ISSN 2079-0570, Advances in Gerontology, 2017, Vol. 7, No. 2, pp. 137–142. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © Ya.V. Gorina, Yu.K. Komleva, O.L. Lopatina, V.V. Volkova, A.I. Chernykh, A.A. Shabalova, A.A. Semenchukov, R.Ya. Olovyannikova, A.B. Salmina, 2017, published in Uspekhi Gerontologii, 2017, Vol. 30, No. 1, pp. 49–55.

The Battery of Tests for Experimental Behavioral Phenotyping of Aging Animals

Ya. V. Gorina[a, b, *], Yu. K. Komleva[a, b], O. L. Lopatina[a, b], V. V. Volkova[b], A. I. Chernykh[c], A. A. Shabalova[a], A. A. Semenchukov[a], R. Ya. Olovyannikova[a], and A. B. Salmina[a, b]

[a] *Voino-Ysenetskii Krasnoyarsk State Medical University, Krasnoyarsk, 660022 Russia*
[b] *Research Institute of Molecular Medicine and Pathobiochemistry, Krasnoyarsk State Medical University, Krasnoyarsk, 660022 Russia*
[c] *Berzon Krasnoyarsk City Clinical Hospital, Krasnoyarsk, 660123 Russia*
**e-mail: yana_20@bk.ru*

Abstract—The purpose of the study was to develop a battery of tests to study social and cognitive impairments for behavioral phenotyping of aged experimental animals with physiological neurodegeneration. Male outbred CD1 mice used for the study were divided into two groups: group 1 consisted of 12-month-old male mice (physiological aging) and group 2 consisted of 2-month-old male mice (control group). A social recognition 5-trial test, elevated plus maze test (EPM), open field test, light-dark box test, and fear conditioning were used for estimation of the neurological state of experimental animals. We found that aged male mice exhibited lower interest in female mice in the social 5-trial task as compared to young male mice. Increased anxiety was observed in the group of aging mice in the EPM and light-dark box tests as compared to the control group. Lower locomotor activity and increased anxiety were found in the aging rats studied in the open field test. Physiological neurodegeneration was related to impaired associative learning and memory in the group of aging mice, which were observed in fear conditioning; particularly, consolidation of fear memory was dramatically suppressed. Analysis of behavioral factors, social interactions, and anxiety confirmed a model of age-related neurodegeneration in the aged mice. We suggest that studies on animal behavior in the open field test, light-dark box test, and fear conditioning is the most informative approach for detection of neurological impairments, including deficits of social contacts and interaction, limitation of interests, and increased anxiety, in aging mice. This allows us to get new basic data on behavioral alterations in a model of age-related neurodegeneration and to develop novel therapeutic strategies for the treatment of age-related brain pathology.

Keywords: behavioral phenotyping, model of physiological aging, cognitive dysfunction.
DOI: 10.1134/S2079057017020060

INTRODUCTION

Mice are preferred rodent species for experiments in many laboratories because their genetic make-up is known in detail [13]. Assessment of mouse behavior has also come a long way to a deeper understanding of its phenotype [3, 11, 23]. The phenotype of mice is complex [4]; therefore, it is important to estimate a wide spectrum of animal behavior, including the general state of health, reflexes, locomotor activity, emotionality, anxiety, affective and social behavior, and learning and memory [10].

In order to tackle this problem, foreign scientists developed for several decades many behavioral tests, which allow us to study sensory and motor functions, the neurophysiological state, emotionality, social and cognitive functions, and others [14, 16]. Performing of a battery of tests in a specific animal model (rodents) allows us not only to understand better the behavioral phenotype, but also to consider some limitations of this model [10].

It should also be noted that studies of animal models not only aim to reproduce any specific human disease or disturbance, but also to provide deeper understanding of the main molecular processes involved in behavior. In its turn, this will allow the development of new therapeutic strategies for treatment of one or more diseases.

Aging is a complex process related to structural and functional alterations in the brain, which result in age-associated behavioral impairments and an increased number of neuro-psychic disturbances [1]. This suggests that these modifications be considered as chronic neurodegeneration. Several studies on the effects of aging on the brain and behavior permitted a better understanding of the bases of underlying behavioral abnormalities in normal and pathological aging [20, 22]. However, despite extensive comparative studies, which are mainly performed in young and old animals, there is a question of an adequate approach in an assessment of social and cognitive functions when

comparing young and aged animals, when processes of physiological neurodegeneration just begin to be manifested in the form of neurological deficits. In other words, behavioral testing of aging animals is specifically interesting for the estimation of brain condition; in rodents, the aging period corresponds to the age of 12 months [8]. Thus, using of a battery of specific behavioral tests provides a more correct interpretation of behavioral phenotypes.

The aim ofthe study was to develop a battery of tests for assessment of social andcognitive impairments under the conditions of physiological neurodegenerationfor behavioral phenotyping of aging experimental animals.

MATERIALS AND METHODS

Male CD1 mice were used for the study. Two groups of animals were formed: group 1 ($n = 10$) consisted of 12-month-old male mice (physiological aging) [8] and group 2 ($n = 10$) consisted of 2-month-old male mice (control group). The animals were housed five per a cage with individual ventilation from the moment of separation from their mothers to the above indicated age. The animals had free access to water and food and were housed under a constant temperature of 21 ± 1°C and a regular light-dark cycle 12-h day/12-h night. The experiments were performed in accordance with the humanitarian principles recommended in the European Community Directive (2010/63/EC).

For neurological assessment, we used the social 5-trial test, elevated plus maze, open field test, dark-light box test, and fear conditioning.

The social 5-trial test was used for assessment of social recognition of a new subject. After repeated contacts, the rodents get accustomed to each other and lose interest in social exploration, which is expressed as a decrease in the duration of a contact. After introducing a new subject, social interest and the duration of social contacts increase [5]. Naive young 8-week-old female mice were used as stimuli. Thirty minutes prior to the test, the male mouse was placed in a testing cage for adaptation. Then, five short sessions were performed. During four sessions, the same female mouse was placed in a testing cage with 10-min intervals and in the fifth session a new female mouse was placed in a testing cage. The duration of each session was 60 s and animal behavior during each session was video recorded. Social interaction was recognized as "head-to-tail" following and the duration of this following was recorded.

For estimation of emotional state, anxiety, fear of height, exploratory and locomotor activity in rodents we used the elevated plus maze test [9]. The maze consisted of two open (50 × 5 cm) and two closed arms (50 × 5 cm) located perpendicularly to each other at a height of 1 m. The animal was placed into the center of the maze and its behavior was video recorded for 10 min. The duration of staying in the closed arms and in the open arms, number of entries into the closed and open arms, and duration of staying in the center of the maze were recorded. Anxiety was estimated as a ratio of time spent in the open arms to time spent in the closed arms and anti-anxiety was estimated as an increase in the time spent in/number of entries onto the open arms.

Free locomotion of animals was studied in the open field test. The test was performed in a round arena with a diameter of 80 cm surrounded with opaque walls with a height of 20 cm. This test allows to study expression and dynamics of elementary behavioral acts in rodents under the stressful conditions in response to placing a laboratory animal into a large arena with large area and intense lighting as compared to a box for routine housing [18]. The test consisted of two stages, which were performed with an interval of 10 min. In the first stage, the animal was placed in the center of the arena and its behavior was recorded for 10 min. In the second stage, a nonsocial object, i.e., a cylinder of 10 × 5 cm, was placed in the center of the arena and the animal was tested again for 10 min. In each session, the distance (m) traveled was recorded.

One of approaches to study individual responsiveness of animals, including anxiety, fear, and depression, is using of a light-dark box [21]. A box consisted of two compartments divided by a wall. Lighted and dark compartments were each 30 × 26 × 20 cm in size and were connected via an opening 3 × 3 cm in size. The top of the dark compartment was closed with a cover, which was able to be opened upward. The mouse was placed into the lighted area and turned by its back to the opening to the dark compartment. The duration of the test was 10 min. The latency of the first entry to the dark compartment, duration of staying in the lighted or dark compartment, and number of entries into the dark compartment were recorded.

Fear conditioning was used in order to estimate associative learning and fear memory consolidation [19]. A device for testing consisted of an acrylic square box 33 × 25 × 28 cm in size with an electrified floor. The device was placed into a sound-protected chamber 170 × 210 × 200 cm in size to minimize external noise during testing. Light-emitting diodes with light intensity of 100 lux and a speaker producing a conditioned stimulus (CS) were located over the box. A grid floor was connected to an electric generator and an electric signal was used as an unconditioned stimulus (US).

On day 1, a conditioning session was performed. For this purpose, the mouse was placed into the experimental box and allowed to freely explore it for 120 s. After this time lapse, white noise, i.e., CS, was presented for 30 s. An electric stimulus with current intensity of 0.3 mA, i.e., US, was presented constantly for 2 s during last 2 s of CS presentation. After a 90-s interval, white noise was presented again for 30 s and a

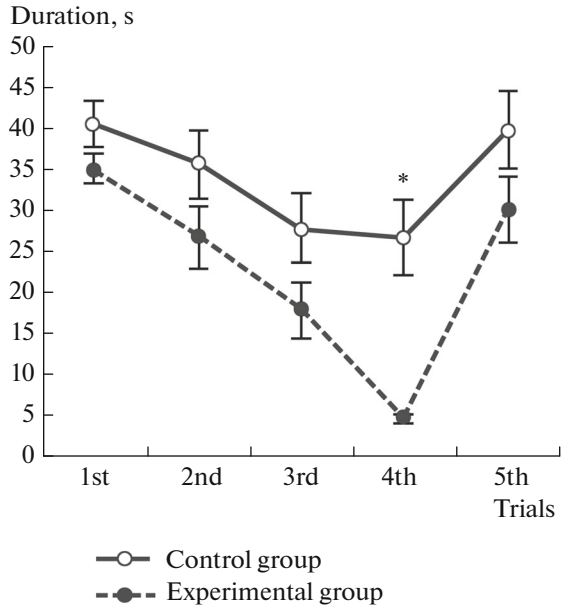

Fig. 1. Duration of "head-to-tail" following studied in a neurobehavioral social 5-trial test. *, $p \le 0.05$ as compared to the control group.

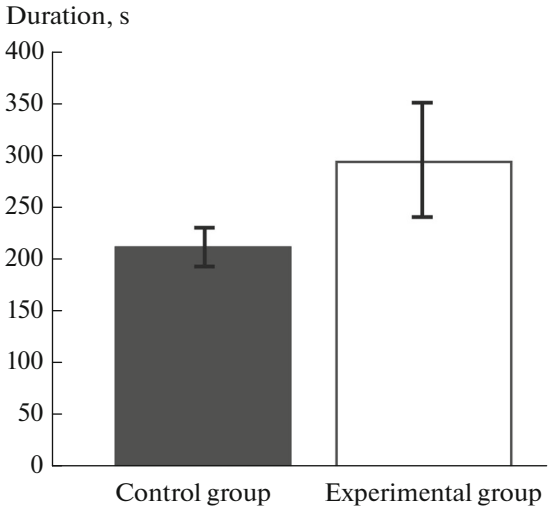

Fig. 2. Animal behavior in the elevated plus maze. Data on the duration of staying in the closed arms are presented.

0.3 mA electric stimulus was presented for the last 2 s of CS presentation. Combinations of white noise and a 0.3-mA electric stimulus were presented thrice during the testing, i.e., 120, 240, and 360 s after the start of the session. After this, the mouse stayed in the box for additional 90 s.

On day 2, the context test was performed. for this purpose, the mouse was placed into the experimental box and allowed to freely explore it for 300 s without presentation of white noise or the 0.3-mA electric stimulus.

On day 3, the cued test was performed. For this purpose, the mouse was placed into the other box with a black floor and black and white walls, which was illuminated with a light intensity of 30 lux. The mouse was allowed to freely explore this box for 180 s. After this time lapse, white noise was presented for additional 180 s, but it was not followed by a 0.3-mA electric stimulus.

After a 3-day interval, i.e., on days 7 and 8, the context and cued tests were performed, respectively.

Statistical analysis of data was performed using the methods of descriptive statistics realized in Statplus 2006 and MS Excel 2010 software. In each sample, the mean value (M) and standard error of mean (m) were calculated. Comparisons of means were performed using the Student's t-test and Mann-Whitney U-test. The level of significance was set as $p \le 0.05$. Data are presented as $M \pm m$.

RESULTS AND DISCUSSION

A statistically significant decrease in interest to a female mouse, i.e., duration of "head-to-tail" following was revealed in the fourth trial of the social 5-trial test in the animals with physiological aging as compared to the control group. Exploration durations were 4.66 ± 0.57 and 26.6 ± 4.52 s, respectively ($p \le 0.005$). Aging animals exhibited less interest in a new female mouse in the fifth trial as compare to the young mice with exploration durations of 30 ± 2.94 and 39.8 ± 3.24 s, respectively ($p > 0.05$; Fig. 1). Thus, the animals of the experimental and control group exhibited memory formation and recognition of a new female mouse; however, the animals of the control group exhibited more interest in a mouse of the opposite sex.

In the elevated plus maze test, the rats of the experimental group exhibited a trend to the increased duration of staying in the closed arms as compared to the control group, i.e., 296.68 ± 54.30 and 213.7 ± 19 s, respectively ($p = 0.118$). This may indicate increased anxiety in the physiologically aging animals (Fig. 2).

Studies on spontaneous locomotor activity in the open field test revealed a statistically significant decrease in motor activity in the aging animals as compared to the control group, i.e., the distances traveled were 30.2 ± 3.8 and 44.4 ± 1.83 m, respectively ($p \le 0.05$), in the first stage of the testing. However, in the second stage of the testing with a nonsocial object (cylinder) located in the center of the arena, the animals of both groups exhibited similar activity, i.e., 26.33 ± 2.24 and 26.47 ± 3.12 m in the experimental and control groups, respectively ($p \ge 0.05$; Fig. 3a). Furthermore, the animals of the experimental group spent statistically significantly more time in the periphery of the arena at both first and second stages of the testing (401.7 ± 16.4 and 176.94 ± 17.5 s, respec-

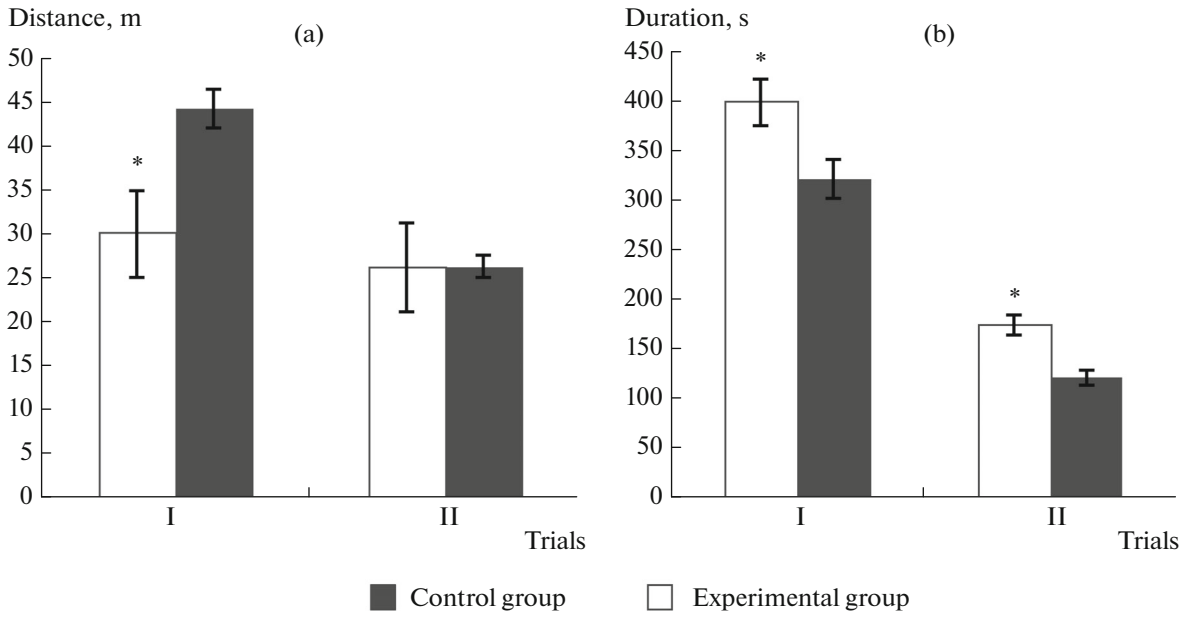

Fig. 3. Data on the behavioral testing in the open field test. (a) distance traveled; (b) time spent in the peripheral circle. *, $p \leq 0.005$ as compared to the control group.

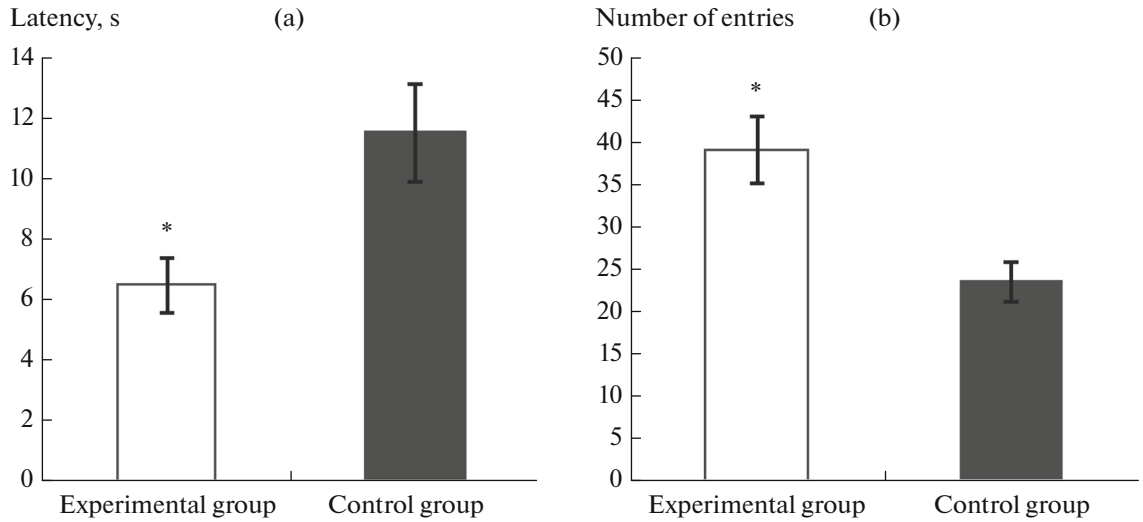

Fig. 4. Data on the neurobehavioral testing in the light-dark box. (a) latency of the first entry into the dark compartment; (b) number of entries into the dark compartment. *, $p \leq 0.005$ as compared to the control group.

tively) as compared to the animals of the control group (323.7 ± 18.7 and 122.9 ± 15.7 s, respectively; $p \leq 0.05$). These data show increased anxiety in the physiologically aging animals (Fig. 3b).

The light-dark box test was used for assessment of specific emotional conditions such as tendency to anxiety, fear, fury, and depression. The animals of the experimental group statistically significantly more rapidly entered the dark compartment as compared to the control animals, i.e., 6.56 ± 1.43 and 11.60 ± 0.96 s, respectively ($p \leq 0.05$; Fig. 4a). A similar trend was observed in the number of entries into the black compartment in the experimental and control groups, i.e., 39.25 ± 4.76 and 23.78 ± 1.89 s, respectively ($p \leq 0.05$). These data indicate increased anxiety in the aging animals (Fig. 4b).

Fear conditioning testing was used for the estimation of associative learning and memory consolidation. Context and cued conditioning are used for testing of a capability to memorize the unpleasant conditioned stimulus and associate it with a specific context. Fear conditioning may be used for studies of hippo-

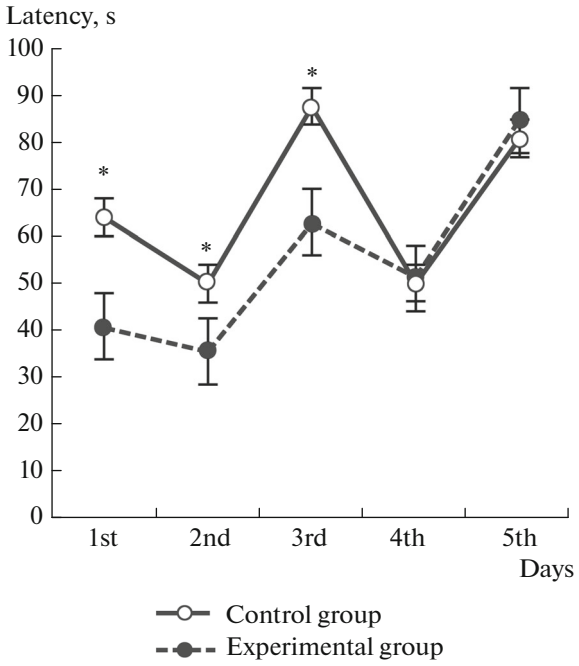

Fig. 5. Results of the fear conditioning test. Data on the number of freezing episodes are presented. *, $p \le 0.005$ as compared to the control group.

campus-dependent and amygdala-dependent emotional memory [2, 12, 15]. It has been shown that the behavioral response, i.e., freezing, during the context testing is formed in the hippocampus whereas the response in the context and cued tests is formed in the amygdala [6, 17]. These data indicate the associative role of the amygdala and the sensory role of the hippocampus in the expression of fear.

We studied the number of freezing episodes because freezing, induced by strong pain or fear, is usually used for quantitative measuring of fear conditioning [7]. The animals of the experimental group exhibited a higher number of freezing episodes as compared to the control group (Fig. 5). Within the testing period, the aging animals did not form a conditioned reflex because of a statistically significant decrease ($p \le 0.05$) in the number of freezing episodes from day 1 (63.7 ± 3.12) to day 7 (50.14 ± 5). However, in the animals of the control group, the number of freezings significantly increased from 40.8 ± 4.44 to 51 ± 3.30 within the same period ($p \le 0.05$).

Thus, physiological aging results in impairments of associative learning in animals, particularly, consolidation of fear memory is significantly inhibited.

CONCLUSIONS

Analysis of behavioral indices, social interaction, and anxiety in the experimental animals supported a model of age-related neurodegeneration in aging animals. We found that behavioral tests such as open field, light-dark box, and fear conditioning are most informative and can reveal typical behavioral features in aging animals, including impairment of social contacts and interaction, limitation of interests, and increased anxiety. This allows one not only to gather new basic data on behavioral indices of age-related neurodegeneration, but also to develop new therapeutic strategies for treatment of age-related brain disorders.

ACKNOWLEDGMENTS

This study was supported by a grant of the President of the Russian Federation for support of leading scientific schools in the Russian Federation, project no. NSh-10241.2016.7.

REFERENCES

1. Bishop, N.A., Lu, T., and Yankner, B.A., Neural mechanisms of ageing and cognitive decline, *Nature,* 2010, vol. 464, no. 7288, pp. 529–535.
2. Brunzell, D.H. and Kim, J.J., Fear conditioning to tone, but not to context, is attenuated by lesions of the insular cortex and posterior extension of the intralaminar complex in rats, *Behav. Neurosci.,* 2001, vol. 115, no. 2, pp. 365–375.
3. Crawley, J.N. and Paylor, R., A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice, *Horm. Behav.,* 1997, vol. 31, no. 3, pp. 197–211.
4. Crawley, J.N., *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice,* New York: Wiley, 2000.
5. Crawley, J., Behavioral phenotyping strategies for mutant mice, *Neuron,* 2008, vol. 57, pp. 809–818.
6. Debiec, J., Díaz-Mataix, L., Bush, D.E., et al., The amygdale encodes specific sensory features of an aversive reinforce, *Nat. Neurosci.,* 2010, vol. 13, no. 5, pp. 536–537.
7. Fitch, T., Adams, B., Chaney, S., et al., Force transducer based movement detection in Fear conditioning in mice: a comparative analysis, *Hippocampus,* 2002, vol. 12, pp. 4–17.
8. Fox, J.G., Barthold, S., Davisson, M., et al., *The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models,* San Diego, CA: Academic, 2007, 2nd ed.
9. Komada, M., Takao, K., and Miyakawa, T., Elevated plus maze for mice, *J. Vis. Exp.,* 2008, vol. 22, p. 1088.
10. Levin, E.D. and Buccafusco, J.J., *Animal Models of Cognitive Impairment,* Boca Raton, FL: CRC, 2006.
11. McIlwain, K.L., Merriweather, M.Y., Yuva-Paylor, L.A., et al., The use of behavioral test batteries: effects of training history, *Physiol. Behav.,* 2001, vol. 73, no. 5, pp. 705–717.
12. Oler, J.A., Ramos, R.L., Penley, S.C., et al., Hippocampal and amygdalar involvement in discriminatory place learning, *Neuroscience,* 2005, vol. 132, no. 1, pp. 1–12.

13. Oliver, P.L. and Davies, K.E., New insights into behavior using mouse ENU mutagenesis, *Hum. Mol. Genet.,* 2012, vol. 21, pp. 72–81.

14. Pennington, C., Hayre, A., Newson, M., et al., Functional cognitive disorder: a common cause of subjective cognitive symptoms, *J. Alzheimer's Dis.,* 2015, vol. 48, no. 1, pp. 19–24.

15. Phillips, R.G. and LeDoux, J.E., Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning, *Behav. Neurosci.,* 1992, vol. 106, no. 2, pp. 274–285.

16. Pogorelov, V.M., Rodriguiz, R.M., Inscol, M.L., et al., Novelty seeking and stereotypic activation of behavior in mice with disruption of the *Dat*1 gene, *Neuropsychopharmacology,* 2005, vol. 30, no. 10, pp. 1818–1831.

17. Rudy, J.W. and O'Reilly, R.C., Contextual fear conditioning, conjunctive representations, pattern completion, and the hippocampus, *Behav. Neurosci.,* 1999, vol. 113, pp. 867–880.

18. Satoh, Y., Endo, S., Ikeda, T., et al., Extracellular signal-regulated kinase 2 (ERK2) knockdown mice show deficits in long-term memory; ERK2 has a specific function in learning and memory, *J. Neurosci.,* 2007, vol. 27, no. 40, pp. 10765–10776.

19. Shoji, H., Takao, K., Hattori, S., et al., Contextual and cued fear conditioning test using a video analyzing system in mice, *J. Vis. Exp.,* 2014, vol. 85. doi 10.3791/50871

20. Shoji, H., Takao, K., Hattori, S., et al., Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age, *Mol. Brain,* 2016, vol. 9. doi 10.1186/s13041-016-0191-9

21. Takao, K. and Miyakawa, T., Light/dark transition test for mice, *J. Vis. Exp.,* 2006, vol. 1. doi 10.3791/104

22. Takao, K., Yamasaki, N., and Miyakawa, T., Impact of brain-behavior phenotyping of genetically-engineered mice on research of neuropsychiatric disorders, *Neurosci. Res.,* 2007, vol. 58, no. 2, pp. 124–132.

23. Yonezaki, K., Uchimoto, K., Miyazaki, T., et al., Post anesthetic effects of isoflurane on behavioral phenotypes of adult male C57BL/6J mice, *PLoS One,* 2015, vol. 10, no. 3. doi 10.1371/journal.pone.0122118

Translated by M. Stepanichev