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Validation of the dimensionality emergence assay for the measurement of innate anxiety in laboratory mice

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Abstract

The open field test is a common tool to measure innate anxiety in rodents. In the usual configuration of this test the animal is forced to explore the open arena and its behavior includes both anxiety and non-anxiety responses. However, the open arena is generally small and allows only limited expression of exploratory behavior. The recently developed dimensionality emergence assay in which an animal is housed in a home cage with free access to a large circular arena elicits graded exploration and promises to serve as a more ethological test of anxiety. Here we examined the predictive validity of this assay for anxiety-related measures in mice. First, we compared their behavior in the presence or absence of access to the home cage and found that mice with access to the home cage exhibited a gradual build-up in exploration of the arena while those without did not. Then we identified behavioral measures that responded to treatment with the anxiolytic drug diazepam. Diazepam altered several classical measures of innate anxiety, such as distance traveled and thigmotaxis, but also led to a dose-dependent acceleration of the build-up as reflected in a significantly reduced latency to attain several exploratory landmarks. Finally, we tested the utility of the dimensionality emergence assay in assessing alterations in innate anxiety reported in mice carrying a knockout allele for the serotonin 1A receptor (Htr1a). Our findings support the validity of the dimensionality emergence assay as a method to extract an expanded repertoire of behavioral measures for the assessment of anxiety in laboratory mice.

1. Introduction

Anxiety is a mental state that is commonly elicited by the anticipation of a threatening experience. Anxiety is associated with a variety of defensive behaviors that can be readily measured in both humans and other animals. One of the most common tests used to measure anxiety-related behavior in rodents is the open field test where the animal is forcibly exposed to an unfamiliar open arena (Crawley, 1985; Stone, 1932). Typical anxiety-related measures in this test include time spent in the center, latency to enter the center, and

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distance travelled in the center relative to total distance. Treatment of rodents with benzodiazepines, GABAergic allosteric agonists with anxiolytic activity in humans, leads to a dose-dependent increase in total distance travelled, time spent in the center, and relative distance in center (Marriott and Smith, 1972) confirming the predictive validity of this test.

However, because the animal is forced to explore the open field in this test its behavior is likely to reflect a complex combination of anxiety and non-anxiety-related factors and discriminating between these is not straightforward. Several modifications to the test have been developed to address this issue. The light-dark test (Crawley and Goodwin, 1980) offers the animal access to a darkened enclosure within the open arena, while the emergence test (Paré et al., 2001; Prickaerts et al., 1996) gives the animal access to a familiar home cage. In the free exploration test (Cigrang et al., 1986; Griebel et al., 1993) the animal is habituated in one part of the test apparatus before being given access to a novel portion. Unlike in the classical open field test, in these tests the animal is offered a clear choice between a more or less safe area of the apparatus, and anxiety-related measures are based on relative time and distance in the exposed or novel compartment. These measures have increased ethological validity compared to those derived from the classical open field, and at least for the light-dark and free exploration tests, these measures have been pharmacologically validated (Chaouloff et al., 1997; Crawley, 1981; Griebel et al., 1993).

Another issue is that both the classical and modified open field tests generally use small open enclosures (typically <50 cm across) and thus provide minimal space for the expression of exploration. Furthermore, exploration of the exposed or novel arena is generally captured as total distance travelled and/or time spent in a particular part of the arena, measures that may be insensitive to more subtle variations in exploratory strategy. In an attempt to address these limitations, Fonio et al. (2009) developed the dimensionality emergence assay. In this assay, the animal is habituated in a home cage from which it is then given access to a large (here 180 cm diameter) circular arena by the opening of a small shutter. Exploratory behavior in the open arena shows a gradual build-up over a period of 20–40 min, with the animal first exploring a small area close to the home cage opening (called "garden") before venturing along the border of the circular arena, and then finally moving away from the walls to enter the center.

The dimensionality emergence assay has several advantages for measuring rodent exploratory behavior. First, the large diameter of the open arena uncovers an expanded exploratory repertoire that follows a stereotyped progression of landmarks (including *cross and retreat*, *exit garden*, *enter center*, *full circle*, *home-related shuttle*, and *cage skip*) that can be readily quantified and have high ethological validity. Second, the large size and circular geometry of the open arena is ideal for applying unbiased videotracking analysis of locomotion using the Software for the Exploration of Exploration (SEE) package (Hen et al., 2004; Drai et al., 2000; Golani et al., 1993). SEE extracts movement episodes and moment-by-moment speed and acceleration data from whole body trajectories in an unbiased, animal-by-animal approach. Using SEE, Lipkind et al. (2004) were able to extract a set of sixteen putative anxiety-related variables from videotracking data of mice in a large (200 cm) forced open field test. Thus, SEE appears to be a promising method to extract additional anxiety-related measures from rodent exploration tests, at least for tests using large circular open arenas.

Here, we present data supporting the validation of the dimensionality emergence assay as a test of innate anxiety in mice. First, we made a direct comparison of exploratory behavior in the forced versus the free version of the test and confirmed the importance of home cage access to the gradual exploratory build-up. Second, we identified exploratory measures that were significantly and dose-dependently modified by treatment with the benzodiazepine diazepam. Third, we examined the impact of lighting on exploratory behavior in the test. Finally, we sought to assess the utility of the dimensionality emergence assay in assessing alterations in innate anxiety reported in mice carrying a knockout allele for the serotonin 1A receptor (Htr1a). Mice lacking Htr1a displayed decreased total locomotion and relative locomotion and time spent in the center in the open field test (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998). These findings demonstrate that the dimensionality emergence assay is a robust and ethological assay of innate anxiety in rodents.

2. Experimental procedures

2.1. Mouse husbandry

Male C57BL/6 J mice (10–12 weeks of age) were purchased from Charles River Laboratories (Calco, Italy) and housed 5 per cage in individually ventilated cages (Thoren Caging Systems, Hazleton, PA) with food and water provided *ad libidum* under controlled temperature (21 ± 0.5 °C), humidity (55–75%), and lighting (lights on: 07:00–19:00) conditions. Htr1a knockout mice (Gross et al., 2002) were backcrossed onto the C57BL/6 J background for 10 generations. Weaning and tail biopsy for genotyping was performed at postnatal day 21 (P21) and after weaning mice were group housed (three to five per cage). Male mice were used for all experiments. Mice were singly housed 8–10 days before the experiment to reduce variation in behavior associated with dominance hierarchies known to form among group-housed male mice.

2.2. Drug treatment

Animals were injected with vehicle (1–2% Tween-80 in saline, i.p.; Sigma-Aldrich, Milan, Italy) or diazepam (0.5 or 1.5 mg/kg, i.p.; Sigma-Aldrich) 15 min before being transferred to the experimental apparatus.

2.3. Dimensionality emergence assay

In our version of this assay we used a large wooden arena (180 cm diameter, 40 cm high, painted white) connected to a wooden shelter (28 cm long, 21 cm wide, 16 cm high, painted black) via a small opening (4.5 cm wide, 4 cm high, inverted-U shape) that could be opened or closed with a removable shutter. The shelter floor was covered with fresh bedding material and the cage top holding food and water as well as some soiled bedding (70% of total) were transferred to the shelter from the home cage together with the animal. The arena was surrounded by a black curtain extending to the ceiling that allowed different lighting in the shelter (<0.1 lux red light) and arena (Low: 2–7 lux red light, High: 25–28 lux white light, periphery to center, respectively). All tests were conducted under Low light conditions unless otherwise specified. Animals were tested during the light period between 10:00 and 18:00. Fifteen minutes after transferring the animal to the shelter the shutter was removed

and exploration of the open arena recorded by a video tracking system (Viewer II, Biobserve, Bonn, Germany).

2.4. Behavioral analysis

Digital tracking files (25 fps, 720×576 pixels) were analysed by SEE Workshop to identify lingering and progression and wall and center segments (Lipkind et al., 2004). Raw data obtained from the tracking system were smoothed using a specialized algorithm implemented in the stand-alone program *SEE Path Smoother* (Kafkafi et al., 2003; Hen et al., 2004). This procedure produces reliable estimates of momentary speeds during motion (momentary speeds during arrests were defined as zero). Rodent locomotor behavior consists of two distinct modes of motion — *progression* and *lingering* (Drai et al., 2000; Golani et al., 1993). During progression segments, the animal traverses large distances and attains high speeds. During lingering episodes the animal remains in a circumscribed location and performs scanning movements. Segmentation of the smoothed path into progression segments and lingering episodes was done using the Expectation Maxima algorithm (Everitt and Hand, 1981) in *SEE Path Segmentor* with a two-Gaussian mixture model. *SEE Path Smoother* and *SEE Path Segmentor* can be downloaded at http://www.tau.ac.il/ilan99/see/help. Finally, *SEE Workshop* was used to calculate cumulative behavioral variables (*distance travelled*, *proportion center time*, *proportion center activity*, *maximum speed*, *lingering speed*, *duration of lingering episode*, *duration of progression segment*, *proportion of time lingering*) in 20 min bins. The center region was defined on an individual-to-individual basis by SEE workshop as described in Lipkind et al. (2004). Latencies to behavioral landmarks were extracted by manual scoring of video files (*cross & retreat*, *exit garden*, *enter center*, *full circle*, *shuttle*, *cage skip*). *Cross and retreat* was scored when the animal retracted to the home cage after placing four paws into the arena; *exit garden* was scored when the animal placed all four paws completely outside the garden area and followed this event with a full excursion into the open arena; the *garden* boundary was individually defined for each animal by looking at the cumulative movement trajectory and outlining the area near the home cage opening where the animal spent the majority of time; *shuttle* was scored when, while returning from an excursion along the wall of the arena, the animal changed direction and moved away from the home cage; *cage skip* was scored when the animal, upon returning from an excursion outside of the garden, did not re-enter the home cage.

2.5. Statistical analysis

Behavioral data were analyzed by analysis of variance (ANOVA) followed by post-hoc comparisons using Duncan's test in cases of significance ($P<0.05$). Variation in landmark latency was analyzed by non-parametric Mann–Whitney test. Significance of differences in order of occurrence of landmarks were assessed by first determining the number of consecutive landmark swaps needed to reproduce the order of a particular animal from the order of the prototypical vehicle treated mouse. Effects of treatment on these non-continuous deviation scores were then analyzed using the non-parametric Kruskal–Wallis test, followed by Dunn's post hoc test in cases of significance. Our approach differs from statistical tests of behavior order used by previous authors (Blois-Heulin and Belzung, 1995).

3. Results

3.1. Effect of shelter access on dimensionality emergence assay

To test if access to a shelter favored a gradual, more stereotyped build-up of exploration in an open arena we compared exploratory behavioral measures between laboratory mice placed in a large unfamiliar open field either with or without access to a home cage-like shelter. The apparatus was a large (180 cm diameter) wooden arena with a small hole on one side that led to a chamber containing food, water, and bedding material. For the first experimental group ("free exploration") a mouse was placed into the shelter with the door closed (see Experimental procedures). Fifteen minutes later the door was opened and the exploration of the animal was recorded with a video tracking system. For the second experimental group ("forced exploration") a mouse was placed at one side of the open field with the door to the shelter closed. As expected, access to the shelter was associated with significantly less total distance traveled in the open arena (Fig. 1A) [repeated measure ANOVA—main effect of group: $F(1,14)=32.0$; $P<0.005$]. Moreover, the total distance traveled by mice in the forced exploration group was maximal in the first 20 min while that for the free exploration group reached a maximum at one hour [repeated measure ANOVA—group×time: $F(3,42)=11.8$; $P<0.005$]. Although the proportion of time spent in the center remained significantly lower in the free exploration group throughout the test (Fig. 1C) [repeated measure ANOVA—main effect of group: $F(1,14)=20.9$; $P<0.005$], the proportion of total distance traveled in the center was significantly lower only during the first 20 min (Fig. 1B) [repeated measure ANOVA—group×time: $F(3,42)=4.29$; $P=0.009$] suggesting increased avoidance of the center in animals with shelter access during the initial phase of testing. Evidence for transient behavioral inhibition in the free exploration group was also seen in the reduced maximum speed attained during progression segments, which eventually reached similar levels in both groups (Fig. 1D) [repeated measure ANOVA—group×time: $F(3,42)=17.6$; $P<0.005$]. These findings confirm that access to a shelter during exploration of a novel open arena leads to a more gradual build-up of exploratory behavior than seen in the forced open field test.

3.2. Effect of diazepam on dimensionality emergence assay measures

The dimensionality emergence assay measures exploration of an unfamiliar environment and elicits a behavioral repertoire that consists of bouts of spontaneous exploratory excursions away from the shelter. Some, but not all of these measures are moderated by the anxiety state of the animal. To identify behavioral measures in this test that reflect anxiety we treated mice with the anxiolytic drug diazepam. During the first 40 min, diazepam induced an increase in total distance traveled (Fig. 2A) [repeated measure ANOVA—group: $F(2,21)=5.61$; $P=0.011$], proportion of time spent in the center (Fig. 2C) [repeated measure ANOVA—group: $F(2,21)=1.932$; $P=0.169$], and maximum speed attained during movement episodes (Fig. 2D) [repeated measure ANOVA—group: $F(2,21)=3.36$; $P=0.05$]. Proportion of total distance traveled in the center (Fig. 2B) was increased throughout the session [repeated measure ANOVA—group: $F(2,21)=2.76$; $P=0.08$]. For total distance traveled and proportion of time spent in the center this effect was dose-dependent, suggesting a specific disinhibitory effect of the drug on this behavior. These findings suggest that total distance traveled as well as time and activity spent in the center of the open arena can be used as predictive behavioral measures of anxiety.

Importantly, the effect of diazepam was most pronounced during the initial 40 min of testing. Given that significant levels of diazepam persist in the rodent brain for at least two hours

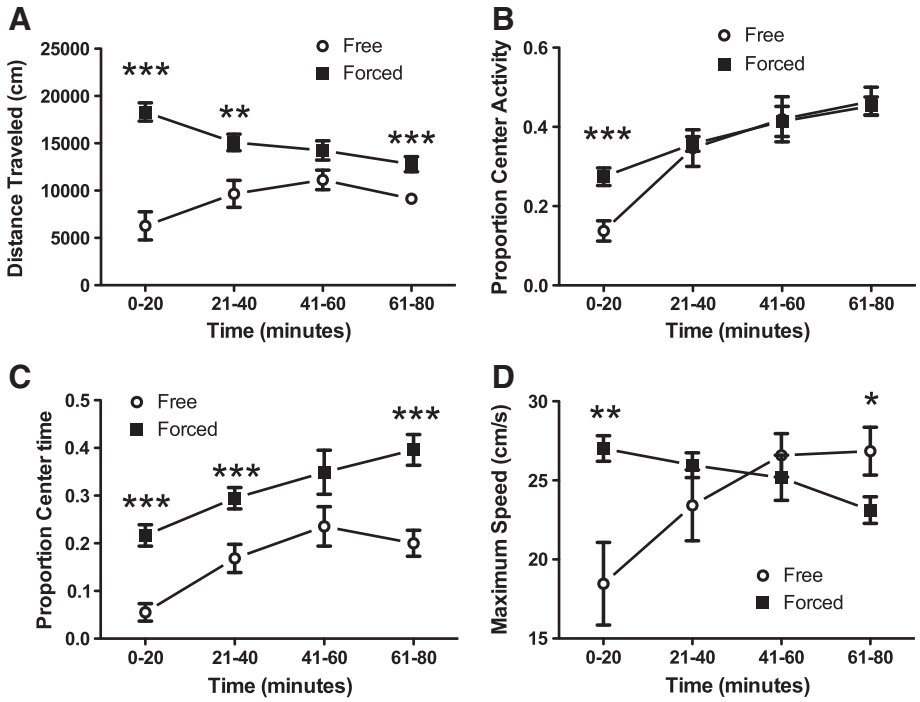

Figure 1 Effect of shelter on exploratory measures in open arena. SEE Workshop was used to analyze and quantify exploratory behavior of mice in a large open arena that either did or did not have access to a shelter connected via a small opening. Mice having access to the shelter exhibited significant reductions in (A) distance traveled, (B) proportion of activity spent in center, (C) proportion of time spent in center, and (D) maximum speed attained during progression segments. Effects of access to shelter were greatest during the initial 20 min period (N = 8, mean ± SEM, *** $P<0.005$, ** $P<0.01$, * $P<0.05$).

following intraperitoneal injection (van der Klejin et al., 1971), this observation suggested that the drug selectively disinhibits behavior during the initial phase of the test when avoidance behavior is most pronounced. To examine this hypothesis in more detail, we extracted latency measures for a set of landmark behaviors that describe the exploratory build-up of mice in the dimensionality emergence assay (Fonio et al., 2009): *cross and retreat*, *exit garden*, *enter center*, *full circle*, *shuttle*, and *cage skip*. Consistent with our hypothesis, diazepam decreased latencies to several behavioral landmarks with significant and dose-dependent reductions seen for *exit garden* and *enter center* (Fig. 3A–D). Notably, diazepam had little effect on latency to *cross and retreat* and its effect on all subsequent landmarks appeared primarily to be the result of a reduction in latency to *exit garden* followed by a relatively unaffected build-up thereafter. This observation suggested that the most salient anxiety landmark measure was the latency to exit the relatively safe area surrounding the shelter entrance.

An alternative approach to analyzing landmarks takes into account their order rather than their latency. While the prototypical order of occurrence of landmarks in vehicle treated mice was: *cross and retreat–exit garden–shuttle–full circle–cage skip–enter center*, diazepam treated mice showed a dose-dependent precocious occurrence of *enter center* and *cage skip* (Table 1). For example, in mice treated with the high dose of diazepam, *cage skip* occurred most often immediately after *exit garden* and was then followed by *enter center*, while in animals treated with the low dose of diazepam only *enter center* was moved to an earlier position in the sequence. These data show that in addition to their effect on reducing the latency to leaving the safe area surrounding the shelter entrance (*exit garden*), anxiety levels also specifically influenced the exploratory strategy of the animal by determining whether the animal would return into the shelter or perform a *cage skip* and venture into the center.

3.3. Effect of lighting on dimensionality emergence assay

Next, we sought to further validate the dimensionality emergence assay by examining the effect of lighting in the open arena on exploratory measures and landmarks. In several studies using the forced open field, lighting intensity has been positively correlated with anxiety behavior (Nagy and Forest, 1970; Nagy and Glaser, 1970). To see if a similar relationship held true for the dimensionality emergence assay we performed the test under conditions of low (2–7 lux, red) or high (25–28 lux, white) light in the open arena. Lighting in the shelter was similar in both experimental groups. An analysis of both cumulative (Fig. S1) and landmark (Fig. S2) measures failed to detect a significant effect of lighting on exploration. These findings suggest that, at least under these relatively dim light conditions, arena lighting did not influence anxiety in the dimensionality emergence assay.

3.4. Effect of Htr1a knockout on dimensionality emergence assay measures

Finally, we sought to assess the utility of the dimensionality emergence assay in assessing alterations in innate anxiety

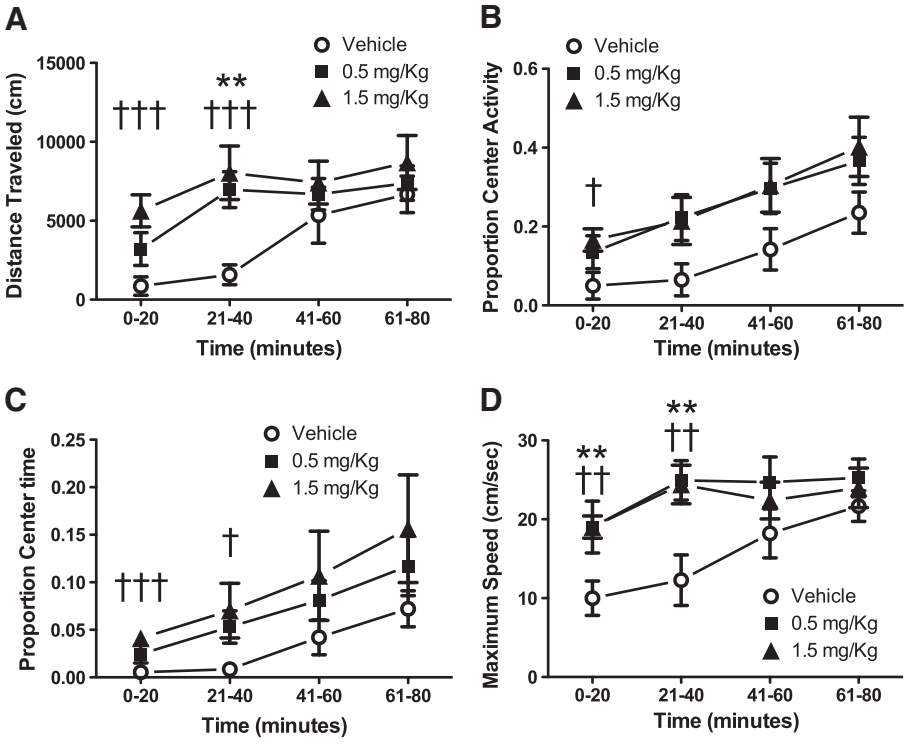

Figure 2 Effect of diazepam on exploratory measures in the dimensionality emergence assay. Treatment of mice with the anxiolytic compound diazepam (vehicle, 0.5, or 1.5 mg/kg, i.p., 15 min before testing) caused a significant and dose-dependent increase in (A) distance traveled, (B) proportion of activity spent in center, (C) proportion of time spent in center, and (D) maximum speed attained during progression segments. The effect of diazepam was greatest during the initial 40 min of testing (N = 8, mean ± SEM, high vs. veh: ††† $P<0.005$, †† $P<0.01$, † $P<0.05$, low vs. veh: ** $P<0.01$).

reported in mice carrying a knockout allele for the serotonin 1A receptor (Htr1a). Mice lacking Htr1a display decreased total locomotion and relative locomotion and time spent in the center in the open field test (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998). To determine whether the dimensionality emergence assay could reveal anxiety-related deficits in mice lacking serotonin 1A receptor allele, groups of Htr1a knockout (KO) and wild-type (WT) littermates were tested under low light conditions. Consistent with their reported phenotype in the open field, Htr1a knockout mice showed a decrease in time spent in the center in the first 20 min [repeated measure ANOVA – group × time: $F(3,99) = 1.19$; $P = 0.31$] and a decrease in total locomotion and relative locomotion in the center of the arena (Fig. 4A–C). However, unlike in the open field test, this deficit was seen only during the initial 40 min of the test, suggesting a normalization of the knockout phenotype with time. Moreover, no difference was seen in the maximum speed attained during progression segments (Fig. 4D) suggesting normal motor function in the knockout mice. Analysis of latency measures revealed a normal latency to *cross and retreat*, but a significant increase in latency to *enter center* and a trend for an increase in latency to *exit garden*, *full circle*, and *cage skip* (Fig. 5A–E).

4. Discussion

Our findings confirm the validity of the dimensionality emergence assay as a behavioral measure of innate anxiety in laboratory mice. Two features of this assay distinguish it from existing tests of anxiety based on spontaneous exploration. First, the large size of the open arena (here 180 cm) and the long duration of the assay (here 80 min) provides ample space and time for the animal to express a wide repertoire of exploratory behaviors. Existing tests typically use small open arenas (<50 cm) and shorter observation periods (<20 min). Measures that can be accurately quantified in a large open arena, such as latency to full circle or shuttle, for example, are likely to be less reliable and informative in a small enclosure where the animal quickly traverses the available space. Moreover, the reduced curvature of a large arena ensures that locomotion along the wall reflect relatively unrestricted movement, a feature that is not assured in a small enclosure.

Second, access to a familiar shelter provides the animal with a safe haven to which it can retire during its exploration of the open arena. The presence of familiar and unfamiliar areas of the apparatus is a feature shared with the free exploration and emergence tests (Cigrang et al., 1986; Paré et al., 2001). However, in these tests the size of the arena is typically small and extensive excursions into the unfamiliar part of the apparatus are difficult to define as they quickly bring the animal back to the familiar area. By directly comparing exploration of the large open arena in the presence or absence of the shelter, we have been able to identify those behavioral components that critically depend on the presence of a safe haven. Our findings demonstrate that exploration of the open arena is slowed significantly in

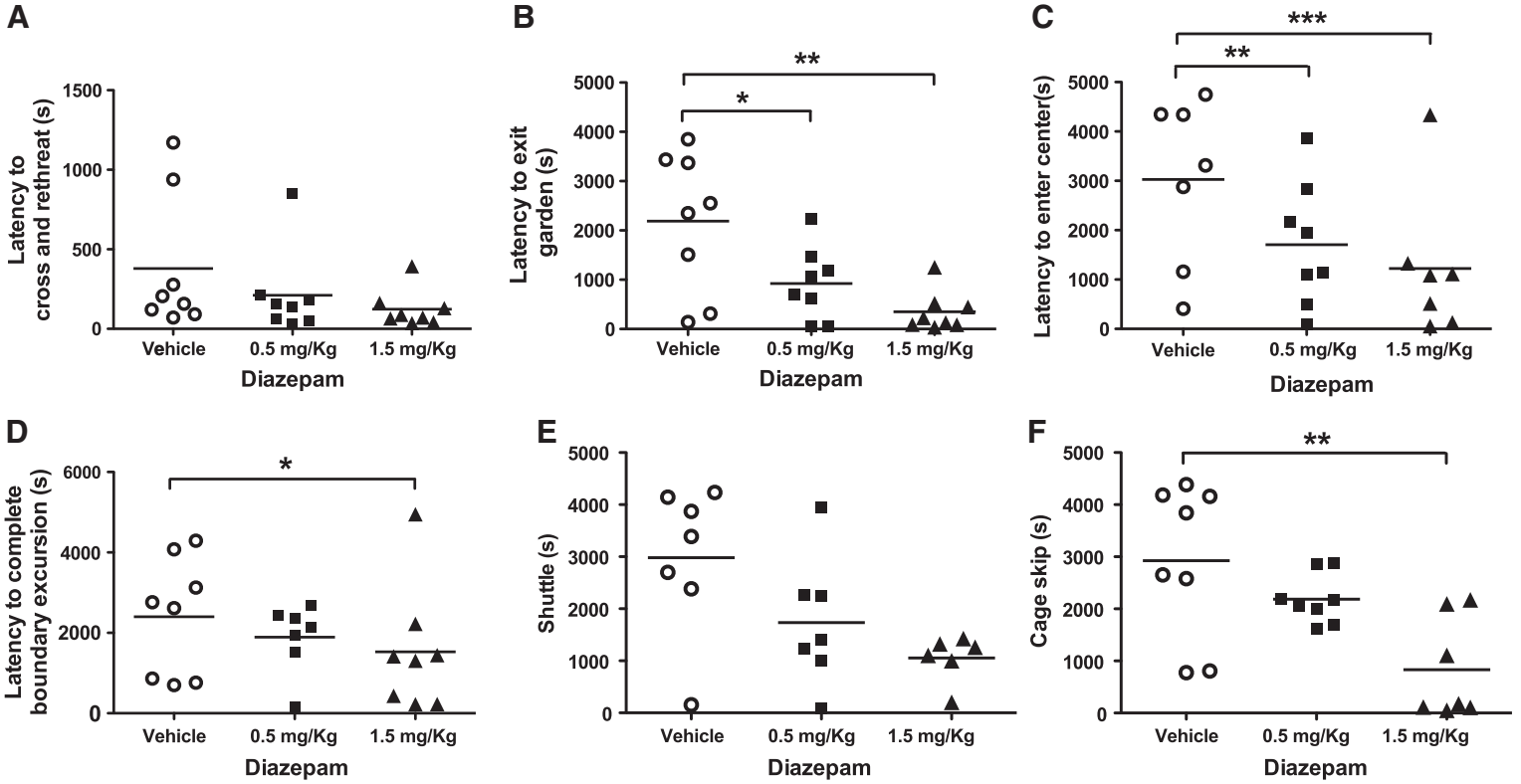

Figure 3 Effect of diazepam on latency to exploratory landmarks in the dimensionality emergence assay. (A–F) Treatment of mice with the anxiolytic compound diazepam (vehicle, 0.5, or 1.5 mg/kg, i.p., 15 min before testing) caused a significant dose-dependent decrease in latency to (B) *exit garden* and (C) *enter center*. Latency to (A) *cross and retreat* was only mildly affected, while latencies to (D) *full circle*, (E) *shuttle*, (F) *cage skip* were reduced as a secondary consequence to a decrease in latency to *exit garden*. Note that some animals did not exhibit all landmarks (N=8, bar indicates mean, *** $P<0.005$, ** $P<0.01$, * $P<0.05$).

Table 1 Effect of diazepam on the order of occurrence of exploratory landmarks in the dimensionality emergence assay. In vehicle treated mice the prototypical order of occurrence of landmarks was: *cross and retreat–exit garden–shuttle–full circle–enter center–cage skip*. Treatment with either a low (0.5 mg/kg), or high (1.5 mg/kg) dose of diazepam led to a significant and dose-dependent re-ordering of the occurrence of exploratory landmarks when compared to vehicle treated mice. Low dose diazepam treatment was associated with the precocious occurrence of *enter center* and high dose diazepam treatment was associated with the precocious occurrence of both *cage skip* and *enter center*. Statistically significant differences (Kruskal–Wallis test, $P=0.0173$) in landmark order were found for the high dose ($P<0.05$), but not low dose ($P>0.05$) of diazepam (Dunn's test).

DZP dose	Order of occurrence					
	1	2	3	4	5	6
Veh	Cross and retreat	Exit garden	Shuttle	Full circle	Center	Cage skip
Veh	Cross and retreat	Exit garden	Full circle	Cage skip	Shuttle	
Veh	Cross and retreat	Exit garden	Shuttle	Full circle	Cage skip	Center
Veh	Cross and retreat	Exit garden	Shuttle	Full circle	Center	Cage skip
Veh	Cross and retreat	Exit garden	Full circle	Shuttle	Cage skip	Center
Veh	Cross and retreat	Exit garden	Cage skip	Full circle	Shuttle	Center
Veh	Cross and retreat	Exit garden	Center	Full circle	Cage skip	
Veh	Cross and retreat	Exit garden	Shuttle	Full circle	Cage skip	Center
Low	Cross and retreat	Exit garden	Full circle	Center	Cage skip	Shuttle
Low	Cross and retreat	Exit garden	Shuttle	Center	Full circle	Cage skip
Low	Cross and retreat	Exit garden	Center	Shuttle	Cage skip	Full circle
Low	Cross and retreat	Exit garden	Shuttle	Full circle	Center	Cage skip
Low	Cross and retreat	Exit garden	Cage skip	Center		
Low	Cross and retreat	Exit garden	Shuttle	Center	Cage skip	Full circle
Low	Cross and retreat	Exit garden	Full circle	Center	Cage skip	Shuttle
Low	Cross and retreat	Exit garden	Shuttle	Full circle	Center	Cage skip
High	Cross and retreat	Exit garden	Cage skip	Center	Shuttle	Full circle
High	Cross and retreat	Exit garden	Cage skip	Shuttle	Full circle	
High	Cross and retreat	Exit garden	Cage skip	Full circle	Shuttle	Center
High	Cross and retreat	Exit garden	Center	Cage skip	Full circle	
High	Cross and retreat	Exit garden	Shuttle	Center	Full circle	
High	Cross and retreat	Exit garden	Cage skip	Center	Full circle	
High	Cross and retreat	Exit garden	Cage skip	Center	Shuttle	Full circle
High	Cross and retreat	Exit garden	Center	Shuttle	Full circle	Cage skip

animals with access to the shelter (Fig. 1). Measures of behavioral inhibition and avoidance are increased during the first 20 min and a more gradual build up can be seen (Fig. 1). These data suggest that the high levels of locomotion seen in the initial period of the forced open field test may reflect a complex mixture of anxiety and non-anxiety measures and should be interpreted with caution.

In our previous report using the dimensionality emergence assay (Fonio et al., 2009) we habituated the animals for 24 h in the shelter before opening the entrance to the open arena. However, overnight habituation to the shelter in the present apparatus did not result in a gradual and consistent build up of exploration in C57BL/6 J mice (A. Jain, unpublished observations) and we adopted the shorter habituation time (15 min) used here. This protocol has the additional advantage of being more compatible with standard pharmacological treatments. The absence of a gradual build up following overnight habituation with our present apparatus compared with our previous results (Fonio et al., 2009) may derive from differences in the inbred strains used (e.g. BALB/c vs. C57BL/6 J; Kafkafi et al., 2003; Flint et al., 1995) or their origin (Harlan, Israel vs. Charles River, Italy). Increased arousal associated with experimenter handling in the short habituation protocol used here is likely to have influenced exploration and the relatively slower build up seen in vehicle injected compared to non-injection mice supports this hypothesis (compare Fig. 2 with Fig. 1).

Our discovery that the latency and order of occurrence of a limited number of exploratory landmarks were sensitive to diazepam treatment confirms our hypothesis that the dimensionality emergence assay provides an improved separation of anxiety and non-anxiety measures. In the commonly used open field test anxiolytic compounds typically alter both thigmotaxis and total locomotion (Treit and Fundytus, 1988; Christmas and Maxwell, 1970) despite claims that total locomotion is not an anxiety measure (Lister, 1990). In support of these earlier studies, we found effects of diazepam both on measures of center activity and total distance traveled (Fig. 2). However, an examination of the exploratory build up revealed selective effects of diazepam on latency to exit the safe area surrounding the shelter entrance (*exit garden*) and the order in which the animal first enters into the center (*enter center*). Importantly, the relative latencies to subsequent landmarks following *exit garden* were not altered by diazepam (Fig. 3) suggesting that these measures reflect aspects of exploration less affected by anxiety. These findings demonstrate that the dimensionality emergence assay can successfully distinguish diazepam-sensitive and insensitive exploratory measures. They also show how the more gradual

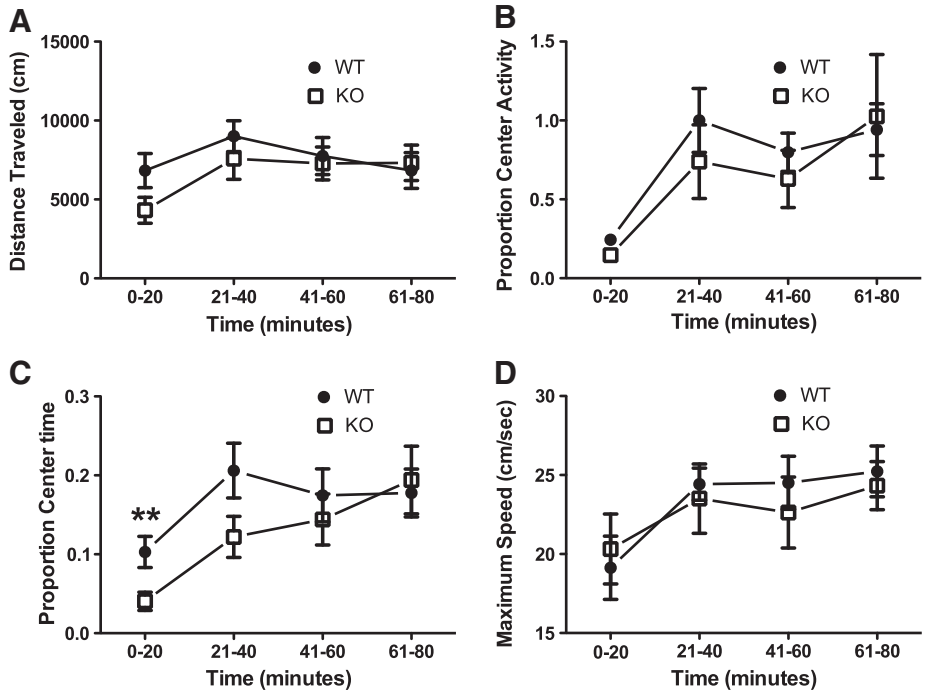

Figure 4 Effect of Htr1a knockout on exploratory measures in the dimensionality emergence assay. Htr1a knockout mice (KO) showed a decrease in (A) distance traveled and (B) proportion of activity spent in center and a significant decrease in (C) proportion of time spent in center and no difference in (D) maximum speed compared to wild-type littermates (WT) during the initial 40 min of the test (N = 14–15, mean ± SEM, WT vs. KO: ** $P < 0.01$).

build up seen in this assay can make latency measures, often found to be highly variable in classical tests, more reliable measures of behavior.

The utility of the dimensionality emergence assay was demonstrated in tests using Htr1a knockout mice. While earlier studies with the open field test indicated both decreased total locomotion and relative locomotion in the center of the arena, the dimensionality emergence assay revealed a deficit in these measures only in the first 40 min, with normal exploration thereafter (Fig. 4). Notably, locomotor capacity did not appear to be altered in the knockout as the maximum speed attained during progression segments was normal (Fig. 4D). The initial exploratory deficit was accompanied by a selective decrease in latency to *exit garden* and *enter center* following a normal latency to *cross and retreat* (Fig. 5A–C). Again, latencies to *full circle* and *cage skip* primarily reflected the delayed exit from the garden seen in these animals and did not appear to be selectively altered in the knockout mice. Importantly, the measures affected in Htr1a knockout mice appear to be a subset of those affected by diazepam opening the possibility that either diazepam has more general, non-anxiety affects on behavioral inhibition, or that Htr1a knockouts exhibit a specific anxiety subtype. These data also suggest that the test is sensitive to both state as well as trait anxiety. An investigation of the neural circuitry affected in each case might help to distinguish these hypotheses. For example, *in vivo* electrophysiology experiments have identified increased theta wave activity and connectivity in the forebrain of Htr1a knockout mice (Gordon et al., 2005; Adhikari et al., 2010) and theta wave activity in these regions is known to be suppressed by diazepam (Hajós et al., 2004). Intriguingly, exploration of the exposed arms of the elevated-plus maze was preceded by a drop in theta wave activity and connectivity (Adhikari et al., 2010), suggesting a role for altered cortical rhythmic synchronization in anxiety-related decision making during exploration.

Finally, we note that the dimensionality emergence assay can be readily adapted for use with SEE Workshop software. This software was developed to aid in the unbiased quantification of whole animal movement during spontaneous exploration (Kafkafi et al., 2003; Hen et al., 2004). SEE Workshop models the distribution of instantaneous speeds exhibited by the animal during the entire recording session as overlapping Gaussians (generally a simple bimodal distribution), determines the theoretical threshold, and then labels each videotracking frame as *lingering* (<threshold) or *progression* (>threshold). Similarly, SEE Workshop calculates the distribution of instantaneous radial distances, calculates a threshold, and labels each videotracking frame as *wall* or *center*. Additional calculations are then based on these categorical, animal-specific assignments. The unbiased method by which the software parses movement ensures that behavioral measures take into account the exploratory style of the individual and provide an ethological assessment of exploration.

5. Conclusions

The dimensionality emergence assay offers several advantages over the classic forced open field test to assess innate

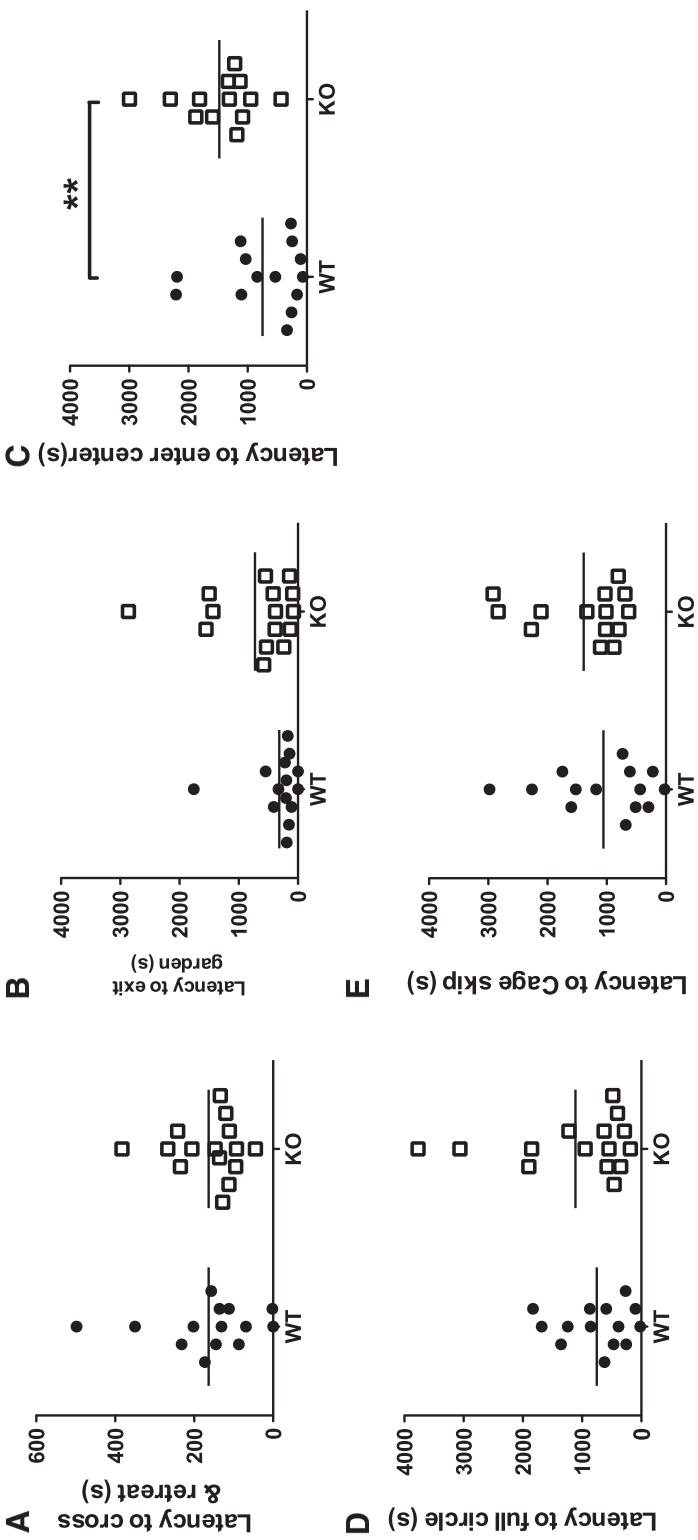

Figure 5 Effect of Htr1a knockout on latency to exploratory landmarks in the dimensionality emergence assay. Analysis of latency measures revealed a trend for an increase in latency to (B) *exit garden*, (D) *full circle*, and (E) *cage skip* and a significant increase in latency to (C) *enter center*, but no change in (A) *cross and retreat* in Htr1a knockout (KO) mice compared to wild-type (WT) littermates (N = 14–15, bar indicates mean, WT vs. KO, ** $P<0.01$).

anxiety behavior in rodents. Most importantly, it offers a wider repertoire of exploratory behavioral measures and thus allows the experimenter to better assess the selectivity of their experimental manipulation. In particular, we have shown that the exploratory build up can be used to distinguish anxiety from non-anxiety behavioral components. These features are likely to be useful in dissecting the neural circuits controlling innate anxiety behavior.

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Contributors

A.J. modified and constructed the testing apparatus according to a prototype developed by U.F. and I.G. A.J. carried out and analyzed all behavioral experiments. U.F. and A.D. provided advice in the use of SEE workshop and generously shared data prior to publication. C.G. and A.J. interpreted the experimental results with the help of I.G. C.G. and A.J. wrote the manuscript.

Conflict of interest

None.

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