Novel Tests of Complex Recognition Memory in Animals and Humans

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ABSTRACT

This thesis sought to address specific methodological issues relating to tasks of recognition memory in animals and humans. Such tasks are very widely used, so the need to reduce variability and improve the translation of animal work to humans is apparent. Study 1 sought to develop a reliable testing method based on the spontaneous recognition paradigm that would reduce the animal numbers required for such tasks. Rats displayed significant performance in multiple recognition tasks carried out in the continual trials apparatus, which allows for multiple trials within a session. Approximately 50% fewer animals were required for statistically meaningful results, compared to studies using the standard one trial a day paradigm. Study 2 sought to further develop the continual trials apparatus for an episodic-like memory task for rodents. This study focussed on the development of an object preference task to investigate the behavioural parameters that would affect recognition in the test phase of the E-maze task. Study 3 aimed to investigate whether the continual trials apparatus could be effectively applied with immediate-early gene imaging during a recognition memory task. Animals tested with novel stimuli showed greater fos expression than animals tested with familiar objects, though not significantly. Finally, Study 4 focussed on the translation of animal models of memory to humans. The analysis of receiver-operating characteristics was used to derive a quantifiable distinction between recollection- and familiarity-based processes of recognition, in a task based on paradigms typically used with rodents.

The key findings of the work in this thesis include evidence of substantial animal reduction using a new behavioural apparatus for assessing recognition memory in rodents, and the successful development of an analogous human task of memory in which processes of recognition can be dissociated and quantified. These two key findings make a significant contribution to the field of recognition memory research as the new rodent behavioural tasks

are a clear improvement on standard tasks with the potential to reduce variance and animal numbers, and reducing the reliance on human subjects' introspective accounts of memory in Study 4 provides a shift away towards better controlled behavioural studies in humans, which more closely reflects the studies carried out with animals, and provides strong validation for particular animal models. Through further validation, the simplicity of the human memory task could make it a useful candidate for assessing different forms of recognition memory with neuropsychological subjects.

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DECLARATION

I confirm that no part of the material offered has previously been submitted by me for a degree in this or any other University. Any material generated through collaboration clearly acknowledges the work of others.

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CHAPTER 1

INTRODUCTION

Recognition memory is commonly impaired in neurodegenerative or brain damaged patients (Aggleton and Shaw, 1996), so it is critical to gain full understanding of brain mechanisms and neural networks that are essential for this memory function in humans. The current review will discuss the behavioural approaches used to assess different forms of recognition memory in non-human animals, and how they can be usefully applied with neuroscientific approaches, such as lesions and immediate-early gene imaging, to inform our understanding of memory function in such animals. In addition, new approaches that address the large animal use in widely used behavioural tasks will be discussed. The implications for animal reduction as well as greater reliability of these tasks are significant, and sit alongside further consideration of the 3Rs (Replacement, Refinement and Reduction), in view of how animal models can be used to inform research on human memory.

A debate which is central to our understanding of recognition memory function is whether it is a single unitary process or two distinct processes. A full discussion is beyond the scope of this review, but has been comprehensively covered elsewhere (e.g. Aggleton and Brown, 2006; Clark and Squire, 2010; Ranganath and Ritchey, 2012), so we shall begin with just a brief introductory overview to provide a basis for the behavioural work to be discussed.

1.1. Recognition memory – two distinct processes?

Recognition and episodic memory are forms of declarative memory whereby memories can be consciously recalled. Recognition memory may be defined as the process of identifying when something (e.g. an object, a person) has been encountered previously. Episodic memory, on the other hand, involves memory for a past experience in one's life.

Researchers have long been interested in the mechanisms underlying recognition memory. Eichenbaum, Otto and Cohen (1994) proposed that recognition is supported by two functionally distinct processes mediated by structures in the medial temporal lobe; the hippocampal formation, supporting recollected associations and relationships amongst stimuli, and the parahippocampal region, supporting recognition of individual items. This functional dissociation of recognition memory was further extended by Brown and Aggleton (2001) when they proposed that the hippocampus is part of an extended circuit specifically necessary for episodic recollection (associated with a feeling of 'remembering'; Tulving, 1985), while the perirhinal cortex is part of a circuit involved in familiarity and recency judgements about an encountered stimulus (associated with a feeling of 'knowing'; Tulving, 1985). Dual-process models, such as those proposed by Eichenbaum et al. (1994) and Brown and Aggleton (2001), are based on recognition processes being functionally distinct, though there is still some debate as to which regions in the medial temporal lobe are necessary to support these processes (Eichenbaum, Yonelinas and Ranganath, 2007). According to these models, the hippocampus, fornix (subcortical fibre pathway connecting to the hippocampus) and anterior thalamus form a neural circuit that is critically involved in the process of recollection but not familiarity. On the other hand, the perirhinal and parahippocampal cortices and the medial dorsal nucleus of the thalamus are necessary for familiarity (Aggleton, Vann, Denby, Dix, Mayes et al., 2005; Bowles, Crupi, Mirsattari, Pigott, Parrent et al., 2007; Brown and Aggleton, 2001; Eacott and Heywood, 1995; Eichenbaum et al., 2007; Fortin, Wright and Eichenbaum, 2004; Langston and Wood, 2010; Ranganath, Yonelinas, Cohen, Dy, Tom et al., 2004; Sauvage, Owens, Yonelinas and Eichenbaum, 2008; Yonelinas, Kroll, Quamme, Lazzara, Sauve et al., 2002). However, other researchers argue that recognition memory is a single process dependent on both the hippocampus and adjacent cortex (Donaldson, 1996; Haist and Shimamura, 1992; Squire, Stark and Clark, 2004; Squire,

Wixted and Clark, 2007). Such models state recognition memory is a process based on familiarity, where 'knowing' reflects weaker memory and 'remembering' is associated with strong memory.

Studies involving human amnesic patients with hippocampal damage have provided useful insight into this debate, with some reporting selective recollection impairment with spared familiarity processing (Aggleton et al., 2005; Bastin, Van der Linden, Chamellet Denby, Montaldi et al., 2004; Gardiner, Brandt, Vargha-Khadem, Baddeley and Mishkin, 2006; Holdstock, Mayes, Roberts, Cezayirli, Isaac et al., 2002; Turriziani, Serra, Fadda, Caltagirone and Carlesimon, 2008; Yonelinas et al., 2002), offering support to the dual-process model, whilst others have found deficits in both recollection and familiarity (Cipolotti, Bird, Good, Macmanus, Rudge et al., 2006; Jenson, Kirwan, Hopkins, Wixted and Squire, 2010; Manns, Hopkins, Reeds, Kitchener and Squire, 2003). To some extent, the inconsistent findings can be attributed to differences in testing measures and/or the specific medial temporal lobe damage varying between patients. If recognition memory is to be convincingly accepted as being supported by dual-processes, then it is necessary to localise the structures within the medial temporal lobe that mediate these processes, and specifically whether the roles of the perirhinal cortex and the hippocampus can be regarded as separate in their support of familiarity and recollection (Aggleton and Brown, 2006; Eichenbaum et al., 2007; Guderian, Brigham and Mishkin, 2011; Montaldi and Mayes, 2010; Montaldi, Spencer, Roberts and Mayes, 2006; Murray, Bussey and Saksida, 2007; Norman, 2010; Squire et al., 2007; Squire and Wixted, 2011; Vann, Tsivilis, Denby, Quamme, Yonelinas et al., 2009; Vann and Albasser, 2011).

The human patient literature goes some way in determining the structures underlying recognition memory, however, a substantial amount of research has, and continues to be, focused on developing animal models of memory which can provide an insight into the

functional neuroanatomy. The importance of such research is evident as animal studies not only allow for impairments after specific and localised lesions to be measured, but they also allow researchers to look at precise genetic and molecular factors involved in memory processes and the effect of pharmacological interventions (Dere, Kart-Teke, Huston and De Souza Silva, 2006), with the aim of developing appropriate treatment for memory impairments in neurodegenerative diseases, and neurorehabilitation for deficits in brain injured individuals.

1.2. Early studies on recognition memory in animals

Subjects with damage to the medial temporal lobe have been reported to experience profound memory deficits (Scoville and Milner, 1957). Early studies on recognition memory in non-human primates sought to reproduce this damage to gain an understanding of the anatomical basis for such deficits. However, the nature of a suitable task to reveal deficits which are analogous to those of patients such as H.M. was not always clear. Gaffan (1974) developed the 'delayed matching to sample' (DMS) task as a one-trial test of visual recognition memory in monkeys. The task consisted of presenting the animal with a single object in the sample phase that had to be displaced for a food reward. In the test phase, the sample object was presented alongside a new object, and the monkey was trained to select /match the object from the sample phase, thus demonstrating memory for that object. The delay between the sample and test phases of the trials could be varied to increase demand on recognition memory, and it was argued that this task was analogous to the yes/no recognition memory tasks used in human memory studies and those used to identify memory impairments in amnesic individuals (Clark and Squire, 2010).

In 1978, Mishkin modified the DMS task so that the monkeys were trained to select the new object in the test phase, rather than the object that had appeared in the sample phase.

Training for this 'delayed nonmatching to sample' task (DNMS) was quicker as it capitalised on the animals' natural preference for novelty (Mishkin, 1978; Mishkin and Delacour, 1975). DNMS has been widely used as a test of recognition memory in both monkeys (e.g. Eacott, Gaffan and Murray, 1994; Mishkin and Delacour, 1975) and humans (e.g. Holdstock, Mayes, Cezayirli, Isaac, Aggleton et al., 2000) in order to understand the neural basis of memory. DMS and DNMS tasks have demonstrated that memory is impaired following rhinal cortex lesions (Eacott et al., 1994; Meunier, Bachevalier, Mishkin and Murray, 1993; Zola-Morgan, Squire and Amaral, 1989), but DNMS performance following selective hippocampal damage has offered inconsistent findings with some studies reporting DNMS deficits (Alvarez-Royo et al., 1995; Beason-Held et al., 1999; Mahut, Zola-Morgan and Moss, 1982; Zola-Morgan and Squire, 1986; Zola, Squire, Teng, Stefanacci, Buggalo et al., 2000), and others reporting no impairment following hippocampal lesions that spare surrounding cortical areas (e.g. Murray and Mishkin, 1998; Nemanic, Alvarado and Bachevalier, 2004). Though questions around the precise role of the hippocampus in DNMS continue to be asked, there is general consensus regarding the importance of the surrounding cortical areas for successful performance.

The DNMS task has been adapted for use in rats using both objects (Aggleton, 1985, Kesner, Bolland and Dakis, 1993; Mumby et al., 1990) odours (Otto and Eichenbaum, 1992a, 1992b; Ramus and Eichenbaum, 2000; Winters, Matheson, McGregor and Brown, 2000) and computer-generated scenes (using the constant-negative paradigm; Simpson, Gaffan and Eacott, 1998) as stimuli in tests of recognition memory. However, there are a number of issues relating to the use of the DNMS task with rats, as a number of lengthy training sessions are required in order for them to acquire the rules of matching or non-matching. It is important to make sure animals have acquired the rules sufficiently prior to testing, so that any deficit in task performance cannot be attributed to failing to apply them (Dix and

Aggleton, 1999). In addition, as animals often receive selective food reinforcement for correct responses in the DNMS task, performance may be confounded through animals acquiring strategies to obtain the food reward; strategies which are not associated with the purpose of the task (Herremans, Hijzen and Slagen, 1995). Due to the issues associated with the DNMS task, it was necessary to find a way of assessing recognition memory in rodents without extensive training procedures or selective food reinforcement.

1.3. Tasks for assessing spontaneous recognition memory in rats

1.3.1. Spontaneous object recognition

Ennaceur and Delacour (1988) developed an alternative way of investigating object recognition in rodents using their spontaneous exploratory activity as a valid measure of recognition memory function. Similarly to the DNMS task, spontaneous object recognition tasks capitalise on the animals' innate preference for novelty as a measure of recognition. Typically, animals are individually placed in an open field with two copies of an object which they can freely explore for a period of time (Figure 1.1.a), often for around three minutes though some tasks end the sample phase when total object exploration has reached a pre-set time threshold (e.g. 25 secs, Winters, Forwood, Cowell, Saksida and Bussey, 2004). Following a delay (of minutes, hours or even days), the animal is returned to the open field arena for the test phase of the trial which contains a copy of the object seen previously and a novel object. The animal's memory for the familiar object from the sample phase is exhibited through preferential exploration of the novel object. As the animal is able to explore the physical objects, behaviour can be driven not only by visual information but also by olfactory and tactile information (Clark and Squire, 2010).

The details of spontaneous object recognition task procedures vary between laboratories and this may influence the conclusions that can be drawn. Typically, the animals

are individually handled when being transferred to and from the open arena, and animals will often only perform one trial a day; a single trial consisting of a sample and a test phase. Animals may perform the task repeatedly over a few days yielding a number of trials per animal (e.g. Norman and Eacott, 2004), but some experiments have tested recognition memory for objects with just a single trial per animal (Dere, Huston and De Souza Silva, 2005). Experimenters often use three minute periods for the sample and test phases (e.g. Norman and Eacott, 2004; Barker and Warburton, 2011); this can, however, be varied with some studies opting for sample phases ranging up to 15 minutes (e.g. Ainge, Heron-Maxwell, Theofilas, Wright, de Hoz et al., 2006). Extending the length of the sample phase may serve to increase the familiarity of the exposed objects, with evidence suggesting that performance on the spontaneous object recognition task can be improved through extending the sample phase period. Albasser et al. (2009) showed that the degree of sample object exploration increased through extending the length of the sample phase, and the degree of sample object exploration was positively correlated with the degree of discrimination between the objects at test. In this study, the test phase duration was five minutes, however, the results were comparable when analysed at two minutes. These results reflect the findings by Dix and Aggleton (1999) in which they reported the most sensitive period of object discrimination with the spontaneous object recognition test phase is in the first two minutes, with object exploration significantly decreasing throughout this period.

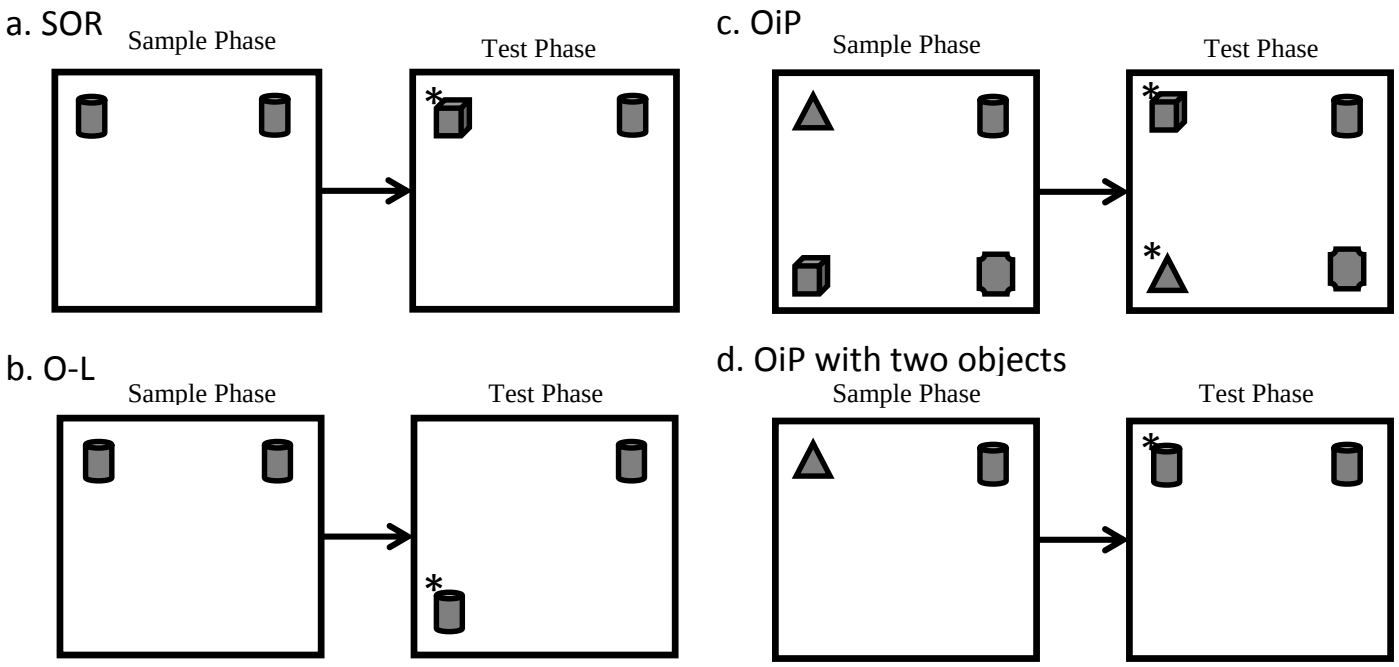


Figure 1.1. Different test procedures for four spontaneous recognition tasks in the open field arena, with figures representing a single trial, consisting of a sample and a test phase. The asterisks indicate the novel object or novel configuration of the object and its spatial location in the test phase that the animals should preferentially explore. a) The spontaneous object recognition (SOR) task. b) The object-location (O-L) task in which one object at test occupies a novel location. c) The object-in-place (OiP) task in which two objects swap locations at test. d) The simplified version of the OiP task in which one object at test occupies a location previously occupied by a different object.

The delay between the sample and test phase is also relevant, as memory strength for the familiar object will decrease with longer delays, thus reducing discrimination performance at test. However, the absolute length over which intact rats can show successful discrimination of novel and familiar objects in this task depends crucially on the nature of the objects, in particular the similarity of the novel and familiar objects (Norman and Eacott, 2004). For example, control animals could successfully discriminate a novel object from one that had been previously explored up to 24 hours ago, when the objects were standard junk objects (e.g. bottles, vases and candlesticks) which differed in many aspects (e.g. material, shape,

size). However, when both novel and familiar objects were made of highly similar material (Duplo) and had been designed to share features in common with each other (e.g. arrangement of blocks into a tower), control animals could only successfully discriminate novel and familiar objects at delays of up to 15 minutes (Norman and Eacott, 2004).

Lesion studies using the spontaneous object recognition task have provided a useful insight into the anatomical basis for recognition memory, with studies demonstrating that the perirhinal cortex is critical for successful performance on this task (Barker, Bird, Alexander and Warburton, 2007; Barker and Warburton, 2011; Bussey, Muir and Aggleton, 1999; Ennaceur and Aggleton, 1997; Ennaceur Neave and Aggleton, 1996; Mumby and Pinel, 1994; Norman and Eacott, 2004; Winters et al., 2004). A large number of hippocampal or fornix lesion studies have reported no detrimental effect on spontaneous object recognition memory (Barker and Warburton, 2011; Ennaceur and Aggleton, 1994, 1997; Ennaceur et al., 1996, 1997; Forwood, Winters and Bussey, 2005; Good, Barnes, Staal, McGregor and Honey, 2007; Langston and Wood, 2010; Mumby, Gaskin, Glenn, Schramek and Lehmann, 2002; Warburton and Aggleton, 1999; Winters et al., 2004), though some studies have found impairment after long delays (e.g. Clark, Zola and Squire, 2000, referred to as the 'visual paired comparison task'; Hammond, Tull and Stackman, 2004). Possible reasons for the inconsistency in findings may be related to the extent of damage to the hippocampus, and/or procedural differences between studies. Ainge et al. (2006) reported that rats with either complete or partial hippocampal lesions were unimpaired on an object recognition task in which exploration of the objects was limited to 30 seconds during the sample phase. However, when the sample phase was defined by 15 minutes of free exposure to the objects, only the animals with the partial hippocampal lesions were unimpaired. Moreover, the complete lesion group showed lower levels of object exploration than the partial or control groups in the second task, suggesting that not only did the extent of lesion size effect object

recognition performance, but this may have also impacted on the exploration of objects at encoding.

The relative simplicity of the spontaneous object recognition task has allowed for widespread use to test recognition memory in rodents, and research suggests that the spontaneous object recognition task is more sensitive to recognition memory deficits than the DNMS task (Clark and Squire, 2010; Nemanic et al., 2004; Pascalis, Hunkin, Holdstock, Isaac and Mayes, 2004). The use of the spontaneous object recognition task across multiple disciplines can be attributed to a number of advantages. The task is very simple to administer and there is consistency of results across species (Clark and Martin, 2005). In addition, issues associated with selective food reinforcement are avoided as the object novelty is sufficient to drive exploration without being associated with a food reward.

There are, however, a number of issues related to administering tasks based on spontaneous exploration. First, as the object exploration, which serves as a measure of the animals' memory, is completely spontaneous with no prior training required, there can be considerable variance in behavioural performance between animals on individual trials. When low numbers of trials are run with each animal, the outcome of these random effects can be marked, resulting in high variability. In addition, influences other than object novelty may drive animal exploration such as particular features of the environment around the testing arena, or initial mis-match of objects in terms of how inherently interesting they are to the animals. These factors may potentially lead to familiar, but inherently salient, stimuli being more attractive for exploration than novel, but inherently relatively unsalient, objects. Careful counterbalancing of objects, both between animals and within the test phases that each animal performs can help to minimise potential exploration differences due to unmatched object salience. The use of D1 and D2 scores as measures of recognition goes some way in reducing potential variability in animal performance (Ennaceur and Delacour,

1988). D1 is calculated through taking the exploration of the novel object at test minus the familiar object exploration. However, D1 takes no account of differences in overall exploration levels and so results may be biased by more active animals. To account for these differences in total exploration at test, the D2 ratio is calculated: the D1 score is divided by the total exploration of both the novel and familiar object at test. The D2 ratio therefore scales the exploration to account for overall differences in total exploration. This ratio can therefore vary from -1 to +1 with anything above zero being indicative of novelty preference.

As the spontaneous tasks rely on free exploration, stress may inhibit or change the nature of such exploration, and so may impair performance on such tasks (Yuan, Long, Liu, Qu, Chen et al. 2009). For example, stress can result in neophobia (Ennaceur, Michalikova and Chazot, 2009), and so even the relatively small amount of stress that may be induced through handling (which may be considerable in these one trial a day tasks as animals are repeatedly taken in and out of the apparatus) may be sufficient to drive behaviour away from the novel stimulus, thereby masking true recognition abilities. Recent evidence supports this view and suggests that particular animal handling procedures can induce aversion and anxiety, which can subsequently influence performance in behavioural tasks (Hurst and West, 2010). In this study, mice demonstrated greater anxiety in an elevated plus maze through reduced entry to the arms without protective walls when they were commonly handled with more anxiety-provoking methods, such as being picked up by the tail.

The spontaneous object recognition task has successfully been used to study memory for objects but the paradigm has also been adapted for testing more complex forms of recognition memory through the use of novel apparatus or task designs. Variants of the spontaneous object recognition task have successfully 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. Dix and Aggleton,

1999; Eacott and Norman, 2004; Ennaceur et al., 1997; Langston and Wood, 2010; Norman and Eacott, 2005). Spontaneous tasks that test different forms of recognition memory are a useful way of investigating the individual components that contribute to episodic memory. If we can understand the role of particular brain structures in these forms of memory then we can begin to form a picture of the potential connectivity of these structures and network interactions.

1.3.2. Recognition memory for the spatial locations of objects

Variants of the spontaneous object recognition task have allowed memory for the object and its spatial location to be investigated. In the object-location task (Save, Poucet, Foreman, and Buhot, 1992), rats are exposed to two different objects in the open field during the sample phase (Figure 1.1.b). At test, one of the objects is moved to a novel location in the open field where an object has never been previously encountered. Intact rats preferentially explore the familiar object occupying a novel location more than the familiar object occupying the familiar location.

An alternative task, known as object-in-place (Dix and Aggleton, 1999), involves exposing rats in an open arena to four different objects during the sample phase (Figure 1.1.c). After a delay, the same objects are present in the test phase but two of them have switched locations in the arena. Therefore, all of the objects in the test phase are equally familiar, and so are the locations that are occupied by objects. However, the specific combination of object and location is novel, and results in greater exploration of an object in a location that it did not previously occupy. Later variants of this task (e.g. Ameen-Ali, Eacott and Easton, 2012; Davis, Easton, Eacott and Gigg, 2013; Eacott and Norman, 2004) have used just two objects in the initial exposure phase, while at test there are two copies of one of these objects both occupying the same locations as the objects in the sample phase

(Figure 1.1.d). Exploration is driven towards the object in the location it did not previously occupy (i.e. novel object-location conjunction). This variant of object-in-place therefore allows the study of memory for object-place conjunctions within a slightly simpler paradigm than that used by Dix and Aggleton (1999). This variant also allows more direct comparison with performance on the spontaneous object recognition task as there are no differences in number of objects present, for example, and so no differential task unrelated loads on memory.

The object-location task has been shown to be hippocampal dependent as rats with dorsal hippocampal lesions (Save et al., 1992) or fornix lesions (Ennaceur et al., 1997) cannot successfully perform the task. Rats with perirhinal cortex lesions, on the other hand, show normal object-location recognition memory (Barker and Warburton, 2011). There is some evidence to suggest perirhinal involvement on the object-in-place task when the task consists of four objects and a delay of five or six minutes between the sample and test phases (Barker et al., 2007; Bussey et al. 2000). However, Eacott and Norman (2004) have reported successful performance on this task when two objects are used, with delays of five minutes. It is possible that the extent of lesion damage may account for the successful performance on the object-in-place task, as the studies by Barker et al. (2007) and Bussey et al. (2000) reported bilateral perirhinal lesions that were almost complete, whereas Eacott and Norman (2004) reported sparing of the caudal part of the perirhinal cortex. It is also possible, however, that a reduced memory load on the perirhinal system in the simplified task used by Eacott and Norman (2004) could also provide some explanation for the differing reports. There are conflicting findings regarding the role of the hippocampus in the object-in-place recognition task with some studies finding impairment after hippocampal or fornix lesions (e.g. Bussey et al., 2000; Mumby et al., 2002) but others finding no impairment (Eacott and Norman, 2004; Langston and Wood, 2010). Procedural differences which result in different

strategies being adopted could account for these conflicting findings; for example, Langston and Wood (2010) have suggested that the procedure adopted in some versions of the object-in-place recognition memory task allow non-hippocampally dependent (Eichenbaum et al., 1990) egocentric strategies to be employed for successful task performance, while others allow only allocentric strategies. For example, in a version of the task in which the entry point into the apparatus differed on each trial, rats with hippocampal lesions were impaired compared to successful performance in the standard version of the task, in which the entry point always remained the same (Langston and Wood, 2010). Only when allocentric strategies are required, therefore, is the task dependent on the hippocampus, which may account for the differing reports on the role of the hippocampus in object-in-place recognition memory. Overall, these findings suggest that the hippocampus may provide necessary spatial information for successful performance of object-location and object-in-place recognition memory within an allocentric framework. The perirhinal cortex is not necessary for successful performance on the object-location task, as there is no geometric change to the objects (Barker and Warburton, 2011; Mumby et al., 2002). The task, therefore, can be solved solely through the spatial information of the object's location provided by the hippocampus (Brown Barker, Aggleton and Warburton, 2012; Dix and Aggleton, 1999). Object-in-place recognition memory has offered conflicting findings with regard to the role of the perirhinal cortex, perhaps an indication of task sensitivity to factors such as lesion size, and the effect of stimuli quantity on memory load.

Work on recognition memory for objects and their spatial locations has extended beyond the use of the open field arena to the use of the radial arm maze and the Y-maze. Some researchers have argued that assessing spontaneous object recognition in the open field can be problematic, as external spatial or contextual factors from the environment external to the arena may contribute to the animal's spontaneous behaviour (Forwood et al., 2005). The

Y-maze minimises these confounding factors as it has high walls and narrow arms for placing the objects to minimise the extent to which animals are influenced by external cues. The object recognition testing paradigm used with the Y-maze is similar to that used with the open field, in that spontaneous behaviour is assessed and one trial a day is performed per animal. In contrast, the 8-arm radial arm maze is designed to assess spatial working memory whereby rats forage from baited arms of the maze and the number of errors (visits to non-baited arms or revisits to arms where food was already retrieved) is recorded. Winters et al. (2004) reported that hippocampal lesioned rats were impaired on a spatial radial arm maze task but showed normal performance on an object recognition task in the Y-maze. Rats with lesions to the perirhinal and postrhinal cortices were impaired on the object recognition task but not on the spatial radial maze task. These findings further support the role of the hippocampus for aspects of recognition that involve memory of spatial information and the perirhinal cortex for object identification. However, in this study, memory was tested using different apparatus, with object recognition tested in the Y-maze rather than the open field to reduce any potential influence of spatial or contextual cues that might influence task performance (e.g. Aggleton and Brown, 1999; Bussey and Aggleton, 2002). It is advantageous to compare both spatial and non-spatial recognition memory tasks using the same paradigm, as noted by Dix and Aggleton (1999), who argue that performance differences can be attributed to different testing procedures. Spatial memory tests continue to be widely used, but when making direct comparisons to non-spatial recognition memory tasks, the spontaneous tasks within an open field arena remain useful due to their simple design, and the potential to develop multiple testing paradigms for various forms of recognition memory in a single apparatus.

1.3.3. Recognition memory for objects in contexts

Spontaneous tasks within the open field have also been useful for assessing the role of context in recognition memory. Contextual cues are necessary for episodic memory, so it is therefore important to first understand the relationship between context and object recognition memory. Dix and Aggleton (1999) investigated memory for objects encountered in particular contexts. In this task, rats were exposed to two copies of an object in an open field during the first sample phase (Figure 1.2.a). In the second sample phase, the rats were exposed to two copies of a different object in a different open field (i.e. different context). During the test phase the rats were placed in one of the open fields with copies of both of the previously encountered objects. The rats preferentially explored the novel configuration of object and context (i.e. the object at test was in a context which differed from its context at sample).

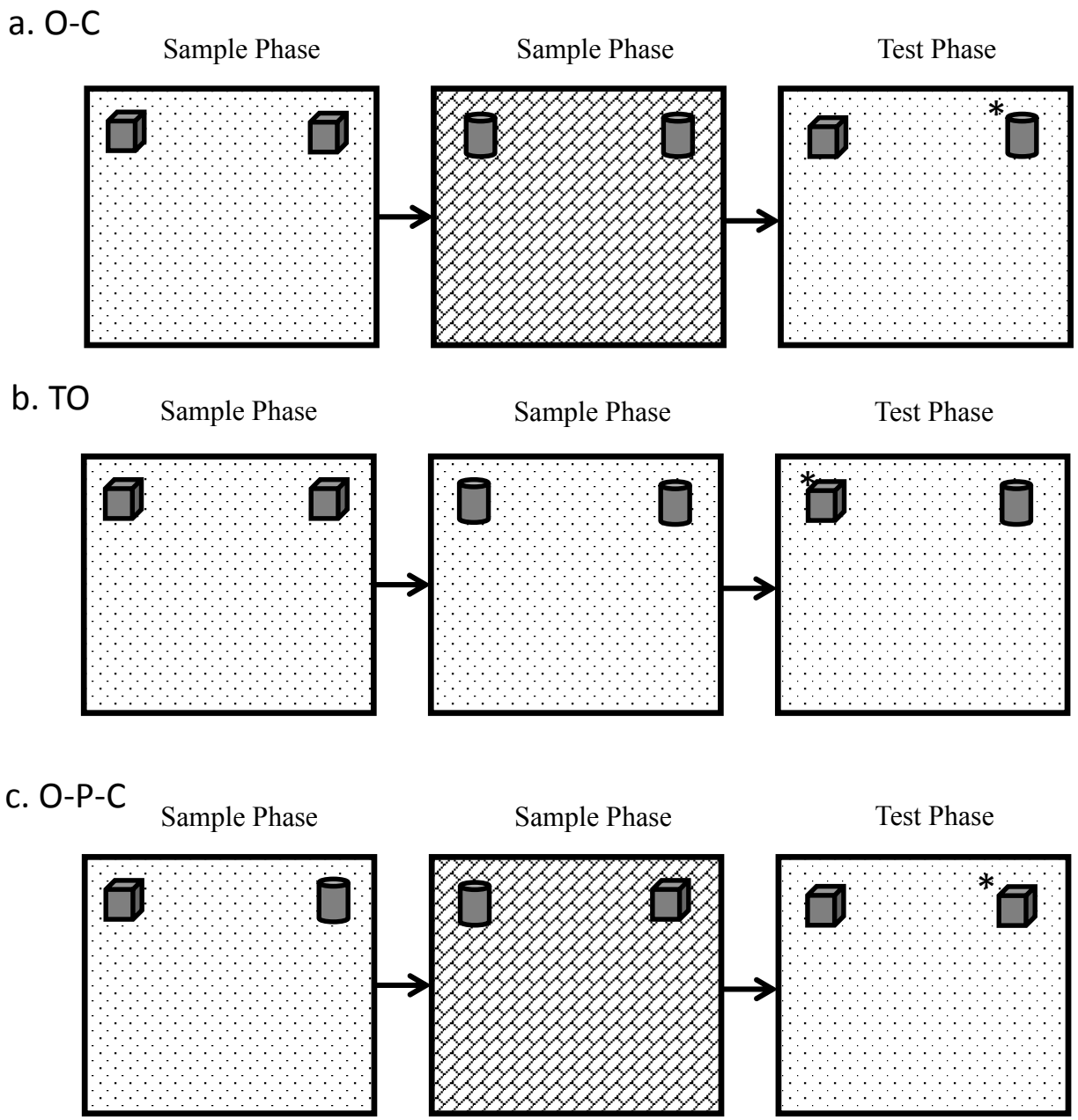


Figure 1.2. Different test procedures for three spontaneous recognition tasks in the open field arena, with figures representing a single trial consisting of sample and test phases. The asterisks indicate the novel configuration at test of the object and an aspect of the environment, such as background context, temporal order of the presented objects, or spatial location and context, in the test phase that animals should preferentially explore. a) Object-in-context (O-C) recognition task consisting of different contexts across the two sample phases. b) Test procedure for the temporal order (TO) recognition memory task illustrating a single trial consisting of two sample phases and a test phase. c) Test procedure for the episodic-like object-place-context (O-P-C/what-where-which occasion) recognition task.

The neural basis of this object-in-context memory was investigated by Norman and Eacott (2005). Severe deficits in task performance were found following postrhinal lesions, even at very short delays of two minutes. In contrast, perirhinal lesioned animals were able to perform the task successfully at these delays, although were impaired at longer delays. Animals with fornix lesions were also able to perform the task at above chance levels although they were mildly impaired in comparison to sham control animals. These findings strongly implicate postrhinal cortex involvement in recognition memory of the configuration of objects and contexts. Together with those using the spontaneous object recognition task, these findings suggest there is a double dissociation between the perirhinal and postrhinal cortices. Animals with perirhinal cortex lesions are impaired on object identification (spontaneous object recognition task; Norman and Eacott, 2004) but not on recognition for the object and context configuration at short delays (object-in-context task; Norman and Eacott, 2005). Animals with postrhinal cortex lesions, on the other hand, are impaired on object-in-context but not spontaneous object recognition tasks (Norman and Eacott, 2005).

The findings by Norman and Eacott (2005) also suggest a lack of critical hippocampal involvement in the object-in-context task, as fornix lesioned animals could perform the task successfully at short delays. Langston and Wood (2010) reported similar findings with hippocampal lesioned animals, but noted that this contrasted with reports by Mumby et al. (2002) who found that animals with lesions to the hippocampus were impaired at object-in-context recognition memory. Langston and Wood (2010) offered an account for the differing reports and suggested that hippocampal involvement may be determined by how the context is defined in the task, e.g. through local features such as the floor and walls of the open field, or through different testing rooms that consist of multiple features that define the environment. The hippocampus may be involved in the recognition of object and context configurations when the task involves different testing rooms to define the context, but it may

not be required when the task involves discrimination between object and context configurations in the immediate environment (Langston and Wood, 2010). Indeed, a recent study by Albasser et al. (2013) demonstrated that hippocampal lesioned rats were able to successfully perform in a biconditional learning task when the correct digging choice was determined by proximal context cues. However, deficits were observed when the correct digging choice was determined by distal room cues (Albasser et al., 2013).

1.3.4. Temporal order/recency memory

Descriptors of episodic memory often include a temporal component (see Section 1.6.1 of this chapter), so it is therefore important to understand how temporal order (or recency) recognition memory is different to other forms of recognition memory before we can conceive of how this process may contribute to episodic memory. In rodents, temporal order recognition memory is often tested in the open field with animals being shown two copies of an object in the first sample phase, which they can freely explore, and two copies of a different object in the second sample phase (Figure 1.2.b). In the test phase, the animals are shown copies of both objects with the expectation that the animals will spend more time exploring the object presented in the first sample phase, as it was seen longest ago and therefore is less familiar than the object seen in the second sample phase. Temporal order recognition memory has been reported as being impaired following hippocampal lesions (e.g. Barker and Warburton, 2011) and the task is also dependent on the perirhinal and medial prefrontal cortices (Barker et al., 2007; Barker and Warburton, 2011; Hannesson, Howland and Phillips, 2004; Mitchell and Laiacona, 1998).

1.4. Multiple trial paradigms for assessing spontaneous object recognition

The spontaneous object recognition task and its variants are useful ways of assessing rodent memory through the animal's spontaneous behaviour. Measuring spontaneous behaviour in the open field with the one trial a day procedure can, however, be time consuming and, as discussed above, there is significant variation in performance between animals. Studies, therefore, often require large animal numbers in order to obtain meaningful results.

One way of addressing some of the issues associated with spontaneous tasks in the open field is through a multiple trial testing paradigm. A new behavioural protocol was developed by Albasser, Chapman, Amin, Iordanova, Vann et al. (2010) using the 'Bow-tie maze' which combines features of spontaneous object recognition tasks with DNMS tasks (Figure 1.3). The Bow-tie maze 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 - one which 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 yields a number of trials for each animal within a single testing session.

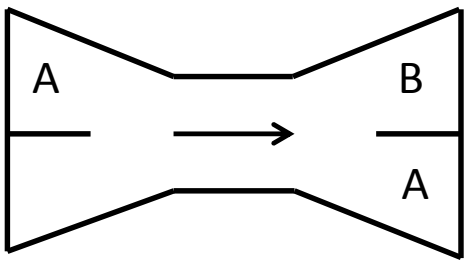


Figure 1.3. Bow-tie maze depicting the general procedure for a single spontaneous object recognition trial, from sample phase (where the animal is exposed to object A on the left), to the test phase (where the animal is shown objects A and B on the right). Spatial locations of the objects are counterbalanced. Arrow denotes the movement of the animal between the two areas of the maze, separated by a guillotine door. Adapted from Albasser, Chapman et al., (2010).

The Bow-tie maze 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 in a single session resulting in faster accumulation of data. Increasing the number of trials run per animal and decreasing potential handling stress reduces the variability in animals' performance which is associated with standard recognition tasks. The Bow-tie maze task provides a useful improvement on the spontaneous object recognition paradigm and it has, for example, been used to investigate perirhinal-based recognition mechanisms (Albasser, Amin, Iordanova, Brown, Pearce et al. 2011). However, developing tasks of more complex forms of recognition memory with the multiple trial method in the Bow-tie maze that, for instance, may rely on a spatial component, would make it difficult to determine whether animals were using egocentric or allocentric strategies, as each trial would involve the animal approaching the objects from the opposite side of the maze. Multiple trial tasks that combine recognition of objects with their spatial location or the context in which they were presented (e.g. Dix and Aggleton, 1999; Eacott and Norman, 2004; Langston and Wood, 2010; Norman and Eacott, 2005) are yet to be demonstrated in the Bow-tie maze, though recent work has successfully demonstrated the use of the Bow-tie maze in assessing recency memory (Kinnavane, Amin, Horne and Aggleton, 2014; Olarte-Sanchez, Kinnavane, Amin and Aggleton, 2014) and the standard object-in-place recognition memory task (Nelson and Vann, 2014).

In light of these issues, Ameen-Ali et al. (2012; see Chapter 2) developed an apparatus 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 continual trials apparatus 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, while the other compartment consists of the object area where the testing takes place (Figure 1.4). 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 with 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 findings from this study revealed that measures of recognition and exploration in spontaneous object recognition, object-location and object-in-context tasks employed with the new continual trials apparatus were comparable with previous studies which have used the one-trial a day paradigm. Importantly, the new design resulted in approximately 50% fewer animals being required to obtain statistically reliable results. As these recognition tasks are very widely used across a number of disciplines, the potential animal reduction in memory research is significant.

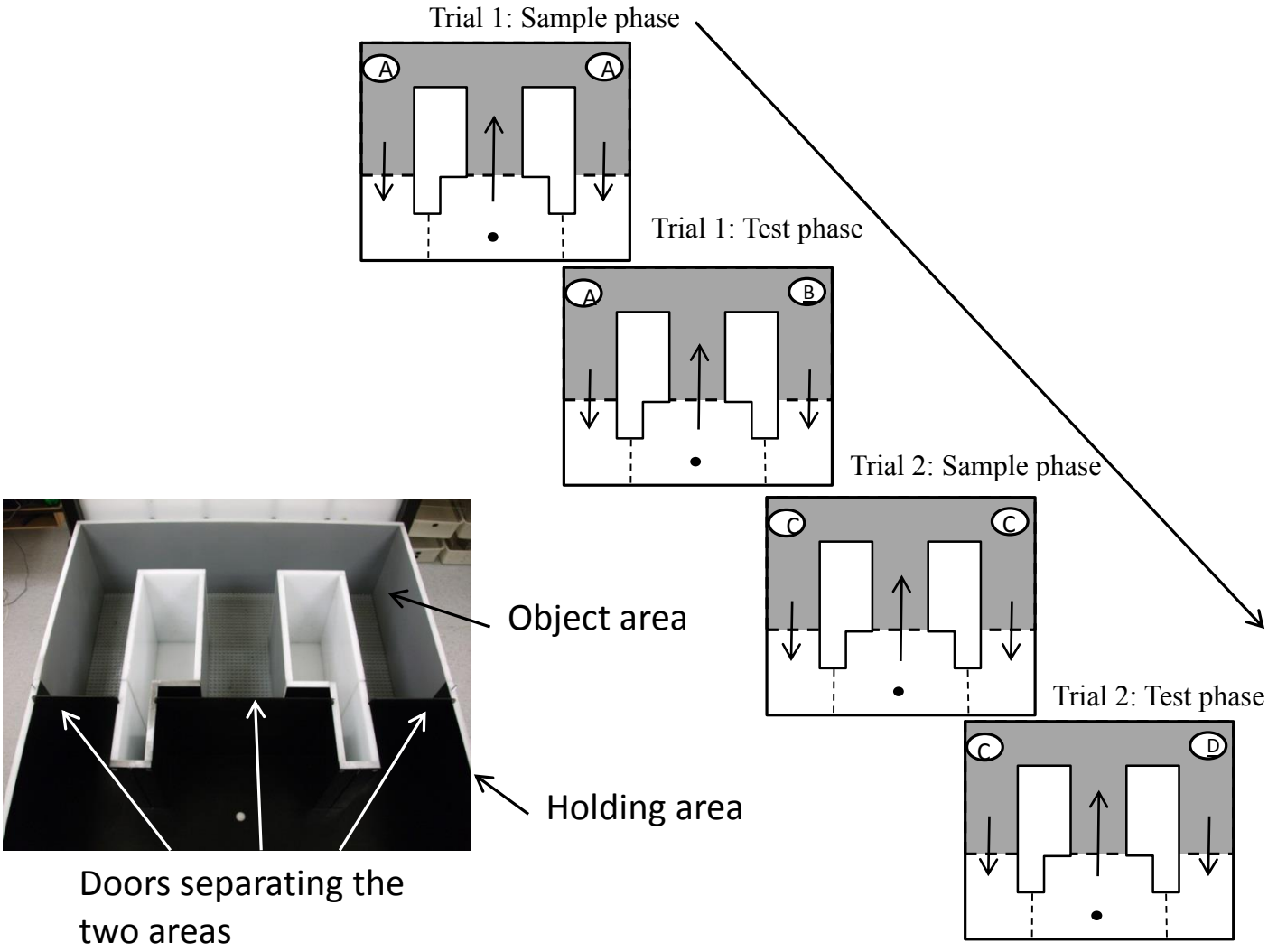


Figure 1.4. Photograph of the continual trials apparatus, from above, with an example of the task procedure for spontaneous object recognition. The animal begins in the holding area (black area on the photograph, white area on the image) and enters the object area (grey area) at the start of the sample phase. The image illustrates the position of the objects. After a period of two minutes, the outer arm doors of the apparatus are opened to allow the animal to return to the holding area. Once the objects have been changed for the test phase of the trial, the animal returns to the object area, again via the central arm door. This procedure continues for each trial. The testing session ceases after the animal has completed a predetermined number of trials, or if the animal fails to shuttle to the next area once the doors have been opened for a period of three minutes. The direction of the rats' movement through the apparatus is indicated by the arrows. The letters represent object presentation from sample to test phase for two trials with novel objects underlined. Adapted from Ameen-Ali et al. (2012).

This multiple trial recognition memory paradigm, like the Bow-tie maze, used food reinforcement of objects in order to encourage animals to continue exploration throughout the testing session, as it is important that the animals do not lose interest in the objects as the testing session continues. Object novelty may not be sufficient on its own to drive exploration in a testing session that may consist of 16 trials or more. It is important to note, however, that objects are not differentially rewarded; all objects, including those in the sample phase, are baited by placing small individual food pellets underneath objects to be displaced (Albasser, Chapman et al., 2010) or immediately in front of objects (Ameen-Ali et al., 2012). Thus the food does not reward exploration of particular objects and is therefore not driving recognition memory performance.

The successful development of multiple trial paradigms for testing recognition memory in rats opens up the potential for immediate-early gene (IEG) imaging as an approach for investigating neuronal activity associated with recognition memory. Fos protein is a product of the immediate-early gene c-fos and a transcription factor associated with neuronal plasticity and learning (Herdegen and Leah, 1998; Herrera and Robertson, 1996; Seoane, Tinsley and Brown, 2012; Tischmeyer and Grimm, 1999). Specifically, fos expression in the perirhinal cortex is deemed to be a reliable marker for changes in neuronal activity associated with recognition memory. Evidence suggests that fos expression in the rat perirhinal cortex increases after viewing novel visual stimuli when compared to viewing familiar visual stimuli during the paired viewing test, in which animals are simultaneously presented with novel stimuli to one eye and familiar stimuli to the other eye (Seoane et al., 2012; Wan, Aggleton and Brown, 1999; Wan, Warburton, Zhu, Koder, Park et al., 2004; Warburton, Glover, Massey, Wan, Johnson et al., 2005; Warburton, Koder, Cho, Massey, Duguid et al., 2003; Zhu, McCabe, Aggleton and Brown, 1996). Although this procedure has provided insight in to neuronal activity during recognition, it can be difficult to interpret

results due to lack of behavioural evidence of recognition. Using the spontaneous recognition paradigm with c-fos imaging would provide the behavioural measure of recognition desired, with animals actively discriminating between novel and familiar objects. This has recently been achieved with the one-trial a day paradigm (e.g. Wilson, Wantanabe, Milner and Ainge, 2013), however, c-fos activity is most readily quantifiable after many trials. C-fos activity has, therefore, recently been assessed using the multiple trial Bow-tie maze (Albasser, Poirier and Aggleton, 2010). C-fos expression in the perirhinal cortex was lower in animals tested in the object recognition paradigm using familiar objects than in animals tested with novel objects. This provides further support of perirhinal involvement in detection of object novelty. Combining behavioural approaches, such as those used in the Bow-tie maze and in the continual trials apparatus, with IEG imaging can provide stronger evidence for not only the neural basis of recognition memory but also the network dynamics involved through the use of structural equation modelling, which can identify the direction of effects between brain structures (Albasser, Poirier et al., 2010). Work is ongoing to explore c-fos activation during more complex tests of recognition involving context (Wilson et al., 2013) and temporal order (Kinnavane et al., 2014; Olarte-Sanchez et al., 2014), but more work is needed to understand processes involved in tasks of object-location and episodic-like memory.

Standard versions of the spontaneous recognition tasks have been widely used by researchers to understand the neural basis of memory. Multiple trial methods also offer a way to reduce the potential variability in these tasks, and in turn reduce the number of animals required in such behavioural studies. Moreover, using multiple trial methods alongside techniques such as IEG imaging demonstrates how these testing paradigms can further our understanding of memory function in the medial temporal lobe. These techniques together could, in some instances, be an alternative to traditional lesion studies. As IEG imaging simultaneously assesses activity of multiple brain regions rather than the function of each

region in separate lesion groups, this again provides potential for further reduction in the number of animals used in this research.

1.5. Cellular correlates of recognition memory

A number of studies have investigated the cellular correlates of recognition memory in order to identify how cells respond in particular brain regions when judgements concerning familiarity are made. Evidence from electrophysiological studies in monkeys and rats have implicated the perirhinal cortex, as cells in this region have been shown to have a reduced response when a stimulus is repeated, relative to the response to a novel stimulus being presented (Brown and Aggleton, 2001; Brown, Wilson and Riches, 1987; Fahy, Riches and Brown, 1993; Riches, Wilson and Brown, 1991; Xiang and Brown, 1998; Zhu, Brown and Aggleton, 1995). Tasks with monkeys have typically required animals to make familiarity-based judgements in receipt of reward. For instance, Xiang and Brown (1998) trained monkeys in a serial recognition task (Gaffan, 1974) to perform a left touch when a novel stimulus was presented, and a right touch when a previously shown stimulus was presented. Tasks with rodents have typically employed the DMS or DNMS tasks. For instance, Otto and Eichenbaum (1992b) used a DNMS task to present rats with a series of odours. For half of the trials, the odour presented did not match the odour from the previous trial, and therefore the animals were trained to respond by accessing the water port as a reward. The other half of the trials contained odours that did not match those from preceding trials, and therefore the animals were trained to respond by not accessing the water port reward. This allowed the researchers to compare neural firing from the novel stimuli and the familiar (repeated) stimuli, whilst the animals were actively discriminating between matched and non-matched odours.

The reduction in neuronal response has been shown to occur after just a single exposure to a stimulus, which is indicative of the one trial learning associated with standard object recognition (Fahy et al., 1993; Winters, Saksida and Bussey, 2008; Xiang and Brown, 1998) and can last with delays of up to 24h (Brown and Bashir, 2002). When several stimuli are required to be remembered, monkeys are still able to demonstrate this reduced response to familiar stimuli, indicating that the mechanisms underlying this process must have a relatively large capacity for maintaining information for a certain period of time (Xiang and Brown, 1998). It is worth noting that some studies have reported enhanced, rather than reduced, neuronal responding in the perirhinal cortex following the repeated presentation of familiar stimuli (Winters et al., 2008), though it is possible that differential task demands, for example, may account for the inconsistent reports. More recent work has investigated the influence of differential reward on neuronal responses in the perirhinal cortex. Thome, Erickson, Lipa and Barnes (2012) presented monkeys with stimuli that differed in terms of their familiarity, and the level of familiarity was not related to reward. The authors reported no differences in neuronal response in the perirhinal cortex related to familiarity, and therefore concluded that when the familiarity of the stimuli is not relevant to the task, no differences in neuronal response relating to recognition memory are found (Banks, Bashir and Brown, 2012).

Strong evidence for the reduced neuronal response in the perirhinal cortex following repeated exposure to familiar stimuli has been reported, however, neurons in the hippocampus have not been shown to have the same effect in monkeys (Brown and Xiang, 1998), rats (Otto and Eichenbaum, 1992), or humans (Rutishauser, Mamelak and Schuman, 2006). Neurons in the hippocampus have only been shown to display a general response, with no specific decreased or enhanced response to familiar stimuli (Eichembaum et al., 2007). Such findings may offer support for the role of the hippocampus in associative recognition

memory, rather than recognition of single items. Indeed, evidence suggests that neurons in the hippocampus are responsible for encoding associations between stimuli and spatial location or context in monkeys (Cahusac, Rolls, Miyashita and Niki, 1993; Wirth, Yanike, Frank, Smith, Brown et al., 2003), and rats (Hampson, Heyser and Deadwyler, 1993, Moita, Rosis, Zhou, LeDoux and Blair, 2003; Wood, Dudchenko & Eichenbaum, 1999; Wood, Dudchenko, Robitsek and Eichenbaum, 2000).

The change in neuronal response following repeated stimulus presentation can be demonstrated using immunohistochemical methods (Zhu, Brown, McCabe and Aggleton, 1995, 1996; Wan et al., 1999). IEGs, such as c-fos, have been shown to be involved in the intracellular cascades that change synaptic strengths and support the mechanisms involved in recognition memory (Aggleton, Brown and Albasser, 2012). A study by Warburton et al. (2005) investigated neuronal activity (using the IEG c-fos as a marker) in the perirhinal cortex following exposure to novel and familiar stimuli, and provided evidence that the differential neuronal responses are dependent on a synaptic plastic mechanism used in long term potentiation (LTP). Through inhibition of the CAMP response element-binding protein (CREB) within the rat perirhinal cortex, not only was recognition memory and perirhinal synaptic plasticity impaired, but there was also evidence of disruption to the differential neuronal response to novel and familiar stimuli. From this work, CREB phosphorylation is strongly implicated in the synaptic plastic processes and differential neuronal responses that underlie familiarity discrimination.

Studies that utilise electrophysiological and immunohistochemical methods are useful for informing researchers on exactly how specific mechanisms and functions work, further highlighting the necessity for important lesion studies for guidance towards suitable regions to be investigated.

1.6. Episodic-like memory tasks

An episodic memory is a representation of a specific event and involves a great deal of contextual information about a specific past event in one's life (Crystal, 2010). In addition, it has been argued that conscious recollection or re-experiencing of the event is necessary for an episodic memory to occur (Tulving, 1972). As such, episodic memory has been considered by some to be unique to humans as it is said to require the ability to subjectively sense time in order to keep track of events that have occurred in one's past, but also for planning things in the future (Dere et al., 2006).

1.6.1. Memory for what happened, where and when

Tulving (1972) defined human episodic memory as remembering what happened, where and when. However, later he added the requirement that the memory included autonoetic awareness (Tulving, 1985). This meant that demonstrating episodic memory in animals may not be possible due to the absence of language (Tulving and Markowitsch, 1998) which is needed to provide an account of subjective experience deemed necessary for assessing autonoetic awareness (Eacott and Easton, 2010; Tulving, 2002). As such, studies on analogous processes of episodic memory in animals are referred to as "episodic-like" memory (Clayton and Dickinson, 1998), which provides a shift away from the phenomenological criteria used when assessing human episodic memory. Episodic-like memory using the what-where-when descriptor has been investigated in both Western scrub-jays and magpies by assessing their natural food caching behaviour to investigate whether they remember what type of food they have cached, and where and when it was cached (Clayton and Dickinson, 1998, 1999a, 1999b, 1999c; Clayton, Yu and Dickinson, 2001, 2003; de Kort, Dickinson and Clayton, 2005; Zinkivskay, Nazir and Smulders, 2009). Demonstrating episodic-like memory in other species that do not have natural food-storing

abilities is, however, considered necessary. Babb and Crystal (2005) devised a task of what-where-when memory in rats using an 8-arm radial arm maze. Animals were trained to remember the arms of the maze in which they had previously encountered food which could be recovered at either short (30 minutes) or long (four hours) delays. When only four of the arms were accessible, just one arm contained the preferred chocolate pellets, however, when all arms of the maze were accessible, the four previously inaccessible arms contained the less preferred food pellets. Chocolate pellets were replenished following the long but not the short delay. Rats learned to use the length of the delay as a cue for whether the chocolate arm had been replenished (and therefore would be worth revisiting), and to avoid other arms that had been previously baited. When the chocolate pellets were paired with lithium chloride (a taste aversion treatment) there was a reduction in the number of visits to chocolate-bearing arms. In combination, the authors argue the rats in this study showed memory for what, where and when, the elements of episodic-like memory. Although this study and others (Babb and Crystal, 2006a, 2006b) present evidence for episodic-like memory in rats, it has been argued that the extensive number of training trials required as part of the testing paradigm could result in rule based learning (Cheke and Clayton, 2010; Clayton and Russell, 2009). Episodic-like memory testing paradigms, such as those by Babb and Crystal (2005), therefore experience the same issues associated with the DNMS task previously mentioned, in that performance may be a result of animals applying differing rules to solve the task.

Consequently, Kart-teke, De Souza Silva, Huston and Dere (2006) devised a testing paradigm based on the spontaneous object recognition paradigm in the open field (Ennaceur and Delacour, 1988) to explore what-where-when memory in rats. As the spontaneous exploratory behaviour of the animal is assessed through their preference for novelty, no procedural training is required. The task used by Kart-teke and colleagues involved two sample phases; for the first, the animals were placed individually in the open field with four

copies of an object in particular locations which they could freely explore. The second sample phase followed a 50 minute delay, and again the animals were placed in the open field with four copies of a different object in different locations to those occupied previously. During the test phase that followed, the animals were exposed to two copies of the objects from each of the two sample phases, with one object from each sample phase occupying the same location it previously occupied ('stationary old' and 'stationary recent' objects), and the other object from each sample phase occupying a different but not completely novel, location than previously occupied ('displaced old' and 'displaced new' objects). The rats showed differential exploration for the displaced objects based on whether they were old or recent, suggesting these components interacted, as the authors suggest, through an integrated episodic-like memory of what (the object), where (location of the object) and when (encountered in the first or second sample phase).

However, there is ongoing debate regarding whether such memory tasks based on what-where-when are really taxing episodic-like memory. It has been noted (Easton and Eacott, 2008; Fortin, Agster and Eichenbaum, 2002; Jacobs, Allen, Nguyen and Fortin, 2013; MacDonald, Fortin, Sakata and Meck, 2014; Roberts, 2002; Roberts, Feeney, MacPherson, Petter, McMillan et al., 2008) that such tasks may in fact be solved by reference to relative memory of 'how long ago' an event occurred by keeping track of relative time elapsed since food was cached or encountered in a particular location, rather than the absolute point in time that the event occurred (Roberts, 2002). This sense of how long ago an event took place can be made via, for example, the storing of circadian oscillators with other event information (Crystal, 2006), although the relative strength of memory traces may also play a role (Staddon, Higa and Chelaru, 1999), with strong traces being associated with more recent events (Roberts et al., 2008). If memory trace is being used to define how long ago objects were encountered in the study by Kart-teke et al. (2006), it is possible that degraded memory

strength trace for the least recent objects may account for why they would be preferentially explored during the test phase (Easton and Eacott, 2008). However, Fortin et al. (2002) have argued that strength of memory trace might not provide a sufficient account for why a least recently seen stimulus may be explored more or selected in a choice test. In their study, rats with hippocampal lesions were impaired on a sequential order task in which they had to select odours presented earlier in a sequence, but they were able to successfully perform discriminations between novel and familiar odours. These findings suggest that the hippocampal lesioned rats still had access to information on trace strength differences, but this was not sufficient for successful performance on the sequential order task. For the control rats, who successfully performed on both tasks, this suggests that in order to make judgements around the sequential order of the presented odours, the relative strength of memory for these items was not required. It is, however, worth noting that although the study by Fortin et al. (2002) demonstrates that memory trace strength may not have been required for the control animals' successful performance, it cannot be inferred that in an episodic-like what-where-when task, animals do not use trace memory strength when it is available.

Nonetheless, the definition of episodic memory includes that the memory of an event should be of an absolute point in time rather than a relative point (see Easton and Eacott, 2008). If episodic-like memory in animals is more accurately defined by how long ago an event took place, then there are fundamental differences between human and animal experiences of these types of memories.

1.6.2. What happened, where and on which occasion

One way of defining a point in time is by reference to its absolute temporal reference (when). However, it has been argued that this definition is too restrictive and should be broadened to include any contextual cue that defines the point in time (or occasion) at which

the specific event occurred (Eacott and Gaffan, 2005; Eacott and Norman, 2004). Multiple contextual cues are often used when remembering the occasion when past events occurred, and these cues are not restricted to the specific time when something happened. Non-temporal information may also be used to indicate the occasion in which something happened; for example we may speak of an event which occurred at your graduation ceremony without reference to the date. As such, Eacott and colleagues (e.g. Eacott and Norman 2004; Easton and Eacott, 2008) have proposed a different description of episodic-like memory in animals defined as 'what-where-which occasion' memory, i.e. memory for an object (what), its location (where) and the occasion or context in which it occurred (which).

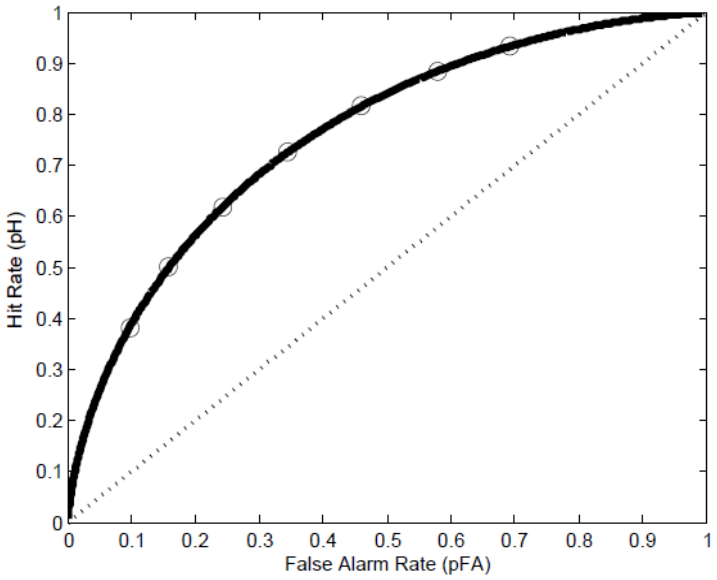
This definition has been used to investigate episodic-like memory in rats (Eacott and Norman, 2004). The authors devised a task in the open field that was a variant on the spontaneous object recognition paradigm (Ennaceur and Delacour, 1988), in which rats were exposed to two objects in a particular background context during the first sample phase (Figure 1.2.c). In the second sample phase, the rats were exposed to copies of the same two objects in switched locations using a different background context. In the test phase of the task, the rats were exposed to two copies of one of the previously seen objects with one of the previously seen background contexts. As such, one of these objects was presented in a location not previously occupied when in that context, resulting in a novel configuration of object-place-context (what-where-which occasion). Intact animals significantly explored this novel configuration more than the familiar one even after a one hour delay. Fornix lesioned rats were impaired on the object-place-context (what-where-which occasion) task even at delays as short as two minutes, though the same animals could perform both object-in-place and object-in-context tasks at the same delays (Norman and Eacott, 2005). This suggests that recognition of a novel configuration of features including objects, locations and contexts is not always hippocampal dependent despite research suggesting that rats with large bilateral

hippocampal lesions are typically impaired in recognition of object and spatial location configurations (Good et al., 2007; Mumby et al., 2002; Save et al., 1992) and of object and background context configurations (Mumby et al., 2002). The study by Eacott and Norman (2004) suggests that the memory processes underlying recognition of object-place-context configurations differ from those required for object-in-place and object-in-context configurations (Eacott and Gaffan, 2005; Langston and Wood, 2010). This task provides a useful measure of episodic-like memory and has been shown to provide insight into the neural correlates of recognition memory. The task does not require any training as with previous episodic-like tasks, and has been successfully used across species (e.g. Davis, Easton, Eacott and Gigg, 2013; Kouwenberg, Walsh, Morgan and Martin, 2009). However, this task remains a recognition task and, despite strong argument that successful performance on this task requires recollection rather than familiarity (Eacott and Gaffan, 2005), it remains difficult to untangle the contributions of familiarity and recall mechanisms to successful performance in the what-where-which occasion task.

1.6.3. Recollection- and familiarity-based processes

One approach to identify the relative contributions of familiarity and recall to recognition tasks has been the analysis of receiver-operating characteristics (ROCs). ROC curves plot hit rate (HR - when a stimulus is correctly identified as being previously encountered) against false alarm rate (FA - misidentifying a novel stimulus as being previously encountered) across a range of response criteria. If a ROC curve deviates upwards from the minor diagonal, this indicates successful recognition (Figure 1.5.a; $p(HR) > p(FA)$).

a.



b.

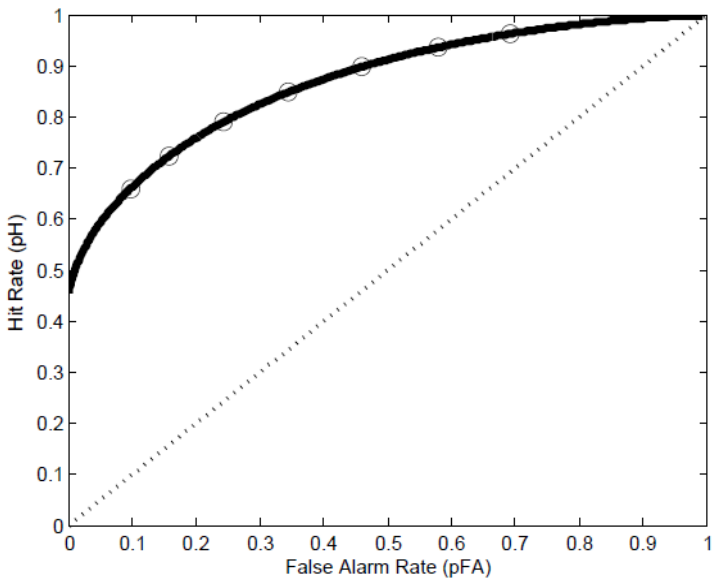


Figure 1.5. Model receiver-operating characteristic (ROC) curves with hit rate plotted against false alarm rate across different criterion levels to illustrate potential performance on a recognition task. a) ROC curve indicates successful recognition as the curve deviates up from the minor diagonal. The curve is symmetrical which can be interpreted as being a result of familiarity-based responses only (dual-process signal detection model) or a result of weak memory (traditional signal detection model). b) ROC curve is asymmetrical with a high y-intercept which can be interpreted as being a result of recollection-based responses (dual-process signal detection model) or a result of unequal variance between old and new item distributions – a sign of strong memory (traditional signal detection model).

Traditional signal detection theory states that recognition responses are based on a single strength variable (Squire et al., 2007), with old items representing high familiarity and new items being low familiarity (all items will have some associated familiarity based on a subjects prior experience). In contrast, dual-process signal detection models (Yonelinas, 1994) state that recognition decisions are based on either recollection- or familiarity-based processes where the shape of an ROC curve can be used to estimate separate measurements of these components. If the curve is asymmetric, with a p(HR) > 0 when p(FA)= 0 (the y-intercept), this can indicate the presence of a linear (all-or-nothing) recollection threshold in addition to a curvilinear familiarity component (Figure 1.5.b). The y-intercept provides a

quantifiable measure of recollection, whereas the measure of familiarity is provided by the degree of curvilinearity in the ROC, equivalent to d' in standard signal detection models.

Fortin et al. (2004) used this approach in an odour recognition task in rats to assess firstly whether there are distinct recollection and familiarity processes in recognition memory, but also to investigate whether the hippocampus is selectively involved in recollection. The ROC for intact rats reflected both familiarity and recollection components, which closely matches the ROC patterns found with human recognition task performance (Yonelinas, 2001). After the animals were split into two groups – one sham group and one group receiving selective hippocampal lesions – the ROC of sham animals did not alter from the previous test. The ROC of the lesion group, however, was fully symmetrical and curvilinear reflecting familiarity-based recognition only. The findings from this study demonstrate not only that recognition memory in this task with intact rats can be based on either recollection or familiarity, but also that the hippocampus appears to be necessary for recollection. The results indicate that animal recognition memory may consist of qualitatively distinct components, as with humans (Morris and Rugg, 2004). ROC analyses can clearly be used to provide evidence of both recollection- and familiarity-based processes, but it is also necessary to obtain behavioural evidence for this dissociation in animals.

To this end, Eacott, Easton and Zinkivskay (2005) developed an episodic-like memory task using the what-where-which occasion descriptor and successfully demonstrated that the task could only be solved using recollection-based rather than familiarity-based processes. Using an E-shaped apparatus, rats were individually exposed to two different objects in particular locations, in a particular background context (Figure 1.6). Rats were then exposed to copies of the previously seen objects in switched locations, and a different background context. The rats were then held in a holding cage with a copy of one of the objects, for the animal to freely explore and become habituated to it. The rats then returned to

the E-maze for the test phase where they were exposed to one of the previously seen contexts, and copies of the two objects presented in the same spatial location as seen in the sample phase that featured that context. When the objects were visible to the animals from the start arm, rats preferentially explored the object that was not presented in the holding cage (i.e. non-habituated object). However, when the objects were no longer visible from the start arm during the test phase (i.e. placed around the corners of the test arms) the animals turned towards the non-habituated (relatively novel) objects at a rate significantly greater than chance. When the objects were visible, the preferential choice for the non-habituated object could be based on relative familiarity alone. The same, however, cannot be said for when the objects were not visible – to make the correct turn towards the non-habituated object the animals need to recollect the prior experience of the object locations in that particular context. The task cannot be solved solely through familiarity mechanisms, but instead rely on memory for what object was found in which spatial location on a particular occasion (represented by the context).

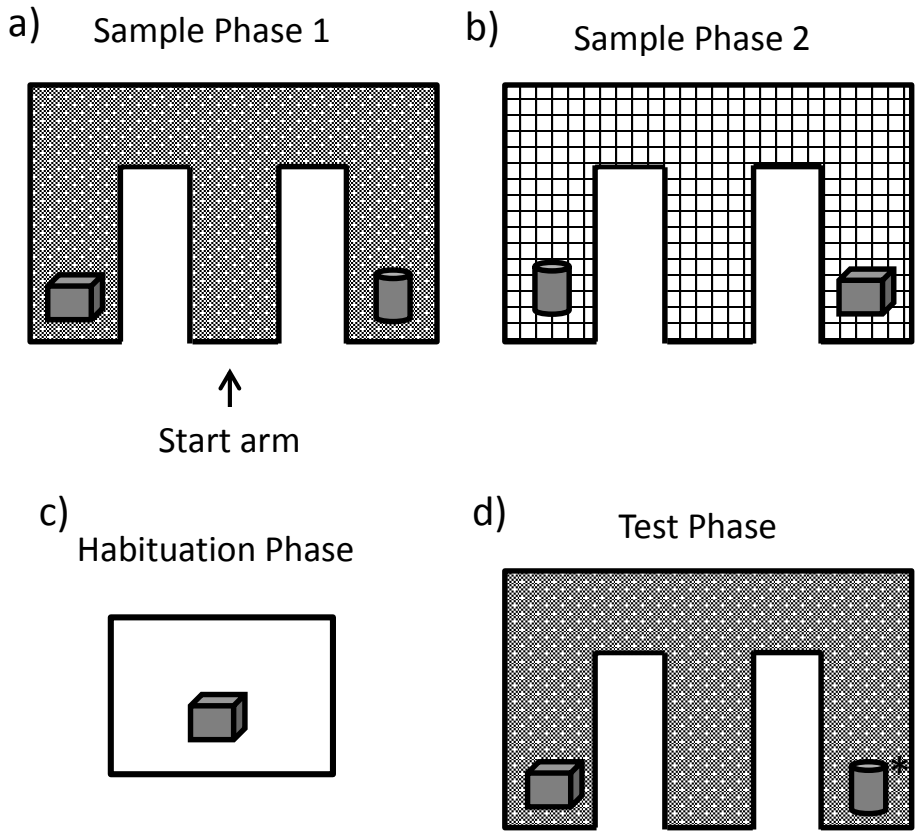


Figure 1.6. Single trial procedure example of the episodic-like memory task, based on what-where-which occasion. In the first sample phase (a), the animals can freely explore the two objects on a particular background context. In the second sample phase (b), a different context is used, and the objects have switched locations. During the habituation phase (c), the animals habituate to one of the objects for a period of eight minutes. In the test phase (d), one of the previously seen contexts is used, with the objects located in the same arms they occupied in the sample phase in which that context was used. In this example, the objects are 'hidden', i.e. not visible to the animals from the start arm. Turn behaviour from the start arm is used as an indicator for recollection, as the animals should preferentially turn towards, and explore, the non-habituated object (indicated by the asterisk). Adapted from Eacott et al. (2005).

In a more recent study, Easton, Zinkivskay and Eacott (2009) investigated performance of fornix lesioned rats on the E-maze task and found that these animals did not significantly seek out the non-habituated object when the objects were not visible from the start arm, in contrast to sham lesioned animals. The fornix lesioned rats were, however, able to demonstrate normal recognition performance measured through discrimination between

the habituated and non-habituated objects at test. These findings suggest that the fornix lesioned rats had impaired recollection (demonstrated through their inability to make correct turns towards objects which are not currently visible), but intact familiarity mechanisms which supported normal recognition performance. This might seem to contrast with the work by Eacott and Norman (2004), in which fornix lesioned rats were reported to be impaired in the open field what-where-which occasion task, whereas animals with the same lesions were not impaired in the recognition measure of the E-maze task. As noted by Easton et al. (2009) these different reports may be accounted for by the procedural differences between the two testing paradigms – in the test phase of the open field task, rats are exposed to two copies of the same objects, and their preferential exploration is based upon memory for what object they have explored, the objects' spatial location and background context. The task can, therefore, only be solved using episodic-like memory. During the test phase of the E-maze task, on the other hand, rats are exposed to two different, previously explored objects, although one has been habituated. Recognition is not reliant upon episodic-like memory in this measure – only object preference is needed. As previously discussed, the hippocampus appears to play a role in spatial recognition memory (Bussey et al., 2000; Mumby et al., 2002; Save et al., 1992). Easton et al. (2009) considered the possibility that the fornix lesion impairment observed in the E-maze task may be a result of a spatial memory deficit (i.e. a single component of what-where-which occasion memory), rather than failure of the integrated episodic-like memory. For instance, it may be possible that no deficit in episodic-like memory occurred following the fornix lesions, but the animals were not able to navigate to the correct object location; the result of a spatial deficit, which is one component that integrates with other components to form an integrated episodic-like memory (Clayton and Dickinson, 1998). However, as noted by Easton et al. (2009), the fornix lesioned animals demonstrated no impairment in object memory, as they displayed normal levels of object

exploration and object preference. When individual components of what-where-which occasion were tested in the open field, animals with fornix lesions (Eacott and Norman, 2004) or hippocampal lesions (Langston and Wood, 2010) were able to successfully perform tasks involving recognition of object and spatial location configurations. It could be therefore inferred that any fornix lesion deficit on the what-where-which occasion open field task (Eacott and Norman, 2004) is not the result of a failure in spatial memory. The spatial demands in the E-maze what-where-which occasion tasks may be higher, but it would be difficult to attribute fornix lesion impairment solely to a spatial deficit, as it may be possible that recall of spatial information can account for poor performance, with recall also being dependent on the hippocampus (Aggleton and Brown, 1999; Yonelinas, 2001).

1.7. Translating recognition memory research to humans

The spontaneous tasks of what-where-when and what-where-which occasion are both tests of episodic-like memory in non-human animals that do not rely on evidence of conscious recollection (autoneotic awareness). Human episodic memory, however, is specifically associated with conscious recollection of an event in one's life, and thus the correspondence between the work with non-human animals and tests of human episodic memory has been questioned. Developing well-controlled behavioural methodology with animals has been necessary due to the inability to question animals about their memory experience. To be able to adopt more well-controlled tasks to study human memory will provide opportunities in some instances, but not all, for human studies to replace animal studies to assess process.

Recent studies have examined human performance on episodic-like tasks using content-based descriptors of what-where-when or what-where-which occasion (Easton Webster and Eacott, 2012; Holland and Smulders, 2011). Such studies are important to

validate the episodic memory models developed from the animal work, and to improve the translation of well-controlled behavioural work in animals to humans.

1.7.1. Episodic-like memory tasks in humans

The episodic memory descriptor of what-where-when (recall of what happened, where and when) has been extensively used in animal memory research when designing behavioural tasks of episodic-like memory. Holland and Smulders (2011) investigated whether human participants use episodic memory in an episodic-like memory task similar to one previously used with animals (Zinkivskay et al., 2009). Participants were asked to hide two types of items on each of two separate occasions and they were then tested for their memory of what was hidden, where and when. Participants were asked how they recalled the information, i.e. did they remember or did they know. Remembering is associated with recollection of an event, reflecting episodic memory, whereas knowing gives a sense of familiarity, which is not episodic (Yonelinas, 2001). Participants in this task mainly reported their recollective experience as being one of remembering, suggesting that episodic memory was used to solve the what-where-when task. However, it is unclear whether task performance was related to the participant's subjective experience of remembering.

A recent study by Easton et al. (2012) investigated performance of human participants on recognition memory tasks used to assess episodic-like memory in animals using both the what-where-when (Clayton and Dickinson, 1998) and what-where-which (Eacott and Norman, 2004) descriptors of episodic memory. Crucially, the study by Easton and colleagues also assessed the subjective experience associated with task performance. The task was closely modelled on those used with non-human animals, and involved viewing two sequentially presented screens that consisted of a number of symbols in different locations on a distinctive background. Locations of the symbols changed between screens. Memory for

either the identity (what) or location of the symbols was tested, with location being prompted by cueing to the first or second screen (what-where-when), or to the distinctive background in which it was presented (what-where-which occasion). Participants also reported their subjective experience for each judgement as being "remember", "know" or "guess". The results suggested that object recognition questions (what) could be answered accurately using either recollection- or familiarity-based processes, but the episodic questions based on what-where-which occasion could only be accurately answered using recollection; episodic questions based on what-where-when could be answered correctly using either recollection or familiarity. This is contrary to reports in the animal literature whereby the what-where-when task is claimed to be dependent on episodic-like memory (e.g. Babb and Crystal, 2005; Clayton and Dickinson, 1998), and therefore only recollection processes. However, as discussed above, what-where-when tasks may be vulnerable to the use of non-episodic strategies such as familiarity-based trace strength information (Roberts et al., 2008). Indeed, a similar dissociation between performance on what-where-which occasion memory and what-where-when memory has also been recently reported in transgenic mice with pathology which selectively affects episodic-like memory (Davis, Eacott, Easton and Gigg, 2013). These results together suggest that what-where-which occasion episodic-like memory tasks for non-human animals may most closely mimic human episodic memory tasks.

1.7.2. Analysis of receiver-operating characteristics

It is still debated as to whether animals remember specific personal experiences in the same way that humans experience memories of retrospective events, or whether they are more simply able to remember the facts relating to an event (in a semantic fashion) without connecting that memory to a personal experience (Roberts, 2002). With current studies on human memory, phenomenology, such as conscious recall of an event, often takes precedent.

In animals, researchers cannot demonstrate such introspection, and therefore cognitive process is inferred from careful control of behaviour. Although the studies by Holland and Smulders (2011) and Easton et al. (2012) are important in promoting the translation of animal work on episodic memory to humans, it is also important to move away from relying on the phenomenological experience of human participants to validate episodic-like tasks.

ROC analysis has been used to distinguish between recollection- and familiarity-based processes in recognition tasks with humans using the remember/know paradigm (Yonelinas, Kroll, Dobbins, Lazzara and Knight, 1998). With this approach, recognition confidence responses are collected alongside the number of correct responses. A recent study from our lab used ROC analysis to distinguish between and quantify the degree of recollection and familiarity components of recognition memory in human participants, in an object recognition memory task consisting of multiple conditions that are analogous to the spontaneous recognition tasks previously used with animals. Using the content-based episodic descriptor of object-location-context (what-where-which occasion) we have been able to show that the degree of recollection is significantly higher when both an object's location and context are congruent across encoding and retrieval phases of the task, relative to when only location (object-location recognition memory) or context (object-in-context recognition memory) is congruent (Ameen-Ali, Norman, Eacott and Easton, unpublished; see Chapter 5). This study is an example of how the behavioural work used in developing animal models can be used to inform human experiments and promote better translation of studying memory process in animals to humans, without relying on phenomenology. The advantage of a task such as ours is that it removes any introspection from the participant, which is often a key component of episodic memory tasks.

1.8. Conclusion

Spontaneous object recognition tasks have contributed greatly to our current understanding of the neurobiological basis of recognition memory, and the value of these tasks is not doubted. Despite some ongoing debate centred around particular methodological issues (Ennaceur, 2010), these tasks are very simple to administer with no required pretraining or reinforcement required. This has allowed much recognition memory research to be carried out with animals without results being confounded by potential rule acquisition or motivational issues.

Studies clearly support the view that the perirhinal cortex is necessary for object recognition memory, and plays some role in the conjunction of objects and their location and context. The role it plays in the conjunction of these features appears to be sensitive to factors such as lesion size and the feature ambiguity of the stimuli used. The contribution of the hippocampus to object recognition memory is not so clear, but evidence seems to indicate that for familiarity-based recognition the hippocampus is not essential. There is a great deal of research supporting the view that the hippocampus plays a critical role in episodic memory (Aggleton and Brown, 1999; Eichenbaum, 2000; Eichenbaum, Dudchenko, Wood, Shapiro and Tanila, 1999; Mishkin, Suzuki, Gadian and Vargha-Khadem, 1997; Morris and Frey, 1997; O'Keefe and Nadel, 1978; Tulving and Markowitsch, 1998). Research is, however, ongoing to investigate how the perirhinal cortex and hippocampus interact along with other brain structures to mediate the integration of information for other more complex forms of memory. The hippocampus may be involved in integrating object information supplied by the perirhinal cortex, and spatial and contextual information processed by the postrhinal cortex. Such integration in the hippocampus may lead to the formation of episodic memories (Bussey and Aggleton, 2002; Eacott and Gaffan, 2005; Eichenbaum et al., 2007).

When considering the animal research dedicated to investigating episodic-like memory in animals, a valid argument is made for defining the content as what happened on a specific occasion rather than a particular time defined by temporal order or time elapsed. Replacing the descriptor 'when' with 'which' allows for both a point in time to be specified in the episodic memory, but also other non-temporal cues to identify that point, which may be just as crucial.

Research on recognition memory continues to encompass work with humans and various animal species, but most notably non-human primates and rodents. Recognition memory tasks continue to develop in terms of how animal behaviour is assessed, but also in the neurobiological techniques that can be applied alongside them. The development of new testing paradigms with the multiple trial approach maintains the advantages of being able to assess an animal's spontaneous behaviour, but reduces the variability in behavioural performance that this is often associated with. The use of such paradigms will allow key questions to be answered about recognition memory function when applied with the lesion approach, IEG imaging or electrophysiology techniques, and research is beginning to look at different forms of memory to see how the multiple trial paradigm can be utilised. In addition, widespread use of the multiple trial paradigm can have significant implications for the 3Rs (Replacement, Refinement and Reduction), which is important for all animal research, as the reduction in animal numbers required using this paradigm has been demonstrated (Ameen-Ali et al., 2012). The use of spontaneous tasks continues to be essential for use in basic and pre-clinical research into the neural basis of memory, and animal studies remain an important contribution for informing recognition memory studies with humans.

1.9. Thesis aims and hypotheses

The main objectives of this thesis centred around improving the methodology used for assessing recognition memory in animals and humans. Firstly, the aim was to develop a reliable testing method for use with rodents, which would reduce the variance often associated with spontaneous recognition tasks, and therefore reduce the number of animals required for statistically meaningful results. It was predicted that animals would significantly explore the novel objects, or novel configurations of objects and an aspect of the environment, more than the familiar objects, or familiar configurations. As the continual trials apparatus allows for multiple trials to be carried out within a single testing session, it was hypothesised that fewer animals (~ n = 6) would be required for significant recognition to be displayed. This is approximately half the number of animals than would be typically used in spontaneous recognition tasks, but should be sufficient, as the animals perform a greater number of trials, the noise in the data is therefore reduced.

A series of behavioural experiments were also carried out to investigate different behavioural parameters on a simplified version of the E-maze episodic-like memory task, using the continual trials apparatus. In this series of experiments, animals' preference for non-habituated objects over habituated objects was assessed. All objects in a test session were either baited on non-baited with food pellets, and the length of time habituating to an object (to induce a preference for the non-habituated object) was varied. It was hypothesised that the strongest indication of object preference (through significant preferential exploration of the non-habituated object) would be apparent when all of the objects were baited with food (as this would encourage exploration of all the objects), and in the condition with the longest habituation period, as this should increase the familiarity of the habituated object, and drive exploration towards the non-habituated object at test.

The second main aim of the thesis was to investigate how effectively the continual trials apparatus could be applied with immediate-early gene (IEG) imaging to offer insights in to the neural mechanisms underlying different forms of recognition memory. A series of behavioural tasks were first carried out to assess performance on spontaneous object recognition and object location tasks when animals were tested on novel or familiar objects. The spontaneous object recognition task was then used with IEG imaging, and it was hypothesised that the group of animals with previous exposure to the test objects (Group Familiar) would have significantly lower c-fos expression relative to the group of animals with exposure to a set of objects different to those at test (Group Novel), and a group of animals with no prior object exposure (Group Naïve). The differences in c-fos expression would be significant in the perirhinal cortex, as research suggests a role in the detection of novelty (Albasser, Poirier et al., 2010).

The final main objective of the thesis was to develop a recognition memory task for humans that was analogous to those used with rodents, with a reduced reliance on subjective experience. The analysis of receiver-operating characteristics (ROCs) was used to dissociate between familiarity- and recollection-based processes across a range of recognition conditions (standard object recognition; object-location; object-context; object-location-context). It was hypothesised that significantly greater recollection would be elicited in the object-location-context condition relative to the other recognition memory conditions, as this condition, based on the animal work, reflects 'episodic-like' memory, which requires recollection.

CHAPTER 2

STUDY 1: REDUCING ANIMAL NUMBERS IN MULTIPLE TYPES OF SPONTANEOUS OBJECT RECOGNITION PARADIGMS

2.1. Introduction

Delayed non match to sample (DNMS) has been widely used as a test of recognition memory in both monkeys (e.g. Eacott, Gaffan and Murray, 1994; Mishkin and Delacour, 1975) and humans (e.g. Holdstock Mayes, Cezayirli, Isaac, Aggleton et al., 2000) in order to understand the neural basis of memory. Whilst versions of DNMS tasks have been used with rodents, difficulties relating to training and performance levels are of concern in these paradigms (Aggleton, 1985; Mumby, Pinel and Wood, 1990; Prusky, Douglas, Nelson, Shapanpoor and Sutherland, 2004; Steckler, Drinkenburg, Sahgal and Aggleton, 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 years between 2007 and 2012, drawn from 31 subject areas (source Web of Science, April, 2012) which include the terms "spontaneous object recognition" or "novel object 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 five 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, Alvarado and Bachevalier, 2004; Pascalis, Hunkin, Holdstock, Isaac and Mayes, 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, Enjuanes, Morillon and Drieu la Rochelle, 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, Long, Liu, Qu, Chen 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, Cho, Kerr, Barredo, Alleyne 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, Chapman, Amin, Iordanova, Vann 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 the 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 (Figure 2.1) 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, while 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 chapter 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, Chapman 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 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.

2.2. Materials and methods

2.2.1. 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-hr light-dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study, except during habituation and testing. 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.

2.2.2. 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 (Figure 2.1). The apparatus was 59cm long and 59cm wide.

Opaque guillotine doors divided the two areas (outer arm doors: 12cm; central arm door: 24cm) 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 (Figure 2.2) approximately 2cm away from the two walls, to allow the animals to get their heads around the objects and explore them fully.

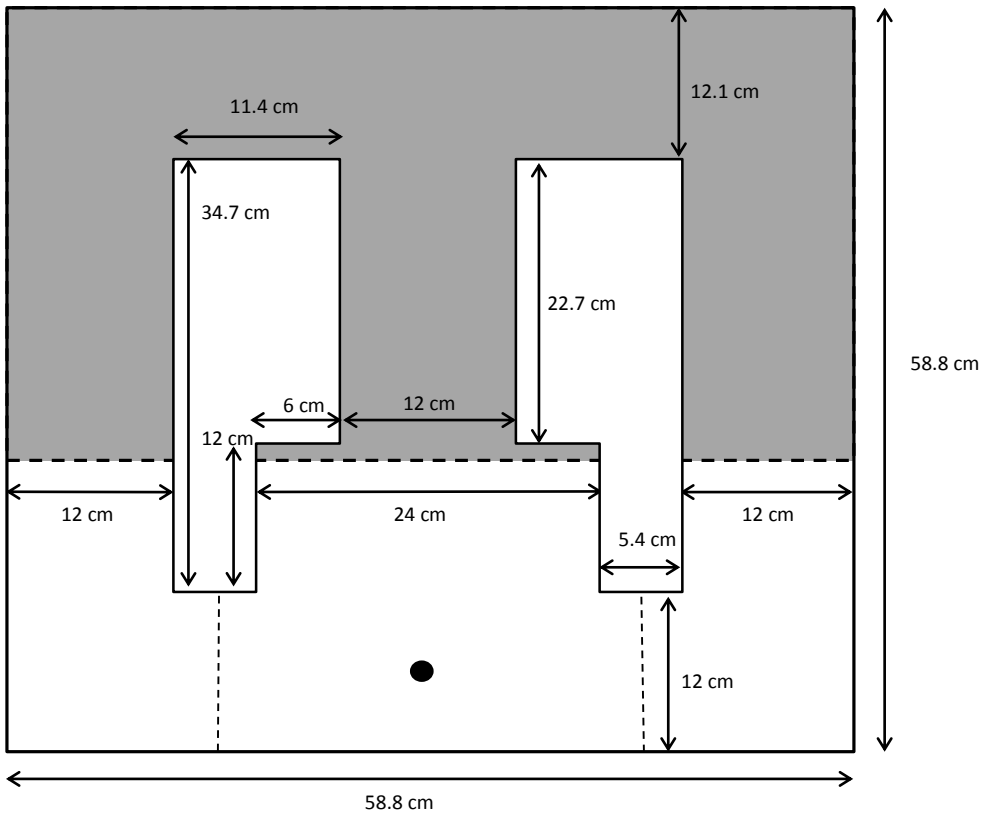


Figure 2.1. The shape and dimensions (in cm) of the continual trials apparatus from the view above (to scale). The grey area represents the object area which is rotatable to reveal different contexts; four in total. The white area represents the holding area, which is stable, with a black circle to represent 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.

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 (see Figure 2.2); context 3- black and white horizontal stripes with a hatched wire surface; context 4- black and white vertical stripes with a hatched wire surface.

2.2.3. Objects

Each experiment used various junk objects of different sizes, shape, colour, and texture. The size of the objects were no larger than ~18cm in height and ~10cm in width (see Figure 2.2. for examples). 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.

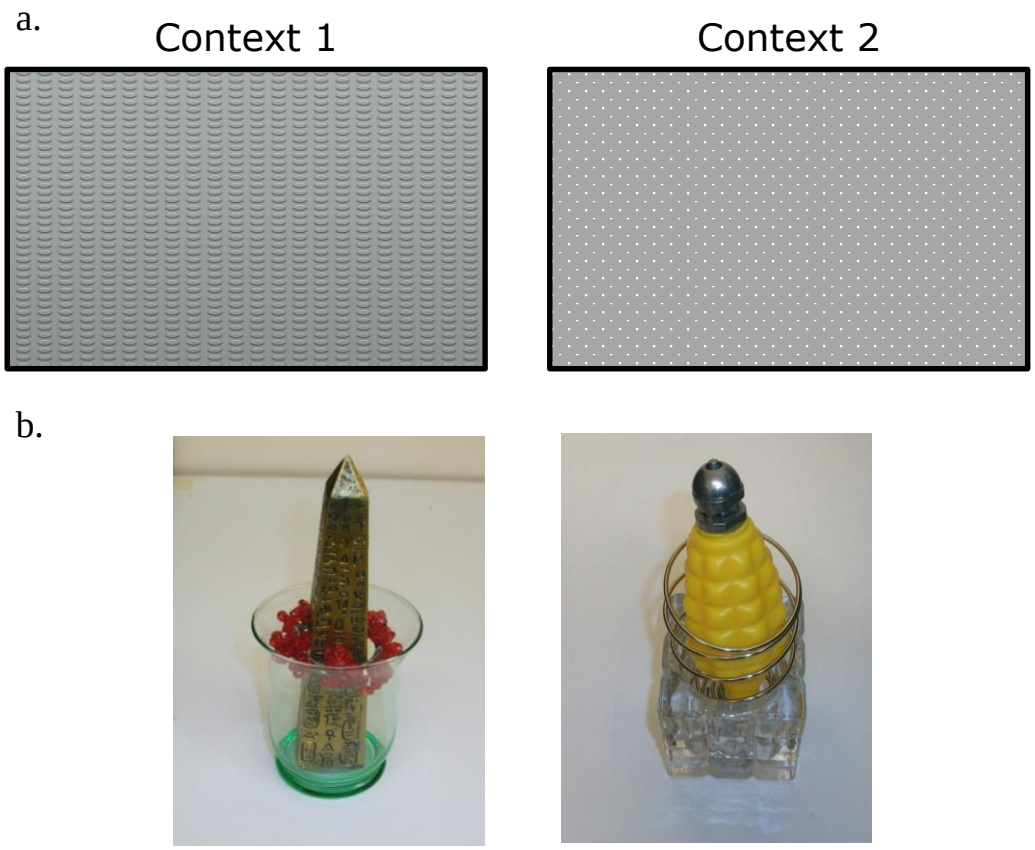


Figure 2.2. a) An illustration of two of the background context patterns used. b) Two examples of objects used in the current study.

2.2.4. 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 minutes per session in order to acclimatise to the room. The light in the test room was produced solely by a 20W 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 lasted approximately five 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 minutes, allowing free exploration. For Phase 2, the animals were placed into the apparatus singly for 20 minutes again for free exploration. For Phase 3, this was repeated but for only 10 minutes. 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 (20mg, Purified Diet; BioServ, Frenchtown, New Jersey, 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 three minutes 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 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.

2.2.5. Behavioural analysis

Exploration of objects was defined as when the nose of the animal was <1cm 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. The novel and familiar exploration times were cumulatively added after each trial for each animal, and the D2 score was recalculated to create 'updated' D2 scores (Albasser, Chapman et al., 2010). Trial by trial D2 scores were also calculated by averaging the novel and familiar exploration times after each trial and recalculating the D2 score. These measures were used to illustrate performance over a session. 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.

2.3. Experiment 1: Spontaneous object recognition

2.3.1. Subjects

Six Lister hooded rats supplied by Harlan UK housed in pairs in diurnal conditions (12-hr light-dark cycle), with testing carried out during the light phase. Water was available ad libitum throughout the study, except during habituation and testing. 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 four months old and weighed from 430-520g.

2.3.2. 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 Figure 2.3.a). 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 two minutes exploring the objects (two copies of object A) in the object area. After two minutes, 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 one minute, 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 two minutes, 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, Chapman 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 three minutes. 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.

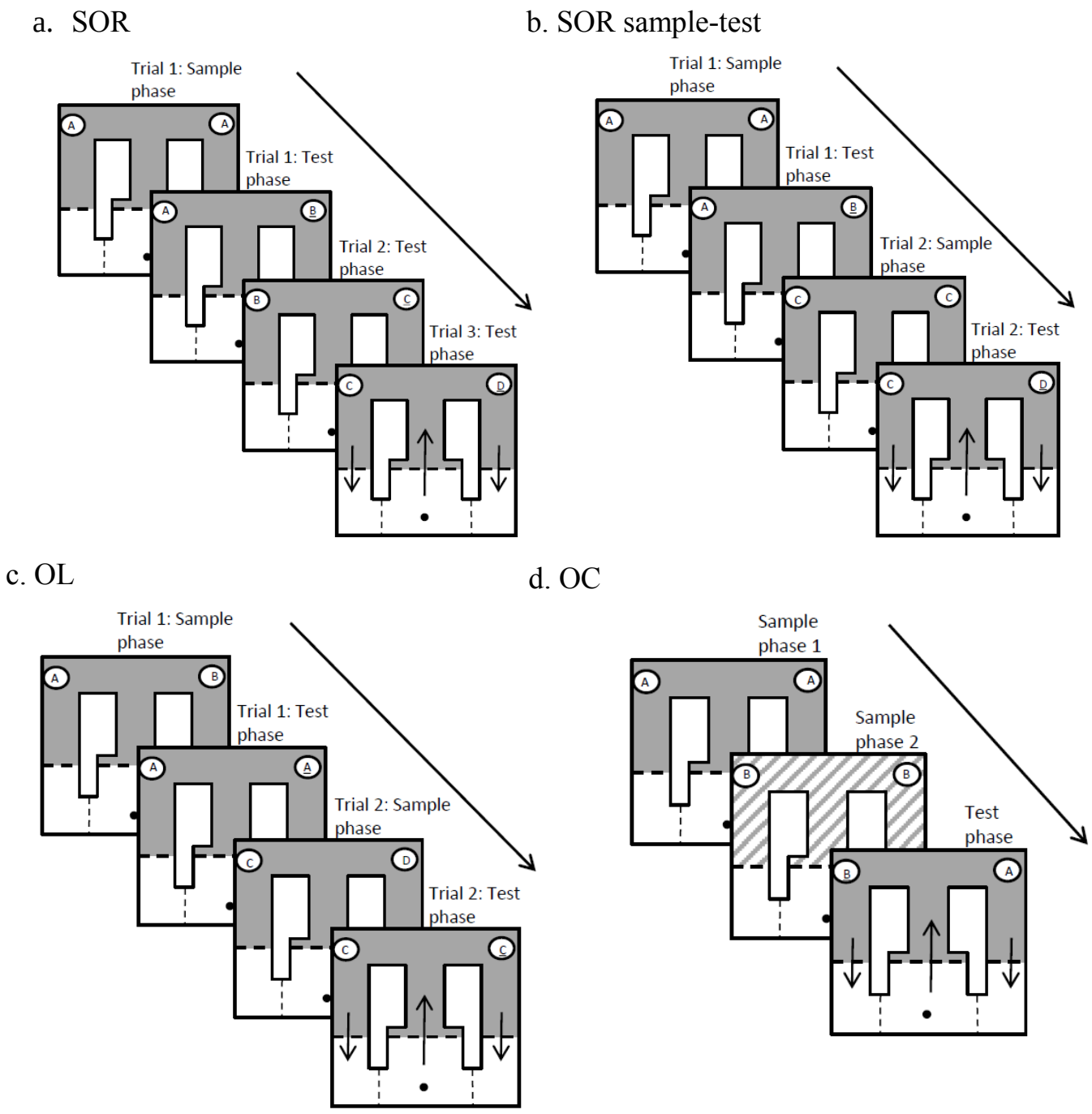


Figure 2.3. An illustration of the test procedures for experiments 1-4 with examples of the order of object presentation. a) spontaneous object recognition (SOR). b) spontaneous object recognition with a sample phase prior to each test phase. c) object-location recognition memory (OL). d) object-in-context recognition memory (OC). The arrows indicate the direction of the rats' movement from the holding area to the object area via the central arm door, and then, two minutes 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.

2.3.3. 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; $t(4) = 9.822$, $p = 0.001$; Figure 2.4) showing clear discrimination of the novel from the familiar stimuli. Figures 2.5.a and 2.5.b illustrate 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 score 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 score: 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 = 27sec; displaced familiar object trials mean total exploration = 30sec), although greater mean D2 scores were shown in trials where familiar object location was static (static familiar object trials mean D2 score = 0.5; displaced familiar object trials mean D2 score = 0.4).

A post-hoc power analysis was conducted with the program *G*Power 3* (Erdfelder, Faul and Buchner, 1996; Faul, Erdfelder, Lang and Buchner, 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 calculated power of 0.99 from a sample size of 11 subjects, thus demonstrating that in the current experiment, the spontaneous object recognition task had a statistical power comparable to a previous study but from a smaller sample.

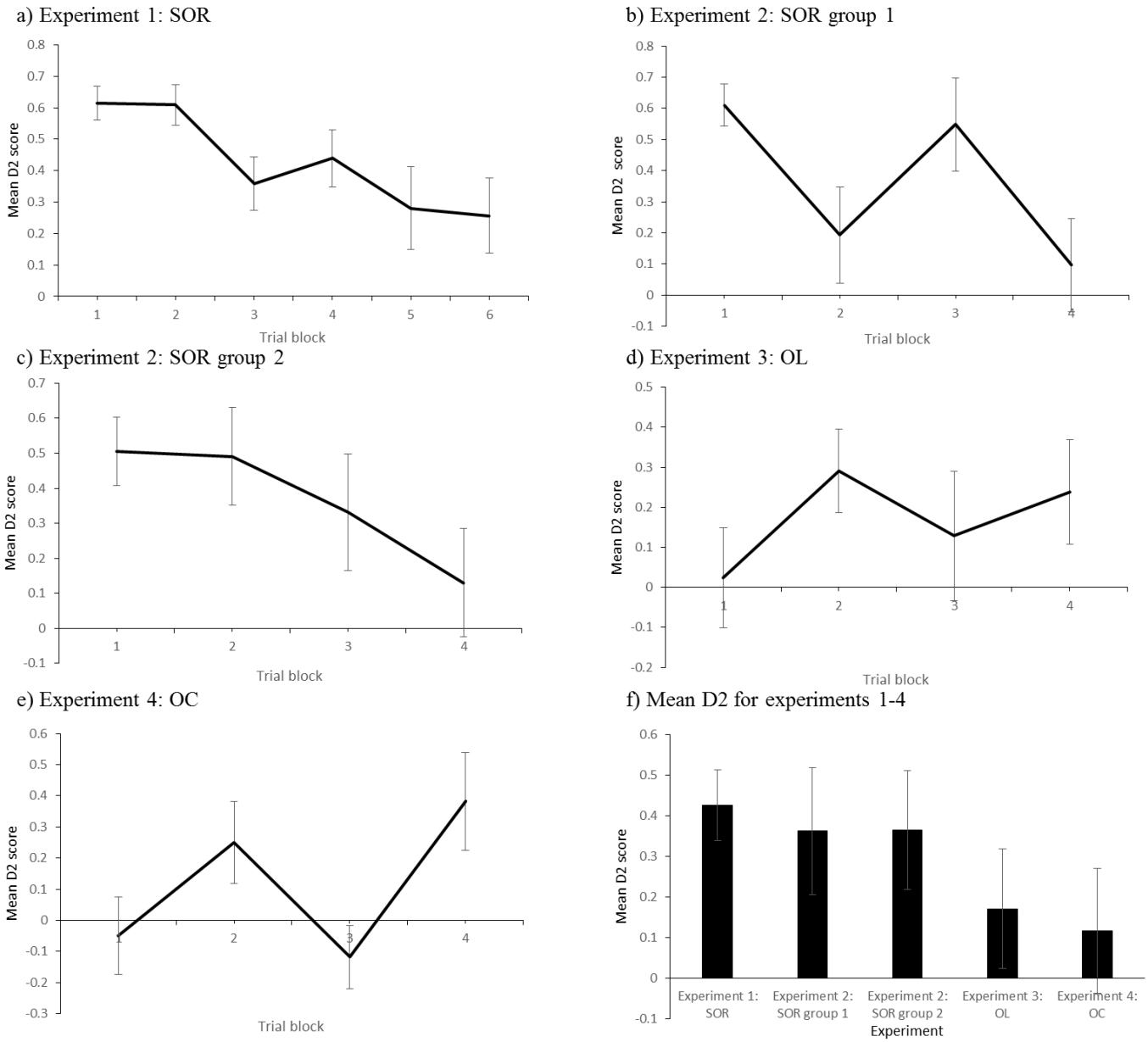


Figure 2.4. Mean D2 scores plotted throughout the testing sessions for experiments 1 to 4. Vertical bars show the standard error of the mean. a) Mean D2 scores for experiment 1 (SOR) across 30 trials, with D2 averaged across 5 trials creating 6 blocks. b) Mean D2 scores for experiment 2 (SOR) group 1 across 16 trials, with D2 averaged across 4 trials creating 4 blocks. c) Mean D2 scores for experiment 2 (SOR) group 2 across 16 trials. d) Mean D2 scores for experiment 3 (Object-Location) across 16 trials. e) Mean D2 scores for experiment 4 (Object-in-Context) across 16 trials. f) Mean D2 scores for experiments 1-4.

2.3.4. Discussion

The current experiment was a replication of the task procedure used by Albasser, Chapman 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, Chapman et al. (2010)'s 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 continued 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 clear evidence that performance considerably changed 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, Chapman et al. (2010), some trials involved the familiar object appearing in a novel location while on others it was seen in the same location as previously. While 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 were naïve.

2.4. Experiment 2: Sample-test object recognition

2.4.1. 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 two months old and weighed from 240-270g.

2.4.2. 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 Figure 2.3.b). 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.

2.4.3. 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 score = 0.4; $t(4) = 5.410$, $p = 0.006$; group 2: mean D2 score = 0.4; $t(3) = 15.603$, $p = 0.001$; Figure 2.4). Figures 2.5.c to 2.5.f illustrate 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 while 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 (mean D2 scores: $p = 0.968$; mean 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).

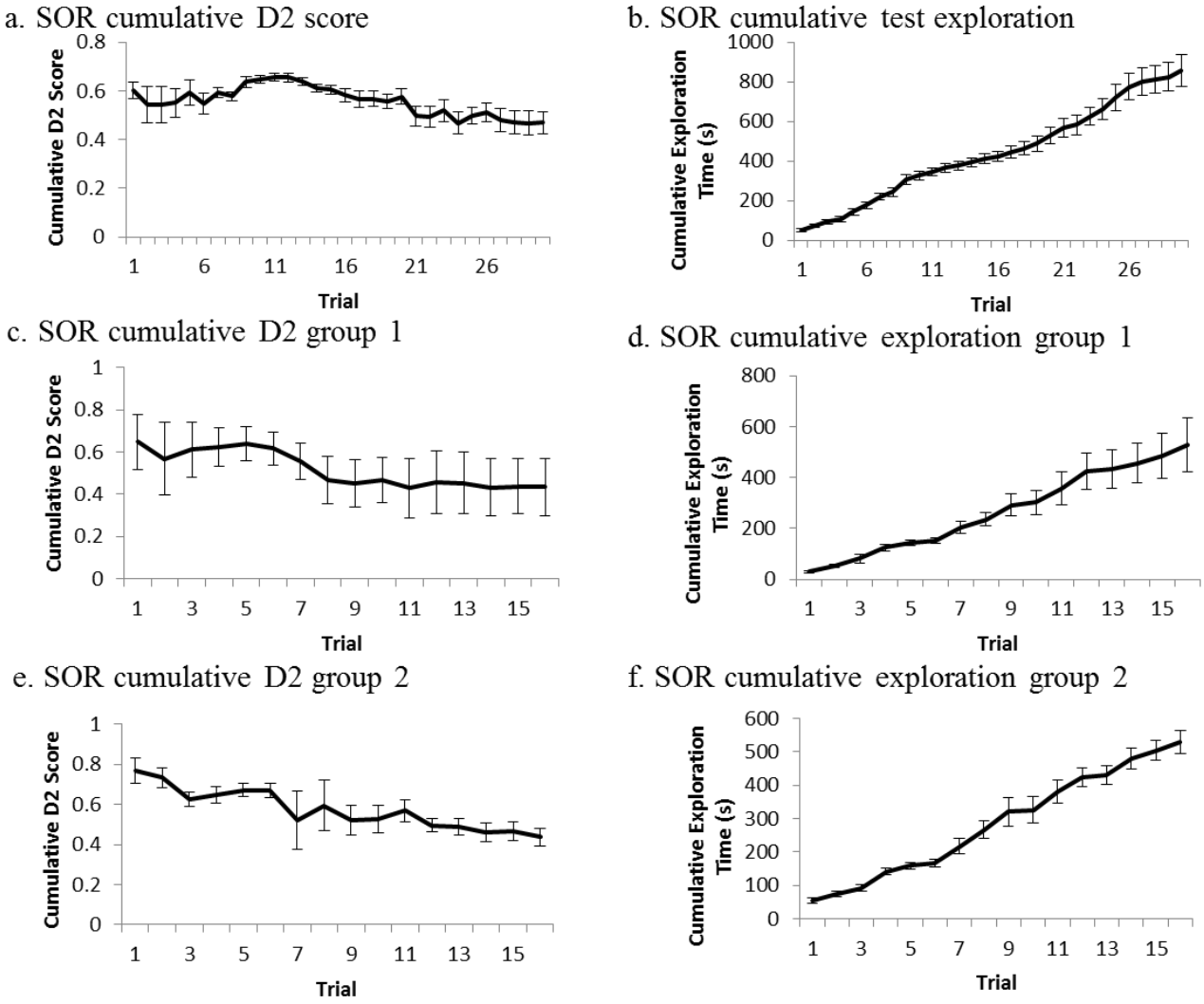


Figure 2.5. Graphs from experiments 1 and 2 depicting animal performance. Vertical bars show the standard error of the mean. a) Cumulative D2 scores for experiment 1 (SOR) across 30 trials. b) Cumulative exploration time for experiment 1. c) Cumulative D2 scores for experiment 2 (group 1) across 16 trials. 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.

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.

2.4.4. 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, Chapman 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 (Figures 2.5.a and 2.5.b) and Experiment 2 (Figures 2.5.c-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.

2.5. Experiment 3: Object-location memory (what-where)

2.5.1. 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.

2.5.2. Pretraining

Animals were habituated to their environment prior to Experiment 1 which lasted approximately five days (see Section 2.2.4). 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 minute session each of shuttling between the two areas of the apparatus and an object training session (see Section 2.2.4 for details).

2.5.3. 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 & B) for two minutes (see Figure 2.3.c). 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 one minute 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 & 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.

2.5.4. 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 score = 0.2; $t(4) = 5.321$, $p = 0.006$; Figure 2.4). Figures 2.6.a and 2.6.b illustrate 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 a very much smaller group size.

2.5.5. 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).

2.6. Experiment 4: Object-in-context memory (what-which)

2.6.1. 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.

2.6.2. Pretraining

Animals were habituated to their environment prior to Experiment 2 which lasted approximately five days (see Section 2.2.4). 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); and object habituation with the two new contexts (phase 3; see section 2.2.4 for details on these procedures).

2.6.3. 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 & A) in a particular context (X) for two minutes (see Figure 2.3.d). 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 one minute, 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 & B) in a different context (Y) for two minutes (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 one minute, 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 & 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.

2.6.4. 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 score = 0.1; $t(4) = 3.03$, $p = 0.039$; Figure 2.4). Figures 2.6.c and 2.6.d illustrate 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 score was calculated for each block derived from the individual D2 scores within that block. Using a 2 (session) x 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: $F(1, 4) = 0.284$, $p = 0.623$).

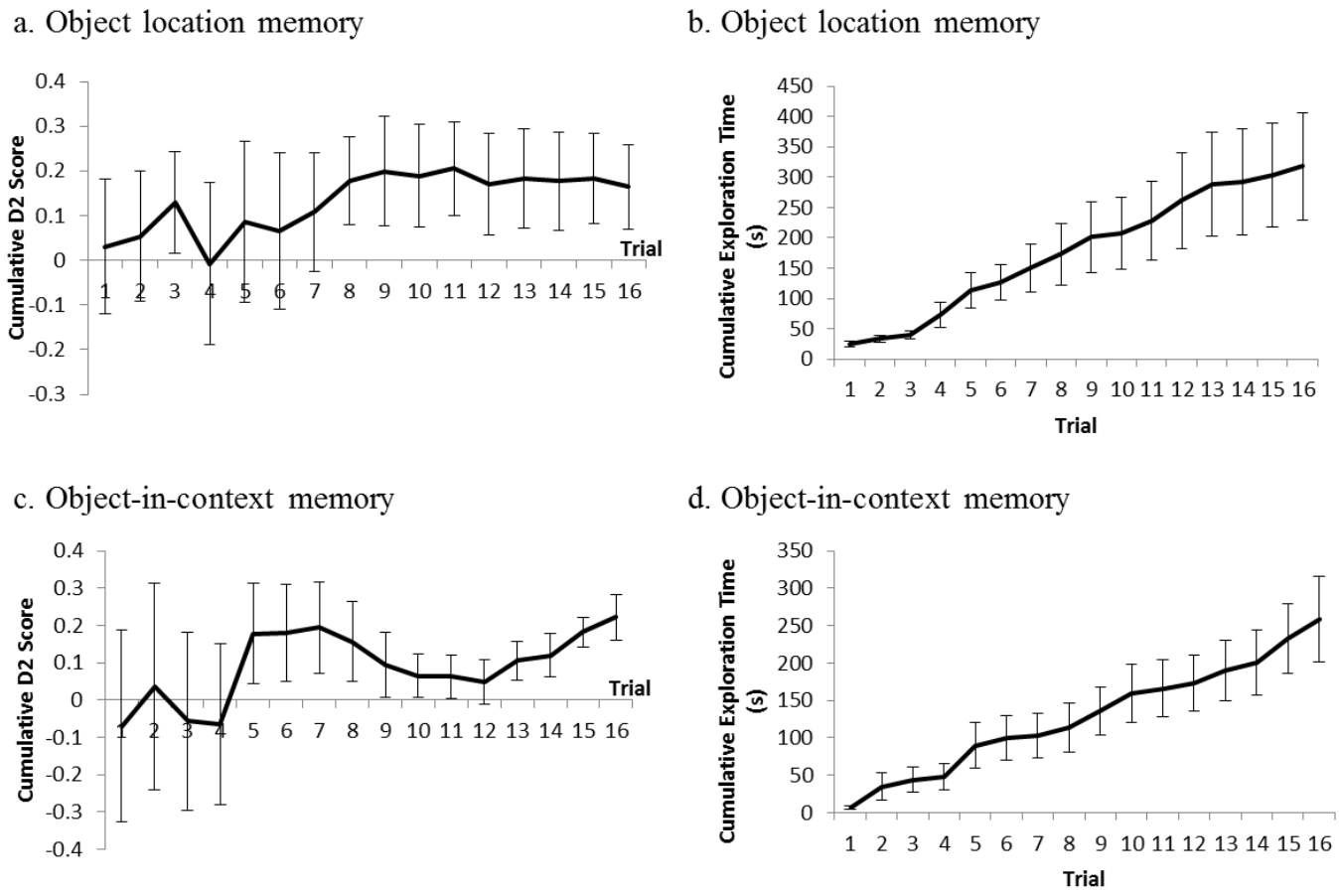


Figure 2.6. Graphs from experiments 3 and 4 depicting animal performance. Vertical bars show the standard error of the mean. a) Cumulative D2 scores for experiment 3 across 16 trials. b) Cumulative exploration time for experiment 3. c) Cumulative D2 scores for experiment 4 across 16 trials. d) Cumulative 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.

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 was 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.

2.6.5. 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 score = 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 performance 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 (Figure 2.6.c) 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 chapter there was a one minute 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 two minute interval was implemented between sample phases, with a two minute 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. While these task differences mean there is potentially scope for further studies improving performance in this task, 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.

2.7. 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 experiments was to develop versions of spontaneous recognition tasks which use fewer animals than the standard versions. While this aim was achieved, in that good results were found with a smaller number of animals analysed, it is true that the results from two of 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), while 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 experiments 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, Chapman et al., 2010). While 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. While 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 five 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 minutes giving a total testing time of 540 minutes. 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 minutes giving a total testing time of 120 minutes 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, Amin, Iordanova, Brown, Pearce 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, Easton and Zinkivskay, 2005; Easton, Zinkivskay and Eacott, 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 chapter 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.

CHAPTER 3

STUDY 2: ELUCIDATING THE BEHAVIOURAL PARAMETERS FOR OBJECT PREFERENCE

3.1. Introduction

Researchers have looked at utilising the spontaneous object recognition paradigm to develop tasks to measure episodic memory; a type of memory that specifically relates to one's past life experiences.

Episodic-like memory tasks based on the what-where-which occasion descriptor have been developed for use with rodents in the open field (Eacott and Norman, 2004), but more recently work has investigated whether processes that are deemed to underlie recognition memory (i.e. recollection and familiarity) can be behaviourally dissociated in an E-maze version of this task. Eacott, Easton and Zinkivskay (2005) developed a task for rodents that could only be solved using recollection-based rather than familiarity-based processes. Using an E-shaped apparatus, the task procedure for a single trial involved placing each animal individually in the central arm before a sample phase commenced. In the sample phase of the trial, each animal was exposed to two different objects, in particular locations on a particular background context. The objects were placed in the left and right corners at the top of the two outer arms and were thus visible to the animals from the start arm. In the second sample phase, the animals were exposed to identical copies of the two previously seen objects but in switched locations, and presented on a different background context. Following this, each animal was held in a holding cage, with no other animals present, with a copy of one of the previously seen objects, for a period of eight minutes. Finally, for the test phase of the procedure, the animals were exposed to one of the previously seen contexts and identical copies of the two previously seen objects, in locations congruent with the sample phase. Rats

preferentially explored the non-habituated object at test, clearly demonstrating recognition for the habituated object that was relatively more familiar. When the objects at test were placed at the end of the outer arms of the maze, so they were no longer visible to each animal from the start arm, the turn behaviour of each animal was recorded, and the animals turned towards the non-habituated (relatively novel) objects significantly more often than what would be predicted by random turning behaviour. When the objects at test were visible from the start arm, preferential choice for the non-habituated object could be based on familiarity for the habituated object (Eacott et al., 2005). When the objects at test were not visible from the start arm, it would not be possible for the animals to make the correct turn towards the non-habituated object purely through familiarity-based mechanisms; it would be necessary for the animals to recollect the prior experience of the previous locations of the objects when they had been presented on that background context in order to make the correct turn (Figure 3.1).

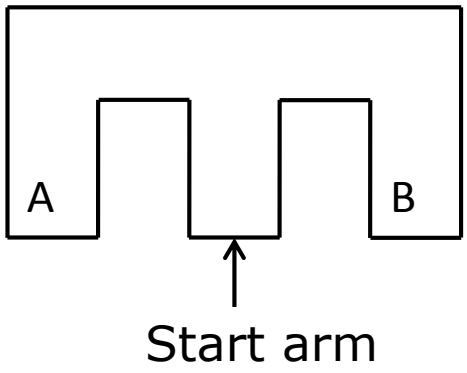


Figure 3.1. E-maze depicting the 'objects hidden' phase of a trial. The animal is placed in the start arm of the maze and the objects at test are placed at the end of the outer arms of the maze, so they are not visible to the animal from the start arm. The turn behaviour of each animal is recorded. Adapted from Eacott et al. (2005).

Fornix-lesioned animals were able to demonstrate normal recognition performance through successfully discriminating between the habituated and non-habituated objects (Easton, Zinkivskay and Eacott, 2009). However, they were unable to successfully seek out

the objects when they were not visible from the start arm, as shown by their turn behaviour being at chance level. These findings further suggest that recollection, rather than familiarity, is needed to solve this task. Moreover, recollection is impaired following lesions to the fornix, an important pathway connecting to the hippocampus, while familiarity is not (Easton et al., 2009). This provides behavioural evidence of a dissociation between recollection and familiarity, which is complemented by analyses of ROC (receiver-operating characteristic) curves that have also suggested a dissociation between recollection- and familiarity-based processes in rodents (e.g. Fortin, Wright and Eichenbaum, 2004). Nonetheless, it is evident that although the animals in the E-maze demonstrated preferential choice of the non-habituated objects, the mean percentage of turns towards the object out of the start arm was quite low at 65.2% (Eacott et al., 2005). As this task relies on object preference as an indicator for recognition, it is possible that relatively low turn accuracy may be a result of weak object preference rather than poor recognition ability. When the fornix-lesioned animals were tested, preferential exploration of the non-habituated object at test indicated that the animals recognised the habituated objects (through preferential exploration of the non-habituated object) possibly through familiarity-based mechanisms. The animals, however, failed to turn towards the non-habituated object significantly greater than chance because this would presumably require recollection (Easton et al., 2009). As the turn behaviour for the control animals was low (but significantly greater than chance), it is possible that the fornix-lesioned animals simply needed stronger object preference to make more accurate turns; the animals clearly recognised the habituated objects at test but this may not have been sufficient to drive their turn behaviour at the start of the test phase.

As recognition in the E-maze task relies on object preference, the current chapter presents a series of experiments designed to investigate the behavioural parameters that could optimise object preference, which could then be utilised in the E-maze task to produce a

stronger behavioural indicator of recollection. These experiments investigated different lengths of habituation time and selective food reinforcement on an object preference task to see how these factors may influence recognition in the test phase of the E-maze task. A single trial typically consisted of a sample phase in which an animal was exposed to two different objects. The animal then spent a period of time habituating to a duplicate copy of one of those objects, before exposure to copies of the same two objects from the sample phase, in the locations they previously occupied, for the test phase. No context changes occurred in the task. As both objects at test have been experienced before, neither are novel; however, one object has become highly familiar through habituation, and as such the non-habituated object is relatively novel. Thus, recognition memory of the habituated object is exhibited through preferential exploration of the non-habituated object.

The experiments in the current chapter were carried out in the continual trials apparatus (Ameen-Ali, Eacott and Easton, 2012; see Chapter 2) which allows for multiple trials within a session, fast accumulation of data and a reduction in the number of animals required for statistically meaningful results. Using the continual trials apparatus will give an insight in to how this type of paradigm could be used in further recognition tasks, with the aim of eventually developing a robust episodic-like memory task for rodents.

The first experiment of the study investigated performance in an object preference task in which the length of the habituation time and the food reinforcement of test objects were manipulated. While previous published studies have used an 8 minute habituation time (Eacott et al., 2005; Easton et al., 2009), a pilot study (Ameen-Ali, Eacott and Easton, unpublished) in the lab had explored recognition performance between trials with habituation times of 3, 5 and 8 minutes, and concluded that greater recognition performance occurred in trails using 3 and 5 minute habituation times. These times were also more practical for the multiple trial method, which would yield more trials with these habituation times. Experiment

1, therefore, investigated 3 and 5 minute habituation conditions, with and without food reinforcement.

3.2. Materials and methods

3.2.1. Subjects

Eighteen male Lister hooded rats supplied by Harlan (Bicester, UK) were used in this series of experiments. All animals were housed in groups of three in diurnal conditions (12-h light-dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study, except during habituation and testing. 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.

3.2.2. Apparatus

The animals were tested in the continual trials apparatus (Chapter 2, Section 2.2.2). During sample and test phases, objects were placed in the top left and top right-hand corners of the object area of the maze approximately 2cm away from the two walls to allow the animals to get their heads around the objects and explore them fully. During the habituation phase of a trial, the objects were placed central in the holding area so the animal could explore the entire object.

The four contexts that constituted the object area were 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. Pretraining and the experiment proper only used context 1.

3.2.3. 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.

3.2.4. Pretraining

All animals underwent the handling and pretraining sessions outlined in Chapter 2 Section 2.2.4.

3.2.5. Behavioural analysis

Exploration of objects was defined as when the nose of the animal was <1cm 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 considered 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 at test (exploration of the novel object minus the exploration of the familiar object) divided by the total exploration time (see Chapter 2, Section 2.2.5).

3.3. Experiment 1: Object-based preference task (three and five minute conditions, with and without food reinforcement)

3.3.1. Subjects

A total of 12 Lister hooded rats supplied by Harlan UK were used in this study. They were housed in threes in diurnal conditions (12-hr light-dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study, except during pretraining and testing. All animals were food deprived to 85% of the free-feeding body weight of age matched controls throughout testing. At the time of testing, six of the animals (used in experiment 1a) were four months old and weighed an average of 300g. A further six animals (used in experiments 1b-d) were two months old and weighed an average of 250g.

3.3.2. Test protocol

3.3.2.1. Experiment 1a: Three minute condition with food reinforcement

Six rats were given a single testing session of 18 trials in which the animals were exposed to a non-habituated object and a habituated object on each trial (Figure 3.2). At the start of each session, the animals were individually placed in the holding area, with the central door opening immediately so they could move through to the object area. Each trial consisted of an initial sample phase in which the animal was exposed to two objects. This was then followed by the habituation phase, in which each animal was exposed to a duplicate copy of one of the objects from the sample phase. The test phase followed with duplicate objects from the sample phase in locations congruent with the sample phase. One object was now highly familiar through habituation, and one was non-habituated. Preferential exploration of the non-habituated object was predicted as it was the object seen longest ago.

For the initial sample phase, each animal spent two minutes exploring the objects (objects A and B) in the object area. After two minutes, the doors on the outer arms of the

apparatus opened and the animals shuttled through to the holding area which contained one of the objects from the sample phase (A) concealing two food pellets in a central food well (habituation phase). After a period of three minutes, the central door opened to allow the animals back into the object area which contained a duplicate copy of the now habituated object A and a non-habituated object B (trial 1). The animals explored these objects for a period of two minutes, after which the doors on the outer arms of the apparatus were opened and the animals could shuttle back through to the holding area containing two food pellets. The central door was then opened for trial 2, allowing the animals back into the object area which contained objects C and D. This procedure then continued for a total of 18 trials.

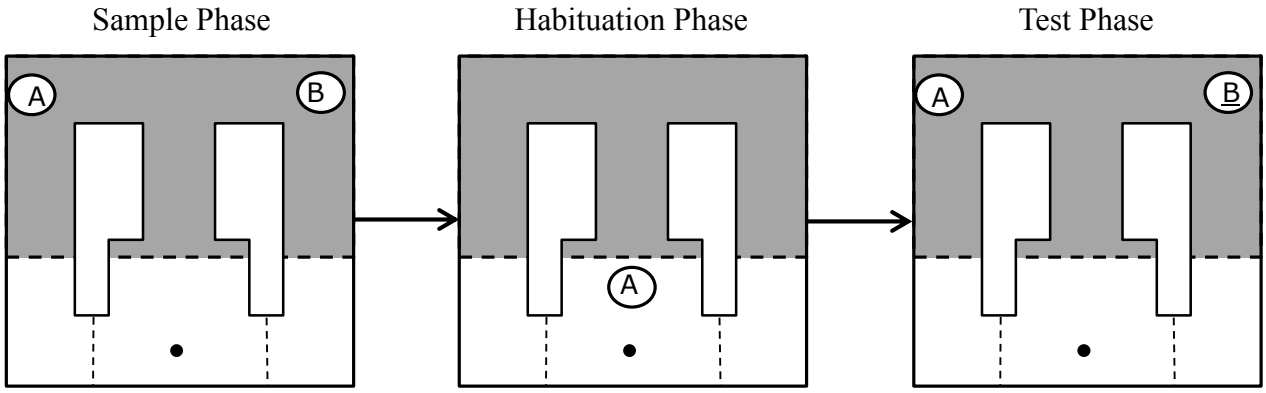


Figure 3.2. General procedure for the object preference task in the continual trials apparatus, depicting a single trial. The animal begins a trial with a sample phase in which it is exposed to two different objects which it can freely explore. After a period of two minutes, the animal returns to the holding area for the habituation phase, in which it can freely explore a duplicate copy of one of the previously seen objects. Following this, the animal returns to the object area for the test phase, where it is exposed to the same objects from the sample phase, though one object is now habituated. The locations of the objects in the test phase are always congruent with the sample phase.

Both the habituated and non-habituated 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, Chapman, Amin, Iordanova, Vann et al., 2010). These food pellets did not differentially reward one object over the other, as both 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 non-habituated 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. However, within each trial, object location was constant, i.e. the location of object A in the sample phase was the same on the subsequent test phase. This was done for all subsequent experiments to replicate the E-maze task.

The criterion for ending a trial was failure to shuttle to the next area of the apparatus after a period of three minutes. This would subsequently cease the testing session and the data for that animal's testing session would not be included in the data analysis for that particular experiment. This applied for all experiments in the current chapter.

3.3.2.2. Experiment 1b: Three minute condition without food reinforcement

Six naïve Lister hooded rats were given a single testing session of 16 trials (reduced from 18 for this and subsequent experiments, apart from Experiment 3, as this number of trials fitted suitably into a three hour maximum testing session) in which the animals were exposed to a habituated object and a non-habituated object on each trial. The test protocol was identical to that used in Experiment 1a, but none of the objects were baited with food

pellets. Experiments 1a and 1b therefore allowed for a direct comparison of exploration with and without food reinforcement in the three minute condition.

3.3.2.3. Experiment 1c: Five minute condition without food reinforcement

The six rats used in Experiment 1b were given a single testing session of 16 trials in which the animals were again exposed to a habituated object and a non-habituated object on each trial. The test protocol was identical to that used in Experiment 1b (none of the objects were baited with food pellets), but the length of exposure during the habituation phase on each trial was increased to five minutes.

3.3.2.4. Experiment 1d: Five minute condition with food reinforcement

The six rats used in Experiments 1b and 1c were given a single testing session of 16 trials in which the animals were again exposed to a habituated object and a non-habituated object on each trial. The test protocol was identical to that used in Experiment 1c (the length of exposure during the habituation phase on each trial was kept at five minutes), but all of the objects were baited with food pellets. Experiments 1c and 1d therefore allowed for a direct comparison of object exploration with and without food reinforcement in the five minute condition.

3.3.3. Results

3.3.3.1. Experiment 1a: Three minute condition with food reinforcement

To determine whether the animals performed above chance, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero. The animals explored the non-habituated objects significantly more than the habituated objects (mean D2 score = 0.2; $t(5) =$

3.049, p = 0.028) showing clear discrimination of the habituated objects from the non-habituated objects (Figure 3.4).

The animals spent on average 33s exploring the habituated objects during the three minute habituation phase with a mean of 6s of exploration occurring in the last minute of habituation. The mean total exploration at test was 13.4s (Table 3.1). A repeated measures ANOVA was carried out to assess overall levels of exploration during the habituation phase, in order to see whether exploration levels increased or decreased minute by minute. There was a main effect of 'minute' ($F(2, 10) = 14.363$, $p = 0.001$) with the significant difference occurring between minute 1 and minute 3 of the habituation phase ($p = 0.009$). This suggests that levels of exploration significantly decreased throughout the habitation phase (Figure 3.3).

Table 3.1. Experiment 1, mean habituation and test exploration.

	Experiment 1											
	1a: 3min with food			1b: 3min without food			1c: 5 min with food			1d: 5 min without food		
	Time	SEM	n	Time	SEM	n	Time	SEM	N	Time	SEM	n
Mean habituation exploration time (secs)	33.0	7.6	6	21.8	4.2	6	24.9	6.5	6	26.8	6.8	6
Mean test exploration times (secs)	13.4	3.4	6	10.6	2.2	6	9.5	2.4	6	8.8	2.9	6

A series of Pearson's correlations was carried out to see whether there was a relationship between either the mean total exploration in the habituation phase or the test phase, with the subsequent D2 scores. No significant correlations were found (mean D2 scores and mean total exploration at habituation: $r = -0.433$, $p = 0.391$; mean D2 scores and mean total exploration at test: $r = 0.368$, $p = 0.472$). A further correlational analysis was carried out to see if there was a relationship between exploration in the last minute of the habituation phase and subsequent D2 scores, but again no evidence was found ($r = -0.453$, $p = 0.367$).

In order to see whether performance changed over the course of the testing session, the D2 scores for each animal were segregated into six blocks, each of three trials. For each animal, a mean D2 score 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 ($F(5, 25) = 1.250$, $p = 0.316$), indicating that performance was relatively stable throughout the testing session.

3.3.3.2. Experiment 1b: Three minute condition without food reinforcement

The animals did not successfully discriminate between the non-habituated objects and the habituated objects (mean D2 score = 0.03; $t(5) = 0.571$, $p = 0.592$). On average, less time was spent exploring the habituated objects during the habituation phase (21.8s; Table 3.1) compared to the animals in Experiment 1a, though not significantly ($t(10) = 1.860$, $p = 0.092$). Both the mean total exploration time at test and the mean D2 score were lower in Experiment 1b than in Experiment 1a, though not significantly (mean D2 score: $t(10) = 2.089$, $p = 0.063$; Figure 3.4; mean total exploration at test = 10.6s: $t(10) = 1.144$, $p = 0.279$). A repeated measures ANOVA was carried out to assess levels of exploration during the habituation phase. There was a main effect of 'minute' ($F(2, 10) = 15.147$, $p = 0.001$) with

the significant difference occurring between minute 1 and minute 3 of the habituation phase ($p = 0.012$). This suggests that levels of exploration significantly decreased throughout the habitation phase (Figure 3.3).

In order to see whether performance changed over the course of the testing session, the D2 scores for each animal were segregated into four blocks, each of four trials. For each animal, a mean D2 score was calculated for each block derived from their individual D2 scores within that block. No effect of block was found ($F(3, 15) = 0.123$, $p = 0.945$), indicating that performance was relatively stable throughout the testing session.

No significant relationship was found either between the mean total exploration in the habituation phase or test phase and the subsequent mean D2 score (mean D2 score and mean total exploration at habituation: $r = 0.029$, $p = 0.956$; mean D2 score and mean total exploration at test: $r = 0.306$, $p = 0.555$).

3.3.3.3. Experiment 1c: Five minute condition without food reinforcement

The animals did not successfully discriminate between the habituated and non-habituated objects at test (mean D2 score = 0.09; $t(5) = 1.693$, $p = 0.151$). The animals spent on average 24.9s exploring the habituated objects during the five minute habituation phase, and the mean total exploration time at test was lower than Experiment 1b (compared because the task procedures differed only in habituation time; Table 3.1), but not significantly so (mean total exploration at test Experiment 1c = 9.5s; $t(5) = 0.867$, $p = 0.426$). No significant difference was found between mean D2 scores for Experiments 1b and 1c ($t(5) = 1.354$, $p = 0.234$; Figure 3.4). A repeated measures ANOVA was carried out to assess levels of exploration during the habituation phase. There was a main effect of 'minute' ($F(4, 20) = 27.146$, $p = < 0.001$) with the significant differences occurring between minute 1 and every other minute of the habituation phase (1 and 2: $p = 0.002$; 1 and 3: $p = 0.001$; 1 and 4: $p =$

0.002; 1 and 5: p = 0.014). This suggests that levels of exploration significantly decreased from the first minute of the habitation phase (Figure 3.3). Performance was relatively stable throughout the testing session, as when the mean D2 scores were blocked together and analysed, no effect of block was found ($F(3, 15) = 1.769$, $p = 0.196$).

a. Three Minute conditions

b. Five minute conditions

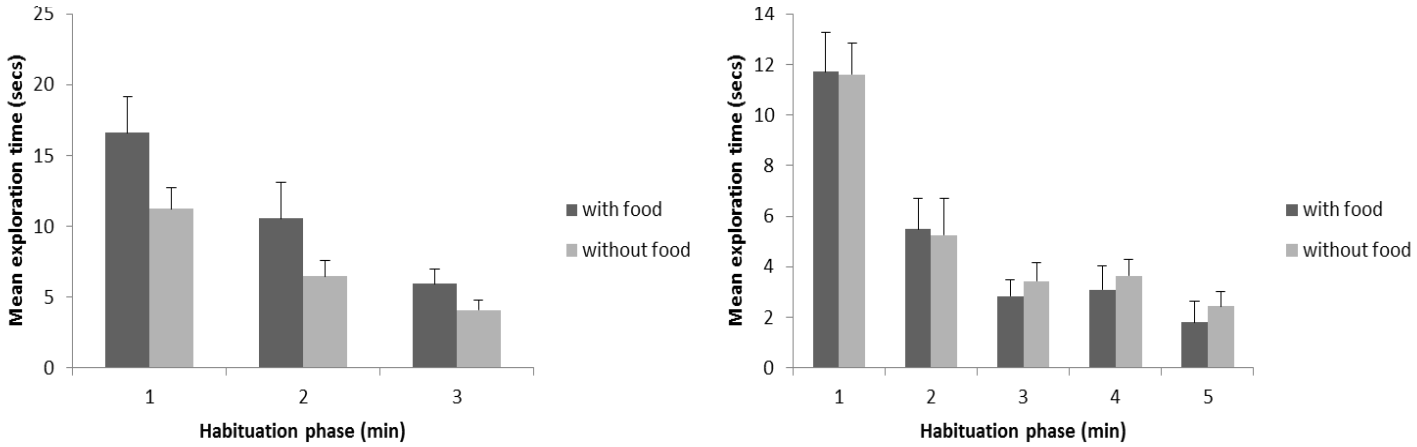


Figure 3.3. Mean exploration times for each minute of the habituation phase. a) Experiments 1a and 1b, three minute conditions with and without food reinforcement, respectively. b) Experiments 1c and 1d, five minute conditions without and with food reinforcement, respectively. Vertical bars show the standard error of the mean.

No significant relationship was found either between the mean total exploration in the habituation phase or test phase, and the subsequent mean D2 score (mean D2 score and mean total exploration at habituation: $r = 0.031$, $p = 0.953$; mean D2 score and mean total exploration at test: $r = -0.295$, $p = 0.570$).

3.3.3.4. Experiment 1d: Five minute condition with food reinforcement

The animals did not successfully discriminate between the habituated and non-habituated objects at test (mean D2 score = 0.01; $t(5) = 0.289$, $p = 0.784$). There was no significant difference between time spent exploring the habituated objects during the habituation phase in Experiment 1d (25s) compared to Experiment 1c (26.8s; $t(5) = 0.513$; $p = 0.630$), which were compared because the task procedures differed only in whether objects were baited or not. The mean total exploration time at test (8.8s; Table 3.1) was lower than Experiment 1a, but not significantly so ($t(10) = 2.000$, $p = 0.073$), and equal to 1c ($t(5) = 0.473$, $p = 0.656$). No significant differences were found on mean D2 scores between Experiments 1a and 1d ($t(10) = 2.134$, $p = 0.059$), and Experiments 1c and 1d ($t(5) = 0.938$, $p = 0.391$; Figure 3.4). A repeated measures ANOVA was carried out to assess levels of exploration during the habituation phase. There was a main effect of 'minute' ($F(4, 20) = 25.951$, $p = < 0.001$) with the significant differences occurring between minute 1 and every other minute of the habituation phase (1 and 2: $p = 0.003$; 1 and 3: $p = 0.006$; 1 and 4: $p = 0.005$; 1 and 5: $p = 0.011$). This suggests that levels of exploration significantly decreased from the first minute of the habitation phase (Figure 3.3). Performance was relatively stable throughout the testing session as no effect of block for the mean D2 scores was found ($F(3, 15) = 1.353$, $p = 0.787$).

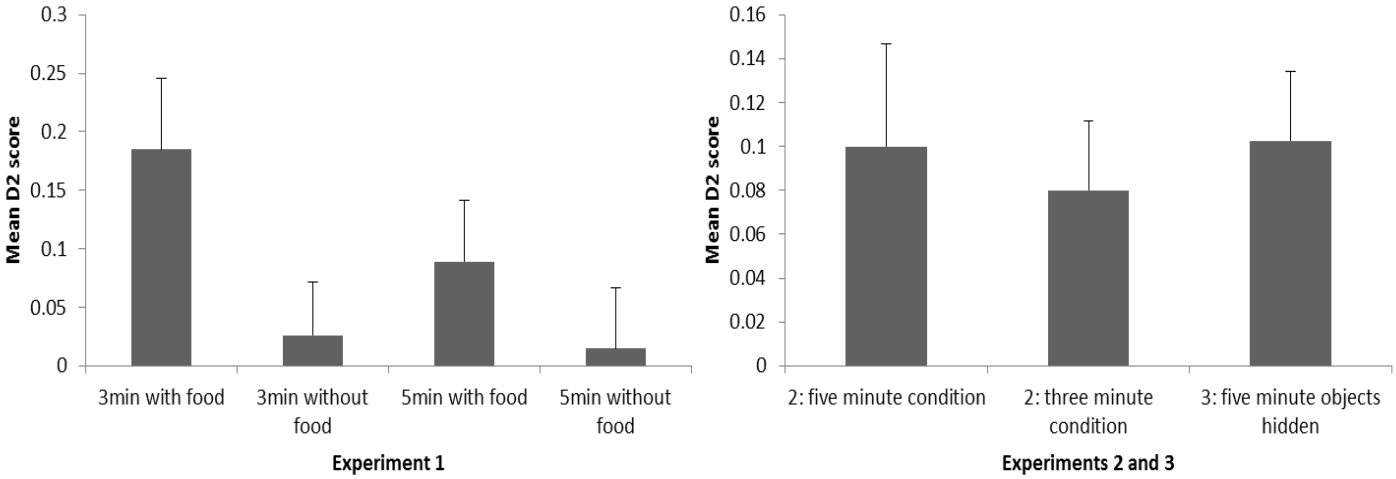


Figure 3.4. Mean D2 scores for Experiments 1-3. Vertical bars show the standard error of the mean.

No significant correlations were found either between the mean total exploration in the habituation phase or test phase and the subsequent mean D2 score (mean D2 score and mean total exploration at habituation: $r = -0.391$, $p = 0.444$; mean D2 score and mean total exploration at test: $r = 0.298$, $p = 0.566$).

3.3.4. Discussion

The current group of experiments aimed to investigate the differential effects of varying the length of the habituation phase and selective reinforcement on recognition. Experiment 1 was divided into four sub-experiments which looked at the effect of different lengths of the habituation phase in each trial, as well as food reinforcement of objects on object preference. Reliable levels of recognition were only found in Experiment 1a, in which the length of the habituation phase was three minutes and the objects were baited. Before the results are discussed in further detail, it is worth noting that as two different groups of animals took part in these four sub-experiments, different learning experiences prior to test may have had an effect on task performance. For instance, the animals in Experiment 1d had

taken part in two previous behavioural tasks (Experiments 1b and 1c), and so may have had a reduced interest in object exploration.

No evidence of correlation between D2 scores and exploration in either the habituation phase or the test phase of each trial was found in any of the four sub-experiments. The mean exploration of the habituated object declined throughout the habituation phase (Figure 3.3), but no evidence was found that this correlated with subsequent D2 scores.

Overall performance within the three minute conditions (Experiments 1a and 1b) did not significantly differ between the two groups. When the objects were baited with food pellets (Experiment 1a), however, higher levels of exploration at test and greater D2 scores (though not significantly) were found compared to when the objects were not baited (Experiment 1b).

Overall performance within the five minute conditions (Experiments 1c and 1d) did not significantly differ, but higher levels of exploration at test were found when the objects were not baited (Experiment 1c) compared to when they were (Experiment 1d). This pattern of results is in contrast to the three minute condition experiments, and initially appears to be an unusual finding. However, it is worth noting that when the animals took part in Experiment 1d they may have had a high familiarity with objects in general due to having taken part in multiple sub-experiments. This may have reduced the animals' interest in the task, but not necessarily their object exploration, as the objects were baited in Experiment 1d. Further analyses were, therefore, carried out to see whether there were any performance differences between the three and five minute condition experiments to determine why object baiting may have had a differential effect within these two time-dependent conditions. When the objects were baited with food (Experiments 1a and 1d, respectively), performance did not significantly differ; however, performance in the three minute condition (Experiment 1a) resulted in higher levels of exploration at test and greater D2 scores. When the objects were

not baited (Experiments 1b and 1c), performance in the three and five minute conditions did not significantly differ, though it was evident that slightly lower levels of exploration at test, but greater D2 scores, were found in the five minute condition. Comparing performance between the two time-dependent conditions indicates that object baiting improved performance in the three minute condition, but when objects were not baited, performance was better in the five minute condition.

Recognition measures were most reliable overall in the three minute condition with object food reinforcement, although it is not possible to conclude overall whether performance was better in this condition. A more controlled between-subjects design with four animal groups (one for each experimental condition), or a counterbalanced repeated measures design with all animals doing each condition, may have given a clearer indication of the condition to yield the most reliable indicators of recognition ability. A between-subjects design, however, would have substantially increased the number of animals required, and the repeated measures design may have been confounded by order effects which would be difficult to control for without a complex design.

The aim of the next series of experiments was to investigate whether the food presented in the holding area had any effect on recognition performance in the object preference task. One possibility that may account for the poor discrimination measures reported in Experiment 1 is that the food presented with the object during the habituation phase may have been positively associated. In turn this may have encouraged exploration of this object at test, even when presented alongside a relatively novel object. Experiment 2 aimed to change the food reinforcement in the holding area so that it was only given when an object was not present, i.e. the food only acted to reinforce shuttling between the areas of the apparatus and not bait the habituated object.

3.4. Experiment 2: Object-based preference task with selective food reinforcement in the holding area (three and five minute conditions)

3.4.1. Subjects

Six naïve Lister hooded rats, supplied by Harlan, were used in this experiment in both conditions. These six animals were housed in groups of three in conditions identical to the previous experiments. At the time of testing, these animals were two months old and weighed an average of 250g.

3.4.2. Test protocol

Six animals performed in both the five minute and three minute conditions, in that order. For both experiments, each of the six rats was given a single testing session of 16 trials for each condition, in which the animals were exposed to a habituated object and a non-habituated object on each trial. The test protocol was identical to that used in Experiment 1, with the one exception that objects during the sample and tests phases were baited with food pellets. The objects used during the habituation phase, however, were not baited. Food pellets were present in the holding area only when shuttling did not directly precede the habituation phase. The aim of this change was to avoid food reinforcement of the habituated object, but to maintain food reinforcement of shuttling through the two areas of the apparatus. As with the previous experiments only context 1 was used. The location of the non-habituated 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 those which were to be non-habituated and those to be habituated, in order to minimise bias for a particular object. However, within each trial, object location was constant, as with the previous experiments.

3.4.3. Results

Performance was first analysed across both conditions before considering how the animals performed in each condition separately. The animals significantly explored the non-habituated objects more than the habituated objects ($t(5) = 3.856$, $p = 0.012$) with a mean D2 score of 0.1, showing that clear object preference for the non-habituated object was established.

3.4.3.1. Five minute condition

The rats significantly explored the non-habituated objects more than the habituated objects (mean D2 score = 0.1; $t(5) = 2.853$, $p = 0.036$; Figure 3.4). The animals spent on average 9.9s exploring the objects during the habituation phase with a mean of 0.6s of exploration occurring in the last minute of habituation. The mean total exploration at test was 12s (Table 3.2).

No significant correlations were found between either the total exploration in the test phase and the D2 scores, or between the total exploration in the habituation phase and the D2 scores (D2 scores and total exploration at test: $r = 0.298$, $p = 0.566$; D2 scores and total exploration at habituation: $r = 0.511$, $p = 0.301$). Performance was relatively stable throughout the testing session, as no effect of block for the mean D2 scores was found ($F(3, 15) = 0.452$, $p = 0.720$).

3.4.3.2. Three minute condition

The rats did not explore the non-habituated objects more than the habituated objects, though the analysis approached significance (mean D2 score = 0.08; $t(5) = 2.547$, $p = 0.051$; Figure 3.4). The animals spent on average 17.1s exploring the objects during the habituation

phase with a mean of 2s of exploration occurring in the last minute of habituation. The mean total exploration at test was 12.7s (Table 3.2).

No significant correlations were found between either the total exploration in the test phase and the D2 scores, or the total exploration in the habituation phase and the D2 scores (D2 scores and total exploration at test: $r = 0.187$, $p = 0.722$; D2 scores and total exploration at habituation: $r = 0.048$, $p = 0.928$). Performance was relatively stable throughout the testing session as no effect of block for the mean D2 scores was found ($F(3, 15) = 0.606$, $p = 0.621$).

Two paired samples t-tests were carried out to see whether performance differed between the three and five minute conditions. Results showed that the animal's performance did not differ significantly on measures of exploration at test and D2 scores (mean D2 score: $t(5) = 0.922$, $p = 0.399$; total test exploration: $t(5) = 0.254$, $p = 0.810$).

Table 3.2. Experiment 2, mean habituation and test exploration.

	Experiments 2 and 3								
	2: Five minute condition			2: Three minute condition			Experiment 3: 5min objects hidden		
	Time	SEM	n	Time	SEM	n	Time	SEM	n
Mean habituation exploration time (secs)	9.9	2.2	6	17.1	4.6	6	6.6	1.4	6
Mean test exploration times (secs)	12.0	2.3	6	12.7	2.5	6	13.2	4.1	6

3.4.3.3. Further analyses

An independent samples t-test was carried out to see whether performance in the five minute condition differed significantly from the group of animals that performed in Experiment 1d, on measures of exploration at test and D2 scores. The only difference between the tasks performed by these two groups of animals was that food was selectively presented in the holding area in this experiment (only present when no object was in the holding area), whereas food reinforcement was always provided in the holding area in Experiment 1d; though it is worth noting that the animals in the current experiment were experimentally naïve, whereas the animals in Experiment 1d had previously taken part in two experiments which may have impacted upon their performance in Experiment 1d. In both experiments the habituation phase time was five minutes and the objects in the sample and test phases were reinforced with food to encourage exploration. Results showed that the two groups of animals did not differ significantly on measures of mean exploration at test or mean D2 scores (mean D2 score: $t(10) = 1.699$, $p = 0.120$; total test exploration: $t(10) = 1.179$, $p = 0.266$), though greater mean D2 scores and mean total exploration time at test was found in the five minute condition of Experiment 2.

An independent samples t-test was carried out to see whether the performance in the three minute condition differed significantly from the group of animals that performed in Experiment 1a, on measures of exploration at test and D2 scores. The only difference between the tasks performed by these two groups of animals was that food was selectively reinforced in the holding area in this experiment, whereas food reinforcement was always provided in the holding area in Experiment 1a; though it is worth noting that the animals in the current experiment had previously taken part in the five minute condition of Experiment 2, which may have impacted upon their performance in the current task. In both experiments

the habituation phase time was three minutes and the objects in the sample and test phases were reinforced with food to encourage exploration. Results showed that the two groups of animals did not differ significantly on measures of mean total exploration at test and mean D2 scores (mean D2 score: $t(10) = 1.530$, $p = 0.157$; total test exploration: $t(10) = 0.240$, $p = 0.815$), though greater mean D2 scores and mean total exploration time at test were found in Experiment 1a, the three minute condition with constant food reinforcement provided in the holding area.

3.4.4. Discussion

Experiment 2 looked at selective food reinforcement in the holding area on recognition performance in three minute and five minute habituation conditions. Reliable levels of recognition were only found in the five minute condition, but performance in the two tasks was only marginally improved in the five minute condition, so there may only be a small advantage to using this length of habituation time.

For both conditions, no significant correlations were found between D2 scores and exploration in either the habituation phase or the test phase. Performance in the three and five minute conditions did not significantly differ, but greater D2 scores were found in the five minute condition despite slightly lower levels of exploration at test.

No significant differences were found between the current five minute condition (selective food reinforcement of the holding area), and the previous baited five minute condition (Experiment 1d; constant food reinforcement of the holding area), on D2 and exploration measures. Performance was, however, slightly improved in Experiment 2 as higher levels of exploration at test and greater D2 scores were observed, which indicates that selective reinforcement in the holding area marginally improved performance. However, this pattern of results for the five minute conditions across Experiments 1 and 2 is in contrast to

the three minute conditions. Performance was greater in Experiment 1a (with constant food reinforcement in the holding area) in comparison to Experiment 2 (with selective food reinforcement in the holding area), suggesting that selective reinforcement of the holding area was not an improvement in the three minute condition. It is important to note, however, that these inconsistencies can be accounted for to some extent by the prior learning experience of the animals in the experimental conditions. For instance, to compare the performance of the animals in the five minute condition of Experiment 2 to those in Experiment 1d is to compare a naïve group (Experiment 2) to a group who had previously performed two behavioural tasks, which could have a significant effect on their task performance. This again highlights the need for well-controlled task design.

Following Experiment 2, which showed that five minutes of habituation without food present at habituation was sufficient to establish a robust object preference, a naïve group of six animals was tested using this habituation condition. However, in this experiment, the objects in the test phase were not visible to the animals from the start arm, i.e. they were placed at the end of the outer arm corridors. This allows turn behaviour (recall) to be measured at test, as well as recognition memory.

3.5. Experiment 3: Object preference task with selective food reinforcement in the holding area (five minute condition with objects not visible)

3.5.1. Subjects

Six naïve Lister hooded rats, supplied by Harlan, were used in this experiment. These six animals were housed in groups of three, in conditions identical to those in the previous experiments. At the time of testing, these animals were two months old and weighed an average of 250g.

3.5.2. Test protocol

Each of the six rats were given a single testing session of 18 trials (increased from 16 used previously to obtain more turn behaviour per animal) in which the test protocol was identical to that used in the five minute condition of Experiment 2, with the only exception that in the test phase, the objects were not visible to the animals from the start arm, i.e. they were placed at the end of the outer arm corridors. Left and right turns were measured when the snout of the animal crossed over a line marked on the lid of the apparatus, which was deemed match the line of sight needed in order for the animal to see the hidden object down the outer arm of the maze.

3.5.3. Results

The animals showed significant preferential exploration for the non-habituated object over the habituated object (mean D2 score = 0.1; $t(5) = 3.255$, $p = 0.023$). The animals spent on average 6.6s exploring the objects during the habituation phase with a mean total exploration at test of 13.2s (Table 3.2).

Performance was also measured through scoring whether the animals first turned left or right when leaving the start arm at the beginning of the test phase. The animals turned towards the non-habituated objects on average 51.8% of the time (S.D. 5.88), which is not significantly above the level expected by chance ($t(5) = 0.764$, $p = 0.479$).

3.5.4. Discussion

Overall, the findings from Experiments 2 and 3 indicate that performance was most reliable when the length of the habituation phase on each trial was five minutes. When compared to the previous five minute conditions in Experiment 1, it is evident that selective reinforcement in the holding area may have contributed to the more reliable measures of

recognition observed, as the objects in the habituation phase were not being positively reinforced. In the previous experiments, constant food reinforcement in the holding area may have been a contributing factor to the poor discrimination measures, in addition to the prior experience of the animals in other behavioural tasks.

The mean percentage of turns towards the non-habituated object in Experiment 3 was at chance (51.8%), but this is not surprising considering that recognition of the non-habituated object was low, although significant, with a D2 score of 0.1. This may reflect weak memory for the habituated object, but is more likely to be due to the strength of the animals' object preference (Eacott et al., 2005).

The difference in performance between the five and the three minute conditions of Experiment 2 was marginal, and for strong object preference to be shown, not only must the habituation to one object be effective, but there must also be sufficient dissipation of habituation from the non-habituation object which was last encountered in the sample phase. The five minute condition presents an opportunity for both object habituation to occur and the longest time (of both conditions) between the sample and test phases for dissipation to occur. However, a common observation in all the object preference tasks from Experiments 1 and 2 was that object exploration during the habituation phase decreased over time (Figure 3.3), and was highest during the first two minutes of habituation. If very little object exploration occurred towards the end of the habituation phase, habituation to the object may have decreased and resulted in poor discrimination in the test phase. Experiment 4 sought to maximise habituation in the five minute condition of the task so that the objects in the habituation phase were only present in the holding area for the final two minutes prior to the test phase. Restricting object exploration to the end of the habituation phase may maximise the level of habituation prior to the test phase and result in more reliable discrimination scores, through allowing objects to become habituated to (but only in the last two minutes of

habituation), and having the longest interval (five minutes) between sample and test phases to increase dissipation of the non-habituation objects, and subsequently make them preferential to explore in the test phase.

3.6. Experiment 4: Delayed habituation object-based preference task

3.6.1. Subjects

Six Lister hooded rats (Harlan) used in Experiment 2 were again used in this experiment, approximately six weeks after previous experiment. Housing conditions were identical to the previous experiments. At the time of testing, these animals were four months old and weighed an average of 300g.

3.6.2. Test protocol

Each of the six rats were given a single testing session of 16 trials in which the animals were exposed to a habituated object and a non-habituated object on each trial. The test protocol was identical to that used in Experiment 2 (five minute condition) with the sole exception that after each sample phase the animal shuttled in to the holding area for the start of the habituation phase which comprised of a three minute delay/rest period, and then two minutes of object habituation (Figure 3.5).

As with the previous experiments, only context 1 was used. The location of the non-habituated 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 non-habituated and which was habituated in order to minimise bias for a particular object. However, within each trial object location was constant (i.e. the location of object A in the sample phase was the same on the subsequent test phase).

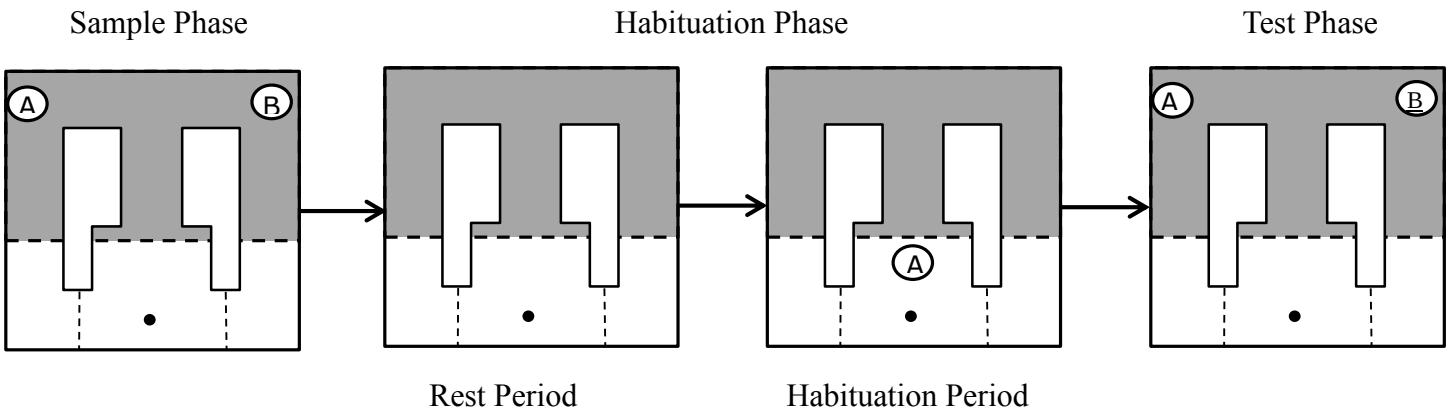


Figure 3.5. Procedure for the object preference task in the continual trials apparatus, with delayed habituation period in Experiment 4, depicting a single trial. The animal began a trial with a sample phase in which it is exposed to two different objects which it could freely explore. After a period of two minutes, the animal returned to the holding area for the habituation phase, which consisted of a three minute rest period and two minute habituation period. For this time the animal could freely explore the habituated object (a duplicate object from the sample phase). Following this, the animal returned to the object area for the test phase, where it was exposed to the same objects from the sample phase, though one object was now habituated. The locations of the objects in the test phase were always congruent with the sample phase.

3.6.3. Results

The animals did not significantly explore the non-habituated objects more than the habituated objects (mean D2 score = 0.06; $t(5) = 1.341$, $p = 0.238$). The animals spent on average 6s exploring the objects during the habituation phase with a mean total exploration at test of 11s.

Two paired samples t-tests were carried out to see whether performance of the group of animals in this task differed significantly from their performance in the five minute condition of Experiment 2. Both tasks involved a five minute habituation phase, but in the current task the habituation phase consisted of a three minute delay before the two minute

exposure to the habituated object, whereas the animals were exposed to the objects in the habituation phase for the full five minutes in Experiment 2. In both tasks, objects in the sample and test phases were baited with food pellets for reinforcement of object exploration, but food reinforcement in the holding area was selectively presented, only when no object was present in the holding area. Results showed that the animal's performance did not differ significantly between the two tasks on measures of mean total exploration at test and mean D2 scores (mean D2 scores: $t(5) = 0.992$, $p = 0.367$; total exploration time: $t(5) = 0.364$, $p = 0.731$), though a greater mean D2 score and mean total exploration time was found in the five minute condition of Experiment 2.

No significant correlations were found either between the total exploration in the test phase and the D2 scores, or the total exploration in the habituation phase and the D2 scores (D2 scores and total exploration at test: $r = 0.486$, $p = 0.329$; D2 scores and total exploration at habituation: $r = -0.229$, $p = 0.468$).

3.6.4. Discussion

Experiment 4 looked at the effect of introducing a delay during the habituation phase prior to object habituation. Reliable levels of recognition were not found in this experiment and no evidence was found of correlation between D2 scores and exploration in either the habituation phase or the test phase of each trial.

Analyses were carried out to see whether there were any performance differences between the current task and five minute condition of Experiment 2, which was a similar task in that the length of the habituation phase was five minutes (so the delay between sample and test phases was matched), but with the exception that the current task had the introduction of a delay in the habituation phase, so that the actual object habituation time was reduced in the current experiment. Performance in the two tasks did not significantly differ, but performance

in Experiment 2 resulted in a greater mean D2 score and mean total exploration at test when compared to the current experiment.

3.7. General discussion

The aim of the current chapter was to elucidate the behavioural parameters to maximise recognition abilities in an object preference task. Overall, the highest measures of recognition and exploration were found in the condition that included a three minute habituation phase, food reinforcement of the sample and test objects and constant food reinforcement in the holding area (Experiment 1a). Reliable measures of recognition were also found, however, when the length of the habituation phase was five minutes, with the objects baited, but with selective food reinforcement in the holding area (Experiment 2). The object preference task was developed with the aim of understanding how habituation may influence recognition in the E-maze episodic-like memory task. Similarly to the E-maze task, a single trial involved habituating to an object from the sample phase, with exploration of the habituated object and a non-habituated, but familiar, object from the sample phase, measured in the test phase. The object preference task only involved one sample phase and no context change, but the location of the objects in the test phase was always congruent with their locations in the sample phase, as with the E-maze task.

The main aim of the current chapter was to investigate different task parameters to improve recognition performance in the E-maze task. However, none of the experiments reported here provided an improvement on either recognition performance or the turn behaviour (measured in Experiment 3). A number of reasons could potentially account for the poor recognition abilities demonstrated in the current chapter, which will be explored in more detail. As previously discussed, some groups of animals took part in multiple experiments, which may have led to performance on later tasks being vulnerable to order effects.

Experiment 2 investigated whether food reinforcement in the holding area of the apparatus inadvertently acted to positively reinforce the habituated object. At test, the habituated object may have been more desirable to explore, even when presented with the relatively novel object from the sample phase. Presenting food selectively in the holding area so that it was never present alongside a habituated object maintained the shuttling between areas of the apparatus, but there did not appear to be any significant improvement in task performance. However, the animals did significantly explore the non-habituated object more than the habituated object in the five minute condition. When this study was replicated (Experiment 3) and the turn behaviour of the animals was measured as an additional indicator of recognition ability, again, the animals showed significant preferential exploration of the non-habituated object over the habituated object, although the mean percentage of turns towards the non-habituated object was only at chance (51.8%).

A final modification was made to the object preference task design in Experiment 4, whereby a delay was introduced during the habituation phase of the five minute condition. The habituated objects were only present for the final two minutes of the habituation phase to maximise the amount of object exploration prior to the test phase, with the aim of obtaining more reliable recognition scores. This task modification proved not to be an improvement, in fact the animals failed to show significant recognition of the non-habituated objects over the habituated objects.

The object preference task design was based on the E-maze task, in which the locations of the objects between the sample and the test phase are congruent (Eacott et al., 2005; Easton et al., 2009). This is unlike other recognition tasks that use the spontaneous object recognition paradigm, as the locations of the sample objects often change when presented in the test phase, sometimes for counterbalancing but other times as part of a specific task design. The poor recognition performance found in some of the current sub-

experiments can be reduced down to two potential reasons: either the object habituation was not effective, and as such there was little difference in terms of recognition of the two objects at test, or there was something additional driving exploration toward the habituated object at test, which meant that the non-habituated objects may still have been preferable, but not significantly so. The task modifications in Experiments 2 and 4 attempted to address these points but with little success. The locations of the objects in the test phase of an object preference trial are congruent with their presentation in the sample phase, but between the sample and the test phase, the habituated object appears in a novel location (the holding area). Studies that have assessed recognition memory for objects and their spatial locations, with the object-location task for instance, have demonstrated that animals preferentially explore the object in the test phase which has been presented in a novel location, more than the object presented in the familiar location, when both objects in themselves are familiar (Barker and Warburton, 2011; Ennaceur et al., 1997; Save, Poucet, Foreman and Buhot, 1992). As the habituated object in the object preference task moves location twice within a single trial (from the object area to the holding area for habituation, and then back to the object area for the test phase), it is possible that these changes in the object's location may drive exploration towards that particular object. Evidently, this added novelty was not sufficient for significant preferential exploration of the habituated object in the test phase, but it may have been sufficient in driving exploration away from the relatively novel non-habituated object, which remained in a congruent location between the sample and test phases of a trial.

The current series of object preference experiments has offered some insight into how object preference could be optimised in the E-maze episodic task. Further work is needed to investigate the specific parameters that can maximise object preference and recognition; however, there are other issues that will need to be addressed before a suitable episodic-like memory task, based on the E-maze task, can be developed. For instance, it is evident from the

work in Chapter 2 that context change within the continual trials apparatus may impact upon performance due to the nature in which contexts are changed whilst an animal remains in the apparatus. It is essential to refine this procedure if any episodic task is to be used in the continual trials apparatus based on the what-where-which occasion descriptor (Eacott and Norman, 2004). As the spontaneous object recognition and object-location memory tasks are the most widely used, the remainder of the animal work in this thesis focussed on adapting these tasks in the continual trials apparatus for use with immediate-early gene imaging, to elucidate recognition memory function in the medial temporal lobe.

CHAPTER 4

STUDY 3: INVESTIGATING RECOGNITION MEMORY USING IMMEDIATE-EARLY GENE IMAGING

4.1. Introduction

When a stimulus is re-encountered, there is evidence to suggest that neurons in the perirhinal cortex reduce their response, and that this process is essential to familiarity discrimination in recognition memory (Brown, Wilson and Riches, 1987; Brown and Xiang, 1998; Brown and Aggleton, 2001; Brown and Bashir, 2002; see Section 1.5). This reduced response following repeated presentation of a stimulus can be imaged in rodents using immediate-early genes (IEGs). IEGs are a specific group of genes that can be activated without previous protein synthesis (Herrera and Robertson, 1996). One group of IEGs, known as 'regulatory transcription factors', influence cell function through downstream genes, and two of these type of IEGs are known as c-fos and zif268. These IEGs are thought to be associated with neuronal plasticity (Herdegen and Leah, 1998; Herrera and Robertson, 1996; Seoane, Tinsley and Brown, 2012; Tischmeyer and Grimm, 1999), with c-fos activity being an indirect, but reliable, marker for changes in neuronal activity associated with recognition memory.

C-fos is widely distributed throughout the brain and can be used to detect differential activation of particular structures in the intact brain (Vann, Brown, Erichsen and Aggleton, 2000). However, it is only possible to measure relative changes in the structures that express the gene, as c-fos is not expressed in every brain region (Chaudhuri, 1997; Vann et al., 2000).

When animals are simultaneously presented with novel stimuli to one eye and familiar stimuli to the other eye in the paired viewing test, fos expression in the perirhinal cortex increases after viewing novel visual stimuli compared to familiar visual stimuli (Seoane et al.,

2012; Wan, Aggleton and Brown, 1999; Wan, Warburton, Zhu, Koder, Park et al., 2004; Warburton, Glover, Massey, Wan, Johnson et al., 2005; Warburton, Koder, Cho, Massey, Duguid et al., 2003; Zhu, McCabe, Aggleton and Brown, 1996), but no changes in hippocampal fos expression have been reported in these studies. Lower fos expression for familiar stimuli compared to novel stimuli is not surprising considering other studies have reported a reduction in single neuron responses when previously novel stimuli are presented again (Brown and Bashir, 2002; Brown, et al., 1987; Brown and Xiang, 1998; Griffiths, Scott, Glover, Bienermann, Ghorbel et al., 2008; Seoane et al., 2012). These findings highlight the importance of the perirhinal cortex in visual recognition memory, with more recent work showing that not only is the imaging of fos expression a reliable marker for neuronal activity associated with recognition memory, but it is indeed necessary for reliable recognition (Seoane et al., 2012). Blocking fos production with antisense fos oligodeoxynucleotide (ODN) in the perirhinal cortex either before or immediately after acquisition, has been shown to produce substantial deficits in recognition memory (Seoane et al., 2012).

Assessing neuronal activity after passively viewing novel or familiar stimuli has provided useful insights into the neural correlates of recognition memory, but it is essential to also have behavioural evidence to demonstrate that animals can distinguish between the novel and familiar stimuli. The spontaneous recognition paradigm (Ennaceur and Delacour, 1988) assesses recognition memory through an animals' preferential exploration of novel over familiar stimuli, thus demonstrating recognition for the familiar stimuli. This behavioural paradigm could provide the measure of recognition necessary; however, in the standard one trial a day version of this task, the animals are only exposed to a limited number of novel stimuli, which is unlikely to be sufficient to yield a detectable neuronal signal. As differential fos expression between groups is often assessed, results may be confounded if it is not

possible to yield a signal that is sufficiently large. Moreover, the task would be vulnerable to confounding factors such as animals being unable to discriminate between the novel and familiar stimuli, and the substantial amount of animal handling required, which would interfere with the neuronal signal (Kinnavane, Albasser and Aggleton, 2015).

New behavioural tasks have been recently devised to measure recognition memory based on the spontaneous paradigm, with the added benefit of being able to carry out multiple trials within a single testing session (Albasser, Chapman, Amin, Iordanova, Vann et al., 2010; Ameen-Ali, Eacott and Easton, 2012). In contrast to the standard version of the spontaneous recognition paradigm, the tasks devised using the Bow-tie maze (Albasser, Chapman et al., 2010) and the continual trials apparatus (Ameen-Ali et al., 2012; see Chapter 2) involve multiple trials within a session, which reduces animal handling and increases the number of trials run per animal. These benefits make multiple trial paradigms ideal for use with IEG imaging, as the likelihood of a detectable neuronal signal and strong behavioural preference of novel over familiar stimuli is increased.

C-fos activity has been assessed using the Bow-tie maze (Albasser, Poirier et al., 2010), whereby increased fos expression in the perirhinal cortex was reported when the animals actively discriminated novel from familiar stimuli in a spontaneous object recognition task. Animals in both the experimental group and the control group were tested using novel and familiar stimuli on each of the 20 recognition trials in the session. The animals in the control group, however, had previously spent a number of sessions familiarising to these objects, so that in the test phase they were relatively novel and relatively familiar, as opposed to being completely novel or completely familiar. This control group allowed the experimenters to identify the c-fos changes associated with recognition memory whilst maintaining comparable visuo-motor demands (Kinnavane et al., 2015). Unlike with the paired viewing test, the spontaneous object recognition task involves

exploring novel and familiar objects at the same time on each trial, so in order to assess differential c-fos expression, it is necessary to have one group of animals highly familiarised to the objects (Group Familiar), so they are ultimately making discriminations based on relative familiarity, and one group of animals highly familiarised to a different set of objects to those used at test (Group Novel). Therefore, the animals are making active object discriminations on each trial, but one group should have a greater novelty response. Albasser, Poirier et al. (2010) reported that the experimental group (Novel) showed significant discrimination between the novel and familiar stimuli, whereas the control group (Familiar) showed no significant discrimination, which is not entirely surprising as the animals were highly familiarised to the objects. This may, however, present an issue as it is possible that differential c-fos expression may have been due to task performance and exploration differences between the two groups of animals. As such, it is important to devise a task that can match performance between animal groups despite their prior familiarisation experience.

The main aim of the experiments reported in the current chapter was, therefore, to devise a behavioural task that could adequately match behavioural performance between animal groups when assessing the effect of relative novelty, but to also see whether similar results could arise from no prior familiarisation, and how these differences would affect c-fos expression. Initially, a series of behavioural tasks were carried out to identify the most suitable test procedure for a spontaneous object recognition task (Experiments 1, 3a and 3b), and for an object-location recognition memory task (Experiments 2, 4a and 4b). The spontaneous object recognition task was chosen because the task is robust and has produced reliable results in the continual trials apparatus (Ameen-Ali et al., 2012; see Chapter 2). The object-location task was also used, as previous studies have investigated object-context recognition memory using the one-trial a day paradigm (Wilson, Wantanabe, Milner and Ainge, 2013), and recency recognition memory using the Bow-tie maze multiple trial

paradigm (Kinnavane, Amin, Horne and Aggleton, 2014; Olarte-Sanchez, Kinnavane, Amin and Aggleton, 2014), but to our knowledge, no studies have yet investigated multiple trial object-location memory paradigms with IEG imaging. Some studies have investigated c-fos activation following spatial memory tasks in the radial arm maze (e.g. Vann et al., 2000), but it is important to devise behavioural tasks which can offer comparable measures of different forms of recognition memory.

Following these behavioural experiments, the most reliable behavioural paradigm was tested using the continual trials apparatus with IEG imaging to investigate whether the network interactions involved in recognition memory, which could arguably be assessed more effectively than if standard paradigms were being used (Experiment 5). It was hypothesised that amongst the three groups of animals tested (Group Familiar, with prior exposure to the test objects; Groups Novel and Naïve, with either no prior exposure to the test objects, or to no objects at all, respectively), all groups would significantly explore the novel (or relatively novel) objects more than the familiar (or highly familiar) objects. It would be important for no significant differences to be found between groups in terms of discrimination performance. With Groups Familiar and Novel matched on prior sensorimotor experience, there should be only small performance differences between these two groups, with Group Novel perhaps demonstrating slightly greater object exploration.

With differential c-fos expression measured, it was further predicted that Group Familiar would have significantly lower c-fos expression in the perirhinal cortex relative to Groups Novel and Naïve, as previous research has implicated this region in the detection of novelty, both when passively viewing novel stimuli (Seoane et al., 2012; Wan et al., 1999, 2004; Warburton et al., 2003, 2005; Zhu et al., 1996), and during a spontaneous object recognition task (Albasser, Poirier et al., 2010). C-fos expression was also quantified in the hippocampus, as research has reported significant fos increases in the CA1 and CA3

hippocampal subfields, but decreased in the dentate gyrus (Albasser, Poirier, et al., 2010), whilst other studies have reported no significant changes in c-fos expression in the hippocampus (e.g. Wan et al., 1999). It was hypothesised that there would not be any significant changes in c-fos in the hippocampus from the current study, as the changes reported in the study by Albasser, Poirier et al. (2010) may be attributed, in some part, to potential spatial demands of the Bow-tie maze, which are not present in the continual trials apparatus.

The first experiment in the current chapter was designed to be a close replication of the Bow-tie maze spontaneous object recognition task (Albasser, Poirier et al., 2010). Two groups of animals were tested on the spontaneous object recognition task in the continual trials apparatus, but one group of animals was tested on a set of objects that they had been previously familiarised to (Group Familiar), and the other group of animals were tested on a set of objects that was different to that which they had familiarised to (Group Novel).

The sample size for this initial experiment was purposefully small, as at this stage it was important to simply investigate recognition performance following familiarisation. The sample size was therefore based on what has been previously used in tasks using the continual trials apparatus (see Chapters 2 and 3). A necessary question that this series of experiments investigated was whether small groups of animals, that could show significant recognition, could also yield statistically meaningful results from the IEG data, or if more animals would be needed for adequate statistical power.

4.2. Materials and methods

Both groups of animals were familiarised to the same set of objects, though in separate sessions, with a familiarisation procedure that was slightly different to that used by Albasser, Poirier et al. (2010). In the Bow-tie maze study, the animals received 12 training

sessions over six days to familiarise them to either a different set of objects than those they would be tested on (Group Novel), or the same set of objects that they would be tested on (Group Familiar). The familiarisation training sessions involved the animals undergoing the same testing procedure that they would receive on the final experimental day. In the experiments in the current chapter, the animals received five familiarisation sessions over five days, which all took place in the open field arena, with testing taking place in the continual trials apparatus. This modification was made so that the animals would solely become familiarised to their object set rather than the objects and the testing procedure.

4.2.1. Subjects

In total, 36 male Lister hooded rats supplied by Harlan (Bicester, UK) were used in this series of experiments. All animals were housed in groups of three in diurnal conditions (12-h light-dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study, except for during times of pretraining, familiarisation or testing. 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.

4.2.2. Apparatus

The familiarisation phase of the task was carried out in the open field which was 1m long and 1m wide. Twenty objects were placed randomly around the apparatus at the start of each familiarisation phase with the same objects being used each time. Each familiarisation phase occurred in two contexts: Context Open Field (OF)1- a hatched wire surface on the floor of the apparatus with coloured circle pattern on the walls; Context OF2- a grey smooth surface on the floor of the apparatus with a pink and white striped pattern on the walls.

The animals were tested in the continual trials apparatus (Chapter 2, Section 2.2.2). During sample and test phases, objects were placed in the top left and top right-hand corners of the object area of the maze approximately 2cm away from the two walls to allow the animals to get their heads around the objects and explore them fully.

4.2.3. 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.

4.2.4. Pretraining

All animals underwent the handling and pretraining sessions outlined in Chapter 2 Section 2.2.4.

4.2.5. Behavioural analysis

Exploration of objects was defined as when the nose of the animal was <1cm 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 considered 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; see Chapter 2, Section 2.2.5).

4.3. Experiment 1: Spontaneous object recognition pilot task

4.3.1. Subjects

Six Lister hooded rats supplied by Harlan UK were housed in threes in diurnal conditions (12-hr light-dark cycle), with testing carried out during the light phase. At the time of testing, the animals were four months old and weighed an average of 300g.

4.3.2. Object familiarisation protocol

Initially the animals underwent a procedure to allow them to become habituated to a set of 20 different junk objects over a period of five days. All six animals were familiarised to objects (Set A), which would later become test objects in the next stage of the experiment (duplicate copies of objects were used) for three of the animals (Group Familiar). The remaining three animals (Group Novel) were to be tested on a different set of objects (Set B; Table 4.1). The animals spent one hour a day, for five days, habituating to objects in the open field arena in two different contexts (30 minutes a day with the objects in context OF1; 30 minutes a day with the objects in context OF2). The animals were placed in the arena with their cage mates and were allowed to freely explore the objects which were randomly placed around the arena.

Table 4.1. Experiment 1, example object presentation for familiarisation phase of spontaneous object recognition task in which 20 objects are randomly placed in an open field for the animals in both groups to freely explore. In the subsequent test phase, Familiar and Novel groups are tested on different object sets (see example test phase object presentation below).

Example familiarisation object presentation									
A	C	E	G	I	K	M	O	Q	S
B	D	F	H	J	L	N	P	R	T

Example test object presentation																			
Animal group	Trial	1		2		3		4		5		6		7		8		9		10	
Familiar	Sample	A	A	C	C	E	E	G	G	I	I	K	K	M	M	O	O	Q	Q	S	S
	Test	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Novel	Sample	1	1	3	3	5	5	7	7	9	9	11	11	13	13	15	15	17	17	19	19
	Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

4.3.3. Behavioural test protocol

On the day that immediately followed the last familiarisation phase, each of the six rats was given a single testing session of 10 trials in the continual trials apparatus, in which the animals were exposed to a novel object (or relatively novel object for the animals in Group Familiar), and a familiar (or relatively familiar) object on each trial, in accordance with the spontaneous object recognition procedure (Ameen-Ali et al., 2012 – see Chapter 2, Section 2.4.2; Ennaceur and Delacour, 1988).

4.3.4. Results

To determine whether the animals performed above chance, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero firstly for both groups combined

and secondly for both Group Familiar and Group Novel separately. The results showed that the animals overall significantly explored the novel objects more than the familiar objects ($t(5) = 2.787$, $p = 0.039$). However, when the groups were analysed separately, neither group significantly explored the novel objects more than the familiar objects (Group Familiar: mean D2 score = 0.29, $t(2) = 2.453$, $p = 0.134$; Group Novel: mean D2 score = 0.22, $t(2) = 1.347$, $p = 0.310$; Figure 4.1), although these analyses have very low levels of power.

Table 4.2. Experiment 1, spontaneous object recognition mean exploration for test phase.

Animal Group	**Exploration (s) for SOR experiment 1**						
	Novel object	SEM	Familiar object	SEM	Total	SEM	N
Novel	6.5	2.0	3.9	1.2	10.4	2.4	3
Familiar	4.6	1.2	2.4	0.6	7.0	1.3	3

The animals in Group Novel spent slightly longer exploring the objects during the test phase when compared to Group Familiar (Table 4.2), though not significantly so (mean total test exploration: $t(4) = 0.903$, $p = 0.417$). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(4) = 0.350$, $p = 0.744$).

4.3.5. Discussion

The current experiment was a spontaneous object recognition task using the continual trials apparatus investigating the effect of prior exposure to objects on task performance. All animals were habituated to a set of objects prior to the testing session, with half of the

animals then being tested with duplicate copies of these objects (Group Familiar), and half being tested with completely novel objects (Group Novel). When both groups were analysed together, it was clear that the animals significantly explored the novel objects more than the familiar objects, showing that the task procedure produced reliable measures of recognition. The mean D2 scores were reasonably high in both groups; however, significant measures of recognition were not found in either group, which is likely to be due to the small sample size with only three animals per experimental group and the resultant loss of power. Both groups displayed comparable discrimination scores with greater mean exploration times found for the animals in Group Novel, though this was not significant.

One of the main aims of the current chapter was to investigate forms of recognition memory with IEG imaging that have not currently been tested. Experiment 2, therefore, investigated the effect of prior exposure to objects on performance in a test of object-location memory, in order to develop an appropriate behavioural measure for use with IEG imaging.

4.4. Experiment 2: Object-location memory pilot task

4.4.1. Subjects

The same six Lister hooded rats (supplied by Harlan) used in Experiment 1 were again used in this experiment. Housing conditions were identical to the previous experiment. At the time of testing, the animals were five months old and weighed an average of 420g.

4.4.2. Object familiarisation protocol

Initially the animals underwent a procedure to allow them to become familiarised to a set of 20 different junk objects over a period of five days. All six animals were familiarised to one of the object sets from Experiment 1 (Set A), which would then become test objects in the next stage of the current experiment for the same animals that were Group Familiar in

Experiment 1. Duplicate copies of objects in the test phase were used. The remaining three animals (Group Novel) had a different set of objects (Set C) in the test stage of the experiment. The animals spent one hour a day (for five days) habituating to objects in the open field arena in two different contexts (30 minutes a day with the objects in context OF1; 30 minutes a day with the objects in context OF2). The animals were placed in the arena with their cage mates and were allowed to freely explore the objects which were randomly placed around the arena.

4.4.3. Behavioural test protocol

On the day that immediately followed the last familiarisation phase, each of the six rats were given a single testing session of 10 trials in the continual trials apparatus, in accordance with the object-location recognition memory procedure (Ameen-Ali et al., 2012 – see Chapter 2, Section 2.5.3; Dix and Aggleton, 1999).

4.4.4. Results

To determine whether the animals performed above chance, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero firstly for both groups combined and secondly for both Group Familiar and Group Novel separately. The results showed that the animals did not significantly explore the novel configurations of the objects and their locations more than the familiar configurations ($t(5) = 1.782$, $p = 0.135$). When analysed separately, the rats in both groups did not significantly explore the novel more than the familiar configurations (Group Familiar: mean D2 score = 0.03, $t(2) = 0.311$, $p = 0.785$; Group Novel: mean D2 score = 0.19, $t(2) = 3.215$, $p = 0.085$; Figure 4.1). Both groups spent a similar total amount of time exploring the objects during the test phase (Table 4.3; mean total

test exploration: t(4) = 0.391, p = 0.716). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: t(4) = 1.430, p = 0.226).

Table 4.3. Experiment 2, object-location recognition mean exploration for test phase.

Animal Group	Exploration (s) for OL experiment 2						
	Novel object	SEM	Familiar object	SEM	Total	SEM	N
Novel	5.0	2.3	2.1	0.6	7.2	2.5	3
Familiar	4.1	1.3	3.8	1.3	7.9	2.4	3

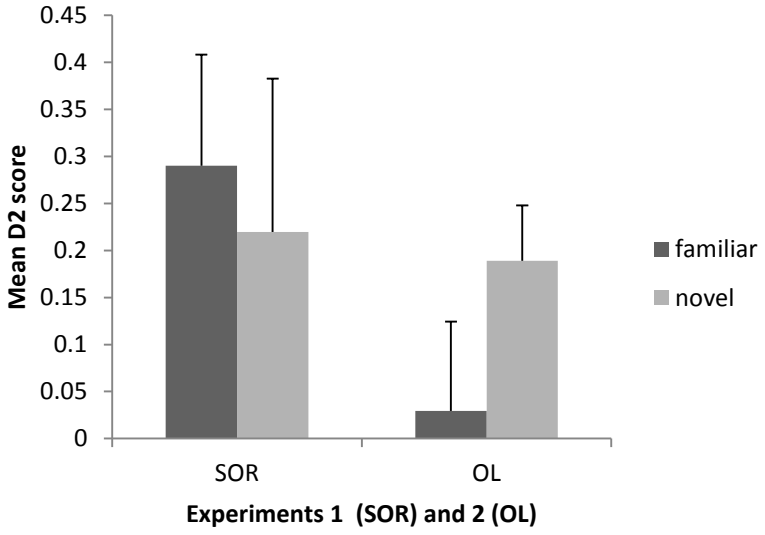


Figure 4.1. Animal performance in Experiments 1 and 2. Mean D2 scores for Experiments 1 (SOR) and 2 (OL). Vertical bars show the standard error of the mean.

4.4.5. Discussion

The current experiment was an object-location memory task using the continual trials apparatus investigating the effect of prior exposure to objects on task performance. All animals were habituated to the same set of objects prior to the testing session, with half of the animals then being tested with duplicate copies of these objects (Group Familiar) and half being tested with completely novel objects (Group Novel). Neither group significantly explored the novel object-location configurations more than the familiar configurations, but this is likely to be attributed to the small sample size and resultant loss of power, at least for Group Novel who displayed reasonable discrimination as indicated through the mean D2 score. Group Familiar had a mean D2 score close to zero indicating no preference, suggesting they were highly familiarised to the objects, and the location novelty at test was not sufficient in this case for the animals to display exploration preferences. No significant differences were found between the two groups on discrimination or exploration measures.

It is possible that exploration differences between the two animal groups in Experiments 1 and 2 could account for differences in fos expression, so although there were no significant group differences in terms of task performance in Experiments 1 and 2 (which may have been due to insufficient power), the protocol was changed so that the sample size was increased, and both groups of animals were tested on the same objects during the test phase. This would hopefully minimise any exploration differences between the two animal groups based on the use of different test objects. The following experiments (3a and 3b) were replications of the spontaneous object recognition task in Experiment 1, but the groups 'Novel' and 'Familiar' were established through familiarisation to two different sets of objects, with one set of objects being used as the test set (the familiarised object set for Group Familiar). Exploration during the sample phase was also analysed to ensure there were no

significant exploration differences at this stage of the task which could account for differential fos expression in the subsequent IEG task.

4.5. Experiments 3a and 3b: Additional spontaneous object recognition pilot tasks

4.5.1. Subjects

Twelve naive Lister hooded rats supplied by Harlan UK were housed in threes in diurnal conditions (12-hr light-dark cycle), with testing carried out during the light phase. Water was available ad libitum throughout the study, except during familiarisation, habituation and testing. 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 two months old and weighed an average of 250g.

4.5.2. Object familiarisation protocol

The animals took part in two spontaneous object recognition tasks. The first task began with the animals undergoing a procedure to allow them to become familiarised to a set of 20 different junk objects over a period of five days. Six animals (Group Familiar) were familiarised to objects (Set A) that would become test objects in the next stage of the experiment (duplicate copies of objects were used). The remaining six animals (Group Novel) were familiarised to a different set of objects (Set B) to Group Familiar, but then tested on the same objects as the other group in the next stage of the experiment (Set A; Table 4.4). The animals spent one hour a day, over five days, familiarising to objects in the open field arena in two different contexts (30 minutes a day with the objects in context OF1; 30 minutes a day with the objects in context OF2). The animals were placed in the arena with their cage mates and were allowed to freely explore the objects which were randomly placed around the arena.

Table 4.4. Experiment 3, example object presentation for familiarisation phase of spontaneous object recognition task. Twenty objects are randomly placed in an open field for the animals in Group Familiar to explore. Animals in Group Novel freely explore a different set of 20 objects. In the subsequent test phase, both groups of animals are tested on the same set of objects, which Group Familiar are highly familiar with.

Animal Group	**Example familiarisation object presentation**									
Familiar	A	C	E	G	I	K	M	O	Q	S
	B	D	F	H	J	L	N	P	R	T
Novel	1	3	5	7	9	11	12	15	17	19
	2	4	6	8	10	12	14	16	18	20

	Example test object presentation									
Trial	1	2	3	4	5	6	7	8	9	10
Sample	A A	C C	E E	G G	I I	K K	M M	O O	Q Q	S S
Test	A B	C D	E F	G H	I J	K L	M N	O P	Q R	S T

4.5.3. Behavioural test protocol

The test protocol was identical to that used in Experiment 1 (see Section 4.3.3*).* On the day that immediately followed the testing session for the spontaneous object recognition task, the animals began the first stage of object familiarisation for the next spontaneous object recognition task through the procedure outlined in Section 4.3.2. The animals which had previously been Group Familiar became Group Novel and vice versa (Table 4.5). This was so that all the animals would experience being tested on both a familiar set and a novel set of objects in the test phase. The new Group Familiar were again familiarised to object Set B, whereas the new Group Novel were familiarised to object Set C. During the testing session that followed familiarisation, both groups were tested using object Set B.

Table 4.5. Animal group progression through Experiments 3 and 4.

Experiment	Group	Animal numbers	Familiarised object set	Test object set
3a: SOR	Familiar	1-6	A	A
	Novel	7-12	B	A
3b: SOR	Familiar	7-12	B	B
	Novel	1-6	C	B
4a: OL	Familiar	1-6	C	C
	Novel	7-12	D	C
4b: OL	Familiar	7-12	D	D
	Novel	1-6	E	D

4.5.4. Results

4.5.4.1. Pooled data

The data from both spontaneous object recognition tasks (Experiments 3a and 3b) were first pooled together and analysed. A one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero for both Group Familiar and Group Novel. The results showed that the Group Familiar animals significantly explored the relatively novel objects more than the relatively familiar objects, and the Group Novel animals significantly explored the novel objects more than the familiar objects (Group Familiar: mean D2 score = 0.19, $t(11) = 5.533$, $p = < 0.001$; Group Novel: mean D2 score = 0.25, $t(11) = 7.053$, $p = < 0.001$).

The animals in both Novel and Familiar groups (Table 4.6) spent a similar amount of time in total exploring the objects during the test phase (mean total test exploration: $t(22) =$

0.527, p = 0.603). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: t(22) = 1.190, p = 0.247).

Table 4.6. Experiment 3, spontaneous object recognition mean exploration for test phase (pooled data).

	Group Familiar Exploration (s)						Group Novel Exploration (s)					
	Familiar object	SEM	Novel object	SEM	Total	SEM	Familiar object	SEM	Novel object	SEM	Total	SEM
	4.1	1.3	6.0	1.3	10.1	1.8	3.7	1.0	7.3	2.0	11.0	2.2
N	12		12				12		12			

4.5.4.2. Separate group analyses

To determine whether the animals performed above chance in the first spontaneous object recognition test, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero for both Group Familiar and Group Novel. The results showed that the Group Novel animals significantly explored the novel objects more than the familiar objects, and the Group Familiar animals significantly explored the relatively novel objects more than the relatively familiar objects (Group Familiar: mean D2 score = 0.21, t(5) = 5.095, p = 0.004; Group Novel: mean D2 score = 0.23, t(5) = 5.438, p = 0.003; Figure 4.2).

The animals in Group Familiar spent more time exploring the objects during the test phase when compared to Group Novel, but not significantly so (Table 4.7; mean total test exploration: t(6.328) = 1.636, p = 0.150).

Table 4.7. Experiment 3, spontaneous object recognition mean exploration for test phase.

		Group Familiar Exploration (s)							Group Novel Exploration (s)					
		Familiar object	SEM	Novel object	SEM	Total	SEM		Familiar object	SEM	Novel object	SEM	Total	SEM
Experiment 3a: SOR	Rats 1-6	4.9	1.4	7.9	1.7	12.9	2.1	Rats 7-12	3.1	0.6	5.8	1.6	8.9	1.7
Experiment 3b: SOR	Rats 7-12	3.3	1.1	4.0	0.9	7.4	1.5	Rats 1-6	4.4	1.4	8.8	2.4	13.2	2.7

The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(10) = 0.207$, $p = 0.840$).

For the second spontaneous object recognition test, again Group Novel animals significantly explored the novel objects more than the familiar objects and the Group Familiar animals significantly explored the relatively novel objects more than the recently familiar objects (Group Familiar: mean D2 score = 0.17, $t(5) = 2.941$, $p = 0.032$; Group Novel: mean D2 score = 0.27, $t(5) = 4.602$, $p = 0.006$). The animals in Group Novel spent significantly more time in total exploring the objects during the test phase when compared to Group Familiar (Table 4.6; mean total test exploration: $t(10) = 3.370$, $p = 0.007$), although the two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(10) = 1.274$, $p = 0.232$).

4.5.4.3. Sample phase analysis

The exploration times during the sample phase of the testing session were analysed to see whether there were any differences in exploratory behaviour between Groups Novel and Familiar (Table 4.8). In both spontaneous object recognition tasks (Experiments 3a and 3b) both groups of animals, on average, spent a similar amount of time exploring the objects in

the sample phase of the testing session (Experiment 3a: $t(10) = 0.544$, $p = 0.598$; Experiment 3b: $t(10) = 0.680$, $p = 0.512$).

Table 4.8. Experiment 3, spontaneous object recognition mean exploration for sample phase.

		Group Familiar			Group Novel	
		Exploration (s)	SEM		Exploration (s)	SEM
Experiment 3a: SOR	Rats 1-6	6.5	1.1	Rats 7-12	5.9	0.4
Experiment 3b: SOR	Rats 7-12	5.6	1.0	Rats 1-6	6.6	1.1

4.5.5. Discussion

The current experiments were two spontaneous object recognition tasks using the continual trials apparatus. Reliable measures of recognition were found with both groups in the first spontaneous object recognition task (Experiment 3a), with both Novel and Familiar groups displaying comparable discrimination scores. Exploration times were, on average, greater for the animals in Group Familiar but no significant differences were found between the two groups on discrimination or exploration measures. Reliable measures of recognition were also found with both groups in the second spontaneous object recognition task (Experiment 3b). Discrimination and exploration measures were, on average, greater for the animals in Group Novel. No significant differences were found between the two groups on measures of discrimination, but mean total exploration time during the test phase was significantly greater for Group Novel when compared to Group Familiar.

When devising a recognition task for use with IEG imaging, it is important to minimise the differences in task performance as it helps to match the sensorimotor experiences of the two groups of animals (Aggleton, Brown and Albasser, 2012). The Novel group in Experiment 3b showed significantly greater object exploration in the test phase than

the Familiar group, but when the data from Experiments 3a and 3b were pooled together, both Novel and Familiar groups showed significant preference for novel (or relatively novel) objects over familiar objects, with no significant differences in task performance or exploration at test between the groups. In addition, analysis of the sample phase exploration times indicated no significant differences between the Novel and Familiar Groups in both Experiments 3a and 3b. This is potentially an important finding in the development of a behavioural paradigm to use with IEG imaging, and an improvement upon the behavioural findings using the Bow-tie maze (Albasser, Poirier et al., 2010). As there were no significant differences in terms of performance between the two groups of animals overall in Experiment 3, it would not be possible to attribute differences in c-fos expression to unmatched behavioural performance if this paradigm was to be used with IEG imaging.

The following experiments (4a and 4b) were replications of the object-location recognition memory task in Experiment 2, but the sample size was increased and the protocol was adjusted so that both groups of animals were tested on the same objects during the test phase, in order to minimise any exploration differences between the two animal groups based on the use of different test objects.

4.6. Experiments 4a and 4b: Additional object-location memory pilot tasks

4.6.1. Subjects

The same 12 Lister hooded rats (supplied by Harlan) used in Experiment 3 were again used in this experiment. Housing conditions were identical to previous experiments. At the time of testing, the animals were four months old and weighed an average of 300g.

4.6.2. Object familiarisation protocol

The animals took part in two object-location tasks. The first task began with the animals undergoing the same object familiarisation protocol as that used in Experiment 3 (outlined in Section 4.3.2), but with an extra object included in each set. A duplicate copy of this additional object would be used for a probe trial (where this object would be paired with a completely novel object) at the end of each testing session in order to see how successful the familiarisation phase had been in inducing object familiarisation. In the previous experiments it is not clear whether the familiarisation of the object sets worked successfully with the current protocol, particularly as the familiar groups performed successfully with minimal performance difference between familiar and novel groups. With a probe trial it is possible to assess whether the familiarisation protocol is successful.

The six animals which were most recently Group Novel in Experiment 3b became Group Familiar, and were again familiarised to objects (Set C) that would become test objects in the test stage of the experiment (duplicate copies of objects were used). The remaining six animals which had most recently been Group Familiar in Experiment 3b became Group Novel, and were familiarised to a set of objects (Set D), and then tested on the same objects as the other group in the test stage of the experiment (Set C). The animals spent one hour a day habituating to objects in the open field arena in two different contexts (30 minutes a day with the objects in context OF1; 30 minutes a day with the objects in context OF2). The animals were placed in the arena with their cage mates and were allowed to freely explore the objects which were randomly placed around the arena.

4.6.3. Behavioural test protocol

The test protocol was identical to that used in Experiment 2 (see section 4.4.3.). On the day that immediately followed the first object-location task the animals then began the

first stage of object familiarisation for the next object-location task, through the procedure outlined in section 4.3.2. The animals which had previously been Group Familiar became Group Novel and vice versa (Table 4.5). The new Group Familiar were again familiarised to object Set D, whereas the new Group Novel were familiarised to object Set E. During the testing session that followed familiarisation, both groups were tested using object Set D.

4.6.4. Results

4.6.4.1. Pooled data

The data from both object-location tasks were pooled together and analysed. A one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero for both Group Familiar and Group Novel. The results showed that the Group Familiar animals significantly explored the relatively novel objects more than the recently familiar objects, but the Group Novel animals did not significantly explore the novel objects more than the familiar objects (Group Familiar: mean D2 score = 0.10, $t(11) = 2.217$, $p = 0.049$; Group Novel: mean D2 score = 0.07, $t(11) = 1.644$, $p = 0.128$). The animals in Group Novel spent slightly more time than Group Familiar (Table 4.9) exploring the objects during the test phase, though not significantly (mean total test exploration: $t(22) = 0.928$, $p = 0.363$). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(22) = 0.399$, $p = 0.694$).

Table 4.9. Experiment 4, object-location recognition mean exploration for test phase (pooled data).

	Group Familiar Exploration (s)						**Group Novel Exploration (s)**					
	Familiar object	SEM	Novel object	SEM	Total	SEM	Familiar object	SEM	Novel object	SEM	Total	SEM
	5.5	1.6	7.1	2.2	13.5	3.2	5	1.3	7	2.5	14	3.1
N	12		12				12		12			

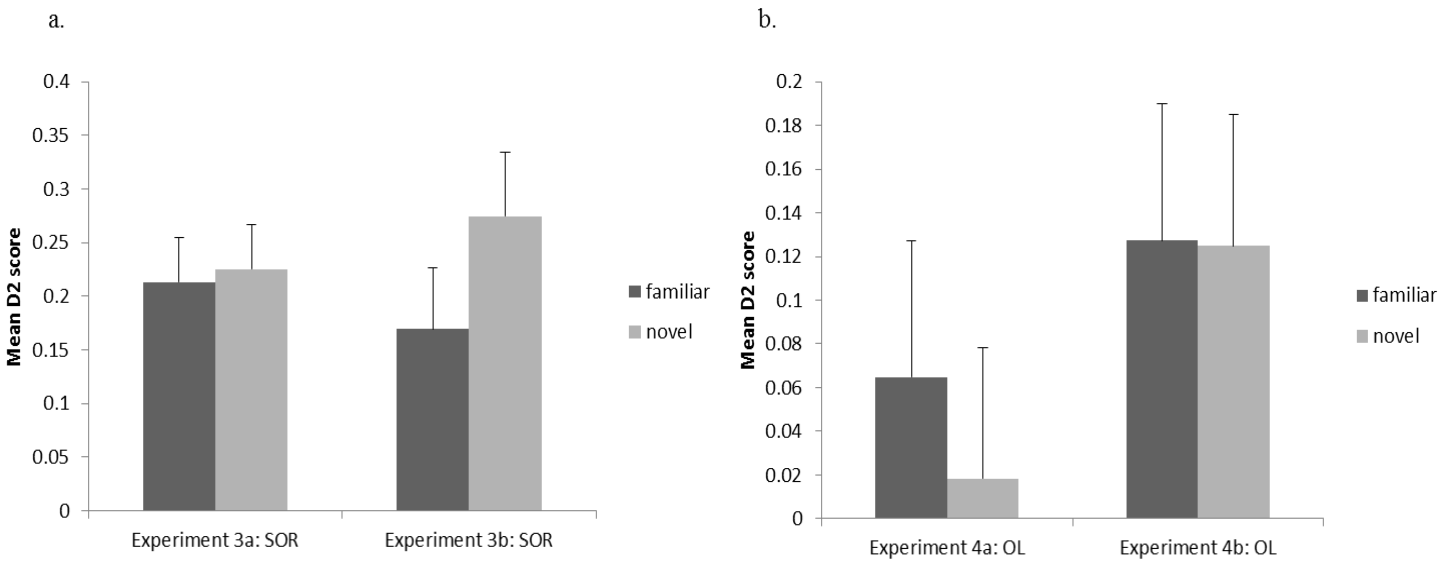


Figure 4.2. Animal performance in Experiments 3 and 4. a) Mean D2 scores for Experiments 3a and 3b: SOR. b) Mean D2 scores for Experiments 4a and 4b: OL. Vertical bars show the standard error of the mean.

4.6.4.2. Separate group analyses

To determine whether the animals performed above chance in the first object-location task, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero for both Group Familiar and Group Novel. The results showed that both Group Novel and Group Familiar failed to significantly explore the novel configurations of objects and their locations more than the familiar configurations (Group Familiar: mean D2 score = 0.06, $t(5) = 1.031$, $p = 0.350$; Group Novel: mean D2 score = 0.02, $t(5) = 0.306$, $p = 0.772$; Figure 4.2). Both groups did, however, spend more time on average exploring the novel configurations over the familiar configurations (Table 4.10). The animals in both Novel and Familiar groups spent a similar amount of time in total exploring the objects during the test phase (mean total test exploration: $t(10) = 1.500$, $p = 0.165$). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(10) = 0.535$, $p = 0.604$).

Table 4.10. Experiment 4, object-location recognition mean exploration for test phase.

		Group Familiar Exploration (s)							Group Novel Exploration (s)					
		Familiar object	SEM	Novel object	SEM	Total	SEM		Familiar object	SEM	Novel object	SEM	Total	SEM
Experiment 4a: OL	Rats 1-6	5.9	1.4	7.6	2.0	13.5	2.6	Rats 7-12	5.0	1.3	6.2	1.8	11.2	2.7
Experiment 4b: OL	Rats 7-12	5.1	1.6	6.7	2.2	11.7	3.5	Rats 1-6	4.0	1.2	7.5	3.2	11.5	3.4

For the second object-location task, both Group Novel and Group Familiar failed to significantly explore the novel configurations more than the familiar configurations (Group Familiar: mean D2 score = 0.13, $t(5) = 2.033$, $p = 0.098$; Group Novel: mean D2 score = 0.12, $t(5) = 2.071$, $p = 0.093$). The animals in both Novel and Familiar groups spent a similar amount of time in total exploring the objects during the test phase (mean total test exploration: $t(10) = 0.086$, $p = 0.933$). The two groups of animals did not differ significantly on mean D2 scores (mean D2 score: $t(10) = 0.030$, $p = 0.977$).

4.6.4.3. Probe trials

At the end of the testing session, a final recognition trial was carried out in which the animals were exposed to an additional familiarised object and a completely novel object. This trial was not used in any other data analyses but rather as a way to see how effective the familiarisation protocol was at familiarising the animals to the objects prior to the test phase. Data for both Familiar and both Novel groups in Experiments 4a and 4b were combined to give an overall mean D2 score for Group Familiar and overall mean D2 score for Group Novel (note – one animal from Group Familiar in Experiment 4b did not complete the probe trial as it failed to shuttle). Both groups significantly explored the novel object more than the familiarised object (Group Familiar: Mean D2 score = 0.22; $t(10) = 2.737$, $p = 0.021$; Group

Novel: Mean D2 score = 0.27; t(11) = 2.691, p = 0.021), suggesting that the objects had been successfully familiarised.

4.6.4.4. Sample phase analysis

The exploration times during the sample phase of the testing session were analysed to see whether there were any differences in exploratory behaviour between Groups Novel and Familiar (Table 4.11). In both object-location tasks the animals in Group Novel spent, on average, more time than Group Familiar exploring the objects in the sample phase of the testing session. This was significant in the first object-location task and approaching significance in the second task (Experiment 4a: t(10) = 2.291, p = 0.045; Experiment 4b: t(10) = 2.183, p = 0.054).

Table 4.11. Experiment 4, object-location recognition mean exploration for sample phase.

		Group Familiar			**Group Novel**	
		Exploration (s)	SEM		Exploration (s)	SEM
Experiment 4a: OL	Rats 1-6	8.0	1.1	Rats 7-12	10.9	0.7
Experiment 4b: OL	Rats 7-12	9.9	1.6	Rats 1-6	13.1	0.7

4.6.4.5. Further analysis

A mixed model three-way ANOVA with a 2x2x2 design was carried out to see whether task performance was affected by 'Group' (Novel or Familiar), the order of the animal groups (whether they were Group Novel first and Group Familiar second or vice versa), or the type of task being performed (spontaneous object recognition or object-location

memory task). An effect of task was found ($F(1, 10) = 14.763$, $p = 0.003$) suggesting that performance differed significantly between the two tasks, but neither an effect of order nor group was found (order: $F(1, 10) = 0.427$, $p = 0.528$; group: $F(1, 10) = 0.306$, $p = 0.592$), indicating that performance in the task was not significantly affected by the order of the animal's groups or indeed the actual group that the animals were in.

4.6.5. Discussion

The current experiments were two object-location memory tasks using the continual trials apparatus. For Experiment 4a, no reliable measures of recognition were found in either group, with no significant difference between the groups on discrimination scores and exploration measures. For Experiment 4b, again no reliable measures of recognition were found in either group, with both groups displaying comparable discrimination scores and mean total exploration times.

When the data from Experiments 4a and 4b were pooled together, only Group Familiar showed significant preference for the novel (or relatively novel) configurations of object and location more than familiar configurations, but this was only marginally significant and there were no significant differences in task performance or exploration at test between the groups. Analysis of the sample phase exploration times indicated no significant differences between the Novel and Familiar Groups in Experiment 4b, but Group Novel explored the sample objects significantly more than Group Familiar in Experiment 4a.

From observing the mean D2 scores for each animal, it is evident that there remains variability in performance within the two animal groups. For the 'Novel' animals, this would have been a standard object-location memory task, as they were tested on objects they had never previously been exposed to. This task has been successfully performed in the continual trials apparatus (See Chapter 2, Section 2.5), so it is questionable why performance was at

chance in the current task. It is worth noting that the familiarisation procedure was successful in familiarising the animals in each group to the object sets, as when a probe trial was carried out at the end of each testing session, in which the animals were exposed to an object from the familiarisation phase (not used in the experiment proper) and a completely novel object, the animals significantly explored the novel object more than the probe object. It is possible that the poor discrimination measures found in these two object-location tasks may be due to the objects at test being explored almost equally, and the location novelty at test not being sufficient to drive exploration to the novel configuration. The animals could clearly distinguish a familiarised object from a completely novel object, as shown in the probe trials, but failed to discriminate between two familiarised objects when one has associated location novelty (Group Familiar), or between novel and familiar items when the familiar item is not highly familiarised (Group Novel). More work is needed to refine this task to see how familiarisation may impact upon recognition, before it can be adapted for use with IEG imaging.

The following experiment (Experiment 5) adapted the spontaneous object recognition task from Experiments 3a and 3b for use with IEG imaging, but a naïve group was included as an addition control that had no prior familiarisation of objects. A naïve group was not previously included in the study by Albasser, Poirier et al. (2010), but is necessary as an additional control, in order to assess the level of c-fos activation from object novelty when the animals do not have matched sensorimotor experience as the other animal groups. If c-fos expression in the naïve group is comparable to the novel group and both are greater than in the familiar group, it can be reasoned that object novelty is important for increases in c-fos expression, and familiarisation has to occur with the objects to be tested on in order for reduced c-fos expression to be found, not just any set of objects. This additional control would therefore inform on c-fos expression when novel objects are used but when there has

been no prior exposure to objects. Following the behavioural test, the animal's brains were processed in accordance with the immunohistochemistry procedure (see Section 4.7.4). The regions of interest were three hippocampal subfields (CA1, CA3 and dentate gyrus) and the perirhinal cortex, as there is ongoing debate around the contribution of these structures to recognition memory (Aggleton and Brown, 1999; Squire, Stark and Clark, 2004; Tulving and Markowitsch, 1998).

4.7. Experiment 5: Spontaneous object recognition c-fos task

4.7.1. Subjects

Eighteen naive Lister hooded rats, supplied by Durham University LSSU in-house breeding colony, were housed in pairs in diurnal conditions (12-hr light-dark cycle) with testing carried out during the light phase. Water was available ad libitum throughout the study, except during familiarisation and testing. All animals were food deprived to 85% of the free-feeding body weight of aged matched controls throughout testing. At the time of testing, the animals were four months old and weighed an average of 300g.

4.7.2. Object familiarisation protocol

The animals took part in a single spontaneous object recognition task which began with the animals undergoing the same object familiarisation protocol as that used in Experiment 3 (outlined in Section 4.3.2). In the current experiment, however, six animals (Group Familiar) were familiarised to objects (Set A; Table 4.12) that would become test objects in the next stage of the experiment (duplicate copies of objects were used); six animals (Group Novel) were familiarised to a different set of objects (Set B) to Group Familiar but were then tested on the same objects as the Familiar group in the next stage of the experiment (Set A); the remaining six animals (Group Naïve) were not familiarised to any

objects but were tested on the same objects as the other two groups in the next stage of the experiment (Set A). The animals in Groups Familiar and Novel spent one hour a day habituating to objects in the open field arena in two different contexts (30 minutes a day with the objects in context OF1; 30 minutes a day with the objects in context OF2). The animals were placed in the arena with their cage mates and were allowed to freely explore the objects which were randomly placed around the arena.

Table 4.12. Object sets for familiarisation and test session for each animal group in Experiment 5 (c-fos experiment).

	Animal Group		
	Familiar	Novel	Naive
Familiarisation	Object set A	Object set B	-
Test session	Object set A	Object set A	Object set A

4.7.3. Behavioural test protocol

On the day that immediately followed the last familiarisation phase, six rats per day (over 3 days) were each given a single testing session of 10 trials in accordance with the spontaneous object recognition procedure. The test protocol was identical to that used in Experiment 3 (outlined in Section 4.5.3) with all animals being tested on object Set A. Two animals per group were tested each day.

4.7.4. Immunohistochemistry

Thirty minutes following completion of the behavioural test protocol, the animals were humanely euthanased with i.p. injections of sodium pentobarbitone (120mg/kg, Pentoject, Animalcare Limited, York, UK). They were then transcardially perfused with 0.1M phosphate buffered saline, followed by 4% paraformaldehyde in 0.1M phosphate

buffered saline. Brains were removed from the skull, postfixed in 4% paraformaldehyde for 24 hours, and then incubated in 25% sucrose solution (made up in 0.1M phosphate buffer).

The brains were cut in the coronal plane into 40μm sections with a cryostat set to -18^{o}c. A series of one in four sections were taken in phosphate buffered saline for subsequent staining and analysis. Sections were washed twice in 0.1M phosphate buffered saline and then processed immunohistochemically to analyse c-fos expression. Sections were placed in blocking solution (25% normal goat serum) for 45 minutes, then washed a further two times in phosphate buffered saline. Sections were then incubated in primary antibody solution at a concentration of 1:4000 (Merkmillipore) overnight on a stirrer plate, at room temperature. Sections were removed from the primary antibody solution and washed five times in phosphate buffered saline. The sections were then placed in secondary antibody solution; biotinylated IgG (anti-rabbit, Vectastain Elite ABC kit) at a concentration of 1:200 for 90 minutes, before a further five washes in phosphate buffered saline. The sections were then incubated in avidin-biotin complex (Vectastain Elite ABC kit) at a concentration of 1:50 for 60 minutes. Following a further five washes in phosphate buffered saline, the sections were reacted with nickel enhanced 3,3-diaminobenzidine tetrahydrochloride (Sigma); the chromogen used to visualise the location of immunostaining. Sections were washed a further five times in phosphate buffered saline before being mounted on to gelatin-coated slides, dehydrated and coverslipped.

4.7.5. Regions of interest

Regions of interest within the c-fos labelled sections were identified with reference to the rat brain anatomy atlas by Paxinos and Watson (2006). Counts were taken from three subregions of perirhinal cortex; rostral (from AP -2.76 to -3.84 relative to bregma; ML -5.6 to -7.2; DV -5.3 to -6.6), mid (AP -3.84 to -4.8; ML -5.6 to -7.4; DV -4.9 to -6.0), and caudal

(from AP -4.8 to -6.3; ML -6.0 to -7.6; DV -4.0 to -5.9). Subfields of the hippocampus (CA1, CA3, and dentate gyrus) were divided into dorsal and intermediate parts (Bast, 2007; Bast, Wilson, Witter and Morris, 2009). The dorsal counts were taken from sections near AP -2.53 from bregma (ML -0.6 to -1.8; DV -2.4 to -4.2), and the intermediate counts were taken from sections near -4.8 and -5.0 from bregma (ML -2.1 to -5.8; DV -2.1 to -7.8).

4.7.6. C-fos quantification

Subregions of the hippocampus and parahippocampal cortices were localised using a light microscope at 5x magnification with photographs taken at 10x magnification under consistent light levels. For each subregion, at least four photographs were taken and images were processed using Scion Image (v4.0.3.2). C-fos expression was identified by taking a mean gray scale of each image and identifying pixels that were 2 standard deviations darker than the mean. C-fos positive neurons were classified as groups of more than 20 and less than 500 adjacent pixels whose gray scale was more than 2 standard deviations greater than the mean gray scale for that image. The density of c-fos expression was calculated by dividing the total count of c-fos positive neurons within each subregion by the total area from which these counts were taken, giving a value of c-fos positive neurons per mm^2. These density scores were then normalised by dividing by the mean count for that subregion, across animal groups, and then multiplying by 100. The process of normalising the cell counts differed from that reported by Albasser, Poirier et al. (2010). In their study, pairs of animals were matched based on group (one from Group Novel, one from Group Familiar), and cell counts were normalised according to the pairs, with the mean number of activated cells in a given animal for a given site divided by the combined mean of the two animals in the pair. As the current experiment involved three groups of animals that were not matched throughout familiarisation, testing, and histology, the normalisation method used by Wilson et al. (2013)

offered a suitable alternative to allow for comparison of subregions with different cell densities. Statistical analyses were carried out using these normalised scores.

4.7.7. Statistical analysis

Normalised c-fos positive counts were analysed in two groupings: hippocampus (dorsal and intermediate portions of CA1, CA3 and dentate gyrus) and perirhinal cortex (rostral, mid and caudal). Counts were analysed using a repeated measures ANOVA with *Group* (Familiar, Novel, and Naïve) as the between subjects factor and *Subregion* as the within subjects factor.

4.7.8. Results

4.7.8.1. Behavioural analysis

4.7.8.1.1. Exploration measures

To determine whether the animals performed above chance in the spontaneous object recognition task, a one-sample t-test (two-tailed) was used to compare the mean D2 scores against zero for all three groups. The results showed that all three groups of animals significantly explored the novel (or relatively novel) stimuli more than the familiar stimuli (Group Familiar: mean D2 score = 0.31, $t(5) = 3.397$, $p = 0.019$; Group Novel: mean D2 score = 0.44, $t(5) = 5.751$, $p = 0.002$; Group Naïve: mean D2 score = 0.37, $t(5) = 5.064$, $p = 0.004$; Figure 4.3). There was a significant difference between groups in the amount of time spent exploring the test objects ($F(2,15) = 9.668$, $p = 0.002$; Table 4.13), but this difference was between Groups Naïve and Novel ($t(10) = 3.575$, $p = 0.005$), and Groups Naïve and Familiar ($t(10) = 3.383$, $p = 0.007$). There was no significant difference in mean test exploration time between groups Familiar and Novel ($t(10) = 0.179$, $p = 0.861$). The three groups of animals did not differ significantly on mean D2 scores ($F(2,15) = 0.592$, $p = 0.565$).

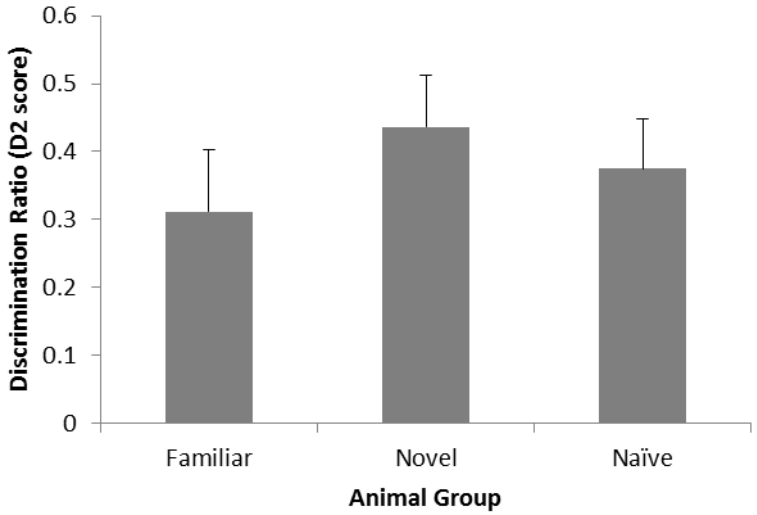


Figure 4.3. Animal performance in Study 4, Experiment 5. Mean D2 scores for the three animal groups. Vertical bars show the standard error of the mean.

4.7.8.1.2. Sample phase exploration

The exploration times during the sample phase of the testing session were analysed to see whether there were any differences in exploratory behaviour between the three groups (Table 4.13). A significant difference was found between the animal groups on their levels of exploration during the sample phase of the testing session ($F(2, 15) = 3.894$, $p = 0.043$), but planned t-tests revealed no significant differences in sample phase exploration level between Groups Familiar and Novel ($t(10) = 0.112$, $p = 0.913$), and marginal differences between Groups Familiar and Naïve ($t(10) = 2.134$, $p = 0.059$), and Groups Novel and Naïve ($t(6.698) = 2.190$, $p = 0.066$) that were also not significant.

Table 4.13. Spontaneous object recognition mean exploration for sample and test phases.

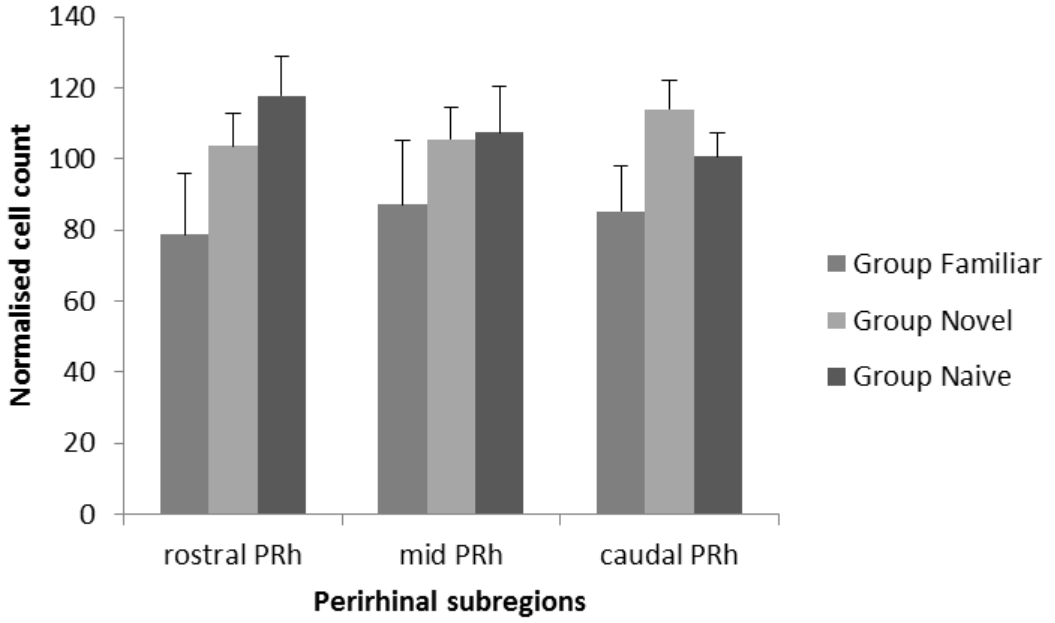
	Group Familiar Exploration (s)	SEM	Group Novel Exploration (s)	SEM	Group Naive Exploration (s)	SEM
Sample phase	6.4	1.0	6.5	0.8	10.8	1.8
Test phase	14.5	3.3	14.2	3.0	23.7	3.6
N	6		6		6	

4.7.8.2. Immediate-early gene results

4.7.8.2.1. Perirhinal cortex

C-fos expression was quantified throughout the perirhinal cortex (Figures 4.4 and 4.5). A repeated measures ANOVA of the perirhinal data revealed no significant *Group* X *Subregion* interaction ($F(4, 30) = 0.518$, $p = 0.723$), but there was a trend towards a significant main effect of *Group* ($F(2, 15) = 2.800$, $p = 0.093$) with increased c-fos counts in Group Novel and Naive, relative to Group Familiar. It is possible that these results may have reached significance with greater statistical power (i.e., another 2-4 animals per experimental group).

a.



b.

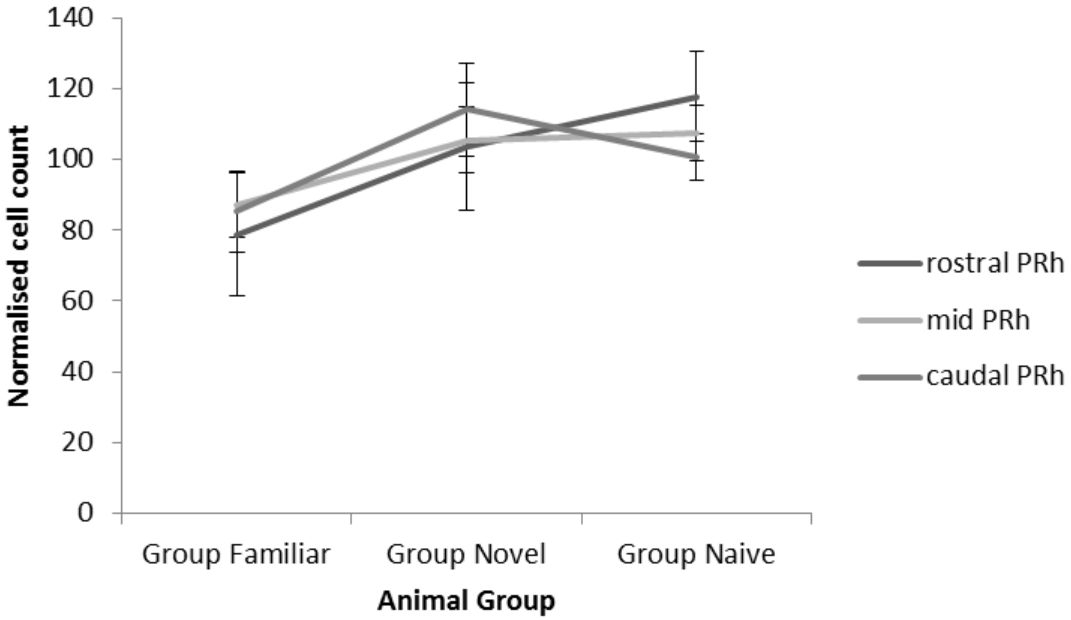


Figure 4.4. Activation in the perirhinal cortex during spontaneous object recognition. a) Normalised c-fos expression in the perirhinal cortex divided into subregions (rostral, mid, and caudal). b) Line graph to further illustrate normalised c-fos expression between the three groups of animals. Vertical bars show the standard error of the mean.

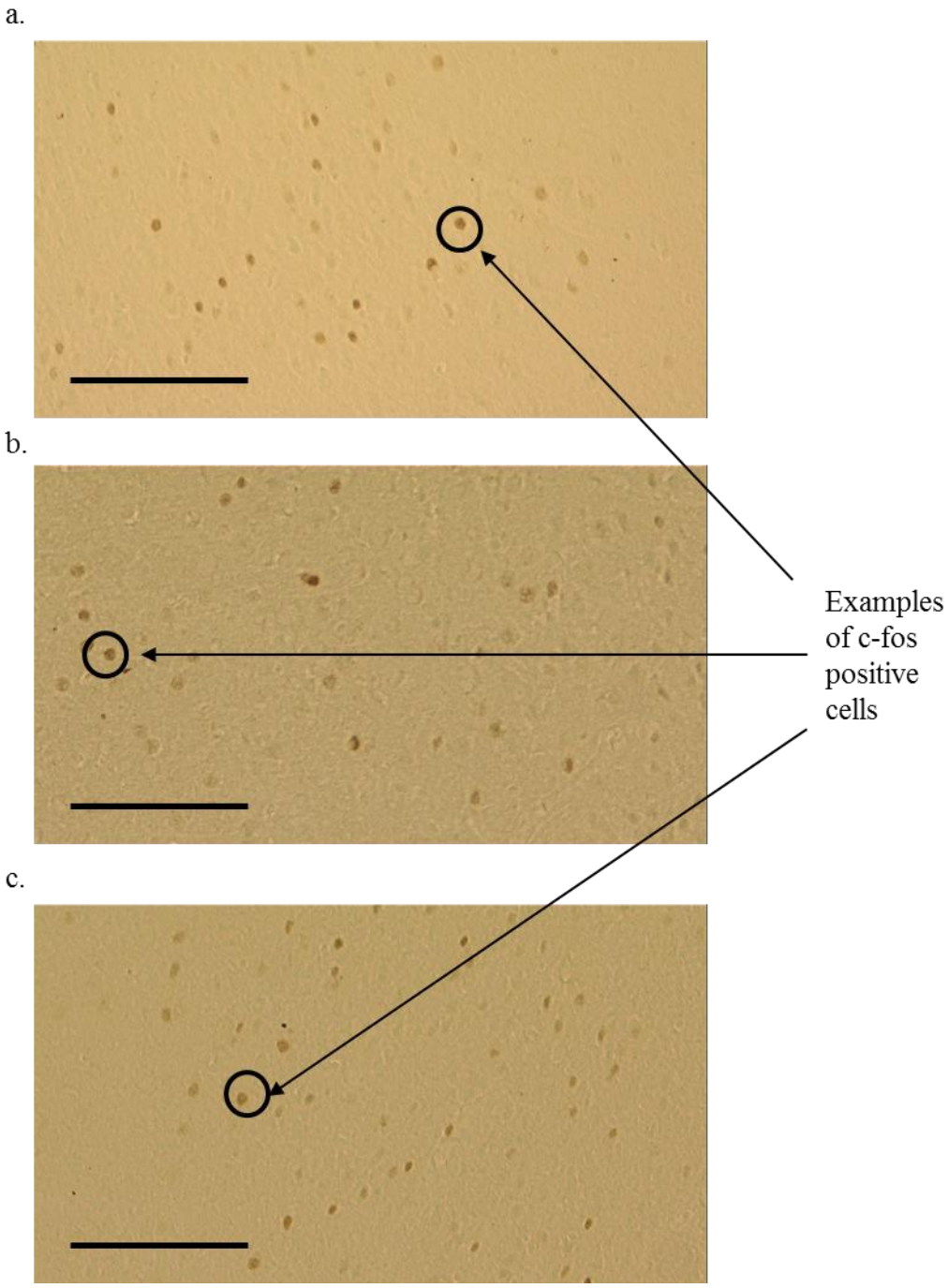
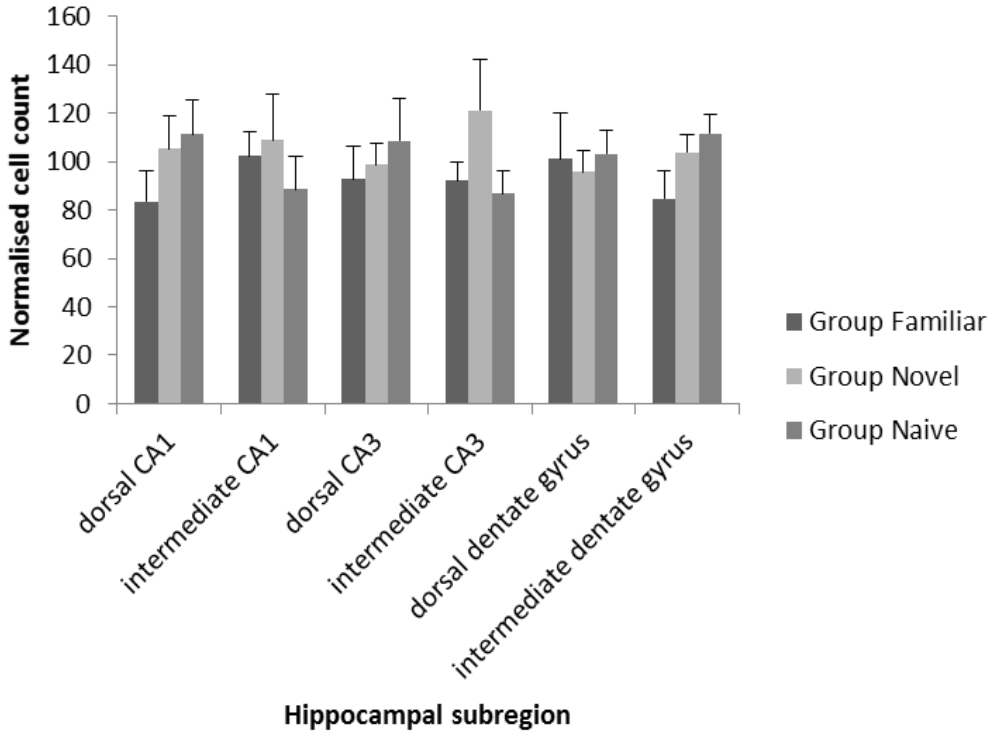


Figure 4.5. Sample photomicrographs of sections stained for c-fos in the perirhinal cortex, taken at 10x magnification, with examples of c-fos positive cells labelled. a) Group Familiar sample, b) Group Novel sample, c) Group Naïve sample. Scale bar, 5μm.

4.7.8.2.2. Hippocampal subfields

C-fos expression was quantified throughout the hippocampus (Figure 4.6). A repeated measures ANOVA of the hippocampal data revealed no main effect of *Group* ($F(2, 15) = 0.538$, $p = 0.595$) and no *Group* X *Subregion* interaction ($F(10,75) = 1.164$, $p = 0.328$).

a.



b.

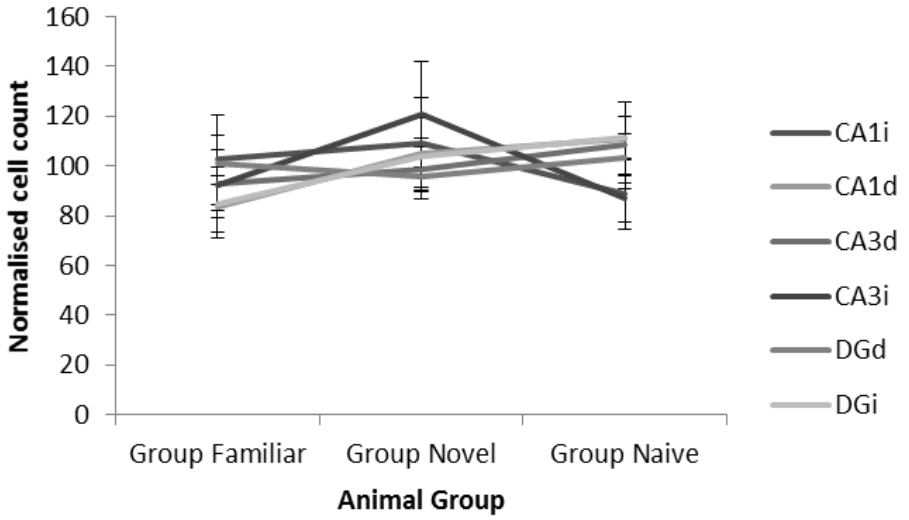


Figure 4.6. Activation in the hippocampus during spontaneous object recognition. a) Normalised c-fos expression in the hippocampus divided into subregions (dorsal and intermediate parts of CA1, CA3, and dentate gyrus). b) Line graph to further illustrate normalised c-fos expression between the three groups of animals. Vertical bars show the standard error of the mean.

C-fos expression in the different hippocampal subfields (CA1, CA3, dentate gyrus) was examined further (Figure 4.7); although increased c-fos counts were found in each subfield for Group Novel relative to Group Familiar, no main effect of *Group* was found ($F(2, 15) = 0.525$, $p = 0.602$), and there was no significant *Group* X *Subregion* interaction ($F(4, 30) = 0.484$, $p = 0.747$).

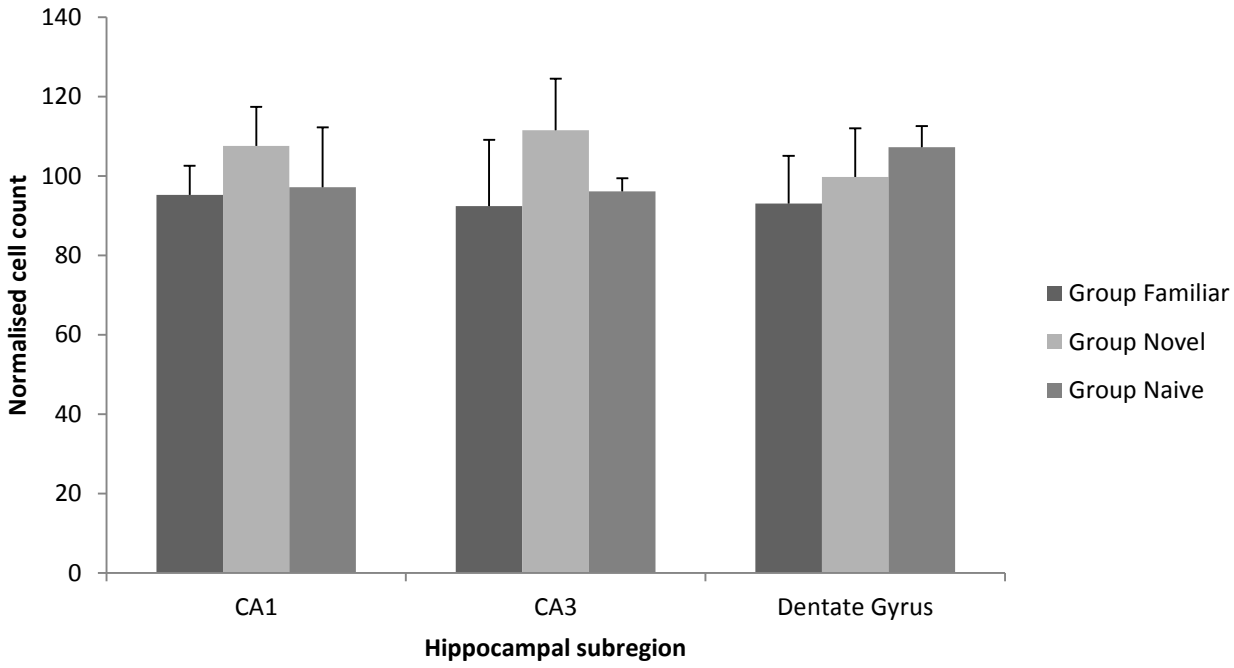


Figure 4.7. Normalised c-fos expression in the hippocampus with dorsal and intermediate counts for each subregion combined. Vertical bars show the standard error of the mean.

Additional analyses compared c-fos expression in the perirhinal and hippocampal regions (Figure 4.8); although c-fos counts were increased in Groups Novel and Naive, no main effect of *Group* was found ($F(2, 15) = 0.900$, $p = 0.184$), and there was no significant *Group* X *Subregion* interaction ($F(2, 15) = 0.992$, $p = 0.394$).

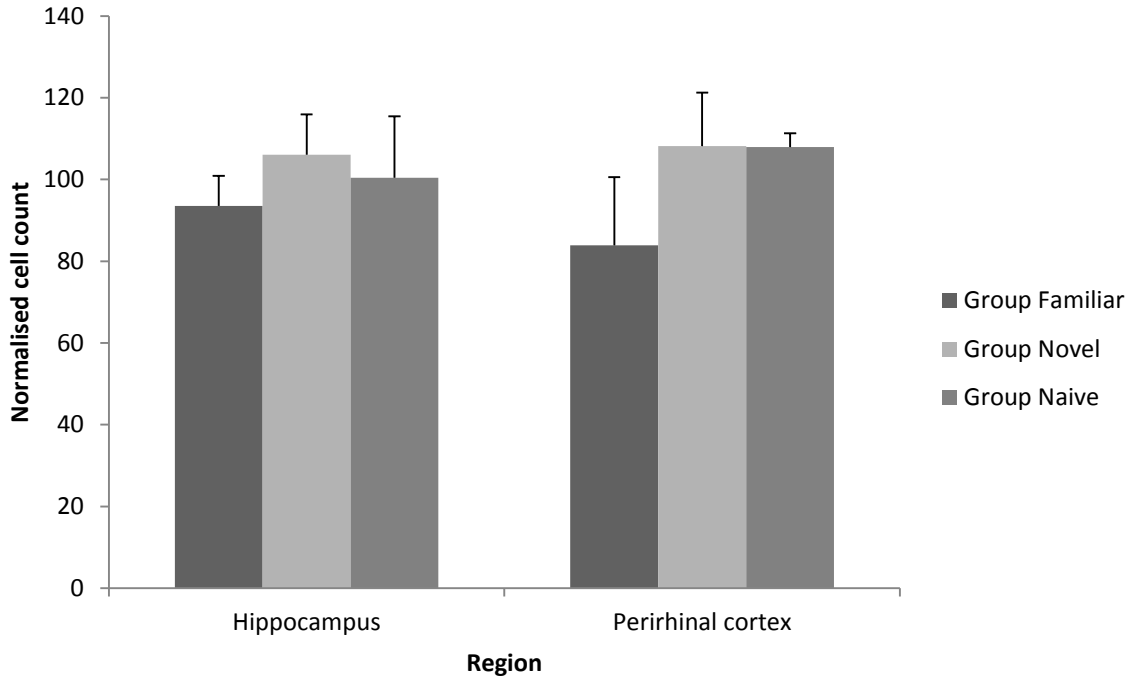


Figure 4.8. Comparison of the normalised c-fos expression in the hippocampus and perirhinal cortex, with c-fos counts for subregions combined. Vertical bars show the standard error of the mean.

4.7.9. Discussion

The current experiment was a spontaneous object recognition task carried out in the continual trials apparatus, whereby rats were tested on either novel objects, or objects that were duplicate objects to the novel ones, but familiar to the animals. The experiment was modified from a previous study by Albasser, Poirier et al. (2010) which looked at c-fos expression in a spontaneous object recognition task carried out in the multiple trial Bow-tie maze. The Bow-tie maze study involved familiarising the animals to objects through a number of recognition trials, whereas the current experiment involved free exploration of objects in the open field arena over a number of sessions prior to the test phase. This modification was made so the animals would only become highly familiarised to the object set they would be tested on (Group Familiar), or an object set they would not be tested on

(Group Novel), rather than an object set and the testing procedure. A further control group was added in the current experiment to include a group with no prior experience of objects (Group Naïve).

Reliable measures of recognition were found in all three groups of animals which was an improvement upon the Bow-tie maze study, whereby reliable measures of recognition were only found in the Novel group. Demonstrating significant levels of recognition across all groups is important because any differences in c-fos expression between groups cannot be attributed to differences in the animal group's ability to perform the task. C-fos expression was analysed as an indirect measure of neuronal activity in the hippocampus and perirhinal cortex. Overall, increased c-fos expression was found in subregions of the perirhinal cortex and hippocampus for Groups Novel and Naïve (Figure 4.8), though this was not significant. Differences in levels of c-fos activation were unlikely to be a result of differences between the groups in terms of the animals' interaction with the objects during the testing session; no significant differences were found between groups Novel and Familiar on total exploration in the test phase of the trials, and there were only marginal exploration differences in the sample phase.

No overall significant activity changes were found in the perirhinal cortex, although increases in c-fos expression were found in all three subregions for Groups Novel and Naive. Albasser, Poirier et al. (2010) reported similar findings as they found significant increases in c-fos expression in the caudal perirhinal cortex and area Te2. Lower fos expression in the perirhinal cortex following exposure to familiar stimuli corresponds to previous studies that have reported reduced neuronal activity (Brown and Aggleton, 2001) or a reduction in the BOLD (blood oxygen-level-dependent) signal (Gonsalves, Kahn, Curran, Norman and Wagner, 2005; Henson, Cansino, Herron, Robb, and Rugg, 2003; Montaldi, Spencer, Roberts and Mayes, 2006). No overall significant activity changes were found in the hippocampus,

which is consistent with the study by Albasser, Poirier et al. (2010) and previous work that has reported no activity changes following exposure to novel visual stimuli (Aggleton and Brown, 2005; Wan et al., 1999, 2004; Warburton et al., 2003; Zhu, Brown, McCabe and Aggleton, 1995). However, Albasser and colleagues did report significant increases in CA1 and CA3 for the Novel group, and significant decreases in the dentate gyrus. In the current experiment, c-fos expression was lower across all hippocampal subfields for Group Familiar, except for the dorsal part of the dentate gyrus (in which it was slightly greater for Group Familiar than Group Novel but still lower than Group Naïve) and the intermediate parts of CA1 and CA3 (in which c-fos expression was slightly greater for Group Familiar than Group Naïve but still lower than Group Novel, though in all cases not significant; Figure 4.6). Activation in the hippocampal subfields overall showed that increased c-fos expression was associated with the group tested on the spontaneous object recognition task with novel rather than familiar objects, though this was not significant. In addition, when animals with no prior exposure to objects were tested on this recognition paradigm, c-fos expression was only slightly increased relative to the Familiar group in the CA1 and CA3 subfields, but was increased relative to both Novel and Familiar groups in the dentate gyrus (though in all cases not significantly). A naïve group was not previously tested in the study by Albasser, Poirier et al. (2010), so these findings suggest that c-fos activation in the perirhinal cortex and hippocampus was similar regardless of whether the animals were naïve to objects in general, or simply tested on objects that were novel.

Although comparisons can be made between the current experiment and the Bow-tie maze study by Albasser and colleagues, it is important to note the procedural differences in the two tasks which may provide some account for why no significant group differences were found in the current experiment. Firstly, in comparison to the study by Albasser, Poirier et al. (2010), the current experiment used approximately half the number of animals per group, so a

lack of power may account for why no significant results were found. Secondly, the familiarisation procedures differed between the two studies, with animals freely exploring objects in an open field arena over a number of sessions in the current study, while animals underwent a series of recognition trials with familiarised objects in the Bow-tie maze study. Although both tasks successfully familiarised the animals to the object sets, the procedural differences may account, at least in some part, for the differences in behavioural results between the two tasks. As the Familiar group in the Bow-tie maze study failed to significantly discriminate between the test objects, and their exploration was lower than the Novel group, it is possible that the familiarisation protocol, which involved a number of recognition trials with the same objects in the same apparatus, led to high familiarisation that ultimately meant that the objects were not sufficiently differentially explored in the test phase. The current chapter familiarised animals to object sets in a different apparatus to the one used in the final testing session, which may account for why the familiar animals differentially explore the test objects, even though they were highly familiar. These procedural differences may be crucial for matching performance between animal groups.

Finally, the Bow-tie maze study reported evidence of a spatial learning element which may correspond to the particular pattern of activation found in the hippocampal subfields (Albasser, Poirier et al., 2010) and may have arisen due to the design of the task, in which the animals approach objects on each trial from one side of the maze and then the other. This pattern of results (i.e. increased c-fos expression in CA1 and CA3, and decreased expression in the dentate gyrus) corresponds to previous work in which novel spatial configurations of familiar stimuli were presented (Wan et al., 1999). These spatial demands are not present with the continual trials apparatus object recognition task and may account, to some extent, for why this pattern of activation was not found in the current experiment.

4.8. General discussion

The series of experiments outlined in the current chapter aimed to investigate whether the continual trials apparatus could be applied with IEG imaging during a recognition memory task. Demonstrating how multiple trial paradigms can be effectively used with IEG imaging is important as it allows researchers to investigate neuronal activation associated with recognition memory, with the behavioural evidence to show that animals can actively discriminate between novel and familiar stimuli. Moreover, multiple trial paradigms such as the Bow-tie maze (Albasser, Chapman et al., 2010) and the continual trials apparatus (Ameen-Ali et al., 2012) increase the likelihood of a detectable neuronal signal.

A series of behavioural experiments was first carried out to assess animal performance on spontaneous object recognition and object-location memory tasks, in which animals were either tested on the objects they had become familiarised to, or a novel set of objects. The study then investigated performance when the animal groups were tested on the same set of objects, but one group had been familiarised to the test objects, and the other group had been familiarised to a different set of objects. The task still consisted of a novel and a familiar group, but the protocol now allowed the animals to be tested on the same set of objects to minimise overall object exploration differences between different object sets, which could impact upon c-fos expression. The most reliable levels of performance were found in the spontaneous object recognition task (Experiments 3a and 3b), and the animal groups did not differ significantly overall on measures of exploration in either the sample or the test phase of the trials. This task therefore became the testing protocol for Experiment 5 which involved IEG imaging.

C-fos expression was investigated following the spontaneous object recognition task in Experiment 5. No overall significant activity changes were found in the perirhinal cortex, although, increases in c-fos expression were found for the Novel group and an additional

Naïve group with no prior exposure to objects. No overall significant activity changes were found in the hippocampus. In contrast, the Bow-tie maze study by Albasser, Poirier et al. (2010) combined a multiple trial spontaneous object recognition paradigm with IEG imaging and reported significant increases in c-fos expression in the caudal perirhinal cortex for the Novel group relative to the Familiar group. No overall differences in activation in the hippocampus between the two groups were found, which is similar to the current experiment. It is possible that procedural differences between the two studies and/or a lack of power due to a small sample size could account for the non-significant results reported in Experiment 5. It is, however, worth noting that in the Bow-tie maze study total exploration of the objects was not well matched between the two groups, as the familiar group displayed less object exploration and the mean discrimination scores were significantly different to Group Novel (Albasser, Poirier et al., 2010). Moreover, as their Familiar group did not perform the task successfully, it is possible that any differences in c-fos expression could reflect differences in ability to perform the task, rather than differences in object novelty. The current experiment demonstrated an improvement in terms of behavioural performance as all three groups of animals displayed no significant differences in discrimination and all showed significant recognition, with the Novel and Naïve groups performing slightly better than the Familiar group. As the current experiment found similar trends in terms of c-fos expression as the Albasser, Poirier et al. (2010) study with groups matched in terms of behavioural performance, this suggests that the findings in the current experiment reflected differences in object novelty, not differences in task performance. Importantly, it is the novelty of the test objects, not just objects in general which result in the increase of fos expression in the perirhinal cortex, as this increase was observed to a similar degree in both the Naïve and Novel groups. As the patterns in c-fos expression were comparable between the current experiment and the study by Albasser, Poirier et al. (2010), even though performance was not

matched in the Bow-tie maze task, their c-fos findings must also reflect differences in object novelty, because even when the Familiar group can perform the object recognition task, as demonstrated in the current experiment, this is not sufficient to increase c-fos activity in the perirhinal cortex for animals in that group. The lower c-fos expression for the Familiar group in the Bow-tie maze task must be due to the high familiarity of the objects and not an inability to do the task.

Generally, IEG imaging can provide a useful insight into the neuronal activity involved in recognition memory, although consideration needs to be given to the temporal resolution. Studies often euthanase animals within 90 minutes of ceasing the test session in order to capture peak fos production. There is, however, a trade off as any activity that the animal engages in during this time could impact on the c-fos expression. In the current experiment, therefore, a 30 minute break following testing was given to be close to peak production, but to also minimise disruption to the activation signal.

With IEG imaging it is not possible to draw causal inferences, as only correlational measures are provided. However, several studies have used structural equation modelling to quantify links between regions based on the relationships between fos counts in different subregions, which allow network dynamics to be assessed (e.g. Albasser, Poirier et al., 2010; Kinnavane et al., 2014). IEG imaging can, therefore, provide a useful alternative to studies which adopt the lesion approach, and fewer animals are required as multiple brain sites can be imaged simultaneously. IEG imaging has been recently used in object-context recognition memory (Wilson et al., 2013) and recency recognition memory (Kinnavane et al., 2014; Olarte-Sanchez et al., 2014), but further work is needed to explore recognition memory with spatial configurations, and episodic-like memory.

The experiments reported in the final experimental chapter of this thesis investigate how the translation of recognition memory research from animals to humans could be

improved. The animal work presented in this thesis so far has attempted to refine procedures of well-controlled behavioural tasks to implicitly measure recognition memory and subsequently reduce animal numbers. Although it is important to validate animals models of memory, human tasks of memory often rely on a subject's introspective account. Improving translation through developing better-controlled behavioural tasks in humans could provide the necessary validation of memory models but also, in some instances, provide opportunity for further animal reduction if human studies can be reliably used to assess memory process.

CHAPTER 5

DISSOCIATING RECOLLECTION- AND FAMILIARITY-BASED PROCESSES USING THE ANALYSIS OF RECEIVER-OPERATING CHARACTERISTICS

5.1. Introduction

Recognition memory is the ability to identify when something has been previously encountered. Testing recognition memory in non-human animals relies on the ability of an animal to display memory of an object (for example) through preferential exploration of a novel object (or novel configuration of an object and an aspect of the environment) over a familiar object (or configuration). Although recognition memory tasks in animals have offered some success, the notion of whether animals possess a specific type of recognition memory that relates to one's past experiences, known as episodic memory, is controversial. This controversy stems from the need to be able to provide a subjective account of an experienced event, which animals are unable to do due to the absence of language (Tulving and Markowitsch, 1998). Animals' 'episodic-like' memory (termed as such due to the lack of subjective experience; Clayton and Dickinson, 1998) can be assessed indirectly through preferential exploration of an object in a specific combination of location and spatial/temporal context. Recent emphasis has been on the development of novel tasks to assess episodic-like memory in animals focussing on the content of the memory itself rather than its associated experiential aspects which cannot be measured.

Research into recognition memory with humans is often carried out very differently to the work with animals. Specifically, in human tests of episodic memory, subjects are able to verbalise their memory experience and classify it as one of 'remembering' or 'knowing'. Tasks that utilise the remember/know paradigm have been used to provide insight on whether recognition memory can be understood as a single process of familiarity (recognition of

individual familiar cues often associated with a feeling of 'knowing') or as a composite of distinct processes of recollection (remembering associations with the stimulus or event, often associated with a feeling of 'remembering') and familiarity.

Researchers have sought to address the methodological differences between tests in animals and humans as a way of improving human testing methods, but also to validate animal models of memory. Work by Holland and Smulders (2011) and Easton, Webster and Eacott (2012), for instance, have investigated whether humans use episodic memory in episodic-like memory tasks previously used with animals, based on either the 'what-where-when' (memory for what happened, where and when; Tulving, 1972) or the 'what-where-which occasion' episodic memory descriptors (memory for an object, its location and background context; Eacott and Norman, 2004). Using the remember/know paradigm, distinctions between recollection- and familiarity-based responses were made to illustrate greater recollection during conditions that reflected episodic-like memory relative to other recognition memory conditions. These findings suggest that tasks of episodic-like memory for non-human animals may closely mimic memory process underlying human episodic memory, however, for a closer reflection, evidence that provides a shift away from reliance on subjective introspection is necessary.

An informative tool for understanding the underlying processes of recognition memory has been to model the patterns of responses in a recognition memory task using signal detection theory (Egan, 1958). Through the analysis of receiver-operating characteristics (ROCs), it is possible to quantify separately the degrees of recollection and familiarity that a subject has in a task, without the need for introspective assessment of the nature of one's memory, therefore allowing human and non-human animal memory to be tested and understood in the same manner. In a typical item recognition task, participants study a list of stimuli (often a list of words) and then discriminate old and new items in a

following test phase. By rating their level of confidence associated with each response, participants' performance can be plotted as an ROC curve with hit rate (HR – when a stimulus is correctly identified as 'old') against false alarm rate (FAR - when a new stimulus is misidentified as being old), as participants' criterion varies from liberal (more likely to respond 'old') to conservative (more likely to respond 'new').

Traditional signal detection theory states that recognition responses are based on a single strength variable whereby old and new items are associated with particular memory strength (Squire, Wixted and Clark, 2007). A response of 'old' would be made if the memory strength for an item exceeds a criterion value (c; Figure 5.1). If this criterion value is not exceeded, the response for the item would be 'new'. This signal detection model (often referred to as the unequal-variance signal detection model – UVSD) is compatible with the view that recognition memory is a single process based on familiarity without the addition of recollection.

An alternative view which supports the notion that recognition memory is based on functionally distinct processes of recollection and familiarity (Brown and Aggleton, 2001; Eichenbaum, Otto and Cohen, 1994) is the dual-process signal detection model (DPSD; Yonelinas, 1994). This model suggests that familiarity reflects a continuous signal detection process (same as the UVSD model) but recollection, on the other hand, is reported to be a threshold process in which items are reported as being remembered if they exceed a memory threshold. It should also be noted that some recent interpretations of the UVSD model support the view of two underlying processes in which memory strength is based on the additive combination of recollection and familiarity (Kelley and Wixted, 2001; Rotello, Macmillan and Reeder, 2004; Wixted, 2007; Wixted and Stretch, 2004).

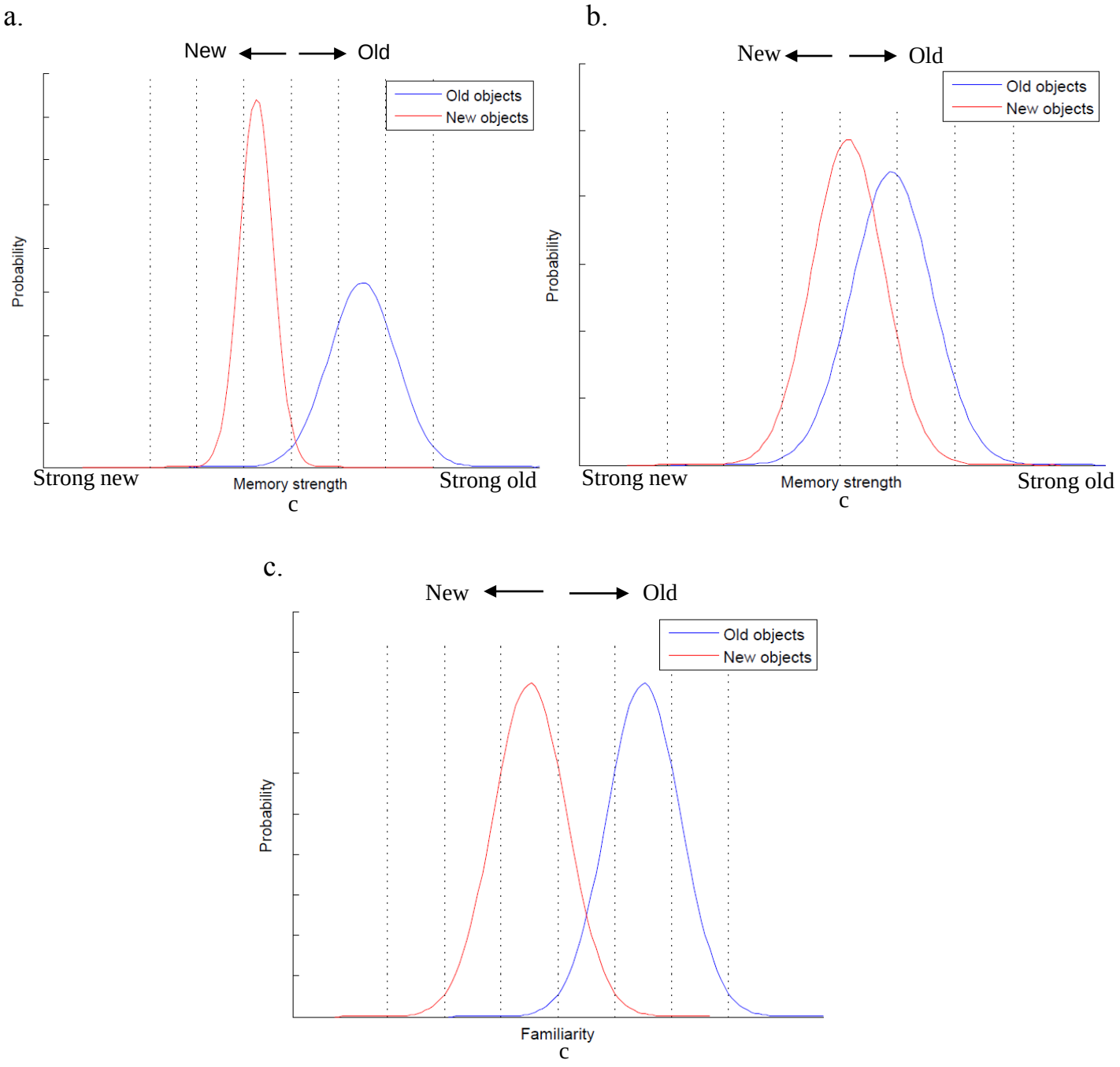


Figure 5.1. The vertical dashed lines indicate the confidence ratings. Items with memory strength to the furthest left are' high confidence new' items, and items with memory strength to the furthest right are 'high confidence old' items. a) UVSD model strong memory condition with unequal variance between the old and new item distributions. A response of 'old' would be made if the memory strength for an item exceeds a criterion value (c). b) UVSD model weak memory condition with unequal variance between the old and new item distributions. c) DPSD model with equal variance between old and new item distributions. Items that are recollected are 'high confidence old' responses. A few of these items may also be based on familiarity. Adapted from Squire et al. (2007).

The degree of asymmetry present in an ROC curve has been taken as an indication of the nature of underlying memory process. The DPSD model states that symmetrical ROC curves result from familiarity-based responses as the old and new items distributions have equal variance, whereas asymmetric ROC curves are a result of recollection-based responses which are assumed to support high-confidence decisions (Yonelinas, 1994). In this case, the ROC curve consists of a continuous curvilinear function reflecting familiarity and a linear function which reflects the recollection component of recognition memory. In contrast, traditional signal detection theory suggests that weak memory, rather than absence of recollection, results in a symmetrical ROC curve. An asymmetrical ROC curve results from unequal variance between old and new distributions, which some regard to be a sign of strong familiarity rather than recollection (Squire et al., 2007).

A number of studies have applied ROC analysis to tasks of recognition memory in humans (e.g. Aggleton, Vann, Denby, Dix, Mayes et al., 2005; Yonelinas, Kroll, Dobbins, Lazzara and Knight, 1998; Yonelinas, Kroll, Quamme, Lazzara, Suave et al., 2002) and animals (Fortin, Wright and Eichenbaum, 2004; Sauvage, Fortin, Owens, Yonelinas and Eichenbaum, 2008) providing support for the DPSD model. Findings report that control subjects have greater asymmetry in their ROC curves than patients or animals with hippocampal lesions, suggesting that the ROC of those with hippocampal lesions reflect familiarity-based recognition only. According to the DPSD model, these findings are not surprising as the hippocampus is deemed necessary for recollection-based recognition, so without intact hippocampal function the ROC curve is likely to reflect only familiarity-based recognition. Furthermore, the estimates of recollection and familiarity correspond well to the estimates derived from the remember/know procedure, wherein ‘remember’ responses are associated with recollection and ‘know’ responses are associated with familiarity (Yonelinas,

2002). There have, however, also been a number of studies which report data suggesting that recognition decisions are based on a single strength variable, supporting the UVSD model (e.g. Rotello, Macmillan, Reeder and Wong, 2005; Slotnick and Dodson, 2005). Both the UVSD and DPSD models can be used to provide convincing accounts of ROC data; as such, it can be problematic when trying to interpret ROC data in favour of one model over the other. One noticeable advantage of the DPSD model, however, is that when the dual process equations are fit to the observed ROC data, quantifiable estimates of recollection and familiarity can be derived from the obtained parameter values whereby recollection is measured as a probability (R) and as familiarity is assumed to be a signal detection process, it is measured in terms of *d'*.

The current experiment devised a task based on recognition memory paradigms typically used with rodents. The first aim of this experiment was to validate methods used in rodent research of recognition memory, which are often used to develop models of human memory. Participants were asked to complete a computer-based memory task, in which they had to discriminate between old and new virtually generated objects. The old objects presented during the test trials were attributed to one of the following conditions: standard object recognition (SOR); object-location (OL); object-context (OC); object-location-context (OLC; see Section 5.2.3). In the SOR condition, the objects at test were presented in a novel location and context relative to their appearance in the encoding phase. This is devised differently to SOR trials with rodents (though based on the same basic principles), whereby objects are presented in the same context, and the location of objects only change for counterbalancing. In such tasks, recognition is signalled by object exploration driven by novelty. Thus, any extraneous factors need to be minimised. With humans, there is no need to rely on novelty preference; as such, for the SOR condition, the only familiar feature is the object. In the OL and OC recognition conditions, the objects are presented either in the same

location but novel context (OL), or the same context but novel location (OC). Again, this is different to the rodent tasks in which novel configurations of object and location, or object and context, drive exploration and signal recognition. With no need to rely on novelty preference, the OL and OC conditions are designed so that familiarity is defined only by the object and location (OL), or the object and context (OC). Finally, in the OLC condition, following the same principles, the objects are presented in the same location and the same context relative to their appearance in the encoding phase, rather than a novel configuration of these features.

ROC analysis was used to distinguish between and quantify the degree of recollection and familiarity across recognition conditions. Familiarity was measured in terms of *d'* as it is thought to reflect a signal detection process and recollection was measured as a probability (R) as it is assumed to reflect a threshold process (Yonelinas, 1994). As the OLC condition, by definition, is akin to the what-where-which occasion descriptor used to infer episodic-like memory in rodents, it was hypothesised that significantly greater recollection would be elicited in this condition relative to the other recognition memory conditions, if this process underlies episodic memories. Therefore, significantly greater ROC asymmetry would be found in the OLC condition, with significantly greater R probability relative to the other recognition conditions.

A second aim of this study was to determine whether the recollection probability observed in the OLC condition could be quantified as the summation of the recollection probabilities observed in the separate object-location and object-context conditions. It was hypothesised that the observed recollection probability would be greater than that predicted by the summation of the constituent components, suggesting that the measure of recollection in the OLC condition reflected the presence of a type of recognition memory distinct from

strong familiarity, lending support to the interpretation of these results in the framework of a dual-process understanding of recognition memory.

5.2. Method

5.2.1. Participants

Twenty-two participants took part in this experiment, all of who were naïve to the purpose of the study. Informed consent was acquired before testing took place. Participants were undergraduate and postgraduate students from the Psychology Department, Durham University and were compensated for their time with either course credit of financial compensation.

5.2.2. Stimuli

A custom set of 64 2D virtual objects were generated using Matlab (MathWorks). Each object was a unique permutation of three components (object back surface, front surface and peripheral feature), of which there were four variations of each. Two background contexts were used which were also generated in Matlab. Context A was a chequered pattern and context B was a granulated surface pattern; both defined by grey-scale variations in luminance. Stimuli were presented on a CRT monitor using a Cambridge Research Systems (Rochester, England) ViSaGe graphics system. The monitor had a resolution of 1280x1024 pixels and ran with a refresh rate of 85Hz. The viewing distance was set at 45cm with participants resting their head on a chin rest. Each object had a width subtending 6.4° of visual angle and each object was presented 12.8° (either to the left or right) from the centre of the screen.

5.2.3. Procedure

A single testing block consisted of encoding and retrieval phases. An encoding phase began with an auditory tone lasting 1s after which a fixation cross would appear in the centre of the screen also lasting 2s (Figure 5.2.a). This was then followed by four objects presented sequentially and separated by periods of 2s fixation. The objects were presented for 2s each. Each of the four objects was presented in a unique combination of location (left/right) and context (context A/context B), such that each context and location was experienced an equal number of times in each encoding phase but no combination was repeated. Participants were instructed to simply move their eyes to the object when it appeared and then back to the fixation cross when the object disappeared. A retrieval phase would then follow, signalled by a tone lasting 1s, in which four objects were shown sequentially – each of these constituting a single trial (Figure 5.2.b). Again, these objects were presented for 2s each and preceded by 2s fixation. The next object in the retrieval phase would not be presented until the participant's responses had been recorded – there was no time restriction for these responses to be made as participants were simply instructed to respond as accurately as possible. The latency between the end of the encoding phase and the beginning of the retrieval phase was the duration of the auditory tone (1s).

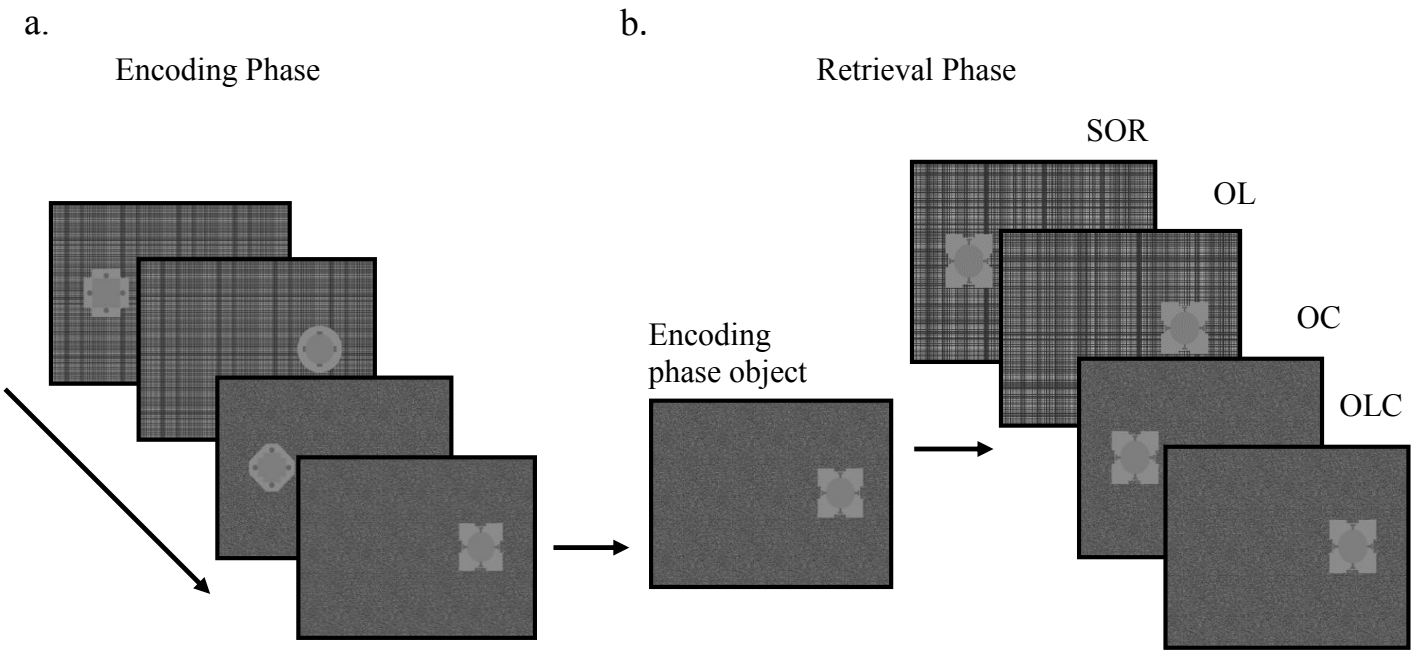


Figure 5.2. Experiment procedure. a) Example object presentation for the encoding phase. Four objects are sequentially presented separated by a fixation cross presented for 1s (not shown in the image). The objects were presented in a unique combination of location and context such that in each encoding phase the left and right locations and contexts A and B would be experienced an equal number of times but presented in a randomly selected order. b) Example object presentation for a retrieval phase demonstrated with an object from encoding. Four objects were sequentially presented in the retrieval phase that may be old or new. New objects would be presented in a random combination of location and context. For old objects, using an example from the encoding phase, the potential locations and contexts are determined by condition. Standard object recognition (SOR); object-location (OL); object-context (OC); object-location-context (OLC).

Each of the objects in the retrieval phase could either be new or old, relative to the immediately preceding encoding phase. If the object was new, it was presented in a random combination of context and location. If it was old, the context and location depended on the condition for that trial. Specifically, in SOR trials, the object was presented in a novel context and novel location relative to its appearance in the encoding phase; in OL memory trials the object was presented in the same location but novel context; in OC memory trials the object

was presented in the same context but novel location; in OLC memory trials the object was presented in the same location and context. Participants had to make two responses after viewing each object – they first had to respond whether the object was old (i.e. it had appeared in the previous encoded phase) or new (i.e. it did not appear in the encoding phase) by pressing one of two buttons. For their second response, participants had to rate how confident they were with that decision through the use of four buttons (1 = guessing; 2 = not very confident; 3 = quite confident; 4 = very confident). The experiment did not advance until both responses had been made. After four trials had been completed, a tone would then signal the start of the next encoding phase.

A single testing block consisted of 10 encoding-retrieval pairs, with participants instructed to respond in the retrieval phase only about objects in the immediately preceding encoding phase. Each testing block, therefore, consisted of 40 trials (four trials per encoding-retrieval phase pair). Of these trials, there was an equal number (8) of novel, SOR, OL, OC and OLC conditions. This design required 48 unique objects for each testing block, which were determined randomly from the 64 available at the start of each block. This meant that some objects would be seen in multiple blocks. In total, participants completed 16 testing blocks over four days (four 10 minute testing blocks per day). This design, therefore, yielded a total of 128 trials per experimental condition.

5.3. Results

5.3.1. Analysis of ROCs

Data from all 16 blocks were analysed collectively for each participant. For each of the five conditions, the response frequencies were tabulated at each of the eight response levels. These data, shown in Table 5.1, show that participants used the entire range of response categories available. These were then converted to cumulative response

probabilities by dividing the frequencies by the total number of responses in each condition, and then cumulatively adding the probabilities from the highest criterion ("definitely old") to the lowest ("definitely new"; see Figure 5.3). False alarm rate (FAR) probabilities were derived from the novel condition and separate hit rate (HR) probabilities were derived from each of the four familiar conditions. As an example, the point with the lowest criterion (the first point on the ROC graph) is equal to the HR and FAR pair in the highest response category only (8; Table 5.1). The next highest criterion is equal to the summation of those HR and FAR from response category 8 and those in response category 7. This is repeated until all categories are cumulatively added. A set of *n* categories, in this case 8, gives n-1 points on the ROC curve, as the cumulative summation of all the categories will always yield both a HR and FAR of 1. There are, therefore, seven points to be plotted on these ROC curves. In total, four individual sets of seven ROC points are derived, each one representing either SOR, OL, OC or OLC memory.

Table 5.1. Mean counts for each confidence response category per task condition.

	Response category							
	New				Old			
	High confidence		Low confidence		Low confidence		High confidence	
	1	2	3	4	5	6	7	8
Novel	35.9	32.8	15.7	12.5	5.3	10.1	9.1	6.6
SOR	5.4	15.2	11.9	8.7	6.3	17.3	26.5	36.7
OL	4.6	11.4	10.4	8.3	6.5	16.7	25.7	44.4
OC	6.1	12.6	10.3	8.7	7.1	17.5	27.4	38.2
OLC	6.1	12.9	9.2	7.7	5.6	16.1	25.3	45.1

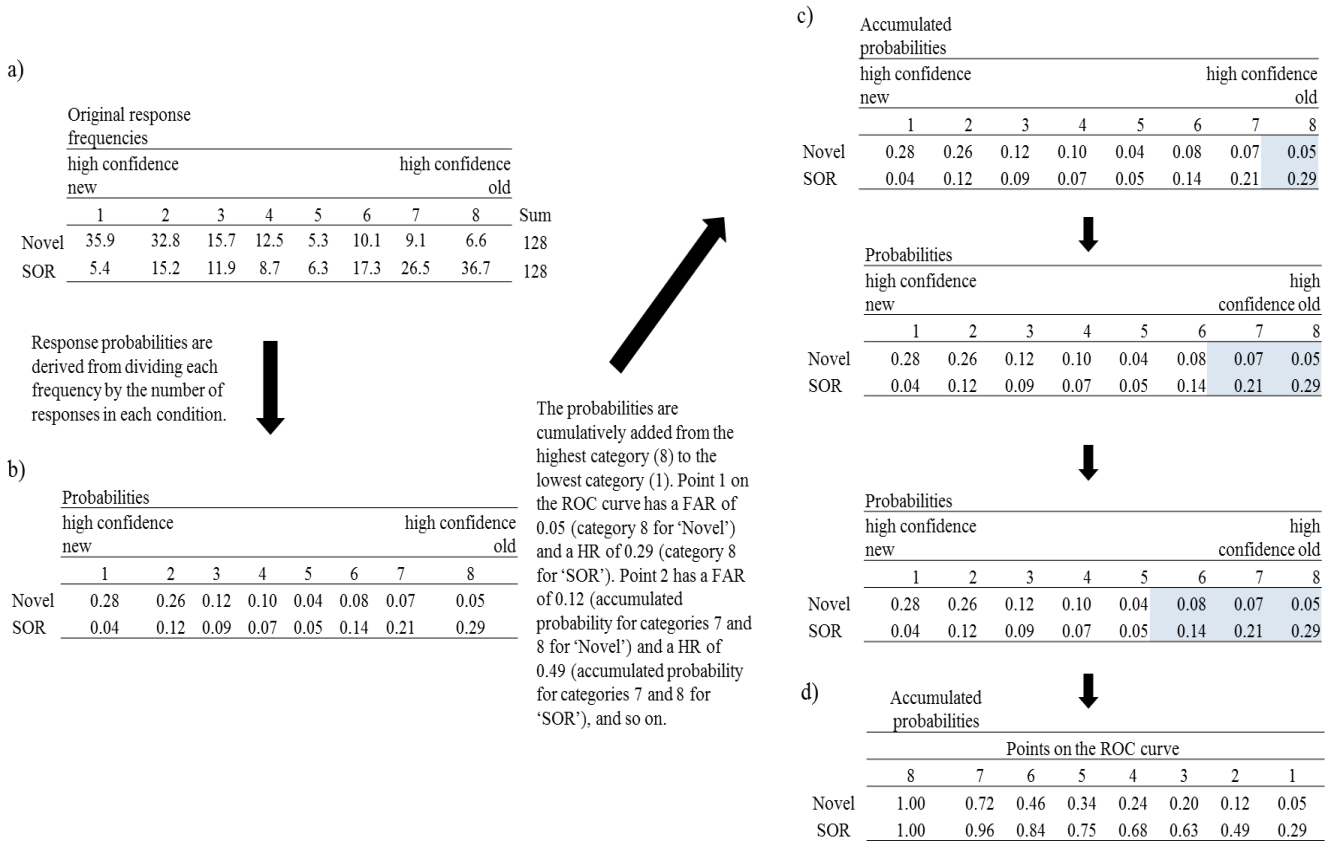


Figure 5.3. Illustration of the data transformation from frequency to accumulated probabilities that represent points on the ROC curve. This illustration represents the SOR condition only. a) Original response frequencies for each criterion, ranging from 'high confidence new' (1) to 'high confidence old' (8). b) Converted probabilities for each response criterion. c) The probabilities are cumulatively added from the highest criterion (8) to the lowest criterion (1). The first three cumulative points are represented. d) After the probabilities are accumulated at each criterion level, this results in eight HR and FAR pairs that represent the points on the ROC curve. Only seven points appear on the ROC curve because the last point is always 1.

The ROC curves were plotted with HR as a function of FAR with the ROC function being fit to the data using a method of least-squares (Figure 5.4). The best fitting ROC curve for each set of points was determined using the dual-process equations (Yonelinas et al., 1998; $HR = R + (1\text{-}R)\ F_{old}$; $F_{old} = \Phi\ (d'/2\text{-}c_i)$; $FAR = F_{new}$; $F_{new} = \Phi\ (-d'/2\text{-}c_i)$) with the free parameters of d', c and R to provide the most suitable account of the data. The only constraint was that $0 \leq R \leq 1$. For each ROC curve the derived parameters of *d'* and R probability were taken as

quantifiable estimates of familiarity and recollection, respectively. To be confident that the best fitting parameters were obtained for each condition and that the ROC curves provided a close fit to the data, the mean sum of squared errors (SSE) between the observed and predicted data was calculated for each condition as a goodness of fit index. The mean sum of squared errors were extremely low and close to zero for each condition indicating a very close fit to the data (SOR: mean SSE = 0.003; OL: mean SSE = 0.002; OC: mean SSE = 0.003; OLC: mean SSE = 0.002).

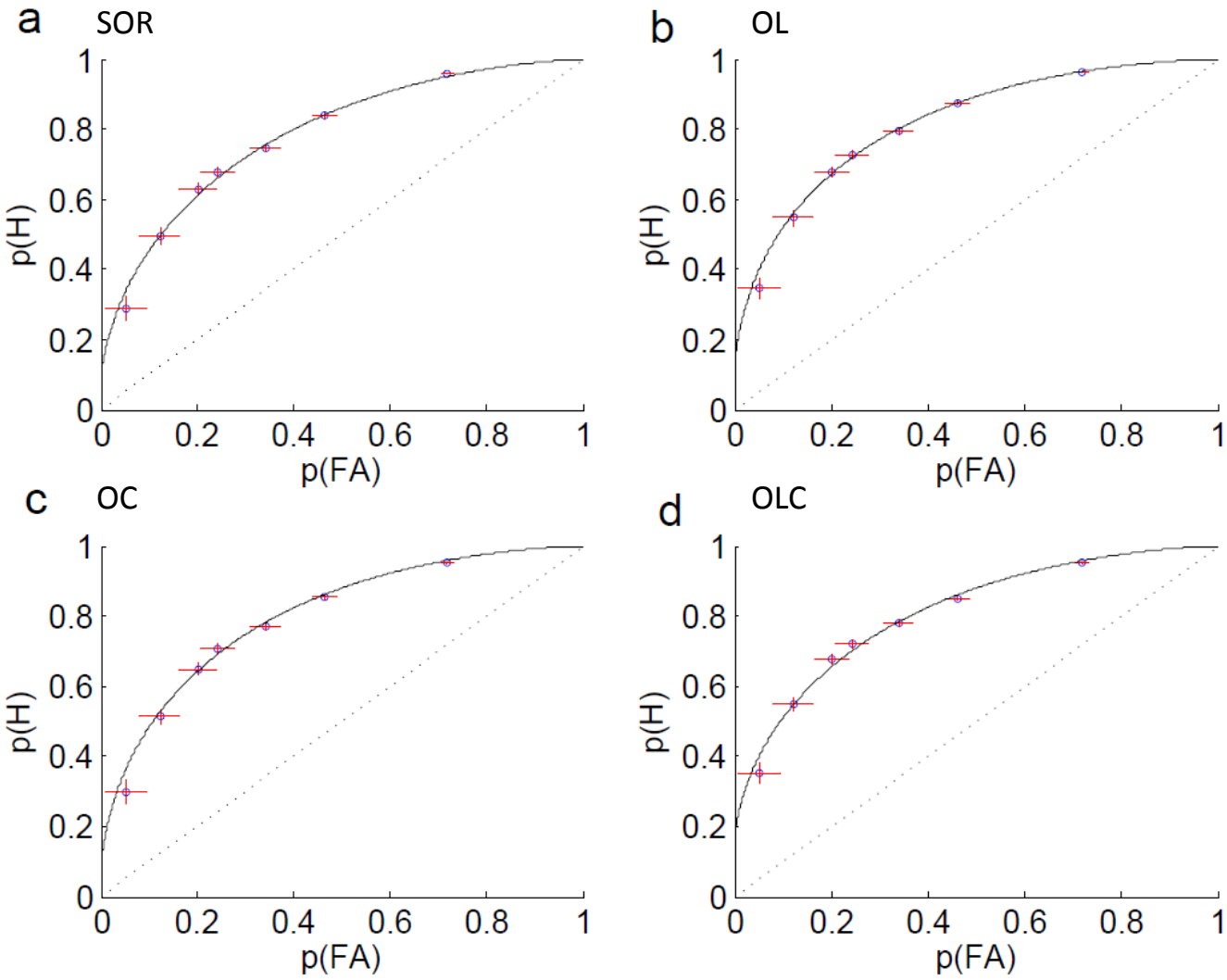


Figure 5.4. ROC curves with hit rate plotted against false alarm rate for all subjects. a) Standard object recognition. b) object-location memory. c) object-context memory. d) object-location-context memory. The horizontal SEM bars show the variance for FAR, and the vertical SEM bars show the variance for HR.

A within-participants analysis of variance was conducted on the mean *d'* and R probability estimates where *d'* was found to vary with task conditions (Figure 5.5.a: SOR = 1.06; OL = 1.21; OC = 1.16; OLC = 1.10; F(3,63) = 5.152, p = 0.003). A pairwise comparison revealed the significant effect to lie between the SOR and OL memory conditions (p = 0.015) with *d'* being highest in the OL condition and lowest in the SOR condition.

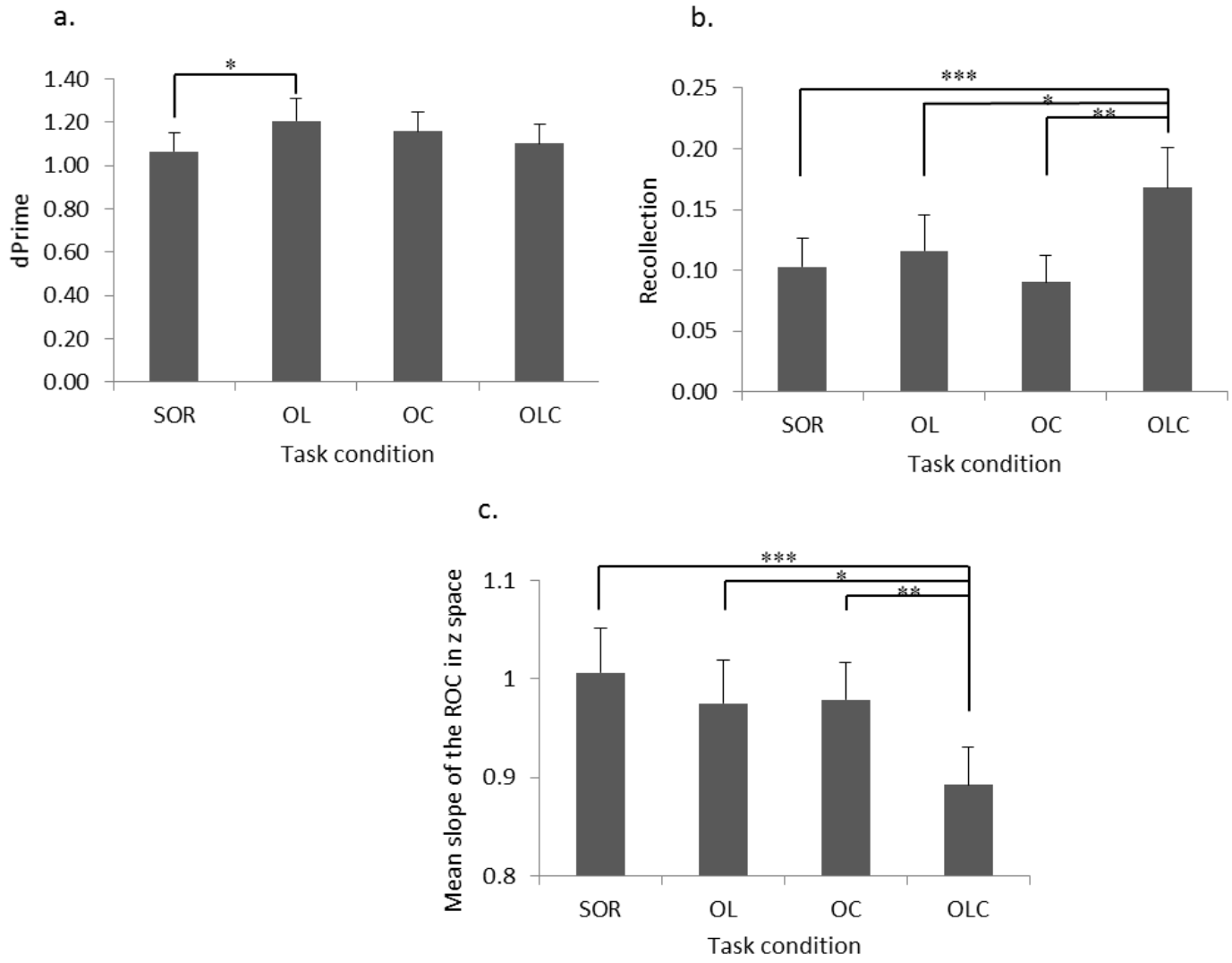


Figure 5.5. a) Mean d' estimates for each recognition condition: Standard object recognition (SOR); object-location (OL); object-context (OC); object-location-context (OLC). b) Mean recollection probability estimates for each recognition condition. c) Mean slope of the ROCs in z-space for each recognition condition. Vertical bars show the standard error of the mean. * = < 0.05; ** = < 0.01; *** = < 0.001.

There was a significant main effect of R probability ($F(3,63) = 5.075$, $p = 0.003$) with the highest probability estimate being found in the OLC condition (Figure 5.5.b; SOR = 0.10; OL = 0.12; OC = 0.09; OLC = 0.17). From planned t-tests, R probability in the OLC condition was found to be significantly greater than the other recognition conditions (SOR and OLC: $t(21) = 3.864$, $p = 0.001$; OL and OLC: $t(21) = 2.238$, $p = 0.036$; OC and OLC: $t(21) = 3.472$, $p = 0.002$), whereas none of the other recognition conditions were significantly different from each other (SOR/OL, SOR/OC, OL/OC all $p = > 0.2$).

5.3.2. Z-transformed ROCs

ROC symmetry can be measured through estimating the slope of the function when the data is plotted following z transformation. A symmetrical ROC along the minor diagonal would produce a z-ROC with a slope of 1.0, with slopes lower than 1.0 indicating more asymmetry. There was significant variation between the four conditions for the z slope measure ($F(3, 63) = 4.118$, $p = 0.010$; Figure 5.6). The slope of the OLC z-ROC (Figure 5.5.c) was significantly less than 1.0 (OLC = 0.89; $t(21) = 2.772$, $p = 0.011$), whereas the slopes of the z-ROCs from the other conditions were not (SOR = 1.0; $t(21) = 0.132$, $p = 0.897$; OL = 0.98; $t(21) = 0.570$, $p = 0.575$; OC = 0.98; $t(21) = 0.567$, $p = 0.577$). In addition, the slope of the OLC z-ROC was significantly more lower than 1.0, compared to the other three recognition conditions (SOR and OLC: $t(21) = 4.310$, $p = < 0.001$; OL and OLC: $t(21) = 2.269$, $p = 0.034$; OC and OLC: $t(21) = 3.401$, $p = 0.003$). A slope significantly lower than 1.0 in the OLC condition indicates that performance may be recollection-based in this condition.

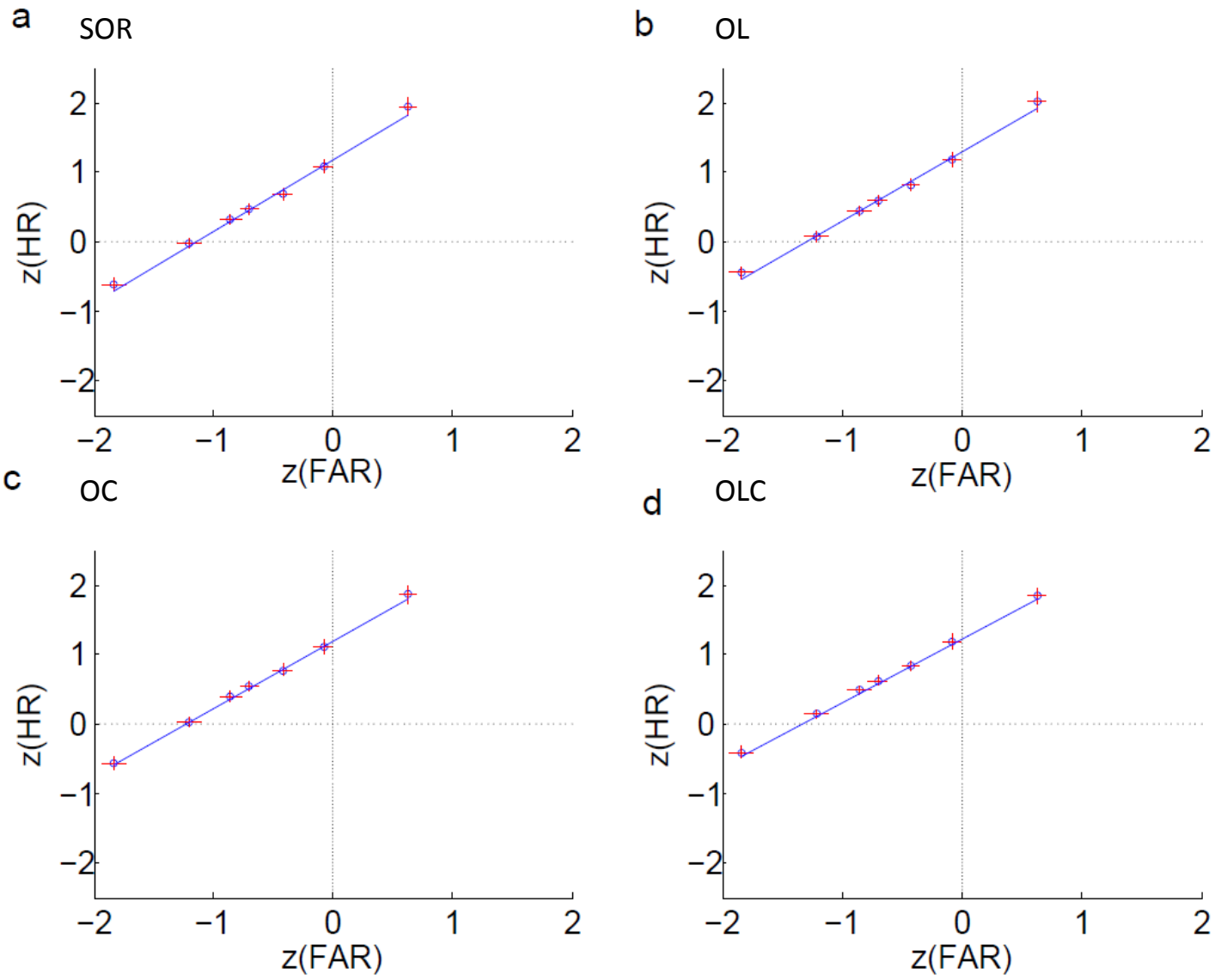


Figure 5.6. ROC curves transformed into z-space for all subjects in each recognition condition. a) Standard object recognition. b) object-location memory. c) object-context memory. d) object-location-context memory. Asymmetrical ROC curves should be linear in z-space, according to standard signal detection theory. The z-slope in the OLC condition is significantly lower than 1, indicating greater asymmetry – a sign of a recollection component. The horizontal SEM bars show the variance for FAR, and the vertical SEM bars show the variance for HR.

5.3.3. Overall measure of performance

As analyses of the ROCs and z-ROCs indicate significant differences between the OLC and other recognition conditions, it was important to consider whether general differences in the difficulty of the task conditions could account for these findings. The area

under the ROC curve (AUC) can be taken as a single measure of performance, ranging from chance (0.50) to perfect performance (1.00), which does not discriminate between different memory processes. To get an indication of subject performance between task conditions the AUC for each task condition was used as a single measure of performance and was calculated using the trapezoidal rule for approximating the definite integral. There was a significant main effect of task condition (SOR = 0.78; OL = 0.81; OC = 0.80; OLC = 0.81; AUC: $F(3,63) = 8.082$, $p = < 0.001$). Pairwise comparisons revealed that the SOR condition was significantly more difficult than the OL ($p = < 0.002$) and OLC ($p = 0.01$) conditions. Crucially, the OLC condition was not significantly less difficult than the OL and/or OC conditions.

An overall measure of *d'* was calculated to get an additional overall measure of performance. This measure was acquired by collapsing the response categories '5-8' and treating it as a single category to represent an 'old' response, and collapsing the response categories '1-4' and treating it as a single category to represent a 'new' response. *d'* was then calculated by subtracting the z(FAR) from the z(HR) associated with these two response categories. There was significant main effect of task condition ($F(3,63) = 6.220$, $p = 0.001$), with pairwise comparisons revealing that the SOR condition was significantly more difficult than the OL ($p = < 0.031$) and OLC ($p = 0.013$) conditions. These results reflect the AUC analysis, further emphasising that the OLC condition was not significantly less difficult than the OL and/or OC conditions; therefore, the differential findings between the OLC condition and the other recognition conditions cannot be attributed to differences in task difficulty.

5.3.4. Predicted OLC performance

An important question to consider is whether the correct combination of location and context in the OLC condition elicited a degree of recollection that is greater than that

predicted by the summation of the separate degrees of recollection associated with location and context alone. If this is the case, this would suggest that the OLC condition reflected the presence of a type of recognition memory distinct from strong familiarity, lending support to the interpretation of these results in the framework of a dual-process understanding of recognition memory.

The observed R probability in the OLC condition was compared to a hypothetical expected value predicted by the combined probability of the location and context components. First the R probability values for just the location (L_R) and just the context (C_R) were calculated. These values are not necessarily equal to the recollection probability values observed in the OL and OC conditions, respectively, because the recollection probability observed in the OL condition, for example, is the combined probability of that found for SOR and some other unknown probability associated solely with presenting the object in the same location. The same is true for the recollection probability associated with context in the OC condition. Following the laws of adding independent probabilities, we can express this in the following way. The recollection probability observed in the OL condition (OL_R) is equal to the recollection probability observed in the SOR condition (SOR_R) plus some unknown degree of recollection probability associated with presenting an object in a familiar location (L_R), minus the intersection of the two. The variables OL_R and SOR_R are known probabilities observed from the experiment, but L_R is unknown and will be derived from the following formula:

$$OL_R = SOR_R + L_R - SOR_R * L_R$$

This equation can be rearranged to find L_R:

$$OL_R - SOR_R = L_R - SOR_R * L_R$$

$$OL_R - SOR_R = L_R (1 - SOR_R)$$

$$(OL_R - SOR_R) / (1 - SOR_R) = L_R$$

$$L_R = (OL_R - SOR_R) / (1 - SOR_R)$$

The recollection probability observed in the OC condition (OC_R) is equal to the recollection probability observed in the SOR condition (SOR_R) plus some unknown degree of recollection probability associated with presenting an object in a familiar context (C_R), minus the intersection of the two. The variables OC_R and SOR_R are known probabilities observed from the experiment, but C_R is unknown and will be derived from the following formula:

$$OC_R = SOR_R + C_R - SOR_R * C_R$$

This equation can be rearranged to find C_R:

$$OC_R - SOR_R = C_R - SOR_R * C_R$$

$$OC_R - SOR_R = C_R (1 - SOR_R)$$

$$(OC_R - SOR_R) / (1 - SOR_R) = C_R$$

$$C_R = (OC_R - SOR_R) / (1 - SOR_R)$$

The probability of observing recollection in the OLC condition was estimated by adding individual probabilities from the three other recognition conditions. This is equivalent to estimating the probability that at least one of the three independent events occurs. The probability of either *A*, *B*, or *C* happening is equal to the addition of the probability of *A*

happening, the probability of *B* happening, and the probability of *C* happening, minus the combined probability of *A* and *B* happening, minus the combined probability of *A* and *C* happening, minus the combined probability of *B* and *C* happening, plus the combined probability of *A*, *B*, and *C* happening. The formula for calculating the probability of at least one of the three independent events occurring is the following:

$$p(AuBuC) = p(A) + p(B) + p(C) - p(AnB) - p(AnC) - p(BnC) + p(AnBnC)$$

We are considering the three factors that can induce recollection (SOR, L, and C) as independent events, and therefore we could substitute them in to this equation to replace *A*, *B*, and *C*, to calculate the probability of at least one of these factors inducing recollection. The derived values for L_R and C_R were used to derive an expected R probability value for the OLC condition (eOLC) using the following formula:

$$eOLC = SOR_R + L_R + C_R - SOR_R*L_R - SOR_R*C_R - L_R*C_R + SOR_R*L_R*C_R$$

The observed *d'* for the OLC condition was compared to a hypothetical expected *d'* derived from summing the *d'* values for the OL and OC conditions (Figure 5.7.a). The observed level of *d'* in the OLC condition (1.10) was in fact significantly lower than the expected value (1.30; $t(21) = 3.133$, $p = 0.005$).

The observed R probability value (0.17; Figure 5.7.b) was significantly greater than that predicted by the summation of the OL and OC probability values (0.11; $t(21) = 2.642$, $p = 0.015$). The combination of OLC thus appears to be something distinct from the simple summation of the individual components of location and context.

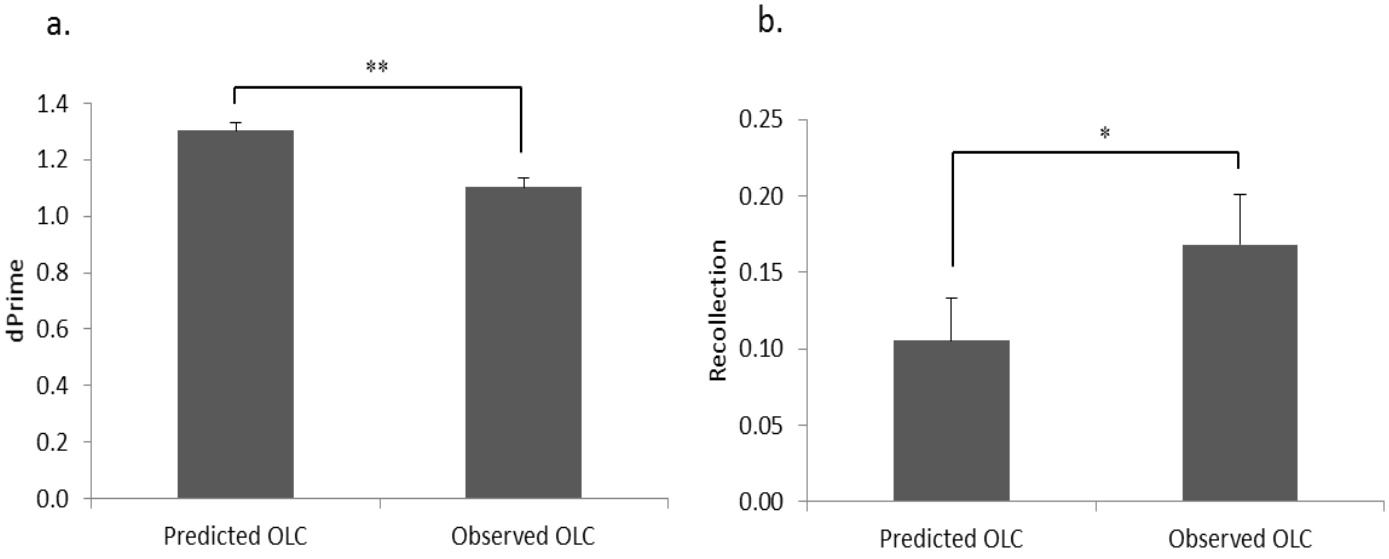


Figure 5.7. a) Predicted and observed *d'* for the object-location-context (OLC) recognition memory condition. b) Predicted and observed R probability values for the OLC condition. Vertical bars show the standard error of the mean. * = < 0.05; ** = < 0.01.

5.4. Discussion

The current chapter offers a unique way of testing recognition memory in humans that provides a shift away from relying on the introspective assessment of the nature of memory. The importance of this is that it allows comparisons between human and non-human animal memory to be made more easily. ROC analysis was used to distinguish between participants' performance in an object recognition task in which multiple conditions were created by presenting the objects in novel or familiar configurations of location and context. Using Yonelinas' (1998) equations for separately quantifying recollection (R) and familiarity (d'), recollection was found to be significantly greater for objects presented in locations and contexts congruent with when they were first encountered (OLC condition), relative to objects presented only in congruent contexts (OC condition) or locations (OL condition), or in a completely different configuration (SOR condition). Importantly, this pattern of results was not found for the measure of familiarity, indicating that the task condition (OLC), that is

deemed to reflect episodic memory, leads to participants being more likely to use recollection, but not familiarity, to successfully recognise the object. These findings were further supported by assessment of the asymmetry of the ROC curves in z-space; the ROC curve for the OLC condition had a slope significantly less than that of the other conditions and this was significantly less than 1.00. Through manipulating the type of recognition memory in the multiple task conditions, it has been possible to vary the degree of recollection elicited during recognition despite participants only being instructed to attend to the objects and respond regarding whether they were 'old' or 'new'. The location and context features associated with the objects must have been incidentally encoded if we are to infer that these features contributed to the differential recollection elicited between the OLC and other recognition conditions.

It could be argued that general differences in task condition difficulty could account for these findings if participants found the OLC condition easier because there are more cues available to help solve the task. This is an unlikely assumption, however, as an increase in measures of both familiarity and recollection should be expected in this case, but indeed only a selective increase in recollection was found. Nonetheless, by taking the area under the ROC curve as a single measure of performance (and thus an index of task difficulty), it was shown that the SOR condition was more difficult than the OL and OLC conditions but, importantly, the OLC condition was not significantly less difficult than either the OL or OC conditions. It is very unlikely, therefore, that differences in the R probability values obtained can be explained by a confounding difference in task difficulty between conditions.

An important question to consider is whether the correct combination of location and context in the OLC condition elicited a degree of recollection that is greater than that predicted by the summation of the separate degrees of recollection associated with location and context alone. Through combining the recollection probabilities associated with the

location and context components alone, a hypothetical expected value of recollection for the OLC condition was derived – this value was found to be smaller than that which was actually observed in the OLC condition. This suggests that the correct combination of object, location and context elicits a degree of recollection-based memory that is distinct from the summation of those individual components. The measure of recollection (R probability) in the OLC condition, therefore, indeed reflected the presence of a type of recognition memory distinct from strong familiarity, lending support to the interpretation of these results in the framework of the DPSD model rather than the UVSD model.

Translating recognition memory research from animals to humans is important not only for validating animal models of memory, but also for developing behavioural experiments in humans that measure separate components of memory (recollection and familiarity) as reliably and accurately as possible. The current chapter presents a human task of recognition memory that is equivalent to that used in animals, providing a shift away from relying on phenomenology (such as the introspective classification of a memory) to distinguish types of memory, and instead using measures derived from signal detection theory. The task therefore avoids the issues often associated with tasks such as the remember/know procedure that are open to participant bias and interpretation of standardised instructions (Yonelinas, 2001), and in turn provides an improvement on previous studies that have investigated models of episodic-like memory in humans using the remember/know paradigm to distinguish between recollection and familiarity (e.g. Easton et al., 2012; Holland and Smulders, 2011). Further work will look at refining the task to focus on whether the OLC condition is a strong representation of the properties of episodic memory and consider how this task can be utilised to further investigate the underlying processes involved in recognition memory, as well as the anatomical basis and network connectivity that allow these memory functions to occur.

CHAPTER 6

GENERAL DISCUSSION

The first objective for this thesis was to address some of the methodological issues associated with recognition memory tasks for rodents, and to develop a more reliable testing method that in turn would reduce the animal numbers required for such tasks. The second aim was to see how effectively the new behavioural paradigms could be applied alongside a technique that would offer insight in to the neural mechanisms underlying different forms of recognition memory. The final aim was to develop a human task of recognition memory that was analogous to the behavioural tasks used with rodents, with a reduced reliance on the subjective human phenomenological experience.

6.1. Summary of findings

Study 1 (reported in Chapter 2) identified a number of methodological issues associated with the widely used spontaneous object recognition task and its variants. The standard version of the task is simple to administer and requires no prior training, as the animals' spontaneous behaviour is measured. These tasks can, however, result in considerable variance in behavioural performance, as influences other than object novelty may drive animal exploration, including any stress induced by handling.

Study 1 investigated animal performance on a series of spontaneous recognition memory tasks using the continual trials apparatus (Ameen-Ali, Eacott and Easton, 2012), which allows for multiple recognition trials to be carried out within a single session. The continual trials apparatus was designed so that animals could perform each trial (consisting of a sample phase and a test phase) in a sequential order, to yield a number of trials in total. This paradigm is similar to that used with the Bow-tie maze (Albasser, Chapman, Amin,

Iordanova, Vann et al., 2010), but the continual trials apparatus offers a unique set up that allows for tasks involving recognition of objects and their spatial location or background context (e.g. Dix and Aggleton, 1999; Eacott and Norman, 2004, Langston and Wood, 2010; Norman and Eacott, 2005). This would be difficult to assess in the Bow-tie maze as, for instance, it would be difficult to distinguish between allocentric and egocentric strategies which an animal may use in a spatial memory task, as they would always have to approach the next phase of a trial from the opposite side of the maze.

As the continual trials apparatus allows more trials to be run per animal, this increases the reliability of the task and reduces the need for large animal numbers. In addition, there is no need to handle each animal before and after each trial, which is necessary with the standard version of the task, thus the new paradigm reduces any potential stress that may impact upon performance. Reliable measures of recognition were found in the multiple trial versions of spontaneous object recognition, object-location and object-in-context recognition. Despite using approximately 50% fewer animals, statistically reliable results were obtained and were comparable to previous studies (Langston and Wood, 2010; Norman and Eacott, 2005). The potential animal reduction is substantial as the spontaneous object recognition task in particular is widely used for both memory research and for detecting potential amnesic properties of new drugs (Bertaina-Anglade, Enjuanes, Morillon and Drieu la Rochelle, 2006). In order for the new apparatus to be implemented across labs and within industry, however, it would be necessary to further demonstrate how the new paradigm could be applied alongside neuroscientific techniques, and how it could be used to assess more complex forms of memory such as those that are episodic in nature.

Study 2 (reported in Chapter 3) investigated different behavioural parameters on a simplified version of the E-maze episodic-like memory task (Eacott, Easton and Zinkivskay, 2005), which assessed animals' preference for non-habituated objects over habituated objects.

Using the continual trials apparatus, different lengths of habituation time and selective food reinforcement were assessed with the aim of determining the most suitable parameters to optimise object preference, which could then be used in the continual trials apparatus to develop an episodic-like memory task based on the paradigm used in the E-maze.

The most reliable measures of recognition in this series of experiments were when the habituation phase on each trial lasted three minutes, the sample and the test objects were baited with food, and there was constant food reinforcement in the holding area, which was also where the habituation phase occurred. None of the current experiments, however, provided an improvement on recognition measures in comparison to those reported in the E-maze task (Easton, Zinkivskay and Eacott, 2009). Experiment 3 assessed the animal's turn behaviour towards the objects at the beginning of the test phase, when the objects were not visible to the animal from the start arm. In the E-maze episodic-like memory task, animals correctly turned towards the non-habituated objects significantly more often than what would be predicted by chance (65.2%; Eacott et al., 2005), which the authors argued could only be solved through recollection-based processes associated with episodic memory. The object preference task in Experiment 3 was not an episodic memory task, but if the animals had a strong object preference for the non-habituated objects then they should turn towards those objects at test, even when those objects were not visible from the start arm (the object locations at test are congruent with their locations in the sample phase. The mean percentage of turns towards the non-habituated object was at chance (51.8%). One possibility for the poor recognition performance in this series of experiments could be related to the number of behavioural tasks that the groups of animals performed in, which may have negatively impacted upon performance in the latter experiments. It may also have been possible that additional location novelty associated with the habituated object may have driven exploration towards it in the test phase, as this object occupies two locations within a single trial. This did

not, however, appear to impact negatively upon task performance in the E-maze task. It is worth noting that in the current object preference task, habituation took place within the test apparatus and multiple trials were carried out within a single session. In contrast, the animals in the E-maze experiment did not perform a succession of trials, and habituation took place in a separate holding box outside of the maze. As such, these differences in task procedure may account for some of the inconsistent findings between the two studies.

Study 3 (reported in Chapter 4) investigated how effectively the continual trials apparatus could be applied with immediate-early gene (IEG) imaging. Combining spontaneous tasks with IEG imaging allows for neuronal activation from recognition memory to be measured whilst also providing the behavioural evidence that the animals can distinguish between novel and familiar stimuli. Demonstrating that multiple trial paradigms can provide an improvement on standard paradigms that have assessed recognition memory using IEG imaging is important because if it is shown to be successful, it may provide an alternative to some, but not all, studies that adopt the lesion approach. IEG imaging allows for multiple brain regions to be imaged simultaneously, which in turn could provide further animal reduction if multiple lesion groups are not required.

The study consisted of a number of behavioural tasks based on the multiple trial spontaneous object recognition and object-location recognition paradigms, in order to determine a suitable task procedure to then apply with IEG imaging and investigate the neural correlates of recognition memory. The tasks which produced the most reliable behavioural measures were the spontaneous object recognition tasks which involved one group of animals becoming highly familiarised to a set of objects that would later become the test objects, and a second group of animals becoming highly familiarised to a different set of objects before being tested on the same objects as the Familiar group (Study 3, Experiments 3a an 3b). In Experiment 5, IEG imaging was carried out following a test protocol based on the

behavioural paradigm used in Experiments 3a and 3b. A naïve group of animals was also included but was not familiarised to any objects prior to test. The results showed no overall significant activity changes in the perirhinal cortex, although increased c-fos expression was found in the Novel and Naïve groups, relative to the Familiar group. No overall significant activity changes were found in the hippocampus. These findings, taken together, share similarities with the study by Albasser, Poirier et al. (2010) that combined the multiple trial Bow-tie maze with IEG imaging and found increases in c-fos expression in the caudal perirhinal cortex, and no overall activity changes in the hippocampus. Such results may reflect a reduction in neuronal activity in the perirhinal cortex when familiar stimuli are viewed again (Brown and Aggleton, 2001; Gonsalves, Kahn, Curran, Norman and Wagner, 2005; Henson, Cansino, Herron, Robb and Rugg, 2003; Montaldi, Spencer, Roberts and Mayes, 2006). Issues relating to procedural differences or lack of statistical power due to a relatively low sample size may, to some extent, account for the non-significant findings in this study, but the potential use of the continual trials apparatus is apparent; further work will be needed to establish a robust protocol.

Finally, Study 4 (reported in Chapter 5) focussed on the translation of animal models of recognition memory to humans. The analysis of receiver-operating characteristics (ROCs) was used to dissociate between the cognitive processes believed to underlie recognition memory in a human task based on the behavioural paradigms typically used to assess recognition and 'episodic-like' memory in animals. Previous human memory tasks have heavily relied on the introspective assessment of a subject's memory, which is not comparable to non-human tasks where memory is often assessed indirectly through preferential exploration of an object, for example (Ennaceur and Delacour, 1988). The primary aim of Study 4 was to validate the methodology of testing recognition memory in animals by showing that humans demonstrate the greatest amount of recollection when test

items are presented in conditions used to infer episodic-like memory in rodents, relative to other recognition memory conditions. In turn, such findings would provide supporting evidence for a dual-process model of recognition memory, with episodic memory supported by recollection- rather than familiarity-based processes (Brown and Aggleton, 2001). In this experiment, familiarity was measured in terms of *d'* and recollection was measured as a probability ('R'; Yonelinas, 1994).

The degree of recollection varied across the recognition conditions with significantly higher recollection being found in the episodic-like memory condition, relative to the other recognition conditions. The results cannot be explained by the addition of more cues to recall (the spatial location and background context that the object was presented in), which suggests that recollection measured in the current experiment must closely reflect a memory process that is distinct from just strong familiarity. If so, these findings would lend support to the dual process signal detection model of recognition memory (Yonelinas, 1994) rather than the unequal variance model, which states that recognition is based on a single strength variable (familiarity; Squire, Wixted and Clark, 2007).

Improving the translation of animal models of memory to humans is necessary and can, in some cases, replace the need for animal studies when assessing memory process. Demonstrating well-controlled human behavioural studies through the use of ROC analysis is one way of minimising the subjective introspection often prevalent in human studies of memory, and allows researchers to measure processes that may closely reflect the memory of animals during spontaneous recognition tasks.

6.2. Recognition memory in animals

One of the main aims of this thesis was to address specific methodological issues relating to spontaneous tasks of memory in animals. As discussed above, the work in this

thesis has successfully established a number of testing paradigms for assessing complex forms of recognition memory in rodents using the continual trials apparatus. Development of an episodic-like memory task in the continual trials apparatus has proved to be more difficult; Study 2 focussed on the object preference aspect of the E-maze episodic-like memory task, with the aim of establishing the exact behavioural parameters to optimise object preference, which could then be utilised in a multiple trial episodic-like memory task. Future work could look to establish an episodic-like memory task based on the open field 'what-where-which occasion' task (Eacott and Norman, 2004) as this task, similarly to other spontaneous recognition tasks, simply relies on differential exploration of objects as an indicator of recognition abilities. The task may, however, need refining to be suitable for the multiple trial paradigm, as when context change has been previously used as part of the task procedure, substantial variance between the animals performance still remained, though performance was significantly above chance (Chapter 2, Study 1, Section 2.6. Experiment 4: Object-in-context memory). To demonstrate that an animal test of episodic memory truly is episodic, however, it would be helpful to provide a behavioural dissociation between recollection- and familiarity-based processes. Although further work may be needed to develop an episodic-like memory task in the continual trials apparatus, the main objective has been achieved in that reliable testing paradigms have been developed and shown to substantially reduce the number of animals required for statistically meaningful results.

Following the successful development of the behavioural paradigms in the continual trials apparatus, it was important to illustrate how these paradigms could be utilised to contribute to our current understanding of the neural basis of recognition memory. Immediate-early gene (IEG) imaging has already been established as a technique to complement multiple trial paradigms with the Bow-tie maze study (Albasser, Poirier et al., 2010). To date, there have been numerous studies that have investigated different forms of

recognition memory using the Bow-tie maze in combination with IEG imaging, which has allowed researchers to elucidate the network interactions during spontaneous object recognition (Albasser, Poirier et al. 2010) and recency recognition memory (Kinnavane et al., 2014; Olarte-Sanchez et al., 2014). IEGs, such as c-fos, can be used as a marker for neuronal activity, which can be enhanced with behavioural paradigms that use multiple trials as they allow for a more reliable detectable signal in comparison to the standard one trial a day recognition paradigm. Moreover, such tasks provide evidence that the animals can distinguish between novel and familiar stimuli which can then be correlated with c-fos expression, and is much improved from paradigms where animals are simply shown novel and familiar stimuli, with no evidence that they can distinguish between them (Seoane, Tinsley and Brown, 2012; Wan, Aggleton and Brown, 1999; Wan, Warburton, Zhu, Koder, Park et al., 2004; Warburton, Glover, Massey, Wan, Johnson et al., 2005; Warburton, Koder, Cho, Massey, Duguid et al., 2003; Zhu, McCabe, Aggleton and Brown, 1996). Studies using the Bow-tie maze have analysed networks of activity through the use of structural equation modelling, which allows for structural relationships to be derived from fos counts so that the strengths and the potential direction of these relationships can be estimated (Albasser, Poirier et al., 2010; Kinnavane et al., 2014; Olarte-Sanchez et al., 2014). If significant differences in the c-fos counts had been found in Study 3, it may have been possible to adopt this approach to assess the network dynamics in the spontaneous object recognition task, which was slightly modified from the procedure used in the Bow-tie maze. Further work will be needed to establish this procedure in the continual trials apparatus, and to develop other recognition task procedures for use with IEG imaging. Of specific interest would be an object-location recognition memory task as this is not currently possible with the Bow-tie maze, but could be carried out in the continual trials apparatus (Ameen-Ali et al., 2012). Establishing these

recognition memory tasks is necessary if an episodic-like memory task is to be developed in the continual trials apparatus.

IEG imaging offers a useful alternative to some studies that may adopt the lesion approach to infer neural correlates, as multiple sites can be imaged simultaneously and could therefore lead to further substantial animal reduction. In addition, assessing the network interactions during an episodic-like memory task would make a significant contribution to the ongoing debate around the structures in the medial temporal lobe that are thought to underlie such memory processes. Of specific interest amongst researchers is elucidating the neural basis for recollection- and familiarity-based processes believed to contribute to different forms of recognition memory, with the hippocampus and perirhinal and parahippocampal cortices being implicated (Aggleton and Brown, 1999; Squire, Stark and Clark, 2004; Tulving and Markowitsch, 1998).

6.3. Recognition memory in humans

The second main aim of this thesis was to improve the translation of animal models of memory to humans. Tests of human memory often rely on subjects' introspective account of whether they judge a presented item to be old or new. For instance, the remember/know paradigm (Tulving, 1985) allows subjects to further categorise their 'old' responses as being one of either 'remember' (associated with recollection-based processes), or 'know' (associated with familiarity-based responses). Yonelinas (2002) further validated this paradigm through arguing that, in accordance with the dual process signal detection model (DPSD), the quantitative estimates of recollection and familiarity derived from this model correspond well with the estimates of recollection and familiarity derived from the remember/know paradigm. Traditional signal detection theorists, however, have stated that different degrees of memory strength can account for the different responses in the

remember/know paradigm, not distinct memory processes (Donaldson, 1996; Dougal and Rotello, 2007; Dunn, 2004, 2008; Hirshman and Henzler, 1998; Slotnick and Dodson, 2005; Shimamura and Wickens, 2009). Wixted (2007) further argued against the use of the remember/know paradigm to provide insight into distinct recollection- and familiarity-based processes, as he argued that remember responses only occur when recollection and familiarity signals are summed together and exceed a particular criterion. The unequal variance signal detection model (UVSD) can therefore be compatible with dual process theories if both recollection and familiarity are considered as continuous processes, in which the degrees of variance are not equal. There is general agreement that familiarity can be regarded as a continuous process but there is no such consensus with regard to recollection. Yonelinas (1994) argued that recognition reflects a Gaussian equal-variance signal detection model, with a continuous familiarity process and a discrete recollection process. Moreover, he argued that a threshold level of memory strength exists whereby recollection occurs as a continuous process above this threshold, and fails if it is below the threshold (Parks and Yonelinas, 2007, 2009). This threshold process has been described as "all or none", but should be interpreted as meaning that memory for an item will only occur if it exceeds the threshold, as opposed to everything about an item will be recollected if the threshold is exceeded (Parks and Yonelinas, 2007).

The experiment carried out in Study 4 used the basic behavioural paradigms used to test spontaneous recognition memory in animals to develop a task of human memory. The analysis of receiver-operating characteristics (ROCs) was used to quantify recollection and familiarity across a number of recognition conditions, including one based on episodic-like memory. Significantly greater recollection was found in this episodic-like memory condition relative to the other conditions, lending support to a dual processes model of recognition memory (Brown and Aggleton, 2001). Could the findings, however, also be explained by a

single process model of recognition? The greater recollection found in this episodic-like memory condition of the current experiment was characterised by significantly greater asymmetry of the ROC curve. The DPSD model states that symmetrical ROC curves result from the familiarity-based responses, and asymmetrical curves are a result of the occurrence of recollection-based responses (Yonelinas, 1994). The UVSD model explains asymmetry in ROC curves in terms of unequal variance between old and new item distributions which may be a sign of strong familiarity, not recollection (Squire et al., 2007). It is, therefore, possible that the greater asymmetry found in the episodic-like memory condition may be a result of strong familiarity rather than recollection-based responses. If strong familiarity was the basis for the responses in the episodic-like memory condition then it would be expected that predicted *d'* and R probability performance based on the summation of the values from the object-location and object-context conditions would be comparable to the observed values, but this was not the case. Significantly greater R probability and significantly lower *d'* values were found, compared to the predicted values which suggests there was something unique about the combination of object, location and context in the episodic-like memory condition, which was more than simply the sum of its parts, and unlikely to simply reflect strong familiarity.

Study 4 offers an improvement on typical human memory tasks including those which have previously attempted to translate animal models to humans (Easton, Webster and Eacott, 2012; Holland and Smulders, 2011), as it does not rely on subjects' introspective account of their memory. There is ongoing debate centred around descriptors of episodic memory, specifically whether it can be defined as consisting of an absolute temporal component or whether it should include a broader contextual descriptor that could be defined by a temporal component, but not exclusively ('what-where-which occasion'; Eacott and Norman, 2004). Future work could look to develop the current experiment to include a what-where-when

condition in order to quantify the degree of recollection in this alternative episodic memory descriptor. Such a task would indicate whether the what-where-when episodic-like memory tasks in animals are measuring something that reflects human experience of episodic memory. Moreover, it would be interesting to test subjects with selective hippocampal damage, because if the hippocampus plays a crucial role in episodic memory, their performance on the current experiment should result in a very different pattern of results that may show poor performance in conditions that strongly require recollection-based responses.

6.4. Conclusion

The work in this thesis has focussed on addressing issues with spontaneous recognition tasks. Specifically, novel approaches centred around the 3Rs (Replacement, Refinement and Reduction) have been developed to improve tasks with animals, which has led to substantial reduction in animal numbers, but also to improve translation to humans. Scope for further research remains, but the work in this thesis has already made a significant contribution to recognition memory research. Further work to implement the methodology established in this thesis is ongoing, although research is still needed to demonstrate the potential use of the continual trials apparatus with neuroscientific techniques, and to further validate the human task reported in Study 4 as being a reliable measure of human episodic memory.

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