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Deficits in memory-guided limb movements impair obstacle avoidance locomotion in Alzheimer's disease mouse model

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Memory function deficits induced by Alzheimer's disease (AD) are believed to be one of the causes of an increased risk of tripping in patients. Working memory contributes to accurate stepping over obstacles during locomotion, and AD-induced deficits of this memory function may lead to an increased risk of contact with obstacles. We used the triple transgenic (3xTg) mice to examine the effects of memory deficits in terms of tripping and contact with obstacles. We found that the frequency of contact of the hindlimbs during an obstacle avoidance task increased significantly in 10–13 month-old 3xTg (Old-3xTg) mice compared with control mice. However, no changes in limb kinematics during unobstructed locomotion or successful obstacle avoidance locomotion were observed in the Old-3xTg mice. Furthermore, we found that memory-based movements in stepping over an obstacle were impaired in these mice. Our findings suggest that working memory deficits as a result of AD are associated with an increased risk of tripping during locomotion.

P atients with Alzheimer's disease (AD) have a higher risk of falling than healthy older people, and one of the common causes of this increased risk is contact with and tripping over an obstacle[1–3]. Most of the investigations of the factors associated with tripping or contact with an obstacle have used obstacle avoidance tasks. When the experiments allow self-selected locomotion, that is, under normal visual conditions and free foot placement, patients with AD have a higher frequency of contact with an obstacle than healthy young or elderly people[4–7]. Previous studies of humans or animals have shown that visual guidance is essential for successfully stepping over an obstacle during locomotion without tripping or falling[8–10]. However, obstacles can also be cleared without immediate visual information during the approach and step-over phases using memory function to perform the appropriate limb movements at the apposite time[8,11–14,16]. The role of memory in locomotion suggests that memory deficits associated with AD may lead to inaccurate limb movements during the process of stepping over obstacles. However, the relative importance of the effects of memory deficits induced by AD in terms of causing tripping over or contact with an obstacle is still unclear.

Quadrupedal animals have been used extensively to examine memory-guided limb adjustments during locomotion and the neural circuits involved in memory-guided limb movements[12–14]. In a study of cats, McVea and Pearson examined the capability of working memory to guide the movement of the hindlimbs[12]. They devised a task in which walking cats were induced to stop for food after only the forelimbs had crossed an obstacle; the cats were induced to straddle the obstacle for a certain range of times. When the cats began to walk forward again, their hindlimbs were required to clear the obstacle on the basis of the memory of the obstacle. It was found that the cats could precisely control hindlimb movements using their memory of the obstacle characteristics, such as height and width. This study demonstrates the importance of memory-guided limb control for stepping over an obstacle and suggests that memory deficits may be an important contributor to coming in contact with and/or tripping over obstacles during locomotion.

In the present study, we used triple transgenic (3xTg) mice that were generated as a model of AD; the 3xTg mice develop age-related memory deficits associated with the accumulation of β-amyloid (Aβ) and neurofibrillary

tangles similar to those found in human AD patients[17]. AD-like characteristics in the brains of the mice are mainly observed in areas related to memory function, such as the neocortex, hippocampus, and amygdalae. In behavioral tests, deficits in long-term reference memory and spatial working memory are manifested by ages of 4 and 7 months, respectively[18,19]. However, the mice do not generally show motor deficits related to muscle strength, balance, or visuomotor coordination even ages of up to 15–18 months[20-22]. In most studies assessing motor deficits in 3xTg mice, conventional motor tasks were used, such as the rotarod, ladder, and beam walk tests. However, as these simple tasks cannot examine the detailed kinematics of limb movements during locomotion, it is unclear whether AD in 3xTg mice affects the kinematics of unobstructed locomotion or obstacle avoidance locomotion.

The goal of this study was to determine whether the working memory deficits that develop in the AD mouse model induce inaccurate movements when stepping over an obstacle during locomotion. To this end, we observed the behavior of freely moving mice as they stepped over an obstacle; in another experiment, we measured the parameters of hindlimb kinematics during unobstructed locomotion and obstacle avoidance locomotion. We found that the leading hindlimb in Old-3xTg mice more frequently hits the obstacle than that in control mice, although we did not find evidence of any change in limb kinematics during unobstructed locomotion or stepping over the obstacle due to AD-like characteristics. However, using an interrupted obstacle avoidance task, which is similar to that described above for cats[12], we found that Old-3xTg mice showed deficits in their working memory. Our findings suggest that the higher contact rate in Old-3xTg mice is a consequence of AD-related working memory dysfunction. This is novel evidence for the concept that working memory deficits associated with AD can affect the process of stepping over an obstacle during locomotion.

Results

Increased frequency of obstacle contact in Old-3xTg mice. We determined whether memory deficits associated with the AD-like phenotype of 3xTg mice increase the risk of tripping or stumbling over an obstacle. The mice were habituated to the runway apparatus and trained to move freely from one end to the other. After training, we recorded and analyzed the locomotor behavior of wild-type (WT), and young and Old-3xTg mice (Young-3xTg and Old-3xTg, respectively) in a series of trials. We counted the number of first contacts of each limb with the obstacle to obtain the contact rate for each of the four limbs. Typical obstacle avoidance behavior in a successful trial compared with that in a trial in which contact occurred is shown in Figs. 1a and 1b. We found that the median (interquartile range) of the interval for the passage of all limbs was not significantly different among the three groups (WT = 595(465−725) ms, Young-3xTg = 535(480−595) ms, Old-3xTg = 505(455−683) ms; p = 0.282; Kruskal-Wallis test). The total number of contacts of all limbs (Fig. 1c) did not differ significantly among the groups ($F_{2,21} = 1.637$, $p = 0.218$; one-way ANOVA). When we examined the behavior of each limb separately, we found no significant difference in the number of contacts among the mice for the forelimbs or trailing hindlimb (Fig. 1d; leading forelimb (LF): $F_{2,21} = 0.626$, $p = 0.544$; trailing forelimb (TF): $F_{2,21} = 0.523$, $p = 0.6$; trailing hindlimb (TH): $F_{2,21} = 0.174$, $p = 0.842$; one-way ANOVA). However, we identified a significantly increased number of contacts of the leading hindlimb of Old-3xTg mice (Fig. 1d; leading hindlimb (LH): $p < 0.001$, Kruskal–Wallis test). We calculated the rate of contact of each limb (Figs. 1e–h) and found that the leading hindlimb of Old-3xTg mice showed the highest rate (Fig. 1g; LH: $p < 0.001$, Kruskal–Wallis test); none of the other limbs showed a significant difference in the rate of contact among the groups (Fig. 1e; LF: $F_{2,21} = 0.626$, $p = 0.544$; Fig. 1f;

TF: $F_{2,21} = 0.572$, $p = 0.573$; Fig. 1h; TH: $F_{2,21} = 0.47$, $p = 0.954$; one-way ANOVA). In this task, there was no evidence of learning effects on stepping behavior after habituation (Fig. 1i; $F_{9,189} = 1.394$, $p = 0.193$; two-way repeated measures ANOVA).

Because the WT and Old-3xTg mice used this experiment were similar in age, we also examined whether age affects the frequency of contact with an obstacle using 17–18 month-old WT mice and whether Aβ accumulation, which is the main pathologic feature of human AD, occurs in the brain in middle-aged and old WT mice (see later section, "Aβ accumulation"). The total number of contacts of all limbs and the number of contacts of each limb in the old WT mice were the same as those in the WT mice at 2–8 months of age (Supplementary Fig. 1). These results may support our hypothesis that the higher contact rate in Old-3xTg mice did not result from normal ageing.

Temporal and spatial parameters of leading and trailing limbs when stepping over an obstacle. We noted that when a stepping limb came in contact with an obstacle, the characteristics of the leading and trailing limb movements differed (Fig. 1b). The difference in kinematics between the leading and trailing limbs of mice when stepping over an obstacle is similar to that observed in the hindlimbs of cats[23] (Figs. 1a,b). Typical toe trajectories in successful trials and those that resulted in contact are illustrated in Fig. 2a. The toe trajectories of the trailing forelimb and hindlimb did not change markedly following contact with the obstacle compared with successful trials (Fig. 2a, see inset). However, the toe trajectories of the leading forelimb and hindlimb were disrupted upon contact with the obstacle; immediately following the contact, the toes appeared to show compensatory elevation (Fig. 2a, see insets). This observation was supported by the finding that the velocity of the leading fore- or hindlimb initially decreased and then increased after obstacle contact (Fig. 2b). No change in the velocity of the trailing limbs after obstacle contact was observed. Our findings indicate that the toe trajectories of the leading limbs are disturbed by the obstacle contact, whereas those of the trailing limbs are unaffected.

Next, we analyzed spatial parameters of the fore- and hindlimbs of mice stepping over an obstacle. In particular, we examined the horizontal distance between the foot placement and the obstacle (toe-obstacle distance) immediately before crossing the obstacle. The toe-obstacle distances of the leading limbs did not differ significantly among the groups (Fig. 2c; LF: among groups, $F_{2,240} = 2.245$, $p = 0.108$; successful trial in which no contact was made with obstacles (hereafter, successful trials) vs trials in which contact was made with obstacles (hereafter, unsuccessful trial), $F_{1,240} = 1.149$, $p = 0.285$: LH: among groups, $F_{2,240} = 2.057$, $p = 0.130$; successful trial vs unsuccessful trial, $F_{1,240} = 0.121$, $p = 0.728$; two-way ANOVA). The trailing limbs of WT and Old-3xTg mice showed shorter toe-obstacle distances in unsuccessful trials than in successful trials (Fig. 2c; TF: among groups, $F_{2,240} = 9.925$, $p < 0.001$; successful trial vs unsuccessful trial, $F_{1,240} = 29.502$, $p < 0.001$: TH: among groups, $F_{2,240} = 2.245$, $p = 0.108$; successful trial vs unsuccessful trial, $F_{1,240} = 1.149$, $p = 0.285$; two-way ANOVA).

We next conducted the rotarod test to determine whether the abilities of balance and motor coordination of Old-3xTg mice were adversely affected compared with those of control mice. Retention time improved in all groups during repeated trials and did not show any significant differences among the groups (Fig. 2d; between retention times, $F_{9,189} = 33.642$, $p < 0.001$; among groups, $F_{2,21} = 0.024$, $p = 0.976$; two-way repeated measures ANOVA). This finding is consistent with previous reports that Old-3xTg mice do not show motor deficits.

Overall, our results suggest that contact with the obstacle by the leading limbs resulted from inaccurate limb elevation, whereas that by the trailing limbs resulted from foot placement too near the obs-
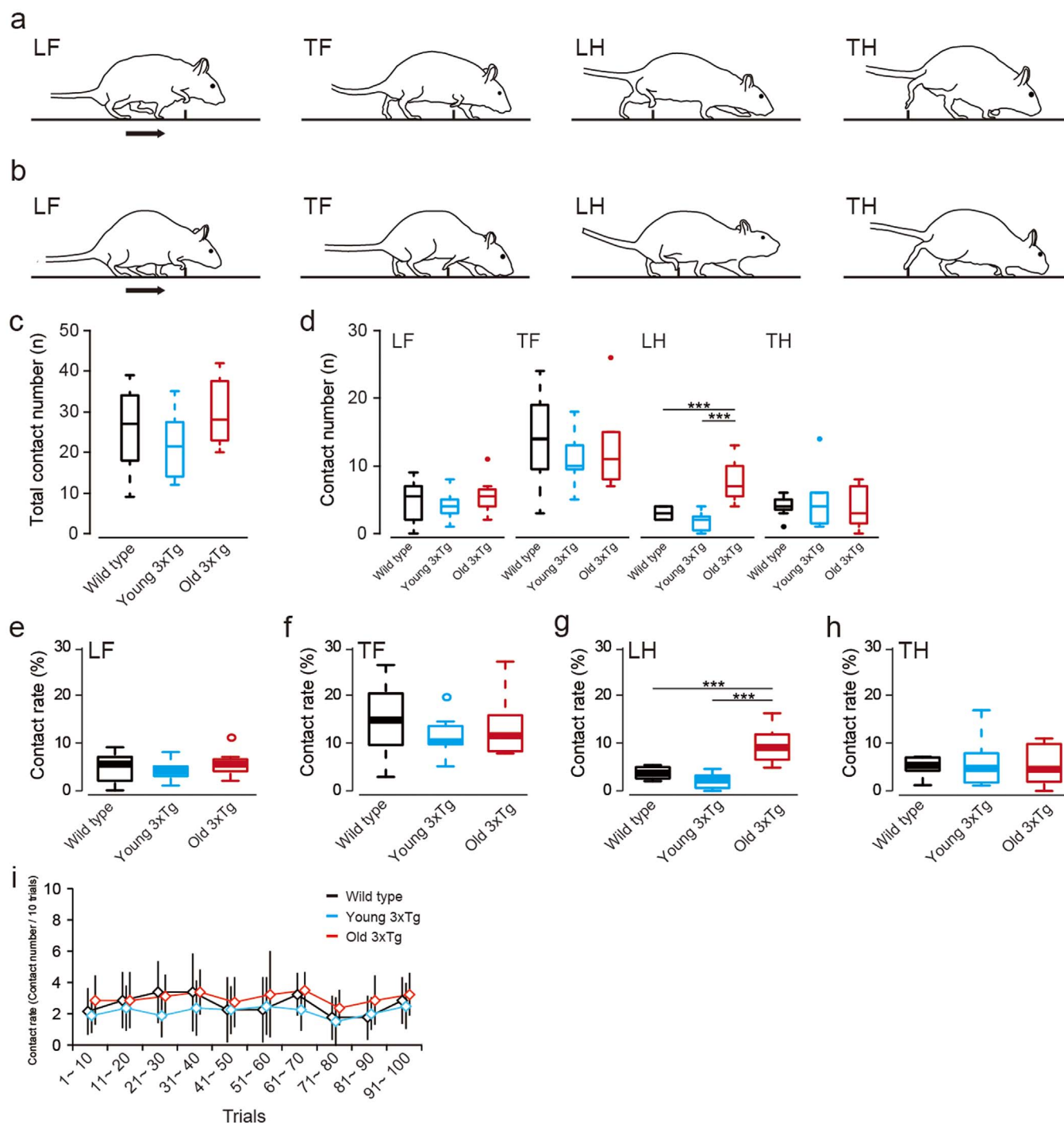
Figure 1 | Increased contact rate in Old-3xTg mice. (a–b) Tracings from a video of typical behavior in accurate stepping (a, successful trials) and contact with obstacle (b, unsuccessful trials) in Old-3xTg mice. (c–d) Box plots showing the total number of contacts (c), number of contacts of each limb (d), and the contact rate for each limb (e–h) in WT, Young-3xTg, and Old-3xTg mice. The horizontal line in the box shows the median number. Whiskers with the box indicate the lowest and highest data within a 1.5-interquartile range of the lower and upper quartiles. Circles indicate the data beyond the whisker ends. LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb. (i) Mean number of contacts in 10 trials. All averages are from 8 animals. Data was analyzed by one-way ANOVA, Kruskal–Wallis test (c-h) and two-way repeated measures ANOVA (i), followed by individual comparison using Bonferroni post-hoc test and Mann–Whitney U-test with Bonferroni adjustment. Values are mean ± s.d. ***$p < 0.001$. The illustration created by S.S. using Adobe Illustrator software.

tacle. Additionally, our results indicate that the increased rate of contact of the leading hindlimb of Old-3xTg mice was not the consequence of disabilities of balance and motor coordination. As our results discounted the effect of motor deficits, we next explored the possibility that the increased rate of contact in Old-3xTg mice is associated with increased gait disturbance.

Kinematics of limb movements during unobstructed locomotion. In previous investigations of motor function changes in aging 3xTg mice, relatively simple tasks were used[20,21]; therefore, we cannot exclude the possibility that the increased rate of contact with an obstacle in Old-3xTg mice resulted from movement and gait disorders associated with the development of AD-like charac-
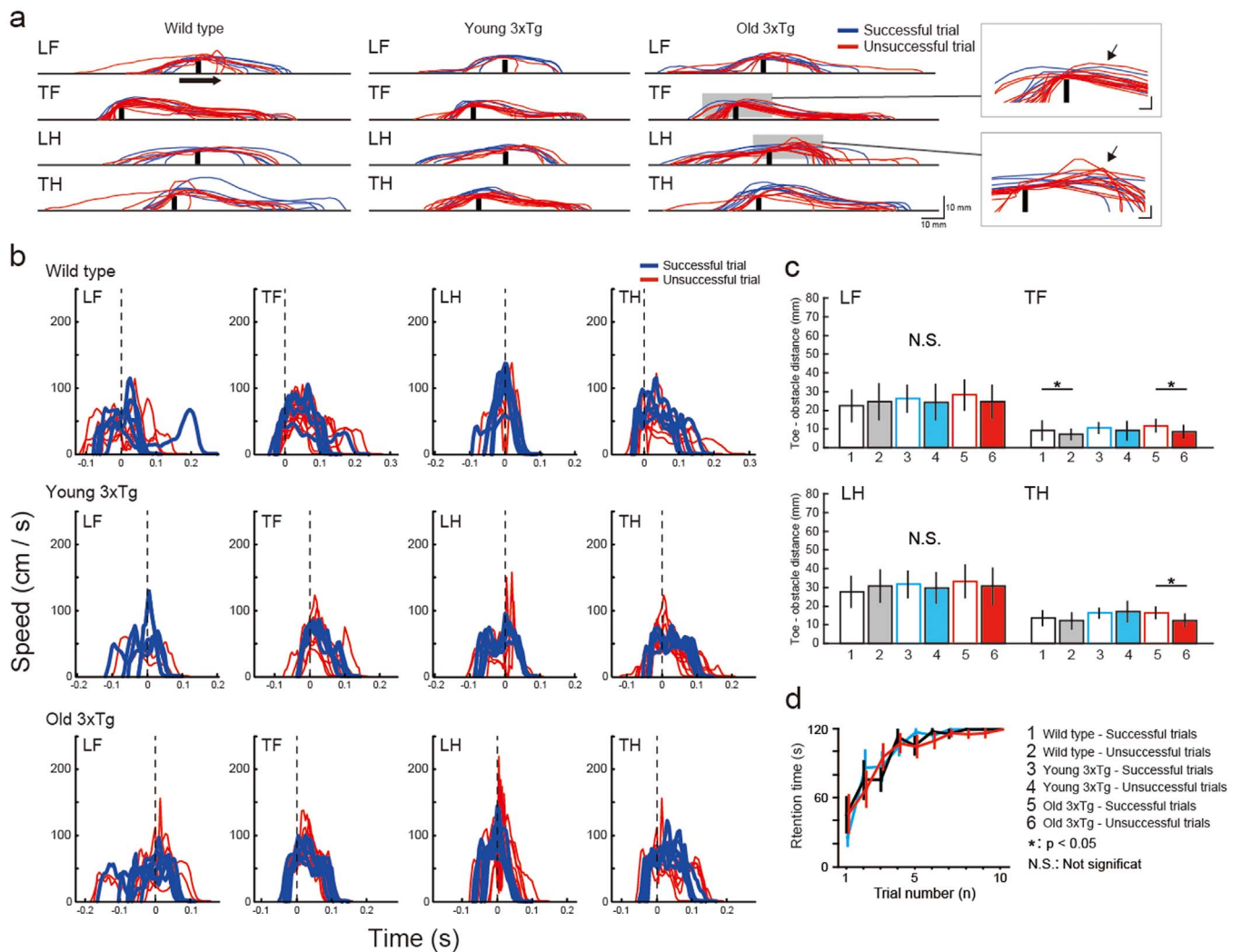
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Figure 2 | Spatial and temporal parameters during stepping over obstacle. (a) Superimposed typical toe trajectories of a WT (left), a Young-3xTg (center), and an Old-3xTg (right) mouse stepping over an obstacle. The blue and red lines indicate successful and unsuccessful trials, respectively. The vertical black bar indicates the obstacle (6 mm). The horizontal black arrow indicates the direction of movement. The insets show enlargements of the shaded areas. In the insets, the black arrows indicate the toe trajectory after making contact with the obstacle. Scale bars indicate 2.5 mm. (b) Superimposed typical toe velocity of a WT, a Young-3xTg, and an Old-3xTg mouse stepping over an obstacle. Blue and red lines indicate successful and unsuccessful trials, respectively. Vertical dashed lines show the time at which their limbs were immediately above the obstacle. LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb. (c) Toe-obstacle distances immediately before stepping over an obstacle. (d) Rotarod retention times for WT, Young-3xTg, and Old-3xTg mice. All averages are from 8 animals. Data was analyzed by two-way ANOVA (c), and two-way repeated measures ANOVA (d), followed by individual comparisons using the Bonferroni post-hoc test and Mann–Whitney U-test with Bonferroni adjustment. Values are mean ± s.d. (c) or s.e.m. (d). *$p < 0.05$.

teristics. To determine whether gait disturbance contributed to the increased rate of contact with the obstacle, we performed detailed analyses of hindlimb kinematics during unobstructed locomotion and obstacle avoidance locomotion (see next section).

Walking speed did not vary significantly between the groups (Table 1). Likewise, maximal toe height and stride length did not significantly differ during unobstructed locomotion (Table 1). The duration of the swing phase also did not show any significant difference among the three groups (Table 1). Thus, our results indicate that the increased rate of contact with an obstacle in Old-3xTg mice was not associated with gait disturbance during unobstructed locomotion.

Kinematics of stepping over an obstacle during locomotion. Next, we investigated whether the higher contact rate in Old-3xTg mice is associated with changes in obstacle avoidance performance. To this end, we analyzed hindlimb kinematics when the animals successfully

stepped over an obstacle during locomotion. The typical kinematics of the hindlimbs is illustrated in Fig. 3a. Walking speed did not vary significantly among the groups nor was it affected by obstacle height (Fig. 3b; among groups, $F_{2,63} = 0.860$, $p = 0.428$; among obstacles, $F_{2,63} = 0.650$, $p = 0.526$; two-way ANOVA). For the leading and trailing limbs, the toe heights immediately above the obstacle in the stepping phase increased significantly with obstacle height, but they were not significantly different among the three groups (Fig. 3c; leading limb: among groups, $F_{2,63} = 3.099$, $p = 0.052$; among obstacles, $F_{2,63} = 39.984$, $p < 0.001$; trailing limb: among groups, $F_{2,63} = 1.902$, $p = 0.158$; among obstacles, $F_{2,63} = 8.816$; $p < 0.001$; two-way ANOVA). The toe-obstacle distance immediately before stepping over the obstacle did not vary significantly among the three groups (Fig. 3d; leading limbs: among groups, $F_{2,63} = 1.398$, $p = 0.257$; among obstacles, $F_{2,63} = 0.250$, $p = 0.780$; trailing limbs: among groups, $F_{2,63} = 2.639$, $p = 0.079$; among obstacles, $F_{2,63} = 0.197$, $p = 0.822$; two-way ANOVA). The swing durations of the

Table 1 | Numerical and statistical parameters for kinematic analysis during unobstructed locomotion

	Wild type	Young 3xTg	Older 3xTg	Group F value	P value
Working speed (mm/s)	174.3 ± 8.9	175.2 ± 6.9	184.7 ± 18.2	$F_{2,21} = 0.347$	0.711
Maximal toe height (mm)	2.7 ± 0.2	3.1 ± 0.2	3.2 ± 0.3	$F_{2,21} = 0.907$	0.419
Stride length (mm)	64.8 ± 2.2	60.0 ± 1.9	65.0 ± 2.4	$F_{2,21} = 1.662$	0.214
Swing duration (mm)	142.7 ± 2.7	137.0 ± 2.4	142.9 ± 3.6	$F_{2,21} = 1.260$	0.304

Numerical and statistical parameters at overground locomotion. All averages are from 8 animals. Data was analyzed by one-way ANOVA. Values are mean ± s.e.m.

leading and trailing limbs when stepping over an obstacle did not differ significantly among the three groups (Fig. 3e; leading limb: among groups, $F_{2,63} = 0.548$, $p = 0.581$; among obstacles, $F_{2,63} = 0.759$, $p = 0.472$; trailing limb: among groups, $F_{2,63} = 2.686$; $p = 0.076$; among obstacles, $F_{2,63} = 0.431$, $p = 0.651$; two-way ANOVA). Our analyses showed that Old-3xTg mice could achieve adaptive control of hindlimb movements during both the approach and stepping phases; thus, the higher contact rate in these animals did

not result from movement and gait disorders. As these disorders did not appear to be of significance with regard to the increased contact rate in Old-3xTg mice, we next investigated whether cognitive deficits are involved.

Do working memory deficits affect memory-guided hindlimb movements?. Previous studies have shown that working memory has an important role in the successful movement of hindlimbs
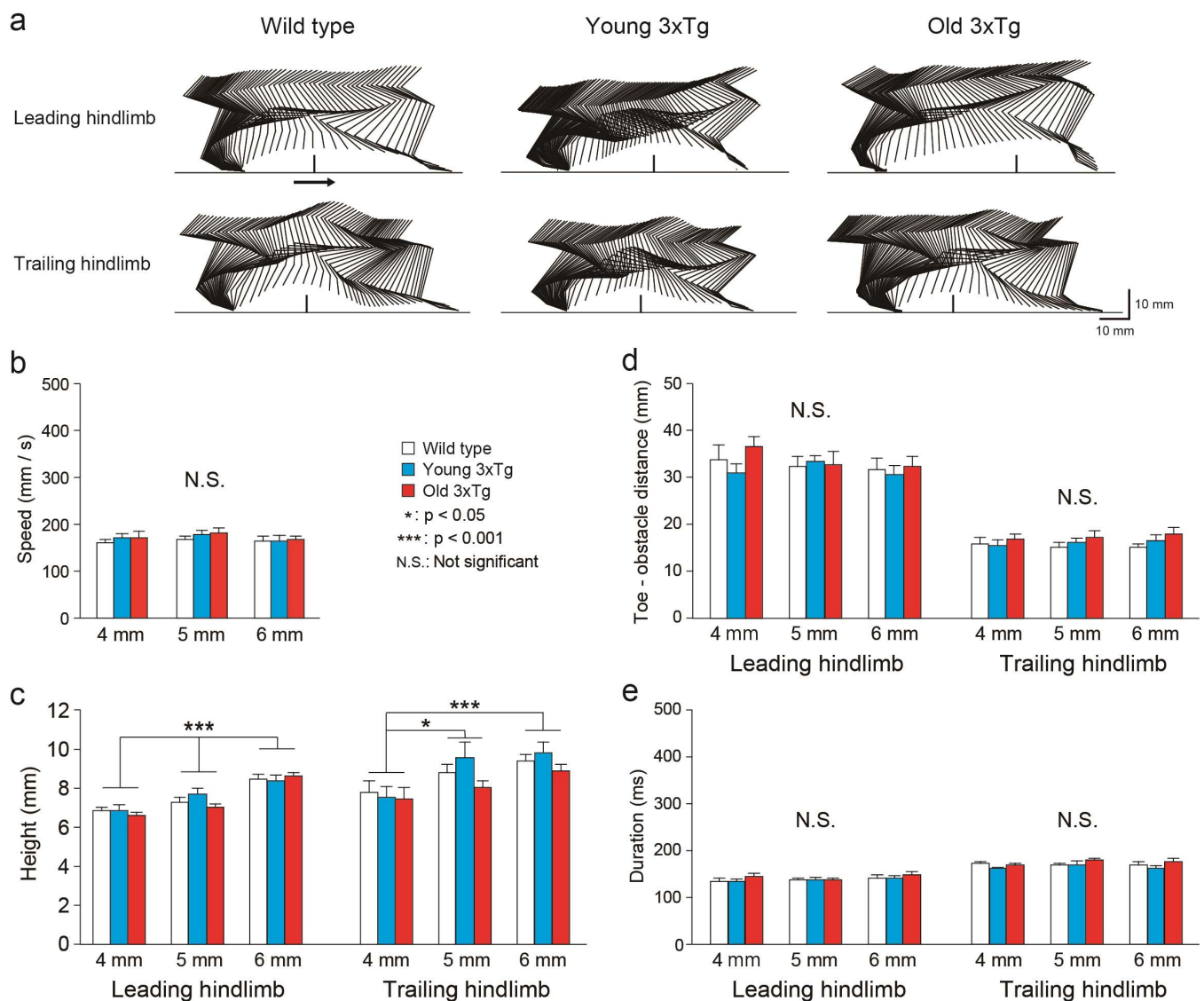

Figure 3 | Kinematic analysis of stepping over obstacle. (a) Stick figures of typical hindlimb movements in WT, Young-3xTg, and Old-3xTg mice during the swing phase of stepping over an obstacle from lift-off to land-on. The vertical black bar indicates the obstacle (6 mm). The horizontal black arrow indicates the direction of movement. (b–e) Spatial and temporal parameters: walking speed (b), maximal toe height during unobstructed locomotion (c), toe-obstacle distance (d) and swing duration (e). All averages are from 8 animals. Data was analyzed by two-way ANOVA, followed by individual comparison using Bonferroni post-hoc test. Values are mean ± s.e.m. *$p < 0.05$, ***$p < 0.001$. N.S = not significant.

when stepping over an obstacle during locomotion. We therefore investigated whether impaired working memory in Old-3xTg mice increases the contact rate owing to the reduced control of the leading hindlimb when stepping over an obstacle. To examine the state of working memory during locomotion, we used the delayed obstacle avoidance task designed by McVea and Pearson[12]. In this experiment, a mouse was induced to stop its forward locomotion by placing food immediately after the obstacle; the mouse only lifted its forelimbs over the obstacle before it ceased walking (see Fig. 4a). The mouse was left in this obstacle-straddling posture for ~30 s (delay period); the obstacle was carefully removed from the runway, and at the end of the designated delay period, the feeding dish was pulled forwards away from the mouse, which then restarted its forward locomotion to chase after the dish (Fig. 4a; Material and Methods). To examine whether animals memorize obstacle characteristics such as height, the maximal toe height of the first step of the mouse after the delay period was measured. For this experiment, we used the same groups of mice as in the experiments above, namely, WT, Young-3xTg and Old-3xTg. Because one of the Old-3xTg mice was excluded as it refused to move from the start position and two 3xTg mice died prior to the end of the experiment, four new Old-3xTg mice were added. The abdominal height of each mouse was measured to examine whether they maintained the appropriate standing posture during the delay period. The abdominal height did not significantly differ among different obstacle heights (Fig. 4b).

For WT mice, the maximal toe height, which exceeded the obstacle height, was largely sustained over the delay period (Fig. 4c), that is, these mice lifted their first stepping hindlimb above the obstacle height and this movement did not change even over a relatively prolonged delay. Moreover, their maximal toe height was accurately controlled for different obstacle heights after different delay periods (Fig. 5a; within 5 s, $F_{2,21} = 15.750$, $p < 0.001$; Fig. 5b; over 5 s, $F_{2,21} = 22.176$, $p < 0.001$; one-way ANOVA). On the other hand, when the mice were stopped immediately before their forelimbs stepped over an obstacle, this memory-guided hindlimb movement was impaired even for short delay periods (Supplementary Figs. 2a,b). These findings are consistent with those of previous studies of cats[12,13,24]. We therefore believe that the delayed obstacle avoidance task can measure the capability of working memory to guide the leading hindlimb of mice stepping over an obstacle.

In contrast to WT mice, the maximal toe height in Young-3xTg mice gradually decreased with the delay period; this effect was particularly evident for the 6 mm obstacle (Fig. 4c) when the delay period was over 10 s. However, the maximal toe height in Old-3xTg mice varied even for short delay periods (Fig. 4c; within 5 s). For the Young-3xTg mice, their maximal toe heights were accurately controlled according to the obstacle height even after longer delay periods (Fig. 5a; within 5 s, $F_{2,19} = 4.153$, $p = 0.032$; Fig. 5b; over 5 s, $F_{2,19} = 11.169$, $p = 0.001$; one-way ANOVA). Although Old-3xTg mice were able to control their toe height when the delay period was less than 5 s, they were unable to maintain the memory of the obstacle height when the delay period exceeded 5 s (Fig. 5a; within 5 s, $F_{2,24} = 5.502$, $p = 0.011$; Fig. 5b; over 5 s, $F_{2,24} = 1.045$, $p = 0.367$; one-way ANOVA). The stride length of Old-3xTg mice was larger than that of WT mice; however, it was not significantly different between the groups at any obstacle height (Fig. 5c; among groups; $F_{2,88} = 4.033$, $p = 0.021$; among obstacles, $F_{2,88} = 2.005$, $p = 0.119$; two-way ANOVA). Our analyses indicate that memory-guided movements based on obstacle height were less accurate in Old-3xTg mice, particularly with longer periods of delay. Moreover, to confirm whether Old-3xTg mice show progressive impairment of spatial working memory, we used the Y-maze test. The total number of arm entries was not significantly different among the groups (Fig. 5d; total entry; $F_{2,21} = 0.321$; $p = 0.729$; one-way ANOVA), but Old-3xTg mice showed significantly reduced spontaneous

alternative behavior (Fig. 5e; % alternation; $F_{2,21} = 4.185$; $p < 0.05$; one-way ANOVA). These results are consistent with previous reports that the spontaneous alternative behavior of 3xTg mice over 7 months of age was impaired[19,25]. Therefore, we conclude from our results that the increased rate of contact of the leading hindlimb of Old-3xTg mice is likely to be associated with deficits in their working memory.

Aβ accumulation. To examine the relationship between the behavioral changes in Old-3xTg mice and AD-like pathologic characteristics, we assessed Aβ accumulation in the cortex (Figs. 6b–d) and hippocampus (Figs. 6f–h). As shown in Figs. 6b and 6f, no Aβ accumulation was observed in the cortex and hippocampus of WT mice. In Young-3xTg mice, mild Aβ accumulation was observed in the cortex (Fig. 6c) but not in the hippocampus (Fig. 6g). However, the cortex and hippocampus of the Old-3xTg mice showed marked Aβ accumulation (Figs. 6d,h). In old WT mice, no Aβ accumulation was observed in the cortex and hippocampus (Supplementary Figs. 1d,e). These findings suggest that there is a relationship between the inaccurate stepping over an obstacle owing to working memory deficits and AD-like pathologic characteristics.

Discussion

In this study, we examined the unobstructed locomotion and obstacle avoidance locomotion in 3xTg mice, an animal model for certain aspects of AD. We obtained three major findings: first, the rate of contact of the leading hindlimb was significantly higher in Old-3xTg mice than in WT and Young-3xTg mice; second, Old-3xTg mice showed no changes in limb movements during unobstructed locomotion or obstacle avoidance locomotion; and third, Old-3xTg mice showed poorer retention of the memory of an obstacle for accurate movement after a delay period than the other groups.

The Old-3xTg mice showed no changes in various kinematic parameters, such as stride length, maximal toe height, or walking speed during unobstructed locomotion (Table 1), nor was their performance in the rotarod test impaired (Fig. 2d). We did find a difference in their behavior in the Y-maze test (Fig. 5e). These findings are in agreement with those previously reported for motor performance and cognitive function in 3xTg mice[20,21,26]. On the other hand, human studies showed that AD patients develop motor deficits such as prolonged reaction times, slow movements, and inaccurate reaching behavior[4,27–29]. Although AD patients, even at an early stage, display gait impairments, such as lower speed, shorter stride lengths, and increased step length variability than healthy individuals[30–32], we were unable to observe these features in Old-3xTg mice. One possible explanation for this apparent interspecies difference comes from lesion studies of rats and cats, which showed that quadrupedal locomotion is restored by compensatory changes in the brain several days after lesioning[33,34]. As neurodegenerative disorders in 3xTg mice occur gradually over several months[35], it is possible that this allows sufficient time to develop compensatory locomotion behavior. Additionally, pathological abnormalities, such as intracellular Aβ accumulation, extracellular amyloid deposits, and tau phosphorylation in the primary motor cortex, are relatively moderate in 3xTg mice compared with changes in brain structures related to memory function, such as the hippocampus and amygdalae[35]. These various factors may explain the absence of motor deficits in the Old-3xTg mice.

When an animal came into contact with an obstacle in the stepping phase, the toe trajectories of the leading fore- and hindlimbs were rapidly elevated, whereas the trailing fore- and hindlimb trajectories remained unaltered (Fig. 2a; see insets). In most instances where contact was made, it seemed that the toe trajectories of the trailing fore- and hindlimbs graze an obstacle (Fig. 2a; see insets). It was reported previously that the toe trajectories and kinematics of
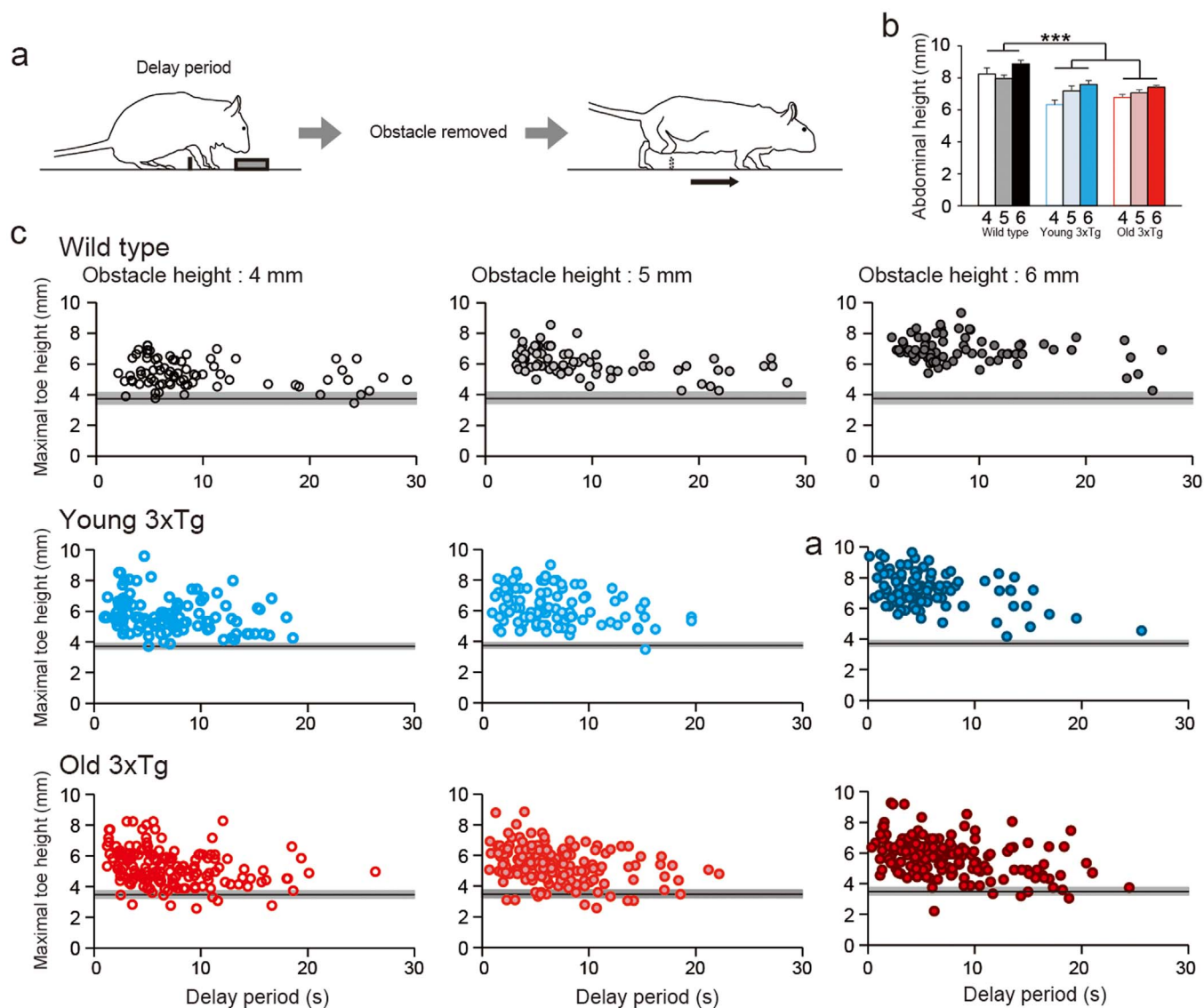
Figure 4 | Delayed obstacle avoidance task. (a) Tracings of typical behavior in delayed obstacle avoidance task in an Old-3xTg mouse. During the delay period, the obstacle was removed. Vertical black and gray bars indicate the obstacle and its original position after removal, respectively. (b) Abdominal heights just before restarting after delay periods: 4, 4 mm; 5, 5 mm; 6, 6 mm. (c) Scatter plots of maximal toe heights of the first step after the delay period. These data show only the leading hindlimb. The horizontal black and shaded lines indicate the maximal toe height of the control and s.e.m, respectively. WT (n = 8), Young-3xTg (n = 8), and Old-3xTg (n = 9) mice were used in this experiment. Data was analyzed by two-way ANOVA, followed by individual comparison using Bonferroni post-hoc test. Values are mean ± s.e.m. ***$p < 0.001$. The illustration created by S.S. using Adobe Illustrator software.

the trailing fore- and hindlimbs of quadrupeds differ from those of the leading fore- and hindlimbs when stepping over an obstacle[23]. The toe trajectory of the trailing limb is controlled by the shoulder and wrist joint[9], and the peak toe position of this limb over an obstacle varies to a greater extent than that of the leading forelimb. In contrast, the toe trajectory of the leading forelimb is controlled mainly by the activity of the elbow flexor and extensors, and is more accurately regulated than that of the trailing forelimb[9]. The velocity profiles of the toes of the trailing limbs did not differ significantly between successful and unsuccessful trials (Fig. 2b; TF and TH). However, the velocity of the leading limbs initially decreased upon contact with the obstacle and then increased (Fig. 2b; LF and LH). Overall, our observation that the rates of contact of the leading fore- and hindlimbs were lower than that of the trailing limb might be due to these different characteristics of kinematic and movement parameters between the leading and trailing limbs. It has been proposed that the leading fore- and hindlimbs require more accurate control

than the trailing limbs[9,33]. Our finding here that the increased contact rate affected only the leading hindlimb supports the interpretation that memory-guided movements during obstacle crossing were impaired in these AD-like mice.

Although the increased contact rate in Old-3xTg mice might be due to deficits in certain aspects of visually-guided movements, we suggest that the present results do not support this. First, the rate of contact of forelimbs in Old-3xTg mice was not affected (Figs. 1e,f). The increased rate of contact of the leading hindlimb in these mice occurred despite the successful movement of the forelimbs. Second, the toe-obstacle distance of the leading forelimb in unsuccessful trials was identical to that in successful trials (Fig. 2c). It was previously reported that limb placement in the final step before stepping over an obstacle is modified to achieve successful crossing on the basis of visual information[36]. Third, in successful trials, the examined spatio-temporal parameters of unobstructed locomotion and obstacle avoidance locomotion did not significantly differ between Old-
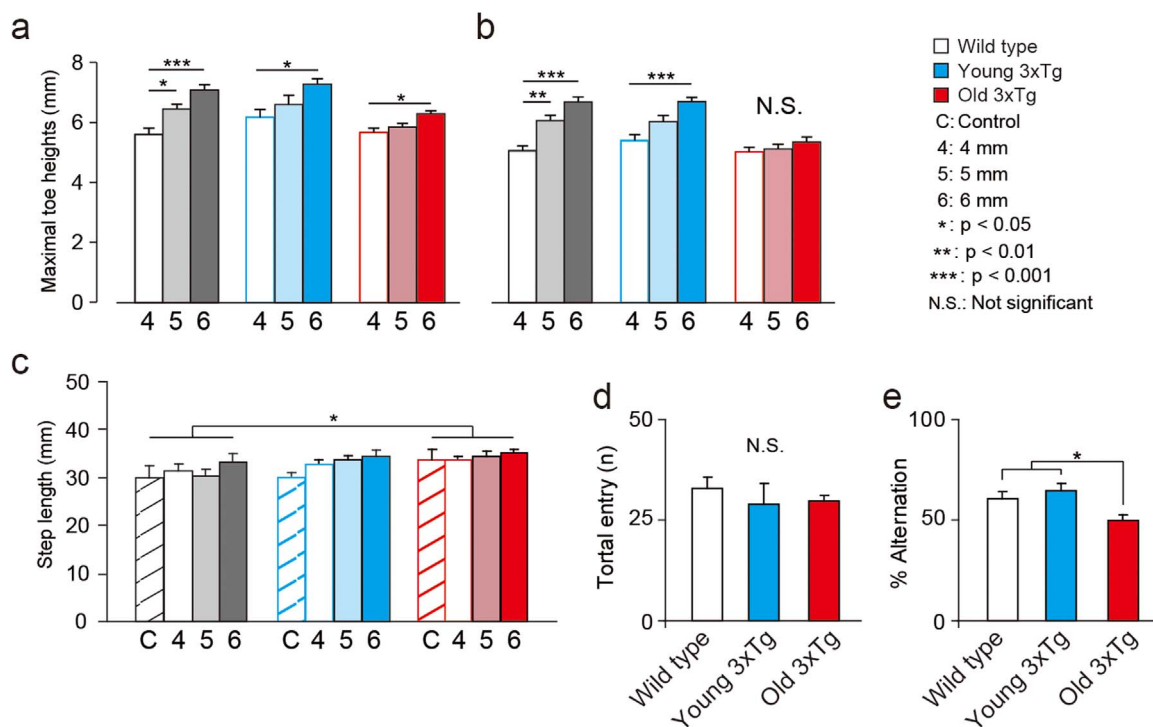
Figure 5 | Working memory deficits in Old-3xTg mice in delayed obstacle avoidance task. (a) Changes in maximal toe height at different obstacle heights. These data show only the leading hindlimb. These graphs show averages of maximal toe heights within 5 s (left) and at 5 s or over (right). (b) Step lengths of the first step after the delay. Data was analyzed by two-way ANOVA, followed by individual comparison using Bonferroni post-hoc test. WT (n = 8), Young-3xTg (n = 7), and Old-3xTg (n = 9) mice were used in this experiment. In these analyses, one Young-3xTg mouse was excluded because no data was available during the ''within 5 s'' period. (d,e) Total number of arm entries (d) and % alternation (e) in spontaneous alternation test using WT (n = 8), Young-3xTg (n = 8), and Old-3xTg (n = 9) mice. Data was analyzed by two-way ANOVA, followed by individual comparison using Bonferroni post-hoc test. Values are mean ± s.e.m. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3xTg and control animals (Table 1 and Fig. 3). These observations suggest that the visuomotor system of Old-3xTg mice has little influence on the planning or execution of adaptive limb movements.

Previous studies of humans and quadrupeds showed that memory functions contribute to the control of limb movements during obstacle avoidance locomotion. For example, during walking, humans can accurately step over an obstacle even when online visual information is blocked several steps before reaching the obstacle[11]. Additionally, recent studies have indicated that accurate stepping movements based on obstacle memory can be performed after a delay period of 2 min or longer[37]. In quadrupeds, hindlimb movements during obstacle avoidance locomotion are assumed to be con-

trolled using stored information related to the obstacle height because these movements occur when there is no visual information available to the animal[15]. Additionally, the toe height of hindlimbs during obstacle avoidance locomotion or after a delay period is modified according to the height of the obstacle[12,36,38]. Our analyses of kinematic parameters yielded results consistent with those of previous studies showing that the hindlimb movements of quadrupeds depend on memory-based limb control (Figs. 4c, 5a, and 5b). The posterior parietal cortex has been suggested to be required for the appropriate movements of the hindlimbs based on working memory during locomotion[13]. Neurons in the posterior parietal cortex of cats show persistent activity when the animals straddle an obstacle during
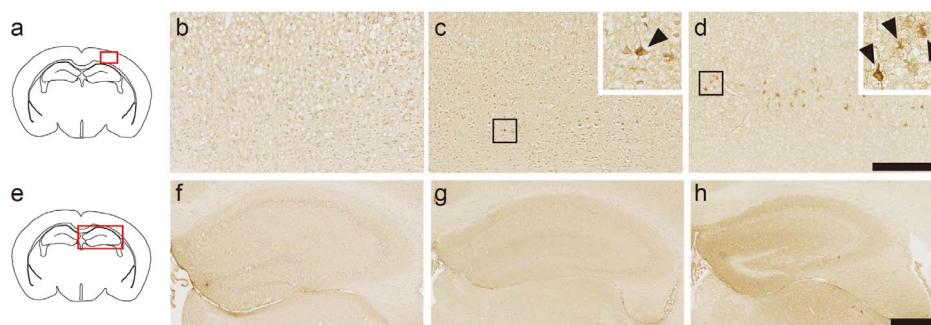

Figure 6 | Amyloid β deposition in cortex and hippocampus. Schematics of coronal mouse brain sections (a,e). Red boxes indicate the region shown in the panels showing the cortex (b–d) and hippocampus (f–h), respectively. (b,f) No Aβ depositions were visible in the cortex (b) or hippocampus (f) of WT mice (8 months old). (c,g) The cortex of Young-3xTg mice (5 months old) has slightly visible amyloid plaques (c), but no plagues were observed in the hippocampus (g). (d,h) The Old-3xTg mice (13 months old) show markedly larger Aβ depositions. Black boxes indicate the region shown in insets (c,d, insets) and arrow heads indicate Aβ deposition. Scale bar, all images: 250 μm (b–d) and 500 μm (f–h). Original magnifications, 10× (b–d), 80× (c,d, insets), 5× (f–h).

normal obstacle avoidance locomotion and when a delay is introduced into the task[39]. Cats with a lesion in the medial part of the posterior parietal cortex show an increased number of contacts of the hindlimbs with an obstacle[40]. These various reports indicate that inappropriate obstacle crossing behavior may occur as a consequence of working memory deficits. However, these studies also showed that inappropriate foot placement prior to an obstacle can result from impaired motor planning due to a lesion in the posterior parietal cortex. Our results do not suggest that deficits in motor planning or execution are associated with the development of AD-like symptoms in 3xTg mice; rather, the leading hindlimb of Old-3xTg mice showed a higher rate of contact with an obstacle than the control group (Figs. 1d,g). The Old-3xTg mice had a clear deficit in their working memory for guiding the leading hindlimb (Figs. 4c, 5a and 5b). These findings were consistent with those of previous studies of AD patients, that is, they have impairments of memory-guided reaching movement but not visually-guided reaching movement[27,28]. From an age of 10 months, 3xTg mice exhibit pathological changes in the extraparietal areas of the cerebral cortex such as the frontal and temporal lobes[41,42]. Further studies are required to elucidate whether these pathological changes are related to alterations in working memory in 3xTg mice that might affect their ability to accurately execute memory-guided limb movements during locomotion.

Methods

Animals. In this study, we used 3xTg-AD mutant mice (n = 20) harboring the familial AD mutations (harboring APP_{Swe} and tau_{P301L} transgenes on a mutant $PS1_{M146V}$ knock-in background)[17] and nontransgenic C57BL/6J (WT) mice (n = 18). The 3xTg-AD mice (MMRRC 034830-JAX) were obtained from the Mutant Mouse Regional Resource Center (MMRRC)-JAX, ME, USA. The WT mice ranged in age from 2 to 8 months at the time of the experiments. The 3xTg mice used were either 2 to 5 months old (Young-3xTg) or 10 to 13 months old (Old-3xTg). The present study was approved by the Ethical Committee for Animal Experiments of the University of Tokyo, and was carried out in accordance with the Guidelines for Research with Experimental Animals of the University of Tokyo and the Guide for the Care and Use of Laboratory Animals (NIH Guide) revised in 1996. All efforts were made to minimize the number of animals used and their suffering throughout the course of the experiments. Excluding the recording periods, all animals were provided with food (CE-2; CLEA Japan; Tokyo, Japan) and water *ad libitum*, and were housed under standard conditions (12 h/12 h light/dark cycle, 22°C). During the experimental periods, the body weights of the mice were maintained at more than 85% of those during the *ad libitum* feeding period prior to the experiments.

Apparatus for locomotion studies. The runway box (length, 40 cm; width, 5 cm) was made of transparent acrylic board (thickness, 3 mm). Three obstacles of black acrylic board were used (heights = 4, 5, and 6 mm; depth = 2 mm). The obstacles were located at the midpoint of the runway (20 cm from both ends).

Obstacle avoidance task in freely moving mice. Animals were habituated to the apparatus, feeding dish, and obstacles over a four day period for up to 1 h/day. After habituation, they were placed at one end of the runway box (start position). A door was then opened to allow the mice access to the runway; they were trained to walk along the runway, with an obstacle present, from the start position to a feeding dish at the opposite end. This training was carried out for two days. In this experiment, stepping behavior was captured from one side in the sagittal plane using a high-speed digital image camera system HAS-220 (DITECT, Inc., Tokyo, Japan). The movements of the mice were recorded at 200 frames/s. After training, we performed the obstacle avoidance task by including a 6 mm obstacle on the runway and recorded the number of contacts with the obstacle and the toe trajectories in the step-over phase in freely moving mice. This task was performed numerous times per day until 100 trials had been completed for each mouse (average 35 ± 9 trials/day). When the mouse stopped to groom itself, or reared up, or returned to the start position before reaching the middle of the runway, the trial was excluded. An intertrial interval of more than 30 s was used. Control trials (successful trials) were pseudorandomly collected, to obtain at least 4 trials.

Kinematic analysis of unobstructed locomotion and obstacle avoidance locomotion. To examine and compare the gait characteristics of the three groups of mice, we carried out a kinematic analysis of the hindlimbs during both unobstructed locomotion and obstacle avoidance locomotion. These experiments were performed after the experiment described above. The mice were partially shaved under 2% isoflurane anesthesia to enable the attachment of circular markers to the hindlimb joints (iliac crest, great trochanter, ankle, and fifth metatarsophalangeal joint). Limb movements during locomotion were captured bilaterally using a high-speed camera (200 frames/s). Temporal (walking speed, swing duration) and spatial (stride length,

maximal toe height, toe-obstacle distance, and angular displacement of joints) parameters were analyzed from the recordings. Obstacle heights of 4, 5, and 6 mm were used. The obstacle avoidance task was performed using the same criteria as those in our previous studies: (1) the trailing limb did not leave the ground until the leading limb reached just above the obstacle; and (2) none of the four limbs came in contact with the obstacle[9,36].

Delayed obstacle avoidance task. The animals were habituated to the apparatus, feeding dish, and obstacles for up to 1 h/day over 4 days. After the habituation period, each mouse was placed at one end of the runway (start position). A door in the apparatus was opened and the mouse was encouraged to walk along the runway and over the obstacle to a feeding dish located at the finish position. This training was carried out for two days. Over the next 4–6 days, the mice were trained to complete the delayed obstacle avoidance task; 4 ± 1 days for WT and Young-3xTg mice and 6 ± 2 days for Old-3xTg mice. Mice walking along the runway were induced to stop when their forelimbs were straddling the obstacle by presenting them with a feeding dish. During the delay period of up to 30 s, the obstacle was removed from the runway while the mice were distracted by the food. After the allotted delay period, the feeding dish was pulled forwards, inducing the mice to follow. In this experiment, obstacle heights of 4, 5, and 6 mm were used. During the training period, obstacles of different heights were randomly used on the runway. After each recording period, the runway was randomly reset with or without obstacles; an intertrial interval of 1–3 min was used. The movements of the mouse limbs were captured in the sagittal plane using a high-speed digital image camera system (100 frames/s). On the day prior to recording, the abdominal pelage of the mice was shaved under 2% isoflurane anesthesia to prevent it from making contact with the obstacle. Recording for about 1 h/day was performed. Data were collected using the following criteria: (1) when the mice stepped over the obstacle, none of the limbs came into contact with the obstacle and (2) the delay period was measured from the moment the mice had all four legs on the runway surface and maintained their abdominal height above the original obstacle height. When its abdominal height was less than the original obstacle height during delay period, the trial was excluded from the analysis.

Rotor rod test. To examine the ability of motor coordination, balance, and motor learning, the mice were tested on a rotor rod (Muromachi Kikai, Ltd.). They were placed on a rod rotating at 8 rpm and tested in 10 trials. Retention time was measured as the duration from the time the mouse was placed on the rod until the time it fell. A cutoff time of 120 s was set.

Spontaneous alternation test. After all the locomotion tasks were performed, a spontaneous alternation test using the Y-maze was performed to examine the spatial working memory of the mice. The apparatus was made of black acrylic board: it consisted of three arms (named A, B, and C) diverging at a 120° angle from the center of the maze and each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. A mouse was placed into one arm of the apparatus for ten minutes to habituate it. It was then returned to its cage for 5 min and the apparatus was cleaned with 70% ethanol. After the 5 min interval, the mouse was placed in arm "A" of the apparatus and the test was run for 8 min. All the tests were recorded using a video camera and analyzed later. A mouse was considered to have entered an arm when the body of the mouse excluding the tail entered the arm. When a mouse entered other arms in the order of "ABCACAB", it was measured for spontaneous alternation behaviors "ABC", "BCA", and "CAB". The spontaneous alternation rate (% alternation) was calculated by subtracting two from the total number of entries into different arms in series then dividing by the total number of arm entries and multiplying by 100.

Immunohistochemistry. The mice were deeply anesthetized with pentobarbital (50 mg/kg body weight) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brain was removed from the skull and cryoprotected in 30% sucrose. Brain sections (20 μm) were obtained using a cryostat. The sections were prepared using an M.O.M kit (Vector) and then immunostained with an anti-human amyloidβ (N) (82E1) mouse monoclonal antibody (IBL, Fujioka, Japan). Immunoreactions were visualized using an ABC kit and 3,3'-diaminobenzidine (Vector Laboratories).

Statistical analyses. Kruskal–Wallis one-way ANOVA, one-way ANOVA, two-way ANOVA, and two-way repeated measures ANOVA were carried out to determine the statistical significance of differences using standard statistical tools (SPSS Japan, Inc., Tokyo, Japan). The Bonferroni post-hoc test and Mann–Whitney U-test with Bonferroni adjustment (adjusted $p = 0.0167$) were carried out as necessary to compare means. The level of statistical significance for variables was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Results are expressed as mean ± s.e.m. or ± s.d.

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Acknowledgments

Author contributions

Conceived and designed the experiments: S.S., D.Y. Performed the experiments: S.S., H.Y., T.A. Analyzed the data: S.S., S.E., D.Y. Contributed reagents/materials/analysis tools: S.S., H.Y., D.Y. Wrote the paper: S.S., S.E., D.Y.

Additional information