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Alternate without alternative: neither preference nor learning explains behaviour of C57BL/6J mice in the T-maze

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Abstract
In rodents, the T-maze is commonly used to investigate spontaneous alternating behaviour, but it can also be used to investigate preference between goods. However, for T-maze preference tests with mice there is no recommended protocol and researchers frequently report reproduction difficulties. Here, we tried to develop an efficient protocol with female C57BL/6J CrL mice for preference tests. We used two different designs, adapting habituation, cues and trial timing. However, in both experiments mice did not show any preference, although we used goods which we knew mice find rewarding. Instead, they alternated choices indicating that exploratory behaviour overruled preference. We argue that this behavioural strategy has evolved as an adaptive trait in saturated conditions where there is no need to take the reward immediately. Therefore, we deem the T-maze unsuitable for preference testing with the procedures we used here.

Keywords
T-maze, Y-maze, preference, mice, reward, choice, alternation.

1. Introduction

The T-maze is a behavioural test using a maze with a start arm (sometimes connected to a start cage) and two choice arms branching off at the same point from the start arm. In the classic design the arms lie exactly opposite

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each other, so that they form a T together with the starting arm. In the Y-maze variation, the arms branch off from the start arm at a steeper angle so that the overall shape of the apparatus is y-shaped. During a T-maze test, an animal is placed either in the start cage or directly inside the maze at the beginning of the start arm. At the end of the start arm, the animal has then to choose between entering the left or the right arm. Depending on the setup, in addition to the spatial position the arms can provide further cues, e.g., visual (mice: Lione et al., 1999; broilers: Buckley et al., 2011), tactile (compare Cunningham et al., 2006) or olfactory cues (Mayeux-Portas et al., 2000). Also, none, one or both arms can contain a reward, which can be food (Crusio et al., 1990; Deacon & Rawlins, 2006; Deacon, 2006), shelter (Pilz et al., 2020) or a platform (in case of the water T-maze, Granholm et al., 2000; Belzung et al., 2001; Guariglia & Chadman, 2013).

The T-maze is an important behavioural test to assess the effect of drugs (mice: Correa et al., 2015; rats: Lohninger et al., 2001), genetic alterations (mice: Granholm et al., 2000; Mayeux-Portas et al., 2000) or diseases (mice: Belzung et al., 2001; rats: Sánchez-Santed et al., 1997; Wu et al., 2018). It is often used to assess spontaneous alternating behaviour, spatial memory and/or discrimination of stimuli (Dember & Fowler, 1958; Wenk, 1998; Belzung et al., 2001; Dudchenko, 2004; Deacon & Rawlins, 2006; Deacon, 2006; Sharma et al., 2010b). Spontaneous alternating behaviour describes the tendency of rodents to choose the arm they did not visit in the preceding trial. This kind of behaviour occurs spontaneously and is not necessarily related to a resource being exploited in the preceding trial (mice: Gerlai, 1998; gerbils: Dember & Kleinman, 1973; rats: Sánchez-Santed et al., 1997). In position discrimination tests (also: spatial memory tests), only one spatial location, either the left or the right arm, is baited (mice: Lione et al., 1999; Granholm et al., 2000; Belzung et al., 2001; Sharma et al., 2010a; Guariglia & Chadman, 2013; Pioli et al., 2014). Thus, the spontaneous alternating is a way to evaluate the working memory (which location was last visited?), while the position discrimination test evaluates the reference memory (Deacon & Rawlins, 2006), similar to the conditioned place preference test (Wenk, 1998; Sharma et al., 2010b; Shoji et al., 2012; Hieu et al., 2020). In a further modification of the position discrimination, the T-maze can also be used as general discrimination test, using additional cues instead of merely the spatial one to provide information on the baited arm (mice: Lione et al., 1999; Granholm et al., 2000; Mayeux-Portas et al., 2000; broilers: Buckley et al., 2011).

Note that with different tasks different memory types are tested: For alternating behaviour, the working memory is important (remembering which arm was last visited). For position or stimulus discrimination behaviour, the working memory is also important (which cue was rewarded?) but between testing days, this information has to be retrieved from the reference memory (Sharma et al., 2010b).

In a modification of the discrimination test, the T-maze is also used as a preference test: The arms are provided with different goods, and the animal is required to choose between them. This form of preference test seems to be easily performed with a variety of animal species (mice: Roder et al., 1996; Correa et al., 2015; Cutuli et al., 2015; wild mice: Nunes et al., 2009; rats: Patterson-Kane et al., 2001; Ras et al., 2002; Denk et al., 2004; van der Plasse et al., 2007; Cunningham et al., 2015; Hernandez-Lallement et al., 2015; Wadhera et al., 2017; Leenaars et al., 2019; pigs: Rooijen & Metz, 1987; hens: Dawkins, 1977; broilers: Buckley et al., 2011; zebrafish: Hieu et al., 2020; fruit flies: Fujita & Tanimura, 2011). Preference is usually assessed by offering the goods in the choice arms of the maze but in some cases, it might be useful to use stimuli which are associated with the to-be-tested goods instead, e.g., in tests for social preference, the real mouse might be replaced by urinary stimuli (Nunes et al., 2009; compare also Fitchett et al., 2006). It also has to be kept in mind that offering the goods itself can lead to saturation and/or influence the choice in the next trial (Kirkden & Pajor, 2006), in the same way as humans might prefer milk after eating something spicy (Nasrawi & Pangborn, 1990).

Preference tests in T-mazes can be performed with discrete or continuous choices: In a discrete measurement task, an animal has to perform multiple trials in which it can choose between the left or the right arm (mice: Tellegen et al., 1969; rats: Patterson-Kane et al., 2001; Ras et al., 2002; van der Plasse et al., 2007; Pioli et al., 2014). In a continuous measurement task, the animal stays in the T-maze for a defined period of time and the time the animal spends in the left or the right arm is used to ascertain preference (mice: Roder et al., 1996; Cutuli et al., 2015; wild mice: Nunes et al., 2009; Correa et al., 2015; compare also Pennycuik & Cowan, 1990; using a U-shaped maze and wild mice).

There are various protocols and recommendations on the conduction of T-maze tests for behavioural measures such as memory and discrimination.

However, there is to date no protocol for T-maze preference tests: The protocols focus either on spontaneous (unrewarded) alternation (Wenk, 1998; Deacon & Rawlins, 2006), rewarded alternation (Deacon & Rawlins, 2006; Shoji et al., 2012; Wenk, 1998) or position discrimination (Deacon, 2006; Shoji et al., 2012). A short comparison of different protocols is given in Table 1.

In general, for spontaneous alternation, no food restriction or habituation is needed. Animals should just be well-habituated to their environment and the handling, before they are placed into the maze. Protocols for rewarded alternation and position discrimination are more complex and differ in their recommendations. Often, food restriction to 85% of free-feeding weight is recommended, although Deacon & Rawlins (2006) at the same time state that well habituated animals should also perform the T-maze without food restriction (Deacon & Rawlins, 2006). For rewarded alternation, forced trials are recommended, in which animal are only allowed to visit one arm by blocking the other. In the following trial, animals get a free choice with both arms accessible. If the animals visit the previously blocked arm, they made an alternating choice. In position discrimination, on the other hand, no forced trials are conducted, and trials are always free choice. Also, rewarded alternation and position discrimination differ with regard to the recommendations made about cleaning: While for rewarded alternation tasks, cleaning seems to be more common, for position discrimination Deacon (2006) explicitly states that not cleaning maximizes the learning potential (Deacon, 2006). However, protocols for both types of tests differ greatly in their recommendations for habituation procedure (individuals or group, duration, free exploration or trials, reward or no reward) and intertrial interval (immediately or more than 10 min). All protocols recommend at least ten trials per day, but depending on the intertrial interval this leads to differing test durations from 50 min (Shoji et al., 2012) to several hours (Deacon, 2006). None of the protocols gives instructions with regard to testing time, and only one of the protocols (Shoji et al., 2012) provides an example for testing time, but only to emphasise that the tests should be repeated in the same time frame (their example is between 9:00 am and 6:00 pm, with lights 7:00 am–7:00 pm). Searching original studies instead of protocols, the time frame of experiments (if stated) varies, e.g., starting 2 h into the dark phase (Locurto et al., 2002), 3 h before the end of the light phase (Guariglia & Chadman, 2013), 3 h into the light phase (Derenne et al., 2014) or in general ‘during the light phase’ (Moy et

Table 1.
Comparison of T-maze protocols by Deacon & Rawlins, 2006; Deacon, 2006; Shoji et al., 2012 and Wenk, 1998.

Protocol	Species	Test	Food deprivation		Habituation			
			Start	% of free-feeding weight	To reward	To test room	To maze: phase 1	To maze: phase 2
Deacon & Rawlins, 2006	Mice	Rewarded alternation	Overnight	>85%, 90–95% is ideal	1 h before dark phase: 2 ml milk/mouse in hc	5–10 min	Whole group, 4 × 3 min with 10 min gaps, 4 days, food in maze	Individuals, ? runs for ? days
Shoji et al., 2012	Mice	Rewarded alternation	1 week before training	80–85%	Daily: 8 sucrose pellets/mouse in hc	>30 min	Whole group, 30 min, 1 day, sucrose pellets in maze	Individuals, 5 × 5 min per maze compartment (30 min), ? days
Wenk, 1998	Rats	Rewarded alternation	During test	85%, allow about 5 g weight gain/week	10 mg food reward/day for a few days before training	?	Pair of animals (cage-mates), for ? min, 3–4 days, reward in maze	Individuals, for ? min, both arms rewarded, 7–10 days
Deacon, 2006	Mice	Position discrimination	Overnight	>85%, 90–95% is ideal	1 h before dark phase: 2 ml milk/mouse in hc	?	Individuals, 6 trials, for ? days, food/drink in maze	Individuals, 1 trial, 1 day, both arms rewarded
Shoji et al., 2012	Mice	Position discrimination	1 week before training	80–85%	Daily: 8 sucrose pellets/mouse in hc	>30 min	Whole group, 30 min, 1 day, sucrose pellets in maze	Individuals, 5 × 5 min per maze compartment (30 min), ? days
Deacon & Rawlins, 2006	Mice	Spontaneous alternation	-	-	-	5–10 min	-	-
Wenk, 1998	Rats	Spontaneous alternation	-	-	-	?	-	-

Table 1.
(Continued.)

	Test					
	Forced trials	Trials	Cleaning	ITI	Goal arm	Cues
Deacon & Rawlins, 2006	(= test) first trial: forced trial, followed by free choice trial	10/day	Optional between trials: soapy water, alcohol solution (10% is common) or other	Repeat after the 10th animal	The arm opposite the arm accessible in the forced trial (first trial); randomized for each trial, session, animal	?
Shoji et al., 2012	(= test) each forced trial followed by a free trial	10/day (max. 50 min)	Between mice: with super hypochlorous water (pH 6–7)	Immediately	The arm not visited during the forced trial	Spatial
Wenk, 1998	(= test) forced trial, followed by free choice trial	10/day	?	0 s to minutes	Randomly varied on each day	Spatial
Deacon, 2006	-	20–40	No cleaning to maximize the learning potential	>10 min (otherwise alternation)	The arm opposite the first arm	Paintwork, floor texture or objects
Shoji et al., 2012	-	10–20/day (max. 50 min)	Between mice: with super hypochlorous water (pH 6–7)	Immediately	Invariable across sessions	Spatial
Deacon & Rawlins, 2006	-	?	Optional between trials: soapy water, alcohol solution (10% is common) or other	?	The arm opposite the arm visited last trial	-
Wenk, 1998	-	10/day	?	0 s to minutes	The arm opposite the arm visited last trial	-

ITI = intertrial interval, hc = home cage, ? = not described.

al., 2008; Shipton et al., 2014). However, day time might influence motivation to gain food (Acosta et al., 2020; Koch et al., 2020) and should therefore be considered carefully.

Thus, there is not 'one perfect test design' with regard to rewarded alternation or position discrimination but various ways to perform it, depending on the research question. However, this makes it difficult to develop a protocol for preference tests. Personal correspondence with other researchers resulted mainly in reports of difficulties in reproduction of the T-maze test, especially when trying to alter the existing protocols for preference tests. In general, varying success rates might be caused by differences in strain performances (Gerlai, 1998; Moy et al., 2008). However, there are various additional factors which might influence results, e.g., differences in handling technique (base of the tail compared to cup or tube handling, Hurst & West, 2010; Gouveia & Hurst, 2017), stress (Mitchell et al., 1985), habituation (Deacon & Rawlins, 2006; Rudeck et al., 2020), level of food restriction (Richman et al., 1986).

One interesting solution for the factor handling is provided by Zhang et al. (2018), who developed an automated T-maze system (Zhang et al., 2018). Here, no handling is involved, and thus, influence of the researcher is reduced. Taking it one step further, Pioli et al. (2014) introduced an automated T-maze which is even home cage based. Here, mice can conduct the test when active and most motivated to work for the reward, which also makes food restriction superfluous (Pioli et al., 2014). However, this automated T-maze is designed for single housing (there is only a companion animal behind a partition), which might not be the desired husbandry condition. In addition, this automated T-maze is meant for spontaneous alternation tasks and it would probably need adjustments for preference tests with regard to, e.g., cue presentation and change of presentation side.

Thus, a working protocol for the conduction of a T-maze preference test is still needed. Here, we performed two experiments in search for such a protocol: In experiment 1, we investigated the preference between two fluids (apple juice vs. almond milk). In experiment 2, we changed the test design and offered one arm containing millet and bedding, and one arm containing only bedding. For both experiments, we used C57BL/6J mice because this is the mouse strain most commonly used; therefore, a working protocol would have the greatest impact for the research community. In addition, we tried to develop a protocol without food or water restriction because this condition itself might change the preference of the mice (see also in the discussion).

2. Material and methods

2.1. Animals

A group of thirteen female C57BL/6J CrL mice was purchased in December 2017 at the age of 3 weeks from Charles River, Sulzfeld. This group was used in experiment 1 ('group 1'). Another group consisting of twelve female C57BL/6J CrL mice was purchased in June 2019 at the age of 4 weeks from Charles River, Sulzfeld. This group was used for experiment 2 ('group 2'). We used females because they show less aggression in groups and we needed these large group sizes for other home cage based experiments.

For both groups applies that all mice within a group had different mothers and different nurses to ensure maximal behavioural variability within the inbred strain. At the age of five weeks, transponders were implanted, a procedure performed under anaesthesia and analgesia (for details see the Appendix). Mice were always handled by tube handling. Both groups took part in multiple other experiments, including the development of an home cage based automated tracking system and conditioned place preference tests. By the time the T-maze test was performed, they were around 12 months (group 1, start in November 2018) or 11 months old (group 2, start in April 2020). In the sense of the 3R, we decided to use these groups despite their rather old age. Especially, because the repeatability of activity measures increases with the age of the mice (Brust et al., 2015), and performance levels of C57BL/6J mice in visual detection, pattern discrimination and visual acuity tasks are not decreased with 12 months (Wong & Brown, 2007). It has to be noted that by the start of the experiment 2, eleven of twelve mice in group 2 at least partly lacked their whiskers. This is important as it might influence their tactile-guided behaviour, for example, novel object recognition or open field activity (Haridas et al., 2018; Tur & Belozertseva, 2018). However, this should not have influenced the mice's ability to perceive visual, olfactory or spatial cues (left or right body turn) and to act on them. In addition, as barbering is a model for a disorder (trichotillomania), it is also important to note that mice which barber show no difference in learning ability itself, with the exception of a extra dimensional shift task (Garner et al., 2011). Here, however, only simple learning was required.

2.2. Housing

One group of mice was kept in two type IV macrolon cages (L × W × H: 598 × 380 × 200 mm, Tecniplast, Buguggiate, Italy) with filter tops. The

two cages were connected via a Perspex tube (40 mm in diameter). This cage system was chosen because of other research purposes, and mice had lived in it since they were around 2 months (group 1) or 3 months old (group 2). Food (autoclaved pellet diet, LAS QCDiet, Rod 16, Lasvendi, Soest, Germany) and tap water (two bottles each cage) were available ad libitum in both cages. Cages were equipped each with bedding material (Lignocel FS14, spruce/fir, 2.5–4 mm, JRS, J. Rettenmaier & Söhne, Rosenberg, Germany) of 3–4 cm height, a red house (The MouseHouse, Tecniplast), papers, cotton rolls, strands of additional paper nesting material, and two wooden bars to chew on. Both cages also contained a Perspex tube (40 mm in diameter, 17 cm long), which was used for tube handling.

Room temperature was maintained at 22 ± 3°C, the humidity at 55 ± 15%. Animals were kept at 12 h/12 h dark/light cycle with the light phase starting at 7:00 am (winter time) or 8:00 am (summer time), respectively. Between 6:30 and 7:00 am (winter time) or 7:30 and 8:00 (summer time) a sunrise was simulated using a Wake-up light (HF3510, Philips, Hamburg, Germany). Once per week, the home cages were cleaned and all mice were scored and weighed. In this context, mice also received a colour code on the base of their tails, using Edding 750 paint markers, to facilitate individual recognition.

2.3. T-maze setup

For the T-maze test, a start cage (type III, L × W × H: 425 × 266 × 155 mm, Tecniplast) filled with 1 cm bedding was connected via a tube to the T-maze. The tube contained an automated door. In experiment 1, the connection between the start cage and the T-maze resembled part of the setup used for habituation so mice were already habituated to it (compare Figure 1a and Figure 1b): a 15 cm tube with an radio frequency identification (RFID) antenna between cage and door, and a 6 cm tube with a light barrier between door and maze. If the mouse interrupted the light barrier in front of the door or was detected by the RFID antenna, the door opened for 5 s. For experiment 2 (without automated habituation), the tube connected to the start cage was 14 cm long and contained an RFID antenna, followed by the automated door and a 1 cm long tube (see Figure 1c). Here, the door also opened for 5 s whenever the transponder of a mouse was detected. There was no light barrier on the other side of the door because this time mice were not allowed to return to the start cage by themselves.

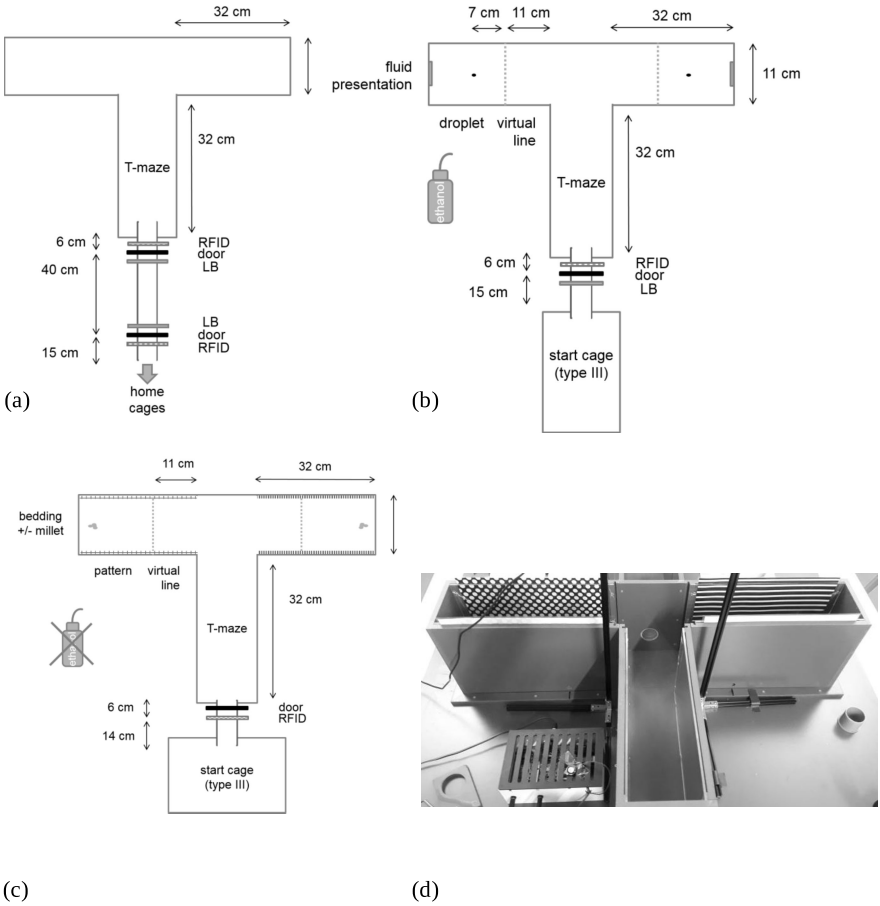

Figure 1. T-maze setup as a schematic drawing for experiment 1 habituation (a) and test (b), and test of experiment 2 (c). (d) Photo of the experiment 2 setup, the box on the bottom left contains the Arduino, which operates the automatic door, the device to its left (with the hole) is an example of the RFID antenna and light barrier constructions. LB, light barrier; door, automatic door; RFID, radio frequency identification antenna.

The T-maze itself consisted of grey plastic and had three arms, each 32 cm long and 11 cm wide, with 20 cm high walls (see Figure 1d). On either side of the arms a mark was made outside the T-maze so that a virtual line could be drawn 11 cm from the central arm during video analysis. If a mouse crossed this line with its whole body (but not yet with its tail), this was defined as a choice being made.

For video recording, in both experiments a webcam (C390e, Logitech, Lausanne, Switzerland) was mounted above the maze on a metal beam construction. The connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

2.4. T-maze test

In the first experiment, the T-maze test was used to compare the preference for two fluids. Mice performed discrete choices between the two arms, which contained a droplet of either almond milk or apple juice. Because insufficient habituation might slow the performance in the maze (Deacon & Rawlins, 2006) and might be one of the main problems, we conducted a thorough habituation phase: For about two weeks, mice had free access to the T-maze via a connection to the home cage. After one week, fluids were presented for 24 h inside the home cage. (As the mice drank extensively from the almond milk bottle during that time, a longer presentation seemed unnecessary.) After thirteen days, mice were moved to the testing room, to habituate to it before the start of the actual T-maze test.

The preference test was then performed on two days, with five test trials per mouse per day (based on the protocol of Deacon (2006) which recommends a larger break approximately after five trials), and a side change after the seventh trial to control for side preference (see Figure 2). The test was conducted between 9:00 am and 7:00 pm (lights 7:00 am–7:00 pm), similar to the example provided by the protocol of Shoji et al., 2012. The mice had the choice between almond milk and apple juice, with 20 μl of fluid as a

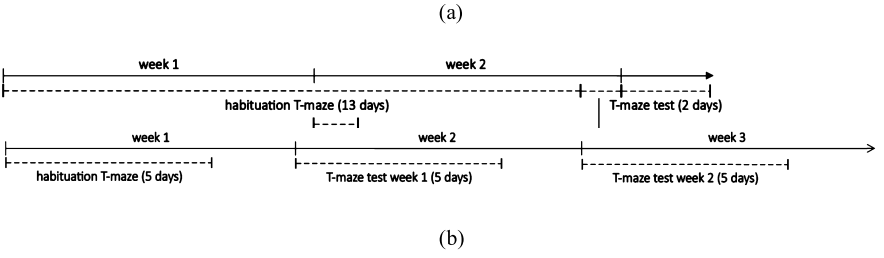

Figure 2. Timeline of experiment 1 (a) and experiment 2 (b). In experiment 2, no habituation to the experimental room was necessary because it took place in the husbandry room. In addition, no habituation to the options (millet with or without bedding material) was necessary because mice were familiar with it from previous experiments.

reward in the respective arm. As an intramaze olfactory cue, we applied some of the fluid onto a cellulose sheet at the end of the arms. In addition, for the first seven trials the spatial intramaze (left/right) and extramaze cues (position in experimental room) remained the same (before presentation side was switched). Between mice, the maze was cleaned with ethanol. During trials, an additional light was added (for more details on the procedure of experiment 1 see Appendix). In this experiment, we expected the mice to prefer the arm with almond milk based on observations made during the initial presentation of the fluids (see Appendix) and results from consumer demand preference tests made in our laboratory (Kahnau et al., data not shown).

In a second experiment we changed the design in several points (see Table 2): active (manual) habituation instead of passive habituation for 3 min on five consecutive days, daily repeated trials instead of block-wise trials, no ethanol disinfection of the maze between mice, no additional light for the T-maze, and intramaze visual cues supplementary to olfactory cues. Also, the choice was now not between two fluids but between millet (0.05 g mixed with bedding material) or no millet (a visually similar amount of bedding material). We changed the reward because we conducted pre-tests in which mice fed more readily on millet than on almond milk outside their home cage. Thus, to increase the likelihood that mice would actually consume their reward, we now used millet. Note that this preference test design now also resembled a learning test because only one arm was baited.

Habituation to the T-maze and the preference test were conducted between 8:00 and 11:00 am (lights 8:00 am–8:00 pm), to keep the test close to the dark phase, and thus, to the active phase, for all animals. To reduce the testing time per day, the preference test was performed on five consecutive days with two trials per mouse per day (leading to the same amount of trials as in experiment 1) and a side change after the sixth trial. Then, after this proved not to show the hoped-for results, a second week was added (see Figure 2b): Again the test was conducted on five consecutive days but this time three trials were conducted per mouse per day (i.e. one trial more than there were options, to have one additional 'test' trial in case the first two function as exploration), and this week, there was no side change. Thus, the visual cues and spatial intramaze (left/right) and extramaze cues (position in experimental room) provided the same information. A comparison of the timeline of both experiments can be found in Figure 2 (for more details on the procedure of experiment 2 see Appendix). In this experiment, we expected

Table 2.
Experimental design of the T-maze tests conducted in experiment 1 and 2.

		Experiment 1	Experiment 2	
			Week 1	Week 2
Habituation procedure	Method	Passive	Active	
	Duration	13 days	5 days	
	Habituation trial	1 (on day 1)	No	
General test setup	Cleaning	With 70% ethanol	No	
	Illumination	171–350 lux	18–50 lux	
	Options	Almond milk vs. apple juice	Millet + bedding vs. bedding	
Test procedure	Duration	2 days	5 days	5 days
	Trials/day/mouse	5	2	3
	Side change	After trial 7 (day 2)	After trial 6 (day 4)	No
	Cue	Odour	Pattern (+ odour)	Pattern + side (+ odour)

Further explanations on the procedures, e.g., on the illumination levels in the T-maze arms, can be found in the Appendix.

the mice to prefer the arm with millet based on observations in pre-tests (see Appendix) and enrichment experiments made in our laboratory, in which mice were willing to work (e.g., lift a flap, turn a flap or move a ball) to get access to millet (Hobbiesiefken et al., data not shown).

2.5. Statistical analysis

In short, for the T-maze preference test video recordings were analysed with the help of BORIS (Behavioral Observation Research Interactive Software, Version 7.9.8; Friard & Gamba, 2016), noting the time points (a) when the mouse was placed into the start cage (only experiment 2), (b) when the mouse entered the maze, (c) when the mouse crossed the virtual line in one of the choice arms, 11 cm into the arm, and (d) when it entered the handling tube to be returned to the start cage or the home cage. Each behaviour was only counted when the mouse had all four paws on the bedding of the start cage (only experiment 2) or the whole mouse (except the tail) had entered the maze, the tube, or crossed the virtual line (both experiments).

All time points and choices were filled into a table and further managed with the help of R studio (experiment 1: Version 1.1.383, experiment 2: Version 1.2.1335, using R 3.4.0 or higher). For each mouse, choices were pooled (experiment 1: for both days, experiment 2: per week), and the percentage of choices for one option was calculated. Examined were side preference (left vs. right), the option preference (almond milk vs. apple juice in experiment 1, millet vs. no millet in experiment 2), alternating choices (same arm as before vs. different) and pattern (only experiment 2, dots vs. stripes). The analysis of alternating was done by labelling the choices according to whether the arm chosen in this trial was also the arm chosen in the trial before. The first day of both weeks, respectively, were excluded from this labelling.

The results from all mice were then used for significance testing: To test for normal distribution, the Shapiro–Wilk test was performed in R. The data was normal distributed ($p > 0.05$); therefore, a t-test was used to compare the percentages of the mice with a random chance level of 0.5. In all statistical tests, significance level was set to 0.05, and result values are given as mean and standard deviation. (For more details on the analysis, especially with regard to the passive habituation of experiment 1, see Appendix.)

2.6. Ethical approval

All experiments were approved by the Berlin state authority, Landesamt für Gesundheit und Soziales, under license No. G 0182/17 and were in accordance with the German Animal Protection Law (TierSchG, TierSchVersV).

The second experiment was preregistered at the Animal Study Registry (DOI: 10.17590/asr.0000213).

3. Results

3.1. Experiment 1

3.1.1. Passive habituation

After one week, all mice except for one visited the T-maze frequently. After nine days, all thirteen mice did so. As in retrospect was noted that the RFID registration system might have had a malfunction (although this was not the case when tested before), some passages might have not been detected. However, as the system could not add additional passages, this only means that there might have been more passages to the T-maze then registered, and habituation might have been even better than the RFID data showed.

3.1.2. Trial duration and intertrial interval in the T-maze

In most cases, mice self-initiated the trials: Only in two out of 143 trials (habituation trials and miss-recorded trials included), a mouse did not start the trial by itself within the set start time and had to be guided by tube handling into the maze.

Habituation trials included a visit in both arms. From the time point when the mice entered the T-maze to the time point when the mice had crossed the virtual line in both arms, on average 17.2 $\pm$ 11.6 s passed (minimum: 7.5 s, maximum: 45.5 s). For the preference test trials, average duration was 4.46 $\pm$ 2.93 s (minimum 1.25 s, maximum: 25.9 s). Note that in this experimental setup, the way back to the start cage was not blocked so mice could return to the start cage and later on re-visit the maze. The numbers given here are only from those times when a mouse entered the maze and actually crossed one of the virtual lines. Mean intertrial interval (ITI), including cleaning time of the maze and the time until the mouse decided to enter the maze once again, was 204.9 $\pm$ 81.8 s (= 3.4 min), ranging from a minimum of 137 s to a maximum of 506 s.

3.1.3. Preference testing

It was not possible to compare the intake of the offered fluid droplet between apple juice and almond milk on the basis of the video recordings as it was only detectable for the opaque almond milk whether it disappeared. Still, we assessed when the animals spent some time investigating the droplet (licking or intensely sniffing it). This was observed in 74 of 139 trials (including only one time during a habituation trial), representing barely more than half of the trials. 75.67% of these observed behaviours were performed towards an almond milk droplet.

Comparing the choices of the mice for the arm with apple juice or the arm with almond milk, mice chose in 52.8 $\pm$ 9.9% of the trials the arm with almond milk. This indicates no preference ($t = 1.028$, df $= 12$, $p = 0.3242$, see Figure 3). Mice showed also no side preference: The left arm was chosen on average in 49.5 $\pm$ 14.1% of trials ($t = -0.13145$, df $= 12$, $p = 0.8976$). As the T-maze test is often used to test for spontaneous alternation (Deacon, 2006), we then analysed the data with regard to alternating choices. Indeed, mice chose in 64.4 $\pm$ 13.5% of trials the arm which they did not choose during the last trial ($t = 3.8442$, df $= 12$, $p < 0.003$).

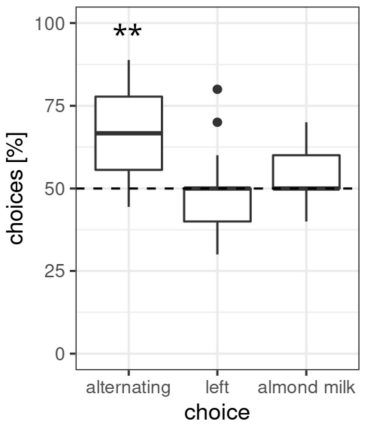

Figure 3. Percentage of choices for the arm not visited in the preceding trial (alternating), the left arm and the arm containing almond milk. Thirteen female mice chose 10 times (5 per day) between an arm containing the odour and a 20 μl droplet of almond milk or apple juice. Presentation side was randomized across the group, and switched after trial seven. $^{**}p < 0.01$.

3.2. Experiment 2

3.3. Active habituation

Mice were familiar with automated doors from previous experiments. However, in this new setup they seemed to experience the door as something new, so that on day one of habituation, only one mouse went into the maze on its own. Nevertheless, on the fifth day of habituation all mice went into the maze by themselves within the time frame of three minutes.

3.4. Trial duration and intertrial interval in the T-maze

Time spent by the mice in the start cage before entering the maze ranged between 1.7 and 159.5 s (on average 21.27 ± 22.71 s). Inside the maze, the mice took only 3.6 ± 1.7 s to make a choice and enter one of the goal arms far enough to cross the virtual line (min 1.4 s, max 14.5 s). There, mice spent about 47.4 ± 33.09 s in the arm before entering the provided tube. After preparing the arms again for the next trial, the mouse was returned to the start cage. This intertrial interval lasted on average 19.4 ± 8.8 s (min 4 s, max 107 s, caused by an error during the preparation), measuring the time between the mice being taken out of the arm and starting the new trial. Including the time between making the choice and leaving the arm would add the approximately 47 s spent in the goal arm.

3.5. Preference testing

In week 1 (two trials per day, side change after trial six), mice chose in 43.3 ± 8.9% the arm containing millet, which meant that they significantly preferred the arm without it ($t = -2.6018$, df = 11, $p < 0.05$, see Figure 4). There was no side preference (left arm chosen in 50.0 ± 12.8%, $t = 0$, df = 11, $p = 1.00$) and no pattern preference (dots chosen in 55.0 ± 10%, $t = 1.7321$, df = 11, $p = 0.11$). However, mice also significantly alternated between arms (63.9 ± 15.8%, $t = 3.0446$, df = 11, $p < 0.05$).

In week 2 (three trials per day, no side change), mice chose in 53.4 ± −11.4% the arm containing millet ($t = 1.0155$, df = 11, $p = 0.33$, see Figure 4b). There was no side preference (left: $t = -0.6603$, df = 11, 47.8 ± 11.7%, $p = 0.52$) or pattern preference (dots: 45.6 ± 10.9%, $t = -1.4062$, df = 11, $p = 0.19$) but mice significantly alternated between trials (67.9 ± 13.4%, $t = 4.5993$, df = 11, $p < 0.001$). When looking at the individual trials (see Figure 5), percentage of alternation was especially apparent in the

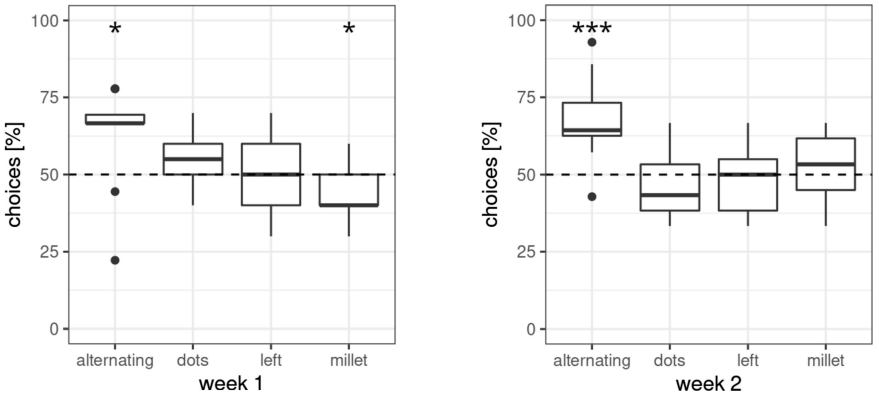

Figure 4. Percentage of choices for the arm not visited in the preceding trial (alternating), the arm marked with dots, the left arm and the arm containing almond milk. One group of 12 female mice chose between an arm containing bedding mixed with millet and an arm only containing bedding. Presentation side and pattern (dots or stripes) was randomized across the group. (a) In week 1, two trials were performed per day (10 in total), and after trial six, presentation side was switched. (b) In week 2, three trials were performed per day (15 in total), and presentation side was kept as last used in week 1. * $p < 0.05$, *** $p < 0.001$.

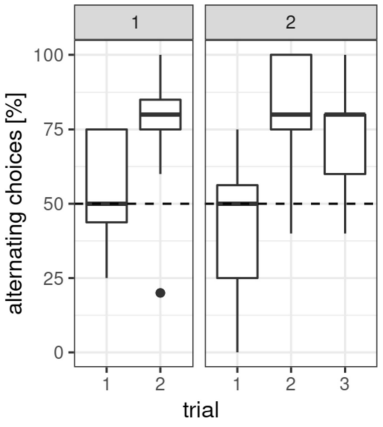

Figure 5. Percentage of choices for the arm not visited in the preceding trial (alternating) across trials for week 1 (left, two trials per day) and week 2 (right, three trials per day). One group of 12 female mice chose between an arm containing bedding mixed with millet and an arm only containing bedding. Presentation side and pattern (dots or stripes) was randomized across the group. In week 1, two trials were performed per day (10 in total), and after trial six, presentation side was switched. In week 2, three trials were performed per day (15 in total), and presentation side was kept as last used in week 1.

second and third trial but not in the first, which was compared to the last trial on the day before (week 1: trial 1 52.1 ± 19.8%, trial 2: 73.3 ± 27.4%; week 2: trial 1 43.8 ± 24.1%, trial 2 81.7 ± 19.9%, trial 3 73.3 ± 17.8%).

4. Discussion

4.1. Habituation

Mice took on average about 10 s (experiment 1) or 4 s (experiment 2) to make a choice after starting the trial. This implies that mice were well habituated: As Deacon & Rawlins (2006) describe, a trial duration longer than two minutes can indicate insufficient habituation, and here, mice were much faster. However, the two minutes Deacon & Rawlins (2006) use as a bench mark usually include the time from placing the animal in the start area of the maze to the actual choice (Deacon & Rawlins, 2006). We here provided the animal the opportunity to self-initiate the test, which probably conduced to a shorter trial time because trials started apparently when the animal itself was motivated.

However, it is possible that animals were not habituated enough for the preference test itself: Judging on the basis of their behaviour in experiment 1, mice tested the fluid drop only in half of the trials. This might be an indication for insufficient habituation, as during pre-tests before the second experiment, mice fed on millet in an unfamiliar surrounding only after several sessions of habituating to it. In addition, we observed during the pre-tests that millet was consumed more willingly in general than almond milk. Therefore, in experiment 2, one week of active instead of passive habituation to the T-maze was conducted, and we used millet as a reward. Here, all mice fed on the millet when choosing the respective arm. Thus, feeding behaviour in the maze seems to be influenced by both the habituation method and the type of reward.

4.2. Lack of preference or reward-aimed behaviour

In preparation of experiment 1, when offering the two fluids in the home cage for habituation, the twelve mice as a group drank nearly 500 ml of the almond milk in 24 h, whereas they drank only about 200 ml of the provided apple juice. This implies a strong preference. However, no fluid preference was found in the T-maze preference test.

In the same manner, mice should have preferred the rewarded arm (bedding and millet) over the unrewarded arm (bedding only). It is not likely that mice did not revisit the arm because they were sated on millet: In maximum, they could have consumed three times 0.05 g, and in another experiment from our research group, mice received about 0.8 g millet per day and were still willing to work for it (e.g., lift a flap, turn a flap or move a ball, Hobbiesiefken et al., data not shown).

There are various possible reasons for this lack of preference, the main ones being the influence of the cues, and the usage of different foraging strategies, which will both be discussed in the following.

4.3. Missing cues

One explanation for the lack of preference might be a missing perceivable cue on where to find the preferred good. In the first experiment, in addition to spatial information (at least during the first seven trials) an odour cue was provided. However, between the trials, the maze was cleaned with ethanol to erase odour cues. This was done because intramaze odour cues of previous decisions might influence the next choice (rats: Means et al., 1992). Nevertheless, the ethanol itself might have left an odour, masking the olfactory cue of almond milk and apple juice.

We investigated this theory by not cleaning the maze between mice in experiment 2. Although we did not provide an additional olfactory cue on a cellulose sheet as in experiment 1, it can be assumed that the options (millet or no millet) naturally include an olfactory cue. In addition, a visual cue (wall pattern) and a spatial cue (no side change in week 2) were provided. Thus, mice should have had the possibility to learn which of the two arms was the rewarded one. However, this also did not lead to a preference for the rewarded arm.

4.4. Foraging strategies

As the setup of experiment 2 is in general similar to simple learning tests (operant conditioning, learning the relationship between behaviour and its outcome), mice should be able to learn the position of the millet. For optimal foraging, animals should adopt in this scenario the win–stay/lose–shift strategy, meaning that they should stay (or return to) where they found food before and change position when they did not find food (Shettleworth, 2010).

However, it seems we observed a similar result here as described in the study by Locurto et al. (2002), in which offspring of a C57BL/6 and DBA/2J

cross easily learned the win–shift strategy but did not exceed chance levels when requested to perform win–stay (Locurto et al., 2002; also Locurto, 2005). This is in contrast to other studies which successfully report using the T-maze for discrimination tests (spatial or visual) which includes learning of the win–stay strategy (Lione et al., 1999; Granholm et al., 2000; Belzung et al., 2001).

4.4.1. Memory dependency

One premise for showing the win–stay strategy would be remembering what was done last time to find food. As trials were performed on multiple days, remembering the last choice made on the day before (which would refer to the reference memory) seemed not possible for the mice, so that the first choice was always based on chance (see analysis of trials, experiment 2, Figure 5). With only two trials per day, a preference based on working memory might also have been disguised in week 1 of experiment 2. However, in week 2, there were always three trials per day. This means even if the mice had not remembered the position of the millet from the day before, after two trials of sampling, the third trial should have been based on a preference. As a result, it could have been expected that a) all third trials were made towards the millet arm, and b) the preference for millet in total was at least in 2/3 of the trials. However, this was not the case as alternation levels in the third trial were similar to the second, and portion of chosen millet arms was about 1/2.

4.4.2. Partial feeding and refilling

Another factor that might prevent the win–stay strategy could be that mice found the reward already lying in the arm, instead of receiving a reward when entering the arm (experiencing the arm as empty but then getting food). As a result, when leaving the arm after eating all the millet, they might have memorised this arm as empty.

This might correspond to the findings of Herrmann et al. (1982), who performed a three-table task with rats (without being previously food restricted): After some exploration time in the apparatus, rats received their reward on one of the three tables. If they were allowed to completely feed on the food, rats were able to learn win–shift but not win–stay. If they were only allowed to feed partially, win–stay behaviour was faster shown than win–shift (Herrmann et al., 1982). This indicates that the animals remember whether the feeding place was emptied or not, and it could explain why mice seldom returned to the arm in which they had experienced food beforehand. Thus,

one way of improving the procedure could be to allow only partial feeding in the goal arms.

Another possibility would be to 'show" the mice that the feeding place is refilled. This is inspired by conditioned place preference tests and the study of Goltseker & Barak (2018): Here, conditioned place aversion was only induced when mice were placed in an empty compartment first, and then experienced the onset of the aversive stimulus (in this case: cold water flooding). Conditioned place aversion was not induced when the mice were placed in an already flooded compartment (Goltseker & Barak, 2018). This implies that timing plays an important role for association formation.

However, experiments like the Lashley III maze (Smith et al., 2017) or the cheeseboard task (Lopez et al., 2010) work without partial feeding or the experience of refilling.

4.4.3. Other motivations

Another factor that might prevent manifestation of the win–stay strategy might be that mice had other motivations than to search for a preferred fluid or a food reward in the maze. To our knowledge, there are no studies investigating this in mice, although this is well-known for birds: As described by Dixon et al. (2013), additional motivations can influence behaviour and the results of preference tests. Here, results of the conditioned place preference test were undermined by the motivation of the birds to search for food or to stay in the more familiar compartment (the one experienced last) (Dixon et al., 2013). This could also be the case here for mice, as further discussed in Section 4.5.4.

However, it cannot be said that mice showed no preference in their behaviour at all. Instead, they showed a clear preference for the arm which they had not visited during the last trial, a behaviour known as 'spontaneous alternation'.

4.5. Influences on spontaneous alternation

Spontaneous alternation behaviour is a common phenomenon in the T-maze (Dember & Fowler, 1958; Deacon & Rawlins, 2006; Sharma et al., 2010b). Although we do not know, what the main cause of the alternation behaviour shown in our experiments is, there are many theories on the factors that influence spontaneous alternation (also reviewed in Richman et al., 1986). In the following we will shortly discuss some of them.

4.5.1. Arrangement of maze arms

In the T-maze goal arms are opposite from each other, forcing animals to make a 90° body turn, while in the Y-maze, the turns are 120°. Some studies use both mazes, assessing alternation in the T-maze, while conducting discrimination tasks with the Y-maze (Shipton et al., 2014). On the other hand, when using the Y-maze for spontaneous alternation, animals are usually placed in a start arm to freely explore the maze without interference of the experimenter or distinct trials (called 'continuous alternation', Hölter et al., 2015).

Alternation decreases when both arms lead towards the same goal (Dember & Fowler, 1958). Also, if the arms are positioned not opposite to each other but in parallel, spontaneous alternation is reduced (Novak et al., 2016a, b). Thus, the setup of the T-maze might not be ideally for preference tests.

4.5.2. Choice of cues

In mice, influence of spatial and non-spatial cues seems to differ between strains and tasks. C57BL/6J, for example, did not exceed chance level in a spatial discrimination task using extramaze cues but were slightly better in a non-spatial proprioceptive task (left vs. right turn). BALB/cByJ, on the other hand, performed well in both tasks (Crusio et al., 1990). In a different experiment, performing a spontaneous alternation task, C57BL/6J mice seemed to rely mainly on extramaze cues, and had in general higher alternation levels than, e.g., DBA/2 (Gerlai, 1998). In addition, in a more recent study with C57BL/6J × Sv129 mice, it was found that distal visual (extramaze) cues might overshadow proximal (intramaze) cues (Hébert et al., 2017).

In our experiments, we used C57BL/6J mice, and we provided several cues: In both experiments for most of the trials (except for those after the side change) the spatial intramaze cues as well as the non-spatial (proprioceptive) cues were the same. In addition, we provided an olfactory (experiment 1) and a visual (experiment 2) intramaze cue. Moreover, in experiment 2 odour trails from previous trials could have functioned as a cue, in which the maze was not disinfected between the trials. However, as the other studies suggest, all intramaze cues might have been overshadowed by extramaze cues. Although we did not artificially add extramaze cues, we did not change the environment, and therefore, extramaze cues (e.g. position and colour of the walls) could have worked also as sufficient cues. However, it is evident that

the mice did not use any of the provided cues to choose the supposedly more rewarding arm.

Instead, the cues might have influenced alternation as it is discussed that animals might be driven to explore the stimulus which is less familiar, i.e., to which they were not exposed last (Richman et al., 1986). Thus, additional motivations during the test might have masked the motivation to gain food reward.

4.5.3. Intertrial interval

In general, spontaneous alternation behaviour seems also to be intertrial interval (ITI) time (and thus, memory) dependent. However, regarding which ITIs support spontaneous alternation and which do not, the literature is mixed. Here, in experiment 2, ITI was about 19 s but never longer than 2 min, and in experiment 1, ITI lasted about 3.5 min. This fits to the description made by Deacon (2006) for mice. We can also confirm that for long ITIs alternation drops to chance level (Durantou et al., 1989; Deacon, 2006): Comparing alternation proportions of individual trials for experiment 2 revealed less alternation behaviour during the first trial of each day. Thus, the last choice of the day before (with an ITI $>$ 21 h) seems not to be relevant for the first choice, reflecting that the behaviour is based on the working memory, not the reference memory (Sharma et al., 2010b).

However, one of the problems of comparing the influence of ITIs might be that studies use different definitions what they exactly consider to be the intertrial interval. For example, Locurto (2005) regards the ITI as the time between two trials but with one trial consisting of two forced choice trials and one free choice trial, meaning the time between the forced choice and the free choice trials is not considered (Locurto, 2005).

4.5.4. Food reward and food deprivation

It is also discussed whether food reward itself influences alternation behaviour, and if so, under which circumstances. Apparently, at least in rats alternation levels are reduced with increasing food deprivation (Richman et al., 1986). This is also implemented in more recent studies with mice, which conduct discrimination tasks with food restriction but alternation tasks without (Shipton et al., 2014). Returning to the topic of the foraging strategies, this implies that the animals switch to win–stay strategy (and away from alternation) only when the motivation to gain food is high enough. In other words: Below a specific food deprivation level, the motivation to explore

what was not experienced in the preceding trial might be higher than the motivation to gain food (Richman et al., 1986). This exploration behaviour could be driven by additional needs, for example, search for shelter (Pilz et al., 2020) or an escape out of the maze (which is commonly used for the Lashley III maze).

In this context, it has also to be kept in mind that it was shown already in the 1960s that conditioned stimuli are not equally effective for all kinds of unconditioned stimuli, for example, gustatory and olfactory stimuli are more easily associated with internal discomfort than audio-visual stimuli (Garcia & Koelling, 1966). This learning phenomenon is probably caused by an evolutionary advantage of facilitated association of specific stimuli. In a similar manner, evolution might have favoured learning mechanisms which cause mice to prefer the win–shift strategy under *ad libitum* food conditions and the win–stay strategy under food restricted conditions. Thus, asking the mice to choose a food rewarded arm over an empty arm might be a completely different question under different feeding conditions.

4.5.5. Arousal

It has to be mentioned that an additional important factor for alternation seems to be fear or stress. Under the key word 'optimal arousal theory' multiple studies can be found, which investigate the effect of a mild stressor (open field test), food shock or water presence (water-escape T-maze instead of dry T-maze) on the alternating behaviour (rats: Means, 1988; Comer & Means, 1989; mice: Mitchell et al., 1984; Mitchell et al., 1985; Bats et al., 2001). In general, this theory suggests that individuals seek the optimal arousal, which is shaped in an upside-down U-curve. Thus, when an animal is not aroused it would seek something arousing, for example, a less familiar environment. When the animal is already 'too much' aroused (behind the peak of the curve), however, it would seek the less arousing stimuli, meaning a more familiar environment. This theory tries to explain why after experiencing a mild stressor, mice perseverated their choices instead of alternating (Bats et al., 2001). Mitchell et al. called it the 'punishment paradox' (Mitchell et al., 1984).

Transferring these observations to our experiments, we could conclude that the procedure before and during the T-maze was probably not stressful as our mice did not perseverate but alternate. What we observed was rather the 'alternating paradox', meaning alternating although perseverating was reinforced.

5. Conclusion

It is obvious that the T-maze as used in this setup was not suitable to investigate preference or reward-aimed learning in C57BL/6J mice. Instead, mice alternated their choices in 60–70% of the trials. Although the main reason behind this alternation behaviour remains unclear, we can at least validate the statement by Deacon & Rawlins that well habituated animals run the T-maze alternation test well without food restriction (Deacon & Rawlins, 2006). It might be possible to increase performance by imposing deprivation on the animals. However, as we were interested in preference under un-restrained conditions, we deem the T-maze as used here not suitable for our research question. Researchers interested in the T-maze as a means for preference assessment should therefore take caution when designing their tests.

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Appendix

A.1. Transponder implantation

At the age of five weeks, transponders (FDX-B transponder according to ISO 11784/85; group 1: Planet-ID, Germany; group 2: Euro I.D., Germany) were implanted under the skin in the neck of the mice. To do so, in group 1 all mice obtained an analgesic (Meloxicam) two hours before the procedure. The transponder implantation itself was performed under isoflurane anaesthesia. RFID (radio frequency identification) transponders were injected directly behind the ears subcutaneously in the neck, so that they were rostrocaudal oriented. After transponder implantation, mice were placed in a separate cage with bedding and sheets of paper, and monitored until they were fully awake again. Then they were returned to their home cage. In group 1, two mice lost their transponders after the first implantation, and for those two mice the transponder implantation was repeated at the age of 8 weeks.

For group 2, the administration time of the analgesic was altered to the evening before the procedure because we hoped to reduce transponder loss this way: By administering the Meloxicam earlier, the analgesic effect was expected to cease before the dark phase after the implantation (active phase), and mice would be more hesitant to focus on the injection side. Implantation of the transponders was performed in the same way as in group 1. In group 2, no transponder was lost.

A.2. Experiment 1

A.2.1. Tested goods

Two fluids ware compared, namely almond milk (3 g sugar per 100 ml; Mandel drink, Alpro, Düsseldorf, Germany) and apple juice (100%, 10 g sugar per 100 ml, made out of concentratel Solevita, Lidl, Kremmen, Germany).

We chose fluids because their odours can work as additional cue without previous conditioning.

During the eighth day of habituation, the two fluids were presented inside the home cage system: One of the usually two water bottles in each cage was replaced by a bottle containing one of the test fluids (500 ml). Originally, it was planned to present the bottles for a few days with randomised positions. However, after the first 24 h the bottle with the almond milk was nearly empty. As there was no leakage of the bottle, we have to assume that the mice drank all of the missing fluid. Health of the mice seemed to be unaffected but we noted excessive urination inside the home cages and the T-maze. Therefore, presentation of the two fluids was immediately stopped.

A.2.2. Habituation to the T-maze

Mice were habituated passively to the T-maze for 13 days, during which they could enter the T-maze whenever they were motivated. To do so, the tube between the two home cages was interrupted by a junction, which had a connection to the T-maze via a tube (40 mm diameter). RFID antennas were installed to receive information on the mice visiting the T-maze. Because mice were too fast for the RFID antennas, they were slowed down by two doors. After a first 15-cm-long tube followed one door, then a 40-cm-long tube, a second door, and a 6 cm long tube leading into the T-maze. Each door was directed by an Arduino micro-controller and surrounded by a light barrier (outer side, leading to the home cage or the maze) and an RFID antenna (inner side, leading to next door). Doors opened for 5 s when the transponder of a mouse was detected by the RFID antenna or a mouse interrupted the light barrier. In addition, with the help of two RFID readers also the direction of movement was reconstructable. In this manner, mice could move freely in and out of the T-maze, while their individual stay time was monitored via the RFID readers and stored onto an SD card by the Arduino.

Every day (except for the weekends), the maze was detached from the cage system, washed with water and then cleaned with 70% ethanol to "reset" odour conditions. After the 13th day of habituation to the T-maze, mice cages were transported from their husbandry room to the experimental room. Here, mice had one day to habituate to the new environment before the start of the experiment. Note that this was also already an extended habituation time as the common T-maze protocols recommend from 10 min up to over 30 min for habituation to the test room.

A.2.3. Preference test

In experiment 1, the T-maze test (not habituation) took place in an experimental room, and an additional light was placed above the T-maze. Light conditions for the left arm were 171 lux, 201 lux for the right arm, 350 lux for the start/central arm, and 264 lux for the spot between the choice arms.

T-maze testing was conducted on two consecutive days. Mice were habituated to the test room before (see above) and performed five test trials per day, with an additional habituation trial beforehand on trial day 1. Test duration was approximately 40 min per mouse, lasting about 9 h per day for the whole group.

The order of tested mice was randomised for both trial days. In addition, presentation side of fluids was randomised for the mice so that for half of the mice almond milk was presented in the left arm and apple juice in the right, and for half of the mice the other way round. For trial day one, presentation side of the fluids did not change between trials. On trial day two, two trials were performed with fluids presented in the same arm as the day before, while in trials three to five presentation sides were reversed to control for a potential side preference.

Before each mouse, 1 ml of the test fluids was administered on a cellulose sheet and stuck to the walls at the end of a choice arm as an odour stimulus. In addition, a fluid droplet of 20 μl was placed on the floor of the respective arm as a reward.

All trials were recorded with a video camera (C390e, Logitech, Lausanne, Switzerland) and iSpy 64 (version 7.0.3.0). Before each mouse, maze and start cage were cleaned with 70% ethanol and bedding in the start cage was replaced by new bedding. Then, the additional light was switched on, and the automated system controlling the door was started.

The first trial of the first day was the habituation trial: A mouse was taken out of the home cage by tube handling and placed into the start cage. The mouse had now 5 min to initiate a trial by going through the tube into the T-maze. If the mouse did not enter the T-maze during this time, it was lifted by the handling tube, allowing the mouse only to leave the tube into the tube leading to the maze by blocking the other tube entry. It was then waited until the mouse had entered both arms of the maze (crossed the virtual line with the whole body but not yet with its tail). After additional 30 s, the mouse was returned to the start cage with the help of the handling tube. Before the start of the next trial, the light and the automated system controlling the door

were switched off; this prevented the mice from entering the maze, while the floor was cleaned with 70% ethanol.

After drying the maze and replacing the droplet on the floor, light and automated system were turned on again and the mouse could re-enter the maze. The mouse had 3 min to do so before it was guided by tube handling into the maze. During the test trials, it was waited until a mouse had entered one of the arms (crossed the virtual line with the whole body but not yet with its tail), before it was returned to the start cage with the handling tube. After the last trial, the mouse was returned to its home cage. Between mice, the whole maze including the walls were cleaned with 70% ethanol.

On day two, there was no habituation trial. In addition, a side switch of the fluids took place after trial two; therefore, between trials not only the floor but the complete maze was cleaned with 70% ethanol. Also not only the droplet on the floor but also the cellulose sheet at the end of each arm was renewed.

For video recording, a webcam (C390e, Logitech) was mounted above the maze on a metal beam construction. The connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

A.2.4. Additional notes on the analysis

During passive T-maze habituation, the two Arduinos automatically saved all RFID detections and additional events (door opened/closed or light barrier interrupted) onto an SD card. Each record included a time stamp (hours, minutes and seconds since start of the Arduino, provided by a real-time clock), milliseconds passed since start of the Arduino, type of event, and the unique RFID transponder number. With the help of R studio (Version 1.1.383), the data sets recorded by the two Arduinos were then tagged with a number for each Arduino and merged. To analyse mouse visits to the T-maze, position changes were extracted (whenever a mouse was detected first by one reader and then by the other), excluding all additional events and RFID detection duplicates (if the same mouse was detected multiple times). Using the time stamps it could then be analysed how long each mouse stayed inside the maze.

In total, 143 trials were analysed, containing 13 habituation trials. Of the 130 preference test trials, five could not be assessed due to camera problems (camera recording stopped unnoticed for one mouse), leaving 125 trials.

Originally, it was planned also to take into account whether the mouse had consumed the reward droplet on the floor or not. However, for apple juice this was not possible: Because of its transparency (in comparison to almond milk), the wet floor left behind looked too similar to the apple juice droplet itself. Therefore, we instead assessed whether the mouse spent some time (>1 s) in which it its behaviour suggested licking or intensely sniffing the droplet.

A.3. Experiment 2

A.3.1. Tested goods

In pre-tests we observed that millet seems to be a better working reward than almond milk: While mice showed no interest in almond milk when offered in a separate cage filled with home cage bedding, mice immediately fed on millet grains. In addition, after a few sessions of habituation, mice also fed on millet in an empty type-III macrolon cage within one minute after entering the cage. We therefore expected mice to do so in the T-maze after the habituation trials as well.

As the aim of this test was mainly to establish a working protocol for the preference test, we decided against comparison of millet and another reward. Instead, we tested millet against "nothing". In this manner, the test design also resembled a simple learning test. To control for the visual (or exploratory) effect, we provided millet mixed with a specific bedding material in one arm and bedding material (without millet) in the other arm. As bedding material we used the same bedding material as in the home cage (Lignocel FS14, spruce/fir, 2.5–4 mm, JRS, J. Rettenmaier & Söhne, Rosenberg, Germany) as this was a definitely neutral (familiar) cue. Mice were already habituated to the millet in the course of other experiments (including the pre-tests).

A.3.2. Habituation to the T-maze

While in the last experiment, mice were habituated passively to the T-maze, in this experiment mice were manually habituated to the maze: Mice were placed individually into the maze setup for a short time period on five consecutive days.

This time, the T-maze was installed in the same room in which the mice were usually kept, so no transportation was necessary. In this husbandry room, no other groups of mice were kept during this experiment. Habituation trials were performed between 08:00 and 11:00 in the morning. After

preparation of the setup, the filter top of the home cage system was removed and mice had 10 min to habituate to the illumination change.

For habituation to the maze, mice were taken individually and in a randomized order out of the cage and placed into a start cage, which contained only bedding material and was connected to the T-maze via a tube with an automated door (similar to the setup in the last experiment). Mice had already experiences with automated doors, thus, no habituation to the door was needed. Starting at the moment the mice entered the T-maze, they had 3 min to explore the whole maze. A return to the start cage was blocked by the automated door. If a mouse did not enter the maze within 3 min, it was retrieved by tube handling and held in front of the connection tube with the end to the start cage closed. If it then again did not enter the T-maze within the next 7 min, it was placed directly inside the maze. After 3 min of T-maze exploration, mice were returned to their home cage.

During habituation, maze arms were empty and without visual cues. The maze was not disinfected between mice but it was cleaned (using paper and water) whenever defecation or urination were observed. The exploration behaviour inside the maze was recorded by a video camera (C390e, Logitech) mounted above the maze on a metal beam construction.

It has to be noted that one mouse of the twelve received only four days of habituation: On day one it showed unusual behaviour which might have been correlated with health issues, and therefore, was excluded. As its behaviour returned to normal within two hours (and the maze test does not cause any severity), and the veterinarian had no objection, we decided to start habituation with this mouse on day two. In the course of the following three weeks (one habituation week and two test weeks) there was no unusual behaviour observed.

A.3.3. Preference test

In experiment 2, the test took place in the husbandry room and no additional light was added. This led to illumination levels of 18 lux minimum at the end of both arms and 50 lux maximum at the start arm. In experiment 2, choice arms of the maze were covered with patterns.

While in the last experiment preference tests were conducted block-wise (two days with five trials each), in experiment 2 preference tests were conducted on five consecutive days, with only two trials (week 1) or three trials (week 2) per day. This test design should enable an improving habituation to the test with every experimental day. It also allowed flexible addition of test

days if necessary (e.g., if mice had not fed on the millet due to still insufficient habituation).

Between habituation trials and test trials, there was a two day break. Just like the habituation, the preference test took place in the same room in which the mice were usually kept. Tests were performed between 08:00 and 11:00 in the morning, taking approximately 6 (week 1) to 8 (week 2) min per mouse. After preparation of the setup (installing laptop and cameras), the filter top of the home cage system was removed and mice had 10 min to habituate to the illumination change.

For the preference test trials, in one of the maze arms 0.05 g millet mixed with bedding material and in the other maze arm a similar amount of bedding material was placed. Walls of both arms were decorated with patterns: either white dots on black ground or white and black stripes. (Patterns are designed according to the description of Cunningham et al. (2006), except that the colour was inverted.) Combination of pattern, treatment and side were randomized across mice. In week 1, presentation side of the millet was kept the same for six trials, and then the side was switched (similar to experiment 1). In week 2, no side change was conducted.

Each experimental day, following a randomized order a mouse was taken out of the cage and placed individually into a start cage. The start cage contained only bedding material and was connected to the T-maze via a tube with an automated door. The mouse now had 3 min to initiate a trial by entering the T-maze. If a mouse had not entered the maze within 3 min, it would have been retrieved by tube handling and held in front of the connection tube with the end to the start cage blocked. Entering the maze, the mouse had the choice between the rewarded (millet and bedding material) and the unrewarded arm (bedding material). As soon as the mouse crossed a virtual line which was 11 cm into the arm, this was considered a choice. The mouse was given time to feed on the millet, while leaving the arm was prevented by the experimenter's hand holding the handling tube. As soon as the mouse entered the tube, it was returned to the start cage and the procedure was repeated. After the second trial (or third trial, week 2) the mouse was returned to the home cage.

Between the two trials of the same mouse, the maze was not cleaned. Between different mice, the maze was not disinfected but it was cleaned (using paper and water) whenever defecation or urination were observed. Both trials were recorded by a video camera (Logitech C390e, Switzerland)

mounted above the maze on a metal beam construction. As in experiment 1, the connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

A.3.4. Additional notes on the analysis

In total, 300 trials were analysed (10 per mouse in week 1, 15 in week 2). Of the 300 preference test trials, one missed the time point of the mouse entering the start cage because the video recording started too late.

Originally, it was planned to also take into account how long the mouse spent eating on the millet. However, as in all but two cases the millet was eaten completely (at least as far as visible) and mice had very different feeding speed, we decided against it.

A.4. Data set

The data sets of both experiments containing the mice's choices for all trials can be found here: https://doi.org/10.5281/zenodo.4621082.