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A novel behavioral paradigm for assessing concept of nests in mice

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Abstract

Abstract concepts in the brain enable humans to efficiently and correctly recognize and categorize a seemingly infinite amount of objects and daily events. Such abstract generalization abilities are traditionally considered to be unique to humans and perhaps non-human primates. However, emerging neurophysiological recordings indicate the existence of neural correlates for the abstract concept of nests in the mouse brain. To facilitate the molecular and genetic analyses of concepts in the mouse model, we have developed a nest generalization test based on mice's natural behavior. We show that inducible and forebrain-specific NMDA receptor knockout results in pronounced impairment in this test. Interestingly, this generalization deficit could be gradually compensated for over time by repeated experiences even in face of the continued deficit in object recognition memory. On the contrast, the forebrain-specific presenilin-1 knockout mice, which have subtle phenotypes, were normal in performing this test. Therefore, our study not only establishes a quantitative method for assessing the nest concept in mice, but also demonstrates its great potential in combining powerful mouse genetics for dissecting the molecular basis of concept formation in the brain.

1. Introduction

Abstract concepts and generalized knowledge are traditionally assumed to be an exclusive hallmark that defines humans and possibly other highly intelligent non-human primates (Warrington and Shallice, 1984; Fodor and British Psychological Society., 1987) Concepts in the brain can allow humans to avoid the burden of remembering and storing each mnemonic detail of a seemingly infinite amount of objects and events. These higher cognitive functions play an essential role in guiding our adaptive behaviors in everyday life and have enabled us to adapt to novel situations with great efficiency (Tranel et al., 1997; Martin and Chao, 2001; Gallese, 2003; Quiroga et al., 2005; Tsien, 2007). For example, when we check into a hotel, the concept of 'bed' in our brains can help us identify the bed effortlessly from various furniture in the room despite the fact that the bed could be drastically distinct in terms of its style or physical shape from the ones that we have in our homes.

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Just like the human's ability to recognize beds, we think that it is likely that non-primate animals such as rodents and birds may also possess some rudimentary levels of high cognitive computations such as the ability to recognize various objects that can be used as their nests. Since a nest or bed can vary widely in its physical appearance (e.g. distinct physical shape, style, color, and textures, etc.), it is conceivable that the internal representations of those objects are likely to require the brain to possess generalized recognition ability into categories beyond specific details of each item. Without such an ability, it would be very difficult for evolutionary and ecological adaptation and survival in natural and complex environments (Squire, 1987). Currently, little is known regarding how the brain actually generates and encodes the abstract concepts of objects such as bed or nest. The hippocampus and its medial temporal lobe have attracted great amounts of attention in the field of memory research (Tsien et al., 1996b; Tang et al., 1999; Tulving and Craik, 2000; Squire et al., 2004). Various clinical studies show that adult patients suffering hippocampal damage had difficulty in forming episodic memories as well as new concepts and general knowledge (Mishkin, 1978; Wilson and Baddeley, 1988; Reed and Squire, 1998; Squire and Zola, 1998; McGuire et al., 2005; Moscovitch et al., 2006). While there are many excellent behavioral paradigms for the measurement of classical learning and memory functions in a variety of animals ranging from humans to mice to fruit flies (Thompson and Krupa, 1994; O'Reilly and Rudy, 2001; Davis, 2005; Sara and Hars, 2006), to the best of our knowledge, there is no behavioral paradigm for the genetic analysis of the molecular basis of abstract concepts in popular genetic models such as mice.

Intriguingly, our recent large-scale recording revealed that some CA1 cells in the mouse hippocampus seem to be involved in extracting commonality and abstracting general features from various episodic events (Squire, 1987; Lin et al., 2006). In support of this notion, we recently reported the neural correlates of the abstract concept of 'nests' or 'beds' in the CA1 region of the mouse hippocampus (Lin et al., 2007). Specifically, we demonstrated the existence of a small number of CA1 neurons which would selectively fire or cease to fire when the mouse perceives nests or beds regardless of these objects' locations and environments (Lin et al., 2007). Parametric analyses indicated that those nest cells' responses seemed to be based on the nest functionality of the various objects, rather than the physical shapes of the objects (Lin et al., 2007). These *in vivo* recording experiments have prompted us to devise a behavioral paradigm that can be used systematically for the genetic analysis of the nest recognition and generalization ability in mice. Here, we report this behavioral method for molecular and genetic analysis of conceptual knowledge of objects in the mouse brain.

Over the past decade, our laboratory has developed a series of conditional gene knockout techniques that allow manipulations of a gene of interest in region-specific and temporally controlled manner (Tsien et al., 1996a; Shimizu et al., 2000; Feng et al., 2001; Wang et al., 2003; Cui et al., 2004). By genetically producing both memory impaired and memory enhanced mice, we have produced strong evidence for the role of the NMDA receptor in the formation of spatial and nonspatial memories (Tsien et al., 1996b; Tang et al., 1999; Rampon et al., 2000; Tsien, 2000). Given the fact that the hippocampus is crucial for not only episodic memory but also semantic memory (memory of concepts and generalized knowledge) (Mishkin, 1978; Wilson & Baddeley, 1988; Reed & Squire, 1998; Squire & Zola, 1998; McGuire et al., 2005; Moscovitch et al., 2006), we hypothesized that the NMDA receptor in hippocampus may also play a crucial role in the formation of semantic memories of facts, concepts, and abstract knowledge. Therefore, we have inducible and reversible, forebrain-specific NMDA receptor knockout mice (Cui et al., 2004), in which the NMDA receptor can be temporarily switched off in the forebrain regions such as the cortex and hippocampus right before the nest behavioral tests. Then by developing this novel

behavioral method we examined the role of NMDA receptor of conceptual knowledge of objects in the mouse brain.

2. Materials and Methods

2.1. Animals

The experiments were carried out on adult mice (BCF background). Generation and genotyping of inducible and forebrain-specific NMDA receptor knockout mice (iFB-NR1-KO) and forebrain-specific PS1-KO mice (FB-PS1-KO) will be the same as described previously (Feng et al. 2001, Cui et al., 2004). In brief, the iFB-NR1-KO mice are homozygous for the floxed-*NR1* gene and heterozygous for the CaMKII-*Cre* transgene, the *NR1-GFP* transgene under control of the tet-O promoter, and the tetracycline transactivator (*tTA*) transgene, which is driven by the β -actin promoter and contains a floxed stop sequence (*fNR1/fNR1*, *Cre*+, *tTA*+, and *NR1-GFP*+). Their littermates served as wild-type control. The FB-*PS1*-KO mice (*fPS1/fPS1*; *Cre*+) were generated by cross homozygous *fPS1* mice with *Cre* transgenic mice (Feng et al., 2001).

The mice used had been housed in standard mouse cages and they had only very limited experience in sleeping inside the natural cotton nests built by their mothers during their nursery period (the first 21 days after birth before weaning). After weaning, they were relocated to a new cage in which only granular bedding materials were layered on the floor (for absorbing urines and occasional dripped water from the drinking bottle). Seven days before experiments, these mice were separated and singly housed. Our mouse facility was on 12 hour light : dark cycles. These mice were not subject to any form of food- and water-deprivation or restriction.

From our previous experiments, inducible knockout of synaptic NMDA receptor function is known to occur in 5 days after we fed the mice with food pellets containing doxycycline at 6 mg/g (Cui et al., 2004). The forebrain-specific knockout was induced by feeding doxycycline (dox) for 7 days or more before the behavioral experiments started, and continuing dox-treatment during the experiments to ensure the NR1 deletion was temporally restricted for the duration of experiments. The ages of mice used in the experiments were between four and seven months old.

2.2. Nest construction

The fundamental feature of abstract concepts and generalized knowledge of nests is that it should not be ascribable to a specific type of shape and physical appearance of the nests. Under the guidance of this criterion, we reasoned that if mice possess the abstract concept of nests, the animals should be able to identify an object as a nest as such they would prefer to sleep in it. We have made a set of nests in various geometric shapes, sizes and texture as well as constructed with different materials. These objects include disposable coffee cups, other cardboard materials, plastic caps from instant coffee bottles or sucrose bottles, or metal caps from phenol containers or Altoids mint tins. The shapes, diameters, and heights of each nest were varied systematically (for examples, see Fig. 1A). In our experiments, all nests which were made from cardboard materials were all newly constructed and were disposed by each use so that we could exclude the odor factor. Similarly, we washed and cleaned the plastic and metal nests after each use to remove any left-over mouse odors.

2.3. Behavioral measurement of generalized ability to recognize nests of mice

The major criterion of concepts is the generalization ability. Therefore, animals should not have encountered the exact same kinds of objects and nests when we present these items to them during each session. Our behavioral paradigms for measuring the conceptual

knowledge of nests in mice are based on the direct behavioral observation of the locations where the animals would sleep or rest after we placed a novel nest inside their cages. The mice had no prior experiences with these artificial nests. Behavioral exploration and interactions with nests were completely voluntary and natural. The mouse's ability to recognize an object as nests were measured by whether or not the animal used it to sleep after the object was introduced into the housing environment for the very first time. In addition, to measure the utility of the object as nest after their first nap, we also continued to track the utility of the object as nest during the subsequent naps. This is based on the observation that mice, just like other rodents, tend to have multiple, short naps during the daytime (in contrast to humans' single sleep epoch each night). Therefore, we time-sampled their sleep/awake behaviors for four hours in one day (typically from 10:00 AM-2:00 PM) by visually checking the mouse behavioral status every 5 minutes. On the day of the experiments, we put a mouse into a new cage with a nest placed inside and then monitor whether the mouse would sleep inside or not. For measuring the occurrence of the very first nap when a new type of nests was presented to mice, we counted the number of mice which sleep in the nest vs. the number of mice which did not sleep in the nest. For a given nest, we can only obtain a single number (e.g. 15 out of 20 mice had their first nap in the nest, therefore, we expressed it as 75% of the first nap inside the nest). As a result, there are no error bars for Figure 1B and Fig 2A. For experiments which required the prolonged measurement over several days, the mice were single-housed throughout the experiments. Since mice tend to have multiple naps during the four hour period, we counted the numbers of naps each mouse took inside the nest vs. outside of the nest. We then averaged it over multiple mice and this is expressed as the percentage of naps inside of the nest (with error bars). Both measurements were used to assess the mice's ability to recognize the objects as nest.

2.4. Novel Object Recognition Task

The experimental protocol was the same as described previously (Rampon et al., 2000). Briefly, mice were individually habituated to an open-field box ($20 \times 20 \times 10$ high inches) for 3 days. During the training sessions, two novel objects were placed in the open field, and the animal was allowed to explore for 15 min. The time spent exploring each object was recorded and found to be no significantly different between genotypes. During the retention tests, the animals were placed back into the same box, in which one of the familiar objects during training was replaced by a novel object, and allowed to explore freely for 15 min. A preference index, a ratio of the time spent exploring any one of the two objects (training session) and the novel one (retention session) over the total time spent exploring both objects, was used to measure recognition memory. A Student's *t*-test was used to determine genotype effects on the behavioral responses.

2.5. Statistical analyses

The conceptual categorization of a given object as a nest by a group of mice can be quantified by counting the number of mice which would spend their first nap either inside of the new object or not once the object was placed inside the cage. For the categorical data in the form of contingency table, the Fisher's exact test was used to assess the significance of genotype effects and nest type effects (the Chi-square test was not used because our sample sizes were small, < 40). Data was presented in percentage of mice which would spend their first nap inside of the new object out of each group. Generalization index was calculated by averaging the percentages of mice which had their first nap inside of the nest over days. A one-way ANOVA and *post hoc* Dunnett's test was used to detect the difference between animal groups. For continuous monitoring of the mice behavior status, we counted the number of naps each mouse took inside the nest vs. outside of nest. The percentage of the naps spent in the nest out of the total number of naps of each mouse was calculated, and

averaged over the mice in each group. The data from the mouse only slept once in that day was excluded. And non-parametric pairwise Mann-Whitney *U*-test was used to determine the genotype effects or nest type effects on the behavioral responses. The novel object recognition data was subjected to a Student's *t*-test. The level of significance was set at $p < 0.05$. Data are presented as mean $\pm$ S.E.M.

Ethic Statement—Experiments were carried out in accordance with the Guidelines laid down by the NIH in the US regarding the care and use of animals for experimental procedures, and was approved by the Animal Use and Care Committee of Medical College of Georgia and East China Normal University. The nest behavioral experiments described in the present work were natural to mice and did not produce any discomfort or pain in mice.

3. Results

3.1. Behavioral measurement of the generalized ability to recognize nests of mice

A variety of nests in different geometric shapes, sizes and texture were made from different materials (Fig. 1A). The mice had no prior experiences with these nests. The mouse's ability to recognize an object as nests was measured by whether or not the animal would use it to sleep or rest in after the object was placed in the home environment for the very first time. The conceptual generalization of a given object as a nest can be quantitatively calculated by the percentage of mice which would sleep inside of the new object once the object was placed inside the cages over total number of mice. Behavioral exploration and interactions with nests were completely voluntary and natural. Our experiments show that although the mice had never encountered these nests prior to the experiment (type #1–6 as shown in Fig. 1B), about 70% of the wild-type mice spent their first nap inside of those nests. From our previous *in vivo* recording study, we showed that the nest cells in the hippocampus would cease to respond if the nest was covered by a glass (Lin et al., 2007). That is, the animals can still explore and run across the covered nest, but the nest cells would no longer change their firing rates, indicating the functionality of the nest is the major determinant. Thus, we also introduced this glass-cover experiment for the behavioral measurement. Indeed, the animals would no longer use the object as their nest if the nest was covered by a glass (nest #7) ($p < 0.05$, Fisher's exact test). This strongly indicates that mice did not randomly treat any object placed in the cage as a preferred place for sleep. In other words, it is the functionality of the object to which the mice seem to be able to recognize.

Since mice would typically undergo multiple wake/sleep cycles during the day time, we continuously monitored the mice for four hours each day (one nest type per day). We asked how consistently the mice would continue to sleep inside the same nest during their multiple sleep cycles. This would give us the measurement of whether and how the mice consistently view the object as their nest. We calculated the number of times that mice slept in the nest over total numbers of naps, and found that the mice spent most of their sleep or nap sessions inside nests #1–6. Since the mice had never seen the nests previously, and were presented a new one on each day, we were able to assess their generalization ability. Indeed, the wild-type mice had consistently slept inside of these nests. Again, once the nest was covered by a piece of glass, the number of times that mice would sleep on it was greatly reduced (nest #7 was nest #6 but covered by a glass floor) ($p < 0.05$, Mann-Whitney *U*-test) (Fig. 1C). These results suggest that the mice possess the generalized ability to recognize and identify various distinct objects as their nests. This ability to generalize can persist despite significant variations in physical shape, color, and texture of these objects, as long as the functionality remains the same.

3.2. Impairment in inducible and forebrain-specific NR1 knockouts but not in forebrain-specific PS1 knockouts

Our previous experiments using region-specific gene knockout technologies have demonstrated that the NMDA receptor serves as the major molecular switch for the formation of memories of spatial maze, fear conditioning, novel object recognition, social transmission of food preference, and conditioned taste aversion, etc (Tsien et al., 1996b; Tang et al., 1999; Rampon et al., 2000). Given the fact that the hippocampus is crucial for not only episodic memory but also semantic memory (memory of concepts and generalized knowledge) (Mishkin, 1978; Wilson and Baddeley, 1988; Reed and Squire, 1998; Squire and Zola, 1998; McGuire et al., 2005; Moscovitch et al., 2006), we hypothesized that the NMDA receptor in hippocampus may also play a crucial role in the formation of semantic memories of facts, concepts, and abstract knowledge. In other words, the knockout of the NMDA receptor may also impair the formation the concept of nest. In order to test our hypothesis, we have produced inducible and reversible, forebrain-specific NMDA receptor knockout mice (Cui et al., 2004), in which the NMDA receptor can be temporarily switched off in the forebrain regions such as the cortex and hippocampus right before the nest behavioral tests. The forebrain-specific inactivation of the NMDA receptor function was induced by feeding doxycycline (dox) for 7 days or more before the experiments started, and then dox-treatment continued during the experiments to ensure the NR1 deletion was temporally restricted to the duration of experiments, thus avoiding any potential developmental effects.

Our experiments suggest that the NR1 knockout mice exhibited the reduced ability to recognize the objects that can be used as nests. For example, only about 40% of the NR1 knockout mice would sleep inside the nests, in comparison to the typical 70–80% of wild-type control mice which would sleep inside the nests (Fig. 2A). On average, the NR1 knockout mice spent 40% of their naps inside of the nests, in comparison to typical 70–80% of napping preference of the other two groups of mice (Fig. 2B).

To further assess the utility of this method in analyzing the molecular mechanisms, we used the forebrain-specific presenilin-1 (PS1) knockout mice (Feng et al., 2001) as another group. Previous study suggested that forebrain-specific knockout of the presenilin-1 gene results in a pronounced deficiency in enrichment-induced adult neurogenesis in the dentate gyrus. While the conditioned PS1 knockout mice were mostly normal in basic forms of memory, these mice exhibited impaired clearance of outdated fear memory traces from the hippocampus. Our experiments here suggest that forebrain-specific presenilin-1 knockout mice had normal nest recognition behavior (Figure 2).

In addition, we calculated the nest-generalization index by averaging the percentage of mice which spent their first nap inside of the new nests across six different types of novel nests (over six days). Our analysis shows that the generalization index of the NR1 knockout mice is significant lower than those of wild-type mice and PS1 knockout mice (Fig. 2C).

As two additional controls, we performed both the glass-cover experiment as well as the capsized nest experiment (with bottom up so that it functioned as a small stage). As expected, the wild-type control mice and PS1 knockout mice no longer slept on the glass-covered-nest or capsized nests (Fig. 2D). That is, their preference levels dropped to the similar level as to those in the NR1 knockout mice.

To exclude the possibility that the generalization deficit was due to the potential difference in sleep behavior, we determined whether the NR1 knockout mice had a normal amount of sleep. Our data suggest that the percentages of the sleep times during the 4 hours of observation period did not significantly differ between groups. On average, the NR1

knockout mice slept for 4.3 ± 1.8 times, whereas the wild-type control mice slept 4.1 ± 1.6 time and 3.8 ± 1.9 for the PS1 knockout mice (mean $\pm$ s.d) during the nest behavior tests.

3.3. Compensation for NR1 knockout deficits by repeated experiences

We have previously shown that the Cre/loxP-mediated NR1 knockout occurred only in about 60% of cortical neurons (and > 98% of hippocampal excitatory neurons) in the forebrain-specific NR1 knockout mice (Cui et al., 2004). Thus, we wondered if the NR1 knockout mice, with the intact NMDA receptors in the remaining cortical neurons, may retain a certain degree of capacity that would allow some kinds of compensation by experience. Therefore, we repeatedly introduced the circular white cardboard nest to mice, four hours a day for four consecutive days. As expected, the wild-type control mice exhibited consistent behavior in spending most of their nap time in the white cardboard nest over four days of testing. Interestingly, we have found that although the NR1 knockout mice slept mostly on the floor for the initial two days, they increased their tendency to sleep inside the nest and reached the comparable level of preference as those of the wild-type control by the day four (Fig. 3A). This indicated that the repeated exposures of the same object can improve the sleep-inside-nest behavior. However, when the same nest was covered by a piece of glass, the sleep-in-nest preference of wild-type mice significantly dropped, as did the preference of the NR1 knockout mice also dropped (Fig. 3B). This suggested that both types of the mice relied on the functionality of the nest for their correct nest recognition performances. With the compensation of nest behavior deficit by experience, we wondered whether the NR1 knockout mice at this later stage also somehow exhibited improved recognition memory for other objects. Therefore, we carried the standard measurement of recognition memory by subjecting the mice to the conventional novel object recognition test. We conducted both the one hour retention and one day retention tests. As expected, the control mice showed a significant exploring preference for the novel object during each retention test (Fig. 4). In contrast, the NR1 knockout mice were severely impaired in both tests and showed only marginal preference for the novel object (Fig. 4). A Student's *t*-test revealed a significant difference in the wild-type mice in retention tests ($p < 0.05$), but not in the NR1 knockout mice. These results indicate profound deficits of novel object recognition memory still persisted in the NR1 knockout mice.

4. Discussion

One of the fundamental questions in neuroscience is to understand how the brain generates and represent abstract knowledge and concepts. Lesions studies in human suggest that the hippocampus and its related temporal lobe are important for generalized object recognition and formation of new semantic memory of concepts and generalized knowledge (Mishkin, 1978; Wilson and Baddeley, 1988; Tranel et al., 1997; Reed and Squire, 1998; Squire and Zola, 1998; Martin and Chao, 2001; Luo and Niki, 2002; Gallese, 2003; McGuire et al., 2005; Quiroga et al., 2005; Moscovitch et al., 2006; Tsien, 2007; Ryan et al., 2008). Here, we developed a behavioral paradigm that can be used for genetic screening and detailed analysis of molecular mechanisms of nest concept formation or representation. By measuring the mouse's natural choice of various distinct objects for sleeping, we described the method for assessing the nest-generalization ability in mice. Our experimental designs were driven by three major criteria regarding the generalization ability in categorizing various objects as nests: the first one is that the animals had no prior encounters with these objects; therefore, the animals can't simply perform this task by recalling and matching with their previously encountered objects. The second criterion is that the animals' ability to recognize objects as nests should be independent of the shape or physical forms of the objects, just like we humans can recognize various beds regardless of shape or style (e.g. the elaborate Louis XVI bed in Versailles palace vs. a Japanese tatami bed). The third criterion

is that as for nests, the properties of such objects are the functionality rather than styles and shapes. The blocking of the nest's functionality should lead to drastic changes in behavioral outcome.

Our results showed that most wild-type mice preferred to sleep in these novel objects as long as these objects functionally served as their nests despite the variations in construction material, style, and shape. The fact that the mice didn't sleep in the nest after they have been covered with glass provides strong evidence for the functionality as the determining factor for categorizing the objects. The importance of the functionality is further illustrated by the capsized nest experiment. These experiments indicate that the generalized nest recognition ability is critically dependent on bodily determination regarding the functionality of these objects for categorization (Davis, 2005).

Due to nest's functionality as its intrinsic property (just like the concept of chairs), the semantic definition for the conceptual categorization of these types of objects is subject to intuitive understanding of the objects. It is likely that some debates may exist as to where one should draw a line for separating a stump from a stool. On the other hand, the three criteria used for the measurement of the nest concept are qualitatively useful. Moreover, our above behavioral results are also highly consistent with our previous neurophysiological observation of nest cells in the mouse hippocampus. In our previous in vivo recording experiments, we have described the hippocampal neurons which would selectively fire or cease to fire when the mouse perceives nests or beds (Lin et al., 2007). By constructing a variety of new nests that the mice had never encountered before, we found that responses of hippocampal nest cells remained invariant over changes in the nests' physical shape, style, color, odor, or texture (Lin et al., 2007). Furthermore, their firing responses were driven by physical determination of the categorical features that would functionally define nests (Lin et al., 2006; Lin et al., 2007). Specifically, nest cells would no longer change their firings if the nest was flipped over. Since it was the same nest (a plastic cap) and placed in the same location, this flip-over experiment represents a stringent control for the shape, color, location, odor etc. The recording experiment demonstrated that when the mouse encountered the inverted nest, the nest cells showed no significant change in its firing. However, when the plastic cap was flipped back to the nest position, the cells fired robustly. Such recording experiments demonstrate that the nest cells are tuned toward the fundamental features that would functionally define the nest: a refuge for the animal to stay in a safe and cozy manner in a given environment. Similarly, the nest cells also lost their responsiveness if the nest was covered by a piece of glass (the glass floor experiment) (Lin et al., 2007). Our present behavioral experiments agree with the in vivo recording data that those covered or capsized nests were also no longer the preferred places for mice to sleep.

To dissect the molecular mechanism underlying concept formation or representation in the mouse brain, we performed the nest tests using inducible and forebrain-specific NMDA receptor knockout mice (cui et al. 2004)) as well as forebrain-specific PS1 knockout mice (Feng et al., 2001). These conditional NR1 knockout mice initially exhibited pronounced deficits in nest recognition test. They were less consistent than their wild-type littermates and PS1 knockouts in terms of identifying various objects as nests. Unexpectedly, after repeated exposure over time, they gradually regarded those objects as nests and their performances improved. This is particularly interesting since their novel object recognition memory (using Lego toys) at such late stage was still impaired, suggesting the existence of additional or remaining pathways which can compensate such generalization deficits by experience. Our current method can be further improved in future to use video-tracking devices for both efficiency and reliability. Clinical studies also reported that three children with hippocampal damage were still able to acquire semantic knowledge and concepts

(although not efficiently, but through many repetitions) despite the profound amnesia in daily episodic memory (Vargha-Khadem et al., 1997; Gadian et al., 2000).

In conclusion, we have devised a behavioral paradigm that would allow the quantitative measurement of the generalization ability to recognize various objects as nests in mice. This novel paradigm may provide an opportunity to dissect the molecular mechanisms underlying concept formation and/or representation in mice. By taking advantage of this behavior paradigm, we show impaired performance in forebrain-specific NR1 knockout mice. Moreover, this generalization deficit could be gradually compensated for repeated experiences. In addition, we also show that the nest generalization ability is independent of forebrain PS1 protein. The similar design strategy can be also extended to study rats or for large-scale genetic screening of mutant mice. It is conceivable that the combination of mouse genetics, optical manipulation, in vivo recordings, and behavioral techniques will likely open the door for studying how the neural circuits in the brain work together to generate abstract concepts and generalized knowledge.

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Fig. 1.

The wild-type mice possess the generalized ability to recognize and identify various distinct objects as their nests. (A) A variety of nests in different geometric shapes and materials have been used for this experiment. These objects, such as disposable coffee cups, other cardboard materials, plastic caps from instant coffee bottles, wooden tray, or metal caps from Altoids candy tins, are shown. The bar scale represents 1 inch. (B) The percentage of mice which spent their first nap inside of six different types of nests as shown in (A). Each nest was presented to a mouse for four hours per day during which we recorded whether or not the mouse spent its first nap inside of the given nest. The Y-axis presents the occurrences of the first nap inside of a nest in the WT group animals ($n=11$). The X-axis

indicates each type (#1–7) of nest (* $p < 0.05$, Fisher's exact test). (C) The occurrences of multiple sleep sessions inside of each type of nests during each day. Each nest was presented to a mouse for four hours per day during which we recorded how many times the mouse slept inside of the nest. Mice typically had multiple sleep-wake sessions during the day (instead of a single sleep session like humans). The Y-axis presents the percentage of the individual sleep sessions spent in nest over the total number of naps. The X-axis indicates each type (#1–7) of nest. Nest #7 was nest #6 covered with glass. This measurement allowed us to determine how consistently the mice viewed the objects as nests. (Error bar: s.e.m., * $p < 0.05$, Mann-Whitney *U*-test).

Fig. 2.

Inducible and forebrain excitatory neuron-specific knockout of the NMDA receptor impaired mice's generalized ability to recognize those objects as nests. (A-B) Only about 40% of the forebrain NR1 knockout mice ($n=23$) would sleep inside the nests when the nest was placed in the cage for the first time. In comparison, the wild-type mice ($n=20$) and forebrain-specific PS1 knockout mice ($n=7$) exhibited a much higher percentage, typically 70–80% in sleeping inside the nests during their first encounters. (C) Generalization index was calculated by averaging the percentage of mice which had their first nap inside of the nest over six testing days (for six different nests). Significant difference was determined by one-way ANOVA and *post hoc* Dunnett's test. (D) The functionality of the nests is

important for determining nest behavior. In the glass-cover experiment (left columns) and the capsized nest experiment (right columns), the wild-type mice ($n=9$) and PS1 knockout mice ($n=7$) no longer preferred sleeping on the objects, while the preference level in the NR1 knockout mice ($n=14$) didn't change much (Mann-Whitney U test). Error bar: S.E.M. ** $p<0.01$, * $p<0.05$.

Fig. 3.

Experience-dependent rescue of the best behavior in inducible NR1 knockout mice. (A) Repeated exposure of the nest (#6) to NR1 knockout mice ($n=23$) can gradually bring the performance close to the wild-type level ($n=20$). There are significant differences in the first day (** $p<0.01$) and the second day (* $p<0.05$ Mann-Whitney U -test). (B) On day five, we further performed the glass-cover experiment using the same nest listed in (A). As expected, the wildtype mice showed a significant drop in their preference to sleep on the glass-covered nest (Error bar: S.E.M., * $p<0.05$, Mann-Whitney U -test), and the sleep-in-nest preference of NR1 knockout mice also dropped but there was no significant difference with the last training day. However, it is statistically significant from the Day 4 performance by the wild-type mice.

Fig. 4. Impaired novel-object recognition memory in inducible NR1 knockout mice despite of improvement in nest generalization performance. Recognition memories for simple objects (such as Lego toys) for wild-type ($n=8$) and NR1 knockout ($n=9$) mice were measured at two different time intervals (one-hour retention and one-day retention). Error bar: s.e.m., $*p<0.05$, $**p<0.01$, determined by a Student's t -test.