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MOUSE PHENOME PROJECT

New replicable anxiety-related measures of wall vs. center behavior of mice in the open field

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Lipkind, Dina, Anat Sakov, Neri Kafkafi, Gregory I. Elmer, Yoav Benjamini, and Ilan Golani. New replicable anxiety-related measures of wall vs. center behavior of mice in the open field. *J Appl Physiol* 97: 347–359, 2004. First published February 27, 2004; 10.1152/japplphysiol.00148.2004.—Anxiety is a widely studied psychiatric disorder and is thought to be a complex and multidimensional phenomenon. Sensitive behavioral discrimination of animal models of anxiety is crucial for the elucidation of the behavioral components of anxiety and the physiological processes that mediate them. Commonly used behavior paradigms of anxiety usually include only a few automatically collected measures; these do not exhaust the behavioral richness exhibited by animals, thus perhaps missing important differences between preparations. The aim of the present study was to expand the repertoire of automatically collected measures in a classical test of anxiety: behavior in relation to the wall in the open field. We present an algorithm, based on the Software for the Exploration of Exploration strategy, which automatically partitions the mouse path into intrinsically defined patterns of movement near the wall and in the center. These patterns are used to design new end points, which provide an articulated description of various aspects of behavior near the wall and in the center. Sixteen new end points were designed with data from C57BL/6J and DBA/2J mice tested in three laboratories. The strain differences in all end points were evaluated on another data set to assess their validity and were found to remain stable. Ten of the sixteen end points were found to discriminate between the two strains in a replicable manner. The entire set of end points can be used on various genetic and pharmacological models of anxiety with good prospects of providing fine discrimination in a replicable manner.

behavioral phenotyping; thigmotaxis; ethological measures; Mouse Phenome Project; C57BL/6J; DBA/2J

ANXIETY DISORDERS ARE AMONG the most common and most studied psychiatric disorder in humans. Animal models of anxiety were developed to facilitate the discovery of the genetic and neurobiological substrates of anxiety and test putative anxiolytic drugs. Evidence from human and animal studies suggests that anxiety is a complex and multidimensional phenomenon at the behavioral, neural, and genetic levels (9, 18, 26, 30, 38). The ability to sensitively discriminate between the behavior of various neurogenetic and pharmacological animal models of anxiety is a key issue in present research because it is necessary for highlighting separate behavioral components of anxiety and the physiological processes that mediate them.

Behavior in the Open Field Test (OFT) is a widely used test of anxiety as well as of exploration and locomotor activity (1, 39) and is one of the most commonly used behavioral tests in genetically engineered mice research (5). As a test of spontaneous (unconditioned) behavior, it allows the animal to exhibit a wide range of behaviors and therefore is highly suitable for the study of complex phenomena such as anxiety. Typically, however, only two spatial measures are collected in this test: the total distance traveled (considered as a measure of general activity) and some measure of the animal's tendency to avoid the arena center. The latter measure is considered anxiety related, based on the assumption that the arena center is more threatening for rodents than its periphery and based on the increase in center occupation seen after administration of anxiolytic drugs (33, 36). These simple measures can be recorded automatically, allowing high-throughput analysis, but they far from exhaust the behavioral richness exhibited by animals in the OFT. More comprehensive analyses of tests of unconditioned behavior are needed (30, 31, 38) that would provide detailed and articulated information about different aspects of the animal's behavior, thus allowing for the reliable interpretation of genetic and pharmacological results. One way to meet this need is to use test batteries, which would include many different behavioral tests (5, 14, 32). Another (not incompatible) approach is to extract more information out of a single test. This is generally done by adding "ethological" measures, collected by human observers (e.g., the frequency of grooming, rearing, stretch attends, and so forth; Refs. 4, 6, 7, 11, but see Ref. 29 for an exception). This type of analysis adds a considerable amount of information but is relatively low throughput, involves subjective judgment, and uses ad hoc criteria.

A complementary high-throughput approach is offered via a software-supported strategy called Software for the Exploration of Exploration (SEE; Refs. 15, 16, 23), which is aimed at the investigation of rodent OFT behavior. SEE includes statistical algorithms that allow a reliable estimation of the animal's locations and momentary speeds at a high spatial and temporal resolution and the segmentation of the continuous x,y-location time series into a string of intrinsically defined, ethologically

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meaningful units. This allows the automatic calculation of multiple behavioral measures (end points) based on the quantification of the various features of these units. SEE end points have been shown to discriminate between inbred mouse strains in a replicable manner across laboratories (22).

Here we present a further development of SEE, which provides a detailed description of mouse behavior near the wall and in the center of the arena. This behavior is commonly quantified by several measures: the time spent in the center (or its proportion out of the entire session), the distance traveled in the center (or its proportion out of the total distance traveled), the number of entries into the center, and the latency to enter the center. Most studies use only one of these measures, but some use several. Sometimes one or more of these measures are quantified separately for a certain time period at the beginning of the session (e.g., first 5 min), based on the assumption that the mouse's anxiety level changes with time.

Obviously, any measure concerned with avoiding the center requires a definition of "being in the center." The arena center is defined arbitrarily as the area at a distance of more than *x* cm from the wall, where *x* is a "cutoff" number ranging between 5 and 20 cm in different studies. The value of the cutoff may be based on ethological considerations (e.g., a distance that enables a mouse to keep its vibrissae in contact with the wall; Ref. 35) but is often determined by technical factors, such as the dimensions of the squares composing a photocell chamber. If behavior in the OFT is stochastic and if the transition between behavior near the wall and in the center is continuous, then the precise definition of the cutoff value (e.g., 5, 10, or 15 cm from the wall) between the two is not of crucial importance, and an arbitrary choice would be justified. This, however, does not seem to be the case. Observation of mouse behavior in the OFT reveals a clear structure: mice typically either run parallel to the wall in close proximity to it or make forays into the center. Furthermore, these forays are of different types: small arc-shaped forays performed near the wall or long forays that cross the arena. A structured behavior demands intrinsic definition of cutoff values, since arbitrarily defined cutoff values might either cut across a continuous structure or indiscriminately lump together distinct structures. Because the behavioral structure is, moreover, likely to differ across preparations (e.g., inbred strains or drug-treated animals), the use of arbitrary criteria might blur important differences between them. Analysis based on intrinsic criteria should therefore considerably enhance the discriminative power of the OFT, and applying such analysis to the behavior near the wall and in the center should increase the discriminative ability of its anxiety-related aspects. In addition, measures based on intrinsic properties of behavior have been shown to provide results that are replicable across laboratories (22), a major concern in present-day research (12).

In this work, we present a new SEE algorithm that automatically separates the path of the mouse into intrinsically defined patterns of movement near the wall and in the center. We also demonstrate its use in the design of new, replicable end points. This is done with data from two inbred strains, C57BL/6J (B6) and DBA/2J (D2), tested across three laboratories. These two strains are widely used in behavioral, genetic, and pharmacological research. Studies that have used the accepted OFT measures of distance traveled and center occupancy usually show B6 to be a more active strain than D2 and to occupy the center more (reviewed in Ref. 13).

After developing new end points of wall vs. center behavior and demonstrating their discriminative power and replicability, we use an independent set of data to assess the validity of these results.

METHODS

Two sets of data from two separate studies were used in the present work. One data set was used for the development of new end points of wall/center behavior. The other set was used for their validation. The data for the first set were collected in a study conducted at three laboratories: The National Institute on Drug Abuse (NIDA; Baltimore, MD), Maryland Psychiatric Research Center (MPRC; Baltimore, MD), and Tel Aviv University (TAU; Tel Aviv, Israel). These data are stored in a publicly available database (http://www.tau.ac.il/~ilan99/see/help) and have already been used in previous studies (22, 24). Data from three of the five experiments available in the database comparing B6 and D2 (one from each laboratory) were used. These experiments had the least differences in experimental conditions between laboratories (the differences are summarized in Table 1).

The second data set is from a study conducted 10 mo later at the same three laboratories; this study included 10 inbred mouse strains (including B6 and D2) and is part of the Mouse Phenome Database (28). In the present study, we used only the data of B6 and D2 mice. The differences between laboratories listed in Table 1 also apply to the second study, except that the sample sizes were 12 mice per strain in each laboratory and the arena's diameter was 250 cm for all laboratories.

The experimental and housing protocols were identical for both studies and are described in detail elsewhere (22). Here we repeat the main points.

Animals

B6 and D2 male mice 9–14 wk old were shipped from Jackson Laboratories.

Housing

Animals were maintained in a 12:12-h reversed-light cycle (lights on from 8:00 PM to 8:00 AM) and were housed two to four per cage under standard conditions of 22°C room temperature and water and food ad libitum. The animals were housed in their room for at least 2 wk before the start of the experiment. All animals were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC, MPRC, and NIDA) or by National Institutes of Health Animal Welfare Assurance Number A5010-01 (TAU). All studies were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Table 1. *Differences in experimental conditions between laboratories*

Laboratory	NIDA	TAU	MPRC
Group	B6 (8) D2 (8)	B6 (9) D2 (9)	B6 (10) D2 (10)
Arena's diameter, cm	250	250	210
Tracking rate	30/s	25/s	30/s
Spatial resolution (pixel size), cm	1.3	1.0	1.0
Brightness, Lux	Not available	350	490

No. of mice are given in parentheses. NIDA, National Institute on Drug Abuse; TAU, Tel Aviv University; MPRC, Maryland Psychiatric Research Center; B6, C57BL/6J mice; D2, DBA/2J mice.

Experimental Procedure

The arena was a large (210- to 250-cm diameter, see Table 1), circular area with a nonporous gray floor and a 50-cm-high primer gray-painted continuous wall. Several landmarks of various shapes and sizes were attached to different locations on the arena wall and on the walls of the room where the arena was located. The arena was illuminated with two 40-W neon bulbs on the ceiling above the center of the arena.

The experiments were conducted during the dark part of the cycle, 1–2 h after its onset. Each experimental animal was brought from its housing room to the arena in a small opaque box and placed within the arena (in a standardized location, near the wall) while still in the box. After 20 s, the box was lifted, and a 30-min session began. The animal's movement was tracked with the Noldus EthoVision automated tracking system (34).

Data Analysis

The raw data obtained from the tracking system were smoothed with the use of a specialized algorithm implemented in the stand-alone program "SEE Path Smoother" (20). This procedure produces reliable estimates of momentary speeds during motion (momentary speeds during arrests were defined as zero).

As was previously shown, rodent locomotor behavior consists of two distinct modes of motion: progression segments and lingering episodes (15, 19). During progression segments, the animals traverse relatively large distances, attaining relatively high speeds. During lingering episodes, the animals stop and perform scanning movements, while staying in a circumscribed neighborhood. Segmentation of the smoothed path into progression segments and lingering episodes was done with the expectation maximum (EM) algorithm (17), using a two-Gaussian mixture model. Stand-alone user-friendly software for smoothing (SEE Path Smoother) and for segmentation (SEE Path Segmentor) can be downloaded at http://www.tau.ac.il/~ilan99/see/help.

We developed the new algorithms and end points described in RESULTS using the Mathematica-based program SEE Package (16) and two recently developed extension programs, "SEE Experiment Explorer" and "SEE Endpoint Manager" (21).

Statistical Methods

Calculation of radial distances and radial speeds. The wall/center separation procedure requires the calculation of the radial distance (the distance from the wall) and the momentary radial speed (the component of the velocity in the direction of the center) for all time points. The computation of these two quantities is based on the arena radius. In a perfectly circular arena, the radius is, of course, a constant. However, in real life, an arena is seldom a perfect circle, often having some dents and irregularities in its shape. Therefore, calculating the radial distance at any time point with a constant radius introduces an error into the computations, and the effect of this error is amplified when the radial velocity (the first derivative of the radial distance) is computed. It was found that even very small dents in the arena shape can cause relatively large errors in the calculation of radial speeds.

To overcome this difficulty, the radial distances and velocities should be calculated with the actual shape of the arena. Because Ethovision (and other tracking systems) does not provide the spatial coordinates of the arena wall directly, an algorithm was developed for the estimation of the arena shape from the movement of the mouse in it. The algorithm, called "Arena Builder," utilizes the fact that most mice spend a large portion of the session running very close to the arena wall (actually touching it). Therefore, their locations can be used to produce an estimate of the arena shape. This estimated arena shape is in turn used to compute the radial distances and the radial speeds at all time points, without the need to use a constant arena radius. The arena shape is calculated separately for each session, thus correcting for small changes in the arena location and small shifts in the position of the camera that might occur between sessions. A detailed description of the algorithm is available online at http://www.tau.ac.il/~ilan99/see/help.

Comparing end point results between strains and across laboratories. To assess the discrimination between strains and the replicability across laboratories of end points generated by the wall/center separation procedure (see RESULTS), we used the linear mixed-effects ANOVA model (25, 27). In this model, the strain is considered as a fixed factor, whereas the effect of laboratory is considered as random. This means that we think of the laboratory effect as being drawn from the population of all possible laboratories' effects. The interaction between strain and laboratory is also considered random. Thus a significant strain difference yielded by the mixed-effects ANOVA model can be regarded as replicable across laboratories. This approach is more conservative than the widely used linear fixed-effects ANOVA model: if a difference between two strains was found to be significant under the mixed model, it will also be significant under the fixed-effects model, but the opposite is not necessarily true. Transformations (listed in Table 2) were used for the analysis of each end point to correct toward approximately normal distributions and to stabilize group variances (which tend to increase with the increase in group means).

As detailed in RESULTS, the end points generated by the wall/center separation procedure are based on measurements performed on discrete segments of the mouse path. To obtain statistically meaningful results, only mice that performed more than 10 segments on which a certain end point was based were included in its analysis.

Correlation between end points. The Pearson correlations between the end points, on the transformed and within-strain normalized data, were computed.

Table 2. *Results for wall/center end points in B6 and D2 (first data set) and the transformations used in their analysis*

End Point	Transformation	First Data Set		*P*
		B6	D2	
MCW	$1/\sqrt{X}$	7.7±0.2	4.3±0.2	0.009
MCC	$1/\sqrt{X}$	8.3±0.4	13.6±1.1	0.03
MLDW	$1/(X+1)$	0.96±0.03	1.4±0.09	0.03
MLDC	$1/(X+1)$	0.58±0.03	0.61±0.05	0.9
NI	None	133.6±7.2	55.8±6.9	0.08
MIL	$\sqrt{X}$	59.7±2.5	20.8±1.6	0.008
MIMWD	None	21.0±0.8	10.6±0.4	0.04
PIBS	Logit	0.15±0.01	0.42±0.02	0.009
ASLI	None	35.9±1.2	16.4±1.1	0.009
OISRI	None	1.03±0.01	0.84±0.02	0.01
MCNW	$1/\sqrt{X}$	9.2±0.6	15.3±1.3	0.03
MCI	$1/\sqrt{X}$	8.3±0.4	13.8±1.0	0.03
MCAC	$1/\sqrt{X}$	8.03±0.4	9.8±1.4	0.68
NNW	$\sqrt{X}$	60.6±5.4	36.0±4.9	0.25
NII	$\sqrt{X}$	58.6±3.8	17.1±2.8	0.03
NAC	$\sqrt{X}$	14.4±1.1	2.7±0.5	0.01

Values are means ± SE (nontransformed). End points are presented in the same order as in RESULTS. End points' names are abbreviated as follows: MCW, median curvature near wall; MCC, median curvature in the center; MLDW, median lingering duration near wall; MLDC, median lingering duration in the center; NI, number of incursions; MIL, median incursion length; MIMWD, median of incursions maximal wall distance; PIBS, proportion of incursions beginning with a stop; ASLI, average segment length in incursions; OISRI, outbound/inbound speed ratio in incursions; MCNW, median curvature in near-wall incursions; MCI, median curvature in intermediate incursions; MCAC, median curvature in arena-crossing incursions; NNW, number of near-wall incursions; NII, number of intermediate incursions; NAC, number of arena-crossing incursions. *P* values for strain differences were obtained with mixed-model ANOVA.

Validation of the new end points using a second data set. Two aspects of the newly developed end points were examined with a second, independent data set. *1*) To assess the stability of the strain differences, we tested the hypothesis that the difference between the two strains, in each end point, is the same as in the first data set. Note that, even within the mixed-model framework, such a difference of differences can be simply tested by using a variation of the two-sided *t*-test because the laboratory and interaction effects drop out. The false discovery rate (FDR) controlling Benjamin Hochberg (BH) procedure (3) was used to control for multiple comparisons. *2*) The first data set was used for the development and selection of potential end points as well as for testing the strain differences. This may hamper the discriminatory value of the subset of end points that were found to be significant. For that reason, we retested end points that were found to be significant in the first data set for their ability to distinguish between strains and across laboratories in the second data set (using the mixed-model and FDR control).

RESULTS

The Wall/Center Separation Procedure

The wall/center separation procedure is an algorithm for separating the path of the mouse into intrinsically defined components of movement in reference to the wall and for defining discrete units of wall/center behavior. It consists of two stages. In the first, movement along the wall is distinguished from movement in the center. In the second, movement in the center is further partitioned into several components.

First stage: distinguishing between movement along the wall and in the center. This stage is based on the observed tendency of mice to run along the arena wall. While a mouse is running along the wall, both its radial speed and its distance from the wall are small. Therefore, to isolate the component of movement along the wall, we classify data points according to both their radial speed and their distance from the wall. Having performed this classification, we divide each class into the following discrete units: segments of progression along the wall, segments of progression in the center, and incursions into the center (forays into the center, which may be composed of several progression segments). Below is a detailed description of this stage of the wall/center separation procedure (also illustrated in Fig. 1).

Separation into two classes of motion. 1) Once the animal's trajectory has been divided into progression segments and lingering episodes (see METHODS), it is now possible to sift out lingering episodes, in which speed is relatively low, irregular, and sometimes erratic, from the data time series, thus obtaining only data of progression segments. Progression segments are characterized by relatively smooth speeds and are therefore more suitable for analysis based on radial speed.

2) With the use of the EM algorithm, a two-Gaussian mixture model is fitted to the frequency distribution of the momentary radial speeds (using square root transformation) of data points belonging to progression segments. The intersec-

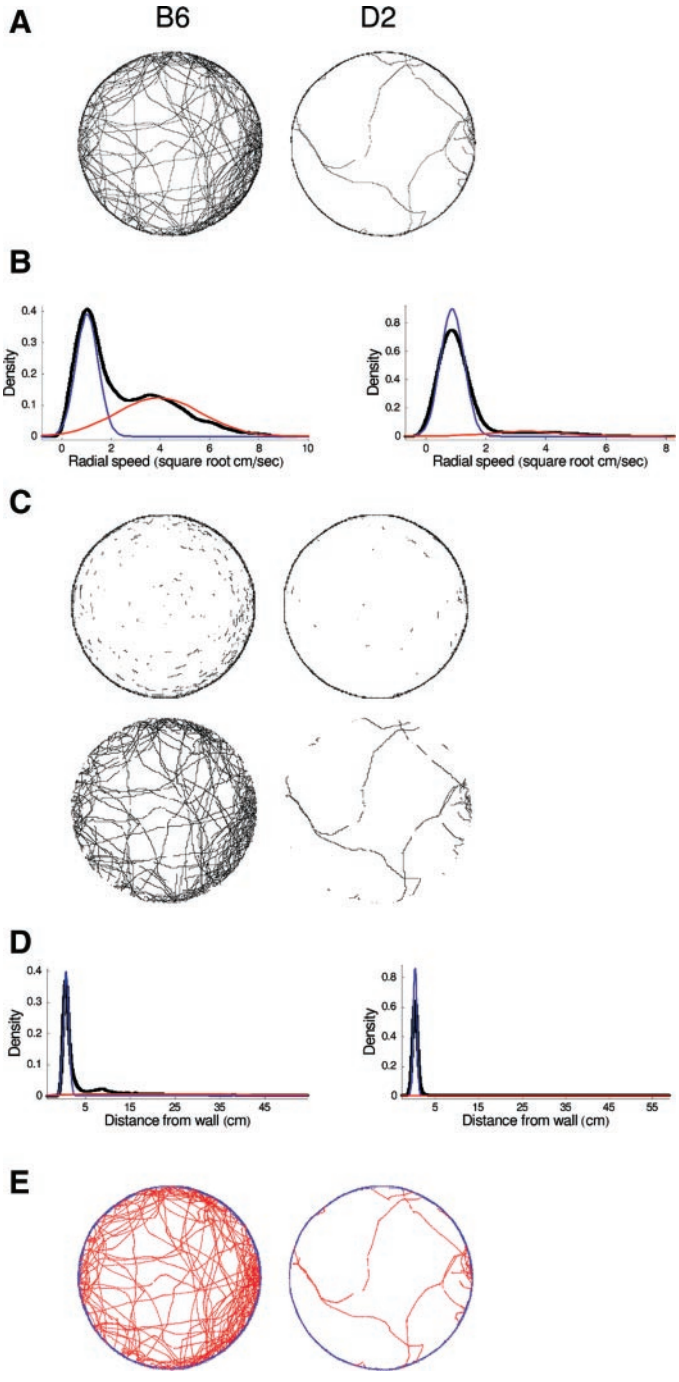

Fig. 1. Demonstration of the wall/center separation procedure using data from 2 mouse sessions. *A*: path plots (plots of the entire path traveled during a session) of a C57BL/6J (B6) mouse (*left*) and a DBA/2J (D2) mouse (*right*). *B*: black lines, density graphs (sliding histograms) of the frequency distributions of radial speeds (square root transformed) in data points belonging to progression segments for the 2 mice; blue and red lines, the 2 Gaussians fitted by the expectation maximum (EM) algorithm to the empirical distributions. The intersection point between the 2 Gaussians is used as a cutoff value for separating the data points into two components: *1*) movement parallel to the wall (low radial speeds), and *2*) movement toward or away from the wall (higher radial speeds). Note that, whereas the first component is very prominent and localized, the second component may be quite flattened. *C*: plots of the locations of all data points that belong to the first (*top*) and the second (*bottom*) components established in *B*. *D*: black lines, density graphs of the frequency distribution of distances from wall in data points belonging to the first component in *B*; blue and red lines, the 2 Gaussians fitted by the EM algorithm to the empirical frequency distribution. The intersection point between the two Gaussians is again used as a cutoff value for separating data points into 2 components. Note that here, too, the first component (of low distances from wall) is localized, whereas the second is flattened and spread out. *E*: plots of all data points belonging to progression segments for the 2 mice. Blue lines, movement along the wall (as defined by the wall/center separation procedure); red lines, movement in the center.

tion point between the two fitted Gaussians serves as the first criterion (or cutoff value) according to which the data points are classified into two groups. As can be seen in Fig. 1*B*, the frequency distributions contain a prominent component of low radial speeds, which is captured by the first Gaussian fitted by the EM algorithm. Figure 1*C* shows that this component contains data points in which the mouse moves in parallel to the wall; the component that is captured by the second fitted Gaussian contains data points in which the mouse either approaches or moves away from the wall.

3) Most of the movement in parallel to the wall is performed along a narrow strip near the wall (as is obvious from Fig. 1*C*, *top*), but some of it is performed at a distance. To isolate the movement along the wall, all data points in which the mouse moves in parallel to the wall (i.e., all data points that belong to the first component in *point 2* above) are further separated into two groups according to their distance from the wall, again using the EM algorithm with a two-Gaussian mixture model. The intersection point between the two fitted Gaussians is used as a second cutoff value. The frequency distributions of the distances from the wall show a salient component of small distances, which is captured by the first Gaussian (Fig. 1*D*), containing data points in which the mouse moves along the wall (i.e., in parallel to the wall and close to it).

Definition of units of behavior. DEFINITION I. A wall segment is defined as any series of successive data points belonging to the wall class. Similarly, a center segment is defined as any series of successive data points belonging to the center class (Fig. 2*A*). A wall or a center segment is bounded by either a lingering episode or a transition between wall and center motion. Note that a progression segment may include more than one wall and/or center segment (as when a mouse progresses along the wall, leaves it, and returns to it again, all without stopping).

DEFINITION II. Center segments are concatenated into higher-order patterns termed incursions. Incursions are bouts of continuous center activity (consisting of one or more center segments) that begin and end near the wall. An incursion is therefore defined as a series of successive center segments.

There are two exceptions for *definition II*. *Exception IIa* is described as follows. It sometimes happens that a series of center segments contains more than one entry into the center, i.e., when a mouse enters the center, returns to the wall, performs a stop there (but does not run along the wall), and then enters the center again. To account for these rare cases, we modify the incursion definition to a series of successive center segments that begins and ends either with a wall segment or with a lingering episode performed near the wall, i.e., a lingering episode of which at least one data point is within the range of the "ring" formed by movement near the wall (i.e., the blue part of the path plots in Fig. 1*E*). To avoid artifacts caused by outliers, this range is defined as the 98th quantile of the distances from wall of all data points belonging to wall segments.

Exception IIb is described as follows. It was found that a small fraction of incursions (as defined above) is performed entirely within the near-wall domain (i.e., their maximal distance from the wall is smaller than the 98th quantile of the distance from wall of wall segments). These are small movements directed toward the center (therefore having high radial speeds), which do not proceed beyond the wall "ring." Despite their high radial speeds, these minute incursions are reclassified as movement near the wall because they are performed in the same distance from the wall as that of most wall segments. Data points that belong to these incursions are pooled with those that belong to wall segments, and new wall segments (i.e., series of successive data points) are defined from the pooled data.

DEFINITION III. Lingering episodes are classified into two groups: *1*) center lingering comprises all lingering episodes that are enclosed within incursions, and *2*) wall lingering comprises all the remaining lingering episodes.

Second stage: classifying incursions into several components. Even a cursory observation of the center component of any mouse's path (Fig. 1*E* and Fig. 2*B*) indicates that incursions are not a homogeneous group: some incursions are performed in relative proximity to the wall, others reach further into the center, and still others even cross the center. These

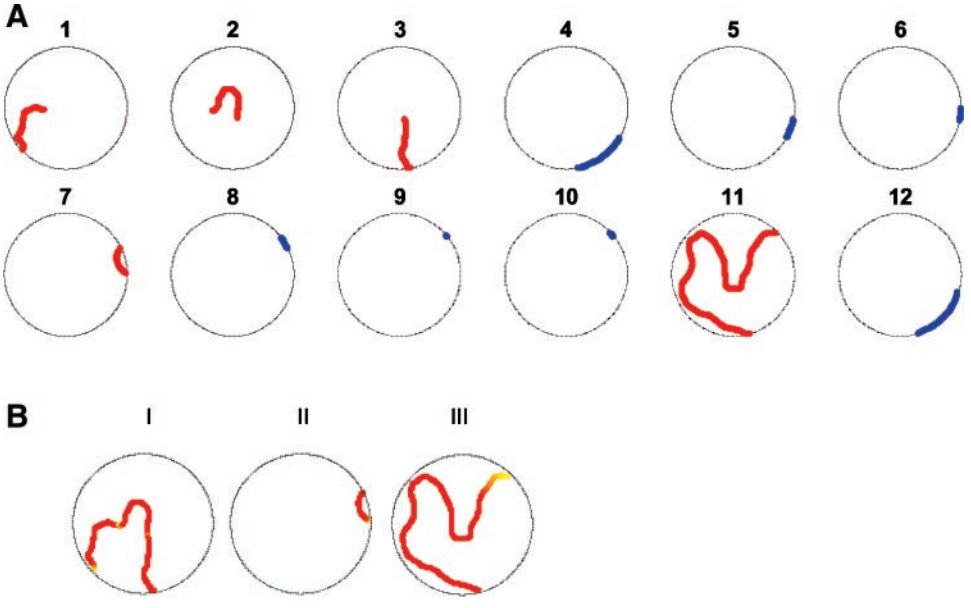

Fig. 2. *A*: plots of 12 successive segments of motion along the wall (blue) and in the center (red) in a B6 mouse session. *B*: plots of 3 successive incursions in the same session. Yellow to red coloring indicates the direction of movement in each segment within an incursion. Note that *incursion I* is composed of 3 center segments (*plots 1*, *2*, and *3* in *A*) and *incursions II* and *III* are each composed of a single center segment (*plots 7* and *11* in *A*, respectively).

different types of incursions can be characterized by the incursions' maximal distances from the wall. However, because the number of incursions in some of the sessions is too small to get a reliable classification, we use the maximal distances of center segments instead (due to there being more center segments than incursions in a session because some incursions are composed of several center segments). With the use of the EM algorithm, a Gaussian mixture model is therefore fitted[1] to the frequency distribution of the maximal distances from wall of center segments (after being log transformed). Figure 3*A* shows the results for a single B6 session, where three Gaussians were fitted by the EM algorithm. The intersection points between the fitted Gaussians are used as cutoff values between separate groups of center segments. These groups are in turn used to classify incursions into types. For example, in the session shown in Fig. 3, all incursions that contain only center segments from *group 1* constitute a single type: near-wall incursions (Fig. 3*B, label 1*). Furthermore, all incursions that contain at least one center segment from *group 2* and no center segments of *group 3* constitute a second type: intermediate incursions (Fig. 3*B, label 2*). Finally, all incursions that contain at least one center segment from *group 3* constitute the third type: arena-crossing incursions (Fig. 3*B, label 3*).

This, however, is not the case in all mice. Sometimes a two-Gaussian or a four-Gaussian mixture model is chosen by the statistical algorithm, identifying two or four incursion types, respectively. However, regardless of the number of fitted Gaussians that characterize the center segments in a mouse session, all mice have a clearly identifiable component of near-wall incursions. This is evident because values of the first (smallest) cutoff value for all mice belong to a rather narrow range (11–28 cm for B6 and 7–17 cm for D2). Furthermore, in all mice that have four-Gaussian components, the values of the largest cutoff value, although their range is not quite narrow, are clearly separated from the range of near-wall incursions (57–96 cm for B6 and 41–94 cm for D2), indicating that all of these mice share a component of large arena-crossing incursions. These two ranges are used to solve the problem of having mice with different number of Gaussian components, by identifying three corresponding components across all mouse sessions. In each mouse session, we choose one cutoff point from the first range (which separates near-wall from intermediate incursions) and another from the second range (which separates intermediate from arena-crossing incursions). If a mouse does not have a cutoff point within the second range (as is the case with some mouse sessions), we use the lower limit of the second range as a cutoff point. Note that, in some mouse sessions, the number of incursions within each type may be small or even zero.

Using the Wall/Center Separation Procedure for Behavioral Phenotyping

The newly defined patterns can now be used to design new end points. One way to do this is to calculate existing end points separately for movement along the wall and in the center. Another way is to characterize features that are relevant only in the newly generated behavior patterns. Using both of these approaches allows the generation of a multitude of new end points of behavior near the wall and in the center. Below, we present several examples of such end points (their values are summarized in Table 2).

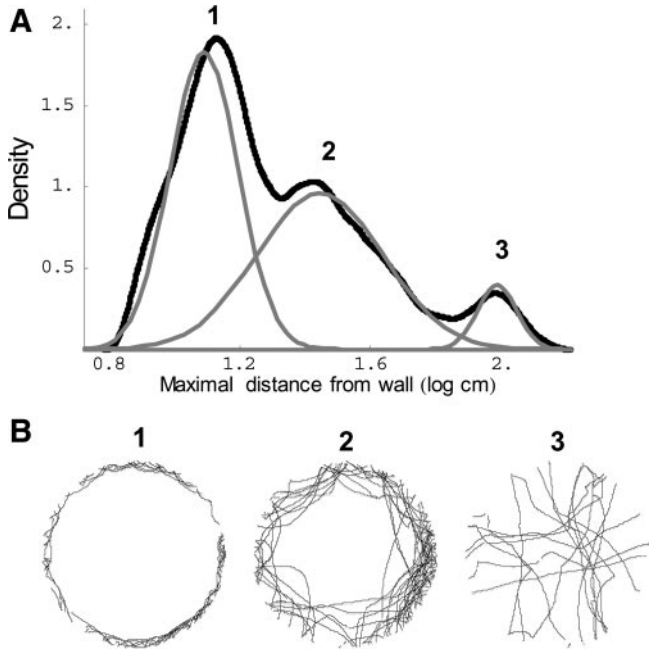

Fig. 3. *A*: black line, a density graph of the frequency distribution of the maximal distances from wall of center segments (log transformed) in a single B6 mouse session; gray lines, 3 Gaussians fitted to the frequency distribution by the EM algorithm. The intersection points between the Gaussians serve as cutoff values for dividing all incursions performed in this session into 3 types. *B*: path plots of the incursions belonging to each type.

Examples of End Points That Are Calculated Separately for Movement Near the Wall and in the Center

Median path curvature during progression. The path curvature is defined as the change in direction between data points located at a fixed distance from each other (5 cm in the present case). This is an improved version, developed by Ehud Fonio (TAU), of the "radius of turn" end point (22). The median curvature was calculated for the pooled data points from all progression segments (Fig. 4*A*) and separately for pooled data points from wall segments only (Fig. 4*B*) and from center segments only (Fig. 4*C*). The results were compared for B6 and D2 mice in the three laboratories.

Figure 4*A* shows that the median curvature calculated for all progression segments is significantly higher for B6 than for D2 mice, meaning that during progression the path of B6 is more curved than that of D2. The same difference between strains (but with a higher statistical significance) is seen when the median curvature is calculated for progression along the wall (Fig. 4*B*). This is presumably because D2 mice run tightly in parallel to the wall, whereas B6 mice progress near the wall along a somewhat meandering path. The strain difference is reversed, however, for median curvature calculated for center segments; the path of D2 is more curved than that of B6 mice during progression in the center (Fig. 4*C*).

Median duration of lingering episodes. The median duration was first calculated for all lingering episodes (Fig. 5*A*) and then

[1] Unlike the first stage of the procedure, in which a two-Gaussian mixture model is used, here the number of components in the Gaussian mixture model is chosen automatically using the likelihood ratio test.

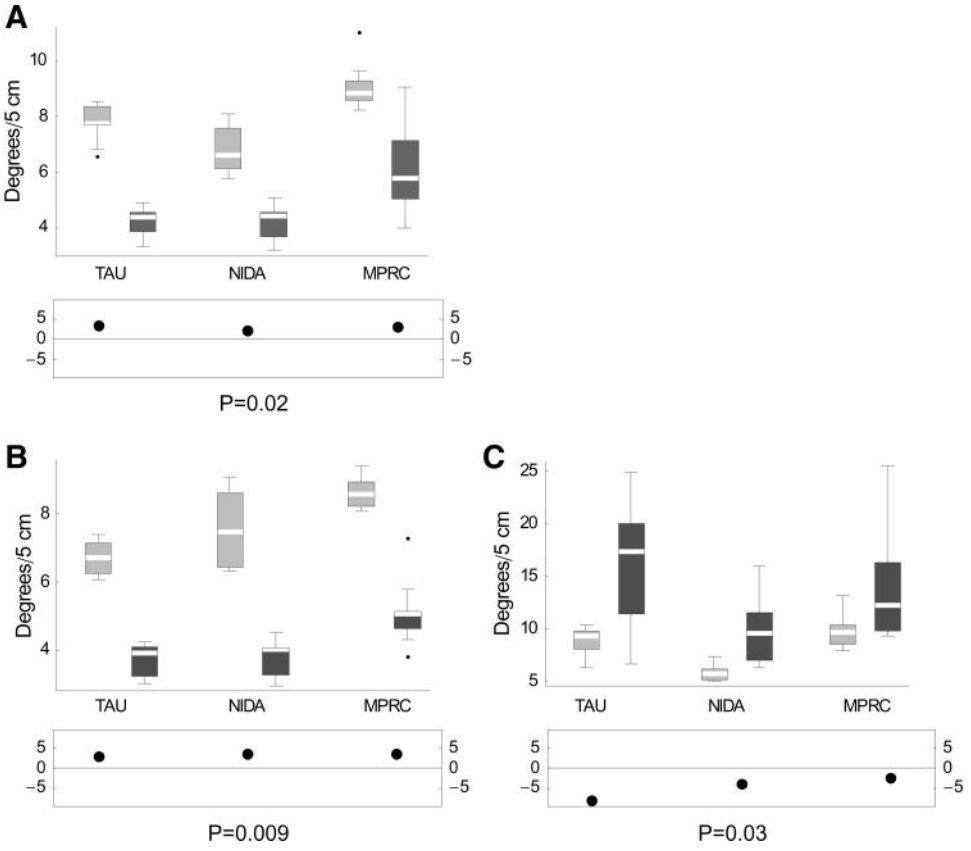

Fig. 4. *A*: median path curvature during progression. *Top* shows box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories for this end point. *Bottom* shows the difference between the strain medians in each laboratory. The same 2 graphs are shown for the median path curvature during progression near the wall (*B*) and the median path curvature during progression in the center (*C*). *P* values are for the strain difference (using mixed-model ANOVA, as discussed in METHODS). TAU, Tel Aviv University; NIDA, National Institute on Drug Abuse; MPRC, Maryland Psychiatric Research Center.

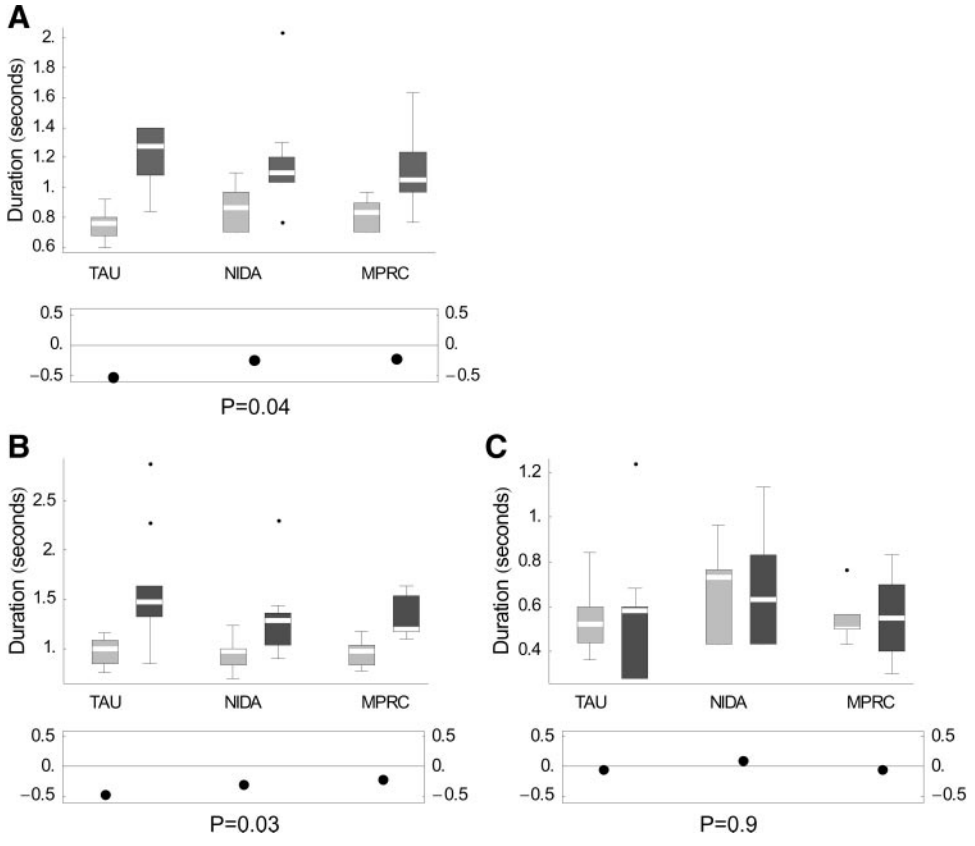

Fig. 5. *A*: median lingering duration. *Top* shows box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories. *Bottom* shows difference between the strain medians in each laboratory. *B*: median lingering duration near wall. *C*: median lingering duration in the center. *P* values are for the strain difference (mixed-model ANOVA).

separately for wall (Fig. 5*B*) and center (Fig. 5*C*) episodes. Figure 5*A* shows that the median duration of lingering episodes is higher in D2 than in B6 mice. Figure 5, *B* and *C*, shows, however, that the strain difference holds only for lingering near the wall. The median lingering duration in the center is similar in both strains.

Examples of End Points Characterizing Wall/Center Units

Number of incursions. Figure 6*A* shows that the number of incursions performed during a session is higher in B6 than in D2 mice in the three laboratories, but this strain difference is not statistically significant.

Median length of incursions. The length of an incursion is calculated as the sum of the path lengths of its constituent center segments (lingering episodes are excluded). Figure 6*B* shows the results for the median length of incursions, which is significantly higher in B6 than in D2 mice.

The median of the maximal distance from wall of incursions. The median of the maximal distance from wall of incursions indicates how far from the wall the mouse tends to venture during its center activity. The results for this end point are shown in Fig. 6*C*. The median maximal distance from wall in incursions is significantly higher in B6 than in D2 mice.

The proportion of incursions beginning with a lingering episode. Sometimes an incursion is directly preceded by a stop (lingering episode); at other times, a mouse proceeds from progressing along the wall to the center without stopping. The proportion of incursions that begin with a stop, out of the total number of incursions, is a measure for the tendency to stop before entering the center. This proportion is significantly higher in D2 than in B6 mice (Fig. 6*D*).

Average segment length in incursions. The average segment length in an incursion is calculated as the length of the incursion divided by the number of center segments included in it. The median of this measure over all incursions was calculated for each mouse. This end point can be regarded as a measure of the tendency to stop during progression in the center. Figure 7*A* shows that, in incursions, the median of the average segment length is significantly higher in B6 than in D2 mice. Another end point previously measured with the same data, the median length of progression segments (i.e., with no distinction between wall and center behavior), which may be regarded as the overall tendency to stop, showed no difference between these two strains (22). D2 mice thus have a higher tendency to stop than B6 mice only when progressing in the center.

Outbound/inbound mean speed ratio in incursions. The data points belonging to each incursion were divided into two groups: data points with positive radial speed (mouse moving away from wall) and data points with negative radial speed (mouse approaching wall). The ratio between the means of the two sets of data points was calculated for each incursion, and the median for all incursions was calculated for each mouse. As shown in Fig. 7*B*, the median outbound/inbound mean speed ratio was significantly higher for B6 than for D2 mice. The value for this end point in B6 was ~1, meaning that B6 mice reach similar mean speeds when moving away as when approaching the wall. In contrast, the value in D2 mice was

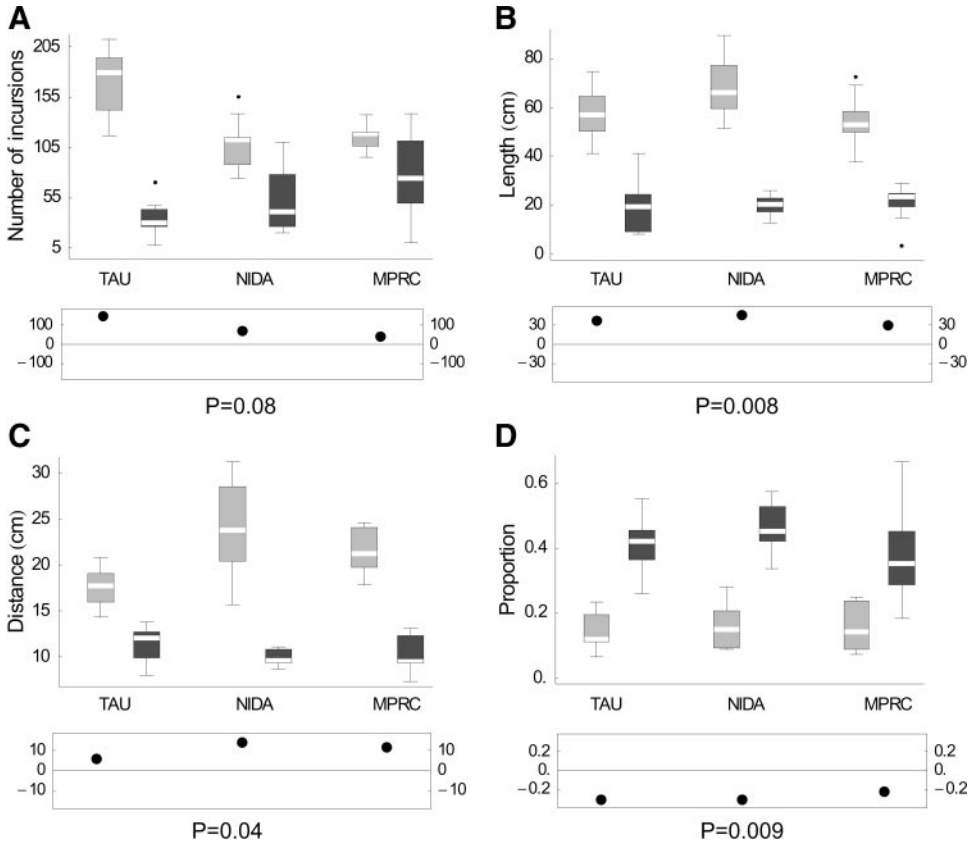

Fig. 6. *A*: number of incursions. *Top* shows box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories. *Bottom* shows difference between the strain medians in each laboratory. *B*: median incursion length. *C*: median of the maximal distance from wall of incursions. *D*: proportion of incursions beginning with a stop (lingering episode). *P* values are for the strain difference (mixed-model ANOVA).

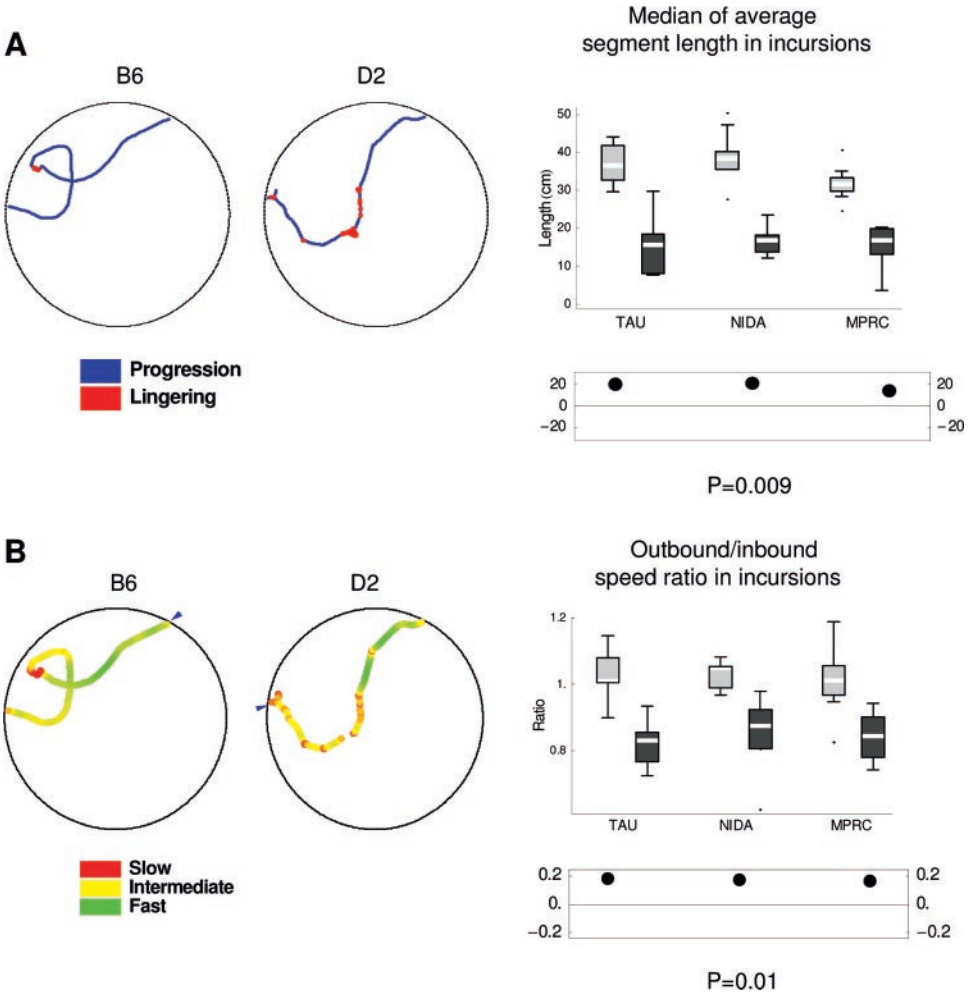

Fig. 7. *A*, *left*: 2 incursions, one of a B6 mouse and one of a D2 mouse. Within each incursion, progression segments are shown in blue and lingering episodes are shown in red. *A*, *right*: average segment length in incursions. *Top* shows box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories. *Bottom* shows difference between the strain medians in each laboratory. *B*, *left*: same 2 incursions of a B6 and a D2 mouse. Blue arrows indicate the start point of each incursion; colors indicate momentary speeds. The D2 mouse, unlike the B6 mouse, moves more slowly when approaching the center than when returning to the wall. *B*, *right*: outbound/inbound mean speed ratio in incursions. *Top* shows box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories. *Bottom* shows difference between the strain medians in each laboratory. *P* values are for the strain differences (mixed-model ANOVA).

<1, meaning that D2 mice reach lower mean speeds when moving away from the wall than when approaching it.

End Points Calculated Separately for Different Incursion Types

The end points presented above were calculated for the entire movement in the center, ignoring its subclassification into incursion types. Any end point characterizing center activity, however, can also be calculated separately for the data in each incursion type. This may potentially accentuate differences between strains but is relatively less useful for strains that are not very active in the center and have a relatively small number of incursions, such as D2 mice. This is because in such strains the number of incursions belonging to each type is often too small for meaningful statistical analysis. Because this was the case with the D2 mice in the present study, there were only two end points that could be calculated separately for each incursion type: *1*) the median curvature (because this measure is not calculated per incursion but for all the pooled data points belonging to incursions) and *2*) the number of incursions per session.

Median curvature. Figure 8*A* shows the results for median curvature during movement in near-wall, intermediate, and arena-crossing incursions. In the first two incursion types, there is a significant strain difference in the same direction as the difference in curvature calculated for overall progression in the center (see Fig. 4*C*), the path of D2 mice being more curved than that of B6 mice. The strain difference disappears, however, during arena-crossing incursions, where both strains have similar (and relatively low) path curvature.

Number of incursions. The results for number of incursions of each type are shown in Fig. 8*B*. The numbers of near-wall incursions are evidently not replicable across laboratories: although there is a large strain difference in TAU, in the other two laboratories B6 and D2 mice have a similar number of near-wall incursions. In contrast, there is a replicable strain difference in the number of intermediate and arena-crossing incursions, with B6 mice making significantly more incursions of both these types than D2 mice in all three laboratories. It now becomes evident that the failure to achieve significant results in the overall number of incursions (see Fig. 6*A*) is due to interlaboratory variation in the numbers of a single incursion type: near-wall incursions.

Correlations Between End Points

The median of all correlations between all the new end points was about zero (0.007); the lower quartile was -0.21 and the upper quartile was 0.23; the lower and upper deciles of the correlations were -0.34 and 0.44, respectively. In addition, 59% of the correlations were statistically indistinguishable

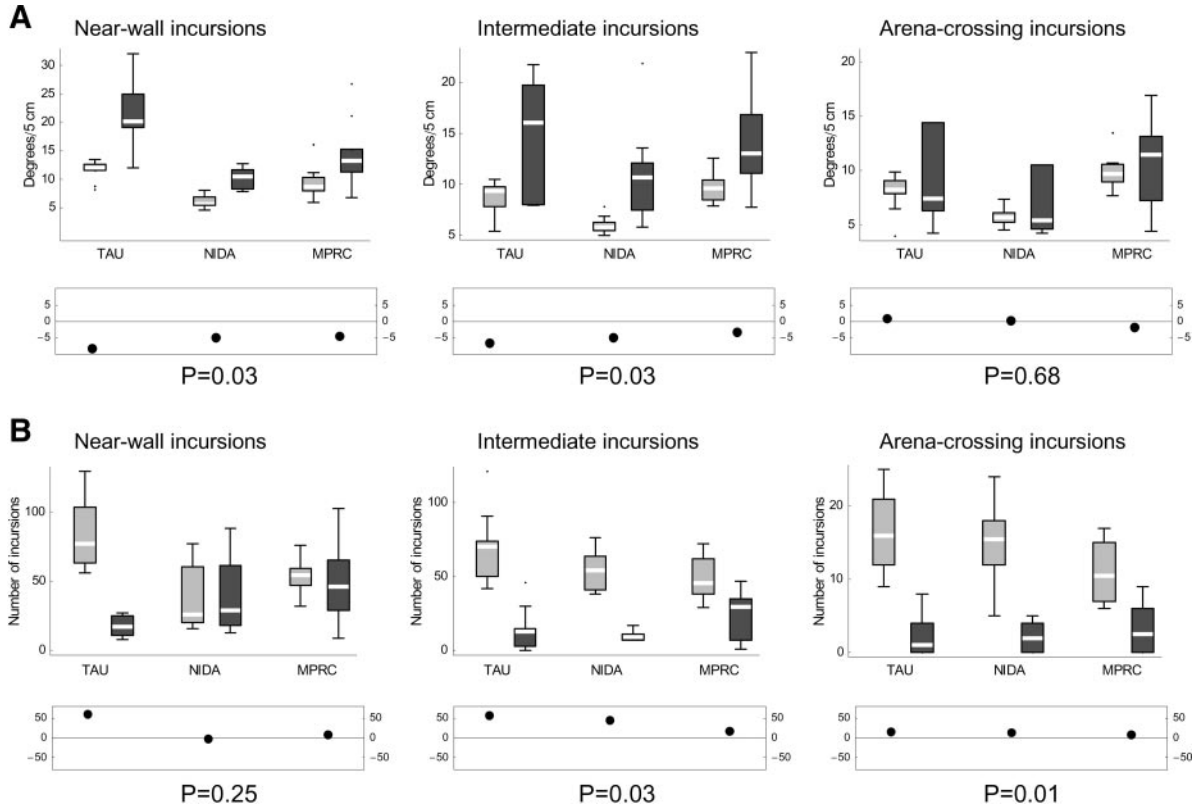

Fig. 8. Median path curvature (*A*) and number of incursions (*B*). Each end point is calculated separately for each of the 3 incursion types: near-wall, intermediate, and arena-crossing. *Top graphs* show box plots for B6 mice (light gray) and D2 mice (dark gray) in the 3 laboratories. *Bottom graphs* show difference between the strain medians in each laboratory. *P* values are for the strain difference (mixed-model ANOVA).

from 0. The pairs of end points with the most extreme correlation (above 0.8 in absolute value) were as follows: number of incursions and number of near-wall incursions (correlation = 0.86), median curvature in the center and median curvature in arena-crossing incursions (−0.89), and median curvature in the center and median curvature in intermediate incursions (−0.86).

Validation of Wall/Center End Points

The results presented so far were calculated on the same data set that was used for the development of the new end points. Having access to a new, publicly available data set contributed by our group to the Mouse Phenome Database (28) and also having access to the raw data of these strains (which are also publicly available at http://www.tau.ac.il/~ilan99/see/help) allow us to validate the results derived on the first data set.

We start by assessing the stability of the strain differences on the 16 newly developed end points. Table 3 summarizes the results (means ± SE) for the two strains in the second data set. In addition, it displays the strain differences in both data sets and the *P* values for the comparison between them. The two *P* values of <0.05 do not remain statistically significant after we controlled for multiplicity using the FDR criterion. Thus there is no evidence for lack of stability in the strain differences across the two data sets in all 16 end points.

Of the 16 end points developed on the first data set, 12 were shown to potentially discriminate between the B6 and D2 strains, with *P* values of <0.05 (using the mixed model). Because of the complex and interactive way by which these 12 end points were developed and selected using the first data set, we validated their discriminatory ability on the second data set. Two end points of the twelve, median lingering duration near wall and median curvature in intermediate incursions, did not

Table 3. *Validation results*

	Second Data Set			First Data Set	
End Point	B6	D2	Strain difference	Strain difference	*P*
MCW	7.1±0.2	3.9±0.1	3.2	3.4	0.59
MCC	7.0±0.2	11.2±0.6	−4.2	−5.3	0.85
MLDW	1.1±0.05	1.4±0.07	−0.3	−0.46	0.22
MLDC	0.57±0.02	0.53±0.05	0.03	−0.026	0.27
NI	127.6±6.5	67.4±5.8	60.2	77.8	0.18
MIL	60.9±2.9	20.1±1.7	40.8	38.9	0.63
MIMWD	21.3±0.9	9.7±0.4	11.7	10.4	0.31
PIBS	0.15±0.02	0.48±0.02	−0.33	−0.27	0.06
ASLI	42.4±1.4	15.7±1.2	26.7	19.4	0.004
OISRI	1.1±0.01	0.84±0.01	0.21	0.19	0.37
MCNW	7.4±0.2	13.0±0.7	−5.66	−6.13	0.64
MCI	7.2±0.2	12.0±0.7	−4.9	−5.54	0.96
MCAC	6.9±0.2	8.3±0.6	−1.44	−1.8	0.84
NNW	61.3±6.1	42.1±3.9	19.2	24.6	0.43
NII	45.3±3.7	20.9±2.6	24.4	41.4	0.008
NAC	21.1±1.5	4.4±0.7	16.6	11.4	0.36

Values for second data set are means ± SE (nontransformed). The difference between the (nontransformed) means of B6 and D2 in each data set is computed for the two data sets. *P* values are data set comparisons, testing the stability of the strain differences between the 2 data sets (performed on the transformed data, see METHODS). None passes false discovery rate.

retain their discriminatory ability, after controlling for multiplicity. The remaining 10 end points are confirmed to discriminate between the two strains.

DISCUSSION

Behavior Phenotyping With the Wall/Center Separation Procedure

In this work, we present a SEE algorithm for the identification of the intrinsic structure of mouse behavior in reference to the wall of the arena. It reveals the existence of four distinct patterns: one of movement along the wall and three of movement in the center. Within these patterns, discrete units are defined: wall and center segments and wall and center lingering. Center segments and center lingering are concatenated into more complex units termed incursions. With the use of this structural basis, new end points characterizing the behavior near the wall and in the center can be designed and tested for discrimination and replicability. Several such end points were calculated on data from two inbred strains, B6 and D2, tested in three laboratories to demonstrate the phenotyping advantages offered by an SEE-based approach to wall/center behavior. One advantage is the ability to compute various parameters separately for different patterns and possibly reveal strain differences that are masked when the same parameters are computed indiscriminately for the entire data. This is demonstrated by the results for path curvature and for lingering duration shown in Figs. 4 and 5. When path curvature is computed separately for progression near the wall and in the center, a previously masked phenomenon is uncovered: D2 mice have higher path curvature than B6 mice during progression in the center (Fig. 4*C*). Consequently, path curvature results during overall progression (showing B6 to have higher path curvature than D2 mice, Fig. 4*A*) actually reflect only the strain difference near the wall. The opposite-direction strain difference in the center is completely obscured and serves only to somewhat reduce the end points' discriminatory powers (hence the higher statistical significance for path curvature near the wall than for the overall path curvature). Similarly, median lingering duration results (Fig. 5*A*) reflect a "mixture" of two phenomena: D2 make longer stops than B6 mice near the wall, and both strains have similar lingering duration in the center (Fig. 5, *B* and *C*). Using the wall/center separation procedure, we identify both phenomena and express these as two distinct end points. A yet finer discrimination is obtained when the behavior in different patterns of center movement is examined separately: path curvature of B6 mice is lower than that of D2 mice during near-wall and intermediate incursions but not during arena-crossing incursions (Fig. 8*A*).

Another fruitful phenotyping approach is demonstrated by the end points shown in Figs. 6 and 7, which describe different features of incursions. In addition to analyzing the entire data during activity in the center as a whole, we can examine separate forays into the center and compute their properties, further increasing the articulation of the behavioral phenotype. The only measure of this kind available in the present research is the number of entries into the (arbitrarily defined) center. Our technique, in addition to providing an intrinsic definition of center entries (incursions), allows the description of many characteristics besides their number. The monitored characteristics of incursions range from rather simple ones, such as length (Fig. 6*B*), to more complex ones, such as outward/inward speed ratio (Fig. 7*B*). An incursion, moreover, is not an indivisible entity; rather, it consists of discrete subunits, center segments and center lingering, thus further increasing the variety of possible end points (e.g., average segment length in incursions, Fig. 7*A*). Here too, the end points can be computed separately for different incursion types (Fig. 8*B*), and it is thus revealed that the strain difference in the number of near-wall incursions is strongly affected by laboratory, whereas the number of intermediate and arena-crossing incursions is not much affected by laboratory; thus there are replicable strain differences.

The fact that all of the 16 end points were validated with a separate data set, collected nearly 1 yr later across three laboratories, attests to the robustness of the end points and the usefulness of this SEE-based methodology for behavior phenotyping.

All of the 16 new end points can be applied to various strains and preparations with good prospects of providing stable results. Ten of these reliably discriminate between B6 and D2 mice.

Beyond Center Avoidance

All presently accepted measures of wall/center behavior assess the degree to which an animal avoids the presumably threatening arena center. Several of the new end points presented in this work are, like the presently accepted measures, also measures of center avoidance. In addition, our analysis includes end points that characterize the activity in the center, the activity near the wall, and the ways in which they differ. The number of incursions, median incursion length, and the median of an incursion's maximal distance from the wall can be considered as a measure of center avoidance. The results with these end points corroborate the results of previous OFT studies (reviewed in Ref. 13), which show B6 as a strain more prone to occupy the center than D2: B6 mice make more incursions into the center than D2 mice (Figs. 6*A* and 8*B*), incursions of B6 mice are longer on average than those of D2 (Fig. 6*B*), and B6 mice get farther into the center during incursions (Fig. 6*C*). However, our results show that, in addition to occupying the center more than D2, B6 mice move in straighter lines while in the center (Fig. 4*C*), are less prone to stop before embarking on a trip to the center (lower proportion of incursions beginning with a lingering episode, Fig. 6*D*), tend to run longer distances in the center without stopping (higher average segment length in incursions, Fig. 7*A*), and have similar speeds while moving away or toward the wall during center activity (whereas D2 mice move faster toward the wall than away from it, Fig. 7*B*). The two strains also differ in some properties of their activity near the wall: the path of B6 mice is more curved than that of D2 mice (Fig. 4*B*), and B6 mice make shorter stops while near the wall (lower median lingering duration, Fig. 5*B*).

Center avoidance is considered as indicative of anxiety, an interpretation that is backed by pharmacological and genetic studies (10, 18, 33). The interpretation of some of the new measures that characterize the activity in the center seems straightforward. Thus it is likely that an anxious mouse will hesitate more before going into the (aversive) arena center than a nonanxious mouse and therefore will have a larger proportion

of incursions beginning with a lingering episode. Likewise, an anxious mouse may be expected to progress more slowly toward the center than away from it (thus having an outbound/inbound speed ratio, which is <1). Other measures, however, are less easy to interpret. For example, when in the center, would an anxious mouse run in a curved or a straight path? Would it tend to stop more or less than a nonanxious mouse? Would it make longer or shorter stops? At present, we are not able to propose an interpretation as to the face validity of these end points as measures of anxiety, and, of course, it is necessary to measure all of these new end points in pharmacological and genetic models of anxiety to discover whether any of them has predictive validity. However, by expanding the behavioral repertoire to include measures that describe behavior in the center in positive terms rather than only in terms of center avoidance, we aim at enhancing the articulateness and comprehensiveness of phenotypes of anxious (vs. nonanxious) mice. Applying the analysis to additional strains, and to various pharmacological and genetic preparations, should provide an answer to the question of which of the new measures are related to anxiety and in what way.

Potential Contribution of the Wall/Center Separation Procedure to the Study of Anxiety

Anxiety is a psychological construct that is thought to have complex behavioral manifestations and a complex genetic basis. The development of advanced tools for behavioral analysis can assist in addressing the complexity of this behavior with a degree of precision sensitive to genetic and pharmacological manipulations; these tools could increase the articulation and richness of behavioral phenotypes. The wall/center separation procedure generates such a phenotype for a classical anxiety-related test: behavior in reference to the arena wall in the OFT.

To obtain a larger number of relatively independent end points characterizing anxiety, behavior geneticists use measures from several tests (14, 38). This methodology has the potential advantage of highlighting properties of anxiety that are stable across situations. It is also possible, however, that the same level of presumed anxiety may be expressed in opposite directions in different stimulus situations. For example, although a dark compartment in the light/dark test may attract the animal to stay away from a lit open space, a bare wall in the OFT may prompt it to rush into the center. By providing many end points that characterize anxiety in the same stimulus situation, SEE has the potential advantage of characterizing the multifaceted nature of anxiety in the very same situation. The two methodologies are, therefore, complementary.

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