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Research Report

Progressive vestibular mutation leads to elevated anxiety

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ABSTRACT

Anxiety disorders are among the most common mental disorders, and are comorbid with balance disorders in a significant proportion of these individuals. Presently, it is unclear whether anxiety and balance disorders are causally related, and what direction this causality may take. We argue that balance disorders may predispose an individual to anxiety and that demonstration of such causality may be informative to the development of preferred treatment for such individuals. To demonstrate that balance disorders may predispose to anxiety, we studied headbanger (*Hdb*) mutant mice in which the balance disorder is due to progressive vestibular impairment and wildtype (Wt) mice. Balance was assessed by swim and tail-hang tests that demonstrated clear behavioral balance deficits in the *Hdb* mice. Anxiety was assessed by open-field and elevated plus-maze tests, which confirmed elevated anxiety in the *Hdb* mice. These findings demonstrate that congenital vestibular genotype predisposes the animal to elevated levels of anxiety in space-related tests. Similar causality in clinics may redirect treatment strategies in afflicted patients.

1. Introduction

Anxiety disorders are among the most common mental disorders (DSM-IV-TR, 2000). Extensive clinical and preclinical research promotes pharmacological, cognitive and behavioral treatments. The basis of these treatments is the conviction that they presumably target the limbic mechanisms involved in the acquisition and expression of the anxiety symptoms (Amaral et al., 1992; Chapman et al., 1954; Davidson et al., 1999). The result is, however, unfortunate for a proportion of individuals that fail to benefit from these treatments. These resilient cases are hard to explain but some failures may be resolved by applying treatments that target some unconventional brain sites other than the limbic system. This strategy may be relevant when anxiety is associated with a neurological disorder that precedes and predisposes the individual to the emergence of anxiety. By neglecting this causal relationship, the treatment may target the allegedly limbic origins of anxiety with little lasting success, instead of targeting the comorbid neurological disorder, the curing of which may also ameliorate the anxiety disorder. To apply this strategy, one has to be absolutely certain that the two disorders are causally related and that the comorbid disorder is the cause of the ensuing anxiety.

Clinical literature has recognized the high prevalence of comorbidity of anxiety and balance disorders (Alvord, 1991; Balaban and Jacob, 2001; Kalueff et al., 2008). The range of the comorbid anxiety disorders is extensive and includes agoraphobia, panic attack and generalized anxiety (Alvord, 1991; Balaban and Jacob, 2001; Balaban and Thayer, 2001; Jacob and

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Furman, 2001). Anxiety is common in acute and chronic vestibular disorders (Eagger et al., 1992; Jacob and Furman, 2001; Matheson et al., 1999; Pollak et al., 2003) and in other proposed clinical entities such as phobic postural vertigo (Brandt, 1996; Huppert et al., 2005), space and motion discomfort (Jacob et al., 2009) and visual vertigo (Guerraz et al., 2001). The above disorders appear following a vestibular deficiency or as a consequence of a deficiency in sensorimotor integration responsible for maintaining balance. The integration involves constant interaction between the vestibular, but also the visual and somatosensory channels, and the motor system.

In spite of the rich literature dealing with the comorbidity between balance and anxiety, it is unclear whether anxiety and balance disorders are causally related, and what direction this causality may take. The three-stage theory of learning can be adopted to conceptualize the prospect that balance disorder may predispose or cause the emergence of anxiety (Erez et al., 2004). The vestibular system is essential for keeping balance, posture, coordination of motor actions and normal eye movements (Highstein, 1991). Impaired vestibular function causes imbalance, nausea, vertigo and abnormal eye movements (Gilman and Newman, 1996) and hinders the acquisition and emission of corrective movements. Such occasions are associated with limbic-related anxiety, perhaps through the parabrachial circuit (Balaban and Thayer, 2001).

Association of imbalance and anxiety has also been demonstrated in animal models. Strains of mice that differ markedly in their level of anxiety also differ in balance skills, with anxious strains showing poorer balance (Lepicard et al., 2000). Highly appropriate for the search of causality are vestibular mutant mice with an early and progressive phenotype. Such is the case of the N-ethyl-N-nitrosourea ENU-generated Headbanger (*Hdb*) mutant mouse, in which stereocilia of the hair cells in the otolith organs are abnormally thin and elongated already at 20 days after birth, with these abnormalities worsening progressively over time (Rhodes et al., 2004). In the present study we tested whether the vestibular *Hdb* mice with impaired balance show increased levels of anxiety.

2. Results

2.1. Balance tests

2.1.1. Swim test and genotyping

In all three replications, 1-month-old mice exhibited dichotomous behavior in the swim test and were classified as either Wt or *Hdb*. Wt mice exhibited normal emergence on the surface of the water and swimming to the ridge of the container. *Hdb* mice exhibited swimming on their back, in circles and diving. Genotyping performed after completion of behavioral tests fully confirmed the swim-based grouping of the mice.

2.1.2. Tail-hang test

Fig. 1 shows the progressive vestibular phenotype in *Hdb* mice along the 3 months of testing, while Wt mice scored "9" at all times. *Hdb* scores were analyzed by one-way ANOVAs which confirmed a significant age effect for each of the three replications [$F(2,18)=156.0$, $p<0.001$; $F(2,18)=384.7$, $p<0.001$; $F(2,18)=324.2$, $p<0.001$, respectively].

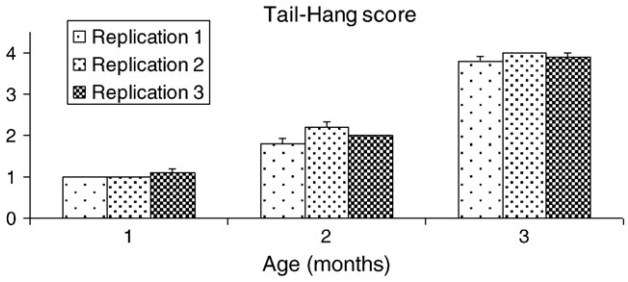

Fig. 1 – Progressive vestibular deficit in vestibular *Hdb* and Wt mice measured by the tail-hang test (mean ± SEM) across three replications performed under identical conditions at age of 1, 2 and 3 months. Scores were in the range from 0—stretching limbs toward the floor, to, 4—curling upward with no stretching toward the floor. Data are presented only for the vestibular mutant *Hdb* mice, as all wild-type (Wt) mice scored "0" on all occasions. In this and subsequent figures, each of the three replications consisted of 10 *Hdb* and 10 Wt mice (total of 60 mice).

2.2. Anxiety tests

2.2.1. Open-field test

Behavioral indices in each of the three replications were analyzed by separate ANOVAs, which included Genotype (*Hdb* vs. Wt) as between subjects variable, and Age (1 and 2 months) and Time (10 periods of 1 min each) as within subjects variables.

First replication: mice were placed in the center of a dimly illuminated OF. Traveled distance increased with time [Time effect; $F(9,162)=17.4$, $p<0.001$; Fig. 2] and with age in Wt but not in *Hdb* mice [Genotype by Age interaction; $F(1,18)=4.5$, $p<0.05$]. Occupancy of the HB was short at the onset and was further shortened with time [Time effect; $F(9,162)=4.7$, $p<0.001$; Fig. 3], with no significant Genotype effect ($p=0.97$). Mice spent a considerably long time in the center of the OF, with *Hdbs* less than Wts at the age of 1 month and more than Wts at the age of 2 months [Genotype by Age interaction; $F(1,18)=8.6$, $p<0.01$].

Second replication: mice were placed again in the center of the OF, which was now brightly illuminated. Traveled distance decreased visibly in comparison to the first replication and the pattern of increased traveling with time was preserved only at the 2-month session [Age by Time effect; $F(9,162)=2.7$, $p<0.01$; Fig. 2]. *Hdb* and Wt mice showed similar travel distance [Genotype effect; $F(1,18)=0.99$, $p=0.33$]. HB occupancy was short at the onset, but increased with time at 1-month-old mice [Age by Time interaction; $F(9,162)=2.5$, $p<0.01$; Fig. 3]. Overall, *Hdb* occupied the HB longer than the Wt mice resulting in only borderline level of significance [Genotype effect; $F(1,18)=3.7$, $p=0.07$]. Mice lingered in the center of the field at the onset but avoided the center toward the end of the session [Time effect; $F(9,162)=29.6$, $p<0.001$]. Avoidance of the center was accelerated with age [Age by Time interaction; $F(9,162)=2.3$, $p<0.05$] with no effect of Genotype ($p=0.38$).

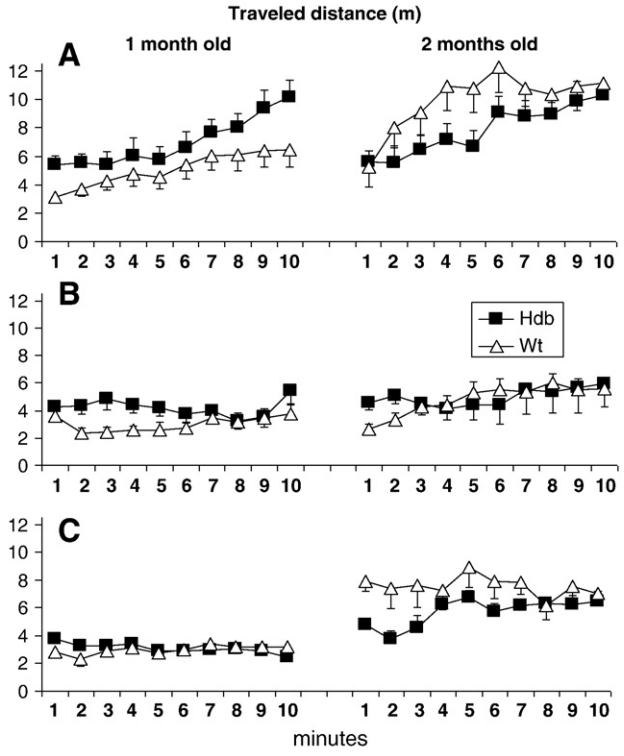

Fig. 2 – Distance traveled by vestibular *Hdb* and Wt mice in the open-field (mean ± SEM) along 10-min sessions at age of 1 and 2 months. A, B and C present the distance at the three replications. Illumination of the open-field and initial positioning of the mice in the open-field were manipulated across the replications. Illumination was low during the first replication and was stronger in the next two replications. Mice were positioned by the experimenter in the center of the open-field during the first two replications and in home-base (HB) corner filled with sawdust during the third replication.

Third replication: mice were tested again in brightly illuminated OF but were placed into sawdust padded corner rather than the center of the field. This manipulation did not affect the traveling distance which was comparable to that measured in the second replication (Fig. 2). Distance increased with age but to less extent in the *Hdbs* at the start of the second month session [Genotype by Age by Time interaction; $F(9, 144) = 2.8$, $p < 0.01$]. *Hdb* occupied the HB longer than the Wt mice [Genotype effect; $F(1,18) = 5.2$, $p < 0.05$; Fig. 3] as observed toward the end of the first month session and at the onset of the second month session [Genotype by Age by Time interaction; $F(9,162) = 6.2$, $p < 0.001$]. All mice, but particularly the *Hdbs*, avoided the center of the OF [Genotype effect; $F(1,18) = 5.8$, $p < 0.05$].

2.2.2. *Elevated plus-maze test*

Indices of open vs. closed-arms preference in the tree replications were analyzed by separate ANOVAs with Genotype (*Hdb* vs. Wt) as between subjects variable, and Age (months) as within subjects variable. Fig. 4 shows the % of distance, visits and time in the open-arms of the elevated plus-maze by the vestibular *Hdb* and Wt mice at the age of 1, 2 and 3 months. The results of the three replications are shown in separate rows.

First replication: low illumination of the EPM was associated with extensive activity. ANOVAs confirmed that the two groups did not differ in their in their preference of the open vs. closed-arms in terms of % traveled distance [Genotype effect; $F(1,18) = 0.4$, $p = 0.56$], % visits [Genotype effect; $F(1,18) = 1.8$, $p = 0.20$] and % time of occupancy [Genotype effect; $F(1,18) = 0.1$, $p = 0.92$].

Second replication: increased illumination decreased mice activity compared to the first replication. ANOVAs confirmed the preferential avoidance of the open vs. closed-arms by the *Hdb* vs. Wt mice in terms of % traveled distance [Genotype effect; $F(1,17) = 12.4$, $p < 0.01$], % visits [Genotype effect; $F(1,17) = 12.5$, $p < 0.01$] and % time of occupancy [Genotype effect; $F(1,17) = 14.4$, $p < 0.01$]. All three indices showed age-related increased behavior in the open-arms by the Wt but not the *Hdb* mice resulting in significant Genotype by Age interaction in the % time of occupancy [$F(2,34) = 4.2$, $p < 0.05$] and borderline significance in the other two indices [$0.06 < p < 0.08$].

Third replication: conditions of the second replication were preserved and ANOVAs confirmed again the preferential avoidance of the open vs. closed-arms by the *Hdb* vs. Wt mice in terms of the % traveled distance [Genotype effect; $F(1,17) = 11.2$, $p < 0.01$], % visits [Genotype effect; $F(1,18) = 10.0$, $p < 0.01$] and % time of occupancy [Genotype effect; $F(1,18) = 11.2$, $p = 0.01$].

3. Discussion

This study investigated whether balance dysfunction due to a vestibular phenotype is associated with elevated anxiety. The model for the balance disorder was an ENU-generated mouse mutant, headbanger (*Hdb*), with a progressive vestibular phenotype compared to Wt mice of the same genetic background. Anxiety was measured in response to exposure to open-field (OF) and elevated plus-maze (EPM). Our results confirmed elevated anxiety in *Hdb* as compared to Wt mice.

The tail-hang test confirmed the presence of a progressive vestibular dysfunction along the 3-month testing period. Based on these findings, we could expect progressive development of anxiety in the *Hdb* mice. Anxiety is typically inferred from avoidance of open space in behavioral tests. In the OF, the preference is for the strip along the walls, particularly the HB corner, compared to the center of the field. In the EPM, the preference is for the closed compared to the open-arms. Regrettably, avoidance of open space may be confounded by motor deficiency in the vestibular mice. The goal of the first replication was therefore to confirm the validity of the OF and EPM as tests of anxiety in vestibular *Hdb* mice. The strategy was to enable the *Hdb* mice to fully express their motor repertoire in the open spaces by reducing the anxiogenic effect of the ambient illumination. Results of the first replication confirmed that under low illumination, mice from both groups were very active in both tests and spent a long time outside of the HB, either along the walls or in the center of the OF, and in the open-arms of the EPM. In fact, 2-month-old *Hdb* showed longer occupancy of the center of the OF compared to Wt mice. These findings indicate that the ability of *Hdb* mice to reach and occupy the open zones of the

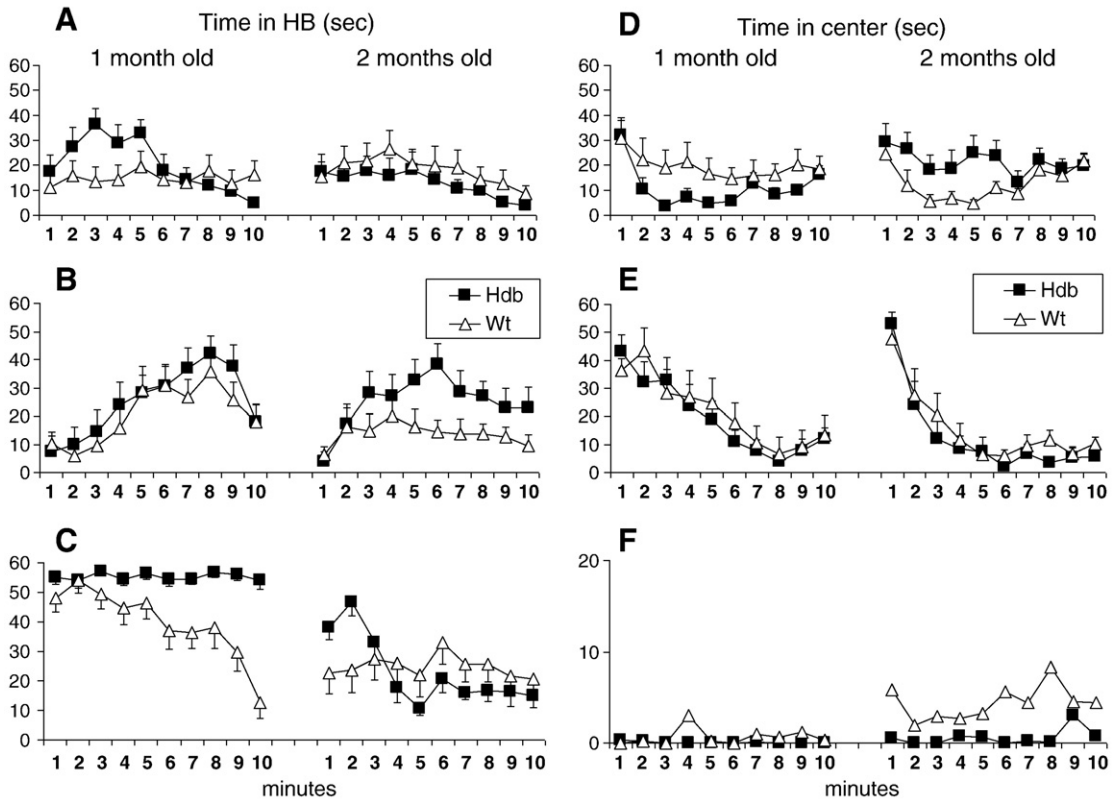

Fig. 3 – Anxiety of the vestibular *Hdb* and Wt mice measured as the time spent in the home-base (HB) and in the center zone of the open-field (mean ± SEM) along 10-min sessions at age of 1 and 2 months. A, B, C present the time in HB, and D, E, F, present the time in the center-zone whereby each row represents successive replication study. See the legend of Fig. 2 for parametric changes introduced along the replications.

OF and EPM is not compromised by the sensory-motor components of the vestibular deficiency. This conclusion is compatible with previous studies in which vestibular mice and rats exhibited an increase in distance traveled in the OF, with a noticeable tendency for increased inner field activity (Avni et al., 2009; Goddard et al., 2008), and in a radial-arm maze (Russell et al., 2003).

After confirming the validity of the OF and EPM tests for vestibular mice, the second replication was performed under brighter illumination with the intention of increasing the anxiogenic effects of the OF and EPM environments. Increased illumination reduced the activity on the two tests, compared to the first replication, and in line with the study hypothesis, revealed elevated anxiety in *Hdb* compared to Wt mice. Preferential anxiety in *Hdb* was reflected in preference of the closed vs. open arms in terms of number of visits, length of traveled distance and length of occupancy. The results of the OF were, however, less convincing, as *Hdb* showed only slightly longer occupation of the HB compared to Wt mice.

The weak signs of anxiety of *Hdb* mice in the OF test motivated the third replication. We argued that the vestibular system is involved in spatial tasks such as exploration, target finding and homing (Russell et al., 2003; Stackman and Herbert, 2002; Wallace et al., 2002). The vestibular *Hdb* mice may therefore experience difficulty in establishing and retaining the location of the HB in the OF test. The immediate outcome of this spatial disorientation may be shortened HB occupancy, erroneously interpreted as a low level of anxiety in *Hdb* mice. Given this reasoning, the purpose of the third replication was to reduce the effects of spatial disorientation on HB occupancy. This was achieved by introduction of the mice into a sawdust-padded corner at the start of the session, instead of the center of the OF, as was done in the second replication. We found that this manipulation accelerated the establishment of the HB, always in the sawdust-padded corner to which the mice were introduced. We also reasoned that the sawdust in the corner serves as a landmark for HB location, thus easing the return to the HB after excursion to the field. Results of the third replication confirmed that all mice occupied the sawdust-padded HB from the very start of the session. At the age of 1 month, *Hdb* mice occupied the HB practically throughout the whole duration of the session, while Wt mice shortened the occupancy along the session. Occupancy of the HB was decreased at the age of 2 months but nevertheless, *Hdb* showed preferred lingering in the HB during the first part of the session. Convincingly, during both sessions, *Hdbs* entirely avoided the center of the OF while the Wt mice showed short but reliable time in the center. Conditions of the EPM were not changed from the second replication and confirmed longer occupancy of the closed-arms by *Hdb* compared to Wt mice. Cumulatively, the two tests demonstrated increased anxiety in *Hdb* mice.

Presently, it is unknown whether anxiety and balance disorders are causally related, and what direction this causality

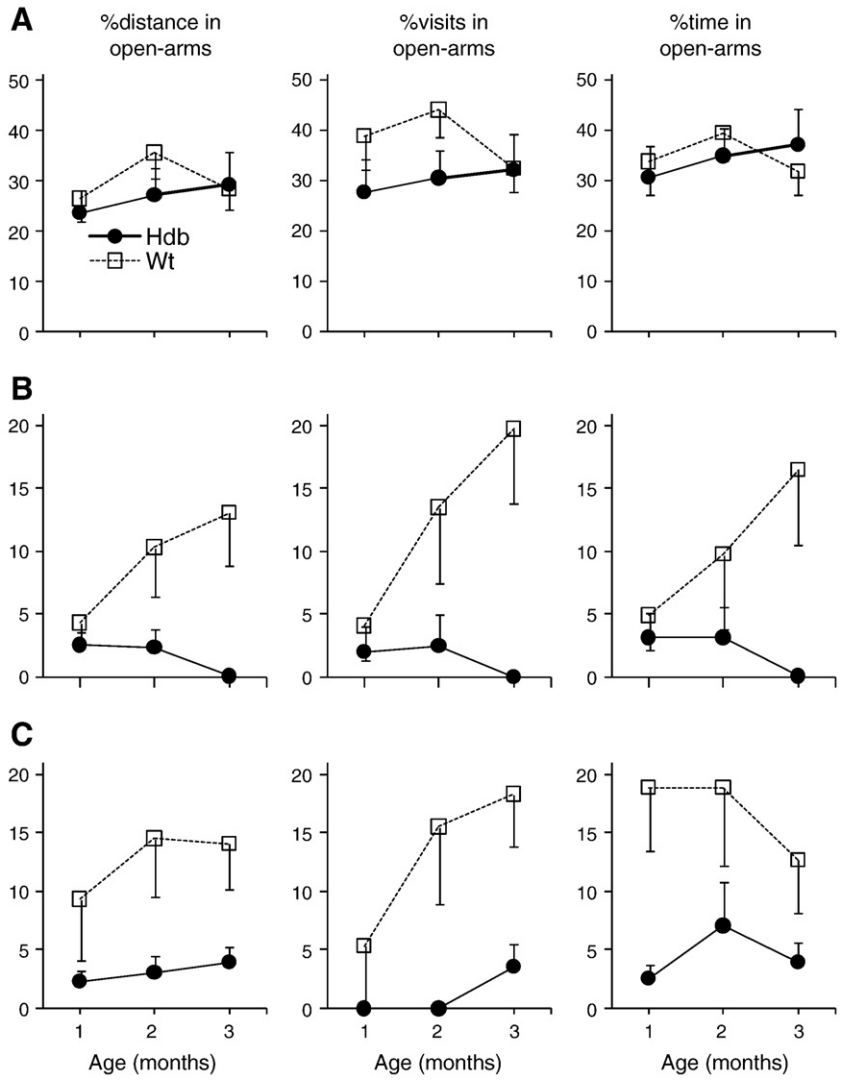

Fig. 4 – Anxiety measured as % of distance, visits and time in the open-arms of the elevated plus-maze by the vestibular *Hdb* and Wt mice (mean ± SEM) during 5-min sessions at age of 1, 2 and 3 months. A, B and C present the three replications. Illumination was low during the first replication and was stronger during the next two replications.

may take. The present experimental methodology provided results that support the view that imbalance associated with progressive vestibular phenotype either predisposes to or causes anxiety disorder. First, the methodology ensured a time order between the genetic manipulation of the vestibular system effective already at a prenatal stage (Rhodes et al., 2004) and the development of postnatal anxiety. Second, findings confirmed a significant association between the balance loss and anxiety. Third, we refuted two alternative causes of elevated indices of anxiety; these were the possible effects of motor disability and spatial disorientation on avoidance of anxiogenic spatial zones. These findings suggest that progressive vestibular mutation predisposes or leads to elevated anxiety. Causal relations between balance and anxiety disorders were expected also to be confirmed by gradual exacerbation of anxiety along the progressive development of the vestibular phenotype in *Hdb* mice. The present results showed only minor signs in this direction. These negative results imply that the progress of vestibular disorder above its level at 1 month of age has little contribution to the development of anxiety, perhaps due to compensatory effects of visual and proprioceptive systems. Alternatively, the negative result may be at least partially explained by the floor effect of the anxiety indices in the PM test, which under the present conditions were very low in *Hdbs* already at the age of 1 month. Further titration of the test conditions, such as level of the ambient light, are necessary to decide on this issue.

The anxiety of *Hdb* mice as reflected by avoidance behavior may resemble some aspects of clinical entities such as phobic postural vertigo (Brandt, 1996), space and motion discomfort (Jacob et al., 2009) and visual vertigo (Guerraz et al., 2001) that are sometimes associated with vestibular disorders. Phobic postural vertigo patients demonstrate dizziness and subjective disturbance of balance in spaces such as bridges, supermarkets or empty rooms, which are accompanied with anxiety. Similar situations trigger discomfort and anxiety in space and motion discomfort and visual vertigo patients. All the above disorders include avoidance of these challenging situations.

We previously suggested a theoretical account for the emergence of anxiety disorder due to poor balance skills under the title of the "three-stage theory of learning" (Erez et al., 2004). The reasoning is based on findings with animals subjected to environmental challenges (Mintz and Wang-Ninio, 2001; Neufeld and Mintz, 2001). Repeated encounters with environmental challenges, including balance or spatial challenges, trigger three stages of adaptive learning. In the first stage, encounters with the challenging events trigger fast acquisition of conditioned-fear responses. In the second stage, additional encounters trigger slow acquisition of conditioned-adaptive motor responses. In the third stage, command of the adaptive motor responses leads to extinction of fear responses. While extinction of fear responses is predicted in normal animals, sustained anxiety is predicted in animals with sensory-motor deficits. Such animals are unable to acquire and/or emit the adaptive motor responses in the second stage of the learning sequence, and therefore the additional encounters with the challenging events trigger repeated anxiety responses. The present findings, demonstrating anxiety-like behavior in *Hdb* mice, are in line with this prediction and suggest that balance deficiency may predispose the animal to an elevated level of anxiety during exploration of unfamiliar areas. Our next attempt will be to demonstrate that anxiety may be ameliorated by corrective treatment of balance with immediate relevance for treatment of clinical cases of comorbidity.

4. Experimental procedures

4.1. Subjects

Subjects were male vestibular mutant Headbanger (*Hdb*; $n=30$) and C3HeB/FeJ wildtype (Wt; $n=30$) mice. *Hdb* is a dominant mutation on a C3HeB/FeJ genetic background. The mutation was mapped to the region of the unconventional myosin VIIa (*Myo7a*) gene and mutation screening revealed an A>T transversion, leading to an isoleucine-to-phenylalanine amino acid substitution in the motor domain (Rhodes et al., 2004). In the auditory system, the *Hdb* mutation is manifested as disorganization of the cochlear hair cells, and in the vestibular system, as elongation of stereocilia of saccular and utricular hair cells. These changes were detected as early as embryonic day 18 in the cochlea and postnatal day 20 in the otoliths, and are progressive in both systems. The functional phenotype in adult mice includes a severe decline in low frequency hearing and imbalance due to progressive peripheral vestibular loss.

Hdb and Wt samples were produced by 16 Wt females housed in 4 cages and 4 *Hdb* males rotated between the cages (one per cage) every four days. The females were separated from the males after 8 days. At P28 litters were weaned and phenotyped using the balance-sensitive swim test and sexed. Samples for each replication study consisted of not more than 2 mice from the same litter. Male *Hdb* and Wt progeny were housed separately, 5 mice per cage (30×15×13 cm) with net top-cover that enabled the mice to experience up-side climbing (Pietropaolo et al., 2007) and free access to food and water. Ambient temperature was kept at 23 °C and light cycle was reversed so that all experimental manipulations took place during the dark phase of the cycle. All experimental protocols conformed to the guidelines of the Institutional Animal Care and Use Committee of Tel Aviv University, and to the guidelines of the NIH (P-04-005).

4.2. Balance tests

4.2.1. Swim test

Grouping was based on the results of balance-sensitive swimming test. The mouse was held by the tail and was released from a height of 5 cm into a plastic container (20×20×20 cm) filled with water, 15 cm high (24–26 °C). Swimming was assessed for up to 10 s following the recommendation to immediately rescue a distressed mouse (EUMORPHIA; http://empress.har.mrc.ac.uk). The score was dichotomous as Wt mice exhibited normal swimming to the surface and then to the ridge of the container, while *Hdb* mice exhibited diving and swimming on their back and in circles (Marshall and Berrios, 1979).

4.2.2. Tail-hang test

Progressive vestibular deficiency was monitored by the tail-hang test (Kiernan et al., 1999). The mouse was held by the distal end of its tail 5 cm above a table for 5 s. Wt mice responded with stretching of their forelimbs toward the surface, while *Hdb* mice curled their trunk toward the tail. Upward curling behavior was graded incrementally from 0—stretching forelimbs toward the floor, with or without curling once towards the tail, 1—stretching forelimbs toward the floor and curling twice towards their tail and so on. Eventually, score "4" was given to a mouse that did not stretch his forelimbs toward the floor at all.

4.2.3. Genotyping

After completion of all the tests, animals were anaesthetized with isofluorene and the distal 0.5 cm of the tail was cut. DNA was extracted and genotyping was performed to confirm the *Hdb* and Wt grouping which was performed initially on the basis of the swimming test. The genotyping assay consisted of PCR followed by either restriction enzyme digestion or direct sequencing to identify the *Myo7a Hdb* mutation (Rhodes et al., 2004).

4.3. Anxiety tests

4.3.1. Open-field test (OF)

The OF was a white plastic rectangular 120×120×25 cm arena, positioned on the floor in an air conditioned (24 °C) room with no windows. One of the corners (~20×20 cm) was padded with clean sawdust to attract the mice as a home-base (HB). Mice were videotaped for 10 min through a vertically positioned camera (Chipset Sony Video; 880 nm). OF was thoroughly cleaned with detergent before introduction of new mice. In the OF test, anxiety is manifested as preference of areas that are distant from the center, i.e., HB as first priority and along the walls as second priority (Carola et al., 2002). Videos were sampled off-line at 25 Hz (Ethovision, Noldus v3) and X-Y coordinates were extracted and analyzed by MATLAB program for the following dependent variables: (i) time of HB occupancy: the field was divided to 6×6 squares, each 20×20 cm, and

HB was defined as the square with highest cumulative time of occupancy. This calculation confirmed that all animals established the HB at the corner padded with the sawdust, (ii) time along the walls in a strip of 20 cm in width excluding the HB corner, (iii) time in the center defined as an area of 40×40 cm in the center of the OF, and, (iv) traveled distance.

4.3.2. *Elevated plus-maze test (EPM)*

The EPM was a plus-shaped maze with 30×5 cm arms creating a center square of 5×5 cm, positioned 45 cm above the floor level. Open- and closed-arms had 0.25 and 15 cm high walls on the sides, respectively. Mouse was placed at the distal end of a closed arm, facing the center of the maze and was videotaped for 5 min. In EPM test, anxiety is manifested as preference of closed- over open-arms (Carola et al., 2002). Videos were analyzed for extraction of the following dependent variables: (i) % time in the open-arms, (ii) % visits in the open-arms, (iii) % distance in open-arms.

4.4. *Procedure*

The study consisted of three successive replications, each including separate samples of *Hdb* ($n=10$) and Wt ($n=10$) mice (total of 60 mice). Consistent with the progressive vestibular phenotype of *Hdb*, mice in each replication were tested repeatedly. To minimize the effect of repeated testing on anxiety scores, the EPM and OF tests were applied with a long intersession interval of 1 month (Walf and Frye, 2007). The tail-hang test was applied repeatedly at P29, P59 and P89, while the swimming test resulted in a dichotomous score already at P28 and therefore was not applied at later stages of the study. EPM test was applied at P33, P63 and P93 while the OF test was applied only at P31 and P61, since at 3 months of age the *Hdb* mice tended occasionally to circle in the OF (but never in the EPM). Procedures of balance tests were kept constant across the three replications while two parameters of the anxiety tests were changed across the replications. The first parameter involved the ambient illumination, which is an important anxiogenic factor in the OF and EPM tests (Bouwknecht et al., 2007; Cosquer et al., 2005). It was set to a low level of illumination of 1 Lux at the first replication, mainly to verify that under such low anxiogenic conditions the *Hdb* mice can reach the different zones of the two arenas to the same extent as the Wt mice. Illumination was set to a higher level of 9 Lux in the next two replications to enhance the anxiogenic effect of the tests. The second parameter involved the initial location of the mouse in the OF. During the first two replications, mice were positioned by the experimenter in the center of the OF, with the intention to induce anxiety, and during the third replication mice were positioned in the sawdust padded HB-corner, a procedure which seems to accelerate the establishment of the HB in rodents and particularly in vestibular mice (Avni et al., 2009).

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