博士論文

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

（アルツハイマー病モデルマウスにおける障害物回避歩行時の記憶誘導性動作の障害）

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Chapter 1.

Introduction

1-1. Significance of the research related to tripping and falling among patients with Alzheimer's disease (AD)

AD is the most prevalent neurodegenerative disease, accounting for 60–80% of dementia cases. The number of AD cases has continued to increase throughout the world, and the number is expected to triple by 2050 compared with current number of AD patients (World Health Organisation, 2012). Previous studies showed that nearly one-third of AD patients have experienced falls from the onset of their illness, and then about 30% of the patients who reported falling lost the ability to walk independently within a few years (Buchner and Larson, 1987, Morris et al., 1987). It is thought that the increased risk of falling among AD patients reduces quality of life and considerably increases costs of social welfare. Therefore, it is important to elucidate the factors that lead to falls among AD patients.

1-2. AD, cognitive function, and falling

The cognitive deficits of AD are reported to be one of the causes of the increased risk of stumbling and falling among AD patients (Nevitt et al., 1991, Holtzer et al., 2007, Liu-Ambrose et al., 2008, Pijnappels et al., 2010). In a longitudinal study during a 4-year period, patients with AD showed a significantly higher frequency of falls than did older people without dementia, even though greater neurologic deficits, such as extrapyramidal signs, were not observed (Morris et al., 1987). Therefore,

deficits of the cognitive functions may contribute to the increased risk of falling in the older people with dementia (Tinetti et al., 1988, Sheridan and Hausdorff, 2007).

Executive function is a component of the cognitive system and includes retention of sensory information for a short period (e.g. working memory), planning of behaviour, inhibition of inappropriate responses, and switching of the prior behaviour to the acquired rule (Gilbert and Burgess, 2008). It is thought that deficits of executive function contribute to the increased risk of falling and to falls among AD patients (Hausdorff and Yogev, 2006, Herman et al., 2010, Mirelman et al., 2012). In a study involving the instrument called Falls Efficacy Scale-International, which assesses the history of falls and self-reported fear of falling, older people with AD and mild cognitive impairment (MCI) showed a higher number of falls than did age-matched cognitively healthy people (Borges et al., 2014). To compare the fall risk among patients at different stages of AD, Coelho et al. (2012) subdivided AD patients into 2 groups based on the Mini Mental State Examination (MMSE) score; this score was used to evaluate cognitive statements (a score of ‘27 and over’ indicated normal cognition, ‘19-26’, ‘18-10’, and ‘9 and below’, indicated mild impairments, moderate impairments, and severe impairments, respectively) (Folstein et al., 1975). They reported that the moderate-AD group is at a higher risk of falls than the mild-AD group is (Coelho et al., 2012). These results suggest that cognitive dysfunction of AD, such as problems with executive function and working memory, is associated with an increased frequency of falls and a higher risk of falling.

1-3. Previous studies on memory-guided obstacle avoidance during locomotion

1-3-1. Obstacle avoidance locomotion in humans

As mentioned above, the deficits in executive function, among other cognitive functions, are associated with falls and fall risk. Previous studies showed that tripping when avoiding obstacle accounts for ~30–50% of all causes of falling (Blake et al., 1988, Berg et al., 1997). The obstacle avoidance tasks have been used to examine the relationship between the causes of tripping on obstacles and cognitive function, such as executive function and working memory. Older people with worsened executive function show an increased number of obstacle contacts during stepping over an obstacle (Di Fabio et al., 2004). These findings show that executive function is important for accurate obstacle clearance without tripping and obstacle contact. In humans, the memory part of executive function is important for successful stepping over obstacles (Lajoie et al., 2012, Shinya et al., 2012, Heijnen et al., 2014). Examination of the fixation points before and during stepping over an obstacle showed that visual fixation on the obstacle during stepping over it is not observed in most cases (Patla and Vickers, 1997, Di Fabio et al., 2003a). Similarly, healthy subjects during forward locomotion can accurately clear obstacles without contact even when their visual information is occluded up to 5 steps ahead (Mohagheghi et al., 2004). In addition, if healthy young people during walking and climbing the stairs are provided with delay periods just before obstacle clearing, they can avoid the obstacles without obstacle contact (Lajoie et al., 2012, Shinya et al., 2012). Moreover, Heijnen et al. (2014) suggested that viewing the obstacles in the approach phase facilitates the appropriate memory function to achieve accurate control of leading and trailing limbs during stepping over an obstacle. These reports suggest that the memory system helps

to execute accurate stepping movements in humans.

When the experiments are performed in a self-selected manner (e.g. normal visual conditions and self-selected foot placement), patients with AD exhibit a higher rate of contacts with obstacles than do young and healthy elderly people (Alexander et al., 1995, Di Fabio et al., 2004, Heijnen et al., 2012, Orcioli-Silva et al., 2012). At present, it is believed that the reason for the higher contact rate during stepping over an obstacle is improper foot placement just before obstacle clearing and not, as it was previously believed, inaccurate foot elevation (Chou and Draganich, 1998, Patla and Greig, 2006). Nonetheless, when healthy young subjects step over an obstacle in a self-selected manner, the rate of contacts with obstacles that results from inaccurate foot elevation is slightly higher than that results from incorrect foot placement (Heijnen et al., 2012). In addition, when patients with AD simultaneously perform the motor and cognitive tasks in order to undertake a cognitive load, toe height of a trailing limb significantly decreases and the number of contacts with obstacles increases but there are no changes in foot placement just before obstacle clearing (Orcioli-Silva et al., 2012). Therefore, it is possible that this inaccurate and decreased toe elevation during stepping over an obstacle is induced by the memory deficits resulting from the development of AD. On the other hand, in studies on humans, it is difficult to understand how changes of performance during stepping over an obstacle are induced by changes in the physiological and pathological background. Therefore, to determine why inaccurate obstacle clearing occurs in AD patients or how the progression of the neurodegenerative disease correlates with the changes in performance during stepping over an obstacle, it is necessary to perform experiments on an animal model in parallel with human studies.

1-3-2. Obstacle avoidance locomotion in animals

Quadruped animals, especially cats, are widely used in studies investigating accurate obstacle clearing during locomotion by means of visually- and memory-guided limb adjustments and/or by neuronal circuits in the brain. The studies in cats have shown that the specific cerebral cortical areas, such as the primary motor cortex (M1) and posterior parietal cortex (PPC), are involved in visually guided locomotion (Beloozerova and Sirota, 1993, Drew et al., 1996, Friel et al., 2007, Drew et al., 2008, Marigold and Drew, 2011). On the other hand, previous studies also showed that quadrupeds utilise not only on-line visual information but also memory function to guide their hindlimbs. For example, it is thought that the control of hindlimbs relies on stored visual information because it is processed after the head and eyes pass over the obstacle (Drew, 1993). Therefore, it is believed that the hindlimbs of quadrupeds are useful as a model for studies of neural structures controlling memory-guided limb movements in mammals.

McVea and Pearson (2006) found that working memory contributes to accurate hindlimb movements in cats during stepping over an obstacle. Briefly, the researchers caused walking cats to stop by food after obstacle clearing by means of forelimbs; the cats were provided with delay periods while their fore and hindlimbs straddled the obstacle, and then, when the cats restarted forward locomotion, their hindlimbs had to step over the obstacle on the basis of the memory of the obstacle. Using this task, it was shown that the hindlimbs of cats are controlled by long-lasting and accurate memory (McVea and Pearson, 2006). Moreover, it was found that lesions of the PPC in cats induce remarkable memory deficits (McVea et al., 2009). In electrophysiological studies, it was found that neurons in the PPC activate before and

during the stepping over an obstacle during walking on a treadmill (Lajoie et al., 2010, Marigold and Drew, 2011). Therefore, it is believed that the PPC contributes to motor planning and retention of working memory during visually and memory-guided locomotion. On the other hand, Sato et al. (2012) showed that hindlimbs of rodents might be controlled in accordance with obstacle height on the basis of stored visual information. Thus, it has been thought that working memory that is similar to the one that was characterised in cats may also contribute to the control of hindlimbs in rodents.

Recently, rodents have become popular animal subjects of research because they make it possible to use the techniques of gene modification and pharmacology (Perrot et al., 2011, Takeuchi et al., 2012). Previous studies on rats demonstrated the contribution of basal ganglia and the cerebellum to stepping over obstacles in locomotion (Perrot et al., 2009, Aoki et al., 2013, 2014). Using transgenic mice lacking the α2-chimaerin gene, Asante et al. (2010) showed that the interaction between M1 and spinal circuits during voluntary movements performs an important function. To date, several types of transgenic-mouse models of AD have been designed to elucidate the mechanisms of cognitive deficits and AD pathophysiology. It is thought that these mouse models can help to answer the question whether AD affects the memory-guided movements during locomotion.

1-4. Mouse models of AD

AD is one of the neurodegenerative diseases for which a standard therapy has not been established yet. The degeneration is characterized by 2 main neuropathological features: accumulation of amyloid-beta (Aβ) and neurofibrillary

tangles (Braak et al., 1999, Jagust and Mormino, 2011). Post-mortem examination of the brain of AD patients shows that the neuropathological features are clearly observed in the hippocampus, amygdala, and cortex (Braak and Braak, 1991). These pathological characteristics induce several cognitive deficits, such as problems with executive function, working memory, attention, episodic memory, and spatial orientation (Perry and Hodges, 1999, Ralph et al., 2003). To date, advances in the genetic-modification technology have produced several types of mouse models of AD for studies of the causes of these deficits and helped to generate a lot of knowledge about the molecular, cellular, and pathological changes associated with AD (Hsiao et al., 1996, Lewis et al., 2000, Oddo et al., 2003, Billings et al., 2005). In addition, as described above, quadrupeds are very useful experimental animals for studies not only of kinematics of limb movements during unobstructed and obstacle avoidance locomotion but also of the physiological parameters related to locomotion (Drew et al., 2008, Asante et al., 2010, Takeuchi et al., 2012, Aoki et al., 2013, 2014). Therefore, the use of mouse models of AD should help to elucidate the causes of inaccurate obstacle clearing caused by the development of AD, e.g. tripping and falling.

The Tg2576 mouse model of AD that overexpresses human APP_{swe} genes (KM670/671NL) shows an increased level of insoluble Aβ and age-dependent Aβ accumulation and memory deficits comparable to those in AD patients (Hsiao et al., 1996, Arendash et al., 2004). However, it has been reported that the Tg2576 mouse model of AD does not exhibit neurodegeneration comparable to that in patients with AD, and a period of ~18 months is necessary for the appearance of Aβ plaques. In addition to APP mutations, mutations in presenilin 1 and 2 have also been identified in patients with familial AD (FAD), which manifests characteristics of AD pathology

earlier than the typical AD. The APP/PS1 mouse model of AD exhibits amyloid plaques at 3–5 months of age (Jankowsky et al., 2001). On the other hand, the JNPL3 mouse model of AD that overexpresses human tau_{P301L} exhibits neurofibrillary tangles (Lewis et al., 2000). The JNPL3 transgenic mice were crossed with the Tg2576 transgenic mice to create double-transgenic mice that develop both Aβ deposits and neurofibrillary tangles (Lewis et al., 2001). These mouse models of AD demonstrate that APP and/or Aβ affect the formation and/or fortification of neurofibrillary tangles. To examine the interaction between Aβ and tau-related pathological features in greater detail, the triple-transgenic (3xTg) mouse expressing APP_{swe}, tau_{P301L}, and $PS1_{M146V}$ was generated (Oddo et al., 2003). The 3xTg mice mimic pathological features of AD, such as Aβ plaques, synaptic dysfunction, and tau tangles; these pathological features are easily detectable in the hippocampus, amygdala, and cortex just as in the brain of humans with AD. This mouse model manifests the memory deficits that occur in conjunction with the progression of pathological characteristics of AD; the memory loss manifests itself as the reference memory loss at 4 months of age (Billings et al., 2005), novel-cognitive-memory loss at 6 months of age (Clinton et al., 2007), and deficits in spatial working memory at 7–8 months of age (Rosario et al., 2006). Therefore, this mouse model is believed to be useful for studies of the relationship between pathological features of AD and cognitive deficits. On the other hand, it is known that the 3xTg mice do not have movement disorders. This result was obtained during the assessment of simple tasks related to motor behaviour, and it is unclear whether the deficits of memory-guided movement are induced by the development of AD.

1-5. The purpose of this study

In previous studies, working memory, including executive function, was implicated in limb movements without on-line visual information during stepping over an obstacle in locomotion. In AD patients, tripping is one of the main causes of falling, and it is possible that the deficits of working memory resulting from the development of AD causes contact with the obstacle during obstacle clearing in locomotion. Nonetheless, the relationship between deficits of working memory and the increased number of contacts is still unclear. Therefore, the goal of this study was to determine whether the working memory deficits that develop in the mouse model of AD increase the frequency of contacts with an obstacle and of inaccurate memory-guided movements during stepping over an obstacle.

1-6. Contents of the present thesis

The present study consists of 2 parts. In Chapter 2, I show that the leading hindlimb in older 3xTg AD mice exhibits a higher rate of contacts with the obstacle compared to wild-type mice and younger 3xTg mice. In Chapter 3, I show that older 3xTg mice exhibit disrupted working memory when performing adaptive control of a hindlimb using a novel obstacle-avoidance task. My findings suggest that the higher rate of contacts results from deficits in memory-guided movements caused by the development of AD.

Chapter 2.

AD caused a higher rate of contacts during obstacle avoidance locomotion

2-1. Introduction

Patients with AD are at a higher risk of falling than healthy older people are, and one of the common causes of this increased risk is contact with and tripping over an obstacle (Blake et al., 1988, van Dijk et al., 1993, Berg et al., 1997). Most of the research into the factors involved in tripping or contact with an obstacle involves obstacle avoidance tasks (Chou and Draganich, 1998, Di Fabio et al., 2003b, Di Fabio et al., 2004, Heijnen et al., 2012). When experiments allow for self-selected locomotion, that is, under normal visual conditions and with free foot placement, patients with AD exhibit a higher frequency of contacts with an obstacle compared with young people or elderly people who are healthy (Alexander et al., 1995, Di Fabio et al., 2004, Heijnen et al., 2012, Orcioli-Silva et al., 2012). Previous studies have shown that executive function and attention play an important role in accurate stepping over an obstacle (Chen et al., 1996, Di Fabio et al., 2005, Harley et al., 2009). Therefore, the increased frequency of obstacle contacts in AD patients may result from a cognitive decline caused by AD. Nevertheless, previous studies have shown that AD patients have impairments in balance as well as gait disturbances (Alexander et al., 1995, Pettersson et al., 2005, Sheridan and Hausdorff, 2007). These motor deficits are associated with falling and have made it more difficult to determine whether cognitive deficits affect the obstacle clearing during locomotion. For these reasons, the

relationship between the cognitive deficits of patients with AD and inaccurate obstacle clearing during locomotion is not well understood.

Quadrupedal animals have been used extensively to examine memory-guided limb adjustments during locomotion and the neural circuits involved in memory-guided limb movements (McVea and Pearson, 2006, 2007a, McVea et al., 2009, Lajoie et al., 2010, Sato et al., 2012). For example, walking cats can maintain forward locomotion for up to 4 steps without contact with an obstacle after sudden cessation of their visual perception (Wilkinson and Sherk, 2005). In obstacle avoidance tasks, it appears that stored visual information that is related to the characteristics of the obstacle, such as obstacle height, is used by rats and cats to adaptively control their hindlimbs in the step-over phase of locomotion (Drew, 1993, Sato et al., 2012). These findings suggest that the memory function plays an important role in the control of the movements of hindlimbs during stepping over an obstacle in locomotion. In addition, it has been reported that lesions in the cerebral cortex that are related to memory function increase the number of contacts with an obstacle during obstacle clearing (Lajoie and Drew, 2007). The hallmark of AD pathology is neurodegeneration and neuronal death caused by accumulation of Aβ and by phosphorylation of the tau protein (Ballard et al., 2011). These pathological changes occur in a wide range of brain regions. Therefore, the progression of the disease may impair the memory-guided limb movements during locomotion.

In the present study, I 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 patients with AD (Oddo et al., 2003). AD-like signs in the brain of the 3xTg

mice are mainly observed in the regions related to memory function, such as the neocortex, hippocampus, and amygdalae. In behavioural tests, deficits in long-term reference memory and spatial working memory are manifested by 4 and 7 months of age, respectively (Billings et al., 2005, Carroll et al., 2007). The mice, however, do not generally show motor disorders related to muscle strength, balance, or visuomotor coordination even up to 15–18 months of age (Gimenez-Llort et al., 2007, Gulinello et al., 2009, Sterniczuk et al., 2010). Therefore, 3xTg mice may be a useful tool for studies of the memory-guided limb movements in locomotion. On the other hand, most studies assessing motor disorders in 3xTg mice have used conventional motor tasks, such as the rotarod, ladder, and beam walk tests. Because these simple tasks cannot assess the detailed kinematics of limb movements during locomotion, it is unclear whether the development of AD in 3xTg mice affects the kinematics of overground locomotion or obstacle avoidance locomotion.

The purpose of this part of experiments was to determine whether mice in this model of AD show inaccurate movements when stepping over an obstacle during locomotion. I found that the leading hindlimb in old 3xTg mice hits the obstacle more frequently compared to control mice, although I did not find evidence of any change in limb kinematics during overground locomotion or stepping over the obstacle because of the development of AD-like signs. My findings suggest that the higher contact rate in old 3xTg mice is the result of memory deficits caused by the development of AD.

2-2. Materials and Methods

Animals

In this study, I used 3xTg-AD mutant mice (n = 16) carrying familial AD mutations (APP_{Swe} and tau_{P301L} transgenes on a mutant $PS1_{M146V}$ knock-in background) (Oddo et al., 2003) and nontransgenic C57BL/6J mice (n = 12). The 3xTg-AD mice (MMRRC 034830-JAX) were obtained from the Mutant Mouse Regional Resource Center (MMRRC-JAX, ME, USA). The C57BL/6J wild-type mice were either 2–8 months old (WT, n = 8) or 18 months old (Old-WT; n = 4). The 3xTg mice were either 2–5 months old (Young-3xTg; n = 8) or 10–13 months old (Old-3xTg; n = 8). The protocol of the present study was approved by the Ethics Committee for Animal Experiments of the University of Tokyo, and was carried out in accordance with the Guidelines on Research on Experimental Animals of the University of Tokyo and the Guide for the Care and Use of Laboratory Animals (NIH Guide) revised in 1996. Every effort was made to minimise the number of animals used and their suffering in the course of the experiments. Outside of 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 weight of the mice was maintained at >85% of that during the *ad libitum* feeding period prior to the experiments.

The apparatus for locomotion experiments

The runway box (length 40 cm, width 5 cm) was made of a transparent acrylic board (thickness 3 mm)(Fig. 2-1). Three obstacles that were made of the black acrylic board were used here (height 4, 5, and 6 mm; depth 2 mm). The obstacles were located

at the midpoint of a runway (20 cm from both ends).

The obstacle avoidance task for freely moving mice

Animals were habituated to the apparatus, feeding dish, and obstacles during a 4-day period for up to 1 h/day. After habituation, they were placed at one end of the runway box (start position). The door was then opened to give 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 2 days. In this experiment, stepping behaviour was recorded 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, I administered the obstacle avoidance task by including a 6-mm obstacle on the runway and recording the number of contacts with the obstacle and the toe trajectories in the step-over phase among 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 a 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 from analysis. An intertrial interval of >30 s was used. Control trials (successful trials) were pseudorandomly collected, to attain at least 4 trials.

Kinematic analysis of overground locomotion and obstacle avoidance locomotion

To examine and compare the gait characteristics of the 3 groups of mice, I carried out kinematic analysis of the hindlimbs during both overground locomotion and obstacle avoidance locomotion. These experiments were performed after the

experiment described above. The mice were partially shaved under 2% isoflurane anaesthesia to enable attachment of circular markers to the hindlimb joints (the iliac crest, great trochanter, ankle, and the fifth metatarsophalangeal joint). Limb movements in locomotion were captured bilaterally using a high-speed camera (200 frames per second). Temporal (walking speed, swing duration) and spatial parameters (stride length, maximal toe height, toe-obstacle distance, and angular displacement of joints) were analysed in the recordings. Obstacle height of 4, 5, and 6 mm was used. The obstacle avoidance task was performed using the same criteria as those used in my 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 4 limbs came in contact with the obstacle (Aoki et al., 2012, Sato et al., 2012).

The 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 boards: it consisted of 3 arms (named A, B, and C) diverging at a 120° angle from the centre 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 1 arm of the apparatus for 10 min to habituate the mouse. The animal was then returned to its cage for 5 min and the apparatus was cleaned with 70% ethanol. After a 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 the video camera and were analysed later. A mouse was assumed to have entered an arm when the body of the mouse excluding the tail fully entered the arm. When a mouse entered other arms in

the order ‘ABCACAB’, I evaluated spontaneous alternation behavioural patterns ‘ABC’, ‘BCA’, and ‘CAB’. The spontaneous alternation rate (% alternation) was calculated by subtracting 2 from the total number of entries into different arms in a series and then dividing by the total number of arm entries and multiplying by 100.

Immunohistochemistry

The mice were subjected to deep anaesthesia with pentobarbital (50 mg/kg of body weight) and were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brain was removed from the skull and cryopreserved in 30% sucrose. Brain slices (20 μm) were prepared on a cryostat. The slices were processed using an M.O.M. kit (Vector) and then immunostained with an anti-human amyloid-β (N) (82E1) mouse monoclonal antibody (IBL, Fujioka, Japan). Immune complexes were visualized using an ABC kit and 3,3′-diaminobenzidine (Vector Laboratories).

Statistical analysis

One-way analysis of variance (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). If the collected date is not normally distributed, Kruskal–Wallis one-way ANOVA was used. The Bonferroni *post hoc* test and Mann–Whitney *U* test with Bonferroni adjustment (adjusted $p = 0.0167$) were carried out as needed to compare the means. The level of statistical significance for variables was set to $p < 0.05$, $p < 0.01$, and $p < 0.001$. Results were expressed as mean ± SEM or ± SD.

2-3. Results

Increased frequency of obstacle contacts among old 3xTg mice

I 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 1 end to the other. After the training, I recorded and analysed the locomotor behaviour of WT and young and old 3xTg mice (Young-3xTg and Old-3xTg, respectively) in a series of trials. I counted the number of first contacts with the obstacle for each limb to obtain the contact rate for each of the 4 limbs. Typical obstacle avoidance behaviour in a successful trial was compared with that in a trial in which contact occurred, as shown in Figures 2-2a and 2-2b. I found that the median (interquartile range) of the interval between the crossings of limbs was not significantly different among the 3 groups (WT 595 [465–725] ms, Young-3xTg 535 [480–595] ms, and Old-3xTg 505 [455–683] ms; $p = 0.282$; Kruskal–Wallis test). The total number of contacts for all limbs (Fig. 2-3a) did not differ significantly among the groups ($F_{2,21} = 1.637$, $p = 0.218$; one-way ANOVA). When I examined the behaviour of each limb separately, I found no significant differences in the number of contacts among the mice for the forelimbs or the trailing hindlimbs (Fig. 2-3b; leading forelimb [LF]: $F_{2,21} = 0.626$, $p = 0.544$; trailing forelimb [TF]: $F_{2,21} = 0.523$, $p = 0.6$; and trailing hindlimb [TH]: $F_{2,21} = 0.174$, $p = 0.842$; one-way ANOVA). However, I identified a significantly increased number of contacts of the leading hindlimb among Old-3xTg mice (Fig. 2-3b; leading hindlimb [LH]: $p < 0.001$, Kruskal–Wallis test). I calculated the rate of contacts for each limb (Figs. 2-3c–f) and found that the leading hindlimb of Old-3xTg mice showed the highest rate (Fig. 2-3e; LH: $p < 0.001$, Kruskal–Wallis test); none of the other limbs

showed significant differences in the rate of contacts among the groups of mice (Fig. 2-3c; LF: $F_{2,21}$ = 0.626, p = 0.544; Fig. 2-3d; TF: $F_{2,21}$ = 0.572, p = 0.573; Fig. 2-3f; TH: $F_{2,21}$ = 0.47, p = 0.954; one-way ANOVA). In this task, there was no evidence of learning effects on the stepping behaviour after habituation (Fig. 2-4; $F_{9,189}$ = 1.394, p = 0.193; two-way repeated measures ANOVA).

Because the WT and Old-3xTg mice used in this experiment were of similar age, I also tested whether age affects the frequency of contacts with an obstacle among Old-WT mice at 17- to 18-month-old (n = 4) and whether Aβ accumulation, which is the main pathological feature of human AD, occurs in Old-WT mice. 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 (Fig. 2-5a–c). In addition, no Aβ accumulation was observed in the cortex and hippocampus (Figs. 2-5d and e). These results seem to support my hypothesis that the higher contact rate in Old-3xTg mice does not result from normal aging.

Temporal and spatial parameters of leading and trailing limbs during stepping over an obstacle

I noted that when a stepping limb came in contact with the obstacle, the characteristics of the leading- and trailing-limb movements differed (Fig. 2-2b). 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 (Lavoie et al., 1995) (Figs. 2-2a, b). Typical toe trajectories in successful trials and those that resulted in contact are illustrated in Fig. 2-6a. The toe trajectories of the trailing forelimb and hindlimb did not change markedly after contact with the obstacle compared with

successful trials (Fig. 2-6a, see inset). Nevertheless, the toe trajectories of the leading forelimb and hindlimb were disrupted upon contact with the obstacle; immediately after the contact, the toes appeared to show compensatory elevation (Fig. 2-6a, see insets). This observation was supported by the finding that the velocity of the leading fore or hindlimb initially decreased and then increased after the obstacle contact (Fig. 2-6b). No change in the velocity of the trailing limbs after obstacle contact was observed. My findings indicated that the toe trajectories of the leading limbs were disturbed by the obstacle contact, whereas those of the trailing limbs were unaffected.

Next, I analysed spatial parameters of the fore and hindlimbs of mice stepping over an obstacle. In particular, I examined the horizontal distance between the foot placement and the obstacle (toe-obstacle distance) immediately before stepping over the obstacle. The toe-obstacle distances of the leading limbs did not differ significantly among the groups (Figs. 2-7a and b; LF: among groups, $F_{2,240} = 2.245$, $p = 0.108$; trials in which no contact was made with obstacles [hereafter, successful trials] versus trials in which contact was made with obstacles [hereafter, unsuccessful trials], $F_{1,240} = 1.149$, $p = 0.285$: 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 smaller toe-obstacle distances in unsuccessful trials than in successful trials (Figs. 2-7a and b; 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).

Overall, my results suggested 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 close to the obstacle. Additionally, my results indicated that the increased rate of contacts for the leading hindlimb of 3xTg mice was not the consequence of disturbances of balance and motor coordination. Because my results refuted existence of the effect of motor deficits, I next explored the possibility that the increased rate of contacts among Old-3xTg mice is associated with greater disturbances of the gait.

Kinematics of limb movements during overground locomotion

In previous studies of motor function changes in aging 3xTg mice, relatively simple tasks were used (Gulinello et al., 2009, Sterniczuk et al., 2010); therefore, I cannot rule out the possibility that the increased rate of contacts with an obstacle in old 3xTg mice resulted from movement (and gait) disorders associated with the development of AD-like signs. To determine whether gait disturbances contributed to the increased rate of contacts with the obstacle, I performed detailed analysis of hindlimb kinematics during overground locomotion and during obstacle avoidance locomotion (see next section).

The typical kinematics of hindlimbs during overground locomotion is illustrated in Figure 2-8b. Walking speed did not vary significantly among the groups (Fig. 2-8c; WT 174.3 ± 8.9 mm/s, Y-3xTg 175.2 ± 6.9 mm/s, O-3xTg 187.4 ± 18.2 mm/s; $F_{2,21} = 0.347$, $P = 0.711$; one-way ANOVA). Likewise, maximal toe height (Fig. 2-8d; WT 2.7 ± 0.2 mm, Y-3xTg 3.1 ± 0.2 mm, O-3xTg 3.2 ± 0.3 mm; $F_{2,21} = 0.907$, $P = 0.419$; one-way ANOVA) and stride length (Fig. 2-8e; WT 64.8 ± 2.2 mm, Y-3xTg 60.0 ± 1.9 mm, O-3xTg 65.0 ± 2.4 mm; $F_{2,21} = 1.662$; $P = 0.214$; one-way ANOVA) did not differ significantly during overground locomotion. The duration of the swing

phase also did not show any significant differences among the 3 groups (Fig. 2-8f; WT 142.7 ± 2.7 ms, Y-3xTg 137.0 ± 2.4 ms, O-3xTg 142.9 ± 3.6 ms; $F_{2,21}$ = 1.260, P = 0.304; one-way ANOVA). Thus, my results indicated that the increased rate of contacts with an obstacle in old 3xTg mice was not associated with gait disturbances during overground locomotion.

Kinematics of stepping over an obstacle during locomotion

Next, I tested whether the higher rate of contacts in old 3xTg mice is associated with changes in obstacle avoidance performance. To this end, I analysed hindlimb kinematics when the animals successfully stepped over an obstacle during locomotion. The typical kinematics of the hindlimbs is illustrated in Figure 2-9a. Walking speed did not vary significantly among the groups nor was it affected by obstacle height (Fig. 2-9b; 4 mm: WT 160.4 ± 5.6 mm/s, Y-3xTg 173.7 ± 8.2 mm/s, O-3xTg 176.8 ± 14.5 mm/s; 5 mm: WT 170.4 ± 12.4 mm/s, Y-3xTg 183.3 ± 8.6 mm/s, O-3xTg 185.2 ± 9.1 mm/s; 6 mm: WT 167.3 ± 10.4 mm/s, Y-3xTg 171.6 ± 11.1 mm/s, O-3xTg 168.4 ± 10.3 mm/s; 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 height just above the obstacle in the stepping phase increased significantly with obstacle height but was not significantly different among the 3 groups (Fig. 2-9c; leading limb: among groups $F_{2,63}$ = 3.099 and P = 0.052; among obstacles $F_{2,63}$ = 39.984 and $p < 0.001$; trailing limb: among groups $F_{2,63}$ = 1.902 and p = 0.158; among obstacles $F_{2,63}$ = 8.816 and $p < 0.001$; two-way ANOVA). The toe-obstacle distances immediately before stepping over the obstacle did not vary significantly among the 3 groups (Fig. 2-9d; leading limbs: among groups $F_{2,63}$ = 1.398 and p = 0.257; among

obstacles $F_{2,63}$ = 0.250 and p = 0.780; trailing limbs: among groups $F_{2,63}$ = 2.639 and p = 0.079, among obstacles $F_{2,63}$ = 0.197 and p = 0.822; two-way ANOVA). The swing duration of the leading and trailing limbs when stepping over an obstacle did not differ significantly among the 3 groups (Fig. 2-9e; leading limb: among groups $F_{2,63}$ = 0.548 and p = 0.581, among obstacles $F_{2,63}$ = 0.759 and p = 0.472; trailing limb: among groups $F_{2,63}$ = 2.686 and p = 0.076, among obstacles $F_{2,63}$ = 0.431 and p = 0.651; two-way ANOVA). My analyses showed that old 3xTg mice could achieve adaptive control of hindlimb movements during both the approach phase and stepping phase; thus, the higher rate of contacts in these animals did not result from movement (or gait) disorders. Because these disorders did not appear to be of significance with regard to the increased rate of contacts in old 3xTg mice, I next tested whether cognitive deficits were involved here.

The spontaneous alternation test in the Y-maze

To confirm that Old-3xTg mice show progressive impairment of spatial working memory, I used the Y-maze test. The total number of arm entries was not significantly different among the groups (Fig. 2-10a; total entry: $F_{2,21}$ = 0.321, p = 0.729; one-way ANOVA), but Old-3xTg mice showed a significant reduction in spontaneous alternating behaviour (Fig. 2-10b; % alternation: $F_{2,21}$ = 4.185, $p < 0.05$; one-way ANOVA). These results were consistent with previous reports showing that the spontaneous alternating behaviour of 3xTg mice (>7 months of age) is impaired (Rosario et al., 2006, Carroll et al., 2007). These results indicated that the Old-3xTg mice used in this study were developing an AD-like disease.

2-4. Discussion

In this study, I examined overground locomotion and obstacle avoidance locomotion in 3xTg mice, the animal model of certain pathological signs of AD. I found that the rate of contacts of the leading hindlimb was significantly greater in Old-3xTg mice than in WT and Young-3xTg mice. Old-3xTg mice, however, do not show worsening of movement deficits during overground locomotion or obstacle avoidance locomotion.

Old-3xTg mice show no changes in various kinematic parameters, such as stride length, maximal toe height, and walking speed, during unobstructed locomotion (Fig. 2-8). I did observe changes in their behaviour in the Y-maze test (Fig. 2-10b). These findings are in agreement with those previously reported on locomotion performance and cognitive function in 3xTg mice (Gulinello et al., 2009, Sterniczuk et al., 2010, Filali et al., 2012). On the other hand, studies on humans showed that patients with AD develop motor deficits such as prolonged reaction time, slow movements, and inaccurate reaching behaviour (Alexander et al., 1995, Ghilardi et al., 1999, Ghilardi et al., 2000, Tippett and Sergio, 2006). Patients with AD, even at an early stage, display gait impairments, such as lower speed, shorter stride, and increased step length variability, compared to healthy individuals (Nakamura et al., 1996, Sheridan et al., 2003, Pettersson et al., 2005); however, I was unable to observe these features in Old-3xTg mice. One possible explanation for this apparent interspecies difference comes from lesion studies on rats and cats, which showed that quadrupedal locomotion is restored by compensatory changes in the brain several days after lesioning (Beloozerova and Sirota, 1993, Aoki et al., 2013). Because neurodegenerative changes in 3xTg mice develop gradually, in the course of several

months (Mastrangelo and Bowers, 2008), it is possible that this situation yields sufficient time for development of compensatory locomotion behaviour. Additionally, pathological changes, such as intracellular Aβ accumulation, extracellular amyloid deposits, and tau phosphorylation in the primary motor cortex, are relatively moderate in 3xTg mice compared to the changes in brain structures related to memory function, such as the hippocampus and amygdalae (Mastrangelo and Bowers, 2008). These various factors may explain the absence of motor deficits in Old-3xTg mice.

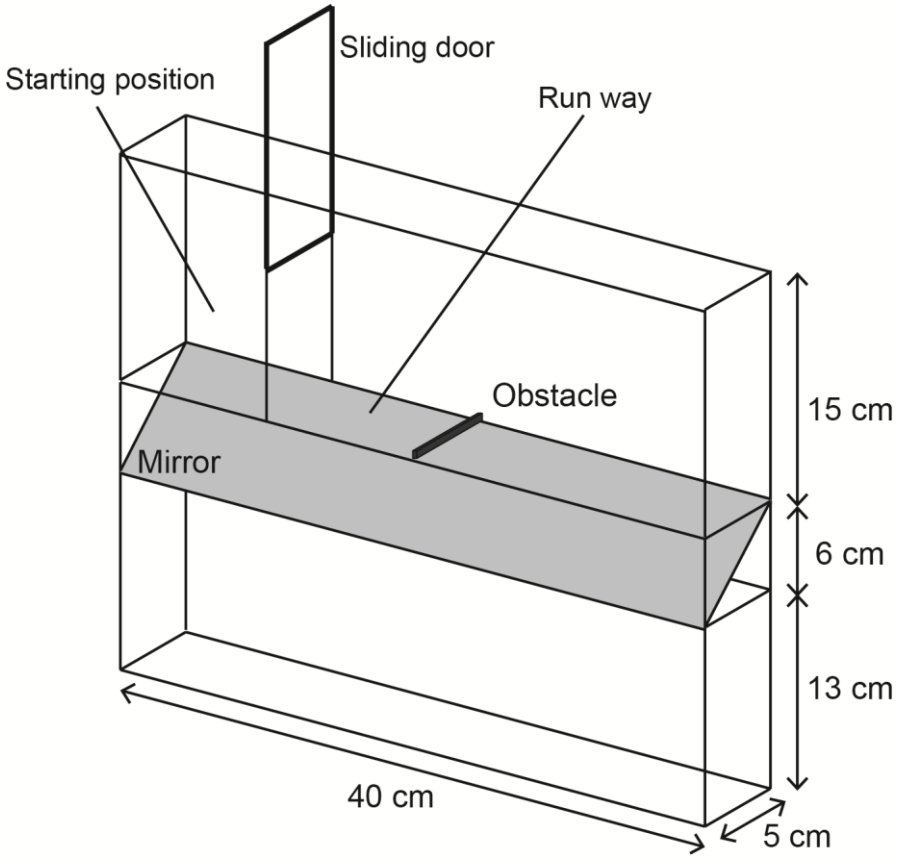
When an animal comes into contact with an obstacle in the stepping phase, the toe trajectories of the leading fore and hindlimbs are rapidly elevated, whereas the trailing fore and hindlimb trajectories remain unaltered (Fig. 2-6a; see insets). In most instances where contact is made, it seems that the toe trajectories of the trailing fore and hindlimbs graze the obstacle (Fig. 2-6a; see insets). It was reported previously that the toe trajectories and kinematics of the trailing fore and hindlimbs of quadrupeds differ from those of the leading fore and hindlimbs during stepping over an obstacle (Lavoie et al., 1995). The toe trajectory of the trailing limb is controlled by the shoulder and wrist joint (Aoki et al., 2012), and the peak toe position of this limb above an obstacle varies to a greater extent compared to 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 regulated more accurately than that of the trailing forelimb (Aoki et al., 2012). The velocity profiles of the toes of the trailing limbs do not differ significantly between successful and unsuccessful trials (Fig. 2-6b; TF and TH). Nonetheless, the velocity of the leading limbs initially decreases upon contact with the obstacle and then increases (Fig. 2-6b; LF and LH). Overall, my observation that the rates of contact of the leading fore and hindlimbs are lower than

those of the trailing limb may be explained by the different values of kinematic and movement parameters between the leading and trailing limbs. It was proposed that the leading fore and hindlimbs require more accurate control than do the trailing limbs (Aoki et al., 2012, 2013). My finding here that the increased contact rate affects only the leading hindlimb supports the notion that memory-guided movements during obstacle clearing are impaired in these mice with an AD-like disease.

Although the increased contact rate in Old-3xTg mice may be due to deficits in certain parameters of visually guided movements, I believe that the present results do not support this idea. First, the rate of contacts of forelimbs in Old-3xTg mice is not affected (Figs. 2-3b, c). The rate of contacts of the leading hindlimb in these mice is increased despite the successful movement of the forelimbs. Second, the toe-obstacle distance of the leading forelimb in unsuccessful trials is identical to that in successful trials (Fig. 2-7a). It was previously reported that limb placement at the final step before stepping over an obstacle is modified to achieve successful stepping over on the basis of visual information (Sato et al., 2012). Third, in successful trials, the examined spatiotemporal parameters of unobstructed locomotion and obstacle avoidance locomotion do not differ significantly between Old-3xTg and control animals (Figs. 2-8 and 2-9). These observations suggest that the visuomotor system of Old-3xTg mice has little or no influence on the planning or execution of adaptive limb movements.

In this study, Old-3xTg mice show no changes in toe-obstacle distance of leading limbs (Figs. 2-7 and 2-9d). Therefore, in Old-3xTg mice, it is likely that the cause of contact with obstacles during stepping over is not inappropriate foot placement just before stepping over but inaccurate toe elevation. McVea and Pearson devised a task to examine the ability of memory to control the movement of the

hindlimbs and reported that a lesion of the PPC (affecting this memory) causes a rapid decline of maximal toe height during obstacle clearing for a short delay period (0.5–5.0 s) (McVea et al., 2009). According to these results of my and/or previous studies, it is likely that memory deficits that result from development of AD-like symptoms may contribute to inaccurate toe elevation and increased frequency of contacts with an obstacle in Old 3xTg mice.



2-1. The apparatus for locomotion experiments

The runway box for mice. The sliding door that can move up and down was located at starting position. Mice walked from the starting position to the opposite end of the box, stepping over an obstacle during locomotion. Unobstructed and obstructed locomotion were captured from the sagittal plane using a high-speed camera.

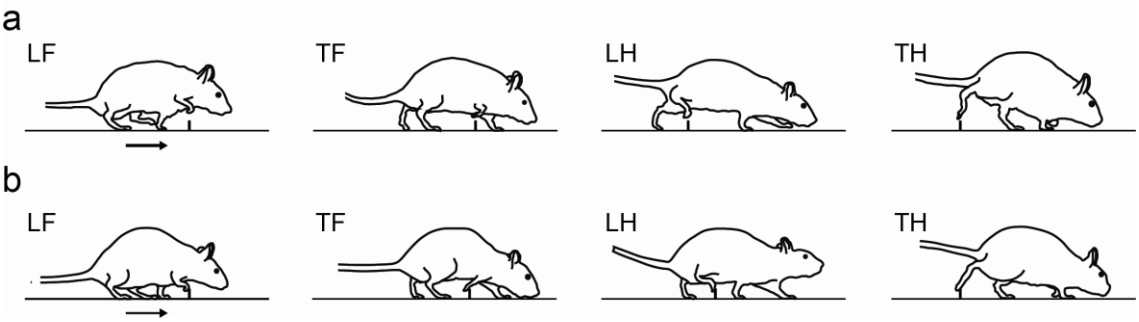


Figure 2-2. Typical behaviour during stepping over an obstacle.

Tracing images from a video of typical behaviour during accurate stepping (**a,** successful trials) and contact with the obstacle (**b,** unsuccessful trials) in Old-3xTg mice. The vertical black bar indicates the obstacle (6 mm). The horizontal black arrow indicates the direction of movement. LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb.

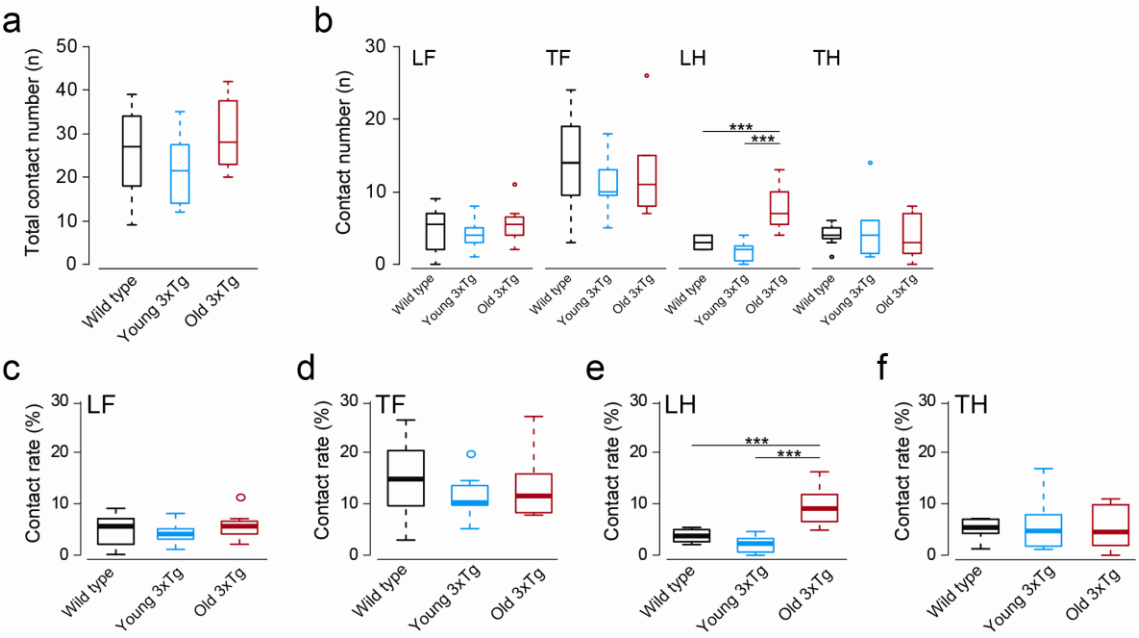


Figure 2-3. The number and rate of contacts with obstacles during stepping over the obstacles.

Box plots showing the total number of contacts **(a)**, number of contacts for each limb **(b)**, and the contact rate for each limb **(c–f)** in wild-type (WT), Young-3xTg, and Old-3xTg mice. The horizontal line in the box shows the median number. Each box indicates the lowest and highest value within a 1.5-interquartile range of the lower and upper quartiles (error bars). Circles indicate the data beyond the above-mentioned range. LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; and TH, trailing hindlimb. All averages are from 8 animals. The data were analysed using two-way analysis of variance and Kruskal–Wallis test (**a**–**f**), followed by individual comparison using the Bonferroni *post hoc* test and Mann–Whitney *U* test with Bonferroni adjustment. ***$p < 0.001$.

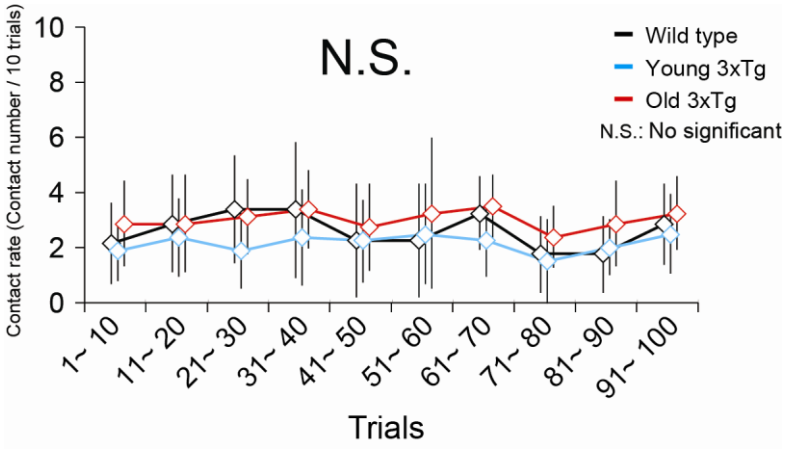


Figure 2-4. Changes in the number of contacts with obstacles throughout the experiment.

Line charts showing the mean number of contacts in 10 trials for all contacting limbs. All averages are from 8 animals. The data were analysed using two-way repeated measures analysis of variance. The data are shown as mean ± SD. N.S. = not significant.

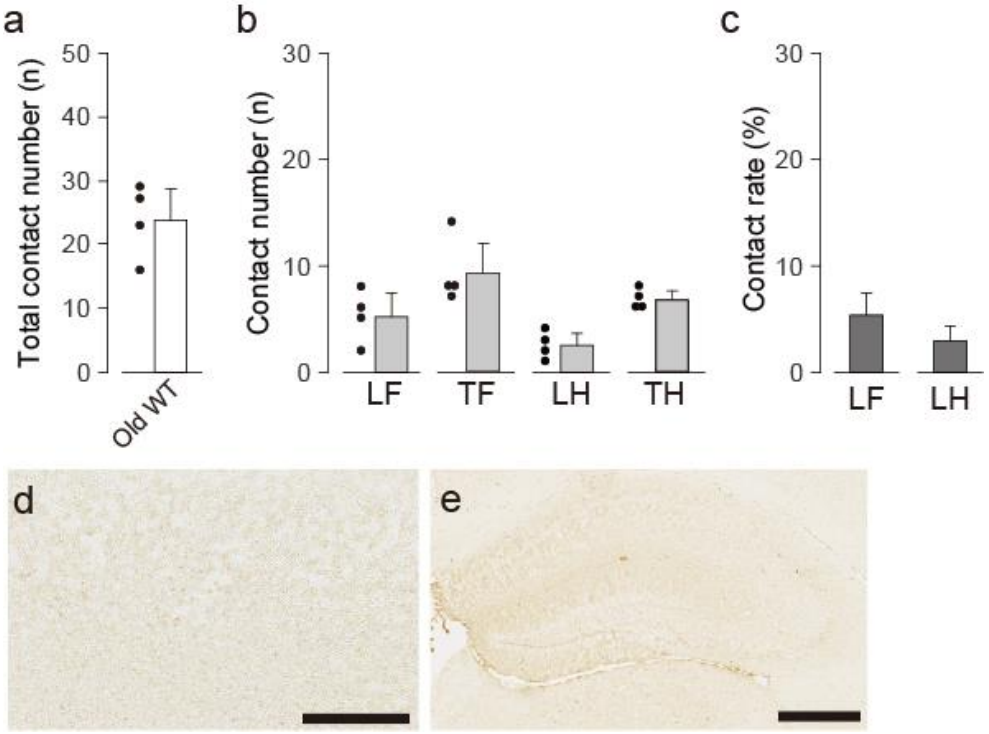


Figure 2-5. The number and rate of contacts with obstacles during stepping over obstacles among Old-WT mice.

Bar graphs showing the mean numbers of contacts for all limbs (**a**) and each limb (**b**) in Old-WT mice. Scatter plots showing data for each mouse (n = 4). LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb. (**c**) Bar graphs showing the contact rate for the leading forelimb (5.3%) and leading hindlimb (3.0%) in Old-WT mice. The data are presented as mean ± SD. (**d**, **e**) No Aβ deposits were observed in the cortex (**d**) or hippocampus (**e**) of Old-WT mice (17 months old). The scale bar, all images: 250 μm (**d**) and 500 μm (**e**). Original magnification: 10× (**d**), 5× (**e**).

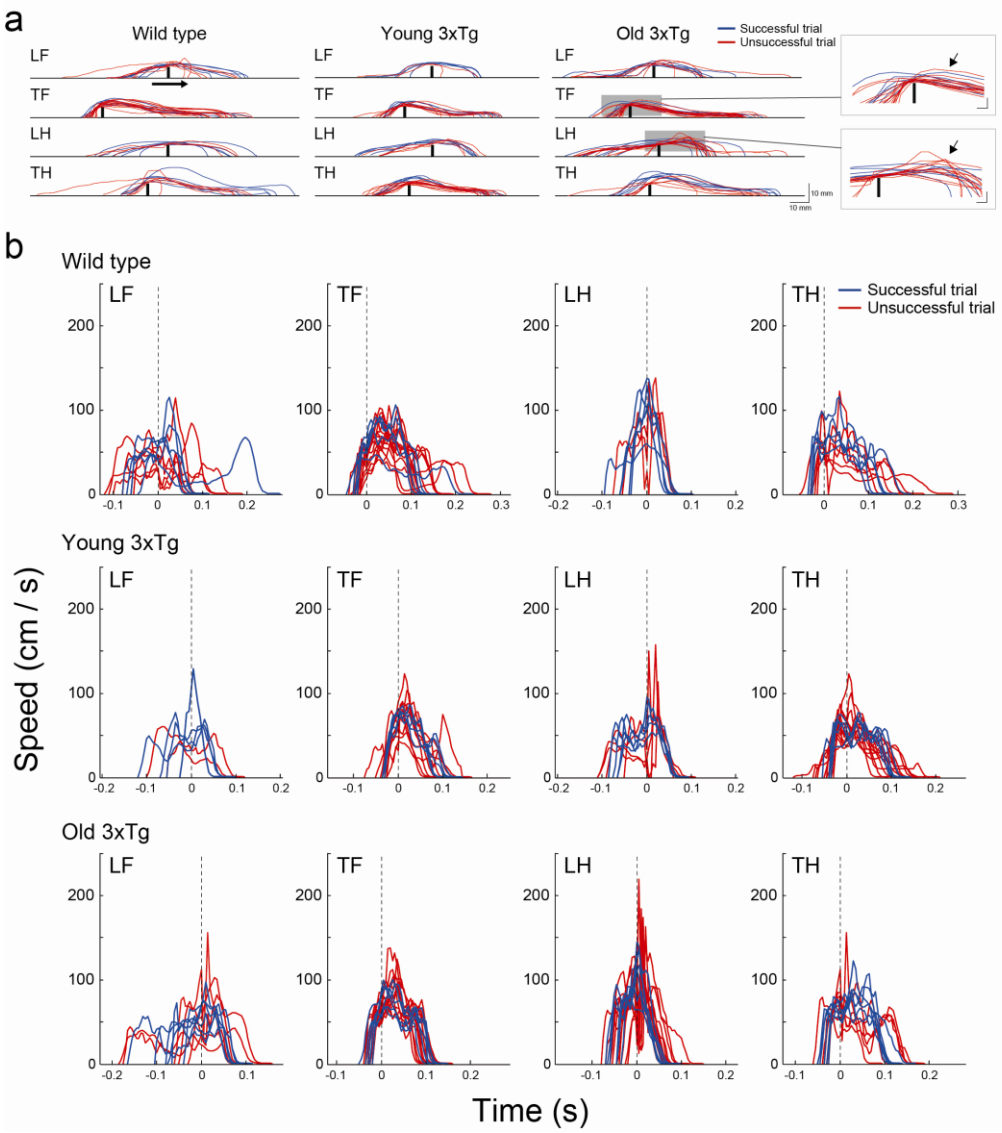


Figure 2-6. Limb trajectories and toe velocity during stepping over an obstacle.

(**a**) Superimposed typical toe trajectories of a wild-type (WT; left), Young-3xTg (centre), and 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 enlargement of the shaded areas. In the insets, the black arrows indicate the toe trajectory after contact with the obstacle. Scale bars are 2.5 mm.

(**b**) Superimposed typical toe velocity of a WT, Young-3xTg, and Old-3xTg mouse stepping over an obstacle. Blue and red lines indicate successful and unsuccessful trials, respectively. Vertical dashed lines show the time points when the limbs were immediately above the obstacle. LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; and TH, trailing hindlimb.

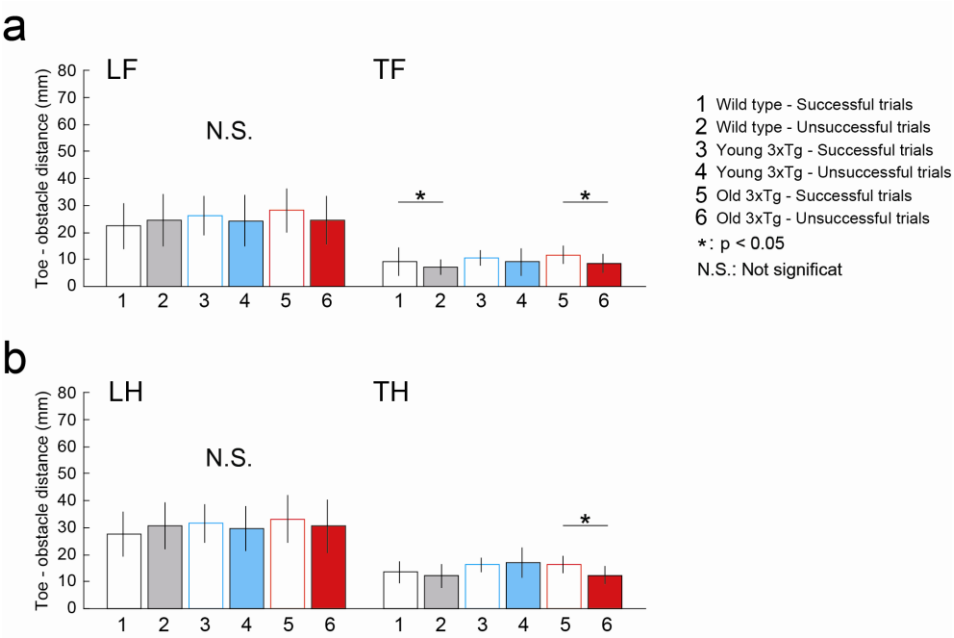


Figure 2-7. Toe-obstacle distance before obstacle clearance.

Bar graphs indicate toe-obstacle distances immediately before stepping over an obstacle (**a**–**b**). LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb. All averages are from 8 animals. Data were analysed using two-way analysis of variance (**c**) followed by individual comparisons in the Bonferroni *post hoc* test. The data are presented as mean ± SD. *$p < 0.05$, N.S. = not significant.

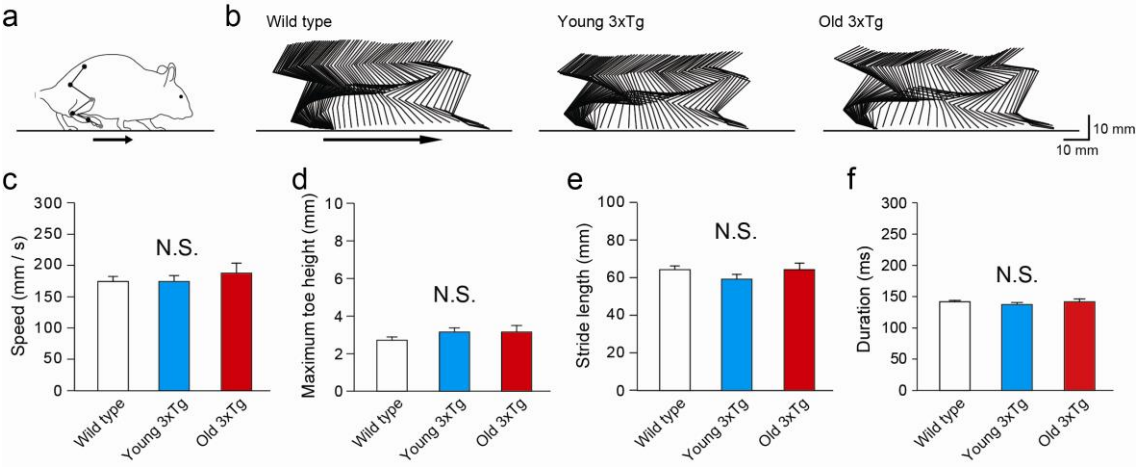


Figure 2-8. Kinematic analysis of overground locomotion.

(**a)** Positions of the markers are indicated. Black circles represent markers attached to the joints and toe. The grey circle indicates the knee position, which was calculated by triangulation from the position of the hip and ankle joints using the measured length of the femur and tibia. The horizontal black arrow indicates the direction of movement. (**b**) Stick figures of hindlimb movements in wild-type (WT), young 3xTg, and older 3xTg mice during the step cycle. The horizontal black arrow indicates the direction of movement. (**c**–**f**) Spatial and temporal parameters: walking speed (**c**), maximal toe height during overground locomotion (**d**), stride length (**e**), and swing duration (**f**). All averages are from 8 animals. The data were analysed by means of two-way analysis of variance, followed by individual comparison in Bonferroni *post hoc* tests. The results are presented as mean ± SEM. N.S. = not significant.

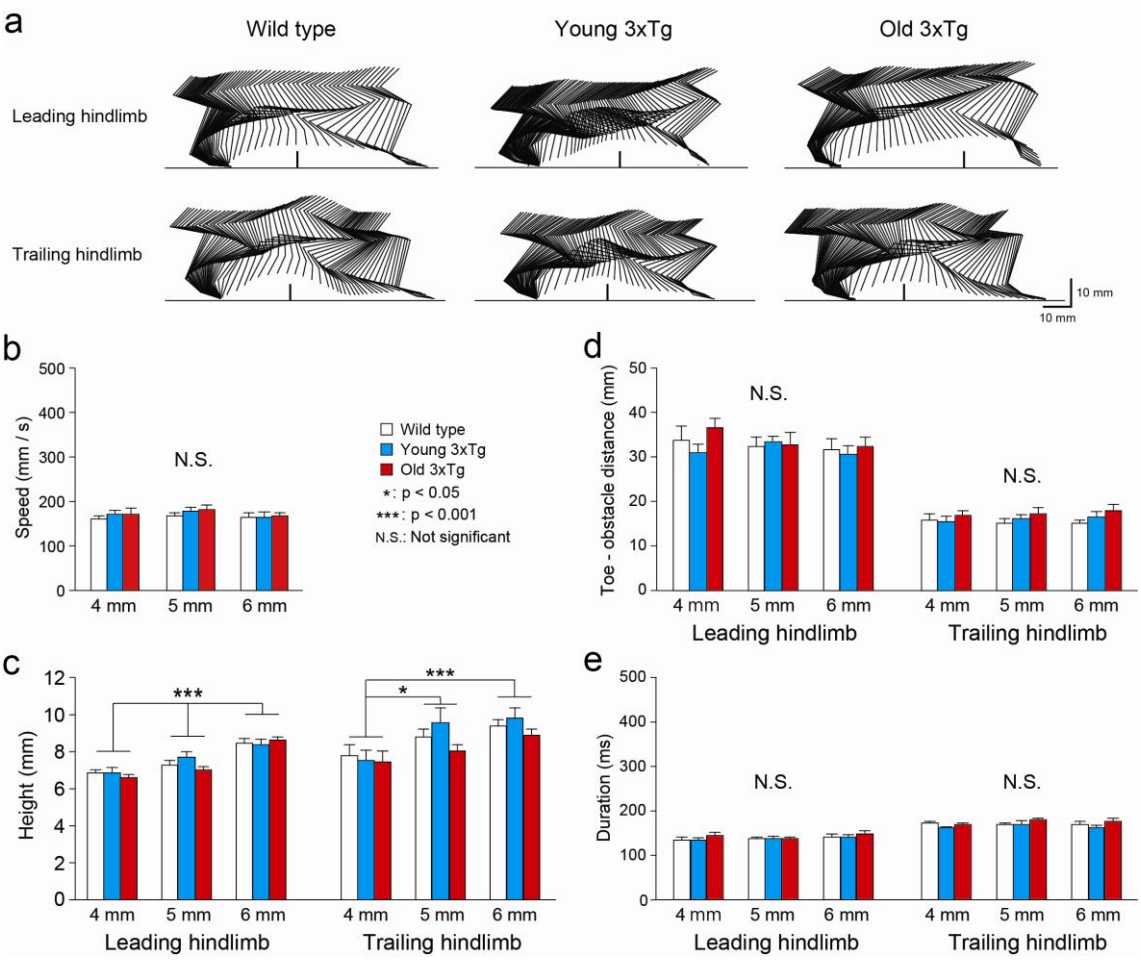


Figure 2-9. Kinematic analysis of stepping over an obstacle.

(**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 landing. 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**), stride length (**d**), and swing duration (**e**). All averages are from 8 animals. The data were analysed using two-way analysis of variance, followed by individual comparison using the Bonferroni *post hoc* test. The results are presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$. N.S. = not significant.

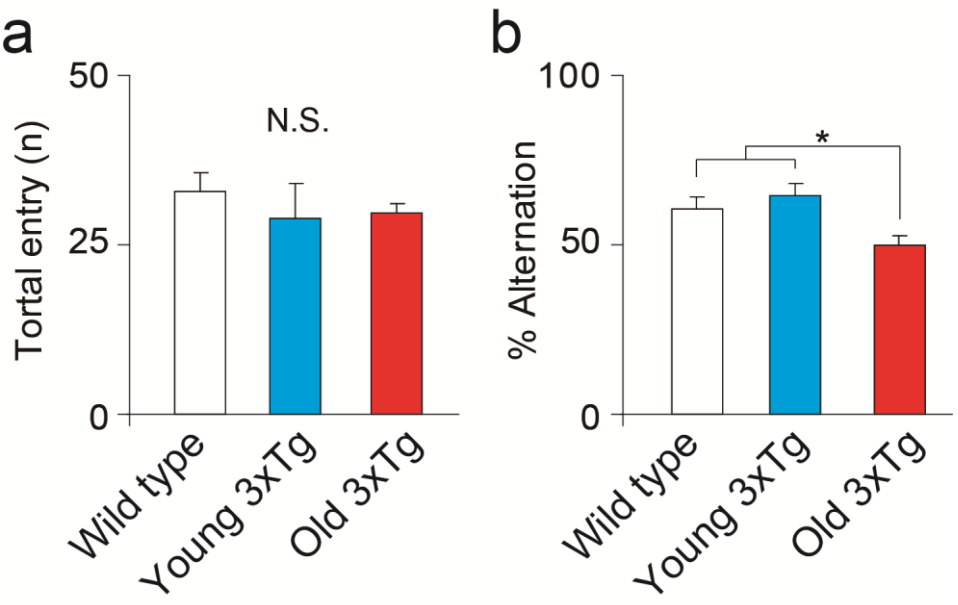


Figure 2-10. The spontaneous alternation test.

These charts show the number of full entries into a maze arm (**a**) and % alternation (**b**) in the spontaneous alternation test using wild-type (WT; n = 8), young 3xTg (n = 8), and older 3xTg (n = 8) mice. The data were analysed using two-way analysis of variance, followed by individual comparison using Bonferroni *post hoc* tests. The results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N.S. = not significant.

Chapter 3.

AD induced working memory deficits that were evident while stepping over obstacles during locomotion

3-1. Introduction

Falling among patients with AD is a serious problem that affects health and quality of life (Sheridan and Hausdorff, 2007). In epidemiological studies, it has been shown that tripping accounts for the highest percentage of causes of falling (Blake et al., 1988, Berg et al., 1997). In walking humans or animals, visual guidance is essential for successful stepping over an obstacle during locomotion without tripping or falling (Patla and Vickers, 1997, Marigold and Drew, 2011, Aoki et al., 2012). However, obstacles can also be cleared without immediate visual information during the approach and step-over phases thanks to the memory function that ensures appropriate limb movements at the apposite time (Drew et al., 1996, Patla and Vickers, 1997, Mohagheghi et al., 2004, McVea and Pearson, 2006, McVea et al., 2009, Pearson and Gramlich, 2010, Lajoie et al., 2012, Shinya et al., 2012). Therefore, AD-induced deficits of this memory function may lead to an increased risk of contact with an obstacle.

In Chapter 2, I showed that the 10- to 13-month-old 3xTg mice (Old-3xTg) exhibit a higher frequency of contacts with an obstacle during locomotion than do control groups (Fig. 2-3a), and that the increased contact rate is observed only in the leading hindlimb (Figs. 2-3b, e) but not in other limbs (Figs. 2-3b–d, f). In addition, the Old-3xTg mice do not show changes of kinematic parameters during unobstructed

locomotion (Figs. 2-8c–d) and obstacle avoidance locomotion (Figs. 2-9b–e). At present, the inappropriate foot placement and inaccurate foot elevation just before and during stepping over an obstacle are considered the factors that cause contact with the obstacle during stepping over in locomotion (Chou and Draganich, 1998, Patla and Greig, 2006, Heijnen et al., 2012). In leading hindlimbs of Old-3xTg mice in successful trials and unsuccessful trials, the distance between obstacle position and toe placement just before stepping over an obstacle does not significantly differ from that of control groups (Figs. 2-7a, b and 2-9d). Therefore, these results suggest that the higher frequency of contacts with an obstacle observed in Chapter 2 results from inaccurate foot elevation. Previous locomotion studies that were focused on the hindlimbs of quadrupeds suggest that working memory is necessary for successful obstacle clearance (McVea and Pearson, 2006, McVea et al., 2009, Whishaw et al., 2009, Lajoie et al., 2010, Sato et al., 2012). Therefore, the increased number of contacts with obstacles of leading hindlimbs in Old-3xTg mice may be caused by the memory deficits associated with AD; these memory deficits may lead to inaccurate limb movements during the process of stepping over an obstacle.

To examine the ability of working memory to guide the movement of hindlimbs during locomotion, McVea and Pearson devised a task where they caused walking cats to stop for food after only the forelimbs had crossed an obstacle; the cats were made to straddle the obstacle for certain periods of time (hereafter, delayed obstacle avoidance task) (McVea and Pearson, 2006). When the cats began to walk forward again, their hindlimbs were required to clear the obstacle based on the memory of the obstacle. The researchers found that the cats could precisely control hindlimb movements using their memory of the obstacle characteristics, such as height and

width. At present, in cats and horses, it is believed that this working memory is involved in the guidance of hindlimb movements in a normal obstacle avoidance task (no delay periods) as well as in a delayed obstacle avoidance task (McVea and Pearson, 2006, Whishaw et al., 2009, Lajoie et al., 2010). Similarly, Sato et al. (2012) showed that stored visual information, involving at least obstacle height, may contribute to the control of hindlimbs during stepping over an obstacle in locomotion (Sato et al., 2012). It is unclear, however, whether the rodents have working memory that is similar to that reported in other quadruped animals.

The 3xTg mice used this study show the development of AD-like signs and progressive cognitive deficits (Oddo, 2003, Oddo et al., 2003, Billings et al., 2005). The interneuronal accumulation of Aβ starts in this model at 4–5 months of age, and then synaptic dysfunction occurs from 6 to 7 months of age. In reference memory, the first deficits are observed at 4 months of age (Billings et al., 2005). Deficits in spontaneous alternation behaviour and in object recognition memory start in this mouse model at 7 months of age and 9–10 months of age, respectively (Rosario et al., 2006, Carroll et al., 2007, Clinton et al., 2007, Guzman-Ramos et al., 2012). Because the Old-3xTg mice used in this study were 10–13 months old, working memory to guide hindlimb movements may also decline because of the development of AD signs and may cause the impairments of memory-guided limb movements.

The purpose of this experimental part was to test whether the impaired working memory in Old-3xTg mice induces deficits of memory-guided limb movements. First, using a delayed obstacle avoidance task that was used in a previous study on cats (McVea and Pearson, 2006), I demonstrated that the maximal toe height of WT mice is sustained above the height of the original obstacle up to 20 s and is

accurately controlled depending on obstacle characteristics. Second, I found that Old-3xTg mice, but not young 3xTg (Young-3xTg) mice, show deficits in working memory. My findings support the hypothesis that the higher rate of contacts among Old-3xTg mice (according to Chapter 2) is a consequence of AD-related working-memory dysfunction.

3-2. Materials and Methods

Animals

In this study, I used 3xTg-AD mutant mice (n = 20) carrying familial AD mutations (APP_{Swe} and tau_{P301L} transgenes on a mutant $PS1_{M146V}$ knock-in background) (Oddo et al., 2003) and nontransgenic C57BL/6J (WT) mice (n = 8). 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 were either 2–5 months old (Young-3xTg) or 10–13 months old (Old-3xTg). The protocol of the present study was approved by the Ethics Committee for Animal Experiments of the University of Tokyo, and was carried out in accordance with the Guidelines on Research on Experimental Animals of the University of Tokyo and the Guide for the Care and Use of Laboratory Animals (NIH Guide) revised in 1996. Every effort was made to minimise the number of animals used and their suffering in 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 weight of the mice was maintained at >85% of that during the *ad libitum* feeding period prior to the experiments.

The apparatus for locomotion experiments

The runway box (length, 40 cm; width, 5 cm) was made of a transparent acrylic board (thickness 3 mm). Three obstacles that were made of the black acrylic board were used (height 4, 5, and 6 mm; depth 2 mm). The obstacles were located at

the midpoint of the runway (20 cm from both ends).

The delayed obstacle avoidance task

The animals were habituated to the apparatus, feeding dish, and obstacles for up to 1 h/day during 4 days. After the habituation period, each mouse was placed at 1 end of the runway (start position). The 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 opposite end. This training was conducted for 2 days. During 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. I caused mice walking along the runway 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 forward, causing the mice to follow. In this experiment, obstacle height of 4, 5, or 6 mm was used. During the training period, obstacles of varying height were used randomly on the runway. After each recording period, the runway was randomly reset with or without an obstacle; the intertrial interval was 1–3 min. The movements of the mouse limbs were recorded in the sagittal plane using a high-speed digital image camera system (100 frames/s). On the day prior to the recording, the abdominal pelage of the mice was shaved under 2% isoflurane anaesthesia to prevent the mice from making contact with the obstacle. Recording was performed for ~1 h/day. The 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 the abdominal height was less than the original obstacle height during the delay period, the trial was excluded from the analysis.

After the delayed obstacle avoidance task, I performed a modified delayed obstacle avoidance task (McVea and Pearson, 2007b). Briefly, I caused the mice walking along the runway to stop by presenting them with a feeding dish before their forelimbs stepped over the obstacle. During the delay period of up to 10 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 forward, inducing the mice to follow. Obstacles of 4, 5, or 6 mm tall were used and were randomly selected for every trial; the intertrial interval was 1–3 min. Before the recording periods, the pelage under the neck was shaved under 2% isoflurane anaesthesia to prevent the neck from making contact with the obstacle. Recording was performed for ~1 h/day. The data were collected using the criteria similar to those for delayed obstacle avoidance task.

The rotarod test

To examine the capacity for motor coordination, balance, and motor learning, I tested the mice on a rotarod (Muromachi Kikai, Ltd.). They were placed on a rod rotating at 8 rpm and tested in 10 trials. Retention time was measured as the period from the time point when the mouse was placed on the rod until the mouse fell. The cut-off time was set to 120 s.

Immunohistochemistry

The mice were subjected to deep anaesthesia with pentobarbital (50 mg/kg of body weight) and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. The brain was removed from the skull and cryopreserved in 30% sucrose. Brain slices (20 μm) were prepared on a cryostat. The slices were prepared using an M.O.M. kit (Vector) and then immunostained with an anti-human amyloid-β (N) (82E1) mouse monoclonal antibody (IBL, Fujioka, Japan). Immunoreactivity signals were visualised using an ABC kit and 3,3′-diaminobenzidine (Vector Laboratories).

Statistical analysis

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 was performed as needed to compare the means. The level of statistical significance for variables was set to $p < 0.05$, $p < 0.01$, and $p < 0.001$. The results were expressed as mean ± SEM.

3-3. Results

Do working memory deficits affect memory-guided hindlimb movements?

Previous studies have shown that working memory plays an important role in the successful movement of hindlimbs during stepping over an obstacle in locomotion (McVea and Pearson, 2006, McVea et al., 2009, Whishaw et al., 2009, Lajoie et al., 2010). I therefore tested whether impaired working memory in Old-3xTg mice causes the increased contact rate owing to the reduced control over the leading hindlimb when stepping over an obstacle. I used the delayed obstacle avoidance task that has been designed to examine the state of working memory during locomotion by McVea and Pearson (2006). In this experiment, I caused a mouse to stop its forward locomotion by placing food immediately after the obstacle; the mouse lifted only its forelimbs over the obstacle before it ceased walking (Fig. 3-1). The mouse was left in this obstacle-straddling posture for ~30 s (delay period); then the obstacle was carefully removed from the runway, and at the end of the designated delay period, the feeding dish was pulled forward away from the mouse; this trick then restarted the mouse's forward locomotion to chase the dish (Fig. 3-1; *Materials and Methods*). During the delay period, the abdominal height of the mouse was measured. When the mouse was induced to move after the delay period, if its abdominal height was less than the obstacle height, then the trial was excluded from the analysis. This task is designed to test whether animals memorise obstacle characteristics, such as height, as was determined by measuring the maximal toe height of the first step after a delay period. For this experiment, I used the same groups of mice as in the experiments above, namely, WT, Young-3xTg, and Old-3xTg. During the habituation period, one of the Old-3xTg mice was excluded from this experiment because it refused to move from the

start position. Although the remaining Old-3xTg mice were able to perform the task, they took longer to be trained (6 ± 2 days) compared with the WT and Young-3xTg mice (4 ± 1 days). After the training, the abdominal height did not differ significantly among different obstacle heights (Fig. 3-4b).

For WT mice, the maximal toe height, which exceeded the obstacle height, was largely sustained during the delay period (Fig. 3-2), that is, these mice lifted their first stepping hindlimb above the obstacle, and this movement did not change even during a relatively prolonged delay. Moreover, the maximal toe height was accurately controlled for different values of obstacle height after different delay periods (Fig. 3-3a; within 5 s: $F_{2,21} = 15.750$, $p < 0.001$; Fig. 3-3b; 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 (Figs. 3-5a, b). These findings are consistent with those of previous studies on cats (McVea and Pearson, 2006, 2007b, McVea et al., 2009). I therefore believe that the delayed obstacle avoidance task can measure the ability 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 length of the delay period; this effect was particularly evident for the 6 mm obstacle (Fig. 3-2), when the delay period was >10 s. The maximal toe height in Old-3xTg mice varied even for short delay periods (within 5 s; Fig. 3-2). In both WT and Young-3xTg mice, their maximal toe height was accurately controlled according to obstacle height even after longer delay periods (Fig. 3-3a; within 5 s: $F_{2,19} = 4.153$, $P = 0.032$; Fig. 3-3b; >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 <5 s, they were unable to maintain the memory of the obstacle height when the delay period exceeded 5 s (Fig. 3-3a; within 5 s, $F_{2,24}$ = 5.502, P = 0.011; Fig. 3-3b; >5 s, $F_{2,24}$ = 1.045, P = 0.367; one-way ANOVA). The stride length of Old-3xTg mice was greater than that of WT mice; however, it was not significantly different between the groups at any obstacle height (Fig. 3-4a; among groups: $F_{2,88}$ = 4.033, P = 0.021; among obstacles: $F_{2,88}$ = 2.005, P = 0.119; two-way ANOVA). My analyses indicate that memory-guided movements based on obstacle height were less accurate in Old-3xTg mice, particularly with longer periods of delay. Therefore, I can conclude that the increased contact rate of the leading hindlimb of Old-3xTg mice was likely associated with deficits in their working memory.

Behavioural tests

I next conducted the rotarod test to determine whether the capacity for balance and motor coordination of Old-3xTg mice is worse than that of control mice. Retention time improved in all groups during repeated trials, and there were no significant differences among the groups (Fig. 3-6; 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 showing that Old-3xTg mice do not exhibit motor deficits (Gimenez-Llort et al., 2007, Gulinello et al., 2009, Sterniczuk et al., 2010).

Aβ accumulation

To examine the relationship between the behavioural changes in Old-3xTg

mice and AD-like pathological changes, I assessed Aβ accumulation in the cortex (Figs. 3-7b–d) and hippocampus (Figs. 3-7f–h). As shown in Figures 3-7b and 3-7f, no Aβ accumulation was observed in the cortex and hippocampus of WT mice. In Young-3xTg mice, mild accumulation of Aβ was observed in the cortex (Fig. 3-7c) but not in the hippocampus (Fig. 3-7g). In contrast, the cortex and hippocampus of the Old-3xTg mice showed marked Aβ accumulation (Figs. 3-7d, h). These findings suggested that there was a relationship between AD-like pathological changes and the inaccurate stepping over an obstacle owing to working memory deficits.

3-4. Discussion

In this study, I established a delayed obstacle avoidance task in mice in order to measure the capacity of working memory for the guidance of leading hindlimb, and I found that in WT mice, working memory could accurately control the toe height of the leading hindlimb. Additionally, using this task, I found that the Old-3xTg mice could not sustain their maximal toe height above the original obstacle height even for short delay periods.

In delayed obstacle avoidance tasks, surprisingly, cats and horses can precisely control the toe height of hindlimbs depending on the original obstacle height, and this phenomenon persists even after 10 min and 15 min, respectively (McVea and Pearson, 2006, Whishaw et al., 2009). Because the period when accurate memory persists is predominantly longer than the duration of working memory (which was examined by classical tasks), this parameter was named ‘long-lasting working memory’ (Pasternak and Greenlee, 2005, Fiset and Dore, 2006, McVea and Pearson, 2006). In my study, when I used WT mice, I often observed that the maximal toe height of their leading hindlimb is sustained above the height of control stepping even for ~25–30 s (Fig. 3-2). In rodents, delay periods of working memory tasks often involve a sensory stimulus, such as visual, odorant, and auditory ones, and are set to small values (range 0.9–5.0 s) (Erlich et al., 2011). The correct rate of delayed visual discrimination task in mice decreases until the chance level when the delay period is >10 s. Therefore, I believe that the results of my study are a long-lasting phenomenon compared to the delay period of sensory-stimulus working memory tasks in mice. Alternatively, McVea and Pearson reported that this long-lasting memory is established via interaction between visual information and the efference copy of a motor command

and/or sensory feedback resulting from forelimbs' stepping (McVea and Pearson, 2007b, Pearson and Gramlich, 2010). In my study, when the WT mice are stopped before a forelimb's stepping over an obstacle (Fig. 3-5a), they cannot control the maximal toe height of their hindlimbs, just as in the previous study on cats, in spite of short delay periods (Fig. 3-5b) (McVea and Pearson, 2007b). Therefore, I concluded that mice also have long-lasting working memory, just as cats and horses do (McVea and Pearson, 2006, Whishaw et al., 2009), and use working memory to guide their hindlimb movements during stepping over an obstacle.

Some researchers suggested that the posterior parietal cortex (PPC) is required for the appropriate movements of the hindlimbs based on working memory during locomotion (McVea and Pearson, 2006). Neurons in the PPC of cats show persistent activity when the animals straddle an obstacle during normal obstacle avoidance locomotion and when a delay is introduced into the task (Lajoie et al., 2010). Cats with a lesion in the medial part of the PPC show an increased number of contacts of the hindlimbs with an obstacle (Lajoie and Drew, 2007). These previous reports indicate that inappropriate obstacle-clearing behaviour may occur because of working memory deficits and suggest that the guidance of hindlimbs in the normal and delayed condition is accomplished by similar neural substrates. These studies, however, also showed that inappropriate foot placement prior to an obstacle clearing could result from impaired motor planning because of a lesion in the PPC. Because the toe-obstacle distance of leading limbs of the walking Old-3xTg mice shows no changes compared to the control groups (Figs. 2-7 and 2-9d), I assumed that the deficits in motor planning or execution are not associated with the development of AD-like symptoms in 3xTg mice; rather, the leading hindlimb of Old-3xTg mice shows a higher rate of contacts with an

obstacle compared to the control groups (Figs. 2-3d, g). The Old-3xTg mice show a clear deficit in their working memory for guiding the leading hindlimb (Figs. 3-2, 3-3a, and 3-3b). Taken together, my results suggest that the inaccurate memory-guided limb movements during locomotion are a consequence of AD-related deficits of working memory.

Previous studies have reported that the PPC of cats is linked not only to memory-guided movements of hindlimbs during locomotion but also to forelimb reaching movements (Fabre and Buser, 1981, Lajoie and Drew, 2007). In reaching tasks with and without delay periods, it was shown that neuronal activities of the parietal lobes in primates are linked to the memory-guided movements of upper limbs (Crammond and Kalaska, 1989, Kalaska and Crammond, 1992). On the other hand, the frontal lobe is also involved in storage of visual and motor information and in execution of accurate memory-guided movements after delay periods (Sawaguchi and Goldman-Rakic, 1991, Funahashi et al., 1993). In obstacle avoidance tasks, the contacts during stepping over an obstacle in locomotion are observed in patients with AD more frequently than in age-matched older subjects (Alexander et al., 1995, Orcioli-Silva et al., 2012). In the studies involving a reaching task in patients with AD, it was shown that the number of movement errors in the condition that requires memory-guided limb movements increases remarkably, but not for the on-line visually guided movements (Ghilardi et al., 1999, Ghilardi et al., 2000). In patients with AD, structural magnetic resonance imaging analysis has revealed changes in the volume of the grey matter even at early stages of AD and showed a decrease in the volume of the frontal and parietal lobes (Schroeter et al., 2009). At ≥10 months of age, 3xTg mice exhibit pathological changes in extensive areas of the cerebral cortex, such as the

frontal and temporal lobes (Nicholson et al., 2010, Feld et al., 2013). Further research is needed to determine whether in 3xTg mice, these pathological changes are related to alterations in working memory that might affect the ability to accurately execute memory-guided limb movements during locomotion.

Figure 3-1. The delayed obstacle avoidance task.

Tracing images of typical behaviour in the delayed obstacle avoidance task in an Old-3xTg mouse. During the delay period, the obstacle was removed. The horizontal black arrows indicate the direction of movement. Vertical black and grey bars indicate the obstacle and its original position after removal, respectively.

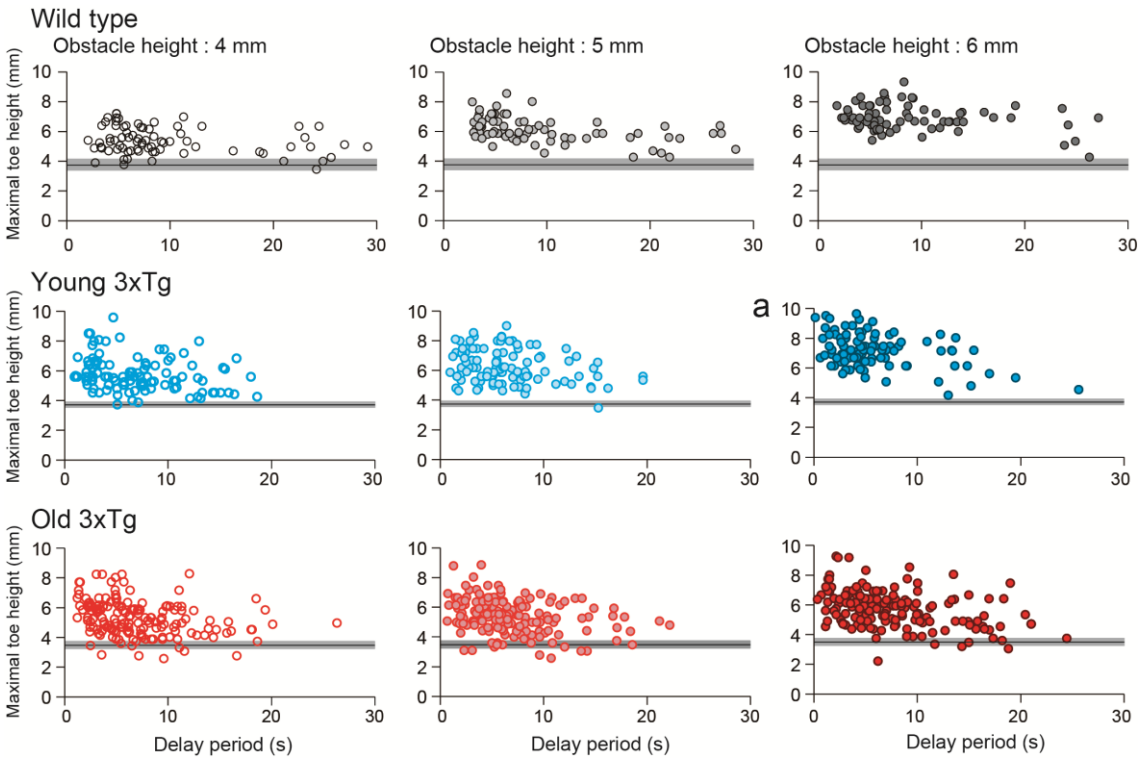


Figure 3-2. Maximal toe height of the first step after the delay periods.

These scatter plots show only the maximal toe heights of the leading hindlimb in the delayed obstacle avoidance task. The horizontal black and shaded lines indicate the average of maximal toe height at unobstructed condition and SEM, respectively. I used WT (n = 8), Young-3xTg (n = 8), and Old-3xTg (n = 9) mice in this experiment.

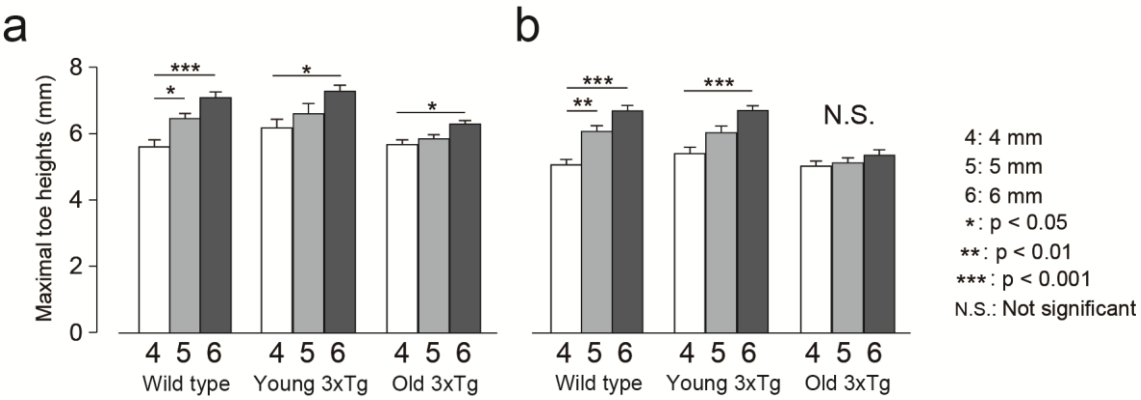


Figure 3-3. Working memory deficits in Old-3xTg mice in the delayed obstacle avoidance task.

Changes in the maximal toe height at varying height of the obstacle (**a**–**b**). These data show only the leading hindlimb. These graphs show averages of the maximal toe height within 5 s (**a**) and at ≥5 s (**b**). WT (n = 8), Young-3xTg (n = 8), and Old-3xTg (n = 9) mice were used in this experiment. The data were analysed using two-way analysis of variance, followed by individual comparison using the Bonferroni *post hoc* test. The results are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001; N.S. = not significant.

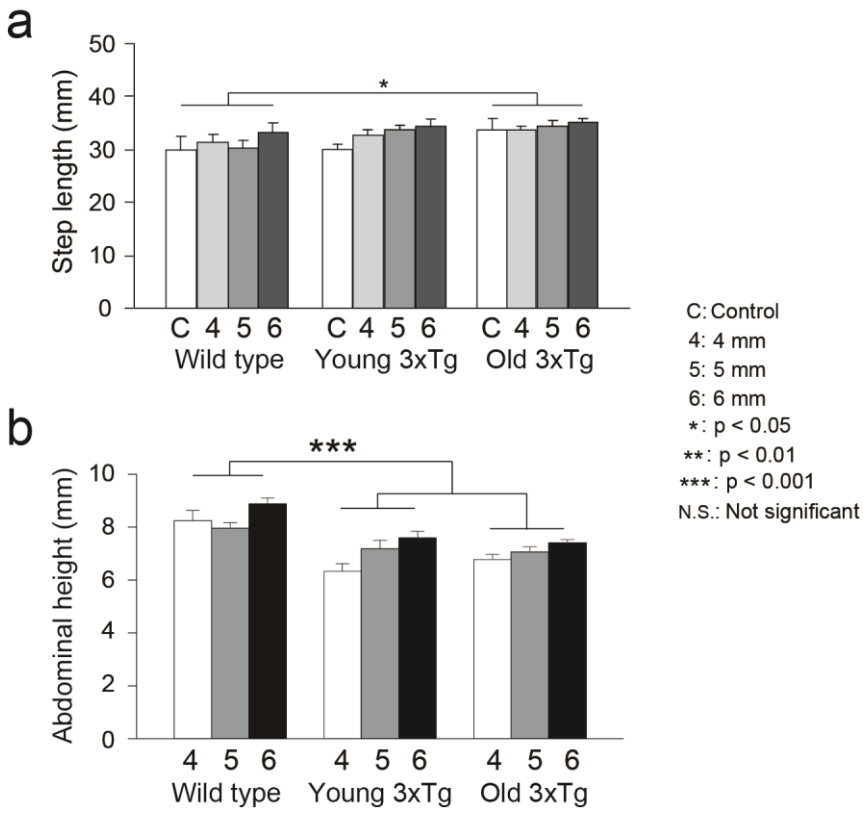


Figure 3-4. Step length and abdominal height in the delayed obstacle avoidance task.

(a) This bar graph illustrates the length of the first step after the delay. (**b**) Abdominal height just before restarting after delay periods: 4: 4 mm; 5: 5 mm; 6: 6 mm. WT (n = 8), Young-3xTg (n = 8), and Old-3xTG (n = 9) mice were used in this experiment. The data were analysed using two-way analysis of variance, followed by individual comparison using the Bonferroni *post hoc* test. The results are presented as mean ± SEM; *$p < 0.05$, ***$p < 0.001$.

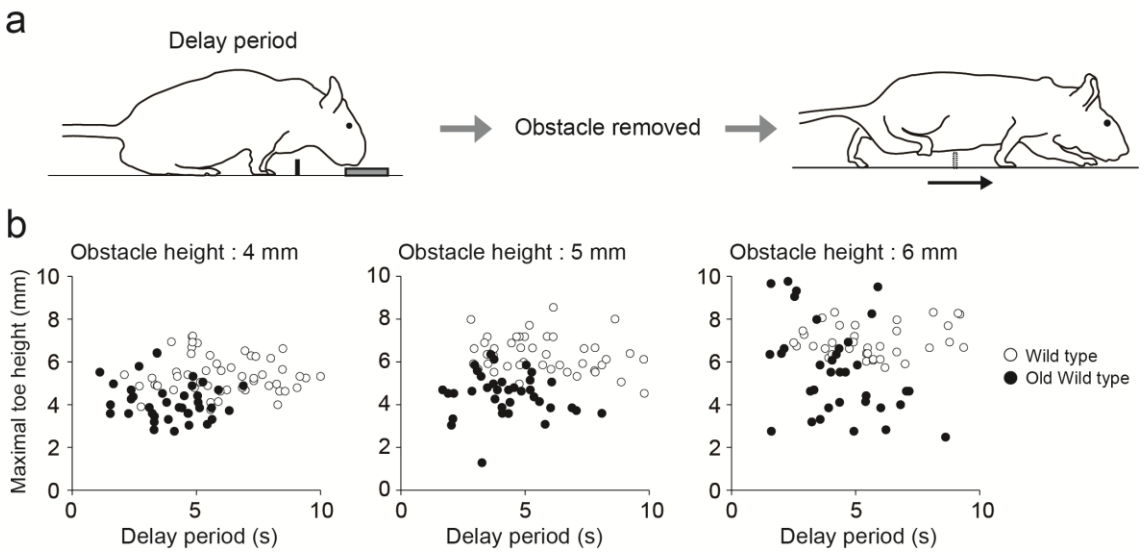


Figure 3-5. The delayed obstacle avoidance task.

(a) Tracing images of typical behaviour in the delayed obstacle avoidance task in a WT mouse. Vertical black and grey bars indicate the obstacle and its original position after removal, respectively. The horizontal black arrow indicates the direction of movement. **(b)** Scatter plots of maximal toe height of the first step beyond the original obstacle position after the delay period (WT at 2–5 months of age, n = 3). These data are only from the leading hindlimb. The white and black circles indicate the maximal toe height after the obstacle was straddled (the same data as in Figure 3-2 for WT) or not straddled, respectively.

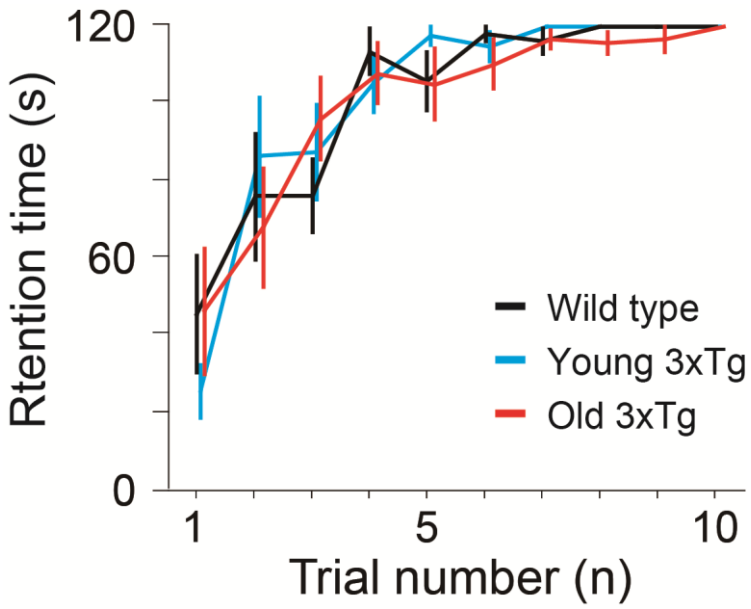


Figure 3-6. The rotarod test.

Rotarod retention periods for WT (n = 8), Young-3xTg, (n = 8), and Old-3xTg mice (n = 9). All averages are from 8 animals. Retention time in every trial did not show significant differences among the groups. The data were analysed using two-way repeated measures analysis of variance, followed by individual comparisons using the Bonferroni *post hoc* test. The results are presented as mean ± SEM.

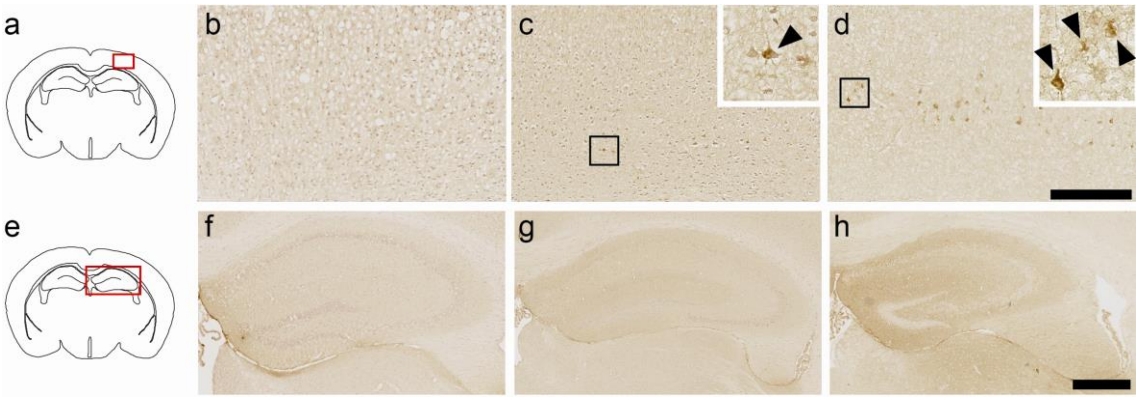


Figure 3-7. Amyloid β deposition in the cortex and hippocampus. Schematics of coronal mouse brain sections (**a**, **e**). Red boxes indicate the region shown in the panels showing the cortex (**b**–**d)** and hippocampus (**f–h)**, respectively. (**b**, **f**) No Aβ deposits were visible in the cortex (**b**) or hippocampus (**f**) of WT mice (8 months old). (**c**, **g**) The cortex of Young-3xTg mice (5 months old) contains 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 deposits. Black boxes indicate the region shown in insets (**c**, **d**, insets) and arrow heads indicate Aβ deposits. The scale bar in all images: 250 μm (**b–d**) and 500 μm (**f–h**). Original magnification: 10× (**b–d**), 80× (**c**, **d**, insets), 5× (**f–h**).

Chapter 4.

General discussion

4-1. Summary of the results

The goal of this study was to determine whether working memory deficits that are caused by the development of AD result in inaccurate memory-guided movements when a mouse stepped over an obstacle during locomotion. Therefore, this thesis consists of 4 chapters including 2 experimental parts.

In Chapter 2, I determined whether the mouse model of AD exhibits inaccurate movements when stepping over an obstacle during locomotion. The leading hindlimb of Old-3xtg mice showed a greater number of contacts with obstacles when stepping over during locomotion compared to WT mice and Young-3xTg mice (Figs. 2-3b, e), but other limbs showed similar rates of contacts with obstacles among the 3 groups (Figs. 2-3b, c, d, and f). Because the toe-obstacle distance of the leading hindlimb did not show changes among the 3 groups (Figs. 2-7a, b and 2-9d), it was likely that the greater number of contacts in Old-3xTg mice results from the inaccurate foot elevation during stepping over an obstacle but not from inappropriate foot placement just before obstacle clearing. Nonetheless, this increased number of contacts of the leading hindlimb in Old-3xTg mice may be the result of motor deficits, such as dysfunction of motor coordination, balance deficits, and gait impairments. Therefore, the kinematics of unobstructed and obstacle avoidance locomotion was examined (Figs. 2-8 and 2-9). During unobstructed locomotion, the changes of kinematic parameters did not show differences among the 3 groups (Fig. 2-8). During obstacle avoidance

locomotion, all groups showed adaptive control of the toe of leading and trailing hindlimbs (Fig. 2-9c), but toe-obstacle distance and swing duration showed no changes (Figs. 2-9b, d, e). In addition, to confirm that Old-3xTg mice show progressive impairment of spatial working memory, I used the Y-maze test. The total number of arm entries was not significantly different among the groups (Fig. 2-10a), but Old-3xTg mice showed a significant reduction in spontaneous alternating behaviour (Fig. 2-10b). According to these results, the increased contact number of the leading hindlimb among Old-3xTg mice is caused by deficits of memory-guided movements that developed because of AD.

To examine the relationship between the deficits of working memory and the inaccurate foot elevation, I used the delayed obstacle avoidance task designed by McVea and Pearson (2006). Although previous studies involving this locomotion task indicated that cats and horses could accurately control the toe height of their hindlimb on the basis of working memory after delay periods, it was not clear whether rodents have these memory systems. In Chapter 3, it was confirmed that WT mice could control toe height of the leading hindlimb depending on obstacle height even if there were forced delay periods (Fig. 3-2). As a result, even when delay periods were forced beyond 10 s, maximal toe height of WT mice was sustained above the original obstacle height (Fig. 3-2) and is adaptively controlled depending on obstacle height (Figs. 3-3a, b). In addition, these types of memory were impaired when the mice were stopped just before forelimb's stepping over the obstacle (Fig. 3-5). The results of the present study are consistent with previous studies, which suggest that in cats, working memory is linked to the control of hindlimbs during stepping over an obstacle.

Next, I determined whether memory-guided hindlimb movements in 3xTg

mice are impaired during stepping over an obstacle after a delay period. In Young-3xTg mice, the maximal toe height, which exceeds the obstacle height, was sustained at least within 5 s, and this height gradually decreases with the delay period when the delay period is >10 s (Fig. 3-2). In this regard, however, the maximal toe height was accurately controlled according to the obstacle height at ≥5 s (Figs. 3-3a, b). On the other hand, the maximal toe height in Old-3xTg mice decreased even for short delay periods: within 5 s (Fig. 3-2). Although Old-3xTg mice could adaptively control their toe height when the delay period is <5 s (Fig. 3-3a), they could not sustain accurate control of the leading hindlimb when the delay period exceeds 5 s (Fig. 3-3b). To confirm that Old-3xTg mice do not have shown balance impairments and dysfunction of the motor coordination, I used the rotarod test. The results did not show differences among the 3 groups (Fig. 3-6). This finding is consistent with the results that did not show gait impairments in Chapter 2 (Figs. 2-8 and 2-9). Moreover, marked Aβ accumulation was observed in the cortex and hippocampus (Figs. 3-7d, h). Taken together, in Chapter 3, the results show that deficits of working memory in Old-3xTg mice lead to inaccurate control of the leading hindlimb when the mice step over an obstacle.

4-2. Integration of the results obtained from the 2 groups of experiments

The goal of this study was to determine whether the development of AD induces inaccurate movements when mice step over an obstacle during locomotion. To this end, the obstacle avoidance locomotion was monitored in freely moving mice, and I measured the number of contacts with an obstacle and the parameters of hindlimb kinematics during unobstructed locomotion and obstacle avoidance locomotion. In

Chapter 2, it has demonstrated that the leading hindlimb in Old-3xTg mice hits the obstacle more frequently compared to control mice (Fig. 2-3b), although I did not find evidence of any changes in limb kinematics during unobstructed locomotion or stepping over the obstacle due to AD-like signs (Figs. 2-8 and 2-9). Nonetheless, using a delayed obstacle avoidance task, I observed the deficit of working memory in Old-3xTg mice (Fig. 3-2). These findings suggest that the increased number of contacts in Old-3xTg mice is the consequence of AD-related working memory dysfunction. This is new evidence supporting the notion that working memory deficits that are associated with AD can affect the process of stepping over an obstacle during locomotion.

4-3. Brain areas that are related to memory-guided movements during locomotion

4-3-1. Memory-guided movements and the posterior region of the cerebral cortex

McVea and Pearson (2009) proposed the conceptual model showing neural possessing that guides the hindlimb movements of cats. According to this model, visual information in terms of obstacle characteristics, such as height and width, is stored in memory for a short period (within several seconds) in area 5 of the PPC. The information that was produced by forelimb stepping movements, such as sensory feedback and/or the efference copy related to the motor programs, returns to area 5 from the motor cortex and from muscle, and these pieces of information are integrated. For these results, the original short-term memory of visual information in area 5 is converted to long-lasting memory (until several minutes). This long-lasting memory is used to guide accurate hindlimb movements during stepping over an obstacle. Among WT mice, the maximal toe height of the leading hindlimb, which exceeds the original

obstacle height, persisted even for delay periods ~20–30 s (Fig. 3-2). These delay periods are longer than the known delay periods in classical working memory tasks, which are based on sensory information (Erlich et al., 2011, Scott et al., 2013, Liu et al., 2014). When the WT mice stopped just before obstacle clearance by forelimbs, long-lasting memory was not formed, in line with a previous study (Fig. 3-5b) (McVea and Pearson, 2007b). These results imply that the control of memory-guided hindlimb movements in WT mice fits the conceptual models proposed by McVea and Pearson (2009).

In primates and rodents, the PPC plays an important role in cognitive functions such as visual attention, motor planning, and working memory (Crammond and Kalaska, 1989, Kalaska and Crammond, 1992, Nakamura, 1999, Lajoie et al., 2010, Erlich et al., 2011, Marigold and Drew, 2011). Thus, are the deficits of long-lasting working memory in Old-3xTg mice caused by the pathological changes in the PPC? As described above, McVea and Pearson (2009) reported that lesions in the PPC specifically induce the deficits of this type of working memory. In their study, the deficits of behaviour in cats whose PPC was destroyed, such as stepping over an obstacle and jumping, were not observed in activities of daily living. In addition, their study does not mention the results on the number of contacts with an obstacle and the limb kinematics in unsuccessful trials, but successful trials in which no contact was made with the obstacle do not show changes in performance during stepping over an obstacle. These results of previous studies are consistent with the results in Chapter 2, where I did not observe deficits in unobstructed and obstructed avoidance locomotion, and with the results in Chapter 3 where I observed the deficits in working memory. On the other hand, lesions of the PPC in cats increase the contact number for forelimbs

and hindlimbs during stepping over an obstacle on a treadmill, and this increased number of contacts result from inappropriate foot placement in most cases; however, it cannot completely rule out the possibility of inaccurate limb elevation (Lajoie and Drew, 2007). The results of previous studies are inconsistent with the results of Chapter 2 where no changes were observed in the toe-obstacle distance in the leading limbs of Old-3xTg mice (Figs. 2-7a, b and 2-9d). Therefore, in Figures 2-3b and 2-3e, it appeared that the increased contact number among Old-3xTg mice was caused by inaccurate foot elevation, not inappropriate foot placement just before stepping over an obstacle. In electrophysiological studies of the PPC in cats, neurons fire in advance of the gait modification and/or during the movement of the 4 limbs in locomotion (Lajoie et al., 2010, Marigold and Drew, 2011). Therefore, it is possible that the same neural substrates are partially involved in locomotor tasks with and without delayed periods. The deficits of visually guided modification and during stepping over an obstacle in locomotion should be observed if the PPC of Old-3xTg mice is impaired by the developing AD. These results, therefore, suggest that the deficits of memory-guided limb movement in Old-3xTg mice also affect the extraparietal cortex.

4-3-2. Memory-guided movements and the frontal region of the cerebral cortex

In primates and humans, it is known that the parietal region, including the PPC and prefrontal regions, are involved in the memory-guided movements (Crammond and Kalaska, 1989, Kalaska and Crammond, 1995, Lacquaniti et al., 1997). It has been reported that frontal motor areas and the PPC have robust reciprocal corticocortical connections (Deiber et al., 1997), and that neural activation of this frontoparietal network is associated with memory-guided arm movements (Johnson et

al., 1996). Therefore, retention of long-lasting memory may require the interaction between the frontal and parietal regions. In addition, neuronal activities of both areas increase during delay periods from the beginning to movement initiation (Funahashi et al., 1993). These persistent activities, which are observed in parietal and frontal regions during the delay period, are associated with the retention of information related to the task. Moreover, one study showed that the neurons in the dorsolateral prefrontal cortex (PFC) exhibit persistent activity related to the retention of motor information, such as motor preparation and movement direction (Kubota et al., 1974). These results are consistent with the participation of the frontal regions as an extraparietal area involved in memory-guided movements during stepping over obstacles. In 3xTg mice at 10 months of age, remarkable Aβ accumulation and formation of plaques are observed in the frontal areas (Hirata-Fukae et al., 2008, Mastrangelo and Bowers, 2008). Using behavioural analysis, Romberg et al. (2011) demonstrated that the progression of AD in 3xTg mice induces not only deficits of memory function but also attentional dysfunction; therefore, those authors assumed that these deficits are caused by functional deficits in the frontal regions of older 3xTg mice. Judging by my present results, it is unclear how attention deficits affect the performance of obstructed avoidance locomotion tasks with and without a delay period. Nonetheless, in the present experiments, the effects of attention deficits seemed to be weak because the toe-obstacle distance (Figs. 2-7a, b and 2-9d) and the contact number for forelimbs in Old-3xTg mice showed no changes (Figs. 2-3b, c, and d). In addition, obstacle contact of the leading hindlimb occurred after forelimbs have successfully cleared the obstacle (Figs. 2-3b, e). Therefore, the results of Chapters 2 and 3 may be explained by the deficits of memory-guided limb movements and of the frontal cerebral region.

Figure 4-1 shows the conceptual model, including the contribution of the frontal region. As described in a previous section (4-3-1), it is likely that both the PPC and PFC perform an important function in memory-guided limb movements during locomotion. The PPC is linked to both visually and memory-guided locomotion and is mainly associated with the control of memory-guided limb movements to accomplish accurate stepping over an obstacle. In addition, it is thought that the retention of long-lasting memory requires both frontal and parietal regions of the brain. In studies on humans, it has been shown that memory-guided reaching movements of patients with AD are impaired even though visual-guide reaching movements are normal (Ghilardi et al., 1999, Ghilardi et al., 2000). In one study, researchers used structural magnetic resonance imaging of the brain of patients with AD; the results showed a decrease in the volume of the frontal and parietal lobes (Schroeter et al., 2009). These pathological changes may be involved in the deficits of memory-guided movements. In a recent study on humans, Shinya et al. (2012) found that short-term visual memory was facilitated by motor interaction with an obstacle during stepping over the obstacle, in line with the results of the study on cats. Therefore, a functional deficit in the PFC and/or PPC that is associated with the development of AD may cause problems with memory-guided stepping over an obstacle in humans.

4-4. Significance and future directions of this research

In the present study, I demonstrated that deficits in memory-guided limb movements impair obstacle avoidance locomotion in a mouse model of AD. The results of this thesis project may improve the understanding of the increased risk of falling and the process of falling in patients with AD. The establishment of an animal

model where it is easy to examine inaccurate limb movements during locomotion may help to elucidate the causes of tripping and falling in humans at the molecular, cellular, and kinematic levels in the future. In addition, this work seems to not only expand the knowledge related to adaptive locomotion but also lead to advancement of the relevant biomedical fields, for example, the development of an exercise program to prevent falling and rational drug design.

4-5. Future directions

As described above, if I compare the results of the present study and the results of previous locomotion studies on cats, the hypothesis that deficits of frontal regions in the cerebral cortex contribute to the increased contact number in the leading hindlimb of Old-3xTg mice can be proposed. Dopamine (DA) neurons play an important role in the execution of memory-guided movements (Sawaguchi and Goldman-Rakic, 1991), and the frontal region in rodents anatomically receives projections from DA neurons (German and Manaye, 1993, Phillips et al., 2004). In studies on humans and other animals, it has been shown that the amount of DA in the brain is reduced by AD (Nazarali and Reynolds, 1992, Guzman-Ramos et al., 2012). In WT mice, a lesion of DA neurons in the ventral tegmental area leads to a greater number of contacts with an obstacle during stepping over the obstacle (Fig. 4-2). Because dopaminergic neurons of the ventral tegmental area mainly project to frontal regions but not the parietal region in the murine cerebral cortex, this observation seems to support participation of the PFC. Therefore, in future studies, it would be worthwhile to examine the relationship between DA neurons and adaptive locomotion.

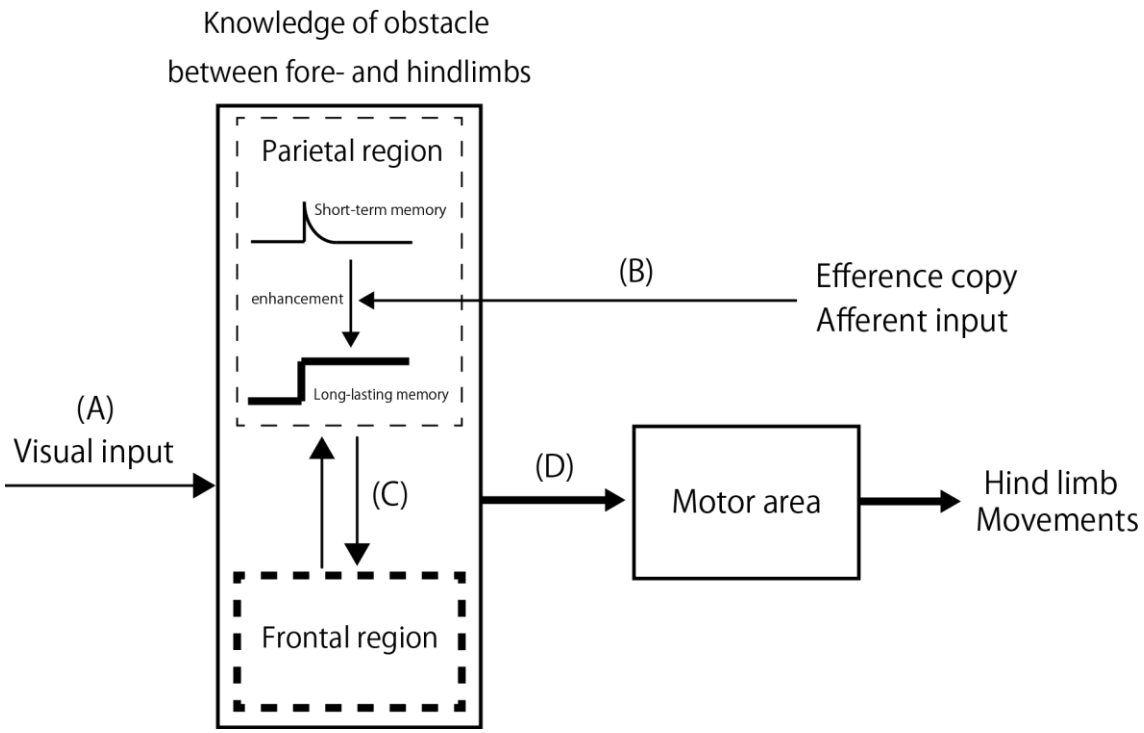


Figure 4-1. A novel conceptual model that includes the frontal region.

This conceptual model was created by modification of the previous model that was proposed by McVea and Pearson (2009). **(A)** The visual information on the obstacle characteristics is stored in both the frontal and parietal regions for a short period as shown in the left box. **(B)** The efference copy related to the motor program and/or afferent information, which is produced by forelimb movements during stepping over an obstacle, is moved (enhanced) from short-term memory to long-lasting memory in the parietal region. **(C)** The retention of long-lasting memory requires the interaction between the frontal and parietal region. **(D)** Long-lasting memory that is derived from the frontal and parietal regions is conveyed to the motor area to guide hindlimb movements during stepping over an obstacle.

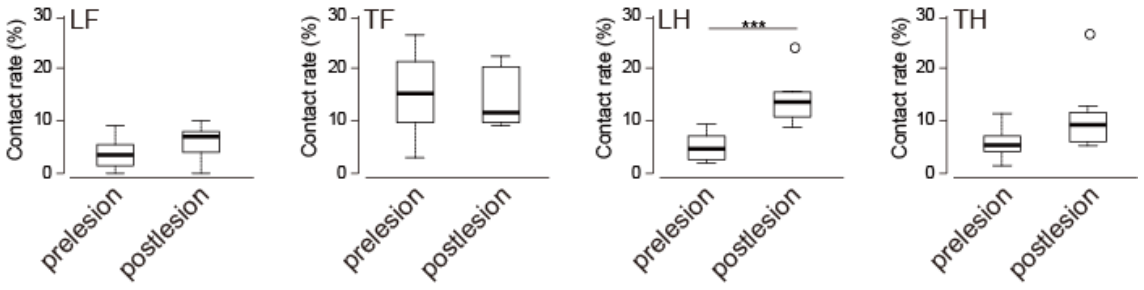


Figure 4-2. Contact rate with obstacles at stepping over obstacles.

Box plots showing the contact rate for each limb in WT. The horizontal line in the box shows the median number. Each box indicates the lowest and highest value within a 1.5-interquartile range of the lower and upper quartiles (error bars). Circles indicate the data beyond the above-mentioned range. The lesions were induced bilateral lesions of the ventral tegmental areas. Pre, prelesion; Post, postlesion LF, leading forelimb; TF, trailing forelimb; LH, leading hindlimb; TH, trailing hindlimb. All averages are from 8 animals. Data was analyzed by Student's t-test and Welch's t-test. ***$p < 0.001$.

Abbreviations

AD = Alzheimer's disease; MCI = mild cognitive impairment; MMSE = Mini Mental State Examination; M1 = primary motor cortex; Aβ = amyloid-beta; FAD = familial Alzheimer's disease; PPC = posterior parietal cortex; PFC = prefrontal cortex; 3xTg = triple-transgenic.

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