$] interaction, the analysis also indicated a significant Trait $\times$ Drug $\times$ Sex [$F(1,64)=4.164, p<0.05$] interaction. Consistent with the findings from the baseline assessment of EPM behavior, HAn rats exhibited higher anxiety-like behavior on the %OA time measure (decreased %OA time) compared to LAn animals. Interestingly, only the HAn males showed a diazepam-induced anxiolytic response (~20%) (Fig. 2A). Separate *t*-tests with Bonferroni adjustments (4 tests, alpha set at $p<0.0125$) were used to compare the %OA time between VEH and diazepam treatments. A significant effect of diazepam to reduce anxious behavior was found only for HAn males ($t(7)=5.637, p=0.0008$).

3.3 Effects of diazepam on %OA entries in the EPM

The percentage of entries into the open arm of the EPM was used as an additional measure of anxiety-like behavior on the EPM. Three-way ANOVA on %OA entries indicated a Sex $\times$ Drug interaction that approached significance [$F(1,64)=3.630, p<0.06$] with no other significant effects. The results indicate that diazepam failed to significantly increase %OA entries in any of the experimental groups (Fig. 2B).

3.4 Effects of diazepam on total entries in the EPM

Total entries (open and closed arms) were counted as an index of general activity in the EPM (Fig. 2C). The three-way ANOVA indicated that HAn animals made more total entries compared to LAn animals as a significant main effect for Trait [$F(1,64)=7.095, p=0.01$] was found. In addition, females rats made more total entries (Sex [$F(1,64)=18.278, p<0.000$]) and diazepam administration increased total entries across groups compared to vehicle (Drug [$F(1,64)=12.667, p<0.001$]).

3.5 Parvalbumin-immunoreactive cells vary by sex and trait anxiety level

Following behavioral assessment, we determined whether GABA interneuron density in the central amygdala, caudate putamen or hippocampus differs between sexes or trait anxiety designation. A two-way ANOVA on parvalbumin (PV)-immunoreactive (IR) cells in the CPu revealed a significant effect of Sex [$F(1,68)=16.04, p<0.001$] as well as a Sex $\times$ Trait interaction [$F(1,68)=7.58, p<0.05$] with females having a greater number of PV-IR cells relative to males for both HAn and LAn traits. LAn animals overall had a greater number of PV-IR cells than their HAn counterparts (Fig. 3A). Analysis of the CeA indicated a significant effect of Sex [$F(1,52)=11.19, p<0.001$] with males showing an increased number of PV-IR cells relative to females (Fig. 3B). Subregions of the hippocampus, CA1, CA2, CA3, and dentate gyrus (DG) were analyzed for reactivity separately. No significant differences were found for DG or CA1 areas (data not shown). The Trait effect approached significance in the CA3 [$F(1,56)=0.12, p=0.0849$] while it reached significance in the CA2 [$F(1,56)=4.27, p<0.05$] with Han animals exhibiting greater PV-IR cells over their LAn counterparts in both areas (Fig. 3C).

3.6 Pearson *r* correlations

To establish whether there is a correlation between baseline anxiety-like behavior, diazepam effects and GABA interneuron density, separate correlations between these measures were conducted. For baseline anxiety-like behavior, Pearson's correlation between PV-IR and the %OA time in the CPu approached significance (Pearson's $r = 0.331$, $p = 0.0644$). Interestingly, %OA entries was significantly positively correlated with parvalbumin immunoreactivity in the CPu (Pearson's $r = 0.354$; $p < 0.05$; two-tailed) and approached significance for %OA entries and PV-IR in the central amygdala (Pearson's $r = 0.305$; $p < 0.08$; two-tailed). Following diazepam treatment, we found significantly positive relation between PV-IR in the amygdala and the %OA time (Pearson's $r = 0.489$, $p = 0.0045$) (Fig. 4A–C).

4. Discussion

An initial finding of our work indicates that animals selectively bred for trait anxiety, after being phenotyped on the elevated plus maze, showed persistent anxiety-like behavioral profiles. In our current design, we exposed the animals to a retest protocol in the elevated plus maze after an inter-trial interval of a minimum of 3 weeks, and found sustained anxiogenic effects across the trials, as has been previously reported by Adamec and Shallow (2000) and Schneider et al. (2011).

The present study found that acute diazepam administration resulted in a significant attenuation of anxiety-like behavior (>%OA time) in HAn males. Overall, LAn females were found to have less anxiety-like behavior as assessed by performance on the EPM (>%OA time, >%OA entries) than HAn females, however, diazepam pretreatment did not reduce anxious behavior in either female group. These findings are supported by previous work showing a 1.0 mg/kg dose of diazepam decreased anxiety-like behavior in males but not females (Wilson et al., 2004). While it is possible that the acute treatment in this study (single 1 mg/kg injection) was too low to elicit an anxiolytic response in females, our work is consistent with a report that found no changes on EPM in females administered an acute dose of diazepam (single 2.5 mg/kg injection) (Singewald, 2007).

As expected, LAn animals showed less anxiety-like behavior relative to HAn animals and this effect was more pronounced in males compared to females. Following the diazepam challenge, LAn animals in general spent more time on OA and made more OA entries but the only significant anxiolytic effect was found in HAn males. However, analysis of total entries revealed that HAn females made significantly *more* total entries than LAn females even though LAn females had greater %OA entries. This suggests that HAn and LAn status is likely reflecting differences in anxiety level as opposed to differences in exploration or locomotor activity in the EPM.

The effects of the estrous cycle on anxiety could explain the observed sex differences as previous work illustrates that during proestrus (i.e., elevated levels of progesterin and plasma allopregnanolone) females show decreased anxiety-like behavior on the EPM (Frye et al., 2000). Allopregnanolone is a direct GABA_A receptor modulator that acts as an anxiolytic with rebound anxiety observed during withdrawal from the treatment (Smith, 2002).

Although we found an increase in total entries after diazepam administration in females, it failed to act as an anxiolytic. Gonadal hormones may have played an important role as estradiol and progesterone have been found to modify the number of GABA receptors in the brain and possibly alter GABA-benzodiazepine binding sites (Maggi and Perez, 1984; Perez et al., 1988). While fluctuations in hormone levels were not a focus of the current work, it is a limitation that we did not measure estrogen levels since they have been shown to interact with the effects of diazepam on avoidance conditioning and locomotor activity in rats (Díaz-Vélez et al., 2000). Analysis of PV-IR cells (PV targets a subpopulation of GABAergic cells that contain this calcium binding protein) in the CPu revealed a significant difference by sex with females exhibiting a greater number of PV-IR cells in both HAn and LAn lines relative to males. It is possible that sex differences in the CPu can be attributed to estradiol acting on GABAergic neurons (Becker 1999). In the current study, LAn animals were found to have an increased number of PV-IR cells compared to HAn animals in the CPu. Consistent with this finding, less anxiety-like behavior was positively correlated with increased GABA neuron density in this region. This finding is in agreement with previous work that has suggested a negative correlation between the number of GABAergic receptors, especially GABA_A and anxiety-like behavior (Rägo, et al., 1988; Yilmazer-Hanke, et al., 2003; Kalueff and Nutt, 2007).

CeA connections to the brainstem and hypothalamus are involved with not only anxiety but physiological responses such as blood pressure and contain calcium-binding parvalbumin proteins (Yilmazer-Hanke et al., 2002). The number of PV-IR cells found in the CeA revealed a significant difference by sex only, with males exhibiting the greater number of cells in this region. Given the EPM results with males showing less anxiety-like behavior, this finding is not unexpected. It is believed that the major GABAergic subtype in the CeA is the alpha2GABA_A subtype, which plays an important role in both anxious states and emotion regulation (Fritschy and Möhler, 1995). The alpha2GABA_A subtype is implicated in diazepam-induced anxiolytic activity after diazepam administration, which was illustrated in this study by an increase in %OA time in males of both anxiety phenotypes although the effect was strongest in HAn males (Löw et al., 2000; McKernan et al., 2000; Crestani et al., 2002).

Although this study did not differentiate between GABA receptor subtypes, the finding that males exhibited a greater number of PV-IR cells in the CeA compared to females could partially account for the differing diazepam-mediated anxiolytic results. This is also supported by the significantly positive correlation between PV-IR in the CeA and diazepam induced behavior on the open arm. Recent work by Urakawa et al. (2013) showed selective increases in the number of PV-IR cells in the basolateral amygdala of animals that received environmental enrichment and these differences correlated with improved responses to anxiogenic stimuli. This supports our current results indicating decreased anxiogenic responses in males that also showed elevated CeA PV-IR. It is possible that circulating estrogens in the females altered the interaction of diazepam on the neurons (Rewal et al., 2005) and / or the GABA_A/benzodiazepine binding site in the CeA (Díaz-Vélez, et al., 2000) as the decreased number of PV-IR cells in the CeA in both HAn and LAn females would suggest an increase in anxiety-like behavior based on previous reports (Rägo, et al., 1988; Crestani et al., 1999) although this pattern was not found currently in our selective outbred

(HAn/LAn) animals. In support of our findings, other work using Roman High and Low Avoidance rats (RHA/RLA) have found that greater PV-IR in the basolateral amygdala of RLA males does not translate into behavioral differences between RHA and RLA males in the EPM (Yilmazer-Hanke et al., 2002).

Previous research has also shown that infusion of benzodiazepines into the hippocampus as well as lesions to this region fail to alter behavior in the EPM (Treit and Menard, 1997; Gonazlez et al., 1998), however, differential fos expression is observed in high and low anxiety lines in the CA3 and DG (Salomé et al., 2004). Currently, significant differences were found in the CA2 region and approached significance in the CA3, however, in both cases the HAn animals illustrated a *greater* number of PV-IR cells compared to LAn animals. Previous work has shown that fos reactivity is attenuated in the CA1 and CA3 regions after administration of the 5HT_{1A} agonist buspirone (Wisłowska-Stanek, et al., 2005) suggesting that these areas contribute to anxiety status independent of GABAergic cells. It is likely that trait anxiety and sex influence other populations of GABA cells (Gulyás, et al., 1991) differently and is worthy of further study.

We report that trait anxiety rats show distinct responses on the EPM and sex-related differences in response to diazepam that correlate with GABA neuron variations along stress regions. Low anxiety animals display less anxiety-like behavior on the EPM and males are more sensitive to the anxiolytic effects of the classic anti-anxiety drug, diazepam. At least a subpopulation of parvalbumin-containing GABA cells vary in accordance with the behavioral and sex distinctions with greater numbers of cells in males correlating with improved sensitivity to diazepam. In light of these findings, potential investigations of individual profiles for treatment might be warranted as well as investigation of brain areas such as the hypothalamus and prefrontal cortex for GABAergic cell differences given their role in anxiety (Singewald et al., 2003; Kalisch, et al., 2004).

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Abbreviations

HAn	high anxiety
LAn	low anxiety
DZ	Diazepam
EPM	elevated plus maze
CeA	central amygdala
PV	parvalbumin
IR	immunoreactivity

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Highlights

- Female Long Evans rats bred for low anxiety phenotypes show *less anxiety* than males
- Male animals were more sensitive to the anxiolytic effects of acute diazepam
- Male rats show greater parvalbumin immunoreactivity (PV-IR) in the central amygdala
- Females show greater PV-IR in the caudate putamen compared to males

Fig. 1.

A–B. Bar graph of baseline behavioral response of female and male rats phenotyped as high (HAn) and low anxiety (LAn) on the elevated plus maze. Data are expressed as group average (A) %OA time (B) %OA entries $\pm$ SEM. There was significant differences between LAn and HAn lines with LAn animals showing significantly more %OA time (** $p < 0.01$) and %OA entries relative to HAn.

Figure. 2.

A–C. Diazepam has distinct anxiolytic sex effects in HAn and LAn female and male rats. **(A)** For %OA time on EPM, female rats from LAn spent more time on OA while male rats were more sensitive to the anxiolytic effects of diazepam (*t*-test with Bonferroni adjustments (alpha set at $p < 0.0125$), $**p = 0.0008$, compared to VEH-treated HAn males). **(B)** The HAn and LAn lines did not show any differences in %OA entries.

Fig. 3.

A–C. GABA neurons containing the calcium binding protein parvalbumin (PV) were counted in several brain areas implicated in anxiety. (A) Females showed a greater number of PV-IR in the CPu, regardless of trait, $\#p < 0.05$. LAn animals consistently showed greater PV-IR relative to HAN, $***p < 0.001$. (B) In the central amygdala, males had the most PV-IR cells. (C) HAN PV-IR counts were greater than LAn in hippocampal regions (CA2 and CA3). Abbreviations: IR, immunoreactive; CPu, caudate putamen; CeA, central amygdala.

Fig. 4.

A–C. Caudate putamen (CPu) parvalbumin-positive GABA neurons moderately correlate while amygdala PV-IR cells significantly correlate with anxiety-like behavior on the elevated plus maze. Plot of CPu PV-IR GABA cells relative to (A) percent open arm time and (B) percent open arm entries on EPM; and amygdala PV-IR relative to (C) percent open arm time. *Abbreviations:* IR, immunoreactive; CPu, caudate putamen; OA, open arm.

* $p < 0.05$, ** $p < 0.01$.