NÖRO PSİKİYATRİ ARŞİVİ
ARCHIVES OF NEUROPSYCHIATRY

RESEARCH ARTICLE

Arch Neuropsychiatry 2022;59:325–329
https://doi.org/10.29399/npa.28099

Repeated Collection of Vaginal Smear Causes Stress in Mice

Aslıhan BAHADIR VAROL[1], Emre Cem ESEN[1], Emine EREN KOÇAK[1,2]

[1]Hacettepe University, Institute of Neurological Sciences and Psychiatry, Ankara, Turkey
[2]Hacettepe University, Faculty of Medicine, Department of Psychiatry, Ankara, Turkey

ABSTRACT

Introduction: Women are more likely to be misdiagnosed in many neuropsychiatric disorders than men. One of the possible underlying reasons for this disparity may be more frequent use of male mice than female mice in neuroscience studies. With the increasing realization of the shortcomings of this approach in understanding the neurobiological basis of these disorders, many funding agencies mandate the inclusion of both male and female subjects in study design. As the behaviors vary with the stage of the estrous cycle, the collection of vaginal smears to identify the estrous stage becomes a widely used procedure. Here we tested whether vaginal smear collection causes similar effects to that of stress by evaluating an increase in depression-like behavior and impairment in memory.

Method: Vaginal smear was collected from Swiss albino mice twice a day for 10 days. In order to test depression-like behavior tail suspension, sucrose preference and splash tests were conducted. Novel object recognition and novel object location tests were performed 1 hour and 24 hours after training to evaluate short-and long-term memory respectively.

Results: The female mice whose vaginal smears were collected demonstrated increased behavioral despair and anhedonia. Vaginal smear group showed deficits in both short-term and long-term memory when compared to the control group.

Conclusion: Our results indicate that the collection of vaginal smear not only increased depression-like behaviors in mice, but also impaired short-term and long-term memory, indicating that the procedure of vaginal smear collection was stressful. We recommend to consider other ways of estrous cycle staging when studying behavior.

Keywords: Depression, estrous cycle, memory, stress, vaginal smear

Cite this article as: Bahadır Varol A, Esen EC, Eren Koçak E. Repeated Collection of Vaginal Smear Causes Stress in Mice. Arch Neuropsychiatry 2022;59:325–329.

INTRODUCTION

The use of laboratory animals has been fundamental for neuroscience research, and it has proven to be crucial in understanding how the brain functions (1,2). However, the translational success of basic neuroscience studies in clinical settings was lower than expected (3–5). One of the reasons is thought to be the use of only male animals in research settings. Neuroscience studies showed the greatest bias towards using male animals among the 10 biological fields investigated by Beery and Zucker (2010), in which male animals were used ~6 times more often than females (6).

It has been increasingly recognized that there is a qualitative difference in male and female behaviors. They adopt different strategies in learning, decision making, responding to threat and spatial navigation (for a detailed review please see reference 7). Therefore, the pitfalls of obtaining the behavioral data from male animals and leaving females out are now clear to the scientific community and grant holders. As a result, several funding agencies and scientific journals across Europe, Canada and the United States of America mandate the inclusion of both sexes in research designs rather than generalizing the results of male animals to both sexes (6–8).

The need to include female animals becomes more evident for studies aimed at investigating the neurobiological basis of psychiatric disorders, given the higher prevalence of psychiatric disorders, such as major depressive disorder and anxiety disorders, among women (9,10). The stage of the estrous cycle during which the experiment was conducted should be taken into consideration in interpreting the research results in females, as the behavior of females shows variability depending on the different stages of the estrous cycle (11,12). An increasing number of studies exploring the behavioral effects of the estrous cycle were conducted in the last two decades. Research suggests that different

Highlights

- **Inclusion of both male and female subjects in study designs is necessary.**
- **Behaviors may vary with the stage of the estrous cycle in females.**
- **Vaginal smear is collected to determine the stage of the estrous cycle.**
- **Repeated vaginal smear collection increases depression-like behavior and disturbs memory.**
- **Visual inspection of vagina is recommended for estrous cycle staging when possible.**

Correspondence Address: Emine Eren Koçak, Hacettepe Üniversitesi Tıp Fakültesi, Psikiyatri Anabilim Dalı, Ankara,Turkey • **E-mail:** emineerenkocak@gmail.com
Received: 08.01.2022, **Accepted:** 30.01.2022, **Available Online Date:** 08.11.2022

aspects of behavior, like unconditioned fear response, social preferences and learning strategy were affected during the estrous cycle (11–13).

To control the effects of the estrous cycle on behavior, researchers determine and follow the stages of the estrous cycle by observing the appearance of external genitalia, examining vaginal cytology, or conducting a biochemical analysis of the urine (14–19). Measuring the electrical impedance of vaginal epithelial cells to determine the stage of the estrous cycle is controversial (14). The observation of the external genitalia is the quickest and the cheapest method, it does not require use of any equipment, it is non-invasive, and it is best for identifying the proestrus or estrus stages. It also necessitates the training of the observer. In cases where identification of all stages of the estrous cycle is required, vaginal cytology is the recommended method (16).

A complete estrous cycle in mice usually takes 4–5 days (20) and documenting at least two consecutive cycles is recommended to detect whether the mice have a steady estrous cycle length (11,21,22). Depending on the requirements of the research design, researchers collect vaginal smears for different periods of time ranging between 10–21 days (11,23–25). In this study, we showed that performing vaginal smears in mice twice a day for 10 days, corresponding to two consecutive estrous cycles, increased depression-like behaviors and caused deficits in both short-term and long-term memory performances. These findings indicate that obtaining vaginal smears can be stressful to animals, leading to changes in their behaviors, which should be taken into consideration when designing experiments.

METHODS

Subjects

Female Swiss albino mice weighing 30–35 grams (n=21) were used. Animals were maintained on a 12/12 hours (h) light/dark cycle at 22±3°C and fed ad libitum. All behavioral experiments were conducted during the light cycle. This research was approved by the Hacettepe University Animal Experimentations Local Ethics Board on 23/10/2019 with the approval number 2019/11-01. Vaginal smears were performed twice a day for 10 days (n=14) for the study group. The control group (n=7) was left undisturbed in their home cages.

Vaginal Smear Test

A blunt sphere pinhead was inserted ~1.7 mm into the vagina to collect the vaginal smear which was then spread onto a slide (26–28). In female mice, the estrous cycle consists of four respective stages; proestrus, estrus, metestrus, and diestrus (25), which usually last for 4–5 days. Documenting at least two consecutive cycles is recommended to detect whether the mice have a steady estrous cycle length (11,21). For this reason, we chose to perform vaginal smears for 10 days, the time window that corresponds to two consecutive cycles. We started behavioral experiments on day 5 as our preliminary findings suggest that the depressogenic effects begin as early as the 5th day of collecting vaginal smears. We performed vaginal smears twice a day as it is a frequently used and well-established method (25). Microscopical differentiation of proestrus and estrus stages is challenging and can be mistaken for each other, therefore conducting vaginal smears once a day may be misleading (29).

Behavioral Experiments

Behavioral experiments started on the 5th day of vaginal smear collection and were performed 1 day apart. Behavioral experiments were conducted in an order of increasing stress (Figure 1).

Sucrose Preference Test

This is a test to evaluate the preference of mice for sucrose over water,

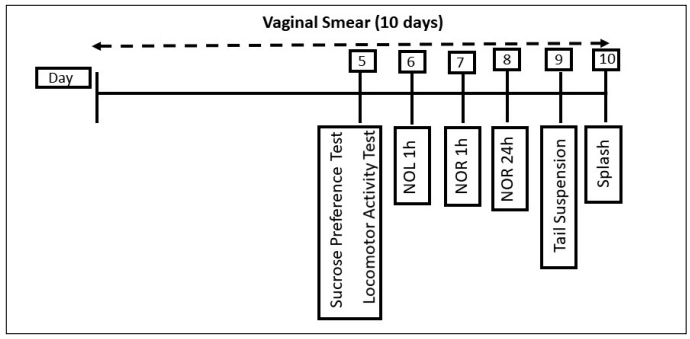

Figure 1. Schematic diagram of experimental design.
h: hour

which is considered a model of hedonic behavior in animals. A decrease in sucrose preference is used as a measure of anhedonia (30).

On the first day, mice were acclimated to consuming sucrose solution by placing two bottles each containing 1% sucrose solution on both sides of their cage overnight for 12 hours. We carried out the test on the 2nd and 4th days, leaving a day off in between during which they had access to only tap water. This was done in order to prevent the mice from getting used to the continuous availability of sucrose, as this may have caused a reduction in the hedonic value of sucrose solution.

On the test days (2nd and 4th days), two identical bottles, one filled with tap water and the other filled with 1% sucrose solution, were placed on either side of the cages. In order to check preference for one side, the bottles containing the sucrose solution were placed on the right side of the cage in half of the animals and on the left side of the cage in the other half; the sides were switched on the second test day (4th day). All the bottles were weighed before and after the 12-hour test period and the difference between pre and post-test weights of each bottle was used as a measure of water or sucrose solution consumed over 12 hours. The preference index was calculated by the following formula: 100 × (sucrose solution consumed/total fluid consumed)

Locomotor Activity Test

Mice were individually placed in a square box (widthxheight: 22.5×30 cm) for 10 minutes. Their activity was tracked and analyzed by Ethovision XT-8. The total distance traveled was calculated as a measure of locomotor activity.

Novel Object Recognition and Novel Object Location Tests

Novel Object Recognition and Novel Object Location Tests (NOR/NOL) were carried out in the same box used for the evaluation of locomotor activity. NOR is more selective to the evaluation of entorhinal functions whereas NOL is more selective to hippocampal functions (31). Two identical objects were placed 6 cm away from two opposite corners of the box and mice were allowed to explore the objects for 10 minutes (training). NOR-1 h was carried out by replacing one of the objects with a novel object, which had a distinctly different shape and was made of a different material 1-hour (1-h) after training. Then, the time spent exploring familiar and novel objects for 5 minutes was tracked and analyzed by Ethovision XT-8. The ratio of the time spent exploring the novel object/time spent exploring both objects was used as a measure of short-term memory. Twenty-four hours after the training, NOR-24 h was performed by replacing the novel object from NOR-1 h with a novel object that is different in shape and material from the previous two objects used. Mice were allowed to explore the objects for 5 minutes and the ratio of the time spent exploring the novel object/time spent exploring both objects was used as a measure of long-term memory. NOL-1 h was carried out similarly to NOR-1 h, but instead of replacing the object, its location was changed and moved to the opposite corner

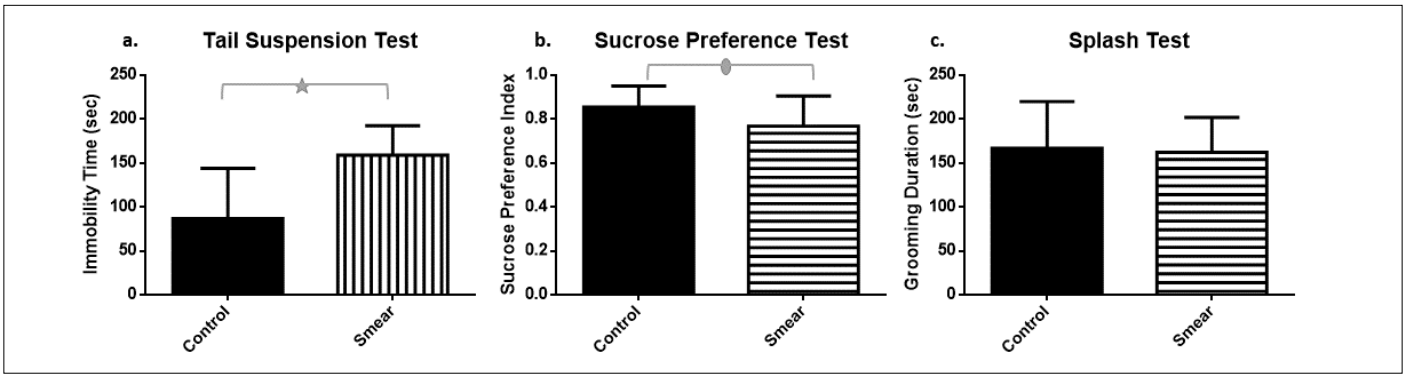

Figure 2. a–c. The results of tail suspension, sucrose preference and splash tests. The vaginal smear group spent more time immobile in tail suspension test **(a).** The sucrose preference index tended to be lower in the vaginal smear group than the control group **(b).** There was no difference in the duration of self-grooming in the splash test between groups **(c).** Black column indicates the control group (n=7), striated column indicates the vaginal smear group (n=14). (* p=0.029, •p=0.132). sec: second

of the box. The ratio of the time spent exploring the relocated object/ time spent exploring both objects was used as a measure of hippocampal short-term memory.

Splash Test

Self-grooming behavior in mice induced by spraying 10% sucrose solution on their back is used as an index of motivational and self-care behavior (32). Time spent grooming to clean their fur from sucrose solution in 5-minutes is manually scored for each animal.

Tail Suspension Test

Mice were individually suspended from their tails with the help of an adhesive electrical tape, 80 cm above the floor. The mice were positioned and aligned with the horizontal plane from the base of their tail. Their movement was recorded for six minutes. The first two minutes of the recording was regarded as acclimation to the test and the data from the last four minutes of the recording was analyzed. Total immobilization time during the last four minutes was calculated. Longer time spent immobilized during the experiment is a measure of behavioral despair (33).

Statistical Analysis

We used SPSS version 23 software (SPSS, Inc., Chicago, IL) for all statistical analysis. Shapiro-Wilk test was performed to test if the data confirms the assumptions for normality. Student's t-test was used to compare groups in terms of their mean sucrose preference index, mean novel object preference and relocated object preference indexes, mean total distance traveled in the locomotor activity test, mean total time spent grooming in the splash test and mean total time spent immobile in the tail suspension test.

RESULTS

Depression-Like Behavior

The total immobility time in the tail suspension test of the vaginal smear group was ~1.7 times higher than the control group (p=0.029) (Figure 2a). The sucrose preference index of the vaginal smear group was 75% whereas it was 85% in the control group and there was a trend towards significance (p=0.132) (Figure 2b). Self-grooming duration was similar in both groups after the application of sucrose solution on their coats (p=0.839) (Figure 2c).

Locomotor Activity

There was no statistically significant difference in the total distance traveled during the locomotor activity test between groups (p=0.499) (Figure 3).

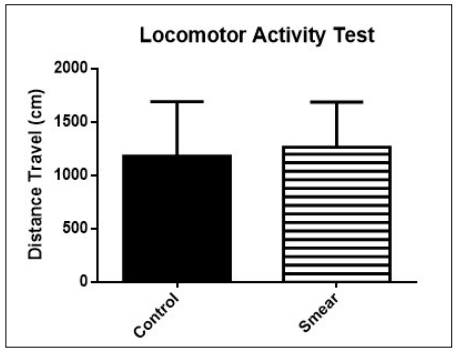

Figure 3. Locomotor activity did not differ between groups. Black column indicates the control group (n=7), striated column indicates the vaginal smear group (n=14).

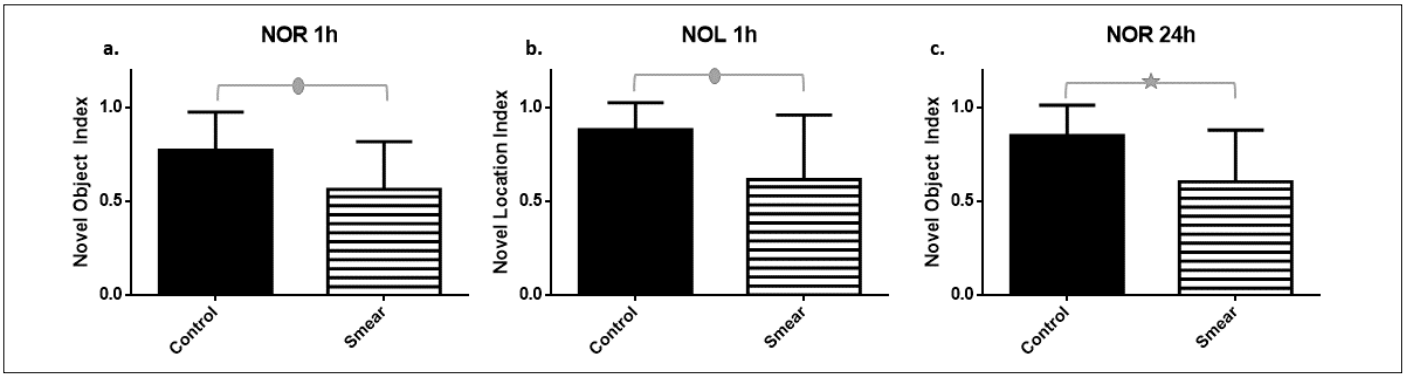

Figure 4. a–c. Short and long-term memory performances. Novel object recognition index **(a)** and novel object location index **(b)** 1-h after the training were lower in the vaginal smear group than the controls. Novel object recognition index 24-h after training was lower in the vaginal smear group than the control group **(c).** Black column indicates the control group (n=7), striated column indicates the vaginal smear group (n=14). (* p=0.025, •p≤0.069); h: hour

Short and Long-Term Memory

Mice in the vaginal smear group spent 21% and 24% less time exploring the novel object or relocated object 1-h after the training, respectively (p=0.061; 0.069 respectively) (Figure 4a, 4b). These findings suggest that collecting vaginal smear decreases short term memory performance. The vaginal smear group spent 25% less time exploring the novel object 24-h after the training (p=0.025) (Figure 4c) indicating a decrease in long-term memory performance.

DISCUSSION

We showed that collecting vaginal smears twice a day for 10 days caused an increase in time spent immobile in the tail suspension test, and a decrease in the sucrose preference test, both of which indicate an increase in depression-like behavior. The anhedonia-like behavior was evident as early as the 5th day of the collection of vaginal smear. There was no difference between the two groups in terms of their locomotor activity. This finding indicates that the increased immobility observed in the tail suspension test was due to an increase in behavioral despair, not due to a decrease in locomotor activity levels. Both behavioral despair and anhedonia were found to be increased by the application of different stressors in the literature (26,34). These findings indicate that the procedure of collecting vaginal smears repeatedly causes stress in mice. We did not find any difference in self-grooming between groups, which is in line with our observation that self-grooming behavior in female mice were resistant to the effects of chronic moderate stress (unpublished data).

The deteriorating effects of chronic stress exposure on memory functions in animals have been well-documented (27,28). Our observation that repeated collection of vaginal smears caused impairments in both short-term and long-term memory further supported the suggestion that the procedure of collecting vaginal smears in mice was stressful. Formation of short and long-term memory engages distinctly different neural mechanisms. Specifically, biochemical alterations of proteins such as kinases, which then leads to increased number of vesicles in the readily releasable neurotransmitter pool or to increased insertion of AMPA receptors are required for short-term memory. Long-term memory, on the other hand, requires new protein synthesis and formation of new synaptic connection (35,36). Our results indicate that repeated vaginal smear collection for 10 days causes stress severe enough to interfere not only with short-term but also with the long-term memory mechanisms.

To summarize, our findings showed that collecting vaginal smear, a procedure to determine the stage of the estrous cycle in rodents, had a significant impact on depression-like behavior and memory performance. We, therefore, recommend using visual inspection of the external genitalia to determine the stage of the estrous cycle whenever possible (16,17). Our observations indicate that even an inexperienced researcher can identify the stage of the estrous cycle with 80% accuracy by observing the appearance of the vagina.

We chose to study the behavioral effects of vaginal smear collection on the 5th and 10th day of the procedure which corresponds to two consecutive estrous cycles. Therefore, we couldn't rule out the possibility of mice getting adapted to the procedure if we continued collecting the smears for more than 10 days. This issue needs to be addressed by future studies, the design of which necessitates the demonstration of vaginal cytology for longer periods of time. Another possible design may be to wait for some period after doing the vaginal smears, however, as there may be unpredictable changes in the estrus cycle, we instead recommend the documentation of vaginal features as a way of staging estrous cycle.

Acknowledgement: *We thank Associate Professor Dr İlknur Pir Yağcı for teaching Aslıhan Bahadır Varol how to collect and interprete vaginal smears from mice.*

Ethics Committee Approval: This research was approved by the Hacettepe University Animal Experimentations Local Ethics Board on 23/10/2019 with the approval number 2019/11–01.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- EEK; Design- EEK, ABV, ECE; Supervision- EEK; Resource- EEK; Materials- EEK, ABV, ECE; Data Collection and/or Processing- EEK, ABV, ECE; Analysis and/or Interpretation- EEK, ABV, ECE; Literature Search- EEK, ABV, ECE; Writing- EEK, ABV, ECE; Critical Reviews- EEK, ABV, ECE.

Conflict of Interest: The authors declared that there is no conflict of interest.

Financial Disclosure: The authors have not received any funding for this study.

REFERENCES

1. Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience, 2nd ed. Boca Raton (FL): CRC Press/Taylor & Francis; 2009.
2. Hoffman KL, editor. Modeling Neuropsychiatric Disorders in Laboratory Animals, 1st ed. Cambridge, UK: Woodhead Publishing; 2015.
3. Harlow HF, Gluck JP, Suomi SJ. Generalization of behavioral data between nonhuman and human animals. Am Psychol. 1972;27(8), 709–716. [Crossref]
4. Shanks N, Greek R, Greek J. Are animal models predictive for humans? Philos Ethics Humanit Med. 2009;4:2. [Crossref]
5. Garner JP. The significance of meaning: why do over 90% of behavioral neuroscience results fail to translate to humans, and what can we do to fix it? ILAR J. 2014;55(3):438–456. [Crossref]
6. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev. 2011;35(3):565–572. [Crossref]
7. Clayton JA. Studying both sexes: a guiding principle for biomedicine. FASEB J. 2016;30(2):519–524. [Crossref]
8. Shansky RM. Sex differences in behavioral strategies: avoiding interpretational pitfalls. Curr Opin Neurobiol. 2018;49:95–98. [Crossref]
9. Piccinelli M, Wilkinson G. Gender differences in depression. Critical review. Br J Psychiatry. 2000;177:486–492. [Crossref]
10. McLean CP, Asnaani A, Litz BT, Hofmann SG. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. J Psychiatr Res. 2011;45(8):1027–1035. [Crossref]
11. Chari T, Griswold S, Andrews NA, Fagiolini M. The Stage of the Estrus Cycle Is Critical for Interpretation of Female Mouse Social Interaction Behavior. Front Behav Neurosci. 2020;14:113. [Crossref]
12. Lovick TA, Zangrossi H Jr. Effect of Estrous Cycle on Behavior of Females in Rodent Tests of Anxiety. Front Psychiatry. 2021;12:711065. [Crossref]
13. Korol DL, Malin EL, Borden KA, Busby RA, Couper-Leo J. Shifts in preferred learning strategy across the estrous cycle in female rats. Horm Behav. 2004;45(5):330–338. [Crossref]
14. Ajayi AF, Akhigbe RE. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. Fertil Res Pract. 2020;6:5. [Crossref]
15. Cora MC, Kooistra L, Travlos G. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol. 2015;43(6):776–793. [Crossref]
16. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse estrous cycle identification tool and images. PLoS One. 2012;7(4):e35538. [Crossref]
17. Champlin AK, Dorr DL, Gates AH. Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. Biol Reprod. 1973;8(4):491–494. [Crossref]
18. Ramos SD, Lee JM, Peuler JD. An inexpensive meter to measure differences in electrical resistance in the rat vagina during the ovarian cycle. J Appl Physiol(1985). 2001;91(2):667–670. [Crossref]
19. Achiraman S, Archunan G, SankarGanesh D, Rajagopal T, Rengarajan RL, Kokilavani P, et al. Biochemical analysis of female mice urine with reference to endocrine function: a key tool for estrus detection. Zoolog Sci. 2011;28(8):600–605. [Crossref]
20. Allen E. The oestrous cycle in the mouse. Am J Anat. 1922;30:297–371. [Crossref]
21. Kopp C, Ressel V, Wigger E, Tobler I. Influence of estrus cycle and ageing on activity patterns in two inbred mouse strains. Behav Brain Res. 2006;167(1):165–174. [Crossref]
22. Wood GA, Fata JE, Watson KL, Khokha R. Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus. Reproduction. 2007;133(5):1035–1044. [Crossref]

23. Wallace JG, Potts RH, Szamosi JC, Surette MG, Sloboda DM. The murine female intestinal microbiota does not shift throughout the estrous cycle. PLoS One. 2018;13(7):e0200729. [Crossref]
24. Caligioni CS, Franci CR. Oxytocin secretion induced by osmotic stimulation in rats during the estrous cycle and after ovariectomy and hormone replacement therapy. Life Sci. 2002;71(24):2821–2831. [Crossref]
25. Yoshinaka K, Yamaguchi A, Matsumura R, Node K, Tokuda I, Akashi M. Effect of different light-dark schedules on estrous cycle in mice, and implications for mitigating the adverse impact of night work. Genes Cells. 2017;22(10):876–884. [Crossref]
26. Vassilev P, Pantoja-Urban AH, Giroux M, Nouel D, Hernandez G, Orsini T, et al. Unique effects of social defeat stress in adolescent male mice on the Netrin-1/DCC pathway, prefrontal cortex dopamine and cognition (Social stress in adolescent vs. adult male mice). eNeuro. 2021;8(2). [Crossref]
27. Tripathi SJ, Chakraborty S, Srikumar BN, Raju TR, Shankaranarayana Rao BS. Inactivation of basolateral amygdala prevents chronic immobilization stress-induced memory impairment and associated changes in corticosterone levels. Neurobiol Learn Mem. 2017;142(Pt B):218–229. [Crossref]
28. Elizabeth A, Adegbuyi A, Olusegun A, Benneth BA, Anthony E, Abayomi A, et al. Morin hydrate attenuates chronic stress-induced memory impairment and degeneration of hippocampal subfields in mice: The role of oxidative, nitrergic and neuroinflammatory pathways. Metab Brain Dis. 2020;35:1145–1156. [Crossref]
29. McLean AC, Valenzuela N, Fai S, Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp. 2012;(67):e4389. [Crossref]
30. Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P. Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. Neuropsychopharmacology. 2004;29(11):2007–2017. [Crossref]
31. Cruz-Sanchez A, Dematagoda S, Ahmed R, Mohanathaas S, Odenwald N, Arruda-Carvalho M. Developmental onset distinguishes three types of spontaneous recognition memory in mice. Sci Rep. 2020;10:10612. [Crossref]
32. Barthas F, Humo M, Gilsbach R, Waltisperger E, Karatas M, Leman S, et al. Cingulate Overexpression of Mitogen-Activated Protein Kinase Phosphatase-1 as a Key Factor for Depression. Biol Psychiatry. 2017;82(5):370–379. [Crossref]
33. Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev. 2005;29(4-5):571–625. [Crossref]
34. Fang X, Jiang S, Wang J, Bai Y, Kim CS, Blake D, et al. Chronic unpredictable stress induces depression-related behaviors by suppressing AgRP neuron activity. Mol Psychiatry. 2021;26:2299–2315. [Crossref]
35. Schacter DL, Wagner AD. Learning and Memory. In: Kandel ER, Schwartz JH, Hudspeth AJ, Sigelbaum SA, Jessell TM. 5th ed. Principles of neural science. New York: McGraw-Hill; 2013. p. 1441–1460.
36. Eric R. Kandel ER, Siegelbaum SA. Cellular Mechanisms of Implicit Memory Storage and the Biological Basis of Individuality. In: Kandel ER, Schwartz JH, Hudspeth AJ, Sigelbaum SA, Jessell TM. 5th ed. Principles of neural science. New York: McGraw-Hill; 2013. p. 1461–1486.