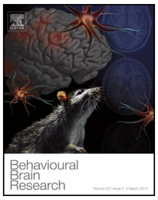
Behavioural Brain Research 270 (2014) 68–74

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Research report

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

Rebecca Ravenelle[a], Nichole M. Neugebauer[b], Timothy Niedzielak[c],
S. Tiffany Donaldson[c,*]

[a] Department of Biological Sciences, Fordham University, Rose Hill Campus, Bronx, NY 10458, USA
[b] Department of Psychiatry and Behavioral Sciences, Northwestern University, Feinberg School of Medicine, 303 E Chicago Avenue, #12-104, Chicago, IL 60611, USA
[c] Developmental and Brain Sciences, Department of Psychology, University of Massachusetts Boston, 100 Morrissey Boulevard, Boston, MA 02125, USA

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.

ARTICLE INFO

Article history:
Received 4 January 2014
Received in revised form 25 April 2014
Accepted 28 April 2014
Available online 6 May 2014

Keywords:
Anxiety
GABA
Diazepam
Elevated plus maze
Striatum
Amygdala

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.

1. Introduction

It is estimated that 15% of the world's population is suffering from an anxiety disorder [1] with lifetime prevalence rates at 19% for males and 31% for females [2]. 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 [3,4]. Although alterations in γ-aminobutyric acid (GABA) transmission have been implicated, the exact mechanisms underlying anxiety disorders remain elusive [5].

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

---

Abbreviations: HAn, high anxiety; LAn, low anxiety; DZ, diazepam; EPM, elevated plus maze; CeA, central amygdala; PV, parvalbumin; IR, immunoreactivity.

* Corresponding author. Tel.: +1 617 287 6249; fax: +1 617 287 6336.
E-mail addresses: Tiffany.Donaldson@umb.edu, Donaldson@gmail.com (S.T. Donaldson).

http://dx.doi.org/10.1016/j.bbr.2014.04.048
0166-4328/

hippocampus [5,6]. Previous studies have illustrated that benzodiazepines also modulate neuroendocrine function [7,8], 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 [9]. 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 [9].

Sex differences have been observed in anxiety disorders with females exhibiting up to three times the prevalence rate for general anxiety disorder [10]. Animal models have also illustrated sex differences in anxiety on several measures including the EPM, open field and social interaction tests [11] 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 [12]. Female ERalpha knockout (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 [13,14]. Circulating gonadal hormones are also believed to influence sensitivity to benzodiazepines in humans [15] 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 [16]. Estrogen has been found to rapidly alter dopaminergic neurotransmission through membrane-bound receptors on GABAergic neurons in the CPu (for review, see [17]), an area that has been implicated in anxiety states in both clinical and animal models [18,19].

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 [16] and acute diazepam administration increases c-fos-immuonreactive (IR) cells in the hypothalamic parventricular nucleus (PVN), CeA and the supraoptic nucleus (SON) [20]. 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 [21]. 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 [22–25]. 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 [26]. 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 [27] amygdala [28] and the CPu [29].

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. We used females and males from the fourth filial generation of selectively outbred high (HAn) and low (LAn) anxiety Long Evans rats, tested in the EPM after both vehicle and diazepam administration and then measured the distribution of GABA neurons within regions implicated in the regulation of anxiety.

2. Materials and methods

2.1. Experimental animals

Sixteen female (250–350 g) and sixteen male (500–700 g) 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 cm × 25 cm × 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 cm × 6.5 cm) without walls and two opposing arms (50 cm × 6.5 cm) with high-enclosed walls (50 cm × 6.5 cm × 15 cm) connected via a central platform (6.5 cm × 6.5 cm). 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 [30]. 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/dH_2O IP (VEH) using a within-subjects design. The diazepam dose was chosen based on previously reported findings of the anxiolytic effects on EPM [5]. 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