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A new behavioural apparatus to reduce animal numbers in multiple types of spontaneous object recognition paradigms in rats

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HIGHLIGHTS

- We explored recognition memory with the new continual trials apparatus.
- Rats performed significantly above chance levels in recognition tasks.
- Results were comparable to standard tasks and maintained statistical power.
- This involved less than a third of the number of animals typically used.

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ABSTRACT

Standard object recognition procedures assess animals' memory through their spontaneous exploration of novel objects or novel configurations of objects with other aspects of their environment. Such tasks are widely used in memory research, but also in pharmaceutical companies screening new drug treatments. However, behaviour in these tasks may be driven by influences other than novelty such as stress from handling which can subsequently influence performance. This extra-experimental variance means that large numbers of animals are required to maintain power. In addition, accumulation of data is time consuming as animals typically perform only one trial per day. The present study aimed to explore how effectively recognition memory could be tested with a new continual trials apparatus which allows for multiple trials within a session and reduced handling stress through combining features of delayed nonmatching-to-sample and spontaneous object recognition tasks. In this apparatus Lister hooded rats displayed performance significantly above chance levels in object recognition tasks (Experiments 1 and 2) and in tasks of object-location (Experiment 3) and object-in-context memory (Experiment 4) with data from only five animals or fewer per experimental group. The findings indicated that the results were comparable to those of previous reports in the literature and maintained statistical power whilst using less than a third of the number of animals typically used in spontaneous recognition paradigms. Overall, the results highlight the potential benefit of the continual trials apparatus to reduce the number of animals used in recognition memory tasks.

1. Introduction

Delayed nonmatch to sample (DNMS) has been widely used as a test of recognition memory in both monkeys (e.g. Eacott et al., 1994; Mishkin and Delacour, 1975) and humans (e.g. Holdstock et al., 2000) in order to understand the neural basis of memory. Whilst versions of DNMS tasks have been used with rodents, difficulties concerning training and performance levels are of concern in these paradigms (Aggleton, 1985; Mumby et al., 1990; Prusky et al., 2004; Steckler et al., 1998). Consequently alternative ways to investigate recognition memory in rodents have been developed.

Spontaneous object recognition tasks capitalise on the animals' innate preference for novelty (Ennaceur and Delacour, 1988) as a measure of recognition: memory of familiar stimuli is exhibited through greater exploration of novel over familiar stimuli at test (Ennaceur, 2010). The animals are able to explore the physical objects meaning that behaviour can be driven by not only visual information but also olfactory and tactile information (Clark and Squire, 2010). The relative simplicity of the spontaneous object recognition task has allowed for widespread use to test recognition memory in rodents: for example there are 534 peer-reviewed papers listed in Web of Science from the past 5 years drawn from 31 subject areas (source Web of Science, April, 2012) which include the terms "spontaneous object recognition" or "novel object

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recognition" with the terms rat or mouse. From this we took a sample of 10 of these papers and calculated that on average, each of these studies involved 80 animals divided into, on average, experimental groups of 10 to often compare different drug effects and different time points of sampling and testing. Subsequently we estimate that approximately 43,000 animals have been used in this type of task and its variants in the past 5 years, although this may be conservative as the estimate does not include animals from non-published studies nor those used in these tasks by pharmaceutical industries.

Evidence suggests that the object recognition task is indeed more sensitive to impairment of recognition memory than DNMS (Clark and Squire, 2010; Nemanic et al., 2004; Pascalis et al., 2004) and variants of the spontaneous object recognition task have been used to provide evidence for functional dissociations within recognition memory with tasks including memory for a novel combination of object and background context or object and location (e.g. Eacott and Norman, 2004; Easton and Eacott, 2008; Langston and Wood, 2010; Norman and Eacott, 2005). Such tasks are also widely used as part of a battery of tests in accordance with the ICH S7A Guideline for Safety Pharmacology Studies to detect potential amnesic properties of new drugs (Bertaina-Anglade et al., 2006).

A number of advantages account for why the spontaneous object recognition task has become so widely used across disciplines to test for recognition memory in favour over DNMS tasks. The most important reasons include the simplicity of administering the task and the consistency of results across species (Clark and Martin, 2005). However, a number of issues are also related to administering spontaneous object recognition tasks. Often these tasks result in considerable variance as the animals' memory is assessed merely through its spontaneous exploration of novel objects. As there is no other form of motivation driving behaviour in these tasks, the animals' behaviour can also be driven by other influences, such as external stimuli or initial mis-match of objects in terms of their inherent interest for animals, potentially leading, for example, to familiar but salient stimuli being more attractive for exploration than novel but relatively unsalient objects. Behaviour can be further influenced through stress induced by external stimuli which can impair performance on memory tasks (Yuan et al., 2009). In addition, stress can make animals neophobic and as such small amounts of stress through handling (which may be considerable in these tasks as animals are repeatedly taken in and out of the apparatus) may drive behaviour away from the novel stimulus, reducing the apparent memory, and masking true recognition abilities. Indeed, recent evidence suggests that particular animal handling procedures can induce aversion and anxiety which can subsequently influence performance in behavioural experiments (Hurst and West, 2010).

Substantial changes to the spontaneous object recognition paradigm have been explored, for instance Furtak et al. (2009) proposed a novel floor projection maze that allows for visual stimuli to be presented on the floor of the apparatus as evidence suggests that horizontal visual information modulates hippocampal place fields more so than vertical visual information (Jeffery and Anderson, 2003). Using three-dimensional junk objects in recognition tasks can naturally lead to problems with object affordances (Chemero and Heyser, 2005; Ennaceur, 2010) which relates to the properties of an object and the ability of an animal to interact with it. Object preference can unintentionally be induced when pairing objects that vary in terms of their texture, shape and size. The use of projected two-dimensional visual stimuli provides a potentially useful solution to this issue which could lead to more reliable findings in recognition tasks.

Albasser et al. (2010) further addressed methodological issues relating to the spontaneous object recognition paradigm. They presented a paradigm which combined features of spontaneous object recognition tasks with DNMS tasks by testing object recognition with a 'Bow-tie maze'. The Bow-tie maze task consists of two compartments which can contain objects. The rat is placed in one compartment of the maze with one object (A). The animal then shuttles to the opposite compartment which contains two objects (A and B) of which one is familiar (A) and one is novel (B). The animal then shuttles back to the first compartment which now contains object B (now familiar) and object C (novel). This sequence continues for the number of trials in that particular session. Each time a rodent shuttles between the two compartments it completes a trial. A trial consists of a duplicate of the novel object from the previous trial (now a familiar object) presented alongside a new novel object.

This new design has the benefits of a spontaneous object recognition task through using preferential exploration of novelty as a measure of recognition, with the advantages of being able to carry out multiple trials per session, resulting in faster accumulation of data. Compared to a standard task of spontaneous object recognition, there is also reduced variance perhaps resulting from both the increased number of trials run per animal and to reduced handling which will reduce stress (Hurst and West, 2010). Thus task performance in this version of the task is a more reliable indicator of recognition abilities.

Although the Bow-tie maze task provides a useful improvement on the spontaneous recognition paradigm, it is not directly comparable with other spontaneous recognition paradigms in the literature, making it hard to compare and interpret data across studies. As previously mentioned, variants of the spontaneous object recognition task have provided a useful insight into recognition memory through developing tasks that combine recognition of objects with their spatial location or the context in which they were presented (e.g. Eacott and Norman, 2004; Easton and Eacott, 2008; Langston and Wood, 2010; Norman and Eacott, 2005). Such tasks are not currently possible in the Bow-tie maze. For instance, developing spatial tasks would be problematic as animals are required to shuttle backwards and forwards between compartments making it difficult to understand what the appropriate spatial location might be on a trial which is essentially a mirror-reflection of the sample event. It would be difficult to discriminate between allocentric and egocentric strategies and may not be comparable to a task in which an animal always experiences objects in the same location in space.

The present study therefore aims to present a new paradigm that adopts the basic concept used for the design of the Bow-tie maze through combining features of the spontaneous object recognition task with features of the DNMS task in a way that allows for further tasks of recognition memory to be tested. Within the new continual trials apparatus (Fig. 1) the paradigm allows for multiple trials per session and measures recognition through preferential exploration of novel stimuli over familiar stimuli. In contrast to the Bow-tie maze, one compartment consists of a holding area, where the animal is initially placed and where it remains before and after each trial, whilst the other compartment consists of the object area where the testing takes place. The object area can be changed to reveal a new context whilst the animal is secure in the holding area. Overall, the apparatus is designed for four contexts making it ideal for testing recognition memory that involves context change within the procedure whilst also being able to conduct multiple trials per session.

The purpose of the current study was to explore how effectively recognition memory could be tested in the new continual trials apparatus with a series of experiments. Experiments 1 and 2 were designed as versions of the spontaneous object recognition task. Experiment 1 was a replication of the task procedure used by Albasser et al. (2010) but with the addition of the animal returning to the holding area in between trials rather than completing a trial every time it shuttles in to the next area. Experiment 2 was

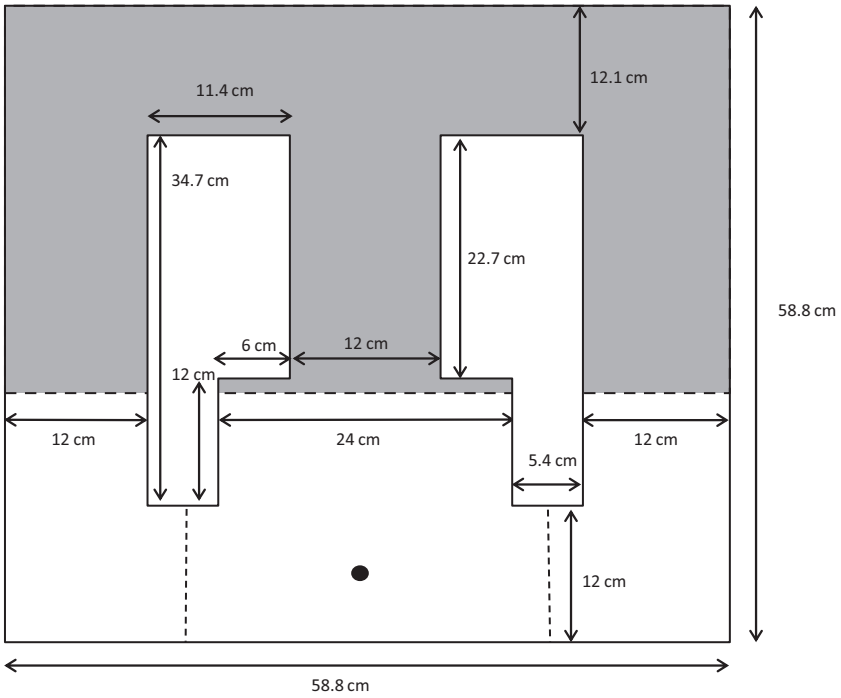

Fig. 1. The shape and dimensions (in cm) of the continual trials apparatus from the view above (to scale). The grey area represents the object area which is rotatable to reveal different contexts; four in total. The white area represents the holding area, which is stable, with a black circle to represent the food well. The dotted lines represent the doors. The rectangular shapes in the apparatus create the central arm and the two outer arms of maze.

similar but included a sample phase prior to each test phase to be more comparable with the standard recognition memory task. In these experiments only one context was used for all trials because it was essential to first determine whether a simple recognition paradigm could be applied successfully to the continual trials apparatus before continuing on to more complex tasks. Experiments 3 and 4 examined performance on more complex recognition tasks of object-location (what-where) and object-in-context (what-which), respectively (Dix and Aggleton, 1999; Eacott and Norman, 2004; Langston and Wood, 2010; Norman and Eacott, 2005).

We propose that as a result of ability to run a great number of trials efficiently and less handling being required with the new apparatus fewer animals will be needed in each experiment in order to obtain measures of exploration and statistical power similar to, or greater than, previous methods employed by researchers.

2. Materials and methods

2.1. Subjects

Twelve naïve male Lister hooded rats supplied by Harlan (Bicester, UK) were used in this series of experiments. Six animals were housed in pairs and six animals were housed in groups of three, all in diurnal conditions (12-h light–dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study. All animals were food deprived to 85% of the free-feeding body weight of age matched controls throughout testing.

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act (1986) and associated guidelines.

2.2. Apparatus

The animals were tested in a square shaped apparatus which comprised of an E-shaped object area, which could be adapted for different contexts, abutting an E-shaped holding area, which was stable (Fig. 1). The apparatus was 59 cm long and 59 cm wide. Opaque guillotine doors divided the two areas (outer arm doors: 12 cm; central arm door: 24 cm) which could be opened and closed by the experimenter. During sample and test phases, objects were placed in the top left and top right-hand corners of the object area of the maze (Fig. 2) approximately 2 cm away from the two wall to allow the animals to get its head around the object and explore it fully.

The four contexts that constitute the object area are as follows: context 1, a grey lego™ surface; context 2, a grey smooth surface with a white polka dot pattern; context 3, black and white horizontal stripes with a hatched wire surface; context 4, black and white vertical stripes with a hatched wire surface.

2.3. Objects

Each experiment used various junk objects of different sizes, shape, colour, and texture. Identical duplicate objects were used within each trial and each animal did not re-encounter the same object within an experiment or on any subsequent experiment.

2.4. Pretraining

All animals were initially given two sessions of handling by the experimenter and two sessions of habituation to the testing room in which they remained in their home cage with their cage mates for a period of 10 min per session in order to acclimatise to the room. The light in the test room was produced solely by a 20 W bulb within a desk lamp positioned to shine on the wall in order to produce a low level diffuse light with no shadows across the apparatus. Constant white noise was played to mask any noises from outside the room. These were the conditions for all subsequent habituation and testing sessions.

Pretraining involved the completion of five phases aimed at habituating the animals to the environment and procedure, which

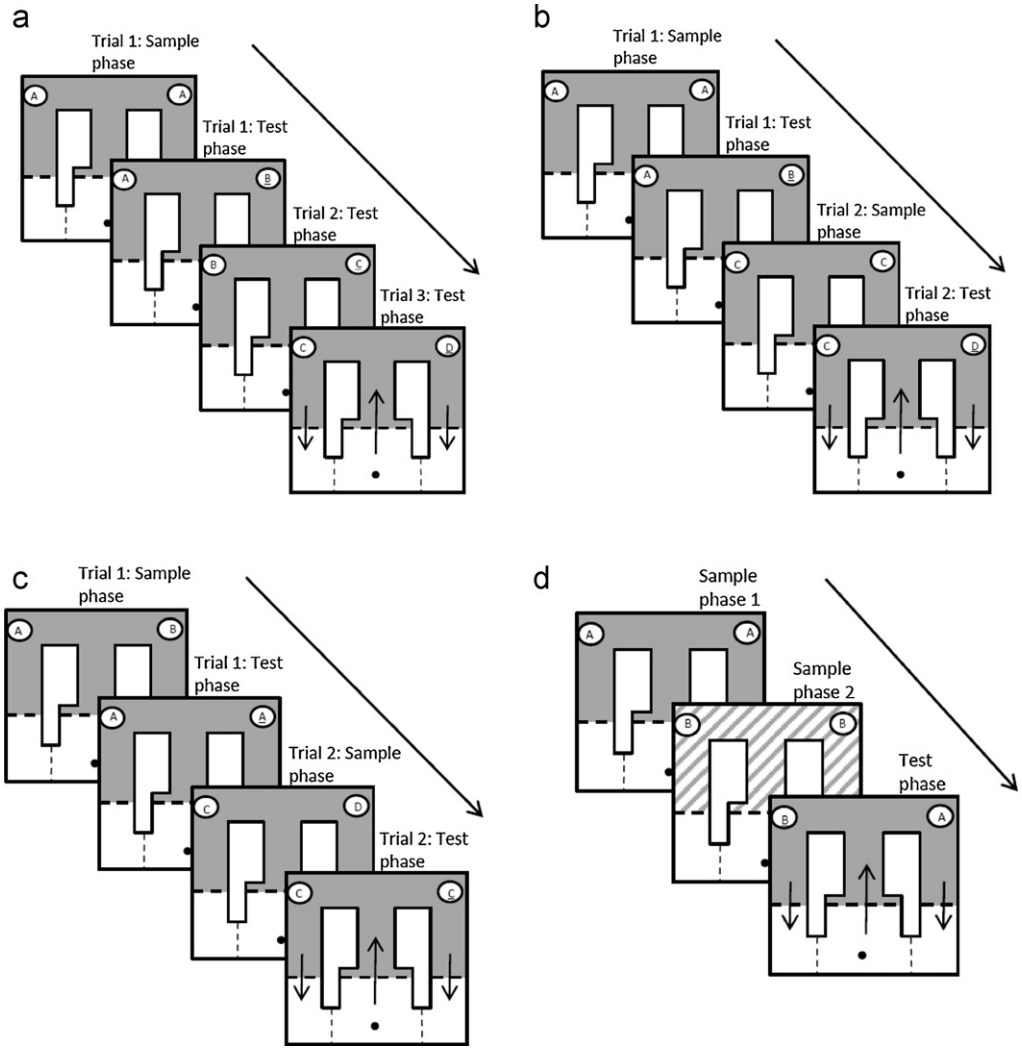

Fig. 2. An illustration of the test procedures for Experiments 1–4 with examples of the order of object presentation. The arrows indicate the direction of the rats' movement from the holding area to the object area via the central arm door, and then, 2 min later, from the object area to the holding area via one of the outer arm doors. The novel objects are represented by the underscored letters.

lasted approximately 5 days. Phase 1 involved placing the rats into the apparatus in pairs or threes (depending on how they were housed) for a period of 30 min, allowing free exploration. For Phase 2, the animals were placed into the apparatus singly for 20 min again for free exploration. For Phase 3, this was repeated but for only 10 min. Phase 4 was aimed at training the animals to shuttle between the two compartments: the holding area and the object area. This phase consisted of three sessions and involved placing dustless precision pellets (20 mg, Purified Diet; BioServ, Frenchtown, NJ, USA) on the floor of the apparatus and using the doors to control the animals' movement between the areas. The food was replenished after the completion of each shuttle. Finally, Phase 5 consisted of the introduction of objects into the apparatus. The animals shuttled into the object area and were exposed for 3 min to two objects which concealed two food pellets per object. Then the doors on the outer arms of the apparatus were opened and the animals shuttled through to the holding area which also contained two food pellets. Once the objects had been changed the central door then opened and the animals shuttled back into the object area. This was done for a total of four different pairs of objects (not re-used in the experiments proper) with pellets available at the object location and back in the holding area once the doors on the outer arms had opened. Pretraining only involved the use of context 1 within the apparatus. Further habituation occurred for animals involved in later experiments that involved context change.

2.5. Behavioural analysis

Exploration of objects was defined as when the nose of the animal was <1 cm from the object or if the object was touched with the animal's nose or paws and where the animal's nose was directed within 45° of the object. Actions such as sitting or climbing on the object were not counted as exploration. Duration of exploration was measured off-line by use of a computerised stop-watch mechanism whilst exploration was observed on a DVD recording. D2 scores were used as a measure of discrimination (Ennaceur and Delacour, 1988) by calculating the difference in exploration time (exploration time for the novel object minus the exploration time for the familiar object) divided by the total exploration time. This was done for each trial resulting in mean D2 scores for each animal which were then used in the data analysis. Cumulative D2 scores were calculated as a 'running total' of the D2 ratio recalculated after each trial within a session and used to illustrate performance over a session (Albasser et al., 2010). The D2 index ranged from −1 to +1 with −1 representing total exploration of the familiar object, +1 representing total exploration of the novel object, and 0 being

indicative of no object preference. Cumulative total exploration was calculated as the sum of the total exploration across the total number of trials.

3. Experiment 1: spontaneous object recognition

3.1. Subjects

Six Lister hooded rats supplied by Harlan UK housed in pairs in diurnal conditions (12-h light–dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study. All animals were food deprived to 85% of the free-feeding body weight of age matched controls throughout testing. At the time of testing, the animals were 4 months old and weighed from 430 to 520 g.

3.2. Test protocol

Each of the six rats were given a single testing session of 30 trials in which the animals were exposed to a novel object and a familiar object on each trial (see Fig. 2a). At the start of each session, the animal was placed in the holding area initially, with the central door opening immediately so they could move through to the object area. The experiment began with an initial sample phase where the animal was exposed to two identical copies of the same object which then acted as the familiar object for the first test trial. Thereafter all runs were test trials. Identical duplicate objects were used for when an object featured in a consecutive trial.

For the initial sample phase, the animal spent 2 min exploring the objects (two copies of object A) in the object area. After 2 min the doors on the outer arms of the apparatus opened and the animal shuttled through to the holding area which contained two food pellets in a central food well. After 1 min the central door opened to allow the animal back into the object area which contained a duplicate copy of the now familiar object A and a novel object B (trial 1). The animal explored these objects for a period of 2 min after which the doors on the outer arms of the apparatus were opened and the animal could then again shuttle through to the holding area. The central door was then opened for trial 2 allowing the animal back into the object area which then contained object B (familiar) and object C (novel). This procedure then continued for a total of 30 trials. Only context 1 was used in this experiment.

Both the novel and familiar objects on each trial were baited with two food pellets each, acting to encourage the animal to explore both objects so that differential exploration could be used as a behavioural measure without compromising validity (Albasser et al., 2010). These food pellets did not differentially reward choices as both the familiar and novel objects were baited. Rather the baiting served to maintain active exploration of the objects over the course of the entire test session. This procedure was also applied to subsequent experiments where all objects (those on both sample and test phases) were baited.

The location of the novel object was counterbalanced to help minimise any bias for left or right exploration within each testing session and also between animals. Objects were also counterbalanced between animals for which was novel and which was familiar in order to minimise bias for a particular object. This was done for all subsequent experiments.

The criterion for ending a trial was if the animal failed to shuttle to the next area of the apparatus after a period of 3 min. This would subsequently cease the testing session and the data for that animals testing session would not be included in the data analysis for that particular experiment.

3.3. Results

One animal was not included in the data analysis for Experiment 1 as shuttling ceased before 30 trials had been completed so only the remaining five animals were included.

To determine whether the remaining animals performed above chance, a one-sample *t*-test (two-tailed) was used to compare the mean D2 scores against zero. The results showed that the rats significantly explored the novel objects more than the familiar objects (mean D2 = 0.4; $t(4) = 9.822$, $p = 0.001$) showing clear discrimination of the novel from the familiar stimuli. Fig. 3a and b illustrates the cumulative values for both discrimination and exploration measures, respectively.

In order to see whether performance changed over the course of a testing session, the D2 scores for each animal were segregated into five blocks, each of six trials. For each animal, a mean D2 was calculated for each block derived from their individual D2 scores within that block. Using a repeated measures ANOVA an effect of block was found ($F(4, 16) = 6.635$, $p = 0.002$). A pairwise comparison revealed the significant effect to lie between trial block 2 and trial block 3 ($p = 0.043$), with performance declining for block 3 before improving in the final block.

Experiment 1 consisted of 30 trials in which there were two potential sources of novelty at test; object novelty (which occurs on every test phase) and familiar object location novelty (which arises when the previously novel object becomes the familiar object on the current trial but changes location due to counterbalancing). Thus, on half of the trials both of the presented objects have some form of novelty which should drive greater overall exploration but could diminish D2 measures of object recognition. However, no significant difference was found on measures of discrimination or exploration between trials with static familiar objects and trials with displaced familiar objects using paired samples *t*-tests (mean D2: $t(4) = 2.052$, $p = 0.109$; mean total exploration time: $t(4) = -1.202$, $p = 0.296$). Despite this, it is evident that mean total exploration is slightly greater for the trials where familiar object location novelty arises (static familiar object trials mean total exploration = 27 s; displaced familiar object trials mean total exploration = 30 s), although greater mean D2 scores were shown in trials where familiar object location was static (static familiar object trials mean D2 = 0.5; displaced familiar object trials mean D2 = 0.4).

A post hoc power analysis was conducted with the program G*Power 3 (Erdfelder et al., 1996; Faul et al., 2007) in order to obtain the statistical power of Experiment 1. Comparisons were made to the statistical power of a previous study which employed the spontaneous object recognition paradigm in a comparable task (Norman and Eacott, 2005) with only one trial carried out per session, a total of two sessions and more than double the number of animals included than the current experiment.

The effect size in Experiment 1 was 4.39 (i.e. a medium effect according to the effect size conventions proposed by Cohen, 1977). The power to detect an effect of this size was determined to be 0.99 with a sample size of five subjects. In comparison, the spontaneous object recognition task carried out in the Norman and Eacott study yielded an effect size of 2.38 with caculated power of 0.99 from a sample size of 11 subjects, thus demonstrating that in the current study the spontaneous object recognition task had a statistical power comparable to a previous study but from a smaller sample.

3.4. Discussion

The current experiment was a replication of the task procedure used by Albasser et al. (2010) with the addition of the animal returning to the holding area between trials rather than completing a trial every time it shuttles into the next area. As in Albasser et al. (2010)'s

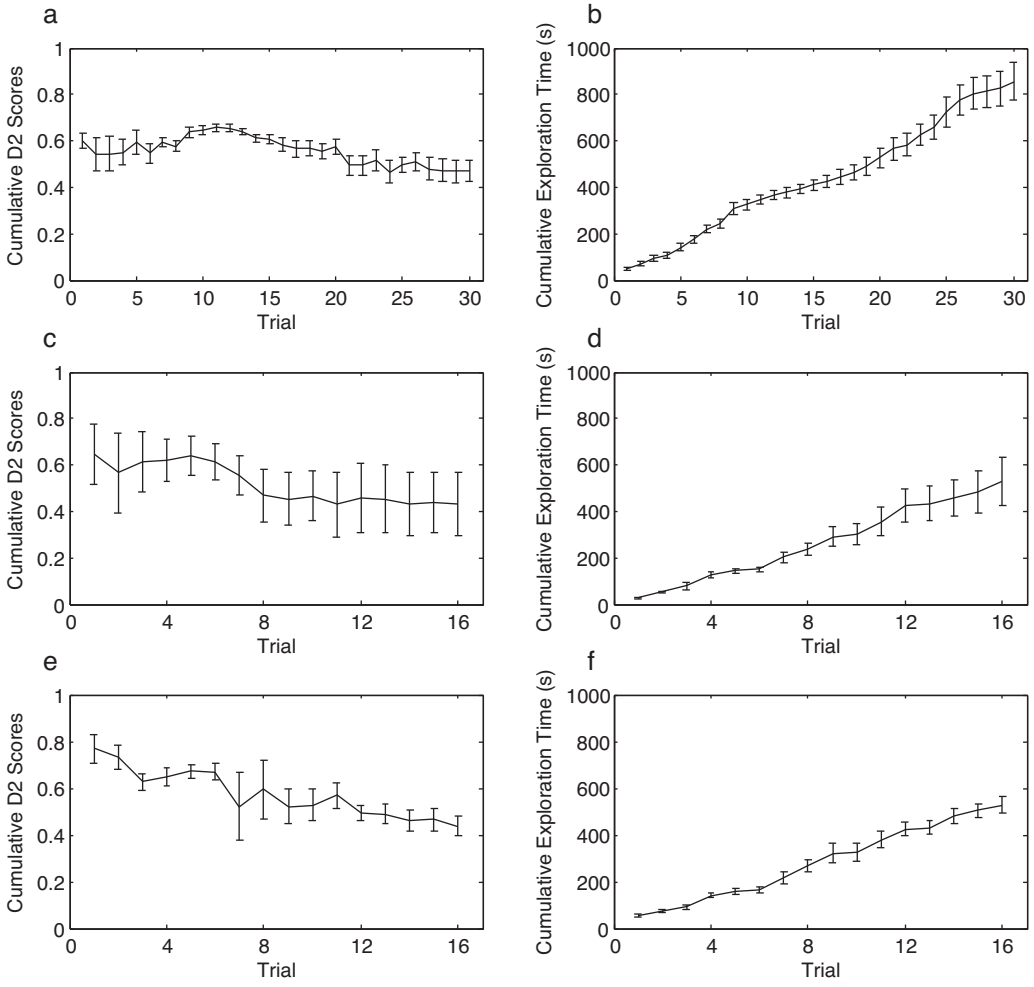

Fig. 3. Graphs from Experiments 1 and 2 depicting animal performance. Vertical bars show the standard error of the mean. (a) Cumulative D2 scores for Experiment 1 across 30 trials. (b) Cumulative exploration time for Experiment 1. (c) Cumulative D2 scores for Experiment 2 (group 1) across 16 trials. (d) Cumulative exploration time for Experiment 2 (group 1). (e) Cumulative D2 scores for Experiment 2 (group 2) across 16 trials. (f) Cumulative exploration time for Experiment 2 (group 2). Cumulative D2 scores were calculated as a 'running total' of the D2 ratio recalculated after each trial within a session. Cumulative exploration was calculated as the sum of the total exploration across the total number of trials.

study, reliable levels of object recognition were found which were comparable to previous studies that have employed the spontaneous object recogintion task (e.g. Dix and Aggleton, 1999; Eacott and Norman, 2004; Ennaceur and Delacour, 1988). It is evident that throughout the 30 trials the animals continue to explore the objects as the cumulative exploration times consistently increased. There was the possibility that the presentation of multiple stimuli throughout the session could result in a build up of interference which could diminish discrimination ratios, particularly for later trials. Despite results suggesting that performance declined slightly (but significantly), performance returned to a high level for the trials grouped in block 5 (trials 19–24) suggesting that this may have been a chance effect. Therefore, overall there is no clearevidence that performance considerably changes across the course of a testing session.

Although Experiment 1 successfully demonstrated recognition memory, the design still has some drawbacks. It was recognised that, as in Albasser et al. (2010), some trials involved the familiar object appearing in a novel location whilst on others it was seen in the same location as previously. Whilst this effect did not significantly affect recognition as measured by D2 scores, there was a non-significant tendency for trials in which the familiar stimuli remained static to show better D2 scores than those in which the familiar stumilus moved locations and so it has the potential to add noise to data. Moreover, the design does not allow direct comparison with spontaneous recogniton tasks in the literature which typically have a sample phase prior to each test phase (e.g. Norman and Eacott, 2005). Thus, Experiment 2 was designed as a spontaneous object recognition task with a sample phase prior to each test phase on each trial to be more comparable with previous spontaneous object recognition tasks in the literature. Two groups were tested; one that had performed in Experiment 1 and thus had experience in a spontaneous object recogntion task and a second group that was naïve.

4. Experiment 2: sample-test object recognition

4.1. Subjects

Group 1: Six Lister hooded rats used in Experiment 1 were again used in this experiment. Housing conditions were identical to Experiment 1.

Group 2: A further six naïve Lister hooded rats also supplied by Harlan were used in this experiment in order to assess the effects of previous testing history on performance. These six animals were housed in groups of three in conditions identical to Experiment 1. At

the time of testing, these animals were 2 months old and weighed from 240 to 270 g.

4.2. Test protocol

Each of the 12 rats were given a single testing session of 16 trials in which the animals were exposed to a novel object and a familiar object on each trial. The test protocol was identical to that used in Experiment 1 with the slight difference that a sample phase occurred prior to every trial where the animal was exposed to two identical copies of the same object which then acted as the familiar object for the test trial (see Fig. 2b). As with the previous experiment only context 1 was used. The location of the novel object was counterbalanced across trials to help minimise any bias for left or right exploration within each testing session and also between animals. Objects were also counterbalanced between animals for which was novel and which was familiar in order to minimise bias for a particular object.

4.3. Results

One animal from group 1 was not included in the data analysis as shuttling ceased before 16 trials had been completed. This was the same animal that failed to shuttle for the duration of Experiment 1 thus the results of the remaining five animals from group 1 were analysed. Two animals from group 2 were not included in the data analysis because although they successfully completed all the trials within the testing session, technical issues with recording meant that their data was lost. Thus, the results from four animals in group 2 were analysed.

To determine whether the animals performed above chance, one-sample *t*-tests (two-tailed) were used to compare the mean D2 scores against zero. The results showed that both groups significantly explored the novel objects more than the familiar objects (group 1: mean D2 = 0.4; $t(4) = 5.410$, $p = 0.006$; group 2: mean D2 = 0.4; $t(3) = 15.603$, $p = 0.001$). Fig. 3c–f illustrates the cumulative values for both discrimination and exploration measures, respectively, for the two groups.

The performance of the two groups of animals in Experiment 2 was compared on measures of exploration and recognition to determine whether performance could potentially be affected by involvment in the previous task. Group 1 had previously taken part in Experiment 1 whilst group 2 were a naïve sample at this stage of testing. Two independent samples *t*-tests (two-tailed) were used to compare mean D2 scores and total exploration times between the experienced (group 1) and the naïve animals (group 2). The results showed no significant difference on either measure (D2 scores: $p = 0.968$; total exploration time: $p = 0.930$) indicating that both groups had similar performance levels despite the different levels of experience with the object recognition task.

In order to see whether performance was maintained across a session, the D2 scores for each animal from both groups combined were segregated into 4 blocks, each of 4 trials. For each animal, a mean D2 was calculated for each block derived from their individual D2 scores within that block. Using a repeated measures ANOVA no effect of block was found ($F(3, 24) = 2.869$, $p = 0.098$; Greenhouse–Geisser corrected).

As with Experiment 1, a post hoc power analysis was conducted in order to obtain the statistical power of Experiment 2 and subsequently make a comparison to the statistical power of the spontaneous object recognition task employed by Norman and Eacott (2005). For the two groups tested in Experiment 2 the effect sizes were 2.42 (group 1) and 7.80 (group 2) with calculated power of 0.98 and 1.0 for sample sizes of five and four subjects, respectively. In comparison to the effect size and caculated power in the Norman and Eacott task (2.38 and 0.99, respectively, from a sample size of 11 subjects) it is evident that the current spontaneous object recognition task in Experiment 2 had a statistical power comparable to a previous study but from very much smaller group sizes.

4.4. Discussion

Experiment 2 was designed to be a continual version of the standard object recognition procedure with a sample phase prior to each test phase on each trial. Two groups were tested: one that had performed in Experiment 1 and thus had experience in a spontaneous object recogntion task and a second group that was naïve. As in Experiment 1, reliable measures of discrimination were found which were comparable to previous studies that have employed the spontaneous object recognition task (e.g. Albasser et al., 2010; Dix and Aggleton, 1999; Eacott and Norman, 2004; Ennaceur and Delacour, 1988).

Experiment 2 used only 16 trials in contrast to Experiment 1 in which continual test trials allowed 30 trials to be run. It is clear that in Experiment 2 performance was maintained across all 16 trials with no evidence found of a build up of interference as a result of the presentation of multiple stimuli within a session. Good levels of both total object exploration and novelty discrimination were maintained throughout the session. Thus the previous suggestion that the fall in performance in one block seen in Experiment 1 was a chance occurance is supported by this data.

There are clear similarities in discrimination and exploration measures between Experiment 1 (Fig. 3a and b) and Experiment 2 (Fig. 3c–f). When performance of the experienced group (group 1) in Experiment 2 was compared to that of the naïve group (group 2) on the same task, no significant difference was found on discrimination or exploration measures demonstrating that both groups performed to a similar degree. This perhaps highlights the potential benefit of using a small batch of animals on similarly designed consecutive tasks as performance in no way appeared hindered and was not significantly different from a naïve batch.

Having successfully demonstrated that object recognition can be conducted in the continual trials apparatus, it was examined whether the paradigm could be adapted to test other spontaneous recognition tasks which are commonly used in the literature (e.g. Eacott and Norman, 2004). Experiment 3 was designed as a test of object-location (what-where) memory.

5. Experiment 3: object-location memory (what-where)

5.1. Subjects

Six Lister hooded rats supplied by Harlan used in Experiment 1 and Experiment 2 (group 1) were again used in this experiment. Housing conditions were identical to previous experiments.

5.2. Pretraining

Animals were habituated to their environment prior to Experiment 1 which lasted approximately 5 days (for details on phases 1–5, Section 2.3). As a number of weeks had passed since the animals took part in Experiment 2, they were re-habituated to the apparatus and procedure with a 10 min session each of shuttling between the two areas of the apparatus and an object training session (see Section 2.3 for details).

5.3. Test protocol

Each of the six rats were given a single testing session of 16 trials. The experiment began with a sample phase where the animal was exposed to two novel objects (A and B) for 2 min (see Fig. 2c). The

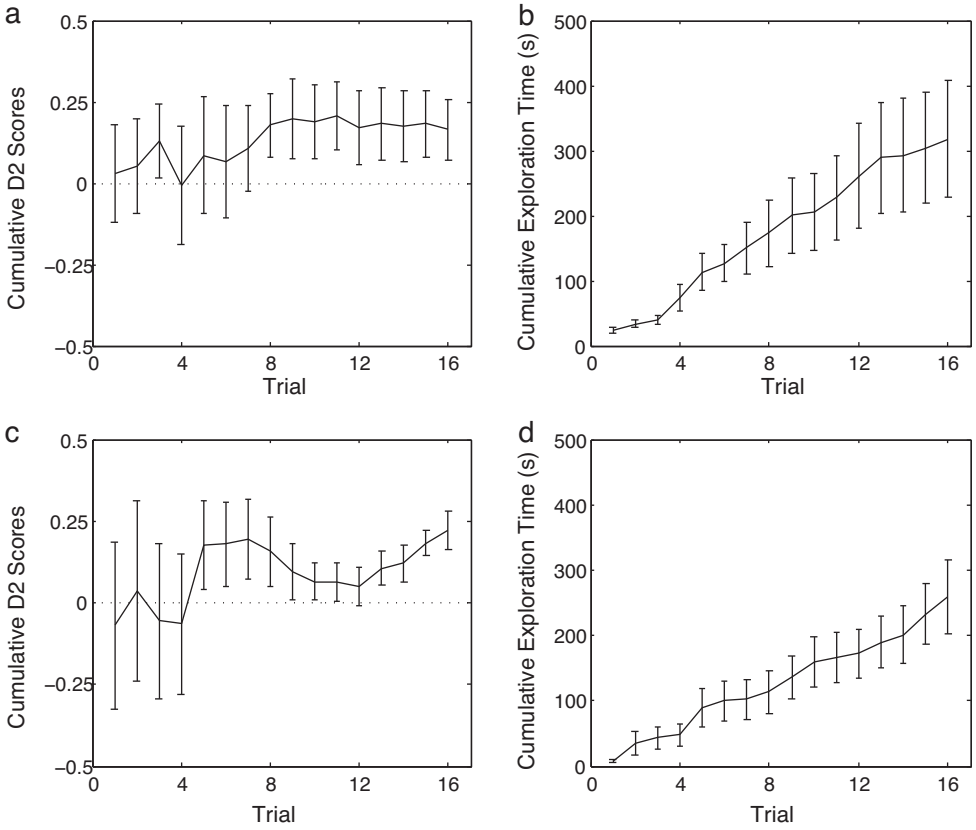

Fig. 4. Graphs from Experiments 3 and 4 depicting animal performance. Vertical bars show the standard error of the mean. (a) Cumulative D2 scores for Experiment 3 across 16 trials. (b) Cumulative exploration time for Experiment 3. (c) Cumulative D2 scores for Experiment 4 across 16 trials. (d) Cumulative exploration time for Experiment 4. Cumulative D2 scores were calculated as a 'running total' of the D2 ratio recalculated after each trial within a session. Cumulative exploration was calculated as the sum of the total exploration across the total number of trials.

outer arm doors of the apparatus were then opened for the animal to shuttle through to the holding area which contained two food pellets. After 1 min the central arm door was opened for the animal to shuttle into the object area for the test phase. The animal was exposed to duplicate copies of one of the objects encountered in the sample phase (e.g. A and A). In this example, object A on the right-hand side is in a novel location for this object and object A on the left-hand side is in a familiar location for this object because object A had not been experienced on the right-hand side during the sample phase. This procedure then continued for a total of 16 trials. Context 1 was used in this experiment.

5.4. Results

One animal was not included in the data analysis for Experiment 3 as shuttling ceased before 16 trials had been completed so the remaining five animals were included in the analysis. This was the same animal that failed to shuttle for the duration of Experiments 1 and 2.

As with the previous experiments, a one-sample *t*-test was used to test whether the animals explored the object in a novel location on each trial significantly more than expected by chance. Analysis of the mean D2 scores showed that the animals preferentially explored the stimuli in novel object-location configurations over those in familiar configurations (mean D2 = 0.2; $t(4) = 5.321$, $p = 0.006$). Fig. 4a and b illustrates the cumulative values for both discrimination and exploration measures, respectively. In order to see whether performance levels changed over the session a repeated measures ANOVA was carried out on blocked data as outlined in Experiment 2. No effect of block was found ($F(3, 12) = 1.026$, $p = 0.416$).

A post hoc power analysis was conducted for Experiment 3 to yield an effect size of 2.38 from a sample size of five. The power to detect an effect of this size was determined to be 0.97. In comparison to the effect size and statistical power of the object-location task employed by Langston and Wood (2010; 1.99 and 0.99, respectively, from a sample size of 12) it is clear that the current object-location task in Experiment 3 had a statistical power comparable to a previous study but from very much smaller group sizes.

5.5. Discussion

Experiment 3 was designed as a test of object-location memory and produced significant levels of novel object-location recognition. In addition, it is evident that the current experiment had high statistical power from a smaller number of animals than is typically used in such tasks.

Similiarly to Experiment 2, no evidence was found of a build up of proactive interference as a result of the presentation of multiple stimuli within a session and good levels of total object exploration and novelty discrimination, not dissimilar to those of Langston and Wood (2010), were obtained. Thus even in this more complex spontaneous recognition paradigm involving association of object and location, there appears to be no disadvantage of running multiple trials within a single session in this apparatus. Therefore, Experiment 4 was designed to test whether the continual trials apparatus could also accommodate tasks involving association of objects and contexts (what-which).

6. Experiment 4: object-in-context memory (what-which)

6.1. Subjects

Six Lister hooded rats (Harlan) from the second group used in Experiment 2 were again used in this experiment. Housing conditions were identical to the previous experiments.

6.2. Pretraining

Animals were habituated to their environment prior to Experiment 2 which lasted approximately 5 days (for details on phases 1–5, Section 2.3). The animals were given three further habituation sessions that consisted of habituating the animals to contexts 2 and 3 (phase 1); encouraging the animals to shuttle between the two areas with each of the two new contexts (phase 2); object habituation with the two new contexts (phase 3) (see Section 2.3 for details on these procedures).

6.3. Test protocol

As this task required two sample phases and a test session, each trial required more shuttling than the previous tasks. For this reason fewer trials were run with each rat each day. Consequently, each of the six rats was given two testing sessions on consecutive days, each session consisting of eight trials. The experiment began with a sample phase where the animal was exposed to two identical copies of the same object (A and A) in a particular context (X) for 2 min (see Fig. 2d). The outer arm doors of the apparatus were then opened for the animal to shuttle through to the holding area which contained two food pellets. After 1 min the central door opened to allow the animal to shuttle back into the object area which would then contain two different identical copies of the same object (B and B) in a different context (Y) for 2 min (second sample phase). The doors on the outer arms of the apparatus would again open for the animal to shuttle to the holding area. After 1 min, the central door would then open for the animal to shuttle into the object area for the test phase. The animal would be exposed to duplicate copies of the objects seen on the previous two sample phases (B and A) in a context also previously seen (X). In this example, object B would be novel and object A familiar because object B had not been experienced in this context (X) during the sample phases. This procedure then continued for a total of eight trials in the first session and a further eight trials in the second session which took place the following day. Contexts 2 and 3 were used in this experiment.

6.4. Results

One animal was not included in the data analysis for Experiment 4 as shuttling ceased before 16 trials had been completed. This was not one of the animals that was excluded from Experiment 2. Thus, data from five animals was analysed for Experiment 4.

Trials from the two testing days for each animal were considered together in this analysis. As with the previous experiments, a one-sample *t*-test was used to see whether the animals explored the object in a novel configuration with context significantly more than what would be expected by chance. Analysis of the mean D2 scores showed that the animals preferentially explored the stimuli in incongruent contexts over those in familiar configurations with context (mean D2 = 0.1; $t(4) = 3.03$, $p = 0.039$). Fig. 4c and d illustrates the cumulative values for both discrimination and exploration measures, respectively.

In order to see whether performance levels changed within and between the two sessions the D2 scores for each animal were segregated into four 4-trial blocks (two blocks per session). For each animal, a mean D2 was calculated for each block derived from the individual D2 scores within that block. Using a 2 (session) $\times$ 2 (block) repeated measures ANOVA, an effect of block was found ($F(1, 4) = 13.761$, $p = 0.021$). A pairwise comparison showed the significant main effect of block to be a result of performance improving in the second block (trials 5–8) of both sessions, however, no significant main effect of session or significant interaction between session and block was found (session: $F(1, 4) = 0.259$, $p = 0.638$; interaction: $F(1, 4) = 0.284$, $p = 0.623$).

A post hoc power analysis was conducted for Experiment 4 to yield an effect size of 1.36 from a sample size of five subjects. The power to detect an effect of this size were determined to be 0.63. Data from an object-in-context task in the Norman and Eacott (2005) study was obtained to make a comparison to Experiment 4. The power to detect an observed effect size of 1.61 was determined to be 0.99 from a sample size of 11 subjects. In comparison to the current experiment, the Norman and Eacott task had higher statistical power but both of the compared tasks had small effect sizes and the current object-in-context task had a reduced sample size yet still demonstrated high statistical power.

6.5. Discussion

Experiment 4 was designed as a test of object-in-context memory and produced an overall mean D2 score of 0.1 which is smaller than that obtained in the object-in-context task of Norman and Eacott (2005) (mean D2 = 0.3). When the statistical power of both tasks was compared it was evident that the current task had lower statistical power than the Norman and Eacott task, however, the statistical power of the current task was still good and involved fewer animals that the Norman and Eacott object-in-context task.

Similarly to Experiments 2 and 3, no evidence was found of a build up of proactive interference in both sessions but evidence did suggest that performace improved in the second block of trials (trials 5–8) in both sessions. The animals appeared to only be performing at chance at the start of each testing session (Fig. 4c) which may be due to insufficient habituation to the context change in the procedure and may have initially disrupted performance in each session. Alternatively, in comparison to the Norman and Eacott task, slight procedural changes may account for differences in performance levels. For instance, in the current study there was a 1 min interval between each of the sample phases and also between sample and test phase on each trial whereas in the Norman and Eacott task a 2 min interval was implemented between sample phases and a 2 min interval between the second sample phase and the test phase. The shorter intervals between exposure phases in the current task may result in the phases being less distinguishable resulting in poorer discrimination when compared to the standard task. Whilst these task differences mean there is potentially scope for further studies improving performance in this task further, it is clear that, as with the previous tasks, significant results with high power can be obtained in this apparatus with a substantially reduced number of animals.

7. General discussion

Overall, the measures of recognition and exploration in tasks employed with the new continual trials apparatus were comparable with studies that have used these tasks with at least double the number of animals except for Experiment 4 which was not directly comparable in terms of the results but nevertheless had good statistical power with fewer animals than previous object-in-context tasks. Being able to offer such a paradigm which is applicable to tasks that are very widely used across a number of disciplines suggests that animal numbers can be substantially reduced and moreover, it is likely that mild potential stress to the animals can

be reduced as less handling and movement of the animal is needed to and from the apparatus during testing (Hurst and West, 2010).

One aim of these studies was to develop versions of spontaneous recognition tasks which use fewer animals than the standard versions. Whilst this aim was achieved in that good results were found with smaller number of animals analysed, it is true that the results from two from 12 animals were not analysed in all experiments entered as the animals failed to reliably shuttle in the apparatus. In one case the animal failed to shuttle in three consecutive tasks (Experiments 1–3), whilst the other animal successfully completed one task (Experiment 2), yet failed to complete sufficient trials in the more complex task of Experiment 4. Peformance in pretraining phases may be indicative of an animal not habituating to the task procedure and in this case further habituation may be required or the decision to drop the animal from testing entirely. However, in this study, the animals that failed to shuttle showed no indication of non-habituation to the task procedure but subsequently failed to perform in the testing sessions of each experiment. The case of the sole animal that failed to shuttle reliably in all of the experiments undertaken (1–3), perhaps suggests that failure to shuttle in at least the one-context studies of Experiments 1–3 is relatively rare in this apparatus (1 from 12 animals). However, where failure to shuttle is seen in one task, it may not be advisable to include that animal in further tasks. This raises the possibility that this procedure may be able to be used prior to surgery in investigations of neural mechanisms of memory using this apparatus, once again allowing the number of animals used in surgical procedures in these experiments to be reduced. However, the case of the animal which failed to shuttle only in Experiment 4 having successfully completed Experiment 2, considered alongside the relatively low D2 scores seen in this study, may again suggest that the task in Experiment 4 requires further refinement.

Little evidence was found in the current studies of a build up of proactive interference diminishing performance within a testing session which is a potential drawback of this type of experimental design (Albasser et al., 2010). Whilst the results from Experiment 1 (spotaneous object recognition) suggested that performance did significantly decline in one block towards the latter end of the session, performance finally improved which is not consistent with a build up of interference. Nor was such an effect seen in any of the subsequent experiments. Indeed in Experiment 4 there was a suggestion of the converse effect, that performance may have been better at the end of testing than in the initial block. Whilst for reasons discussed above, Experiment 4 may need further refinement which could possibly remove this effect, there is certainly very little evidence of a deleterious effect of running multiple trials within a day in any of the current experiments.

The new apparatus shows potential for considerably reducing the number of animals used in memory tasks designed to detect potential amnesic properties of new drugs (Bertaina-Anglade et al., 2006). The spontaneous object recognition task and the object-location task are the most widely used memory tasks for screening new drugs and with the implementation of the continual trials apparatus, the use of animals in such studies can potentially be considerably reduced. As previously mentioned, approximately 43,000 animals have been used in these tasks in the past 5 years but with the application of the continual trials apparatus we estimate that this could have been reduced to 26,000. This further illustrates how animal numbers can be reduced but in addition to this, data accumulation occurs at a faster rate. If we take Experiment 1 as an example, we ran six animals that each could have completed 30 trials in approximately 90 min giving a total testing time of 540 min. This results in a total of 180 trials. In comparison, a standard task may involve 12 (or more) animals each completing a single trial in approximately 10 min giving a total testing time of 120 min but yielding only 12 trials. If we compare the rate of data accumulation (data/time) of the two tasks it is evident that the rate of data accumulation with the new paradigm is in fact three times faster than the standard paradigm. It is also worth noting that the approximated time for the standard paradigm does not include the time taken to handle the animals before and after each trial so the estimate is likely to be conservative. It is important to stress that the new paradigm offers a good balance between reliability through repeated trials in a single animal and the time taken to run an experiment, and thus it is a great improvement on the standard recognition paradigm and it can be applied to multiple recognition memory tasks.

There are further benefits of using this new type of paradigm some of which are illustrated in published studies. For instance, Albasser et al. (2011) demonstrated how, using the Bow-tie maze, it was possible to look at the manipulation of the sample phase of a trial to systematically affect recognition during the test phase. Such tasks can prove useful in understanding perirhinal-based recognition mechanisms. Additionally, using the continual trials apparatus it may be possible to develop tasks of episodic-like memory, particularly those which provide evidence for recollection-based processes (Eacott et al., 2005; Easton et al., 2009).

Although the current design of the apparatus includes multiple contexts and so allows object-in-context (what-which) designs, this is not necessary for the more common object and object-location tasks (Experiments 1–3) which require only a single context. Thus, the apparatus can be simply adapted to have one context if experimental designs did not require context change and this would be easy to construct in any laboratory situation.

In summary, the current study has presented a novel apparatus that has provided reliable measures of recognition on a number of tasks commonly used in the literature with rodents. In comparison to previous studies that have employed such tasks, it is evident that with the new paradigm the number of animals needed to obtain reliable results and maintain the statistical power of the tasks is greatly reduced. This has implications for research that employs recognition tasks in rodents as potentially great reductions in animals numbers can be made and data accumulation is rapid.

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