

Protocol

# The Suok (“ropewalking”) murine test of anxiety

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## Abstract

In the present study, we suggest that long elevated horizontal rod (Suok test, ST) and its light–dark modification (LDST) may be used for behavioral characterization in mice, including simultaneous assessment of their anxiety, activity, and neurological phenotypes. To establish the ST and the LDST as murine models of anxiety, we used several different mouse strains which differ markedly in their anxiety and activity (C57BL/6, 129S1/SvImJ, NMRI, and BALB/c). Here we show that our tests are able to ethologically discriminate between high and low anxiety mouse strains, as assessed by horizontal and directed exploration, stops, and defecation boli. The spatial distribution of the LDST behaviors is also sensitive to these strain-specific anxiety phenotypes, showing clear avoidance of the brightly lit part of the test in stressed (rat exposed) vs. control NMRI mice. In addition, we validated the ST in 129S1/SvImJ and BALB/c mice by assessing the behavioral consequences of acute stress such as rat exposure. Finally, we showed that our test is able to detect high anxiety and poorer motor coordination in 129S1/SvImJ (vs. C57BL/6) mice. The results of our study show that the ST emerges as an experimental tool to analyze anxiety, motor-vestibular anomalies, as well as anxiety-induced motor impairments in mice. Overall, we suggest that the ST can be a useful protocol in neurobehavioral stress research including modeling stress-evoked states, pharmacological screening of potential anti-stress drugs, or behavioral phenotyping of genetically modified animals.

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## 1. Type of research

Novel behavioral test to perform ethological analysis of anxiety and motor activity in mice.

### 1.1. Introduction

Several classical theories have attempted to describe the behavior of animals in novel environment. For example, Gallup and Suarez suggested that animal behavior in the open field test represents a conflict between the need to reinstate contact with co-specifics (affiliation) and evade possible

predation [21], while other studies [44] postulated the role of the balance between two natural motivations—the tendency to explore (curiosity, novelty-seeking) and the initial tendency to avoid the unfamiliar (fear, anxiety)—in guiding animal responses to novelty. Collectively, these and other subsequent behavioral studies underline the complex nature of animal exploration in different experimental situations and the need to further examine them in detail [28,61,62].

Since anxiety has long been known to be induced by novelty, it can be assessed by the intensity of behavior of mice in an unknown environment [7,44,48]. In rodents, traditional anxiety tests include the elevated plus maze, the light–dark test, the open field test, holeboard, mirrored chamber, and free exploratory paradigm [9,25,31,48,49,58,61,62]. NMRI, C57BL/6 (C57), BALB/c (BC), and 129S1/SvImJ (S1) mouse strains are commonly used in neurobehavioral anxiety

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research [13,17,22,25,34,37,39,45,60], demonstrating marked strain differences in their behavioral phenotypes (summarized in Table 1). Overall, NMRI mice are considered to be a “moderate activity” non-anxious strain compared to the high-activity non-anxious novel-seeking C57 strain, active anxious neophobic BC mice, and anxious hypoactive S1 mice [10,17,29,46,47,60,65]. Together, these behavioral strain differences in anxiety underlie the fact that unconditioned fear of novelty is a genetically determined animal response, which can be used to dissect animal anxiety in different experimental situations.

The main goal of our study was to establish a fast combined behavioral test for profiling anxiety and motor function in mice. Our test, the murine “ropewalking” Suok test (ST) of anxiety, is named after a brave little ropewalker girl in Yu Olesha’s “The three fat men” (1927) and consists of placing an animal on a long horizontal metal rod (2–3 m)

elevated above the floor (“ropewalking”) in a dimly lit room (Fig. 1). It evokes two obvious threats—the fear of height and the fear of the novel rod—and is based on an ethological analysis of animal exploratory activity. In addition, the light–dark ST modification (LDST) was also established in the present study (Fig. 1), with the aversion to brightly lit environment representing an additional anxiogenic factor. These tests combine principles of several different traditional behavioral models, including the beam, elevated plus maze, open field, holeboard, and light–dark tests [5,52,61,62], allowing us to evoke and assess animal anxiety and motor performance simultaneously.

To establish the ST, we first used this method to assess anxiety in three mouse strains widely used in neuro-behavioral research and markedly different in their anxiety phenotypes (anxious BC vs. non-anxious NMRI, C57 mice), and showed that our protocol is able to detect strain

Table 1  
Summary of the behavioral strain differences between C57BL/6 (C57), BALB/c (BC), NMRI, and 129S1/SvImJ (S1) mice

Test	Behavioral measure	Strain ranking	References
Activity			
Home cage	Baseline horizontal and vertical activity (total, light, dark)	BC > C57 > S1 C57 > NMRI	[17,26,45a,b,55]
Anxiety			
Open field	Horizontal and vertical exploration Time in the center	NMRI > BC; C57 > BC > S1 NMRI > BC	[1,6,40,55] [40]
	Defecation	NMRI > BC > C57	[1,11,55]
Elevated plus maze	Closed time	C57 > BC	[1]
	Total entries	NMRI > BC	[23]
	Defecation, urination	BC > NMRI	[66]
EZM	Time in open quadrant	C57 > BC; C57 > S1	[12,45h,55]
Modified holeboard	Time spent, entries, holes	BC = C57	[46,47]
	General locomotion	C57 > BC	[46,47]
Light–dark test	Transitions between compartments	NMRI > BC	[23,40]
	Time in light	NMRI > BC	[23,40]
	Chronic stress sensitivity	BC > C57	[32]
MC	Exploratory activity	C57 > S1	[31]
Rat exposure	SAP, freezing, defensive burying	C57 > BC	[65]
Food neophobia	Latency to explore familiar food	BC = C57	[46,47]
	Latency to explore unfamiliar food	BC > C57	[46,47]
	Food intake after exposure	C57 > BC	[46,47] <sup>a</sup>
Cat exposure	Vertical activity drop	NMRI > BC	[4,5]
Free exploratory test	Neophobic response	BC > NMRI	[4,5]
	Horizontal and vertical activity	NMRI > BC	[4,5]
Motor functions			
Horizontal rod (beam) test	Missteps crossings (hindleg slips)	S1 > C57	[14,45c]
	Anxiety-induced missteps	BC > C57	[37]
	Motor deficits	No: NMRI, C57	[34,45d]
Other behaviors			
Startle	Acoustic startle response	BC > S1 > C57	[45e,63]
FST	Immobility (floating)	S1, BC > NMRI = C57	[17,45d,60]
TST	Immobility	BC > NMRI	[40]
Nociception	Hindleg hot plate response	S1 > C57 > BC	[45f]
	Hot plate, tail-flick latency	BC > S1 > C57	[45b,f]
Maternal	Maternal care	C57 > BC	[9]
Grooming	Novelty and social grooming bouts	C57 > S1	[26,29]
Burying	Overall burying activity	C57 > BC	[65]
Wildness	Wildness and biting	C57 ≥ S1 > BC > NMRI	[45g] <sup>a</sup>

MC, mirrored chamber; EZM, elevated zero maze; FST, Porsolt’s forced swim test; TST, tail suspension test. The Mouse Phenome Database [45] projects: (a) Seburn; (b) Mogill; (c) Crabbe and Wahlsten; (d) General MPD strain information; (e) Willot; (f) JaxWest; (g) Wahlsten; (h) Flatherty.

<sup>a</sup> Own unpublished data.

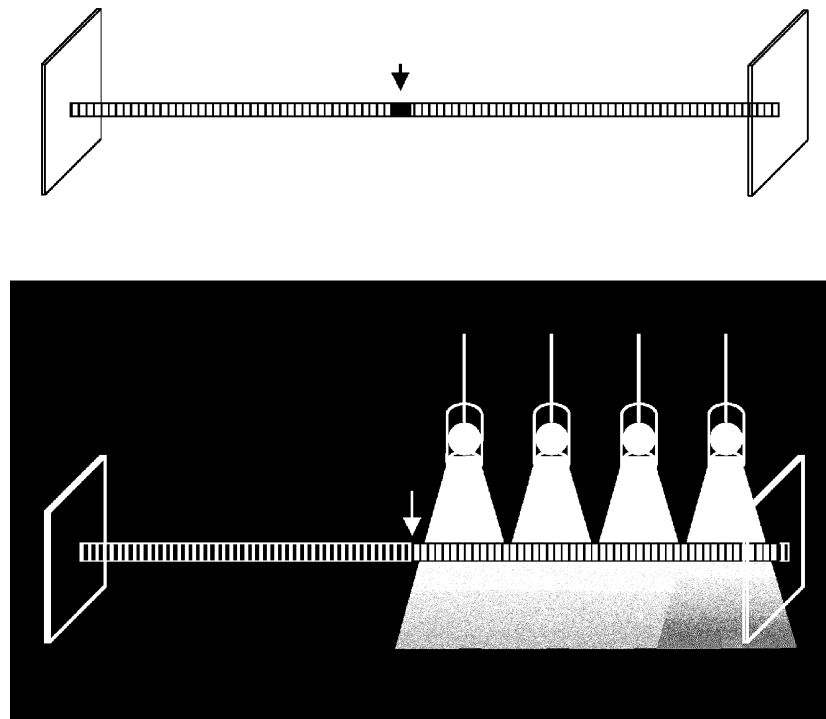


Fig. 1. The Suok test (upper panel) and its light–dark modification (lower panel).

differences in anxiety in these mice. We then applied the LDST to NMRI and BC strains, and demonstrated that mice of the more anxious BC strain spent predictably more time in the dark part, traveled less distance, and made more stops than did their less anxious NMRI counterparts.

In the third experiment, we used the ST to compare anxiety in two groups of mice with different levels of evoked anxiety. Since rats are natural predators of mice, and mice exposed to rats demonstrate high anxiety and/or fear [65], we exposed NMRI mice to a rat (stressed group) vs. unexposed controls and showed that our method is able to detect different levels of stress in these two mouse groups.

Finally, since many mouse strains display abnormal locomotion, we wanted to know if our protocol can be used to assess motor abnormalities in mice. For this, we analyzed motor performance in the ST in two mouse strains markedly different in their baseline motor-coordination abilities: C57 (unimpaired motor functions) vs. S1 (locomotor problems) [45]. In order to assess anxiety-induced sensory-motor deficits in mice in the ST, we also compared rat-exposed S1 and BC mice to their non-stressed counterparts. Using these strains as an example, we demonstrated that our protocol is able to detect the strain differences in both baseline and stress-induced motor performance in mice.

Overall, our results show that the protocol allows modeling anxiety by assessing mouse ST behaviors and could be extensively used in neurobehavioral stress research and behavioral phenotyping of genetically modified animals. ST may be used to assess anxiety, activity, and motor anomalies in mice as a fast, simple, one-trial procedure which is based on animal spontaneous behavior, uses natural

stimuli, and does not need prior training. The protocol assesses a wide range of behaviors and may be a rich source of behavioral information for neuroscience research. Taken together, this suggests that our method may represent a useful tool in neuroscience research, including behavioral genetics and experimental modeling of various stress disorders.

## 2. Time required

The time required for this protocol was calculated taking into account standard experiments with 8 animals per group and two groups:

- (a) Handling of naive animals: 3–4 days, 5 min/mouse/day.
- (b) Rat exposure requires 5 min.
- (c) Assessment of animal behavior in the ST or LDST requires 5 min.
- (d) Animals are to be allowed at least 7 days between the tests if a battery of behavioral tests is used.
- (e) Analyses of the ethological data: 2–4 days depending on the amount of data collected.

## 3. Materials

### 3.1. Animals

Experiments were carried out on 16 C57, 32 NMRI, 24 S1, and 40 BC adult male mice (3 month old, 25–30 g, University of Tampere, Finland;  $n = 8$  in each group). All the animals

used in this study were experimentally naive, housed 2–3 per cage (dust-free soft wood sawdust bedding), and kept in a controlled environment maintained at a constant temperature ( $24 \pm 1^\circ\text{C}$ ) and humidity ( $50 \pm 5\%$ ) with free access to food and water. The animals were maintained on a 12:12 h light/dark cycle (lights on at 6.00 h and off at 18.00 h). Behavioral testing was always conducted between 14.00 and 18.00 h. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives. The procedures used in this study were in strict accordance with the European legislation and the guidelines of the National Institutes of Health on the use and care of laboratory animals. All animal experiments reported here were approved by the Ethical Committee of the University of Tampere.

### 3.2. Pre-test manipulations and equipment

For the rat exposure test, we used male Wistar rats placed in a small Plexiglas box ( $20 \times 20 \times 20$  cm) divided into two equal compartments by a wire net (1-cm mesh), allowing visual, olfactory, auditory, and even tactile communication between the animals (to avoid direct attacks, in our experiments we used relatively young non-aggressive 200-g rats). Mice were placed individually in the empty compartment next to the rat compartment for 5 min prior to testing in the ST. The apparatus was cleaned thoroughly between subjects (wet and dry cloths). The lighting in the experimental room was similar to that in the holding room during these procedures.

### 3.3. Special test equipment

The ST was a 2.6-m aluminum tube 2 cm in diameter, elevated to a height of 20 cm from the cushioned floor (Fig. 1). The rod was separated into 10-cm segments by line drawings and fixed to two Plexiglas side walls ( $50 \times 50$  cm; 1 cm thick) preventing the mice from escaping sideways. The experimental room was dimly lit during this test. The LDST consisted of the same aluminum rod, with four 60-W bulbs 40 cm above the rod (NB: directed light!) to illuminate the “light” part of the test, providing the only lighting in the experimental room (Fig. 1). All the equipment used in our study (3.2 and 3.3) was constructed by a local manufacturer (TAU Workshops) according to our specifications.

### 3.4. Behavioral measures

Summarized in Table 2.

## 4. Detailed procedure

### 4.1. Testing protocol

(a) Transport mice from their holding room to the experimental room and leave undisturbed for 1 h prior to testing.

- (b) Expose the mice to different stressors for 5 min (3.2), and then place mice individually in the middle part of the ST (snout facing either end) or LDST (snout facing the dark end). Support the animals by hand during the initial placement (up to 5 s), if necessary, to avoid a fall due to incorrect positioning. All test apparatus is thoroughly cleaned (wet and dry cloth) before each animal.
- (c) Observe the animal ST or the LDST behaviors for 5 min. During observations, the experimenter (inter-rated reliability  $>0.90$ ) always sits in the same place, 2 m away from the apparatus. Score animal anxiety-related measures, as summarized in Table 2, using a specially designed register. Horizontal and directed exploration, stops, and defecation scores are crucial measures in this test. In all experiments, the latency measures are reckoned as total observation time (300 s) in the mice not showing the respective behaviors.
- (d) Identify and register separately animal motor behavioral parameters (see [14,33] for details), as summarized in Table 2. Falls and hindleg slips are critical measures in this test.
- (e) In view of the importance of light/dark stimuli for LDST, differentiate behavioral measures as a function of their occurrence in the light or dark parts of the test (Table 2) similar to the standard light–dark paradigm protocol [7,15].
- (f) Statistics. All results are expressed as mean  $\pm$  SEM. Data are analyzed by Mann–Whitney test for comparisons between experimental groups. A probability of less than 0.05 is considered statistically significant.

### 4.2. Brief summary of the experimental design, tests, and functions tested

1. ST (Anxiety): BC vs. C57 mice (Experiment 1); BC vs. NMRI mice (Experiment 2).
2. LDST (Anxiety): BC vs. NMRI mice (Experiment 3).
3. ST (Anxiety): Stressed (rat-exposed) vs. non-stressed NMRI mice (Experiment 4).
4. ST (Motor deficits, Anxiety): S1 vs. C57 mice (Experiment 5).
5. ST (Anxiety-evoked motor deficits): Stressed (rat exposed) vs. non-stressed S1 and BC mice (Experiment 6).

## 5. Results

### 5.1. ST performance in mouse strains with different anxiety phenotypes

The ST was first applied to two mouse strains (BC and C57) spontaneously expressing highly contrasting anxiety phenotypes (Table 1). Predictably, the more anxious BC group showed less exploration and more anxiety than did

Table 2  
Summary of behavioral parameters measured in the Suok test (ST) and the light–dark Suok test (LDST)

Measures	Description	ST	LDST
General behavioral measures			
H	Horizontal activity	Number of segments visited (4 paws)	T, L, D
V	Vertical activity <sup>a</sup>	Number of vertical rears (occur relatively rare in this test, may be more frequent in some strains)	T, L, D
S	Stopping activity	Number of stops (complete cessation of movement (>1 s) except breathing)	T, L, D
D	Head dips	Down-directed exploration, number of exploratory looks down	T, L, D
O	Orientation	Side-directed exploration (visual and olfactory scanning of environment and whisking, with body in a stretched position)	T, L, D
TS	Time spent	Time spent (s) in each compartment of LDST	–
DA	Displacement activity <sup>a</sup>	Usually short bouts of paw/nose grooming: frequency (DF) or duration (DD), s	T
L	Leaving latency	Latency (s) to leave a 20-cm virtual central zone around the placement point (4 paws)	T
Tr	Transitions	Number of crossing the center (4 paws) <sup>b</sup>	T
B	Defecation boli	Number of defecation boli deposited	T, L, D
LD	Latency to defecate <sup>a</sup>	Latency (s) to the first defecation boli	T
U	Urination <sup>a</sup>	Number of urination episodes	T
Ethologically derived parameters			
AS	Average speed <sup>a</sup>	Number of segments visited divided by the time spent	T, L, D
%T	% Time	Percent of time in the light and dark parts of LDST	–
%H	% Horizontal activity	Percent of segments (of total segments visited) visited in light and dark parts of LDST	–
%S	% Stops	Percent of stops in the light and dark parts of LDST	–
ID	Inter-stop distance <sup>a</sup>	Average distance between two stops (the number of segments visited divided by the number of stops)	T, L, D
Additional measures			
F	Falls	Number of falls from the rod	T
LF	Latency to fall <sup>a</sup>	Latency to the first fall from the rod (s)	T
SB	Stops near the border <sup>a</sup>	Number of stops before entering the light zone of the LDST (visual system index <sup>c</sup> )	–
HS	Misssteps	Number of hindleg slips	T
Brief summary of anxiety measures			
Increased in anxiety: S, L, B, %T(D), %H(D), %S(L), ID, DA (DF, DD), F, HS			
Decreased in anxiety: H, V, D, O, Tr, LD, AS, %T(L), %A(L), LF			
Unaltered/Mixed/Unclear: UR			

Behavioral measures: T, total; L, in the light part of the LDST; D, in the dark part of the LDST.

<sup>a</sup> Optional measures.

<sup>b</sup> In LDST = the number of light–dark transitions.

<sup>c</sup> This measure may also indirectly assess anxiety (usually higher in more anxious mice).

their non-anxious C57 counterparts (Fig. 2), as assessed by horizontal activity, stops, directed exploration episodes, latency to leave the center, and defecation scores. Average inter-stop distance was also different in these strains (Fig. 2): the more anxious mice showed shorter inter-stop distance.

Since these mice have also been known to display contrasting activity phenotypes (BC > C57, Table 1), in a separate experiment we wanted to test our protocol in mouse strains with the same anxiety strain ranking but different activity strain ranking. For this, we repeated the same experiment in NMRI and BC mice, markedly different in their anxiety (BC > NMRI) and activity levels (NMRI > BC) (Table 1). Again, despite strain differences in motor activity, the more anxious BC group showed less explora-

tion and more stops and defecation in the ST compared to the less anxious NMRI mice (Figs. 2 and 3), confirming the strain profiles obtained in the free-exploratory paradigm and several other anxiety tests [3,55].

In the third experiment, the BC and NMRI mice were subjected to LDST. Although both groups can freely explore both parts of the test, the highly anxious BC mice showed a clear preference for the dark part. In contrast, the less anxious NMRI mice showed only slight aversion to the light part of the test, also demonstrating lower average speed and shorter inter-stop distance (Fig. 2). These results are in line with earlier findings that BC mice exhibit a very robust response in the light–dark paradigm [32], suggesting their high sensitivity to light–dark situations, including the LDST. Moreover, our findings show a striking similarity

to other recent reports [23,40] showing that NMRI mice produce more light–dark transitions and spend more time in the light part of the light–dark test compared to BC mice.

Taken together, these findings confirm that (1) our protocol works consistently with several mouse strains widely used in behavioral research, (2) the test is sensitive to the strain differences in anxiety, and (3) its results are generally consistent with strain-specific profiles demonstrated in other behavioral tests. In addition, our data show a striking parallelism between the LDST and the light–dark test, as assessed in NMRI and BC mice, suggesting that both

tests of anxiety may target essentially the same behavioral domain(s).

5.2. ST performance in different stressful situations

In order to further show that our protocol is sensitive to different levels of anxiety, we compared the ST performance in stressed and non-stressed mice (Experiment 4; Fig. 2). For this, we used active non-anxious NMRI mice and assessed their ST behavior following a 5-min exposure to a young male Wistar rat. Fig. 2 shows that stressed NMRI

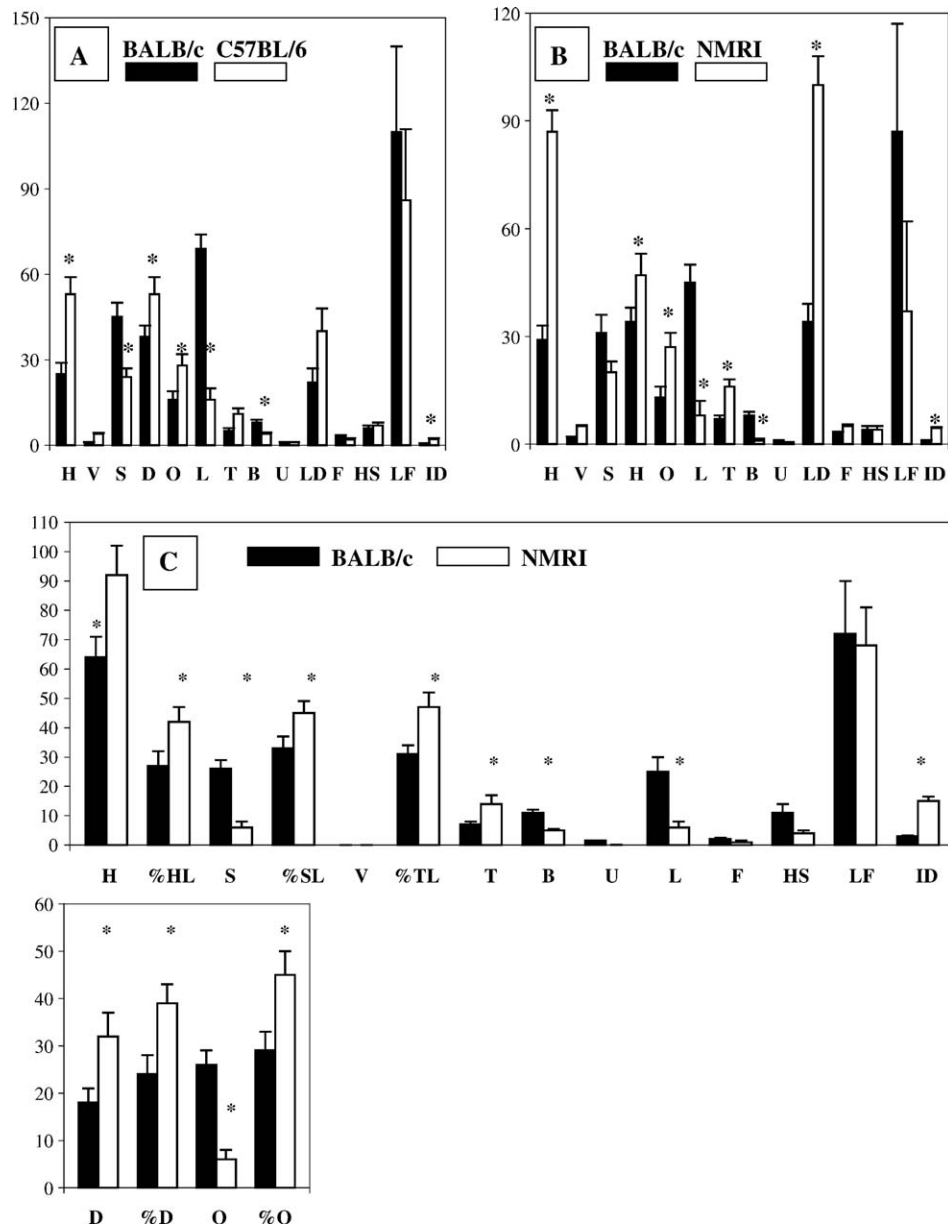


Fig. 2. Behavioral profiles of NMRI, BALB/c, 129S1, and C57BL/6 mice tested in the Suok and the light–dark Suok tests for 5 min. Behavioral measures are as in Table 2. Data are expressed as mean ± SEM.  $P < 0.05$  between groups ( $U$  test). Suok test: A, B, D, E, F, G: H–horizontal activity (segments), V–vertical activity, S–stops, D–head dips, O–orientation, L–latency to leave center, T–number of transitions, B–defecation boli, U–urination episodes, LD–latency to defecate, F–falls from the rod, HS–hindleg slips, LF–latency to fall, ID–average inter-stop distance. Light-dark Suok Test: C (additional behavioural indices): %HL–% horizontal activity in the light, %SL–% stops in the light, %TL–% time spent in the light, %D–% head dips in the light, %O–% orientation in the light. \* $P < 0.05$  ( $U$  test) difference between strains (A, B, C, E) and groups (D, F, G).

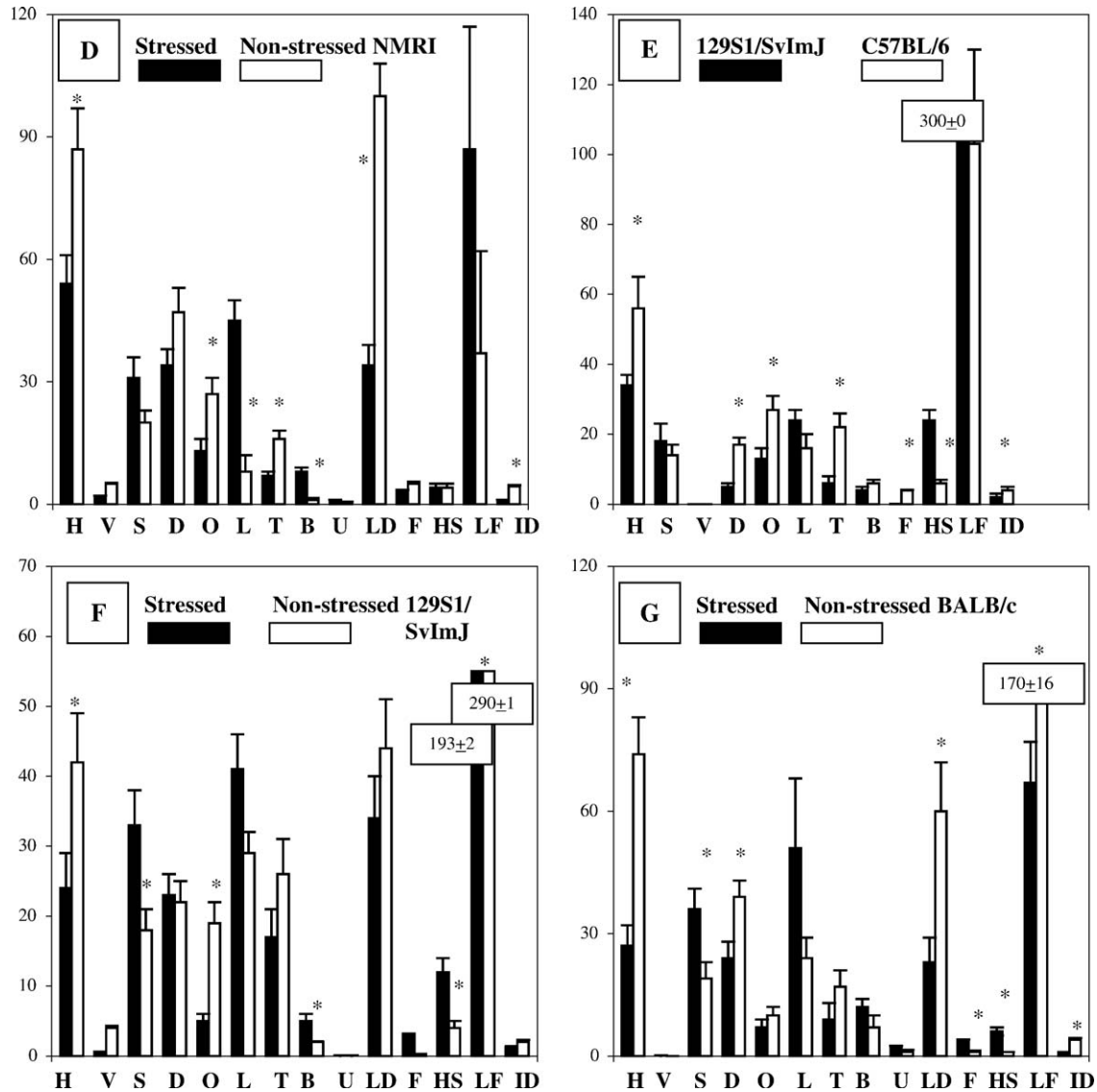


Fig. 2 (continued).

mice predictably display more anxiety in the ST compared to their non-stressed controls.

Overall, the fact that our protocol is equally sensitive to both genetic-dependent (strain-specific, e.g., BC vs. NMRI or C57 mice; ST) and test-dependent (e.g., stressed vs. non-stressed NMRI; LDST) variations in anxiety shows its utility for modeling both state and trait anxiety subtypes in mice (see Refs. [3–5] for a detailed discussion of their clinical importance).

### 5.3. Analysis of motor impairments in ST

In order to show that our protocol is able to detect both strain-specific and anxiety-induced motor dysfunctions, we analyzed motor coordination in the mice in this test. The test was first applied to two mouse strains known to display markedly different baseline motor coordination (normal: C57; impaired: S1, Table 1). As can be predicted

based on the similarity between the ST and the beam test, the C57 mice showed less anxiety and fewer hindleg slips compared to the S1 mice (Fig. 2). Our results clearly show that this protocol is able to detect baseline motor-coordination deficits in certain mouse strains and can therefore be a useful method for neurological screening of mice, in addition to testing anxiety in the same animals.

Finally, we used our method to analyze possible anxiety-evoked motor impairments in BC and S1 mice (Fig. 1) following a 5-min “anxiogenic” exposure to a rat. In general, these strains show similar motor-sensory skills, neurological reflexes and muscular strength, and share similar brain anomalies (agenesis of the corpus callosum) [38,39,59,64]. As can be predicted, rat exposure produced marked motor coordination deficits in both strains compared to non-exposed controls (Fig. 2). However, this response was more pronounced in S1 mice, possibly due to the low sensitivity of BC in models based on rat exposure [65]. In general,

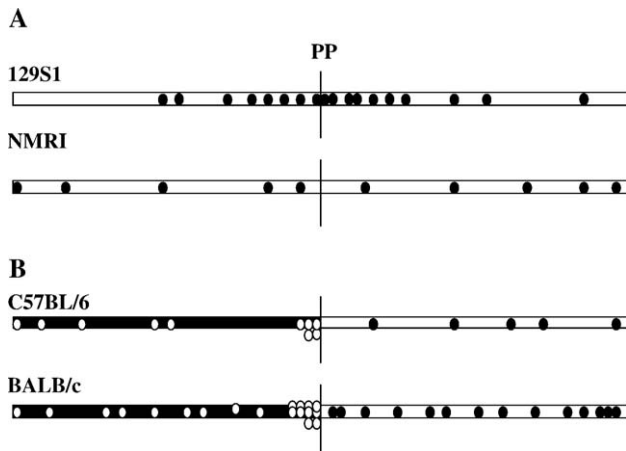


Fig. 3. Schematic drawing of the spatial distribution of stopping activity (circles) performed in the Suok tests by one representative animal from each mouse strain. (A) Suok test: note robust strain differences in anxious 129S1 mice (more stops, clustered near the placement point) vs. non-anxious NMRI mice (fewer stops, distributed evenly). (B) light-dark Suok test: note that anxious BALB/c mice produced more stops in the dark part of the test, also showing frequent stops near the border (before entering the aversive light part of the test), compared to non-anxious C57BL/6 mice. PPplacement point.

these results are in line with previously published studies showing the link between anxiety and motor-vestibular-coordination deficits in mice [35–37]. However, our data indicate a clear advantage of our approach over previously used tests, since the ST is a useful method allowing parallel assessment of both anxiety and motor coordination in the same experiment.

There have been remarkably few studies assessing anxiety in mice from the sensory-motor integration viewpoint (see discussion in [37,52]). Consistent with the important role of such integration for spatial orientation, spatial anxiety, and motor/balance control [52,57], our data show that anxious mice tend to display impaired balance control in the ST compared to their non-stressed controls or less anxious strains. Our results show that a detailed analysis of the ST performance appears to be a particularly useful tool for the study of mouse anxiety and motor-sensory functions (including balance control) in the field of stress research and behavioral neurogenetics.

## 6. Discussion

Overall, the major questions in the present research were (1) Is our protocol able to detect anxiety in mice tested in different stress situations? (2) Can the protocol be effective in detecting behavioral differences in different strains with markedly different anxiety profiles? (3) Can the present ST detect anxiety-evoked and strain-specific motor impairments, in addition to measuring anxiety? The results presented here answer all these questions positively, and we will next discuss how this protocol can be used in neurobehavioral research.

### 6.1. General assessment of the protocol

- (i) As can be seen in Figs. 2 and 3, there was a general pattern of behavior demonstrated in the ST by more anxious animals. This includes (1) lower horizontal exploratory activity, (2) more stops, (3) lower levels of directed exploration (head dips, orientation), (4) slower average speed (5) shorter inter-stop distance, and (6) more defecation boli deposited. Urination scores appear to be a weak index of anxiety in this test. In the LDST, there were additional behavioral markers of anxiety, such as (1) lower percentages of horizontal and directed exploration in the more aversive light part of the test, and (2) higher percentages of horizontal and directed exploration in the dark part of the test. In addition, we noted that more anxious mice generally display more stops and defecation boli in the aversive light part of the test (Fig. 3), although this phenomenon may be masked by a decrease in the time spent in the light part.
- (ii) Another aspect to consider here is the difference in brain anatomy reported for the mouse strains used in the present study. It has long been known that S1 and BC mice suffer from agenesis and dysplasia of the corpus callosum [38,60], a structure connecting the two brain hemispheres and integrating motor, sensory, and cognitive functioning [41]. Interestingly, some impairments in motor coordination have been reported in humans and mice with abnormal corpus callosum [38,39,41,59]. Thus, acallosal mice may display abnormal ST behaviors due to loss of communication between brain hemispheres. Since this structure may be crucial for the transcallosal passage of motor signals and feedback sensory signals controlling movements in the ST, it was possible to assume that callosal anomalies in S1 and BC mice may affect our results compared to C57 and NMRI mice. However, both acallosal S1 and “callosal” C57 mice showed good performance in the ST (Fig. 2), thus demonstrating no overt motor and coordination deficits (except hindleg slips, robust in S1 mice but not affecting their ST retention). In contrast, acallosal BC mice and “normal” NMRI mice showed similar poor retention in this test. Collectively, these data negate the notion that strain differences in the corpus callosum may affect the animals’ ST performance, also suggesting that the ST may be a useful tool for an in-depth analysis of the link between certain brain anomalies and their impact on mouse behaviors.
- (iii) In novel areas (such as the open field test), mice frequently establish a pronounced home base (HB, a “protected area” where they spent most of their time, and from which they perform exploratory excursions) [19]. Although the HB behavior may be an important part of rodents’ exploration, and an interesting behavior to measure [19,28], there are marked strain



differences in mouse HB behaviors which may dramatically affect their exploration, leading to behavioral artefacts and incorrect data interpretation. For example, anxious BC and S1 mice [45–47] frequently build a HB near the starting point (usually at the center of the apparatus), which can be mistaken for an “anxiolytic” athigmotaxis. We noted that the ST does not allow HB behaviors in mice (due to its shape) even if tested over a long period of time (15 min), suggesting that the test is less prone to incorrect data interpretations related to HB behaviors and representing its additional advantage for ethological analysis of mouse anxiety. One additional advantage of the test is its one-dimensional nature, allowing more adequate and easy assessment of animal locomotion (distance traveled  $\approx$  number of segments visited) and spatial distribution of behaviors in both ST versions (compared, for example, with the open field test, where this relation is not linear, and additional sophisticated equipment is needed to calculate the distance traveled).

- (iv) Overall, altered animal ST performance can reflect higher anxiety, altered activity levels, impaired motor-sensory functions, or a combination of these three. We note, however, that our protocol simultaneously controls for these factors. For example, altered defecations, stops, and exploration will detect anxiety; spatiotemporal distribution of horizontal and stopping activity will control activity levels, while motor impairments parameters (falls, paw slips) will detect possible motor impairments in mice.
- (v) In general, an animal model has to meet face and construct validity criteria in order to be a good model of human disorder [28], i.e., to reflect phenomenological similarities between animal and human pathologies, and share a neurobiological rationale behind the pathology in question. Our study suggests that both ST modifications show good face validity, possessing a marked behavioral similarity between “ropewalking” anxious mice in this test and humans with anxiety spectrum disorders. Moreover, both ST versions possess good construct validity, as they have a clear underlying theory and biopsychological mechanisms. Finally, the test seems to have good predictive validity, since it is sensitive to genetically or stress-evoked anxiety phenotypes in mice already established in other well validated behavioral paradigms (Table 1).
- (vi) In conclusion, the ST has a clear neurobiological rationale since it is based on the innate aversion of mice to novelty, open spaces, height (and brightly lit environment for the LDST), and the natural exploratory (curiosity) drive. The test may be used to assess anxiety, activity, and motor anomalies in mice, and has the advantage of being quick and easy to perform. It combines the principles of several popular and well-validated anxiety models, allowing us to study

distinct subtypes of anxiety simultaneously (including state and trait anxiety, which can be both evoked and measured in the ST). Moreover, the procedure is one-trial, easy to automate, based on animal spontaneous behavior, uses natural stimuli, and does not necessitate prior training of mice or food/water deprivation. The ST is easy to standardize, since (unlike many other behavioral paradigms [60]) it has only 4 intrinsic variables (length, diameter, height, and surface texture). Importantly, the test examines a wide range of behaviors (from locomotion and exploration to displacement and vegetative behaviors), and, together with parallel assessment of several principally distinct classes of measures (gross, spatiotemporal, and ethologically derived characteristics of behavior, Table 2), may be a rich source of behavioral information for neuroscience research. Finally, the strain differences detected in this test suggest that it may be a valuable tool in the search for the genetic bases of anxiety-related disorders. Taken together, this suggests that our method may be a useful tool in neuroscience research, including behavioral genetics and experimental modeling of various stress disorders.

## 6.2. Potential applications

- (i) Since the analysis of animal behavior is easier when one has some point of comparison, the problem of reference animals is crucial in behavioral neuroscience. Our data clearly show that S1, BC, NMRI, and C57 mice, due to their marked strain behavioral differences, represent excellent reference strains for anxiety-oriented studies using the ST or LDST. In addition, BC and S1 mice seem to represent the ideal reference strains for the assessment of specific anxiety-induced motor-sensory dysfunctions in mice (see also Refs. [36,37]). Finally, given their marked strain differences in motor functions, S1, BC, and C57 mice may be recommended as reference strains in the ST used in animal motor coordination research.
- (ii) Given the impact of age on mouse exploration [24,42], although not directly tested in this study, it is possible to assume that the ST may detect age-related alterations in mouse anxiety and motor-sensory functions. Indeed, since the LDST has many features in common with the light–dark paradigm, it is possible to assume that the age factor may play a similar role in both tests. Given the high sensitivity of the light–dark test to age of mice [24], we suggest that the ST and LDST may also be sensitive to the age of the mice tested. Clearly, this promising research direction requires further investigation and may represent an important potential application of our method given the high percentages of age-related anxiety and motor disorders in the elderly.

- (iii) Since BC mice are traditionally known as a neophobic strain and given their clear neophobic profile in the ST (Fig. 2), we suggest that analysis of behaviors of these mice in the ST may lead to interesting models of human generalized anxiety. In contrast, our findings in NMRI and S1 mice exposed to a rat suggest the utility of the ST or LDST to assess acute anxiety/fear in these strains as a potential model of human panic disorder. Finally, our present data show high sensitivity of S1 and C57 mice to the ST (Figs. 2, 3), which may be particularly useful for psychopharmacology or behavioral genetics, given the fact that these strains are commonly used in psychopharmacology research and represent common genetic backgrounds for gene-targeting in mice [29,39,45,58,60].
- (iv) Overall, the protocol can be extensively used in psychopharmacology research in the search for novel anxiolytic anti-stress drugs. For example, the fact that the mice reliably re-entered the more aversive light part of the LDST indicates that the test may be able to detect both anxiogenic and anxiolytic states produced by various stressors and drugs, as does conceptually “similar” the light–dark test [20,24]. Given the results of our study, the ST and LDST will be especially useful in achieving a more accurate interpretation of the behavioral data, especially in situations when conflicting data have been obtained in other anxiety tests, and there is a need for more detailed and in-depth behavioral analysis. For example, both tests may be extensively used when studying different manipulations with mixed or unclear effects, interpreting the behavior of novel mutant mice with unknown or unclear behavioral phenotype, or screening drugs with unclear profile. Finally, given the similar nature of novelty exploration in mice and rats [19], we may suggest that the ST, after some modifications, may also be suitable for use with rats and other small laboratory rodents.

### 6.3. Troubleshooting

(i) Our observations show that animal ST performance is sensitive to stressors, suggesting good predictive validity for the current protocol as a powerful tool to detect anxiety in animal behavior. However, some specific stress-evoked behaviors (stereotypies, displacement activity) may be a confounding factor in this test, because alterations in these behaviors may reciprocally affect animal ST exploration, see [27,29,30] for discussion. Since such behaviors are more obvious when mice are first exposed to ST, their performance is easily affected by minimal changes in environmental conditions. Thus, it is recommended that the experimenters handle the mice for 5 min/day for 3–4 days before the first experiment in order for them to become familiar with environmental stimuli, also see [51]. This will also habituate mice to possible pre-testing procedural stressors, such as

removal from the cage, weighing, injection, and placement on the rod. In addition, transportation of mice from their holding room to the experimental room has to be as minimal as possible and at least 1 h. Acclimation time is necessary.

(ii) Since animal anxiety response is known to habituate after repeated exposures to stress, we note that experimentally naive animals in Test 1 usually show clearer and more robust results. If, however, a battery of several stressors has to be used on the same animals (which is nowadays the most common situation in behavioral research [15,16,43]), the mice should be allowed at least 7 days for acclimation between the tests (BC, C57, NMRI). For some mouse strains (such as S1, showing robust habituation to novelty [60]), re-testing in the ST has to be avoided. In some other strains, the acclimation period may be extended to 10–14 days, depending on their memory phenotypes. We have also noted that the ST works better if the battery includes 3 or fewer tests. Moreover, since the ST shares construct similarity with the elevated plus maze, open field, hole-board, and light–dark tests, it is recommended not to include these tests in the test battery with the ST to avoid animal habituation to similar stressors.

(iii) The timetable and intensity of the experiments and even experimenter identity may also be critical for obtaining correct and reliable data [2,11,16,28]. Since rodents are very sensitive to the rhythm of activity of the researchers and animal house personnel, all their behaviors may be affected by this factor. Thus, although this constitutes a common problem in all behavioral studies [2], it is advisable to consistently avoid, or, alternatively, prefer, weekends when scheduling the experiments aiming to assess animals’ ST behaviors. Another behavioral problem with the ST is that individual mice from well-performing strains (<5%) sometimes display almost immediate fall from the rod (possibly, due to fear-evoked stupor-like reactions). To solve this “initial falling” problem, it is recommended to gently support these mice by hand for 5–10 s (to allow the animal to get a firm grip) during the initial placement. If, however, these mice would still display unusually poor ST retention, it is necessary to exclude them from the experiments.

(iv) Another related common behavioral problem is that mice sometimes display an unusually low or unusually high activity level in anxiety tests, including the ST. For example, some mice may have unusually long latencies to leave the central zone or show abnormal freezing at one point, while others may display atypical non-stop locomotion in the test. Although the usually low percentage of these animals (5–10%) does not affect the results of experiments, this potential problem should be considered when performing analysis of animal performance in the ST. As one possible solution, it can be recommended to carefully check home cage behaviors before testing and, if necessary, exclude mice with abnormally low or high general motor activity from the experiments.

(v) Mice tested in the ST or LDST frequently display whisking behavior (especially robust during head dips and

side-directed exploration) underlining the potential role of whiskers in animal behavior in this test, and the need to control the status of whiskers in mice. Notably, the Dalila effect (whisker barbering), commonly observed in socially housed laboratory mice [54], is more common in certain mouse strains (e.g., C57 [54]), suggesting a strong genetic component. In the present study, we observed this effect in 25–30% of C57 and NMRI (but not BC and S1) mice, ranging from snout whiskers shortening and/or removal (C57) to complete snout denuding (NMRI) always performed by a dominant male (whose whiskers remained intact), similar to that reported in earlier studies [54]. Although this factor is not specific to the present test and may play a role in any other exploratory-based anxiety model [15,16] as a part of the rodent complex information-gathering system in unfamiliar elevated spaces [8], it is recommended to control the whisker status of mice prior to the ST to allow more accurate inter-strain comparisons and use more homogenous groups (for example, by including similar numbers of mice with normal, fully or partially removed whiskers in each experimental group). In addition, to minimize whisker barbering in mice, it may also be recommended to improve animal housing/dietary conditions and use more environmental enrichment [54].

(vi) Since animal exploration is generally dependent on locomotor activity [31], the behavioral alterations may not necessarily reflect changes in anxiety per se. Indeed, like many other exploration-based ethological models, the ST may be sensitive to possible confounding alterations in locomotion (for example, induced by drugs or genetic manipulations). We stress, however, that this problem is rather common to all existing animal models of anxiety [20] and is not limited to the ST. Therefore, it is necessary to control possible treatment-induced alterations in locomotor activity when assessing mouse performance in the ST to eliminate false-positive or false-negative results. For example, it is recommended to perform a screening of possible locomotor alterations in the open field test in addition to assessing mouse activity in ST. Moreover, expression of the LDST data as percentages of total time spent and segments visited (Table 2) helps to reduce possible activity-related artefacts in this version of the test.

(vii) In spite of the apparent simplicity of this test, the animal behavior in the ST seems to be influenced by many factors. For example, it is possible to assume that visual, olfactory, vestibular, and tactile stimuli may influence the ST performance, as can be seen by the large number of directed exploration in this test (Fig. 2). Therefore, it is necessary to control for possible alterations in all major sensory functions in mice before assessing mouse performance in the ST, according to standard behavioral phenotyping protocols [15,16]. Interestingly, however, the ST itself allows detection of vestibular anomalies in mice, as discussed above. Moreover, the LDST can also be used to indirectly assess visual anomalies in mice, as assessed by the ability of mice to stop before entering the light part of the test. Indeed, all the mice

used in this study clearly distinguished the border between the two parts of the LDST, making frequent stops near the virtual border between the two parts of the test (Fig. 3). This suggests, for example, that albino mice such as BC may not have visual problems (see Ref. [50] but [15,16]).

Moreover, like many other anxiety tests [2,11,20,28,53], the ST appears to be sensitive to environmental factors, such as observation room conditions (ventilation, temperature, humidity, soundproofing, etc.). Therefore, these factors have to be carefully controlled for in the experiments. Special attention has to be paid to lighting conditions, since the overall behavioral performance of some mouse strains (e.g., C57, BC) is particularly affected by this factor [11]. Furthermore, the lighting conditions of both holding and experimental rooms have to be controlled, since it is likely that animals tested in the ST under light that is brighter than in their holding room will exhibit altered baseline activity and/or more anxiety. Overall, dimly lit experimental room appears to be the best testing environment for the ST.

(viii) Finally, in contrast to other (less rigorous) exploration-based tests, the ST performance may be affected by physical factors, such as the body size and weight of the animals, representing a technical problem for the ST, since bigger mice may have more difficulties in keeping their balance. Since some mouse strains as well as mutant and wild type mice of the same age markedly differ in their body size, weight, or both (e.g., [18]), it is recommended to use the ST to test mice of similar body size/weight to allow more accurate comparison between the groups. In addition, although all the mouse strains used in this study performed relatively well on the present aluminum ST rod, it is possible to assume that some other mouse strains (for example, with motor-vestibular or coordination anomalies) may require a less slippery surface of the apparatus. For this, adding a layer of masking tape (e.g., [33]) may be recommended to provide the mice with a firm grip. Alternatively, a more textured material (e.g., wood) may be used in this test. In addition, the diameter of the rod may be increased for such mice, to enable them to get a better grip.

## 7. Quick procedure

- (a) Expose the mice to different stressors for 5 min (if necessary) and then assess their activity in the ST or LDST for 5 min.
- (b) Identify and register separately the ST or LDST behaviors (see Table 2 for a complete list of behavioral measures).

## 8. Essential literature references

Original papers [33,37,40,50]; reviews [2,3,16,39,56]; On-line Mouse Phenome Database [45].

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