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Activation of amygdalar metabotropic glutamate receptors modulates anxiety, and risk assessment behaviors in ovariectomized estradiol-treated female rats

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Abstract

Anxiety disorders are more prevalent in females than males. The underlying reasons for this gender difference are unknown. Metabotropic glutamate receptors (mGluRs) have been linked to anxiety and it has been shown that interaction between estrogen receptors and mGluRs modulate sexual receptivity in rats. We investigated the role of mGluRs in anxiety-related behaviors in ovariectomized (OVX) female rats with (OVX+EB) or without (OVX) estradiol implants. We centrally infused (s)-3,5-dihydroxyphenylglycine (DHPG), a group I mGluR agonist, into the basolateral amygdala (BLA) of OVX+EB and OVX rats at 0.1 and 1.0 μ M. Male rats that normally have low estradiol levels were used to compare with OVX rats. Generalized anxiety, explorative activity and detection and analysis of threat were analyzed in the elevated plus maze (EPM) and risk assessment behaviors (RABs). DHPG (1.0 μ M) increased the percentage of time spent in- and entries into- the open arms in OVX+EB, but not in OVX females or male rats. Flat-back approaches and stretch-attend postures, two RABs, were significantly reduced by DHPG (0.1 and 1.0 μ M) in OVX+EB female rats only. DHPG did not modulate rearing- and freezing, behaviors related to exploration and fear-like behavior, respectively. However, DHPG (1.0 μ M) increased head dipping and decreased grooming behaviors in OVX female rats, suggesting a weak explorative modulation. The effects of DHPG observed in OVX+EB, were blocked by 50 μ M of (RS)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), a mGluR1 antagonist. AIDA and/or estradiol did not modulate anxiety and or RABs. Our results show that intra-BLA infusion of DHPG exerts an anxiolytic-like effect in OVX+EB, but not in OVX or male rats. This effect seems to depend upon mGluR1 subtype activation. Our findings led us to suggest that the effects observed in OVX +EB rats might be due to an interaction at the membrane level of estrogen receptors with mGlu1 within the BLA.

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Keywords

estrogen; basolateral amygdala; metabotropic glutamate receptors; anxiety-related behaviors; ovariectomized female rats

1. Introduction

Anxiety disorders are among the most common forms of mental illness in the United States, affecting nearly 18% of the American population yearly. These disorders include social phobia, generalized anxiety, obsessive-compulsive, panic, and post-traumatic stress disorders, each of which is characterized by different symptoms. Anxiety disorders affect females more than males (Kendler and Prescott, 2001), raising the possibility that gonadal hormones play a role in the etiology and prevalence of anxiety-related behaviors. In humans, changes in circulating levels of estrogen throughout the female life span have been linked to fluctuations in anxiety levels and mood disorders (Goldstein et al., 2005; Walf and Frye, 2006). Specifically, peri- and post-menopausal women experience higher anxiety and depressive symptoms in comparison to pre-menopausal ones (Tangen and Mykletun, 2008), supporting relation of gonadal hormones with female mood disturbance.

Anxiety-related behaviors are also found in rodents, in whom estrogen can induce anxiolytic effects (Frye and Wawrzycki, 2003; Hill et al., 2007; Nomikos and Spyrali, 1988; Walf and Frye, 2005), anxiogenic (Morgan and Pfaff, 2002), or no significant effects (Diaz-Veliz et al., 1997; Mora et al., 1996). These effects depend upon dosage concentration, task, age and/or reproductive stage (Nomikos and Spyrali, 1988). The mechanisms underlying estrogen modulation of anxiety remain unknown, however.

The amygdala is a complex of brain nuclei that plays a key role in modulating anxiety-related behaviors, fear conditioning, and emotional memory (Anglada-Figueroa and Quirk, 2005; Davis and Whalen, 2001; File, 2000; LeDoux, 2003; Vazdarjona and McGaugh, 1999). The basolateral (BLA), lateral (LA), and central (CeA) amygdala are the primary amygdala nuclei. Threat-related sensory information is processed within the BLA nucleus. The BLA then modulates the activity of the medial part of the CeA nucleus by direct and indirect glutamatergic projections to orchestrate behavioral- and physiological- anxiety-related responses. The BLA has, thus, been associated with the anxiolytic actions of drugs, such as the benzodiazepines (File, 2000; Pesold and Treit, 1995).

Metabotropic glutamate receptors (mGluRs) are found in different amygdala nuclei including the BLA and CeA (Romano et al., 1995), and have been implicated in anxiety and depression (Chojnacka-Wójcik and Pilc, 2001; Palucha and Pilc, 2007; Spooren et al., 2010). Eight mGluR subtypes have been identified and divided into three groups, based on amino acid sequence, pharmacology and transduction mechanisms (Conn and Pin, 1997). For instance, group I receptors (mGluR1 & 5 genes) are coupled to phospholipase C (Conn and Pin, 1997), which together modulate fear and anxiety in a task dependent manner (Busse et al., 2004; Pérez de la Mora et al., 2006; Steckler et al., 2005). Further evidence that mGluR5 has an anxiolytic effect includes attenuation of hyperthermic response to stress in male mGluR5-knockout mice (Brodin et al., 2002), and anxiolytic effects elicited

following systemic administration of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a selective mGluR5 antagonist (Tatarczynska et al., 2001). In contrast, few experiments link anxiolytic-like effects to mGluR1 α (Klodzinska et al., 2004), and these effects appear to be largely dependent upon the experimental procedure. The interaction between membrane estrogen receptors and mGluR1 α has been shown to modulate sexual receptivity (Dewing et al., 2007), and intracellular signaling (Boulware et al., 2005), in female rats. Whether group I mGluR plays a role to modulate female anxiety, and whether estrogen can, in turn, modulate mGluR activation in the BLA remains unclear, however.

The main scope of our study was to investigate the contribution, if any, of amygdala group I mGluRs to the regulation of female anxiety. We used ovariectomized (OVX) rats, half receiving implants containing estradiol benzoate and the other half containing empty implants, to avoid confounding effects produced by cyclic changes of ovarian hormones. Since estradiol modulates sexual receptivity through mGluRs (Meitzen and Mermelstein, 2011), we aimed to determine whether estradiol modulates anxiety through group I mGluR activation. Male rats that normally have low estradiol levels were used to compare with OVX female rats that did not receive estradiol implants and, therefore, have male-like estradiol levels. We hypothesized that infusion of DHPG will exert an anxiolytic-like effect in ovariectomized rats receiving estradiol implant (OVX+EB), without modulating OVX and/or male rats. Note that the impact of estradiol on castrated males is beyond the scope of the current study. Bilateral infusion of (S)-3,5-dihydroxyphenylglycine (DHPG), a group I mGluR agonist, was administered into the BLA. Animals were submitted to the elevated plus maze (EPM) to analyze open arms explorative activity associated to generalized anxiety. We also assessed novel ethological-oriented behaviors, including risk assessment behaviors (RABs), to evaluate behaviors related to threat detection and analysis, emotional reactivity, and explorative/locomotor activity.

2. Methods

Experimental procedures were conducted according to the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico Medical Sciences Campus (MSC) and the National Institutes of Health guide for the care and use of laboratory animals. The MSC animal care facility is registered with the United States Department of Agriculture and accredited by the American Association for Laboratory Animal Care (AALAC).

2.1 Animals and Housing

Naïve adult female and male Sprague-Dawley rats, weighing 220-280g, were purchased from Charles River Laboratories (Wilmington, MA). Same-sex pairs were housed in acrylic cages (26.7 × 48.3 × 20.3 cm) and were exposed to a light-controlled room (12:12-h light:dark cycle; lights on at 08:30 h) at a room temperature of 18 – 26 °C, relative humidity of 30-70%, with food and water made available ad libitum. Animals were acclimatized to laboratory conditions for one week prior to surgeries.

2.2 Surgical Procedures

Animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg, i.p.), and fixed in a stereotaxic apparatus (Kopf Instruments, Tujunga CA). Two guide cannulas (14 mm long, 23-gauge; Small Parts, Miami FL) made of stainless steel were bilaterally implanted 2 mm above the BLA (coordinates from bregma of Paxinos and Watson's 1998 atlas, AP = -2.80mm, ML = $\pm$ 5.0mm, DV = 6.0mm). The cannulas were anchored to the skull with four stainless steel screws and fixed in place with dental acrylic cement (Stoelting, IL). A 14-mm long stainless steel stylet (14-mm long, 30-gauge, Small Parts, Florida) was inserted into each cannula. The stylets were removed, cleaned and/or replaced periodically in order to prevent cannula occlusion and to reduce the stress induced by handling, which may increase adrenal steroid levels (Erskine and Kornberg, 1992). Immediately after brain cannulation, the females were ovariectomized and a 6 mm silastic implant (1.47 mm i.d. $\times$ 1.96 mm o.d.), with (OVX+EB) or without (OVX) estradiol benzoate replacement, was placed subcutaneously in the posterior neck, as described previously (Pérez-Acevedo et al., 2006). The 4 mg amount of estradiol benzoate was sufficient to maintain constant proestrous-like estradiol blood levels (Febo et al., 2002; Pérez-Acevedo et al., 2006). Animals received 1.0 ml of 0.9% saline (s.c.) and were injected with an analgesic (buprenorphine 0.05-0.1 mg/kg, i.m.) to prevent dehydration and distress, respectively, during recovery. After surgery, animals were housed individually and allowed to recover for one week prior to behavioral testing. All animals were handled daily throughout the recovery period to provide the same pre-testing experience.

2.3 Drugs

The (S)-3,5-dihydroxyphenylglycine (DHPG; Tocris Cookson, Ellisville MO) was dissolved in 0.9% sodium chloride. The solution was stored in tightly sealed 1.0 mM aliquots at -20°C for up to one month. The day of testing, the DHPG was diluted to 0.1 or 1.0 μ M, using sterile 0.9% saline as a vehicle. These concentrations were chosen based on previous experiments (Holy and Wisniewski, 2001). Specifically, DHPG activates group I mGluRs with EC₅₀ values below 10 μ M (mGluR1a=6.6 and mGluR5a=2), although in some cell lines the EC₅₀ values are closer to 30 μ M. In males, 10, 100, and 1000 μ M intracerebroventricular (icv) injections of DHPG into the lateral cerebral ventricle do not produce any effect when tested in the EPM (Nadlewska et al., 2002), but 1.0 μ M icv does produce anxiogenic responses (Holy and Wisniewski, 2001). To establish whether DHPG effects were mediated by mGluR1 α subtype activation, 1-aminoindan-1, 5-dicarboxylic acid (AIDA) (Tocris Cookson, Ellisville MO), a selective mGluR1 α subtype antagonist was used. AIDA selectively antagonizes mGluR1 α subtype at concentration between 10-300 μ M (Kingston et al., 2002). AIDA was also dissolved in 0.9% sodium chloride and stored in 1.0 mM aliquots at -20°C. On the test day, AIDA was diluted to 50 μ M in 0.9% sodium chloride, a concentration previously tested with non-behavioral effect after intra-BLA infusion in the EPM (data not shown). The DHPG was then diluted in 50 μ M AIDA to 1.0 μ M and micro-infused bilaterally to OVX+EB animals.

AIDA has an in vitro IC₅₀ of 360 μ M and has been used to selectively antagonize the mGluR1 subtype at concentrations in the range of 10-300 μ M (Kingston et al., 2002). In order to determine AIDA's working dose we performed a pilot experiment where we tested

the effect of AIDA at two concentrations, 50 and 100 μM . We selected these concentrations based on previous experiments (Kingston et al., 2002). For our pilot study, we used ovariectomized females treated with estradiol (OVX+EB) since this was the experimental group in which AIDA exerted an anxiolytic effect. At both concentrations, AIDA did not alter the percentage of open time ($F(2,16)=0.29$; $p=0.75$) and entries ($F(2,16)=0.61$; $p=0.56$) as reflected by a One way ANOVA. Likewise, it did not change the number of flat-back approach ($F(2,16)=1.03$; $p=0.38$) and stretched-attend posture ($F(2,16)=0.68$; $p=0.52$), two of three risk assessment behaviors recognized by Rodgers as more sensitive index of anxiety. However, AIDA at 100 μM , but not at 50 μM , showed a tendency to increase the total number of entries ($F(2,16)=1.20$; $p=0.33$), yet this effect was not significant. Thus, for experiment 2 we selected AIDA's working concentration of 50 μM to avoid any confounding effect that might alter changes in locomotor activity.

2.4 Infusion Procedure

The experiments were conducted one week after surgery. Animals were transferred from the animal facility to the behavioral testing room and habituated to this new environment for at least one hour prior to the experiment. Five minutes prior to behavioral testing, the animals were restrained manually and then the BLA was bilaterally infused with either the vehicle (0.9% saline), DHPG (0.1 or 1.0 μM), AIDA (50 μM) or DHPG+AIDA (1.0 and 50 μM , respectively). Brain infusions were performed with stainless steel injectors (16 mm long, 30-gauge; Small Parts, Miramar FL), using an infusion pump (Harvard Apparatus, Holliston MA). The final volume infused into the BLA was the same for all animals (0.5 μL /side over a period of one minute). Injectors remained in place for an additional minute to prevent back flow of the fluid, and then removed and replaced with stainless steel stylex (14 mm long, 30-gauge; Small Parts, Miramar FL). The injectors were cleaned between animals using a hot bead sterilizer.

2.5 Experimental Design

An animal can display different anxiety-related behaviors, which have been associated to specific types of anxiety (Crawley, 2007). To discriminate between generalized-anxiety and behaviors-related to threat- detection and analysis, each animal was assessed in the EPM, and conventional spatiotemporal measures and RABs were analyzed. In experiment 1, ovariectomized females receiving (OVX+EB) and not receiving (OVX) estradiol replacement, and godanal intact males were micro-infused five minutes prior to the test with either vehicle or DHPG (at 0.1 or 1.0 μM). Animal behavior was recorded and analyzed by blind observers. A different batch of animals was used for experiment 2. The OVX+EB animals were micro-infused with vehicle, DHPG (1.0 μM), AIDA (50 μM) or DHPG (1.0 μM) co-infused with AIDA (50 μM). Five minutes after micro-infusion, we followed the experimental procedures as previously described. At the end of the behavioral testing, animals were euthanized by rapid decapitation.

2.6 Elevated Plus-Maze (EPM)

The EPM was used to measure open arms explorative activity, as described previously (Pérez-Acevedo et al., 2006). The test was monitored during the dark phase of the light-dark cycle under dim red-light illumination (20W). The maze, which is elevated 79 cm from the

floor, consisted of a cross-shaped, opaque, white acrylic platform with two 50×10 cm arms without walls (open) and two $50 \times 10 \times 48$ cm arms with walls (closed). The arms were oriented perpendicular to each other and connected by an open central area (10×10 cm). To minimize olfactory cues, the maze was cleaned with a mild detergent (Quatricide PV-15) between experimental sessions. The animals were placed in the center of the maze facing one of the open arms. Behaviors were videotaped for five minutes for subsequent analysis. Conventional spatiotemporal measures such as the percentage of time spent in the open arms, (time in open arm / total time in open and closed arms), and the percentage of entries into the open arms, (open arm entries / total entries into the open and closed arms), were recorded and analyzed as an index for generalized-anxiety. In addition, we also analyzed the animal's general motor activity using three different parameters: 1) total number of entries; 2) frequency of closed entries; and 3) frequency of rearing (the animal stands bipedally) (Bananej et al., 2011; Cruz et al., 1994; Espejo, 1997; Pellow et al., 1986).

2.7 Behavioral Postures during EPM Performance

During the five minute test, several ethological-oriented measures were analyzed. Risk assessment behaviors (RABs) included the flat back approach (the trunk elongates while approaching stimuli), stretch-attend posture, (the trunk extends and then flexes back to original position), and head dipping, (the head flexes below the edge of the open arm). These behaviors have been associated to detection and analysis of threats or threatening situations. In addition, we analyzed rearing, an exploratory behavioral posture, freezing, (no movement other than respiration, for periods of 15 seconds or longer), related to fear, and grooming, (animals licks themselves using their forelimbs, mouth, and hind limbs, often in a orderly sequence), related to non-exploratory behavior. Anxiolytic drugs decrease the number of RABs during the EPM. These parameters are a more sensitive index for anxiety-related behaviors than conventional measures obtained from the EPM alone (Rodgers and Cole, 1993).

2.8 Histology

Following euthanasia by rapid decapitation, the brains were quickly removed, stored at -80°C and then sectioned at $50\text{ }\mu\text{m}$ using a microtome (Leica CM 1900, on, Meyer Instruments, Houston TX). Coronal sections were stained with cresyl violet and analyzed using a light microscope. Cannula placement was verified histologically and identified in diagrams modified from the Paxinos and Watson atlas (1998), by an individual who was blind to the treatment condition (Fig. 1). Animals in which the cannulas were placed incorrectly were not included in the analysis, but used as anatomical controls (see supplementary data).

2.9 Statistics

Data are presented as mean $\pm$ standard error of the mean (SEM) values. For experiment 1, female data was analyzed using a two-way analysis of variance (ANOVA) to determine: 1) drug; 2) estradiol treatment effect; and 3) possible interaction of estradiol treatment and drug administration. Male data was analyzed using a one-way ANOVA to determine whether DHPG modulates anxiety depending upon drug concentration. Post hoc multiple comparison analyses were performed using Student-Newman-Keuls Tests. In female rats, we analyzed OVX+EB-vehicle vs. OVX-vehicle rats to assess whether estradiol modulates behavior by

itself. For experiment 2, a two-way ANOVA was performed to analyze data of OVX+EB animals micro-infused with vehicle, DHPG or AIDA+DHPG effects. Kruskal-Wallis one way ANOVA on ranks, followed by Dunn's method, were used to analyze data with unequal variance. Student's *t*-test was used to analyze gender differences between male and ovariectomized female rats infused with vehicle. Differences were considered statistically significant when *p* values were < 0.05 and the power of the test was > 0.80.

3. Results

3.1 Experiment 1

3.1.1 Effects of DHPG on Generalized Anxiety—To assess generalized anxiety, conventional spatiotemporal parameters of anxiety were measured in the EPM. Following intra-BLA infusion of either vehicle or DHPG (0.1 and 1.0 μ M), a two-way ANOVA revealed a significant effect of the drug on the percentage time spent in the open arms (Fig. 2A; $F(2,77)=3.71$; $p=0.03$). Estradiol treatment effect (Fig. 2A; $F(1,77)=1.18$; $p=0.28$) and an estradiol treatment $\times$ DHPG interaction (Fig. 2A; $F(2,77)=0.65$; $p=0.52$) were not detected. Post hoc comparisons revealed that DHPG at 1.0, but not at 0.1 μ M increased the percentage time spent in the open arms in OVX+EB (Fig. 2A; $p=0.02$ and $p=0.71$, respectively), but not in OVX (Fig. 2A; $p=0.51$ and $p=0.64$) female rats. In male rats, regardless of dosage, DHPG did not modulate the percentage time spent in the open arms (Fig. 2B; $F(2,39)=1.00$; $p=0.37$). A two-way ANOVA revealed a significant effect of the drug on the percentage entries into the open arms (Fig. 2C; $F(2,77)=3.65$; $p=0.03$). Estradiol treatment effect (Fig. 2C; $F(1,77)=0.28$; $p=0.60$) and an estradiol treatment $\times$ DHPG interaction (Fig. 2C; $F(2,77)=0.88$; $p=0.42$) were not detected. Post hoc comparisons revealed that DHPG at 1.0, but not at 0.1 μ M increased the percentage entries into the open arms in OVX+EB (Fig. 2C; $p=0.01$ and $p=0.18$, respectively), but not in OVX (Fig. 2C; $p=0.34$ and $p=0.58$, respectively) female rats or male rats (Fig. 2D; $F(2,39)=1.80$; $p=0.18$). Reduction of percentage of time spent in- and entries into open arms by DHPG, were not observed in OVX+EB misplaced animals (Fig 2; A' and C'; $p>0.05$).

3.1.2 Effects of DHPG on behaviors-related to detection and analysis of threat, explorative, fear-like and non-exploratory behaviors—Ethological measures related to risk assessment behaviors (FBA, SAP, and head dipping), to exploration (rearing), fear-like (freezing), or non-exploratory (grooming), behaviors were analyzed during the EPM. DHPG significantly decreased the number of FBA and SAP, in OVX+EB, but not OVX female and male rats (Fig. 3). A two-way ANOVA revealed a significant effect of the drug on FBA behavior (Fig. 3A; $F(2,77)=4.74$, $p=0.01$). Estradiol treatment effect (Fig. 3A; $F(1,77)=0.00$; $p=0.99$) and estradiol treatment $\times$ DHPG interaction (Fig. 3A; $F(2,77)=2.74$; $p=0.07$) were not detected. DHPG, at both 0.1 and 1.0 μ M, decreased the number of FBA in OVX+EB (Fig. 3A; $p=0.005$ and $p=0.003$, respectively), but not in OVX female (Fig. 3A; $p=0.85$ and $p=0.22$, respectively) and male rats (Fig. 3B; $F(2,39)=0.13$; $p=0.88$, respectively). A significant effect of the drug was also detected on SAP behavior (Fig. 3C; $F(2,77)=3.32$, $p=0.04$). Estradiol treatment effect (Fig. 3C; $F(1,77)=0.02$; $p=0.89$) and estradiol treatment $\times$ DHPG interaction (Fig. 3C; $F(2,77)=2.42$; $p=0.10$) were not detected. DHPG, at both 0.1 and 1.0 μ M, decreased the number of SAPs in OVX+EB (Fig. 3C;

$p=0.01$ and $p=0.04$, respectively), but not in OVX female (Fig. 3C; $p=0.96$ and $p=0.26$, respectively) and male rats (Fig. 3D; $F(2,39)=0.31$; $p=0.74$). Reduction in the number of FBAs and SAPs by DHPG, were not observed in OVX+EB misplaced animals (Fig 3; A' and C'; $p>0.05$). A two-way ANOVA revealed a significant effect of the drug on head dipping behavior (Table 1; $F(2,77)=4.66$, $p=0.01$). Estradiol treatment effect (Table 1; $F(1,77)=0.17$; $p=0.68$) and estradiol treatment $\times$ DHPG interaction (Table 1; $F(2,77)=1.27$; $p=0.29$) were not detected. DHPG at 1.0, but not at 0.1 μM increased the number of head dipping in OVX (Table 1; $p=0.02$ and $p=0.98$, respectively), but not in OVX+EB females (Table 1; $p=0.66$ and $p=0.11$). In males, regardless of drug concentration, DHPG did not modulate head dipping (Table 1; Kruskal-Wallis one way ANOVA on Ranks; $H=2.72$; $p=0.26$).

In terms of exploratory behaviors, intra-BLA infusion of DHPG did not affect rearing in OVX+EB and OVX female rats (Table 1; $F(2,77)=1.15$; $p=0.32$); or male rats (Table 1; $F(2,39)=2.22$; $p=0.12$). In females, estradiol treatment effect (Table 1; $F(1,77)=0.02$; $p=0.89$) and estradiol treatment $\times$ DHPG interaction (Table 1; $F(2,77)=1.98$; $p=0.14$) on rearing behaviors were not detected. In terms of fear-related behaviors, DHPG did not modulate freezing in female (Table 1; $F(2,77)=1.51$; $p=0.22$) and male (Table 1; $F(2,39)=0.97$; $p=0.39$) rats. In females, estradiol treatment effect (Table 1; $F(1,77)=0.01$; $p=0.93$) and estradiol treatment $\times$ DHPG interaction (Table 1; $F(2,77)=0.50$; $p=0.61$) on freezing behaviors were not detected. In terms of non-explorative grooming behavior, a two-way ANOVA revealed a significant effect the drug (Table 1; $F(2,77)=4.13$; $p=0.02$) and estradiol treatment (Table 1; $F(1,77)=7.13$; $p=0.01$), in female rats. Estradiol treatment $\times$ DHPG interaction (Table 1; $F(2,77)=0.56$; $p=0.57$) in grooming behaviors were not detected. DHPG at 1.0, but not 0.1 μM reduced the number of grooming events in OVX (Table 1; $p=0.03$ and $p=0.33$, respectively), but not in OVX+EB (Table 1; $p=0.36$ and $p=0.58$, respectively) female rats, while estradiol by itself reduced this behavior (Table 1; $p=0.02$). In male rats, grooming behavior was not altered by DHPG (Table 1; $F(2,39)=0.79$; $p=0.46$). Sex differences were only found for non-explorative behavior where OVX female rats displayed more grooming behavior than male rats (Table 1; t -test, $p=0.04$).

3.2 Experiment 2

3.2.1 The anxiolytic-like effect of DHPG in OVX-EB female rats is blocked by AIDA—Based on previous findings (Boulware et al., 2005), we assumed that the anxiolytic-like effects of DHPG in OVX+EB female rats can be mediated by mGluR1 α subtype. If this is true, then a co-infusion with the specific mGluR1 α antagonist, AIDA, will block the observed DHPG effect on generalized-anxiety and risk assessment behaviors (RABs). Therefore, OVX+EB females were micro-infused with either vehicle, DHPG (1.0 μM), AIDA (50 μM) or co-infused with DHPG plus AIDA within the BLA. In generalized anxiety, two-way ANOVAs revealed a significant effect of DHPG on percentage time spent in open arms (Fig. 4A; $F(1,46)=5.42$; $p=0.02$), and no effect of AIDA or AIDA $\times$ DHPG interaction (Fig. 4A; $F(1,46)=0.22$; $p=0.64$ and $F(1,46)=1.98$; $p=0.17$, respectively). Post hoc comparisons detected differences between DHPG and vehicle animals ($p=0.02$), but not between DHPG and AIDA or AIDA+DHPG ($p=0.20$ and $p=0.57$, respectively). For the percentage of entries into open arms, two-way ANOVAs revealed a significant effect of

DHPG (Fig. 4B; $F(1,46)=5.53$; $p=0.02$), and no effect of AIDA or AIDA $\times$ DHPG interaction (Fig. 4B; $F(1,46)=0.40$; $p=0.53$ and $F(1,46)=2.00$; $p=0.16$, respectively). Post hoc comparisons detected differences between DHPG and vehicle animals ($p=0.02$), but not between DHPG and AIDA or AIDA+DHPG ($p=0.16$ and $p=0.57$, respectively).

In RABs, two-way ANOVAs revealed a significant effect of DHPG (Fig. 4C; $F(1,46)=8.34$; $p=0.006$), and no effect of AIDA or AIDA $\times$ DHPG interaction on FBA (Fig. 4C; $F(1,46)=0.66$; $p=0.42$ and $F(1,46)=0.62$; $p=0.44$, respectively). Post hoc comparisons detected differences between DHPG and vehicle animals ($p=0.01$), but not between DHPG and AIDA or AIDA+DHPG ($p=0.27$ and $p=0.20$, respectively). Two-way ANOVAs failed to detect significant effect of DHPG (Fig. 4D; $F(1,46)=1.73$; $p=0.20$) and AIDA, or AIDA $\times$ DHPG interaction on SAP (Fig. 4C; $F(1,46)=2.31$; $p=0.14$ and $F(1,46)=0.64$; $p=0.43$, respectively). No effects were observed in the number of head dipping, rearing and grooming (Table 2).

In terms of locomotor activity, two-way ANOVAs revealed no effect of DHPG (Fig. 5A; $F(1,46)=0.41$; $p=0.53$) and AIDA, or AIDA $\times$ DHPG interaction in the total number of entries (Fig. 5A; $F(1,46)=0.03$; $p=0.87$ and (Fig. 5A; $F(1,46)=0.41$; $p=0.53$, respectively). A two-way ANOVAs detected no effect of DHPG (Fig. 5B; $F(1,46)=3.73$; $p=0.060$) and AIDA, or AIDA $\times$ DHPG interaction in the number of closed entries (Fig. 5B; $F(1,46)=0.15$; $p=0.70$ and Fig. 5B; $F(1,46)=1.53$; $p=0.22$, respectively). In the number of open entries, a two-way ANOVAs detected no effect of DHPG (Fig. 5C; $F(1,46)=0.63$; $p=0.43$) and AIDA, or AIDA $\times$ DHPG interaction (Fig. 5C; $F(1,46)=0.014$; $p=0.90$ and Fig. 5C; $F(1,46)=0.06$; $p=0.81$, respectively).

4. Discussion

Overall, the goal of this study was to evaluate the role of amygdalar group I mGluRs in female anxiety and explorative/locomotor behaviors. In our opinion, experiments using only males provide an incomplete understanding of the neurobehavioral system and a too generalized interpretation of the findings into the anxiety-pharmacological treatments. In fact, gender has been argued to play an important role in the etiology and potential pharmacotherapeutic treatment of anxiety disorders (Wilson, 1996; Wilson and Biscardi, 1997). Thus, we focused our study on determining the role of amygdalar group I mGluRs in anxiety using a female animal model. To avoid potentially confounding hormonal variations during the estrous cycle, we used ovariectomized female rats, with (OVX+EB) and without (OVX) estradiol replacement, to assess the impact of estrogens on group I mGluR activation during the EPM and the display of behaviors-related to threat- detection and analysis, explorative, fear-like and non-exploratory behaviors. Group I mGluR receptors (Romano et al., 1995), as well as estrogen receptors (ERs) (Blurton-Jones and Tuszynski, 2002), are expressed within the BLA; therefore, we expected to find effects of estradiol or through mGluR activation in female anxiety.

The elevated plus-maze (EPM) is an unconditioned test that is prone to more variable baselines than conditioned behavioral tests. To minimize anxiety baseline levels and the high variability baselines within the EPM, we performed the experiments taking into

consideration the following factors: housing and testing conditions, light levels, time of testing, prior handling and experience, and olfactory cues. After controlling all variables, we obtained baseline levels ranging from 45-50%, which is a little higher than baselines reported from other laboratories. However, these baselines are within the range of previous studies conducted in our research facility (Jorge et al., 2005; Pérez-Acevedo et al., 2006; Rojas-Ortiz et al., 2006) and by others assessing the BLA region (File et al., 1998). Notice that these measures were obtained by dividing the open arm exploration (open arm time or entries) by the *total arm* exploration (time in or entries into the open plus closed arms, respectively). Therefore, our measures excluded the time spent in the central platform (square), the time spent in the arm transitions and the attempts to enter to a specific arm (animals in the center platform with anterior paws inside a maze arm). In fact, the exclusions of central platform activity not only get rid of the ambiguity of the plus-maze, but also decrease the denominator of the equations: open arm time or entries/(open + closed time or entries) $\times$ 100. Reduction in the denominator number will produce a higher ratio value. Thus, the equation denominator must be cautiously considered since some studies use the total maze exploration (e.g., 300s, the session duration), reporting percentages of open time around 10% (Rodgers et al., 2010, 2011).

The BLA plays a role during the acquisition of specific phobias associated to the EPM one trail tolerance (File et al., 1998). Whether the surgery and/or intra-BLA infusion protocol might underlay some closed arm phobia and/or an increase in open arm exploration in naïve animals, is unclear and must be considered by separated studies. Nevertheless, it is clear that the pharmacology sensitive of EPM is highly dependent upon behavioral baselines. For example, chlordiazepoxide, a potent benzodiazepine, might or not produce anxiolytic-like effects depending upon the level of baseline avoidance in different background mice strains (Rodgers et al., 2002). Low open arm aversion makes difficult the detection of anxiolytic drugs. In our study, intra-BLA infusion of DHPG, at 1.0, but not at 0.1 μ M, increased the percentage of time spent in- and entries into- open arms in OVX+EB female rats, near to one quarter, without affecting OVX females and/or intact male rats. Likewise, intra-BLA infusion of anxiolytic drugs, such as benzodiazepines and ethanol, reduces open arm fear driven by thigmotaxis, a natural defensive response in which the animals remain close to the vertical surface (Burghardt and Wilson, 2006). DHPG effects were induced exclusively by local drug action within the BLA region and depended upon mGluR1 α activation as it was blocked by AIDA. We expected to find no differences between DHPG alone and DHPG plus AIDA, if the effects were induced by mGluR1. Note that DHPG is a selective group I mGluR agonist that acts through both, mGluR1 and mGluR5 subtypes. AIDA, however, is a selective mGluR1 antagonist. Therefore, differences between DHPG alone and co-infusion of DHPG plus AIDA could be explained by activation of mGluR5 and not through mGluR1 subtype.

Consequently, our results suggest that activation of amygdalar mGluR1 α decreased the conflict resulting from the approach/avoidance duality to explore the open arms, suggesting anxiolytic-like effects in generalized anxiety. These effects were dependent upon sex and estradiol treatment in female rats. Given that the effects of DHPG are exclusively observed in OVX+EB, but not OVX female rats can be explain by the fact that different types of

anxiety-related disorders depend upon specific brain regions and/or circuitries, which are not equally sensitive to specific estradiol regimens.

In the EPM, the anxiolytic-like effects of DHPG in OVX+EB females were confirmed by a reduction in the frequency of novel ethological behaviors, named by Cole and Rodgers, (1993) as risk assessment behaviors (RABs). These behaviors are valuable in identifying anxiolytic-like actions of drugs not detected by conventional spatiotemporal measures (Cole and Rodgers, 1994; Griebel et al., 1998). Under a potential threat (e.g., elevated and open spaces), rodents cease ongoing exploratory behaviors and display RABs as a mechanism to get threat-related sensory information and to optimize the most adaptive behavioral strategy (Blanchard et al., 1993, 2011; Rodgers and Cole, 1993). Intra-BLA infusion of DHPG decreased the number of flat-back approach (FBA) and stretch-attend posture (SAP), two RABs, in OVX+EB, but not in OVX female or male rats. Interestingly, in OVX female rats only, DHPG increased head dipping, an exploratory-RAB. Reduction in the frequency of FBA, SAP, as well as the increase in head dipping behaviors, suggest an anxiolytic-like profile (Blanchard et al., 2011; Griebel et al., 2000). Supporting, our hypothesis that ovariectomized rats receiving estradiol implants to modulate group I mGluRs will show less anxiety than ovariectomized females not receiving estradiol implants or intact male rats.

Intra-BLA infusion of DHPG in female or male rats did not modulate rearing and freezing, explorative- and fear-like behaviors, respectively. However, DHPG decreased grooming, a non-exploratory behavior, in OVX female rats only. Diazepam, as well as other anxiolytic drugs, decrease grooming behavior during exploratory-based-anxiety tests (Casarrubea et al., 2011; Jászberényi et al., 2009; Rogel-Salazar and López-Rubalcava, 2011). The effect of DHPG observed on grooming behavior led us to suggest that amygdalar group I mGluR plays an indirect role on exploration during the EPM affecting females, but not male rats. In addition, the lack of DHPG effect on the frequency of rearing, total entries, as well as closed entries, rule out any sedative-like action of the drug as it did not change the animal's locomotor activity.

Estradiol treatment by itself did not modulate classical spatiotemporal measures related to generalized anxiety and RABs during the EPM. This finding was unexpected, knowing that anxiolytic effects occur after 3-days (Nomikos and Spyraiki, 1988) and 6-7 days (Koss et al., 2004), of estrogen administration. In addition, in OVX rats long-term weekly administration (14 weeks of estradiol), showed anxiolytic-like effects in the EPM (Walf and Frye, 2010). These studies find support in cyclic experiments, wherein females in proestrous exhibit less anxiety-related behaviors than estrus and diestrus female rats in most, but not all of the tasks tested (Frye et al., 2000). In our study, estradiol by itself only decreased grooming behavior in female rats. Reduction in the frequency of this non-exploratory behavior might suggest a weak anxiolytic-like effect of estradiol.

Note that estradiol effects on anxiety may depend upon concentration, administrative protocol, and the assessed task (Frye and Wawrzycki, 2003; Koss et al., 2004; Nomikos and Spyraiki, 1988; Walf and Frye, 2006, 2008). For example, acute estradiol administration does not modulate EPM behavior in OVX rats (Walf and Frye, 2008), whereas, chronic administration decreases EPM anxiety (Walf and Frye, 2010). That might explain why

estradiol failed to modulate spatiotemporal and other ethological measures besides grooming during our study. Nevertheless, despite estradiol-effect discrepancies, all the aforementioned studies consistently support our interpretation that the ovarian hormonal milieu, might modulate the neural circuitry underlying anxiety.

At present, only few experiments analyze the spatiotemporal measures in female and male rats during the EPM. Naïve females exhibit less anxiety-like behaviors than male rats during the test (Xiang et al., 2011; Zúena et al., 2008). However, no differences have been documented as well (Vaglenova et al., 2008). In our study, statistical differences upon sex were not found for open arm explorative activity and RABs. However, OVX females show more grooming behavior than male rats, suggesting that OVX females are more anxious than males during EPM performance.

To our knowledge, this study is the first to report an anxiolytic-like effect after DHPG (a group I mGluR agonist) infusion within the BLA in OVX+EB female rats. Several studies have investigated DHPG in males, but the target organ could not be clearly identified because infusions were intracerebroventricular. In males, DHPG infusions at different doses (nanomolar range), produce different effects depending upon the concentration and animal treatment. Some studies indicate that DHPG exerts anxiogenic-like effects (Holy and Wisniewski, 2001), whereas, others reveal anxiolytic-like effects (Nadlewska et al., 2002), and some other report no effects (Car and Wisniewski, 2000). In combination, these studies suggest that the results obtained depend upon task and drug concentration (Pietraszek et al., 2005; Spooren et al., 2000; Tatarczynska et al., 2001). In fear conditioning, DHPG at a 5-tens higher concentration into the BLA region enhances the formation of fear-like memories (Rudy and Matus-Amat, 2009). Furthermore, the selective blocking of intra-amygdala mGluR5 reduces anxiety levels in the EPM (Pérez de la Mora et al., 2006). DHPG, however, activates not only mGluR5, but mGluR1 α . This group I subtype can explain, at least in part, female anxiety through the modulation of estradiol at the membrane level.

Estradiol might act through membrane- and nuclear-initiated mechanisms to produce BLA responsiveness to DHPG in OVX+EB rats. First, estradiol modulates the activation and/or membrane insertion of glutamate receptors (for review see Moura and Peterson, 2010). Recent studies have reported a novel mGluR1 α -estradiol receptor (ER) interaction at the membrane level (Boulware and Mermelstein, 2005; Boulware et al., 2005; Dewing et al., 2007; Meitzen and Mermelstein, 2011). Specifically, in the hippocampus striatum and arcuate nucleus of hypothalamus, ER α activation stimulates group I mGluR signaling (Grove-Strawser et al., 2010). Estradiol acts on ERs and activates mGluR1 α , inducing CREB phosphorylation via signaling cascade that includes Gq, PLC, IP₃R and MAPK. Further reports evidence that ERs and mGluR1 α interaction is sex dependent, occurring in female, but not in male rats (Dewing et al., 2007; Kuo et al., 2009). Additionally, although estradiol nuclear effects are not the scope of the present study, is highly expected the modulation of anxiety-related genes by estradiol in OVX+EB rats. Precisely, phosphorylated CREB induced by estradiol-mGluR1 α interaction might enhance the transcription of different genes including brain-derived neurotrophic factor (BDNF) and neuropeptide Y (NPY), two well-known genes that are in turn associated with drug mechanisms for antidepressant- and anxiolytic-like effects, respectively (Nair and Vaidya, 2006).

Furthermore, estradiol might modulate mGluR1 α gene expression within the BLA enhancing the region response to DHPG micro-infusion. The present study evidences that partial activation of mGluR1 α reduces anxiety in a sex and estradiol dependent manner. Thus, ERs and mGluR1 α interaction at membrane and nuclear levels might underlie a potential signaling mechanism for DHPG anxiolytic-like effects in OVX+EB female rats. However, further experiments are necessary to confirm ER and mGluR1 α co-localization and their interaction within the BLA neurons.

Based on the neuroanatomical organization and neurochemical profile of the amygdala (Sah et al., 2003; Tye et al., 2011), we suggest two possible mechanism by which group I mGluR activation mediates anxiolysis in the EPM in OVX+EB rats. In the first pathway, group I mGluR-estradiol interaction within the BLA may elicit activation of non-pyramidal class II cells, which are GABAergic interneurons. These cells form local networks around the initial segments of the BLA glutamatergic pyramidal cells, raising the possibility that their activation elicits a tight inhibitory control over BLA output (Aroniadou-Anderjaska et al., 2007). In the second pathway, intra-BLA excitation of glutamatergic terminals on the lateral part of CeA (CeL) may modulate indirectly the activity of the medial part of CeA (CeM), producing a feed forward inhibition of CeM outputs. Recently, Tye and colleagues, (2011) found that direct activation of BLA terminals to CeL reduces anxiety in the EPM and open field test. However, it is important to emphasize that different subpopulations and projections of BLA neurons can be activated by intra-BLA infusion of DHPG. BLA nucleus is well positioned to receive and/or send projections of diverse brain regions, including, amygdalar intercalated cells (ITCs) (Paré et al., 2004), bed nucleus of the stria terminalis (BNST), hippocampus (Vianna et al., 2004), and prelimbic and infralimbic cortices (Sierra-Mercado et al. 2011). The discrete activation of these projections might provide multiple anatomical mechanisms to modulate BLA activity and/or outputs, regulating anxiety-related responses. Finally, BLA anatomical organization might be modulated by structural changes induced by estradiol. In vitro, estradiol treatment promotes rapid increase in the rate of new spine formation and their transformation into new synapses (Mendez et al., 2011). Therefore, estradiol treatment might enhance specific pre-existing connections in order to facilitate DHPG anxiolytic-like response.

In summary, we demonstrate that in female rats intra-BLA infusion of DHPG exerts an anxiolytic-like effect in an estrogen-dependent manner. We interpret our results as indicating that estradiol receptors within the BLA are likely to interact with mGluR1 α at the membrane level to modulate the activation of specific neural circuitries involved in generalized-anxiety and in the display of anxiety-related behaviors associated to detection and analysis of threats or threatening situations. Differential exposure to ovarian hormones may affect neural circuits and/or receptors underlying anxiety-related disorders. Therefore, further investigation is mandatory to facilitate the understanding of this complex issue.

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Highlights

Anxiety disorders are more prevalent in females.

Metabotropic glutamate receptors (mGluRs) are linked to anxiety.

We examined the role of amygdala group I mGluR in ovariectomized rats with and without estradiol replacement in female anxiety.

Activation of amygdala group I mGluRs in estradiol-treated rats produces anxiolytic-like effects.

Fig.1. Schematic diagram of injector tip localization within the basolateral amygdala (BLA) of ovariectomized female rats (A) with estradiol replacement (OVX+EB), (B) without estradiol replacement (OVX); and (C) male rats. Only data from animals in which the injection sites were bilaterally localized in the BLA were used. Numbers correspond to position or distance of coronal sections posterior to bregma. Diagram adapted from *The Rat Brain in Stereotaxic Coordinates* (Paxino and Watson, 1998). White circles = Vehicle-treated animals; Gray

circles = DHPG (0.1 μ M); Black circles = DHPG (1.0 μ M); Gray and white squares = AIDA (50 μ M); and Gray and black squares = DHPG (0.1 μ M) plus AIDA (50 μ M).

Fig. 2.

Effects of DHPG in the elevated plus maze (EPM). (A) Intra-BLA infusion of DHPG at 1.0 μ M, but not at 0.1 μ M increased the percentage time spent in open arms by ovariectomized female rats with estradiol replacement (OVX+EB), but not by ovariectomized female rats without estradiol replacement (OVX) or (B) male rats. (C) DHPG at 1.0 μ M but not at 0.1 μ M increased the percentage entries into the open arms by ovariectomized female rats with estradiol replacement (OVX+EB), but not by ovariectomized female rats without estradiol replacement (OVX) or (D) male rats. Effects of DHPG (1.0 μ M) were not observed in misplaced OVX+EB rats (A' and C'). Values represent mean $\pm$ SEM. * = $p < 0.05$. OVX +EB rats: vehicle, $n=20$; DHPG (0.1 μ M), $n=10$; DHPG (1.0 μ M), $n=15$. OVX rats: vehicle, $n=14$; DHPG (0.1 μ M), $n=10$; DHPG (1.0 μ M), $n=14$. Male rats: vehicle, $n=15$; DHPG (0.1 μ M), $n=12$; DHPG (1.0 μ M), $n=15$.

Fig. 3.

Effects of DHPG on flat-back approach (FBA) and stretch-attend posture (SAP) $n=15$.

Effects of DHPG on flat-back approach (FBA) and stretch-attend posture (SAP) behaviors analyzed in the plus maze. (A) Intra-BLA infusion of DHPG (0.1 or 1.0 μM) significantly reduced the number of FBA only in ovariectomized females with estradiol replacement (OVX+EB), but not in ovariectomized female rats without estradiol replacement (OVX) or (B) male rats. (C) DHPG (0.1 or 1.0 μM) significantly reduced the number of SAP only in OVX+EB, but not in OVX or (D) male rats. Effects of DHPG (1.0 μM) were not observed in misplaced OVX+EB rats (A' and C'). Values represent mean $\pm$ SEM. * = $p < 0.05$. OVX +EB rats: vehicle, $n=20$; DHPG (0.1 μM), $n=10$; DHPG (1.0 μM), $n=15$. OVX rats: vehicle, $n=14$; DHPG (0.1 μM), $n=10$; DHPG (1.0 μM), $n=14$. Male rats: vehicle, $n=15$; DHPG (0.1 μM), $n=12$; DHPG (1.0 μM), $n=15$.

Fig. 4.

The effects of DHPG in OVX+EB were blocked by co-infusion of AIDA, an mGluR1 antagonist. (A) In OVX+EB, intra-BLA infusion of DHPG (1.0 μ M) increased the percentage time spent in- and entries (B) into- the open arms in OVX+EB. AIDA (50 μ M) or the co-infusion of DHPG (1.0 μ M) plus AIDA (50 μ M) did not alter the percentage time spent in- and entries (B) into- the open arms. (C) In OVX+EB, intra-BLA infusion of DHPG (1.0 μ M) decreased FBAs and (D) SAP behaviors. AIDA (50 μ M) or the co-infusion of DHPG (1.0 μ M) plus AIDA (50 μ M) did not alter FBA (C) or SAP (D) behaviors. Values represent mean $\pm$ SEM. $p > 0.05$. Values represent mean $\pm$ SEM. * = $p < 0.05$. OVX+EB female rats: vehicle, $n=23$; DHPG (1.0 μ M), $n=11$; AIDA (50 μ M), $n=7$; and DHPG (1.0 μ M) plus AIDA (50 μ M), $n=9$).

Fig. 5.

Effects of DHPG and/or AIDA on locomotor activity of OVX+EB rats during the EPM.

BLA-micro infusion of DHPG and/or AIDA did not modulate the total number of entries

(A) and frequency of closed entries (B). Values represent mean $\pm$ SEM. $p > 0.05$. Values

represent mean $\pm$ SEM. * = $p < 0.05$. OVX+EB female rats: vehicle, $n=23$; DHPG (1.0 μ M),

$n=11$; AIDA (50 μ M), $n=7$; and DHPG (1.0 μ M) plus AIDA (50 μ M), $n=9$).

Table 1

Effects of intra-BLA infusion of DHPG on behaviors-related to detection and analysis of threat, explorative, fear-like and non-exploratory behaviors

Treatment	Behavioral Response			
	Exploratory	Non-exploratory		
	Head Dipping	Rearing	Freezing	Grooming
OVX+EB				
Vehicle	11.48 ± 1.78	6.50 ± 0.98	1.28 ± 0.50	2.18 ± 0.44 ^ψ
DHPG (0.1 μM)	7.05 ± 2.07	5.30 ± 1.39	2.00 ± 0.71	1.60 ± 0.63
DHPG (1.0 μM)	12.53 ± 1.69	5.50 ± 1.14	0.83 ± 0.58	1.23 ± 0.51
OVX				
Vehicle	7.54 ± 1.75	4.75 ± 1.18	0.61 ± 0.60	3.82 ± 0.53 ^{#ψ}
DHPG (0.1 μM)	7.45 ± 2.07	4.65 ± 1.39	2.05 ± 0.71	3.00 ± 0.63
DHPG (1.0 μM)	14.07 ± 1.75 [*]	8.32 ± 1.18	1.32 ± 0.60	1.82 ± 0.53 [*]
MALE				
Vehicle	8.53 ± 1.69	3.97 ± 1.14	1.13 ± 0.58	2.07 ± 0.51 [#]
DHPG (0.1 μM)	9.25 ± 1.09	6.88 ± 1.27	2.08 ± 0.65	2.13 ± 0.57
DHPG (1.0 μM)	13.80 ± 1.69	6.40 ± 1.14	1.03 ± 0.58	2.87 ± 0.51

Note. Data was analyzed using a two-way ANOVA for female rats and one-way ANOVA for male rats. Statistical significant:

Data are show as mean ±SEM

* drug within group effect

differences between OVX female vs male rats

^ψ estradiol effect.

Table 2

Effects of intra-BLA infusion of DHPG and/or AIDA on behaviors-related to detection and analysis of threat, explorative and non-exploratory behaviors

	Behavioral Response		
	<i>Exploratory</i>	<i>Non-exploratory</i>	
OVX+EB	Head Dipping	Rearing	Grooming
Vehicle	12.61 ± 1.22	6.35 ± 0.90	2.46 ± 0.39
DHPG (1.0 µM)	11.59 ± 1.77	6.18 ± 1.30	1.54 ± 0.56
AIDA (50.0 µM)	8.14 ± 2.21	6.36 ± 1.62	2.78 ± 0.70
DHPG + AIDA	11.57 ± 1.96	7.27 ± 1.43	1.89 ± 0.62

Note. Data was analyzed using a two-way ANOVA for OVX+EB female rats. Data are show as mean ± SEM