A novel developmental rodent model to investigate the neural basis of episodic memory

By

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Abbreviations

ADS - Antibody diluting solution

AP-1 - Activator protein 1

CA1 - Cornu Ammonis 1

CA2 - Cornu Ammonis 2

CA3 - Cornu Ammonis 3

DG - Dentate gyrys

DI - Discrimination index

DLE - Dorsalateral entorhinal cortex

DMTS - Delayed mating to sample

E1 - Episode 1

E2 - Episode 2

EC - Entorhinal cortex

GABAa - γ-Aminobutyric acid

HLA - How long ago

IEG - Immediate early gene

IHC – Immunohistochemistry

ITI – Intertrial interval

LH - Lutenizing hormone

LTP - Long term potentiation

MTL - Medial Temporal Lobe

NMDA - N-methyl-D-aspartate

NOR - Novel object recognition

OC - Object context

OP - Object place

OPC - Object place context

PBS - Phosphae buffered saline

PFA - Paraformaldehyde

PRM - Pattern recognition memory

SPI - Serial parallel independent

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Declarations

I hereby declare that the following thesis is based on the results of investigations conducted by myself and that this thesis is of my own composition. Work other than my own is stated with reference to the researcher or their publications. This body of work has not previously been presented for a higher degree.

Miss Stephanie Lyon

I certify that Stephanie A. Lyon has completed nine terms in experimental research in the Division of Neuroscience, School of Medicine, University of Dundee. She has fulfilled the conditions of the Ordinance General No. 39 of the University of Dundee and is qualified to submit this thesis in application for the degree of Doctor of Philosophy.

Dr Rosamund Langston

Abstract

Episodic memory is the recollection of unique autobiographical events, including rich detail of times, places and associated contextual information. In neurodegenerative diseases, such as Alzheimer's disease, episodic memory is greatly impaired. The ability to recall episodic memories is thought to develop later in childhood than other types of memory such as novelty recognition, however this ontogeny in animals is so far unconfirmed due to a lack of suitable behavioral tests. The aim of this thesis was to develop a protocol suitable for testing the development of memory in juvenile rats, and to understand if rats show a similar ontogenic profile of memory as humans.

A novel protocol was developed and first tested in adult rats. The protocol was compressed into two days of testing using a battery of spontaneous object recognition tasks to allow testing of narrow critical developmental time windows. The tasks were then used to assess the ontogeny of different types of memory in rats from p25 to adulthood. The Object Place Context (OPC) task was used as a model of episodic memory and used alongside the Novel Object Recognition (NOR), Object Context (OC) and Object Place (OP) tasks, all of which were tested in two days.

It was discovered that all ages tested could perform basic object recognition (NOR task). Associative contextual memory (OC task) developed between p38 and 42 and both spatial (OP task) and episodic memory (OPC task) developed between p46 and 48. Further investigations pinpointed this spatial and episodic memory development to overnight between p47 and p48. Immunohistochemistry allowed the investigation of the anatomical locus of the episodic memory task, and showed subtle increases in the CA3 in p48 rats. It was concluded that rats do show a similar ontogenic profile of memory a humans, with recognition memory developing before episodic memory. This information will be invaluable when studying disease during development, and may also be extrapolated to understand disease of old age.

Chapter 1

Introduction

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1.1 Memory

1.1.1 Why is memory important?

Memory has allowed us to evolve into the successful species we are today; we are able to learn from the past and use the information to create a more successful future. It allows us to interact socially and professionally, and creates our personal experience of the world. Therefore, when memory is impaired or affected by disease, injury or pharmacological agents, the results are distressing to everyone involved. It is still not fully understood how memories are formed, and why they are so susceptible to manipulation by drugs or disease, however the development of animal models of memory are enabling researchers to make great progress in the understanding of the neurobiology of memory.

Memory has been studied since Ebbinghaus in 1879 who was the first person to attempt to model higher learning processes experimentally. At the time it was thought that higher mental processes could not be studied experimentally, and therefore when he developed a way of studying memory in humans he was somewhat of a pioneer. He understood that learning and memory would be affected by prior knowledge and experience, therefore he generated a list of “words” which could be easily memorized but which had no prior associations. To do this he generated a range of “pseudowords” which consisted of three syllables in a consonant-vowel-consonant pattern. These nonsense words had no prior meaning,

and any that did have a prior meaning (such as dot, or cat) would be excluded. He generated a list of words such as dax, box and yat. From his study where he would say aloud a list of these words and then try to repeat them by memory, he determined that humans impose meaning even on nonsense syllables to make them more meaningful (Ebbinghaus, 1885)

The field of research has evolved since then with the ability to model memory in a number of different animals in the laboratory being common practice. Deficits in memory are implicated in a number of diseases for example Dementia, a disease which affects 850,000 people in the UK (Alzheimer's Research UK, 2015).

1.1.2 Different subtypes of memory

Memory is the process in which information is encoded, stored and then retrieved, and it has long been thought of not as a single process but instead memory has been characterised into a number of sub types. The first segregation was made in 1965 when memory was defined as having two types, primary (short term) and secondary (long term) (Waugh and Norman, 1965). However primary memory is now, more aptly, known as working memory (Baddeley et al., 1974). An example of working memory, devised in 1956, long before the term “working memory” was introduced, is to repeat a list of numbers immediately after studying them, without referring back to the original reference (Miller, 1956). By seeing how many numbers a person can immediately repeat, it has become a measure of “information processing capacity”. Crucially, working memory is only successful if the person is not interrupted, for example a phone number is

often repeated or rehearsed in order to remember it long enough to dial or write it down. This is known as the “phonological loop” and is based around a short term storage of the information, which is quickly forgotten after the loop is broken (Baddeley et al., 1974).

Secondary memory has since been sub-defined further into procedural and declarative. Procedural can be thought of as “skill learning”, exemplified by the ability to carry out a motor task such as riding a bicycle or tying shoe laces. These skills become almost automatic once they have been acquired, however explaining in words how to perform them is far more difficult. Declarative memory, however, requires the ability to recall facts or personal experiences which we have acquired, usually through language in humans. Declarative memory can then be subdivided into two further categories, semantic and episodic. This distinction between the two types of declarative memory was first defined in 1972 by Tulving, however it was based on information which had been available, but largely ignored, for a number of years. Semantic memory is knowledge which has been acquired, and repeated a number of times, such as “the queen lives at Buckingham palace” or “Christmas is on the 25th December”. It is easy to communicate these facts to someone else through verbal communication, and it is not necessary to remember the context in which the knowledge was first acquired. It becomes a known fact. However, memory for specific unique events including their temporal and spatial factors, is known as episodic memory. For example, “When I visited Buckingham I watched the changing of the guards whilst drinking a bottle of juice” or “I remember I got this scarf from my sister when I visited her in Bristol for Christmas last year”

are both examples of episodic memories. When recalling subtle details about the event one usually relives the whole experience in the mind.

1.1.3 How is episodic memory defined?

The definition of episodic memory has grown in complexity over the decades in which it has been studied. The landmark paper published by Tulving in 1972 provided the first definition and this has since acted as the basis for all further definitions. He stated that “episodic memory receives and stores information about temporally dated episodes or events, and temporal-spatial relations among these events” whereas semantic memory is “a mental thesaurus, organized knowledge a person possesses about words” (Tulving, 1972). The examples used of episodic memory demonstrate personal experiences that are remembered in “temporal-spatial relation to other experiences” (Tulving, 1972).

In 1983 Tulving’s original definition was updated to include a requirement for autonoetic consciousness, i.e., a possession of self-awareness, and the ability to realise that the event happened to you personally, in the past (Tulving, 1983).

In 1993 Tulving updated his previous distinctions between semantic and episodic memories to include his belief in the presence of different kinds of conscious awareness. He claimed that episodic memory involves conscious memory, and a conscious act of recollection, whereas semantic memory involves a greater feeling of familiarity (Tulving, 1993).

It is thought that there are five levels of awareness in humans, which develop at different ages during childhood (Rochat, 2003). These levels

have been coined by Rochat from a study carried out where a “Post-It” piece of paper was placed onto a child’s head. The child is then engaged in play to ensure that it has no awareness of the note, before it is then placed in front of a mirror and it’s actions noted. Level zero is a complete lack of self-awareness (and therefore is not counted as one of the five levels of self-awareness). At this stage the individual has no understanding that the mirror is a reflection of the world, and instead treats it as an extension of the environment. This level characterizes the moments of absence that adults have when we sometimes frighten ourselves for a short instant when we may catch our reflection unexpectedly and perceive it as another human. There are some animals which also show this level of self-awareness. Budgerigars (*Melopsittacus undulates*) are often kept as pets, and owners sometimes give the bird a mirror, to which the bird will sing courtship songs and treat the reflection as another bird (Rochat, 2003). Level 1 is known as “Differentiation” and is where the child is no longer oblivious to the fact that mirrors are a reflection and there is an understanding that there is a contingency between movements seen and felt. Level 2 is “Situation”, where the individual is now capable of exploring the link between the seen movements on the mirror surface and what is proprioceptively perceived of the own body. During this level, the individual is aware that the mirror surface is spatially situated in relation to the body and is a solid surface. Level 3, “Identification”, sees the child recognise itself and understand that the reflection in the mirror is “me” rather than another individual. In terms of the “Post-It” experiment, this is the stage where the child reaches for it, to touch or remove it. Level four is known

as “Permanence” where the child is able to identify itself beyond the present time, and can identify itself in pictures and movies taken in the past, even when the self is younger, at a different location or in different clothes. This is when the identification of self is not tied to a temporal or spatial locus and is not tied to mirror experience. Finally, level five is full self-consciousness or “meta” self-awareness. Individuals with full self-consciousness are aware of what they are and how they are perceived by others, as well as displaying emotions such as pride or shame (Rochat, 2003).

It is thought that babies are not born with self-obliviousness (level zero), and instead they are capable of demonstrating a sense of their own body (level 1). When touching the cheek of newborn babies, they tend to orientate their head towards the stimulation, as well as understanding self and non-self touch (Rochat and Hespos, 1997). By the end of two months of age, infants reach level two, showing signs that they have a clear sense of how their body is situated in relation to the environment, for example reaching for objects that they see (Rochat, 2003). Level three (recognised themselves in a mirror) is not reached until eighteen months of age. However at this age children still show a degree of confusion regarding the identification of self. When the child looks in the mirror, it sees a reflection of itself and although the mirror might be “Me”, it is also typically what others look like. In the 1960’s, the French psycholigst Jean Piaget studied his own children and made observations of their development. He noted that at 23 months his daughter, Jacqueline, announced to him, when returning from a walk, that she is going to see her father, her aunt and

Jacqueline in the mirror. At this age she is perfectly capable of understanding that it is “Me” in the mirror, however she refers to herself in the third person (Piaget, 1962). At 35 months of age he showed his daughter a photograph of herself, to which she said “It’s Jacqueline”. He then asked her “Is it you or not?”, to which she replied “Yes it’s me, but what has Jacqueline in the photo got on her head?”. More recently Povienelli reported the commentary of a three year old viewing herself on a television screen with a sticker on her forehead, where she said “it’s Jennifer, it’s a sticker, but why is she wearing my shirt?” (Povinelli, 2001). Together, these observations demonstrate the me-but-not-me dilemma in which children struggle with between the age of eighteen months and four years. It is thought that full self-awareness (level 5), develops between four and five years of age (Rochat, 2003), however it is a complex and dynamic phenomenon which is difficult to demonstrate unambiguously.

In animal models of episodic memory, self-awareness has been one of the most difficult things to demonstrate. Self-awareness has been shown in chimpanzees (Gallup, 1970, Hirata, 2007), however both protocols used involved a great deal of training which suggests that self-awareness may not be implicitly present in non-humans. However, one may be able to use and apply the five levels of self-awareness as defined by Rochat to demonstrate that animals have a degree of self-awareness, even if it is not “level five” on Rochat’s scale.

1.1.4 How are memories encoded?

In 2001 Tulving introduced the importance of the Serial Parallel Independent (SPI) model of relations between episodic, semantic and perceptual memory (Tulving, 2001). Perceptual memory allows the identification of the content of a memory (e.g. “this looks like porridge”), the semantic memory allows the knowledge of the facts surrounding the memory (e.g. “porridge is usually eaten for breakfast”), and episodic memory contains the unique spatiotemporal aspects of the event (e.g. “I ate porridge for breakfast on Tuesday whilst watching the news in the lounge”). The SPI model of the relations between these three memory types (perceptual, semantic and episodic) states that the encoding of the information is serial (S), therefore it must be known what porridge is and what it looks like in order to know that it is usually eaten for breakfast and to recall eating it for breakfast on Tuesday. Storage of the memories is parallel (P) which means that the perceptual information is stored separately to the semantic which is stored separately to the episodic, all with their own storage systems. However, retrieval of the memories is independent (I) between the systems, therefore it could be remembered that porridge was eaten for breakfast on Tuesday without consciously remembering what porridge looks like and when it is usually eaten. Tulving highlighted some of the evidence supporting this model, which he stressed was not a neurological model, rather a more psychological model, although he hinted at the fact that there may be an underlying neurological system that maps on to the SPI model. The evidence surrounding the model is two-fold. The first is that “memory” can operate adequately at lower levels,

independent of episodic memory existence (through damage to the system, or through lack of ontogeny of episodic memory). For example, children can learn the alphabet, or a new word, or the 3 times table, however they do not, and have no need to remember the episodic events surrounding the moment when they learnt a specific fact. Secondly, the model allows for the changes seen in patients with retrograde amnesia (loss of memories formed before the amnesic event), where impairment is seen in only episodic memory, or only in semantic memory, or both. Whereas anterograde amnesia (the loss of the ability to generate new memories after the event which caused the amnesia), according to the SPI model, cannot occur in semantic memory alone.

1.2 Models of Episodic Memory

1.2.1 Testing episodic memory in humans

Episodic memory was thought to be a uniquely human experience when it was first defined by Tulving in 1972. The use of spoken language enables humans to communicate freely, and indeed many researchers have investigated episodic memory using interview, self-reporting and free recall. One such task, known as the method of unconstrained search, involves the participant inspecting a word until an association to it is made based on a personal memory which is able to be dated (Crovitz and Schiffman, 1974) . Another method is the Autobiographical Interview, where participants are asked to recall events from five life periods, events in which they were personally involved in and for which they have a

memory of being personally involved in, providing as much detail as possible (Levine et al., 2002)

Tests that don't have to involve language or free recall can also be used to measure episodic memory in humans, examples of which are paired associate learning (PAL) and delayed matching to sample (DMTS). PAL is a well-used test of episodic memory that involves the pairing of items, sometimes words or patterns. One example shows the user a number of boxes on a screen, each with a pattern in it. After a short delay the participant is shown one of the patterns and they are asked to touch the screen where they originally saw the pattern (Cambridge, 2015b). A DMTS is similar to the PAL task but this time with a delay between the sample and the test. For example the participant is shown a complex visual pattern (the sample) and then after a delay is shown four similar patterns, only one of which matches the original sample pattern. The participant must select the matching pattern (Cambridge, 2015a).

1.2.2 Scrub jays as a model for episodic-like memory

There has been great debate as to whether or not animals possess episodic memory at all and due to the lack of language in non-human animals, it is impossible to demonstrate autonoetic consciousness, one of the requirements of episodic memory as defined by Tulving in 1983. Therefore the term "episodic-like" memory was coined by Clayton in 1998 to describe behaviour observed in scrub jays.

Figure 1.1 A scrub jay caching food in an ice cube tray filled with sand. *Note the complex visual cues in the environment which enable the bird to remember where it stored the food (Clayton et al., 2003).*

Western scrub jays (*Aphelocoma californica*) instinctively cache (store) food in the wild. The food storing behaviours of these birds in the wild suggest that they possess a highly developed memory system in order to remember where and when they stored their food. Clayton *et al.* (1998) developed a lab based trial in order to test scrub jays' memory for the what, where and when of episodic-like memory (Clayton et al., 2003, Clayton and Dickinson, 1998, Clayton et al., 2001). The trial was based around teaching the animals about the perishability of food, i.e., when food becomes inedible over time. Scrub jays will preferentially choose mealworms over peanuts, however they were trained that mealworms will degrade 4 hours after caching whereas the peanuts will still be fresh after 124 hours. The "degrading" was done by the experimenter whilst the bird was absent and involved soaking the worms in washing-up liquid and leaving them to rot. After the birds were taught this, they were allowed to store food as normal. If they were allowed to recover the food after 4 hours, they retrieved the

preferred mealworms, however if they weren't allowed to retrieve the food until 124hours after caching, they did not bother searching for the mealworms and instead retrieved only the peanuts. These results were obtained even after the food was removed before recovery and the tray was filled with fresh sand, eliminating the possibility that the birds relied on olfactory cues. This task is a good demonstration of the "what, where and when" components of episodic memory – the animals must remember what food they stored, where in the tray they stored the food and when they stored it, in order to prevent them searching for food which will have degraded.

Can scrub jays mentally travel backwards or forward in time?

Conscious recollection is one of Tulving's criteria for episodic memory (Tulving, 1983) and involves the process of mentally travelling back in time to the event. However this phenomenon of "mental time travel" can also be thought of as a way of planning for the future, imagining a future event for which one must react to in the present. The Bischof-Köhler hypothesis states that non-human animals can only react to events which will immediately affect them, and cannot plan for the future. It is well known that animals will carry out a task in order to gain an immediate reward, for example a rat will press a lever for a food reward. Food caching alone is not an example of future planning if the animal does it instinctively without any thought for the future consequences. However, in the wild, caching

between scrub jays is competitive, and the individual relies on the fact that it is the only one who knows where the food is stored.

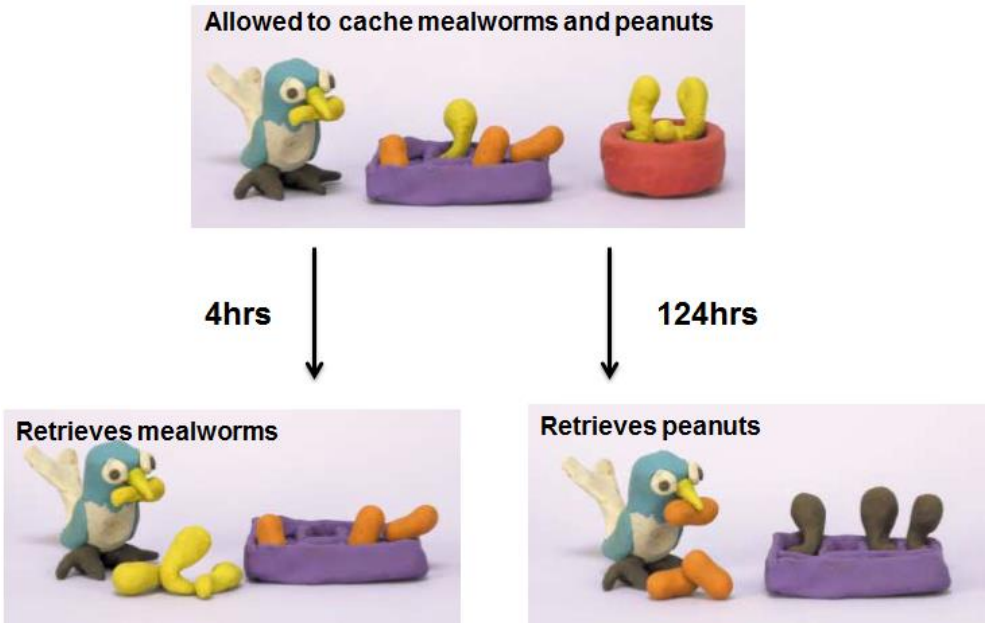

Figure 1.2 Demonstrating episodic-like memory in Scrub Jays (Clayton et al. 2003). *By teaching scrub jays that their favourite food, mealworms, degrade after 124 hours, Clayton et al demonstrated that the animals can remember what food was stored where and when.*

Clayton adapted the test above to attempt to prove that animals will in fact alter their behaviour in order to benefit them at a future time, and will act in a way that is independent to their current needs, desires and feelings. Clayton *et al*. allowed a scrub jay to cache food either in private, or whilst being watched by another bird. After 3 hours the birds were then allowed back in to the arena where the caching tray was, always in private, and this time with a new tray as well as the old tray. If they had been allowed to originally cache their food in private, they did not move the food, however if they had been observed caching the food, they re-cached the food into the new tray (Clayton et al., 2003). This goes some way to demonstrating that the birds are able to remember the past, plan for the future and alter their behaviour accordingly.

1.2.3 Rat models of episodic memory

Rats are an immensely valuable resource within the field of neuroscience due to their ability to learn and perform complex behavioural tasks. Behavioural tasks can be used to further understand the psychological and neurological basis of certain behaviours and functions. Once they are robust, the behavioural paradigms can then be used to investigate the effects of disease or pharmacological agents. There are a number of different tasks to test different types of memory and this section will outline some of them and discuss their uses and limitations.

There has been a shortage of work proving that rats can plan for the future. However one study in 2008 went some way to demonstrating that rats are aware of events which may happen (Roberts et al., 2008). Four arms of an eight-arm maze contained a small food reward, which the rats were able to retrieve immediately if they wished, however, after a longer delay, the rats were then able to retrieve a reward from the other 4 arms, which contained a much larger reward. The rats were only allowed to receive the larger reward if they did not retrieve the initial smaller reward. The rats soon learned this rule and it goes some way to showing that rats are able to alter their behaviour (i.e. not retrieve the initial reward), in order to benefit them in the future. If rats are able to plan for the future, this suggests that they have the ability to form episodic-like memories, which include “mental time travel”.

Unlike scrub jays, most laboratory animals do not instinctively store food therefore Clayton’s test cannot be easily manipulated for use in rodents.

Bird et al. attempted to replicate Clayton's scrub jay test with Norway rats, and succeeded in part, by demonstrating that rats will carry two types of food (cheese and pretzels) down arms of an 8-arm maze, and store it at the end of the maze (Bird et al., 2003). They will then return to the preferred cheese first before the less-preferred pretzels. This demonstrates the "what" and "where" components of episodic memory, but not the "when" component. Any attempts to teach the rats about degradation of food by Bird *et al*. have failed and proves the difficulty in demonstrating the temporal component of episodic memory.

There has been some success by another group, Babb and Crystal, in designing a task for rats which involves a temporal component and food retrieval (Babb and Crystal, 2005). An eight arm radial maze was used for the task, and in the sample phase rats were given access to four of the eight arms. Rats were trained to visit all four of these arms, three of which contained normal rat chow and one which contained the preferred chocolate flavour chow. After a delay, rats were placed back in the maze and had access to all eight arms and the length of this delay was the cue as to where the rats would find food. They were trained that after a short time delay of thirty minutes they were to visit the four arms that had not contained food during the sample phase (a delayed non-matching to sample) to find food, whereas after four hours the four locations where food would be found in the test phase were the same as in the sample phase, including the location of the chocolate flavoured chow. The measure of whether or not the rats remembered what food was found when and where was based on how many of their initial four arm choices in the test phase

was directed towards the arm which contained the chocolate chow. After the short, thirty minute delay rats chose the chocolate arm approximately 20% of the time (i.e. chance level)(Babb and Crystal, 2005). However, after the longer, four hour delay rats chose the arm containing chocolate approximately 50% of the time, suggesting that they were able to utilise the length of time passed, and their memory of locations and identity of food, to make a decision (Babb and Crystal, 2005). However, these results must be taken lightly, as although they look promising, the rats received a large amount of training (approximately eighty training sessions per rat) which was very methodical, and also that the rats had previously been used for another behavioural task on the same radial arm maze. This may suggest that instead of true episodic-like memory which is trial unique, the rats instead possess a long term semantic-like memory for the maze and the procedure.

There have been a wide range of studies conducted which claim to model episodic memory by using odour-based tasks. One such study was carried out by Veyrac *et al*. in 2015. This study combined an odour cue with the delivery of either sucrose or quinine solutions. The testing arena was rectangular in shape with four odour ports. When the rat nose poked at an odour port, an odour was delivered and a vacuum system ensured that the odour remained confined to the port. A hole in the wall just below the odour port allowed the introduction and withdrawal of a drinking pipette, and each lick to the pipette triggered the delivery of a calibrated volume of solution. The arena could be configured to a number of different contexts using different arena floors, a video projector that projected visual patterns onto

the floor, and two loudspeakers which provided different sound environments.

In order to use this task to assess episodic memory, extensive training is required. The group describe a “shaping” phase where the animal is initiated to the introduction of the pipette when the odour port is nose poked, as well as habituation to receiving odour stimulation associated with sugar or quinine to “give the animals a hint that the odour might be an indicator for finding a sweet or avoiding a bitter drinking solution which otherwise could not be predicted”. The shaping phase took, in total, thirteen days. During the following three days, “routine” sessions were carried out. These “routine” sessions consisted of neither visual nor auditory enrichment of the context, and no odours or solutions were introduced. The aim of these sessions were to “enhance the salience of the following episodes”. Therefore each episode was characterised by a unique combination of odour, place and context positively rewarded by sucrose solution.

Next, the rats took part in two different episodes, Episode 1 (E1) and Episode 2 (E2), separated by a one day “routine” session. In each of these episodes, a non-overlapping configuration of only two ports was available, each of which released two different odours which were unique to each episode. (E.g. odours A and B at ports two and three in E1, odours C and D at ports one and four in E2). In each episode, one of the ports delivered quinine solution regardless of the odour, whereas the other port delivered quinine solution with one odour, and sucrose solution with the other odour. Each episode session consisted of twenty four trials, combined in a

pseudorandom sequence (six repetitions of four different configurations). Finally, twenty four hours, or twenty four days depending on the experimental group, after the last episode, rats were tested on a “test” session, which consisted of twelve trials in the E2 context. Firstly the two-port test was carried out which matched the set up in the episode in terms of context, available ports and odours, except that no sucrose reward was given, only water regardless of place and odour. Secondly, the four-port test was carried out in which, for the first time, all four ports were available to the rat (therefore two ports were in context and two ports were out of context), with all odours from the two episodes presented at their respective ports. Again, only water was presented and no sucrose reward was given.

By analysing the behaviour of the rats in terms of nose pokes and licks, the authors utilised this protocol to demonstrate that rats could correctly remember the unique combination of odour, place and context in order to gain a sucrose reward (Veyrac et al., 2015). In order to prove that the rats are carrying out this task in an episodic-like way, they temporarily inactivated the hippocampus using the γ-aminobutyric acid A ($GABA_A$) receptor agonist muscimol and demonstrated that this disrupts the animal’s capacity to recollect the memory (Veyrac et al., 2015). Furthermore, c-Fos imaging revealed that there was specific activation within the hippocampal-prefrontal cortex network, which correlated with the accuracy of the recollection performance (Veyrac et al., 2015).

Arguably this study is an exceptional example of a well-controlled and very elegant study. They ensured that rats could hear and discriminate the

environmental sounds that they had highly contrasting black and white visual patterns for the contexts, and that odour pairs used were easily distinguishable by the rats and each had similar preference by the rat when allowed to spontaneously explore the odours. However, they state that this is a task which requires minimal training, and this is something which could be argued against. The protocol is long and requires the rats to be water deprived. Furthermore, the presence of a sucrose reward means that the episodic memory will have an emotional component and will involve the amygdala and does not model the basic what-where-which of episodic memory, instead it models a more complex form of reward learning.

Instead of food storage behaviour like the scrub jays or the study by Bird *et al,* or odour-based tasks like Veyrac *et al*., many behavioural tasks with rats have exploited the fact that rats will spontaneously explore a novel aspect of their environment (Ennaceur and Delacour, 1988). These tests depend on the fact that a rat can detect a novel object within a familiar environment and are usually designed to be simple to use in untrained animals.

In 2006 Kart-Teke et al. attempted to design such a behavioural task for rats which included the notoriously difficult temporal component of episodic memory. The task requires the rat to integrate both object identity, object location and when the object was seen. The protocol requires no training or rule learning by the rat, and is not reliant on the use of rewards. The test involved three days of habituation to the testing arena (an open field) followed by one day of the task consisting of two samples and a test phase. Each phase was five minutes with a fifty minute interval between phases.

Each phase contained four objects, with all objects in sample phase one identical to each other, and all objects in sample phase two identical to each other but different from the objects encountered in sample phase one. There were eight possible locations for objects within the box and the four locations used in sample phase one were chosen at random. In the second sample phase, two objects occupied positions which were used in the first sample phase, whereas the other two objects occupied locations that had not had an object previously. Finally, in the test phase, two objects from each phase were presented; “old” from sample phase one, and “recent” from sample phase two. One of each object was in a familiar location, whereas the other was in a novel location where that particular object had never been seen, but the rat had encountered an object there previously. This task aimed to understand whether the exploratory behaviour of the rats would indicate an interaction between recency of an object and spatial displacement. One might predict that the rat would preferentially explore the “old displaced” object (A_2 in figure 1.3), if the rat is able to remember “what, where and when”. However what the authors actually found was that rats spent the most time at the “recent displaced” object (B_2 on figure 1.3), followed by the “old stationary” (A_1 on figure 1.3). On average, rats spent more time exploring “old stationary” objects than “recent stationary” objects, however this was not the case for displaced objects where rats spent more time exploring “old stationary” than “old displaced”. This task demonstrated the fact that rats can remember the order in which objects

were presented, however it fails to demonstrate clearly that rats can form a truly temporal memory and integrate it into an episodic memory.

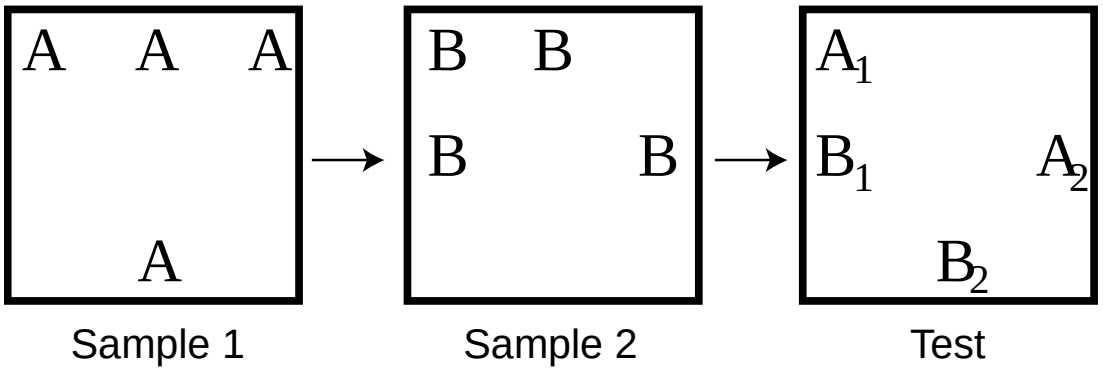

Figure 1.3 Behavioural task for rats developed by Kart-Teke et al. in 2006. *Objects are represented by the letters A and B and arrows indicate a fifty minute delay (Kart-Teke et al., 2006).*

Roberts *et al* attempted to understand whether if, in food caching studies, animals instead of remembering when an event happened animals may be instead keeping track of how much time has elapsed since the event. This could be done by either circadian timers, their own behaviour or strength of a decaying memory trace (Roberts et al., 2008). Rats were assigned to one of two groups, a “When” group, or a “How Long Ago” (HLA) group. An eight-arm maze was used, and in the sample phase rats were allowed to enter four randomly chosen arms. This sample phase could occur at 9am or 12.30pm, and three of the arms contained reward pellets with the fourth arm containing the highly preferred cheese cube. The test phase was conducted at either 9.30am (half an hour after the sample), 1pm (either half an hour or four hours after the sample) or 4.30pm (four hours after the sample phase) where all eight arms were open. The four arms closed during the sample phase now contained reward pellets and the three arms containing reward pellets in the sample phase were now empty. On the arm where the rat consumed cheese in the sample phase, another piece

of cheese was placed on the same arm to replenish it, or the cheese was not replenished and the arm was empty. Rats assigned the “When” group had to learn that the sample phase time was important, with one half of the rats learning that when the sample phase was at 9am the cheese would always be replenished, whereas when the sample phase was at 12.30pm the cheese would never be replenished (and vice versa for the other half of the group), regardless of the delay between sample and test phases. Rats in the HLA group were taught that the delay between the sample and test phases (either half an hour or four hours) was the cue for whether the cheese was replenished or not. Again half the rats learnt that the short phase implied replenished cheese and the other half learning that a long delay implied replenished cheese.

As per a lot of studies involving food rewards with rats, the training was extensive. There was sixteen days in which the cheese was always replenished followed by 16 days in which the cheese was always pilfered, followed by forty days of randomised trials where the cheese may be replenished or pilfered. Performance was measured by the probability of the rat returning to the cheese arm in the first four arm visits, and the mean rank of entry into the cheese arm (ie, early return to the cheese arm was indicated by a high probability of visiting it in the first four choices, and a low rank of entry)

Performance was measured over the last twenty trials and it was shown that rats used how long ago the sample phase was as a cue to figure out if the cheese would be replenished or not, and did not use the time of day as a cue. When the long interval was increased to twenty eight hours, the

same pattern was seen, rats used the elapsed time to predict whether the cheese would be replenished or not. This could be due to circadian oscillators, the animals own behaviour or the strength of the memory trace (Roberts et al., 2008)

The difficulty in modelling the temporal component of episodic memory in rodents was partly addressed by Eacott and Norman in 2004, when they devised a task based on the definition of episodic memory that Tulving introduced – “A unique spatiotemporal event”. This definition can be thought of as “What (unique event), where (spatial) and when (temporal)”. However, some may argue that the “when” component of episodic memory is in fact less of a definitive time stamp, and more of a contextual component of the event. As an example of this, I can recall my breakfast this morning, I can picture it in my minds eye and I could recall the details of it. However I am unlikely to remember that it was exactly 8.10am, instead I would use semantic inputs to suggest that it was probably morning as I was eating porridge and porridge is usually eaten for breakfast, I hadn’t left the house yet to get to the lab and I leave at 8.30am every day, therefore it was probably between 8am and 8.30am. Another example would be an episodic memory of a birthday party as a child. One could ascertain what year it was in by which house the party was in, which group of friends were there or the types of presents that were given.

Eacott and Norman (2004) exploited the fact that in humans the temporal aspect of episodic memory is notoriously poor (Friedman, 1993), and that it is not necessary to remember the exact time of an event, instead just the cues surrounding the specific event which allow calculation of the time or

date. They rephrased “what, where, when” to “what, where, which”, ie on which occasion, with which contextual information. This was the basis of the task that can arguably be thought of as one of the most elegant and useful models of episodic memory in terms of novelty detection for rodents, and is the basis of the behavioural tasks used in this thesis.

The task consists of three phases, with each phase lasting until the rat had spent at least 15 seconds exploring each object for a minimum of two minutes and a maximum of five minutes. Between each phase rats were placed in a holding box for two minutes. The first sample phase was conducted in “context one”, and the rat was presented with two different objects. The second sample phase saw copies of the objects seen in sample phase one used, however they had switched position within the box and also the context had changed to “context 2”. Finally in the test phase, two more copies of one of the previously encountered objects are presented in one of the contexts, in the same locations as previously. Only one of these objects has not been encountered in its current location and context, although both the context and the location are familiar in themselves (figure 1.4). Therefore the “what”, “where” and “context” (ie “which”) combination is novel. Eacott and Norman proposed that if rats possess episodic-like memory then they will be able to remember that one of the objects has been seen in that context and in that location, and therefore can recognise that the other object is in a novel configuration. Indeed, at delays of up to one hour between the sample and test phases, rats will preferentially explore the object which is in a novel configuration.

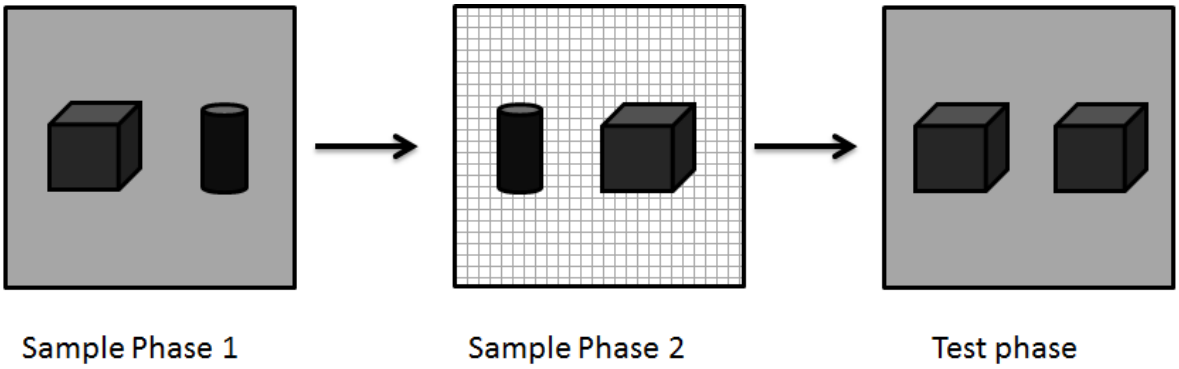

Figure 1.4 Eacott and Norman's "what, where which" task as a model of episodic memory for rats. *Each object is encountered in both locations within the arena, and in both contexts, however in the test phase the object on the right hand side has never been encountered on the right in the grey context. Due to the fact that rats preferentially explore novelty over familiarity, the rat will explore this object more in the test phase. (Eacott and Norman, 2004)*

Eacott and Norman demonstrated that this task is episodic-like in nature by showing that fornix lesions hindered the ability of the rat to correctly identify the object in the novel configuration. This deficit in the object-place-context (OPC) task was present at very short delays (2mins and 5mins), compared to both intact and sham operated rats which were unimpaired at delays of up to 60mins. The fact that lesioned rats were unable to perform the OPC task but were able to perform the object-context (OC) task (where the only novel aspect is the combination of object and context, the location (left or right) is not involved) at the same delays, accentuates the importance of the hippocampus in the OPC task and helps to validate it as a test of episodic memory.

In 2005, Eacott et al. published a study based on the Eacott and Norman OPC task, however they made a number of changes to try to overcome the fact that the task may be able to be solved by familiarity alone, not recollection. At least, there is no real way of understanding if the rat is performing the task using familiarity, recollection or both. Eacott et al.

devised a task which must be solved using recollection alone. They did this by carrying out the test in an E-maze shown in figure 1.5. In this maze the rat could see all objects A and B from the starting position (S) when they were placed in the locations shown in A. However when the objects were placed in the locations shown in C, the rat could no longer see the objects from the starting position.

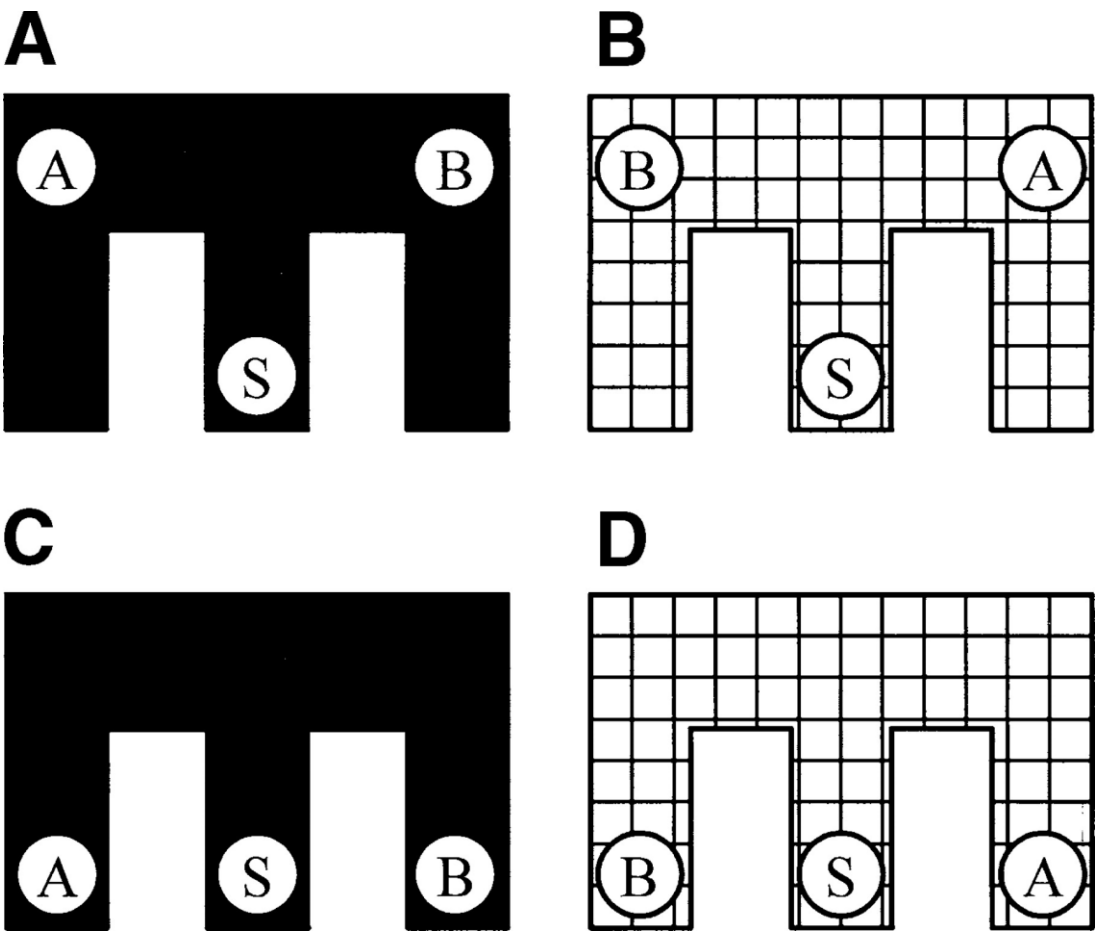

Figure 1.5 E-maze apparatus for rats designed by Eacott et al.
A) E-maze with black context and objects A and B visible from the start arm (S). B) E-maze with mesh context and objects A and B visible from the start arm and objects in opposite position to the black context. C) E-maze with black context and object not visible from the start arm. D) E-maze with mesh context and objects not visible from the start arm (Eacott et al., 2005).

Following on from extensive habituation and pre-training sessions the rats were tested. Testing consisted of two “sample phases” as shown in figure 1.5 . Then the rat was then given one of these objects in the holding box

for eight minutes, before being placed back into the testing maze in one of the contexts. Due to the fact that rats prefer a novel object over a familiar one, if the rat can remember on which side of the maze it saw the objects previously, it would select the arm which contains the object it did not see in the holding box (ie the least recent and therefore most novel object). The fact that in this test phase the objects were not visible from the starting position, means that the rats could not solve the task by familiarity and instead had to recall the previous episode. This is another example of a what-where-which task which relies solely on the innate preference of rats for novelty, and requires no reward or aversive stimuli.

1.3 How powerful are novel object recognition based tasks?

As has just been discussed, the use of novel object based recognition tasks is widespread within neuroscience. However novel object recognition (NOR) tasks are subject to a lot of debate about how to perform them. In the previous section it was described how small changes to the apparatus or protocol can result in an entirely different behavioural response and outcome. Therefore this section will focus on some of the issues that must be addressed when undertaking a novel object based recognition task.

1.3.1 The effect of objects used in novel object recognition based tasks

All laboratories that use NOR based tasks have their own library of objects from which to choose. There are no standard objects which all investigators use. As such, one must implement a great deal of thought when selecting objects to use. In this section I am going to discuss a number of variables

that must be addressed; object affordances, odour cues and rodent visual perception of objects.

The definition of affordance is the relation between an object (or an environment) and an organism, that affords the opportunity for that organism to perform an action. For example a knob affords twisting, a door affords pushing. Affordances can be thought of as “relations between the abilities of animals and the properties of objects”(Chemero and Heyser, 2005). In the study in which object affordances were described, Chemero and Heyser described how in an investigation they undertook to understand the effect of ethanol withdrawal on memory, they realised that object affordances have a great influence on the behaviour of the animal. They observed that the ethanol withdrawal did not impair recognition memory, however this result was inaccurate due to massive object biases of the rats. They showed that rats preferred objects that afforded climbing, ie those objects which the rats could climb onto. When critiquing this study, Ennaceur explored the fact that the study was quite probably ill-designed in the fact that the objects were not counterbalanced in such a way to overcome any biases which the rats may have (Ennaceur, 2010). The tall objects which the rats could not climb on were always given as the novel object to the control group, whereas the short objects which the rats could climb on were given to the withdrawal group and there was no alternation of objects between the two groups. This resulted in the withdrawal group having an “advantage” as they naturally preferred the identity of the novel object.

As well as object identity, odour cues have also been given a large degree of importance in the majority of studies conducted, with multiple copies of the same object used, and all objects cleaned between phases and between rats in order to prevent any biasing based on odours (Ennaceur, 2010). Interestingly, different researchers have different opinions on how to prevent odour having an effect on the test. For example in the study described previously by Kart-Teke et al. they wrote *"Since the objects were made of the same material (glass), they could not be distinguished by olfactory cues during the test trial".* This suggests that they feel that the material of the object affects how much of an odour trail is left by the animal, which I would argue is incorrect due to the fact that the material of the object is not the *source* of the olfactory cues, although it is worth noting that different material will retain olfactory cues different. Regardless of whether this is correct or not, it is just one case which highlights the lack of consistency to odour-based issues between groups studying novel object based recognition tasks.

The visual perception of objects by rodents is something that also must be taken into consideration when planning and executing a NOR based task. Various features of an object such as its colour, brightness and shape can be involved in the discrimination and memory of it. Most studies which utilise this task rely heavily on the visual aspects of the objects, with testing carried out in the light phase. About 97% of the rat retina consists of rods (used for low-light vision and are responsible for peripheral and night vision) and the remaining 3% consists of cones (used for bright-coloured vision and perceive and recognize colours when light falls on them) (Carter-

Dawson and LaVail, 1979, Jeon et al., 1998). Humans have trichromatic vision with three types of cones, whereas rats and mice have dichromatic vision due to only two types of cones, which results in limited colour vision (Bowmaker, 2008, Jacobs, 1993, Jacobs et al., 2001). Furthermore, rats can perceive ultraviolet light and can discriminate it from visible light (Jacobs et al., 2001). Based on this information it is understandable that rats and mice can discriminate between stimuli that differ in brightness but cannot be trained in colour discrimination (Jacobs et al., 2001). Therefore, it is possible that objects with the same shape and brightness, but with different colours to the human eye, may appear the same to a rat or a mouse. The visual abilities of rodents must also be kept in mind when designing the testing arena, especially when the NOR based task is investigating contextual memory. It is imperative that the contexts differ in ways other than the colour of the walls or floor, and instead could have different patterns and tactile elements also.

As well as the colour and brightness of an object, the spatial orientation of an object must also be taken into consideration in NOR based tasks. When an object is not symmetrical in it's three dimensional shape, the investigator must be meticulous in ensuring that it is placed in the environment in the same orientation every time. If an asymmetrical object is not replaced in the same spatial orientation as it was previously seen, some of it's parts may appear in a novel orientation and therefore the task may be compromised. Until recently, it was presumed that rodents could not fully utilise the 3-dimensional structure of an object if it was placed in a different orientation, due to their lower visual acuity compared to primates

(Zoccolan, 2015). However this may not be the case as recently a review has been published which outlines an array of expeiments which demonstrates that rats are in fact capable of invariant visual object recognition (Zoccolan, 2015). This suggests that object orientation may not actually not have an effect on object recognition experiments, but regardless of this it is good practice to always ensure that object's spatial orientation remain consistent throughout an investigation.

1.3.2 The effect of anxiety in novel object recognition based tasks

One could argue that the fact that the rat prefers a novel object is possibly due to an active avoidance of the familiar object due to a stress induced during the sample phase, for example the fact that the animal was contained within the inescapable testing arena could be thought of as stressful, despite the fact that all animals are habituated to the box. However, this theory can be disproved somewhat by a study carried out in 1999 that looked at the effects of anxiolytic drugs on novelty-induced place preference (Klebaur and Bardo, 1999). They aimed to prove or disprove the alternative interpretation of novelty seeking as an active avoidance of the familiar, by administering the benzodiazepine anxiolytic diazepam and the nonbenzodiazepine anxiolytic gepirone at a number of doses. The animals did not show a decrease in the novelty seeking behaviour, in some cases even when the dose administered decreased locomotor activity. These experiments indicate that preference for novelty reflect the positive aspects of the novel rather than the negative aspects of the familiar.

1.3.3 How are object recognition tasks scored?

The fundamental theory of NOR based tasks is that the exploration of the novel object implies a memory for the familiar object, and that this exploratory behaviour of the rodent can be translated into a quantitative score to be compared between or within groups. However, according to Ennaceur and Delacour (1998) there are two ways in which this score can be generated. The first measure D_1 is the difference between time spent at the novel object (T_N) and time spent at the familiar (T_f) object [$D_1 = T_N – T_F$]. The second measure, discrimination index (DI), is D_1 divided by the total exploration at both novel and familiar objects [$DI = (T_N – T_f) / (T_N + T_f)$. DI has a value between negative one and positive one, with positive scores generated when more time is spent at the novel object compared to the familiar, negative scores when more time is spent at the familiar object compared to the novel and a score of zero generated when there is equal time spent at both novel and familiar objects indicating no preference. This measure is an effective way of adjusting for any imbalance in exploration time across a population, however when it is used as a measure of memory exploratory activities should also be analysed in case the experimental conditions (i.e. a drug or lesion) cause a change in exploratory behaviour, which might otherwise be missed when using a score like this.

Over time there have been further measures developed and used such as the percentage of time spent exploring the novel object relatively to the total time spend exploring both objects, sometimes known as a “preference index (Benice et al., 2006, Broadbent et al., 2010, Oliveira et al., 2010, Wang et al., 2007, Hammond et al., 2004). A preference index of above

50% indicates a preference for the novel object, below 50% a preference for the familiar object and a preference index of 50% indicates no preference for either the novel or familiar object.

Throughout this thesis, DI will be used as a performance measure on all NOR based tasks, due to it's ability to normalize the data in a way where groups of rats can be directly compared, regardless of overall exploratory activity. It must be noted that although a positive discrimination index is representative of a preference for the novel object, a negative discrimination index may also be indicative of a "memory" as it merely suggests the rat has a preference for the familiar object. These tasks rely on the fact that rats "prefer" novelty, however if rats produce a negative score it may be due to the fact that they simply do not prefer the object, it may not be due to the inability to remember the sample phase.

1.4 The hippocampus

1.4.1 Episodic memory and the hippocampus

The hippocampus has been at the centre of neuroscience research for decades due to its distinctive anatomical layout of unidirectional excitatory pathways. It is an elaborate, but highly organised, collection of neurons which are connected in an organised way which suggests a very specific function. The role of the hippocampus was brought to light by Milner and Scoville in their landmark 1957 paper, where they documented the case of "patient HM". Patient HM received a bilateral removal of parts of the medial temporal lobe (MTL) in a somewhat experimental attempt to cure his epilepsy. Removal of the ventral frontal lobe was often used to treat

psychosis, and although he was not psychotic, his epileptic seizures were so common that they left him incapacitated. After the surgery, his epilepsy did indeed improve dramatically, however it was the side effects of his surgery which Milner and Scoville documented, along with nine other patients who had undergone similar surgery, which were interesting. All patients showed dramatic memory loss, however no loss of intelligence (tested using the Wechsler Memory Scale and the Wechsler Adult Intelligence Scale). Upon further investigation of the patients, it was found that the two patients who showed no memory deficits at all in fact did not receive bilateral damage to the hippocampus. One only received unilateral damage to the hippocampus, and the other received bilateral removal of the amygdaloid complex and the uncus, but the hippocampus was left undamaged. This helped to prove that the memory deficit seen in patient HM was due to bilateral damage to the hippocampus, despite also having damage to the amygdaloid complex and the uncus.

Further testing of HM's memory deficit demonstrated that he showed a complete loss of the ability to form new episodic memories (anterograde amnesia), but also showed a degree of retrograde amnesia by failing to recall any episodic events during the 19 months prior to surgery. However he would frequently talk about events during his childhood, suggesting that he was able to recall the episodic memories from his youth. As the definition of episodic memory has developed over the past 50 years, it has come to light that HM probably possessed no episodic memory at all. So how was he able to recall complex events from childhood? This was probably due to the fact that the events which would constitute an episodic

memory were repeated to him over and over by friends and family, such that the memory is likely to be semantic in nature, rather than a true episodic memory. The current thinking about HM's condition was that he lacked all episodic memory but he retained semantic memory. This supports Tulving's distinction between the two memory subtypes, and also provides evidence that there are different brain regions involved. This is further supported by the work of Vargha-Khadem whose research was conducted on patients with brain injuries that occurred at birth or early childhood. These individuals had bilateral hippocampal damage and had pronounced amnesia for episodes in everyday life, however all patients attended mainstream schools, had normal development of speech and language and showed normal literacy and factual knowledge. They were able to form and recall semantic memories but not episodic, and her findings provide support for the view that episodic and semantic memory utilise different brain regions and it is only the episodic component that is fully dependent on the hippocampus (Vargha-Khadem et al., 1997).

The Nadel and Moscovitch multiple trace theory of memory states that memories are stored in the cortex but an index is kept in the hippocampus (Nadel and Moscovitch, 1997). Because the episodic memories are unique events which happened only once, there will only be one index entry, whereas semantic memories which have been encountered on multiple occasions, will have multiple index entries in the hippocampus. Thus, patient HM showed relative sparing of semantic memory because each episode in which the semantic memory is experienced, creates a new index entry and increases the chance that it will remain after part of the

hippocampus has been damaged; there are more indices spread throughout the hippocampus for each semantic memory compared to each episodic memory. This is in contrast to Marr's thoughts in 1971 who hypothesised that all memories are eventually "copied over" to the cortex and will no longer rely on the hippocampus.

1.4.2 Hippocampus anatomy

Despite thousands of research articles on the hippocampus, and decades of studies on it, the scientific community fails to agree and remain consistent on the nomenclature of it. It is not consistently agreed upon amongst researchers which areas constitute the hippocampus and which constitute surrounding areas. However, within the book "The Hippocampus Book", the authors adopt a system which I will adopt throughout this thesis – The hippocampus proper has three subdivisions, CA3, CA2 and CA1, where CA is derived from the Latin *cornu ammonis*. The hippocampal formation includes the hippocampus proper, as well as the surrounding regions of dentate gyrus (DG), subiculum, presubiculum, parasubiculum and entorhinal cortex (EC).

The hippocampal formation is unlike any other brain region due to it's unique set of unidirectional, excitatory pathways. The perforant path is the major input into the hippocampus with axons arising from the EC. Projections from layer II of the EC input into the granule cells of the DG and projections from layers III and V input into the pyramidal cells of the CA1 as well as the subiculum via the temporoammonic pathway. The perforant pathway can be separated into the lateral perforant pathway

which arises from the lateral entorhinal cortex, and the medial perforant pathway which arises from the medial entorhinal cortex.

The mossy fibre pathway is the pathway that runs from the DG to the CA3. The axons of the DG granule cells are called mossy fibres and they extend from the DG to the CA3 pyramidal cells, forming their major input. These pyramidal cells are then the source of the major input to the CA1 via Schaffer collateral axons, a pathway called the Schaffer collateral pathway. The CA1 field of the hippocampus then projects to the subiculum, which closes the hippocampal loop by projecting to the deep layers of EC. This CA1-Subiculum-EC pathway is the major output of the hippocampus however the EC also projects to presubiculum, parasubiculm, perirhinal and postrhinal cortices.

1.4.3 Regions of the hippocampal formation

Dentate gyrus

The dentate gyrus is a characteristic V or U shape and has three layers – molecular, granular and polymorphic. The granular layer is the most prominent and contains granule cells that project to interneurons of the CA3 but also to pyramidal cells. The granule cells of the DG have a distinguishable late time of formation during brain development, and in rats approximately 85% of granule cells are generated after birth (Bayer and Altman, 1974)

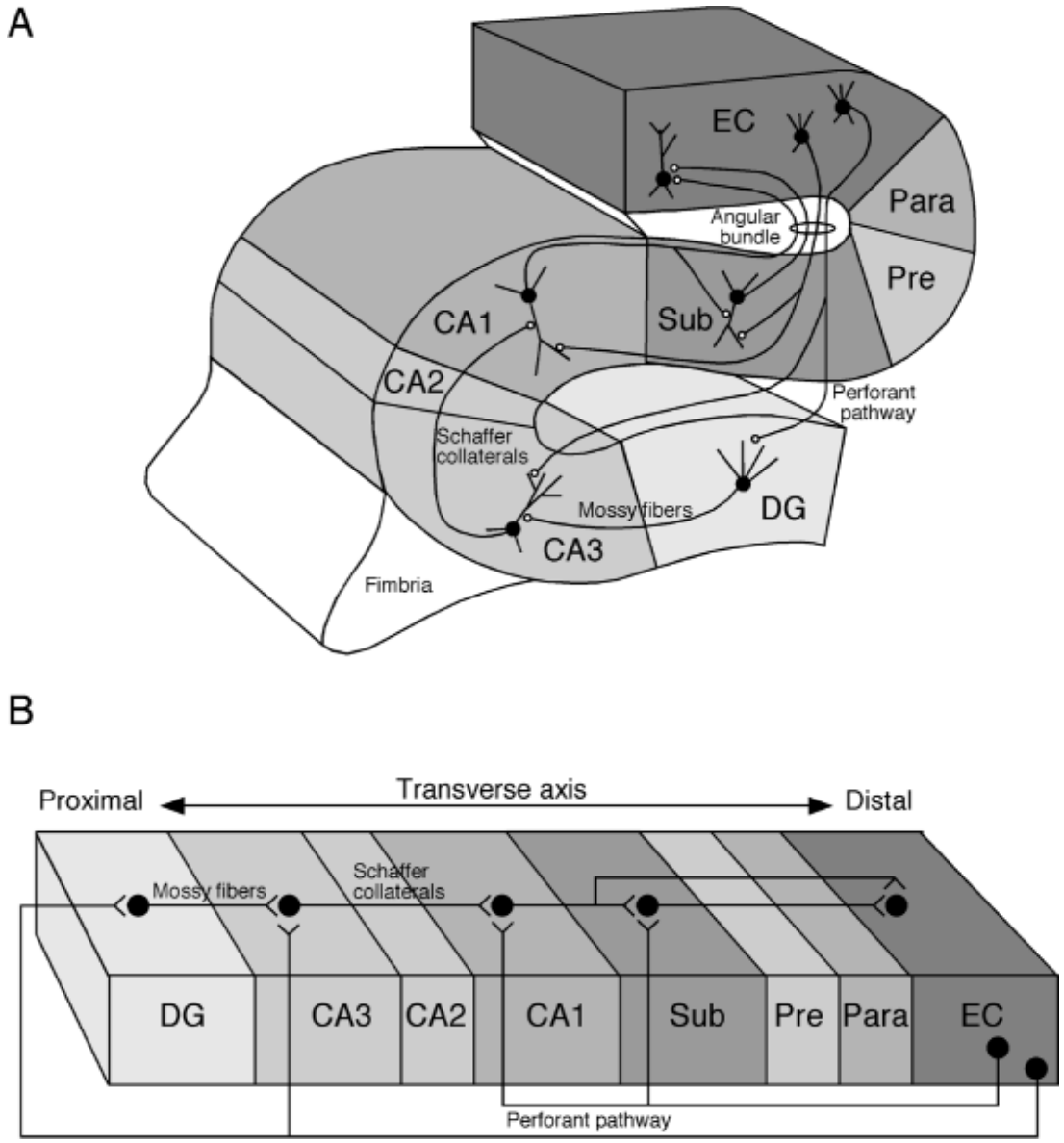

Figure 1.6 The hippocampal formation (Anderson et al., 2007). *A) Connections between the different regions of the hippocampus. B) Projections along the transverse axis of the hippocampal formation. EC- Entorhinal cortext, Para – Parasubiculum, Pre – Presubiculum, Sub – Subiculum, CA1 - Cornu Ammonis 1, CA2 – Cornu Ammonis 2, CA3 – Cornu Ammonis 3, DG – Dentate gyrus.*

Entorhinal Cortex

The EC is the major gateway between the hippocampal formation and the neocortex. Its name entorhinal derives from the fact that it is partially enclosed within the rhinal sulcus. In 2014 May-Britt and Edvard Moser received the Nobel Prize for Physiology and Medicine for discovering that the medial EC contains grid cells. Grid cells fire at distinct spatial locations, with their firing “map” encoding a tessellate pattern and forming a spatial map of the environment.

The lateral EC has been shown to be critical for contextual processing (Wilson et al., 2013a, Wilson et al., 2013b). It is possible that contextual features of the environment are integrated with object identity in the lateral EC, and this information may then be combined with spatial information from the medial EC to contribute to episodic memory in the hippocampus.

Subiculum

The subiculum has a range of electrophysiological and functional properties which distinguish it from other hippocampal regions (O'Mara et al., 2001). Given the range of neuroanatomically distinct regions which the subiclum receives input from and sends input to, it’s not surprising that it is able to influence a wide range of cortical and subcortical regions. The subiculum receives inputs from the CA1, presubiculum, parasubiculum, EC, perirhinal cortex, retrosplenial cortex and projects back to CA1, perirhinal cortex, retrosplenial cortex, prefrontal cortex, anterior cingulate cortex (O'Mara et al., 2001). The function of the subiculum is not well understood due to the fact that it is a region of the brain that is relatively

understudied, however it is thought to play a role in spatial navigation (O'Mara et al., 2009).

Pre- and para subiculum

In regards to cytoarchitecture, the pre and parasubiculum are more similar to the EC than to the hippocampus. The hippocampus proper is allocortex with three layers, whereas the EC is multilaminate typically having six layers similar to neocortex. Both presubiculum and parasubiculum receive inputs from the subiculum, however neither region receives direct inputs from the hippocampus, in contrast to the subiculum.

Perirhinal and post-rhinal cortices

The perirhinal and postrhinal cortices are part of the cortical region that surround the hippocampal formation, lying adjacent to the hippocampus within the temporal lobe. These cortices receive sensory information from the visual, olfactory and somato-sensory cortices, and the connectivity within these areas is complex. This connectitivy can be segregated into two loops; perirhinal-LEC-hippocampus and postrhinal-MEC-hippocampus. The first of these loops involves projections from the perirhinal cortex to the LEC, which then projects to the CA1-subiculum junction as part of the perforant pathway. The second of these loops has projections orininating in the postrhinal cortex which project to the MEC, again projecting to the CA1 via the performant pathway.

1.4.4 The amygdala and its connections with the hippocampus

As is the case with the hippocampus, the amygdala is a complex structure consisting of a number of distinct nuclei, resulting in it sometimes being

referred to as the “amygdaloid complex”. These nuclei can be classified into three groups: The deep or basolateral group, the superficial or cortical-like group and the centromedial group.

The amygdala has projections to and from a wide range of brain regions including the thalamus, the prefrontal cortex and the hypothalamus to name only a few. However the connections between the amygdala and the hippocampus are of most interest to researchers working on learning and memory. The prominent source of basolateral afferent projections is the hippocampal formation, arising from the CA1, the ventral subiculum and the EC. Furthermore, the amygdala sends efferent projections to the hippocampal formation. The basolateral nuclei send projections to the EC, CA3, CA1, subiculum and parasubiciulm, the accessory basal nucleus projects to the EC, CA1 and parasubiculum and finally the lateral nucleus sends projections to the EC and parasubiculum (Sah et al., 2003).

1.5 The development of the rat, and how this relates to the neurological development

1.5.1 Rat sexual development

The rat pubertal period starts at p28 with rising circulating gonadal hormone levels in both sexes. (Spear, 2000, Gabriel et al., 1992). From p35-40 the females show vaginal opening and irregular ovarian cycling (Gabriel et al., 1992) with males showing a gradual increase in testosterone (Gabriel et al., 1992). From p46-49 females exhibit regular ovarian cycles and males are capable of producing fertile sperm (Gabriel et al., 1992). By p60 rats are generally considered to be sexually mature, however male

testes continue to develop with testosterone levels peaking at p70 before falling to adult levels

1.5.2 Puberty, adolescence and the brain

Puberty is the period of life in which an individual becomes capable of sexually reproducing, whereas adolescence is the time between childhood and adulthood, including puberty, but also cognitive, social and emotional maturation (Sisk and Zehr, 2005). Puberty is marked by the increase in secretion of gonadal steroid hormones, whereas adolescence is marked by a remodelling of cortical and limbic systems, which in turn leads to adult-like cognition, social behaviours and decision making (Sisk and Zehr, 2005). This remodelling of the adolescent brain is accomplished by a number of mechanisms, many of which are also responsible for the development of functional neural circuits in early brain development. These mechanisms include neurogenesis (Pinos et al., 2001), apoptosis (Nunez et al., 2001), growth of axonal projections and axon sprouting (Benes et al., 2000, Cunningham et al., 2002) and dendritic elaboration and retractions (Goldstein et al., 1990, Meyer et al., 1978), These structural changes are sex and brain region specific, and are influenced by a number of pubertal hormones.

1.5.3 Neurotransmitter system changes during rat development

N-methyl-D-aspartate (NMDA) receptors are the classic learning and memory receptors (Riedel et al., 2003). NMDA receptors are activated by glutamate, and play a key role in hippocampal long term potentiation (LTP), hypothesised to be the cellular correlate of learning and memory (Lynch,

2004). NMDA receptor number peaks in early adolescence (p21) followed by a loss of one third of receptors by p60 (Insel et al., 1990) This pruning leads to a reduction of excitatory glutamatergic input into the nucleus accumbens (Frantz and Van Hartesveldt, 1999).

Dopaminergic transmission is thought to contribute to reward and attention, amongst other things and these are critical behaviours during the adolescent period. The remodelling of the dopaminergic system varies between brain region and between receptor subtypes. Dopamine receptors D1, D2 and D4 in the frontal cortex and the hippocampus rise steadily to adult levels from p7 to p60 (Tarazi and Baldessarini, 2000), D1 and D2 dopamine receptors are initially overexpressed in the striatum during early adolescence which are then pruned later in adolescence (peak levels at p40 then decreasing until p80 for D2 receptors and p100 for D1 receptors) (Teicher et al., 1995, Andersen et al., 2000). In the nucleus accumbens dopamine receptor levels rise from the beginning of puberty (p28) and peak at p40, however the number of receptors does not decrease after this and remain at the same level into adulthood (Teicher et al., 1995).

1.5.4 Physical and electrophysiological changes in the rat brain during development

Longitudinal MRI studies of male Wister rats between p21 and six months characterised that cortical thickness reaches final value at one month, whilst the volume of the cortex, striatum and whole brain continue to increase up until two months of age (Mengler et al., 2014). Myelin accumulation is pronounced until three months of age, however after this

time myelination increases in cortex are still seen using histological analysis (Mengler et al., 2014).

There is a progressive increase in the density of projections between the amygdala and prefrontal cortex from the second postnatal week and p100 (Cunningham et al., 2002), maturing around the same time as dopamine receptor numbers in the prefrontal cortex.

There are also changes that have been noted in long term potentiation (LTP) in *ex-vivo* slices of different ages of rodent. It has been shown that protein kinase A is necessary for LTP before p27, and after p49 but not in between these ages (Lu et al., 2007). Furthermore, it has been demonstrated that Glu2R-lacking AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are required for LTP induction in p14 and p56, but not at p21(Lu et al., 2007). The requirement of different sub-types of of AMPA receptors in LTP is a debated topic in the field, however it has been suggested that the discrepancies in the literature may be explained by the lack of consistency in the age of animal used (McCutcheon and Marinelli, 2009). A review in 2009 highlighted these inconsistencies, with 34% of the papers studied using adults, 59% using animals less than p70, and 7% not specifying any age of animal at all (McCutcheon and Marinelli, 2009). Furthermore, the variation was also seen within tests, with 30% of experiments using animals whose ages varied by three to four weeks, often spanning puberty.

1.5.5 Contextual fear conditioning development in rats

One of the major foci of studies looking at development of memory in rats has been fear conditioning, including both cued fear conditioning and contextual fear conditioning. Cued fear conditioning involves delivering an aversive stimulus to the animal (e.g. a small electrical shock delivered through a metal grid on the floor) that is paired to a cue such as a tone or a light. Contextual fear conditioning pairs the aversive stimuli to an environmental context. By testing the freezing behaviour of the animal when presented with the cue or when placed in the context, without the aversive stimuli, it is possible to understand if the animal has been able to associate the cue or the context with the aversive stimuli and if it is able to recall the aversive stimuli event. It is well documented in the literature that contextual fear conditioning develops later (p23) than cued fear conditioning, and this in turn has led to two related theories – that the hippocampus is slow to develop (Rudy, 1993), or that the interactions between the hippocampus and other structures are slow to develop (Stanton, 2000).

In order to address this issue, in 2010 Foster and Burman utilised a fear paradigm at a number of different ages. The crucial part of this protocol was that it had three phases (Foster and Burman, 2010). Phase one introduced the rat to the context for five minutes with no shock, in phase two the rat was given a foot shock within five seconds of being placed in the context, and was then immediately removed, and finally phase three was the test phase where the rat was given the same length of time in the context as phase 1 and was not given a foot shock. Freezing behaviours

were recorded and used as a measure of fear. In order to investigate whether pups aged p17 were able to process contextual information, they tested rats in the following way – Phase one on p17, phase two on p18, phase three on p19 (17/18/19) and a second group of rats had phase one on p17, phase two on p24, phase three on p25 (17/24/25) See figure 1.8 for diagram of groups and ages.

	Group				
Age	17/18/19	17/24/25	23/24/25	-/24/25	23/30/31
17	**Pre**	**Pre**			
18	**Shock**				
19	**Test**				
20					
21					
22					
23			**Pre**		**Pre**
24		**Shock**	**Shock**	**Shock**	
25		**Test**	**Test**	**Test**	
26					
27					
28					
29					
30					**Shock**
31					**Test**

Figure 1.7 Grouping procedure for Foster and Burman's fear conditioning task. *Ages are in postnatal days, with each box representing a stage of the behavioural protocol. Pre – Five minutes of pre exposure, Shock – Immediate footshock, Test – Five minutes of context exposure in the absence of a shock.*

Group 17/18/19 had the test phase before the age at which contextual fear conditioning emerges, and group 17/24/25 had the test phase after the age at which contextual fear conditioning. The major finding of this paper was that pups aged p25 displayed fear conditioned freezing in the test phase when they had been exposed to the context on p17 but had received the foot shock on p24 (the 17/24/25 group). This freezing was equivalent to pups in older age groups of 23/30/31 and 23/24/25. Furthermore, control groups were used where the pups received no pre-exposure to the context

at any age, and were just exposed to phases two and three of the paradigm on p25 and 25 (-/24/25), and these rats demonstrated no freezing behaviour, showing that the pre-exposure to the context is required for learning and that immediate shock does not support contextual fear conditioning in the absence of contextual pre-exposure. A final control was used to assess if the p17 rats were performing the task in a hippocampus-independent manner. This experiment showed that hippocampus lesioned animals failed to exhibit freezing in phase three when tested at 17/24/25.

The authors suggest that this demonstrates that rats exposed to an environment on p17 were able to form and maintain a memory of the contextual information, but are unable to integrate this contextual information with the fear-inducing stimulus they received on p24. One can think of this as an inability to associate the fear memory with the contextual memory and they hypothesised that this demonstrates that a functional connection between the hippocampus and the amygdala has not developed until p23-24.

This paper adds to a growing body of literature that suggests that rats around p16-18 are able to encode and store contextual information but are as yet unable to retrieve that information (Gershman et al., 2010, Yap and Richardson, 2005, Yap and Richardson, 2007)

1.5.6 Development of spatial firing in the hippocampus of rats

In the adult brain, the hippocampus and entorhinal cortex are key components of the neurological network representing space and orientation. Within this hippocampal-parahippocampal circuit, there are

populations of cells which fire selectively when an animal is in a certain location or orientation. CA1 Place cells fire action potentials selectively when an animal visits a specific part of the environment (O'Keefe and Dostrovsky, 1971), an area known as the cell's place field. Grid cells are neurones in the medial entorhinal cortex which fire action potentials when a freely moving animal moves through specific small regions which are equal in size and arranged in a tessellate pattern covering the entire available environment (Fyhn et al., 2004, Hafting et al., 2005). There is also a population of cells in the medial entorhinal cortex and the adjacent pre- and parasubiculum which only fire as an animal approaches the edge of the environment, known as border cells (Solstad et al., 2008). Finally, adding to the array of spatial representative cells in the medial entorhinal context, there are a population of cells called head-direction cells, which fire when the animal are facing a particular direction (Sargolini et al., 2006).

Each of these cell types show different development in juvenile rats, with head direction cells in the pre- and parasubiculum having adult-like properties from the first navigation of the environment after eye-opening at p15 (Langston et al., 2010, Wills et al., 2010), and parasubiculum head directions cells present from p11, before eye-opening (Bjerknes et al., 2015). Border cells also show a similar development, with stable recording fields found at the first exploration of the environment (Bjerknes et al., 2014). Hippocampal place cells are also present from eye opening, but show a slight increase in number up to p35 (p16-24 compared to p25-35) (Langston et al., 2010). Although grid cells are also present at p16, they are the slowest of the spatial cell types to develop. The grid cells present

at eye opening are rudimentary and lack strict firing fields. The grid cells continue to mature until p33 at which point they meet adult like criterion (Langston et al., 2010).

1.5.7 Comparison of key time points in humans and rats

Figure 1.8 is taken from Semple et al. review paper published in 2013, which highlights some of the benchmarks of brain maturation in both humans and rodents, and compares the two species. By reviewing the literature in this way, they have demonstrated that although the time scale of development is different, the order of key events in brain maturation are largely consistent between humans and rodents. Interestingly, the physical brain development does not stop until 20 years of age in humans, and p60 in rodents, both ages at which the individual is considered “adult”.

1.6 Memory development and hippocampal changes during childhood in humans

Infantile amnesia, also known as childhood amnesia, is the phenomenon where adults have very few memories from their childhood. This is in contrast to the fact that infants can recall memories, however these memories appear to be lost over time. For example, we may have no memory of our second birthday, however we can probably remember our 21st birthday. This amnesia cannot be explained as a result of greater forgetting, as adults can recall fewer memories before the age of five as might otherwise be expected from the extrapolation of the mathematical function that describes forgetting from the age of five onwards (Eacott, 1999). Quite often adults may have a “snapshot” memory of an event such

as “I just remember riding a bicycle”, however this memory lacks contextual information or any story surrounding the memory. Other early memories that we retain may have strong emotional components, such as the birth of a sibling or the death of a relative.

Interestingly, it seems that the inability of adults to recall memories from childhood is not due to the inability to form memories as a child. In fact, children up to the age of eighteen months can form memories lasting many months (Hartshorn et al., 1998). However, the ability of children to form episodic memories is debated, and it’s thought that they cannot distinguish between semantic “knowing” something, and episodic “recalling” it. It is thought that children under the age of four years old cannot form episodic memories (Perner and Ruffman, 1995) and that this may contribute to the lack of memories that adults can recall from childhood.

One theory that has emerged in the literature that provides an explanation as to why infantile amnesia as a phenomenon exists, is based on the fact that the DG undergoes neurogenesis throughout the developmental period and right into adulthood. This in stark contrast to most brain regions, where neurogenesis is largely completed at birth (Josselyn and Frankland, 2012). It has been stated that levels of neurogenesis and memory stability are inversely related.

> *“The inability to form stable, persistent memories in early life coincides with a period of high neurogenesis, whereas the ability to form stable, persistent memories only emerges at later developmental periods as the rate of neurogenesis declines”*
>
> *(Josselyn and Frankland, 2012)*

It has been suggested that increasing neurogenesis in adults may destabilize existing hippocampus-dependent memories, a category in which episodic memories fall. Shortly after the new cells are “born” in the DG, they migrate to the granule cell layer of the DG, and around two weeks after this they begin to make functional connections with other brain regions (Zhao et al., 2006). This establishment of new synaptic connections perhaps alters the existing architecture of the DG-CA3 networks and therefore possibly leading to degradation or loss of information stored in these connections. By four weeks of age the cells are thought to be able to contribute to hippocampal memories, and have more excitable and plastic synapses than the older cells (Josselyn and Frankland, 2012) and therefore may “take over” from the other cells.

The ability to retrieve specific episodes continues to improve throughout childhood, in particular the ages at which the child is at primary school. This is based on evidence from a number of studies in which children are asked to complete tasks such as recalling associations between events and their context, or memorizing objects presented against various backgrounds (Ghetti and Bunge, 2012). The substantial improvement of children on these tasks during this “middle childhood” age could be explained by more efficient use of strategies based on semantic knowledge and organisation, improvement, however there are neural changes that occur that could underlie the development of episodic memory, as opposed to the development of supporting types of memory. The hippocampus develops rapidly in the first few years of life, with hippocampal volumes doubling in the first year of life (Gilmore et al., 2012). However, the structure of the

hippocampus continues to develop well beyond the early years of childhood, with evidence that while the overall hippocampal volume remains relatively stable, the anterior hippocampus loses mass and the posterior hippocampus gains mass between the ages of four and twenty five (Gogtay et al., 2006). It is thought that this is due to synaptic pruning in the anterior hippocampus and neurogenesis or myelination in the posterior hippocampus (Ghetti and Bunge, 2012). In a fMRI study looking at the differences in activation between anterior and posterior hippocampus during an episodic memory task, it was found that in adults there was an increase in activation of the left anterior hippocampus during an episodic memory task. Eight to eleven year olds did not show this activation, whereas fourteen year olds showed the same pattern as adults (Ghetti and Bunge, 2012), causing the authors to hypothesise that anterior hippocampus becomes functionally specialised for episodic memory around this age.

1.7 Modelling the ontogeny of episodic memory in rodents.

Animal models of behaviour are invaluable to the scientific community. They provide a valuable tool for investigating the neural basis of behaviour and disease. An animal model of memory ontogeny would be useful in understanding how memory develops during childhood, and what the neural basis is of this development. Furthermore, once it is understood how memory develops in infancy, it could possibly provide an understanding of how memory declines in non-pathological age related memory loss.

This thesis aims to develop a behavioural model of memory development in rats which can be used in a short time window to enable testing at precise time points. This behavioural protocol will utilise novel object recognition based tasks, tested at a number of ages from post weaning to adulthood. Furthermore, it will use immunohistochemistry to understand the anatomical locus of the development of episodic memory in juvenile rats.

Human	Rodent	Developmental milestones
23–32 wk gestation (pre-term infant)	pnd 1–3	Oligodendrocyte maturation state changes—pre-dominance of mitotically active pre-OLs[a]. Immune system development. Establishment of the blood-brain barrier.
36–40 wk gestation (term infant)	pnd 7–10	Peak brain growth spurt. Peak in gliogenesis. Increasing axonal and dendritic density. Oligodendrocyte maturation state changes–switch to a pre-dominance of immature OLs. Consolidation of the immune system.
2–3 year old	pnd 20–21	Brain reaches 90–95% of adult weight. Peak in synaptic density at 50% > adult levels. Peak in myelination rate. Neurotransmitter and receptor changes.
4–11 year old	pnd 25–35	Fractionation/specialization of prefrontal cortex neural networks (structural maturation). Maximum volume of grey matter and cortical thickness.
12–18 year old	pnd 35–49	Reduced synapse density, reaching a plateau at adult levels. Refinement of cognitive-dependent circuitry. Ongoing myelination; increasing white matter volume and fractional anisotrophy.
20 years +	pnd 60+	Adult levels of neurotransmitters. Adult levels of synaptic density. Ongoing myelination and declining grey matter.

[a] OL: oligodendrocyte.

Figure 1.8 Key anatomical brain development time points. Comparisons between human and rodent (Semple et al., 2013)

Chapter Two

A longitudinal study of the physical development of a litter of Lister Hooded rats.

2.1 Introduction

Within the current literature there is a lack of information about the postnatal growth of rats. Charles River Laboratories have an average growth curve for Lister Hooded rats published on their website (Figure 2.1), however this only starts from 3 weeks of age therefore pre-weaning growth is not shown.

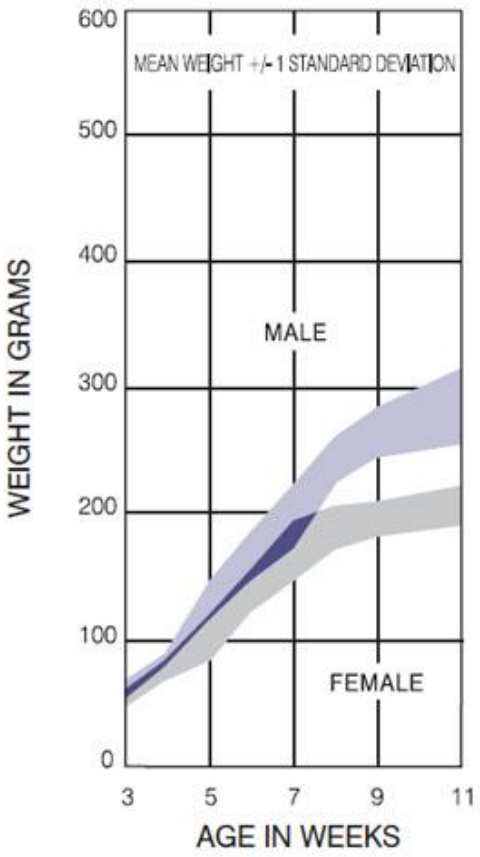

Figure 2.1 Charles River Laboratories growth curve for a Lister Hooded rat. (Charles River Laboratories)

As this thesis is focused on understanding the normal development of the Lister Hooded rat, it is imperative that the basic growth is understood. The breeding conditions used throughout all experiments within this thesis were kept constant and therefore by understanding the growth of a typical litter, it can be assumed that all litters follow the same trends. Litters were culled to

eight pups (four male and four female or as close to as possible), within 3 days of birth and all housing conditions were kept unchanged. In a substantial study (241 litters) conducted in 2009, it was found that increased litter size has a detrimental effect on pup body weight, fur development, incisor eruption and eye opening (Chahoud and Paumgartten, 2009). By strictly controlling the environment and litter size, the variability was reduced and it could be assumed that all pups were developing physically in the same way. Female rats have on average twelve teats, however some rats have only ten (AFRMA, 1998). Therefore with eight pups there will always be at least one teat available for feeding. Furthermore, for behavioural tasks conducted in this thesis, eight animals per group meant that tasks could be correctly counterbalanced across rats (see section 3.2.3 for explanation of counterbalancing).

2.2 Method

For this investigation, one litter was used that was born to a breeding pair that had had six previous litters which were all used for behavioural experiments. The litter used for this investigation received no behavioural testing. Adult rats for breeding were purchased from Charles River Laboratories, and pups were weaned from their parents at 21 postnatal days (p21). Pups were then kept in same sex groups of 4 in cages with opaque white plastic bases measuring 31cm x 50cm x 19cm (width x length x height) fitted with wire mesh lids (8cm high) bringing the total height of each cage to approximately 27cm. Rats were kept on a 12 hour light/dark cycle (light phase 0500 – 1700) at 22-24°C and 45-55% humidity. All animals had unrestricted access to food (Special Diets Services (SDS) Rat and Mouse (RM) No.1 Expanded Maintenance for all post-weaning animals, and SDS RM No.3 Breeding Expanded for breeding pairs)

and water at all times, except for the small period of time where they were being weighed. The maintenance and testing of laboratory animals complied with national (Animals [Scientific Procedures] Act, 1986) and international (European Parliament and Council Directive of 22 September 2010 [2010/63/EU]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

The litter of rats used for this particular investigation were born early morning on a Sunday, and that day was taken as p0. The litter had 11 pups in it, and on p3 the litter was culled to 8, with 4 males and 4 females kept. Every day from p1 to p60 pups were weighed using a Sartorius Practum 5101-1S weighing scale. Weighing was carried out between 0800 and 1300. Rats' tails were marked using coloured Sharpie marker pens in order to distinguish them from one another. On some days photographs and videos were taken of pups, and notes were made of any behavioural changes seen. Cages were changed once per week and always after the weighing on that day.

2.3 Results

2.3.1 Growth curve

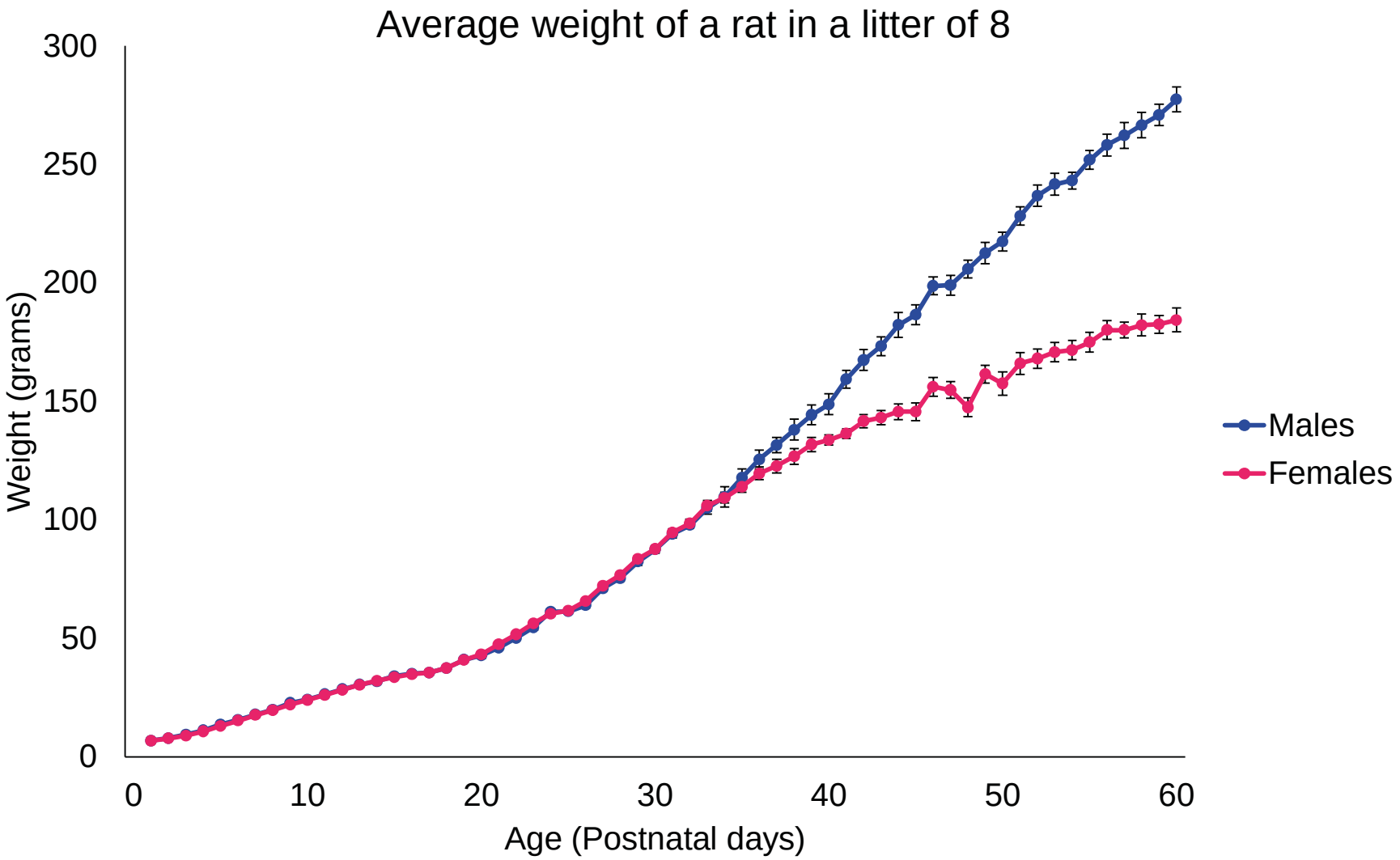

Figure 2.2 Mean weights of rats across 60 days from birth in a litter of eight rats. *Error bars represent SEM*

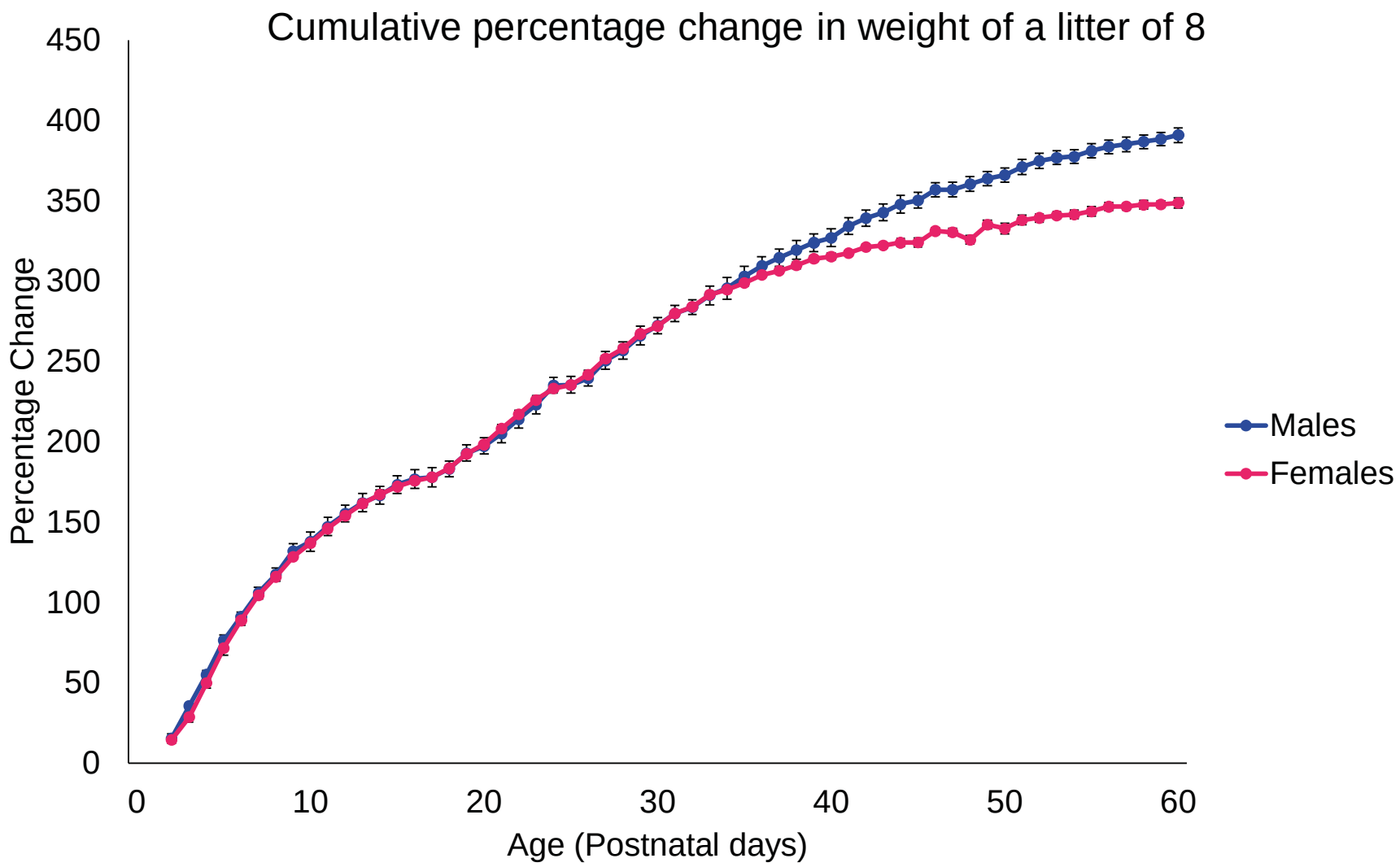

Figure 2.3 Cumulative percentage change across 60 days from birth in a litter of eight rats. *Percentage change was calculate d between each day and the previous day. Error bars represent SEM.*

2.3.2 Statistical analysis

A repeated measures ANOVA was carried out on the raw weights as shown in figure 2.2 with sex as between subjects factor and day as within subjects factor. There was a significant effect of day ($F_{(59,354)}$ = 3122.73, $p<0.05$), a significant effect of sex ($F_{(1,6)}$ = 4908.36, $p<0.05$) and a significant interaction between day and sex ($F_{(59,354)}$ =133.99, $p<0.05$).

A repeated measures ANOVA was carried out on the cumulative percentage change as shown in figure 2.3. with sex as between subjects factor and day as within subjects factor. There effect of day was approaching significance ($F_{(58,348)}$ =1.360, $p = 0.051$), no significant effect of sex ($F_{(1,6)}$ = 1.398, $p>0.05$) and an interaction between day and sex approaching significance ($F_{(58,348)}$ = 1.359, $p = 0.051$).

2.3.3 Photographic documentation of growth

p1

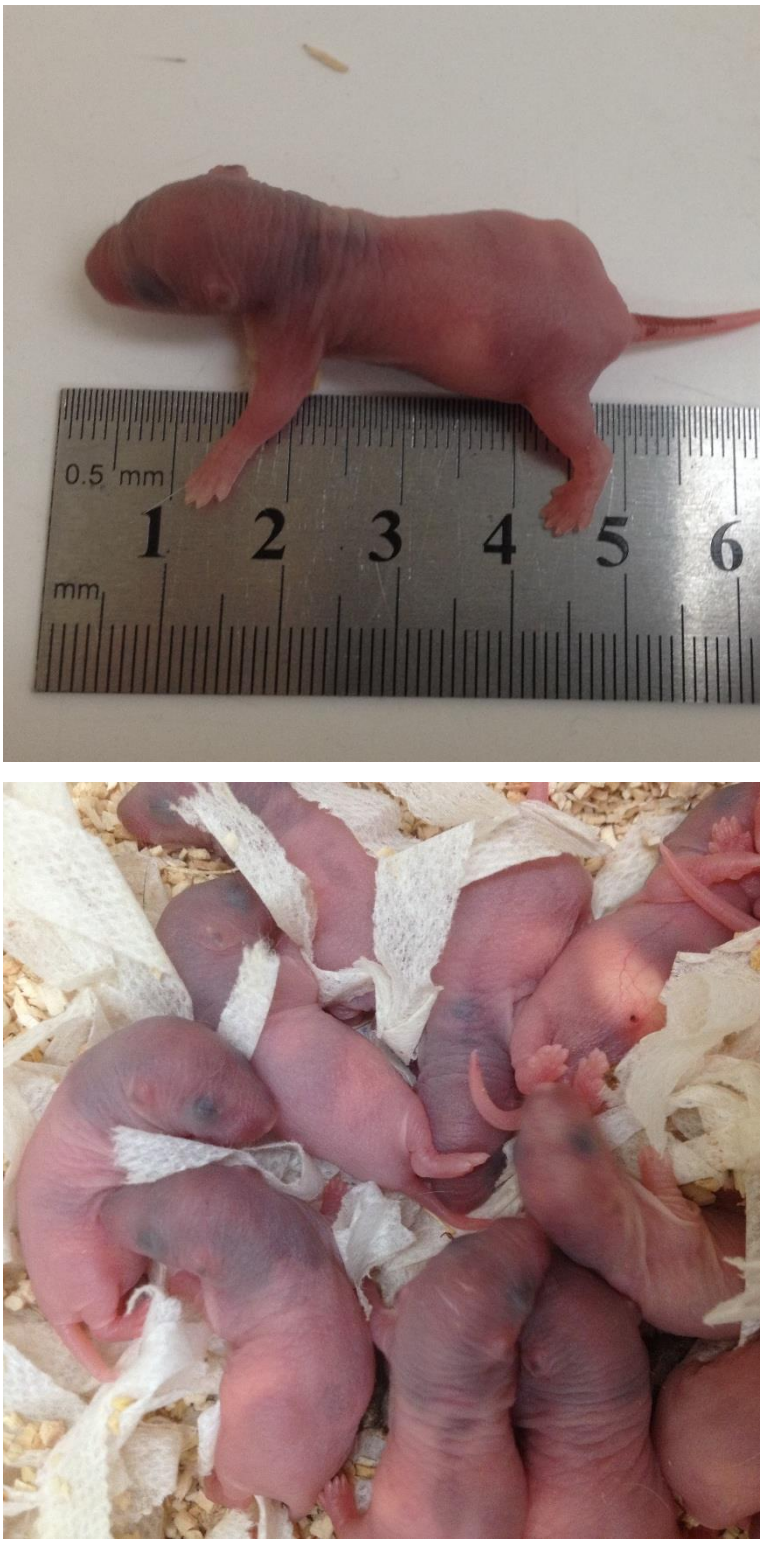

Figure 2.4 Lister hooded rats aged p1. *Eyes and ears are closed. The skin is translucent and milk can be seen in the stomach.*

p4

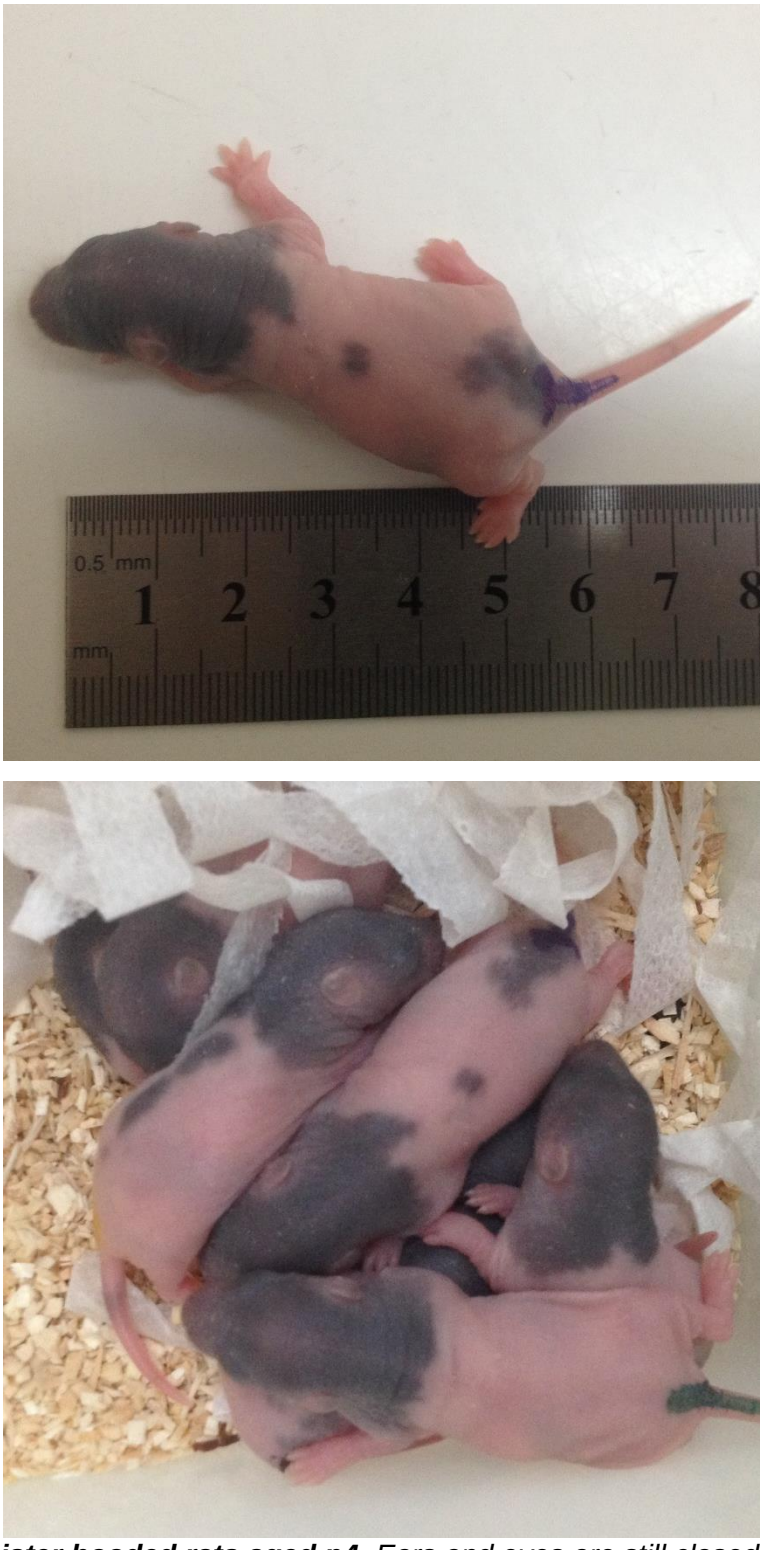

Figure 2.5 Lister hooded rats aged p4. *Ears and eyes are still closed however the skin has thickened and the black markings are now visible.*

p7

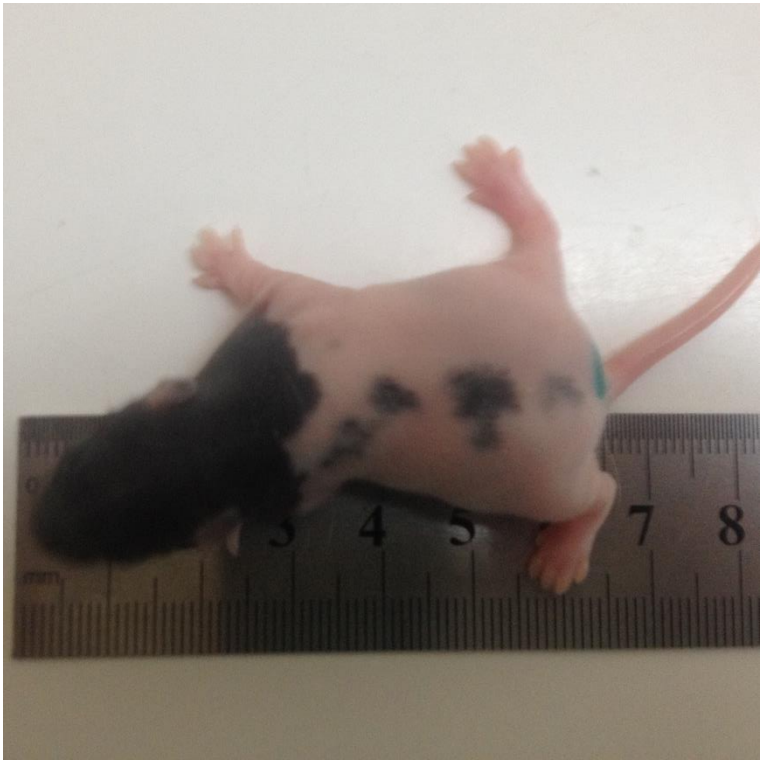

Figure 2.6 Lister hooded rat aged p7*. The rats have gained weight and grown in length, the ears have started to open.*

p9

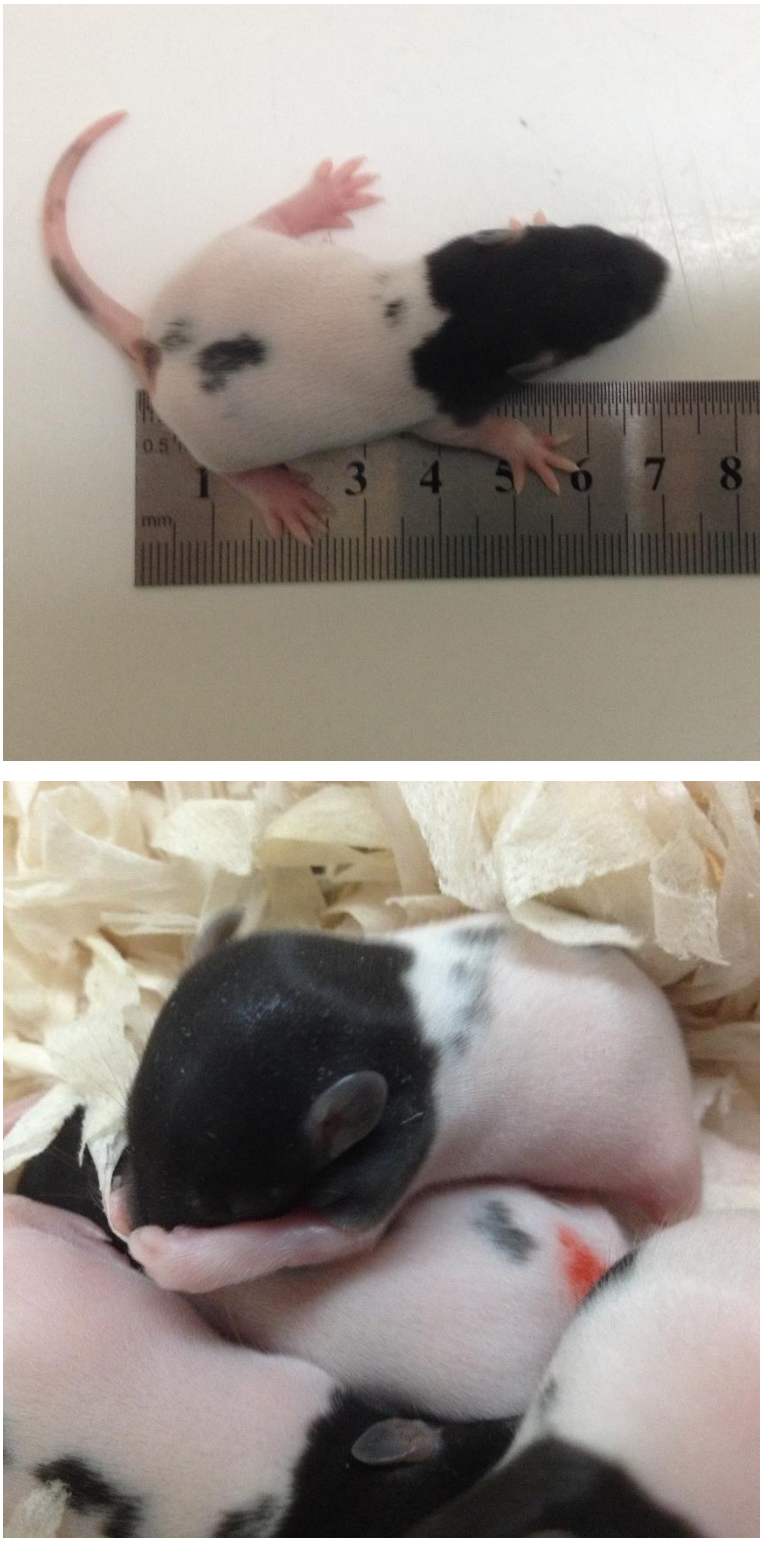

Figure 2.7 Lister hooded rat aged p9. *Fur has now grown considerably, ears are fully open and the rats are starting to show grooming-like behaviours.*

p11

Figure 2.8 Lister hooded rat aged p11. *Considerable increase in length and density of the fur.*

p15

Figure 2.9 Lister hooded rat aged p15. *Rats eyes are now open.*

p16

Figure 2.10 Lister hooded rats aged p16. *Rats are now eating solid food.*

p20

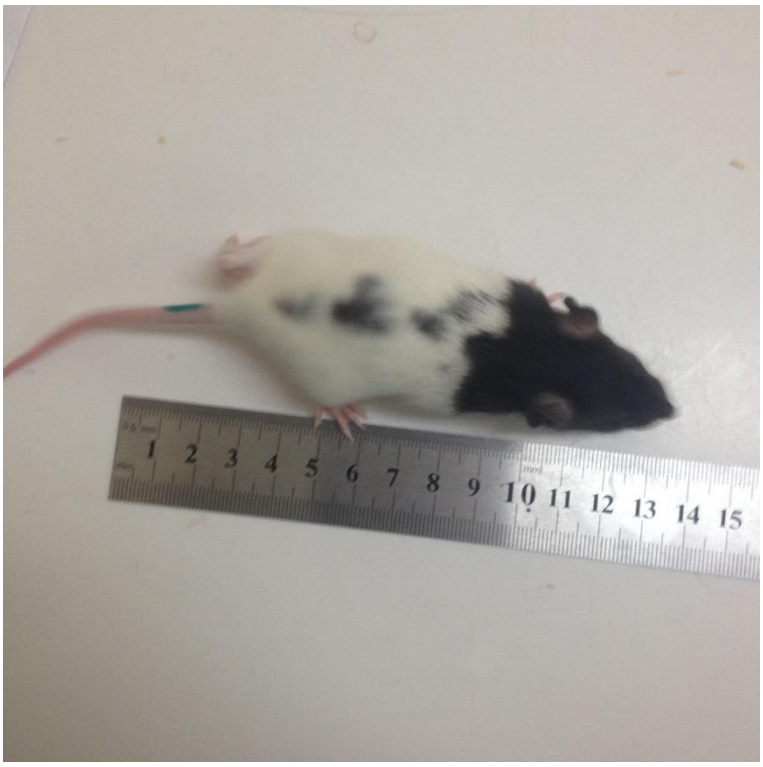

Figure 2.11 Lister hooded rat aged p20. *Rats are now ready to be weaned and have grown to 12cm in length (without tail).*

2.3.4 Behavioural development

Throughout the investigation, the pups developed a range of different behaviours. On p8 the pups were starting to try to walk. This was noticeable when they were placed onto the weighing scales or next to the ruler for photographing. On p9 the pups showed some grooming-like behaviours and were using their front paws to wipe their faces. On p14 the pups were weighed at 0950 and it was noted that the eyes were still closed, whereas at 0845 on p15 the eyes were open. As soon as the eyes were open the pups were actively exploring the cage, chewing bedding and showing an interest in food pellets. On p16 the pups were observed eating food pellets.

2.4 Discussion

This investigation has successfully tracked and documented the development of a litter of Lister Hooded rats from birth to p60.It has shown that at around p40 the growth curve for the males starts to become increasingly different from the females' growth curve. This is the age at which female rats have their first oestrus cycle (Gabriel et al., 1992) therefore this may be an influential factor in their growth and may prove to be an age at which cognitive changes are seen. Furthermore there is no effect of cage changing on the growth of the animals, as shown by the fact that there are not consistent decreases or increases in the growth curve after the cage changing event.

One initial difficulty that had to be overcome was recognising which rat was which. The rate of growth, combined with the mother's grooming meant that the coloured marker came off between weighing sessions. This only posed a problem until around p7 when the pups had a small amount of fur which held

onto the colour. From p1 it could be distinguished which pups were male and which were female, therefore even if individual rats were indistinguishable, each pup remained in its sex group throughout the investigation. Furthermore, 4 of the pups had very distinct markings which made them easy to distinguish from their littermates.

Pups were actively attempting to walk before their eyes were open. It has been documented in the literature that Parahippocampal head direction cells are present at p11 (although they've not been investigated before this age) (Bjerknes et al., 2015, Tan et al., 2015). The pups have a form of a spatial map at p11, and although it's not adult-like in its stability, the directional tuning stabilized after eye opening (Bjerknes et al., 2015, Tan et al., 2015). In this study the pups were demonstrating an attempt to walk at p8, three days earlier than the current published work on head direction cell development. It would therefore be interesting to investigate whether the head direction cells were present even earlier, however this may prove difficult due to the nature of the surgery required to record from the cells.

The righting reflex of rats placed supine on a flat surface is present from birth, whereas righting when falling in the air from a supine position emerges at the end of the first postnatal week, and is not mature until the end of the third week (Pellis and Pellis, 1994). The directional sensitivity of head direction cells is abolished when the vestibular input is lesioned (through injection of sodium arsanilate into the inner ear), suggesting that vestibular inputs are involved in the normal function of head direction cells (Stackman and Taube, 1997). When righting from lying there are vestibular inputs and proprioceptive-tactile information, whereas when righting from falling there is only vestibular

information available, providing the eyes are still closed and therefore there is no visual information available (Pellis and Pellis, 1994). The fact that rats have a righting reflex from falling, albeit an immature one, from p7 suggests that the vestibular input to the brain is present, and therefore may be providing information to the head direction cells. This provides a further indication that head direction cells may be present before the current literature has investigated.

This investigation has provided a comprehensive overview of the growth and physical development of a litter of Lister Hooded rat pups. As the breeding and postnatal housing conditions were kept constant throughout all experiments, it is assumed that all rat pups used in this thesis follow the same pattern of growth and development.

Chapter 3

Developing a novel protocol in adult rats to test memory subtypes over a shortened time period.

3.1 Introduction

As discussed in chapter one, Eacott and Norman's object place context (OPC) task is a valuable model of episodic memory that can be used in rats. It is based on the novel object recognition (NOR) task, which can be manipulated in a number of ways to assess different memory types. Episodic memory can be thought of as what happened, where it happened and on which occasion or in which context. NOR models the "what" component of episodic memory and OPC models the "what, where, which", however the NOR task can be manipulated to provide two more tasks which model the "where" component of episodic memory, and the "which" component of episodic memory. These tasks, object place (OP) for "where" and object context (OC) for "which" are valuable as they model spatial and contextual memory respectively.

A number of other episodic memory tasks for rats were discussed in chapter one, however all the tasks used extensive habituation and training, even the spontaneous detection tasks. The four tasks described here (NOR, OP, OC and OPC), have been used together in previous studies (Langston and Wood, 2010) where there was eight days of habituation (four to the empty arena and four to object locations) followed by a three day break, four days of NOR, four days of OP, four days of OC and four days of OPC. However there is not currently a protocol in the literature that uses all four of these tasks in a short enough timescale to investigate development of rats. Chapter two provided evidence that developmental changes in rats occur over a timescale of days, rather than weeks and months like humans. Therefore any development in behavioural tasks are hypothesised to develop over the course of days, and a protocol like Langston and Wood would be unsuitable to detect these changes.

The aim of this investigation was to design a protocol where all four tasks could be completed in a short period of time, with each task only completed once with a vision to utilising it in juvenile rats to test the development of these tasks. The protocol first had to be tested in adult rats to see if adult rats could perform well in these tasks when they are only tested once.

3.2 Methods

3.2.1 Subjects

Eight male and eight female Lister hooded rats, bred in house, were used at 6 months old. Rats were housed in same sex groups of at least two in cages with opaque white plastic bases measuring 31cm x 50cm x 19cm (width x length x height) fitted with wire mesh lids (8cm high) bringing the total height of each cage to approximately 27cm. Rats were kept on a 12 hour light/dark cycle (light phase 0500 – 1700) at 22-24°C and 45-55% humidity, with behavioural testing carried out in the light phase. All animals had unrestricted access to food (Special Diets Services (SDS) Rat and Mouse (RM) No.1 Expanded Maintenance for all post-weaning animals) and water at all times, except for the small periods of time when they were being behaviourally tested (10-15 minutes, twice per day). The maintenance and testing of laboratory animals complied with national (Animals [Scientific Procedures] Act, 1986) and international (European Parliament and Council Directive of 22 September 2010 [2010/63/EU]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

3.2.2 Apparatus

Testing was carried out within a 40cm x 58cm rectangular arena with 47cm high walls. The arena could be configured to make two different contexts. Context 1 had blue walls with a rubber mat floor, whereas context two had white and black walls with a plastic grid placed on lino tiles for the floor (see figure 3.1 for photos of the contexts). All walls and floors were made of plastic which could be easily wiped clean. The arena sat 65cm above the ground in the corner of the room (see Figure 3.2 for the layout of the experimental room), with prominent extra-maze cues placed in the Northwest and Northeast corners of the arena, suspended 40cm above the arena floor. These cues were constantly present irrespective of the contextual configuration of the arena.

Objects used were easily cleanable household objects made of plastic, metal, ceramic or glass and varied in size from 12cm x 3cm x 3cm to 16cm x 13cm x 12cm. They were fixed to the floor of the arena using 3M Dual Lock™ Velcro located centrally within the north-east and north-west quadrants. Exploration was assessed via an overhead video camera linked to a recorder and monitor. Between testing, all areas of the arena and all objects were wiped clean using Citroxx Bio surface wipes

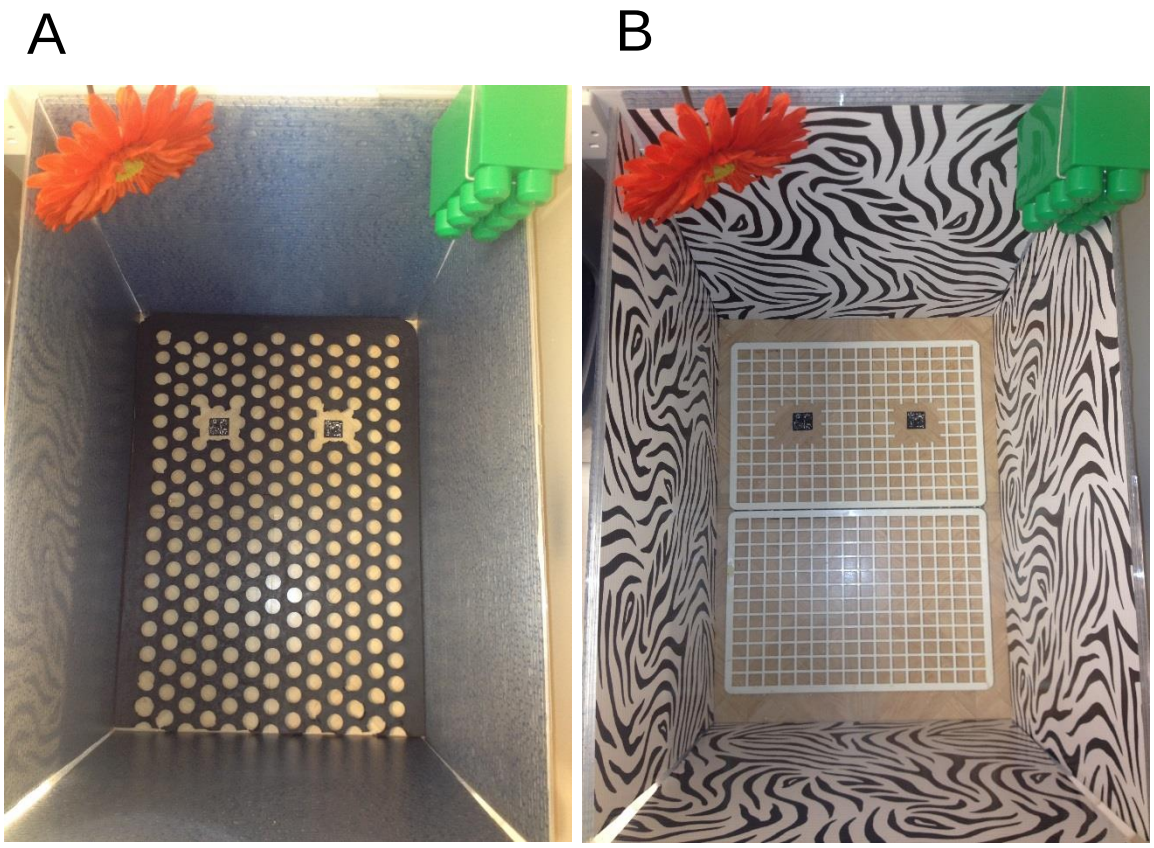

Figure 3.1 Photographs of the testing arena. *Context 1 (A) has blue walls with a rubber mat floor, context 2 (B) has black and white walls with white plastic grid floor.*

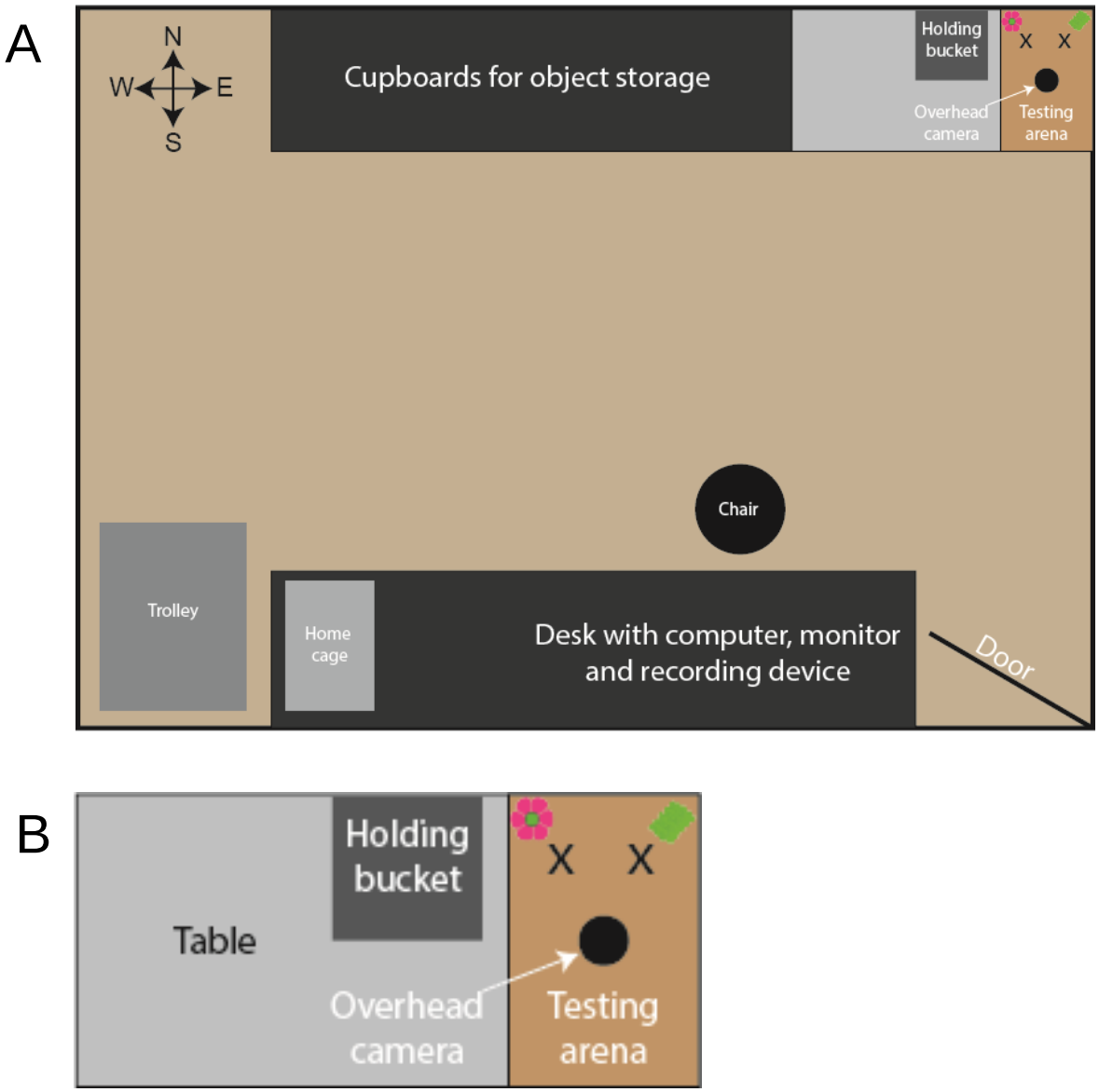

Figure 3.2 Schematic showing the layout of the experimental room from above. *A - The testing arena is shown in the Northeast corner of the room, with the two extramaze cues illustrated as a flower and green lego block. The trolley used to transport the animals, along with the home cage of the animals undergoing testing, was placed in the corner furthest from the testing arena.*
B - A close up view of the Northeast corner of the testing room where the testing arena and holding bucket are located. The crosses represent object location left and right as viewed from above.

3.2.3 Behavioural Testing

Rats were handled for 3 minutes, once per day for five days prior to testing. Handling was carried out in the room where the testing would be. Rats were transported there from their home room in a fully covered trolley. Once in the room the home cage was always placed in the corner of the room furthest from the testing arena. Rats were then habituated to the testing arena twice a day for two days. On the first habituation day they were given 10 minutes in each context in their cage groups in the morning. In the afternoon they were given 3 minutes in each context individually. Between context changes rats were placed in an opaque holding box (54cm x 22cm x 23cm) which had a small amount of standard bedding in the bottom and a lid with ventilation holes. On the second day of habituation, they were given 3 minutes in each context in the morning and afternoon with unique novel objects in the locations where objects would be presented during testing. During this habituation day, objects were changed between contexts and between the morning and afternoon sessions, familiarising the rat with the fact that objects will change between phases. Each object was only presented once during habituation and did not appear at any later stage of testing. Care was taken to always ensure that the rat was placed in the arena facing the south wall, away from the objects, as would happen in the testing. This was to ensure that the spatial information was processed in an egocentric manner. Spatial processing can be either allocentric or egocentric. Allocentric refers to the encoding of information about the location of objects with respect to other objects or landmarks, whereas egocentric refers to the encoding of information about the location of objects with repect to the self. Egocentric spatial memory

is not dependent on the hippocampus, whereas allocentric spatial memory is dependent on the hippocampus due to the need to create a “map” (Langston and Wood, 2010).

On the two testing days, animals were taken to the testing room in the same way and at the same time of day as the habituation days. On day one NOR was carried out in the morning and OC in the afternoon with day two consisting of OP in the morning and OPC in the afternoon. See Figure 3.3 for an explanation of each task.

On a given trial the rat to be tested was removed from the home cage and placed in the holding box. The testing arena walls and flooring along with the objects to be used were cleaned using Citroxx bio wipes, and then the arena was set up in the appropriate configuration. The rat was placed in the arena from the south side, facing the south wall. Both sample and test phases were 3 minutes long, and were filmed using an overhead camera connected to a monitor and recording device at the opposite side of the room to the testing arena. Exploration was timed using a simple timer programme on a computer, with the observer pressing one button for each object to indicate the start and end of exploration. Exploration was defined as sniffing, chewing or climbing on the object, however sitting on, or next to an object without any signs of active exploration (whisking or nose movement) was not included. Between the phases the rat was placed in the holding bucket while the arena was cleaned and reconfigured for the next phase. After the test phase the rat was returned to its home cage and after all rats had been tested the home cages were returned to the housing room using the covered trolley.

	Day 1	Day 2	Day 3	Day 4
Am	**Habituation to empty arena in cage groups (10 minutes in each context)**	**Habituation to object locations individually (3 minutes in each context)**	**Novel object recognition (NOR) task (3 minutes per phase, 1 minute inter-trial interval)**	**Object place (OP) task (3 minutes per phase, 1 minute inter-trial interval)**
pm	**Habituation to empty arena individually (3 minutes in each context)**	**Habituation to object locations individually (3 minutes in each context)**	**Object context (OC) task (3 minutes per phase, 1 minute inter-trial interval)**	**Object place context (OPC) task (3 minutes per phase, 1 minute inter-trial interval)**

Table 3.1 Timetable of testing. *After five days of testing each rat goes through two days of habituation (Days one and two) and then two days of testing (Days three and four)*

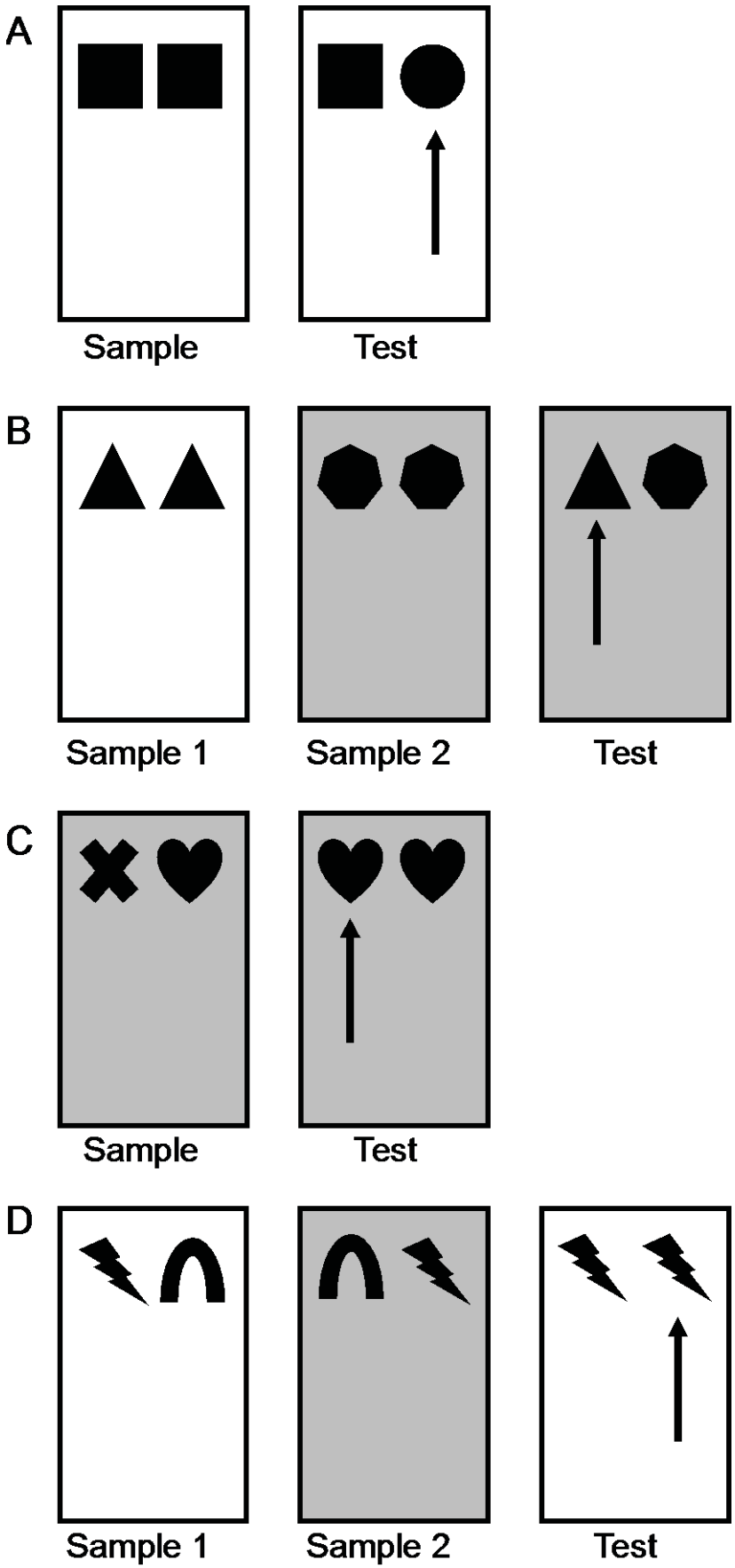

Figure 3.3
Diagrams showing the configuration of the four tasks used.

For all tasks, each phase was three minutes long and Inter-trial interval between sample and test phase was one minute. Black arrows indicate the object in a novel configuration

A - Novel Object Recognition (NOR) task. The sample phase consisted of two identical objects and the test phase had a single copy of the sample objects, and one object which had not been previously encountered.

B – Object Context (OC) task. The first sample phase has two identical objects in one of two contexts, the second sample phase has two different, but identical to each other, in the opposite context. The test phase is in one of the two contexts, with one copy of each of the previously seen objects. Both objects have been previously encountered but only one will have been seen in that particular context.

C – Object Place (OP) task. Two different objects are presented in the sample phase, with two identical copies of one presented in the test phase. Although the object is familiar, the position within the box (left or right) is novel for that object.

D – Object Place Context (OPC). Two different objects are presented in one context, then then again in the other context, but in the opposite position to the first sample phase. In the test phase, two identical copies of one of the objects are presented in one of the contexts. One object is in a novel configuration with regard to the location and context combination in which it has previously been seen.

3.2.4 Counterbalancing

All rats saw the same pair of objects in each task, and never saw the same object in more than one task. However in order to control for any overall bias towards particular objects and locations, within task and within individual rats the object identity, object location, and context were counterbalanced.

For NOR and OP there were three factors that may influence the rats behaviour – the context (one or two), the object identity (A or B) and the position in which the novel object was presented (left or right). In order to minimise the effect of any innate preference for object, context or place, half the rats carried out the test in context 1, and half in context 2. Within each of these two groups, half the rats saw object A as the familiar object, and B as the novel object, with the other half of the rats seeing B as the familiar and A as the novel. Within each half of *these* groups, half saw the novel object on the right and half saw the novel object on the left.

For OC and OPC the counterbalancing had an extra factor; the order in which the contexts were presented. For example, two rats partaking in the OC task may have the following set up.

Rat 1 – Sample phase 1 in context 1 with two copies of object A, sample phase 2 in context 2 with two copies of object B, test phase in context 1 with one copy of A and one copy of B.

Rat 2 - Sample phase 1 in context 1 with two copies of object A, sample phase 2 in context 2 with two copies of object B, test phase in context 2 with one copy of A and one copy of B.

For rat 1, the “novel” object in the test phase is object B, however this object was seen more recently (in sample phase 2) than the familiar object. Therefore if it did not prefer object B, the novel object, it would be difficult to interpret whether that was because it didn’t recognise that it had not seen object B in context 1 before, or whether it preferred object A due to the fact that it hadn’t seen it since sample phase 1.

For rat 2, the “novel” object in the test phase is object A. This version of the task could be considered “easier” than the version that rat 1 had, due to the fact that the novel object is also the least recently encountered object, having been seen in sample phase 1 but not 2. Therefore if the rat explored the novel object more than the familiar object, it would be difficult to interpret whether or not the rat was recognising that it had not seen the object in that context before, or whether it was just more interested in it because a longer time had passed since it last saw it (Kart-Teke et al., 2006).

In order to control for these factors, the order of contexts was counterbalanced over the population of rats, as well as the object identity, object location, and test phase context. Therefore an equal number of rats had the novel object as the most recently encountered object, compared to the number of rats that had the familiar object as the most recently encountered object.

For the OPC task, the recency of the objects in the test phase was not a factor to be considered as two identical objects were presented in the test phase. However a rat which had the same context in sample phase 2 and the test phase, arguably had an “easier” test than a rat which had the test phase context as that which was seen in context 1 as it need not even remember

sample phase 1 in order to “solve” the task. Therefore, the order in which the contexts were presented was counterbalanced across the population of rats used, along with the object identity, object location, and test phase context.

The final thing to consider with regard to counterbalancing is the within rat counterbalancing. Rats will spontaneously alternate if given the choice between two arms on a T-maze (Lalonde, 2002) therefore between tasks the novel object should not be presented on the left, then right, then left, then right in the test phase. Furthermore the novel object should not always be the same side across trials. Therefore rats saw the novel object in the order of left, right, right, left or right, left, left right across the four tasks.

Tables 3.1 – 3.4 show examples of the counterbalancing, where letters a-h represent objects.

Counterbalancing Tables

NOR		Sample phase		Test phase	
Rat	Context	Left	Right	Left	Right
1	1	a	a	b	a
2	1	b	b	a	b
3	2	b	b	a	b
4	2	a	a	b	a
5	2	a	a	a	b
6	2	b	b	b	a
7	1	b	b	b	a
8	1	a	a	a	b

Table 3.2 Counterbalancing for the NOR task. *Object identities are represented by the letters a and b*

OC	Sample phase 1			Sample phase 2			Test phase		
Rat	Context	Left	Right	Context	Left	Right	Context	Left	Right
1	1	c	c	2	d	d	1	c	d
2	2	c	c	1	d	d	1	d	c
3	2	d	d	1	c	c	2	d	c
4	1	d	d	2	c	c	2	c	d
5	2	c	c	1	d	d	2	d	c
6	1	c	c	2	d	d	2	c	d
7	1	d	d	2	c	c	1	c	d
8	2	d	d	1	c	c	1	d	c

Table 3.3 Counterbalancing for the OC task. *Object identities are represented by the letters c and d*

OP		Sample phase		Test phase	
Rat	Context	Left	Right	Left	Right
1	2	e	f	e	e
2	2	f	e	f	f
3	1	f	e	f	f
4	1	e	f	e	e
5	1	f	e	e	e
6	1	e	f	f	f
7	2	e	f	f	f
8	2	f	e	e	e

Table 3.4 Counterbalancing for the OP task. *Object identities are represented by the letters e and f*

OPC	Sample phase 1			Sample phase 2			Test phase		
Rat	Context	Left	Right	Context	Left	Right	Context	Left	Right
1	1	g	h	2	h	g	2	g	g
2	2	g	h	1	h	g	2	h	h
3	2	h	g	1	g	h	1	h	h
4	1	h	g	2	g	h	1	g	g
5	2	h	g	1	g	h	1	g	g
6	1	h	g	2	g	h	1	h	h
7	1	g	h	2	h	g	2	h	h
8	2	g	h	1	h	g	2	g	g

Table 3.5 Counterbalancing for the OPC task. *Object identities are represented by the letters g and h*

3.2.5 Data analysis

All phases of every task were recorded using an overhead camera and exploration times at each object recorded (see section 3.2.3 for explanation of exploration). 10% of all videos were blind scored by an independent observer to confirm that no investigator bias was present. For the test phase of each task, each animal's raw scores were converted into a discrimination index (DI) using the following equation.

$$\boldsymbol{DI} = \frac{(\boldsymbol{exploration\ at\ novel\ object\ -\ exploration\ at\ familiar\ object})}{(\boldsymbol{exploration\ at\ novel\ object} + \boldsymbol{\ exploration\ at\ familiar\ object})}$$

When a mean was calculated, standard error of the mean (SEM) was also calculated as follows where

σ = standard deviation
n = number of observations

$$SEM\ \frac{\sigma}{\sqrt{n}}$$

Statistical analysis was carried out in SPSS or R.

3.3 Results

3.3.1 Discrimination index for each task

A repeated measures ANOVA on the data shown in figure 3.4 with task as the within subject factor showed a significant effect of task ($F_{(3,42)} = 4.803$, $p < 0.05$) On average rats were able to perform all four tasks at a level significantly above chance; (NOR - $t(15) = 10.478$, $p < 0.05$, OC - $t(15) = 3.517$, $p < 0.05$, OP - $t(15) = 5.725$, $p < 0.05$, OPC - $t(15) = 3.389$, $p < 0.05$) with the highest performance shown on the NOR task. OC showed the lowest performance with a DI score of 0.2, however this was still significantly above chance. These scores remained significant when Bonferonni corrections for multiple comparions were applied which lowered the p value required for significance to 0.0125.

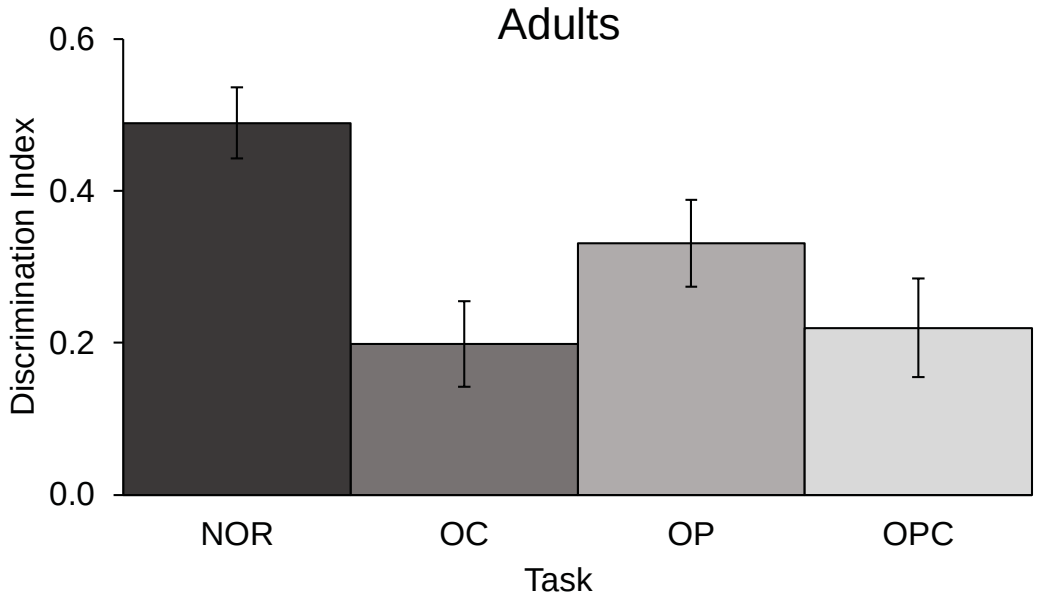

Figure 3.4. Mean adult rat performance on the four tasks performed over two days. *Adult rats (n=16) could perform significantly above chance on all four tasks. Rats had the highest score on novel object recognition (NOR) task with all other tasks also having a score significantly above chance*
Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).

3.3.2 Effect of sex on discrimination index

A repeated measures ANOVA was conducted on the data shown in figure 3.5 task as the within subjects factor and sex as the between subject factor. There was a significant effect of task ($F_{(3,42)} = 4.651$, $p < 0.05$), a significant effect of sex ($F_{(1,14)} = 235.677$, $p < 0.05$) but no significant interaction between task and gender ($F_{(3,42)} = 0.524$, $p > 0.05$).

A one way ANOVA was conducted for each task with sex as the between subject factor. There was no significant effect of sex on any task (NOR - $F_{(1,14)} = 0.565$, $p > 0.05$, OC - $F_{(1,14)} = 0.839$, $p > 0.05$, OP - $F_{(1,14)} = 0.214$, $p > 0.05$, OPC - $F_{(1,14)} = 1.470$, $p > 0.05$)

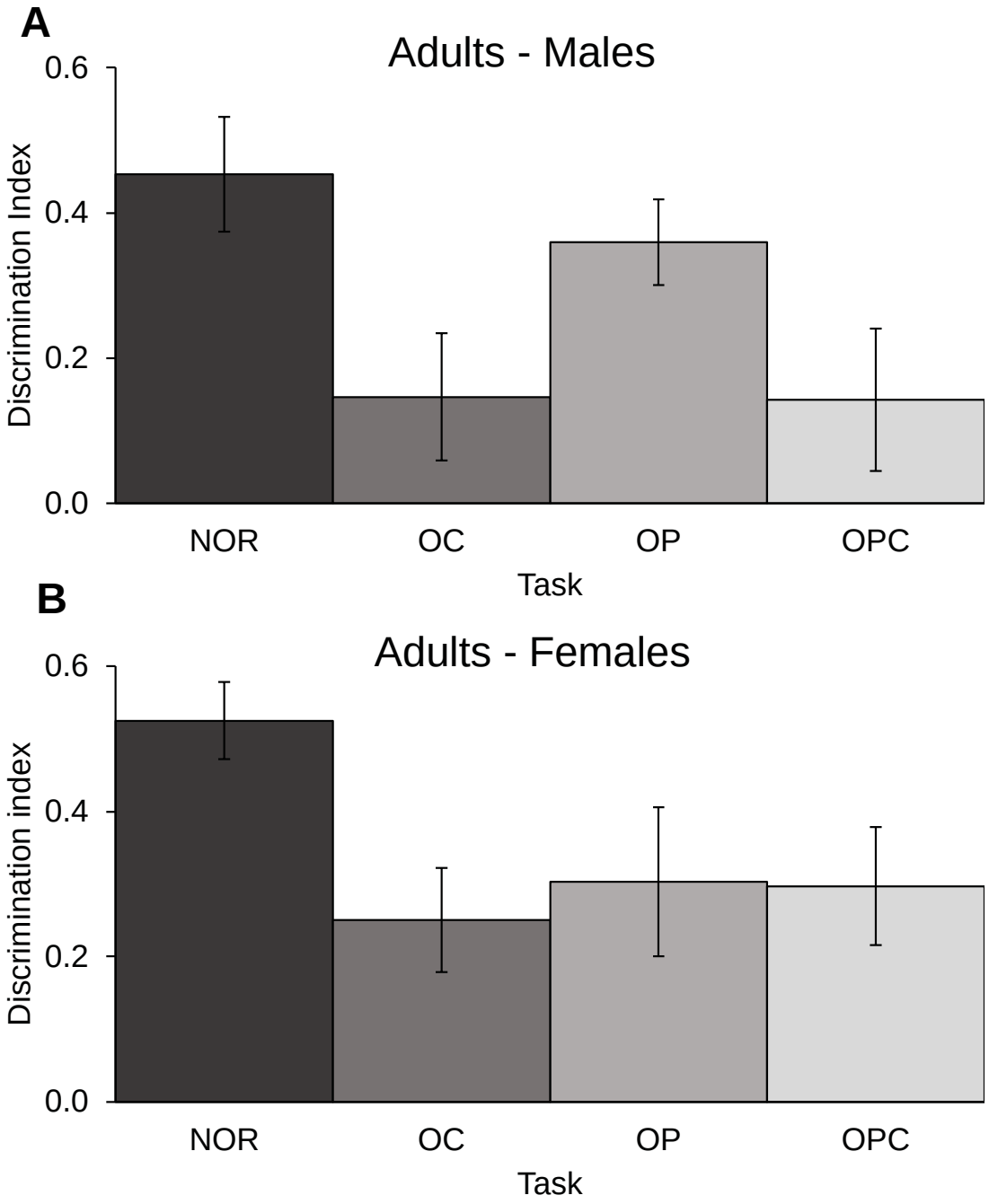

Figure 3.5 Discrimination index on four tasks performed over two days in adult rats, split into males (A) and females (B).
A repeated measures ANOVA showed a significant effect of task and no interaction between task and age
Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).

3.3.3 Effect of counterbalancing on discrimination index

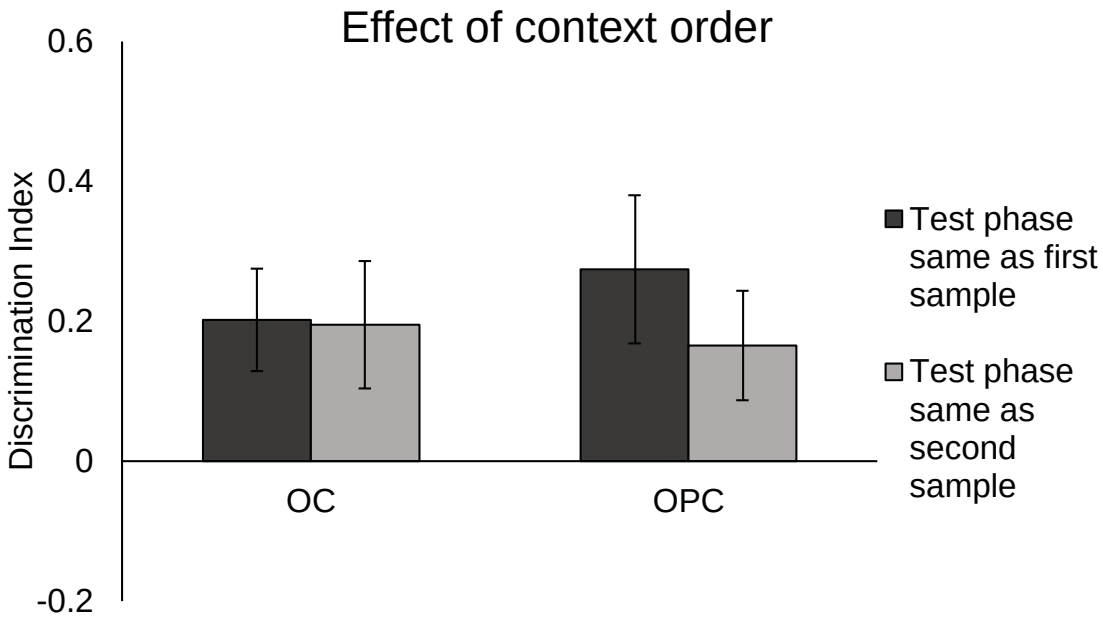

Figure 3.6 The effect of context order on OC and OPC tasks. *There was no effect of context order on performance as measured by discrimination index in either the object context (OC) or object place context (OPC) tasks.*

A one way ANOVA was conducted on the data shown in figure 3.6 to understand the effect of context order on DI. There was no significant difference between rats whose test phase was the same as the first sample phase and rats whose test phase was the same as the second sample phase, in neither OC ($F_{(1,14)} = 0.004$, $p > 0.05$) nor OPC ($F_{(1,14)} = 0.691$, $p > 0.05$).

3.3.4 Average exploration

In order to understand if the exploration levels of the rats changes across the tasks, total average exploration was investigated. The sum of exploration at both objects in each phase was generated, before an average was taken of all phases and this can been seen in figure 3.7.

A repeated measures ANOVA showed a significant effect of task ($F_{(3,45)}$ = 12.841, p <0.001) with bonferonni posthoc tests showing a significant difference between NOR and OP (p < 0.001), OC and OP (p < 0.01) and OPC and OP (p < 0.001).

Exploration in the sample phase alone was also investigated by using the total exploration at both objects in the sample phase. In OC and OPC where there are two sample phases, an average was taken of all the sample phases across all rats (32 sample phases across 16 rats).

A repeated measures ANOVA showed a significant effect of task ($F_{(3,45)}$ = 13.296, p <0.001) with bonferonni posthoc tests showing a significant difference between NOR and OP (p = 0.01), NOR and OPC (p<0.05), OC and OP (p < 0.01) and OPC and OP (p < 0.001).

There was no significant correlation between sample phase exploration and performance in any of the tasks. Correlation was tested by a 2-tailed pearson product correlation test in SPSS; NOR - r = -0.017, p >0.05, OC sample phase 1 - r = -0.353, p >0.05, OC sample phase 2 - r = -0.270, p >0.05, OC mean of both sample phases - r = -0.096, p >0.05, OP- r = 0.136, p >0.05, OPC sample phase 1 - r = -0.171, p >0.05, OPC sample phase 2 - r = -0.301, p >0.05, OPC mean of both sample phases - r = -0.331, p >0.05.

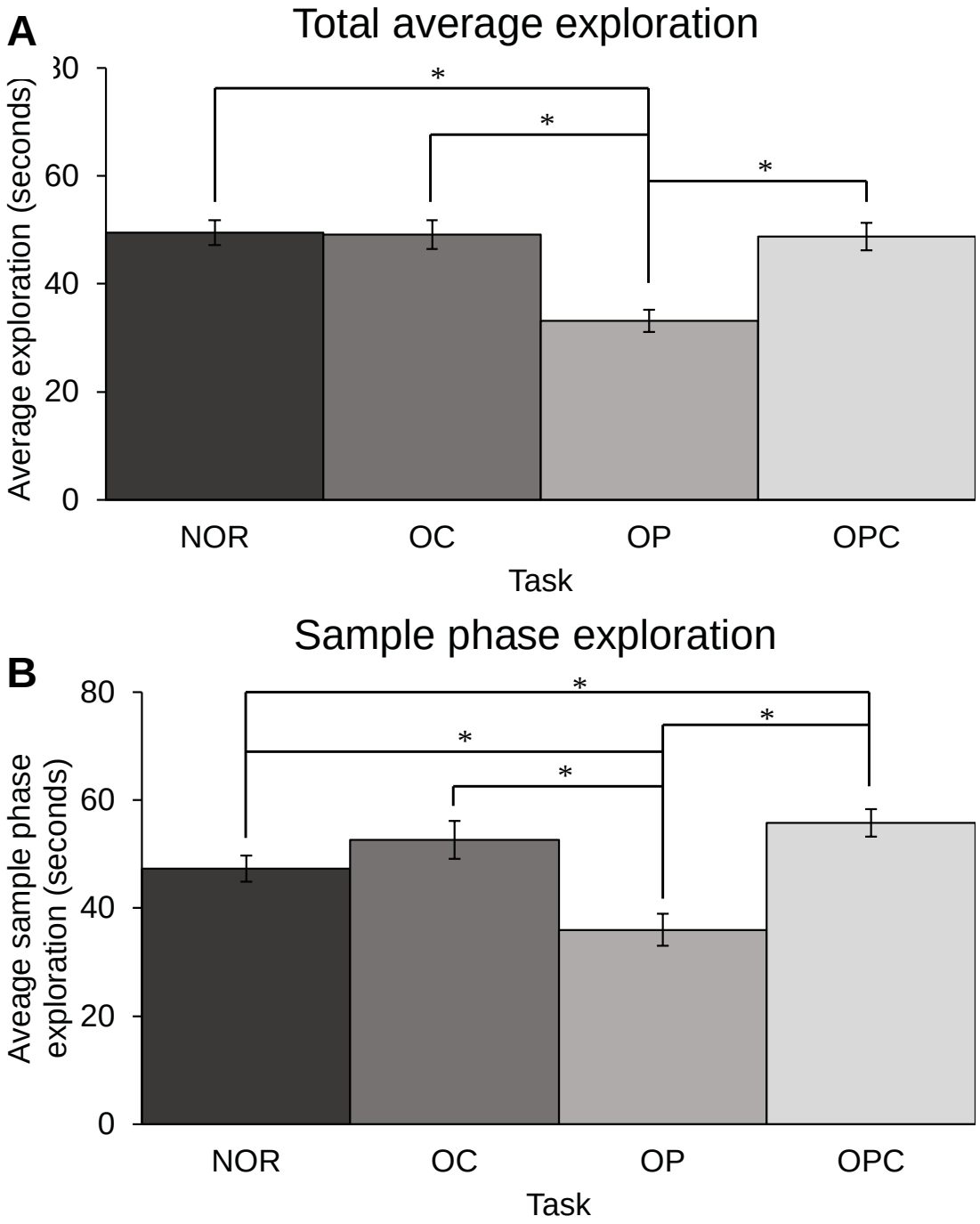

Figure 3.7 Exploration activity of adult rats in four tasks over two days.
Stars denote significance with at least a 95% confidence interval.
A – Total average exploration across all phases of the test show that rats explored significantly less in OP task than in the other three tasks.
B – In sample phase alone exploration was significantly less in OP compared to all other tests, and in NOR compared to OPC.
Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).

3.3.5 Effect of sex on exploration time

To assess the relationship between sex and total exploration time, a repeated measures ANOVA with task as within subject factor and sex as between subjects factor was performed on the data shown in figure 3.8. This showed a significant effect of task ($F_{(3)}$ = 12.398, P <0.01), no effect of sex ($F_{(1)}$=2.327, P > 0.05) and no interaction between task and sex ($F_{(1,3)}$ = 0.483, P >0.05).

To assess the relationship between sex and sample phase exploration time, a repeated measures ANOVA with task as within subject factor and sex as between subjects factor was performed on the data shown in figure 3.9. This showed a significant effect of task ($F_{(3)}$ = 13.025, P <0.01), no effect of sex ($F_{(1)}$=3.036, P > 0.05) and no interaction between task and sex ($F_{(1,3)}$ = 0.694, P >0.05).

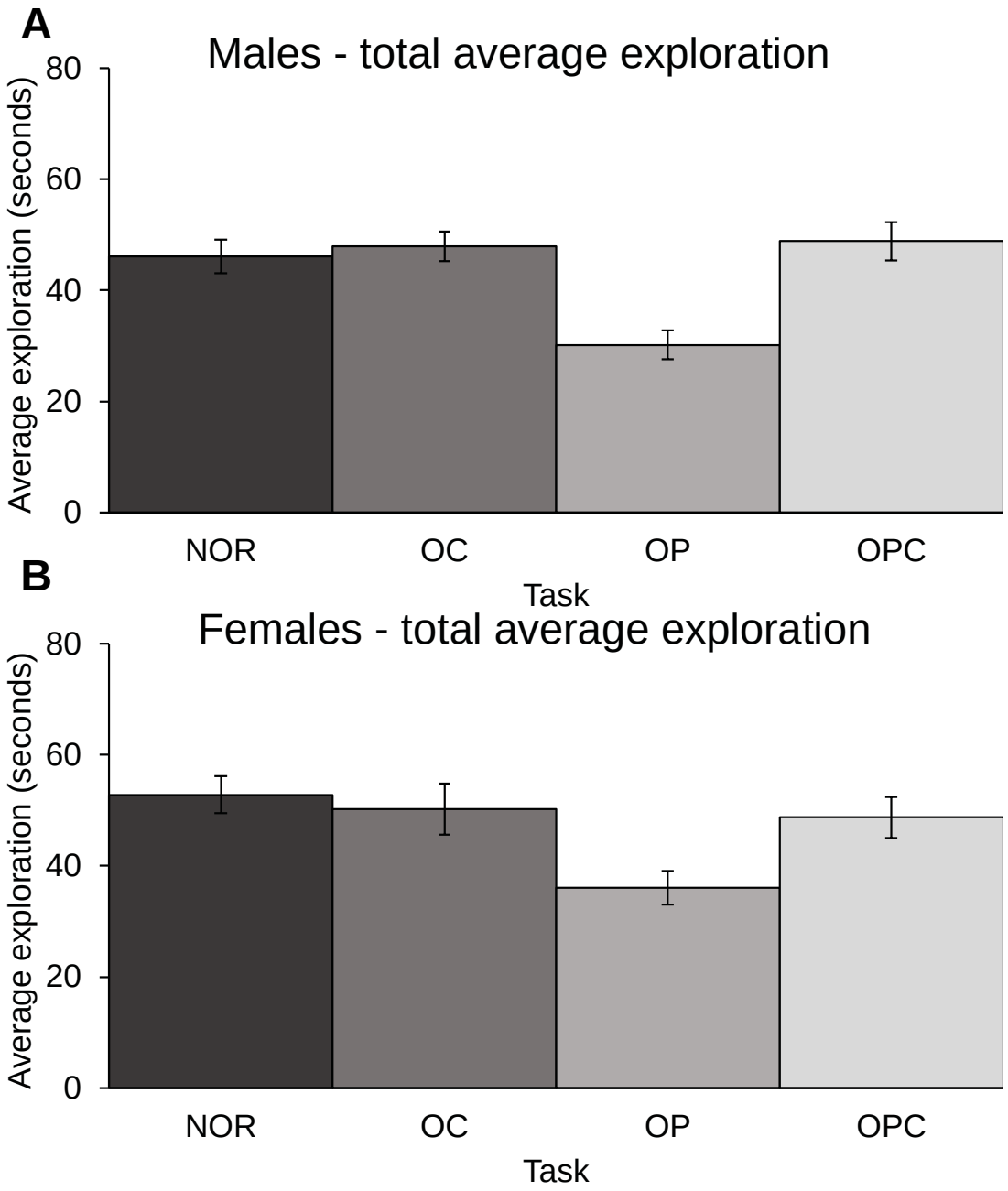

Figure 3.8 Exploration of adult rats in four tasks over two days, split into males and females.

There was a significant effect of task), no effect of sex and no interaction between task and sex.

Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).

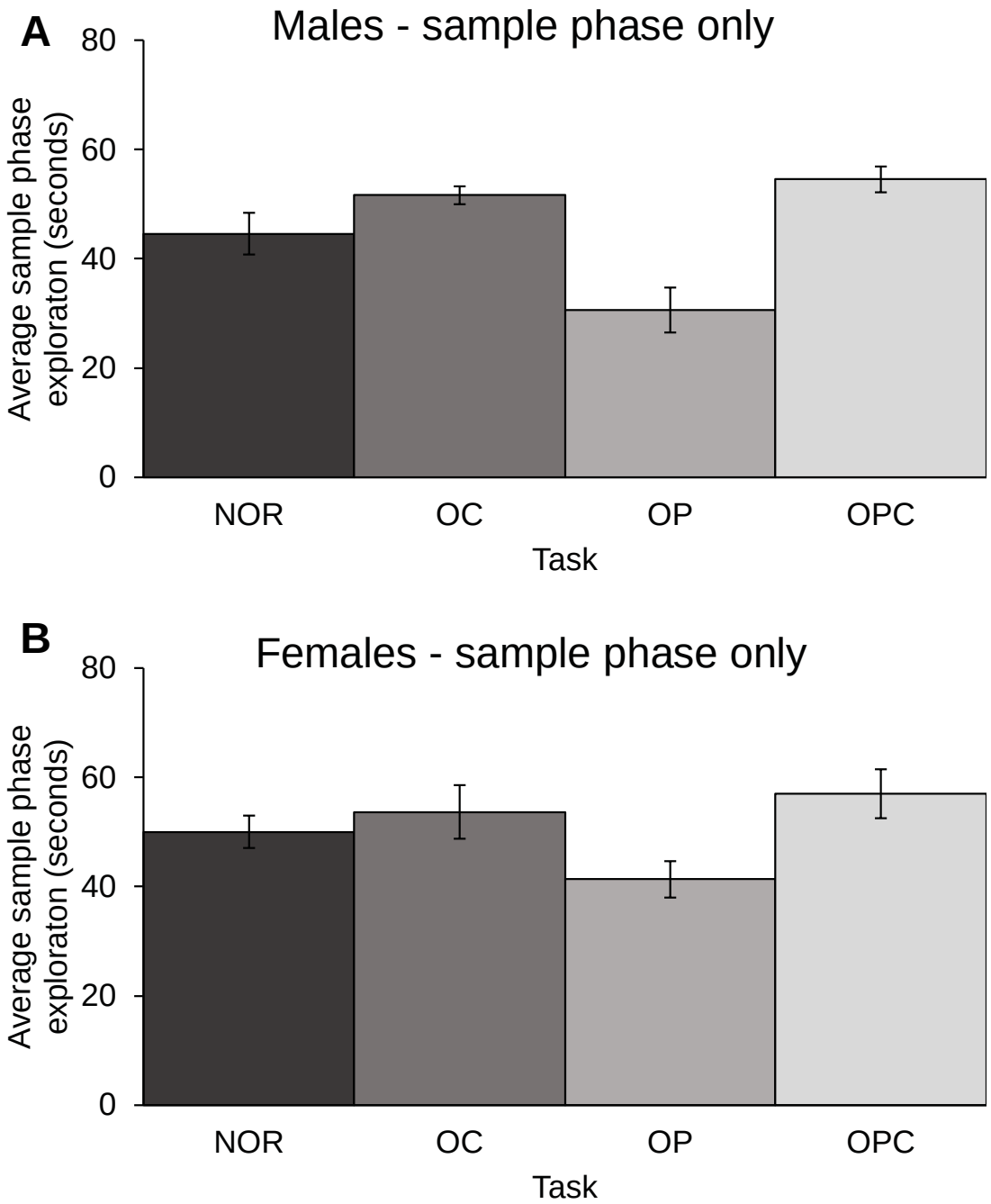

Figure *3.9 Sample phase exploration of adult rats in four tasks over two days, split into males and females.* *There was a significant effect of task, no effect of sex and no interaction between task and sex.*
Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).

3.3.6 Correlations between discrimination index and a number of measurements across tasks.

Pearson product moment correlation tests were conducted to investigate if there was a correlation between the performance as measured by DI and a number of different measurements including exploration within sample phase 1, sample phase 2 (when present) and test phase, as well as the time taken for the rat to explore the first object within each phase (ie the latency to the first object). The only significant correlations were seen in the OPC task where there was a significant negative correlation between the exploration in the second sample phase and DI, and a significantly positive correlation between the latency to the first object in the sample phase and DI. See table 3.6 for results of this.

	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	-0.292	>0.05	-0.411	>0.05	0.351	>0.05	0.134
Exploration sample phase 2			>0.05	0.034			<0.05	-0.547
Exploration test phase	>0.05	-0.081	>0.05	-0.014	>0.05	0.313	>0.05	0.284
Latency to first object sample phase 1	>0.05	0.094	>0.05	0.088	>0.05	-0.485	<0.05	0.569
Latency to first object sample phase 2			>0.05	0.133			>0.05	0.171
Latency to first object test phase	>0.05	-0.186	>0.05	0.025	>0.05	-0.118	>0.05	0.397

Table 3.6 Correlations between discrimination index (DI) and a number of measurements across tasks. *Significant results are shown in bold. The only significant correlation was seen in the OPC task and was between DI and exploration in sample phase 2, and latency to the first object in sample phase 1. Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).*

A Welch Two Sample t-test was used to investigate if there was a difference in performance between rats that explored the novel object first in the test phase and rats that explored the familiar object in the test phase. Results are shown in table 3.7

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.47	0.52	$F_{(15)} = 1.20$, $p > 0.05$
OC	0.18	0.25	$F_{(15)} = 0.45$, $p > 0.05$
OP	0.36	0.30	$F_{(15)} = 0.45$, $p > 0.05$
OPC	0.19	0.28	$F_{(15)} = 0.51$, $p > 0.05$

Table 3.7 Differences in discrimination index (DI) across tasks between rats that explored the novel object first, compared to those which explored the familiar object first. *There were no significant differences in this measure in any tasks. Abbreviations – Novel object recognition (NOR), Object Context (OC), Object Place (OP) and Object place context (OPC).*

3.4 Discussion

This investigation has successfully designed and implemented a protocol using four novel object recognition based tasks that can be used in a shortened time window enabling testing during development at critical time points. All four tasks were performed over the course of two days, with each rat completing two tasks per day. Previously a protocol like this has never been developed and implemented in such a way and will provide as a valuable tool for researching recognition, spatial, contextual and episodic-like memory in rats during restricted time periods.

The fact that adult rats were successfully able to perform all four tasks significantly above chance, with females showing a slightly, however non-significant increase in performance in comparison to the males, is a success. It demonstrates the value of the tasks in that they require no training or rewards and are completely reliant on the innate behaviour of the rat.

The fact that there was a significant effect of task as shown in figure 3.4, shows that the rats are performing differently on each task. This may reflect the differences in complexity of the tasks, although we cannot say whether the rats find a task “difficult” or not as there is no reward and no real “question” being asked of the rat. However, it is clear that adult rats have different levels of performance in each of the tasks, with NOR showing the highest level of performance. This must be kept in mind when moving on to carry out this task in younger animals, as it’s clear that adult animals produce differing scores on the different tasks.

In the literature, there are some studies conducted where animals have been excluded from analysis based on a lack of exploration in the sample phase

(Langston and Wood, 2010). In other studies, including the study where the tasks were originally presented, the sample phase length was determined by the animal itself and was brought to an end when the animal reached 15 seconds exploration at each object in a 2-5minute window (Eacott and Norman, 2004, Langston and Wood, 2010). In order to understand if a lack of exploration in the sample phase is a valid reason to exclude an animal, the correlation between sample phase exploration and performance in the test phase was investigated. Overall, no correlation was found between sample phase exploration and performance. Of course, correlation does not equal causation however within this group of animals it suggests that exploration levels did not affect performance. No rat ever had an exploration score of zero seconds, therefore it cannot be said that animals do not need to actively explore the objects to encode the memory, rather the animals used in this investigation all spend enough time exploring the objects to encode the memory.

However, it is comprehensible that object exploration is not the only part of the task that allows the animal to encode a memory given that three of the four tasks have elements other than object identity. The OC task requires the animal to encode the contextual information and it's plausible that the animal is encoding this the entire time it's in the testing arena. The same argument could be applied to the OP task, where the spatial component of episodic memory is tested. The rat need not explore the object to encode it's location relative to itself (egocentric) or relative to extramaze cues (allocentric). Finally for the OPC task, the animal must combine object identity, object location and associated contextual information in order to encode the episode. I propose

that a low level of object exploration in the sample phase does not necessarily correlate with the animal failing to encode the episode, or being at a disadvantage to other animals within the group. In light of this finding, in all subsequent experiments in this thesis no animals were excluded from analysis due to a lack of exploration in the sample phase. However due to the fact that no adult rats had an exploration score of zero seconds and therefore it is unknown how this affects performance, any rat in subsequent experiments with an exploration score of zero seconds in the sample phase will be excluded from analysis. Finally, any rat which has an exploration score of less than ten seconds in the test phase will be excluded as there is no way to analyse memory if there is no exploration.

In the OPC task it was found that there was a positive correlation between the latency to explore the objects in the first sample phase, and the performance of the animal. This suggests that the longer the animal takes to start exploring in the first sample phase, the higher the performance as measured by DI. As this is only seen in one sample phase of one task, it is not possible to draw any real conclusions from this. If the correlation was seen in the test phase one might be able to hypothesise that the rats are using the time before exploration to recall and to “figure out” which object is in a novel configuration, however this correlation is not seen.

In this investigation no difference in performance was seen between genders, which is interesting considering there are some studies within the literature that have demonstrated a role of the oestrus cycle of females in cognition. It has been shown that hormone fluctuations during the oestrus cycle can have a detrimental effect on spatial memory (Sutcliffe et al., 2007, Frye, 1995), as

well as having an effect on the learning strategy of female rats. When ovarian hormones are high rats were more likely to solve a spatial task in an allocentric manner, whereas when the hormones levels dropped at oestrus the rats implemented an egocentric strategy (Korol et al., 2004) It has also been shown that during the four to five day oestrus cycle of the female rat, oestrogens and progestins regulate synaptogenesis in the CA1 region of the hippocampus (McEwen et al., 1997). In the present study there was no investigation of the stage of the oestrus cycle in the female rats, and therefore it cannot be said whether the female rats may have been effected by fluctuating hormone levels. In future it would be interesting to note at which stage of the cycle the female rats were at during testing, and correlate this to performance. This can be carried out by a vaginal lavage where a small volume of saline is inserted into the vagina, removed and examined on a microscope slide under a microscope, where cell type can be quantified and correlated to hormone level fluctuations. The appearance of the external genitalia can also help to establish the stage of the oestrus cycle.

This investigation has successfully demonstrated that adult rats can perform NOR, OC, OP and OPC tasks using a novel shortened protocol with only two days habituation and in a single trial.

Chapter 4

Using a novel protocol to assess memory ontogeny in juvenile rats

4.1 Introduction

Following on from the previous chapter (chapter 3) where a successful protocol was developed to assess four types of memory (object recognition, associative context memory, spatial memory and episodic-like memory) in a short time period, the focus of this investigation was to understand the ontogeny of these memory types in juvenile rats. Four tasks were used based on Eacott and Norman's original "What, Where, Which" task (Eacott and Norman, 2004), and used alongside other control tests of novel object recognition, spatial and associative memory (Langston et al., 2010). The tasks used were compressed into a two day testing window, so as to enable the precise development of the tasks to be investigated. Until now, behavioural studies looking into memory ontogeny in rats have not been done in such a manner.

Within the current literature, in the field of memory development, there is very little on OPC memory development, and instead a lot of the focus of the literature is on contextual fear conditioning. In humans we know that different types of memory develop differentially in childhood, with simple novelty detection developing before associative memory with the last type of memory to develop being episodic memory (Gathercole, 1998). An animal model of this differential memory development would be invaluable. There are a number of conditions where memory development is affected such as autism (Goddard et al., 2014), attention deficit hyperactivity disorder (ADHD) (Klingberg et al., 2002) and Down's Syndrome (Chapman and Hesketh, 2001). An understanding of how memory develops in healthy individuals may provide an insight into ages which may be important to test in developmental disease. In

order to model the memory development changes in disease in rodents, there must be a standard protocol to use, and data from healthy animals to compare to. Once it is understood how different memory subtypes develop in a healthy animal, models of disease can utilise the same protocol and observe any changes. This chapter aimed to understand this development in healthy rats.

4.2 Method

4.2.1 Subjects

Male and female lister hooded rats, bred in house, were housed in groups of at least two. Adult rats for breeding were purchased from Charles River Laboratories. Litters were culled to 8 pups shortly after birth, keeping as close to equal number of males and females as possible. If the litter was born during the day (between 8am and 5pm) then that day was taken as p0, and if the litter was born overnight then the following morning was taken as p0. Pups were weaned from their parents at 21 postnatal days and were then kept in same sex groups of at least 2 in cages with opaque white plastic bases measuring 31cm x 50cm x 19cm (width x length x height) fitted with wire mesh lids (8cm high) bringing the total height of each cage to approximately 27cm. Rats were kept on a 12 hour light/dark cycle (light phase 0500 – 1700) at 22-24°C and 45-55% humidity, with behavioural testing carried out in the light phase. All animals had unrestricted access to food (Special Diets Services (SDS) Rat and Mouse (RM) No.1 Expanded Maintenance for all post-weaning animals, and SDS RM No.3 Breeding Expanded for breeding pairs) and water at all times, except for the small period of time when they were being behaviourally tested. Breeding pairs were culled after having 6 litters and the

maintenance and testing of laboratory animals complied with national (Animals [Scientific Procedures] Act, 1986) and international (European Parliament Directive of 22 September 2010 [2010/63/EU]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

4.2.2 Apparatus and behaviour

All apparatus and behaviour was the same as in chapter 3. The protocol was kept exactly the same from the study using adult rats, using the same size of objects and testing arena throughout, regardless of age and size of rat. Rats were handled for five days, followed by two days of habituation and two days of testing on the four tasks, NOR, OC, OP and OPC. Juvenile rats only completed this protocol once (i.e. a cross-sectional study rather than longitudinal) and each age group consisted of two litters of rats. Counterbalancing was once again implemented to prevent any biases. Exploration was timed and DI calculated in the same way as the adult study.

4.2.3 Statistical analysis

Once the DI had been calculated for each rat, statistical analysis was carried out. In order to do this rats ages were defined in two ways, firstly their true age in postnatal days, e.g. p25, and secondly defined as either “young” or “old”. The true age in postnatal days allowed a repeated measures ANOVA to be carried out as well as the more complex statistical analysis described here. This “young”/”old” definition was further split into categories, where each age category varied on the definition of “young and old”. Categories were generated which allowed each age to be considered young and old, apart from

the youngest age (p25/26) which was always defined as young, and the oldest age (p50/51) which were always defined as old.

Statistical general linear models were fitted to the data for each age category, and models were checked for constancy of variance and normality of errors. All analyses were conducted in R (R core development team 2014)

Deviation is the difference between the observed value and the mean, and statistical errors and residuals are two closely related measures of deviation. The difference between the observed value in the sample and the unobservable *population* mean is a statistical error whereas the difference between the observed value in the sample and the observable *sample* mean is a residual.

The Residual sum of squares (RSS) is the sum of the squares of the residuals. We square the residuals to make all values positive so that when they are summed, the negative values are included in the sum as well as the positive. RSS tells us how much of the variation in the dependent variable our model has not explained. A small residual sum of squares (RSS) indicates a tight fight of the model to the data.

The Explained sum of squares (ESS) measures how much variation there is in the modelled values and this is compared to the total sum of squares and the RSS. It is the sum of the squares of the deviations of the predicted values from the mean value of a response variable, in a standard regression model. ESS tells us how much of the variation in the dependent variable our model has explained. In general the total sum of squares = ESS + RSS

Regression analysis is a process in statistics that allows the relationship among variables to be estimated and understood. Usually the focus is on a relationship between a dependent variable (in this case DI) and one or more independent variables (in this case age). Regression analysis can be used to understand which independent variables are related to the dependent variable, and to explore their relationships. Linear regression is an approach for modelling the relationship between a scalar (one dimensional physical quantity that can be described by a single real number – unlike vectors) dependent variable and one or more independent variables.

In the results tables presented in this chapter, the sum of squares is presented as well as the mean squares which is calculated as sum of sum of squares divided by the degrees of freedom. Mean squares are an estimate of population variance. In our models we searched for the situation where the RSS mean square was at the lowest point, therefore this is the model which explains more of our data than it does not explain – it maximises the explained variance. There is no family wise error as it is a simple pairwise comparison that minimises the residual sum of squares, the models were not directly linked or compared to one another in any way.

4.2.4 Other measures analysed

Exploration was timed live by the experimenter as the camera fed to a television screen which allowed DI to be calculated. Information about which object the rat approached first, and the latency to approach the first object, was gathered after the experiment by watching the recorded footage again.

4.2.5 N numbers

N numbers for each age group and task are shown in table 4.1. Occasionally rats were excluded from analysis due to an object falling over, zero exploration in the sample or test phase, or interruption of the task by someone entering the testing room, however these events were uncommon. Finally in the OPC task on p35, one video failed to record and therefore the first object analysis could not be conducted, however all live scoring was still conducted.

	NOR	OC	OP	OPC
P25/26	18	18	18	17
P31/32	15	15	15	15
P34/35	16	14	15	15 (one video not recorded therefore 14 for 1st object analysis)
P38/39	16	15	16	15
P42/43	16	16	16	13
P45/46	16	17	17	15
P47/48	16	14	16	15
P50/51	14	15	15	16

Table 4.1 n-numbers for each task at each age group. *NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object Place Context*

4.3 Results

4.3.1 Novel object recognition

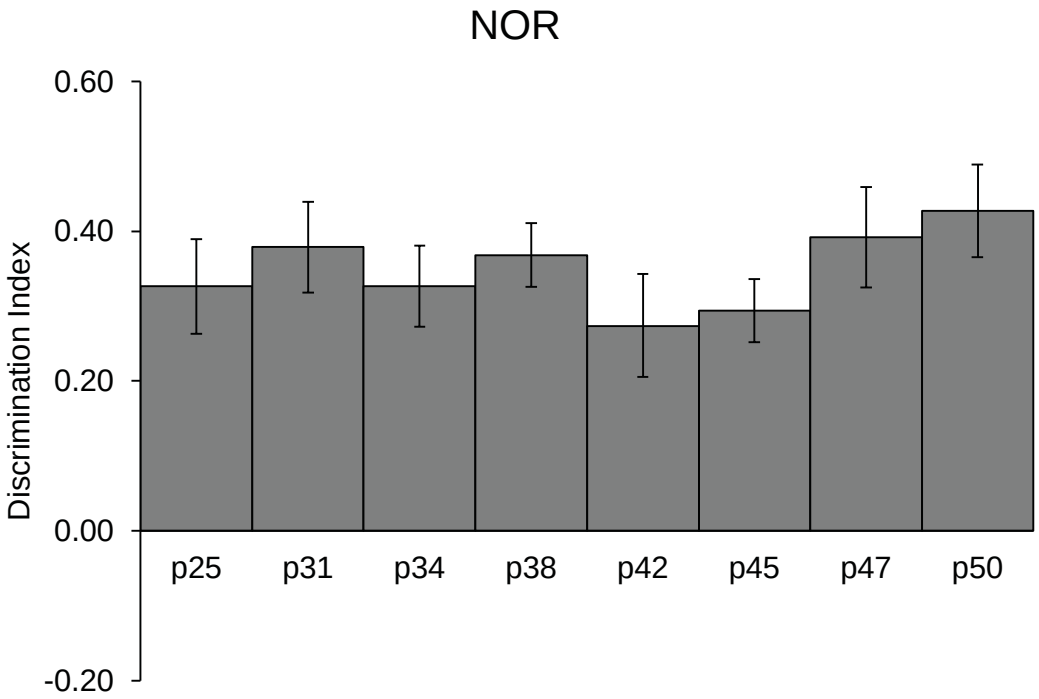

Figure 4.1 Performance of rats on a novel object recognition (NOR) task across a number of ages
Performance on novel object recognition (NOR) task across a number of ages from postnatal day 25 (p25) to p50. No significant change was seen in performance across the ages.

Model	Young	Old		Df	Sum sq	Mean Sq	Age	
							F	p
1	All ages		Age	7	0.296	0.042	0.773	0.611
			Residuals	120	6.566	**0.055**		
2	25	31	Age	1	0.009	0.009	0.167	0.683
			Residuals	126	6.853	**0.054**		
3	31	34	Age	1	0.000	0.000	0.008	0.930
			Residuals	126	6.862	**0.055**		
4	34	38	Age	1	0.002	0.001	0.033	0.930
			Residuals	126	6.861	**0.055**		
5	38	42	Age	1	0.000	0.000	0.006	0.936
			Residuals	126	6.862	**0.055**		
6	42	45	Age	1	0.038	0.038	0.707	0.402
			Residuals	126	6.824	**0.054**		
7	45	47	Age	1	0.156	0.156	2.933	0.089
			Residuals	126	6.707	**0.053**		
8	47	50	Age	1	0.109	0.109	2.032	0.157
			Residuals	126	6.756	**0.053**		

Table 4.2 Statistical output for novel object recognition (NOR)
Rats ages were defined in two ways, firstly their true age in postnatal days, eg p25, and secondly defined as either "young" or "old". This "young"/"old" definition was further split into categories, where each age category varied on the definition of "young and old". Categories were generated which allowed each age to be considered young and old, apart from the youngest age (p25) which was always defined as young, and the oldest age (p50) which were always defined as old. Statistical general linear models were fitted to the data for each age category, and models were checked for constancy of variance and normality of errors. Across all models there was no change in the residual mean square RSS values and no significant effect of age.

For NOR there was no change in any of the RSS values and there was no significant effect (the p value was never below 0.05) therefore there was no change between the groups at any age.

4.3.2 Object context

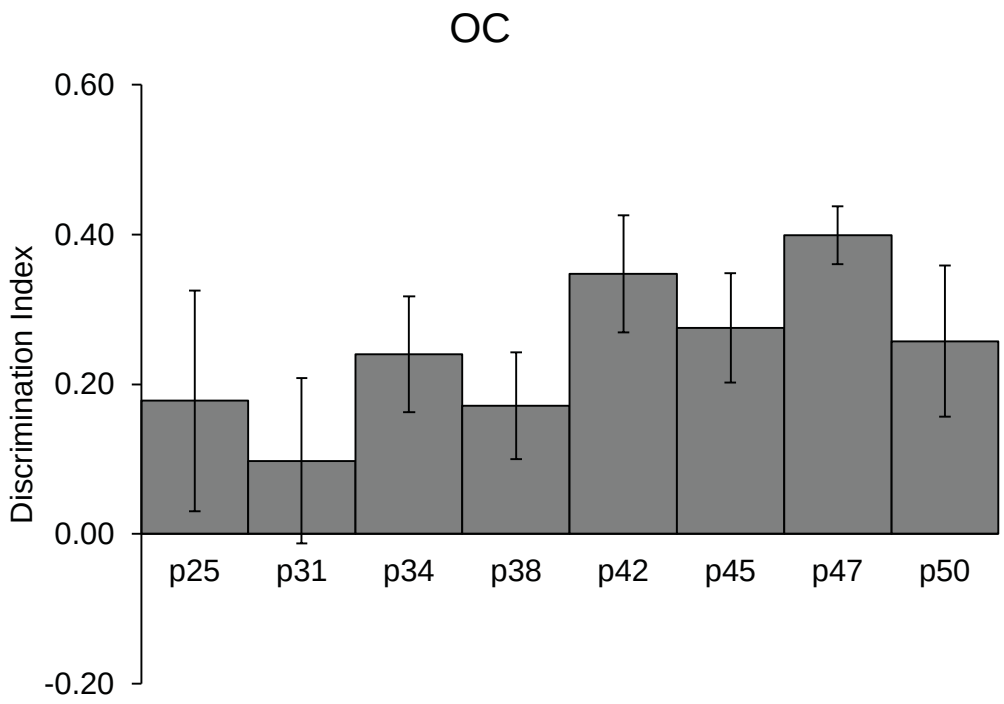

Figure 4.2 **Performance of rats on an object context (OC) task across a number of ages**
Statistal analysis demonstrated that when <=p38 is categorised as "young" and >=p42 is categorised as old, there is a significant effect of age, where age is either young or old.

Model	Young	Old		Df	Sum sq	Mean Sq	Age	
							F	p
9	All ages		Age	7	1.006	0.143	0.987	0.444
			Residuals	116	16.900	**0.146**		
10	25	31	Age	1	0.093	0.093	0.634	0.427
			Residuals	122	17.814	**0.146**		
11	31	34	Age	1	0.473	0.473	3.311	0.071
			Residuals	122	17.434	**0.143**		
12	34	38	Age	1	0.416	0.416	2.899	0.090
			Residuals	122	17.491	**0.143**		
13	38	42	Age	1	0.673	0.673	4.763	**0.031**
			Residuals	122	17.234	**0.141**		
14	42	45	Age	1	0.294	0.2940	2.037	0.156
			Residuals	122	17.613	**0.144**		
15	45	47	Age	1	0.254	0.254	1.758	0.187
			Residuals	122	17.652	**0.145**		
16	47	50	Age	1	0.003	0.003	0.021	0.885
			Residuals	122	17.904	**0.147**		

Table 4.3 Statistical output for object context (OC)
Rats ages were defined in two ways, firstly their true age in postnatal days, eg p25, and secondly defined as either "young" or "old". This "young"/"old" definition was further split into categories, where each age category varied on the definition of "young and old". Categories were generated which allowed each age to be considered young and old, apart from the youngest age (p25) which was always defined as young, and the oldest age (p50) which were always defined as old. Statistical general linear models were fitted to the data for each age category, and models were checked for constancy of variance and normality of errors. When <=p38 is categorised as "young" and >=p42 is categorised as old, there is a significant effect of age, where age is either young or old.

For OC the model which showed the lowest RSS was model 13 where <=p38 is young and >=p42 is old. This model was also the only model which had a significant effect of age.

4.3.3 Object place

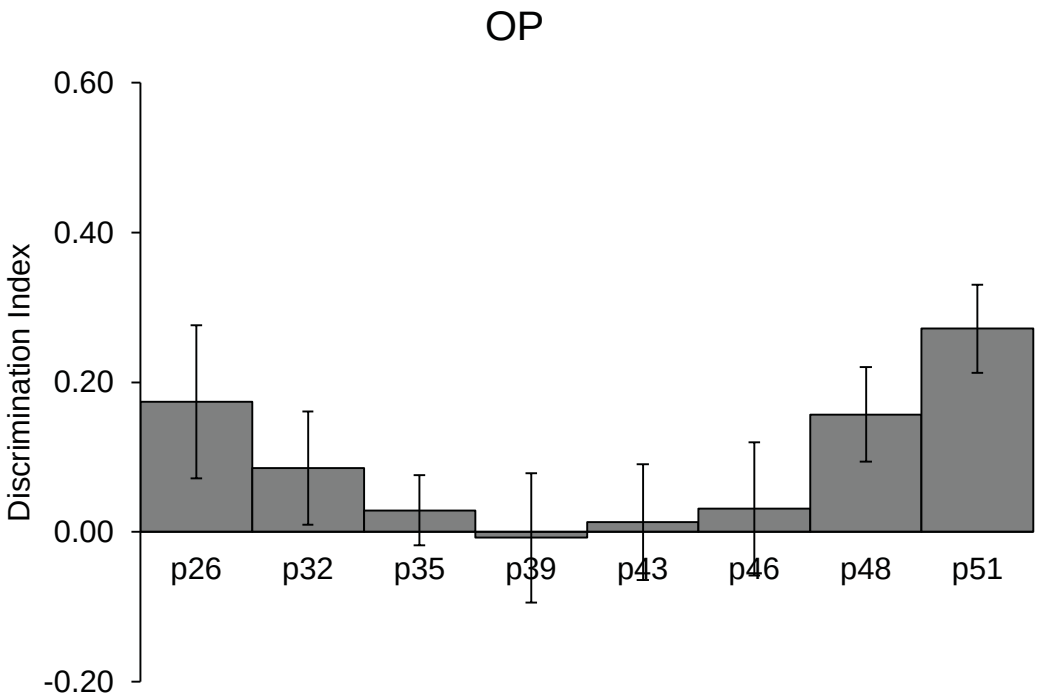

Figure 4.3 **Performance of rats on an object place (OP) task across a number of ages**
Statistical analysis demonstrated that when <=p46 is categorised as "young" and >=p48 is categorised as old, there is a significant effect of age, where age is either young or old.

Model	Young	Old		Df	Sum sq	Mean Sq	Age	
							F	p
17	All ages		Age	7	1.052	0.150	1.51	0.169
			Residuals	120	11.922	**0.099**		
18	26	32	Age	1	0.134	0.134	1.31	0.254
			Residuals	126	12.841	**0.101**		
19	32	35	Age	1	0.070	0.070	0.68	0.411
			Residuals	126	12.900	**0.124**		
20	35	39	Age	1	0.004	0.004	0.03	0.852
			Residuals	126	12.971	**0.103**		
21	39	43	Age	1	0.053	0.053	0.51	0.475
			Residuals	126	12.922	**0.103**		
22	43	46	Age	1	0.224	0.224	2.21	0.140
			Residuals	126	12.751	**0.101**		
23	46	48	Age	1	0.571	0.571	5.80	**0.018 ***
			Residuals	126	12.404	**0.098**		
24	48	51	Age	1	0.534	0.534	5.41	**0.022 ***
			Residuals	126	12.440	**0.099**		

Table 4.4 Statistical output for object place (OP)
Rats ages were defined in two ways, firstly their true age in postnatal days, eg p25, and secondly defined as either "young" or "old". This "young"/"old" definition was further split into categories, where each age category varied on the definition of "young and old". Categories were generated which allowed each age to be considered young and old, apart from the youngest age (p25) which was always defined as young, and the oldest age (p50) which were always defined as old. Statistical general linear models were fitted to the data for each age category, and models were checked for constancy of variance and normality of errors. When <=p46 is categorised as "young" and >=p48 is categorised as old, there is a significant effect of age, where age is either young or old.

For OP the model which showed the lowest RSS was model 23 where <=p46 is young and >=p48 is old. This model also had a significant effect of age. Despite there being two models which had a significant effect of age (23 and 24), model 23 explains more of the variation in the data due to a lower RSS.

4.3.4 Object place context

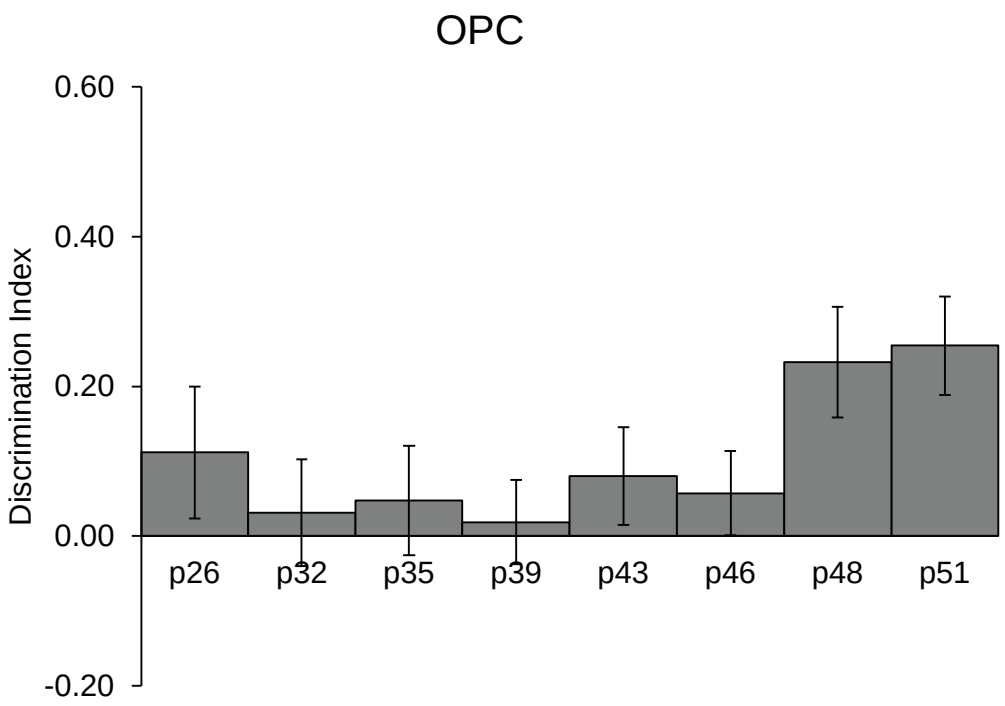

Figure 4.4 **Performance of rats on an object place context (OPC) task across a number of ages**
Statistical analysis demonstrated that when <=p46 is categorised as "young" and >=p48 is categorised as old, there is a significant effect of age, where age is either young or old.

Model	Young	Old		Df	Sum sq	Mean Sq	Age	
							F	p
25	All ages		Age	7	0.891	0.127	1.62	0.135
			Residuals	114	8.929	**0.078**		
26	26	32	Age	1	0.001	0.001	0.01	0.915
			Residuals	120	9.819	**0.082**		
27	32	35	Age	1	0.042	0.042	0.51	0.476
			Residuals	120	9.778	**0.081**		
28	35	39	Age	1	0.125	0.125	1.55	0.215
			Residuals	120	9.695	**0.081**		
29	39	43	Age	1	0.334	0.334	4.22	**0.042 ***
			Residuals	120	9.486	**0.079**		
30	43	46	Age	1	0.465	0.465	5.96	**0.016 ***
			Residuals	120	9.355	**0.078**		
31	46	48	Age	1	0.802	0.801	10.6	**0.001 ***
			Residuals	120	9.019	**0.075**		
32	48	51	Age	1	0.413	0.413	5.26	**0.023 ***
			Residuals	120	9.407	**0.078**		

Table 4.5 Statistical output for object place context (OPC)
Rats ages were defined in two ways, firstly their true age in postnatal days, eg p25, and secondly defined as either "young" or "old". This "young"/"old" definition was further split into categories, where each age category varied on the definition of "young and old". Categories were generated which allowed each age to be considered young and old, apart from the youngest age (p25) which was always defined as young, and the oldest age (p50) which were always defined as old. Statistical general linear models were fitted to the data for each age category, and models were checked for constancy of variance and normality of errors. When <=p46 is categorised as "young" and >=p48 is categorised as old, there is a significant effect of age, where age is either young or old.

For OPC the model which showed the lowest RSS was model 31, where <

=p46 is young and >=p48 is old. This model also had a significant effect of age. Despite there being four models which had a significant effect of age (29, 30,31, 32), model 31 explains more of the variation in the data due to a lower RSS.

4.3.5 Effect of counterbalancing on discrimination index

As described previously in section 3.2.3, the order of contexts for OC and OP were counterbalanced so that across the population of rats there was an equal number of rats which saw each condition.

The effect of context order was analysed by a repeated measures ANOVA for each task with age as within subjects factor and the test phase context (test phase context the same as the first sample phase, or test phase context the same as the second sample phase) as the between subjects factor.

In the OC task there was a significant effect of the test phase context ($F_{(1)}$ = 22.776, $p < 0.05$) but no significant effect of age ($F_{(7)}$ = 1.316, $p > 0.05$) and no interaction between test phase context and age ($F_{(1,7)}$ = 1.601, $p > 0.05$)

In the OPC task there was a significant effect of the test phase context ($F_{(1)}$ = 20.56, $p < 0.05$) but no significant effect of age ($F_{(7)}$ = 1.515, $p > 0.05$) and no interaction between test phase context and age ($F_{(1,7)}$ = 0.637, $p > 0.05$)

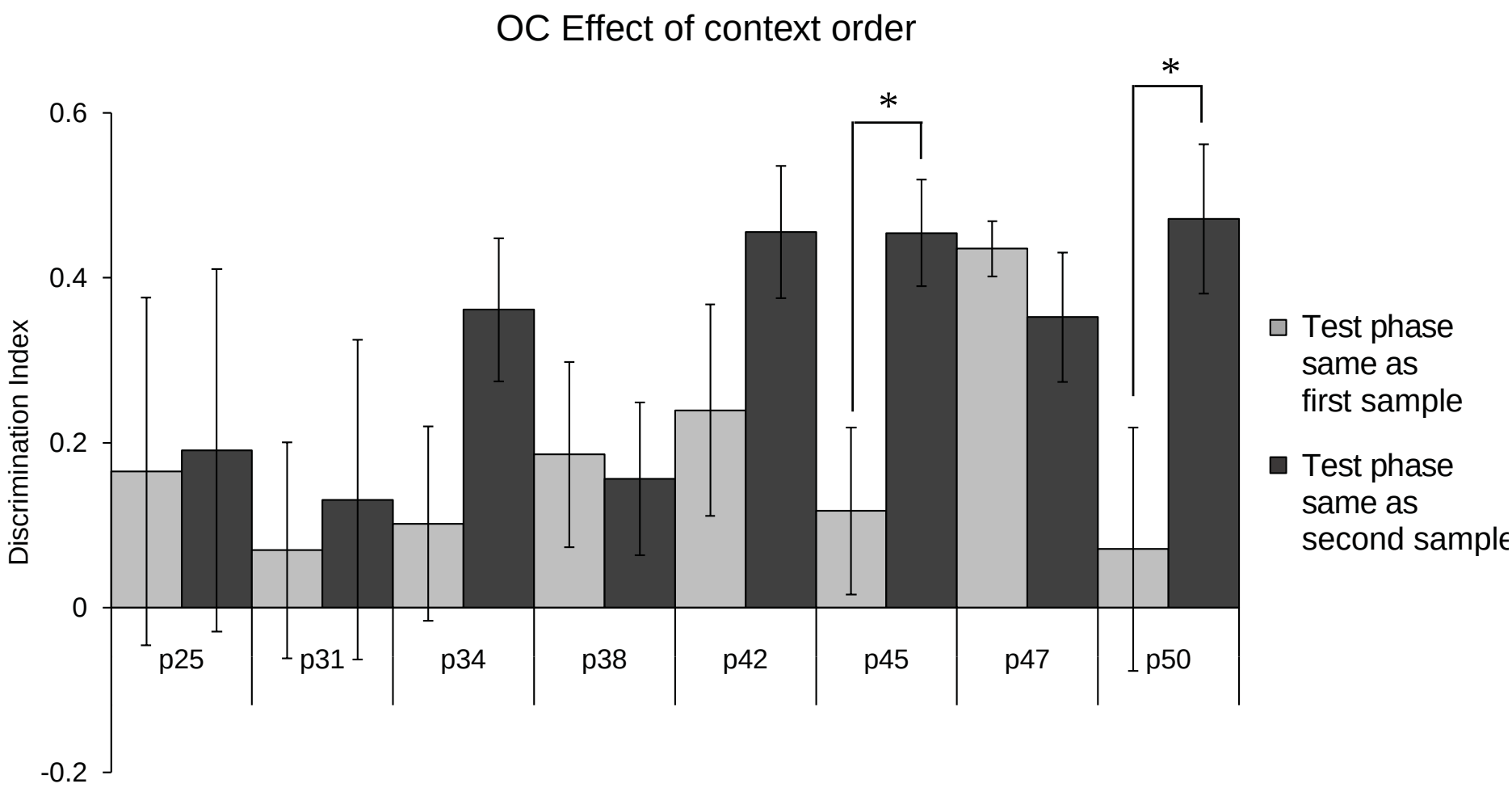

***Figure 4.5 Effect of context order on the object context (OC) task**. Stars denote significant difference (p<0.05) between the two conditions.*

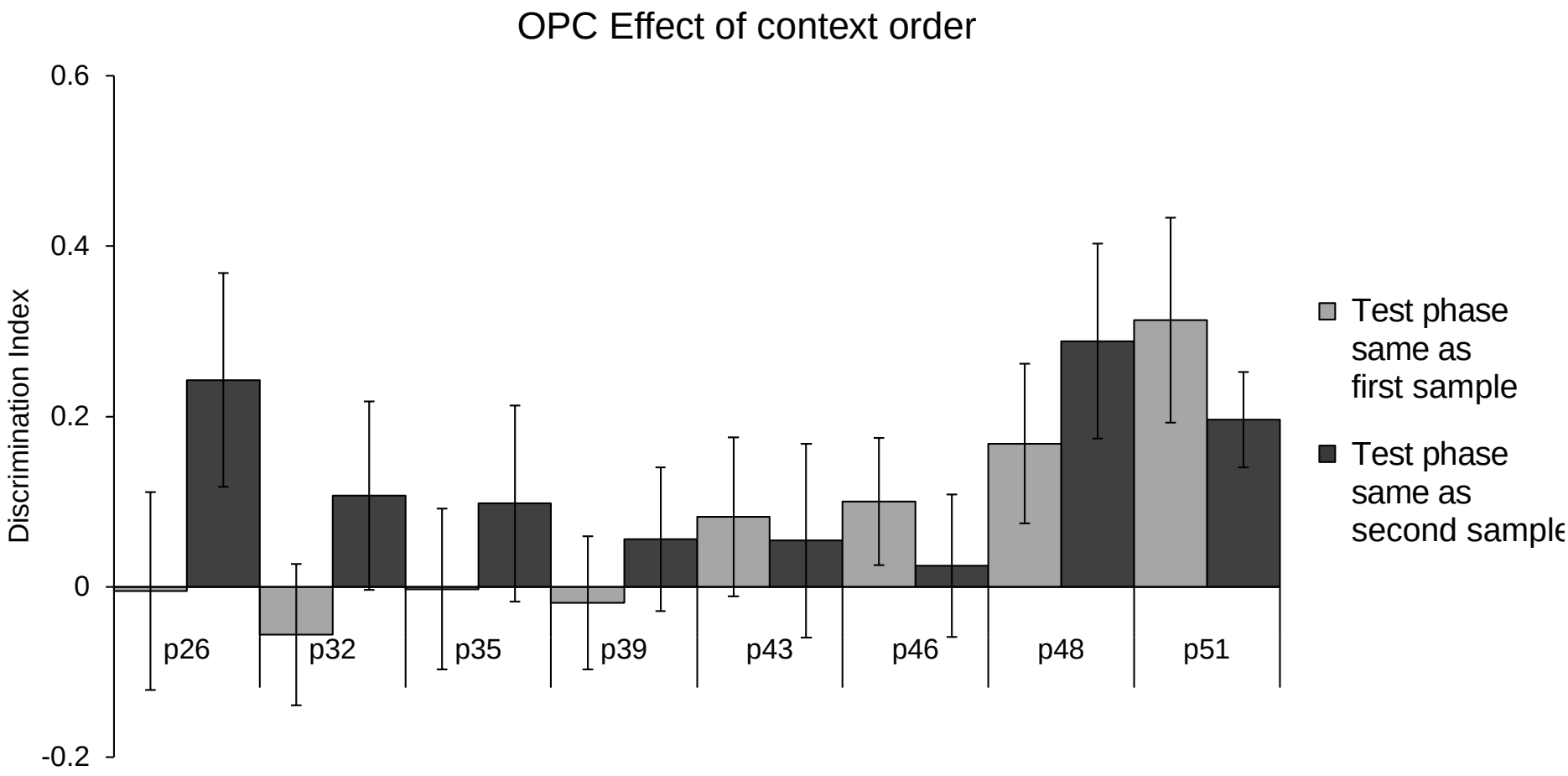

Figure 4.6 Effect of context order on the object place context (OPC) task*. There were no significant difference between the two conditions at any age.*

4.3.6 Further analysis across tasks

4.3.6.1 All ages and tasks combined

Correlation tests were carried out on DI with a number of different measurements. These were exploration in sample phase 1, exploration in sample phase 2 (where applicable, i.e. for OC and OPC), exploration in test phase, latency to first object (time it takes for the animal to explore an object) in sample phase 1, latency to first object in sample phase 2 and latency to first object in test phase. Furthermore, t-tests were used to analyse the difference in performance between sex, and between animals who approached the novel object first and animals who approached the familiar object first in the test phase.

From this analysis we were able to conclude that there was a significant positive correlation between the amount of object exploration in the test phase and performance as scored by DI, as well as a significant improvement on performance when the animals approached the novel object first in the test phase. However there was no significant correlation between performance, as measured by DI, and exploration in the sample phases or latency to the first object in either sample phases or test phase. There was also no significant effect of sex.

Discrimination index (DI) vs	**p**	**Corr**
Sample phase 1 exploration	> 0.05	-0.017
Sample phase 2 exploration	> 0.05	-0.041
Test phase exploration	**0.001 ***	**0.143**
Latency to first object – sample 1	> 0.05	-0.044
Latency to first object – sample 2	> 0.05	0.122
Latency to first object – test	> 0.05	-0.057

Table 4.6 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, within each phase across all tests and ages.
There was a significant positive correlation between performance as measured by discrimination index, and the amount of exploration in the test phase.

Average DI males	Average DI females	result
0.192	0.207	$t_{(474)}$=0.654, p > 0.05

Table 4.7 Average discrimination index (DI) of males and females across all ages and tasks.
No significant difference in performance as measured by discrimination index between males and females.

Average DI when 1st object was novel	Average DI when 1st object was familiar	result
0.2603	0.0884	$T_{(363)}$= -5.78, **p < 0.001**

Table 4.8 Comparison of performance based on which object was approached first within the test phase across all ages and tasks.
Rats that approached the novel object first in the test phase performed significantly better as measured by discrimination index (DI) than rats that approached the familiar object first.

4.3.6.2 Further analysis – individual ages

In order to further understand how the rat's behaviour changed over the ages tested, the analysis conducted in section 4.3.5.1 was repeated however instead of group all ages and tasks together, the rats were split into their age groups and tasks..

P25/26

P25/26	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	0.126	>0.05	0.18	>0.05	0.325	>0.05	-0.471
Exploration sample phase 2			>0.05	-0.145			>0.05	0.074
Exploration test phase	>0.05	-0.08	>0.05	0.134	>0.05	0.059	>0.05	-0.152
Latency to first object sample phase 1	>0.05	-0.157	>0.05	0.172	>0.05	-0.104	>0.05	0.368
Latency to first object sample phase 2			>0.05	0.344			>0.05	0.054
Latency to first object test phase	>0.05	-0.194	>0.05	-0.064	>0.05	-0.138	>0.05	-0.015

Table 4.9 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p25/26

There were no significant correlations between DI and any measures.
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI males	Average DI females	result
NOR	0.39	0.19	$T_{(17)}$= 1.71 , p >0.05
OC	0.24	0.05	$T_{(17)}$= , p >0.05
OP	0.22	0.07	$T_{(17)}$= , p >0.05
OPC	0.10	0.13	$T_{(16)}$= , p >0.05

Table 4.10 Average discrimination index (DI) of males and females in each task at p25/26. *There were no significant differences between males and females in any tasks at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	Only 1 familiar therefore unable to do statistics		
OC	0.34	0.04	$T_{(18)}$ = 1.02, p >0.05
OP	0.28	-0.1	$T_{(17)}$ = 1.60, p >0.05
OPC	0.29	-0.05	$T_{(16)}$ = 2.15, p <0.05

Table 4.11 Comparison of performance based on which object was approached first within the test phase at p25/26.
Rats that approached the novel object first in the test phase of OPC performed significantly better as measured by discrimination index (DI) than rats that approached the familiar object in OPC. No other tasks showed a significant difference between the two groups.
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Once Bonferonni corrections for multiple comparisons were applied to each of these tables, none of the scores remained significant. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P31/32

P31/32	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	0.058	>0.05	0.338	>0.05	-0.269	>0.05	-0.004
Exploration sample phase 2			>0.05	0.139			>0.05	-0.207
Exploration test phase	>0.05	-0.147	>0.05	0.038	>0.05	0.168	>0.05	-0.333
Latency to first object sample phase 1	<0.05	-0.568	>0.05	-0.002	>0.05	0.292	<0.05	0.545
Latency to first object sample phase 2			>0.05	-0.131			>0.05	-0.162
Latency to first object test phase	>0.05	-0.097	>0.05	-0.144	>0.05	0.190	>0.05	-0.220

Table 4.12 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p31/32 *There was a significant negative correlation between latency to explore first object in sample phase one in NOR, and a significant positive correlation between latency to explore the first object in sample phase one in OPC.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI males	Average DI females	result
NOR	0.35	0.40	$T_{(14)} = 0.37$, p >0.05
OC	0.08	0.11	$T_{(14)} = 0.15$, p >0.05
OP	0.10	0.05	$T_{(14)} = 0.32$, p >0.05
OPC	-0.02	0.09	$T_{(14)} = 0.80$, p >0.05

Table 4.13 Average discrimination index (DI) of males and females in each task at p31/32. *There were no significant differences between males and females in any tasks at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	Only 1 familiar therefore unable to do statistics		
OC	0.11	0.04	$T_{(14)} = 0.31$, $p > 0.05$
OP	0.13	0.02	$T_{(14)} = 0.69$, $p > 0.05$
OPC	0.24	-0.11	$T_{14)} = 3.02$, $p < 0.05$

Table 4.14 Comparison of performance based on which object was approached first within the test phase at P31/32.

Rats that approached the novel object first in the test phase of OPC performed significantly better as measured by discrimination index (DI) than rats that approached the familiar object in OPC. No other tasks showed a significant difference between the two groups.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Once Bonferonni corrections for multiple comparisons were applied to each of these tables, none of the scores remained significant. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P34/35

P34/35	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	<0.05	0.533	>0.05	0.212	>0.05	0.418	>0.05	-0.186
Exploration sample phase 2			>0.05	0.100			>0.05	0.038
Exploration test phase	>0.05	0.099	>0.05	0.281	>0.05	0.04	<0.05	-0.554
Latency to first object sample phase 1	<0.05	-0.058	>0.05	0.274	>0.05	-0.347	<0.05	-0.074
Latency to first object sample phase 2			>0.05	-0.009			>0.05	-0.322
Latency to first object test phase	>0.05	-0.064	>0.05	-0.054	>0.05	0.222	>0.05	-0.120

Table 4.15 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p34/35. *There was a significant negative correlation between DI and exploration in the sample phase of NOR and exploration in the test phase of OPC.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI males	Average DI when females	result
NOR	0.33	0.31	$T_{(15)} = 0.11$, p >0.05
OC	-0.01	0.10	$T_{(13)} = 1.31$, p >0.05
OP	0.24	0.21	$T_{(14)} = 0.13$, p >0.05
OPC	0.04	0.04	$T_{(14)} = 0.002$, p >0.05

Table 4.16 Average discrimination index (DI) of males and females in each task at p34/35. *There were no significant differences between males and females in any tasks at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.34	0.18	$T_{(15)} = 2.02$, $p > 0.05$
OC	0.17	0.35	$T_{(13)} = 1.07$, $p > 0.05$
OP	0.05	-0.01	$T_{(14)} = 0.72$, $p > 0.05$
OPC	Only 1 familiar therefore unable to do statistics		

Table 4.17 Comparison of performance based on which object was approached first within the test phase at P31/32.

There were no significant differences in discrimination index (DI) between rats that approached the novel object first in the test phase and rats that approached the familiar object in any tasks.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Once Bonferonni corrections for multiple comparisons were applied to each of these tables, none of the scores remained significant. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P38/39

P38/39	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	-0.148	>0.05	0.019	>0.05	0.280	>0.05	-0.501
Exploration sample phase 2			>0.05	-0.133			>0.05	0.372
Exploration test phase	>0.05	0.145	>0.05	0.349	>0.05	0.272	>0.05	-0.424
Latency to first object sample phase 1	<0.05	-0.110	>0.05	0.109	>0.05	-0.245	<0.05	-0.018
Latency to first object sample phase 2			>0.05	-0.279			>0.05	0.263
Latency to first object test phase	>0.05	-0.155	>0.05	-0.265	>0.05	-0.332	>0.05	-0.101

Table 4.18 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p38/39. *There were no significant correlations between DI and any measures. (NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)*

	Average DI males	Average DI when females	result
NOR	0.37	0.35	$T_{(15)}$ = , p >0.05
OC	0.10	0.30	$T_{(14)}$ = 1.82, p >0.05
OP	-0.18	0.28	$T_{(15)}$ = 3.44, p <0.05
OPC	0.02	0.01	$T_{(14)}$ = 0.03, p >0.05

Table 4.19 Average discrimination index (DI) of males and females in each task at p38/39. *Females performed significantly better than males as measured by DI in OP.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.37	0.36	$T_{(15)} = 0.05$, $p > 0.05$
OC	0.15	0.09	$T_{(14)} = 0.28$, $p > 0.05$
OP	0.09	0.13	$T_{(15)} = 1.45$, $p > 0.05$
OPC	0.10	-0.10	$T_{(14)} = 2.14$, $p > 0.05$

Table 4.20 Comparison of performance based on which object was approached first within the test phase at P38/39.

There were no significant differences in discrimination index (DI) between rats that approached the novel object first in the test phase and rats that approached the familiar object in any tasks.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Oncee Bonferonni corrections for multiple comparisons were applied to each of these tables the significant difference between males and females on the OP task remained true. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P42/43

P42/43	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	0.096	>0.05	0.017	>0.05	0.222	<0.05	-0.653
Exploration sample phase 2			>0.05	0.206			>0.05	-0.252
Exploration test phase	>0.05	-0.148	>0.05	0.260	>0.05	0.370	>0.05	-0.413
Latency to first object sample phase 1	<0.05	-0.084	<0.05	-0.564	>0.05	0.057	<0.05	0.304
Latency to first object sample phase 2			>0.05	0.294			>0.05	0.136
Latency to first object test phase	>0.05	-0.108	>0.05	-0.374	<0.05	0.517	>0.05	-0.7

Table 4.21 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p42/43. *There was a significant negative correlation between the latency to the first object in OC and DI.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI males	Average DI when females	result
NOR	0.34	0.20	$T_{(15)} = 0.097$, p >0.05
OC	0.25	0.44	$T_{(15)} = 1.25$, p >0.05
OP	-0.12	0.14	$T_{(15)} = 1.89$, p >0.05
OPC	-0.02	0.17	$T_{(12)} = 1.45$, p >0.05

Table 4.22 Average discrimination index (DI) of males and females in each task at p42/43. *There were no significant differences between males and females on any task at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.35	0.03	$T_{(15)} = 2.52$, $p < 0.05$
OC	0.38	0.28	$T_{(15)} = 0.62$, $p > 0.05$
OP	0.20	-0.13	$T_{(15)} = 2.72$, $p < 0.05$
OPC	0.09	-0.01	$T_{(12)} = 0.59$, $p > 0.05$

Table 4.23 Comparison of performance based on which object was approached first within the test phase at P42/43.
Rats that approached the novel object first in the test phase of NOR and OPC tasks performed significantly better as measured by discrimination index (DI) compared to rats that approached the familiar object first in the test phase.
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Oncee Bonferonni corrections for multiple comparisons were applied to each of these tables, none of the scores remained significant. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P45/46

P45/46	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	-0.345	>0.05	-0.047	>0.05	-0.003	>0.05	-0.169
Exploration sample phase 2			>0.05	0.192			>0.05	-0.171
Exploration test phase	>0.05	0.155	>0.05	0.192	>0.05	-0.154	>0.05	0.412
Latency to first object sample phase 1	<0.05	0.268	>0.05	0.006	>0.05	-0.434	<0.05	-0.155
Latency to first object sample phase 2			>0.05	0.294			>0.05	0.431
Latency to first object test phase	>0.05	-0.04	>0.05	0.149	>0.05	-0.078	>0.05	-0.503

Table 4.24 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p45/46. *There were no significant correlations between DI and any measures. (NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)*

	Average DI males	Average DI when females	result
NOR	0.24	0.38	$T_{(15)} = 1.78$, p >0.05
OC	0.22	0.34	$T_{(16)} = 0.80$, p >0.05
OP	0.11	-0.08	$T_{(16)} = 1.16$, p >0.05
OPC	0.13	-0.05	$T_{(14)} = 1.47$, p >0.05

Table 4.25 Average discrimination index (DI) of males and females in each task at p45/46. *There were no significant differences between males and females on any task at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.23	0.30	$T_{(15)} = 0.59$, $p > 0.05$
OC	0.33	0.22	$T_{(16)} = 0.76$, $p > 0.05$
OP	0.16	-0.08	$T_{(16)} = 1.40$, $p > 0.05$
OPC	0.08	0.02	$T_{(14)} = 0.39$, $p > 0.05$

Table 4.26 Comparison of performance based on which object was approached first within the test phase at P45/46.

There were no significant differences in discrimination index (DI) between rats that approached the novel object first in the test phase and rats that approached the familiar object in any tasks.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

P47/48

P47/48	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	0.109	>0.05	-0.034	>0.05	-0.170	<0.05	0.606
Exploration sample phase 2			>0.05	-0.02			<0.05	0.535
Exploration test phase	>0.05	0.236	>0.05	0.323	>0.05	-0.116	>0.05	0.101
Latency to first object sample phase 1	>0.05	0.04	>0.05	0.013	>0.05	0.3	<0.05	-0.449
Latency to first object sample phase 2			>0.05	0.204			>0.05	0.074
Latency to first object test phase	>0.05	0.051	>0.05	0.180	>0.05	-0.025	>0.05	-0.128

Table 4.27 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p47/48. *There were significant positive correlations between DI and both first and second sample phases in OPC, as well as a significant negative correlation between DI and the latency to the first object in sample phase one of OPC.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI males	Average DI when females	result
NOR	0.45	0.31	$T_{(15)} = 1.16$, p >0.05
OC	0.40	0.38	$T_{(13)} = 0.31$, p >0.05
OP	0.08	0.24	$T_{(15)} = 1.43$, p >0.05
OPC	0.29	0.15	$T_{(14)} = 0.89$, p >0.05

Table 4.28 Average discrimination index (DI) of males and females in each task at p47/48. *There were no significant differences between males and females on any task at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.40	0.36	$T_{(15)} = 0.32$, $p > 0.05$
OC	0.37	0.43	$T_{(13)} = 0.87$, $p > 0.05$
OP	0.20	0.07	$T_{(15)} = 1.06$, $p > 0.05$
OPC	0.32	0.12	$T_{(14)} = 1.34$, $p > 0.05$

Table 4.29 Comparison of performance based on which object was approached first within the test phase at P47/48.

There were no significant differences in discrimination index (DI) between rats that approached the novel object first in the test phase and rats that approached the familiar object in any tasks.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

Oncee Bonferonni corrections for multiple comparisons were applied to each of these tables, none of the scores remained significant. Bonferonni corrections reduced the p value required for significance to 0.0125 when four comparions were made (as in the tables of sex and first object identity) and to 0.00125 when fourty comparisons were made (as in the table for exploration and latency to first object).

P50/51

P50/51	NOR		OC		OP		OPC	
DI vs ;	P	Corr	p	Corr	p	Corr	p	Corr
Exploration sample phase 1	>0.05	-0.479	>0.05	0.045	>0.05	0.418	>0.05	-0.073
Exploration sample phase 2			>0.05	0.081			>0.05	0.175
Exploration test phase	>0.05	-0.394	>0.05	0.286	>0.05	0.256	>0.05	0.291
Latency to first object sample phase 1	>0.05	-0.330	>0.05	0.154	>0.05	0.324	>0.05	-0.05
Latency to first object sample phase 2			>0.05	0.251			>0.05	-0.315
Latency to first object test phase	>0.05	0.473	>0.05	0.379	>0.05	-0.017	>0.05	0.067

Table 4.30 Correlation tests on the relationship between discrimination index (DI) and exploration times and the latency to explore the first object, for each test at p50/51. *There were no significant correlations between DI and any measures. (NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)*

	Average DI males	Average DI when females	result
NOR	0.50	0.33	$T_{(13)} = 1.40$, p >0.05
OC	0.18	0.34	$T_{(14)} = 0.80$, p >0.05
OP	0.25	0.28	$T_{(114)} = 0.27$, p >0.05
OPC	0.34	0.16	$T_{(15)} = 1.36$, p >0.05

Table 4.31 Average discrimination index (DI) of males and females in each task at p50/51. *There were no significant differences between males and females on any task at this age.*
(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

	Average DI when 1st object was novel	Average DI when 1st object was familiar	result
NOR	0.44	0.39	$T_{(13)} = 0.25$, $p > 0.05$
OC	0.34	0.17	$T_{(14)} = 0.82$, $p > 0.05$
OP	0.29	0.24	$T_{(14)} = 0.49$, $p > 0.05$
OPC	0.28	0.21	$T_{(15)} = 0.62$, $p > 0.05$

Table 4.32 Comparison of performance based on which object was approached first within the test phase at P50/51.

There were no significant differences in discrimination index (DI) between rats that approached the novel object first in the test phase and rats that approached the familiar object in any tasks.

(NOR – Novel object recognition, OC – Object context, OP – Object Place, OPC – Object place context)

4.3.7 Comparison of exploration levels between adults and juvenile rats

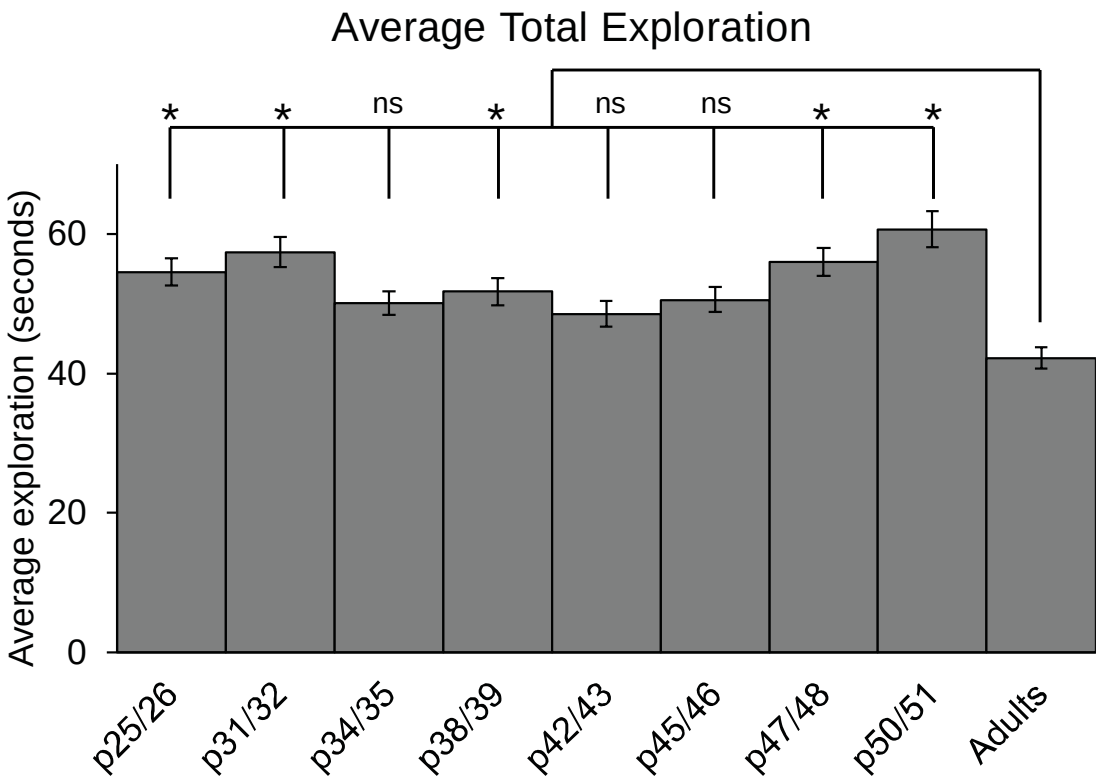

Figure 4.7 Comparison of average exploration levels between adult and juvenile rats across all four tasks.
Stars indicate significance where p<0.05. Non-significant results are indicated by "ns".

A one-way ANOVA was conducted which showed a significant effect of age ($F_{8,136)}$ = 6.524, p <0.05) with Bonferroni posthoc analysis showing that ages that had exploration levels significantly different to adults were p25/26, p31/32, p38/39, p47/48 and p50/51.

4.4 Discussion

Following on from the development of the two day / four task protocol in adult rats in chapter 3, this study successfully implemented the same novel protocol on juvenile rats at a wide range of ages. Not only was the protocol implemented for the first time in such a manner, it provided an insight into memory ontogeny in juvenile rats.

The youngest age tested was p25, four days after weaning. At this age rats were able to recognise when an object identity had changed. This was demonstrated using the NOR task, which is a short-term recognition memory task. The performance of the rats on this task throughout all ages tested remained constant. This consistent ability of the rats to perform NOR is fundamental to the other three tasks, and suggests that when the rats cannot perform OC, OP or OPC, it is not because they cannot recognise object identity.

The statistical analysis performed for the OC task suggest that this task is developing between p38 and p42. This is relatively late compared to much of the development that takes place in rats and considering that they possess the ability to recognise object novelty from at least p25.

The statistical analysis for OP and OPC suggest that these tasks develop between the ages of p46 and p48, even later than the OC task. These tasks are the only two that develop at the same time, however this may not be too surprising given that the OPC task requires the rat to recognise object identity, object location and the contextual information associated with the object. Therefore if the rat lacks to ability to recognise one of these components, it

would be unable to put all the information together and perform the task successfully.

Further analysis conducted suggested that overall there are no obvious relationships between performance and sex, exploration time, the latency to explore the objects when placed in the arena or the identity of the first object explored. However, on some tasks and ages some correlations were seen but the majority of these scores did not remain significant once Bonferonni corrections for multiple comparions was applied.

The only age at which there was a significant difference in performance between genders was on p39 where females performed significantly better than males on the OP task. It has been shown that low luteinizing hormone (LH) levels can produce an increase in performance on a spatial memory task in female rats (Ziegler and Thornton, 2010). On the day before oestrus, proestrus, there is a large surge in LH, which then plummets on the day of oestrus, coinciding with vaginal opening and ovulation. It is known that rats experience this first oestrus cycle between p35-40 (Gabriel et al., 1992), with the exact date varying between individuals as in humans. Although there is a study conducted which shows that adult rats do not synchronise their oestrus cycles like humans (Schank, 2001), it is possible that the female rats used in this investigation have the same onset of oestrus at this age, due to the fact that they are from the same litter and have been kept under controlled housing conditions. Given that on p39 female rats show an increase in performance on the spatial task, OP, it is possible that this may be the age at which the rats used in this investigation experience oestrus for the first time. This could be confirmed by monitoring plasma levels and vagina opening.

In conclusion, this investigation has demonstrated that rats, similar to humans, show differential development of different memory subtypes, with recognition memory and associative contextual memory developing before spatial and episodic memory.

Chapter 5.

Investigating the development of long term recognition memory in the juvenile rat.

5.1 Introduction

In chapter 4 it was shown that rats as young as p25 possessed the ability to recognise a novel object after a 1 minute delay. When the task is conducted using a short inter-trial interval such as this, the perirhinal cortex is required (Ennaceur et al., 1997, Bussey et al., 2000). When rats are given lesions in other brain regions such as hippocampus or prefrontal cortex, their performance is unimpaired (Barker and Warburton, 2011). However it has been shown that at a longer delay of twenty four hours, hippocampal lesioned animals cannot perform the task (Hammond et al., 2004).

This investigation aimed to assess whether twenty four hour NOR develops differently compared to the two minute NOR task in rats, and what this might demonstrate in terms of brain development.

5.2 Method

Equipment used was the same as in previous testing seen in chapters three and four, including the testing arena, the object types and the recording equipment. Rats were bred, housed and transported to and from the testing room as described in previous chapters. The same group of adult rats that were tested in chapter three were used as a control for this experiment alongside three litters (n=12 for p19/20, n=8 for p29/30 and p34/35). Rats were handled for five days, and habituated for two days in the same way as described previously, however only one context was used for this task and

therefore rats were only habituated to that particular context. The context was “context two” from chapters three and four (figure 3.1), which consisted of

black and white zebra print walls with a white plastic mesh floor. The room layout was the same as chapters three and four and can be seen in figure 3.2.

This task was a variation on the NOR task used previously with the following manipulations. Firstly the rats were allowed to explore the sample phase for ten minutes, and the test phase for five minutes rather than three minutes in the previous investigations. Secondly, the time between the two phases, the inter-trial interval (ITI), was either two minutes or twenty four hours. All rats were tested on both ITIs, with half the rats taking part in the two minute NOR task in the morning of day one, followed by the sample phase of the 24hr NOR in the afternoon of day one and the test phase of the 24hr NOR in the afternoon of day two. The other half of the rats saw the sample phase of the 24hr NOR task in the morning of day one, followed by the test phase of the 24hr NOR task in the afternoon of day two, and the two minute NOR task in the afternoon of day two.

5.3 Results

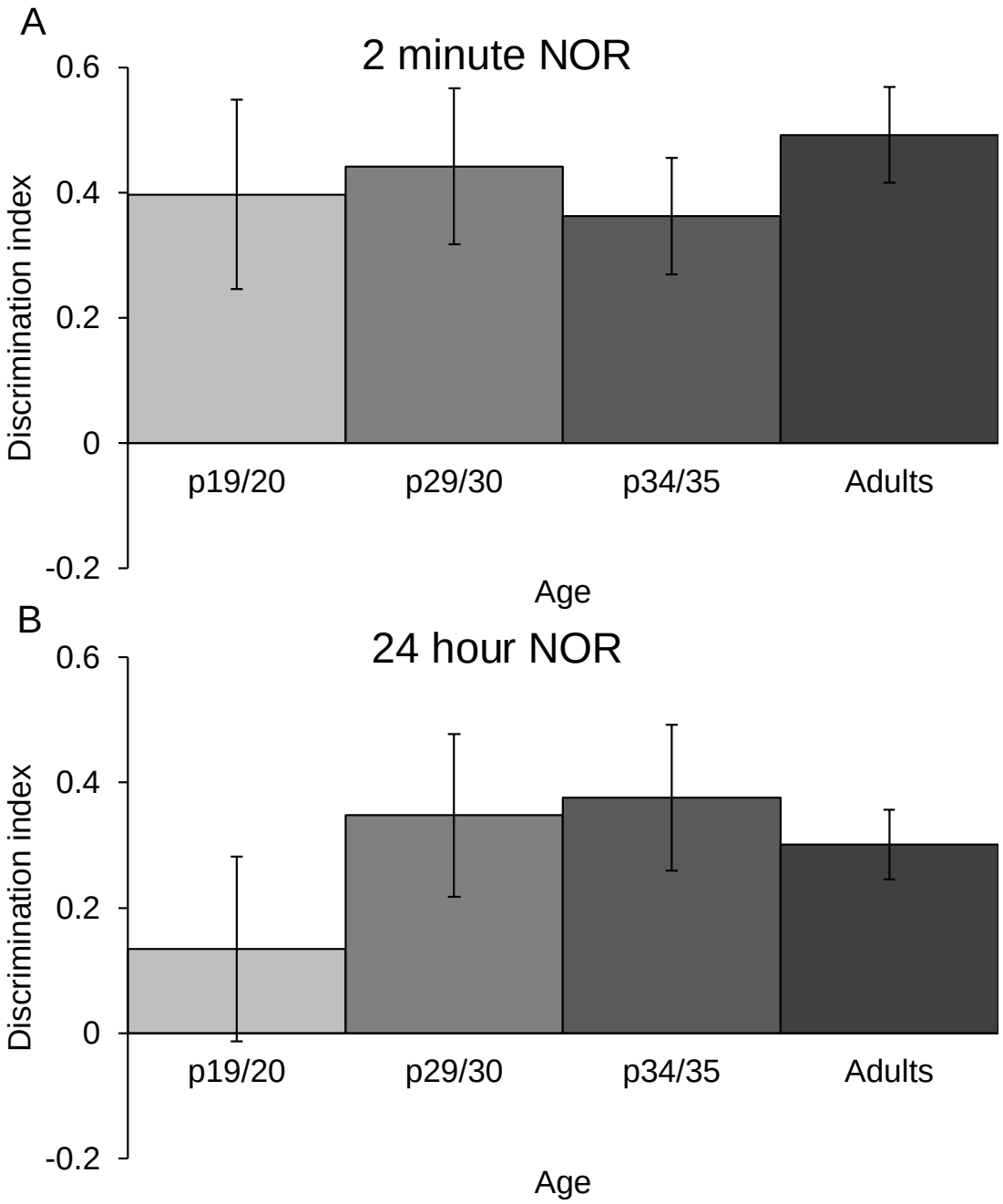

Figure 5.1 Performance of rats at four different ages on two versions of the novel object recognition (NOR) task.

A – No significant difference in performance across ages as measured by discrimination index (DI), with all age groups performing significantly above chance (0).

B - No significant difference in performance across ages as measured by (DI), with only the three older age groups performing significantly above chance

A one way ANOVA across ages on the two minute NOR task showed that there is not a significant effect of age ($F_{(3,39)} = 0.251$, $p>0.05$). Furthermore there was not a significant effect of age in the twenty four hour NOR task ($F_{(3,39)} = 0.921$, $p>0.05$).

Paired t-tests at each age between two minute NOR and twenty four hour NOR showed that the only age at which there is a significant difference in performance as measured by DI is the adult age group ($t_{(14)} = 2.625$**,** $P<0.05$). All other ages were not significant (p19/20 – $t_{(11)} = 1.029$, $P>0.05$, p29/30 - $t_{(7)} = 0.423$, $P>0.05$, p34/35 - $t_{(7)} = -0.076$, $P>0.05$)

Finally, t-tests against chance (0) showed that all age groups were performing at a level significantly above chance for the two minutes NOR task (p19/20 - $t_{(11)} = 2.620$, $p <0.05$, p29/30 $t_{(7)} = 3.564$, $p <0.05$, p34/35 $t_{(7)} = 3.914$, $p <0.05$, Adults - $t_{(14)} = 6.439$, $p <0.05$). However for the twenty four hour NOR task, only the three older age groups could perform the task significantly above chance (p29/30 $t_{(7)} = 2.681$, $p <0.05$, p34/35 - $t_{(7)} = 3.226$, $p <0.05$, Adults - $t_{(14)} = 5.416$, $p <0.05$), with the p19/20 age group not producing a DI which is above chance level (p19/20 - $t_{(11)} = 0.914$, $p >0.05$).

5.4 Discussion

The main finding of this investigation was that at p29/30 rats are able to perform the twenty four hour NOR task. If the hippocampus is required for the successful recollection of an object after a twenty four hour delay, we may be able to conclude from this experiment that the hippocampus is functional at p29/30.

A previous study looked at three age groups, p20-23, p29-40 and p50+, finding that the youngest age group could only perform NOR at ITIs of fifteen minutes and one hour, and could not perform the task when there was a twenty four hour ITI, agreeing with the data presented here (Reger et al., 2009). However this study used Sprague-Dawley rats with variable n numbers (n numbers of 13, 17 and 26) in each group. Furthermore, rats were not bred on site and therefore would have undergone transportation which could be argued is somewhat stressful, and rats were cross fostered onto different dams therefore were not littermates. The investigation carried out here used Lister Hooded rats, had comparable n numbers in each group, bred all animals in house therefore eliminating transportation and never cross fostered any pups. Furthermore the Reger et al study used different apparatus for each age group, arguing that objects and arena had to be “down sized” for the youngest age group. These arenas varied in size considerably, with the smallest arena approximately half the size of the largest. Finally, Reger *et al* provided the youngest age group with longer sample phases than the older age group. The authors argue that these measures are improving the task, however it could be argued that the different conditions of the task prevent the direct comparison of results between ages. Therefore the current study, which uses the same time periods and apparatus regardless of age, may provide a more comprehensive understanding of the ontogeny of twenty four hour NOR.

Although a one way ANOVA across the ages on the twenty four hour NOR task showed that there was no effect of age on performance as measured by DI, the youngest age tested in this investigation, p19/20, had a performance score which was not significantly above chance level. This level was not

significantly different from their performance on the two minute task at the same age, but still suggests that at this age the rats are not able to recognise a novel object after twenty four hours.

The failure of the animals to perform the twenty four hour NOR task is probably not due to the lack of development of the hippocampus. This can be said based on other work within the literature that suggests that at p19 rats can perform the hippocampus dependent task, the Morris Water Maze (Rudy et al., 1987). Instead, it is possible that the ontogeny of this task may be due to the development of hippocampal connections with other brain regions. It has been shown that there are connections from the perirhinal cortex to the CA1 region of the hippocampus (Naber et al., 1999), and it may be possible that these connections are required for twenty four hour NOR based on the fact that the perirhinal cortex has been implicated in short delay object recognition (Ennaceur et al., 1997, Bussey et al., 2000) and the hippocampus has been implicated in long delay but not short delay (Hammond et al., 2004). It may be that at this age, p19/20, the perirhinal cortex inputs into the hippocampus are not functional or mature, but by p29 when the animals can perform the long delay task, these inputs are functionally mature.

This task utilised a ten minute sample phase for both two minute and twenty four hour NOR. This was based on the idea that the twenty four hour task may be more “difficult” and therefore an increase in the length of the sample phase may allow the animal to encode more of the sample phase. However, when one looks at the performance score for the adult rats in the two minute task, it can be seen that it is similar to the score in the NOR task in chapter three, when four tasks were performed over two days and the sample phase was

only three minutes in length. The fact that performance did not increase with this extra time for encoding suggests that either a ceiling level has been reached at a score of around 0.5, or that an increase in the length of the sample phase does not improve performance. It would therefore be interesting to test the twenty four hour NOR task with a three minute sample phase to see if the length of time the animal has to encode the memory has an effect on the performance as measured by discrimination index.

The only age group at which there was a significant difference between the two minute and the twenty four hour task was the adults age group, where the performance at the long delay was significantly lower (although still above chance) than at the small ITI. This suggests that this version of the task is more “difficult”, however one must be careful not to anthropomorphise the behaviour.

To conclude, this investigation has demonstrated that at p29/30, rats are able to perform the NOR task with a twenty four hour ITI, a task which has been suggested to be dependent on the hippocampus. Prior to this age rats are unable to perform the twenty four hour NOR task, but are able to perform other hippocampus dependent tasks (Rudy et al., 1987), suggesting that the development of the hippocampus itself is not the crucial factor in the development of the twenty four hour NOR task. Instead, it is hypothesised that the development of inputs from the perirhinal cortex to the hippocampus may be the crucial factor.

Chapter 6

Further investigation of the ontogeny of context processing using a context dissociation task

Practical work for this chapter was carried out by Mr Brian O'Dioluin as part of his BMSc intercalated undergraduate degree, under the supervision of Miss Lyon who was responsible for experimental design and interpretation. All data was reanalysed by Miss Lyon for the use in this thesis.

6.1 Introduction

The aim of this experiment was to build on the OC task from chapter 4, some of the results of which are shown in figure 6.1. In that task the development of the OC task was not completely clear, although the statistics indicated that the development was between p38 and p42.

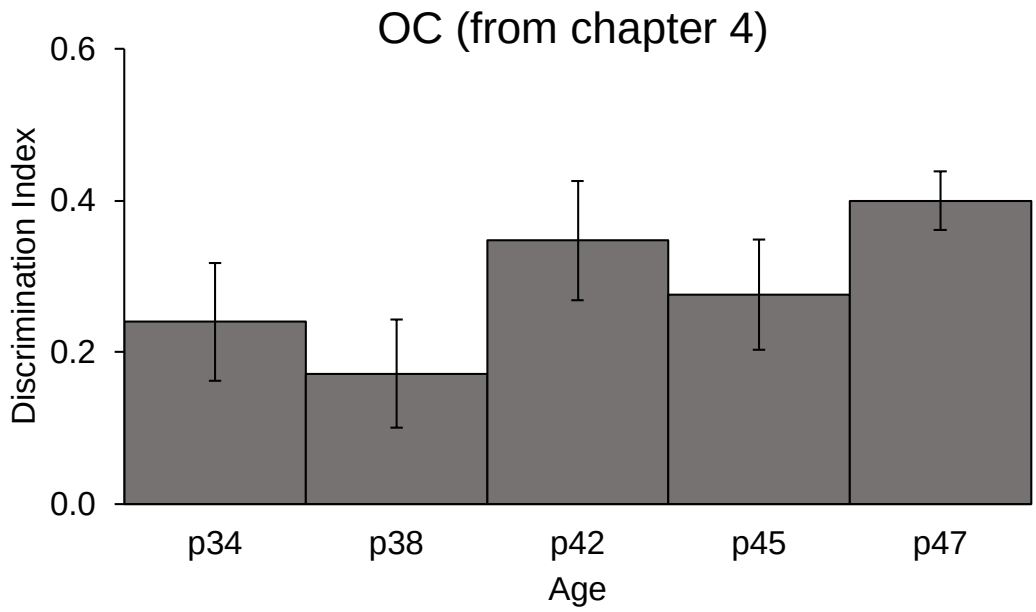

Figure 6.1 Results from object context (OC) task from ages p34-p47 as shown in chapter 4. *Statistical analysis showed that between p38 and p42 the ability to perform OC developed.*

Whether or not the hippocampus is necessary in contextual processing and memory is a debated topic. The OC task used so far in this thesis is one of the most commonly used spontaneous object recognition tasks when testing memory for contexts. Mumby et al showed that rats with HPC lesions cannot perform the OC task, however Langston and Wood found that OC was not affected by HPC lesions. When looking at the methodology of these papers, there is one main difference, and that is the contexts themselves. In Langston and Wood the contexts are in the same testing arena, within the same experimental room and with the same extramaze cues (Langston and Wood, 2010). However in Mumby et al the contexts are in entirely different rooms,

with the rat having to be transported from one room to another between contexts (Mumby et al., 2002).

Allocentric spatial coding is when one understands where something is with respect to other objects, whereas egocentric spatial coding is when the location of objects in space is understood by referring to the position of oneself. Allocentric spatial representation of an environment has been shown to be dependent on the hippocampus (Holdstock et al., 2000, Morris et al., 1986, Morris et al., 1990, Ramos, 2013), whereas egocentric spatial mapping can be hippocampus independent. (Zaehle et al., 2007).

This may explain why Langston and Wood and Mumby et al had different conclusions on whether the OC task requires the HPC. The test is extremely sensitive to changes, and by having the extramaze cues remain the same between contexts, as well as always putting the rat in the testing arena facing the same direction, Langston and Wood allowed the rats to process the space egocentrically and therefore may have avoided the use of the hippocampus.

A variation on the NOR task has been used by O'Brien et al in an attempt to understand under which conditions HPC lesions affect object recognition in rats (O'Brien et al., 2006). The task they used was three variations of the NOR task with OC influences. The first task was basic NOR, where the context did not change between the sample and the test phases. The second task was NOR but where the context changed between the sample and test phase and both the first and the second contexts were familiar, which the rat had encountered before but not within this task. The third task was NOR with changing contexts again, but this time the second context was an unfamiliar,

entirely novel context which the rat had never encountered. The logic behind the experiment is based on the fact that rats with HPC lesions typically display good object recognition and that the sensitivity of normal rats' object recognition is affected by contextual changes (Dellu et al., 1997). They hypothesised that a change in context should have little effect on object recognition in HPC lesion rats if they cannot remember the context in which they encountered an object, however intact rats would be more sensitive to a context change. This is because the contexts which O'Brien et al used were more like the Mumby et al experiment, as the contexts were in entirely different rooms with different extra-maze cues, therefore possibly making the contextual processing hippocampal dependent in this scenario.

The results from this investigation were that control rats recognised novel objects in all three test conditions whereas HPC lesioned animals could only do the basic NOR task and did not correctly identify and explore a novel object if the context had changed. O'Brien et all suggests that this shows that the hippocampus is not required for recognising an object if it is in the same context as previously encountered, however it is required if it is seen out with the context in which it was encoded.

However, some representation of the context must have been acquired and retrieved otherwise the HPC lesioned rats should have treated the tasks in which the contexts changed as simple NOR and would have produced a positive score. The HPC lesioned rats actually produced a score that was more indicative of chance performance, ie they did not prefer either the novel or the familiar objects, which suggests that they either did not recognise either object as familiar, or they did not recognise that either object had changed.

Further evidence for the idea that HPC lesioned animals can recognise contextual change can be found when looking at a study by Wilson et al which investigated the effect of Lateral Entorhinal Cortex (LEC) lesions on performance of rats in the OC task. This study used extramaze cues which were consistently present between context, therefore possibly making the task hippocampal independent. They demonstrated that LEC lesioned animals were unable to recognise object-context associations yet showed normal object recognition and context recognition, suggesting that contextual information is integrated with object identity in the LEC.

The entorhinal cortex is the main input into the HPC, therefore if the HPC is lesioned as in O'Brien et al, the object-context association cannot be utilised in a normal way and therefore the animal may show a deficit in the task. The LEC is still intact therefore the contextual information is still acquired, however it cannot proceed down the pathway to the hippocampus, which is required for O'Brien's hippocampus-dependent, allocentric context changes.

This chapter utilises the task that O'Brien et al used, however only the basic NOR and NOR with a context change to a familiar context; named Context Dissociation Task (CDT). The task where the test phase was entirely novel was not used.

As previously described, the OC task is a test of the associations between context and object, however we are using this task to test the ability of rats to dissociate an object from its context. This allows us to approach the question of context memory development from a different angle.

6.2 Methods

Equipment used was the same as in previous testing seen in chapters three and four, including the testing arena, the object types and the recording equipment. Rats were bred, housed and transported to and from the testing room as described in previous chapters.

Following on from five days handling and two days habituation as per previous chapters, the same rats were tested at a number of ages on two tests. On each day the rats performed both tests, with half the rats tested on NOR in the morning, and CDT in the afternoon (See figure 6.2 for explanation of the tasks), and the other half of the rats performing CDT in the morning and NOR in the afternoon. Objects and contexts were counterbalanced between and within rats as described previously.

Two litters of rats were used, with one litter consisting of 2 male and 6 female, with the other litter 4 male and 4 female. All rats were bred in house under the same conditions as previously described.

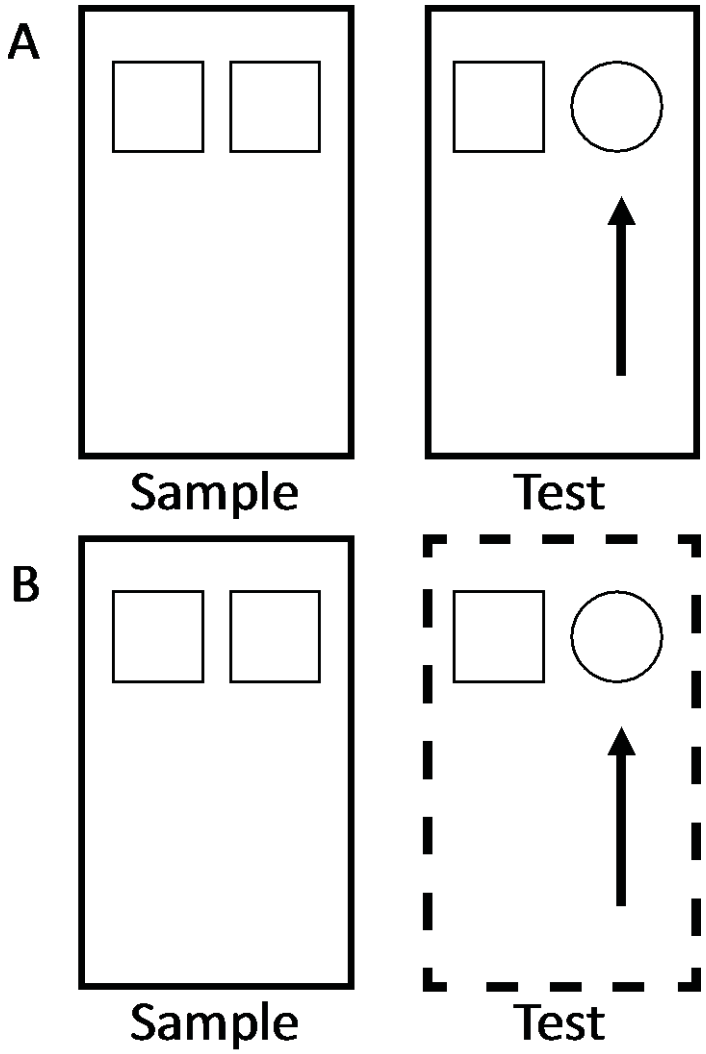

Figure 6.2 Diagrams showing the object configurations for the Context Dissociation Task (A) which was test alongside Novel Object Recognition (B)
A - Novel Object Recognition (NOR) task. Two identical objects are placed in the centre of the northwest and northeast quadrants for the 3 minute sample phase. After a two minute interval, the rat is placed into the same context containing a copy of the sample phase object, and an entirely novel object.
B - Context Dissociation Task (CDT). Two identical objects are placed in the centre of the northwest and northeast quadrants for the 3 minute sample phase. After a two minute interval, the rat is placed into a familiar but different context containing a copy of the sample phase object, and an entirely novel object.

6.3 Results

As shown in figure 6.3, on p34 the rats can perform both NOR and CDT tasks, on p40 their performance in CDT decreases, on p41 their performance in both tasks is low, and then on p47 the performance of the rats is improved back to the level it was at on p34.

A one-way ANOVA across the ages for each task showed that there was a significant effect of age for NOR ($F_{(3,68)} = 7.146$, $p < 0.05$) and CDT ($F_{(3,68)} = 3.034$, $p < 0.05$). Bonferroni multiple comparison posthoc tests showed that for NOR rats' scores on p41 was significantly different to p34, p40 and p47. For CDT rats' scores were never significantly different between ages.

In order to understand if the exploration levels of the rats changes across the tasks, total average exploration was investigated. The sum of exploration at both objects in each phase was generated, before an average taken of all phases.

A repeated measures ANOVA was conducted on total exploration times with age as within-subjects factor and task as a between subjects factor. There was a significant effect of age ($F_{(3,65)} = 3.089$, $p < 0.05$), no effect of task ($F_{(1,30)} = 1.362$, $p > 0.05$) and no interaction between task and age ($F_{(3,65)} = 2.763$, $p > 0.05$).

A repeated measures ANOVA was conducted on DI with age as within-subjects factor, and time and task as between subjects factors. There was a significant effect of age ($F_{(3,84)} = 7.239$, $p < 0.05$) but no significant effect of time ($F_{(1,28)} = 0.025$, $p > 0.05$) or task ($F_{(1,28)} = 2.121$, $p > 0.05$). There was a significant interaction between age and time ($F_{(3,84)} = 4.007$, $p < 0.05$), but no

significant interactions between age and task ($F_{(3.84)}$ = 1.463, p > 0.05) or between age, time and task ($F_{(3,84)}$ = 0.208, p > 0.05).

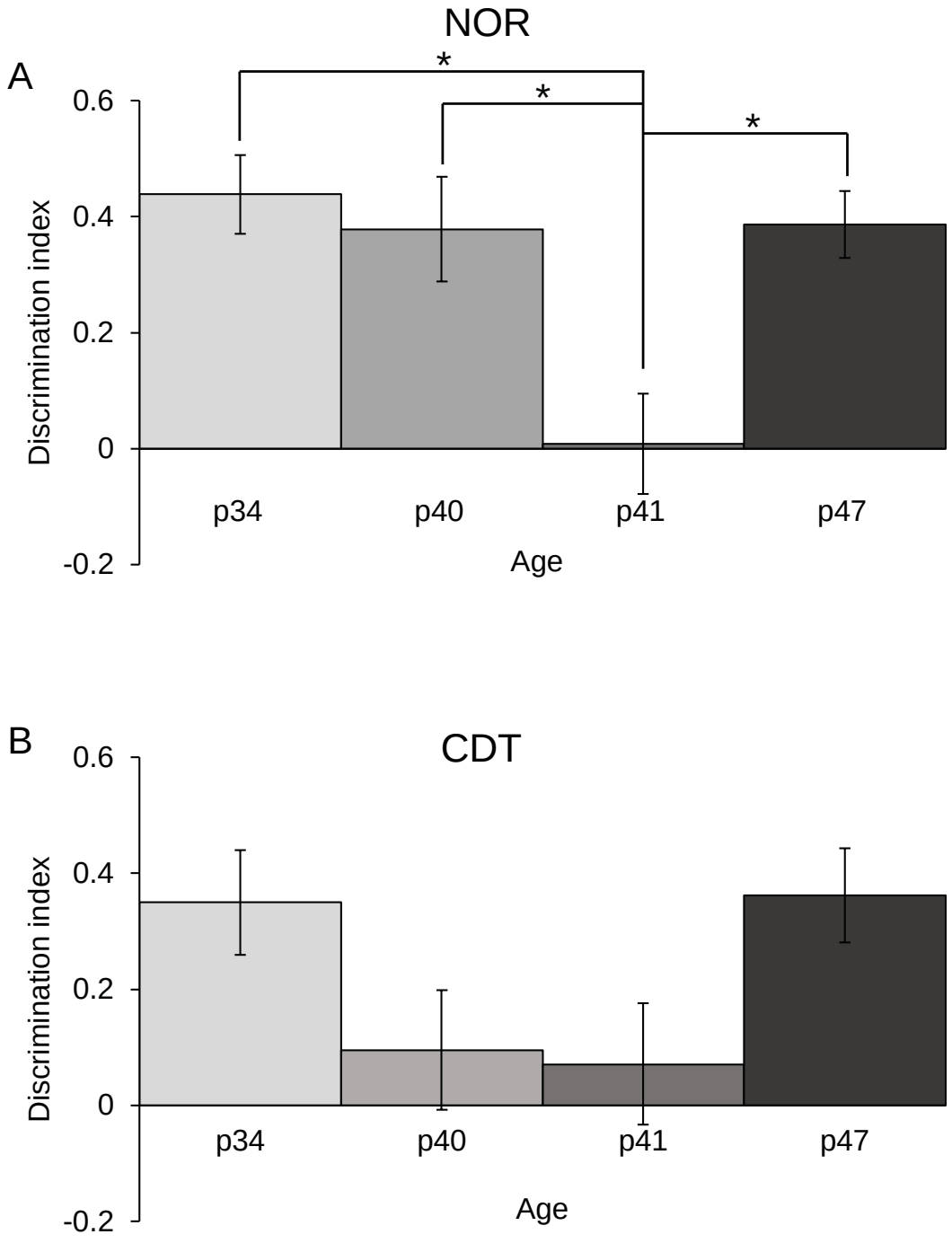

Figure 6.3 Results from A) novel object recognition (NOR) and B) context dissociation task (CDT). *Stars denote significance (p<0.05) in an independent sample t-test.*

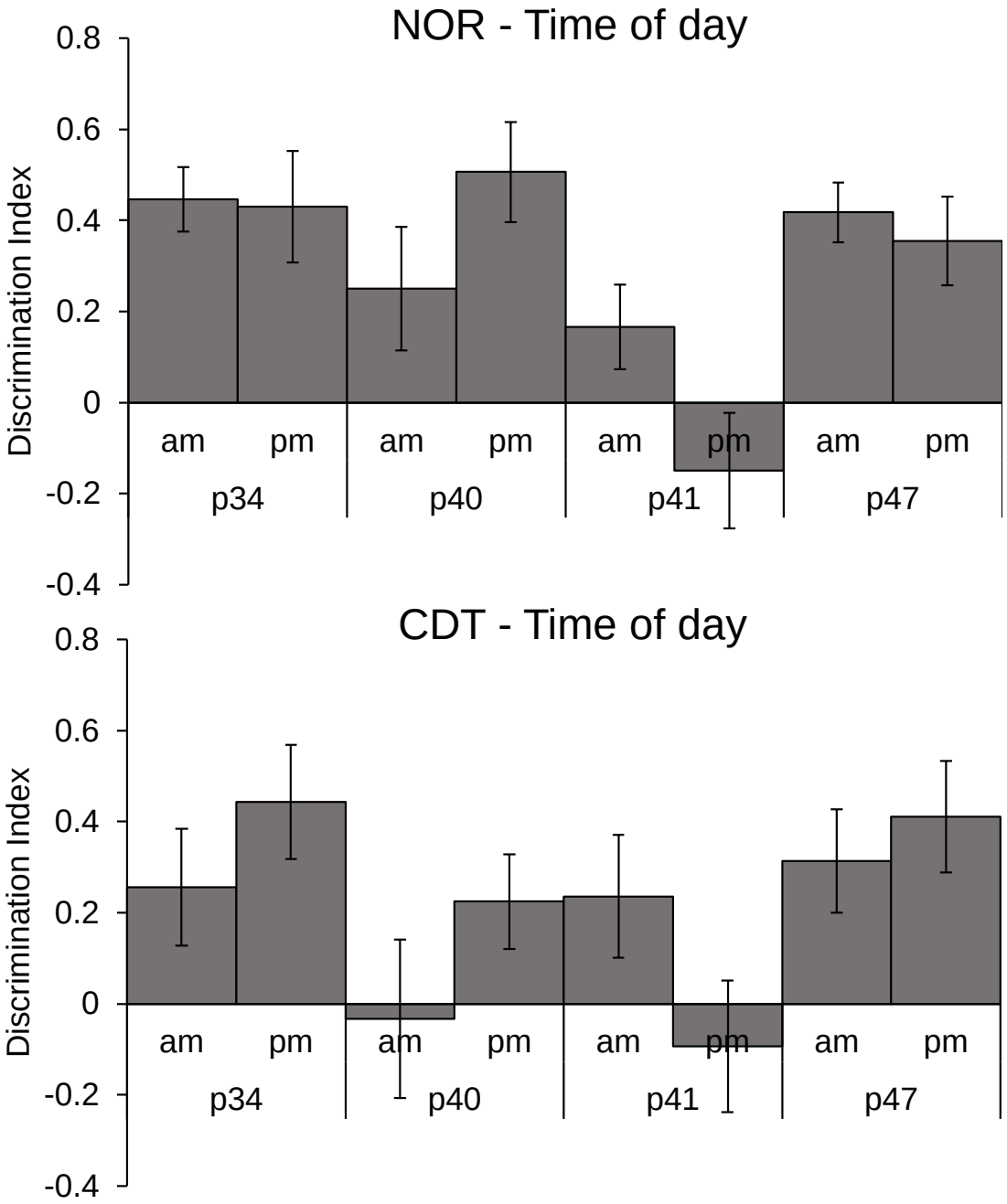

Figure 6.4 Mean discrimination index on both novel object recognition (NOR) and context dissociation task (CDT) on ages p34 to p47, separated into morning and afternoon.

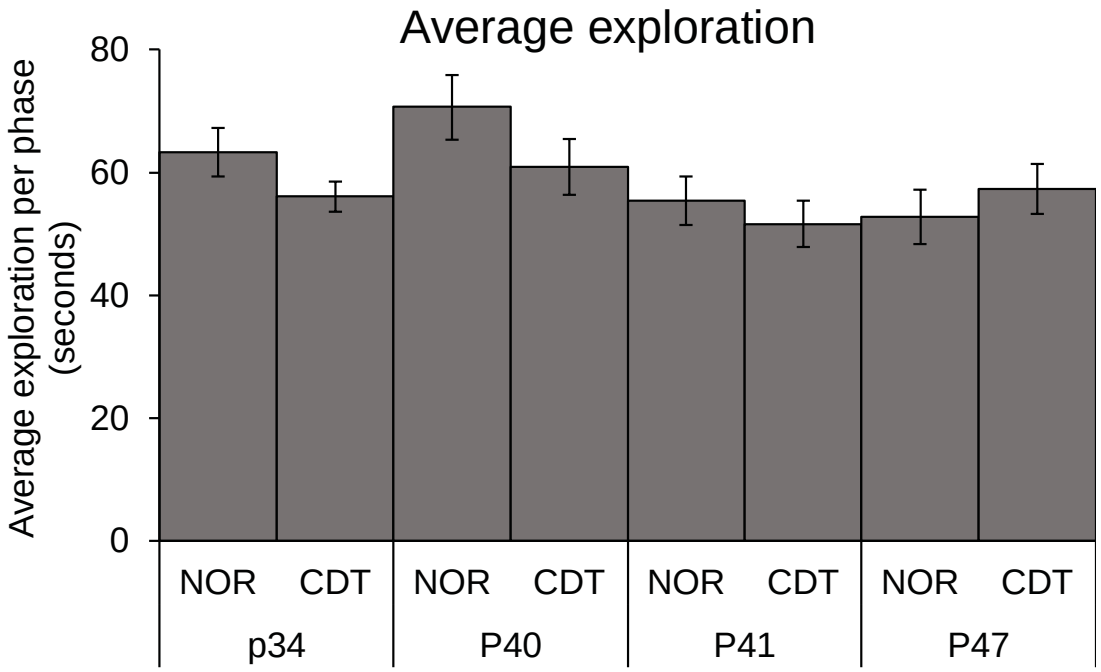

Figure 6.5 Mean exploration on both novel object recognition (NOR) and context dissociation task (CDT) on ages p34 to p47.

6.4 Discussion

When interpreting the data the following hypothesis is proposed. In order to perform the CDT task correctly, rats encode contextual information in the sample phase as well as object identity and spatial information. Then in the test phase they must dissociate the object from the context in which they last saw the object, in order to recognise that one of the objects is familiar and the other is novel. In other words, if they are unable to dissociate the object from the context, they may see a completely different scene when the object is presented against a new background. If this is the case, and the animal cannot dissociate the object from the context, one would assume that it would spend an equal amount of time at both objects in the test phase as both objects would appear novel. However, there is another circumstance where the animal may not be able to perform CDT but is still able to generate a positive discrimination

index. In this scenario the rat is unable to encode contextual information but is able to process object identity. Therefore when the context changes in the test phase, the animal is unable to recognise that the context has changed but can recognise that one object has changed, therefore shows a preference for the novel object and generates a positive discrimination index. I hypothesise that this is the scenario that is happening on p34 when the rats generate a positive discrimination index in both NOR and CDT.

On p40, the rats can perform NOR however when they are tested on CDT they are unable to recognise the novel object in the test phase. The fact that the rats were able to recognise object identity in NOR, suggests that it is not the case that they aren't performing at CDT due to a lack of object encoding. Rather, it is possible that at this age the rats are able to process, encode and retrieve contextual information but cannot dissociate the objects from their context. The "familiar" object in the test phase may appear novel to the rat because the rat is unable to dissociate it from the sample phase context., if this was the case one might think that there would be overall an increase in exploration in CDT compared to NOR, if the rat was treating both objects in the test phase as novel. Figure 6.5 shows the mean average exploration across both sample and test phase and no difference is seen between any task or age. To establish if there is an increase in exploration in CDT compared to NOR on p40, one must look only at the test phase exploration, which can be seen in figure 6.6, and again there is no difference in exploration between the two tasks. The fact that there is no increase in exploration in CDT compared to NOR suggests that either the familiar object is not perceived as novel by the rat, or that the exploration level has reached a "ceiling point".

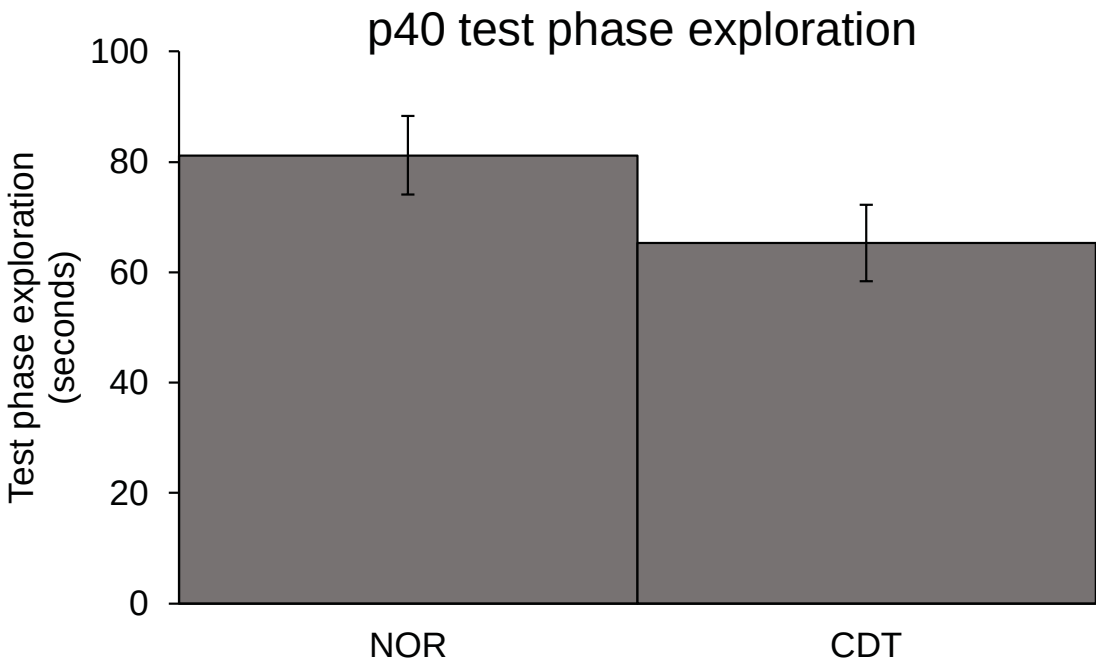

Figure 6.6 Mean exploration within the test phase of novel object recognition (NOR) and context dissociation task (CDT) on p40. *There is no significant difference between the two tasks,*

On p41 the rats are unable to generate a positive discrimination on either NOR or CDT, ie they cannot distinguish between the novel and familiar object in the test phase on either task. The hypothesis generated for this behaviour is as follows; - The day before this was the first day tested where the rats were able to utilise the contextual information in their memory formation of the sample phase. However, they may be as yet unable to dissociate an object from its surrounding context. In the afternoon of p40, the rats seem to suddenly be able to utilise contextual information for the first time. This contextual change may be so salient that when they tested on NOR the following morning on p41, the novel object in the test phase is not salient enough to overcome the saliency in the context change. The perirhinal cortex is required for NOR (Bussey et al., 2000, Ennaceur and Aggleton, 1997) and perirhinal cortex lesions produce only mild, delay dependent impairments of OC (Norman and Eacott, 2005). The perirhinal cortex sends projects to the EC (Burwell and

Amaral, 1998) which in turns inputs into the hippocampus. Furthermore there is a direct pathway between the perirhinal cortex and the CA1 of the hippocampus (Naber et al., 1999).

It is possible that when the LEC connections with the hippocampus mature on or before p40, the input into the hippocampus is so strong that it overpowers the input from the perirhinal cortex and therefore the object recognition ability of the animal is compromised. However, it is unlikely that naïve rats tested on a single day of p41 are unable to perform NOR having never been tested on CDT, although this has not been tested so cannot be dismissed. Instead it is more likely that the experience of the rat on p40 had an effect on it's performance on p41. It is possible that the LEC input into the hippocampus was utilised for the first time in a task on p40 in CDT, and therefore the following day the input from the perirhinal cortex recognising the object change, was not strong enough to overcome the LEC input that it experienced the day before.

The next time the rats were tested was at p47, and at this age the rats can generate a positive discrimination score on both tasks. As previously discussed there are two ways in which a rat can generate a positive discrimination index on CDT – either by performing the task "correctly" and recognising both the object change and the contextual change and dissociating the object from the context, or by "ignoring" the context change and treating the task as a basic NOR task. By examining the discrimination indices it may be possible that it has been understood how the rats are performing the CDT task. However I hypothesise that because the rats showed a decreased performance on p40 and 41, on p47 when they generate a

positive score, they are possibly performing the task in an adult like way, ie dissociating the familiar object in the test phase from the sample phase context and recognising it as familiar even though it's in a mismatched context.

Further support for this theory comes when one looks back to the data generated in chapter 4 of this thesis. The "object context" (OC) task used there was one in which the rat had to associate the object with a context and therefore remember which object it saw in that context previously. However the "novel" object is not entirely novel in this task, it was previously encountered in another context within the task. Therefore in order to correctly identify which object is in a novel configuration in the test phase of OC, the rat must first associate the familiar object with the context, but also dissociate the novel object from the context in which it saw it previously. The results generated from the OC task show less of an abrupt development as the OP and OPC task. If the rats are able to recognise contextual information at p40 and 41 but are unable to dissociate an object from its context, as shown by the CDT task, then the OC task will be somewhat difficult. If they can associate the familiar object with its context, but not dissociate the "novel" object from its context then both objects will appear similar in their saliency resulting in a discrimination index close to 0.

Figure 6.1 shows the results from the OC task in chapter 4, at the days tested in this chapter. The rats show a general trend of improving across the days, however the statistics used showed that between p38 and p42 there was a significant change in discrimination index. This age coincides with the behavioural changes seen in this chapter, and together they suggest this this age, p38 to 42, is an important time point in the development of adult-like

contextual processing. Based on this behavioural evidence, it is possible that this is the age at which the LEC inputs into the hippocampus develop. As discussed in section 6.1, the EC is the main input into the hippocampus, and therefore if the input is not present as in lesion studies, or not mature as in development, the animal will show a deficit in the ability to utilise contextual information in its formation of memories.

It was discussed in section 1.5.5 that in a fear conditioning paradigm there is a difference in the age at which the animal can form and maintain a memory of contextual information, and the age at which they can associate this memory with a fear-inducing stimulus (Foster and Burman, 2010). This supports the hypothesis that in our task the rats are able to recognise the change in context in CDT at an age before they can utilise that information to form a memory. Interestingly, contextual fear conditioning develops in rats at p23, with cued fear conditioning, where there is no context memory required develops at p16 (Foster and Burman, 2010). This is much earlier than this investigation has demonstrated that context memory develops. This may be due to the fact that in contextual fear conditioning there is a strong input from the amygdala to the hippocampus (Sah et al., 2003). This input from the amygdala which develops at p21-24 (Chareyron et al., 2012) and may provide an explanation as to why contextual information is encoded and utilised much earlier in tests where there is a strong emotional element. In all the behavioural tests conducted in this study, there are no obvious emotional elements such as fear or positive reinforcement such as food. Therefore the amygdala input is likely to be less strong and the task may rely more upon the LEC input to the hippocampus to provide the contextual processing ability.

In future, it would be beneficial to test naïve rats on p41 to clarify whether the lack of performance on p41 is due to the experience that they had the day before, as well as testing naïve rats on CDT at a number of ages including p40 and 41. This would allow us to understand if the deficits seen in the CDT and NOR tasks on these days in this investigation were due to experience or if the deficits are also seen in rats which have never been tested previously.

Finally, this investigation has shown that on p42 rats do not perform well on NOR nor CDT tasks, yet on p46 they perform well on both tasks therefore it would be interesting to uncover how the animals behave in this task during the ages of p42-46 as so far this has not been carried out.

Chapter 7

Narrowing down the critical period for episodic memory ontogeny

7.1 Introduction

In chapter four it was demonstrated that the ability to perform OP and OPC develops between p46 and p48. The rats performed OP in the morning and OPC in the afternoon of either p46 or p48 and the nature of the protocol meant that rats were never tested on p47, and OP was never in the afternoon and OPC never in the morning. All rats had experienced NOR and OC tasks on the previous day (See figure 3.3 for explanation of the tasks).

In order to understand when exactly the OP and OPC task develop between p46 and p48 this chapter utilised a slightly different protocol. It aimed to understand

- if time of day had an effect,
- how the animals behaved on p47
- whether the fact that they had performed NOR and OC on the previous day had any effect on their ability to perform the task on p48.

In order to test for any effect of time of day, the order in which the tasks were performed was reversed. Testing animals on p46, p47 and p48 allowed the understanding of how the animals perform on OP and OPC on the previously un-tested p47 as well as the conformation of the behaviour on p46 and p48. Finally, to understand whether previous testing either on NOR or OC as per chapter four, or on OP and OPC as per this chapter, rats were tested solely on p48.

7.2 Methods

7.2.1 Three consecutive day testing

Equipment used was the same as in previous testing seen in chapters three and four, including the testing arena, the object types and the recording equipment. Rats were bred, housed and transported to and from the testing room as described in previous chapters. This task varied from the previous testing where rats were never tested on the same task twice. Instead one set of rats were tested on OP in the morning and OPC in the afternoon for three consecutive days at ages p46, p47, and p48, and one set of rats tested at the same ages, but with OPC in the morning and OP in the afternoon. Different objects were used for each task and each day, and each rat never saw the same set of objects more than once. All objects, locations and context orders were counterbalanced in the same manner as described in chapter three,

On each of the three testing days, the rats were taken into the testing room in the morning. The rat to be tested was put into the holding box briefly while the testing arena was configured for the sample phase. The rat was then placed into the arena and allowed to explore the sample phase objects for three minutes, after which it was then placed back into the holding box while the arena was configured for the test phase. Finally it was then allowed to explore the test phase for three minutes. In the afternoon of the test days for the 3 day consecutive testing, the rats were tested in a similar way but this time on the other task. For all testing, the rats were recorded using an overhead camera and the exploration was timed by the experimenter.

7.2.2 Object place (OP) and object place context (OPC) on p48 only

Testing was carried out in the same arena, with the same cues and object types as the two day/ four task protocol in chapters three and four. Rats were handled, transported and habituated in the same way as the two day / four task protocol, and the room layout was identical. In this task the rats were only ever tested on one day, at p48, on the OP task in the morning and OPC task in the afternoon. All testing procedures such as the holding box, recording and timing were all carried out in an identical manner to all other tasks.

7.3 Results

7.3.1 Three consecutive day testing on OP and OPC

A repeated measures ANOVA was conducted with task as within-subject factor, age as between subjects factor and time of day as a covariate.

There was a significant effect of age ($F_{(2,41)}$ = 16.807, p <0.05), but no significant effect of task ($F_{(1,41)}$ = 1.653, p >0.05), interaction between task and time of day ($F_{(1,41)}$ = 1.990, p >0.05) nor interaction between task and age ($F_{(2,41)}$ = 0.983, p >0.05).

Despite the lack of significant effect between age and task, there was an effect of age, therefore one sample t-tests against chance were conducted for each age on each task. This showed that on p46 and p47 rats did not perform above chance, however on p48, both in the morning and afternoon, rats performed each task significantly above chance (OP - p48am; $t_{(7)}$ = 2.935, p<0.05, p48pm; $t_{(7)}$ = 2.228, p<0.05. OPC - p48am; $t_{(7)}$ = 5.988, p<0.05, p48pm; $t_{(7)}$ = 5.439, p<0.05).

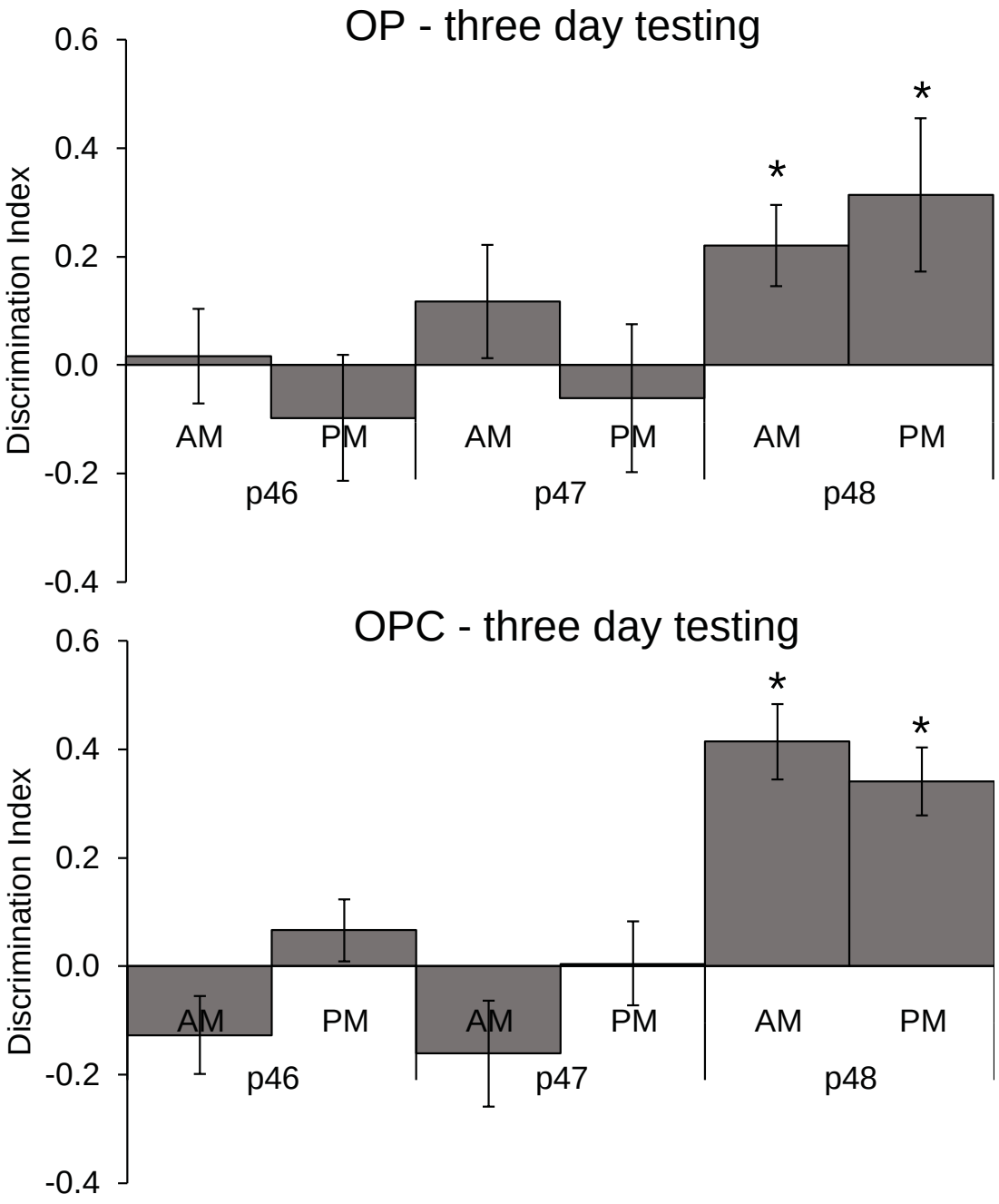

Figure 7.1 Three consecutive day object place (OP) and object place context (OPC) testing results. *Rats performed significantly above chance on both tasks on p 48 only, with no difference in performance between morning and afternoon.*

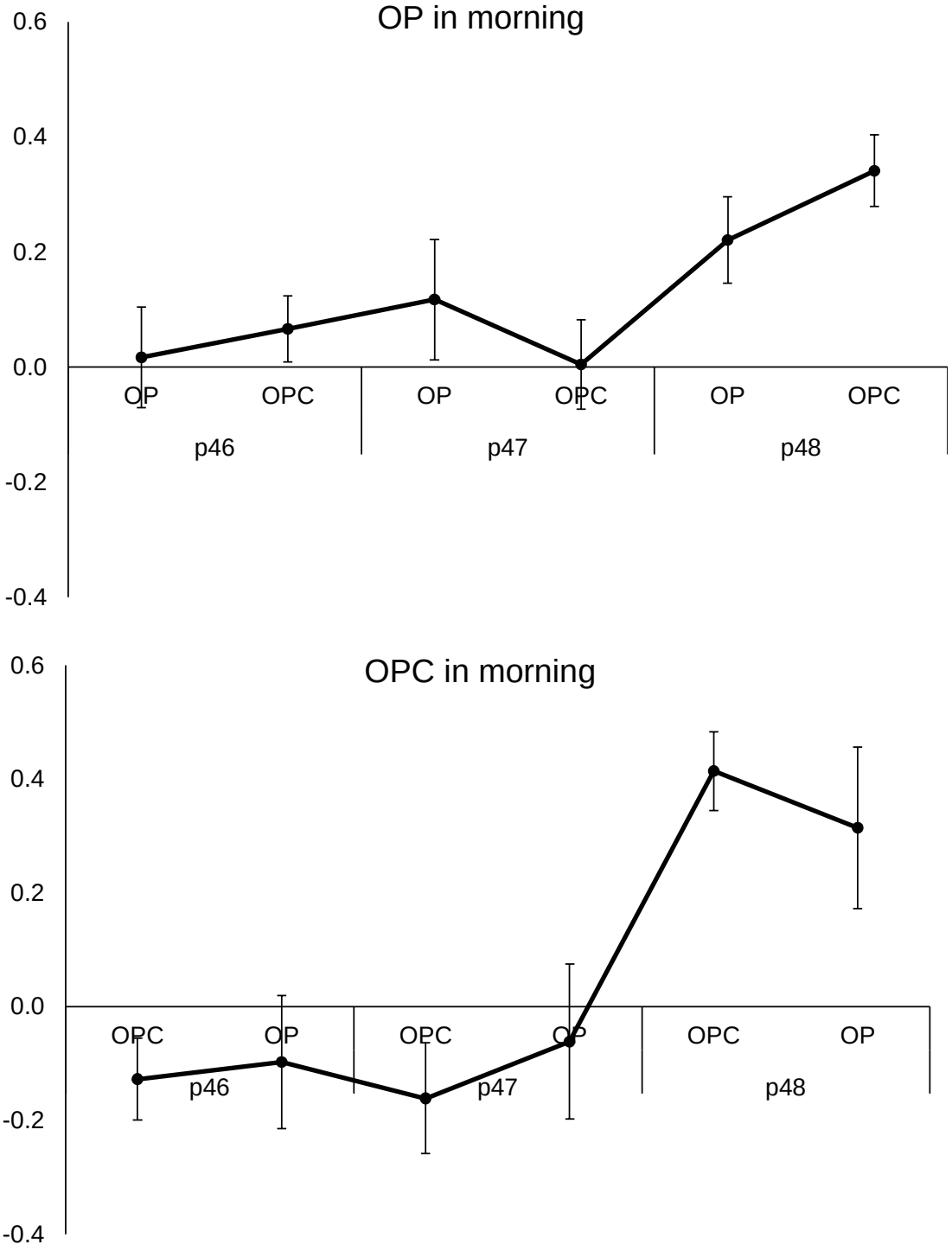

Figure 7.2 Line graphs separating rats which completed Object Place (OP) first from rats which completed Object Place Context (OPC) first. *Rats which completed OPC first showed a lower performance on p46 and p47 compared to rats which completed OP first, however the time of day effect was not significant.*

7.3.2 OP and OPC on p48 only

A one way ANOVA between the tests show that there was a significant difference between the performance in OP and OPC ($F_{(1,14)} = 27.061$, $p < 0.05$). Furthermore a one sample t-test against chance (0) showed that rats performed significantly above chance on both tasks (OP – $t_{(7)} = 4.122$, $p < 0.05$, OPC - $t_{(7)} = 7.590$, $p < 0.05$).

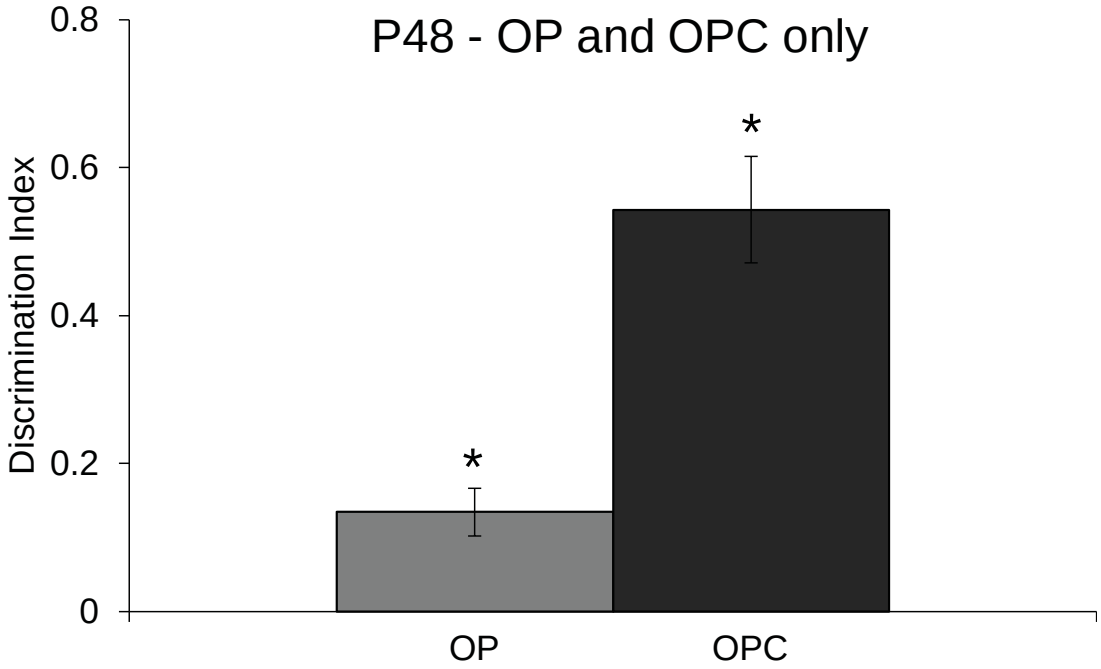

Figure 7.3 Object place (OP) and object place context (OPC) results on p48 only. *Rats could perform both tasks significantly above chance, with performance on OPC significantly better than performance on OP.*

7.4 Discussion

In chapter four it was shown that the juvenile rats had a sharp increase in performance between p46 and p48. This chapter aimed to narrow down the exact time at which episodic memory and spatial memory developed by testing rats multiple times around this time. It was shown that rats which are tested on OP and OPC on three consecutive days show the same development as the rats that took part in the two day/ four task protocol. In chapter four p47 rats were never tested on these two tasks as they instead were tested on NOR and OC due to the two day protocol. Therefore it was interesting to show that on p47 rats are unable to perform the OPC task. Furthermore, the ability to perform both tasks develops on the same day. This task also investigated the effect of time of day, as the abrupt development raised questions about whether or not the time of day would have an effect on performance. It was shown that rats performed the same regardless of whether they were tested in the morning or the afternoon. This suggests that any physical or neurological changes that may be occurring at this age are not developing gradually throughout the day, and instead may develop during the dark phase between p47 and p48. Furthermore, naïve rats tested on p48 only on the OP and OPC tasks show that they can perform both tasks without previously being tested on any task, demonstrating that previous experience is not necessary for this development.

As shown in figure 7.2, rats that were tested on OPC in the mornings tended to show a lower performance on p46 and p47 than rats that were tested on OP in the mornings, however this effect was not significant. The OPC task involves a change of context which may interfere with learning, however within the

literature there is evidence that context changes in fact do not interfere with memory (Rosas et al., 2013, Leon et al., 2012).

This age, p47/48, has not previously been implicated in any specific developmental changes, however this is a time point which is during adolescence, after puberty but before full adulthood. As has previously been discussed, the OPC task is dependent on the hippocampus whereas the OP task is not (Langston and Wood, 2010, Eacott and Norman, 2004). One might assume that these tasks are developing due to the maturation of the hippocampus, however if that was the case the rats may already have the ability to perform the non-hippocampal OP task prior to this day. The OP task has been shown to rely on the entorhinal cortex, specifically the lateral entorhinal cortex (Wilson et al., 2013b), and is not dependent on the hippocampus. Furthermore the perirhinal cortex is required for the NOR task (Norman and Eacott, 2005), and the postrhinal and lateral entorhinal cortices are required for the OC task (Wilson et al., 2013b, Wilson et al., 2013a, Norman and Eacott, 2005). It could be hypothesised that on this day the entorhinal cortex may mature and allow the animal to perform the OP task, and therefore the hippocampus may be able to integrate the information from all components of the OPC task.

A study looking at the development of spatial memory in children between eighteen months and five years of age showed results which may support this hypothesis, or at least demonstrate that something similar may occur in humans (Ribordy et al., 2013). Testing involved asking children to find rewards which were hidden under a number of cups within an open field arena. Entry points to the arena could be varied, as could local cues. The results of the

experiment showed that a basic form of allocentric spatial memory is present by two years of age (Ribordy et al., 2013). However, the ability to distinguish and remember closely related spatial locations improves between the ages of two and three and a half years old, which also coincides with an increase in episodic memories in children (Ribordy et al., 2013). The authors suggest that the initial development of spatial memory at two years of age coincides with the functional maturation of the CA1 region of the hippocampus, and the improving memory capacity up to the age of three and a half coincides with the maturation of the dentate gyrus and the trisynaptic pathway of the hippocampus (entorhinal cortex to dentate gyrus, dentate gyrus to CA3 via mossy fibre projections and CA3 to CA1 via Schaffer collateral pathway) (Ribordy et al., 2013). This pathway may also be involved in the processing of our OPC task and if the entorhinal cortex is the “rate limiting step” then once it matures the pathway can input into the hippocampus.

In future it would be interesting to reverse the light cycle to see if the development of OP and OPC tasks change, as the data generated here suggest that the development of these tasks occurs during the dark phase as all testing was condicted in the light phase.

Chapter 8.

Spatial memory ontogeny in juvenile rats; allocentric vs egocentric and intramaze vs extramaze cues

8.1 Introduction

Previous chapters have demonstrated that the OP task develops at p48 when conducted either as part of the two day, four task protocol, when it's tested three times on consecutive days at p46, p47 and p48 or when it is tested on p48 only. Both these protocols include cues which hang just inside the arena in the Northwest and Northeast of the arena which are intramaze to some extent but are not within reach of the rats. This task is presumed to be egocentric in nature due to the fact that the rats enter the arena at the same place and orientation every time, and that object locations remain constant throughout and are marked by prominent 3-dimensional cues as well as the fact that hippocampal lesioned rats can also perform this task (Langston and Wood, 2010). An allocentric version of the OP task is the novel place task, where rats are exposed to two objects in the test phase, with one in an entirely novel location and the rat is placed equidistant from the objects, therefore in a novel starting location (see figure 8.1 for a comparison of the egocentric task used previously in this thesis, and the allocentric task used here). This task is allocentric due to the requirement of the animal to create a spatial map of the environment, and if the rat codes space egocentrically it may look like nothing has changed in terms of object locations (due to the starting position being still equidistant from the objects). Furthermore, this version of OP, so called novel place, has been shown to be dependent on the hippocampus, rats with hippocampal lesions cannot recognise which object has moved location (Mumby et al., 2002, Langston and Wood, 2010).

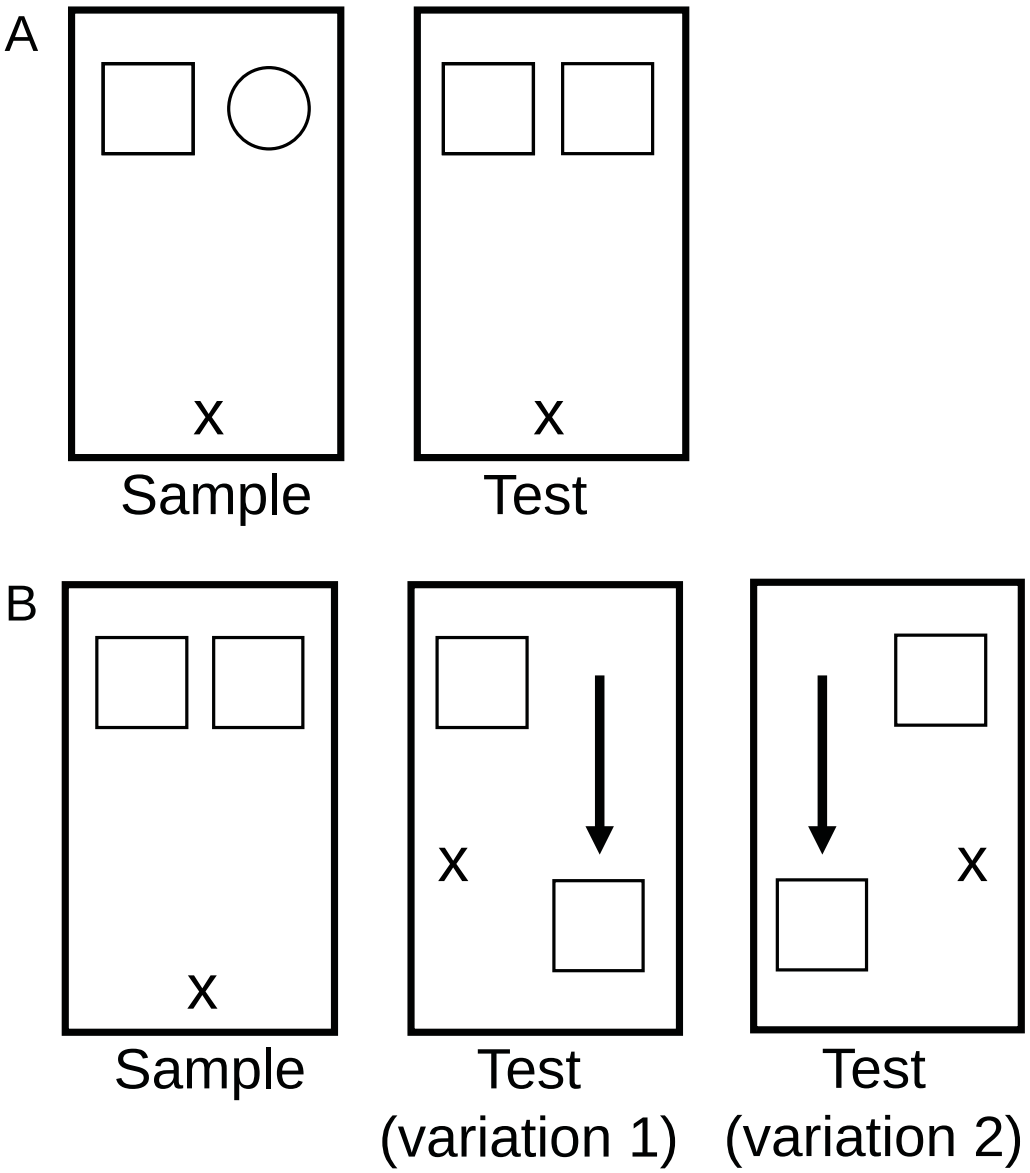

Figure 8.1 Comparison of egocentric (A) and allocentric (B) versions of the OP task. A - *Egocentric object place task where x represents the location at which the rat is placed in the box facing the south wall. B - Allocentric novel place task where x represents the location at which the rat is placed in the box facing the West wall in variation 1 of the test phase and the right wall in variation 2 of the test phase.*

8.2 Method

8.2.1 Novel Place Task

Equipment used was the same as chapters three and four, including the testing arena and the recording equipment. Rats were bred, housed and transported to and from the testing room as described in previous chapters. The arena was configured with the walls from Context 2 (zebra print) but with a different floor, a smooth sticky-back plastic with a woodgrain effect stuck onto cardboard. This was because the original floor used previously had velcro stuck on the floor which would be visible when the objects were moved to the novel location. Objects were stuck down with blu-tack allowing them to be moved to the novel position without leaving any mark as to where they were previously.

Following on from five days handling and two days habituation as per previous chapters, the same rats were tested on p46 and p49. Rats were given two identical objects in the sample phase placed in the centre of the Northwest and Northeast quadrants of the arena. Rats were allowed to freely explore the objects for three minutes before being placed into the holding box for one minute, during which the arena was cleaned and rearranged. The test phase consisted of two more copies of the sample phase object, with one object moved to a novel location. The novel location was the centre of either the Southwest or Southeast quadrants, with each rat only seeing one of these locations before returning to the home cage. The two variations of the test phase were counterbalanced across all the rats. Figure 8.2 shows a diagram of the task with the x marking location where the animal was placed into the

arena. This location changed between sample and test phase to ensure that the rat was placed in equidistant to both objects in the test phase thus following an allocentric strategy.

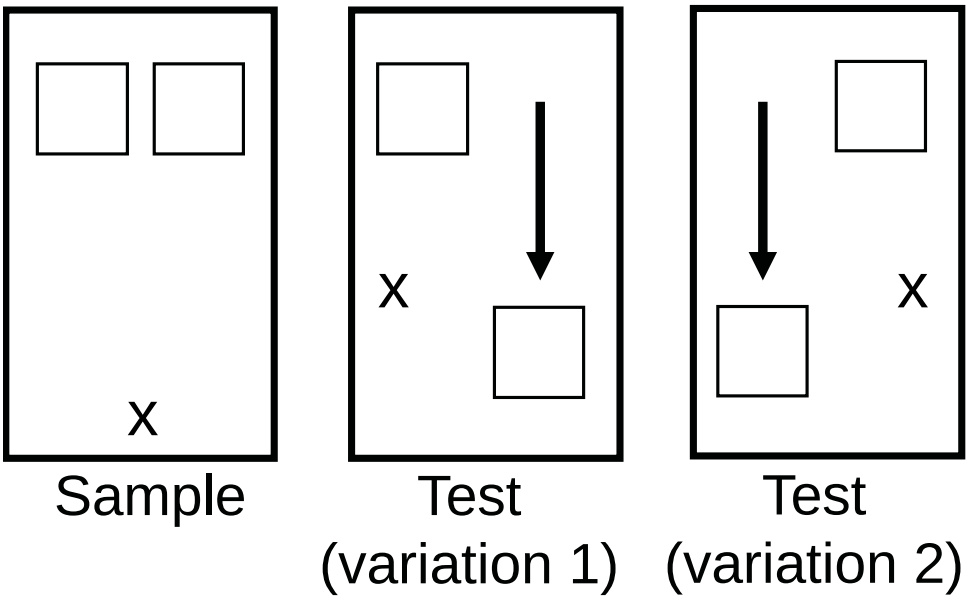

Figure 8.2 Diagram showing the object configurations for the Novel Place Task *Two identical objects are placed in the centre of the Northeast and Northwest quadrants for the 3 minute sample phase. Rats are then presented with either one of the two test phase variations, where two more copies of the objects are presented, but where one of the objects has moved to a location in which an object has never been seen before.*

8.2.2 Object place with alternative cues

The testing arena used for these variations of the OP task was the same arena used for the 2 day / 4 task protocol. The only exception to this was the identity and/or position of the cues. In the original testing in chapter three, and for all other tasks in the thesis except this one, two cues were placed at the top of the walls of the arena, just inside the arena, at the northeast and northwest of the arena. These cues were an artificial orange flower in the northwest of the arena, and a green Lego Duplo block in the northeast of the arena. However, in these versions of the OP task we have two variations of this. The first of the cue variations was moving the original flower and Lego Duplo block to the

middle of the east and west walls, as opposed to at the north end of each wall. The second variation was to have two black stickers in the shapes shown in figure 8.3 on the wall behind each object, with the flowers and lego block no longer visible.

For this task, rats were tested at ages p38 and p45 with the cues at the side of the arena, and p39 and p46 with the stickers on the north wall. On these tests the rats were given 3 minutes in the morning with random objects, never previously seen and never used for testing, in order to habituate the animals to the new cues. In the afternoon of each testing day the rats were tested on OP in the same way as the 2 day/4 task protocol. On p48 the rats were tested on both sets of alternative cues with the cues at the side in the morning and the stickers in the afternoon. Half of the rats were tested with the walls and floor from context 1 throughout, and half the rats tested with the walls and floor from context 2 throughout. Due to a technical complication with the recording equipment, recording of the sample phases for one litter on p48 was not completed and therefore the exploration data for the sample phase for these rats is not available, however all test phase data was recorded as were the sample phases for all other ages and rats

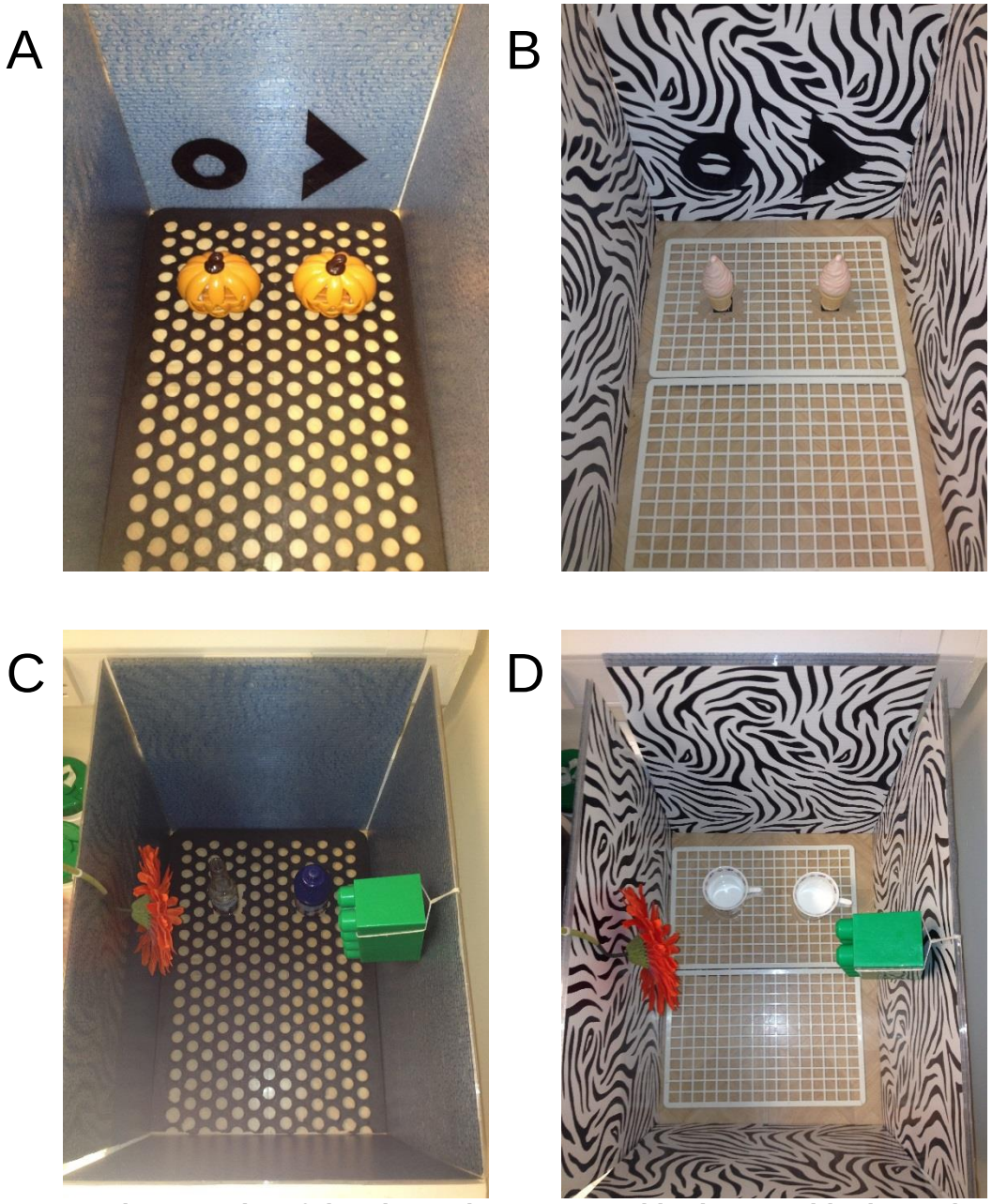

Figure 8.3 Photographs of the alternative cues used in the OP with alternative cues task. *A) Context one with stickers cues. B) Context two with stickers cues. C) Context one with cues at the side. D) Context two with cues at the side, with example objects shown in all photographs.*

8.3 Results

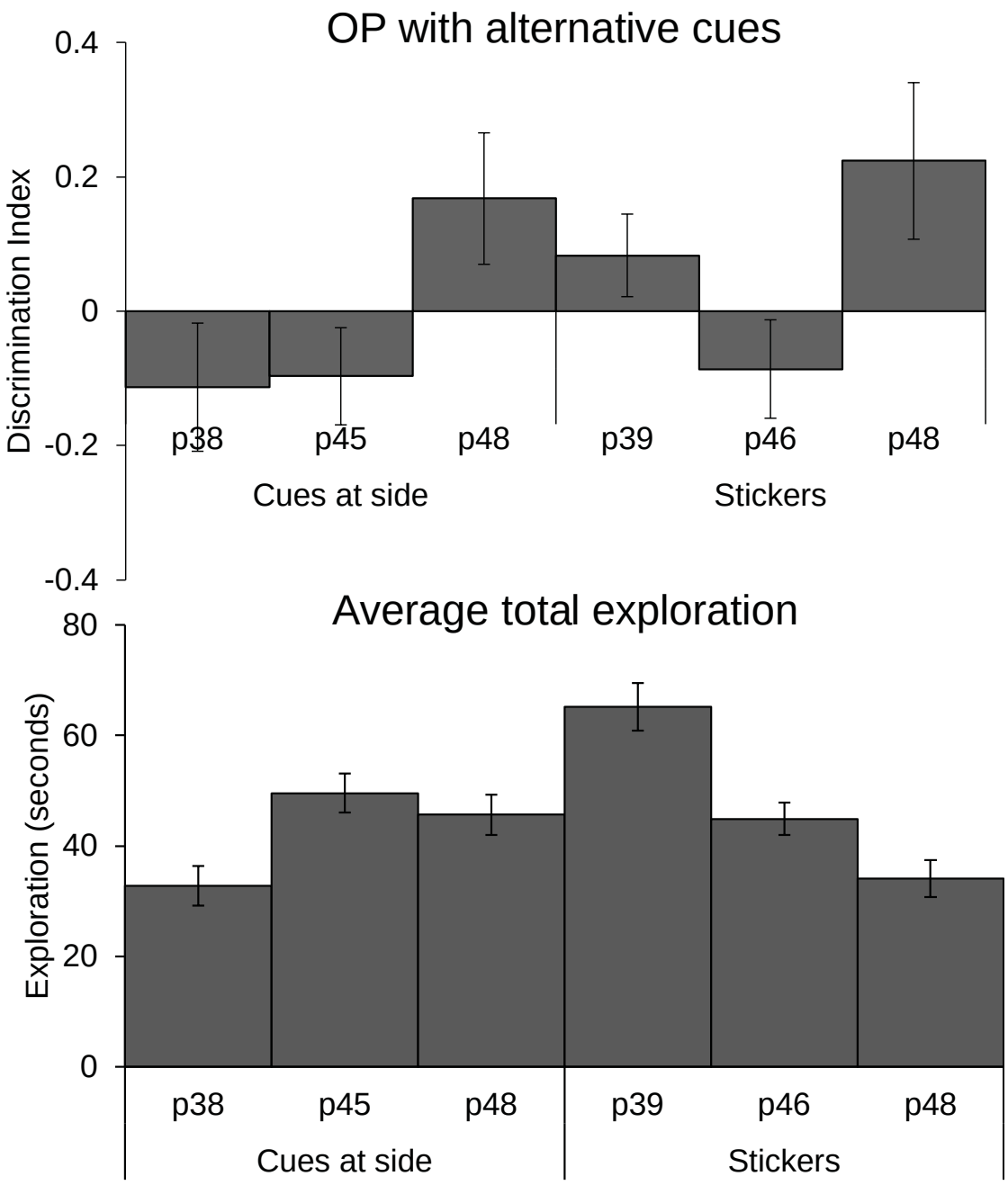

Figure 8.4 Object place (OP) with alternative cues results*. There was no significant effect of age on performance as measured by discrimination index when the cues were at the side of the arena and there was a significant effect of age when stickers were used as cues. Bonferroni posthoc tests showed that there was a significant difference in performance between p46 and p48.*

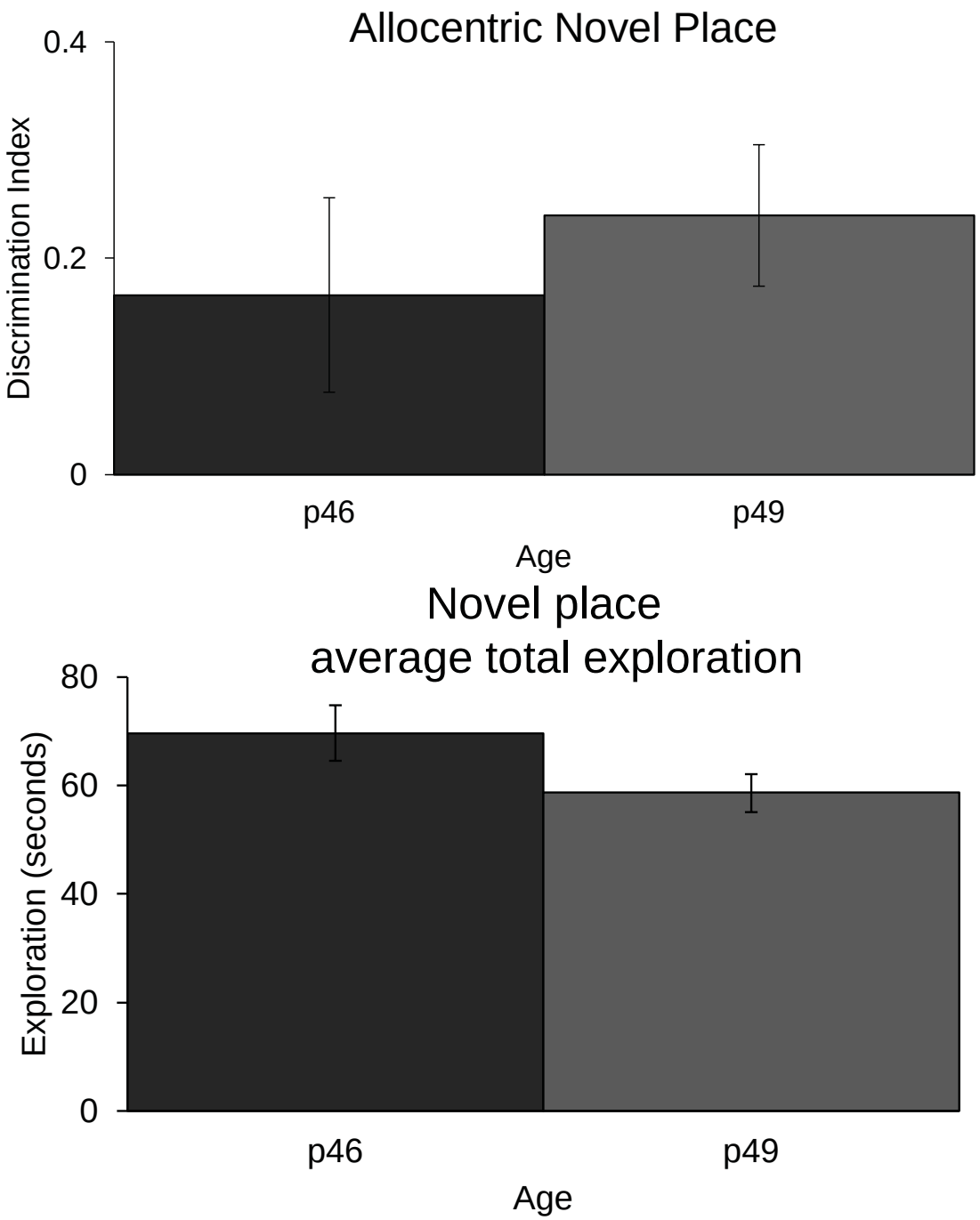

Figure 8.5 Allocentric novel place task results. *There was no significant difference in performance between p46 and p49 but rats age p46 did not perform the task significantly above chance and rats aged p49 did perform significantly above chance.*

8.3.1 Allocentric novel place task

A one way ANOVA between the ages showed that there was no significant difference in performance between p46 and p49 ($F_{(1,16)}$ = 0.440, p>0.05). However a one sample t-test against chance (0) showed that p46 rats did not perform the task significantly above chance ($t_{(8)}$ = 1.853, p >0.05) whereas at p49 rats performed significantly above chance ($t_{(8)}$ = 3.670, p <0.05).

8.3.2 Object place with alternative cues

A one way ANOVA with bonferonni posthoc tests for each of the two conditions (cues at the side and stickers) was conducted. There was a significant effect of age when the stickers were used as cues ($F_{(2,44)}$ = 3.263, p <0.05), with Bonferroni posthoc tests showing that there was a significant difference in performance between p46 and p48.

When the cues were placed at the side of the arena, there was not a significant effect of age, however this was reaching significance ($F_{(2,42)}$ = 3.081, p = 0.056).There were no significant differences in performance between any ages with this condition.

8.4 Discussion

In chapters four and seven it was demonstrated that the OP task develops between p47 and p48. This chapter aimed to investigate whether this remained true when the OP task was manipulated, and what insight that might give to how the rats are performing the OP task. It was found that when the OP task was conducted with different cues, and when an allocentric version of the task was used, the development remained the same. Rats at age p47 could not perform the task whereas rats at p48 could.

Spatial representation of the environment can be either allocentric or egocentric. Allocentric memory encodes information about the location of an object, or its parts, with respect to other objects. However, egocentric memory encodes information about the location of an object in space relative to oneself. Taking the example of our OP task used in chapters three, four and seven, if the rat is encoding the object location in an allocentric way it may remember the location of an object relative to the location of the extramaze cues, whereas if it was encoding the location in an egocentric way it would remember the location of an object relative to itself.

A study carried out in 2000 looked at the spatial memory of a patient that had selective bilateral hippocampal damage (Holdstock et al., 2000). It was shown that this patient, known as patient YR, had selective impairment in allocentric but not egocentric memory, suggesting a role for the hippocampus in allocentric memory processing. Four years later a study was conducted by another group which looked at the spatial memory of thirty patients, however these patients had unilateral hippocampal damage (Feigenbaum and Morris,

2004). This study found that only the patients with damage to the right hippocampus showed deficits in allocentric spatial memory, suggesting a role for the right hippocampus but not the left hippocampus in allocentric spatial memory. This was further supported by an MRI study in humans conducted in 2010 which drew the same conclusion (Igloi et al., 2010). There is also evidence that indeed the right hippocampus in rats is more involved in spatial processing than the left (Klur et al., 2009).

The novel place task used here forces the rats to encode the space in an allocentric manner due to the fact that the starting position of the rats change. The fact that it develops at the same time as the OP task used in other chapters suggests that it may be the case that the rats are performing both in a similar manner, they may be performing the OP task in an allocentric way. This hypothesis is further supported by the fact that when the cues are manipulated the task still shows development at p47/48, with a similar level of performance as measured by DI to previous testing of these tasks, suggesting that the animals may not be using the cues to encode the spatial location of the objects. It may be that if the rats are using the right hippocampus more than the left due to the allocentric nature of the task, then the development of the ability to perform the task possibly indicates an anatomical or functional development in the right hippocampus at this age. If this is the case, that the right hippocampus is developing at this age, what is functionally different between the two halves that may be developing? There have been asymmetries noted in a number of measures including synaptic strength (Shipton et al., 2014), gene expression (Moskal et al., 2006, Klur et al., 2009) and noradrenaline levels in response to stress (Spasojevic et al., 2013).

Furthermore it has been noted that there are differences in hippocampal CA1 pyramidal cell synapses between the two halves of the hippocampus. CA1 synapses receiving neuronal input from the right CA3 are larger and have twice as much expression of the GluR1 subunit of the AMPA receptor as those receiving input from the left, and CA1 synapses receiving neuronal input from the left CA3 are smaller and have comparatively higher levels of the NR2B subunit of the NMDA receptor (Shinohara et al., 2008).

Further work to fully understand the differences between the right and left hippocampi of the rats performing the OP task would most likely include unilateral lesions of the hippocampus, mRNA analysis to quantify gene expression, Western Blot analysis of levels of receptor expression and immunohistochemistry to look at localisation of receptors. These studies would need to be carried out at ages before and after the development of this task.

The premise behind changing the cues was to provide alternative extramaze cues (this time the cues were in front of the object from the perspective of the rat when placed in at the south wall), and to provide a set of intramaze cues (stickers on the north wall). On a radial arm maze task it has been shown that extramaze cues are not necessary for accurate performance, and instead adult rats can effectively rely on intramaze cues to discriminate between visited and unvisited arms (Kraemer et al., 1983). Adult rats also learn faster with intra-maze cues but interestingly when they solve tasks with extra-maze cues they do so by referencing the whole set of cues, rather than using them as local cues or to guide their learning (Diez-Chamizo et al., 1985).

In humans it has been shown that adults navigate around an enclosed space using distal cues, whereas children up to the age of eleven are biased towards using intramaze cues (Buckley et al., 2015). However this doesn't seem to be the case in juvenile rats as results showed that throughout all of the cue changes in this investigation rats showed the same pattern of development as in the two day four task protocol, regardless of cue type. If the rats were able to utilise intramaze cues more effectively then it is possible that the development of the task may occur earlier. However, this investigation indicates that the rats may be performing the tasks in a similar way and do not show a clear bias as to preferring either intramaze or extramaze cues.

Chapter 9

Visualising c-fos expression to anatomically localise episodic memory

9.1 Introduction

Previous chapters in this thesis have all used behavioural techniques to understand the ontogeny of memory in juvenile rats. It was shown that the OPC task, a model of episodic memory, develops between p47 and p48. Due to the fact that this task has been shown to be dependent on the hippocampus, it was hypothesised that the ontogeny of this task is due to the development, of hippocampal or parahippocampal regions. The aim of this investigation was to use immediate early gene (IEG) imaging to define anatomically what is developing at this abrupt developmental time window.

9.1.1 Immediate early genes, AP-1 and c-Fos

Within the field of neuroscience IEG imaging has been commonly used as a method of visualising and quantifying neuronal activity. IEGs are activated at the transcription level as a first response to a stimuli, before any protein has been synthesized (Perez-Cadahia et al., 2011). IEGs are rapidly and transiently induced within minutes by extracellular stimuli such as action potentials (Perez-Cadahia et al., 2011). When the cell is stimulated, a first messenger (eg a neurotransmitter or pharmacological agent) interacts with receptors on the cell surface. This receptor then activates second messenger systems either through a G protein or through the influx of ions. The second messenger system consists of protein kinases, which phosphorylate a range of neuronal proteins, which then allow the cell to carry out appropriate action to the response.

IEGs encode many functionally different products such as secreted proteins, cytoplasmic enzymes, ligand-dependent transcription factors and inducible

transcription factors (Perez-Cadahia et al., 2011). One of these inducible transcription factors is c-Fos, which acts via Activator Protein 1 (AP-1).

AP-1 is a collective term referring to a family of dimeric transcription factor proteins which are composed of proteins belonging to the Fos, Jun and ATF (Activating Transcription Factor) sub families, which recognise either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP (cyclic adenosine monophosphate) response elements (CRE, 5'-TGAC/GTCA-3') (Shaulian and Karin, 2002). The Fos proteins cannot form homodimers and therefore form stable heterodimers with Jun proteins. Levels of AP-1 are regulated by changes in Jun and Fos gene transcription, mRNA turnover, protein turnover, post-translational modifications of Jun and Fos and interactions with other transcription factors (Shaulian and Karin, 2002). AP-1 is a transcription factor of a range of genes which are involved in cellular proliferation, transformation and death.

C-fos protein is used as a marker for neuronal activity in object recognition tasks (Barbosa et al., 2013, Zhu et al., 1995, Wilson et al., 2013b). It has also been shown to be necessary for long-term memory and NMDA-receptor dependent synaptic plasticity (Fleischmann et al., 2003) and is usually visualised using immunohistochemistry. C-fos expression is highest after sixty minutes (Bertaina-Anglade et al., 2000) and therefore by sacrificing the animal at this point after a behavioural task, it allows the visualisation of active brain areas during the task.

9.1.2 Immunohistochemistry

Immunohistochemistry is the process of detecting antigens in cells of a tissue using antibodies. Visualising an antibody-antigen interaction can be accomplished by conjugating the antibody to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction.

The first step in an immunohistochemistry protocol is to block all epitopes on the tissue sample in order to prevent nonspecific binding of the antibodies. If this step does not occur the antibodies may bind to any epitopes within the tissue, regardless of specificity. Normal serum (generated before any immune response) is a common blocking agent due to the fact that it contains large amounts of antibodies that bind to reactive sites and therefore prevent the antibody of interest from binding to the nonspecific sites. Sufficient washing after the blocking step is crucial to remove excess protein in order for adequate detection of the target antigen.

After the blocking step, the tissue is incubated with primary antibody. Primary antibodies are raised against the antigen of interest and are usually unlabelled. After washing away excess, unbound primary antibody, a secondary antibody is added. The secondary antibody is raised against the immunoglobulins of the primary antibody species and binds to the primary antibody whilst still retaining one free antigen-binding site. A biotinylated enzyme (usually either alkaline phosphatase or horseradish peroxidase) is pre-incubated with free avidin to form large avidin-biotin-enzyme complexes. This enzyme solution is then added to the tissue sample and the complexes bind to the secondary antibodies. Avidin is a glycoprotein found in the egg white and tissues of birds,

reptiles and Amphibia and has an extremely high affinity for biotin. It allows biotin-containing molecules in a complex mixture to be specifically bound to avidin. It has four identical subunits, each of which binds to one biotin molecule, allowing the binding of four biotin molecules to every one avidin. By increasing the number of biotin molecules it will allow more of the reporter molecule to bind. The reporter molecule binds to the enzyme and is coloured or fluorescent, therefore allowing the visualisation of the location of the target antigen. Figure 9.1 shows a schematic representation of the avidin-biotin complex (ABC) staining method.

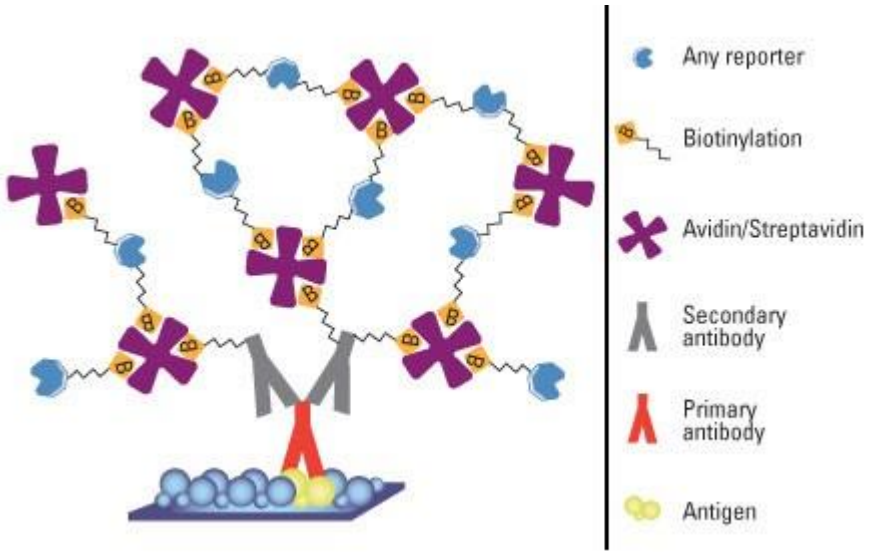

Figure 9.1 Schematic representation of the avidin-biotin complex (ABC) staining method. (ThermoFisher, 2015)

9.1.3 Quantifying c-fos expression after immunohistochemistry

It was first observed that c-fos is expressed after neuronal activation in 1987 (Morgan et al., 1987) and it has since been used as a marker for neuronal activity in investigations to understand the anatomical locus of a behaviour. Traditional methods of quantifying c-fos expression in the brain after

immunohistochemistry have involved manually counting cells by hand by the experimenter. However one major downfall to this method is the fact that it relies on the consistency of one person to select cells which are stained and not select cells which are not. In figure 9.2 an example of staining is shown from the dorsolateral entorhinal cortex (DLE) of a rat brain which has undergone staining for c-fos. As is typical for this type of experiment there is a range of staining intensities and defining a cell as positively stained may vary between experimenters. Therefore this method of selecting which cells are positively stained is not reliable and not easily reproducible. Experimenters may produce different cell counts from the same image.

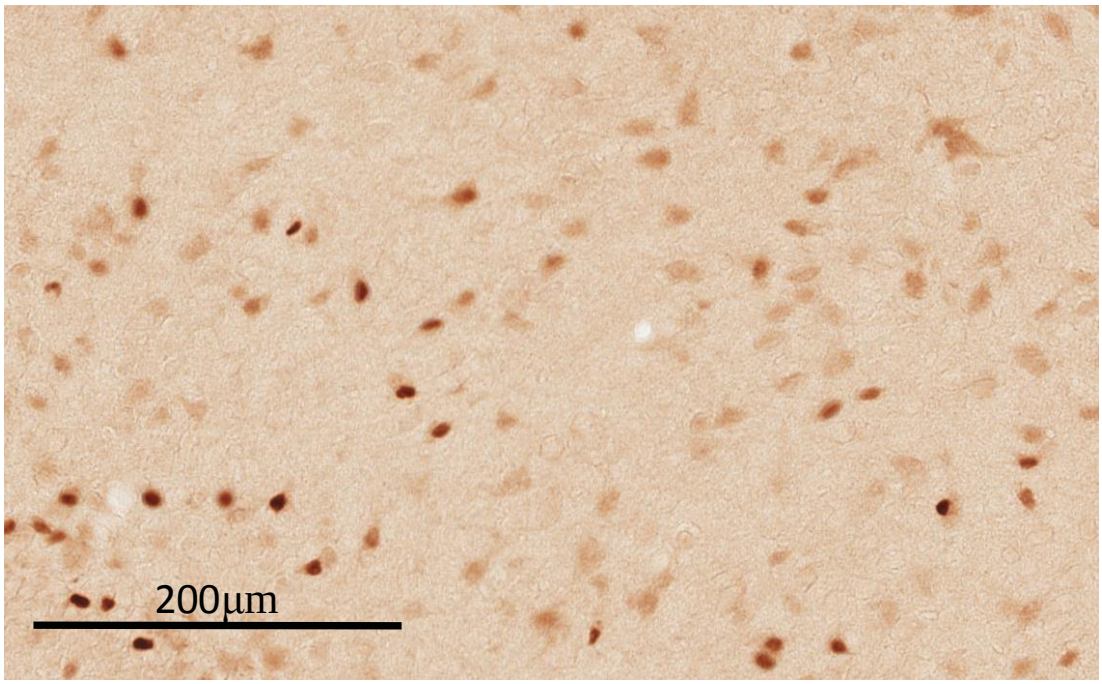

Figure 9.2 An example of c-fos visualisation after immunohistochemistry using DAB (3,3'-Diaminobenzidine) in the lateral entorhinal cortex of a rat.

A computer programme has been developed by Aperio Technologies Ltd called ImageScope which is used to view, annotate and analyse images generated from a slide scanner. Slide scanners are devices which can be used to make digital copies of glass slides. Aperio Technologies Ltd provides an online database to store digital copies of slides (eSlides) which can then be

opened in ImageScope for further analysis. This software is widely used in pathology labs for analysing tumour cells, and has a number of different analysis algorithms that it can run, including one called “IHC (immunohistochemistry) Nuclear Image Analysis”. This algorithm has a number of settings which can be manipulated in order to achieve the best detection of cells within a sample. Once it detects a cell it puts it into one of three categories, light staining, medium staining or dark staining. It then provides a “markup” image showing which cells it has detected and which category it has placed it in. The parameters of this algorithm, such as the red, green, blue thresholds for the stain for each category, the minimum and maximum nuclear size, as well as many others, can be changed by the experimenter to ensure maximum detection of cells and exclusion of artefacts. The markup image from the image shown in figure 9.2 is shown in figure 9.3, where red is dark staining, orange is medium staining and yellow is light staining. The blue staining is not counted as the software is also programmed to pick up negatively stained cells but does this based on a counterstain.

Therefore if no counterstain is present it is unable to pick up negatively stained cells and the blue areas on the markup image are not counted.

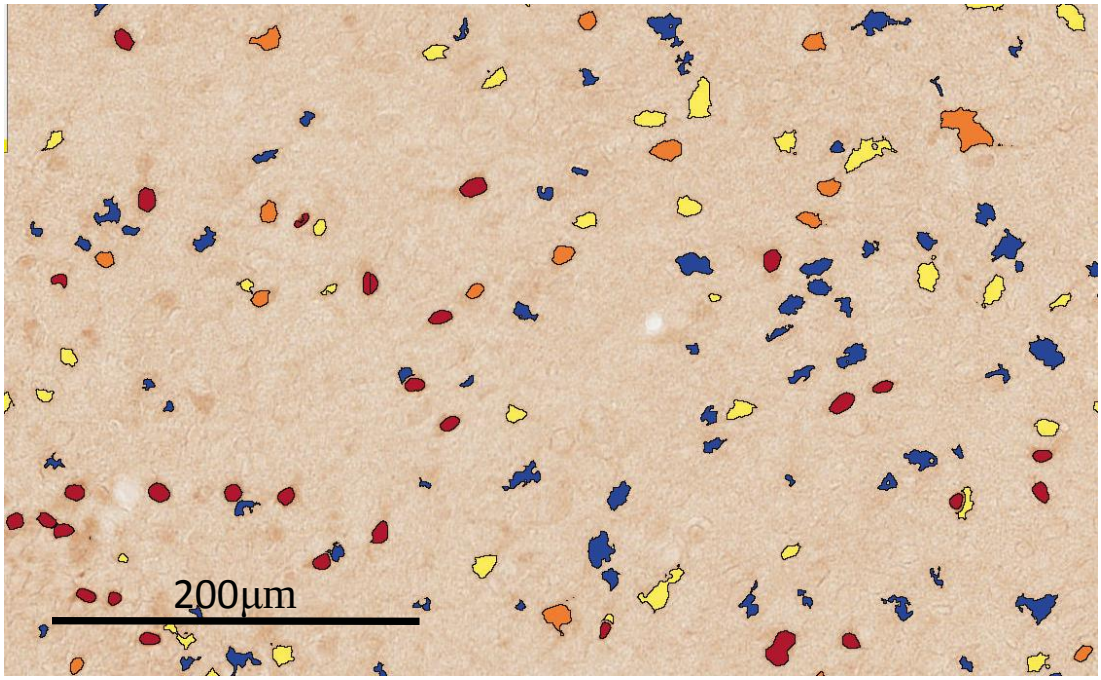

Figure 9.3 Markup image generated from ImageScope after Immunohistochemistry Nuclear Image Analysis algothrm has been applied. *This example is from the lateral entorhinal cortex of a rat after c-fos visualisation using immunohistochemistry with DAB (3,3'-Diaminobenzidine).*

9.2 Method

9.2.1 Subjects

Lister Hooded rats were used for this investigation, bred in house and maintained in the same conditions as previously described, including culling litters to eight within days of birth. Eleven rats were used in total, from two litters, five at p47 (three males and two females) and six at p48 (three males and three females). One female at p48 was excluded during the image analysis phase due to uneven staining of brain slices.

9.2.2 Behaviour

Rats received five days of handling and two days of habituation as per the two day four task protocol in chapters three and four. Following habituation the rats

were tested on the OPC task. Rats were sacrificed 60 minutes after the completion of the test phase of the task.

9.2.3 Preparation of tissue

The tissue was fixed using a transcardial perfusion method. This method involved giving the rat a lethal dose (0.5ml) of sodium pentobarbital (Euthatal, 200mg in 1ml) via intraperitoneal injection. Once the rat has lost all reflexes the chest cavity is opened, cutting the diaphragm to give access to the heart. A butterfly needle that is attached to the solution to be perfused is inserted into the left ventricle and the right atrium is cut. Phosphate buffered saline (PBS) is then flushed through the animal until no blood remains within the vascular system. The perfusing solution is then switched to 4% paraformalde in phosphate buffer until the animal is fully fixed. The brain is then removed from the skull and placed into a cryoprotective solution until cutting (see “9.2.4 optimising immunohistochemistry protocol” section).

Before the brain was cut using a cryostat it was removed from the cryopotection solution and the cerebellum cut off. Cryomatrix glue was used to stick the cut side down so that the front of the brain was facing upwards. The brain was then frozen to -50°C and cut within the cryostat chamber which is kept at -20°C. All brains were cut at 48µm coronal slices, with all slices from hippocampus ventrally collected into four series, allowing up to four sets of immunohistochemistry to be carried out. The slices were then stored in antifreeze (150g sucrose in 150ml Ethylene glycol made up to 500ml with 0.1M phosphate buffer) at -20°C.

9.2.4 Immunohistochemistry

Immunohistochemistry for c-fos protein was carried out using the following steps.

1. Wash in PBS for 5 minutes x 2
2. Blocking Solution for 45 minutes
3. Wash in PBS for 3 minutes x 2
4. Primary Antibody in ADS overnight (1 in 4000 dilution of ADS to antibody)
    - Primary antibody – Anti-c-fos (AB-5) (4-17) Rabbit pAb, Calbiochem PC38
5. Wash 3 minutes x 5
6. Secondary Antibody in ADS for 90 minutes (1 in 200 dilution)
    - Secondary antibody in Vectastain ABC kit (Rabbit IgG), Vector Labs pk-6101
7. Wash 3 minutes x 5
8. ABC for 1 hour
9. Wash 3 minutes x 5
10. 3,3'-Diaminobenzidine (DAB) for 10 minutes
    - Sigmafast™ 3,3'-Diaminobenzidine tablets, Sigma D4168
11. Wash 3 minutes x 5
12. Store in PBS and mount within 5 days

- Blocking solution
    - 1 part 10% Triton
    - 20 parts normal goat serum
    - 80 parts PBS

- Antibody diluting solution (ADS)
    - 1 part normal goat serum
    - 1 part triton
    - 80 parts PBS

After IHC the brains were mounted onto glass slides and covered with glass coverslips and DPX mountant.

9.2.5 Optimising immunohistochemistry protocol

In order to achieve the most revealing results, the protocol was altered a number of times as information was gathered about the best ways to optimise the protocol. The method described above (sections 9.2.1 – 9.2.4) were carried out following a number of prior experiments. The first experiment took rats aged p47 and p48 and transcardially perfused them one hour after the completion of the OPC task. Brains were removed from the skull immediately after the perfusion and placed into 20% buffered sucrose solution. Brains were left in the same sucrose for 48 hours before being frozen on the -40°C plate or with the cryospray and then sectioned into 48µm slices. This method however resulted in poor cutting of the brains. It was hypothesised that this was due to a sub-optimal fix and therefore the experiment was repeated, with the same behavioural protocol, however this time the brains were left in the skull for 10 minutes after the perfusion was completed. The cryoprotection protocol was also changed so that the brains were put in half 4% PFA and half 30% sucrose (final concentration 2% PFA and 15% sucrose) for 24 hours and then into 30% sucrose alone for 24 hours. These brains were then frozen using

cryospray only and cut at 48µm, collecting from hippocampus ventrally into four series. Immunohistochemistry was carried out on this set of brains.

9.2.6 Image analysis

Slides were scanned on an Aperio ScanScope XT slide scanner at x20 magnification producing an electronic image known as an eSlide which was viewed, annotated and analysed using Aperio ImageScope software. Annotations were drawn on the eSlide outlining each region of interest for each slice. Once each region of interest had been outlined, the IHC Nuclear Image Analysis algothrithm (supplied by Aperio) was applied. Before running the algorithm it was tuned to ensure that it was able to identify cells and put them into the correct staining category (light, medium and dark staining). After the analysis was complete the data was exported into excel.

9.2.7 Analysis of data

Data was analysed in excel and statistical analysis conducted in SPSS. For each rat the total number of cells counted and the total area analysed was produced by the software. This was then converted into cells per mm^2 for each brain region by dividing the number of cells by the total area analysed. This number was known as the raw cell count per mm^2. To allow for comparison of regions with different cell densities, raw counts were normalized (Wilson et al., 2013a). This normalization was performed by dividing each rat's raw cell count for a given brain region by the average across all rats (in both age groups) for that brain region, then multiplying by 100.

9.3 Results

9.3.1 Behavioural results

Rat	*Age*	*Sex*	*DI*	*Mean*	*SEM*
1	*p47*	*Male*	*0*	*-0.03*	*0.09*
2	*p47*	*Male*	*0.1*		
3	*p47*	*Male*	*0.04*		
4	*p47*	*Female*	*-0.4*		
5	*p47*	*Female*	*0.1*		
6	*p48*	*Male*	*0.2*	*0.40*	*0.07*
7	*p48*	*Male*	*0.45*		
8	*p48*	*Male*	*0.49*		
10	*p48*	*Female*	*0.62*		
11	*p48*	*Female*	*0.25*		

Table 9.1 Object-place-context task results from rats used in c-fos investigation.
DI – Discrimination index, SEM – standard error of the mean.

9.3.2 Normalized cell counts

A repeated measures ANOVA was conducted on normalized cell counts shown in figure 9.4, with brain region as within subjects factor and age as between subjects factor showed that all three staining levels showed the same pattern of statistical significance. All three had no significant effect of region (Dark - $F_{(12)}$=0.144, p >0.05, Medium - $F_{(12)}$=0.098, p >0.05, Light - $F_{(12)}$=0.230, p >0.05), no effect of age (Dark - $F_{(1)}$=0.029, p >0.05, Medium - $F_{(1)}$=0.015, p >0.05, Light - $F_{(1)}$=0.232, p >0.05) and no interaction between region and age

(Dark - $F_{(1,12)}$=0.809, p >0.05, Medium - $F_{(1,12)}$=0.730, p >0.05, Light - $F_{(1,12)}$ = 0.522, p >0.05).

9.3.3 Effect of sex – combined ages, normalized cell counts.

A repeated measures ANOVA was conducted on the normalized cell counts shown in figure 9.5 with brain region as within subjects factor and sex as between subjects factor. All three levels of staining had no significant effect of region (Dark - $F_{(12)}$=0.234, p >0.05, Medium - $F_{(12)}$=0.234, p >0.05, Light - $F_{(12)}$=0.439, p >0.05). Dark and medium staining showed a significant effect of sex (Dark - $F_{(1)}$=8.152, p <0.05, Medium - $F_{(1)}$=8.379, p <0.05) and light showing no effect of sex ($F_{(1)}$=3.020, p >0.05). Finally, there was no interaction between region and age at any level of staining (Dark - $F_{(1,12)}$=0.462, p >0.05, Medium - $F_{(1,12)}$=0.416, p >0.05, Light - $F_{(1,12)}$.0.828, p >0.05).

9.3.4 Effect of age on males only

A repeated measures ANOVA was conducted on the normalized cell counts shown in figure 9.6 for males only with brain region as within subjects factor and age as between subjects factor. All three levels of staining had no significant effect of region (Dark - $F_{(12)}$=1.122, p >0.05, Medium - $F_{(12)}$=0.914, p >0.05, Light - $F_{(12)}$=0.538, p >0.05), no effect of age (Dark - $F_{(1)}$=0.000, p >0.05, Medium - $F_{(1)}$=0.001, p >0.05, Light - $F_{(1)}$=0.003, p >0.05) and no interaction between region and age (Dark - $F_{(1,12)}$=1.078, p >0.05, Medium - $F_{(1,12)}$=1.340, p >0.05, Light - $F_{(1,12)}$ = 1.187, p >0.05).

9.3.5 Effect of age on females only

There was only one female in p48 with cell counts from DIE, VIE and ME therefore these regions could not be compared with p47 rats. A repeated

measures ANOVA was conducted on the normalized cell counts for females only as shown in figure9.7 with brain region as within subjects factor and age as between subjects factor. All three levels of staining had no significant effect of region (Dark - $F_{(12)}$=0.220, p >0.05, Medium - $F_{(12)}$=0.111, p >0.05, Light - $F_{(12)}$=1.049, p >0.05), no effect of age (Dark - $F_{(1)}$=0.172, p >0.05, Medium - $F_{(1)}$=0.099, p >0.05, Light - $F_{(1)}$=0.495, p >0.05) and no interaction between region and age (Dark - $F_{(1,12)}$=0.905, p >0.05, Medium - $F_{(1,12)}$=0.495, p >0.05, Light - $F_{(1,12)}$ = 1.094, p >0.05).

9.3.6 Collapsed data into one staining level

Combined staining counts from all three levels of staining was calculated to create a single score for each region as shown in figure 9.8. This score was normalised as previously described and a repeated measues ANOVA conducted with brain region as within subjects factor and age as between subjects factor. There was no significant effect of region ($F_{(12,72)}$ = 0.163, p > 0.05), no significant effect of age ($F_{(1,6)}$ = 0.09, p > 0.05) and no significant interaction between brain region and age ($F_{(12,72)}$ = 0.574, p > 0.05).

A repeated measures ANOVA across regions within the hippocampus (FC, Dorsal CA1, Dorsal CA2, Dorsal CA3, Ventral CA1, Ventral CA3 and Dentate Gyrus) was carried out with brain region as within subjects factor and age as between subjects factor. There was no significant effect of region ($F_{(6,48)}$ = 0.001, p > 0.05), no significant effect of age ($F_{(1,8)}$ = 3.059, p > 0.05) and no significant interaction between brain region and age ($F_{(6,48)}$ = 1.897, p > 0.05).

A repeated measures ANOVA across regions outwit the hippocampus (Perhrihinal cortex, Subiculum, DLE, DIE, VIE and ME) was carried out with

brain region as within subjects factor and age as between subjects factor. There was no significant effect of region ($F_{(5,30)}$ = 0.083, $p > 0.05$), no significant effect of age ($F_{(1,6)}$ = 0.028, $p > 0.05$) and no significant interaction between brain region and age ($F_{(5,30)}$ = 0.587, $p > 0.05$).

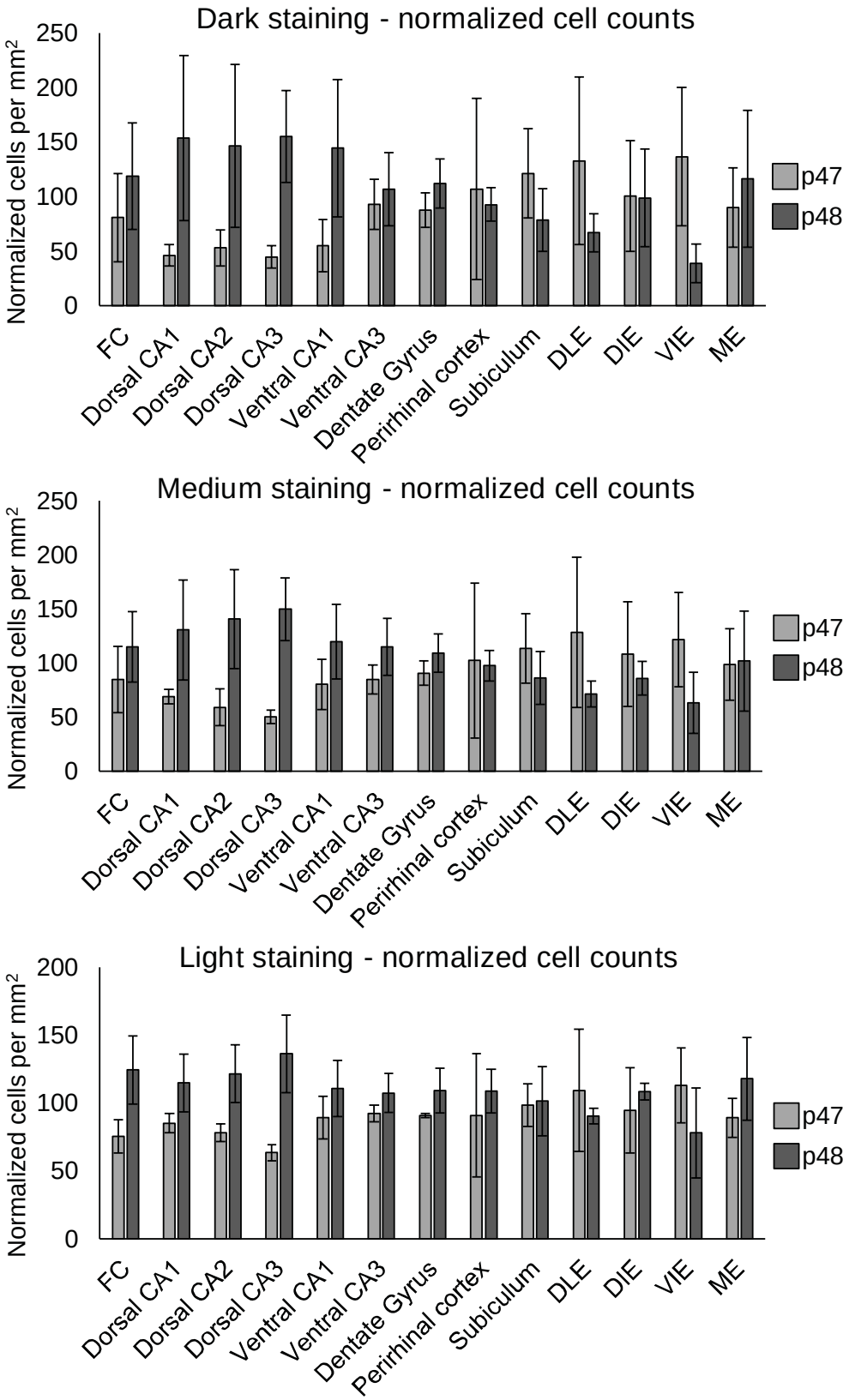

Figure 9.4 Normalized counts of positively stained cells in hippocampal and parahippocampal regions after completion of an OPC task. *There was no significant effect of region, no effect of age and no interaction between age and region. FC – Fasciolarum cinereum, CA – Cornu ammonis, DLE – Dorsolateral entorhinal cortex, DIE – Dorsal-intermediate entorhinal cortex, VIE – Ventral-intermediate entorhinal cortex, ME – Medial entorhinal cortex*

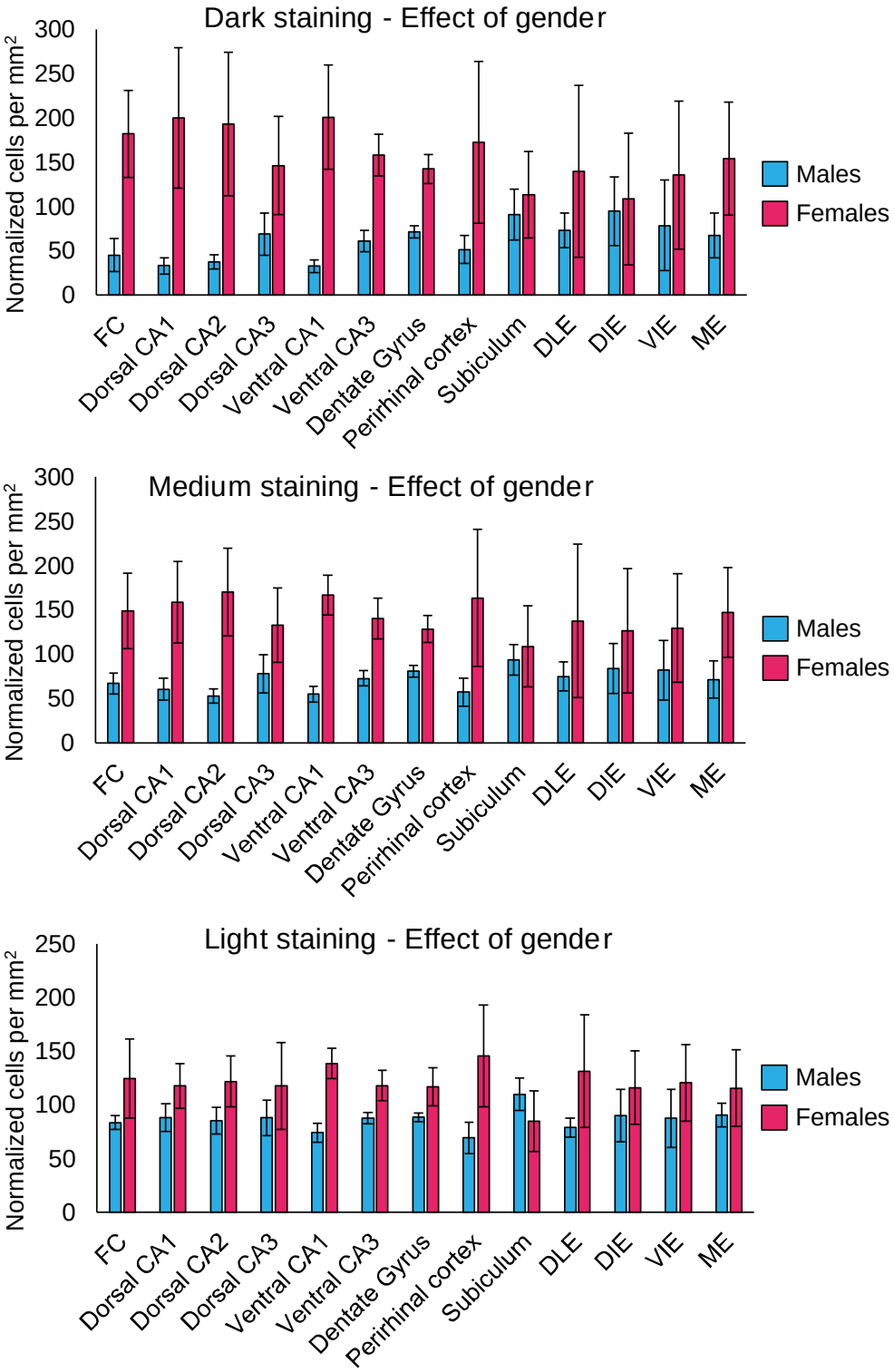

Figure 9.5 Normalized counts of positively stained cells in hippocampal and parahippocampal regions after completion of an OPC task – effect of sex. *There was no significant effect of region, dark and medium staining showed an effect of sex and no interaction between age and region at any staining level.*
FC – Fasciolarum cinereum, CA – Cornu ammonis, DLE – Dorsolateral entorhinal cortex, DIE – Dorsal-intermediate entorhinal cortex, VIE – Ventral-intermediate entorhinal cortex, ME – Medial entorhinal cortex

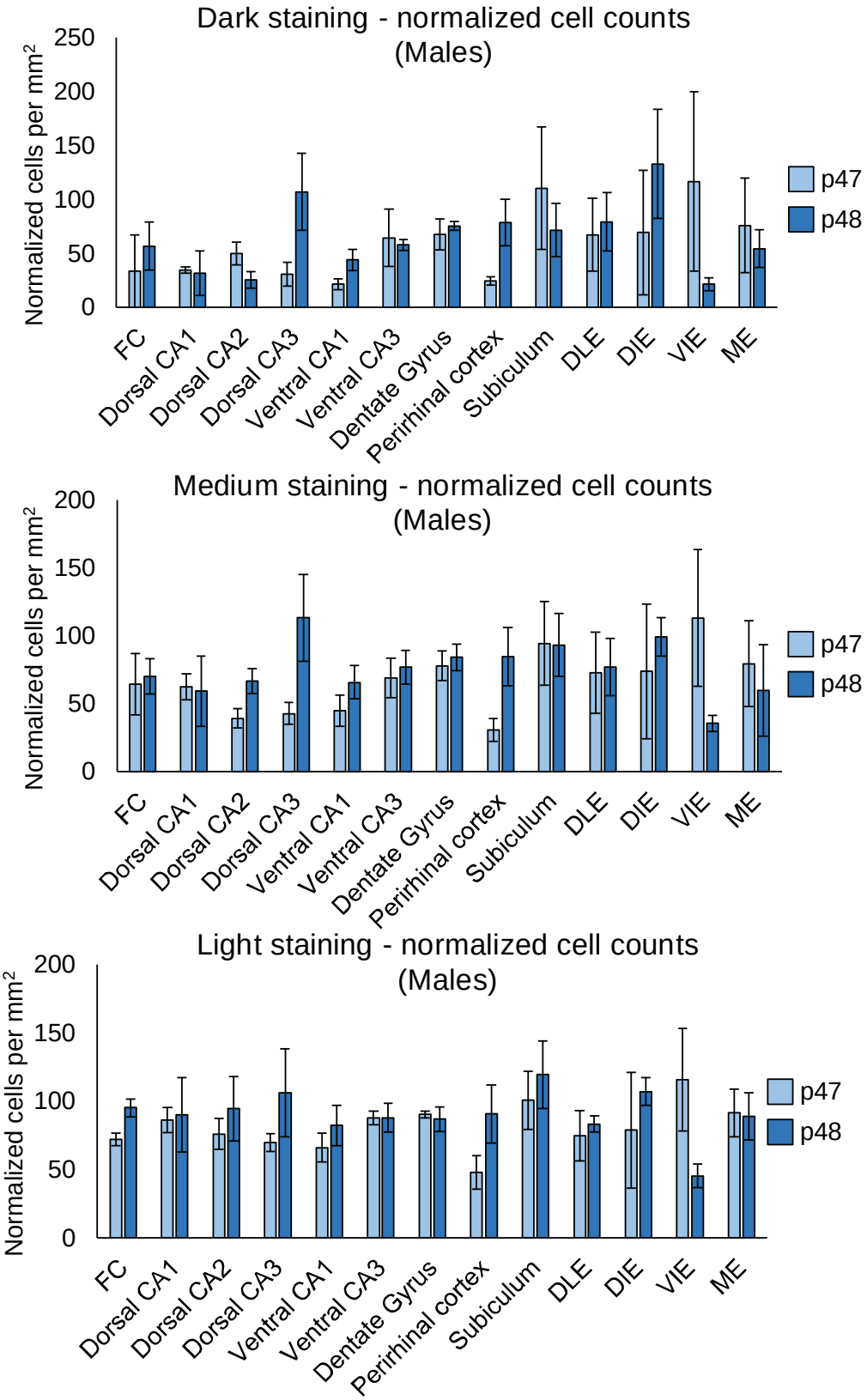

Figure 9.6 Normalized counts of positively stained cells in hippocampal and parahippocampal regions after completion of an OPC task –males only.
There was no significant effect of region, no effect of age and no interaction between age and region. FC – Fasciolarum cinereum, CA – Cornu ammonis, DLE – Dorsolateral entorhinal cortex, DIE – Dorsal-intermediate entorhinal cortex, VIE – Ventral-intermediate entorhinal cortex, ME – Medial entorhinal cortex

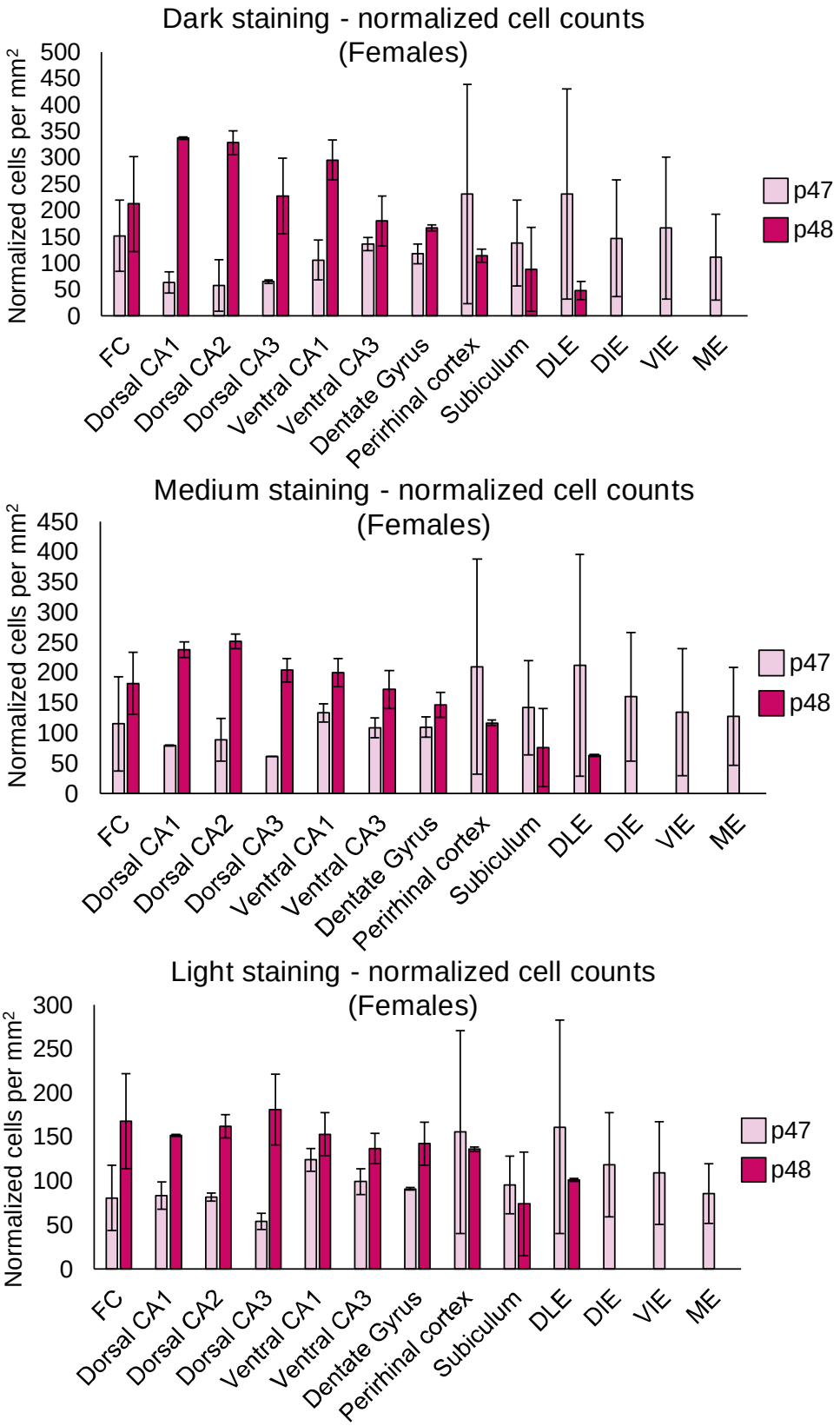

Figure 9.7 Normalized counts of positively stained cells in hippocampal and parahippocampal regions after completion of an OPC task – females only.
There was no significant effect of region, no effect of age and no interaction between age and region. FC – Fasciolarum cinereum, CA – Cornu ammonis, DLE – Dorsolateral entorhinal cortex, DIE – Dorsal-intermediate entorhinal cortex, VIE – Ventral-intermediate entorhinal cortex, ME – Medial entorhinal cortex

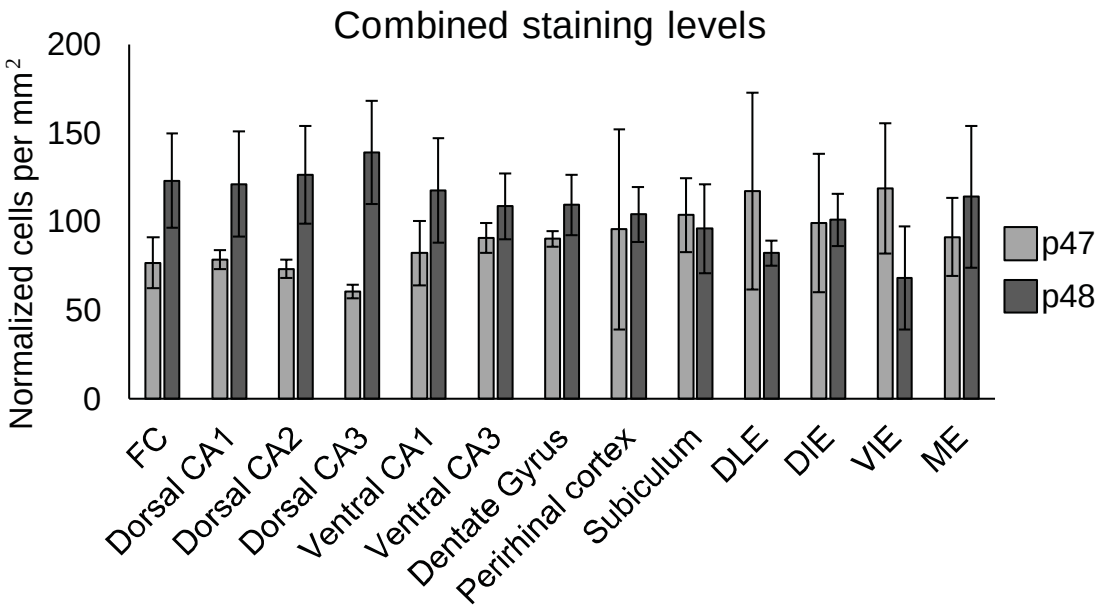

Figure 9.8 Normalized counts of positively stained cells in hippocampal and parahippocampal regions after completion of an OPC task – combined staining levels.

There was no significant effect of region, no effect of age and no interaction between age and region. FC – Fasciolarum cinereum, CA – Cornu ammonis, DLE – Dorsolateral entorhinal cortex, DIE – Dorsal-intermediate entorhinal cortex, VIE – Ventral-intermediate entorhinal cortex, ME – Medial entorhinal cortex

9.4 Discussion

This investigation investigated the changes in c-fos expression in the brains of rats after completion of an OPC task. Despite the fact that the lack of significant interaction in a repeated measures ANOVA prevents any further statistical tests from being carried out, it is possible to look at the data and try to gain a better understanding into it for future work.

To allow for comparison of regions with different cell densities, raw counts were normalized by the method described in section 9.2.7. When the raw counts were normalized the dorsal CA3 region consistently showed more staining at p48 than p47 in all three levels of staining. This is the only region where it looks as if there would be a significant difference between levels of staining on the two dates due to the difference in bar sizes and the fact that the error bars do not overlap between ages. In this region there is a smaller variation between rats at p47 than at p48. Interestingly, in an optogenetic study using mice, silencing of the CA3 area of the left hippocampus impaired associative spatial long-term memory (elevated Y-maze task involving the use of extramaze spatial cues) whereas silencing of the CA3 in the right hippocampus did not (Shipton et al., 2014). Acute silencing of either the left or right CA3 regions also impaired performance on a hippocampus dependent short-term memory task (spontaneous alternation in a T-maze) (Shipton et al., 2014). The study also found that high frequency stimulation-induced LTP is present at CA3-CA1 synapses when afferents originate in the left CA3, but not when they originate in the left (Shipton et al., 2014). The authors suggest that these data implicate the left CA3 as a key component in the hippocampal circuitry that supports long term memory (Shipton et al., 2014). It may be that

the functional asymmetry relates to the synaptic asymmetry due to the fact that LTP has been suggested as a cellular model for learning and memory (Martin et al., 2000).

It could be concluded from the immunohistochemistry data in this investigation that the increase in CA3 c-fos expression in rats aged p48 after the OPC task is due to the functional maturation of this brain region. It has been discussed that this is a region with differences between the hemispheres with the left CA3 suggested as a key component in hippocampal memory tasks. The development seen here may also be unilateral which may account for the increase in variability seen at p48 compared to p47. The method used here did not allow for any differences between left and right hemispheres to be analysed but in future it may be possible to “mark” one half before cryosectioning so that when mounted it is easy to see which hemisphere is which.

An optogenetic study which temporarily silenced the CA3 region may provide an idea as to whether the CA3 firing is allowing the rats to develop the ability to perform the OPC task, or whether the increase in CA3 firing is *because* of the development of the ability to perform the task. Until such a study is carried out it is not possible to conclude whether the increase in CA3 firing is the cause of the ontogeny of episodic memory.

This investigation also looked at the differences in c-fos staining between ages in males and females separately. Females consistently showed more staining than males for all three staining levels, especially dark and medium, and also showed more variation than males (figure 9.5). This is most prominent in

hippocampal regions except for dorsal CA3. Males showed differences between p47 and p48 in dorsal CA3 and perirhinal cortex in dark and medium staining levels (figure 9.6) and females had increased c-fos levels in hippocampal regions (CA1, CA2, CA3) between p47 and p48.

As discussed previously, female rats experience their first oestrus between p35 and p40 (Gabriel et al., 1992). In chapter four it was hypothesised that the female rats in our investigation may all experience the onset of their first oestrus cycle at the same time due to strictly controlled housing and breeding conditions, despite the fact that there is evidence that adult rats do not synchronise their cycles (Schank, 2001). The oestrus cycle of a rat is four or five days long and the first few cycles can be irregular (Goldman et al., 2007). As was also discussed earlier, the oestrus cycle can have an effect on spatial memory (Korol et al., 2004) and synaptogenesis (McEwen et al., 1997). If the oestrus cycle is having an effect on the cognition of the female rats in this study, and each rat is at a slightly different stage of their cycle, it is possible that this may account for the variability seen in the data generated from the female rats in this investigation.

It could be argued that within this study there is a lack of a proper control. The p47 age group can be thought of as a control as this is the age at which the rats cannot perform the task, however in future it would be beneficial to include one or more futher control groups. These controls would be carried out in the same way as the OPC test group, however they would allow a “baseline” level of c-fos expression to be quantified. The first of these would be a single context control with no objects where the animal would be allowed into the arena three times as in the OPC task, but no objects are present and nothing changes

between phases. This would control for the act of the rat entering, spending time in and being removed from the arena, as well as time spent in the holding arena and the exploration of the arena itself. The second would be a multiple context control with no objects, where the context changes between phases as in the OPC task, but no objects are present. Thirdly a single context control with objects present which do not change between phases would control for the object exploration, and finally a multiple context control with objects which do not change between phases would control for the changing contexts and the object exploration. If all four of these controls were carried out at each age, alongside the OPC task, it would allow the c-fos signal which is solely due to the OPC memory to be eluded. This would involve a large cohort of animals and would require a substantial amount of time, however it may provide more of an insight into the neuronal activity which underlies the OPC task development at p48.

Chapter 10

Final summary and conclusions

This thesis has combined a number of behavioural tasks and an immunohistochemistry investigation to develop a way of studying memory ontogeny in juvenile rats, and to understand the neural basis of episodic memory.

First a developmental timeline of body weight from birth to p60 was generated, with notes made on key physical developmental time points. Pups were actively attempting to walk before the eyes were open and it was hypothesised that this may suggest an even earlier development of head direction cells than is currently in the literature (p11), possibly at p8. Head direction cells receive vestibular input, and rats have a righting reflex from falling at p7 (Pellis and Pellis, 1994) which may provide a further indication of the presence of head direction cells before the age at which the current literature suggests.

In the next chapter a novel protocol for investigating memory in a shortened time window was successfully designed and implemented in adult rats. The protocol tested the rats on four tasks testing object recognition (NOR), associative contextual memory (OC), spatial memory (OP) and episodic memory (OPC). This protocol can be carried out in two days and follows on from five days of handling and two days of habituation. In this investigation there was no correlation found between sample phase exploration and performance and there was no overall difference found between genders. Within the literature there is evidence for hormone fluctuations during the oestrus cycle having a detrimental effect on spatial memory and on the learning strategy of rats (Sutcliffe et al., 2007, Frye, 1995), however it was unclear as to whether there was an effect during this study.

This protocol, four tasks within two days, has never before been tested in rats and it's crucial to the current literature. Firstly it demonstrates that adult rats do not need to "practice" these tasks in order to do them, and secondly that positive performance scores can be generated after one trial. In the original study in which the OPC task was developed, rats were tested four times on each trial and an average score generated across the four tasks (Eacott and Norman, 2004) and this is commonplace in laboratories that utilise these task. Multiple trials of rats are beneficial in some situations due to the fact that a lot of data can be collected from one animal. However, on occasions where the animal is only available for a short period of time, for example during development, a critical phase of a disease or after pharmacological intervention, the knowledge that rats can perform these tasks the first time they are tested is crucial.

This protocol was then carried out on juvenile rats at a number of ages. Each litter of rats was only tested on the protocol once and therefore the study was of a cross sectional nature rather than a longitudinal one. Complex statistical analysis was carried out, allowing the age at which the task developed to be understood. The youngest age tested was p25, at which point the only task the rats were able to complete successfully was the NOR task which tested object recognition memory. This is an interesting observation that, an age at which rats are mature in many other ways, they are unable to perform the OC, OP and OPC tasks, suggesting a lack of associative contextual, spatial and episodic memory. Novelty is the consistent element of all four tasks, and the fact that the performance of rats on the NOR task remained consistent across the ages, with all rats performing at a high level is crucial to the rest of the

investigation. This is because it allows us to make the conclusion that if a rat cannot perform one of the other three tasks, it is not because they lack the ability to recognise novelty. The OC task which tested associative contextual memory showed a developmental profile which suggested that the ontogeny of this memory type was between p38 and p42. This is a relatively late timepoint given the fact that contextual fear conditioning has been shown to develop at p23 (Rudy,1993) and that this is a task which involves the association between contextual information and an event. This development of contextual fear conditioning is thought to coincide with the development of the amygdala, as the fear conditioning paradigm has a strong emotional component. The OC task used here is thought not to stress the animal in any way, and it's possible that the early amydala development does not play a role in the ability to perform the OC task.

The spatial OP and the episodic OPC tasks both developed between p46 and p48 and this timepoint was investigated further in chapter seven where these tasks were tested over three consecutive days. It was found that the ability to perform both tasks develops at the same time, overnight between p47 and p48. This development was found to be independent of experience and it was hypothesised that that this may coincide with the maturation of the trisynaptic pathway (EC - DG - CA3 - CA1).

This sudden development of the ability to perform these tasks is a crucial part of the investigation. Firstly it had not been demonstrated previously in the literature at what age rats could perform these tasks. Secondly this age has not been implicated in any other developmental event within the current literature. It is relatively late within the rat's life as this is a time when the rat

has been weaned and could have mated if given the chance. What's more, this is an age at which many scientific investigators would not class as immature or juvenile. This is a fact that has been highlighted by a review in 2009 by McCutcheon and Marinelli. They took LTP as a subject matter and investigated the ages of animals used for each study, finding that 30% of papers used animals whose ages were during this crucial timewindow of puberty and adolescence (McCutcheon and Marinelli, 2009). They also found that often animals' ages within a single study varied by up to four weeks and yet their data were pooled together and they were treated as part of the same group. This study has demonstrated that one day within a rat's life is enough time for substantial behavioural changes to occur and provides an argument for the fact that the scientific community needs to be more stringent in the ages at which animals are used for investigations, and that the age of an animal should not be overlooked.

Within chapter four, where the novel two day testing protocol was used in juvenile rats, it was shown that there were no overall relationships between performance and sex, exploration time, latency to explore the objects when placed in the arena or the identity of the first object explored. Although there was no overall difference between males and females in the investigation, there was a difference seen on the OP task on p39, with females performing significantly better than males. It was hypothesised that this marked the day on which the rats experienced their first oestrus and therefore showed an increase in performance due to an increase in LH (Gabriel et al., 1992, Ziegler and Thornton, 2010).

The NOR task was manipulated to look at the ontogeny of long term object recognition memory. A twenty four hour delay was introduced between the encoding and recall phases and it was found that rats aged p29/30 rats were able to perform this task, whereas rats aged p19/20 were not. It was hypothesised that this development was due to the maturation of perirhinal cortex inputs into the hippocampus. This task utilised a ten minute sample phase rather than a three minute sample phase as in the previous chapters and was conducted alongside a control task with the same length of sample phase but instead with a two minute delay. Regardless of the increase in time available for encoding in comparison to the three minute sample phase in previous chapters where the NOR task was utilised, no increase in performance was found. This allowed the speculation that the rats had reached a “ceiling level” of performance with a score of around 0.5, and that an increase in the length of the sample phase does not improve performance in an object recognition task.

The next investigation that was conducted utilised a variation of the OC task. The CDT task was used alongside NOR in a longitudinal study around the days at which the previous OC task had suggested were important for contextual memory, p38-42. On p34 rats were able to generate a positive score on both NOR and CDT, on p40 rats can perform NOR but not CDT, on p41 rats cannot generate a positive score on either NOR or CDT and finally on p47 rats can perform both tasks with a positive score. It was hypothesised that on p34 the positive score generated in the CDT task was not because the rats were performing the task in a mature way, but instead were not recognising the change in context and instead were treating the task like NOR.

On p40 when the rats could not generate a positive score on CDT task it was hypothesised that this was due the development of the ability to process, encode and retrieve contextual information but the rats were as yet unable to dissociate an object from it's associated context. On p41 when the rats could not perform above chance on either CDT nor NOR it was hypothesis that this correlated with the development of LEC connections with the hippocampus. It may be that these connections are so strong on this day (possibly the first day at which the animals can use the contextual information in their memory formation) that the LEC input overpowers the input from the perirhinal cortex. This possible lack of saliency of perirhinal cortex inputs may explain the lack of ability of the rats to perform the NOR task as seen in this investigation. It was hypothesised that by the next time the rats were tested, p47, they were performing both tasks in an adult like way and therefore could generate a positive score on both tasks. This overall hypothesis was supported by literature on fear conditioning and by the data generated in the previous chapter where the OC task was used.

The final behavioural task conducted in this thesis was investigating the ontogeny of spatial memory, and was based on the OP task used in both the cross sectional protocol and the three day consecutive testing. It was found that an allocentric version of the OP task showed the same pattern of development as the OP task, thought to be an egocentric version of the task. It was suggested that because of this rats in pervious chapters tested on OP may also be completing it in an allocentric way. It was also hypothesised that the rats were utilising the right hippocampus more than the left due to the fact that the right hippocampus is more involved in allocentric processing in

humans and rats. This investigation also looked at spatial memory with both intramaze and extramaze cues and found that using extramaze cues in a different position or using intramaze cues did not affect the development of the OP task.

The final experiment carried out in this thesis was an immunohistochemistry study of c-fos expression in the hippocampus and parahippocampal regions of a rat brain after completion of the OPC task. This investigation showed no overall changes in c-fos expression between rats aged p47 and rats aged p48, however subtle increases were noted in the CA3 of p48 rats. It was hypothesised that this region may be important in this task, and that in particular the left hippocampus may be of interest in future studies. This study also found that female rats had much more variability in c-fos expression than males, and it was discussed that this could be due to discrepancies in the oestrus cycle.

Gathering all the information together from this thesis, it is clear that the hippocampal regions, and in particular the LEC, perirhinal cortex and CA3, are important regions in the development of memory in the juvenile rat. The CA3 has been implicated as an area which may show hemispheric differences, however it is unclear which side is the key one here. Literature has suggested that the right hippocampus is important for allocentric memory (Feigenbaum and Morris, 2004, Igloi et al., 2010, Klur et al., 2009) and the data here suggests that the rats are performing some of the tasks in an allocentric manner. However there have been studies which suggest that the left hippocampus is important in hippocampal long term memory (Shipton et al., 2014). Future work should aim to address this issue and to understand this

further. The final thing that must be addressed in future is the stage of the oestrus cycle that female rats are in during behavioural testing. There is clear evidence in the literature as well as data in this thesis which suggests that this is a factor which is having an effect on behavioural data. Excluding female rats entirely from behavioural studies is one option, and one which a lot of laboratories take, however I feel that it is important to utilise female rats but with caution and with appropriate extra measures in place.

Within this investigation the sample size for experiements was selected based on the practicalities of the study and the constraints that working with live animals apply. In particular, behavioural experiments in juvenile animals is something which relies heavily on the practical aspects of testing. When a litter is born it is up to the experimenter to figure out, based on the date of birth, if and when the rats can be used and tested. Furthermore, occiasionaly an animal was excluded from analysis due to an object falling over, jumping out of the testing arena or chewing and consequently pulling down one of the arena walls, therefore reducing the stastical power further. In the future it would be highly beneficial to conduct a power analysis which would allow the sample size required to detect an effect of a given size with a given degree of confidence to be understood. If the sample size is too low, the experiment will lack the ability to provide reliable answers, however if the sample size is too large it wastes time and resources.

This thesis has successfully demonstrated that rats show a similar ontogenic profile of memory subtypes as humans, with recognition memory developing before episodic memory. The juvenile brain provides a useful tool for understanding memory and it may be that the future of research into

neurodegeneration in old age may rest on our knowledge of the construction of the brain during development.

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