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Genetic ablation of the mammillary bodies in the *Foxb1* mutant mouse leads to selective deficit of spatial working memory

Konstantin Radyushkin,[1,2,*] Konstantin Anokhin,[2,*] Barbara I. Meyer,[1] Qiuhong Jiang,[3] Gonzalo Alvarez-Bolado[1,3] and Peter Gruss[1]
[1]Max-Plank Institute of Biophysical Chemistry, Department of Molecular Cell Biology, Am Fassberg 11, Göttingen 37077, Germany
[2]P.K. Anokhin Institute of Normal Physiology, Department of Systemogenesis, Mokhovaya str. 11/6, Moscow 103009, Russia
[3]Max-Planck Institute, Department Molecular Embryology, 30625 Hannover, Germany

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Abstract

Mammillary bodies and the mammillothalamic tract are parts of a classic neural circuitry that has been implicated in severe memory disturbances accompanying Korsakoff's syndrome. However, the specific role of mammillary bodies in memory functions remains controversial, often being considered as just an extension of the hippocampal memory system. To study this issue we used mutant mice with a targeted mutation in the transcription factor gene *Foxb1.* These mice suffer perinatal degeneration of the medial and most of the lateral mammillary nuclei, as well as of the mammillothalamic bundle. *Foxb1* mutant mice showed no deficits in such hippocampal-dependent tasks as contextual fear conditioning and social transmission of food preference. They were also not impaired in the spatial reference memory test in the radial arm maze. However, *Foxb1* mutants showed deficits in the task for spatial navigation within the Barnes maze. Furthermore, they showed impairments in spatial working memory tasks such as the spontaneous alternation and the working memory test in the radial arm maze. Thus, our behavioural analysis of *Foxb1* mutants suggests that the medial mammillary nuclei and mammillothalamic tract play a role in a specific subset of spatial tasks, which require combined use of both spatial and working memory functions. Therefore, the mammillary bodies and the mammillothalamic tract may form an important route through which the working memory circuitry receives spatial information from the hippocampus.

Introduction

Severe anterograde amnesia in patients with Korsakoff's psychosis has often been attributed to injury of the mammillary bodies (MB) (Kahn & Crosby, 1972; Mayes *et al*., 1988; Kopelman, 1995), a group of nuclei located at the caudal end of the hypothalamus. Because MB have a strong anatomical connection via the fornix to the hippocampus, together they are believed to form an extended hippocampal–diencephalic memory system (Tonkiss & Rawlins, 1992; Sziklas & Petrides, 1993; Aggleton & Saunders, 1997; Aggleton & Brown, 1999; Sziklas & Petrides, 2000). Moreover, MB also form part of another cerebral circuit that involves the anterior thalamic nuclei and the medial prefrontal cortex (mPFC) (Gonzalo-Ruiz *et al*., 1992). Because of these connections of MB with the hippocampal formation and the mPFC, this diencephalic structure has been considered to play a crucial role in memory processes (Sziklas & Petrides, 2000; Vann & Aggleton, 2003). Available data suggest that disruption of the MB does not necessarily lead to impairments in non-spatial learning and memory (visual discrimination and recognition) (Sziklas & Petrides, 1998). However, significant impairments after damage of the MB have been observed on the different tasks requiring spatial learning and memory (Saravis *et al*., 1990; Sziklas & Petrides, 1998). The severity of the impairment appears to depend on the level of task difficulty and the amount of damage to the region (Sziklas & Petrides, 1993). The specific functions of MB within different memory systems, however, remain controversial, and the role of MB in either spatial or working memory is under debate (Sziklas & Petrides, 1993, 2000; Santin *et al*., 1999; Vann & Aggleton, 2003).

Previous studies have analysed different aspects of mammillary function mainly through various surgical methods (Sutherland & Rodriguez, 1989; Sziklas & Petrides, 1993; Vann & Aggleton, 2003). Although such studies have supplied a wealth of useful information, they bear common disadvantages characteristic of acute surgical injury such as damage to adjacent fibre tracts or surrounding nuclei (Sutherland & Rodriguez, 1989; Aggleton *et al*., 1991; Sziklas & Petrides, 1993). Here we adopted a different experimental approach based on the use of *Foxb1* mutant mice with a genetic ablation of the medial mammillary (MM) nuclei and mammillothalamic tract (MTt). These mice carry a targeted mutation in the transcription factor gene *Foxb1*, which is normally expressed in the diencephalon, midbrain and spinal cord (Kaestner *et al*., 1996). *Foxb1* null mutant fetuses develop an MB but between embryonic day (E)18.5 and postnatal day (P)4 the size of the mutant mammillary region is dramatically reduced through an abnormally high abundance of cell death due to failure of MTt fibres to enter the target region of the dorsal thalamus (Alvarez-Bolado *et al*., 2000b). Consequently, adult *Foxb1* mutant mice have no MM nuclei, substantially reduced lateral mammillary nucleus (LM) and no MTt at all.

Correspondence: Dr P. Gruss, as above.
E-mail: Peter.Gruss@mpg-gv.mpg.de

*K.R. and K.A. contributed equally to this work.

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Our behavioural analysis of these mutants revealed that genetic lesioning of MB produces a highly specific memory deficit, restricted to the performance of spatial working memory tasks with no impairment of spatial reference memory. These results demonstrate that memory impairments produced by ablation of MB in *Foxb1* mutant mice differ from the effects of hippocampal lesions (Olton *et al*., 1979; Morris *et al*., 1982) and indicate that, despite the close anatomical connections between MB and hippocampus, they underlie different aspects of spatial memory.

Materials and methods

Animals

For the targeted mutation of *Foxb1* gene, embryonic stem cells from the mouse strain 129sv were used [see Wehr *et al*. (1997) for details of genetic construction and subsequent genotyping]. Behavioural experiments were performed after 16 backcrosses to C57/Bl6 mouse strain. Behavioural testing was done in wild-type ($n = 12$, males = 7, females = 5), $Foxb1^{-/-}$ ($n = 11$, males = 6, females = 5), and $Foxb1^{+/-}$ ($n = 10$, males = 5, females = 5) age-matched littermates. Given that no morphological and cellular abnormalities were found in the brain of the $Foxb1^{+/-}$ mice (Wehr *et al*., 1997; Alvarez-Bolado *et al*., 2000b), heterozygous animals were used as an additional control group only in some of the tests. Because *Foxb1* mice are poor breeders we used the same individual mice (males and females) in different tests. The order of testing was as follows: spontaneous alternation, social transmission of food preference, Barnes circular maze, eight-arm radial maze, cued and contextual fear conditioning. The order of testing was set from the least invasive to the most invasive to minimize the possible influence of experimental history (McIlwain *et al*., 2001). The interval between tests was 3–5 days. Given that no morphological and cellular abnormalities were found in the region of mamillary bodies in the $Foxb1^{+/-}$ mice (Wehr *et al*., 1997; Alvarez-Bolado *et al*., 2000b), heterozygous animals were used as an additional control group only in some of the tests. Mice were housed 4–5 per cage in a room with a 12-h light/dark cycle (lights on at 06:00 h) with *ad libitum* access to food (except for the period during which the radial maze and social transmission of food preference tests were conducted; see below) and water. Behavioural tests were conducted during the light phase of the day from 10:00 until 17:00 h. All experiments were performed with permission of the Bezirksregierung Braunschweig in accordance with the German Animal Protection Law.

Histology

After completion of behavioural testing, homozygotes, heterozygotes and wild-type mice used in the tests were anaesthetized and intracardially perfused with 4% paraformaldehyde in PBS. Brains were dissected out, fixed overnight at 4 °C, then dehydrated and processed for paraffin embedding using standard methods (Bancroft & Gamble, 2001). Sagittal sections, 20 μm thick, were stained with cresyl violet according to the Nissl procedure and examined under a light microscope.

Behavioural tasks

Spontaneous alternation

Spatial working memory was assessed by recording spontaneous alternation behaviour during an 8-min session in a Y-maze. The spontaneous alternation test in a Y-maze is based on the natural trait of mice to enter the arm of a Y-maze that had not been recently explored, i.e. the arm that was not entered in the previous choice. The maze was made of grey plexiglass, each arm was 40 cm long, 20 cm high and 10 cm wide, and the arms converged to an equilateral triangular central area. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded manually. Arm entry was considered to be completed when the hind paws of the mouse had completely entered into the arm. Alternation was defined as successive entries into the three arms, in overlapping triplet sets. The percentage of alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2) multiplied by 100%.

Social transmission of food preference

Hippocampus-dependent (Bunsey & Eichenbaum, 1995) non-spatial (olfactory) learning and memory were studied using the social transmission of food preference task, as previously described (Kogan *et al*., 1997; Wrenn *et al*., 2003). The experiment was conducted in three phases: (i) habituation to powdered food, (ii) interaction between 'demonstrator' and 'observer' mice, and (iii) test of the food preference in the 'observer' mice.

To reduce neophobic avoidance of unusual food, mice were habituated to eat powdered rodent food from metal cup (9 cm in diameter, 5 cm in depth) assemblies that minimized spilling of the food from the cup and defaecation/urination in the cup. Habituation was performed in the home cage over a period of 3 days. Two cups with powdered food were placed in each cage, and the usual food pellets were removed.

For the interaction, a 'demonstrator' mouse was chosen from each cage. Wild-type animals were used as the demonstrator mice. Demonstrators were food deprived for 12 h with *ad libitum* access to water. After food deprivation, each demonstrator was presented with a cup of 1% cinnamon or 2% cocoa. To avoid a potential bias for one scent, half the demonstrators received cocoa-flavoured food and the other half received cinnamon-flavoured food. The demonstrators were allowed to eat the flavoured food for 1 h. The cups were weighed before and after presentation to the demonstrators. The criterion for inclusion in the experiment was consumption of at least 0.2 g. All demonstrator mice tested met this criterion. Immediately following scented food consumption, each demonstrator was placed back into their home cage and allowed to interact with the littermate observer mice for 10 min. After the interaction period, the demonstrator mouse was removed from the interaction cage and returned to its individual cage.

In the third phase of the experiment, the food preference of the observer mice was tested 24 h after the end of the interaction with the demonstrator. Twelve hours before the preference test, observer mice were food deprived and caged individually with free access to water. The 1-h preference test consisted of presenting each observer mouse with a pair of weighed food cups in the individual cage. One cup contained the flavour of food eaten by the demonstrator (cued) and the other contained the novel flavour of the pair (novel). The location of the cups in the test cages was balanced by placing the cued flavour cup at the front of the cage in half the cages and at the back of the cage in the other half. The novel flavour cup was placed at the opposite end of the cage. After 2 h, both food cups were removed and weighed to quantify the food preference of the observer mice. The criterion for inclusion in the experiment was consumption of at least 0.2 g in total. All mice met this criterion. The ratio of the weight of the cued food eaten and the weight of food eaten totally was used as a measure of food preference.

Barnes circular maze

The Barnes maze test for mice was performed as previously described (Bach *et al*., 1995). The maze consisted of a grey disc (120 cm in diameter) with 40 holes (5 cm in diameter) around the perimeter. A dark escape tunnel was located under one of the holes. White noise (120 dB) and bright light (1900 Lux) were used to motivate the search for the escape tunnel. An overhead video camera was used to record the behaviour of mice during the test. The disc was elevated 90 cm above the floor and situated in a room with white walls carrying a number of prominent extra-maze cues. These distal cues were hidden during the cued (visible) version of the test. The experiment started with a pretraining session (four trials per day) in which a black cylinder was situated around the hole of the escape tunnel. The mouse was placed in the cylinder and the aversive stimuli were turned on. The mouse was given a maximum of 3 min to escape into the tunnel and was allowed to stay in the tunnel for 1 min with the buzzer turned off. If the mouse failed to escape, it was placed in the tunnel. Training trials (four trials per day, 5 min intertrial interval) were started after 3 days of pretraining. At the beginning of the trial, each mouse was put in a starting chamber in the centre of the maze for 10 s and a buzzer was turned on. The starting chamber was then removed and the mouse was allowed to explore the maze. The trial ended when the mouse entered the tunnel or after 5 min had elapsed. The buzzer was then turned off and the mouse was allowed to stay in the tunnel for 1 min. In the cued version of the maze, the cue (an aerosol can) was placed directly behind the hole of the escape tunnel, which was randomly determined for each trial. In the spatial version of the maze, the spatial location of the tunnel was kept constant, whereas the disc with holes was rotated; this was to prevent the use of odour cues. The order of hole exploration, the search strategy employed and the number of errors were manually recorded by an experimenter. Hole exploration was defined as nose pokes and head deflections over a hole. An error was defined as searching a hole that did not have the tunnel beneath it. The strategy was determined by observing the overall search pattern. If the mouse made numerous crosses over the centre of the maze and visited non-adjacent holes, then a random strategy was recorded. If the mouse made sequential hole searches, then a serial strategy was noted. If the mouse explored fewer than three holes from the location of the goal, then a spatial strategy was recorded.

Eight-arm radial maze

The floor of the maze was made of grey plexiglas, and the wall (6 cm high) consisted of transparent plexiglass. Each arm (6 × 30 cm) radiated from an octagonal central starting platform (perimeter 8 × 8 cm). Manually controlled guillotine doors closed the entrance into each arm. Identical food wells (2 cm deep and 2 cm in diameter) were placed at the distal end of each arm. The maze was elevated 30 cm above the floor and placed in a room with a number of extra-maze cues, including the experimenter. During the experiment, the maze was maintained in a constant orientation. One week before pretraining, animals were deprived of food until their body weight was reduced to 80–85% of the initial level and pretraining started on the eighth day. Each mouse was placed in the central starting platform and was allowed to explore and to consume food pellets (20 mg, rodent precision pellets, Bio-Serv, Frenchtown, USA) scattered around the whole maze for a 5-min period (one session per mouse). At the end of each arm was an inaccessible compartment that contained a large quantity of food pellets to exclude the possibility of orientation by food odour. In subsequent pretraining sessions the number of pellets was decreased until only one pellet was placed in each food well. After these pretraining trials, actual maze acquisition trials were performed. In the pure working memory version of the maze all eight arms were baited with food pellets. Mice were placed on the central platform and allowed to consume all eight pellets within 15 min. The mice were confined in the centre platform for 5 s after each arm choice. This delay forces the mouse to use a spatial strategy to remember the arms visited instead of adopting a simpler serial searching strategy. Each trial was terminated immediately after all eight pellets were consumed or 15 min had elapsed. An 'arm choice' was defined as travelling more than 5 cm from the central platform. The animals performed one trial per day (seven trials in total), and for each trial choices of arms and latency to retrieve all pellets were recorded by the experimenter. Working memory errors were defined as revisits to the arms.

In the version of the maze that simultaneously tested spatial working and reference memory (Olton & Samuelson, 1976), a subset of four arms was baited with food and the other four arms were left unbaited. Each animal was placed in the central arena and allowed to visit arms until all four baited arms had been visited or 15 min had elapsed. Mice were tested for seven trials (one trial per day). The spatial position of baited arms was constant for each individual mouse. Arms were randomly relocated after each session to prevent animals from using intra-maze cues. Working memory errors were defined as the number of revisits to any of the arms and reference memory errors were defined as the number of visits to the never-baited arms.

Cued and contextual fear conditioning

These experiments were performed using a computerized fear-conditioning system (TSE Gmbh, Bad Homburg, Germany). The computer was connected to a control unit containing a shock and a tone generator. Training took place in an apparatus consisting of a box (58 cm × 30 cm × 27 cm) with a simple grey interior and a 12-V light attached to the ceiling. The pre-exposure and conditioning context consisted of a plexiglass chamber (36 cm × 20 cm × 20 cm) placed on a removable shock grid made of stainless steel rods (4 mm in diameter, spaced 6 mm apart). The shock grid was connected to a shocker-scrambler unit delivering shocks of defined duration and intensity. The grid, the tray below the grid and the chamber were cleaned with 70% ethanol between each animal. For both contextual and cued fear conditioning, mice were trained within the same session, with the following protocol: the pre-exposure time of 2 min in the conditioning box was followed by a 30-s tone (conditioned stimulus, 10 kHz, 75 dB). Immediately after the end of the tone, the shock (0.5 mA, 2 s) was delivered, and after 15 s the procedure was repeated once more. During the pretrial period, freezing behaviour was monitored. The contextual memory test was performed 24 h after training. Mice were monitored for freezing for 2 min in the same context (chamber and interior) as used for training. The tone-dependent memory test was performed 2 h later. Visual context (the interior of the experimental box) was altered by white paper printed with different colour and black-and-white patterns (horizontal and diagonal stripes, squares and triangles). A different chamber (25 × 20 × 15 cm) was used for this test. The floor texture was changed by replacement of the grid floors with smooth plexiglass floors. The chamber was cleaned with 1% acetic acid between animals. Freezing was monitored in the same mice for 2 min in the absence of tone (preconditioned stimulus freezing) and for 2 min in the presence of a continuous tone (conditioned stimulus freezing). Freezing behaviour, defined as the absolute lack of movement (excluding respiratory movements), was scored by the experimenter every 10 s. The data were converted to the percentage of samples scored as freezing.

Statistical analysis

Experimental data were analysed by analysis of variance (ANOVA) using STATISTICA (StatSoft, Inc., Tulsa, OK, USA) software. Because initial between-subjects ANOVA analyses including sex and genotype as factors revealed no main effect of sex and no genotype–sex interaction, data from both sexes were pooled for subsequent analysis. Repeated-measures ANOVA was used to analyse the within-subjects data where appropriate (escape latency in the Barnes maze, number of errors in the eight-Arm maze). Tukey's test was used for *post hoc* analyses. Threshold of significance in all tests was $P < 0.05$. In description of the results data are presented as the mean ± SEM.

Results

Foxb1 *adult homozygotes lack the medial and most of the lateral mammillary nuclei*

To assess precisely the neuroanatomical defect in individual *Foxb1* mutants that had been used in the behavioural experiments, we examined the caudal hypothalamus of adult homozygous, heterozygous and wild-type brains on Nissl-stained histological sections. At lateral levels, the LM was easy to recognize in wild-type mice (Fig. 1A), forming a characteristic protrusion on the caudal–ventral surface of the brain. In mutant mice, however, the LM was noticeably smaller and caused no 'bulge' on the outer surface of the brain (Fig. 1B). At the level of the MTt, wild-type mice showed a robust and easily identifiable mammillary axonal tree [premammillary nuclei (pm) and MTt] (Fig. 1C), together with the dorsal (PMd) and ventral (PMv) premammillary nuclei and the lateral subdivision of the MM nucleus (MMlat) (Fig. 1C). The PMd is considered to be a part of the MB and contributes bifurcated axons to the anterior thalamus (through the MTt) and to the periaqueductal grey (through the MTt) (Canteras & Swanson, 1992; Alvarez-Bolado *et al*., 2000b). In the mutant mice, the PMd and PMv were of normal size, but the MMlat was absent and the pm (containing axons from the remnants of the LM and from the PMd) was dramatically reduced in size (Fig. 1D). A third latero-medial level was observed in the wild type mice, the pm, the beginning of the mammillotegmental tract (MTGt) and a large MMlat (Fig. 1E), whereas in the mutant mice the axonal tracts were substantially reduced and the MMlat was absent (Fig. 1F). At medial levels (Fig. 1G and H), the large mass of the medial (MMm) plus median (MMn) subdivisions of the medial MB, sharply protruding from the caudal surface of the brain (Fig. 1G), formed a clear contrast with the mutant hypothalamus, where these cell groups were conspicuously absent (Fig. 1H). Inspection of the sections at high magnification confirmed that in the mutant mice, the MM nucleus was not present (Fig. 2A–D). The disappearance of this nucleus during development left a smaller, cell-sparse space between the supramammillary nucleus and premammillary nuclei (asterisk in Fig. 2B and D).

The hippocampal region of the mutant mice, however, appeared normal (Fig. 3). Brains of heterozygous animals were indistinguishable from the wild-type samples (data not shown).

Foxb1 *mutants have selective deficit in the spatial version of the Barnes circular maze*

We studied spatial learning and memory functions of *Foxb1* mutant mice in the Barnes circular maze. This test for spatial learning and navigation has the advantage of being much less reliant on motor functions than is swimming in the water maze (Barnes, 1979; Mansuy *et al*., 1998).

To exclude possible non-cognitive deficits in $Foxb1^{-/-}$ mice, we initially conducted a cued version of the Barnes maze, in which the escape hole was directly marked. This version of the task does not depend on spatial memory and navigation (Barnes, 1979). $Foxb1^{-/-}$ mice were as proficient as wild-type and heterozygous controls (Fig. 4A) in the acquisition and performance of the cued version of the Barnes maze (main effect of group $F_{2,33} = 2.15$, $P > 0.05$; sessions $F_{6,198} = 6.78$, $P < 0.001$; interaction $F_{12,198} = 0.38$, $P > 0.05$).

We then trained mice in a spatial version of the Barnes maze (i.e. the escape hole was not marked). The results of this test are presented in Fig. 4B. Repeated-measures ANOVA revealed a significant main effect of genotype ($F_{2,33} = 5.24$, $P < 0.01$) and day of experiment ($F_{3,99} = 17.83$, $P < 0.001$) on escape latency, whereas no genotype–day interaction was detected ($F_{6,99} = 1.16$, $P > 0.05$). To achieve a better understanding of the nature of these differences between the mutant and control animals, we analysed the search strategies used by mice at different stages of the experiment. The analysis showed that at the beginning of training the homozygous animals, as well as heterozygotes and wild-type mice, used a random search strategy (Fig. 4C). Then, during the next 2 weeks of training, all mice acquired a more efficient serial search strategy (Fig. 4D). Finally, by the end of training, $Foxb1^{+/+}$ and $Foxb1^{+/-}$ mice had developed a spatial strategy (the most efficient one) that was used by 78 and 82% of these animals, respectively (Fig. 4E). However, only 18% of the $Foxb1^{-/-}$ mice showed a spatial strategy. These data indicate that $Foxb1^{-/-}$ mice have a specific impairment in spatial learning and memory in the Barnes maze.

Foxb1 *mutants show no deficit in classical fear conditioning and in social transmission of food preference*

We investigated the performance of *Foxb1* mutants in a contextual fear conditioning task, sensitive to hippocampal lesions (Kim & Fanselow, 1992). *Foxb1* homozygotes, as well as heterozygous and wild-type littermates, showed a high level of freezing behaviour in a context test 24 h after the conditioning (56.4 ± 5.7, 53.2 ± 5.0 and 62.4 ± 3.1% receptively; Fig. 5A). As a control, we tested the mice in a hippocampus-independent cued fear conditioning task. No significant differences were detected between *Foxb1* homozygotes, heterozygotes and wild-type mice, as all of them showed a high level of freezing (69.2 ± 2.9, 67.4 ± 2.4 and 72.7 ± 2.1%, respectively; Fig. 5B). Thus, $Foxb1^{-/-}$ mice showed no deficit in contextual and cued fear conditioning tests.

We then tested *Foxb1* mutant mice in another hippocampus-dependent but non-spatial cognitive task – the social transmission of food preference test (Bunsey & Eichenbaum, 1995). No significant differences in the total amount of eaten food were found between $Foxb1^{-/-}$ (0.69 ± 0.1 g) and wild-type (0.67 ± 0.09 g) animals. Performance of $Foxb1^{-/-}$ animals did not differ from the behaviour of the wild-type mice in this task (72 ± 6.4 and 68 ± 5.6% food preference, respectively).

Working memory is selectively impaired in Foxb1 *mutants*

We first tested the behaviour of *Foxb1* mutants in a spontaneous alternation task, which is a preliminary test for unbiased working memory. During the 8-min session, wild-type and $Foxb1^{+/-}$ control mice showed 69 ± 3.5 and 64 ± 4.1% of spontaneous alternation, respectively, whereas $Foxb1^{-/-}$ mice showed a significant impairment of spontaneous alternation behaviour (50 ± 4.2%, main effect of the genotype $F_{2,29} = 7.39$, $P < 0.05$). *Post hoc* analysis revealed no

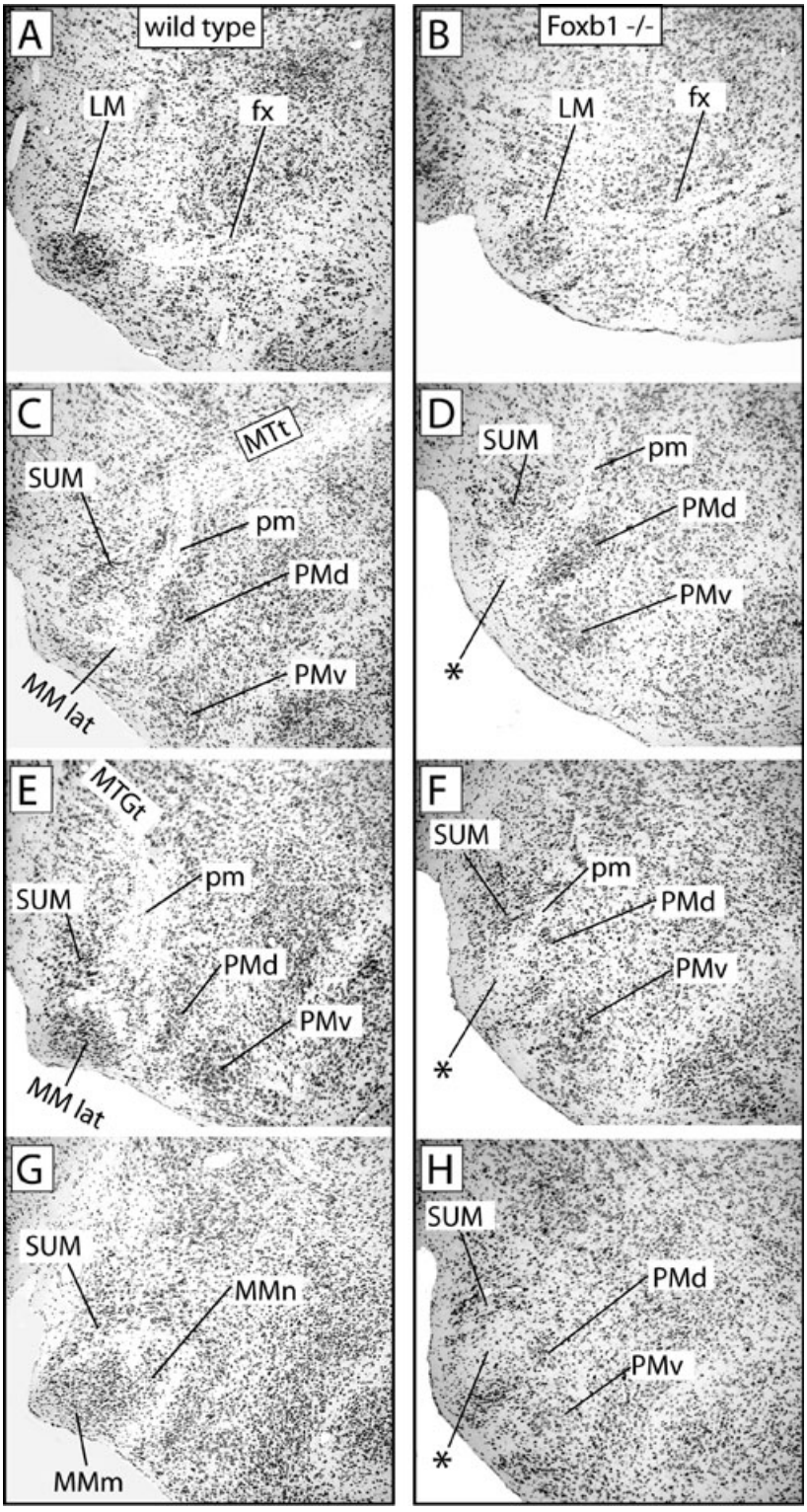

FIG. 1. *Foxb1* homozygotes lack the medial mammillary and most of the lateral mammillary nuclei. Lateral to medial (top to bottom) sagittal sections of P56 male wild-type (left) and *Foxb1* homozygous (right) mouse brains, showing the mammillary region in the caudal hypothalamus. Each left–right pair of images shows comparable latero-medial levels in wild-type and mutant brains, respectively. (A and B) Level: fornix reaches MB; the mutant (B) shows reduced fornix and LM. (C and D) Level: principal mammillary tract and MTt; in the mutant (D), PMd and PMv are intact, the lateral subdivision of the MM cannot be detected, a very reduced principal mammillary tract can be seen (pm) and the place of the lateral subdivision of the MM has become a cell-sparse area populated by some small cells of glial appearance (asterisk). (E and F) Level: principal mammillary tract and MTGt; asterisk as in previous level. The characteristic prominence of the MB (particularly visible in E and G) has disappeared in the mutant. (G and H) Level: medialmost. Abbreviations: asterisk, cell-sparse, glial-filled area in the presumptive position of the MM in the mutant; fx, fornix; LM, lateral mammillary nucleus; MM lat, lateral subdivision of the medial mammillary nucleus; MMm, medial subdivision of the medial mammillary nucleus; MMn, median subdivision of the medial mammillary nucleus; MTGt, mammillotegmental tract; MTt, mammillothalamic tract; pm, principal mammillary tract; PMd, dorsal premammillary nucleus; PMv, ventral premammillary nucleus; SUM, supramammillary nucleus.

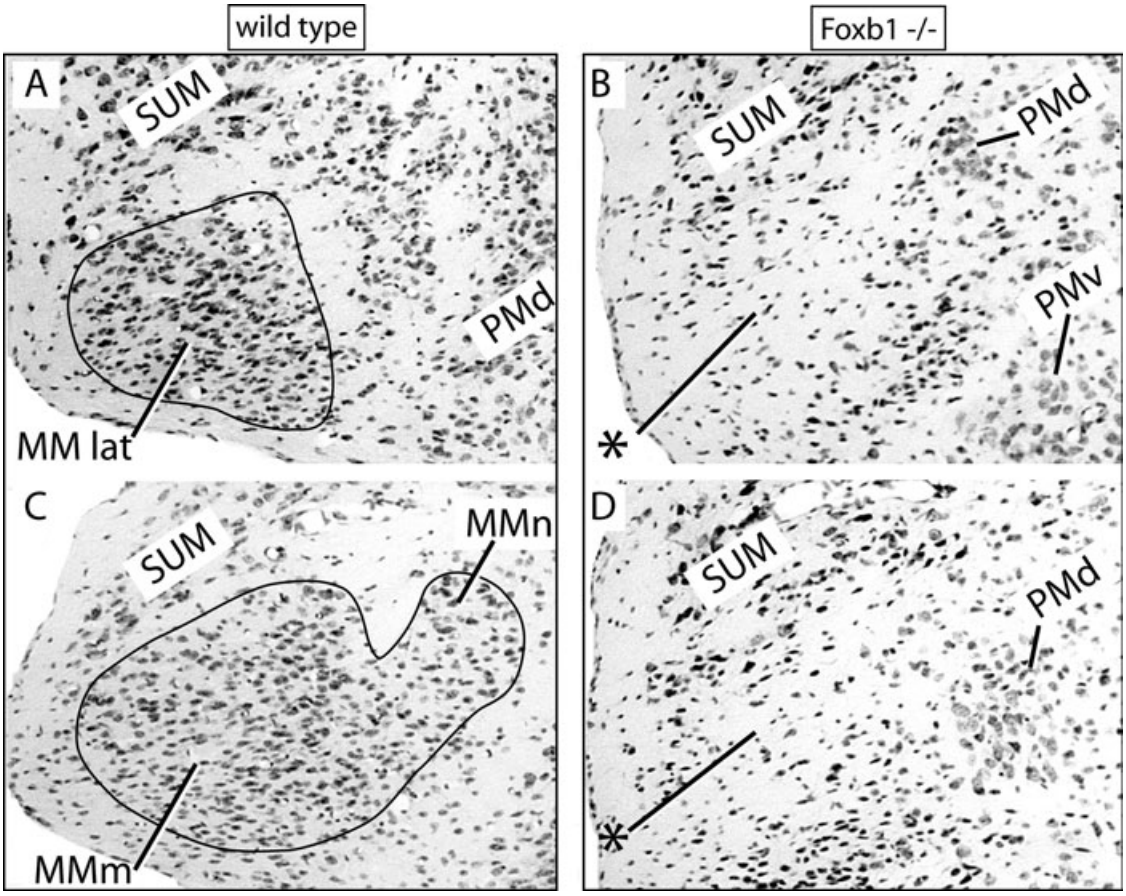

FIG. 2. Confirmation of anatomical defects at high magnification. Details of Fig. 1E and F at high magnification show that the large mass of the lateral subdivision of the MM (A) is missing in the mutant (B). The medial (MMm) and median (MMn) subdivisions (C) are also missing in corresponding sections of mutant brain (D). The place formerly occupied by the MM is filled by a cell-sparse neuropil (asterisks in B and D).

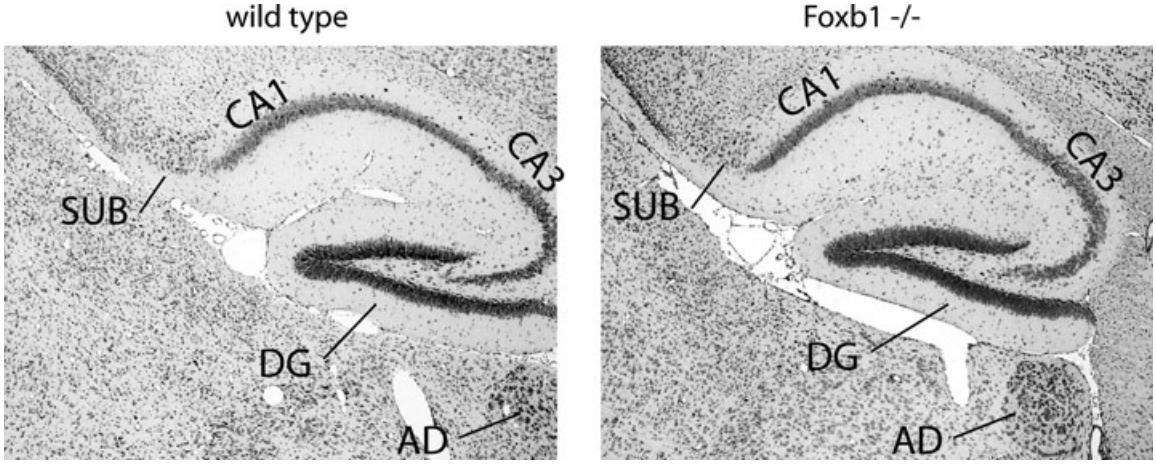

FIG. 3. The hippocampus shows normal morphology in *Foxb1* homozygotes. Nissl-stained sagittal sections of wild-type (left) und mutant (right) adult brains showing the distinctive morphological traits of the hippocampal region. No differences could be observed in this region between the two. AD, anterodorsal thalamic nucleus; CA, Ammon's horn; DG, dentate gyrus; SUB, subiculum.

significant difference between wild-type and $Foxb1^{+/-}$ mice but did reveal a significant difference between $Foxb1^{-/-}$ and wild-type as well as $Foxb1^{+/-}$ mice. We found no significant difference in the total number of arm visits between wild-type (13.8 ± 1.4), $Foxb1^{+/-}$ (14.0 ± 1.2) and $Foxb1^{-/-}$ (14.5 ± 1.0) animals.

In order to corroborate these results, we tested our mutants in the eight-arm radial maze task, a classical test for working memory in rodents (Becker *et al.*, 1980). Mice were trained with one trial per day for 7 days (Fig. 6). Repeated-measures ANOVA revealed a significant effect of genotype ($F_{2,29} = 98.45$, $P < 0.001$) and day of experiment

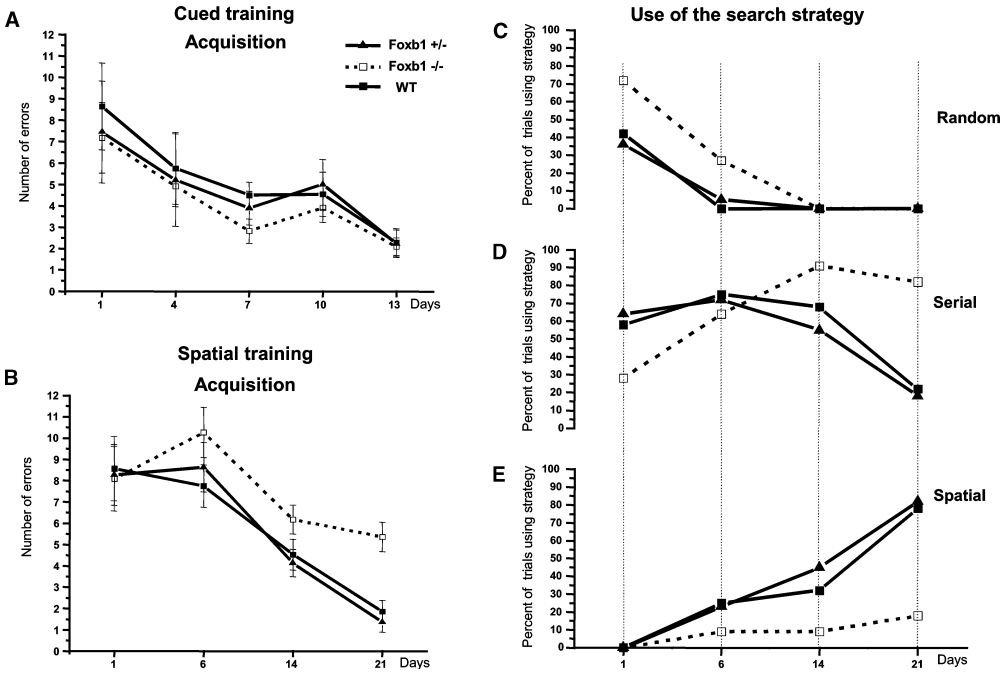

FIG. 4. *Foxb1*$^{-/-}$ mice are impaired in acquisition of the spatial memory version of the Barnes circular maze but have normal memory in a cued version of the maze. (A) Mean number of errors (± SEM) during acquisition of a cued version of the maze for wild-type (black squares, $n = 12$), *Foxb1*$^{-/-}$ (white squares, $n = 11$) and *Foxb1*$^{+/-}$ mice (black triangles, $n = 10$). (B) Mean number of errors (± SEM) during acquisition of a spatial version of the maze for wild-type (black squares, $n = 12$), *Foxb1*$^{-/-}$ (white squares, $n = 11$) and *Foxb1*$^{+/-}$ mice (black triangles, $n = 10$). (C–E) Use of random search strategy (C), of serial search strategy (D) and spatial search strategy (E) by wild-type (black squares, $n = 12$), *Foxb1*$^{-/-}$ (white squares, $n = 11$) and *Foxb1*$^{+/-}$ mice (black triangles, $n = 10$).

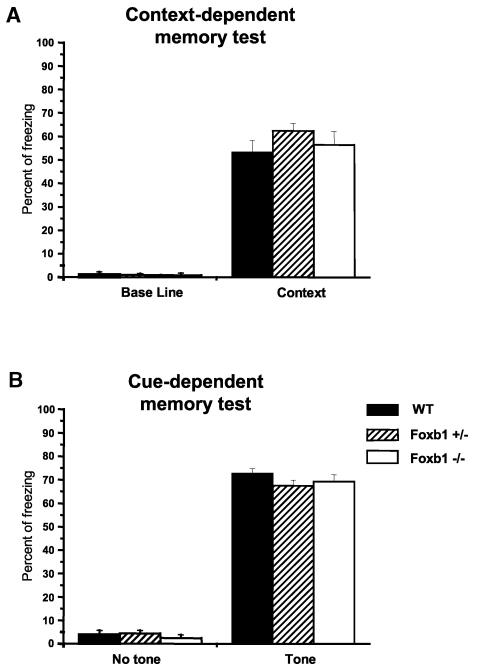

FIG. 5. *Foxb1*$^{-/-}$ mice acquired cued and contextual fear conditioning task without deficit. Mean percentage of freezing (± SEM) during testing in the fear conditioning task for wild-type (black bars, $n = 12$), *Foxb1*$^{-/-}$ (open bars, $n = 11$) and *Foxb1*$^{+/-}$ mice (striped bars, $n = 10$). (A) The contextual memory test was performed 24 h after training. (B) The tone-dependent memory test was performed 26 h after training.

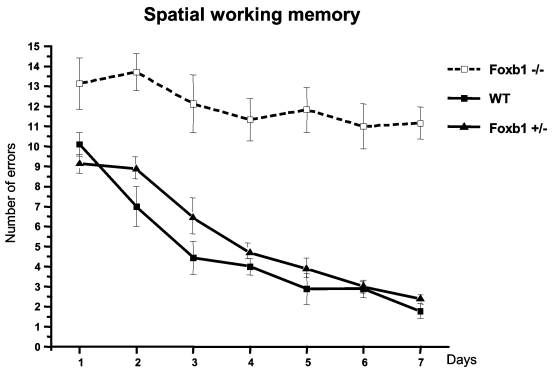

FIG. 6. *Foxb1*$^{-/-}$ mice are impaired in acquisition of the spatial working memory on the eight-arm radial maze task. Mean number of errors (± SEM) during acquisition of the working memory task on the eight-arm radial maze for wild-type (black squares, $n = 12$), *Foxb1*$^{-/-}$ (white squares, $n = 11$) and *Foxb1*$^{+/-}$ mice (black triangles, $n = 10$).

($F_{6,174} = 29.15$, $P < 0.001$) on the number of working memory errors, as well as a significant genotype–day interaction ($F_{12,174} = 2.89$, $P < 0.05$). *Post hoc* analysis determined that control mice significantly improved their performance over training, whereas mutant mice did not learn the task and made significantly more revisiting errors than the controls throughout the complete period of training.

Finally, we used a version of the eight-arm maze task that allows the simultaneous evaluation of both spatial reference memory and spatial working memory (Olton & Samuelson, 1976). Repeated-measures ANOVA revealed a significant main effect of genotype ($F_{1,13} = 171.89$, $P < 0.001$) and day of experiment ($F_{6,78} = 24.67$, $P < 0.001$) on the number of working memory errors, whereas no genotype–day interaction was detected ($F_{6,78} = 0.32$, $P > 0.05$; Fig. 7A). However, repeated-measures ANOVA of reference memory errors showed no significant main effect of genotype ($F_{1,15} = 2.72$, $P > 0.05$) but a

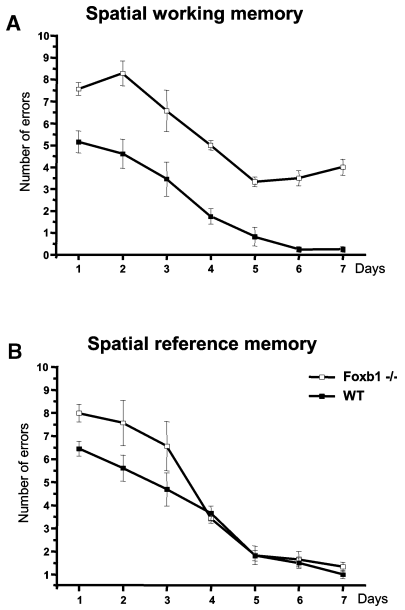

FIG. 7. *Foxb1*$^{-/-}$ mice are impaired in a spatial working memory task but not in a spatial reference memory task on the eight-arm radial maze. (A) Mean number of spatial working memory errors (± SEM) during acquisition of the eight-arm radial maze task for wild-type (black squares, $n = 12$) and *Foxb1*$^{-/-}$ mice (white squares, $n = 11$). (B) Mean number of spatial reference memory errors (± SEM) during acquisition of the eight-arm radial maze task for wild-type (black squares, $n = 12$) and *Foxb1*$^{-/-}$ mice (white squares, $n = 11$).

significant main effect of day of experiment ($F_{6,90} = 44.15$, $P < 0.001$) on the number of working memory errors, whereas no genotype–day interaction was detected ($F_{6,90} = 1.45$, $P > 0.05$; Fig. 7B).

Discussion

Diencephalic morphological phenotype of the Foxb1 *mutant*

The present study examined effects of a targeted mutation of the *Foxb1* gene on learning and memory functions in mice. *Foxb1* codes for a forkhead homeotic gene specifically expressed in portions of caudal diencephalon, midbrain, hindbrain and spinal cord (Kaestner *et al*., 1996; Wehr *et al*., 1997; Alvarez-Bolado *et al*., 1999, 2000a). Our *Foxb1*$^{-/-}$ mice lacked medial mammillary nuclei and a substantial part of the lateral mammillary neurons. The place in the posterior hypothalamus previously occupied by the medial mammillary nuclei had become in the adult a smaller area filled with cell-sparse neuropils (Fig. 2). This is in agreement with our previous observation that the medial mammillary nuclei are absent in *Foxb1* mutant mice (Wehr *et al*., 1997), and the lateral mammillary nucleus is reduced to 45% of its earlier size (Alvarez-Bolado *et al*., 2000b). The mammillary axonal tree in *Foxb1*$^{-/-}$ mutants was severely reduced, consisting of a thin principal mammillary tract and almost undetectable mammillotegmental tract, as well as a small group of rostrally directed axons ending around the ventral thalamus and representing probably the aborted remnants of the mammillothalamic tract (Alvarez-Bolado *et al*., 2000b). Tuberomammillary and ventral premammillary nuclei lay in the same brain region but they do not express *Foxb1* and they were not affected by the *Foxb1* mutation (Alvarez-Bolado *et al*., 2000b). Our analysis has also shown that the hippocampus neuromorphology was not affected in the *Foxb1*$^{-/-}$ mutant mice. The presence of a still relatively large and clearly visible fornix bundle in the mutants is presumably due to the survival of a part of the LM. The supramammillary nuclei, which do not express *Foxb1*, also remained intact in *Foxb1*$^{-/-}$ mice. This is an important factor for their behavioural analysis because these nuclei set hippocampal theta rhythm (Kirk, 1998) and injury to them occurs during most surgical lesions of MB (Sutherland & Rodriguez, 1989; Aggleton *et al*., 1991).

Thus mutation of the *Foxb1* gene results in damage to diencephalic structures, which are considered to play a critical role in Korsakoff syndrome (Mayes *et al*., 1988; Kahn & Crosby, 1972; Kopelman, 1995). However, the mechanism of this lesioning is different from traditional surgical techniques and has no typical side-effects such as damage to adjacent fibre tracts or surrounding nuclei (Sutherland & Rodriguez, 1989; Aggleton *et al*., 1991). It was therefore valuable to study the learning and memory functions in *Foxb1*$^{-/-}$ mice as a novel genetic model of MB lesions.

Memory alterations in Foxb1 *mutants*

Foxb1$^{-/-}$ mice had impaired learning in the Barnes circular maze test. Specifically, they were not able to learn the spatial version of the task although they acquired the cued non-spatial navigation task. *Foxb1*$^{-/-}$ mice did not differ in performance of the cued version of the task from their heterozygous and wild-type littermates. This shows that these mutants have no visual or motivational impairments that preclude finding an escape hole and that their motor deficit (Dou *et al*., 1997) does not influence their general performance in the Barnes maze. The motor disturbances in *Foxb1* mutants due to their spinal cord phenotype have been described previously (Dou *et al*., 1997). In particular, Dou *et al*. (1997) found that the number of motoneurons in the lateral motor column at the lumbar level was reduced by nearly 50% in the *Foxb1* (*TWH* according to their nomenclature) mutants. Moreover, mutant mice had a reduction in strength and abnormal hind limb clasping reflex (Dou *et al*., 1997). Taking these findings into consideration, we selected the learning and memory tasks that make minimal demands on the motor abilities of mice: spontaneous alternation, eight-arm radial maze, fear conditioning and social transmission of food preference. Performance of mice and the criteria used to measure learning in these tasks (number of error choices, number of freezing events, amount of eaten food) are relatively little altered by motor impairments. As was previously shown, the Barnes maze is a suitable task to study spatial learning and memory in mice with motor impairments (Mansuy *et al*., 1998; Paylor *et al*., 2001). Therefore, the deficit that was observed in mutants in the spatial version of this maze provides clear evidence of specific spatial learning and memory impairment in *Foxb1*$^{-/-}$ mice.

A deficit in spatial memory and navigation is commonly explained by a deficiency in hippocampal function (Olton *et al*., 1979; Morris *et al*., 1982). Because the MM nucleus is indeed the target of a major efferent bundle from the hippocampus (Swanson & Cowan, 1977; Meibach & Siegel, 1977), ablation of MM and MTt nuclei in *Foxb1*$^{-/-}$ mice may lead to a functional insufficiency of the whole hippocampal memory system. To test this hypothesis we used two other learning and memory tasks that require integrity of the hippocampus: contextual fear conditioning (Kim & Fanselow, 1992) and social transmission of food preference (Bunsey & Eichenbaum, 1995). It was found, however, that performance of *Foxb1*$^{-/-}$ mice was not impaired in either task. These results suggest that genetic ablation of the MB and mammillothalamic tract in *Foxb1*$^{-/-}$ mice produces learning and memory deficits that are not equivalent to the behavioural impairments caused by lesions of the hippocampus. This conclusion is in agreement with studies showing that lesioning of the MB in rats does not impair passive avoidance learning (Langlais & Savage, 1995; Savage *et al*., 1997), a task that is

also dependent on hippocampal function (Stubley-Weatherly *et al*., 1996; Izquierdo & Medina, 1997).

Thus *Foxb1* mutant mice are impaired in some of the hippocampus-dependent tasks (Barnes maze) but have no deficit in other hippocampus-dependent tasks (contextual fear conditioning and social transmission of food preference). This might be possible if learning and memory in the Barnes maze depends on the participation of MB in a neural circuitry, which is implicated in this task but not in the other two. MTt links MB to the anterior thalamic nuclei (ATn), which projects to the medial prefrontal cortex (Gonzalo-Ruiz *et al*., 1992). This cerebral circuitry is known to be important for successful performance in spatial tasks, including the Morris water maze (Kolb *et al*., 1983; Sutherland *et al*., 1988; Granon & Poucet, 1995). Moreover, lesion studies in rats have shown that the ATn and mPFC play a crucial role in working memory (Granon *et al*., 1994; Boehm *et al*., 1996; Byatt & Dalrymple-Alford, 1996; Kesner *et al*., 1996). Therefore, we next investigated whether *Foxb1* mutant mice have impairment in working memory functions.

The results from spontaneous alternation and the eight-arm maze tests indicate that *Foxb1* mutants with lesions of MB and MTt are indeed impaired in the spatial tasks involving working memory. However, ablation of these brain structures in $Foxb1^{-/-}$ mice obviously does not impair their ability to learn the spatial location of baited arms, as shown in the reference memory task in the eight-arm maze (Fig. 7B). That fact that *Foxb1* mutants decrease the number of working memory errors during training in the version of the radial arm maze, when only four arms were baited (Fig. 7A), is explained by a reduction in the number of appealing arms due to acquisition of reference memory rather than by an improvement of their working memory. At the beginning of the experiment mice visit both baited and unbaited arms at random, but in the course of training animals from both groups learned the spatial location of unbaited arms and stopped visiting them. However, the $Foxb1^{-/-}$ mutants repeatedly visited unbaited arms due to impairment of working memory. Because the number of baited arms was half the total number of arms, improved reference memory resulted in a simultaneous two-fold reduction in the mean number of working memory errors (Fig. 7A).

Thus, our results showed that although MB and MTt were not critical for the memorization of allocentric spatial information, they were essential for behaviour that required extended navigation using this spatial information (as in the Barnes maze). This conclusion is in agreement with data showing that electrolytic lesions of MB in rats do not disrupt learning in allocentric spatial conditional associative tasks (Sziklas *et al*., 1996; Sziklas & Petrides, 2000), but do impair spatial working memory in the radial arm (Saravis *et al*., 1990; Sziklas *et al*., 1996; Neave *et al*., 1997) and performance in the spatial version of the Morris water maze (Santin *et al*., 1999). Thus our results suggest that the role of MB and MTt is restricted to a subset of spatial tasks that rely on working memory to navigate to a memorized spatial location.

Working memory is defined as the ability to retain and manipulate mnemonic information to guide ongoing behaviour (Baddeley, 1986) and consists of at least two important components. One is the short-term storage of information regarding recent trials to make decisions about future actions (Goldman-Rakic, 1995). Such retrospective working memory is implicated in tasks dependent upon choices made in previous trials (for instance, making choices of arms in spatial alternation and radial-maze tests). This behaviour is obviously impaired by the genetic ablation of MB and MTt (present study) as well as by cytotoxic and electrolytic lesions of MB (Aggleton *et al*., 1995; Sziklas *et al*., 1996), which constitutes evidence for the implication of MB in this first component of working memory.

Another component of working memory is short-term storage and manipulation of information necessary to guide goal-directed actions (prospective working memory) (Fuster, 1989), including spatial navigation and route planning (Granon & Poucet, 1995). The implication of MB and MTt in this component of working memory would explain the dissociation observed in our mutants between the deficits in spatial learning in the Barnes maze and the preservation of spatial reference memory in the eight-arm maze. The Barnes maze requires the remembered location of a hidden goal to be stored in a working memory buffer in order to reach it from a distant position. This type of memory, however, is not required for selecting baited arms in the eight-arm radial maze: at the point of choice in the radial arm maze the mouse had simply to select the entrance into a correct arm based on its allocentric reference memory.

Another potential cause of the spatial navigation deficit in $Foxb1^{-/-}$ mice may be the anatomical defects in the lateral mammillary nuclei in these mice. A number of studies have demonstrated the existence of head direction neurons in the LM (Blair *et al*., 1998; Stackman & Taube, 1998). Furthermore, the LM may be critical for driving the head direction neurons in the anterior thalamic nucleus (Blair *et al*., 1999). The head direction neurons are thought to be involved in computing direction during spatial navigation (Taube, 1998) and disruption of this circuitry in the $Foxb1^{-/-}$ mice might impair finding of the escape location in the Barnes maze. Such computation, however, may not be essential for selecting a correct arm in the radial maze.

The role of MB in spatial navigation and working memory may be mediated by its connection through MTt to anterior thalamic nuclei and further to the mPFC (Gonzalo-Ruiz *et al*., 1992). The prefrontal cortex has been suggested to be the key structure of a working memory system in rodents (Kesner *et al*., 1996), non-human primates (Fuster, 1989) and humans (Cohen *et al*., 1997; Courtney *et al*., 1997). Moreover, lesion studies have shown that the mPFC is required for successful performance in the water maze (Kolb *et al*., 1983; Sutherland *et al*., 1988; Granon & Poucet, 1995). Rats with lesions in the mPFC show impaired spatial working memory (Shaw & Aggleton, 1993; Granon *et al*., 1994) but have normal spatial reference memory (Joel *et al*., 1997; Quirk *et al*., 2000). Our results obtained in mice with genetic ablation of MB are similar to these effects of mPFC lesions.

Our experiments suggest that MB and the MTt contribute to spatial learning and memory, but this contribution is different from that of the hippocampus. Although the hippocampus is important in a broad set of tasks implicating spatial information (Morris *et al*., 1982; Nadel, 1991) and representation of allocentric space (O'Keefe & Nadel, 1978), our results show that the role of MB and MTt is limited to the recruitment of spatial information in the working memory tasks.

The finding that genetic ablation of MB leads to prominent and selective spatial working memory deficits suggests that at least one component of the memory deficit in patients with Korsakoff's syndrome may be due to disruption of neural structures in and around the MB typical of this syndrome. Further studies of *Foxb1* mutant mice may validate this animal model for diencephalic amnesia and contribute to the explanation of memory disturbances in humans with neuropathology in the region of the MB.

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Abbreviations

ATn, anterior thalamic nuclei; E, day of embryonic development; LM, lateral mammillary nucleus; MB, mammilary body; MM, medial mammillary nucleus; MMlat, lateral subdivision of the medial mammillary nucleus; MMm, medial subdivision of the medial mammillary nucleus; MMn, median subdivision of the medial mammillary nucleus; mPFC, medial prefrontal cortex; MTt, mammillothalamic tract; P, day of postnatal development; pm, principal mammillary tract; PMd, dorsal premammillary nucleus; PMv, ventral premammillary nucleus.

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