Chem. Senses 26: 663–672, 2001

A Simple and Reliable Test of Olfactory Learning and Memory in Mice

Heather M. Schellinck, Catherine A. Forestell and Vincent M. LoLordo

Department of Psychology, Dalhousie University, Halifax, Nova Scotia, Canada

Correspondence to be sent to: Heather Schellinck, Department of Psychology, Dalhousie University, Halifax, Nova Scotia, Canada. e-mail: heathers@is.dal.ca

Abstract

The present paper describes a quick and efficient method for assessing olfactory discrimination learning in mice. In training mice received trials in which one odor (CS+) was paired with sugar and another odor (CS–) was paired with no sugar. When the mice were subsequently placed in a chamber with CS+ odor at one end and CS– odor at the other, they spent more time digging in CS+ than in CS– odor. In Experiment 2 mice trained with this procedure and tested after 60 days also spent more time digging in CS+ than CS– in the test phase, indicating that this olfactory discrimination task is effective for assessing long-term memory. In addition to the outbred strain of CD1 mice used in Experiments 1 and 2, C57Bl/6NCr/BR and DBA/2NCr/BR mice used in Experiment 3 also acquired this learned odor discrimination. Moreover, Experiment 4 showed that DBA animals were capable of acquiring this odor discrimination after receiving only two training trials (one exposure each to CS+ and CS–) per day for 4 days.

Introduction

The adoption of the mouse as the animal of choice by behavioral geneticists has accentuated the need for behavioral tests which adequately measure mouse behavior (Brown *et al.*, 2000). In the past most tasks were designed for rats and some of those tasks have simply been downsized for use with mice (Crawley, 2000). While some tests have been successfully adapted (Mondadori and Weiskrantz, 1993; Crawley and Paylor, 1997), simple modifications may fail to take into account inherent differences in the behavior of the two species that could affect task performance. For example, it has recently been reported that mice fail to perform as well as rats in the Morris water maze (Gerlai and Clayton, 1999). This difference in performance could be related to physical and or physiological differences between the two species based upon their choice of habitat in the wild. Rats prefer to live in wet swampy land whereas mice are likely to be found in dry forested areas or farmlands (Nowak, 1999).

Odor cues are known to be important for rodents in many contexts (Brown and MacDonald, 1983; Schellinck and Brown, 1998) so tests of olfactory learning and memory for mice would be useful. Indeed, there has been renewed interest in the use of olfactory paradigms to study memory processes in rodents (Reid and Morris, 1993; Eichenbaum, 1998; Galef and Whiskin, 1998; Galef *et al.*, 1999). Rats, for example, have been shown to demonstrate primate-like learning characteristics; they can develop a learning set (Slotnick and Katz, 1974; Slotnick *et al.*, 2000) and appear to understand the relational organization among a series of odor items, i.e. behave on the basis of transitive inference (Bunsey and Eichenbaum, 1996). Recently several tests have been adapted to assess olfactory memory in mice (Berger-Sweeney *et al.*, 1998; Bodyak and Slotnick, 1999; Zagreda *et al.*, 1999; Mihalick *et al.*, 2000a). We too have recently developed a reliable test of olfactory memory in mice. It is an ethologically relevant procedure that takes advantage of the mouse's tendency to use olfactory cues to forage for food. The task involves a simple odor discrimination in which mice learn to associate one odor (CS+) with a sugar reward and a second odor (CS–) with no reward. During the test phase both odors are presented simultaneously in the absence of sugar. The animals demonstrate that they have learned that the CS+ odor signals the availability of sugar by digging in that odor.

Experiment 1

In Experiment 1 we assessed the effectiveness of the methodology for demonstrating discrimination learning in outbred laboratory mice. We have included very detailed methods so as to provide sufficient information for those investigators wishing to adopt the test for their own purposes.

Materials and methods

Subjects

Twenty-four male adult CD1 mice (Charles River, Quebec), weighing 20–30 g, were maintained on a 12:12 reverse light/dark schedule with lights on at 19:00 h. The animals

were weighed daily at 15:00 h and fed sufficient Purina rat chow (~4 g each) to maintain ~80–85% of their free feeding weight. Water was available *ad libitum*. The mice were housed individually in polyethylene cages (30.5 × 6.2 × 6.2 cm) containing pine chip bedding. All procedures and methods were reviewed and authorized by the University Committee on Laboratory Animal Care at Dalhousie University.

Apparatus

Odor stimuli. Phenyl acetate (Aldrich Chemicals) and linalool (Aldrich Chemicals) were diluted to a concentration of 15% in propylene glycol (Caledon Chemical Co.). Phenyl acetate has a rose-like odor and will be referred to as rose throughout the manuscript. Linalool has a lemon-like odor and will be referred to as lemon. For convenience, a number of odor samples were prepared in advance and stored in 1.5 ml centrifuge tubes (Eppendorph) at –80°C until needed. Odor pots (Figure 1) were constructed in such a way as to ensure that the animals could come into contact only with the odor vapor. An odor (0.05 ml) was presented on filter paper (Whatman no. 1), 55 mm in diameter, which had been placed on the bottom of a plastic beverage cup. The top of the cup was cut so that the pot was ~1.5 cm high. A plastic Petri dish cover containing 10 pre-drilled holes (0.5 mm) kept the filter paper in place. Pine chip bedding was placed on top of the Petri dish cover about level with the top of the odor pot. If an odor stimulus was paired with sugar reinforcement, the sugar (~0.05 g) was buried in the pine chips.

Training apparatus. Mice were exposed to the odor stimuli in polycarbonate opaque cages with stainless steel tops (30.0 × 13.0 × 11.5 cm) identical to their home cages. The odor pots were placed in the center of the back third of the cage.

Test apparatus. Discrimination tests were conducted in a 69 × 20 × 20 cm box constructed from 3 mm clear acrylic. Two pieces of acrylic divided the chamber into three equal compartments. Each dividing wall had a 6 × 5.5 cm opening so that the mouse could move from the center of the box to either end. Acrylic doors (7.5 cm^2) could be slid into place to block the openings. An ~1000 ml beaker of pine chip bedding was distributed over the floor of the chamber. Four new odor pots identical to those described previously were used in the test phase. Two stopwatches (Fisher Scientific) were used to record digging behavior.

Procedures

Training. Four days prior to training the mice were placed on a food restriction schedule. They were assigned to groups as follows: group R+/L–, six animals received rose paired with sugar and lemon alone; group L+/R–, six animals received lemon paired with sugar and rose alone; group R–/L–, six animals received both odors alone, i.e. neither was paired with sugar; group NO, six animals were not exposed to either lemon or rose odors at any time during training. The

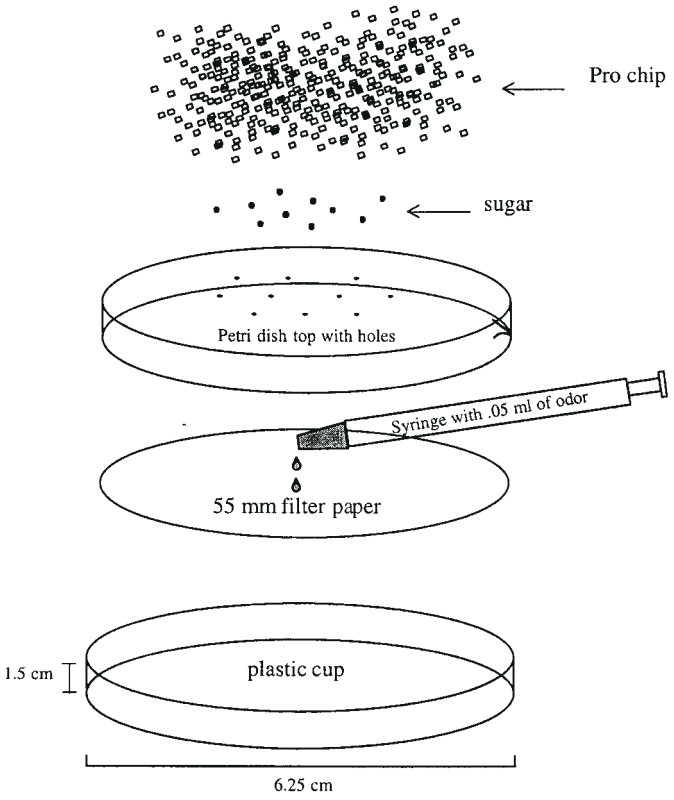

Figure 1 A diagram of the odor pot used to present the odors in training and test.

mice were trained 2 h after lights off, and 3 h after training were fed their daily food ration. During training subjects received eight 10 min trials per day, which consisted of four trials of rose and four trials of lemon. Intervals between training trials were ~10 min. Training lasted for 4 days, thus animals received 32 trials in total. The order of odor presentation was randomized across days.

For efficiency we used five separate training cubicles to present the odors: in two cubicles either rose or lemon was presented with sugar, in another two cubicles the odors were presented without sugar and in the fifth cubicle no odors were presented. Each room always contained the same odor pots and cages. The rooms were illuminated with dim red light. All rooms were in use simultaneously. For example, while the R+/L– animals were receiving rose paired with sugar, the L+/R– animals were receiving lemon paired with sugar, control R–/L– animals were receiving either lemon or rose without sugar and control NO animals were receiving neither odors nor sugar. At the end of each trial the mice were returned to the colony room for 10–15 min and the filter paper containing the odors and the bedding were discarded. The odor pots and cages were not washed between trials.

Testing. Discrimination tests were performed on the day following the last training trials at approximately the same

time as training, i.e. several hours after lights off. We used a vacant animal colony room for testing because the air exchange in this room was more efficient than in other laboratory areas. Moreover, since animals received the CS+ and CS– stimuli in different rooms in training, the use of another room for testing ensured that any contextual cues that became associated with sugar would not influence behavior in the test phase. Prior to testing the animals were habituated to the apparatus. Odor pots containing pine chip bedding but no odors were placed in the middle of each end compartment. The mouse was placed in the center of the apparatus and the doors leading to the end compartments were removed to give the mouse the opportunity to explore the apparatus. After 2 min the mouse was returned to its home cage for ~5 min prior to the test phase.

The rose and lemon odors were then placed in the odor pots. Sugar was never present during discrimination testing. Following placement of the odor pots in the discrimination chamber the mouse was again placed in the center of the chamber and the doors to the end compartments removed. The time the mouse spent digging in both odor pots during a 2 min period was recorded with two stopwatches by an observer blind to the conditions of the experiment. Digging was defined as digging with forepaws or 'nosing' in the bedding, but only if the bedding moved during the investigation. At the end of each trial the test chamber was washed with hot water and dried prior to habituating and testing another subject. Particular care was taken to wash the corners of the box and the door openings to minimize the odor of a previous subject. The discrimination chamber was rotated 180° between subjects to counteract the influence of any room cues.

Data analysis

The following non-parametric statistics were used to look for differences between or among the groups: Wilcoxon's matched-pairs signed-ranks test used for two related samples, the Mann–Whitney *U* statistic for two independent groups, the Kruskal–Wallis one-way ANOVA for three or more independent groups and Friedman's rank test, a repeated measures ANOVA (Siegel, 1956).

Results and discussion

Mice that had received one of the odors paired with sugar in training dug almost exclusively in the presence of CS+ odor in the test phase (Figure 2). A Kruskal–Wallis one-way ANOVA revealed significant differences in the amount of time digging in the lemon odor during the test phase between the L+/R–, R+/L–, R–/L– and NO groups during training ($H = 13.74$, $P < 0.01$). Only the L+/R– animals dug in the lemon. Similarly, a Kruskal–Wallis one-way ANOVA revealed significant differences in the amount of time spent digging in the rose odor during the test phase between the R+/L–, L+/R–, R–/L– and NO groups during training ($H = 10.86$, $P < 0.01$). With one exception, only the R+/L– animals dug in the rose odor (Table 1).

These data, along with those collected previously (Brennan *et al*., 1998), indicate that this paradigm provides extremely consistent results. The advantages of this test are clear; it is easy to administer and requires no elaborate or costly apparatus. As described here, although several hours a day are devoted to the experiment, 12–16 animals can be trained and tested within a week.

Experiment 2

It is clear from the data presented in Experiment 1 that mice

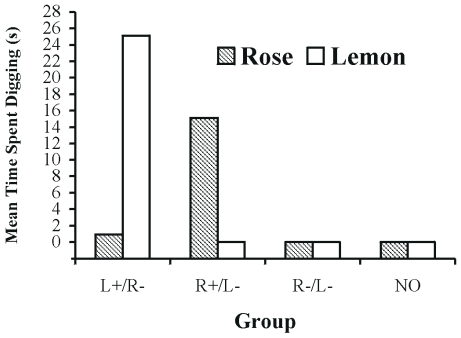

Figure 2 Mean time spent digging in rose and lemon during the tests in Experiment 1 for the experimental groups, i.e. those for whom rose was paired with sugar (group R+/L–) or lemon was paired with sugar (group L+/R–) in training, and control groups, i.e. those for whom neither rose nor lemon was paired with sugar (group R–/L–), or who received no odors or sugar (group NO) in training.

Table 1 Time spent digging in lemon and rose during the test phase for individual animals in Experiment 1

Exp'l group[a]	Lemon	Rose	Prefer. for CS+[b]	Control group	Lemon	Rose
L+/R–	70.00	0.00	1.00	R–/L–	0.00	0.00
	13.10	5.61	0.70		0.00	0.00
	20.00	0.00	1.00		0.00	0.00
	11.55	0.00	1.00		0.00	0.00
	26.90	0.00	1.00		0.00	0.00
	9.15	0.00	1.00		0.00	0.00
R+/L–	0.00	6.15	1.00	NO	0.00	0.00
	0.00	42.00	1.00		0.00	0.00
	0.00	0.00	N/A		0.00	0.00
	0.00	16.05	1.00		0.00	0.00
	0.00	16.05	1.00		0.00	0.00
	0.00	19.54	1.00		0.00	0.00
	0.00	5.97	1.00		0.00	0.00

[a]Group treatments were as follows: group L+/R– received lemon paired with sugar and rose alone; group R+/L– received rose paired with sugar and lemon alone; group R–/L– received lemon and rose alone; group NO was not exposed to any odors or sugar in training.

[b]Proportion of total digging time spent digging in CS+ in the tests.

can quickly learn to discriminate between two odors. If this paradigm is to be used to study the processes involved in long-term memory it is necessary to determine whether the mice can retain the information for longer than 1 day following training. Long-term odor memory has been studied primarily in rats [but see Laska *et al*. for a study in primates (Laska *et al*., 1996)]. In experiments with rats the time between training and testing generally ranged from several minutes to several days (Roman *et al*., 1989; Ravel *et al*., 1992, Larson *et al*., 1995), although there have been several reports of rodents remembering odor discriminations for periods up to 1 month (Beauchamp and Wellington, 1984; Slotnick and Risser, 1990).

In this experiment we assessed the ability of CD1 outbred mice to remember the relationship between an odor and reward for 14, 30 and 60 days following training. We also tested the mice for 3 days in succession in each time period to determine whether the discrimination would extinguish rapidly.

Materials and methods

Subjects

Thirty-two adult male CD1 mice (weight 20–25 g) were obtained from Charles River Canada (St Constant, Quebec, Canada). They were housed and food restricted as described for Experiment 1.

Apparatus

The odor stimuli and testing and training apparatus were the same as used in Experiment 1 except that Prochip (PWI Industries, St Hyacinthe, Quebec, Canada) rather than pine chip bedding was used in the training cages, test apparatus and odor pots. For those animals receiving sugar reinforcement for the first odor–sugar pairing the sugar was placed on the top of the Prochip in the odor pot; for the remaining CS+ trials the sugar was buried in the Prochip. The pine chips used previously were replaced with Prochip as it is uniform in size and has very little odor.

Procedure

As in Experiment 1, 4 days prior to training the mice were placed on a food restriction schedule to maintain them at 80–85% of their free feeding weight. Half of the animals received rose as CS+ and lemon as CS–; the remainder received lemon as CS+ and rose as CS–. The animals were trained in batches of eight per week. In this experiment the mice were given three CS+ and three CS– trials per day in a random order for 4 days rather than the four of each trial type for 4 days given in Experiment 1. Following training mice were randomly assigned to one of four groups: group 1 was tested for their odor discrimination on days 1, 2 and 3 after training; group 14 was tested on days 14, 15 and 16; group 30 was tested on days 30, 31 and 32; group 60 was tested on days 60, 61 and 62. The habituation and testing procedures were as described in Experiment 1, except that the mice were only habituated to the apparatus on the first of the three test days. The mice in groups 14, 30 and 60 were given food *ad libitum* until 4 days prior to testing; the mice in all groups were food restricted during the 3 test days.

Results and discussion

The animals tested 1–3 (group 1), 14–16 (group 14), 30–32 (group 30) and 60–62 days (group 60) following training learned and remembered the odor discrimination task. Wilcoxon's matched-pairs signed-ranks tests indicated that the mice in all four groups spent more time digging in CS+ odor than in CS– odor on the first day of testing (group 1, $T = 3$, $P < 0.05$; group 14, $T = 0$, $P < 0.05$; group 30, $T = 1$, $P < 0.05$; group 60, $T = 0$, $P < 0.05$; Table 2). There were, however, significant differences in the amount of time

Table 2 Time spent digging in CS+ and CS– stimuli for individual animals in groups 1, 14, 30 and 60 in the test phase of Experiment 2

Group		CS+	CS–	Preference for CS+
1	R+/L–	13.99	1.08	0.93
		14.40	0.00	1.00
		4.33	0.00	1.00
		18.76	0.00	1.00
	L+/R–	13.98	0.00	1.00
		13.50	0.00	1.00
		0.00	0.00	N/A
		0.00	5.69	0.00
14	R+/L–	48.40	0.00	1.00
		17.97	0.00	1.00
		0.00	0.00	N/A
		41.85	0.00	1.00
	L+/R–	28.28	1.27	0.96
		27.39	0.00	1.00
		36.26	0.54	0.99
		19.27	0.00	1.00
30	R+/L–	0.00	7.60	0.00
		41.15	1.84	0.96
		26.86	0.00	1.00
		38.37	0.29	0.99
	L+/R–	36.35	0.00	1.00
		26.96	0.00	1.00
		30.48	0.00	1.00
		19.15	0.00	1.00
60	R+/L–	15.96	0.00	1.00
		28.13	0.00	1.00
		35.71	0.00	1.00
		26.12	0.87	0.97
	L+/R–	21.59	16.32	0.57
		23.28	0.00	1.00
		19.70	7.71	0.72
		12.80	0.00	1.00

Group treatments were as follows: group 1 was tested on days 1, 2 and 3 after training; group 14 was tested on days 14, 15 and 16 after training; group 30 was tested on days 30, 31 and 32 after training; group 60 was tested on days 60, 61 and 62 after training.

spent digging in CS+ between groups as revealed by Kruskal–Wallis one-way ANOVA (H = 20.7, P < 0.01). Mann–Whitney U analyses revealed that group 1 dug less than group 14 [U (8,8) = 8, P < 0.01], group 30 [U (8,8) = 7, P < 0.01] and group 60 [U (8,8) = 6, P < 0.01] (Figure 3).

The mice showed a marked decline in digging in CS+ over the 3 days of testing (Figure 4). Friedman's rank test revealed significant differences in time spent digging in CS+ odor across days for the subjects in group 1 (Xr^2 = 10.78, P < 0.01), group 14 (Xr^2 = 10.57, P < 0.01) group 30 (Xr^2 = 6.82, P < 0.05) and group 60 (Xr^2 = 10.75, P < 0.01). A Wilcoxon's matched-pairs signed-ranks test revealed that the subjects in group 1 (T = 0, P < 0.05), group 14 (T = 1, P < 0.05), group 30 (T = 2, P < 0.05) and group 60 (T = 2, P < 0.05) spent less time digging on day 2 than on day 1. There were no differences in digging between days 2 and 3 in any of the groups (all Ps < 0.05).

One of the goals in testing this paradigm is to provide a method for assessing the long-term positive and negative effects of genetic and pharmacological manipulations on memory. Overall, the data indicate that this odor discrimination task is appropriate for assessing long-term olfactory memory in mice. Of the 32 animals tested, only two mice (one in group 1 and one in group 14) dug more in CS– than in CS+. Moreover, all of the mice tested 60 days following training remembered the discrimination. This latter finding demonstrates the enduring nature of an olfactory memory, especially considering that the mice received only 12 trials with each of the two odors.

The mice tested immediately following training spent significantly less time digging in CS+ than did the mice in groups tested 14, 30 or 60 days after training. This was not an expected result and it is not clear how to interpret it. One might hypothesize that those animals tested 1 day after training would have a stronger memory for the odor–sugar association than those animals tested several weeks following training. Consequently, one might expect them to dig longer in CS+ than the other groups. The group differences in time spent digging on the first test could be related to differences in contextual cues between the training and testing rooms. The visual environment was clearly different between the testing and training chambers. Differences in auditory and olfactory cues were more subtle but nonetheless also present. Perhaps the details of these contextual cues were progressively forgotten with time since training such that group 1 experienced more generalization decrement than the other groups (Riccio *et al*., 1984; but see Bouton *et al*., 1999a,b; Riccio *et al*., 1999). If this were true, the test context would have been a more effective retrieval cue after a longer retention interval, resulting in more digging in the CS+ odor pot.

For the mice in all groups the lack of reinforcement on the initial test trial was followed by a significant reduction in digging in subsequent trials (Figure 4). Whether extinction would be so rapid if it were conducted in the training context remains to be seen.

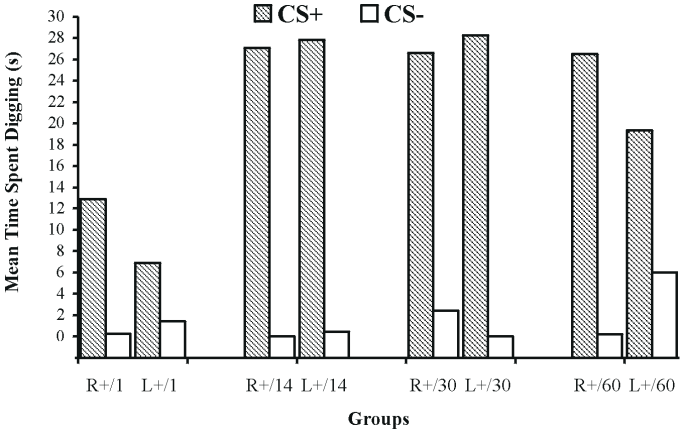

Figure 3 Mean time spent digging in CS+ and CS– in test 1 of Experiment 2 for mice that were tested 1 (group 1), 14 (group 14), 30 (group 30) or 60 days (group 60) after training.

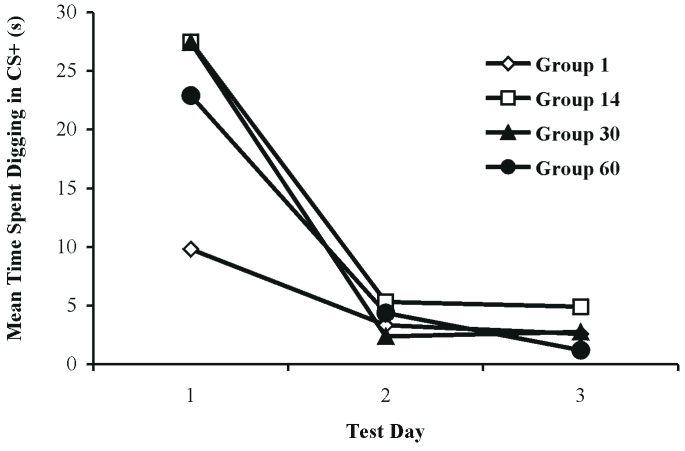

Figure 4 Mean time spent digging in CS+ odor on three consecutive test days beginning 1 (group 1), 14 (group 14), 30 (group 30) or 60 days (group 60) after training in Experiment 2.

Experiment 3

The data presented in Experiments 1 and 2 revealed that outbred mice can learn and remember an odor discrimination for a long period of time after only 4 days training. In Experiment 3 we tested the ability of two inbred strains of mice to learn the odor discrimination task. The C57Bl/6NCr/BR (C57) and DBA/2NCr/BR (DBA) strains were chosen as they are often used in transgenic and gene targeted research (Rogers *et al*., 1999). Strain DBA has been found to perform poorly in tests of spatial memory compared to other strains, including C57 (Upchurch and Wehner, 1988; Ammassari-Teule *et al*., 1993; Paylor *et al*., 1993; Petrie, 1995; Arns *et al*., 1999). Their poor performance has been attributed to differences in hippocampal

morphology (Crusio *et al.*, 1987; Schwegler *et al.*, 1990), hippocampal biochemistry (Wehner *et al.*, 1990, Fordyce *et al.*, 1995) and hippocampal neurophysiology (Bampton *et al.*, 1999). Their memory for olfactory stimuli has not been extensively investigated. In a report that appeared while the present paper was in revision, Mihalick, Langlois and Krienke (Mihalick *et al.*, 2000b) found that in a simultaneous odor discrimination task, although the strains did not differ in original learning, DBA completed a first reversal significantly faster than C57 and also made fewer incorrect responses while learning the new discrimination.

The performance of C57 has been tested extensively in a number of memory tasks (Castellano *et al.*, 1999; Gould and Wehner, 1999; Rogers *et al.*, 1999) including an olfactory discrimination task (Yamazaki *et al.*, 1982). In this task C57 were trained in a Y maze to discriminate between the urinary odors of congenic mice which differ only at the major histocompatibility complex. The animals took as many as 300 trials to learn the task (Yamazaki *et al.*, 1982). This difficulty in learning may have been related to the complexity of the task. Strain C57 has been found to have a cholinergic deficiency in the forebrain when compared with DBA (Bentivoglio *et al.*, 1994). Whether this would affect their ability to learn an olfactory discrimination is unknown. In this experiment we compared the ability of strains C57, DBA and outbred CD1 to acquire an olfactory discrimination.

Materials and methods

Subjects

CD1 adult male mice weighing ~25 g and DBA and C57 mice weighing ~20 g were obtained from Charles River Canada. Their living conditions were as described for Experiment 1.

Apparatus

The odor stimuli and testing and training apparatus were the same as used in Experiment 1 except that clear acrylic tops were used instead of the stainless steel wire cage tops used during the training phase in Experiment 1.

Procedure

As in Experiment 1, 4 days prior to training the mice were placed on a food restriction schedule to maintain them at 80–85% of their free feeding weight. Eight animals of each strain were assigned to groups as follows: group R+/L–, four animals per strain received rose paired with sugar and lemon alone; group L+/R–, four animals per strain received lemon paired with sugar and rose alone. The animals were run in two equal sized batches, with 12 animals run each week and a random order of odor presentation. The training procedure was identical to that described in Experiment 2. Following training the mice were first habituated to the test apparatus and then tested as described for Experiment 1. The test occurred on the day after the last training session.

Results and discussion

The C57 and DBA mice dug exclusively in CS+ odor in testing and six of eight of the CD1 mice dug only in CS+ odor. Two of the CD1 mice did not dig in either CS+ or CS– odor (Table 3 and Figure 5). Analysis of the time spent digging in CS+ odor revealed differences between the three

Table 3 Time spent digging in CS+ and CS– stimuli by CD1, C51 and DBA strains in the test phase of Experiment 3

Strain	Group	CS+	CS–	Preference for CS+
DBA	R+	20.44	0	1.00
		20.64	0	1.00
		34.39	0	1.00
		25.53	0	1.00
	L+	26.35	0	1.00
		17.08	0	1.00
		16.54	0	1.00
		22.33	0	1.00
C57	R+	35.22	0	1.00
		29.43	0	1.00
		16.13	0	1.00
		20.38	0	1.00
	L+	14.54	0	1.00
		14.57	0	1.00
		11.52	0	1.00
		24.27	0	1.00
CD1	R+	18.51	0	1.00
		12.67	0	1.00
		0	0	N/A
		0	0	N/A
	L+	7	0	1.00
		23.04	0	1.00
		12.9	0	1.00
		20.24	0	1.00

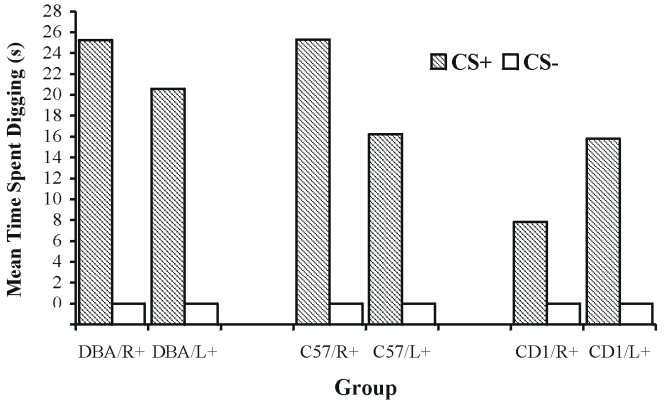

Figure 5 Mean time spent digging in CS+ and CS– odors during testing of DBA, C57 and CD1 mice in Experiment 3.

strains [Kruskal–Wallis $H(2) = 6.84$, $P < 0.05$]. Subsequent Mann–Whitney U analyses revealed that CD1 mice dug less than DBA mice [U (8,8) = 7, $P < 0.01$] and marginally less than C57 mice [U (8,8) = 12, $P < 0.038$]. The DBA and C57 groups did not differ from each other [U (8,8) = 22, $P > 0.05$].

Body mass differences between groups may have accounted for the between-group differences in the test phase. The CD1 animals weighed ~5 g more than the DBA and C57 mice on average, therefore food restriction may have been less effective for CD1 mice compared with the other two strains. If this is true, perhaps the CD1 animals were less motivated to express their conditioned discrimination than C57 and DBA mice. We have found that in this paradigm lack of appropriate food deprivation may lead to lack of digging during preference testing (Forestell *et al.*, 2001).

In contrast to the results of previous reports in which spatial learning was assessed (Upchurch and Wehner, 1988; Ammassari-Teule *et al.*, 1993; Paylor *et al.*, 1993; Petrie, 1995; Arns *et al.*, 1999), DBA performed as well as C57 in our olfactory task. This suggests that olfactory memory as measured in this task does not rely upon the same hippocampal substrate as that required for spatial learning [but see Mihalick, Langlois and Krienke (Mihalick *et al.*, 2000b)]. Other investigators have suggested that odor recognition memory may be independent of hippocampal function (Dudchenko *et al.*, 2000), whereas relational memory (Bunsey and Eichenbaum, 1996) and short-term memory are associated with hippocampal function (Staubli and Lynch, 1995). The role of various subcortical and cortical structures in the acquisition and retention of olfactory learning clearly requires further investigation.

Experiment 4

This paradigm was developed to create a more rapid method of assessing olfactory learning in mice. As the data in Experiments 1–3 indicate, the mice can learn and remember the task exceedingly well. It may be that the number of trials needed to learn the task is much fewer than we originally considered when developing the paradigm. In Experiment 4 we assessed the ability of DBA mice to learn the task when given only one or two trials of each kind per day for 4 days instead of the three or four trials of each kind per day given in the previous experiments.

Materials and methods

Subjects

Nine DBA adult male mice, weighing ~20 g, were obtained from Charles River Canada. Their living conditions were as described for Experiment 1.

Apparatus

The odor stimuli and testing and training apparatus were the same as used for Experiment 1. Prochip was used in the test apparatus and odor pots.

Procedure

As in Experiment 1, 4 days prior to training the mice were placed on a food restriction schedule to maintain them at 80–85% of their free feeding weight. They were assigned to groups as follows: group 1, three animals received rose paired with sugar and lemon alone and two animals received lemon paired with sugar and rose alone, with one training trial per day for 4 days; group 2, two animals received rose paired with sugar and lemon alone and two animals received lemon paired with sugar and rose alone, with two training trials per day for 4 days. The training and testing procedures were identical to those described for Experiment 1.

Results and discussion

The mice learned the odor discrimination whether they were trained for one trial per day or two trials per day. A Wilcoxon matched-pairs signed-ranks test [T (9) = 0, $P < 0.05$] indicates that during testing the mice spent more time digging in CS+ odor than in CS– odor (Figure 6 and Table 4). Animals that were trained for one trial of CS+ and CS– per day dug significantly more in CS+ than animals tested for two trials of each odor type per day [U (4,5) = 1, $P < 0.05$]. It is possible that the animals that had more training trials were more efficient at finding sugar and, thus, when there was none in the test they stopped digging sooner.

In this experiment, unlike in Experiments 1–3, ~50% of the mice dug in CS– odor. The mice that received less training dug more in CS– odor than mice that were trained more [U (4,5) = 2.5, $P < 0.08$). Thus, even though the mice with fewer training trials learned to discriminate between CS+ and CS–, it appears that with more training mice learned to further inhibit digging in CS–. This would suggest

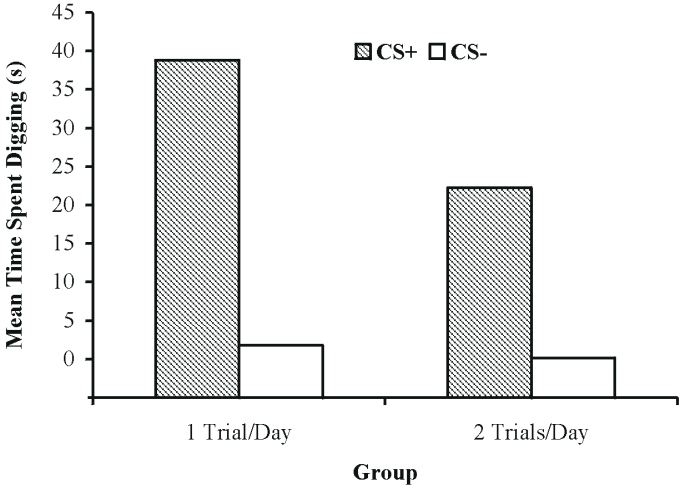

Figure 6 Mean time spent digging in CS+ and CS– in testing of DBA mice that received 1 (group 1) or 2 (group 2) training trials/day in Experiment 4.

Table 4 Time spent digging in CS+ and CS– stimuli by groups 1 and 2 in the test phase of Experiment 4

Group[a]		CS+	CS–	Preference for CS+
1	R+/L–	44.00	3.00	0.94
		26.00	0.00	1.00
		32.00	3.00	0.91
	L+/R–	32.00	1.00	0.97
		60.00	2.00	0.97
2	R+/L–	16.00	0.00	1.00
		23.00	0.00	1.00
	L+/R–	23.00	0.00	1.00
		27.00	0.68	0.98

[a]Group 1 received one training trial per day for 4 days and group 2 received two training trials per day for 4 days.

that the strength of discrimination became stronger with more training.

General discussion

The data presented in Experiments 1–4 indicate that this test provides an appropriate measure of olfactory learning and memory. Outbred mice can learn and remember an odor discrimination between two odors for 60 days. This result provides us with a background to assess the role of interference in the retention of long-term odor memories. Two inbred strains of mice (DBA and C57) also learned the task. The known hippocampal anomalies of strain DBA (Crusio *et al*., 1987; Schwegler *et al*., 1990; Wehner *et al*., 1990, Fordyce *et al*., 1995; Bampton *et al*., 1999) did not appear to influence their ability to perform the task nor did the cholinergic forebrain deficiencies of strain C57 (Bentivoglio *et al*., 1994) prevent these animals from learning the discrimination. It is possible, however, that a more complex task, such as one involving multiple odor discriminations, would reveal memory deficits in these inbred strains of mice (Mihalick *et al.*, 2000b)

We have used a preliminary version of this test to investigate the neurochemistry of olfactory learning (Brennan *et al*., 1998) and to understand the neural pathways involved in olfactory discrimination learning (Forestell *et al*., 1999). Over time we have made a number of successful modifications to the task. For example, we have greatly reduced the number of training trials. In Experiment 4 we demonstrated that one trial per day for 4 days is sufficient for strain DBA to learn the task. It is possible that the mice could learn the odor discrimination in only one trial with each CS. Our analysis of the amount of time spent digging during acquisition of learning does not support this claim (personal observation). We have noted that animals do not always dig on the second CS+ trial.

Unlike in many other published reports with mice, our mice were housed individually in these experiments. Because of their social nature it is generally recommended that mice be housed in groups (Brain, 1975), however, based upon our experience in this experiment it is our conclusion that it may be more appropriate to house food-restricted mice individually. When housed in pairs the mice appeared to compete for food. This led to one mouse acquiring more food than the other, making it difficult to maintain both at 80–85% of their free feeding weight. To prevent variations in the degree of food deprivation becoming an issue when using this paradigm, housing the animals individually would seem to be worthwhile. We would recommend housing the mice in pairs prior to the 5 day experimental period and if possible following the experiment if they are to be kept for further memory testing at a later date.

The current interest in learning and memory processes in mice has led to the development of a number of paradigms to assess olfactory learning, in particular. Both the methodology and task requirements have varied. Several laboratories (Berger-Sweeny *et al*., 1998; Zagreda *et al*., 1999; Mihalick *et al.*, 2000b) have successfully modified Bunsey and Eichenbaum's simultaneous odor discrimination task in which the subject is required to dig in odor-scented sand for reward (Bunsey and Eichenbaum, 1995). Depending upon the qualitative and quantitative properties of the odors used, preventing mixing of the odors can be difficult in these tasks. Bodyak and Slotnick used an operant task and a computer controlled odor delivery system to overcome this problem of stimulus control (Bodyak and Slotnick, 1999). The latter paradigm should be particularly useful in the study of complex learning processes such as those involved in delayed matching to a sample. Nonetheless, it can involve a substantial training time. In the task described here the mice can be trained and tested within 5 days. As the animals are given successive odor discrimination training, the problem of odor stimuli mixing is eliminated.

The overall objective of our experiments was to determine whether an associative odor discrimination paradigm could be developed which would reliably and efficiently assess learning and memory in mice. Of the 77 experimental animals tested in the four experiments 70 showed a preference for CS+ odor, two showed a preference for CS– and five did not show a preference for either odor. Very few mice spent any time digging in CS– odor. The consistent nature of the results obtained in the experiments reported here confirms that this paradigm can be reliably employed to assess olfactory discrimination and long-term memory in mice.

Acknowledgements

Patti Guidry and Mary Elizabeth MacIsaac assisted with data collection. M. MacIsaac was the receipient of the Laing Summer Undergraduate Research Award at Dalhousie University. This research was supported by Natural Sciences and Engineering Research Council of Canada research grants to H.M.S. and V.M.L.

References

Ammassari-Teule, M., Hoffman, H.-J. and **Rossi-Arnaud, C.** (1993) *Learning in inbred mice: strain-specific abilities across three radial maze problems*. Behav. Genet., 23, 405–412.

Arns, M., Sauvage, M. and **Steckler, T.** (1999) *Excitotoxic hippocampal lesions disrupt allocentric spatial learning in mice: effects of strain and task demands*. Behav. Brain Res., 106, 151–164.

Bampton, E., Gray, R. and **Large, C.** (1999) *Electrophysiological characterisation of the dentate gyrus in five inbred strains of mouse*. Brain Res., 841, 123–134.

Beauchamp, G. and **Wellington, J.** (1984) *Habituation to individual odors following brief, widely spaced presentations*. Physiol. Behav., 32, 511–514.

Bentivoglio, A., Altavista, M., Granata, R. and **Albanese, A.** (1994) *Genetically determined cholinergic deficiency in the forebrain of C57BL/6 mice*. Brain Res., 637, 181–189.

Berger-Sweeney, J., Libbey, M., Arters, J., Junagadhwalla, M. and **Hohmann, C.F.** (1998) *Neonatal monoaminergic depletion in mice (*Mus musculus*) improves performance of a novel odor discrimination task*. Behav. Neurosci., 112, 1318–1326.

Bodyak, N. and **Slotnick, B.** (1999) *Performance of mice in an automated olfactometer odor detection, discrimination and odor memory*. Chem. Senses, 24, 637–645.

Bouton, M.E., Nelson, J.B. and **Rosas, J.M.** (1999a) *Stimulus generalization, context change, and forgetting*. Psychol. Bull., 125, 171–186.

Bouton, M.E., Nelson, J.B. and **Rosas, J.M.** (1999b) *Resolution now! Reply to Riccio, Richardson, and Ebner (1999)*. Psychol. Bull., 125, 190–192.

Brain, P. (1975) *What does individual housing mean to a mouse?* Life Sci., 16, 187–200.

Brennan, P., Schellinck, H., De La Riva, C., Kendrick, K. and **Keverne, E.** (1998) *Changes in neurotransmitter release in the main olfactory bulb following an olfactory conditioning procedure in mice*. Neuroscience, 87, 583–590.

Brown, R.E. and **MacDonald, D.W.** (eds) (1985) Social Odours in Mammals, Vols 1 and 2. Clarendon Press, Oxford, UK.

Brown, R.E., Stanford, L. and **Schellinck, H.** (2000) *Developing standardized behavioral tests for knockout and mutant mice*. ILAR J., 41, 163–174.

Bunsey, M. and **Eichenbaum, H.** (1996) *Conservation of hippocampal memory function in rats and humans*. Nature, 379, 255–257.

Castellano, C., Cabib, S., Puglisi-Allegra, S., Gasbarri, A., Sulli, A., Pacitti, C., Introini-Collison, I.B. and **McGaugh, J.L.** (1999) *Strain-dependent involvement of D1 and D2 dopamine receptors in muscarinic cholinergic influences on memory storage*. Behav. Brain Res., 98, 17–26.

Crawley, J. (2000) *Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests*. ILAR J., 41, 136–143.

Crawley, J. and **Paylor, R.** (1997) *A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice*. Horm. Behav., 31, 197–211.

Crusio, W., Schwegler, H. and **Lipp, H.** (1987) *Radial-maze performance and structural variation of the hippocampus in mice: a correlation with mossy fibre distribution*. Brain Res., 425, 182–185.

Dudchenko, P., Wood, E. and **Eichenbaum, H.** (2000) *Neurotoxic hippocampal lesions have no effect on odor span and little effect on odor recognition memory but produce significant impairments on spatial span, recognition, and alternation*. J. Neurosci., 20, 2964–2977.

Eichenbaum, H. (1998) *Using olfaction to study memory*. Ann. NY Acad. Sci., 855, 657–669.

Fordyce, D., Clark, V., Paylor, R. and **Wehner, J.** (1995) *Enhancement of hippocampally-mediated learning and protein kinase C activity by oxiracetam in learning-impaired DBA/2 mice*. Brain Res., 672, 170–176.

Forestell, C.A., Schellinck, H.M., LoLordo, V.M., Brown, R.E. and **Wilkinson, M.** (1999) *Olfactory conditioning and immediate early gene expression in mice*. Chem. Senses, 24, 618.

Forestell, C.A., Schellinck, H.M., Dreumont, S. and **LoLordo, V.M.** (2001) *Effect of food restriction on acquisition and expression of a conditioned odor discrimination in mice*. Physiol. Behav., 72, 559–566.

Galef, B. and **Whiskin, E.** (1998) *Determinants of the longevity of socially learned food preferences of Norway rats*. Anim. Behav., 55, 967–975.

Galef, B., Whiskin, E. and **Horn, C.S.** (1999) *What observer rats don't learn about foods from demonstrator rats*. Anim. Learn. Behav., 27, 316–322.

Gerlai, R. and **Clayton, N.** (1999) *Analysing hippocampal function in transgenic mice: an ethological perspective*. Trends Neurosci., 22, 47–51.

Gould, T. and **Wehner, J.** (1999) *Genetic influences on latent inhibition*. Behav. Neurosci., 113, 1291–1296.

Kogan, J., Frankland, P., Blendy, J., Coblentz, J., Marowitz, Z., Schutz, G. and **Silva, A.** (1996) *Spaced training induces normal long-term memory in CREB mutant mice*. Curr. Biol., 7, 1–11.

Larson, J., Lieu, T., Petchpradub, V., LeDuc, B., Ngo, H., Rogers, G. and **Lynch, G.** (1995) *Facilitation of olfactory learning by a modulator of AMPA receptors*. J. Neurosci., 15, 8023–8030.

Laska, M., Alicke, T. and **Hudson, R.** (1996) *A study of long-term odor memory in squirrel monkeys (*Saimiri sciureus*)*. J. Comp. Psychol., 110, 125–130.

Mihalick, S.M., Langlois, J.C., Krienke, J.D. and **Dube, W.V.** (2000a) *An olfactory discrimination procedure for mice*. J. Exp. Anal. Behav., 73, 305–318.

Mihalick, S.M., Langlois, J.C. and **Krienke, J.D.** (2000b) *Strain and sex differences on olfactory discrimination learning in C57BL/6J and DBA/2J inbred mice (*Mus musculus*)*. J. Comp. Psychol., 114, 365–370.

Mondadori, C. and **Weiskrantz., J.** (1993) *NMDA receptor blockers facilitate and impair learning via different mechanisms*. Behav. Neural Biol., 60, 205–210.

Nowak, R. (ed.) (1999) Walker's Mammals of the World, 6th Edn., Vol. 2. Johns Hopkins University Press, Baltimore, MD.

Paylor, R., Baskall, L. and **Wehner, J.** (1993) *Behavioral dissociations between C57BL/6 and DBA/2 mice on learning and memory tasks: a hippocampal-dysfunction hypothesis*. Psychobiology, 21, 11–26.

Petrie, B. (1995) *Learning set spatial navigation performance in three mouse strains*. Psychol. Rep., 77, 1339–1342.

Ravel, N., Vigouroux, M., Elaagouby, A. and **Gervais, R.** (1992) *Scopolamine impairs delayed matching in an olfactory task in rats*. Psychopharmacology, 109, 439–443.

Reid, I. and **Morris, R.** (1993) *The enigma of olfactory learning*. Trends Neurosci., 16, 17–20.

Riccio, D.C., Richardson, R. and **Ebner, D.L.** (1984) *Memory retrieval*

deficits based upon altered contextual cues: a paradox. Psychol. Bull., 96, 152–165.

Riccio, D.C., Richardson, R. and **Ebner, D.L.** (1999) *The contextual change paradox is still unresolved: comment on Bouton, Nelson, and Rosas (1999)*. Psychol. Bull., 125, 187–189.

Rogers, D., Jones, D., Nelson, P., Jones, C., Quilter, C., Robinson, T. and **Hagan, J.** (1999) *Use of SHIRPA and discriminant analysis to characterize marked differences in the behavioral phenotype of six inbred mouse strains*. Behav. Brain Res., 105, 207–217.

Roman, F., Han, D. and **Baudry, M.** (1989) *Effects of two ACTH analogs on successive odor discrimination learning in rats*. Peptides, 10, 303–307.

Schellinck, H.M. and **Brown, R.** (1998) *Searching for the source of urinary odors of individuality in rodents*. In Johnston, R.E. *et al.* (eds), Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Press, New York, pp. 267–280.

Schwegler, H., Crusio, W. and **Brust, I.** (1990) *Hippocampal mossy fibres and radial-maze learning in the mouse: a correlation with spatial working memory but not with non-spatial reference memory*. Neuroscience, 34, 293–298.

Siegel, S. (1956) Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

Slotnick, B. and **Katz, H.** (1974) *Olfactory learning set formation in rats*. Science, 185, 796–798.

Slotnick, B. and **Risser, J.M.** (1990) *Odor memory and odor learning in rats with lesions of the lateral olfactory tract and mediodorsal thalamic nucleus*. Brain Res., 529, 23–29.

Slotnick, B., Hanford, L. and **Hodos, W.** (2000) *Can rats acquire a learning set?* J. Exp. Psychol. Anim. Behav. Process., 26, 399 –415.

Staubli, L.T. and **Lynch, G.** (1995) *Variants of olfactory memory and their dependencies on the hippocampal formation*. J. Neurosci., 15, 1162–1171.

Upchurch, M. and **Wehner, J.** (1988) *Differences between inbred strains of mice in Morris water maze performance*. Behav. Genet., 18, 55–68.

Wehner, J.M., Sleight, S. and **Upchurch, M.** (1990) *Hippocampal protein kinase C activity is reduced in poor spatial learners*. Brain Res., 523, 181–187.

Yamazaki, K., Beauchamp, G., Bard, J., Thomas, L. and **Boyse, E.** (1982) *Chemosensory recognition of phenotypes determined by the T1a and H-2K regions of chromosome 17 of the mouse*. Proc. Natl Acad. Sci. USA, 79, 7828–7831.

Zagreda, L., Goodman, J., Druin, D.P., McDonald, D. and **Diamond, A.** (1999) *Cognitive deficits in a genetic mouse model of the most common biochemical cause of human mental retardation.* J. Neurosci., 19, 6175–6182.

Accepted March 9, 2001