

Olfactory based spatial learning in neonatal mice and its dependence on CaMKII

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Spatial learning and memory involves the ability to encode geometric relationships between perceived cues and depends critically on the hippocampus. Visually guided spatial learning has been demonstrated in adult animals. As infant animals rely heavily on olfaction, olfactory based spatial learning was assessed in infant mice. When 12-day-old pups were displaced

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from their nest, they learned within a few training trials to use the spatial pattern of odor cues to move back to the nest. However, mouse pups that over-expressed Ca²⁺/calmodulin-dependent protein kinase (CaMKII) in hippocampal neurons were impaired in olfactory based spatial learning. *NeuroReport* 11:1051–1055 © 2000 Lippincott Williams & Wilkins.

INTRODUCTION

An animal's movements within its habitat are under strong selection pressure [1]. Learning about routes to ecologically important locations allows the animal to minimize costs such as predation or energy expenditure. A variety of species have the ability to learn the arrangement of surrounding cues and use such spatial representations to navigate within the landmarks from any position to a specific location [2,3]. In standardized tests such as the Barnes maze or the Morris water maze, adult rodents learn spatial constellations of visual cues [4,5], but in preweaning altricial mammals olfactory cues play an important role in orienting responses. In contrast to the visual or auditory systems, the olfactory system involved in orienting responses such as locating the mother's nipple is functional earlier, in the first postnatal week [6], and pups of this age are able to learn olfactory associations [7]. We therefore determined the abilities of mouse pups to learn the spatial constellation of odor cues. A spatial learning task was developed in which mouse pups had to navigate back to their nest when displaced. Spatial learning and memory depend critically on longterm potentiation (LTP) in hippocampal neurons. Genetically altered adult mice that over-express a constitutively active form of Ca²⁺/calmodulin-dependent protein kinase (CaMKII) in hippocampal neurons do not show low frequency LTP and fail to learn the spatial version of the Barnes maze [4,8,9]. To determine whether olfactory spatial learning is disrupted by an over-expression of the constitutively active CaMKII-Asp²⁸⁶

transgene, as in visual spatial learning in adult mice, homing of mutant pups was investigated. We compared olfactory based navigation back to the nest in wild-type and mutant mouse pups.

MATERIALS AND METHODS

Subjects: C57B6/CBA female mice were mated with males that expressed a mutant form of CaMKII [9]. Mice were housed under standard laboratory conditions. Cages were checked twice daily, at ~09.00 h and 18.00 h. Pups found at either time were termed 0 days of age.

Litter size ranged between 9 and 11 pups. Litters were not culled and contained both wild-type and mutant pups. Treatments were approved by the Institutional Animal Care and Use Committee.

Spatial learning test: On postnatal day 12, all pups of a litter were tested in succession, 1 min apart. Five to six pups, whose genetic backgrounds were not known, were put in a Petri dish (diameter 9 cm), containing soiled home cage bedding, in the test cage lateral to the middle of the cage. The test cage was a plastic tub (46 × 25 × 21 cm) with clean wood-chip bedding. In each of the four cage corners, the tip of a cotton applicator was fixed 8 cm above the floor and was impregnated with either lemon oil, orange oil (both from Humco Laboratory), vanilla extract or peppermint extract (both from Ehlers). After 2 min of acclimatization, one pup was placed facing the wall in one of the two corners of the cage most distant from the dish. As the

animal moved about, the locomotion path was drawn on a paper facsimile of the plastic tub and the latency of crossing the lip of the dish with the two forepaws was recorded by stop watch. As reinforcement, the animal was left in the huddle for 1 min. If the pup did not reach the dish after 150s, it was put back into the dish by the experimenter. The animal was then started three more times alternating from the two corners. After each trial, the bedding of the test cage was thoroughly mixed and smoothed. Even though most of the pups (60%) had not yet opened their eyes, the cage was rotated 180° twice during the four trials to exclude guidance from any distal cues. After the fourth training trial, and after the pup had spent 1 min in the huddle, the test trial was carried out. The dish containing the littermates was taken out of the test cage. The animal was again put in the corner, opposite the site of last

training trial. The path and latency of crossing the indentation left by the dish with the two forepaws were recorded. Because the odor cues remained constant, the above procedure is called fixed. A similar procedure was used for the control pups except that between each trial the four odors were randomly exchanged between the corners to avoid the same constellation twice, and thus eliminate the ability of the pups for use these cues to navigate to the goal. This procedure is called altered. Pups of a litter were tested alternating between fixed and altered odors.

Genotyping: Mice were screened for the CaMKII transgene after testing by PCR for routine typing. After genotyping, homing data from three groups were analyzed: wild-type pups trained and tested with either fixed (wild-fixed, 33 pups from 13 litters) or altered (wild-altered, 37

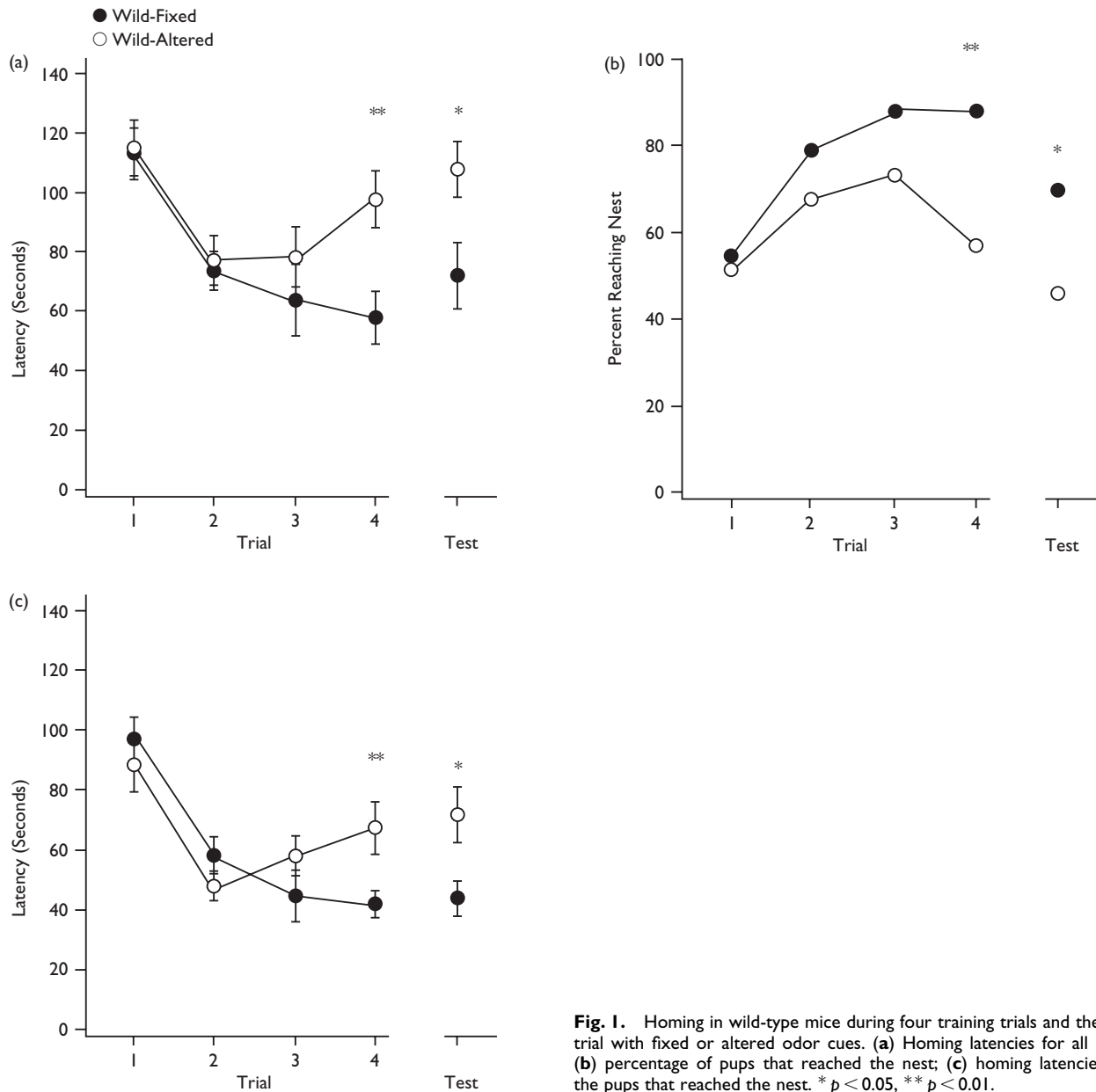


Fig. 1. Homing in wild-type mice during four training trials and the test trial with fixed or altered odor cues. (a) Homing latencies for all pups; (b) percentage of pups that reached the nest; (c) homing latencies for the pups that reached the nest. * $p < 0.05$, ** $p < 0.01$.

pups from 12 litters) odors, and mutant pups with fixed (mutant-fixed, 15 pups from eight litters) odors. In preliminary analyses no sex differences were found for any of the measures and data of littermates were pooled.

In situ hybridization: To determine expression of the transgene, *in situ* hybridization was performed as described previously [9]. Cryostat sections were hybridized to an oligonucleotide probe that specifically detects the CaMKII-Asp²⁸⁶ mRNA.

Odor discrimination test: In order to demonstrate that mutant pups were not impaired in olfactory or motor function, an odor discrimination test was carried out. The test chamber consisted of a plastic tub (14 × 9 × 8 cm) with a wire mesh floor and was placed in a transparent plastic container to control for outside odors. The floor of the test chamber was divided into three areas of the same size (4.6 × 9 cm). Home cage bedding was placed under the mesh at the end of one side of the test chamber; in the center was an area without shavings, the neutral zone; under the mesh of the opposite side was clean bedding. On the day of testing, on postnatal day 12, a pup was taken out of the home cage and placed in the neutral zone. The pup faced one of the two walls along the neutral zone. The test procedure consisted of four 2 min trials in which the time the pup spent in each of the three areas and the number of crossings between the areas was recorded. A crossing was defined as when all four paws of the pup crossed the line dividing two areas. The orientation of the pup was alternated, on the start of each trial. The test chamber was rotated 180° between trials 2 and 3. After testing, the genotype of the pups was determined as described above. A total of 87 pups from nine litters were tested: 67 pups from nine litters were wild-type, and 20 pups from five litters were mutant. No sex differences were found and the data from littermates were pooled.

RESULTS

To determine whether mouse pups use a spatial pattern of odor cues when homing, we first compared wild-type pups tested with a fixed pattern to wild-type pups tested with an altered pattern. Homing latencies changed during the four training trials across both groups (repeated measure ANOVA, $F(3,69) = 14.09$, $p < 0.001$), but the groups significantly differed across trials (trial × group: $F(3,69) = 2.88$, $p < 0.05$). Both wild-fixed and wild-altered pups decreased homing latencies between the first and the second training trial ($p < 0.01$, Fig. 1a). However, the wild-fixed pups kept short latencies during the remaining trials, but wild-altered pups increased latencies, which were significantly longer on the last training trial than the latencies of the wild-fixed ($p < 0.01$). When the nest was removed during the test trial, wild-fixed pups reached the former nest site as fast as during the fourth training trial. Moreover, the wild-fixed pups reached the nest site significantly faster than the wild-altered pups ($p < 0.03$, Fig. 1a). Not only were the latencies shorter, but a greater number of wild-fixed pups reached the nest site during the fourth training trial ($\chi^2 = 8.28$, $p < 0.01$) and in the test trial ($\chi^2 = 4.02$, $p < 0.05$, Fig. 1b). To determine whether the overall latencies of the wild-altered pups were longer due to there being fewer of

these pups reaching the nest, the latencies of only the pups reaching the nest during the training and test were compared. For these analyses, individual data rather than litter means were used. Again, the latencies of the wild-fixed pups were significantly shorter on the fourth training trial (t -test, $t(48) = 2.79$, $p < 0.01$) and during the test ($t(38) = 2.66$, $p < 0.02$, Fig. 1c).

On postnatal day 12, the transgene was over-expressed in mutant pups throughout the forebrain (Fig. 2).

To determine the spatial learning abilities of mutant pups, we compared their homing latencies to the latencies of the wild-type pups, both with a fixed odor pattern. In both groups, homing latencies changed across the training trials (ANOVA, $F(3,57) = 16.56$, $p < 0.001$), but there was no difference between groups ($F(3,57) = 0.91$), although mutant pups had longer latencies during the first trial ($p < 0.05$ Fig. 3a). The number of animals reaching the nest did not differ between groups (Fig. 3b), neither did the latencies of the pups that reached the nest (Fig. 3c). When the nest was removed during the test trial, however, wild-fixed pups reached the former nest site significantly sooner than the mutant-fixed pups ($p < 0.001$, Fig. 3a), and significantly fewer mutant pups reached the nest ($\chi^2 = 10.26$, $p < 0.002$, Fig. 3b). As during the test only three of 15 mutant pups reached the nest site no statistical analysis was performed on latency data for pups reaching the nest.

In order to show that mutant pups were not impaired in olfactory or motor function, we carried out the odor discrimination test. All pups preferred home cage bedding over clean bedding (home: wild-type 259.2 ± 24.7 s, mutant 235.1 ± 35.6 s; clean: wild-type 45.8 ± 9.9 s, mutant 56.9 ± 23.7 s; ANOVA, $F(1,12) = 48.1$, $p < 0.001$) and wild-type did not differ from mutant pups. There was no difference in locomotion (rate of crossing: wild-type 9.2 ± 1.9 , mutant 7.3 ± 1.9).



Fig. 2. *In situ* hybridization showing the over-expression pattern of the CaMKII-Asp²⁸⁶ transgene in the 12-day-old mouse. Coronal brain section with hippocampus.

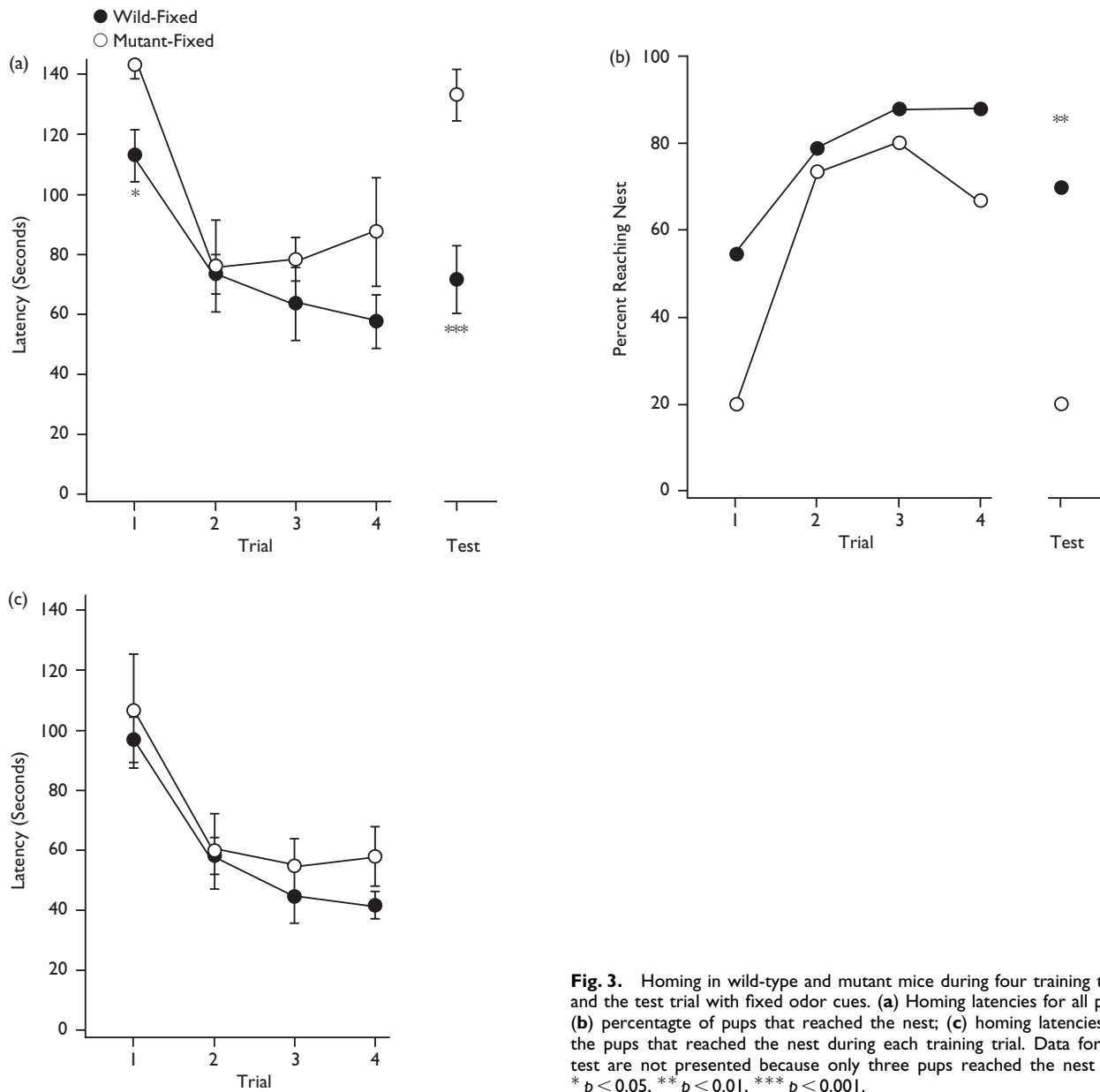


Fig. 3. Homing in wild-type and mutant mice during four training trials and the test trial with fixed odor cues. (a) Homing latencies for all pups; (b) percentage of pups that reached the nest; (c) homing latencies for the pups that reached the nest during each training trial. Data for the test are not presented because only three pups reached the nest site. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

Mammals use different orientation mechanisms when homing. They may use guiding cues such as odor trails or beacons [10,11], integrate their path through sensory feedback [12], or learn the spatial configuration of landmarks [4]. In our experiment, mouse pups moving within a spatial pattern of odor cues were faster in reaching their nest site when the pattern was stable. During training, they may have learned to use the nest as a beacon and follow an olfactory, thermal or acoustic gradient to the nest as it has been shown in kittens [10]. In the test trial, when the nest was removed, they were not able to rely on such associative memory. We suggest that in the test trial pups used spatial memory of surrounding odor landmarks. Our experimental design controlled for other orienting mechanisms such as following an odor trail, using distal cues, or

using route-based information. A possible mechanism could involve learning about variations in odor gradients in relation to the nest location. Mouse and rat infants are capable of visual spatial learning in the third postnatal week as assessed in the Morris water maze [13,14]. Learning in the Morris water maze requires elaborate training schedules, as swimming in infant mice and in mice in general is a rather species-atypical behavior pattern [15]. In contrast, young rodents are often translocated from their nest under natural conditions, and successful homing is adaptive. Our study shows that in an ecologically relevant situation, mouse pups show spatial learning and memory within a few training trials at a very earlier age.

To determine the role of the hippocampus in olfactory based spatial learning, homing of mutant pups was investigated. Infant mice expressing the CaMKII-Asp²⁸⁶ trans-

gene in the hippocampus were not impaired in olfactory discrimination or locomotion but failed to use a spatial pattern of odors when homing. The pups may have learned to follow nest cues during the training as some pups were repeatedly successful in reaching the nest, but when the nest was removed, they were, in contrast to the wild-type pups, not able to use the spatial pattern of surrounding odor cues to home. CaMKII normally converts to a Ca²⁺-independent kinase upon activation through autophosphorylation at a single threonine residue [16–18]. This conversion to the Ca²⁺-independent state is required for the induction of LTP and for normal spatial learning in adult mice [19]. Increasing the level of Ca²⁺-independent CaMKII in adult mice through expression of the CaMKII–Asp²⁸⁶ transgene in the hippocampus impairs LTP in response to theta frequency (5–10 Hz) stimulation and impairs visually guided spatial learning [4,8,9]. In contrast to the visual or auditory systems, olfactory receptors synapse directly onto the cerebral cortex without relaying in the thalamus [20]. In particular, they project to the hippocampus and thereby have a rather unique access to this structure that is key to spatial learning. Little is known about the hippocampus and olfactory spatial learning in mammals, although the hippocampus has been shown to encode short-term and long-term memories of non-spatial odor patterns [21,22]. Pigeons, however, seem to use odor cues for homing [23]. Ablations of the hippocampus [24], which is homologous to the hippocampus of mammals [2], and of the piriform cortex [25] both disrupted homing. Our results extend these findings and demonstrate a role of the hippocampus in olfactory spatial learning in infants of a rodent species.

CONCLUSION

Twelve-day-old mice used olfactory based spatial memory to navigate back to their nest. Hippocampal CaMKII plays

an important role in such short-term spatial learning and memory that relies on odor cues. Adult mice of the line tested in the present study fail in the spatial version of the Barnes maze, which requires the acquisition of the spatial pattern of distal visual cues to locate the home [4]. Therefore, we suggest a common neural mechanism mediating spatial learning across ages and sensory modalities.

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