Investigation of Changes in Anxiety and Learning Behavior During Oestrous Cycle in the Rat

Sıçanda Östrus Siklusunda Anksiyete ve Öğrenme Davranışlarındaki Değişikliklerin Araştırılması

Kübra AKILLIOĞLU,[a]
Sayad KOCAHAN,[b]
Emine BABAR MELİK,[c]
Enver MELİK[c]

[a]Department of Physiology,
Division of Neurophysiology,
Çukurova University Faculty of Medicine,
[c]Retired Faculty Member of
Çukurova University, Adana
[b]Adıyaman University
School of Health Sciences, Adıyaman

Geliş Tarihi/*Received:* 30.09.2011
Kabul Tarihi/*Accepted:* 20.03.2012

Yazışma Adresi/*Correspondence:*
Sayad KOCAHAN
Adıyaman University
School of Health Sciences, Adıyaman,
TÜRKİYE/TURKEY
skocahan@posta.adiyaman.edu.tr

ABSTRACT Objective: Ovarian steroids modulate brain mechanisms such as learning, memory and novel object recognition. However, the influence of oestrous cycle on the selection of behavioural strategies in response to novelty and to partial or complete fearful stimuli has not yet been studied. In this study, we aimed to show the effect of oestrous cycle on learning, memory and selection of coping strategies in the face of anxiety. **Material and Methods:** We examined novelty-induced anxiety responses in female Wistar rats during the oestrous cycle in the open-field test and during the presentation of fearful partial or whole cues during the passive avoidance (PA) apparatus test. The open-field and passive avoidance behaviours of rats were analyzed during three phases of the oestrous cycle- oestrus, pro-oestrus and dioestrous. **Results:** The female rat in pro-oestrus phase spent less time in the centre of the open field ($p<0.001$) and showed decreased locomotor activity and exploratory behaviour in the apparatus ($p=0.003$). In the passive avoidance test, freezing behaviour increased in the pro-oestrus phase compared with the oestrus and dioestrous phases ($p<0.001$). **Conclusion:** The results of this study showed that the phases of the oestrous cycle could affect cognitive performance and behavioural coping strategies with novel stimuli and fearful cues.

Key Words: Anxiety; learning

ÖZET Amaç: Over kaynaklı steroidlerin, beynin öğrenme, bellek, yeni nesne tanıma mekanizmalarını ve anksiyete-korku davranışlarını düzenlediği bilinmektedir. Östrus siklusunun, duygusal ve bilişsel işlevler üzerindeki etkisi birçok çalışmada gösterilmesine rağmen, deneysel araştırmalarda elde edilen bulgular çelişkilidir. Bu çalışmada, östrus siklusunun anksiyete karşısında seçilen savunma stratejisi ve öğrenme-bellek üzerindeki etkilerinin araştırılması amaçlanmıştır. **Gereç ve Yöntemler:** Bu çalışmada, yetişkin Wistar dişi sıçanlarda, östrus siklusunun yenilik ile tetiklenen anksiyete cevapları, açık alan testinde, korku uyandıran ipuçlarına karşı savunma davranışları ve öğrenmeleri ise pasif sakınma testinde değerlendirilmiştir. Hayvanların açık alan ve pasif sakınma davranışları, östrus siklusunun östrus, proöstrus ve diöstrus evrelerinde incelenmiştir. **Bulgular:** Proöstrus evresindeki dişi sıçanların, açık alanın merkezinde geçirdikleri zaman ve düzeneği araştırmaları anlamlı ölçüde azalmış ($p=0,001$); düzenekteki lokomotor aktivitede de azalma olduğu gözlenmiştir ($p=0,003$). Pasif sakınma testinde, östrus ve diöstrus evresi ile karşılaştırıldığında, proöstrus evresinde donma davranışının arttığı ($p<0,001$) ve deneğin, korku uyandıran ipuçlarını daha iyi anımsadığı gösterilmiştir. **Sonuç:** Bu çalışmanın sonuçları, östrus siklus fazlarının, yeni uyaranlar ve korku uyandıran ipuçlarına karşı davranışsal savunma stratejilerini etkileyebileceğini göstermektedir.

Anahtar Kelimeler: Anksiyete; öğrenme

Turkiye Klinikleri J Med Sci 2012;32(5):1307-13

doi: 10.5336/medsci.2011-26719

The finding from epidemiological studies that the rapid fluctuation of the apparent emotional state of females seems to markedly increase with menarche and decrease with menopause has prompted investigation of the effect of ovarian steroids (oestrogen and progesterone) on emotional state and behaviour. Thus, the effect of ovarian steroids on cognitive and behavioural performance in humans and experimental animals has been the subject of recent research. Rodents have oestrous cycles, which are analogous to the human menstrual cycle. In studies involving rodent oestrous cycle phase (pro-oestrus, oestrus and dioestrous), ovarian steroids were shown to affect emotional state fluctuation, learning, memory and motor activity.[1-3]

Important physiological changes during the pro-oestrus phase of the oestrous cycle, which is the period in which the quantity of ovarian steroids in the brain peaks, were reported in a number of studies.[1,4,5] Increases in synaptic plasticity and synaptic density in the hippocampus and changes in Long Term Potentiation (LTP) are some examples.[4-6] Decreases in hippocampal synaptic density and the number of synapses were detected during the oestrus phase, in which the quantity of ovarian steroids in the brain decreases.[7,8] The structural changes that take place during the oestrous cycle were reported to cause significant behavioural changes in test animals.[9]

Experimental studies found a relationship between the levels of oestrogen and progesterone and anxiety-related behaviour.[9,10] Studies on novelty-induced anxiety that employed the open-field test showed both decreased and increased anxiety with high oestrogen levels.[11,12] Other studies showed no change in anxiety level.[13] In ovariectomised subjects treated with progesterone, a decrease in anxiety in the open-field test was reported.[14]

Clinical research revealed a direct relationships between ovarian steroids and protection of neuronal networks, memory formation and memory storage.[15,16] In studies by Kimura et al. including females with high oestrogen levels and low levels of other ovarian steroids, who are in the early follicular phase, comparisons of verbal capability and muscular coordination showed that oestrogen levels could affect these capabilities in a negative way.[16] In studies of oestrogen replacement therapy on females in the menopausal phase, beneficial effects on cognitive function were shown in schizophrenic and Alzheimer patients.[17-20]

Females were reported to select emotion-focused strategies to cope with stress.[21] The use of emotion-focused strategies were attributed to the perception of females that the cause of stress is unchangeable and that they have to tolerate the situation. The relationship between female selection of stress-coping strategies and ovarian steroids cannot be clarified perfectly yet. Although there are many studies showing the effects of ovarian steroids on emotional and cognitive functions, there are conflicting findings among the experimental research results.[11-13] In this study, we aimed to evaluate behavioural strategy selection in the open-field test during novelty-induced anxiety and learning and memory performance and behaviour in response to partial or complete acquired fearful cues in a passive avoidance test in the female Wistar rat, during the pro-oestrus, oestrus and dioestrous phases of the oestrous cycle.

MATERIAL AND METHODS

This experiment was approved by the Çukurova University Medical Sciences Experimental Research and Practice Center Local Ethics Committee. The procedures in the study were performed according to the National Institute of Health Guide for Care and Use of Animals.

ANIMALS

Wistar rats bred in Çukurova University Faculty of Medicine Physiology Laboratory were used. Female rats 12-13 weeks old (all groups n=10) weighing 250-270 g were used in the behaviour test. Food and water were given without restriction. The room temperature was fixed at 21±2°C; the behaviour tests and animal breeding were performed at this temperature. The light-dark cycle of the room in which the animals were housed was fixed at 12 hours of light followed by 12 hours of dark ($05^{:00}$-$17^{:00}$ light, $17^{:00}$-$05^{:00}$ dark). The behaviour tests took place at a controlled time, from $10^{:00}$-$12^{:00}$. The animals were allowed to adapt to hands and to the room before the behaviour tests.

DESCRIPTION OF THE OESTROUS CYCLE

The oestrous cycle of female rats was tracked using vaginal smear starting one week before the behaviour test. Vaginal smear was performed from 8:00-9:00 before the behaviour test. Animals in the pro-oestrus, oestrus and dioestrous phases of the oestrous cycle were used in the behaviour tests. Only those rats that had at least two regular 4-day estrous cycles were used for the experiments.

APPARATUS

Open Field

The open-field apparatus used in the behaviour test had dimensions 103x103x51 cm, was made of black plexiglass and had an open top. The base was divided into twenty-five squares and was enclosed by a 2 cm thick wall in the shape of a square box. The region of the open-field apparatus next to the square wall was called the periphery and the rest was called the centre field. Rats naturally prefer the safe periphery, and they avoid the anxiety-inducing centre field. In the open-field apparatus, spending time in the centre and peripheral fields was considered a criterion of novelty-induced anxiety.[22-24] The distance travelled in the apparatus in the centre and peripheral fields represents locomotor activity. The frequency of rearing (vertical activity) was considered an exploratory behaviour.[23] At the time of the test, the animals were placed in the apparatus randomly and their behaviour was recorded for five minutes by a video camera. The apparatus was cleaned with 5% alcohol between the experiments.

Passive Avoidance

The animals underwent a passive avoidance test one hour after the open-field test. The passive avoidance apparatus had two parts, one made of black plexiglass (dark part, 27x27x27 cm) and the other transparent (light part, 25x17x17 cm), which were connected to one another by a hole. The light part of the apparatus was lit by a 60 W lamp located at a height 36 cm above the ground. At the time of test, the rat was placed in the light part of the apparatus and was expected to move by instinct to the dark part of the apparatus. A 1 mA electric shock was applied to the grid floor of the dark part for 2 sec. Shortly after the electric shock application to the foot, the rat was taken from the apparatus and was placed in the light part of the apparatus again after 1 min. The percentage of time animals spent exhibiting freezing behaviour in the light part of the apparatus during 300 sec was recorded.[25] The freezing behaviour of rats was considered a behavioural criterion of learning and memory.[26] The apparatus was cleaned with 5% alcohol between the experiments.

STATISTICAL ANALYSIS

The data were expressed as median (min-max). For statistical analysis, the statistical software SPSS 11.5 (Chicago, IL.) was used. The Kruskal-Wallis test was used to make comparisons between the groups. When significant differences were observed, two group comparisons were made by Mann-Whitney U test. The significance level was determined to be $p<0.05$.

RESULTS

OPEN-FIELD TEST

In the open-field test, the effect of the oestral cyclus on time spent in the centre and periphery of the apparatus, the novelty-induced anxiety criterion was significant ($p=0.001$). Compared with rats in the oestrus phase, rats in the pro-oestrus ($p=0.001$) and dioestrous ($p=0.002$) phases spent less time at the centre of apparatus (Figure 1). There were no significant differences between the rats in the pro-oestrus and dioestrous phases ($p=0.224$).

Regarding locomotor activity, there were significant differences in the distance travelled in the apparatus ($p=0.008$). The rats in the pro-oestrus ($p=0.003$) and dioestrous ($p=0.015$) phases travelled significantly less distance in the apparatus compared with rats in the oestrus phase (Figure 2). There was no significant difference in distance travelled in the apparatus between the rats in the pro-oestrus and dioestrous phases ($p=0.820$).

In the open-field apparatus, there were significant differences in the frequency of rearing, which is an exploratory behaviour (p<0.001). The rats in the pro-oestrus (p<0.001) and dioestrous (p<0.001) phases had significantly decreased frequencies of rearing compared with rats in the oestrus phase (Figure 3). There was no significant difference between the rats in the pro-oestrus and dioestrous phases (p=0.650) (Table 1).

PASSIVE AVOIDANCE TEST

The passive avoidance test revealed significant differences between the groups in freezing time (%) in the light part of the apparatus (p<0.001). The rats in the pro-oestrus phase had significantly increased freezing times compared with rats in the dioestrous and oestrus phases (p<0.001) (Figure 4). There were no significant differences between the rats in the oestrus and dioestrous phases (p=0.634) (Table 2).

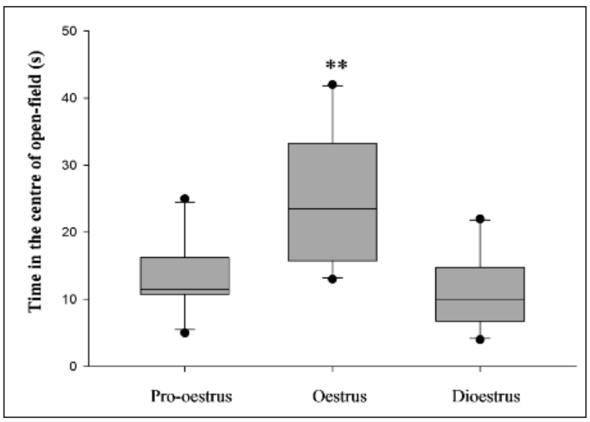

FIGURE 1: Time spent at the centre area of the open field (s).
$n_{pro-oestrus}=n_{oestrus}=n_{dioestrus}=10$.
**p<0.01 compared to pro-oestrus and dioestrous.

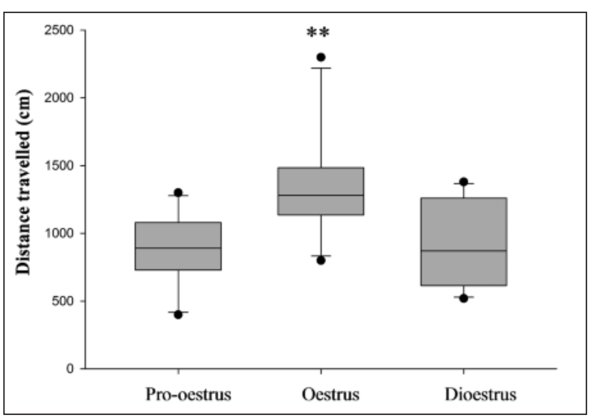

FIGURE 2: Distance travelled in the open field (cm).
$n_{pro-oestrus}=n_{oestrus}=n_{dioestrus}=10$.
**p<0.01 compared to pro-oestrus and dioestrous.

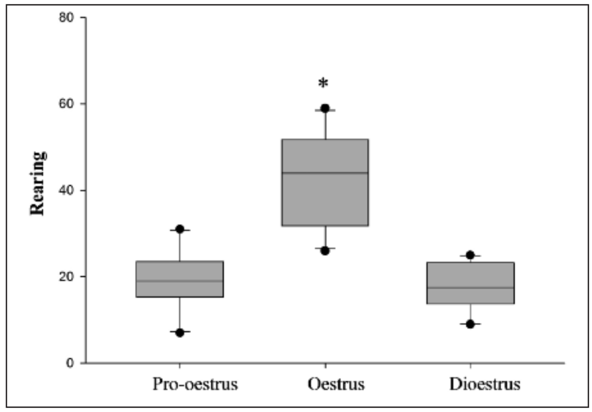

FIGURE 3: Frequency of rearing in the open field.
$n_{pro-oestrus}=n_{oestrus}=n_{dioestrus}=10$.
*p<0,001 compared to pro-oestrus and dioestrous.

DISCUSSION

In our study, the rats in the pro-oestrus and dioestrous phases showed decreased locomotor activity, exploratory behaviour and time spent in the centre of the apparatus in the open-field test. Other studies on behaviour in response to novel anxiety-inducing stimuli in the open-field test, also reported significant differences between the phases of the oestrous cycle.[11-13] While some studies report increases in time spent at the centre of the apparatus in the open-field test in the pro-oestrus and dioestrous phases, others show decreases.[11,12]

In studies, differences in locomotor activity and exploratory behaviour have been shown.[11-13] Increased time spent at the centre of the apparatus in the open-field test was suggested to indicate decreased novelty-induced anxiety.[23] In our study, animals adapted to the anxiety-inducing centre of the open-field apparatus, spent more than expected time at the centre field and showed increases in locomotor activity and exploratory behaviour, which may be interpreted as an active behaviour strategy in response to novelty-induced anxiety. While the rats in the pro-oestrus and dioestrous phases exhibited a passive behaviour strategy in response to novelty-induced anxiety in the open-field test, the

TABLE 1: Behaviours in the open-field test.

	Pro-oestrus (n=10) median (min-max)	Oestrus (n=10) median (min-max)	Dioestrus (n=10) median (min-max)	(p)		
Time spent at the centre (s)	11.5(5-25)	23.5(13-42)	10(4-22)	0.001	Oestrus-prooestrus	0.001
					Oestrus-dioestrus	0.002
					Prooestrus-dioestrus	0.224
Distance travelled (cm)	890(400-1300)	1280(800-2300)	870(520-1380)	0.008	Oestrus-prooestrus	0.003
					Oestrus-dioestrus	0.015
					Prooestrus-dioestrus	0.820
Rearing	19(7-31)	44(26-59)	17.5(9-25)	<0.001	Oestrus-prooestrus	<0.001
					Oestrus-dioestrus	< 0.001
					Prooestrus-dioestrus	0.650

rats in the oestrus phase displayed an active behaviour strategy.

In the pro-oestrus phase, levels of ovarian steroids (oestrogen, progesterone) rise. They then drop in the oestrus phase and rise again in the dioestrous phase.[27-29] Studies have shown that the Hypothalamic-pituitary-adrenal (HPA) axis is more sensitive to environmental stressors in the presence of oestrogen.[30] Ovariectomised rats treated with estradiol have greater HPA responses than those not treated.[31] Higher levels of ACTH and corticosterone are produced in response to stress in female rats when levels of oestrogen were high during the early pro-oestrus phase.[32] This is compatible with the results of our study; the passive behaviour strategy for coping with novelty-induced anxiety observed in the pro-oestrus (the period of peak oestrogen levels) and dioestrous (the period when oestrogen levels begin to increase) phases may be attributed to high levels of oestrogen that increases sensitivity to environmental stressors.

In the passive avoidance test, the evaluation of the freezing behaviour of animals in the pro-oestrus, dioestrous and oestrus phases revealed that the animals in the pro-oestrus phase displayed much more freezing behaviour than the animals in the dioestrous and oestrus phases. This shows that animals in the pro-oestrus phase have a better memory and display a passive behaviour strategy in response to fearful stimuli. In the passive avoid-

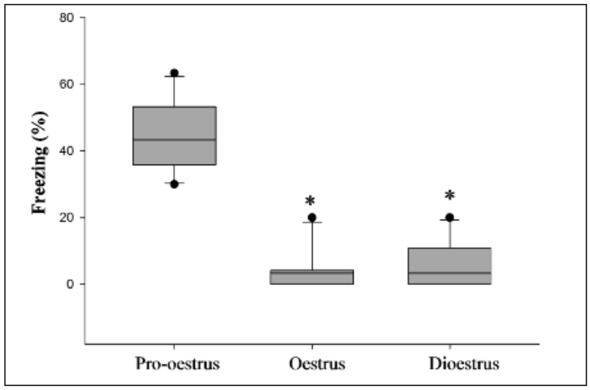

FIGURE 4: Freezing (%) in the passive avoidance test.
$n_{pro\text{-}oestrus}=n_{oestrus}=n_{dioestrus}=10$.
*p<0.001 compared to pro-oestrus.

ance test, animals in the oestrus phase displayed an active behaviour strategy in response to fearful stimuli and had a poorer memory, consistent with the results of the open-field test.

The finding that freezing behaviour decreased in response to fearful cues suggested that the animals in the dioestrous phase displayed an active behaviour strategy in the passive avoidance apparatus and showed decreased memory. While the female rats in the dioestrous phase selected a passive behaviour strategy in response to novelty in the open-field test, selecting an active behaviour strategy in the passive avoidance test may be attributed to the ovarian steroid profile of the rats in this phase (low progesterone level and oestrogen level beginning to rise).

TABLE 2: Freezing (%) in passive avoidance test.

	Pro-oestrus (n=10) median (min-max)	Oestrus (n=10) median (min-max)	Diestrus (n=10) median (min-max)	(p)		
Freezing (%)	43.3(30-63.3)	3.3(0-20)	3.3(0-20)	<0.001	Oestrus-prooestrus	<0.001
					Oestrus-dioestrus	0.634
					Prooestrus-dioestrus	<0.001

Increased memory in the pro-oestrus phase in the passive avoidance test may be associated with high levels of oestrogen in this phase. In rats, changes in hippocampal plasticity have been shown to respond to the cyclic changes in oestrogen and progesterone that occur in the oestrous cycle.[8] Maximum increases in synaptic branching in CA1 pyramidal cells were observed during the pro-oestrus phase, during which oestrogen peaks; in the oestrus phase, during which hormone levels are low, there was a 30% decrease in synaptic density and the number of synapses.[8,19] Ovarian steroids were shown to cause electrophysiological changes in the hippocampus.[5] While oestrogen had excitatory effects, the effects of progesterone and its metabolites were inhibitory.[33] However, the effect of ovarian steroids on the formation and storage of memory was suggested to be related to the neurotransmitter systems (GABA, glutamate, acetylcholine).[34] The fact that these changes in the hippocampus have important impacts on learning and memory are consistent with the results of our study.

The results of our study suggest that in the pro-oestrus phase, the effects of oestrogen are the selection of passive behaviour strategies to fearful cues and novelty-induced fear and positive effects on cognitive performance.

CONCLUSION

The results of this study showed that phases of the oestrous cycle might affect cognitive performance and behavioural coping strategies to novel stimuli and fearful cues.

REFERENCES

1. Walf AA, Rhodes ME, Frye CA. Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. Neurobiol Learn Mem 2006;86(1): 35-46.
2. Gouveia A Jr, dos Santos UD, Felisbino FE, de Afonseca TL, Antunes G, Morato S. Influence of the estrous cycle on the behavior of rats in the elevated T-maze. Behav Processes 2004;67(2):167-71.
3. Llaneza DC, Frye CA. Progestogens and estrogen influence impulsive burying and avoidant freezing behavior of naturally cycling and ovariectomized rats. Pharmacol Biochem Behav 2009;93(3):337-42.
4. Korol DL, Unick K, Goosens K, Crane C, Gold PE, Foster TC. Estrogen effects on spatial performance and hippocampal physiology in female rats. Soc Neurosci Abstr 1994;20(1): 1436.
5. Warren SG, Humphreys AG, Juraska JM, Greenough WT. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res 1995;703(1-2):26-30.
6. Woolley CS, McEwen BS. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 1992;12(7):2549-54.
7. Sutcliffe JS, Marshall KM, Neill JC. Influence of gender on working and spatial memory in the novel object recognition task in the rat. Behav Brain Res 2007;177(1):117-25.
8. Woolley CS. Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. Horm Behav 1998; 34(2):140-8.
9. Byrnes EM, Bridges RS. Reproductive experience alters anxiety-like behavior in the female rat. Horm Behav 2006;50(1):70-6.
10. Marakaoğlu K, Özdemir S, Çivi S. [Postpartum depression: review]. Turkiye Klinikleri J Med Sci 2009;29(1):206-14.
11. Meziane H, Ouagazzal AM, Aubert L, Wietrzych M, Krezel W. Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. Genes Brain Behav 2007;6(2):192-200.
12. Morgan MA, Pfaff DW. Effects of estrogen on activity and fear-related behaviors in mice. Horm Behav 2001;40(4):472-82.
13. Diaz-Veliz G, Soto V, Dussaubat N, Mora S. Influence of the estrous cycle, ovariectomy and estradiol replacement upon the acquisition of conditioned avoidance responses in rats. Physiol Behav 1989;46(3):397-401.
14. Fernandez-Guasti A, Picazo O. Changes in burying behavior during the estrous cycle: effect of estrogen and progesterone. Psychoneuroendocrinology 1992;17(6):681-9.

15. Hampson E. Estrogen-related variations in human spatial and articulatory-motor skills. Psychoneuroendocrinology 1990;15(2):97-111.
16. Kimura D, Hampson E. Cognitive pattern in men and women is influenced by fluctuations in sex hormones. Curr Dir Psychol Sci 1994; 3(2):57-61.
17. Amantea D, Russo R, Bagetta G, Corasaniti MT. From clinical evidence to molecular mechanisms underlying neuroprotection afforded by estrogens. Pharmacol Res 2005; 52(2):119-32.
18. Henderson VW, Paganini-Hill A, Emanuel CK, Dunn ME, Buckwalter JG. Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects. Arch Neurol 1994;51(9):896-900.
19. Birzniece V, Bäckström T, Johansson IM, Lindblad C, Lundgren P, Löfgren M, et al. Neuroactive steroid effects on cognitive functions with a focus on the serotonin and GABA systems. Brain Res Rev 2006;51(2):212-39.
20. Kulkarni J, Riedel A, de Castella AR, Fitzgerald PB, Rolfe TJ, Taffe J, et al. A clinical trial of adjunctive oestrogen treatment in women with schizophrenia. Arch Womens Ment Health 2002;5(3):99-104.
21. Cotton DHG. Stress and gender. Stress Management-An Integrated Approach to Therapy. 1st ed. New York: Brunner/Mazel, Inc; 1990. p.1-3.
22. Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav Brain Res 2002;134(1-2):49-57.
23. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463(1-3):3-33.
24. Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R. Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. Brain Behav Immun 2007;21(4):458-66.
25. Melik E, Babar E, Ozen E, Ozgunen T. Hypofunction of the dorsal hippocampal NMDA receptors impairs retrieval of memory to partially presented foreground context in a single-trial fear conditioning in rats. Eur Neuropsychopharmacol 2006;16(4):241-7.
26. Fanselow MS. Conditioned and unconditional components of post-shock freezing. Pavlov J Biol Sci 1980;15(4):177-82.
27. Takeo Y. Influence of continuous illumination on estrous cycle of rats: time course of changes in levels of gonadotropins and ovarian steroids until occurrence of persistent estrus. Neuroendocrinology 1984;39(2):97-104.
28. Hawley WR, Grissom EM, Barratt HE, Conrad TS, Dohanich GP. The effects of biological sex and gonadal hormones on learning strategy in adult rats. Physiol Behav 2012;105(4):1014-20.
29. Pompili A, Tomaz C, Arnone B, Tavares MC, Gasbarri A. Working and reference memory across the estrous cycle of rat: a long-term study in gonadally intact females. Behav Brain Res 2010;213(1):10-8.
30. Carey MP, Deterd CH, de Koning J, Helmerhorst F, de Kloet ER. The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. J Endocrinol 1995;144(2):311-21.
31. Le Mevel JC, Abitbol S, Beraud G, Maniey J. Dynamic changes in plasma adrenocorticotrophin after neurotropic stress in male and female rats. J Endocrinol 1978;76(2):359-60.
32. Smith MD, Jones LS, Wilson MA. Sex differences in hippocampal slice excitability: role of testosterone. Neuroscience 2002;109(3):517-30.
33. Cahill L. Why sex matters for neuroscience. Nat Rev Neurosci 2006;7(6):477-84.
34. Dohanich G. Ovarian steroids and cognitive function. Curr Dir Psychol Sci 2003;12(1): 57-61.