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Dinah L. Ramos-Ortolaza

Raura J. Doreste-Mendez

John K. Alvarado-Torres

Annelyn Torres-Reveron

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Dinah L. Ramos-Ortolaza[a], **Raura J. Doreste-Mendez**[b,c], **John K. Alvarado-Torres**[b], and **Annelyn Torres-Reveron**[b,d,*]

[a]Department of Biology, Pontifical Catholic University of Puerto Rico, Ponce, PR, USA

[b]Department of Basic Sciences: Physiology and Pharmacology, Ponce Health Sciences University/Ponce Research Institute, Ponce, PR, USA

[c]School of Brain and Behavioral Sciences, Ponce Health Sciences University/Ponce Research Institute, Ponce, PR, USA

[d]Dept. of Biomedical Sciences, University of Texas at Rio Grande Valley School of Medicine, Edinburg TX USA

Abstract

Chronic social isolation could lead to a disruption in the Hypothalamic-Pituitary-Adrenal (HPA) axis, resulting in anxiety and depressive-like behaviors but cycling estrogens could modify these behaviors. The aim of this study was to determine if changes in ovarian hormones during the normal cycle could interact with social isolation to alter anxiety and depressive-like behaviors. In parallel, we examined the expression of glucocorticoid receptor (GR) and synaptic vesicle protein synaptophysin in the hippocampus and hypothalamus of Sprague Dawley normal cycling female rats. We assigned rats to either isolated or paired housing for 8 weeks. To assess anxiety and depressive-like behaviors, we used the open field test and forced swim test, respectively. Female rats were tested at either diestrus, estrus, or proestrus stage of the estrous cycle. After behaviors, rats were perfused and brains collected. Brain sections containing hippocampus and hypothalamus were analyzed using immunohistochemistry for synaptophysin and glucocorticoid receptor (GR) levels. We found an increase in depressive-like behaviors for isolated animals compared to paired housed rats, regardless of the estrous cycle stage. Interestingly, we found a decrease in anxiety behaviors in females in the estrus stage accompanied by a decrease in GR expression in hippocampal DG and CA3. However, no changes in synaptophysin were observed in any of the areas of studied. Our results support the beneficial effects of circulating ovarian hormones in anxiety, possibly by decreasing GR expression.

Keywords

Estrous cycle; Social isolation; Depression; Anxiety; Glucocorticoids; Hippocampus

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[*]Corresponding author at: Dept. of Biomedical Sciences, Division of Neurosciences, University of Texas at Rio Grande Valley- School of Medicine, 1214 W. Schunior St. Edinburg, TX, 78541, USA.

Conflict of interest

None declared.

1. Introduction

Women are twice as likely than men to suffer from depression and anxiety [1–4]. They may also respond differently to treatment compared to men [5,6]. Even though there has been an increase in the inclusion of women in clinical studies of these disorders, many of them have not examined the outcomes by sex [6]. For this reason, it is necessary to further evaluate sex differences contribution in the development of depression and anxiety.

A useful approach to evaluate depression and anxiety is to use an animal model of social isolation. In humans, social isolation and loneliness can contribute to depression and anxiety [7,8]. In animals, it has been shown that loss of bonding with other animals can lead to physiological problems, stress, anxiety, and depressive-like behaviors [9,10]. Therefore, we chose this model to evaluate how ovarian hormones may modulate the development of depression and anxiety.

Behavior in female animals is commonly associated with fluctuations in ovarian hormones. Several studies have, in fact, established a link between these hormones and the incidence of depression and anxiety disorders [11–14]. It has been suggested that the higher prevalence of these disorders in women may be related to hormonal fluctuations, particularly those characterizing menopause transition [15–17]. There has been a particular interest in estrogen, as fluctuations and decrease in its levels might lead to these disorders in women [18–20]. Estrogen administration has proven to be successful in treating depression under certain circumstances [21], but not others [22], suggesting that the mechanisms underlying gender differences are not solely based on estrogenic mechanisms.

Given the stressful nature of social isolation, the associated development of anxiety and depression could be due to dysregulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis. Some animal studies using social isolation have found sex-differences in the response of glucocorticoid receptors (GR), which regulate the HPA axis. For instance, in females, isolation stress increases GR levels in the hypothalamus and prefrontal cortex, while no sex differences are found in the pituitary or hippocampus [23]. In a posterior study by Mitic et al. [24], they found an increase in nuclear GR for both sexes after 6-week isolation stress. This suggests a region- and sex-specific involvement of GR in social isolation that could potentially be associated with differential development of anxiety and depression in females.

On the other hand, synaptophysin is a protein important for vesicle fusion to the presynaptic membrane and it has been correlated to synaptic plasticity [25]. In a study on sex differences after chronic social isolation in adolescent rats, female rats showed more anxiety and depressive-like behaviors with reduced levels of synaptophysin in prefrontal cortex [26], while in males, a decrease in overall levels of synaptophysin in the hippocampus has been reported [27]. These findings suggest different synaptic plasticity changes in females after chronic isolation stress. In spite of this, correlations to anxiety and depressive-like behaviors to the expression of these proteins across estrous cycle stages are largely lacking.

The aim of this study was to evaluate anxiety and depressive-like behaviors to synaptophysin and GR after chronic social isolation stress in female rats across the estrous cycle. We hypothesized that females in the diestrus (low hormone) phase would display more anxiety

and depressive-like behaviors, as well as an increase in GR and a decrease in synaptophysin compared to proestrus (high hormone) females. We tested anxiety and depressive-like behaviors in normal cycling females after being socially isolated during 8 weeks as compared to pair housed counterparts living on mildly enriched housing conditions. Also, we measured the expression of hippocampal synaptophysin as a marker of synaptic plasticity and GR in the hippocampus' CA1, CA3, dentate gyrus (DG), and ventromedial hypothalamus (VMH). Our animal model mimics some of the basic traits of isolation in humans, providing us with a foundation for understanding sex differences, which will help explain potential brain mechanisms involved in anxiety and depression depending on hormone levels.

2. Materials and methods

2.1. Animals

Adult (60 days old) female (n = 41) Sprague-Dawley rats (from the animal facilities at the Ponce Health Sciences University, Ponce Research Institute, PR) were housed under standard conditions and maintained on a 12-h light/dark cycle, with food and water available *ad-libitum*, unless otherwise specified. All experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee at Ponce Health Sciences University.

2.2. Estrous cycle determination

The estrous cycle phase of female rats was determined for 8 consecutive days at the beginning of the housing period and during 10 consecutive days prior to behavioral testing using vaginal smear cytology [28–30]. Length of estrous cycle and cyclicity was determined by the frequency of proestrus presentations within the time measured. We also measured levels of estrogen from blood samples collected before the perfusion procedure to verify the phases of the estrous cycle. Plasma serum levels for estradiol were determined by enzyme-linked immunosorbent assay (ELISA) using a mouse/rat estradiol kit (Calbiotech, cat#ES180s), using an antibody raised in rabbit against estradiol. The sensitivity of this kit is 3 pg/ml; previously used to measure plasma levels of estradiol in Sprague-Dawley rats [31].

2.3. Blood corticosterone measurements

Plasma serum levels for corticosterone were determined by enzyme-linked immunosorbent assay (ELISA) using a mouse/rat estradiol kit (Calbiotech, cat#ES180s), using an antibody raised in rabbit against corticosterone following the kit protocol instructions.

2.4. Behavioral evaluation

2.4.1. Social isolation protocol—All rats were housed in standard polycarbonate cages with metal lids. Control rats were housed in pairs with a nylabone and a small PVC cylinder inside the cage for the mild enrichment condition. The isolated rats were housed individually and were not handled, except during bedding changes twice a week. Rats were kept under these conditions for 8 consecutive weeks. Even though both isolated and paired animals were in the same room and could see each other, isolated animals did not have contact with

other animals. Isolation conditions such as those used in this study have been shown to induce depressive-like behavior in rodents [32].

2.4.2. Open field test—The open field test (OFT) was performed to measure anxiety and locomotor activity after social isolation or paired housing. The apparatus consisted of a black square wood box (interior dimensions: W 94.5 × L 94.5 × H 38.1 cm). The test was performed in an isolated room with dim red light and white noise. During the test, the animal was placed in the center of the apparatus and allowed to move freely for 10 min. The apparatus was cleaned with 70% ethanol solution after each test. Distance travelled was measured as a general locomotion indicator. Cumulative time spent in the center was recorded in 5-min intervals. Total time spent in the center was used as a measure of anxiety, where higher values are related to less anxiety. All behavior was recorded using a video camera located above the apparatus and analyzed with EthoVision XT 8.5 (Noldus, The Netherlands).

2.4.3. Forced swim test—The forced swim test (FST) was performed to measure depressive-like behaviors [33,34] after social isolation or paired housing. The apparatus consisted of a glass cylinder (40 cm height and 30 cm diameter) filled to 30 cm with 30 ± 1° C water. Water level prevented the rats from escaping or touching the bottom of the cylinder. Rats were subjected to one 10-min session of the FST immediately after the OFT. Although standard FST protocols for rats require two sessions, we decided to do one 10-min session as previously described [35] to prevent possible learned immobility [36,37]. Three types of behaviors were analyzed: struggling, defined as a rapid movement in which the rat breaks the surface of the water with the forepaws; swimming, defined as horizontal movements across the surface of the water; and immobility defined as the absence of movement, except for those required to breathe and keep the head above the water. Depressive-like behaviors are characterized by an increase in immobility time and a decreased latency for immobility [38]. Behavior was recorded using a video camera located in front of the apparatus and analyzed with AnyMaze Software (Stoelting, Wood Dale, IL).

2.5. Brain immunohistochemistry

2.5.1. Synaptophysin expression—Rat brains were fixed by aortic arch perfusion with 4% paraformal-dehyde. The brains were removed and post-fixed for 24 h in 30% sucrose/10% buffered formalin. Coronal sections were obtained on a Leica Vibratome (40 μm) and stored in cryoprotectant until fluorescent immunohistochemical processing. Prior to immunofluorescence, coronal sections of each treatment groups were coded with hole punches and pooled into single crucibles, with paired rats receiving one punch and isolated animals not punched. Tissue sections were rinsed in phosphate buffer (PB) followed by Phosphate-buffered saline (PBS; pH 7.4) and incubated in 10% Normal Goat Serum (NGS) in PBS for one hour. Tissue sections were incubated for 48 h at 4 °C in mouse monoclonal anti-synaptophysin (1:400; Sigma, S5768, St. Louis, MO, USA) in 0.1% NGS in PBS. This antibody has been previously characterized for specificity by Devoto and Barnstable [39]. Sections were then rinsed in PB and PBS and incubated with donkey anti-mouse Alexa Fluor 488 (1:400, Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. Sections were mounted on slides, dehydrated, and cover slipped. For percent

area labeled, images of regions of interest (CA3, CA1, and DG subregions of the hippocampus) were captured using a CCD Camera on a Nikon 200 microscope at the same illumination level for all images.

2.5.2. Glucocorticoid receptor (GR) expression—Tissue was processed in the same way after brain removal as described for the synaptophysin immunohistochemistry. Sections were rinsed in phosphate buffer (PB) followed by Tris-buffered saline (TBS; pH 7.6) and incubated in 0.5% bovine serum albumin (BSA) in TBS for 30 min. Tissue sections were incubated for 24 h at room temperature and for 24 h at 4 °C in rabbit monoclonal antiserum raised against human/mouse/rat GR (1:500; Santa Cruz Biotechnology, SC-1004, Santa Cruz, CA, USA) in 0.1% BSA in TBS. This antibody has been previously used to identify GR antigen in male and female Sprague-Dawley rats' hippocampus [24]. Sections were then rinsed in TBS and incubated with peroxidase–avidin complex (at twice the recommended dilution; Vector, Burlingame, CA, USA) for 30 min followed by development in 3,3'-diaminobenzidine (DAB) and H_2O_2 in TBS for 3.5 min. Sections were mounted on slides, dehydrated, and cover slipped.

2.5.3. Percent area analysis—For percent area occupied calculation, images of regions of interest (e.g., ventromedial nucleus of the hypothalamus [VMH]; in the hippocampus, the CA1, CA3, and DG subregions) were captured using a CCD Camera on a Nikon 200 microscope at the same illumination level for all images within a comparison group. Images were thresholded to account for positive labeling over background, using a black background in Image J. The region of interest was outlined and the resulting image converted to a binary frame. The corresponding labeled area from the whole region of interest outlined was calculated by the program and output was shown as percent area stained. Average values for each animal from 2 separate experimental runs were used to determine the mean group average. Values from control and experimental tissue processed together were statistically compared to determine the differences in immunohistochemical-area labeled.

2.6. Statistical analyses

Behaviors in the open field (distance traveled, time in the center) and in the forced swim test (immobility and latency to immobility) were analyzed by two-way ANOVA with social condition and by estrous cycle stage. Post hoc analyses were done using Tukey HSD or Sidak's Multiple Comparison Test, when appropriate, using SPSS V. 21 (IBM). This analysis was done also for synaptophysin expression in females with social condition and estrous cycle stage as factors.

3. Results

As an indicator of general health, we monitored the weight gain of the animals during the study. By 8-weeks, isolated females gained on average $29 \pm 1.7\%$ while paired housed females gained on average $33 \pm 1.7\%$ from their respective start weight. There were no differences between paired or isolated females ($F_{(1,30)} = 2.10$, $p = 0.19$) or by estrous cycle ($F_{(2,30)} = 1.36$, $p = 0.27$).

In addition to weight, we monitored the estrous cycle of female rats during the first and last 10 days of the housing period. Only 5 out of 36 rats (13.8%) did not follow a regular estrous cycle. These non-cycling animals showed either a persistence on estrus stage, or a continuous switch between estrous and diestrus without entering into proestrus stage. Regularly cycling rats had an average cycle length of 4.8 days showing normal transitions between estrus, metestrus, diestrus and proestrus. Since we were interested in determining the extent to which hormonal changes during the estrous cycle could affect anxiety and/or depressive-like behavior after social isolation, only those animals with a regular estrous cycle were included in the behavioral experiments. ELISA analysis of a subset of serum samples to confirm our smear readings revealed a 23% increase in the levels of serum estradiol in rats in proestrus over rats in diestrus.

3.1. Corticosterone levels after chronic social isolation did not change by treatment or estrous cycle

Corticosterone was measured from blood serum samples at the time of sacrifice. After 8 weeks of isolation, corticosterone levels did not vary by estrous cycle or treatment, as shown in Table 1.

3.2. Locomotor activity was enhanced in isolated animals

By the end of 8-weeks in social isolation, animals were tested for anxiety and locomotor activity using the Open Field Test (OFT) for 10 min. Since the animals showed a more exploratory behavior at different time points of the test, we analyzed the OFT data in time bins of 5 min. First, we measured the distance travelled by the animals in the OFT apparatus to determine whether social isolation would affect general locomotor activity. For the 0–5 min bin, isolated animals showed equal amount of locomotor activity as paired housed animals ($F_{(1,30)} = 0.009$, $p = 0.93$; Fig. 1A) as well as female rats for all estrous cycle stages ($F_{(2,30)} = 0.699$, $p = 0.50$; Fig. 1A). However, from 5 to 10 min, isolated animals showed a hyperlocomotor response that was significantly different from the paired housed counterparts ($F_{(1,29)} = 5.514$, $p = 0.03$; Fig. 1B). Also, a significant main effect for the estrous cycle was found, with paired females in the proestrus stage showing less locomotion than isolated females in diestrus ($F_{(2,29)} = 3.64$, $p = 0.04$; Fig. 1B).

3.3. Anxiety was reduced in female rats during the estrus stage of the cycle

We also measured the amount of time the animals spent in the center of the apparatus to determine whether social isolation would be anxiolytic or anxiogenic. Our analysis showed no differences in time spent in the center of the OFT apparatus between socially isolated and paired animals for the 0–5 min time-bin ($F_{(2,30)} = 0.01$, $p = 0.96$; Fig. 2A). However, a significant main effect of estrous cycle stage was observed during the 0–5 min time-bin ($F_{(1,30)} = 8.63$, $p = 0.001$; Fig. 2A) for which females tested during the estrus stage showed a significantly more amount of time spent in the center of the apparatus as compared to females in diestrus or proestrus stages. By the 5–10 min time-bin, there were no differences in time spent in the center of the apparatus (Fig. 2B), suggesting that the rats have adapted to the environment and were moving freely across the cage. No interactions were observed between estrous cycle and housing, as depicted in Table 2.

3.4. Depressive-like behavior was enhanced in isolated animals

In addition to the effects in anxiety, we wanted to determine the extent to which social isolation could lead to depressive-like behaviors and their onset by using the FST. We first measured the total amount of time the animals spent immobile during the test. We found no statistically significant differences in the total amount of time spent immobile by treatment $F_{(1,31)} = 0.049$, $p = 0.83$, however a tendency was observed when analyzed by estrous cycle ($F_{(2,31)} = 2.70$, $p = 0.08$; Fig. 3A). We then measured the latency to immobility, which is the amount of time it takes an animal to stop moving for the first time. We found a significant main effect of treatment, with isolated animals showing a decreased latency to immobility, which means an earlier onset of depressive-like behaviors ($F_{(1,31)} = 4.48$, $p = 0.04$; Fig. 3B). When analyzing the data by estrous cycle stage, no statistical significant differences were observed in latency to immobility between isolated and paired animals ($F_{(2,31)} = 2.577$, $p = 0.09$ Fig. 3B), although a tendency to significance can be observed. Paired estrus showed an increase in latency to immobility when compared to isolated proestrus ($p = 0.04$). No interaction was observed between estrous cycle and housing for immobility or latency to immobility, as depicted in Table 2.

3.5. GR expression was reduced in estrus females corresponding to a decrease in anxiety

Glucocorticoid receptor expression was measured in DG, CA3, CA1, and the ventromedial nucleus of the hypothalamus (VMH) in females. Average percent area occupied was calculated using three sections per animal. No effects were found in isolated versus paired animals in CA3 ($F_{(1,17)} = 0.18$, $p = 0.67$; Fig. 4A). However, we found a significant main effect for estrous cycle stage in this area ($F_{(2,17)} = 7.22$, $p = 0.01$; Fig. 4A). Post-hoc analysis showed that in isolated females in the estrus stage presented a significant decrease in CA3 GR receptor expression compared to proestrus ($p = 0.02$). A similar trend of decreased GR expression in the estrus stage compared to the proestrus was observed in the paired housed females but it falls short of significance ($p = 0.06$; Fig. 4A). In the DG, a significant main effect for estrous cycle stage was found (Table 3; $F_{(2,12)} = 4.70$, $p = 0.03$; Fig. 4B). Diestrus females showed a tendency to an increase when compared to estrus in the isolated group ($p = 0.05$). No differences were found either in CA1, DG or VMH in isolated versus paired animals.

3.6. No changes in synaptophysin were observed either by treatment or estrous cycle stage

Hippocampal synaptophysin expression was measured in three subareas; DG, CA3 and CA1. Percent area occupied was calculated using three sections per animal, as described for GR. Again, no interaction between housing treatment or estrous cycle was found (Table 4). No differences in percent area occupied by synaptophysin for isolated versus paired animals were found either in DG ($F_{(1,25)} = 0.001$, $p = 0.98$; Fig. 5A), CA3 ($F_{(2,25)} = 0.45$, $p = 0.64$; Fig. 5B), or CA1 ($F_{(1,22)} = 0.60$, $p = 0.45$; Fig. 5C). We found no changes by estrous cycle in any of the subareas. However, a tendency was observed for CA1 ($F_{(2,22)} = 2.88$, $p = 0.08$; Fig. 5C). Post-hoc analyses did not reveal any significant differences.

4. Discussion

The aim of this study was to examine anxiety and depressive-like behaviors in comparison to synaptic and GR markers after chronic social isolation stress across the estrous cycle in female rats. The main finding was a decrease in GR in the DG and CA3 area of the hippocampus corresponding to a decrease in anxiety for the paired female rats in this stage compared to proestrus and diestrus. In terms of locomotion, using the open field test, which we used to assess anxiety behaviors, isolated rats showed a hyperlocomotion response for the last 5–10 min interval of the test. For anxiety behaviors, we observed an increase in time in the center for the paired rats in the estrus stage, suggesting an anxiolytic effect of housing compared to proestrus and diestrus. In the forced swim test, isolated rats showed a decrease latency to immobility compared to paired rats, with no changes in total immobility time. Contrary to our hypothesis, no changes were observed in synaptophysin either by estrous stage or treatment.

Regarding the effects of social isolation in anxiety, our results showed no differences in anxiety between isolated and paired rats, as measured in the open field test. These results do not rule out the possibility of social isolation leading to anxiety, as it has been suggested that the effect of social isolation on anxiety can vary depending on the selected behavioral test and/or animal strain [40,41] Interestingly, we found a significant reduction in anxiety for the paired estrus females compared to the diestrus and proestrus. Our results show a decrease in anxiety for the mid-levels of estrogen (estrus) instead of in the peak in the gonadal hormones (proestrus). This result was surprising since high levels of estrogen appear to be protective in terms of cognition, brain plasticity, and behavior [42,43]. A previous study by Sayin et al. [44] found that high levels of estrogen exert an anxiolytic effect. These effects appear to occur in a short-term period of time, or by the non-genomic effects of estrogen, lasting from minutes to hours. However, our results suggest the involvement of classical or long-term effects of estrogen, which occur in a period lasting from hours to days [45]. The decrease in GR in DG and CA3 was only seen for the isolated rats in estrus, although anxiety changes were seen in this stage but for the paired rats. These results suggest that for socially isolated rats, anxiolytic effects are less dependent on GR and/or GR circuits are dampened by this housing condition [46]. Other possible circuits mediating this effect remain to be investigated.

Some studies have found a hyperlocomotion state in novel environments after chronic isolation [41,47]. A possible mechanism for this state involves the impairment of the monoamine system, specifically dopamine and noradrenaline axons in the medial prefrontal cortex (mPFC), the basolateral nucleus of the amygdala, and the hippocampus [48]. Another possible mechanism could be associated with changes in dendritic arborizations in the mesocorticolimbic system, leading to a dysregulation in dopamine in the ventral tegmental area (VTA), which projects to mPFC, amygdala, and nucleus accumbens [49]. In females, increase in locomotor activity is known to be estrogen dependent and may serve as a mechanism to ensure reproduction [50].

As expected, isolated rats showed a sooner onset to depressive-like behaviors as measured by their latency to immobility. Interestingly, paired female rats in the estrus stage showed a

higher latency to immobility compared to proestrus. In a previous study by Carrier and Kabbaj [51], ovariectomized females did not respond to either testosterone or imipramine for depressive-like behaviors after chronic isolation stress. These support the idea that female treatments for depression should be further investigated in terms of its interactions with ovarian hormones. Grippo et al. [9] studied possible mechanisms for depressive-like behaviors in male and female prairie voles. They found that the hypothalamic oxytocin and the corticotropic-Rreleasing Hhormone (CRH) systems might be involved, which we did not study here and may be possible pathways mediating the onset of depression in females.

We did not observe any changes in synaptophysin, although it has been implicated for the development of anxiety and depressive-like behaviors. This may be due to changes in synaptic plasticity depending on age, since Leussis and Andersen [26] found a decrease in this protein in the hippocampus in isolated adolescent rats, but our current study is in adults. Another possibility for this lack of changes is differences in isolation paradigms, since some studies have done this type of housing for less time. Giving rats more time under chronic stress in the same environment, could serve as a habituation period for synaptic plasticity changes thus possibly explaining the lack of differences observed here.

5. Conclusion

Our data add to an increasing body of evidence showing that ovarian hormones exert modulatory activity on anxiety and depressive-like behaviors. However, ovarian hormones effects were independent of the social isolation treatment effects as no interactions were observed. Ovarian hormones also decrease glucocorticoid receptors, as quantified in the hippocampus. Further research on the role of ovarian hormones modulation of hippocampal glucocorticoid systems and thus behaviors is deemed necessary to eventually generate sex-specific treatments that address the higher incidence of depression and anxiety in women.

Acknowledgments

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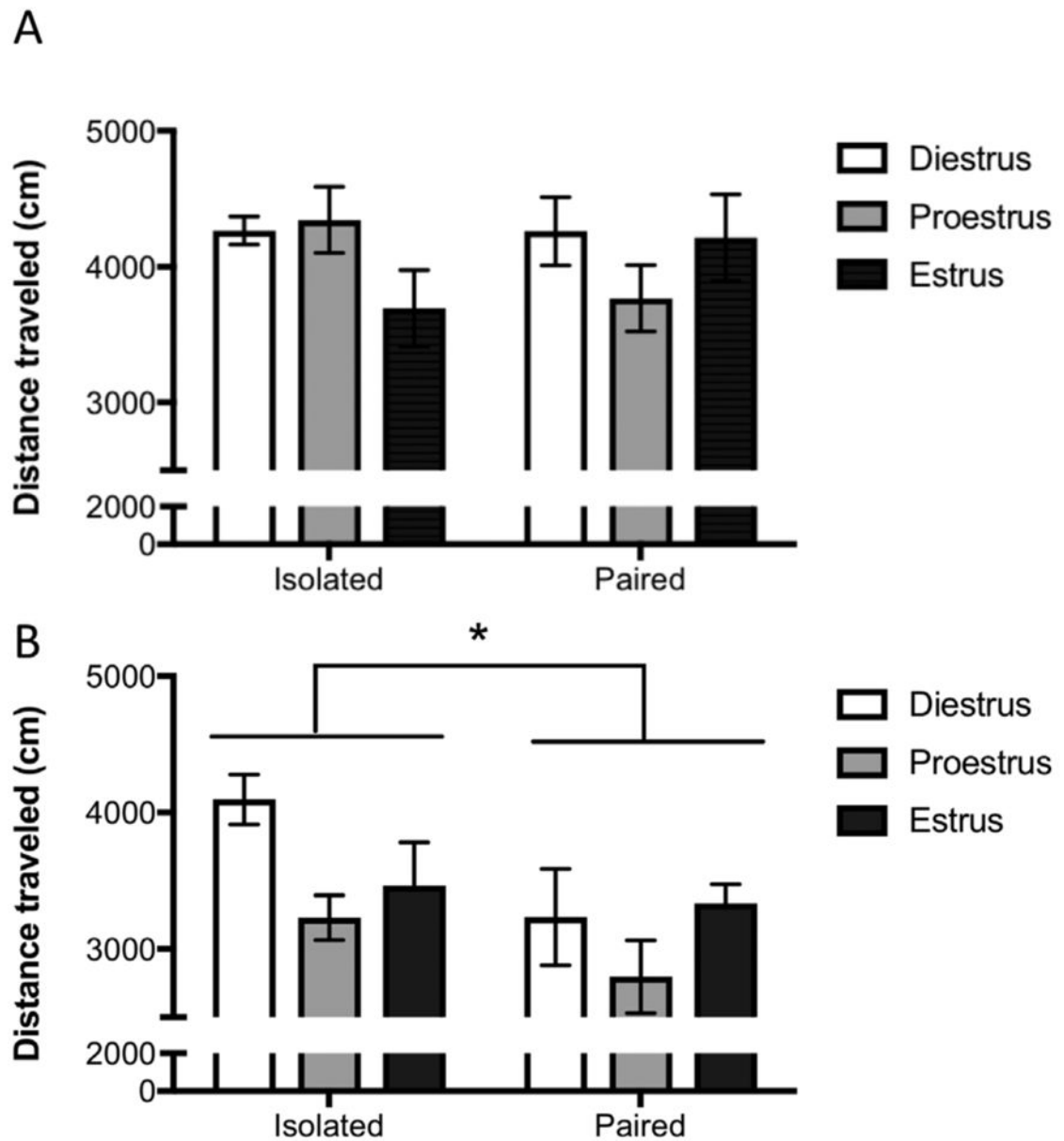
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Fig. 1.
Effects of social isolation and estrous cycle stage (A, B) in locomotion after social isolation or pairing measured by total distance travelled in the Open Field Test. No significant differences were found between isolated and paired rats in locomotion in the 0–5 min interval (A). However, isolated rats showed an increase in locomotion in the 5–10 min interval (B). Data are presented as mean ± SEM distance (cm). * $p < 0.05$.
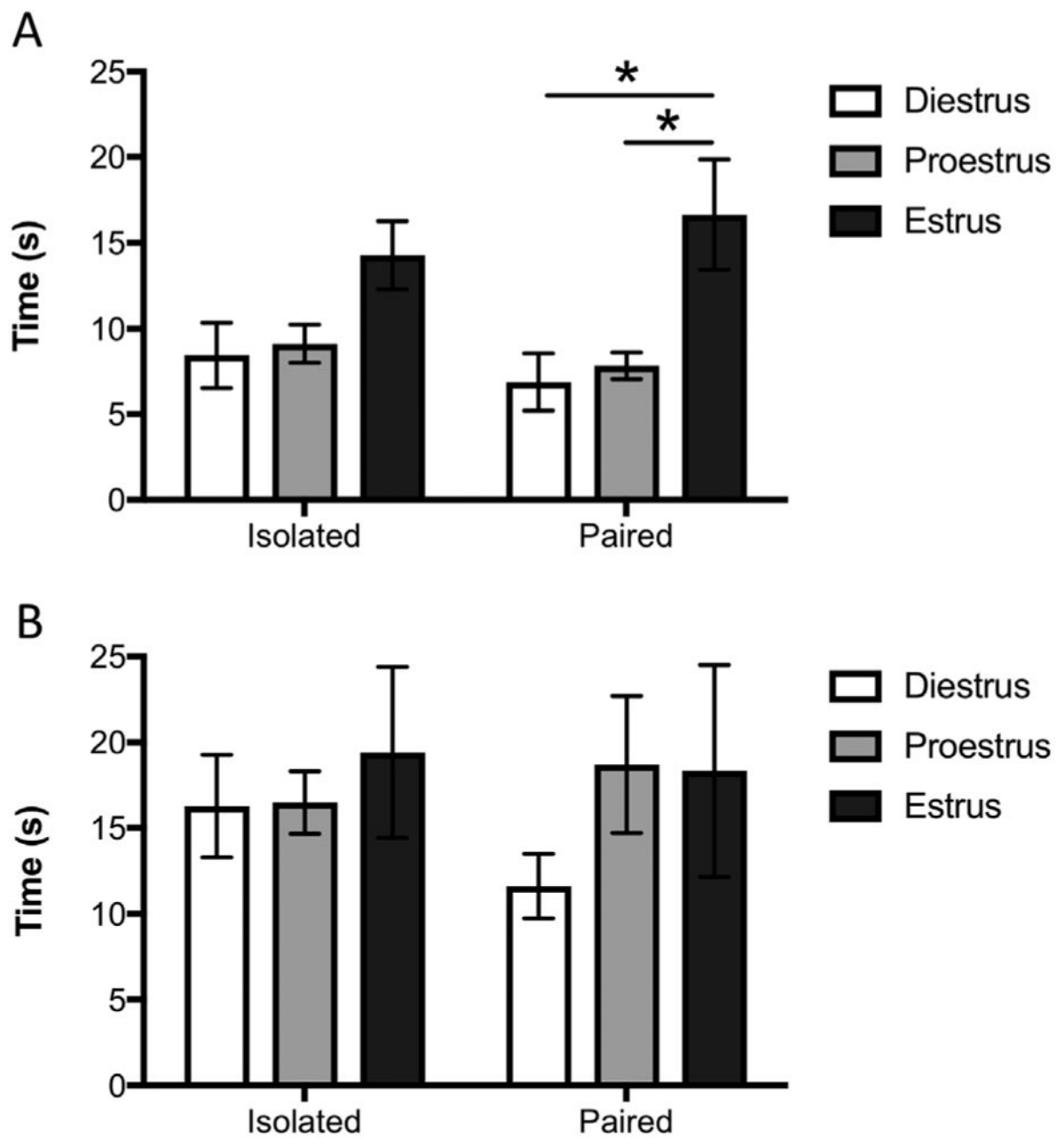
Fig. 2.
Effects of social isolation and estrous cycle stage in anxiety behaviors measured by time spent in the center in the Open Field Test. Paired females in estrus stage showed an increase in time in the center compared to proestrus and diestrus, suggesting a decrease anxiety (A). For the last five minutes of the test, there were no differences in time spent in the center of the apparatus, suggesting that the rats have habituated (B). Data are presented as mean ± SEM time (s). * $p < 0.05$.
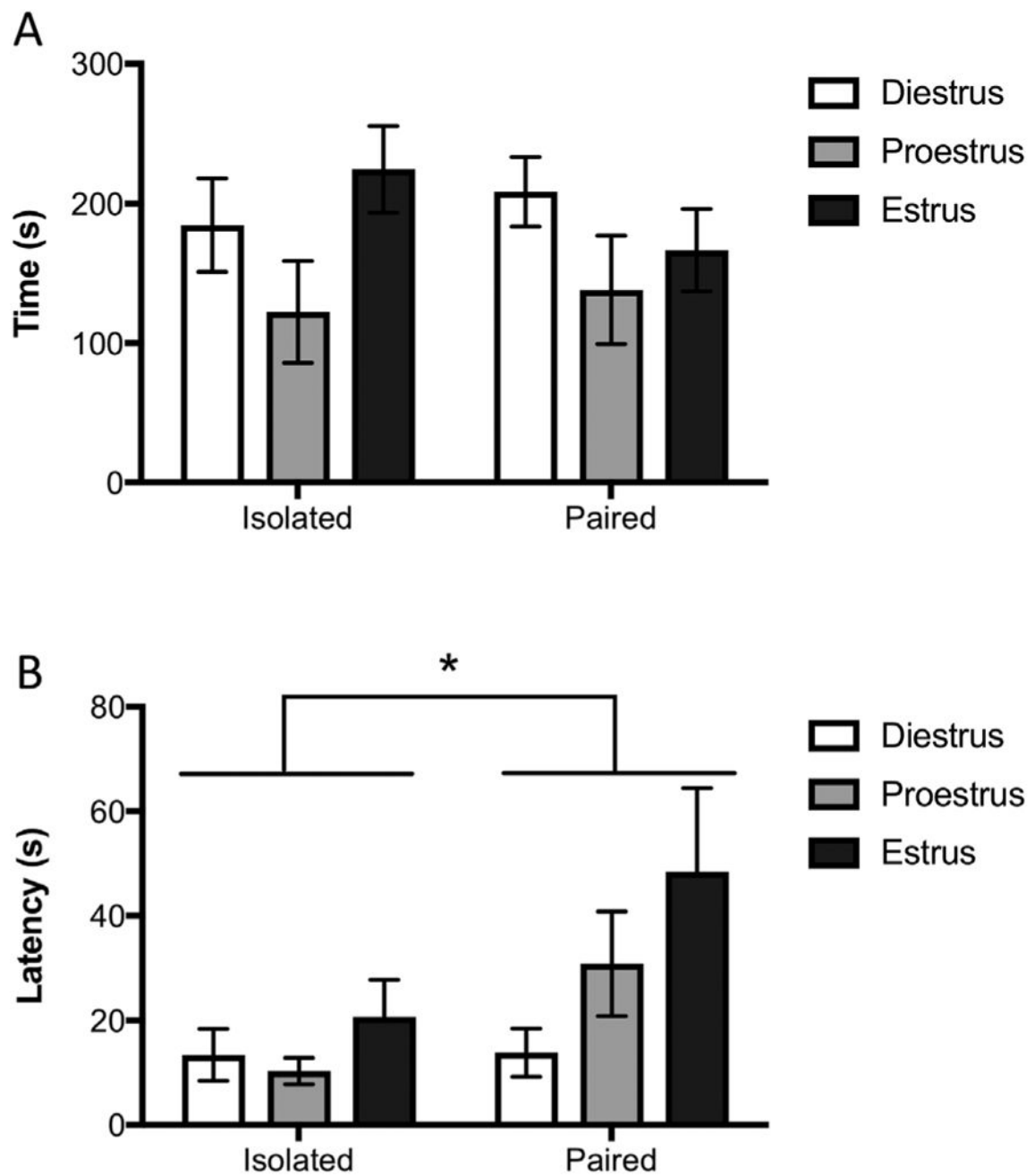
Fig. 3.
Effects of social isolation and estrous cycle stage in depressive-like behaviors measured by total immobility and latency to immobility in the Forced Swim Test. No significant differences were observed in the total immobility time by either isolation or estrous cycle stage (A). However, a main effect of isolation was found, where isolated rats displayed less latency to immobility compared to paired rats. Paired estrus showed an increase in latency to immobility when compared to isolated proestrus (p = 0.04) (B). Data are presented as mean ± SEM time (s). * p < 0.05.
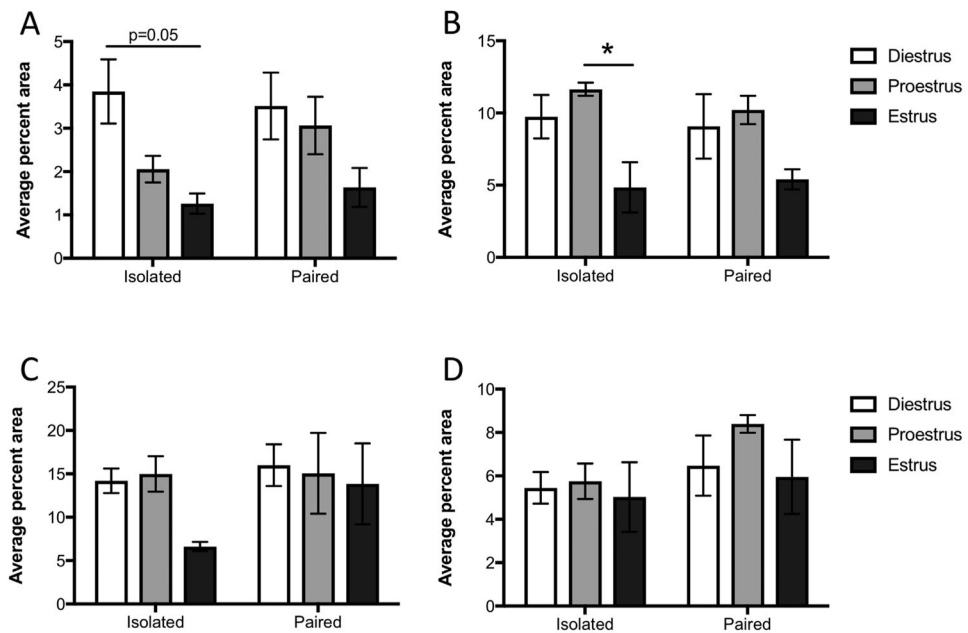
Fig. 4.
Effects of social isolation and estrous cycle stage in Glucocorticoid Receptor (GR) expression as measured by average percent area occupied in DG (A), CA3 (B), CA1 (D) and VMH (D). No significant differences were observed in the GR expression for isolated versus paired rats. A main effect for the estrous cycle was found in the DG. Isolated females in diestrus showed a trend to an increase in GR when compared to estrus (A; $p = 0.05$). Females in the estrus stage showed a decrease in GR expression compared to proestrus in the isolated group and a trend to a decrease for the paired group (B). No differences were found between isolated or paired rats or by estrous cycle stage for the other subareas (C, D). Data are presented as mean $\pm$ SEM percent area. * $p < 0.05$.
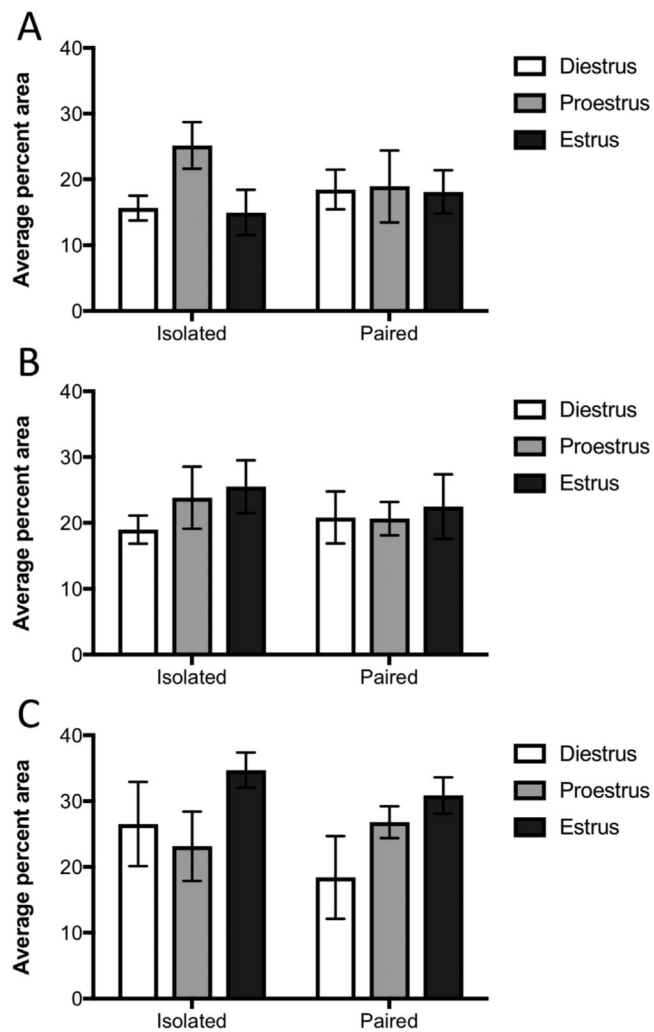
Fig. 5.
Effects of social isolation and estrous cycle stage in Synaptophysin expression as measured by average percent area immunolabeled in DG (A), CA3 (B), and CA1 (C). No differences were found between isolated or paired rats, or by estrous cycle stage for any subarea of the hippocampus (A, B, C). Data are presented as mean ± SEM of the percent area.

Table 1

Sources of variation in the effect of cycle and treatment for Corticosterone levels.

Corticosterone	Effect of Cycle	Effect of Treatment	Interaction
	$F_{(2, 29)} = 0.2536$, $p = 0.77$	$F_{(1, 29)} = 1.615$, $p = 0.21$	No

Table 2

Sources of variation in the effect of estrous cycle and treatment for Isolated and Paired female rats in Anxiety- and Depressive-like behaviors.

Behavior	Effect of Cycle	Effect of Treatment	Interaction
Distance 0–5 min	$F(2, 30) = 0.699$, p = 0.50	$F(1,30) = 0.009$, p = 0.93	No
Distance 5–10 min	$F(2, 29) = 3.636$, **p = 0.04**	$F(1, 29) = 5.514$, **p = 0.03**	No
Time center 0–5 min	$F(2, 30) = 8.630$, **p = 0.001**	$F(1, 30) = 0.009$, p = 0.96	No
Time center 5–10 min	$F(2, 29) = 0.821$, p = 0.45	$F(1, 29) = 0.134$, p = 0.72	No
Latency to Immobility	$F(2, 31) = 2.577$, p = 0.09	$F(1, 31) = 4.476$, **p = 0.04**	No
Immobility	$F(2, 31) = 2.700$, p = 0.08	$F(1, 31) = 0.049$, p = 0.83	No

Table 3

Sources of variation in the effect of estrous cycle and treatment for Isolated and Paired female rats in the expression of hippocampal and hypothalamic Glucocorticoid Receptor (GR).

Region	Effect of Cycle	Effect of Treatment	Interaction
DG	$F (2, 12) = 4.701$, **p = 0.03**	$F (1, 12) = 0.360$, $p = 0.56$	No
CA3	$F (2, 17) = 7.215$, **p = 0.01**	$F (1, 17) = 0.183$, $p = 0.67$	No
CA1	$F (2, 17) = 1.179$, $p = 0.33$	$F (1, 17) = 1.215$, $p = 0.29$	No
Ventromedial Hypothalamus	$F (2, 17) = 0.971$, $p = 0.40$	$F (1, 17) = 2.501$, $p = 0.13$	No

Table 4

Sources of variation in the effect of cycle and treatment for Isolated and Paired female rats in the expression of hippocampal synaptophysin.

Region	Effect of Cycle	Effect of Treatment	Interaction
DG	F (2, 25) = 1.303, p = 0.29	F (1, 25) = 0.001, p = 0.98	No
CA3	F (1, 25) = 0.185, p = 0.67	F (2, 25) = 0.453, p = 0.64	No
CA1	F (2, 22) = 2.880, p = 0.08	F (1, 22) = 0.596, p = 0.45	No