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Research report

Mice with vestibular deficiency display hyperactivity, disorientation, and signs of anxiety

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ABSTRACT

Previous studies revealed that vestibular cues are crucial for exploration in the absence of visual cues. The working hypothesis of this study was, accordingly, that mice with vestibular dysfunction would become disoriented or unable to globally explore an unfamiliar environment. In 2- and 3-month-old mutant headbanger (*Hdb*) mice, stereocilia of hair cells are abnormally elongated, yet maintain partial staircase arrangement, suggesting some spared vestibular function at these ages. Here we tested a group of 3-month-old mutant *Hdb* and a group of non-mutant mice obtained from the same litters (Wt mice). Each individual mouse was introduced into a dark 120 cm × 120 cm arena and its behavior was followed for 10 min. *Hdb* mice were hyperactive and appeared to engage in local exploration, traveling in a restricted zone for a while and then shifting to travel in another zone. In contrast, Wt mice traveled across zones incessantly with fewer visits to recently entered zones. Thus, *Hdb* seemed to display local compared with the global exploration of Wt mice, indicating that they were less oriented in the global environment. In addition, *Hdb* exhibited numerous stretch-attends, which is suggested as a sign of elevated anxiety. Altogether, the three comorbidities of hyperactivity, anxiety, and disorientation can be presented as a syndrome associated with vestibular deficiency in this animal model, and serve in studying vestibular deficiency in humans.

1. Introduction

Animal studies are essential for revealing causal relations between pathology and behavioral phenotypes. Since clinical studies in children revealed frequent cases of comorbidity between balance dysfunction and another disorder, such as spatial disorientation [1], elevated anxiety [2,3] or hyperactivity [4], we set out to study how a dysfunctional vestibular system affects spatiotemporal and perhaps emotional behavior in rodents. For this we used the *Hdb* mutant mouse [5], in which stereocilia of hair cells are abnormally elongated. Since the vestibular system is normally involved in performing spatial tasks such as homing, radial maze exploration and target finding [6–8], we assumed that vestibular dysfunction would affect the spatiotemporal structure of navigation and exploration and that vestibular *Hdb* mice might not be able to explore an unfamiliar environment, as do normal mice. Moreover, considering the feeling of discomfort that humans feel once the mishap of being disoriented occurs [9], it was reasonable to assume that impaired capacity to navigate due to vestibular dysfunction would result in elevated anxiety. Indeed, clinical literature recognized frequent occurrence of comorbidity between balance and anxiety disorders [10]. Even more specific are studies suggesting that it is the vestibular deficiency that is associated with anxiety ([11]; see also [12] for possible mechanism). Causality of this form of comorbidity is not yet known, and we therefore sought for anxiety in *Hdb* vestibular mice, which, in line with the above findings, may provide a model to study the clinical form of comorbidity.

In order to study the spatiotemporal structure of behavior under vestibular dysfunction, we tested *Hdb* mice in a dark open-field. We chose the open-field since this is a sensitive test in which behavior is commonly regarded as a fundamental index of general behavior and as predictive of locomotor scores in other novel environments [13]. Past studies of vestibular rodents in the open-field revealed hyperactivity [7,14], but it was not clear whether hyperactivity is a reflection of disorientation in the environment, or whether the vestibular animals displayed a different form of exploration and progression. Indeed, vestibular rats tested for homing and goal-finding in the open-field [6,8,15] used curvilinear (less direct) homing paths than normal rats [6], thus matching the notion

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that the vestibular system provides internal cues that allow animals to orient and keep track of their location [16]. In the same vein, assessing anxiety of vestibular rats in an open-field surprisingly indicated reduced anxiety compared to controls [17], as measured by reduced avoidance of central zones [18]. Altogether, these past studies lead us to study how *Hdb* mice organize their spontaneous behavior and to search for indicators of stress in their open-field behavior. In the open-field, where animals move freely, stress indicator may be sticking to the walls and avoiding the center [19], or increased incidence of stretch-attends [20].

In spatial tasks, rodents may relay on visual, olfactory or self-generated cues [21]. Among the latter are vestibular and proprioceptive information [8,15,22,23]. Accordingly, in the present experiment, mice were tested in a completely dark open-field in order to maximize their dependence on internal cues. Comparing vestibulary dysfunctioning *Hdb* mice with non-mutant *Wt* mice could thus emphasize the possible role of vestibular information in spatial tasks. We also performed a detailed ultrastructural analysis of hair cell stereocilia in order to correlate vestibular defect with the behavioral phenotype at age of testing. Specifically, we posed the following three questions: (i) Do 3-month-old *Hdb* mice suffer from apparent anatomical vestibular pathology? (ii) Are 3-month-old *Hdb* mice limited compared with Wt mice in the capacity to explore a dark open-field, where exploration depends more on self-generated cues? and (iii) Do 3-month-old *Hdb* mice display indicators of elevated stress such as clinging to the walls (avoiding the central area) or increasing the incidence of stretch-attends?

2. Materials and methods

2.1. Animals

Headbanger (*Hdb*) is a dominant ENU-induced mouse mutant with a low frequency hearing loss and vestibular dysfunction, both of a progressive nature. The mutation was mapped to the region of the unconventional myosin gene myosin VIIa (*Myo7a*), and mutation screening revealed an A > T transversion in a conserved region in the motor-encoding domain of the gene [5]. The vestibular utricular hair cells of 5-month-old *Hdb* heterozygotes are unusually long with thin stereocilia and lack the normal staircase arrangement of the hair bundle. C3HeB/FeJ *Hdb*/+ males were mated with wild type (+/+; Wt) C3HeB/FeJ females. At the age of 60 days, male mice from resulting litters were phenotyped using both a swimming test and reaching response test (http://empress.har.mrc.ac.uk/). After completion of all behavioral tests, the phenotype was confirmed by a restriction enzyme genotyping assay or direct sequencing to identify the *Myo7a Hdb* mutation, as previously described [5]. Male mice were divided into two groups, a mutant *Hdb*/+ group ($n = 11$) and a Wt +/+ group ($n = 13$). Each group was kept in standard 12 cm × 32 cm × 12 cm plexiglas cages, with 2–4 mice per cage, under reversed 12:12 h light cycle and 23 °C room temperature, and with *ad lib* standard rodent pellets and water. All behavioral tests were conducted during the dark phase of the light cycle. Additional mice were used for scanning electron microscopy and immunohistochemistry. All experiments were carried out in full compliance with the Tel Aviv University Animal Care and Use Committee (M-06-071 and M-05-058).

2.2. Swimming test

When released to water, rodents with an intact vestibular system swim to the water surface whereas those with abnormal vestibular function either swim in circles or on their side, or are unable to swim to the water surface and present underwater tumbling [24,25]. This differential behavior in the swim test was used here to distinguish between *Hdb* and Wt mice. The swimming apparatus was comprised of a plastic tub (20 cm × 20 cm × 20 cm) filled with 15 cm high water at a temperature of 24–26 °C. Each mouse was held by its tail and released into the tub from a height of 5 cm, and its swimming ability was assessed for up to 30 s. The mice exhibited dichotomous behavior since they either swam vigorously to the water surface or tumbled under water, and were therefore classified as Wt or *Hdb*, respectively.

2.3. Reaching response test

Each mouse was held by its tail and lowered toward a table. The mice exhibited dichotomous behavior, either presenting a reaching response (stretching their forelimbs toward the table surface), or curling their trunk toward their tail [26]. Accordingly, they were classified as Wt or *Hdb*, respectively.

2.4. Open-field test

The open-field was a 120 cm × 120 cm arena with 24 cm high walls, made of white vinyl, located in an air conditioned (~24 °C) room with no windows. All light sources in the testing room were eliminated or covered (including indicator lights of camera and VCR) to prevent any source of visible light. Animals were tested in the open-field at the age of 3 months. This age was set since spatial behavior cannot be assessed in fully mature *Hdb* mice that already at the age of 5 months show frequent interruptions of exploration by bouts of high-speed circling (informal observations by S. Shefer and M. Mintz). Three-month-old *Hdb* mice show only sporadic bouts of circling and only in response to stressing stimuli. For open-field testing, an individual mouse was removed from its home cage into an identical carrying cage, and placed for 3–5 min in a room adjacent to the testing room. The arena was thoroughly cleaned with detergent, and when dried, the mouse was placed gently in the middle of the open-field and videotaped for 10 min. Testing took place under infrared illumination (wave length of 880 nm) not visible to mice, emitted by a Chipset Sony video camcorder (TRV23e) located above the open-field providing a top view.

2.5. Behavioral analysis

Analysis was carried out during playback of the video records onto a tracking system (*Ethovision 3.1* by Noldus, NL). This program identifies the body surface of the mouse, calculates the location of the center of that surface and provides as output the time and *x–y* coordinates of that location. *Ethovision* was set to sample the location of the mouse at a rate of 5 Hz throughout the 10 min observation period. For analysis, the open-field was divided to a matrix of 36 square zones of 20 cm × 20 cm each. Data from *Ethovision* were exported to *Microsoft Excel* for further analyses. The following parameters were measured or calculated for each mouse for the 10 min observation period:

- Total traveling distance (m).
- Total traveling time (s) defined as time when traveling speed was greater than zero (see [27], for discrimination between walks and stops).
- Total time spent in each zone (s).
- Momentary turn angle (degree/s) calculated as the change in direction of traveling for each successive sample of coordinates.
- Total number of stops, with a stop being defined as pause longer than 1 s in progression. This parameter therefore included only relatively long pauses, and disregarded brief pauses in progression. A filter was used in order to discriminate stops from walks as described in Avni et al. [27].
- Average speed (m/s) calculated as total traveling distance (m) divided by total traveling time (s).
- Number of successive returns to a recently visited zone: A successive return was defined as revisiting a most recently visited zone. For example, a mouse traveled from zone A to zone B and returned to A. Another example, the mouse traveled from zone A, to B, to C, to B and finally to A, in this case the successive return is scored for zone B but not for zone A.
- Distance between successive returns (m) defined as the distance a mouse traveled until it began a new successive return.
- Time between successive returns (s) defined as the duration of time a mouse traveled until it began a new successive return.
- Number of stretch-attends: When traveling, mice may pause, stretch their body forward while their hind legs remain rooted in the same place and then retreat back by arching their trunk. This behavioral pattern was described as 'stretch-attend' and served as indicative of anxiety ([28–32]; see however, [33]). In the present study we applied a semi-automatic detection of stretch-attends with *Ethovision*. We first manually sampled stretch-attends and analyzed the *x–y* coordinates in terms of *distance* (amplitude of the stretch) and *turn angle* (change in direction of traveling after the retreat from the stretch). According to these parameters, criteria for stretch-attends were defined as follows: a change of at least 0.85 cm in position (forward traveled distance recorded due to the stretch of the mouse's body) followed by at least 135° change in direction (turn angle).
- Length of stretch-attend (cm). The change of location by stretching during stretch-attends.

2.6. Scanning electron microscopy (SEM)

Whole inner ears were dissected from 3-month-old mice (+/+, $n = 4$; *Hdb*, $n = 4$). The middle ear ossicles were removed to assure fast penetration of the fixative through the oval window into the inner ear labyrinth. Fixation was done in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 4 h on ice. After three washes with 0.1 M phosphate buffer, further fine dissections were made to expose the surface of the vestibular sensory organs. The samples were run through the OTOTO procedure [34]. In brief, three 1 h incubations in 1% osmium tetraoxide were done, separated by 20 min incubations in a saturated solution of thiocarbohydrazide. The samples were dehydrated in gradients of ethanol, critical point dried, and coated with gold for 45 s at 20 mA. Images were acquired with a JEOL JSM-6701F scanning electron microscope.

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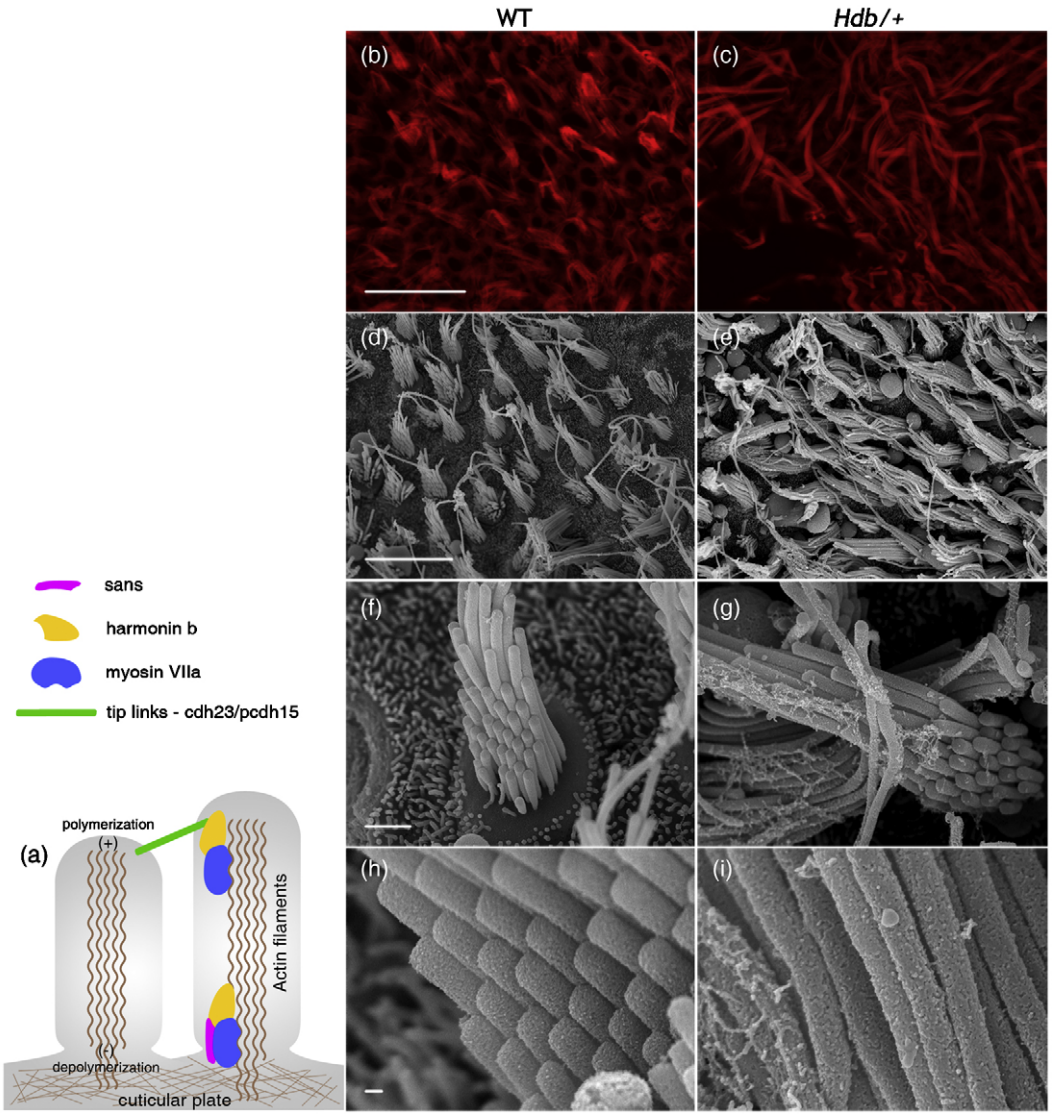

Fig. 1. The morphology of stereociliar bundles is affected in *Hdb* mutant mice. (A) Schematic diagram of two adjacent stereocilia composed of F-actin filaments (modified from [69]). Sites of myosin VIIa protein localization and two of its interactors, harmonin and sans, are shown, as well as sites of depolymerization and polymerization. (B and C) Confocal images of utricular whole mounts from 2-month-old Wt (B) and *Hdb* (C) mice. Whole mounts were immunostained with phalloidin to visualize the actin component of stereocilia. In the mutant, stereocilia are elongated. (D–I) SEM of stereociliary bundles from 3-month-old Wt (D, F and H) and *Hdb* (E, G and I) mice. At low magnification (D–E), the stereocilia are seen to be longer in the mutant. The staircase pattern is seen in a bundle derived from a Wt mouse (F), but this pattern is only observed at the bottom of the mutant bundle (G). In order to photograph the bundle at the same scale, the tip of the bundle could not be observed for the mutant (G). At high magnification, the staircase pattern is seen in the bundle derived from a Wt mouse (H), but is completely lost in the upper region in the mutant (I). Scale bars: (B and C) 20 m; (D and E) 10 m; (F and G) 1 m; (H and I) 100 nm.

2.7. Whole mount immunohistochemistry

Whole inner ears were dissected from 2-month-old mice. Prior to fixation in 4% paraformaldehyde overnight at 4 °C, the middle ear ossicles were removed to assure fast penetration of the fixative through the oval window into the inner ear labyrinth. After washes with PBS, further fine dissection was performed in order to reveal the utricle. Dissected samples underwent permealization in 0.5% triton for 1.5 h at room temperature and then blocking with 10% NGS and 1% BSA for 1 h at room temperature. After additional washes with PBS, samples were stained with rhodamine phalloidin (red) (Invitrogen, Carlsbad, California) to visualize the actin cytoskeleton. Images were acquired with a Zeiss LSM 510 META confocal microscope with LSM Image Browser Rel. 4.2.

2.8. Statistical analysis

Open-field data were checked for normal distribution using Kolmogorov–Smirnov continuous distribution test. A Student's *t*-test for independent measures was used to compare *Hdb* and Wt mice on above-mentioned behavioral indices using STATISTICA 6.0 program.

3. Results

3.1. Vestibular phenotype

Thirteen mice that presented both normal swimming and reaching response were classified as Wt, and 11 mice that presented both abnormal swimming and abnormal reaching response were classified as *Hdb*. Genotyping fully confirmed this subgrouping of the mice. Scanning electron microscopy (SEM) was previously performed for the vestibular system only on P20 and 5-month-old mice [5]. We expanded our analysis to 3-month-old mice at a higher resolution, as well as immunofluoroscence at 2 months of age to observe F-actin filaments of the stereocilia. Analysis included utricle, saccule and cristae that showed similar findings and here we present results from the utricle. Fig. 1A shows a schematic diagram of the mammalian hair bundle with stereocilia-containing

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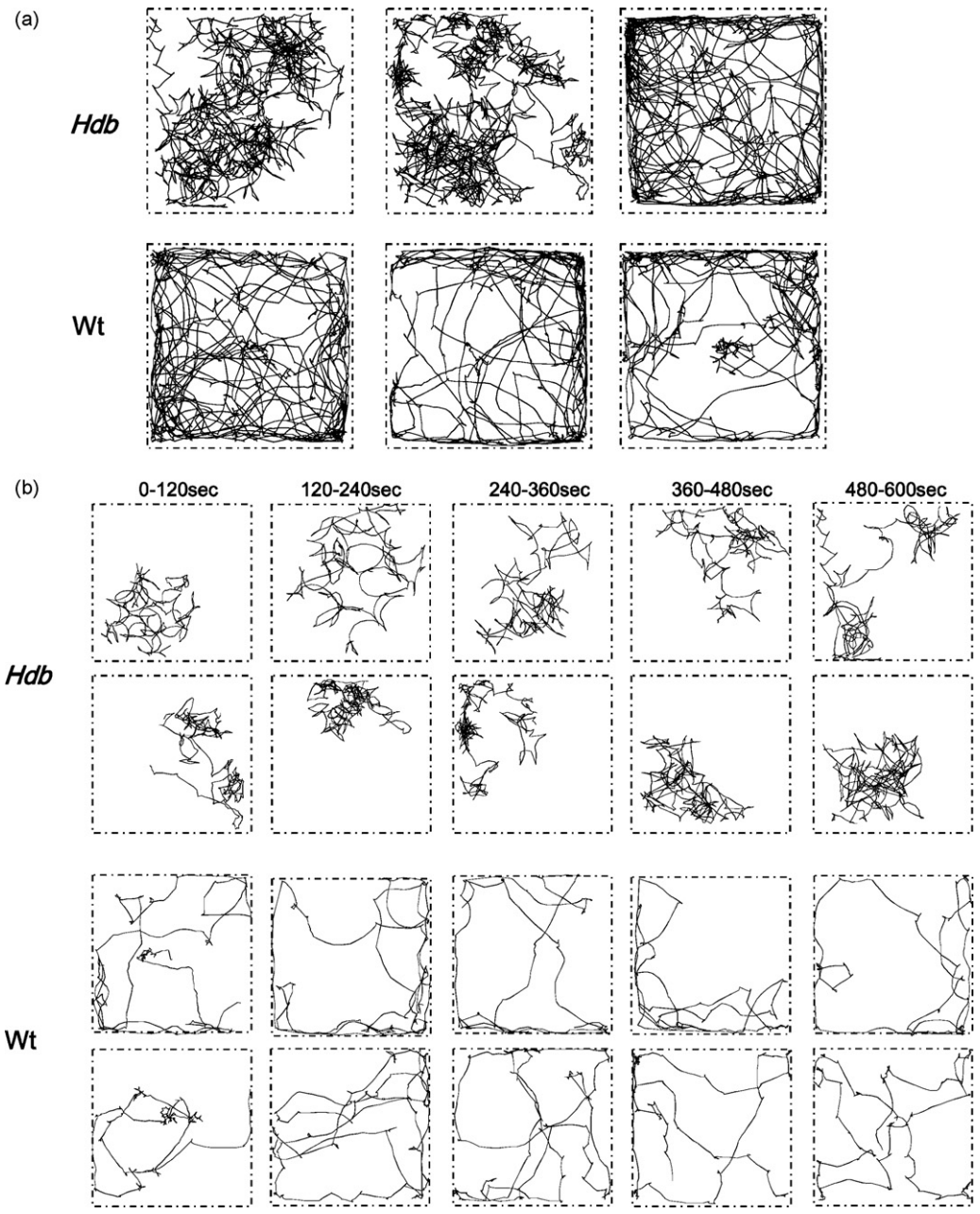

Fig. 2. (A) Exemplary 10 min traveling routes of *Hdb* (top) and Wt (bottom) mice. The walls of the open-field are marked with a square. Both groups traveled in the center and perimeter of the open-field. However, while Wt mice progressed from one zone to the next thus covering most of the open-field, *Hdb* mice concentrated their activity momentarily in a certain zone. Yet, note an exceptional traveling route of a single *Hdb* mouse that seemed to travel similarly to Wt mice (upper row, right). (B) Traveling routes of *Hdb* (two top rows) and Wt mice (two bottom rows), presented for successive periods of 120 s. Traveling routes of *Hdb* mice were composed of clusters of activity: the mice progressed from a certain zone and then retreated back to it, and repeated this behavior several times thus forming compact path in form of an asterisk, they then left that zone, progressed to a new one and again repeatedly retreated to the new zone. Wt mice however, did not exhibit clusters of activity rather they progressed fluently from one zone to the next. As a consequence, for the same time interval Wt mice reached more zones in the open-field compared with *Hdb* mice.

actin filaments. Immunostaining of actin filaments with phalloidin revealed longer actin-filled stereocilia in *Hdb* compared to Wt mice (Fig. 1B and C). SEM confirmed that stereocilia were more elongated in *Hdb* hair bundles compared to stereocilia of Wt mice, similar to our previous observations in younger and older *Hdb* mice [5]. However, bundles at 3 months of age appeared to be at an intermediate stage between P20 and 5 months. The bundles were no longer perpendicular to the utricular surface, as at P20, but were not yet lying along the surface, as seen at 5 months (compare Fig. 1D and E in the present study with Fig. 4 in [5]). The staircase pattern is seen in a bundle derived from a Wt mouse (Fig. 1F), as well as in the lower portion of the mutant bundle (Fig. 1G), but this pattern is lost in the upper part of the mutant bundle (Fig. 1H and I).

3.2. Open-field behavior

Locomotor behavior of *Hdb* mice significantly differed from that of Wt mice. The *Hdb* traveled greater total distance, spent more time traveling, paid more visits to the various arena zones, and had fewer stops of more than 1 s (Table 1). These parameters reflect a higher activity of the *Hdb* compared with Wt mice.

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Table 1
Parameters of activity, path shape and anxiety.

	Wt (mean ± SEM)	*Hdb* (mean ± SEM)	$t_{(22)}$	p
Parameters of activity				
Traveled distance (m)	61.4 ± 4.4	87.6 ± 10.8	2.5	**<0.021**
Traveling time (s)	462.8 ± 13.0	534.89 ± 17.54	3.5	**<0.002**
Speed (m/s)	0.13 ± 0.01	0.16 ± 0.02	1.9	0.077
Total number of stops (>1 s)	45.1 ± 3.7	18.3 ± 3.8	5.3	**<0.001**
Number of visits at zones	303.0 ± 22.4	472.4 ± 50.7	3.4	**<0.003**
Parameters of path shape				
Number of successive returns	47.8 ± 2.7	103.55 ± 0.25	6.5	**<0.001**
Time between successive returns (s)	6.7 ± 0.4	2.4 ± 0.25	9.0	**<0.001**
Distance between successive returns (m)	0.8 ± 0.05	0.4 ± 0.07	4.5	**<0.001**
Parameters of anxiety				
Number of stretch-attends	193.4 ± 6.6	277.5 ± 15.8	5.5	**<0.001**
Length of stretch-attends (cm)	1.54 ± 0.11	1.96 ± 0.15	2.4	**<0.023**

Significant difference between Wt and *Hdb* mice is marked by bold letteres.

In addition to their higher activity level, *Hdb* mice differed from Wt mice in the spatiotemporal structure of exploratory behavior, as can be seen in their traveling routes (Fig. 2A). While Wt mice traveled over most of the open-field, *Hdb* mice traveled as if they were momentarily confined to a certain sector of the open-field, and from time to time shifted to another sector, where they again were active for a while before shifting to yet another sector. Within a sector, exploration was comprised of short bouts of forward progression. Occasionally, the *Hdb* mouse had a longer bout of forward locomotion, which resulted in traveling to another sector where it reinstated the local exploration pattern (Fig. 2B). Thus, locomotor behavior of *Hdb* mice comprised localized and tangled clusters of progression, in contrast to Wt mice that traveled in relatively long-distance bouts of progression (see video clips). This difference in the form of progression was manifested in *Hdb* as a significantly higher number of successive returns in predefined zones (see Section 2) compared with Wt mice (Table 1). That is, *Hdb* mice traveled short distances and repeatedly retreated back, whereas Wt mice traveled from one zone to the next, progressing to remote parts of the arena. Accordingly, *Hdb* had a shorter elapsed time and a shorter traveled distance between successive returns to zone, compared with Wt mice (Table 1). In all, the higher amount of successive short bouts and retreats in *Hdb* mice seems to reflect a different form of progression compared to the Wt mice. This difference in path shape, however, was not reflected in the cumulative time spent at the various arena zones, which was similar in *Hdb* and Wt mice (Fig. 3). Cumulatively, the Wt mice pattern of exploration supported relatively long-distance scanning of the entire open-field, giving the impression of global exploration of the open-field. In contrast, the *Hdb* mice pattern of exploration supported prolonged short-distance and repetitive scanning of few local zones at one time, and then shifting to other local zones.

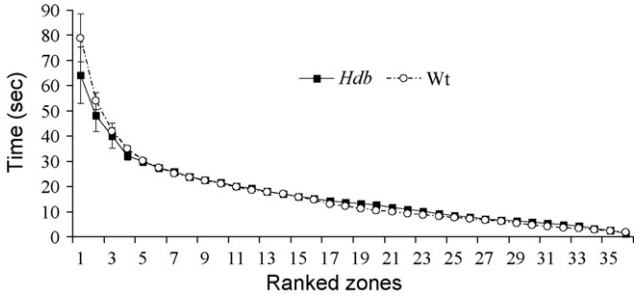

Fig. 3. Time (mean ± SEM) spent in the 36 zones of the open-field. The zones were ranked from maximum to minimum time spent in the zone for the *Hdb* ( ) and Wt (◯) groups. As can be seen, both groups exhibited similar distribution of time across zones, without concentration of time spent in one zone. No significant difference was found in the maximum time spent in one zone (rank of 1st zone) between the groups (t_{22} = 1.05, p = 0.304).

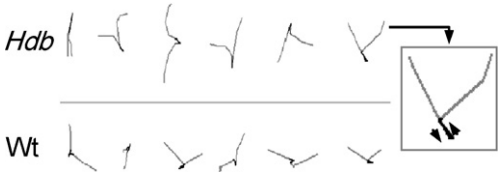

Fig. 4. Exemplary traveling segments containing stretch-attends of *Hdb* (top) and Wt (bottom) mice. Within a segment, progressing is marked with grey and stretching in black (see enlarged example on the right with the arrows indicating direction of stretch and unstretch). The number and the length of the stretch were greater in *Hdb* compared with Wt mice (p < 0.001 and p < 0.023, respectively).

When traveling, *Hdb* mice paused, stepped forward with only the forelegs while their hindquarters remained rooted thus stretching their body forward. They then unstretched to the normal body posture and resumed traveling. This behavioral pattern is known as 'stretch-attend' (see Section 2), and in *Hdb* mice it was usually followed by a change in direction of progression. The number of stretch-attends was significantly higher in *Hdb* mice compared with Wt mice (Table 1). Also, stretch-attends in *Hdb* were more conspicuous since they were longer compared with those of Wt mice (Fig. 4 and Table 1). Since stretch-attends implicated a pause and change in the direction of progression, *Hdb* appeared to travel along irregular short paths compared with relatively longer and straighter paths in Wt mice.

4. Discussion

The present study demonstrated how vestibular dysfunction altered the spatiotemporal structure of open-field exploration, a behavior that is commonly regarded as a fundamental index of general behavior and a predictive of locomotor scores in other novel environments [13]. We tested 3-month-old headbanger (*Hdb*) mutant mice, an animal model of progressive vestibular dysfunction due to abnormally elongated stereocilia of hair cells. We found that *Hdb* mice were hyperactive and appeared to engage in local exploration, traveling in a restricted sector of the open-field for a while and then shifting to another restricted sector. In contrast, non-mutant Wt mice traveled across sectors incessantly with fewer visits to recently entered zones. Thus, *Hdb* seemed to display local compared with the global exploration of Wt mice. In addition, *Hdb* exhibited numerous stretch-attends, which is suggested as a sign of elevated anxiety [20]. In the following discussion we first describe the anatomical characteristic of the tested vestibular dysfunction, we then discuss the impact of such dysfunction on the spatiotem-

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poral structure of motor behavior, and finally we scrutinize the possibility that vestibular dysfunction is involved in elevated anxiety.

4.1. What is anatomically wrong in Hdb mice?

On a structural level, the vestibular system is comprised of the utricle and saccule organs that control the sense of linear acceleration and gravity, and of three semicircular canals that are responsible for sensing angular acceleration. An actin-rich stereocilia bundle lies on the apical surface of the hair cells and deflection of this region elicits the opening of mechanotransduction channels [35]. A comparison of vestibular stereocilia across age in *Hdb* mutant mice implied that these mice exhibit progressive vestibular dysfunction. In the vestibular organ, mature stereocilia are supported by a rigid paracrystalline array composed of actin filaments [36], whose length is tightly regulated through an actin turnover process of paracrystal disassembly and actin filament depolymerization at the base of the stereocilia and actin polymerization at the tip of the stereocilia, allowing stereocilia to maintain a constant length [36]. Another study of myosin VIIa-deficient mice, generated by mosaic complementation, suggested that myosin VIIa regulates establishment of a set-point for stereocilia heights [37]. In *Hdb*, a threshold of myosin VIIa might be required for regulation of stereocilia length, and reduced or absent levels in stereocilia associated with abundant pointed end directed force, would lead to excessive growth of stereocilia, which would only stop due to finite supply of G-actin. Regardless of the exact mechanism, the observed changes in the structure of the hair bundle in *Hdb* mice (Fig. 1) may directly or indirectly lead to aberrant mechanoelectrical transduction that ultimately leads to a reduction in vestibular function. The 3-month old *Hdb*/+ mice used in this study presumably still maintain some vestibular function, since, for example, the staircase arrangement is still present and is perpendicular to the cell layer in the lower part of the hair bundle, suggesting that vestibular transduction will occur at least partially.

The vestibular system is involved in performing spatial tasks (e.g., homing, radial maze, target finding), especially in the absence of external cues [6–8]. Furthermore, the vestibular system is involved in maintaining correct spatial and angular coding by place and head-direction cells, respectively [38–40]. Accordingly, vestibular dysfunction involves altered spatiotemporal organization of behavior, as described in the present results (e.g., Fig. 2 and Table 1).

4.2. What is behaviorally wrong in Hdb mice?

Hyperactivity in *Hdb* compared with Wt mice, as demonstrated in the present study, is in line with previous reports of hyperactivity in variety of vestibular mice [41,42] including *Hdb* [5] and in rats following bilateral vestibular deafferentation [43]. Further research may explain the clinical association between vestibular and hyperactivity disorders and advance the attempts to ameliorate hyperactivity by vestibular stimulation [44]. Despite the hyperactivity, both *Hdb* and Wt mice traveled over the entire open-field, yet utilized distinct modes of progression. *Hdb* progressed in short bouts, repeatedly revisiting the same or nearby zones, whereas Wt mice typically progressed in long bouts to farther zones (Fig. 2). In other words, Wt mice traveled from one sector to the next, whereas *Hdb* mice repeatedly scanned a limited sector before traveling to an adjacent limited sector, such that *Hdb* and Wt mice seemingly exhibited local and global exploration, respectively. An interpretation of this local exploration in *Hdb* mice could be that they were more cautious and explore sector-by-sector, rather than traveling across sectors.

It is not yet known why *Hdb* mice were engaged in local exploration rather than utilizing alternative strategies such as thigmotaxic wall-following [45,46]. However, hippocampal rats were also found to alter their spatial behavior from traveling along the perimeter to traveling over the entire area [47]. Perhaps this behavior of *Hdb* mice is a result of two mechanisms. First, *Hdb* are mutants of Wt C3H/HeJ mice that have a weak tendency to travel along walls compared with other strains [48]. Second, at age of 3 months, *Hdb* mice may still retain a limited ability of using internal cues to calculate their position in relation to a specific location by, for example, path-integration, which is based on proprioceptive sensation and efferent-copy of motor commands [49]. In absence of both visual and vestibular information, accuracy of navigation is probably limited to short excursions [50]. For accuracy, frequent resetting of path-integration is required [49], which could be carried by returning to the start point of traveling [51–54]. Indeed, we found that *Hdb* mice displayed short bouts of progression followed by retreats to the start of the bout. It is not likely that this pattern of progression is a result of poor locomotion since *Hdb* mice were hyperactive. Rather, it might be interpreted as a reset of path-integration during exploration. This suggestion should be further assessed by experiments designed to test the path-integration ability of *Hdb* mice.

In what concerns the emergence of disordered exploration, several lines of evidence support direct relation to vestibular disorder. Spatial functions of the hippocampus [55], involve place cells [56], which activity is modulated by idiothetic vestibular information generated by self-movements [57]. Vestibular lesions disrupt spatial firing of place cells [39,58]. Similarly, vestibular information modulates hippocampal EEG theta waves [59] and electrical stimulation of the vestibular organ modulates hippocampal field potentials [60]. Accordingly, the progressive vestibular deficiency of *Hdb* mice might distort the functioning of the hippocampus in spatial tasks that require exploration based on idiothetic cues. Indeed, hippocampectomized rodents are impaired in orientation based on self-movement cues [61,62] confirming the suggestion that vestibular information might be essential for hippocampal computation of idiothetic cues. Nonetheless, there are also conflicting studies suggesting that neuronal circuits sufficient for computing a homing vector using path-integration are located outside the hippocampus [63].

4.3. What might be psychologically wrong in Hdb mice?

Forward progression in *Hdb* mice was frequently interrupted with 'stretch-attends', a behavior which was previously noted in the open-field [18] and plus-maze [28,30], and was commonly considered as sign of elevated anxiety toward the space ahead of the animal ('risk assessment'; [64]). Accordingly, the increased rate of stretch-attends in *Hdb* mice implies that vestibular deficiency could be associated with anxiety, an association well recognized under the title of 'comorbidity between balance and anxiety disorders' [3,65,66]. This conclusion of elevated anxiety should be, however, taken with a grain of caution, as one could also expect *Hdb* mice to display other characteristics of elevated anxiety, such as confining locomotion to the periphery of the open-field, which was clearly not the case in the present study.

At this stage, it is only prudent to state that progressive deterioration of vestibular skills predispose the organism to elevated anxiety. Erez et al. [3] suggested a theoretical account of the emergence of anxiety disorder in individuals with poor balance skills under the title of 'three-stage theory of learning'. The reasoning is based on experiments with animals subjected to environmental challenges [67,68]. Repeated encounter with any environmental challenge, including balance or spatial challenge, triggers three stages of adaptive learning. First, initial encounters with the challenge trigger fast acquisition of conditioned-fear responses. Second,

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additional encounters trigger slow acquisition of conditioned-protective motor responses. Third, command of the adaptive motor responses leads to extinction of fear responses upon the subsequent encounters with the challenge. While extinction of fear responses is predicted in normal animals, sustained anxiety is predicted in animals with sensory-motor deficit. In the first stage of initial encounters with the challenge, these animals, like normal animals, acquire conditioned-fear response. In subsequent encounters, these animals experience difficulty in acquisition of adaptive motor responses necessary to facing the challenge. Finally, if the challenges are frequent as may happen to animals with balance and spatial disorder, they may present a sustained state of anxiety. The present results confirm this prediction and suggest that vestibular deficiency may predispose the animal to an elevated level of anxiety during exploration of unfamiliar area. However, this line of reasoning does not prove that disordered balance is an immediate precursor of anxiety.

In conclusion, we show here that progressive vestibular disorder leads to a syndrome composed of changes in spatial behavior, hyperactivity and perhaps also increased anxiety. Some evidence supports the suggestion that vestibular deficiency leads directly to spatial disorder, which separately or in composition with distorted balance leads to anxiety. The emergence of hyperactivity in this syndrome is presently least understood. Altogether, the three comorbidities of hyperactivity, anxiety, and disorientation can be presented as a syndrome associated with vestibular deficiency in this animal model, and may serve as a tool to study the consequences of vestibular deficiency in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2009.03.033.

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