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Sex and estrous cycle influence diazepam effects on anxiety and memory: Possible role of progesterone

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ABSTRACT

Studies with rodents and humans show the relationship between female sex hormones and cognitive/emotional tasks. However, despite the greater incidence of anxiety disorders in women, the data are still inconclusive regarding the mechanisms related to this phenomenon. We evaluated the effects of a classical anxiolytic/amnestic drug (diazepam; DZP) on female (at different estrous cycle phases) and male rats tested in the plus-maze discriminative avoidance task (PMDAT), that allows the concomitant evaluation of memory and anxiety-like behavior. Further, in order to investigate the role of progesterone and its metabolites in the effects of DZP in the PMDAT, female rats were pre-treated with the progesterone receptor antagonist mifepristone or the 5-alpha-reductase inhibitor finasteride. The main findings were: (1) DZP caused memory impairment and anxiolysis in both sexes, but only the highest dose induced the anxiolytic effect in females; (2) females in proestrus did not present the amnestic and anxiolytic effects of DZP (at 2.0 and 4.0 mg/kg, respectively) and (3) the co-administration of mifepristone reestablished both amnestic and anxiolytic effects of DZP, while finasteride reinstated the amnestic effect in proestrus female rats. These results suggest that changes in the endogenous levels of progesterone and its metabolites are important in the modulation of emotional/cognitive behavior in female rats. Based on the influence on different aspects of DZP action, the mechanisms related to this modulation are probably linked to GABAergic transmission, but this point remains to be investigated. Further, the variation in therapeutic and adverse effects of DZP depending on sex and hormonal state is of great relevance considering the higher prevalence of anxiety disorders in women.

1. Introduction

One-third of the world population is likely to have at least one anxiety episode in their lifetime. These disorders were estimated to be more prevalent than substance-use disorders and mood disorders in some countries like Brazil, Canada, Netherlands and Turkey (WHO, 2000). Also, there is a higher prevalence of anxiety disorders among women (Bekker and Van Mens-Verhulst, 2007; McLean and Anderson, 2009; Toufexis et al., 2006).

The benzodiazepines (BDZ) are widely used since the 70s in order to treat human anxiety (e.g. diazepam, DZP; Valium®) (Möhler, 2011; Hollingworth and Siskind, 2010). They are positive allosteric modulators of the -aminobutyric-acid-type-A ($GABA_A$) receptors. Its widespread use has some limitations due to their potential side effects such as anterograde amnesia (Uzun et al., 2010). A recent Brazilian research study has shown that women use more BDZ than men in all age groups (12–65 year-old) (Galduróz et al., 2005). Moreover, adverse effects in response to BDZ vary across the menstrual cycle (Sundström et al., 1997). Further, sex differences in pharmacokinetics and pharmacodynamics in this class of drugs have been reported (Franconi et al., 2007; Gandhi et al., 2004; Kashuba and Nafziger, 1998; Schwartz, 2003; Waxman and Holloway, 2009; Whitley and Lindsey, 2009).

The plasticity of $GABA_A$ receptors fluctuates across the estrous cycle of female rats, and this fluctuation has been associated with levels of neurosteroids (Lovick, 2008; Maguire and Mody, 2009). Díaz-Véliz (2000) suggested a possible relationship among sex, stage of the estrous cycle, BDZ and the effects of neurosteroids on rat's behavior. Some

Abbreviations: AV, aversive enclosed arm; AO, open arms; FIN, finasteride; MIF, mifepristone; NAV, non-aversive enclosed arm; PMDAT, plus maze discriminative avoidance task; %TAV, percentage of time spent in aversive arm; %TOA, percentage of time spent in open arms.

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neurosteroids are active metabolites of progesterone, which modulate $GABA_A$ receptors through specific sites (Carver et al., 2014; Lambert et al., 1995, 2009). This mechanism is believed to contribute to the effects of progesterone on anxiety (Bitran et al., 1995; Gomez et al., 2002). Further, the classic progesterone receptor is associated with decreases in anxiety-related behaviors in male rats (Auger and Forbes-Lorman, 2008).

Despite the aforementioned evidence in humans, studies on female animals have been rare in behavioral neuroscience (Cahill, 2006). So far, scientific research using rodents as animal models is predominantly held with male animals (Berry and Zucker, 2011), what seems to be a contradiction. Part of the explanation for not using females in behavioral studies comes from the fact that females have hormonal fluctuations across their estrous cycle. The estrous cycle in female rats lasts 4- or 5 days throughout the reproductive life of the rat, except during pregnancy, pseudo pregnancy, and lactation (Becker et al., 2005). Estrous cycle is characterized by four stages: diestrus, proestrus, estrus and metestrus (Marcondes et al., 2002; Pompili et al., 2010). These hormonal fluctuations may have a role in activation of regions and neuronal subunits necessary for the behavioral expression of fear responses and anxiety, as showed in previous studies (Chen et al., 2009; Díaz-Véliz et al., 1997; Frye et al., 2000; Galeeva and Tuohimaa, 2001; Gouveia et al., 2004; Izídio et al., 2011; Korol et al., 2004; Marcondes et al., 2001; de Macêdo Medeiros et al., 2014; Molina-Hernández et al., 2013; Mora et al., 1996; Pompili et al., 2010; Sadeghipour et al., 2007; Toufexis et al., 2006; Walf et al., 2009).

Recently, a report from our group showed differences in the performance between naive male and female rats in the plus-maze discriminative avoidance task (PMDAT) (Ribeiro et al., 2010). This task evaluates memory and anxiety-like behavior concomitantly and is useful for the study of drugs that have effects on both phenomena (Silva and Frussa-Filho, 2000; Kameda et al., 2007). A previous study in this paradigm, with male mice, showed that both the anxiolytic and amnestic effects of chlordiazepoxide are related to each other (Silva and Frussa-Filho, 2000). However, this association between the effects of BDZ on anxiety and memory on the task has not been studied in females yet.

Given the potential sex differences in the effects of BDZ, as well as the greater incidence of anxiety disorders in women, we evaluated the effects of the classical anxiolytic/amnestic drug DZP on female and male rats tested in the PMDAT. A possible influence of the estrous cycle phases in the effects of DZP on memory and anxiety was also investigated. Moreover, as variations of DZP actions occurred across the estrous cycle, we also tested proestrus female treated with an antagonist of progesterone receptors (mifepristone) and an inhibitor of the enzyme 5-alpha-reductase (finasteride), the enzyme responsible for one of the pathways of progesterone metabolism.

2. Materials & methods

2.1. Animals

A total of 182 female and male three- to four-month-old Wistar rats from our colony were used. Male rats were used only in experiment I. The animals were allocated in plastic cages (30 × 37 × 16 cm, five per cage), and kept under controlled temperature (22–24 °C) and a light-dark cycle of 12/12 h (lights on at 6:30 am). Food and water were available ad libitum throughout the experiments. The procedures described here were approved by the local ethical committee (CEUA/UFRN no. 009/2013) and are in accordance to the Brazilian law for the use of animals in scientific research (Law Number 11.794).

2.2. Drugs

Diazepam (DZP, Valium®, Roche Brazil) was diluted in saline solution (0.9% NaCl) and given at 1, 2 or 4 mg/kg intraperitoneally (i.p.). Mifepristone (MIF, Tocris, USA) was diluted in a solution with absolute ethanol, sesame oil and tween 80 and given at 30 mg/kg. The dose of mifepristone was chosen based on the study of Milad et al. (2009) that showed an effect on memory extinction in female rats. The same procedure was used to dilute finasteride (FIN, Sigma-Aldrich, USA), which was given at 25 mg/kg (Concas et al., 1998). Both drugs were administered subcutaneously (s.c.). Saline (SAL) or the vehicle (VEH) solution with absolute ethanol, sesame oil and tween 80 were administered to control animals for DZP or MIF and FIN treatments, respectively. All treatments were administered in a volume of 1 ml/kg of body weight.

2.3. General procedures

The rats were handled by the experimenter during 10 min for five consecutive days before the beginning of behavioral experiments. The estrous cycle was determined by vaginal smears. Briefly, plastic pipettes were kindly introduced in the vagina with distilled water in a volume of approximate 0.1 ml. The bulb of the pipette was slightly pressured and when this pressure ended a liquid with vaginal cells enters the interior of the pipette. This material was stained with a diluted solution of methylene's blue (5 mg/ml) and analyzed in an optical microscope. This procedure was repeated daily during two regular cycles, between 12 and 1 p.m. Only female rats with regular estrous cycle were used in the experiments. Estrus phase is characterized by a predominance of cornified cells, proestrus by a predominance of epithelial nucleated cells, diestrus by predominance of leucocytes and metestrus by the same proportion of nucleated cells, leucocytes and cornified cells (Becker et al., 2005; Marcondes et al., 2002; Pompili et al., 2010).

2.4. The plus-maze discriminative avoidance task (PMDAT)

The apparatus employed was an adaptation of the conventional elevated plus-maze, made of wood, with two open arms (OA, 50 × 15 cm) opposed to two enclosed arms (50 × 15 × 40 cm). An incandescent lamp (100 W) and a speaker (80 dB) were placed above one of the enclosed arms (aversive arm). The animals were placed individually in the center of the apparatus facing the intersection between the open arms. During a 10-min length training session, each time the animals entered the aversive enclosed arm (AV), they received an aversive stimulation (light and noise). The test session occurred 24 h later, when the rats were again placed in the apparatus for 10 min, without receiving the aversive stimulation (non-aversive enclosed arm — NAV, with the lamp and the speaker still present over the aversive arm, but turned off). The apparatus was cleaned with a 5% alcohol solution after each behavioral session, in order to eliminate any olfactory cues. All sessions were recorded with a digital camera placed over the apparatus and the behavioral tracking was held with the use of the video-tracking software Anymaze® (Stoelting, USA). All behavior experiments were performed between 2:00 and 5:00 p.m.

In order to evaluate learning and memory we investigated the percentage of time spent in aversive arm (%TAV = time in AV / (time in AV + NAV) × 100) in the training and test sessions, respectively. Anxiety/emotionality levels were evaluated by the percentage of time spent in open arms (%TOA = time in OA / (time in AV + NAV + OA) × 100). The anxiety parameter was considered for analysis only in the training session, because the reliability of anxiety-like behavior in the test session would be jeopardized by a lower motivation to explore the apparatus and absence of novelty, according to File (1990). Spontaneous locomotor activity of the animals was measured through distance traveled in the apparatus (m).

2.5. Experimental designs

2.5.1. Experiment I: DZP dose-response curve in the PMDAT

Female and male rats received SAL or 1, 2 or 4 mg/kg DZP (n = 9–10/group). After 30 min, the animals were submitted to the training in the