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A Behavioural, Molecular and Lesion Examination of Hippocampal Contributions to Morris Water Maze Acquisition.

Mairéad Diviney, B.A. (Hons)

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Head of Department: Dr. Fiona Lyddy
Research Supervisor: Dr. Seán Commins

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Abstract

The Morris water maze (MWM) is a widely known, simple and effective task in the examination of spatial learning and memory. Successful acquisition of the task is thought to rely on retained representations of allocentric spatial relations, whereby animals learn to associate the location of a hidden platform with surrounding distal cues and subsequently use this information to navigate towards the hidden goal. As the distal cues are critical in this process, features of the cues, such as location, are an important factor to consider in examining how the task is solved. It has also been well documented that the hippocampus is a critical structure in the processing of allocentric representations. However, there has been debate surrounding the exact nature of this involvement, with suggestions that hippocampal damage leads to deficiencies in navigational aspects of the task rather than purely spatial processing impairments. To assess this, we adopted novel methods of analyses which include sub-second monitoring of each individual animal's behaviour as they navigate during a training trial. From this analysis we initially determine that positioning of the distal cues around the maze can impact on intact animals' performance. Specifically, we noted that animals with cues positioned close to their goal are more efficient in reaching the target and use more view-dependent strategies, over animals whose cues are in a position further away, who, instead, are more reliant on view-independent behaviours in order to reach their goal. Molecular examinations of both groups of animals reveal higher BDNF expression in the dorsal hippocampus in the group whose cues are positioned further away from their goal, which we suggest reflects the Far cue groups need to infer their position more than the Near cue group. Following this, assessment of animal behaviour following lesions to the dorsal hippocampus indicated that both the Near and Far lesioned groups were

significantly impaired in the MWM. Behavioural analysis highlighted lesioned animals' deficits in accurately monitoring and adapting their motor movements in response to task demands, suggesting that the impairments seen in the maze are due deficits in integrating exploratory behaviours, rather than a purely spatial memory impairment. While there were few differences in performance of the Near and Far lesioned animals, further assessment of the intact hippocampus using immunohistochemical procedures revealed increased c-Fos expression in the Far cue group in area CA1 of the hippocampus. Further to this, subregional assessment using lesion and IEG methodologies led to the distinction that the dentate gyrus, in particular, is critical in performance in the water maze.

Together, the behavioural, molecular and lesion data assessing hippocampal contributions to acquisition of the MWM are discussed in terms of models of navigation. From this, we suggest that the water maze task is solved using a vector-model of navigation, rather than the widely reported, and accepted, cognitive mapping theory of spatial learning. The behavioural lesion data also supports a role for the hippocampus in this model, specifically as lesioned animals' display clear impairments in the accurate judgement of distance and direction to their goal when in the maze; a critical feature of the vector-model.

Chapter 1

Literature Review
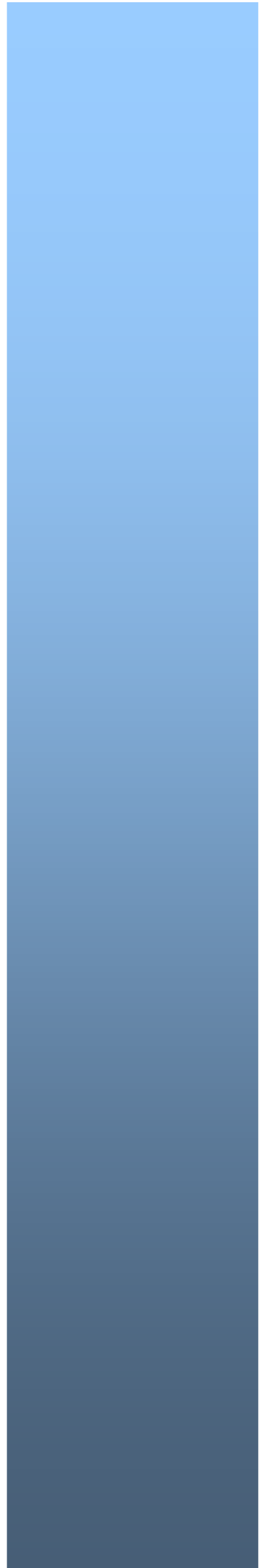
1.1 Introduction

Memory and learning are concepts that have been visited and revisited time and again in attempts to understand the encoding, storage and retrieval of information. One of the most common approaches used in studying learning and memory is the examination of animal abilities in specifically designed tasks. In particular, navigation tasks have taken a key role in this investigation, largely due to the ease at which they can be manipulated to enable thorough examination of the processes involved in learning and also due to the recognition of navigation as a crucial component of intelligent behaviour (Olton, 1977). For instance, all animals must learn to make meaningful, planned and accurate movements in order to source water, food or a mate. As well as this, all animals who venture away from their home base, in search of these resources, face the impending task of returning home. This can be accomplished in numerous ways with several sources of information available to the navigating animal, for example magnetic fields (Gould, 2011), scent (Reinhard et al., 2004; Wallace et al., 2002a), ultraviolet light (Sakura et al., in press; von Frisch, 1960) and the orientation of stars (Emlen, 1970; Mauck et al., 2008), to name a few. However, in the rodent, the two most widely reported and examined navigational strategies include the processes of egocentric and allocentric navigation (Squire, 1992; Whishaw & Tomie, 1997b).

1.2 Navigational Strategies

The first, and more spatially simple form of navigation reported in the literature is egocentric navigation. Navigation using this strategy requires the subject to use itself as its own point of reference, where all external cue points are encoded and processed in relation to the navigator. Egocentric navigation, therefore, involves the navigator

using information from bodily cues such as idiothetic information originating from vestibular and kinaesthetic systems (Allen, 2004; Burgess et al., 2004). These body-centred based systems incorporate changes in the navigator's movements of their muscles, joints and tendons, which together allow for the calculation and estimation of movement behaviour, such as alterations in acceleration, providing critical information on the subject's current position while they move (Etienne et al., 1996). This type of egocentric navigation has also been referred to as path integration (Benhamou, 1997; Etienne & Jeffery, 2004) and importantly, does not require the presence of external cues. Therefore, this type of information is of critical importance when an animal cannot depend on external information to guide them to a goal, for example when landmarks are unstable or uninformative. However, not only has egocentric navigation been defined as the incorporation of movements while navigating, it has also been categorised as the use of a single beacon cue, where the navigator does not need to encode the spatial relationship of the cue to the target, but needs only the knowledge that certain movements towards the beacon will lead them directly to their goal.

An allocentric strategy, on the other hand, depends on information from spatial cues alone, irrespective of the navigator's location in an environment. In this instance, multiple available cues can be used, whereby the navigator processes the spatial relationship between the cues and the goal in order to memorise the target's position (Allen, 2004; Benhamou & Poucet, 1998). It has been suggested that there are two components to this process. First, the establishment of spatial relationships between landmarks in the environment occurs, and second, the updating of distance and direction from start to goal by reference to information external to the navigator. Using an allocentric strategy, the navigator, if disoriented, should readily be able to recall

how cues from the surrounding environment are related to each other and so have little difficulty in finding their location in space again. By using and processing this type of environmental information, it has been suggested that a 'map' of the layout of an environment can be developed (O'Keefe & Nadel, 1978; Tolman, 1948).

One such widely known and examined 'map'-like representation is O'Keefe and Nadel's (1978) cognitive map. This map is developed from the spatial information gained from a previously encountered environment and has been defined as a stable Euclidean representation of the distances and directions between landmarks and locations. This theory is mostly in keeping with the idea of allocentric spatial processing and is not dependent on the viewer's location; instead all of the elements within the 'map' are arranged according to their location to each other irrespective of the navigator (O' Keefe & Nadel, 1978). A critical feature of the 'map', which allows for highly efficient and flexible navigation, is that the moving animal gains information from its surroundings that are also beyond their direct field of perception, allowing for short cuts and novel paths to be taken (Poucet, 1993). O'Keefe and Nadel (1978), the key proponents of the cognitive map theory, proposed that for the map to be successfully generated, the animal must firstly thoroughly explore their environment; the acquired details of this exploration would then become integrated in the map-like system, subsequently reducing the need for further exploration once it is established.

Cognitive mapping theory also proposed that the hippocampus is the neural structure dedicated to creating these map-like representations of space (see Section 1.4). Within the theory, both egocentric and allocentric representations are also accounted for. When the navigating animal can head directly for landmarks in the

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environment, they are thought to be using 'taxon' navigation, which is deemed a form of response learning, not reliant on the hippocampus. Animals can also use a 'locale' system, which requires an intact hippocampus and supports allocentric learning and the development of the cognitive map. Gallistel (1990) further added to O'Keefe and Nadel's (1978) definition of a cognitive map, postulating that such maps are constructed using path integration processes within which animals rely on body-centred signals to keep track of their position *in relation* to an allocentric reference frame. However, while cognitive map theory has received much attention, some of the findings reported to confirm these ideas are somewhat imprecise and thus not easily understood (Eichenbaum et al., 1999). Furthermore, there may be simpler explanations to account for animal navigation.

One such position on this comes from associative learning theory, which is a more recent alternative to the cognitive mapping model of spatial representation. It postulates that allocentric space may be simply represented as an associative mechanism (Miller & Shettleworth, 2007), where one factor (be it object or action) can be learned only through the association with a separate, pre-occurring factor. So rather than building up an overall representation of the layout of an environment, which may be cognitively taxing, the navigator need only associate individual items in the environment as required. Evidence has been provided for this type of learning in spatial navigation, where a target location is learned by associating elements in the environment with actions made by the navigator (see below). Specifically, two key elements of associative learning theory influencing spatial learning, are blocking and overshadowing.

In blocking, once an association has been made between two elements, it weakens the formation of any new possible associations. In other words, the associative capacity of an element in the environment is limited, which can result in competition between elements that become newly introduced to already familiar surroundings (Kamin, 1968). For instance, in spatial domains when an environment is originally learned with a single defined cue (1), and later with an additional cue added to the arrangement (1, 2) which the animal can also learn about, the navigator is significantly impaired in the task when only cue 2 is presented to solve the task. Therefore, it can be said that the original learning with cue 1, blocked any further learning about a new cue in the environment. A good example of this was reported by Rodrigo et al. (1997) when they followed a similar pattern of cue manipulation during training in the Morris water maze (MWM), which resulted in impairment in the navigating animal when only the second or 'blocked' cue was available.

Similarly, overshadowing is based on the same assumption, but in this instance both stimuli are presented at the same time, with one being much more salient to the viewer than the other (Pavlov, 1927). Specifically, when a number of cues or landmarks are available, associative theory predicts that, when learning, the animal will weight some cues as more important than others. So rather than constructing a 'map' that incorporates all of the environmental stimuli in an all-or-none manner as described by O'Keefe and Nadel (1978), the animal weights the importance of specific individual cues or landmarks with goal finding. This was clearly demonstrated by Chamizo et al. (2006), when a landmark placed close to the platform in the MWM was better learned than landmarks that were present at the same time but were positioned farther away. While these findings suggest a role for associative learning theory in

spatial learning and memory, the definitive nature of associative learning has come under question, as unlike cognitive mapping theory, no clear neural underpinnings for such a mechanism have been well established. Doeller and Burgess (2008) have provided some evidence for the role of the striatum in associative learning however, more investigation beyond purely behavioural studies needs to be conducted on this.

1.3 Morris Water Maze Navigation

A task widely used to examine the many facets of spatial learning and memory is the MWM. It is often favoured over other spatial tasks as unlike land based navigational paradigms, such as the radial-arm maze (Olton & Samuelson, 1976), or Y-mazes (Wright & Conrad, 2005), there is no need to introduce measures such as food deprivation prior to training, and the possible use of non-spatial olfactory and auditory cues are also eliminated by the presence of water (D'Hooge & De Deyn, 2001; Paul et al., 2009). Furthermore, it also appeals to the natural propensity of rats as swimmers and to their innate curiosity in new environments; this particularly is of key importance as the animal must search the entire maze to have a chance of finding their goal, which is often submerged in water (Morris, 1984; Figure 1.1). The task has been adapted in multiple ways to examine the different components of spatial learning and memory, from the examination and treatment of neurocognitive disorders (Kenney & Gould, 2008; Porsolt et al., 2010), to drug treatments (Cunningham & Sanderson, 2008; Hoane, 2007), and the underlying neuropharmacology of learning and memory (Adams et al., 2008; Yan et al., 2007).

Figure 1.1: Schematic and photographic images of a standard version of the Morris water maze [adapted from http://pnf.ruhosting.nl/MorrisWaterMaze.htm., 28/07/2011]

While the MWM appears to be a relatively straight forward task in examining spatial navigation, there are numerous and conflicting interpretations of how the task is acquired; for example can the maze be learned using associative mechanisms or can it only be solved using a map-like representation? Manipulations to the MWM paradigm, however, have proven useful when assessing the different types of learning used by the navigator to solve the task. The most common differentiation is between spatial and non-spatial training paradigms. For example, in the standard, spatial reference version of the water maze (often referred to as place learning), animals are placed in the pool from a number of random start points to locate a goal hidden (at a fixed location) beneath the surface of the water, with only external cues available to guide their

search. In a non-spatial version of the maze, the platform is usually made visible or is directly marked using a prominent beacon, to allow direct finding of the goal. Many other manipulations including length of training (Kealy et al., 2008; Pouzet et al., 2002), start-position manipulation (Kealy et al., 2008; Tamara et al., 2010b), cue number (D. Harvey et al., 2009), and cue location (proximal or distal) alterations during training (Chamizo & Rodrigo, 2004; Chamizo et al., 2006; Hamilton et al., 2004; Timberlake et al., 2007; Wortwein et al., 1995), and retention, (McGauran et al., 2004), have all been used to assess specific aspects of spatial learning. In particular, they enable assessment of when and under what circumstances a spatial strategy will be preferred over another.

In particular, under conditions where the cues are placed in a proximal position, or directly mark the goal, it has been found that the most frequently used navigational strategy is egocentric navigation (Carman & Mactutus, 2002; Cheng & Spetch, 1995). This more direct form of *cued* (egocentric) navigation is one of the simpler forms of searching in the maze as the navigator is only required to know an appropriate response to the stimulus (i.e. should it approach it or not; Jeffrey, 2003). Rodents, time and again, have been shown to use this navigational strategy with much success (Lindner et al., 1997; Morris, 1981; Sutherland & Dyck, 1984; Timberlake et al., 2007). Linder et al. (1997) for example, used a cued version of the MWM, with the platform raised above the surface of the water acting as a cue itself. As expected, rats acquired the task relatively quickly, a result also demonstrated in the original Morris (1981) study, and later by Sutherland and Dyck (1984). Similarly, beaconed navigation where a cue is attached to the goal returns equivalent findings (Martin et al., 2005; Roberts & Pearce, 1999). While an efficient method of goal locating when available, it

is not necessarily spatial in nature, as the navigator can locate their goal without making reference to any other features of the pool and it can also only be used under these cued environmental conditions.

It is also rare that landmarks are provided in isolation or positioned conveniently beside the desired goal. Most often, cues are located some distance away from the target and thus require some form of spatial processing to allow for successful navigation. Interestingly, however, when a cue is moved away from a goal so it no longer directly marks its position but remains in close proximity (approx. 50cm; Chamizo & Rodrigo, 2004), it remains possible for the navigating animal to locate their target by using distance information alone provided by the beacon. However, beyond a certain distance (approx 110cm; Chamzio & Rodrigo, 2004) animals must also know the direction from the landmark to the goal, in order to refine their search to accurately locate their target (Chamizo et al., 2006; Mackintosh, 2002). When the distance between goal and cue increases, the navigating animal must have a mechanism to enable successful learning, and it has been suggested that this system relies on the animal's ability to learn to use and integrate information from a number of cues in the environment independent of the animal's own location. This is most often observed, for example, in a hidden platform, *place* (allocentric) version, of the MWM, where rats must learn the spatial relationship of several distal cues to one another and the goal itself, as no cues explicitly mark the goal directly (Aggleton et al., 2000; Commins et al., 1999; Harvey et al. 2008; McGauran et al., 2004; Morris, 1981). This is in keeping with O'Keefe and Nadel's (1978) proposal of the development of a cognitive map using allocentric representations of an environment.

It has been proposed, however, that place and cued learning are not mutually exclusive strategies, with evidence indicating that rats can make use of both types of learning concurrently and interchangeably (Redhead et al., 1997; Whishaw & Mittleman, 1986; Whishaw, 1998b). Whishaw (1998a) found evidence for this when they trained rats to swim to a visible platform (cued), while also having distal cues present in the training environment, and later found that they could search in the accurate goal location (place response) even when it was no longer visible, indicating the concurrent learning of place while being trained to go to cue. Similarly, Hamilton et al. (2004), in a study examining which type of cue (proximal or distal) controlled navigation in a water maze, found that when both are available they can be used sequentially to navigate during a cued task. They suggested that the initial segment of the swim trial is controlled by distal cues, with animals using these cues to orient in the general direction of the goal. However, as the animal moves closer to the vicinity of the platform, they then switch and become more reliant on proximal cues that overtly indicate the platform's location for greater accuracy in reaching the goal. A similar strategy switch was also observed by Harvey et al. (2008) in a standard place MWM.

1.3.1 Acquiring the Morris Water Maze

Despite the advantages to using the MWM, there remains inconsistency regarding the interpretation of how the task is solved. The majority of MWM studies report general measures such as escape latency, path length and swimming velocity (Baldi et al., 2003; Maurer & Derivaz, 2000; Morris, 1981) and while useful in providing an overview of the animal's general performance in the maze (Gallagher et al., 1993), they may not be sufficient to resolve the issues in navigation. In agreement with this,

Hamilton et al. (2004) have suggested that a detailed moment-to-moment examination of swimming behaviours may provide a more sensitive analysis of how stimuli control behaviour. The utility of such an assessment was highlighted when detailed examination of swimming behaviour, including kinematics, heading direction and accuracy of movement revealed subtle variations, within a single training trial, in how animals solved the MWM (Hamilton et al., 2004). This level of detailed, bottom-up, investigation enabled a clear differentiation of behavioural methods employed by animals within individual trials, when in the MWM. Further attempts have been made to assess the changing behaviour of animals in the MWM. Graziano et al. (2003) adopted a slightly different method of detailed behavioural analysis to that described above. Instead of examining a small portion of behaviour within a trial, they grouped behaviours of the navigating animal into a number of overall categories and applied the most prominent behaviour to an entire trial. This novel method of analysis also characterised each behaviour in terms of successive levels of learning and familiarity of a goal location, reflecting yet extending, the standard criteria of acquisition.

While useful, these behaviours do not give a detailed account of the subtle variations of movement within and between trials. Harvey et al. (2008), however, recently took this examination further suggesting that meticulous examination of the movements of the animal while swimming enables visualisation of distinct patterns of behaviour. For this, the authors carried out a sub-second behavioural analysis as animals swam in the maze and from this, a number of individual behaviours were established. A number of the observed behaviours were based on behaviours seen in other species as more detailed accounts of navigation have previously been conducted on species other than the rodent. Stability and approach behaviours, for example,

where the animal keeps the distal cue stable on their field of vision is similar to behaviour seen by navigating wood ants (Harris et al., 2007; Judd & Collett, 1998) and blowflies (Campbell, 2001). As the insect approaches an object initially, they hold the image steady on their retina, and when reintroduced to the environment or when an object is revisited, they match the current image on the retina with the previous preferred retinal position. If there is a mismatch in view the animal turns to rectify the discrepancy (Collett et al., 1992), another behaviour seen in the navigating rat (Harvey et al., 2008). Similarly, scanning seen in rats in the MWM (turning; Graziano et al., 2003; Harvey et al., 2008), has also been reported as a method of landmark sampling in wasps (Jeanson et al., 2003). Critically, and unlike Graziano et al. (2003), Harvey and colleagues (2008) examined each behaviour individually within and between trials, rather than grouping behaviours for an entire trial. The findings from this method of behavioural analysis were subsequently applied to the more widely reported egocentric and allocentric navigational strategies. This then enabled clear differentiation of strategy use across training within a single standard MWM procedure, a finding that would not have been elucidated by the examination of escape latency alone.

Traditionally the analysis of such behaviours has been regarded as being too complex, time-consuming and subjective (Graziano et al., 2003; Martin et al., 2003; Tchernichovski & Benjamini, 1998) and is often ignored in favour of easier measurements. The importance of such behavioural investigation, however, cannot be underestimated (D. Harvey et al., 2008; 2009). In particular, a study conducted by D. Harvey et al. (2009) examining the effect of 1 versus 3 cues in solving a place MWM highlights the importance of examination beyond escape latency. Initial examination, using these basic measurements of performance revealed no differences between the

groups trained under the two distinct cue conditions, suggesting the task was learned in a similar manner by both training groups. However, further detailed inspection of the subjects swimming behaviours revealed differences in the animals' searching, where exposure to a single cue led to a simple beacon searching strategy being employed (D. Harvey et al., 2009). This detailed approach to quantifying water maze performance, therefore, appears to be crucial in identifying differences in how the task can be learned with subtle modifications to experimental design (Hardt et al., 2009). It also leads to more accurate representation of strategies being used, the details of which often lead to confusion when analysed using broad measures (Moghaddam & Bures, 1996; Timberlake et al., 2007; Whishaw & Brooks, 1999). Together these findings highlight the importance of developing new measures of assessment within the MWM, as the standard measures used over the past 30 years appear not to be sufficient in determining subtle variations in behaviour as an animal learns.

1.4 The Hippocampus and Spatial Navigation

All of the studies that have been described thus far highlight the quick and efficient learning of intact animals in both a cued and place versions of the MWM (Hamilton et al., 2004; Kealy et al., 2008; Tamara et al., 2010b; Timberlake et al., 2007). Specifically, in the more difficult place version of the task, unlesioned animals can readily locate a hidden goal from multiple start positions when only extramaze, distal cues are available to do so (Morris, 1981), highlighting their ability to use information gained from the environment flexibly to find a goal that cannot be seen. However, when damage to the brain is incurred it can lead to substantial interruption in such tasks, and is particularly detrimental when the acquired damage is to the hippocampus

(D'Hooge & De Deyn, 2001; Morris et al., 1982; O'Keefe & Nadel, 1978). In particular, the hippocampus is thought to play a pivotal role in allocentric processing which is required particularly during place navigation in the MWM (O'Keefe & Nadel, 1978; see Figure 1.2).
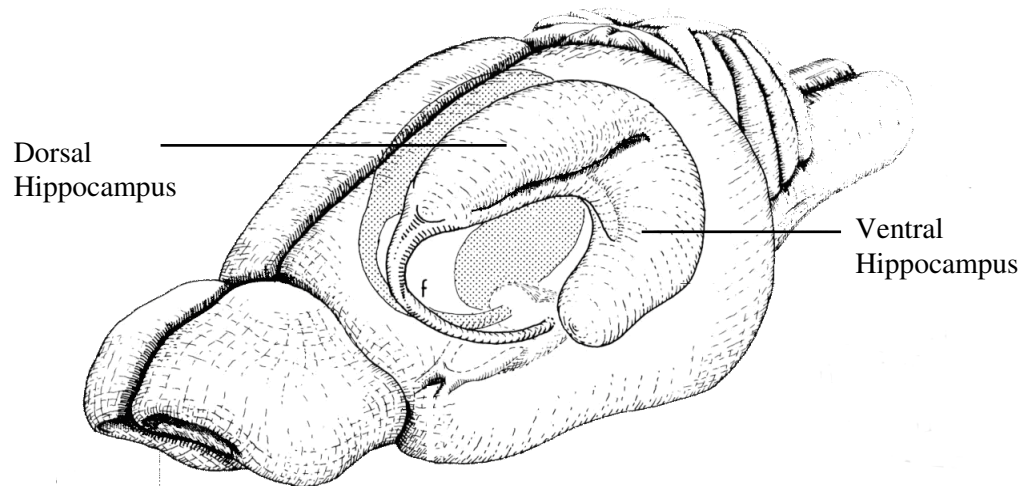
Figure 1.2: Diagram of the rat hippocampus including distinction between the dorsal and ventral hippocampus [Adapted from Amaral and Witter (1995), with permission from Elsevier].

However, prior to animal navigation studies, human studies, with patients that had temporal lobe damage, initially brought attention to the hippocampus as a brain structure that is critically important in learning and memory. A key case is that of patient HM. HM suffered from intractable epilepsy and in 1953 underwent a surgical resection of his medial temporal lobes, losing approximately two-thirds of his hippocampus, parahippocampal gyrus, and amygdala and as a result suffered from severe anterograde amnesia, where he was unable to acquire long-term memory for new facts or events. Importantly, however, it was also revealed that HM could form long-term procedural memories, providing an early indication of a dissociation

between brain regions and memory type (Scoville & Milner, 1957). While HM provided a wealth of information regarding learning and memory, in general, he also contributed to the examination of spatial memory. In contrast to his inability to form new declarative memories, he remained capable of drawing the layout of an apartment which his family had moved into after his operation, meaning he retained some spared spatial memory for the environment (McClelland et al., 1995). It was posited that through HMs repeated exposure to the house and his locomotion between the rooms, HM built an allocentric representation of this environment (Corkin et al., 2002). This retained ability was later attributed to the sparing of a number of other structures external to the hippocampus (Corkin et al., 2002). However, while he learned the layout of this environment after repeated exposure, he remained unable to learn other spatial information, for example the correct sequence of turns in a visual maze (Milner, 1965).

While some of HM's ability to learn new spatial information remained intact other hippocampal damaged patients (Rosenbaum et al., 2000) have shown a persistent inability to form new memories for the spatial layout of an environment. King et al. (2004), for example, studied patient Jon, who had experienced developmental amnesia as a result of bilateral hippocampal damage. Jon presented with memory impairment including spatial deficits; forgetting where he put things, and being unable to find his way around certain surroundings. By using a virtual reality task, the investigators examined the accuracy of Jon's spatial memory. For this, the authors employed two conditions; a shifted-view test and a same-view test which relied on either the allocentric or egocentric navigational systems, respectively. As expected, Jon was impaired in the shifted-view condition, displaying an impaired ability to flexibly

reconstruct accurate representations underlying the shifted-view recognition condition, implicating the hippocampus is needed to enable flexible representation of an environment (King et al., 2004). Similar deficits have been demonstrated in tests involving table-top environments (e.g. Holdstock et al, 2000). Hartley et al. (2007), similarly, examined the hippocampal contribution to memory for spatial and non-spatial information in visual scenes. They tested patients with focal hippocampal lesions, including Jon and patients KC, VC, RH and MH. All patients showed impaired spatial and spared non-spatial processing, with those with greater damage (e.g. patient MH) including the parahippocampal area, displaying the worst performance. Bartsch et al. (2010), similarly, examined 14 patients with acute transient global amnesia which resulted in focal CA1 lesions of the hippocampus. They assessed these patients in a virtual MWM and found that they displayed significant impairments in a place version of the task. These results again support the role of the hippocampus in allocentric processing.

So while patient data initially provided invaluable insight into the brain regions involved in spatial tasks, particularly the hippocampus and parahippocampus, assessment of activated brain regions in intact human participants has also provided valuable insight into spatial navigation processes (Maguire et al., 1997; 2000; Ghaem et al., 1997), with evidence, again, strongly implicating the hippocampus in spatial navigation. A study conducted by Maguire et al. (1997), involving London taxi drivers with detailed knowledge of London, showed increased activation of the right hippocampus during a recall task. In addition to increased activation of the hippocampus, the taxi drivers also showed navigation-related structural change, with the posterior hippocampi of taxi drivers being significantly larger than control

participants (Maguire et al., 2000). However, furthering this study, Maguire et al. (2006) later assessed a London taxi driver (patient TT) who had sustained bilateral hippocampal damage. From this, they found that the hippocampus was not required for general orientation in the city or for knowledge of the spatial relationships between landmarks, or even for active navigation along some routes. Rather, Maguire et al. (2006) concluded that perhaps the hippocampus is only necessary for enabling efficient navigation in places learned long ago, particularly where large-scale environments are concerned, and successful navigation requires access to detailed spatial representations. So while there is evidence for hippocampal involvement in spatial learning and memory, particularly allocentric learning, there remains some ambiguity as to its exact function and whether it is always required for navigation.

In addition to the navigational research in humans, animal studies into spatial memory have further contributed valuable information to our overall understanding of spatial processing and memory. The data on human patients is often difficult to entirely attribute to the hippocampus as often there is external damage to other medial temporal lobe (MTL) structures, leading to an overall interpretation of MTL function, rather than specifically hippocampal function (Squire & Zola-Morgan, 1991). While, imaging and electrophysiological studies have attempted to overcome this problem (Law et al., 2005; Maguire et al., 2006), animal studies allow for further, more localised assessment of multiple brain regions in easily manipulated tasks (Dudchenko et al., 2000; Morris et al., 1982; Chang & Gold, 2003a, b). Lesions, in particular, are a classic technique used to examine the function of different brain regions and enable the evaluation of whether a number of functions can or cannot be carried out normally following damage to a specific region (see Section 1.4.1 below). However, as with

human studies, animal lesion techniques also have some drawbacks (Finger et al., 2004; see also Morris, 2007), however they remain an important and widely used technique in behavioural neuroscience. In addition, other means of examination including molecular and electrophysiological mechanisms have also been employed in the assessment of the role of the hippocampus in learning and memory (see below) and allow for the examination of activation within an intact structure at a molecular level. Importantly, animal studies also allow for the use of experimental designs that allow for the assessment of physical movement within tasks; a method often not practical in human studies where virtual reality and table top tasks are, instead, widely applied. The importance of this ease of task manipulation is apparent in the debate surrounding the function of the hippocampus in spatial navigation (see Section 1.4.2).

As mentioned, animal studies provide a number of sources to assess the role of the hippocampus in spatial navigation. Firstly, assessment of the hippocampus at a neuronal level has implicated it as playing a specific role in the processing of space. Specifically, activity within the hippocampus, in the pyramidal cells (also referred to as 'place cells'), are location-specific, the activation (firing) of which are highly correlated to where the animal is in its environment (O'Keefe & Dostrovsky, 1971; Wilson & McNaughton, 1993). Moreover, their firing stability and location is dependent on the retained and constant configuration of distal cues or landmarks in the environment (Muller et al., 1987; O'Keefe & Burgess, 1996; Save et al., 2005). Adding to this, Cressant et al. (1997) found that distal peripheral cues play a critical role in fixing the orientation of place cell fields; for example, rotating cues in the environment, when the animal was not present, resulted in equal rotation of firing fields when the animal was reintroduced back into the environment (Bostock et al.,

1991; Cressant et al., 1997; Gothard et al., 1996; O'Keefe & Burgess, 1996). Therefore, it appears that the role of the place cell is to enable the animal to successfully navigate in the environment. The fact that place cells appear to encode the animal's location based on distal cues and are independent of its heading as it moves and navigates, suggests an important role for the hippocampus in spatial processing.

An important and widely accepted model of learning and memory, which has also proven useful in the assessment of different brain regions and the molecular mechanisms underlying the process of spatial learning, is long-term potentiation (LTP). LTP is a form of long-term synaptic plasticity which refers to changes in the strength of the synapses between two different populations of neurons. LTP is the rapidly induced and relatively enduring increase in synaptic strength following an electrophysiological event, such as high-frequency stimulation, and was first observed in the rabbit hippocampus (Bliss & Lømo, 1973). LTP can last anything from an hour up to a year (Abraham, 2003) and is widely considered the major cellular mechanism that underlies learning and memory (Cooke & Bliss, 2006). Specifically, LTP follows the requirements for a physiological mechanism for memory formation as set out by Hebb (1949), which posits that repeated activation of a neuron by another, leads to a strengthening of the connection between the two neurons which is commonly paraphrased as 'cells that fire together, wire together'. LTP has, therefore, been repeatedly examined in learning and memory research. Some further evidence to implicate synaptic plasticity as being a suitable model for learning and memory comes from a number of areas. Firstly, there are a number of behavioural paradigms that can affect both synaptic plasticity and learning and memory. Environmental enrichment (Leggio et al., 2005) and exercise (Vaynman et al., 2004), for example, have been

shown to enhance performance in learning and memory tasks and they have also been shown to enhance changes in synaptic plasticity (O'Callaghan et al., 2007). Furthermore, molecular evidence has indicated that molecules that are involved in synaptic plasticity have been shown to also be necessary in learning and memory processes. The expression of the immediate early gene c-Fos, for example, has been shown to increase following LTP induction (Jeffery et al., 1990). It has also been shown that knockout mice for c-Fos show impairments in LTP induction and learning (Fleischmann et al., 2003). In addition, alterations in BDNF signalling, for example, are observed following induction of LTP (Gooney et al, 2002) and learning (Mizuno et al., 2000). The assessment of a number of markers of neuronal activation have, therefore, proven useful when studying brain regions involved during spatial learning and memory and LTP (Anohkin & Rose, 1991; Kelly & Deadwyler, 2002).

Immediate early genes (IEG), for example, are good markers of activity as their expression results in long-term structural changes to the neuron by encoding transcription factors, growth factors and proteins involved in signal transduction (Lanahan & Worley, 1998). Such factors may include, brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 and 4 (NT-3 and NT-4), and activation of extracellular signal-regulated kinase (ERK), amongst others (see below). There are thought to be up to 40 IEGs that have been previously investigated, of which the most commonly examined ones in learning and memory include c-Fos, Egr-1, Arc, c-Jun and jun-B (Amin et al., 2006; Guzowski et al., 2005; He et al., 2002). The examination of these following learning have also implicated the hippocampus in spatial tasks such as the MWM, with increases in c-Fos, Egr-1 and Arc expression, seen in all hippocampal subregions following spatial training

(Guzowski et al., 2001; Teather et al., 2005). Even more interesting, is the finding that IEG expression in the hippocampus is altered in relation to the spatial complexity of a task, with increases in expression corresponding with increases in spatial demands. Fletcher et al. (2007), for instance, found no increase in Arc expression in the hippocampus following cued learning in the MWM, however, when task demands changed and animals were trained in a place MWM, Arc expression increased significantly. Genetic manipulations of IEGs in knock-out studies have also highlighted a role for the hippocampus in spatial learning (Korte et al., 1996). An example of this was highlighted by Granado et al. (2008) who reported that mice lacking the D1 dopamine receptor resulted in an inability to express Egr-1 in area CA1 of the hippocampus, and these mice were subsequently shown to be impaired during both the acquisition and retention of the MWM (Granado et al., 2008).

Other biochemical markers have also been implicated in learning, memory and LTP. NGF, for example, has been implicated in the induction of LTP (Castren et al., 1993) and NT-3 and NT-4 have similarly been shown to be involved in spatial learning and memory (Liu et al., 2009; Shimazu et al., 2006). Additionally, BDNF has shown to be required for LTP induction (Korte et al., 1995) and learning (Linnarsson et al., 1997). Downstream BDNF signalling has also been shown to occur with synaptic plasticity and learning; with evidence of increased phosphorylation of ERK observed after learning (Crow et al., 1998; Maguire et al., 1999). In particular, BDNF, a neurotrophin originally thought to be critical in neuronal development and survival (Barde, 1994; Davis & Squire, 1984), has been more recently implicated in learning and memory, particularly its expression in the hippocampus (Gooney et al., 2002; Mizuno et al., 2000). Hall et al. (2000) demonstrated the rapid induction of BDNF in

hippocampal area CA1 following contextual learning, while learning in the water maze has also been shown to increase the expression of BDNF in the hippocampus (Harvey et al., 2008; Kesslak et al., 1998). Additionally, while BDNF knockouts are often fatal (with most animals only surviving a few weeks after birth) a prominent role for this protein in synaptic plasticity has been elucidated (Ernfors et al., 1995). BDNF-knockout mice, for example, showed impaired induction of LTP in the area CA1 (Korte et al., 1995; 1998) and impaired spatial memory (Heldt et al., 2007). Similarly, selective removal of BDNF from the dorsal hippocampus also impairs spatial learning and discrimination (Gorski et al., 2003) and has been shown to alter LTP in the hippocampus (Korte et al., 1996; Pozzo-Miller et al., 1999). From the above evidence, it can be seen that BDNF may underlie several important processes in synaptic plasticity and learning and memory involving the hippocampus.

1.4.1 Hippocampal Lesions

While neural markers may provide correlating evidence for the role of the hippocampus in spatial learning and memory, more direct evidence comes from lesion studies. Numerous tasks have shown that animals with hippocampal damage are most severely impaired in spatial tasks that specifically rely on allocentric navigation, such as place-learning in the water maze, performing correct choice in the radial-arm maze, spatial object recognition tasks, and T-maze alternation paradigms (Clark et al., 2000; Dudchenko et al., 2000; Jarrard, 1978; Morris et al., 1982, 1990; Olton et al., 1978; Sutherland et al., 1983). Egocentric processing, on the other hand, is thought to be processed by other brain structures and systems (e.g. the striatum, Chang & Gold, 2003a, b; McDonald & White, 1993; vestibular system, Semenov & Bures, 1989).

In early studies examining the impact of damage to the hippocampus, Jarrard and colleagues (1986; 1989) found that hippocampal lesioned rats were largely impaired in spatial tasks in which they were dependent on extramaze cues, such as when learning to locate the correct arm in the radial-arm maze. Even when the hippocampus was temporarily inactivated using lidocaine, it led to significant impairment in place learning in a cross-maze. The critical importance of a functioning hippocampus was further highlighted in this study, as when the effect of the lidocaine diminished, hippocampal function returned, leaving animals again capable of accurately responding to place (Packard & McGaugh, 1996).

In addition to land-based spatial tasks, in a seminal study, Morris et al. (1982) examined the effect of hippocampal lesions in the Morris water maze, providing one of the earliest reports that lesioned animals were unable to locate a hidden goal using only distal cues in the water-based task. Since then, it has been widely established that lesioned animals required to use allocentric navigation are significantly impaired when compared to controls (Cain et al., 2006; Dolleman-van der Weel et al., 2009; Sutherland et al., 1983). Further, more elaborate, variations of the MWM, used to assess hippocampal removal on spatial learning and memory, have reported similar findings. Hollup et al. (2001), using an annular water maze, where the animal (and hidden platform) is confined to a corridor at the periphery of the pool, found that lesioned animals could not recognise the goal location using only distal information, despite the restricted exploratory conditions.

While the focus of hippocampal involvement has been placed on its role in spatial, allocentric processing, there have also been assessments carried out examining its contribution to egocentric-based tasks (de Bruin et al., 2001; Dolleman-van der

Weel et al., 2009). These studies often reveal lesioned animals to be unimpaired in comparison to controls, suggesting the animal remains capable of using a similar egocentric strategy as that employed by intact animals. Recent studies have confirmed these findings, for example, Mogensen et al. (2005), trained fimbria-fornix lesioned animals from a fixed start position to a fixed goal, excluding extramaze cues and thus removing the possibility of using any external or allocentric-based information to complete the task. Lesioned animals, although initially impaired, quickly learned the task to the same level as sham-controls indicating that hippocampal destruction has no detrimental effect on this type of navigation.

These findings suggest that as spatial demands increase, so too does the impairment in performance of the hippocampal lesioned animals (Save & Poucet, 2000). Therefore, while it is generally agreed that lesioned animals are most impaired when a task can only be solved using allocentric information some debate has evolved and centred around the mechanisms involved in this process; particularly whether place learning is, in fact, critically dependent on a functioning hippocampus (Cain et al., 2006; Morris et al., 1990; Save & Poucet, 2000; Whishaw & Tomie, 1997a). To try to tease apart how hippocampal damage can affect different aspects of spatial processing, we review a number of different experimental methodologies that have been used to assess hippocampal functioning.

1.4.2 Hippocampal lesions; Impairments in navigating or place finding?
While many lines of research suggest the hippocampus' importance in using the relations between distal cues to locate places (Guzowski et al., 2001; Heldt et al., 2007; Morris et al., 1982; Sutherland et al., 1983), there has also been evidence to the

contrary (Cain et al., 2006; Keith & Galizio, 1997), which has caused some debate around the exact nature of hippocampal involvement in learning and remembering spatial tasks. Cognitive map theory posits that there are two components involved in spatial learning, the first of which involves learning and processing movement information (taxon) and the second requiring learning about the spatial relationships of environmental features (locale; O'Keefe & Nadel, 1978). Put simply, spatial navigation comprises two separable components; 1) navigation to a location and 2) learning and recognising the location of the goal upon arrival. To determine this distinction, specific measures of acquisition and performance have been used and detailed across studies.

Morris et al. (1990) provided initial evidence in support of the retained ability of hippocampal lesioned animals to learn place following training in an adapted MWM paradigm. Specifically, lesioned animals were trained with both a hidden and visible platform in the same position but interchanged between training trials. Surprisingly, lesioned animals showed clear evidence of place learning of the goal location, displaying accurate place finding when only the hidden platform and use of distal cues was available. This slight modification of training in the task enabled hippocampal ablated animals to learn the location of place using allocentric information. However, lesioned animals displayed some inaccuracies in the precise localisation of behaviour along with an inability to carry out appropriate reattempts to search for the goal when compared to controls. It has since been shown, that with only minor changes to a training procedure, hippocampal lesioned animals can learn to acquire a place response in the MWM (Whishaw & Tomie, 1997a; Whishaw, 1998a),

Whishaw and Jarrard (1996) also noted the ability of animals to learn the location of place. Specifically, animals that were initially trained with a visible platform in a modified water maze and then later had the platform removed from, or submerged in the pool, showed accurate and localised searching at the spatial location of place. However, when trained in a new, hidden platform version of the task, behavioural examination of the training period revealed a clear deficit in the performance of the lesioned group when compared to controls, such as inappropriate circling. This indicated that perhaps hippocampal lesions may, in fact, disrupt navigational aspects of spatial tasks. Similarly, pre-training trials have also been incorporated into training procedures to reduce the need for lesioned animals to learn, both, the correct behavioural strategies to get to goal (i.e. learning to navigate to place), while also attempting to learn the spatial location of the goal (i.e. learning location of place). Cain et al. (2006) noted that animals given pre-training in the water maze, prior to hippocampal lesions, performed significantly better than non-pre-trained lesioned animals, suggesting that the reduction of behavioural demands enabled lesioned animals to successfully reach place and thus show evidence for intact place learning.

The evidence from the modified procedures suggests that perhaps, the impairments seen by lesioned animals in spatial tasks are an inability to compute movement information, such as distance and direction. A palpable example demonstrating this, involved testing hippocampal lesioned animals in a visual discrimination task, where they were required to encode allocentric spatial information but without the involvement of movement control. The lesioned group successfully learned about the allocentric cues, moreover, their performance was enhanced by the

allocentric encoding in the visual, computer generated Y-maze, again implicating that hippocampal ablated animals can learn about allocentric spatial cues when the need to encode navigation movements is removed (Gaffan et al., 2000). The above adapted training indicates hippocampal animals' ability to learn about allocentric cues to locate place but only when they were not required to attend to idiothetic cues. Together these findings suggest that the hippocampus plays a role beyond allocentric processing and may, instead, be involved in monitoring behaviour in order to reach place.

1.4.3 Hippocampal lesions; Impairments in integrating behaviours?

With clear evidence of lesioned animals' retained ability to learn about place, the observed difficulties in spatial tasks have been termed as navigational difficulties in 'getting there' (Gaffan et al., 2000; Whishaw et al., 1995, 2001). This difficulty has been further suggested as effecting path integration aspects of navigation where the animal must continuously monitor internal, idiothetic movement information.

To assess this idea, Whishaw et al. (1995) examined lesioned animals' performance in an adapted MWM procedure, where instead of training animals from random positions at the edge of the pool, they were initially placed on a visible platform at the beginning of training and gradually moved away to a start position at the edge of the pool. As the placing of the animal from the edge of the pool has often been seen to result in the adoption of non-effective behaviours, such as circling and thigmotaxis (Morris et al., 1982; Leggio et al., 2006; Wright et al., 2004), the restrictive training procedure aimed to reduce the animals' use of inefficient behaviours, and successfully resulted in accurate acquisition of the task in the lesioned group. Further confirmation of this, comes from Whishaw (1985a, b), who found that

after 31 days of training in a standard MWM, fimbria-fornix lesioned animals showed preference for the hidden platform target quadrant; a finding not observed after only 5 days of training. Interestingly, while escape latency data confirmed that they could locate place, a detailed examination of behavioural movements indicated a significantly altered pattern when compared to controls. For example, it was reported that if they missed the platform, the lesioned animals had great difficulty in reorienting themselves and were unable to update and correct any errors made. The observed alterations in behaviour led Whishaw (1985a) to conclude that perhaps the hippocampus is necessary in monitoring movements and integrating movement paths. These findings demonstrate the intact ability of lesioned animals to localise a spatial location when movement demands are initially reduced. The reported behavioural differences between groups, and the lesioned animals' inability to correct their path trajectories following errors, has added further weight to the suggestions that the hippocampus may be involved in providing an accurate route to a goal's location.

While convincing, the above evidence does not provide unambiguous support for the hippocampus' exclusive involvement in path integration. Confirmation of this would further require the use of a task that specifically assesses path integration and the definite use of idiothetic information in task completion. Allen et al. (2007) provide evidence from such a task, where hippocampal lesioned rats were trained in either a light or dark version of the radial-arm maze. In the light condition, where either visual *or* idiothetic information could be used, the lesioned animals successfully acquired the task. In the dark condition, however, where only idiothetic cues were available the lesioned animals remained severely impaired, unlike intact animals whose performance improved over time. This assessment, along with evidence from modified

allocentric tasks (Whishaw & Jarrard, 1996), lends support to the suggestion that the hippocampus is needed to process information based on idiothetic inputs. An additional feature to the above is that perhaps there is interplay between path integration and external cues that affects lesioned animals' abilities to solve place tasks. This also ties in with the suggestion that navigating animals can use both path integration and visual landmark information in tandem when exploring (Gallistel, 1990; Etienne et al., 1996).

While lesion evidence has implicated the hippocampus in the organisation and integration of movements, additional evidence has also come from the examination of head direction (HD) cells. HD cells are found in the postsubiculum, anterior thalamus, and dorsal striatum (Ranck, 1984; Taube, 1995; Wiener, 1993) and fire only when the animal points its head in a particular direction (Taube, 1998). They are of key interest as these cells, unlike the previously described place cells, do not fire in response to environmental cues and are thus thought to be responsive to the same information that support the development of path integration. Golob and Taube (1999) found evidence to implicate the hippocampus in the organisation and integration of movements through the examination of HD firing stability following hippocampal lesions. Specifically, once the hippocampus was destroyed, HD cell firing became unstable; a result not expected if the hippocampus was not, somehow, involved in path integration processing. This cellular and the abovementioned lesion data, together, strongly place the hippocampus in the role of monitoring and integrating movements in spatial tasks.

1.4.4 Hippocampal lesions: Impairments in flexibility or perseveration?

Adding to the already complex body of literature on the role of the hippocampus in spatial navigation, a final suggestion has been made to implicate the structure in processes beyond just spatial or idiothetic cue processing. Further interpretation, rather, has suggested that the deficits seen following hippocampal damage are as a result of animals' perseveration in maladaptive behaviours, which results in the often reported presence of rigid search patterns and subsequent inability to complete spatial tasks (Day et al., 1999; Galani et al., 1998; Jarrard & Bunnell, 1968). Wright et al. (2004) provide evidence for this employing a standard place MWM. From observations of behaviour, they deduced that lesioned animals' impairment in the task was due to their inability to habituate to the environment, which led to ineffective and stilted exploration. Specifically, the lesioned animals swim path distances indicated persistent but ineffective searching. In addition they could not adapt their search in the maze during a retention trial towards the specific target quadrant, instead retaining a random search pattern in all areas of the maze. These results fit in with a number of studies linking habituation and the control of inhibitory responses to the hippocampus (Kimble, 1968), with evidence from lesion studies reporting attenuated habituation of exploratory behaviours (Galani et al., 1998; Gray & McNaughton, 1983). As habituation is readily demonstrated in intact animals (Kimble, 1975), it is perhaps, a critical feature in enabling animals to reach their goal.

Alternatively, it has been suggested that the deficits observed in spatial tasks are due, not just to an inability to habituate, but also to a difficulty in flexibly using and altering swimming patterns. Whishaw and Mittleman (1986), for example, illustrated lesioned animals' ability to learn place when a fixed strategy could be used,

however when required to adapt their strategy and use the already learned information flexibly to navigate from new start positions, they were unable to do so (see also Eichenbaum et al., 1990). This, therefore, may indicate the hippocampus' role in the flexible application of already learned information to new environments.

A more detailed interpretation has suggested that the deficits seen are due to a difficulty with pliancy and strategy switching throughout training, which can manifest as perseveration in ineffective non-spatial strategies. Assessment of intact animals' behaviour highlights this difficulty, as normal animals alter their behaviours and exploratory strategies with ease as they become familiar with an environment (Harvey et al., 2008). Although no studies of hippocampal lesions have adapted the level of behaviour analysis as Harvey et al. (2008) to determine changes in strategies across training, Day and Schallert (1996) attempted to test whether the use of a single strategy, without the need to alter exploratory behaviours during training, could enable the successful acquisition of the task in lesioned animals. By using an innovative training protocol, they trained animals initially with a large hidden platform occupying most of the pool, gradually reducing it in size as training progressed, thereby removing the need for the animal to spontaneously alter their search strategy. By adopting this method of training, lesioned animals were successfully able to locate the goal at its smallest diameter, using only external distal information to guide their search. These findings illustrate a retained ability in lesioned animals to learn and find place when only one strategy was available to do so, removing the need to choose or change between searching strategies.

This evidence provides an alternative explanation beyond the idea that the hippocampus is essential for place learning. The above adapted training procedures

facilitate lesioned animal's acquisition of place, as they prevent errors being made initially, thus, encouraging behavioural flexibility (Eichenbaum et al., 1990). Perhaps the hippocampus is specifically involved in adapting navigational strategies when response requirements change in spatial tasks, i.e. to respond to the environment with pliancy (Day et al., 1999).

1.5 Neuroanatomy of the Hippocampus

The lesion data presented above is a major source of information regarding hippocampal function and its role in solving spatial tasks. However, the location and size of lesions to the hippocampus can also be used to help identify the role played by specific subregions in spatial memory. Here we will provide a brief overview of the neuroanatomy of the hippocampus, however, see Witter and Amaral (2004) for a more detailed review. In the rat brain, the hippocampus lies under the medial temporal lobe bihemispherically. It is an elongated C-shaped structure with the long axis of the hippocampus referred to as the septotemporal/dorsoventral axis and the orthogonal axis as the transverse axis. Anatomically the hippocampus can be divided into a number of distinct fields; the dentate gyrus (DG), and areas CA1, CA2 and CA3; *CA Cornu Ammonis*. All hippocampal subregions share a characteristic three layered appearance. The granule and molecular layers of the DG form either a distinct V or U shape, depending on the septotemporal level, and comprises two blades: the suprapyramidal blade and infrapyramidal blade. Both CA3 and CA1 are comprised of a principal cell layer called the pyramidal cell layer (Witter & Amaral, 2004).

The hippocampus receives its main input from layers II and III of the entorhinal cortex (EC) via a pathway of fibres known as the perforant path (PP). The inputs from

the EC terminate in all subdivisions of the hippocampus (Kohler, 1985; Witter et al., 1989; Ruth et al., 1988) and also, critically, provide the main route by which neocortical inputs reach the hippocampus. Specifically, cells located in layer II of the EC project to the DG granular cells and to the pyramidal cells in CA3, whereas cells in layer III project to the CA1 and subiculum (Witter & Amaral, 1991; Witter & Groenewegen, 1984). The DG provides only one major connection within the hippocampus, projecting to CA3 through mossy fibres. A similar unidirectional pattern holds for projections from CA3 to CA1 and similarly CA1 projections to the subiculum. However, CA3 pyramidal cells give rise to divergent projections to a number of parts of the hippocampus with projections to other cells within CA3, while also making up the Schaffer collaterals that project to CA1 (Ishizuka et al., 1990). Cells in the CA1 project, in turn, to cells in the subiculum and to the deep layers of the EC (layer V; Naber et al., 2001). These projections from the EC have been recognised as the trisynaptic circuit pathway (EC→DG→CA3→CA1; see Figure 1.3). Further cortical-hippocampal connections have been described in the disynaptic circuit, where EC layer II projects to CA3 directly (Tamamaki & Nojyo, 1993), which then projects onto area CA1 (EC→CA3→CA1). EC also projects directly to CA1 (Monosynaptic circuit EC→CA1; Steward & Scoville, 1976).

Apart from the entorhinal input, the DG and CA cells receive few direct inputs from the cortex. The differing pathways projecting into the hippocampus from cortical structures suggests that each subregion is not entirely dependent on the preceding region for input indicating that each region can act both independently and/or in conjunction with each other (Marr, 1971; McNaughton & Morris, 1987; Nakazawa et al., 2002; Steffenach et al., 2002). Further examination of the contribution of the

different structures within the hippocampus to spatial navigation and memory has been

assessed by means of IEG and lesion methodologies (see Section 1.5.2).
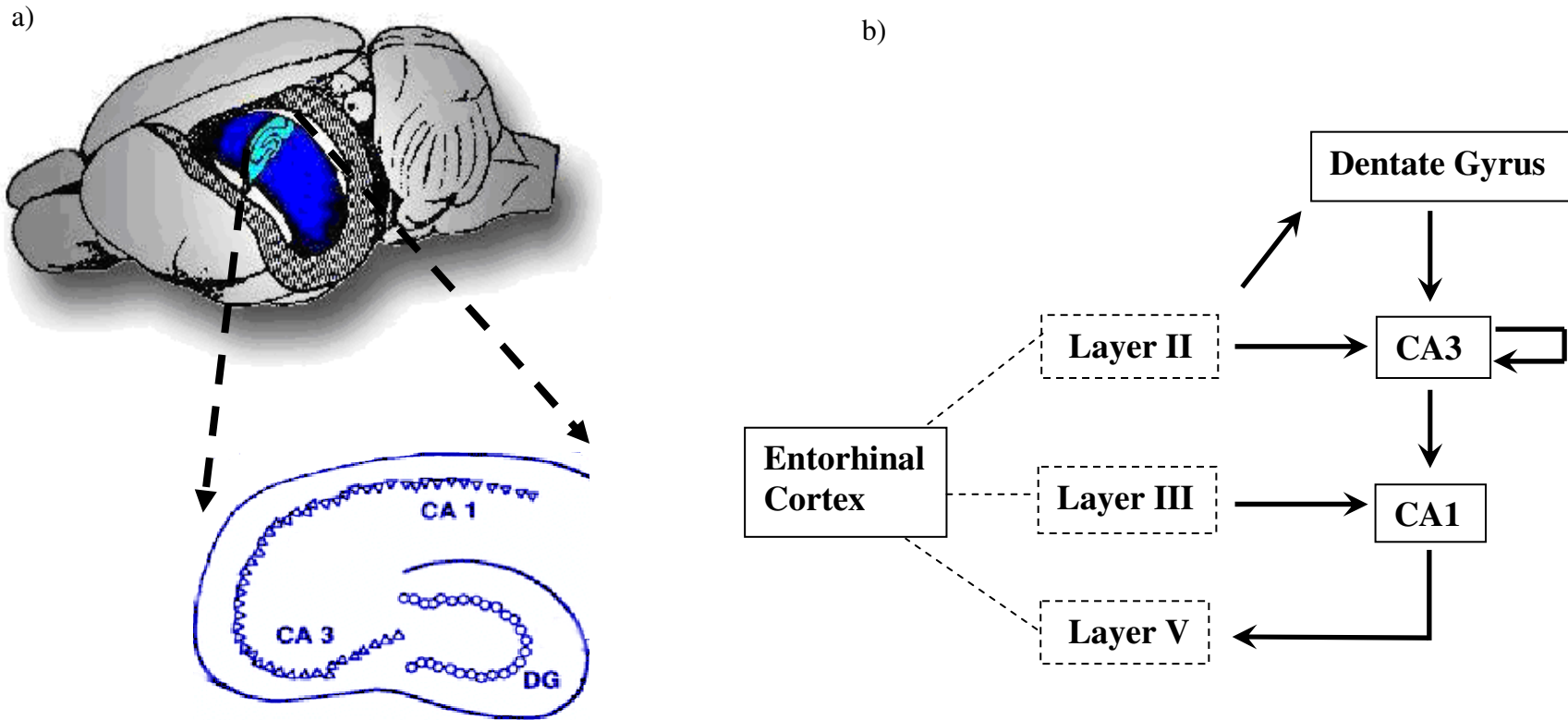
Figure 1.3: a) Diagram of the position of the hippocampus within the brain and a magnification of a schematic coronal slice of the dorsal hippocampus, highlighting the sub-regions which include CA1, CA3 and the DG [Adapted from Amaral and Lavenex (2007) with permission from Oxofrd University Press]. b) The trisynaptic circuit showing the connections between the entorhinal cortex layers II and III and the sub-regions of the hippocampus.

1.5.1 Function of the Dorsal and Ventral Hippocampus

Evidence from the majority of animal and human studies has implicated the hippocampus in memory (Scoville & Milner, 1957; Squire & Zola-Morgan, 1988) and in acquiring spatial tasks (Morris et al., 1982), however, it has also been suggested that the hippocampus does not solely play a role in these spatial oriented cognitive processes (Honey et al., 1998; Ross et al., 1984; Sinden et al., 1986; Zola-Morgan & Squire, 1985). Rather, more detailed examination of the afferent and efferent projections of the hippocampus along the septotemporal/dorsoventral axis suggests that there are several distinct processes within the structure, with the dorsal portion of the hippocampus having a more specified role in spatial processing, whereas the ventral pole is associated with emotion-related processing (Deguchi et al., 2011; Moser et al., 1995).

This suggestion that the dorsal hippocampus is more involved in spatial processing is in line with the anatomical organisation of its cortical-subcortical connections. Specifically, through the topographic organisation of the PP projections, the information from the lateral portion of the EC projects to, and therefore has a greater influence on, the dorsal portion of the hippocampus. Conversely, information from the medial portion of the EC is relayed to the ventral aspects of the hippocampus. Critical in this process is the information that is initially received by the EC. Specifically, the lateral EC receives information from other neocortical areas, such as the visual, auditory and somatosensory cortices, which in turn would suggest that the receiving dorsal level of the hippocampus be highly involved in processing of this external sensory information (Burwell & Amaral, 1998; Dolorfo & Amaral, 1998; Witter et al., 1989; Witter & Groenewegen, 1984). The ventral subregion noticeably

differs from the dorsal hippocampus in its anatomical connections. It receives projections from the medial portion of the EC, which initially obtains information from the amygdala (Petrovich et al., 2001; Pitkanen et al., 2000) as well as other subcortical structures that are associated with the hypothalamic-pituitary-adrenal axis (Jacobsen & Sapolsky, 1991; Witter & Amaral, 2004). Consequently, the ventral aspect of the hippocampus has been associated with fear, anxiety and emotion-related processing (Kohler et al., 1985).

Behavioural examination has also provided convincing support for separate mnemonic roles of the subregions of the hippocampus, reflecting the neuroanatomical projections and connectivity along the dorsoventral axis. Firstly, place cell firing patterns implicates a diversity of function within the structure. In particular, while place cells have been found in both the dorsal and ventral hippocampus (Poucet et al., 1994), the proportion of these cells is lower in the ventral hippocampus and the place fields are generally much larger and less selective than in the dorsal region, suggesting greater specialisation of location in the cells of the dorsal hippocampus (Jung et al., 1994). In addition, Moser et al. (1995) first detected a diversity of functions behaviourally when lesions ranging from 20-100% of the hippocampus revealed that when the dorsal portion of the hippocampus was completely ablated, leaving up to 60% of the ventral hippocampus intact, animals were significantly impaired in solving the MWM. Conversely, when equally large portions of the ventral hippocampus were destroyed, animals completed the water maze without any negative consequence. These results provided some of the first indications of the critical importance of the dorsal but not ventral hippocampus in spatial learning.

Extending the original findings of Moser et al. (1995), Bannerman and colleagues (1999) noted a double dissociation of function along the dorsoventral axis of the hippocampus, initially finding a role for dorsal but not ventral hippocampus in the MWM following respective lesions of the hippocampus. This has also been seen across a number of spatial tasks, including the T-maze and radial-arm maze (Bannerman et al., 1999; McHugh et al., 2008; Pothuizen et al., 2004; Zhang et al., 2004). In a companion study, Richmond et al. (1999) examined the effect of differential hippocampal lesions on a freezing task where the animal received an anxiety provoking foot-shock; a task that naturally elicits a freezing related fear response. Critically, lesions to the ventral, but not the dorsal hippocampus, disrupted the animals' ability to develop a freezing response, with only ventral lesioned animals unable to process fear/anxiety related information, a finding since reported in a number of studies (Bannerman et al., 2002; Czerniawski et al., 2009; Escalassen et al., 2009; Kjelstrup et al., 2002; Maren, 1999; McHugh et al., 2011). Furthermore, the examination of corticosterone in rats that had been placed in a brightly lit Perspex box for 10 minutes for 6 days, a task which would normally elicit anxiety in animals, revealed a lowered neuroendocrine stress response in ventrally lesioned animals only (Kjesltrup et al., 2002). Further weight has been added to these findings by measuring the level of brain tissue oxygen from either the dorsal or ventral hippocampus under a spatial radial maze task and a non-spatial anxiety task. Increased signals during anxiety were observed in the ventral but not the dorsal hippocampus, whereas increased dorsal hippocampal and not ventral hippocampal signals were recorded during spatial processing (McHugh et al., 2011), again suggesting a clear differentiation of function between the dorsal and ventral hippocampus.

1.5.2 Function of the Hippocampal Subregions

As previously described, the hippocampus consists of structurally dissimilar processing subfields that are interconnected serially as well as directly with the EC (Witter & Amaral, 2004). This arrangement suggests that individual subfields may subserve discrete functions. At a genetic level, immunohistochemical investigations have shown a differential expression of a number of IEGs within the hippocampus. Specifically, French et al. (2001) found increased IEG expression in the DG following induction of LTP but IEG expression in area CA1 did not, however, parallel this increase. Teather et al. (2005), similarly, reported augmentation of c-Fos in area CA1, but not CA3, following training in the MWM. These results imply that even at a neuronal level, there appear to be differences between the specific subregions of the hippocampus.

1.5.2.1 The Dentate Gyrus

The examination of behaviour following removal/inactivation of specific hippocampal regions also informs us of distinctive functions within the hippocampal structure. The DG is of special interest in many investigations as it receives and processes the first projections from the cortex in the trisynaptic circuit, therefore being in a key position to control the flow of information within the hippocampus. Interestingly, in a thorough lesion study, Okada and Okaichi (2009) revealed that lesions to the DG caused an equal level of impairment as animals with entire hippocampal ablation in a reference MWM. In addition, while lesions to CA1 resulted in some deficit in the task, it was not as extensive as that seen in the complete and DG groups. More striking, was the finding that inactivation of CA3 did not result in any deficits in the acquisition of the

water maze. The pronounced impairment seen in the DG lesioned animals indicate that it may be preferentially more involved than the other subregions in processing spatial information, a finding that has been described, particularly in tasks requiring allocentric spatial processing (Jeltsch et al., 2001; Sutherland et al., 1983; Walsh et al., 1986; Xavier et al., 1999). In keeping with this, the DG has been well placed in the role as an encoder of newly acquired spatial information (Lee & Kesner, 2004). Interestingly, a more detailed account of the dentate gyrus' role in encoding of information indicated that it may be critical in the process of habituation and reducing perseveration, allowing for appropriate modification of behaviours and therefore further processing of information in the environment (Xavier et al., 1999).

While there is abundant lesion evidence supporting the role of DG in spatial processing, it has been further proposed that it is particularly involved in pattern separation aspects of encoding and learning. This is seen under circumstances where DG lesioned animals have been required to differentiate between spatially similar environments. Specifically, DG animals tend to show robust impairment when required to detect spatially displaced objects; a finding not seen in CA1 and CA3 lesioned animals (Gilbert et al., 1998; 2001; Goodrich-Hunsaker et al., 2008). This difficulty in pattern separation could also go towards explaining the impairments seen in acquiring the MWM, as during the encoding of the task the animal is required to learn a goal location from many start points, with each of these containing partially overlapping, subsets of spatial cues (Rolls & Kesner, 2006).

Whilst the dentate gyrus has had much attention with respect to spatial processing due to its position within the hippocampal-cortical circuitry, spatial information is also transmitted to the *Cornu Ammonis* areas directly via the EC

41

(Yeckel & Berger, 1990; Jones, 1993). Although not entirely clear, CA1 and CA3 may be functionally independent, with anatomical projections suggesting the possibility of different functions (i.e. EC projections directly to CA3 and also to CA1; Witter & Amaral, 2004). Evidence in support of this from electrophysiological studies has revealed differing responses in place cell activation in area CA1 and CA3 following inactivation of the DG, with CA1 place field firing remaining unchanged, whereas CA3 firing was significantly disrupted. This, again, may reflect the anatomical distribution of connections with the removal of DG perhaps leading to disruption of any information being projected through mossy fibres to CA3 (Mizumori et al., 1999), while the connection between EC and CA1 was spared (Brun et al., 2002).

1.5.2.2 Cornus Ammonis 3 (CA3)

CA3 appears to play a different role in the processing of spatial information as unlike the DG, does not seem to be critical in the acquisition of the MWM (Brun et al., 2002; Nakazawa, et al., 2002; Okada & Okaichi, 2009; Steffenach et al., 2002; Sutherland et al., 1983). More detailed accounts of CA3 involvement in spatial processing, rather, have suggested a specific role for the structure in the retrieval, rather than encoding, of memory (Nakawzawa et al., 2002; Rolls & Kesner, 2006). Lee and Kesner (2004), for example, found that while lesioned animals were unimpaired in acquiring a Hebb-William maze, they were unable to accurately recall the task when assessed during a retention trial. More specifically, there have been suggestions for a role of CA3 in spatial pattern completion, particularly in the recalling of spatial information when there is an incomplete cue set during the retrieval phase of a task. Nakazawa et al. (2002) supported this claim demonstrating impaired retention in genetically-modified

mice that had a deletion of the NMDA receptor subunit NR1 in area CA3 and found that while they retained normal acquisition of a spatial reference memory paradigm, they showed significant deficit when required to recall the task when only a subset of the original cues was available to navigate (Nakazawa et al., 2002; also Gold & Kesner, 2005). Area CA3, therefore, appears critical in retrieving learned spatial information after an alteration has been made to the environment; a feature that is crucial to the navigating animal as variations are often made during retention testing (Kealy et al., 2008; McGauran et al., 2004) and indeed in most situations where an animal must navigate.

However, there have also been suggestions of a role for area CA3 beyond the retrieval of allocentric spatial information which stems from suggestions of an overall role of the hippocampus in path integration processes (Save et al. 2005; Whishaw et al., 2001). A number of studies have proposed that a suitable and likely area of the hippocampus to process this information is CA3, due to the CA3-CA3 recurrent collaterals, which automatically lends itself to the processing of internally generated associative memory (Rolls & Kesner, 2006). Consistent with this, place cells have been shown to update idiothetically, in the dark (Mizumori et al., 1999), with the CA3-CA3 network specifically showing evidence of continued activation without direct input from external stimuli for a short period of time after exposure to an environment (Hampson et al., 2000; Wirth et al., 2003).

1.5.2.3 Cornus Ammonis 1; CA1

The function of CA1, however, is more difficult to define. Initial evidence from lesion studies examining separate DG, CA3 and CA1 lesions in allocentric spatial tasks, have

shown that DG and CA1 result in similar impairment (Nunn et al., 1998; Stubley-Weatherly et al. 1996), however damage to the DG tends to lead to more extensive impairments (Okada & Okaichi, 2009). As damage to CA1 does not disrupt spatial learning to the same degree as DG ablation, proposals have been put forward implicating it, instead, in very specific aspects of acquisition, such as enabling the flexible and effective alteration of behaviour during spatial navigation (Dillon et al., 2008). Support for this proposal comes from the study of CA1 lesioned animals' successful acquisition in a Y-maze. Specifically, when the exact search strategy employed by the lesioned animals was assessed, it was found that CA1 animals could only use a serial (egocentric) strategy throughout training, and were unable to effectively change this to a more efficient allocentric strategy; a behavioural transition that was easily and flexibly made by the control group (Dillon et al., 2008).

In a more navigationally complex water star-maze task, forebrain specific NMDA-receptor knockout mice (NR1-KO), lacking CA1 NMDA receptors, displayed overall impairment in acquiring the task (Rondi-Reig et al., 2006). However, through assessment of intact animals' behaviour the deficit in CA1 animals was easily enlightened, as unlike control counterparts, they were unable to readily switch from a single sequential egocentric strategy to an allocentric spatial search strategy. This may suggest CA1 is needed for the flexible alteration of strategies in a spatial task, a requirement particularly essential in the MWM to ensure the most efficient navigation to the target goal. Interestingly, Reisel et al. (2002), noted intact performance in the place version of the MWM in mice lacking the AMPA receptor subunit GluR1, which results in a deficit in hippocampal CA3-CA1 LTP. However, the GluR1-KO mice showed profound impairments in a spatial working memory T-maze and Y-maze. So

while impaired hippocampal CA3-CA1 LTP did not result in an impairment in the place MWM as expected, the poor performance in the hippocampal-dependent, spatial working memory tasks does indicate a hippocampal dysfunction as a result of the lack of LTP and suggests that GluR1 synaptic plasticity is required for flexible, working memory components of spatial learning. So while the results from Rondi-Reig et al. (2006) and Reisel et al. (2002) appear to be at odds, it must be clarified that Rondi-Reig et al. adopted a novel version of the water maze, based on a combination of the standard MWM (Morris et al., 1982) and Y-maze (Packard & McGaugh, 1996), and as such did not adopt the same place MWM procedure as Reisel et al. (2002). In addition, findings from both studies lend themselves to the suggestion that CA1 may play a role in the flexible adaptation of spatial behaviour; be that changing strategy (Rondi-Reig et al., 2006) or rapidly adapting to changing task demands (Reisel et al., 2002). Furthermore, Reisel et al. (2002) suggest that perhaps the lack of impairment in the standard MWM in GluR1-KO mice is due to multiple types of synaptic-plasticity in the hippocampus, namely early- and late-onset LTP. These different forms of LTP may contribute differentially to hippocampal information processing. Specifically, it has been noted that late- but not early-onset LTP is intact in GluR1-KO mice (Hoffman et al., 2002), which might support the learning of a fixed, hidden platform location over several days. This would also account for the impairment in the MWM in these animals when they received hippocampal lesions, removing the possibility for any form of LTP to occur, thus resulting in significant delays.

In contrast to the acquisition aspects of CA1 involvement, it has also been suggested that CA1 may be involved in the retrieval of memories, playing a particular function in recalling intermediate-term memories (Hunasker & Kesner, 2008; Kesner

et al., 2004; Rolls & Kesner, 2006). In particular, Lee and Kesner (2002) trained CA1 lesioned animals in a spatial delayed non-matching to place task with delays ranging from 10 seconds to 5 minutes and noted that lesioned animals were only impaired when they were presented with the 5 minute delay (Lee & Kesner 2002). Remondes and Schuman (2004) further assessed the idea of a role for CA1 in the retrieval of spatial memories by examining the effect of temporoammonic (TA) lesions, which disrupted the direct projections from the EC to CA1, on both short- (24 hours) and long-term (4 weeks) recall of a learned MWM task. They found that animals with TA lesions displayed accurate, localized searching in a target area during a probe test conducted 24 hours after training, however, this effect disappeared when re-tested 4 weeks later, with TA animals searching in random positions around the pool. However, to further elucidate whether the disruption caused by the TA lesion was as a result of disrupted consolidation or retrieval, intact animals were initially trained in the MWM and then received TA lesions 24 hours or 3 weeks following training. Animals that received their lesions 24 hours after training, displayed a significant impairment when tested 4 weeks later. However, animals that received lesions 3 weeks post-training showed a significant preference for the target quadrant, indicating that the memory had been adequately consolidated at the time of the lesion. While previous evidence has indicated that damage to area CA1 results in impairments in the retrieval of spatial memories (Lee & Kesner, 2002), evidence from Remondes and Schuman (2004), showing the retained ability to retrieve a short-term spatial memory, or to retrieve the memory when the lesion was made 3 weeks after training, argues against a simple retrieval deficit and instead highlights the retained importance of the cortical

input from the EC to the CA1 via the TA path in the establishment of long-term memories.

Anatomically, as CA1 is the output region of the hippocampus, projecting the already highly processed information from the DG and CA3 onto other cortical and subcortical regions, it can appear to have similar functions as CA3 and DG. Detailed methodological analysis, as reviewed however, reveals subtle, although numerous, functions of each of the different areas. While a single role for each region remains unclear, evidence suggests that the hippocampal subregions likely serve complimentary but computationally distinct roles in spatial processing.

1.6 Objectives of this Thesis

The overall aim of this thesis is to assess the importance of detailed behavioural examination in the Morris water maze and in particular the utility of this type of analysis in identifying the role played by the hippocampus in spatial navigation. These aims will be achieved through a series of experiments that will employ behavioural, biochemical and lesion techniques to assess specific behavioural and neural components involved in learning in the water maze.

It has been generally accepted that when the platform is hidden and only distal cues are available to solve the task, that allocentric place navigation is being employed and also definitively assessed by the experimenter. However, the conflict within the literature regarding the presence of parallel and/or sequential use of different strategies in the maze suggests otherwise (Burgess, 2008; Chamizo & Rodrigo, 2004; McGauran et al., 2005; Moghaddam & Bures, 1996). Specifically, a number of adaptations to the water maze have led to multiple interpretations of how the task can be accomplished.

One example includes the position of environmental cues which has resulted in controversy surrounding the theoretical underpinnings of MWM acquisition (Chamzio & Rodrigo, 2004; Morris et al., 1982). Therefore, in an initial experiment (Chapter 2) we extend previous studies and assess the strategies used by animals in the acquisition and retention of the water maze under conditions where a number of distal cues are placed in either a near or far configuration respectively.

However, earlier attempts at quantifying the MWM have generally failed to examine the behavioural changes that occur when learning in any great detail, with many reports on the acquisition of the task merely recording escape latencies, distances and velocity (Dalm et al., 2000; Lopez et al., 2008). Therefore, in a second experiment (Chapter 3a) we examine, in detail, the swimming behaviours of animals during the trial, as well as analysing the platform behaviour of the animals, to ascertain what strategies are being employed in the task. As we also expect that one of the cue conditions carries a higher spatial demand, detailed assessment of behaviour will reveal the use of different learning strategies under the near and far cue conditions.

As the hippocampus has also been widely reported as playing a critical role in learning the MWM (Eichenbaum et al. 1990; O'Keefe & Nadel, 1978; Whishaw et al., 1995) and to ascertain if cue positioning leads to differential neural changes in the structure, we assess the expression of the neurotrophin BDNF, following training in the maze (Chapter 3b). Chapters 4 and 5 expand on our molecular investigation by performing dorsal hippocampal lesions and examining the resulting effect on the acquisition of the task under the two cue conditions. Due to the controversy surrounding the hippocampus' role in spatial processing, which ranges from allocentric involvement to path integration and inhibitory responding, basic measures of

acquisition will not be sufficient to differentiate between these areas. Therefore, we apply our method of in-depth behavioural analysis to lesioned animals' performance in the maze. In addition, assessment of behaviour coupled with comparison under both cue conditions will allow us to determine if the hippocampus is preferentially more involved in spatial or navigation components of the task (Chapter 6). As there appears to be a differentiation of function across the subregions of the hippocampus reported in the literature, we also aim to further quantify hippocampal involvement in spatial processing by examining subregional activation within the intact hippocampus using IEG immunohistochemical methods and assess c-Fos immunoreactivity following MWM training (Chapter 7).

Therefore, overall we hypothesise that the Far cue training position will result in slower learning in the MWM than the Near cue position, and that behavioural analysis will highlight the use of different learning strategies between these groups; specifically, we predict that the Far trained group will display more allocentric behaviours than the Near trained group. We also predict higher hippocampal activity in the Far trained group and suggest that the dorsal hippocampal lesioned animals will display greater impairments in the Far training condition than in the Near training condition.

Chapter 2

Evidence of allocentric learning in both Near and Far trained animals following cue rotation in the Morris water maze.

Abstract

Animals can use a number of navigational strategies to locate their goal in an environment. However, there remains conflict over the individual strategies used in the Morris water maze, with some authors suggesting animals solve the task using either a purely allocentric or purely egocentric strategy, whereas others suggest a combination of both. Recently, focus on specific features of environmental cues, such as size, shape, number and location has come to the fore and there have been suggestions that such features may influence the strategies used to solve spatial tasks. Therefore, here we investigate the importance of distal cues, and attempt to separate out differences in strategies used by navigating animals through the examination of the effect of cue location on water maze performance. For this, we trained two groups of rats for 5 days, 4 trials per day, with a cue configuration located in either a position near the hidden platform (Near trained n=14) or with cues located far from the platform (Far trained; n=14). Seven days post-acquisition, animals in both training groups were randomly assigned to one of two groups; Control (cue position as in training) and Cue-rotated (cues rotated 180°). Animals trained under both conditions followed the distal cues during retention, even when cues were relocated 180 degrees from the original training position. Our findings suggest animals acquire a strong platform-cue association irrespective of the distance and location of the cues.

2.1 Introduction

The Morris water maze, an extensively used apparatus in spatial learning and memory, has seen widespread interpretations of how the task is solved, with a number of designs adopted to examine the strategies used in the task (Chang & Gold, 2003a; Epp et al, 2010; Gerlai et al., 2002; Hamilton et al., 2009; Vorhees & Williams, 2006). The most commonly used paradigm involves training animals to locate a hidden, unmarked platform in a consistent area known as place (Bures et al., 1998; Morris, 1981).

To solve a place task, a navigating animal generally has two sources of information with which it can locate itself within an environment; egocentric and allocentric (Aggleton et al., 2000; Begega et al., 2001; Pearce et al., 1998; Valerio et al., 2010; Wang & Spelke, 2000). In egocentrically-based navigation, animals can use view-dependent information whereby any available external information is represented in relation to the animal without spatial relations to other markers being developed (Brown, 1992; Maurer & Derivaz, 2000). Alternatively, animals can rely on information gained from their own motor movements, in relation to a specific point in their journey, to get to their goal (Whishaw et al., 2001). Therefore, solving the water maze can be done satisfactorily without any extramaze cues (Baldi et al., 2003). Moghaddam and Bures (1996), for example, successfully trained animals to rely solely on an egocentric strategy in the place version of the MWM by training animals in the dark with no available distal cues. A probe trial, where the start and goal positions were rotated, further confirmed that animals were indeed solely relying on egocentric navigation to find a target location. Similarly, learning to directly approach a distinct, proximal or beacon environmental cue, with little information on the spatial relationship between it and other cues, is also sufficient to locate a concealed goal

within the MWM (de Bruin et al., 2001; Save & Poucet, 2000). Despite evidence suggesting that egocentric strategies can successfully be employed in the MWM, conflicting data has shown that egocentric navigation is often not an initial strategy adopted by navigating animals (Maaswinkel & Whishaw, 1999; McGauran et al., 2004). Kealy et al. (2008), for example, found that despite overtraining animals from a fixed start to a fixed goal position, they did not adopt an egocentric, procedural strategy when the start position was altered during a probe trial. Instead they continued to rely on the available distal cues in the environment. In a second experiment, when the probe trial was performed in the dark, removing all visual access to external stimuli, animals, once again, did not rely on an egocentric strategy, instead swimming in a thigmotactic fashion at the pool edge. These findings questioned the ready reliance on egocentric navigation in a place water maze task.

Rather, allocentric navigation has been strongly implicated in solving more complex navigational tasks (Etienne et al., 1990; McGauran et al., 2004; Warburton et al., 1997). This strategy involves defining a place relative to another location or to another object (e.g. remote landmarks) and is independent of the viewer (Bures et al., 1998). The distal cues remain stable and are at some distance away from the platform, thus, allowing the animal to locate its goal in relation to those cues (Whishaw et al., 2001). It is thought that stable distal cues are most frequently used by animals in solving the MWM (Brandeis et al., 1989; Maurer & Derivaz, 2000; Prados & Trobalon, 1998). Certainly, rodents have been shown to successfully navigate a spatial maze using several external cues, with later retention of the task dependent on the association between the cue configuration and the location of the goal (Cohen & Bussey, 2003; Ethier et al., 2001; Morris, 1981). McGauran et al. (2004) demonstrated

that by rotating the distal cues during retention in the MWM, the animals rotate their search in response to this change, indicating a retained reliance on distal cues. It appears that the distance and direction information gained from the cues enables animals' successful and accurate finding of the goal during both learning and recall.

However, there remains some ambiguity as to the exact methods used to solve the water maze with suggestions that a combination of both strategies can be used. The concurrent use of both ego- and allocentric navigation has been described in a hierarchical manner, where allocentric navigation is primarily relied upon, switching to egocentric guidance if allocentric information is unreliable (Hamilton et al., 2004; Lavenex & Schenk, 1995; Maaswinkel & Whishaw, 1999; Packard & McGaugh, 1996). Nonetheless, distal cues recurrently play a crucial role in the animal's ability to locate a goal and are an important factor to consider when examining the strategies used to navigate (Morris, 1981; Sutherland et al., 1983). Recently, the saliency of available visual cues (Lopez et al., 2008; Rodrigo et al., 1997; Young, et al., 2006), the number of cues (Della-Chiesa et al., 2006; D. Harvey et al., 2009; Prados, 2000; Prados & Trobalon, 1998) and the location of distal landmarks (Chamizo & Rodrigo, 2004; Chamizo et al., 2006; Kamil & Jones, 2000) have been investigated in relation to their relative effects on spatial performance. Particularly, the effect of cue location on how the MWM is solved has been questioned, with suggestions that different strategies will be used, with the possibility that the task may be learned through associative mechanisms, when distances differ in cue positions. Chamizo and Rodrigo (2004), for example, have shown that the distance a landmark is from the goal is a critical factor in an animal's ability to learn the task. They showed that rats trained with a single cue were impaired when the cue was suspended at the pool wall furthest from the hidden

goal with performance only increasing when the cue was located closer to the goal. Consequently, the authors concluded that the superior performance of the 'near' group was due to the cue being used as a beacon to locate the goal, suggesting a cue-focused strategy was adopted by animals. Others have shown similar findings, where the spatial proximity of a landmark is a predictor of how well the animal will solve a task (Chamizo et al., 2006; Spetch et al., 1996; Tamara et al., 2010a).

While it is well established that distal cues are most often used as a primary way of locating a hidden goal, it is difficult to determine the exact nature of this use. Since evidence has suggested specific elements of the cues are influential in task solving, it is worth examining if cue location, in particular, effects the establishment of cue relationships and goal localisation. Retention trials using a displacement paradigm are a useful means to examine distal cue use in the water maze (McGauran et al., 2004; Shettleworth & Sutton, 2005), and can provide crucial information about the formation of memories for the spatial layout of an environment (Nadel et al., 2000). It is suggested that if an association is established between the distal cues and the platform position during acquisition, this information should be stored and subsequently used during recall in the task (Harvey et al. 2008; Kealy et al., 2008). Here we aim to dissociate the types of stored spatial memory and determine if different strategies are employed when learning the task under differing training designs. We trained one group of animals with a distal cue-configuration located near to the hidden goal and a second with distal cues located far from the goal. Following a 7-day retention phase, a single probe trial was carried out where animals in both groups were divided into a control- or cue-rotated group. The control group's cues were in the same position as during training. The cue-rotated group, however, had their cues shifted 180 degrees.

2.2 Method

2.2.1 Subjects

Male Wistar rats (n=29) obtained from Harlan Laboratories, UK, served as subjects in the current study. Subjects were approximately three months old and weighed 200-300g at the beginning of experimentation. All animals were housed 3 per cage, in a temperature-controlled environment ($21\pm1^{o}C$), which was maintained on a fixed 12:12 hr light-dark cycle (0700-1900hr). All rats were given *ad libitum* access to food and water. Experimentation took place during the light phase and all subjects were well handled before experimentation began. The rats had no prior exposure to the maze and were experimentally naïve.

2.2.2 Apparatus

The Morris water maze (MWM) consisted of a uniformly black, circular, fibreglass pool (170cm in diameter; 36cm deep) resting on a table 70cm above the ground (Figure 2.1). The maze was filled with water to a depth of approximately 21cm and kept at a temperature of $20\pm1^{o}C$. A removable black concrete platform (11cm diameter, 19cm height) located in the North East quadrant of the pool was used by animals to escape the water. The platform was submerged 2cm below the water surface, rendering it invisible to the rats when swimming.
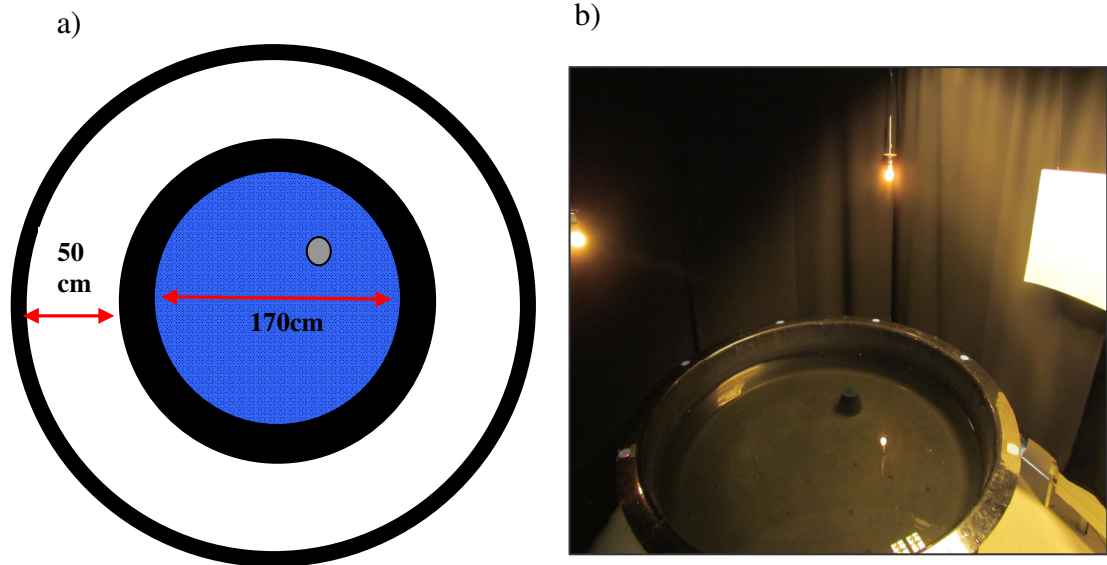
Figure 2.1: a) Aerial view (schematic) of the layout of the standard MWM used in experiments throughout this thesis. b) Photographic image showing the platform located below the surface of the water, with three representative cues attached to the surrounding black curtain.

The pool was surrounded by a black curtain which was located approximately 50cm from the pool wall. This provided a uniform background around the entire pool. Three distal cues were located at fixed positions around the maze. The distal cues included two 25W light bulbs suspended from the ceiling. Both lights were located on the inside of the surrounding curtain at a distance of 75cm from the pool at an angle of approximately 60°. A rectangular sheet of white paper (55cm x 81cm) was also attached to the curtain for use as a cue. The position of the distal cues, as well as the hidden platform, remained fixed throughout acquisition of the task. An overhead camera positioned in the laboratory ceiling, above the centre of the maze, captured all of the animal's movements throughout experimental trials and relayed this to a connected computer for later analysis, using EthoVision (Noldus Information Technologies, Wageningen, Netherlands). This digital tracking system recorded escape latencies, distance travelled and swimming velocity of each animal on all trials.

2.2.3 Procedure

2.2.3.1 Acquisition

The first training condition had three distal cues located around the pool, including a light cue positioned in the North West (NW) quadrant and the North East (NE) quadrant and a white sheet of card (55 x 81cm) at the East (E) of the pool. The hidden platform was located in the NE quadrant. This condition was subsequently called the Near training condition (NT) with the nearest cue (NE light cue) positioned 120cm from the platform. A second condition in this experiment involved the distal cues being placed in a location further away from the hidden platform. In this condition a light cue was position in the South West (SW) quadrant, and in the South East (SE) quadrant, and a white sheet of card was located to the West (W) of the pool. The hidden escape platform was located in the NE quadrant. This condition is the Far training condition (FT) with the furthest cue (SW light cue) positioned 220cm from the hidden platform (Figure 2.2).

Animals, in both conditions (NT; n=14 and FT; n=15), were trained for 5 consecutive days (4 trials/day). Each acquisition trial consisted of the animal being placed into the water maze for 60 seconds, facing the wall, at one of four pseudo-random points around the pool (N, S, E and W), with the stipulation that each release point was used once during a session. The animals' task during this time was to locate the hidden escape platform in the centre of the NE quadrant. If, after the maximum allocated time, the animal had not found the escape platform they were guided to its position by the experimenter, using a ruler. The rat was allowed to remain on the platform for 15 seconds followed by an inter-trial interval (ITI) of 10 seconds, where they were placed in an open topped container outside the pool's vicinity. Animals were

carried to and from the maze in this rectangular container (30 cm x 40 cm x 30 cm). The three distal cues, for both groups, were visible throughout all of the acquisition trials and platform intervals. To ensure consistency and correct identification of each animal a simple tail marking system using a non-toxic marker was employed.
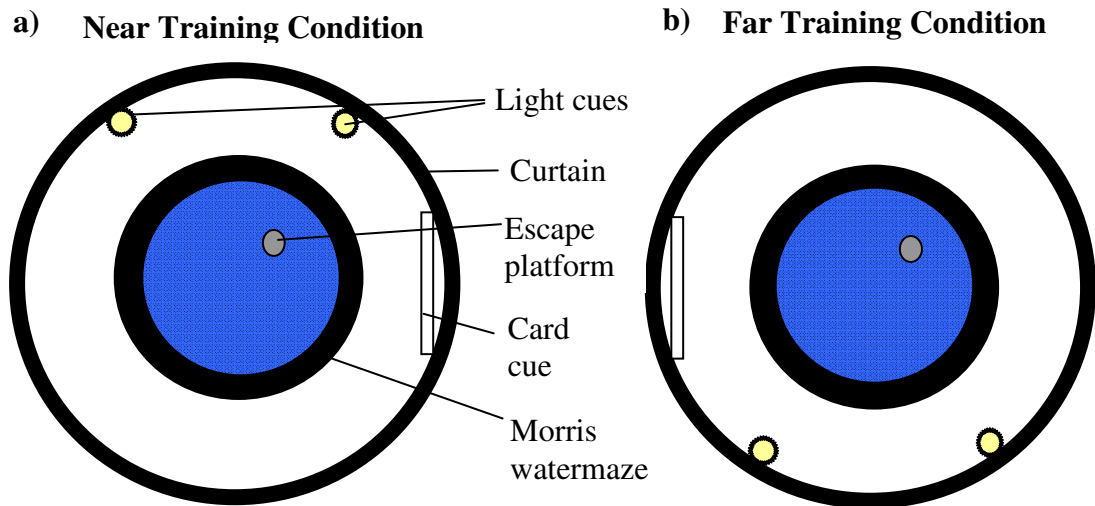
Figure 2.2: Morris water maze apparatus; Aerial view of the layout of the MWM and cue location for a) Near and b) Far groups used in this experiment.

2.2.3.2 Retention

Retention of the task was assessed 7 days post-acquisition. Subjects in both training conditions were randomly assigned to one of two groups; a control group or a cue-rotated group. For animals trained in the NT condition, the control group (NCT, n=7) had their distal cues located in the same place as they were during training (i.e. in the Near position). The cue-rotated group in this condition (NCR, n=7), had the distal cues rotated 180° during the retention trial. Within the FT group, the control group (FCT, n=8) had their distal cues located in the Far position, as in training. The cue-rotated group in this condition (FCR, n=7) had their distal cues rotated 180° for retention (see Figure 2.3). Retention for both groups was assessed in a single 60 sec trial with the hidden platform removed from the maze and all animals starting from the NW.

a) **Near Training Condition** b) **Far Training Condition**

NCT NCR FCT FCR
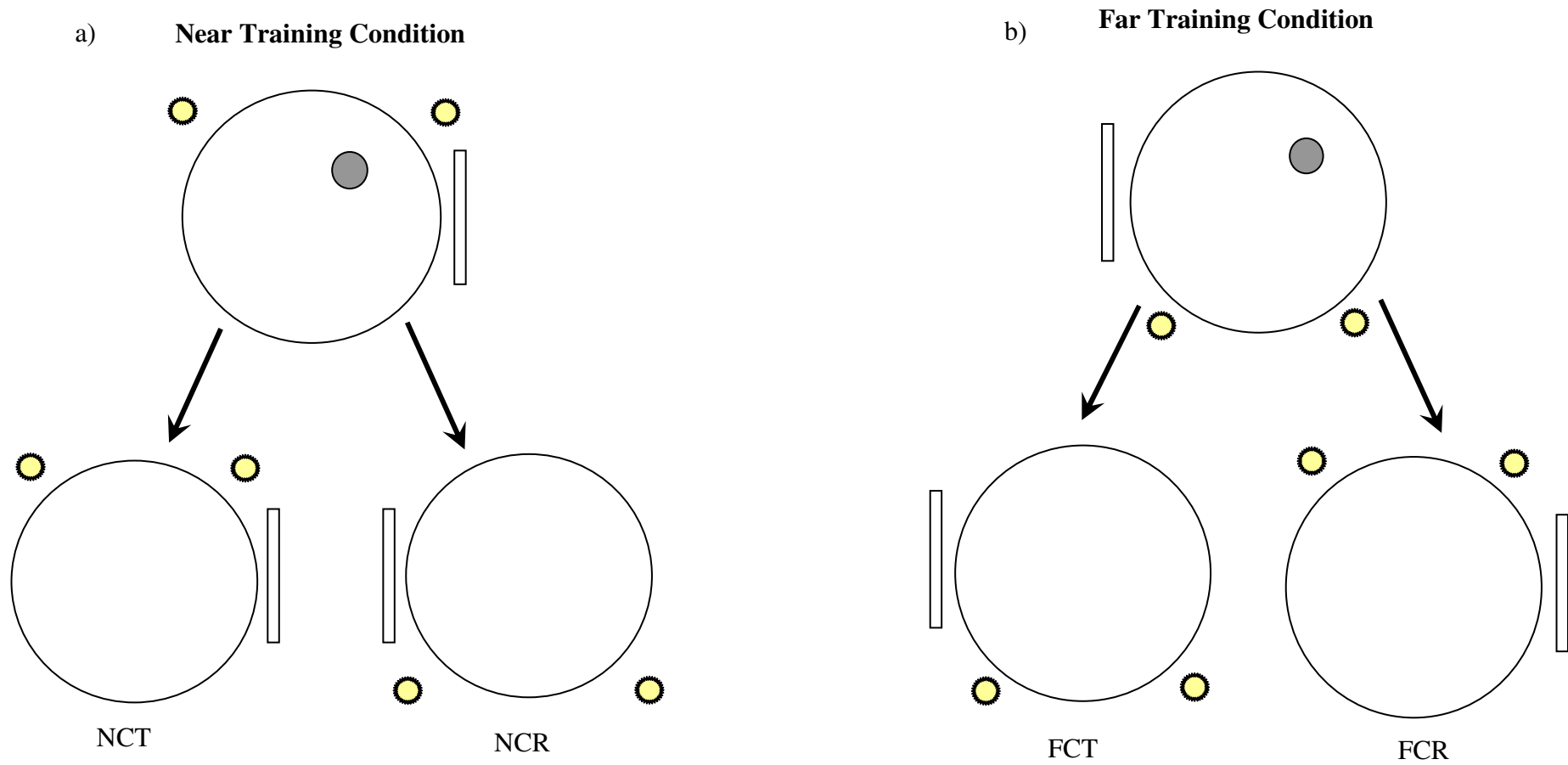
Figure 2.3: Representation of the training and retention cue layout in the a) Near training condition and b) Far training condition.

For statistical analysis of retention trials, the water maze was divided into a number of predefined sections (McGauran et al., 2005). These sections consisted of quadrants, platform areas, platform corridors and outer corridors (Figure 2.4). The first section was one of four equal quarters (NE, NW, SE and SW) of the entire water maze labelled "quadrant". The "platform area" was defined as a circular region of approximately 27cm in radius and centred where the platform was located during the acquisition period. There was a similar "platform area" symmetrically positioned in each of the four quadrants. The "platform corridor" was defined as a circular passage way, approximately 20 cm in width, encompassing the four "platform areas" in its centre. The "outer corridor" was defined as a corridor 20 cm in width around the inside wall of the pool. These predefined sections were combined to give a maze map (McGauran et al., 2005). The percentage of time spent (of 60 seconds) by animals in each of these sections was examined for the retention probe trial assessment.
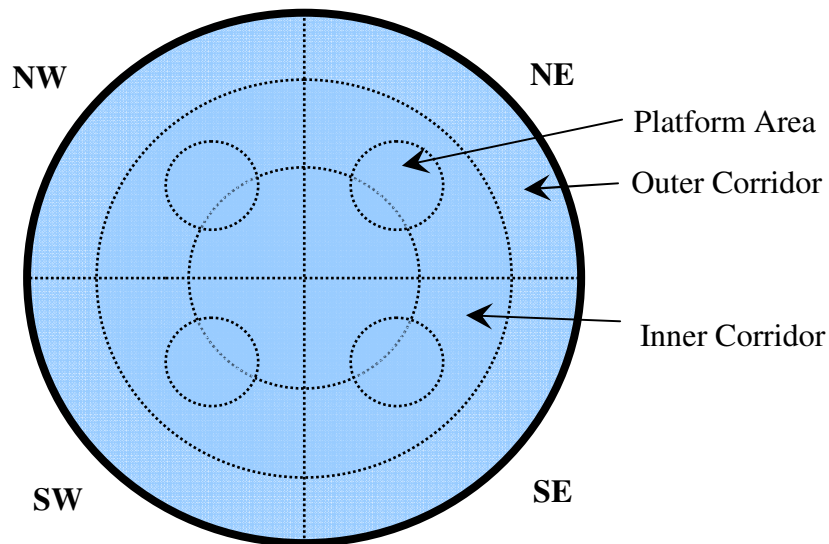
Figure 2.4: Aerial-view (schematic) of a maze map (adapted from McGauran et al., 2005) of the predefined zones of the water maze.

2.2.4 Statistics

A series of repeated-measures analysis of variance (ANOVA), with appropriate Bonferroni corrected comparisons, were conducted on the data collated for each swim trial. Independent t-tests were also calculated where required. All statistical analysis was conducted using the SPSS package (Version 17 for Windows). A star-based system representing the significance level of p-values was used throughout; $*p<0.05$, $**p<0.01$, $***p<0.001$. Error bars, where present, show standard error of the mean, which is in turn denoted by S.E.M. and the symbol ±.

2.2.5 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

2.3 Results: Near Training Condition

2.3.1 Acquisition

All animals in the Near training condition (NT) acquired the water maze over 5 days of training. The mean escape latencies decreased from 31.86±2.85 sec on Day 1 to 10.68±1.78 sec on Day 5 (see Figure 2.5) with a repeated measures ANOVA confirming a significant decrease across days [$F_{(4, 52)}$ = 18.75, p<0.001]. Subsequent Bonferroni-corrected t-tests demonstrated that the mean on Day 5 was significantly shorter than Days 1 (p<0.001) and 2 (p<0.05; see Figure 2.5).
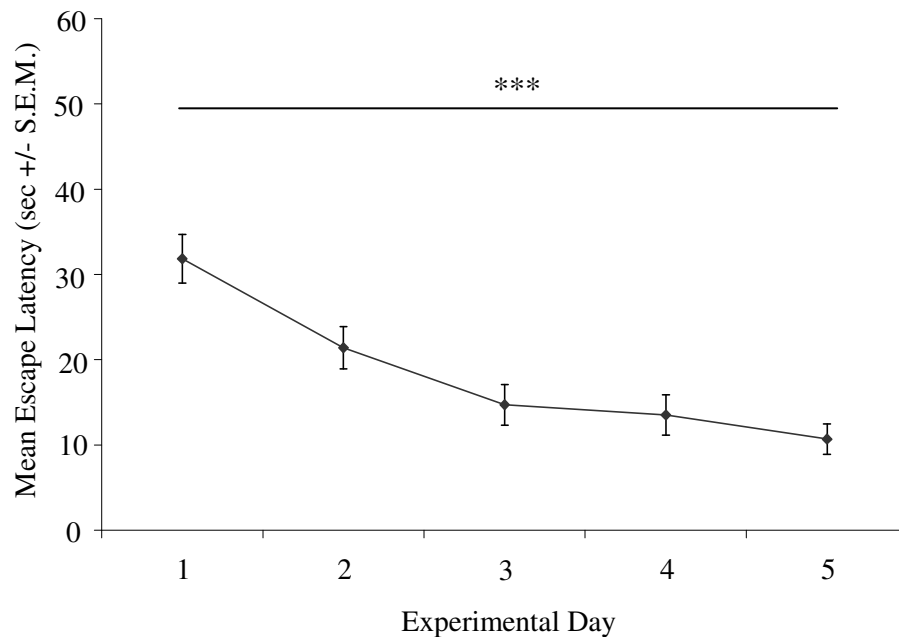
Figure 2.5: Mean escape latencies (sec. ± S.E.M.) of NT animals throughout water maze training.

Distance travelled was also examined to determine animals' successful acquisition of the task overall. The mean distance travelled decreased across training from 744.82±72.86 cm on Day 1 to 254.36±43.83 cm on Day 5 (see Figure 2.6). A repeated measures ANOVA revealed a significant decrease in the distance swam throughout training [$F_{(4, 52)}$ = 20.44, p<0.001] with further Bonferroni-corrected t-

tests demonstrating the shortest distance travelled was on Day 5 when compared to Days 1 (p<0.001) and 2 (p<0.05). The mean velocity was also assessed to examine successful acquisition. The animals' swimming speed remained relatively stable throughout training in the task with a mean on Day 1 of 23.17±0.81 cm/sec and Day 5 of 24.58±1.68 cm/sec. However, a repeated measures ANOVA revealed a significant change in velocity as training progressed [$F_{(4, 52)} = 4.73$, $p<0.01$]. Subsequent Bonferroni-corrected t-tests demonstrated that the mean velocity was significantly faster on Day 5 (M: 24.58±1.68 cm/sec) than Day 3 (M: 20.35±0.97 cm/sec; p<0.05).
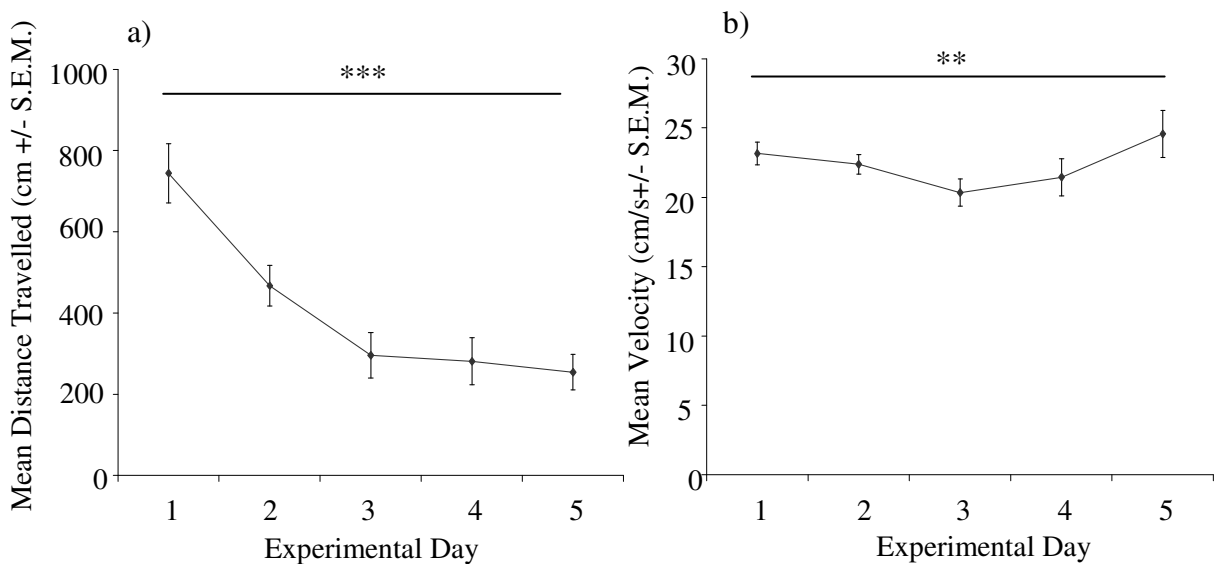
Figure 2.6: a) Mean distance travelled (cm ± S.E.M.) and b) Mean velocity (cm/s ± S.E.M.) of NT animals throughout water maze training.

2.3.2 Retention

Retention of the task was assessed 7 days post-acquisition. For this, animals were randomly assigned to two groups; Near Control (NCT, n=7) and Near Cue-Rotated (NCR, n=7). NCT animals' cues remained in the same location as in training (i.e. the Near position) whereas animals in the NCR condition had their cues rotated 180° from their original position during the acquisition period. To assess retention the pool was

divided into a number of zones (see Methods section 2.2) and percentage time spent in these areas was examined. Maze maps (adapted from McGauran et al., 2005), were used initially to determine where animals in both groups spent the majority of their time searching during the 60 second probe trial. Figure 2.7 illustrates differences between the groups in the mean percentage time spent in the different areas of the maze.
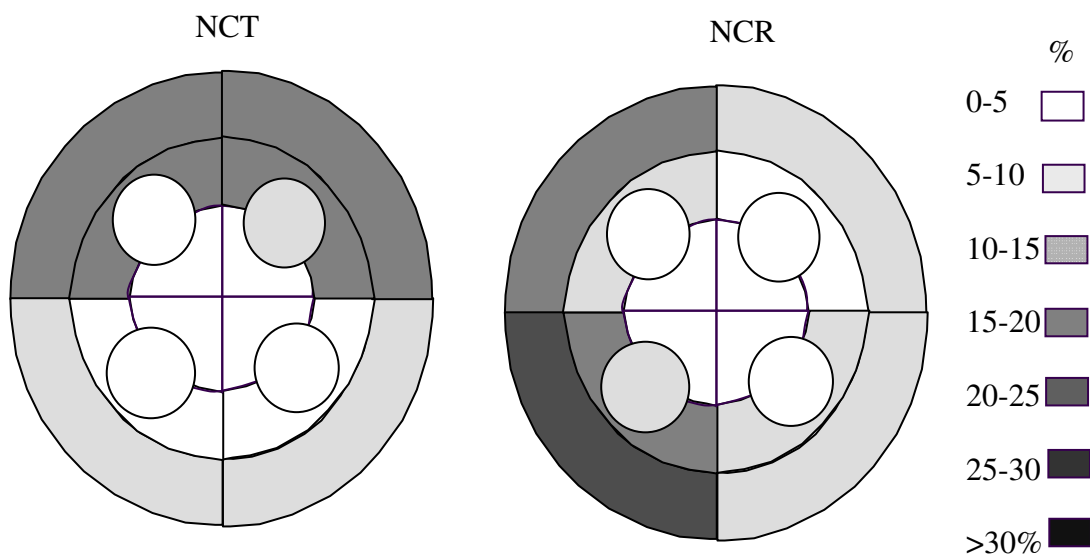
Figure 2.7: Maze maps illustrating the mean percentage time spent by the Near Control and Near Cue-Rotated groups in the different zones of the maze during the retention trial.

The time spent by both groups in each of the quadrants of the maze was examined initially to determine if there were any differences in search patterns between the groups (Figure 2.8). A repeated measures ANOVA revealed a significant pattern of searching during the retention trial for the NCT group [$F_{(3, 18)}$ = 9.67, p=0.001] with subsequent Bonferroni-corrected pairwise comparisons revealing that they spent significantly more time searching in the NE quadrant (M: 38.05±3.4%) than in the SE (M: 18.76±1.74%) or SW (M: 15.24±2.82%) quadrants (p<0.05). The NCR group also displayed a significant pattern of searching [$F_{(3, 18)}$ = 18.56, p<0.001],

particularly in their expected SW goal quadrant (M: 40.33±2.93%) than in the NE (M: 10.29±1.07%) and SE quadrants (M: 17.14±1.97%; Bonferroni-corrected $p<0.05$). Both groups of animals spent high percentages of their time in the NW quadrant, where they were placed in the pool initially, however as described above, the animals do move away from this area into other quadrants of the arena.
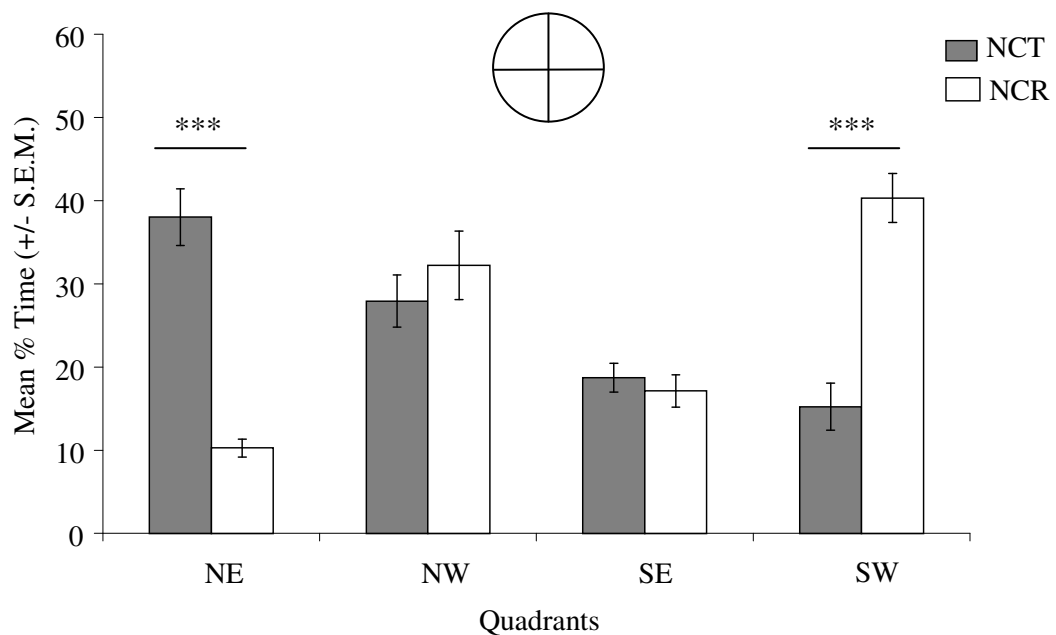
Figure 2.8: Mean percentage time spent by the Near Control and Near Cue-Rotated groups in the NE, NW, SE, and SW quadrants during the retention trial.

Examination of each of the goal quadrants was subsequently carried out to examine the specific areas in which the animals searched during the retention trial. The NE quadrant was the goal quadrant for the NCT animals as this was the location the animals were trained to search for the platform during acquisition. The SW was also examined as, if the animals used the cues to learn the location of the platform, the NCR animals would be expected to follow the rotated cues and search in the SW quadrant of the pool. Comparison of the goal quadrants overall revealed that the NCT

group (M: 38.05±3.4%) spent significantly more time in the NE quadrant than the NCR group (M: 10.28±1.07%; t(12) = 7.78, p<0.001). In addition, when the SW quadrant was assessed a significant difference between the groups was revealed (t(12) = 6.17, p<0.001) with the NCR group (M: 40.33±2.93%) spending significantly more time here compared to the NCT group (M: 15.24±2.82%; see Figure 2.8). Furthermore, assessment of the NE outer corridor revealed that the NCT group (M: 16.43±1.89%) spent significantly more time searching there than the NCR group (M: 5.38±0.52%; t(12) = 5.64, p=0.001). Whereas, the NCR group (M: 20.33±3.87%) spent significantly more time in the SW outer corridor than the NCT group (M: 8.24±2.75%) during the retention trial (t(12) = 2.55, p<0.05; Figure 2.9).
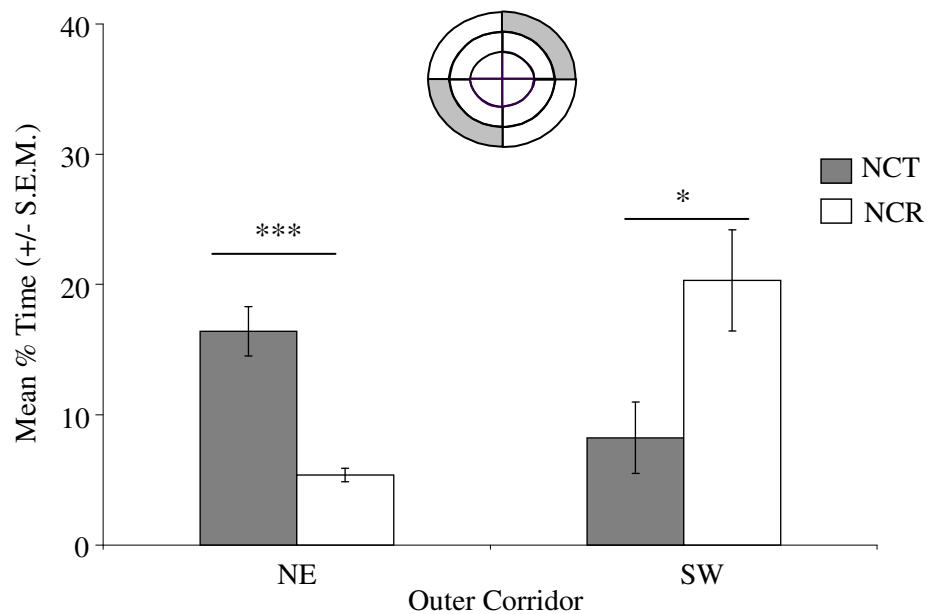
Figure 2.9: Mean percentage time (±S.E.M.) spent by the Near Control and Near Cue-Rotated groups in the NE and SW Outer Corridors, during the retention trial.

The platform corridor, which is a corridor encompassing the expected platform location was also assessed for the NE and SW quadrants. Independent t-tests revealed a significant result, with the NCT group (M: 16.38±2.34%) spending more time searching in the NE platform corridor than the NCR group (M: 3.67±0.72%; t(12) = 5.19, p=0.001). Assessment of the SW platform corridor similarly revealed that the NCR animals (M: 15.8±8.1%) spent significantly more time searching in this area when compared to the NCT group (M: 4.67±1.0%; t(12) = 3.46, p<0.01; See Figure 2.10).

Figure 2.10: Mean percentage time spent by the Near Control and Near Cue-Rotated groups in the NE and SW Platform Corridors, during the retention trial.

Finally, we assessed the platform area; this was examined due to its precise position with regard to the expected hidden platform location (Figure 2.11). It was found that the NCT group spent significantly more time searching in the NE platform

area when compared to the NCR group (t(12) = 5.38, p<0.001; NCT, M: 9.67±1.2%; NCR, M: 2.29±0.66%). Meanwhile the NCR group (M: 9.57±4.7%) spent a significantly higher percentage of time searching in the SW platform area than the NCT group (M: 3.48±1.04%; t(12) = 2.95, p<0.05).

Figure 2.11: Mean percentage time spent by the Near Control and Near Cue-Rotated groups in the NE and SW Platform Areas, during the retention trial.

The results suggest that the NT animals rely on the distal cues to learn the water maze task, and later remain reliant on the cue configuration during a retention probe trial. This is evident as both the control and cue-rotated animals search in their respective goal quadrants with quite accurate searching in all regions of these particular quadrants.

2.4 Results: Far Training Condition

2.4.1 Acquisition

Animals in the Far Training condition (FT) also successfully learned the task over 5 days of training. FT animals had a mean escape latency on Day 1 of 37.07±2.89 sec which decreased with training to 14.29±1.67 sec on Day 5 (see Figure 2.11). A repeated measures ANOVA confirmed a significant difference in escape latencies across training days $[F_{(4, 56)} = 26.09, p<0.001]$. Further Bonferroni-corrected comparisons demonstrated that the mean on Day 5 was significantly faster than Days 1 (p<0.001), 2 (p<0.001), and 3 (p<0.01; see Figure 2.12).
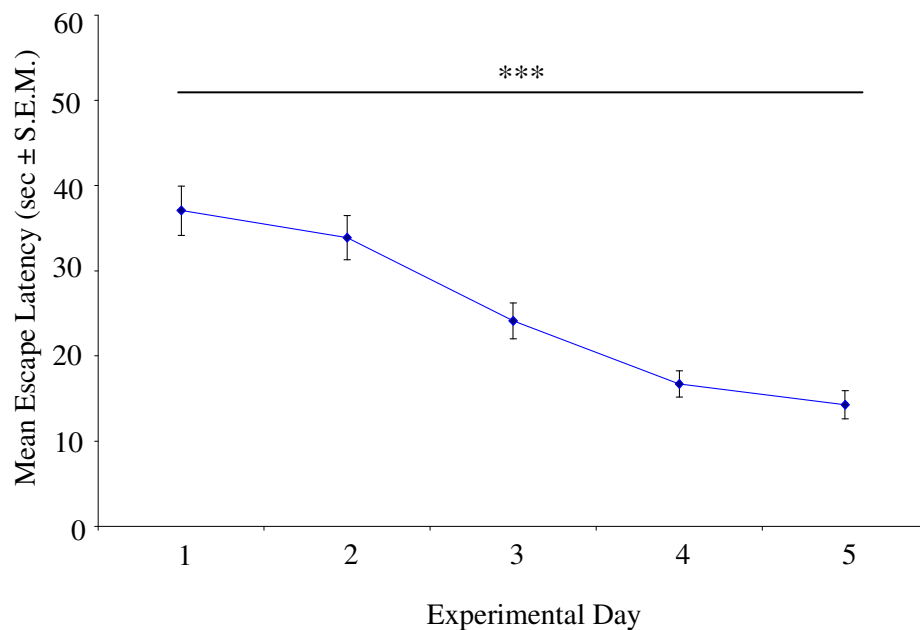
Figure 2.12: Mean escape latencies (sec ± S.E.M.) of FT animals throughout water maze training.

Distance travelled also indicated successful learning of the task. On Day 1 FT animals travelled a mean distance of 821.47±74.28 cm which decreased with continued training to 318.98±36.25 cm on Day 5 (see Figure 2.13). A repeated measures ANOVA verified a significant decrease in distance travelled throughout training [F(4,

56) = 21.56, p<0.001]. The mean daily velocity was also assessed, however, no significant change was seen across the training period [F(4, 56) = 2.12, p>0.05].
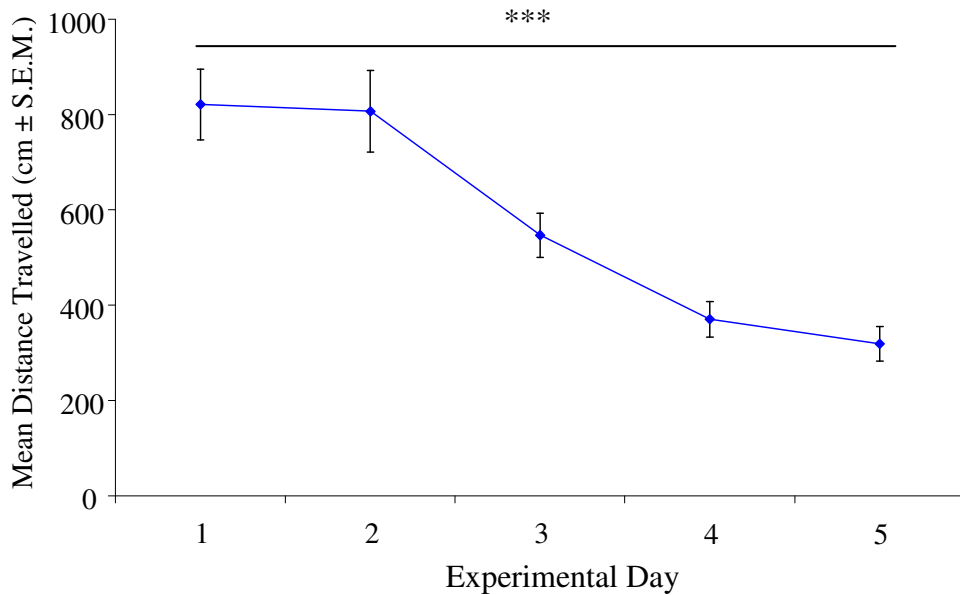
Figure 2.13: Mean distance travelled (cm ± S.E.M.) of FT animals across water maze training.

2.4.2 Retention

For the retention trial, which was assessed 7 days post-acquisition, animals were randomly assigned to two groups; Far Control (FCT, n=8) and Far Cue-Rotated (FCR, n=7). FCT animals' cues remained in the same location as in training (i.e. the Far position). Animals in the FCR condition, however, had their cues rotated 180° from their original position during acquisition. For purposes of analysis, the pool was divided into zones as previously described (see Methods section 2.2). Once again, maze maps were used initially to determine where animals in both groups spent the highest percentage of time searching during the retention trial. Figure 2.14 illustrates differences between the groups in the time spent in the different areas of the maze.
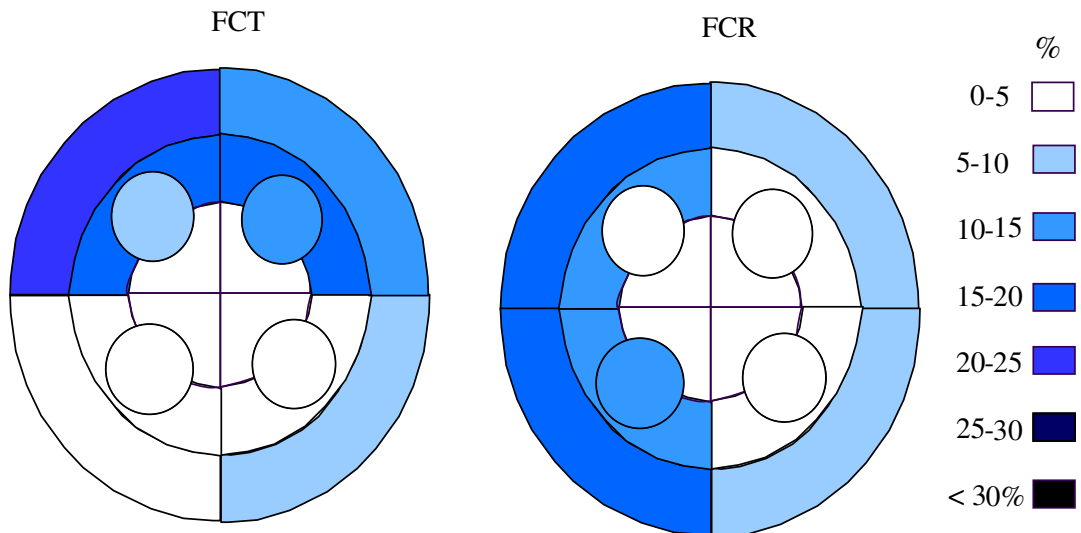
Figure 2.14: Maze maps illustrating the mean percentage time spent by the Far Control and Far Cue-Rotated groups in the different zones of the maze during the retention trial.

Initially the mean percentage time spent in the four quadrants of the pool was examined for both groups (Figure 2.15). A repeated measures ANOVA revealed overall significant effects for time spent in the different quadrants of the maze for the FCT group [$F_{(3, 21)} = 9.41$, $p<0.001$]. Further Bonferroni-corrected pairwise comparisons indicated that the FCT group spent the majority of time searching in the NW quadrant (M: 42.88±5.89%) when compared to the SW (M: 12.01±2.84%; $p<0.01$) and SE quadrants (M: 10.08±2.96%; $p<0.05$), and also spent a higher percentage of time searching in the NE quadrant (M: 35.04±5.9%) than in the SE ($p<0.05$) and SW quadrants ($p<0.05$). The FCR group also had a significant pattern of searching during the retention trial [$F_{(3, 18)} = 5.17$, $p<0.01$]. Overall the FCR group appeared to spend a higher percentage of time in the NW (33.8±4.51%) and the SW of the pool (34.38±5.60%), however following Bonferroni-corrected comparisons this was just outside statistical significance. Further assessment of the goal quadrants (i.e. NE for FCT, and SW for FCR) highlighted differences between the groups, with the

FCT group (M: 35.04±5.9%) spending significantly more time in the NE quadrant than the FCR group (M: 14.33±3.89%; t(13) = 2.84, p<0.05).
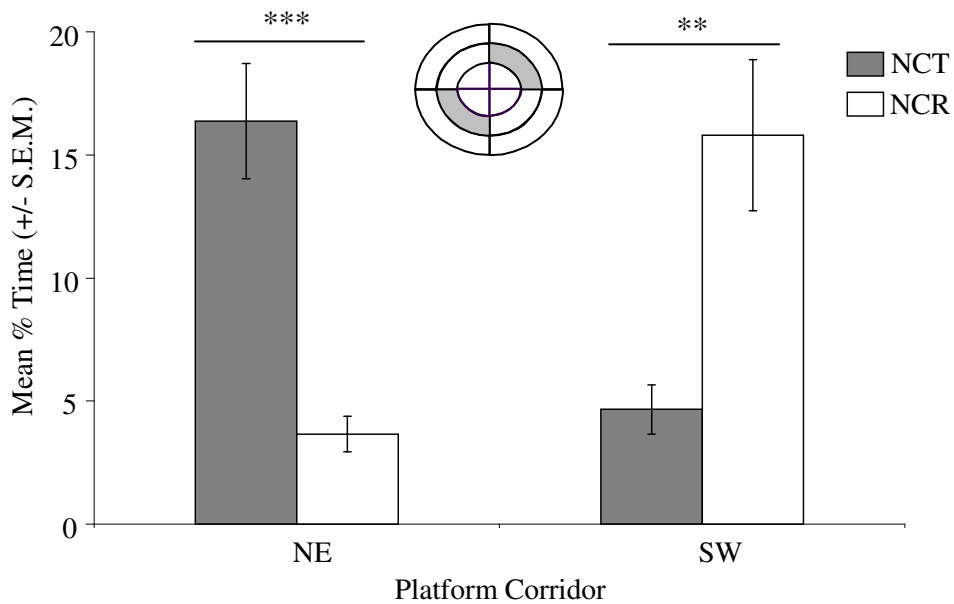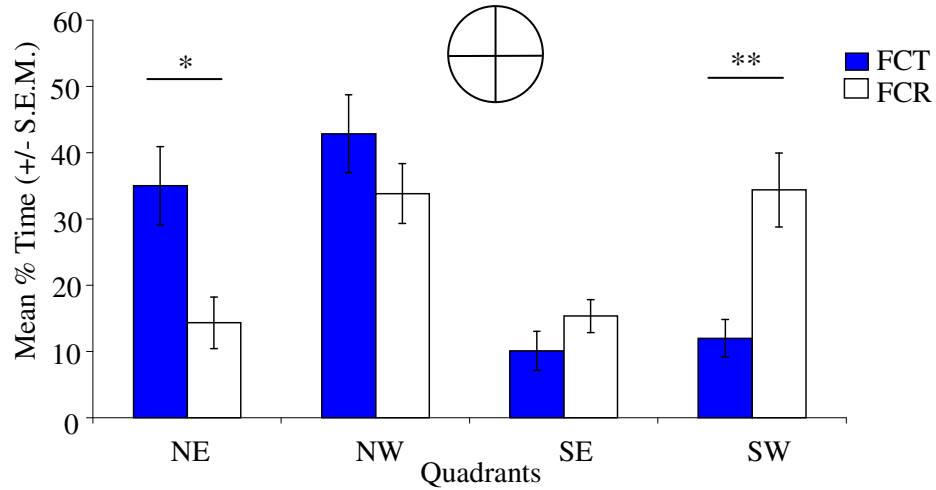

Figure 2.15: Mean percentage time spent by the Far Control and Far Cue-Rotated groups in the NE, NW, SE, and SW quadrants during the retention trial.

To further examine the search patterns of the animals during retention, the zones within each of the goal quadrants were assessed. Initial examination of the time spent searching in the NE outer corridor revealed no differences between the groups (t(13) = 0.95, p>0.05). However, the FCR group (M: 18.09±3.40%) were found to spend significantly more time searching in the outer corridor of their SW target quadrant than the FCT group (M: 7.21±2.46%; t(13) = 2.64, p<0.05; Figure 2.16).
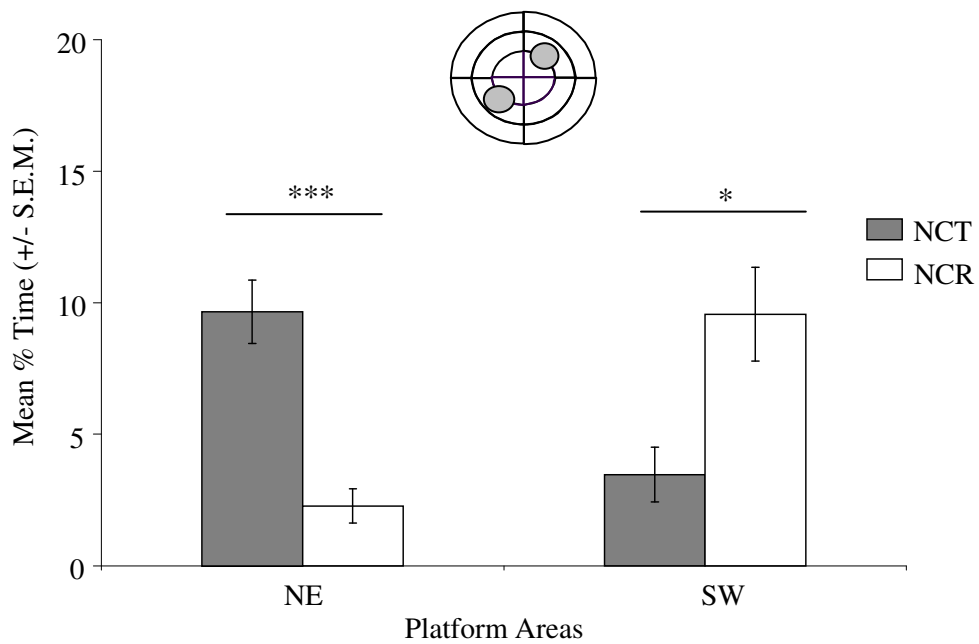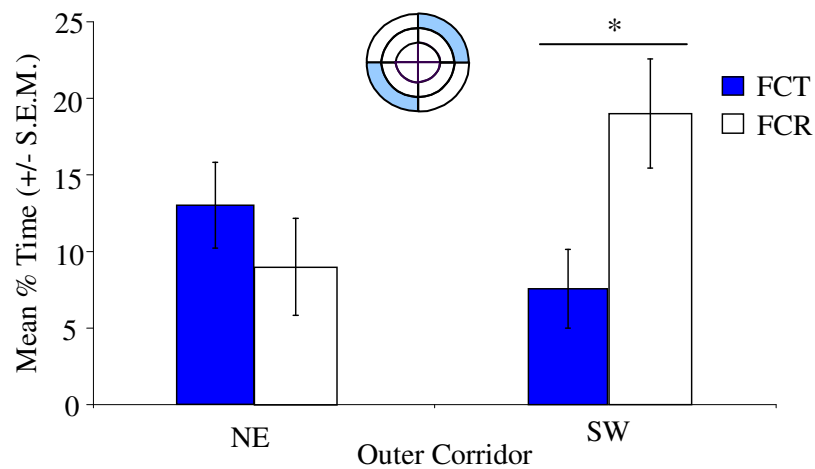

Figure 2.16: Mean percentage time spent by the Far Control and Far Cue-Rotated groups in the NE and SW Outer Corridors, during the retention trial.

Following this examination, time spent in the platform corridor was assessed to determine if the groups searching was in closer vicinity to the expected platform location. Independent t-tests revealed a significantly higher percentage time was spent by the FCT (M: 15.08±2.50%) than the FCR (M: 4.76±1.00%) group in the NE platform corridor (t(13) = 3.82, p<0.01). Similarly, the FCR group (M: 14.85±1.98%) spent significantly more time in the platform corridor of their target SW quadrant than the FCT group (M: 4.5±0.92%; t(13) = 4.74, p=0.001; see Figure 2.17).
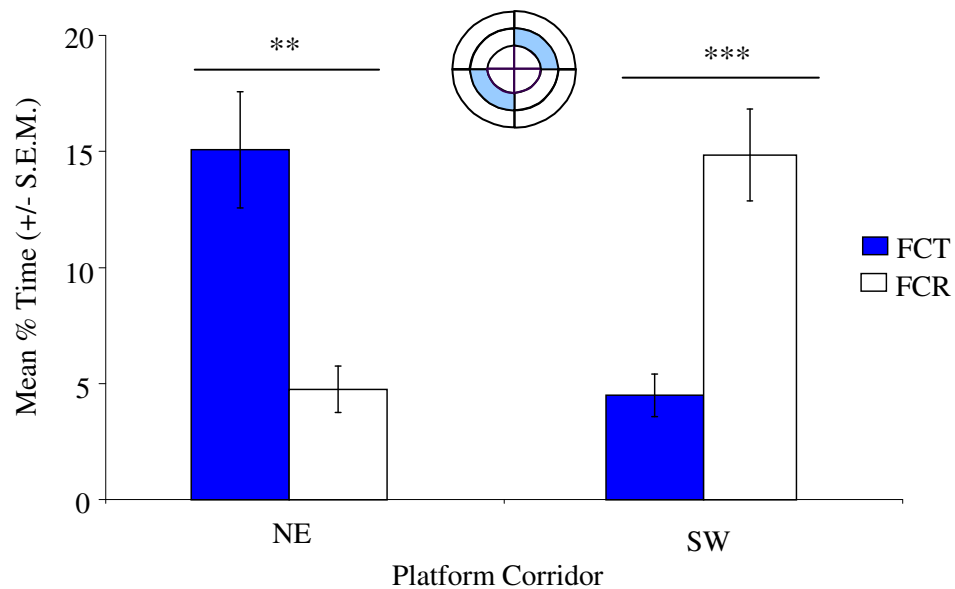
Figure 2.17: Mean percentage time spent by the Far Control and Far Cue-Rotated groups in the NE and SW Platform Corridors, during the retention trial.

Time spent in each of the platform areas was next examined as this is the expected area where the platform should be for each group of animals (Figure 2.18). Independent t-tests revealed that the FCT group (M: 10.63±3.16%) spent significantly more time searching in their expected platform area in the NE quadrant than the FCR group (M: 2.48±0.54%; t(13) = 2.54, p<0.05). Whereas, the FCR group (M: 11.57±1.42%) spent more time swimming in the SW platform area than the FCT group

(M: 2.58±0.67%) during the probe trial (t(13) = 5.95, p=0.001), indicating that the rotation of cues led to a parallel change in their searching location.
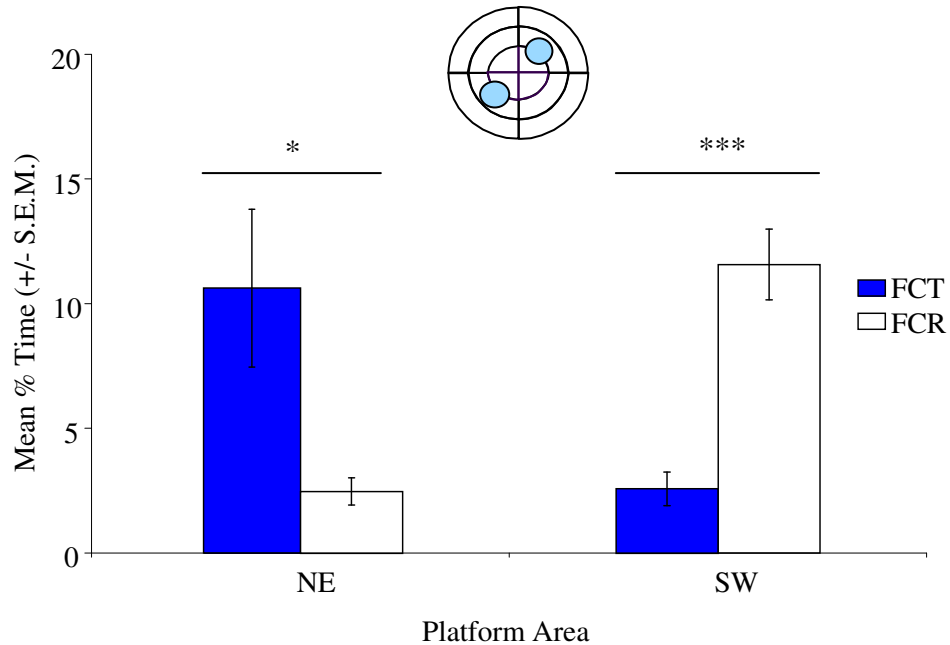
Figure 2.18: Mean percentage time spent by the Far Control and the Far Cue-Rotated groups in the NE and SW Platform Areas, during the retention trial.

Initial analysis of the overall quadrants of the pool during the retention trial did not reveal differences in the search strategies employed by each group. However, when detailed examination of the zones within each quadrant was carried out, specific differences and patterns of searching emerged, with all animals directing their searching in the areas closest to where the platform would be located for each respective group.

2.5 Results: Comparison of Near Training and Far Training Retention Trials

To fully determine that animals trained with Near and Far cues remained reliant on the cues, we assessed if, during retention, the control (CT) and cue-rotated (CR) animals searched in their respective target areas, irrespective of cue-position during training (i.e. Near or Far position). For this, a two-way ANOVA was conducted to explore the impact of cue-position (NT and FT) and cue-rotation (CT and CR) on the percentage time spent searching in the target NE platform area and the SW platform area. Assessment of time spent in the NE platform area (i.e. the CT target region) revealed that there was a significant difference between CT and CR animals, overall, irrespective of cue-position [$F_{(1, 25)} = 16.73$, $p<0.001$], with the CT group spending $10.14\pm1.32\%$ of time in the NE platform area and the CR group spending a mean of $2.38\pm1.36\%$ in this region of the pool. However, there was no difference between the NT and FT groups [$F_{(1, 25)} = 0.09$, $p>0.05$] in the time spent in the NE platform area overall and no interaction effect between cue-position and cue-rotation [$F_{(1, 25)} = 0.04$, $p>0.05$]. Similar assessment was carried out to examine the SW platform area, as this was the target area for the CR group during the retention trial. Overall, there was a significant difference found between the CT (M: $3.03\pm0.88\%$) and CR (M: $10.57\pm0.91\%$) animals in the time spent searching in the SW quadrant [$F_{(1, 25)} = 35.48$, $p<0.001$]. However, there was no effect of cue-position [$F_{(1, 25)} = 0.19$, $p>0.05$] and no interaction effect between cue-position and cue-rotation [$F_{(1, 25)} = 1.3$, $p>0.05$]. These results further confirm that animals, irrespective of cue-position during training, remain reliant on the distal cues during the retention trial.

2.6 Discussion

The effect of cue rotation during a retention trial in the MWM was assessed in two groups of trained animals; one trained with cues close to the hidden platform and one trained with cues located at a distance further away from the goal. We attempted to examine both groups ability to avail of an allocentric strategy during a retention trial conducted one week after training. We suggest that if, following displacement of distal cues, a concurrent switch in searching occurred, it would indicate a learned and retained reliance on the external distal cues.

The findings of the study show that animals in both the NT and FT groups successfully acquired the task as evidenced in escape latencies, distance travelled and velocity. Further to this, examination of the retention trial revealed that, for both groups of animals, a general rotation of searching behaviour occurred that was directly in line with the 180° shifted distal cues. NCT animals spent the majority of their time searching in the expected NE quadrant than in any other quadrant of the water maze, and also spent significantly more time searching in NE areas when compared to the NCR group. NCR animals retention trials also suggest that they altered their searching pattern in line with the rotation of the distal cues as evidenced by the high percentage time spent in the SW quadrant (i.e. the rotated "platform position"). Similarly, retention of the FT group had a comparable pattern to that of the NT animals. Specifically, the FCT group spent more time searching in the NE platform area than the FCR group, whereas the FCR group appeared to follow the rotated cues in their searching, spending a high percentage of their time swimming in the SW quadrant, where the expected "rotated" platform should be than the FCT group.

The results, in support of previous research, initially demonstrate that animals in both groups can learn and subsequently retain knowledge of the water maze task seven days after training has ended, regardless of cue position during acquisition (McGauran et al., 2004; van Groen et al., 2002). The findings of this study are also in line with that of others, indicating a reliance on the distal cues when locating a hidden goal; the animals in both training groups altered their searching and followed the cues even when cues had been rotated from their original position (Collett et al., 1986; Harvey et al., 2008; Kealy et al., 2008; Kelly et al., 2010; Morris, 1981). This also implies that the association made between the distal cues and the platform location during training is retained and later required during retention of the maze. This dependence on the stable relationship of the cues to the platform suggests an allocentric strategy was used by both groups during learning and later recall.

While there is evidence for the importance of distal cues, many authors would suggest that a number of strategies are used to solve the MWM (Aggleton et al., 2000; Moghaddam & Bures, 1996; Timberlake et al., 2007). Findings have shown that in a beacon version of the task, for example, where an obvious marker for the goal is available, rats will solely rely on this to complete the task and ignore any other available information (Manteiga & Chamizo, 2001; Roberts & Pearce, 1999). However, if such a proximal cue is neither available nor accurate for locating a goal, a configuration of distal cues will instead be used if accessible. Finally, if these external cues are misleading or uninformative animals will revert to self-movement cues to navigate (Maaswinkel & Whishaw, 1999; Stackman & Herbert, 2002; Vanderwolf, 2001). Maaswinkel and Whishaw (1999) support the suggestion that multiple navigational strategies are employed in the water maze, and suggest that animals can

flexibly combine and switch between strategies when necessary. A critical point in determining the relative contribution of each strategy, however, is the experimental design and although it is apparent that a number of methods can be used to solve the task, our findings support the idea that when external cues are available, be they in a position close to or far away from the hidden platform, the place water maze paradigm is primarily solved relying on the association between the external cues and the target location. While this is critical, and highlights the remained dependence on distal cues in locating a hidden goal when they are available (Harvey et al., 2008; Maurer & Derivaz, 2000), there were no proximal cues available within the pool during the training period in the current study. As previous research suggests, if such proximal cues were included in the environment, animals would likely avail of the cues in a hierarchical manner, relying initially on closer, more proximal cues as they allow for more efficient and localised searching (Maaswinkel & Whishaw, 1999). However, when only distal cues are available, our findings suggest that animals remain reliant on external cues following cue rotation, confirming the importance of external information alone in the ability to locate a goal.

However, there were some anomalies in the search patterns in both the NT and FT groups. Both groups, for example, appeared to spend a high percentage of time in the peripheral region of the maze, particularly at the start location (NW). Previous research has suggested that accurate searching during a retention trial is time dependent, with failure to initially locate the platform leading to searching in other quadrants. In addition, it has been shown that animals often search at the location they were initially placed in the pool (Mabry et al., 1996; McGauran et al., 2004; 2005). This may be due to increased stress as there is no available escape route and so animals

revert to safe behaviours (i.e. swimming close to the pool edge; Johansson et al., 2002). Overall, however, the animals did spend the majority of their time searching in their expected platform quadrants indicating successful recall of the goal location.

The aim of this chapter was to establish if animals build up cue-platform associations during acquisition and subsequently retain them for later use. This appears to be the case, as both groups of animals searched in cue-relevant quadrants. However, how the animals learn and establish associations between the cues is not fully revealed through the assessment of the retention trial alone. To determine if there are subtle differences between the groups learning of the task that is dependent on cue location, we will attempt, in the next Chapter, an in-depth examination of how cue associations are formed during the acquisition phase of the Morris water maze under the different cue conditions.

Chapter 3a

Learning the Morris water maze; the effect of cue location on swimming behaviours and navigational strategies.
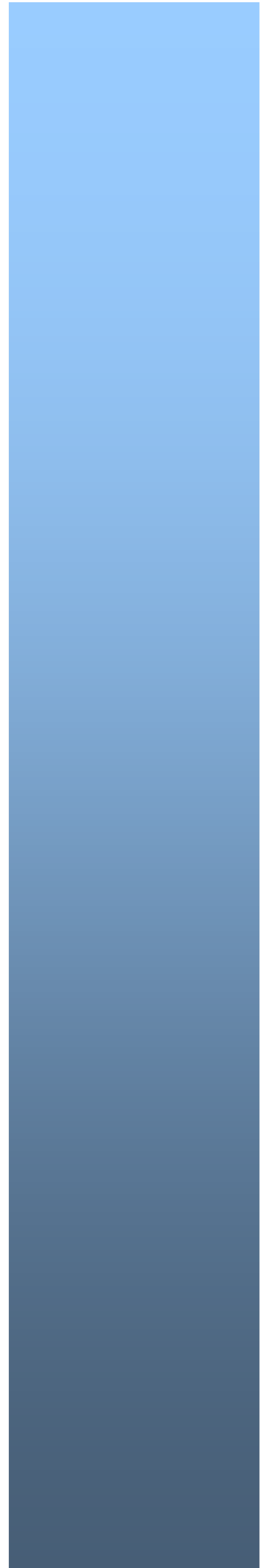
Abstract

In the previous Chapter it was confirmed that animals develop a strong platform-cue association in the MWM, remaining reliant on distal cues regardless of distance, and rotation during a retention trial. However, as there has been contention over the exact methods used to learn this task, and with the increasing knowledge of the importance of cues in solving the maze, we investigated the effect of cue location on how animals acquire the MWM. Through in-depth behavioural analysis we attempted to separate out differences in allocentric and egocentric navigational strategy use, in the maze, under differing cue conditions. For this, male Wistar rats (n=14) were divided into two groups: Near trained (NT; n=7) and Far trained (FT; n=7) as in Chapter 2. All animals were trained in the MWM for 5 consecutive days receiving 4 trials per day. Sub-second behavioural analysis of the animals' swimming tracks throughout training revealed significant differences between the groups, particularly in their thigmotactic and turning behaviours and also in how they appear to use the cues overall. The behavioural data suggests that when cues are located in close proximity to the goal animals use the cues directly, in a view-dependent strategy, to find the submerged platform. However, when cues are located at a distance further away from the goal, animals must infer more to locate the platform position and so use a view-independent inferring strategy.

3a.1 Introduction

As confirmed in Chapter 2, animals have a propensity to rely on visual cues to guide them to their goal location. In our place version of the task, animals appear to use the distal cues throughout training, with retention of the task reliant on the stable configuration of the external cues. Similar support for these findings have been noted in numerous studies examining the role of distal cues in water maze acquisition (Harvey et al., 2008; Kealy et al., 2008; McGauran et al., 2004; Morris, 1981). However, we also noted some discrepancies between animals in the Near trained and Far trained groups (e.g. preliminary observation of slower acquisition for the FT group compared to the NT group in Chapter 2), indicating a possible effect of cue location in learning the task. This would suggest that the precise way in which animals solve the task warrants further investigation. However, until recently, acquisition has only been generally explained using overarching navigational strategies such as egocentric or allocentric guidance. These terms, however, allude only to the fact that animals rely on a configuration of distal cues when solving allocentrically or alternatively, use a learned set of motor movements or follow a visible beacon or proximal cue, when solving a task egocentrically (Leggio et al., 1999; Moghaddam & Bures, 1996; Morris, 1981; O'Keefe & Nadel, 1978; Tamara et al., 2010a; Timberlake et al., 2007). Disagreement also remains regarding the strategies employed in the water maze (Chang & Gold, 2003a; Hamilton et al., 2004; Packard & McGaugh, 1996; Whishaw & Mittleman, 1986) and under what conditions specific strategies will be employed (Burgess, 2008; Rodrigo, 2002; Redish, 1999).

To try to determine this, gross measures of acquisition alone have been relied upon such as escape latencies, distance travelled and velocity (Chamizo et al., 2006;

Lopez et al., 2008; Morris, 1981; Whyte et al., 2009). However, this often results in an oversimplified description of rodent behaviour. Some attempts have been made at more precise examination, however these have generally only included details of overall day behaviours in the maze, such as thigmotaxis or circling (Baldi et al., 2003; Brandner & Schenk, 1998; Hamilton et al., 2004). Further, more comprehensive accounts outlining how rodents behave, from trial to trial, during acquisition have provided detailed categorisation of overall swimming behaviours based on dominance within a trial and have been applied to animals' performance in attempts to explain how animals learn the task across days (Graziano et al., 2003; Leggio et al., 2003; Petrosini et al., 2003).

However, with controversy surrounding the exact strategies used by animals in solving the MWM, general classifications of behaviours are not sufficient to resolve this issue. Recently, evidence has emerged illustrating the importance of examination beyond basic measures, highlighting the specific influence of distal cues on a place MWM on more discrete behaviours. Specifically, Harvey and colleagues (2008) carried out an in-depth, second by second, examination of swimming patterns and found that multiple behaviours occurred within a single trial. Specifically, the authors illustrated a change in behaviours across days with a reduction in egocentric behaviours and an increase in more allocentric related movements as the task is learned. Hamilton et al. (2004) and Korz (2006) have also suggested that more detailed examination of behaviours will allow for a sensitive analysis of how stimuli control behaviour over examination of standard measures alone. This approach may, therefore, identify differences in swim patterns that could highlight subtle changes in spatial learning over a training period.

In line with strategic concerns, there has also been disagreement over the methods in which animals use the external cues to locate a hidden goal in a number of spatial tasks. A variety of factors relating to environmental cues have been shown to effect how an animal will learn a spatial task (Prados, 2000; Chamizo & Rodrigo, 2004; Lopez et al., 2008; Young, et al., 2006). However, cue location, in particular, appears to influence an animal's ability to solve spatial tasks with the distance a landmark is from the goal being a critical factor (Biegler & Morris, 1996; Chamizo et al., 2006; Spetch & Wilkie, 1994; Vallortigara et al., 1990). For example, Morris (1981) showed that rats' use of an allocentric strategy in the water maze varied with the accuracy with which local and distal cues predicted the location of the hidden platform. Similarly, when a number of cues were available in a touch screen spatial task, a learned response was not controlled by the overall configuration of landmarks, rather it was controlled by the proximity of the goal to an individual landmark (Spetch & Wilkie, 1994; Spetch, 1995). Spetch (1995) also noted that control over a pecking response in pigeons that was acquired by a landmark at a given distance from the target was later overshadowed by the presence of another landmark that was in a position closer to the target. Similar overshadowing of distal cues by proximal cues has also been seen in object exploration tasks (Craig et al., 2005).

In relation to the MWM, overshadowing of cues has also been reported, whereby a navigating animal will not avail of external cues to locate a hidden goal when a proximal beacon is available instead (Roberts & Pearce, 1999). Similarly, Morris (1981) found that rats that received prior training with a beacon in the MWM were more accurate in locating the correct platform location in a subsequent place version of the task, than animals trained without a local beacon. Equally, Chamizo and

Rodrigo (2004) found that rats were most impaired in learning the MWM when a single available cue was suspended at the pool wall furthest from the hidden goal, and only when the cue was located closer to the platform did performance improve, with optimal performance reached when the cue acted as a beacon, further highlighting the impact of cue positioning on learning the task. However, in this instance an individual distal cue may not be sufficient in examining if an allocentric or egocentric strategy is used by animals in the maze, as when locating a hidden goal allocentrically, animals require a number of sources of external information in order to accurately calculate the distance and direction of their position (Rodrigo et al., 1997; Kubie & Fenton, 2009).

As animals appear to learn the water maze task differently under diverse cue conditions, specifically in relation to cue proximity from a goal (Chamizo & Rodrigo, 2004), and with subtle changes in behaviour seen throughout training in a standard MWM (D. Harvey et al., 2008; 2009), we aimed to examine the effect of location of a distal configuration of cues on animals' discrete swimming behaviours and on their ability to successfully learn the task. As proposed by Hamilton et al. (2004) in-depth behavioural analysis would be well suited for examining the role of cues in task acquisition and as some differences emerged between NT and FT groups in Chapter 2, we predict that these animals will display different swimming behaviours. Specifically, we predict that animals trained with near cues will display more cue-direct, egocentric behaviours which will be performed at or towards the cues, as their cues are positioned close to their goal and may, therefore, be used in a beacon-like manner. We predict that the Far trained animals will display more cue-independent, allocentric behaviours including movements away from the cues to infer the goal location, as they will not have the same access to their cues while approaching their goal.

3a.2 Method

3a.2.1 Subjects

Male Wistar rats (n=14) served as subjects in this experiment. All were housed an handled in the same manner as described in detail in Chapter 2 (section 2.2.1).

3a.2.2 Apparatus

The Morris water maze (MWM) paradigm as described previously (Chapter 2) was again used in this experiment. Three distal cues (two 25W bulbs and a white sheet of card) were used in this experiment and the position of the cues remained stable throughout training. The platform position was also held constant throughout training and was located in the NE quadrant.

An overhead camera positioned in the laboratory ceiling, above the centre of the maze, captured all of the animals' movements throughout experimental trials and EthoVision tracking system recorded escape latencies, distance travelled and swimming velocity of each animal on all trials. In addition to the gross measures of performance during acquisition, the swimming behaviour of the animals, and the behaviour of the animals while on the platform, following the swimming phase of the trial, were also recorded for later analysis. This was achieved using a second camera (Sanyo hi-resolution b/w CCD camera 1-1.3, 5-50mm) placed directly above the platform in the laboratory ceiling (see Figure 3a.1). This camera provided an aerial view of all of the rats' actions throughout training trials (i.e. swimming behaviours and head direction while on the platform). These images were relayed in motion picture format to a second connected computer for recording and later assessment. Therefore, all head orientations during the platform interval were monitored, in addition to their movements and behaviour during swimming in the pool itself.
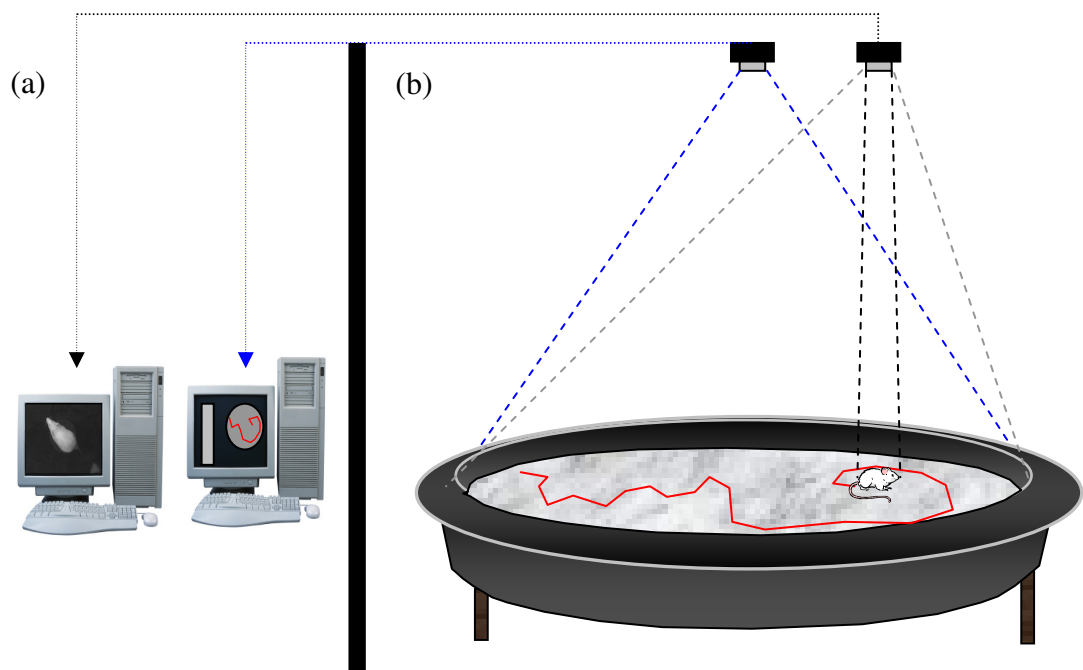
Figure 3a.1: Schematic representation of the experimental set-up. (a) Two computers, located in a separate room, recording all information pertaining to the acquisition trial and platform interval, via connected cameras. (b) Water maze arena, including camera positions.

3a.2.3 Procedure

Animals (n=14) were randomly assigned to one of two groups with cues in either the near (Near trained (NT); n=7) or far position (Far trained (FT); n=7). Animals assigned to the NT group had three distal cues available to them. The distal cues included two 25W light bulbs suspended from the ceiling; one near the North West (NW) quadrant of the pool and the other near the North East (NE) quadrant. A rectangular sheet of white paper (55 cm x 81 cm) was also attached to the curtain on the east side of the pool for use as a cue. Animals in the FT group had the same distal cues available to them, however the cues were located at a position opposite from the platform with one light cue suspended at the SW quadrant and a second from the SE quadrant. The white cue card was now situated on the western side of the pool (see Figure 2.3 Chapter 2,

and Figure 3a.4). Both groups followed the same training procedure in the maze as described in Chapter 2 (i.e. 4 trials per day for 5 days). The three distal cues were visible throughout all of the acquisition trials and platform intervals.

3a.2.4 Assessment of the platform interval

Digital recordings of animal movements during the 15 second platform interval were saved to a connected computer using a video capture software package (VirtualDubMod 1.5 10.2). Each platform interval produced a 15 second motion picture of the animal's head movements. However, to enable analysis of head movements, each video file was divided into 15 still digital photographs (Figure 3a.2) each a second in length, using a video segmenting program (TMPDEnc 2.5, Hiroyuki Hori/Pegasys Inc.). Therefore, this produced one digital photograph for each consecutive second spent on the platform during an interval trial. In total, for the current study, there were 4200 digital stills (i.e. 14 animals x 4 trials x 15 seconds x 5 days) of animal head directions.

Figure 3a.2: A digital photograph example of an animal's head direction during the platform interval with line drawn from midpoint of the animal's eyes to the snout. (NT008; Day5-trial 1, 2-3sec).

Using Adobe Photoshop 5.0 the head direction of the animal was determined in each still shot on the platform. For this, a line was manually drawn between the midpoint of the subject's eyes running to the tip of the snout using the computer mouse (Campbell, 2001; Harvey et al., 2008). Photoshop calculated the angle of this line in degrees, on a scale ranging between $0\pm180^{\circ}$ which then converted to $0\text{-}360^{\circ}$, with the south of the platform taken as 0°.

3a.2.5 Definition of searching strategies used during in-trial locomotion

The swimming behaviours of all rats were also examined for each training trial across all experimental days. To examine behaviours, swim tracks from each animal during each trial were provided by EthoVision. The tracks comprised of *x, y* coordinates for the animal's position throughout the entirety of each trial with each coordinate being 0.2 second increments apart. From visual inspection and detailed analysis a number of distinct behaviours emerged. These observed behaviours were analysed for every training trial, under a number of categories including: percentage time spent (of total time in the pool) of each behaviour, and the frequency of each behaviour at individual locations in the maze (i.e. $0\text{-}360^{\circ}$).

The first behaviour, referred to as **thigmotaxis**, is characterised by the animal moving almost exclusively at the periphery of the maze (see Figure 3a.3 for a representative track illustrating swimming behaviour). Within this category two sub-classes are evident: **parallel thigmotaxis** (Figure 3a.3(i)) and **vertical thigmotaxis** (Figure 3a.3(v)). Parallel thigmotaxis refers to animal movements alongside the maze wall, with the animal remaining within 10 cm of the pool's edge. Vertical thigmotaxis is characterised by animal movements facing the pool wall; the animal makes direct

contact with the wall of the arena while continuing to move around the periphery of the maze.

The second behaviour, **direct behaviour**, is characterised by animal movements in a straight, definite direction over a minimum period of 1 second. This behaviour is comparable to the actions of the wood ant (*Formica rufa*; Nicholson et al., 1999) when approaching a landmark. More specifically when detailing rodent behaviour, Graziano et al.'s (2003) definition of direct finding and approach swimming behaviours and Harvey and colleagues (2008) direct-approach in the Morris water maze has close similarities to this style of swimming. Figure 3a.3 (ii) illustrates a period of direct movement.

The final behaviour identified is characterised by a **turn**. A turn is considered a whole body turn and not just the animal's head. For this, the animal moves in one direction followed by an obvious change in orientation ($>50^{o}$) and movement in a second direction. Therefore, turning is the incident of observable angular change between two periods of movement in different directions (Figure 3a.3 (iii) & (iv)). Turns are also divided into a number of categories; **turns towards** the cues (Figure 3.3(iii)) and **turns-away** from the cues (Figure 3a.3(iv)). Turn-towards the cues include an animal moving in one direction and turning (change in direction $>50^{o}$) and heading in a different direction towards a distal cue (range $\pm30^{o}$ either side of the cue). A turn-away typically involves an animal performing a whole body turn and change in direction ($>50^{o}$), that is not towards a distal cue, rather the animals perform turns in a direction away from the distal cues. Other movements such as scanning and large rotational movements were also noted (see Harvey et al., 2008), however these had very low levels of occurrence and, therefore, were not analysed further.
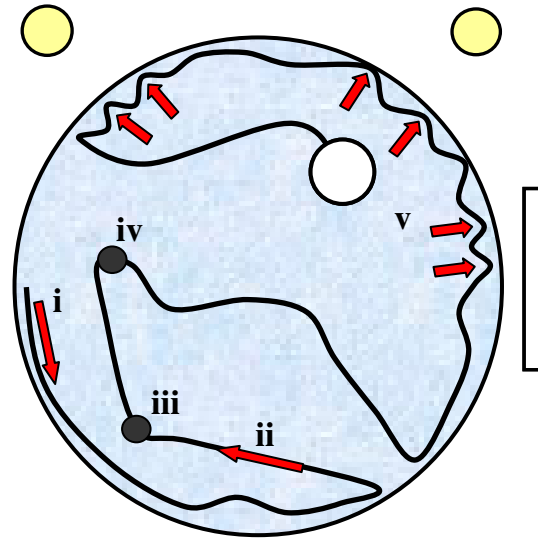
Figure 3a.3: Representative swim track displaying some of the behaviours seen within a training trial in the MWM including i) parallel thigmotaxis, ii) direct, iii) turn towards a cue, iv) turn-away from cue, v) vertical thigmotaxis.

3a.2.6 Zones of the pool

Furthermore, in order to fully examine turning behaviour, each external distal cue was colour-coded either blue, red or green, in the results for ease of analysis. See Figure 3a.4 for coded cues for both groups' cue arrangements. To examine the location of turns made in the maze, the pool was divided into three zones of equal area, based on the location of each turn. For each turn point, a line was drawn between the centre of the platform, the turn position and the pool edge using Adobe Photoshop 5.0. This program measured the length of each line and then normalised the location of the turn on each respective line, giving a percentage position on that line of where each turn point lay (i.e. pool wall was at 100% and platform edge was at 0%). This accounted for unequal distances from the platform to the side of the pool. Three zones were used in order to categorise the location of turn positions in the pool; turns within 0-33% were in the "near" zone. Turns within 34-66% were in the "middle" zone. Turns within 67-

100% were in the "far" zones. The mean number and location of turns for each animal was then assessed (adapted from Harvey et al., 2008).
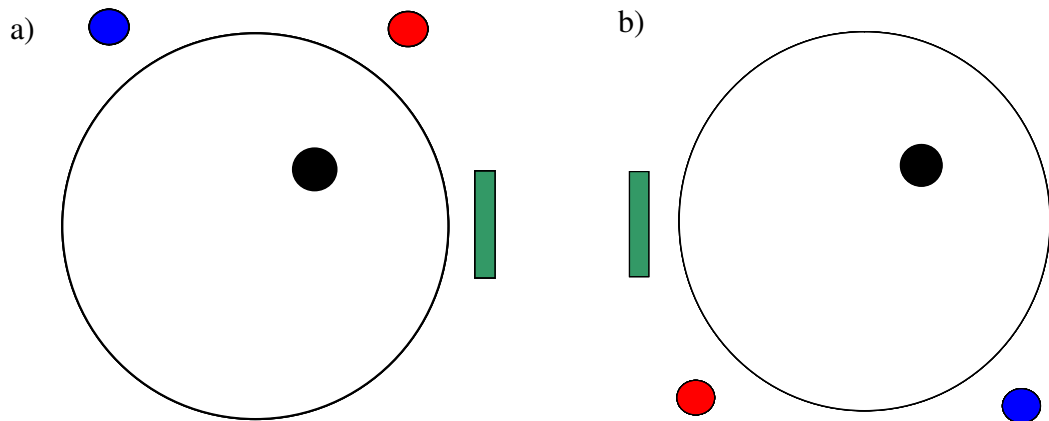
Figure 3a.4: Colour coded cues for a) the Near trained group; NW light cue coded blue, NE light cue coded red, and sheet of card at E coded green and b) the Far trained group; SE light cue coded blue, SW light cue coded red, and W sheet of card coded green.

3a.2.7 Statistics

A series of mixed-factorial and repeated-measures ANOVAs with appropriate Bonferroni-corrected comparisons were conducted, where appropriate, on the data collated for each swim trial. Independent and dependent t-tests were also calculated where required on all linear data.

Statistical assessment of the raw data concerning the animal's head direction and all data in circular format was conducted using circular statistics (Oriana Version 2.0, Kovach Computing Services, UK) that are equivalent to linear statistical procedures. Descriptive statistics including the mean angle, standard deviation and error of the mean were calculated for the animal's orientation during the platform interval. Angular variance (r) was also noted, and refers to the spread of the data set

(ranging between 0 and 1, where 1 represents all head directions that are in a unified direction). Rayleigh Uniformity tests were also employed to assess the significance of data distribution around the mean vector length (mean vector r). Watson-William's F-tests were also used in determining if the mean angles in two or more data sets differed significantly. The resulting F statistic is the same as Fisher's variance ratio statistic used in analysis of variance. A star-based system for significance representing p-values of $*<0.05$, $**<0.01$, $***<0.001$, respectively, was used throughout. Error bars and the symbol $\pm$ were employed throughout to indicate standard error of the mean (S.E.M.).

3a.2.8 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC.

3a.3 Results

3a.3.1 Basic Measures of Acquisition

Overall acquisition was initially assessed using standard measures including escape latency, total distance moved and mean velocity travelled. The mean escape latencies (EL) decreased across the five days of training for the Near trained group from 39.56 ± 5.32 sec on Day 1 to 10.22 ± 1.68 sec on Day 5. The Far trained group's ELs also decreased across acquisition from 42.19 ± 5.65 sec on Day 1 to 14.12 ± 2.76 sec on Day 5 (see Figure 3a.5). A 2 x 5 mixed factorial ANOVA confirmed that there was a significant decrease in EL across training days with a main effect for acquisition day $[F_{(4, 48)} = 24.04, p<0.001)]$. Bonferroni-corrected t-tests revealed that ELs on Day 4 and 5 were significantly faster than Day 1 ($p<0.001$) and Day 2 ($p<0.01$). There was also an overall difference between the groups $[F_{(1, 12)} = 5.27, p<0.05]$, whereby the FT group had slower ELs (M: 25.49 ± 3.89sec) than the NT group (M: 19.31 ± 2.70 sec) throughout training. No interaction effects were revealed between the groups across days $[F_{(4, 48)} = 0.66, p>0.05]$.
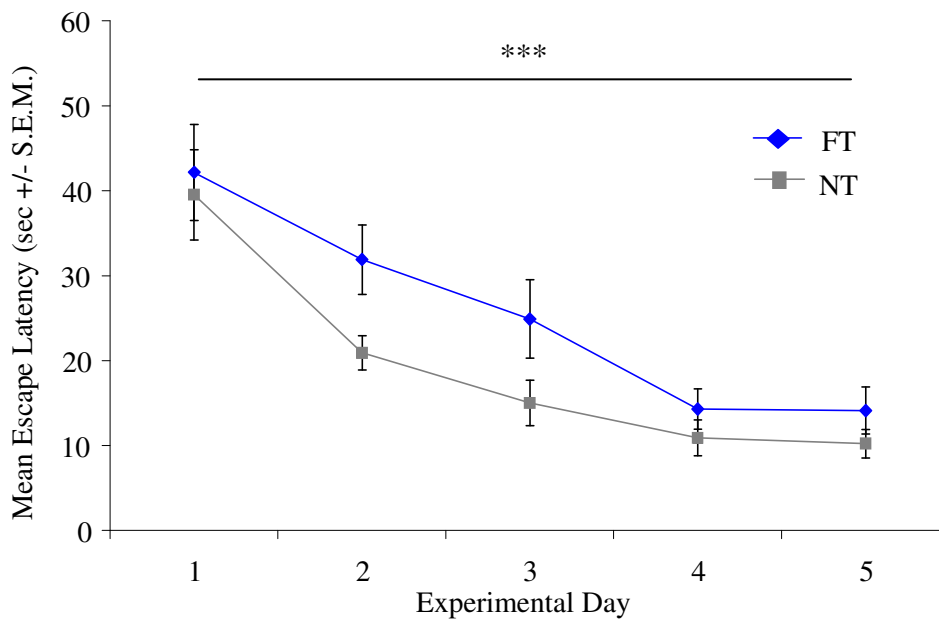
Figure 3a.5: Mean escape latency ($\pm$ S.E.M.) during acquisition for the NT and FT groups.

Further basic measures of acquisition, including distance and velocity, were also evaluated across training days. The total distance travelled showed an overall significant decrease with acquisition [$F_{(4, 48)}$ = 18.23, $p<0.001$]. Bonferroni-corrected t-tests revealed that the distance travelled on Days 4 (M: 312.6±54.2 cm) and 5 (M: 281.97±53.65 cm) was significantly shorter than Day 1 (M: 841.22±108.26 cm; $p<0.001$) and Day 2 (M: 627.52±72.52 cm; $p<0.001$). However, there was no main effect for group [$F_{(1, 12)}$ = 3.95, $p>0.05$] or interaction effect between day and group [$F_{(4, 48)}$ = 0.77, $p>0.05$] indicating both groups travelled similar distances throughout the training period. Similar assessment of the mean velocity travelled revealed a significant main effect for day [$F_{(4, 48)}$ = 7.38, $p<0.001$]. Bonferroni-adjusted t-tests indicate that Day 1 velocities (M: 21.17±1.10 cm/sec) were significantly slower than Day 2 (M: 25.41±1.20 cm/sec, $p<0.01$), Day 3 (M: 24.83±1.08 cm/sec, $p<0.05$) and Day 4 (M: 25.86±1.05 cm/sec, $p<0.001$). No main effect for group [$F_{(1, 12)}$ = 1.52, $p>0.05$] or interaction between group and day [$F_{(4, 48)}$ = 1.16, $p>0.05$] were noted. Therefore, from the standard criterion of acquisition, it would appear that there were slight differences between the groups' learning of the task, with the FT group slower than the NT group at acquiring the water maze.

3a.3.2 Behavioural Analysis; Platform behaviour

We first examined each animals' behaviour while on the platform (15 seconds after each trial). This evaluation would show, firstly, if information, namely cue associations, are established during this period in training (D. Harvey et al., 2009; Devan et al., 2003) and secondly, if differences between the groups would emerge. The

range of head movements made, as well as head orientations while on the platform, were the main measures examined.

3a.3.2.1 Range of Head Movement

The mean range of head movement was first examined to determine if subjects use the platform interval to learn the location of the environmental cues. We initially examined if a preference to take in several views or a more focused orientation of a particular region, when on the platform, emerged. Any potential differences between group behaviours while on the platform would also be revealed from this analysis.

For this, each training day was divided into the 4 respective training trials and the mean range of head movements was calculated for each group, across the 5 acquisition days. A 2 x 5 x 20 mixed factorial ANOVA revealed that there was no change in head movements across days [$F(4, 48) = 0.78$, $p>0.05$]. There was also no overall effect for group [$F(1, 12) = 0.01$, $p>0.05$], or interaction effect between day and group [$F(4, 48) = 0.45$, $p>0.05$]. However, a significant decrease in the range of head movements across trials was found [$F(3, 36) = 26.50$, $p<0.001$]. However, there was no interaction effect between trial and group [$F(3, 36) = 0.27$, $p>0.05$] or trial and day [$F(12, 144) = 1.70$, $p>0.05$; Figure 3a.6]. Subsequently, to further examine the main effect of trial, a series of repeated measures ANOVAs with Bonferroni-corrected t-tests were conducted. From this, trial differences on a number of days were revealed, with an overall pattern of greater movement on trial 1 of each day over subsequent training trials. Specifically, on Day 1 [$F(3, 39) = 15.49$, $p<0.001$] the greatest range of movement was found on trial 1 (M: 88.15 ± 7.75^{o}) compared to trials 2 (M: 58.13 ± 7.31^{o}, $p<0.05$), 3 (M: 40.56 ± 5.81^{o}, $p<0.001$) and 4 (M: 39.49 ± 6.55^{o}, $p<0.001$).

A similar finding was observed on Day 2 [F(3, 39) = 8.96, p<0.001] with differences between trial 1 (M: 66.73±6.66°) and trials 2 (M: 41.88±9.23°, p<0.05), 3 (M: 43.55±9.34°, p<0.05) and 4 (M: 41.71±7.98°, p=0.001). Significant differences between trial 1 (M: 77.15±6.0°) and trials 2 (M: 40.39±7.11°, p<0.01), 3 (M: 43.05±6.6°, p<0.001) and 4 (M: 36.88±7.81°, p<0.001) were found on Day 3 [F(3, 39) = 8.84, p<0.001]. Similarly, a significant effect for trial was found on Day 4 of training [F(3, 39) = 5.56, p<0.01], with trial 1 (M: 69.29±7.24°) differing significantly from trial 3 (M: 39.96±6.47°, p<0.05) and 4 (M: 34.77±6.39°, p<0.05). However, by Day 5, no differences between trials were noted [F(3, 39) = 0.53, p>0.05].
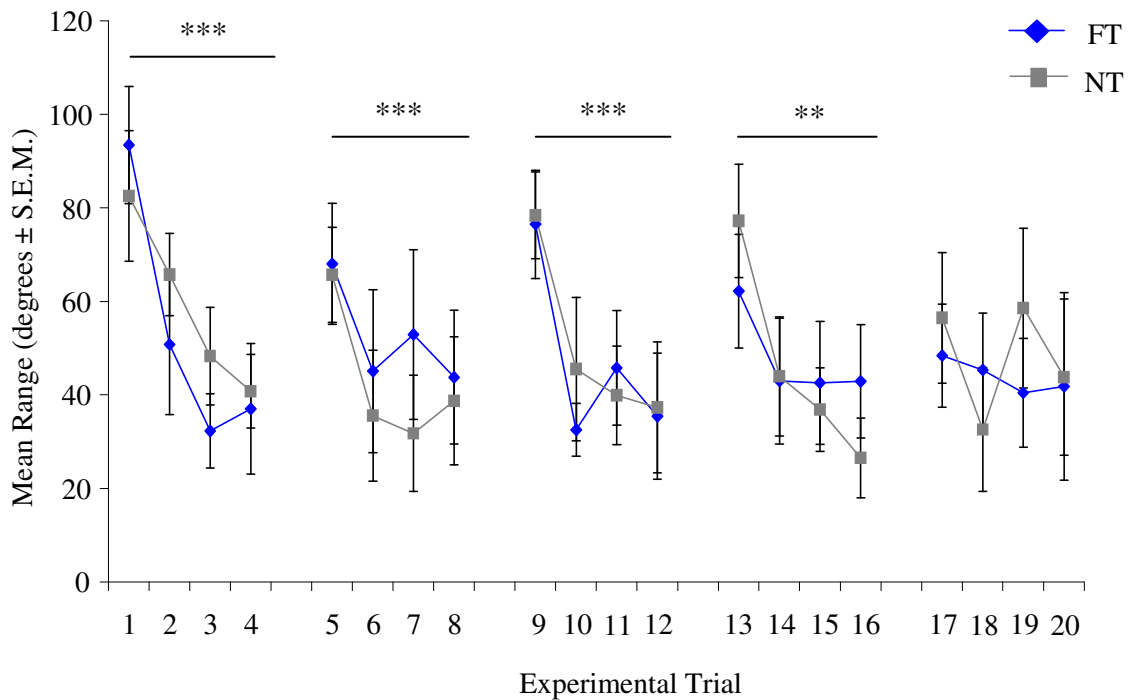
Figure 3a.6: Mean range of head movement (±S.E.M.) made by animals in the NT and FT groups during the platform interval on each trial across 5 training days.

3a.3.2.2 Mean Head Direction

Consequently, as it was revealed that animals look around while on the platform, it could be suggested that they were acquiring information about the platform's spatial

relationship to the distal visual cues during this period. To determine if animals were oriented in a specific direction or towards the relative distal cues available to each group, during the platform interval, mean head directions were statistically examined.

Initial descriptive analysis for each group was conducted to investigate if either group showed preferences for the distal cues while on the platform. In the NT group, the mean head direction on the first day of training was 65.19 ± 24.0^{o} with changes in direction across training (see Table 3a.1). However, when investigated further Rayleigh Uniformity tests revealed no significant preferred head-direction for the NT group on the first four acquisition days, with a significantly preferred heading revealed on Day 5 only (see Table 3a.1). As the majority of days did not reveal that the NT animals had a significantly preferred heading direction, no further tests were conducted. For the FT group a mean head direction towards the NE of the pool of 143.86 ± 78.19^{o} on Day 1 was revealed, however there was a change in head-direction from Day 1 to Day 2 with a further shift in the angular orientation of head-direction on Days 3, 4 and 5 (see Table 3a.1). Rayleigh tests of Uniformity returned no significantly preferred head direction on any of the acquisition days for the FT group.

The data suggests that animals in the NT group did not spend the majority of their time looking towards the cues at the North of the pool during the platform interval, as was expected. Similarly, the FT group did not direct their attention to their respective cue arrangement to the South of the pool across training.

Table 3a.1: Mean head direction (± S.E.M.) during training for the NT and FT groups. (* denotes significant orientation).

	Near Trained			**Far Trained**		
Day	Mean±S.E.M.	Rayleigh Uniformity	Compass Location	Mean±S.E.M.	Rayleigh Uniformity	Compass Location
D1	65.19 ± 24.0^o	Z=2.57, p>0.05	E	143.86 ± 78.19^o	Z=0.7, p>0.05	NE
D2	334.2 ± 30.0^o	Z=1.93, p>0.05	S	32.45 ± 40.17^o	Z=0.22, p>0.05	SE
D3	173.09 ± 34.7^o	Z=0.38, p>0.05	NE	167.67 ± 35.21^o	Z=1.56, p>0.05	NE
D4	338.71 ± 43.6^o	Z=0.08, p>0.05	S	180.04 ± 32.30^o	Z=1.75, p>0.05	N
D5	224.9 ± 21.48^o	Z=2.95, p<0.05*	NW	220.16 ± 36.38^o	Z=0.45, p>0.05	NW

3a.3.3 Behavioural Analysis; Swimming behaviour

As there were few differences in head directions on the platform between the groups, we suggest that perhaps animals gain more information during the locomotion phase of the task, and propose that differences in how groups learn the task will emerge through further analysis of this period in training. For this, a number of behaviours (see Methods Section 3.2) were analysed by examining the mean time spent in a particular behaviour and the mean frequency of the behaviour at a given location in the maze.

3a.3.3.1 Thigmotaxis

From examination of video recorded tracks, and tracks produced by EthoVision providing x, y coordinate data points every 0.2 seconds of the animal in the maze, a number of behaviours were elucidated, the first including thigmotactic-like behaviours i.e. the amount of time spent at the side of the pool. The mean percentage time spent by the NT group in thigmotactic behaviour was first examined. NT animals spent 51.32±7.01% of their total time swimming in the pool on Day 1 in thigmotaxis. As

training continued they spent less time in thigmotaxis displaying a mean of 40.10±3.99%, 24.44±2.97%, 21.37± 4.96%, and 15.09±7.40%, on Days, 2, 3, 4, and 5, respectively. FT animals spent 47.61±3.67% of their time in thigmotaxis on Day 1 of training, with a mean of 40.95±5.15% on Day 2, 26.92±5.34% on Day 3, 34.26±4.96% on Day 4 and 31.84±7.4% on Day 5 (see Figure 3a.7a). A 2 x 5 mixed factorial ANOVA confirmed an overall effect for day [$F_{(4, 48)}$ = 10.97, $p<0.001$], with Bonferroni-corrected t-tests revealing an overall significant difference between Day 1 and Days 3 ($p<0.01$), 4, ($p<0.05$) and 5 ($p<0.05$). However, no group [$F_{(1, 12)}$ = 2.4, $p>0.05$] or day x group interaction [$F_{(4, 48)}$ = 1.63, $p<0.05$] was found.

Following analysis of general thigmotaxis, two types of thigmotactic behaviour emerged: vertical and parallel thigmotaxis (see Methods section 3.2). The mean percentage time spent in each of these behaviours was also examined (see Figure 3a.7). A 2 x 5 mixed factorial ANOVA examining differences in parallel thigmotaxis revealed no effect for day [$F_{(4, 48)}$ = 2.06, $p>0.05$] and no effect for group [$F_{(4, 48)}$ = 0.39, $p>0.05$]. However, there was an interaction effect between day and group [$F_{(4, 48)}$ = 3.7, $p<0.05$]. Further, individual t-tests showed the time spent in parallel thigmotaxis was significantly different between the groups on Day 3 of training only ($t_{(12)}$ = 2.51, $p<0.05$; Figure 3a.7b). The percentage time in vertical thigmotaxis was also assessed and an overall effect for day was noted [$F_{(4, 48)}$ = 21.37, $p<0.001$]. Bonferroni-adjusted t-tests revealed a higher percentage of time was spent in this behaviour on Day 1 compared to Days 2, 3, 4 or 5 (all $p<0.001$, see Figure 3a.7c). However, there was no group effect [$F_{(1, 12)}$ = 2.69, $p>0.05$] or day x group interaction noted [$F_{(4, 48)}$ = 0.41, $p>0.05$], indicating a similar level of performance between the groups on this behavioural measure.
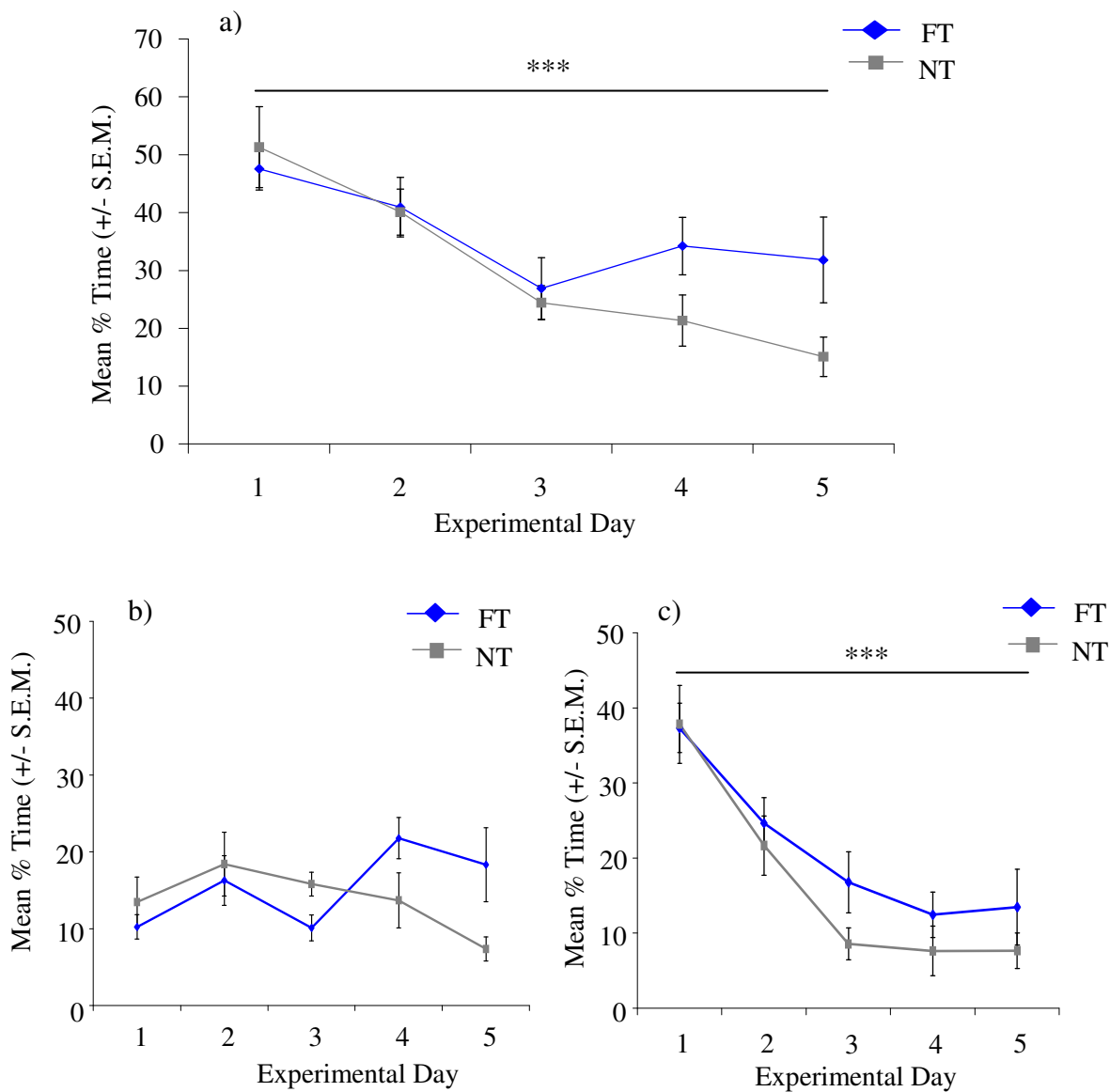
Figure 3a.7: Mean percentage time spent by the NT and FT animals in a) total thigmotaxis b) parallel thigmotaxis and c) vertical thigmotaxis.

As parallel thigmotaxis was only performed approximately 10-20 % of the time across the training period (see Figure 3a.7b), the location at which this behaviour was made was not further assessed in detail. The location around the maze, at which the animals performed vertical thigmotaxis was, however, assessed to determine if animals had a preferred position in performance of this behaviour. This was done by recording the mean number of times the behaviour was executed at a particular location. Results of this for the NT and FT groups are presented in the histogram below (see Figure 3a.8). From visual inspection of vertical thigmotaxis, further differences in where the groups searched were revealed. The NT group's searching focused between 160-230°, encompassing the NE and NW light cues on Days 1 and 2 of training, peaking at approximately 180°. Day 3 of training onwards had no specific peaks as animals became more familiar with the task, reducing the time spent in thigmotaxis overall. The FT group also focused on their respective cues with vertical thigmotactic behaviour performed at the cue card to the east of the pool and the SW light cue on Day 1, peaking at 330°. Day 2 and 3 saw a slight shift of focus towards all respective cues with peaks at 0/360° for Day 2 and 300° on Day 3. No predominant peaks were observed on Day 4 or Day 5 of training in vertical thigmotaxis for the FT group as the level of performance decreased with continued training.
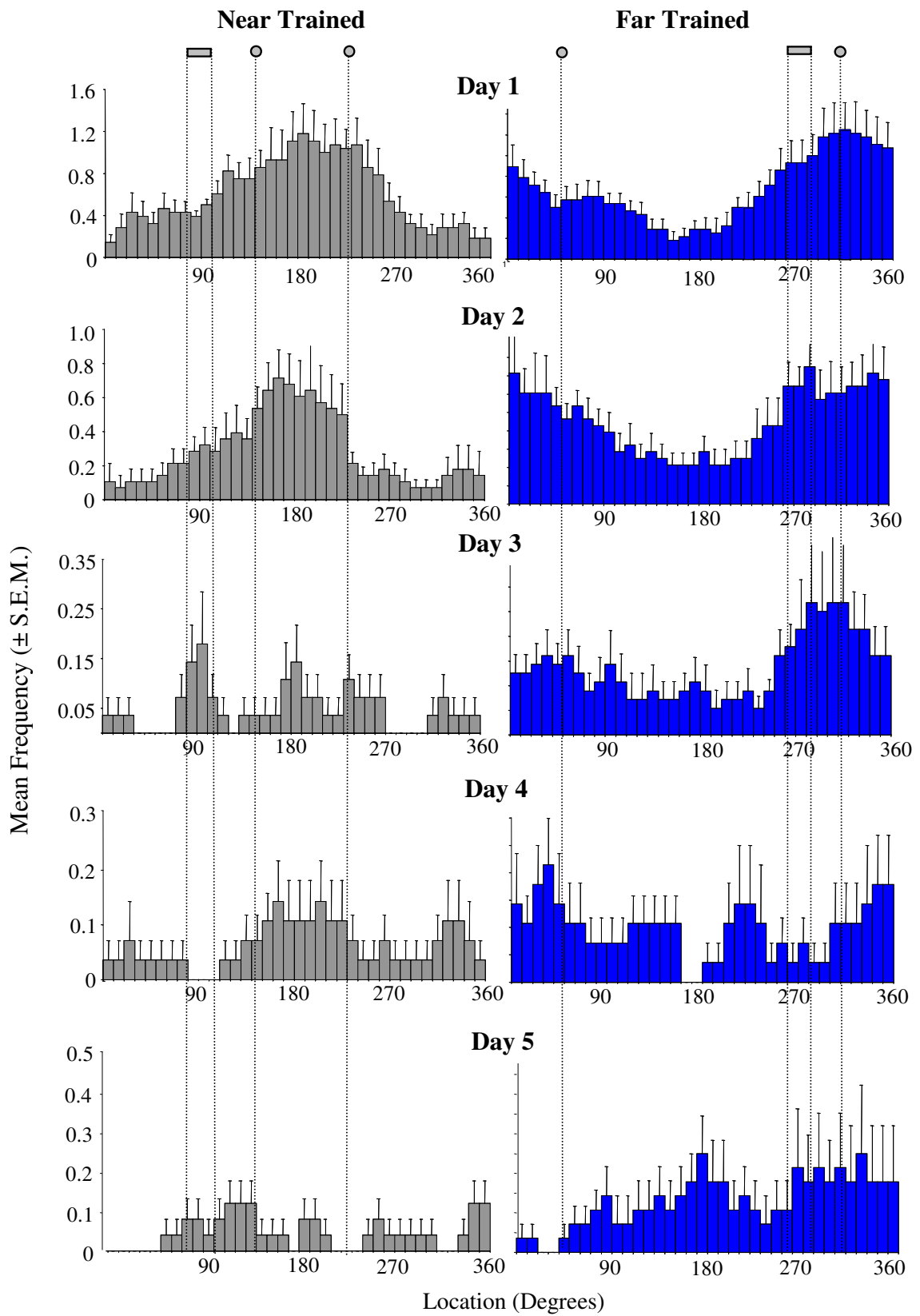
Figure 3a.8: Mean frequency (+/- S.E.M.) spent in vertical thigmotaxis behaviour at each location (degrees) around the water maze for the NT and FT groups.

3a.3.3.2 Direct

Following examination of thigmotactic behaviours, the next behaviour investigated was direct swimming, on each training day. This was defined as movement in a straight, definite direction for at least 1 second. The mean percentage time, of total time in the pool, spent in this behaviour was initially assessed. The NT group spent, on average, 22.61±3.86% of time in direct behaviour on Day 1 and the time spent performing this behaviour continued to increase across training; Day 2 M: 35.26±3.63%; Day 3 M: 38.28±2.41%; Day 4 M: 49.11±6.29%; Day 5 M: 51.96±4.33%. The FT group also appeared to spend more time in direct behaviour with continued training; Day 1, M: 23.66±1.92%; Day 2, M: 25.04±1.28%; Day 3, M: 33.11±1.32%; Day 4, M: 37.53±3.07% and Day 5: 33.78±5.55%. A 2 x 5 mixed factorial ANOVA revealed an overall effect for day [$F_{(4, 48)} = 10.54$, $p<0.001$], with Bonferroni-corrected t-tests indicating the lowest percentage time spent in direct behaviour was on Day 1 in comparison to Day 3, 4 (both $p<0.001$) and Day 5 ($p<0.05$). A group effect was also found [$F_{(1, 12)} = 12.14$, $p<0.01$], with NT animals (M: 39.44±4.1%) spending more time in this behaviour than FT animals overall (M: 30.63±2.87%; see Figure 3a.9). No interaction effect between day and group was found [$F_{(4, 48)} = 1.85$, $p>0.05$].

We also examined the direction towards which animals swam when performing direct movements. The mean number of times each group spent directly swimming in a certain direction or towards a defined region of the pool was calculated. As this behaviour was not performed as readily as thigmotaxis, the pool was divided into two segments, with one segment containing cues and the other containing no cues. The cue containing area for the NT group encompassed the region from 60-240° and for the FT

group encompassed the area from 240-60° (see Figure 3a.10). The non-cued area for the NT group encompassed the region from 240-60°, and for the FT group included the area from 60-240°. Statistical analysis revealed that the NT animals had no significant difference in their heading direction towards the cue containing region compared to the non-cue region of the pool on Day 1 (t(6) = 2.32, p>0.05). However, it was revealed that the NT animals spent significantly more time heading towards the cues than towards the non-cued area of the maze on Day 2 (t(6) = 2.32, p<0.01), Day 3 (t(6) = 4.47, p<0.01), Day 4 (t(6) = 5.62, p<0.001), and Day 5 (t(6) = 11.6, p<0.001). Similarly, FT animals' data was analysed in the same manner. Dependent samples t-tests revealed no differences between segments on any day of training [Day 1: t(6) = 1.46, p>0.05; Day 2: t(6) = 1.19, p>0.05; Day3: t(6) = 0.75, p>0.05; Day4: t(6) = 0.40, p>0.05; Day 5: t(6) = 0.77, p>0.05]. The FT animals did not appear to have any significantly preferred heading direction across training (Figure 3a.10).
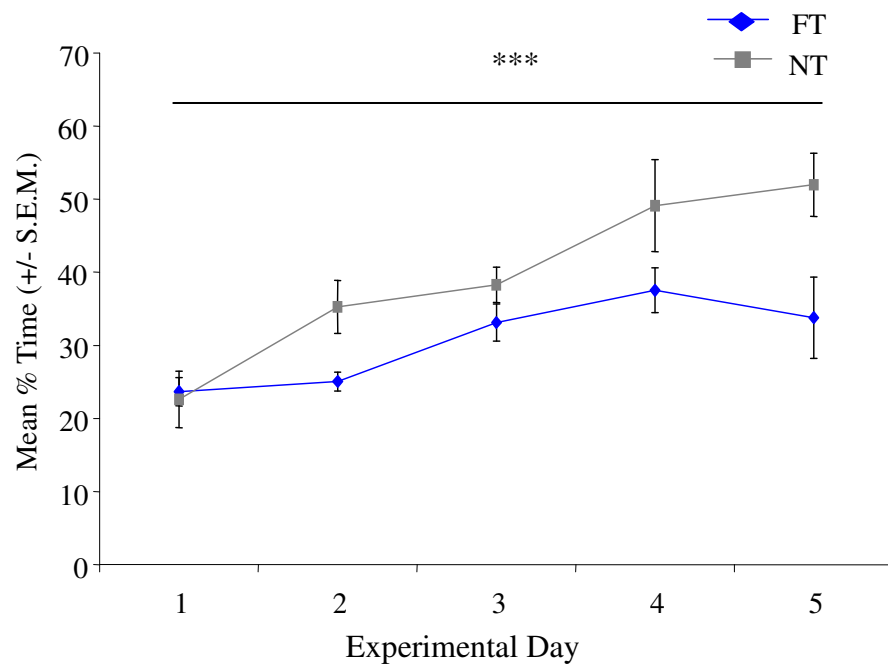
Figure 3a.9: Mean percentage time (±S.E.M.), of total time swimming, spent in direct behaviour, on all training days for the NT and FT groups.
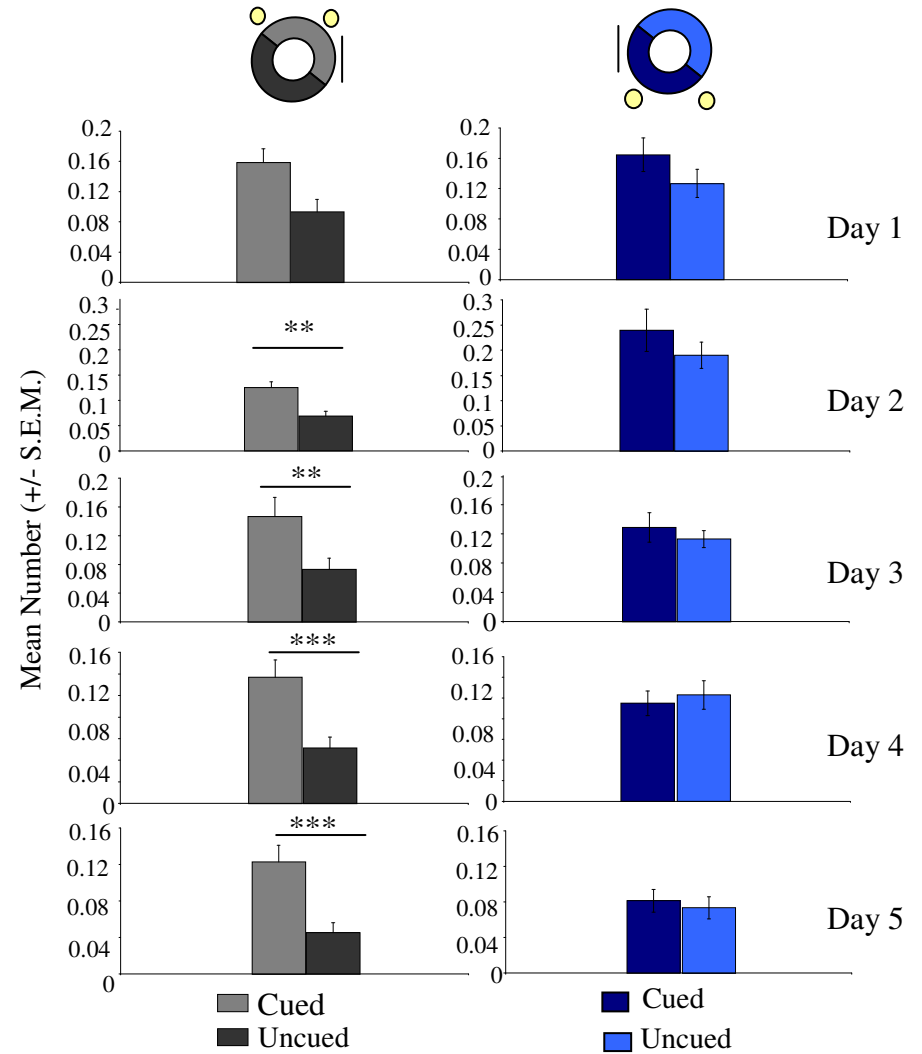
Figure 3a.10: Mean number of occurrences of direct behaviour towards the cued and uncued sections in the maze for the NT (grey) and FT (blue) groups across training days (see insert).

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3a.3.3.3 Turning Behaviour

Following examination of initial prominent swimming behaviours throughout trials, further subtle behaviours emerged and were subsequently explored in detail. The first of these were turning behaviours in the pool. For this, we examined the mean number of turns overall on each training day. We also examined the location of turns in relation to the position of the platform, including the location of turns made towards each of the distal cues (colour-coded see Figure 3a.4 in Methods Section 3a.2). We also recorded and analysed turns made away from the distal cues.

Initially, the mean number of overall turns (including both turns towards and turns-away from the cues) were examined (Figure 3a.11). A 2 x 5 mixed factorial ANOVA revealed no overall significant difference in the mean number of turns made across training days $[F(4, 48) = 2.34, p>0.05]$. However, a main effect for group was found $[F(1, 12) = 7.88, p<0.05]$, where the FT group (M: 20.97 ± 2.68) made significantly more turns overall than the NT group (M: 16.71 ± 2.34). However, no interaction effect between day and group was revealed $[F(4, 48) = 0.74, p>0.05]$. Further observation of the number of turns and turn locations can be seen in the spatial distribution diagrams (Figure 3a.12a).
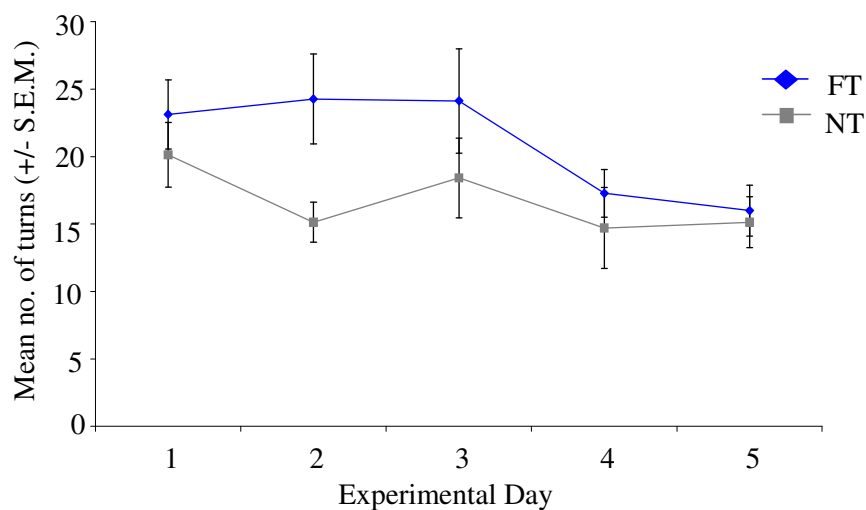
Figure 3a.11: Mean number of turns made (+/- S.E.M) by the NT and FT groups across 5 days of training.

The mean number of turns made towards the cues on each acquisition trial was then assessed for each animal, producing an overall mean number per day (see Figure 3a.12a for spatial distribution of all turns). A 2 x 5 mixed factorial ANOVA revealed no main effect for day [$F(4, 48) = 1.89$, $p>0.05$]. However, there was a main effect for group overall [$F(1, 12) = 4.91$, $p<0.05$], with the NT group performing more turns towards the cues (M: 12.17 ± 1.43) than the FT group (M: 10.17 ± 1.55; see Figure 3a.12b). However, there was no day x group interaction effect [$F(4, 48) = 1.04$, $p>0.05$]. When the distributions of turn positions were evaluated for each cue in the NT group, it was revealed that turn location for each respective cue were within defined locations and not randomly dispersed throughout the pool. In fact, Rayleigh Uniformity tests identified significantly preferred locations for turns towards each cue on all days in the NT group (see Table 3a.2). The relative stability of turn locations for each cue can be seen in the mean daily location of turns towards each cue relative to the platform (Figure 3a.13). Watson William F-tests were used to examine any change in turn location throughout training for each cue. There were few changes throughout training with significant differences in the mean orientation of turns towards the blue cue seen only between Days 1 and 5 [$F(1, 67) = 4.51$, $p<0.05$], and Days 4 and 5 [$F(1, 56) = 4.6$, $p<0.05$]. Similarly, the angular location of turns for the red cue on Day 2 was revealed to be significantly different to Day 1 [$F(1, 51) = 4.65$, $p<0.05$]. When the mean locations of turns for the green cue were examined there were no statistically significant differences in the mean location of turns across acquisition days (all $p>0.05$).

When the directional position of turns from the platform were assessed for the FT group, it was found that turn positions were generally in significantly preferred

locations. Table 3a.3 displays all Rayleigh Uniformity data on the locations of turns towards each cue throughout training. Following several Rayleigh Uniformity tests, the mean daily direction of turns for the blue and red cue were shown to be in significantly preferred directions on all days of training. However, turns for the green cue were only in significantly preferred directions on Days 1, 2 and 4 (see Table 3a.3). Overall, turn locations in the FT group were generally stable on all days.

The relative stability of turn locations for each cue can be seen in the mean daily location of turns towards each cue relative to the platform (Figure 3a.14). Watson William F-tests showed significant differences in the mean orientation for turns towards the blue cue with Day 1 being significantly different to Days 3 [$F_{(1, 64)}$ = 8.08, $p<0.01$], 4 [$F_{(1, 49)}$ = 4.11, $p<0.05$] and 5 [$F_{(1, 52)}$ = 15.58, $p<0.01$]. Similarly, Day 2 was significantly different to Day 3 [$F_{(1, 68)}$ = 4.32, $p<0.05$] and Day 5 [$F_{(1, 56)}$ = 10.27, $p<0.01$]. The angular location of turns for the red cue on Day 1 was significantly different to Day 3 [$F_{(1, 54)}$ = 5.48, $p<0.05$], Day 4 [$F_{(1, 52)}$ = 43.58, $p<0.001$] and Day 5 [$F_{(1, 49)}$ = 36.77, $p<0.001$]. Day 2 preferred direction was significantly different to Day 4 [$F_{(1, 44)}$ = 20.08, $p<0.001$], and Day 5 [$F_{(1, 41)}$ = 19.22, $p<0.001$]. In addition, the mean preferred angular location on Day 3 was different to Day 4 [$F_{(1, 42)}$ = 28.32, $p<0.001$] and Day 5 [$F_{(1, 39)}$ = 22.50, $p<0.001$]. The greater change in position between days for the FT group may reflect the overall slower rate of acquisition, as reported by basic acquisition measures, with the changes in turn positions across days further suggesting a refinement in searching as the task and goal location becomes more familiar.
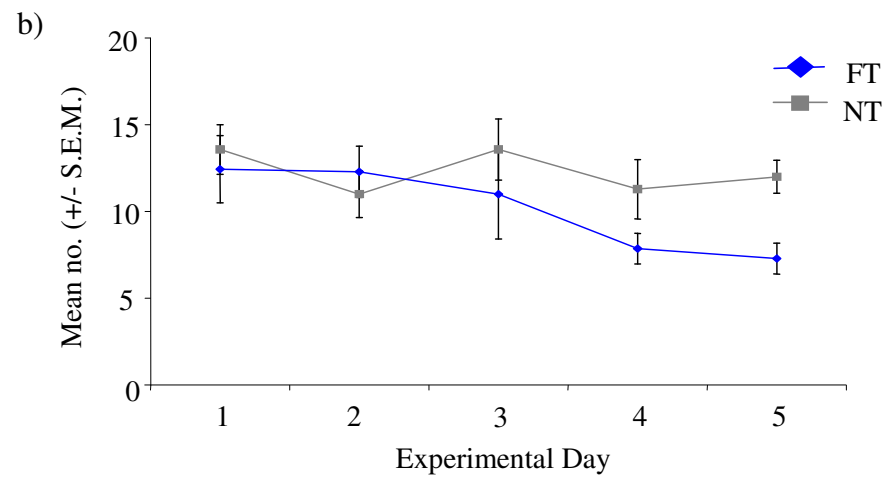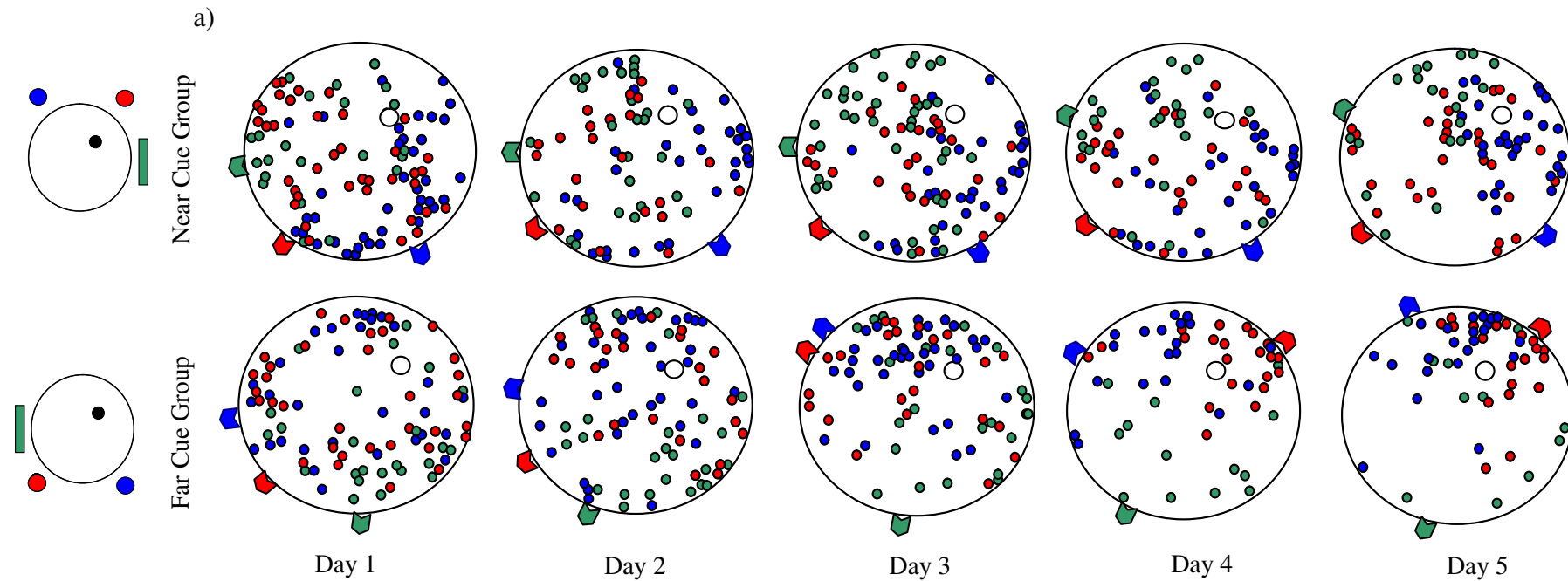
Figure 3a.12: a) Location of turns made towards the cues (colour coded) on all experimental days. Mean location of turns towards specific cues is denoted by corresponding coloured block arrows. Insert shows the location of cues around the maze. Mean location of turns towards specific cues is denoted by corresponding coloured block arrows b) Mean number of turns made towards the cues (+/- S.E.M) in the NT and FT group across 5 days of training.
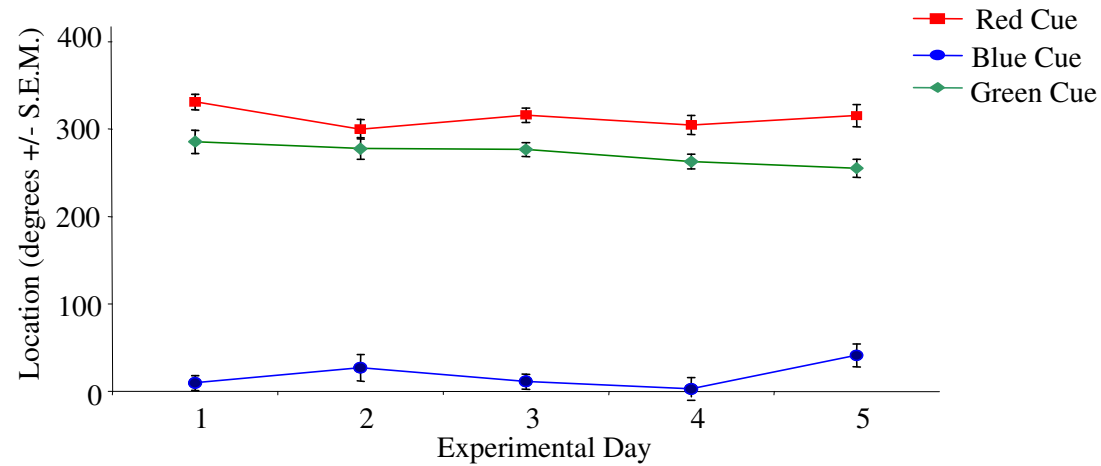
Figure 3a.13: Location of turns (degrees +/- S.E.M.) towards the cues in the NT group.

Table 3a.2: Rayleigh Uniformity test results of the mean position of turns towards each cue over 5 days, for the NT group.

Near Trained Group

Cue	Day 1			Day 2			Day 3			Day 4			Day 5		
	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green
Mean (degrees)	9.47	331	285.54	27.08	299.9	277.8	11.05	316.02	276.5	2.78	304.6	263.1	41.17	315.5	255.2
Z Score	17.18	14.95	7.43	6.26	9.82	8.74	15.2	16.37	17.9	8.22	9.99	13.4	8.22	8.55	10.3
p	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
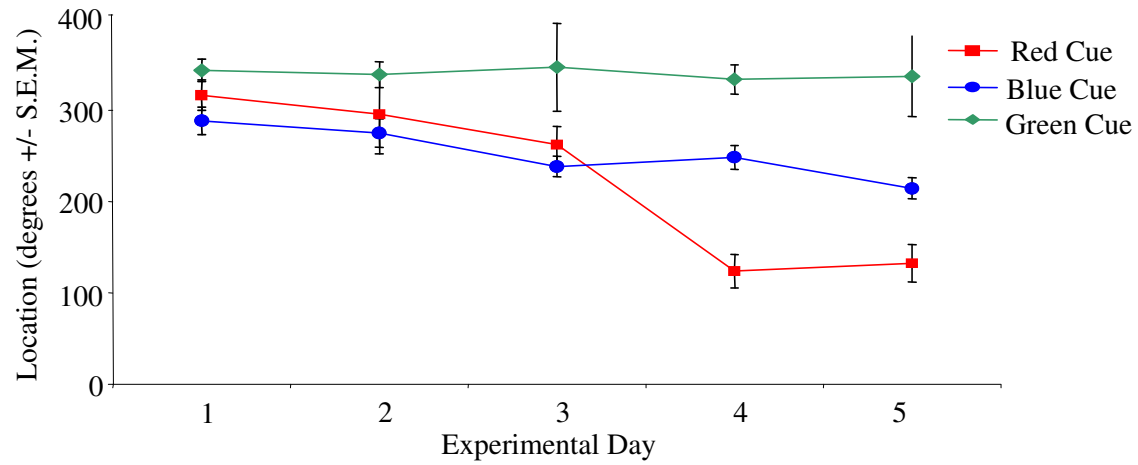
Figure 3a.14: Location of turns (degrees +/- S.E.M.) towards the cues in the FT group.

Table 3a.3: Rayleigh Uniformity results of the mean position of turns towards each cue for the FT group.

Far Trained Group

Cue	Day 1			Day 2			Day 3			Day 4			Day 5		
	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green
Mean (degrees)	288.5	316.4	343.6	275.2	295.8	339.2	238.4	262.4	347.03	248.5	124	333.9	214.6	132.6	337.1
Z Score	6.58	5.43	8.79	6.12	0.86	6.92	10.7	3.69	0.69	7.42	4.43	5.17	9.44	3.5	1.25
p	***	***	***	**	-	***	***	*	-	***	**	**	***	*	-

3a.3.3.4 Turns-Away

We also examined turns-away from the cues (colour-coded black). The overall daily
mean number of turns-away was initially compared in each group (Figure 3a.15). A 2
x 5 ANOVA revealed no overall significant difference across days [$F_{(4, 48)} = 2.01$,
$p>0.05$]. However, a main effect for group was found [$F_{(1, 12)} = 67.46$, $p<0.001$],
where the FT group (M: 10.8±1.33) made significantly more turns-away from the cues
than the NT group (M: 4.54±1.04; see Figure 3a.15b). There was no interaction effect
between day and group found [$F_{(4, 48)} = 0.91$, $p>0.05$]. Rayleigh Uniformity tests
examining the location of turns-away in the NT group revealed a significantly
preferred direction on Day 1 only (see Table 3a.4). Watson-William F-tests examining
the differences in the mean orientation of turns-away across training, for the NT group,
revealed significant differences between Day 1 and Days 4 and 5 [$F_{(1, 70)} = 25.96$,
$p<0.001$; $F_{(1, 64)} = 6.84$, $p<0.05$, respectively], Day 2 and Days 3 and 4 [$F_{(1, 63)} =
7.58$, $p<0.01$; $F_{(1, 57)} = 12.56$, $p<0.001$, respectively], and between Day 3 and Days 4
and 5 [$F_{(1, 62)} = 18.92$, $p<0.001$; $F_{(1, 56)} = 12.75$, $p<0.001$, respectively]. The mean
location of turns-away from the cues for the FT group revealed significiantly preferred
turn locations on all days for this group (see Table 3a.4) perhaps indicating a greater
reliance on this behaviour than the NT animals. Watson William F-tests returned no
differences in turn locations across days for this group (all $p>0.05$).

Table 3a.4: Mean turn away locations (± S.E.M.) during training for the NT and FT groups

	Near Trained			**Far Trained**		
Day	Mean±S.E.M.	Rayleigh Uniformity	Compass Location	Mean±S.E.M.	Rayleigh Uniformity	Compass Location
Day1	326.18±13.8°	Z=7.74,p<0.001	SW	337.09±8.17°	Z=21.04, p<0.001	S
Day2	282.41±36.84°	Z=1.18, p>0.05	W	328.18±9.18°	Z= 17.41, p<0.001	SW
Day3	13.82±54.49°	Z=0.55,p>0.05	SE	338.33±8.64°	Z=19.54, p<0.001	S
Day4	167.93±99.94°	Z=1.34,p>0.05	NE	335.28±10.05°	Z=14.37, p<0.001	S
Day5	238.31±53.35°	Z=0.57,p>0.05	NW	337.52±17.22°	Z=5.29, p<0.01	S
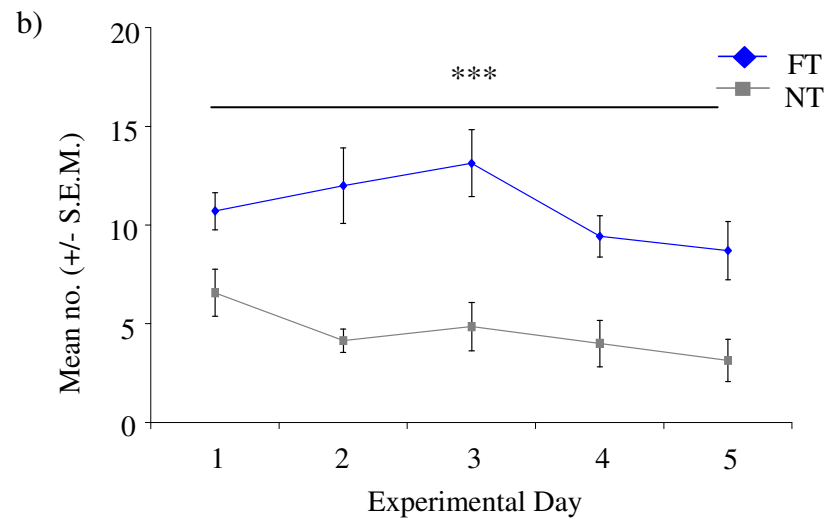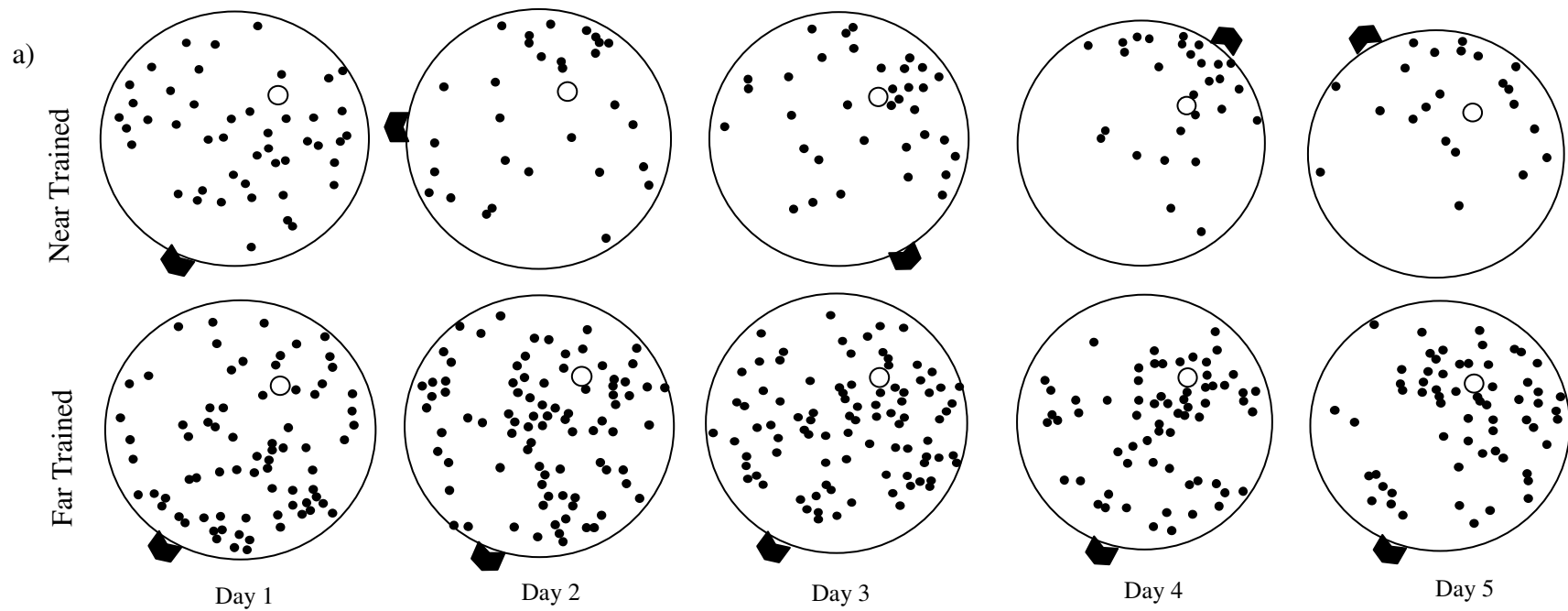
Figure 3a.15: a) Spatial representation of location of all turns-away from the cues for the NT and FT groups across experimental days. Mean location is denoted by black block arrow. b) Mean number of turns-away made across training for the NT and FT groups.

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3a.3.3.5 Turns in Zones

Finally, we examined whether animals were making turns closer to the platfrom as training progressed. For this, the mean number of turns for the NT group in each zone of the maze was analysed (Figure 3a.16a). A 5 x 3 repeated measures ANOVA, examining turn location, revealed no significant main effect for day [$F(4, 24) = 1.07$, $p>0.05$], however a significant main effect for zone was revealed [$F(2, 12) = 20.33$, $p<0.001$] where the overall mean number of turns in the far zone was significantly higher (M: 8.17 ± 1.27) than the middle (M: 5.2 ± 1.21, $p<0.01$) and near zones (M: 4.15 ± 1.11, $p<0.01$). A significant interaction effect between day and zone [$F(8, 48) = 3.30$, $p<0.01$] was also found. Further analysis using daily one-way repeated measures ANOVAs revealed a significant effect for zone on Day 1 [$F(2, 12) = 15.23$, $p<0.001$] with significant differences between the near and far ($p<0.001$), and the middle and far zones ($p<0.05$), following Bonferroni corrected t-tests. By Day 5, however, there were no differences between the zones [$F(2, 12) = 0.24$, $p>0.05$], with a reduction of turns in the far zone and an increase in turns in the near zone (see Figure 3a.16a).

The mean number of turns made in each zone for the FT group was also assessed using a 5 x 3 repeated measures ANOVA. Overall, no main effect for day was noted [$F(4, 24) = 2.54$, $p>0.05$]. However, a main effect for zone was found [$F(2, 12) = 66.31$, $p<0.001$] with subsequent Bonferroni-corrected t-tests illustrating the highest mean number of turns were in the far zone, which was significantly different to both the near ($p<0.001$) and middle ($p<0.01$) zones overall (Figure 3.16b). In addition, an interaction effect between zone and day was also noted ($F(8, 48) = 18.95$, $p<0.05$). When examined in more detail using daily one-way repeated measures ANOVAs a significant difference between zones on Day 1 [$F(2, 12) = 20.15$, $p<0.001$] was

revealed, with a significantly higher number of turns made in the far zone than in either the near ($p<0.01$) or middle zones ($p<0.05$). Analysis on Day 5 revealed similar results [$F(2, 12) = 9.16$, $p<0.01$], with significant differences between the near and far ($p<0.01$) and middle and far zones ($p<0.01$), with the highest number of turns remaining in the far zone even by the last day of training (see Figure 3a.16b).
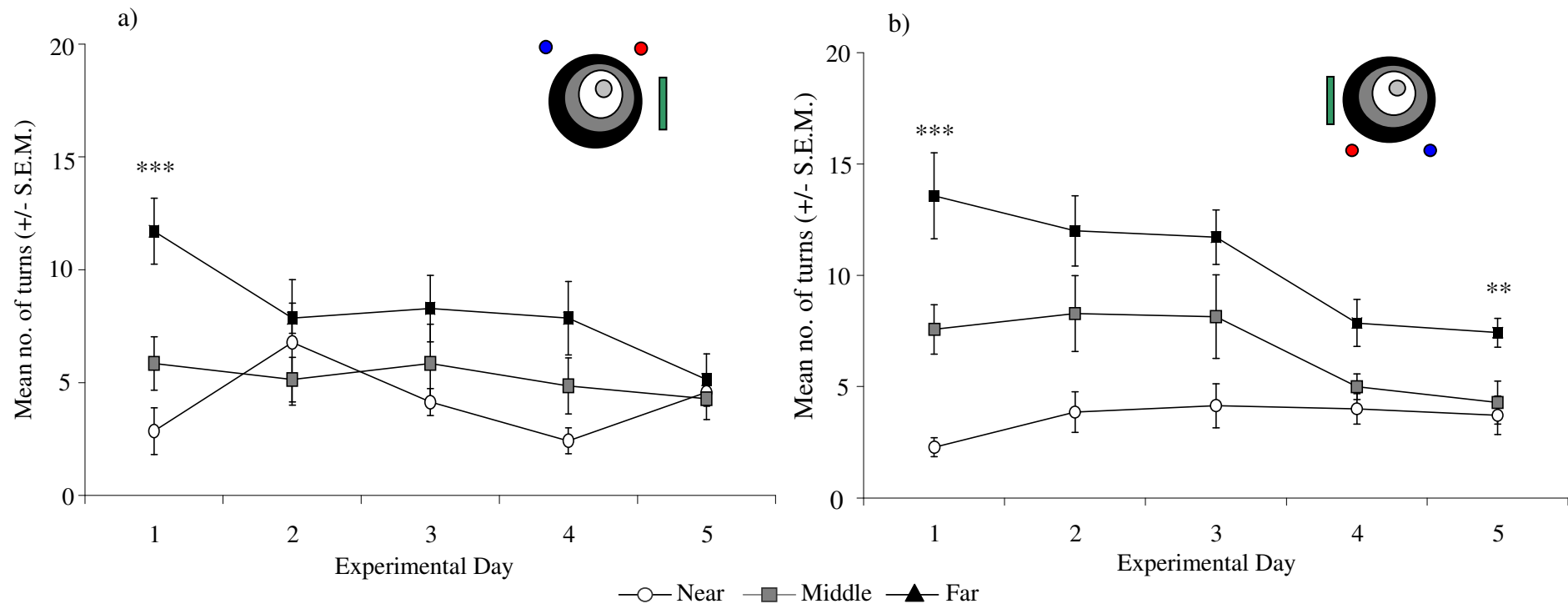
Figure 3a.16: Mean number (± S.E.M.) of turns in the near, middle and far zones for a) the NT and b) FT groups over 5 days of training. Inset; schematic representation of the three zones used for analysis.

3a.3.4 Summary

Analysis of the data indicates that the animals use a number of swimming behaviours in combination to solve the task. In particular, they initially avail of thigmotactic behaviour, swimming at the side of the pool, particularly at the cues. Later in training they appear to use more direct behaviour followed by turns to accurately locate the platform. The NT group perform more direct behaviours, which are focused on the cues and are carried out for a longer percentage of time in the pool. Complimenting this, they also perform less turns-away from the cues suggesting a direct reliance on the cues throughout training. Critically, it was also noted that NT animals perform increasingly more turns closer to the platform as training progressed illustrating increased and accurate learning of the goal position throughout the training period. The FT group also performed similar turns closer to the goal throughout training, however this alteration in behaviour did not reach the same level as the NT group, with a higher number of turns remaining in the outer area of the pool, suggesting a slower and less accurate pattern of searching. In addition, the FT group spend less time performing direct behaviours which are also less focused, and not directed towards the distal cues. Alongside this, they also performed more turns-away from the cues than the NT group. Together these findings suggest that the FT animals, while performing some cue-focused behaviours must also move away from the cues to successfully locate their goal.

3a.4 Discussion

The aim of this Chapter was to assess the effect of distal cue positioning on acquisition of a place version of the MWM. Studies have demonstrated that the location of cues will have an impact on an animal's ability to learn the task (Chamizo & Rodrigo, 2004; Chamizo et al., 2006), with some suggesting that different strategies may be employed when the position of the cues are altered by distance (Cook & Tauro, 1999; Tamara et al., 2010a). Here we attempted to determine if different strategies were used by each group of animals by examining the animals' swimming tracks in each individual training trial.

Basic measures of analysis revealed overall differences in learning between the groups with the NT group learning the task more rapidly than the FT group. This result is similar to that demonstrated by Chamizo and Rodrigo (2004) who showed that when an available distal cue is in a position far from the goal, the animals are slower at learning the task than when the cue is located nearer the goal. From this, they propose that closer landmarks have better control in guiding the animal than more distant landmarks as they use it in a beacon-like manner. A similar level of control has also been seen to aid accurate searching in navigating insects when local landmarks are available (Graham & Collett, 2002). Chamizo and Rodrigo (2004), however, had only one cue available to the navigating animals, so it is difficult to fully extrapolate their findings to ours. However, it has been suggested that the salience of a landmark within a configuration of landmarks, also depends on its location to the goal. Therefore, when a number of external cues are available, they may compete among themselves, with the closest cue to a goal, overshadowing the other available cues (Rescorla, 1976). The examination of escape latencies alone, however, merely suggests that the animals are slower at learning when

cues are far away from the goal but does not indicate if this is due to different information being acquired or distinct strategies being used. Rather, as it has been proposed that only through extensive examination can subtle navigation processes be understood in the water maze (Cain, 1998), we next investigated the swimming tracks of the animals to determine the strategies employed when acquiring the task.

Firstly, to establish if different cue conditions led to alterations in animals' exploration of their environment, we first examined the platform behaviour of the animals. Head scanning on the platform was initially inspected as it has been established as being an important instinctual behaviour for exploring animals (Gharbawie et al., 2004; Petrosini et al., 2003), and it is also during the platform interval that the animal has ample time to examine its surroundings, including the distal cues, from the goal position (Keith & Mc Vety, 1988). This interval has also been recognised as an incorporated method used by insects to remember a goal location in a large environment which is done by taking and remembering views of the surrounding environment from the goal position (Akesson & Wehner, 2002; Cartwright & Collett, 1983). Assessment of the platform interval in the current study revealed that there were no differences between the groups' mean daily head movements while on the platform, indicating that they took in similar views from the surrounding environment. However, when the mean head movement within trials was investigated, it revealed the highest range occurred for both groups on Day 1 of training, which would reflect the highest level of curiosity of the new environment. Indeed by Day 4 there was a decrease in head movement while on the platform with no differences in the range across trials on Day 5 for either the NT or FT group. These results make intuitive sense, as when first placed in a novel situation, there would be an increase in the drive to

explore, leading to searching behaviour (Montgomery & Monkman, 1955), with later familiarity of the environment resulting in a decrease in an animal's need to observe their environment. A similar finding was also reported by B. Clark et al. (2005) with animal head movements in an open field task decreasing by Day 4 of exposure to the environment. Furthermore, the direction in which animals from both groups looked did not differ and neither group showed a preference for looking at (or at least facing) specific distal cues.

Although the platform behaviour of both groups indicates it is used as an initial point to observe the cues and surroundings in relation to the platform, it does not reveal any differences in how the NT and FT animals primarily use the external stimuli. Therefore, the processing of the distal visual cues during the swimming phase of the task was subsequently examined. The first, most prominent, behaviour to emerge from this was thigmotactic behaviour. Although it has previously been suggested as being a response behaviour in situations of anxiety (Barnett, 1963; Devan et al, 1999; Mendez et al., 2008), Jeanson et al. (2003) previously highlighted the presence of non-random patterns of thigmotactic movement in the cockroach at the edge of an arena. It has also been found that animals can orient themselves by making physical contact with the border of an environment (Creed & Miller, 1990), with Lipp and Wolfer (1998) similarly arguing that thigmotaxis is driven by instinct rather than anxiety and that it has also evolved as an escape response from water. Thigmotactic behaviour in the rodent, therefore, may be performed in a systematic pattern and as such be useful to the navigating animal, particularly at the beginning of training; this appears to be the case in our findings. Overall there was a general reduction in thigmotaxis throughout the training period, as would be

expected with both familiarity and more effective strategies emerging to solve the task. However, the FT group did not appear to reduce their time spent in this behaviour to the same extent as the NT group, possibly indicating an initial alteration in search patterns between the groups, however this difference did not reach statistical significance. Interestingly, when thigmotaxis was examined in more depth, two specific types of the behaviour emerged. Parallel thigmotaxis, firstly, appeared to be performed at most points around the maze and by both groups to a similar level. The more interesting of these swimming behaviours, however, was vertical thigmotaxis. Here, specific patterns emerged with peaks of this behaviour at the respective cue locations for both groups, suggesting the behaviour is being used initially in a visually-guided, egocentric manner to orient in the pool and familiarise themselves with the external information. The occurrence of this cue-focused behaviour may indicate the development and early understanding of a cue-escape association, however, the accurate location of this escape has not yet been established at these early stages and is likely not possible from this behaviour alone. Graham and Collett (2002) also posited that such direct use and attention towards the distal cues can facilitate in the initial acquisition of a new route.

Conversely, NT animals spent more time in direct behaviour than the FT group. The NT group also had preferred heading directions on all days with the focus of the behaviour towards the distal cues, whereas the FT group spent less time in direct behaviour with heading directions dispersed evenly across the pool. This type of direct behaviour has been suggested as being an egocentric, view-dependent, behaviour with animals heading in a specific direction, often in relation to a cue or beacon (Graziano et al., 2003; Harvey et al., 2008). Collett (2010) posits that animals learn a set of heading

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directions, a type of behaviour seen in insects, where they focus their view to particular cues and later will reorient themselves, matching their current image to the earlier retinal positions when the environment was first encountered. Here, it appears that as NT animals gain knowledge of the importance of the cues they begin to perform more direct behaviours, with this becoming specifically directed towards the cues as training progresses and the importance of the cues is comprehended. Therefore, using this behaviour animals may learn to associate views of the distal cues directly with rewarded motor actions (i.e. move towards cues to reach goal; Sheynikhovich et al., 2009). The NT animals appear to use their cues in this manner as they are relying on immediate visual information to navigate and locate their goal (Fey et al., 2011).

In addition, it may be more cognitively efficient for the NT group to use near cues directly to locate the goal in a beacon-like fashion, rather than building up relations between all available cues (Collett, 2010). D. Harvey et al. (2009) noted that under a reduced cue arrangement, animals displayed more direct behaviours towards the available cue than animals with multiple cues. This type of direct cue use has been shown to facilitate learning in rodents and insects and results in more accurate searching in spatial tasks (Graham & Collett, 2002; Hines & Whishaw, 2005; Pearce et al., 2001). Consequently, animals using direct behaviour merely need to remember the correct cue(s) to approach in order to make contact with the platform. This method would not be reliable for animals in the FT group, however, as the goal is not in a direct line to the cues. If the FT animals were to rely on this method to locate the goal they would, more often than not, miss the goal, as accuracy would be impaired as a result of the increased distance of the goal from the cue, supporting our findings here (Chamizo & Rodrigo, 2004; Spetch &

Wilkie, 1994). Therefore, they must rely on other methods to locate their goal, which we suggest may be through the use of turning behaviour away from the cues.

So while exploration of the peripheral areas of the maze initially, evidenced in thigmotactic behaviour, along with a shift to more goal-directed swimming, indicates improved learning of the task, these behaviours alone do not reveal key differences in the strategies being used; rather they are only indicators of emerging differences between the groups. Here we suggest that turning behaviour, rather, may signify increased knowledge and understanding of the layout of an environment (Collett, 2010; Harvey et al., 2008; Tchernichovski et al., 1998). Interestingly, the FT group made more turns on the whole in training. When looked at more carefully, however, turns made towards specific cues were performed more by the NT group. We suggest that this behaviour is representative of the animals need to turn towards the cues to orient themselves in the pool. This is often seen in insect navigation where, as they are familiarising themselves within an environment, they include more turns in their searching in order to locate the goal (Collett, 2010). This may be interpreted as being a more view-independent, allocentric strategy (Harvey et al., 2008), however, this behaviour remains reliant on direct use of the external cues individually, rather than as a spatial array whose relationship to one another is of significance. As training progressed, the NT animals turn positions shifted from outer segments of the pool to the area closest to the platform, indicating a refinement in the animals' learning as they search in closer vicinity to the goal. Korz (2006) demonstrated a similar finding, suggesting a preference for central parts of the maze indicates a more strategic swimming pattern. Similarly, Brudzynski and Krol (1997) state that animals will perform more turns when in a familiar area. The FT group, however, performed the lowest

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number of turns towards the cues on Day 5 when compared with the NT group, and the majority of these turns remained in the outer area of the maze, being performed in the far zone.

While useful for familiarisation of an environment, the above cue-focused behaviours are not an optimal method for creating an allocentric map-like representation of an environment (Kubie & Fenton, 2009). However, turns-away from the cues may suggest a more allocentric strategy being used as the animals can not solely avail of direct contact with individual cues and instead must associate the cues with the hidden goal in order to complete the task. By avoiding and turning away from the cues, the animals illustrate their overall knowledge of the environment and their ability to infer the location of the goal without making direct reference to the cues. As suggested by Harvey et al. (2008) this strategy represents the animals need to confirm their position based on the cues spatial relations, whereby information gained from turning towards and then away from the cues is used to subsequently reorient in the pool. We propose that this highlights the FT groups' ability to deduce both direction and distance information from the distal cues enabling them to locate a hidden goal without direct use of the distal cues. In addition, as the FT animals must move away from direct cue use, this may clarify the longer time taken by them to locate the goal. Fey et al.'s (2011) computational model of rat behaviour also supports this idea, and demonstrates that when cues are in a position on the opposite side of the pool to the platform, it is more difficult to learn the task relying on a cue based strategy.

From the observed behaviours it appears that both the NT and FT groups employ a view-dependent, cue-guided strategy for much of the training period in the water maze

and it is not until later days that more viewer-independent inferring movements begin to fully emerge. This supports Burgess (2006) suggestion that many trials are required in order for the accurate location of a hidden platform to be remembered and that it is often not until late in training that an allocentric-based representation becomes apparent. Our results also somewhat reflect Redish's (1999) suggestion that animals navigating in a maze with cues can avail of a number of behaviours, the first two of which are egocentrically based and the last a more allocentric oriented behaviour. These are taxon movements where the animal moves towards a specific cue (comparable to our thigmotaxic and direct behaviours), route where the animal associates direction with each sensory view it obtains (as seen in our turning towards cues), and finally locale navigation where the animal learns a map on which the location of the goal is located (somewhat evidenced in our turning away behaviours; Redish, 1999; Leggio et al., 2003). We say 'somewhat' here, as inferring movements are never presented alone, with cue-directed behaviours also being performed within the same trial. Therefore, although, thought to be a viewer-independent (allocentric) task, the MWM may be solved using more viewer-dependent behaviours than once thought. Overall, our behavioural analysis provides information regarding subtle differences in how animals solve the water maze under different cue conditions that cannot be discriminated by parameters such as escape latencies. In addition, our findings also highlight the importance of taking cue location into consideration when examining the strategies used by navigating animals in the MWM.

Chapter 3b

Learning the Morris water maze; the effect of distal cue location on hippocampal BDNF expression.

Abstract

One brain structure involved in allocentric learning and thought to be particularly involved in solving the place version of the water maze is the hippocampus (Morris, 1981; O'Keefe and Nadel, 1978). A neurotrophin found in high abundance in this structure is brain-derived neurotrophic factor (BDNF). BDNF has been generally implicated in synaptic plasticity and spatial learning and memory and, as a result has been considered a useful marker for the examination of hippocampal activation during tasks such as the Morris water maze. Behavioural analysis from Chapter 3a suggests that when the cues are located in a position that is far away from the goal, the animals will rely on a more view-independent, allocentric searching strategy, whereas when cues are located in a position near the platform, the animals tend to use the cues in an egocentric, cue-directed manner. To identify whether the hippocampus is differentially involved in water maze learning under near and far cue conditions, we examined dorsal hippocampal BDNF expression following five days of training in the water maze under the respective cue conditions. In addition, as exercise is known to augment BDNF levels, two exercise yoked-control groups were also included in this study. Results indicate a higher expression of BDNF in the Far trained group when compared to the Near trained group, an increase not observed between the yoked-control groups. These findings lend support to our behavioural findings that when cues are located at a distance away from the goal, animals must infer more to locate the platform position and so use a viewer-independent, inferring strategy.

3b.1 Introduction

As evidenced in Chapter 3a, cue location has an effect on how the animal searches in the maze and subsequently solves the task. We suggest that the differences in behaviour between the groups indicate the use of different strategies in the maze and propose that the Far trained (FT) animals rely on a more view-independent, allocentric strategy to deduce the platform location, whereas the Near trained (NT) group can use the external cues more directly to orient themselves. However, beyond behavioural examination, investigations of the neural and molecular mechanisms involved in the formation of memories can also be informative to our understanding of how spatial tasks are learned (McGaugh & Izquierdo 2000). Lesion studies, for example, have made a significant contribution to understanding the neural underpinnings of learning (Eichenbaum et al., 1992; Winocur et al., 2010; Zhang et al., 2004; Zheng et al., 2003), with results from such studies leading to the suggestion that there is a complete dissociation of allocentric and egocentric strategies in the brain (O'Keefe & Nadel, 1978; Whishaw & Jarrard, 1996). Specifically, allocentric navigation is thought to be dependent on the hippocampus (Alverhne et al., 2008; Morris et al., 1982), whereas more egocentric systems are dependent on the caudate nucleus and striatum (Cook & Kesner, 1988; Packard & McGaugh, 1996).

Investigation of molecular activation in different brain regions has also been informative in understanding general learning and memory mechanisms (McGaugh & Izquierdo, 2000; Chang & Gold, 2003a, b). Neurotrophins, in particular, are essential for neuronal growth, differentiation, maintenance and survival in the central and peripheral nervous systems and have also been implicated in the process of learning and remembering (Leibrock et al., 1989; Tyler et al., 2001; Yamada et al., 2002). In

particular, brain derived neurotrophic factor (BDNF), a member of the neurotrophins family, is an important molecular marker in learning and memory and has been implicated in a range of learning and memory paradigms in mice (Horger et al., 1999), zebra finch (Wade, 2000) and chicks (Johnston & Rose, 2001). There is also much evidence to support its role in synaptic plasticity and long-term memory processes (Bekinschtein et al., 2007; Figurov et al., 1996; Lynch et al., 2007; Poo, 2001). While, it is one of the most widely distributed neurotrophins in the brain (Hofer et al., 1990), it is of particular interest in the hippocampus as this region has the highest expression of BDNF and its receptor tropomyosin receptor kinase B (TrkB), compared to other brain regions (Chao, 1992; Murer et al. 2001; Schmidt-Kastner et al., 1996; Yamada et al., 2002).

Adding to this, BDNF has also been strongly implicated in hippocampal-dependent spatial learning (Hall et al., 2000; Kesslak et al., 1998; Mizuno et al., 2000; Yamada et al., 2002). Harvey et al. (2008), for example, noted increased expression of BDNF in a group of animals trained in the place version of the MWM over that of exercise controls. The same authors also showed that continued training in the maze subsequently led to a higher expression of BDNF in the hippocampus but not in the entorhinal cortex. Similarly, elevated levels of BDNF mRNA have been shown in the hippocampus but not other structures including the cerebellum, striatum or neocortex following training in the MWM (Kesslak et al., 1998). Furthermore, when BDNF was genetically knocked-out in mice, it was subsequently shown that these mice displayed impaired long-term potentiation (LTP) and had poorer performance levels in spatial tasks when compared to wild-type, control mice (Korte et al., 1995). Conversely, enhancement of BDNF expression, by administering a single intra-hippocampal BDNF

injection, has been shown to result in significantly better performances in the place MWM over control animals (Cirulli et al., 2004; Falkenberg et al., 1992), again highlighting BDNF involvement in learning and memory.

However, the examination of BDNF as a marker of learning during spatial tasks needs careful control and interpretation as physical movement and exercise alone have been shown to result in a number of molecular changes, such as increased neurogenesis, and enhanced synaptic plasticity in the hippocampus (Adlard et al., 2004; Albeck et al., 2006; Ding et al., 2006; Vaynman et al., 2003). Specifically, exercise has been linked to increasing activation in the BDNF-TrkB signalling pathway (Knaepen et al., 2010; Widenfalk et al., 1999; Wu et al., 2011), with a number of authors noting increased levels of BDNF following exercise in both spatial and non-spatial tasks (Griffin et al., 2009; Hopkins & Buccie, 2010; Vaynman et al., 2003). Berchtold et al. (2010), for example, showed that mice that had been placed on an exercise regime prior to training in a radial-arm water maze, showed both improved performance and increased hippocampal BDNF when compared to sedentary animals. Equally, Griffin et al. (2009) demonstrated that 1 week of exercise prior to training increased performance in an object displacement task, which was associated with a concurrent increase in hippocampal BDNF expression relating to the level of exercise. Therefore, physical activity alone, such as swimming, is sufficient to increase BDNF levels in the hippocampus (Cotman & Berchtold, 2002; Neeper et al., 1995).

As BDNF is expressed rapidly during or soon after learning (Bekinschtein et al., 2007), it makes it an ideal marker for the investigation of hippocampal activation during spatial learning in the MWM. Therefore, to ascertain if the behavioural changes observed between the NT and FT groups in the previous section are reflected in the

brain, we looked at levels of BDNF in the dorsal hippocampus following training in the MWM. We specifically looked at the dorsal hippocampus as it has been implicated in spatial learning and memory processing over the ventral hippocampus (Bannerman et al., 1999, 2004; Kjelstrup et al., 2002; McHugh et al., 2004; Pothuizen et al., 2004). We suggest that as the FT animals displayed more view-independent, allocentric, inferring behaviours to locate their goal, they may be using a more hippocampal-dependent learning mechanism and therefore predict that animals in the FT group will have higher levels of dorsal hippocampal BDNF expression when compared to the NT group. We also examined BDNF levels of two yoked-exercise control groups to determine the relative effect of spatial learning and exercise on BDNF activation.

In addition to this, we noted specific attention towards the cues in all animals' performances of thigmotaxis in Chapter 3a, with both the NT and FT groups swimming at the cues. A question that may arise from these findings is whether animals are naturally attracted to the light of the cues or if they are indeed using them to find the platform, as we suggested in the previous section. Having yoked-controls (primarily to examine BDNF) gives us an ideal opportunity to further examine this and determine whether animals will still swim at the cues despite not being able, or required, to learn the task. Thus, our aims in this section are three-fold. First, we aim to examine whether the FT group show greater BDNF expression than the NT group. Second, to examine whether learning groups show greater BDNF expression than exercise yoked-controls, and third, whether the exercise group are instinctively attracted to the cues, despite having no need to learn an escape from the maze.

3b.2 Method

3b.2.1 Subjects

Male Wistar rats (n=14) that served as subjects in Chapter 3a were used for BDNF analysis in this experiment. In addition, a further 10 male Wistar rats were used as exercise controls. These were treated and housed in similar conditions to those described previously (see Chapter 2, section 2.2.1).

3b.2.2 Apparatus

The Morris water maze (MWM) paradigm as described (Chapter 3a) was again used in this experiment. All settings and experimental protocols were identical to those in the previous chapter. The distal cue configurations for the Near trained (NT) and Far trained (FT) animals were located in the same respective positions around the maze as previously described. Cue location for both yoked-control groups matched the Near and Far trained animals.

3b.2.3 Procedure

The NT (n=7) and FT (n=7) animals all received identical training to that outlined in Chapter 3a; 4 trials for 5 consecutive days in the MWM task, commencing from one of the four pseudo-random start positions (N, S, E, W). The motor control groups (Near control (NC), n=5 and Far control (FC), n=5) were placed in the pool for the same time as their learning counterparts, for 4 trials per day for 5 days (without a platform present). The length of time each group spent swimming was determined by their spatial equivalent group's mean time spent swimming on each respective training day. All animals were sacrificed immediately after the experiment and the dorsal

hippocampus was dissected and frozen in Krebs-CaCl$_2$/dimethyl sulfoxide (DMSO) for molecular analysis.

For analysis of the acquisition trials for the NT and FT groups, escape latencies, distance and velocity were defined. For further analysis on the yoked-control groups and to determine if the animals were responding to the distal cues despite no platform being present, an area in the maze called the outer corridor was assessed. This was defined as a circular area 20cm in width at the periphery of the pool wall (see Figure 3b.1). This corridor was divided into North and South sections. These sections were divided according to the location of the cues for both groups, i.e the North outer corridor encompassed the Near cues and the South outer corridor encompassed the Far cues (see Figure 3a.4, Chapter 3a). The mean percentage time spent by the Near and Far control groups was assessed in each of these corridors.
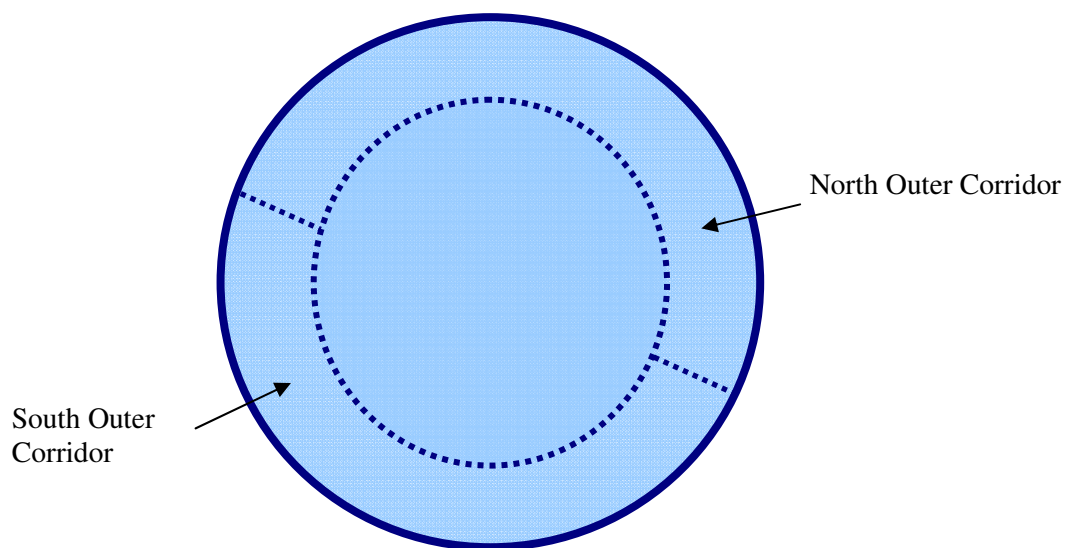
Figure 3b.1: Aerial-view (schematic) of a maze map of the North outer corridor and the South outer corridor of the water maze.

3b.2.4 BDNF Enzyme-Linked Immunoabsorbant Assay (ELISA) Protocol

Brain-derived neurotrophic factor protein was assessed using the BDNF E_{max}™ ELISA kit (Promega, UK) according to the manufacturer's recommendations. All animals were sacrificed following their final trial on the last day of acquisition and brains were immediately extracted and dissected on ice. The brain region dissected and examined was the dorsal portion of the hippocampus. The tissue sample was placed into 1ml Krebs-$CaCl_2$ solution (containing 2mM $CaCl_2$ and DMSO [1:10]) and stored at -20°C for later BDNF analysis. For protein extraction, dissected dorsal hippocampal sample tissues were homogenised in ice-cold Krebs solution 50 times. Protein was measured using a Bradford Assay, following which samples were diluted with Krebs solution to give equal protein concentrations, and stored at -20°C. For the ELISA, flat-bottomed 96-well plates were incubated overnight at 4°C, with 100µl of carbonate coating buffer (0.025M sodium bicarbonate, 0.025M sodium carbonate, pH 9.7) containing anti-BDNF monoclonal antibody (diluted 1:1000) in each well.

Following this overnight incubation, excess antibody was removed from the plates using one wash of Tris-HCL wash buffer (TBST; 20mM Tris-HCL (pH 7.6); 150mM NaCl; 0.05% Tween v/v). Plates were then blocked for 1h at room temperature for non-specific binding with block and sample buffer. This was followed by another wash before 100µl of dorsal hippocampal sample and standards were added to the wells for a 2h incubation at room temperature. Plates were washed five times with TBST, followed by a 2h incubation (room temperature) with anti-human BDNF pAb (diluted 1:500; 100µl/well), five washes with TBST, and a 1h incubation (room temperature) with anti-immunoglobulin Y horseradish peroxidase (1:2000 dilution; 100µl/well). Enzyme solution (TMB one), was brought to room temperature in

advance and subsequently incubated on the plate (100µl/well). The plate was left for 15 minutes (room temperature) until samples turned blue. This reaction was stopped by adding 100µl of 1M HCL to each of the wells. Plates were read at 450nm, using a 96-well automated plate reader, and BDNF concentrations were estimated for the standard curve.

3b.2.5 Statistics

Repeated-measures ANOVAs with appropriate Bonferroni-corrected t-tests (if required) were conducted on the data collated for the swim trials. BDNF data was analysed using independent samples t-tests. Error bars and the symbol **±** was employed throughout to indicate standard deviation from the mean, which is in turn denoted by S.E.M. A star-based system for significance representing p-values of *<0.05, **<0.01, ***<0.001, respectively, was used throughout.

3b.2.6 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

3b.3 Results

3b.3.1 Basic Measures of Acquisition

From our analysis presented in Chapter 3a differences were observed between the NT and FT groups when basic measures of acquisition were assessed (e.g. escape latency). Here we present distance travelled data, which highlight subtle, but non-significant differences between the training groups [$F(1, 12) = 3.95$, $p>0.05$; Figure 3b.2).
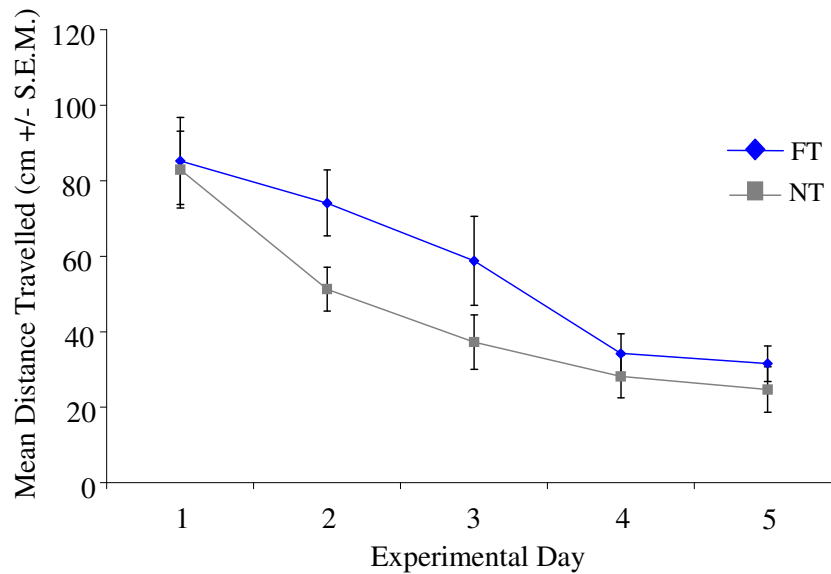
Figure 3b.2: Total distance travelled (cm ± S.E.M.) during acquisition in the NT and FT groups.

3b.3.2 Percentage Time in Outer Corridor during Acquisition

As evidenced from the previous chapter, we found differences between the NT and FT animals' swimming behaviours following in-depth analysis. The first behaviour displayed was thigmotaxis and we specifically noted differences in the position at which both groups performed vertical thigmotaxis, with each group's attention focused towards their respective external cues. However, as suggested in the introduction, a question that may arise is that the animals may be only visually attracted to the light of the cues and may not be displaying learning from the cues. To assess this, the yoked-

control group were an ideal group to assess, as thigmotaxis has been described as an automatic behaviour in response to fear (Barnett, 1963; Devan et al., 1999), and as they have no platform to escape from the pool, they would likely spend the majority of their time searching in the outer periphery of the maze. Therefore, to discern whether control animals, despite having no platform, would swim in a region influenced only by the cues, we examined the time spent by both control groups in specific areas of the outer corridor of the maze during the retention trial.

3b.3.2.1 Outer Corridor

Examination of the full outer corridor suggests that the animals in both the NC and FC groups spent a high percentage of their time swimming in this area, as expected (Figure 3b.3). Specifically, on Day 1 animals in the NC group spent $77.20\pm2.94\%$ of their time in the outer corridor, with the FC group spending $75.69\pm6.73\%$ of their time in this area of the maze. Statistical analysis revealed no differences between the groups on Day 1 ($t(8) = 0.21$, $p>0.05$). Similarly, on Day 5 the NC group spent $89.90\pm2.99\%$ of time in this area, with the FC group spending $92.29\pm2.88\%$ of their time in the outer corridor. Statistical analysis again revealed no differences between the groups on Day 5 ($t(8) = 0.575$, $p>0.05$).
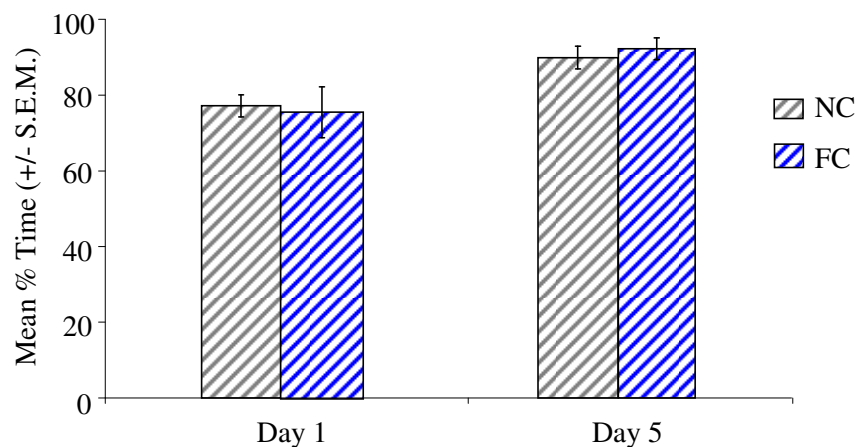
Figure 3b.3: Mean percentage time spent in the outer corridor on Day 1 and 5, for the NC and FC groups.

3b.3.2.2 North and South Outer Corridors

The outer corridor was subsequently divided into North and South sections as determined by the near and far cue locations. The North section encompassed all of the near cues and the South section encompassed all of the far cues. Assessment of the North section of the pool on Day 1 revealed that the NC group spent 47.3±3.78% of their time swimming in the area, whereas the FC group spent only 36.17±3.61% of their time searching in the North outer corridor. Furthermore, when the South section of the pool was assessed, it was noted that the mean percentage time spent by the NC animals was 31.71±2.20% and 40.98±3.51% for the FC group (see Figure 3b.4). A 2 x 2 mixed factorial ANOVA, however, revealed no main effect for area [$F(1, 8) = 2.98$, $p > 0.05$]. There was also no difference between the groups in the time spent in each area [$F(1, 8) = 0.07$, $p > 0.05$]. However, an interaction effect between area and group was found [$F(1, 8) = 10.68$, $p < 0.05$]. Further independent t-tests, however, revealed no differences between the groups in either the North ($t(8) = 2.13$, $p > 0.05$) or South sections of the corridor ($t(8) = 2.27$, $p > 0.05$), indicating no preferred swimming area for either group on Day 1.

The percentage time spent by each group in the North and South sections on the final day in the pool was also assessed to determine if either group's searching had altered throughout exposure to the pool. In the North section of the pool it was found that the NC group spent 33.50±9.31% of their time swimming in the area and the FC group spent 30.86±5.48% of their time searching in the North outer corridor. Analysis of the South section of the pool revealed that the mean percentage time spent searching in this area by the NC animals was 56.0±9.58% and 61.49±3.94% for the FC group (Figure 3b.5). Further analysis using a 2 x 2 ANOVA revealed a main effect for area

[F(1, 8) = 6.55, p<0.05], whereby a higher percentage of time was spent in the South area (M: 58.75±6.76%) than the North area (M: 32.17±7.39%) on Day 5 of training. However, there was no main effect for group [F(1, 8) = 0.49, p>0.05] nor interaction effect between area and group [F(1, 8) = 0.15, p>0.05].
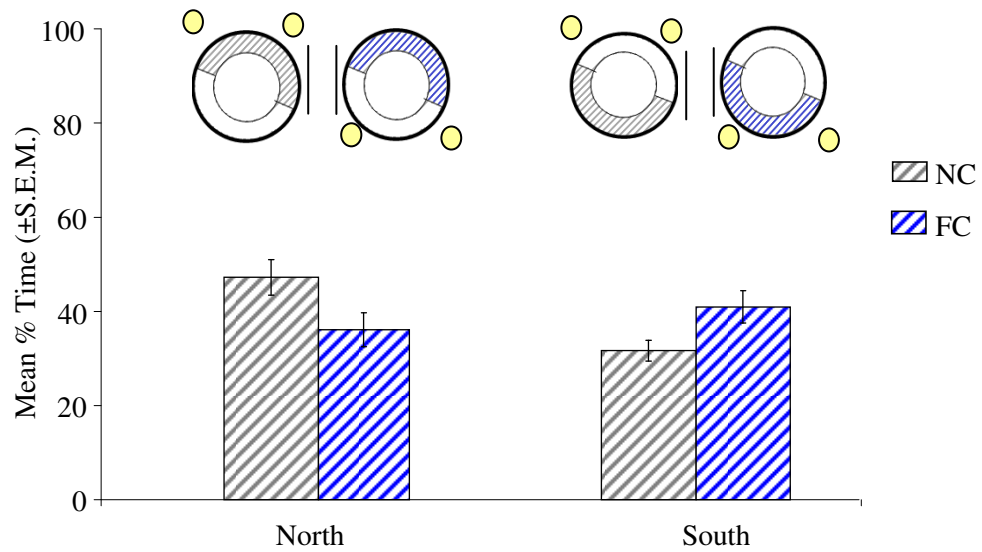
Figure 3b.4: Mean percentage time spent in the North and South outer corridor on Day 1, for the NC and FC groups.
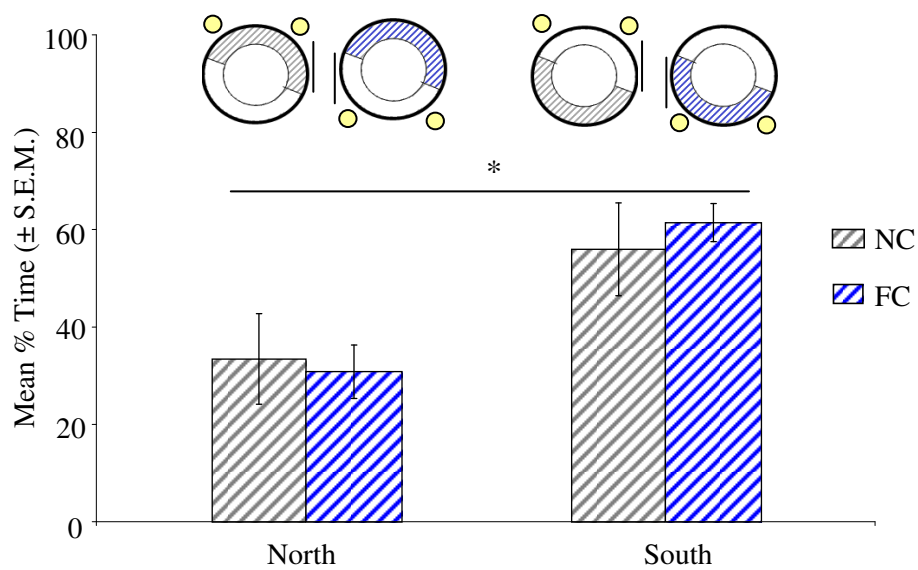
Figure 3b.5: Mean percentage time spent in the North and South outer corridor on Day 5, for the NC and FC groups.

3b.3.3 BDNF expression and learning

To determine if the observed behavioural changes associated with learning of the task (as reported in Chapter 3a) were reflected in the underlying molecular changes within the dorsal hippocampus we monitored BDNF expression during acquisition (Figure 3b.6). An independent samples t-test revealed significant differences in the expression of BDNF between the NT and FT groups ($t(12) = 6.37$, $p=0.001$) with the FT group having a higher mean level of hippocampal BDNF expression in the dorsal hippocampus (M: 316.56±13.91 pg/mg) than the NT group (M: 250.16±4.89 pg/mg). To ensure length of time in the maze alone was not a factor in the increase in BDNF in the learning group, a t-test comparing the Near and Far control groups was conducted. Results found no difference between these two groups ($t(8) = 1.24$, $p>0.05$) indicating that physical exercise was not a factor in the increase in BDNF levels between the training groups (see Figure 3b.6).
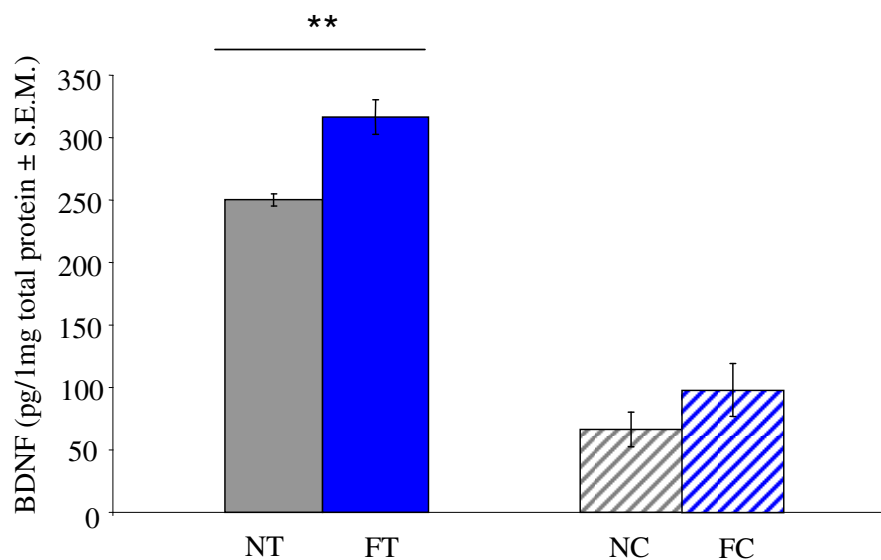
Figure 3b.6: Mean level of BDNF expressed of total protein in the dorsal hippocampus following training in the MWM task in the NT and FT groups and in the yoked control Near and Far groups.

3b.4 Discussion

Behavioural analysis of the NT and FT groups showed that path length to the goal is similar for both groups, indicating both are capable of locating the hidden goal. In-depth analysis, reported in Chapter 3a, however highlighted differences in how the task is solved, with thigmotaxis, direct and turning behaviours all suggesting the FT group solves the task using a more view-independent, inferring strategy than the NT group. As an inferring, allocentric strategy has a higher cognitive load attached to it, requiring development of multiple viewpoints, we suggested that this would take longer to develop over training and may also lead to increased activation of the hippocampus (Suthana et al., 2009). Thus, in this chapter we suggested that there would be an increase in hippocampal BDNF expression in the FT group. Our hypothesis was supported with a higher level of hippocampal BDNF found in the FT group.

These results are consistent with other reports implicating the importance of hippocampal BDNF in allocentric learning. McGauran et al. (2008), for example, showed increased hippocampal BDNF following spatial learning in a place version of the MWM. Furthermore, an increase in BDNF mRNA has also been reported in animals spatially trained in a number of allocentric tasks including the water maze (Falkenberg et al., 1992) and radial-arm maze (Mizuno et al., 2000) when compared to animals that were untrained. Additionally, Minichiello et al. (1999) demonstrated that when Trk-B knock-out mice were required to learn either a cued or place version of the MWM, they demonstrated more robust impairments when assessed in the more demanding place task (see also Heldt et al., 2007; Linnarsson et al., 1997; Mu et al., 1999), again implicating a specific role for BDNF in allocentric spatial processing. These observed, spatial learning related increases in BDNF have also been shown to be

region specific, with higher activation seen only in the hippocampus and not in other regions, such as the hypothalamus or striatum (Kesslak et al., 1998; Silhol et al., 2007). Findings from the current study also compliment previous reports from studies mapping hippocampal BDNF expression throughout a training period. Specifically, Harvey et al. (2008) found that BDNF expression paralleled the observed increase of inferring, allocentric-related behaviours recorded on later training days in the MWM. This parallel pattern of molecular and behavioural data lends further support to the role of the hippocampus in later allocentric processing of information, which is in line with suggestions that allocentric strategies often do not fully emerge until later in training in spatial tasks (Burgess, 2006). To this end we propose that it is the increased allocentric inferring abilities that FT animals engage in to locate the goal that is reflected in the increased hippocampal BDNF levels.

The observed increases in BDNF may, however, be due to a number of other behavioural factors rather than learning. It is known, for example, that exercise alone can increase BDNF (Griffin et al., 2009; Huang et al., 2006; Neeper et al., 1995). We controlled for this by including two groups of matched, activity yoked-controls who swam in the maze with the platform removed. However, before the effects of exercise on BDNF could be determined, we verified that the rats were solely exercising and demonstrating no learning behaviours, such as swimming in the regions influenced by the light from the external cues. All the control animals displayed thigmotaxis, as expected, due to no escape platform available in the maze. Moreover, our results demonstrate that the animals were not influenced by the cues as they did not spend significantly more time at the cue locations; instead they displayed passive swimming with no clear direction in their behaviours. This confirms our control groups were not

displaying learning behaviours during this phase of the task and contrasts with the trained groups, who, when performing thigmotactic behaviours, did so at their respective cue locations (see Chapter 3a). This further supports our suggestion that thigmotactic behaviour may provide support for an early learning association of the importance of cues to help direct an escape, rather then purely a fear-related response. Furthermore, we also noted no difference between NC and FC groups' BDNF levels, suggesting that the higher expression of the FT over the NT group was due to learning and not as a result of differences in length of time in the pool. Therefore, while motor movements and exercise may, somewhat, increase BDNF, spatial learning enhances hippocampal BDNF expression further.

While the yoked control group is a widely used control in studies examining the molecular underpinnings of learning in spatial tasks, it has inherent problems, in that the free-swimming animals may be exposed to uncontrollable stress resulting in learned helplessness in the maze, as there is no escape available. This pattern of behaviour is also a model for depression and may result in lowered BDNF expression (Greenwood et al., 2007). In particular, BDNF has been suggested to be involved in stress-induced hippocampal adaptation and pathogenesis of depression in the adult animal (Duman et al., 1997), with decreases in BDNF mRNA levels in the hippocampus observed following stress (Lee et al., 2008; Smith et al., 1995; Xu et al., 2004). Others have also found that antidepressant treatment ameliorates stress-induced reduction of BDNF mRNA in the hippocampus (Nibuja et al., 1995). Therefore, there has been speculation that the lack of increase in BDNF levels following an exercise yoked-control group may be as a result of stress, which can result from forced swimming in an inescapable version of the MWM (Shi et al., 2010). This may explain

the lower levels of BDNF seen in both the NC and FC groups here in comparison to the NT and FT groups. However, we suggests that stress, alone, would not fully account for the differences in the BDNF levels, as the animals were only in the pool for a minimal length of time on Day 5, which is when BDNF was analysed. Marmigere et al., (2003) also suggest that it is only at longer exposures to stress that hippocampal BDNF mRNA is shown to decrease. Furthermore, it has also been shown that increases in corticosterone levels during MWM training do not affect hippocampal BDNF mRNA expression (Schaaf et al., 1999).

Overall, it was shown that spatial learning augmented BDNF levels in the hippocampus. Specifically, learning using cues that are located at a position further away from the goal led to significantly higher increases in its expression, suggesting a greater reliance on the hippocampus. This further supports our behavioural data that indicates a more view-independent, inferring strategy is being adopted (Chapter 3a). Furthermore, by assessing the activity yoked-controls we also confirmed the importance of thigmotactic behaviour in spatially trained animals. BDNF analysis of the exercise control animals also indicates that BDNF is expressed more highly following learning than physical activity. Although we have shown hippocampal BDNF is increased in the FT compared to the NT group, we cannot definitively say that the hippocampus alone is directly involved. Therefore, our next two Chapters attempt to address this question by examining the effect of dorsal hippocampal lesions on learning the water maze.

Chapter 4

Dorsal hippocampal lesions alter exploratory behaviour in the Morris water maze under Near cue training conditions.

Abstract

A critical structure thought to be involved in learning the MWM is the hippocampus, with evidence from lesion studies strongly implicating its importance in the processing of allocentric representations. However, in light of our previous findings revealing differences in behaviour and BDNF expression in intact animals trained under diverse cue conditions, and with ongoing debate surrounding the precise nature of hippocampal involvement in spatial tasks, it is unclear how exactly the hippocampus is involved. Therefore, to assess this, we adopted our novel method of behavioural analysis, as used in Chapter 3a. Male Wistar rats were divided into two groups; sham (n=8) and dorsal hippocampal lesion (DH, final n=8). DH lesioned animals received injections of NMDA (10mg/ml) in 8 hippocampal sites bilaterally. Animals were then trained in the water maze with distal cues in the near position, as previously described. Results indicate that DH animals are impaired in the task generally, however, they demonstrate a significantly different pattern of acquisition when compared to sham animals. This includes a reduction in Near DH animals' time spent in navigationally complex behaviours, such as direct and turning behaviour, and an increase in time spent in more basic movements such as thigmotaxis, in comparison to shams. Changes in these behaviours throughout training also highlighted impairments in the DH animals' exploration, generally manifesting in a delay in alternating behaviours to that of a more useful strategy. Lesioned animals also displayed inaccurate judgement of distance and direction to the hidden platform. From our comprehensive account of animals' movements in the water maze, we suggest that perhaps the impairment seen in place finding following hippocampal damage is due to a deficit in integrating exploratory behaviours, rather than a purely spatial memory impairment.

4.1 Introduction

As confirmed by earlier Chapters, animals have a propensity to rely on external visual cues during place navigation in the MWM (Chapter 2). We have also demonstrated that when animals have cues located in a position close to their goal they will perform view-dependent, cue-directed behaviours to locate the submerged platform, with a limited amount of inference needed (Chapter 3a). Animals with cues far away from the goal, however, must move away from the cues, resulting in more turns-away and less cue-focused behaviours. Consequently, a higher level of inferring appears to be required to reach the goal under Far cue conditions. We have also shown an increased level of hippocampal BDNF corresponding with spatial learning in the maze but particularly when cues are located far from the goal (Chapter 3b). This may indicate that with cues in a position further away a more hippocampal-dependent strategy is needed to locate the platform. To investigate this idea, we examined the effect of dorsal hippocampal lesions on an animal's ability to locate a hidden goal under the respective cue conditions (see also Chapter 5).

It is widely accepted that the hippocampus is of particular importance in spatial learning and memory (Jarrard, 1993; Morris et al., 1982; Morris, 1984; O'Keefe & Nadel, 1978). Cognitive mapping theory, in particular, suggests that the hippocampus is essential in forming relationships between environmental stimuli and is required for the storage and updating of those relationships (O'Keefe & Nadel, 1978). Support for the hippocampus' role in spatial memory comes from many lines of investigation including human and animal research. In humans it has been found, using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), that hippocampal activation is stronger during memory-guided spatial navigation (Ghaem

et al., 1997; Maguire et al., 1998; 2006). In animals, cellular (place cells; C.D. Harvey et al., 2009; O'Keefe & Dostrovsky, 1971), molecular (BDNF; Harvey et al., 2008; Messaoudi et al., 1998; Mizuno et al., 2000; IEG expression; Guzowski et al., 2001; Vann et al., 2000; Lipid levels; Köfeler et al., 2010) and lesion data (Cain et al., 2006; Ramos, 2010) have all implicated the hippocampus in spatial tasks, with particular emphasis on the dorsal hippocampus' role in allocentric spatial processing (Bannerman et al., 1999, 2004; Hock & Bunsey, 1998; Pothuizen et al., 2004).

In relation to the MWM, animals with hippocampal lesions often exhibit an inability to learn the spatial location of a hidden goal submerged in the maze (Morris et al., 1982; Sutherland et al., 1983). These impairments, however, appear to augment as spatial demands increase, with alterations to different aspects of MWM training paradigms highlighting this division. For example, in tasks where spatial demands are minimal, such as when the platform is made visible (de Bruin et al., 2001; Dolleman-van der Weel et al., 2009; Maglakelidze et al., 2010), or when a locale or beacon cue is present (Trullier et al., 1999), hippocampal lesioned animals are generally unimpaired in reaching their goal. Furthermore, when the platform is always at the same distance and direction from a visible landmark, lesioned animals can successfully locate their goal, as they are likely relying on an egocentric, procedural strategy (Mogensen et al., 2005; Schenk & Morris, 1985; Whishaw, 1985a; Whishaw & Tomie, 1997a). Extending this, Save and Poucet (2000) examined the effect of hippocampal lesions in solving a MWM when a number of proximal cues were used in the maze. This modification of the task is of particular interest, as although the cues were close to the goal none directly marked its position, suggesting the need for spatial processing of the cues. Interestingly, lesioned animals were relatively unimpaired in this task, suggesting

that despite having a damaged hippocampus, lesioned animals could still process spatial information to some degree. As the cues moved further away, however, impairments in the maze became pronounced, with lesioned animals' ability to locate a hidden goal markedly reduced (Save & Poucet, 2000). Numerous studies have reported similar deficits when only distal cues are available to locate the goal (de Bruin et al., 2001; Morris et al., 1982; 1990).

There has, however, been some debate over the exact nature of hippocampal involvement in spatial tasks with suggestions of hippocampal involvement in the monitoring of behaviour while swimming in the maze (see Chapter 1 for more detailed discussion). This stems from observations of significant alteration and sometimes extinction of exploratory behaviours following hippocampal ablation (D'Hooge & De Deyn, 2001; Leaton, 1965; Morris et al., 1982; Wallace et al., 2002b; Whishaw et al., 1994). Cognitive mapping theory accounts for this, maintaining that animals would lose the ability to explore efficiently as they would not be able to learn or retain information about the spatial features around them, leaving exploration redundant (O'Keefe & Nadel, 1978). Others, however, have proposed that the impairments displayed by lesioned animals are not a purely spatial learning deficit but rather are directly resulting from the behavioural alterations that occur following hippocampal damage (Day et al., 1999; Eichenbaum et al., 1990; Whishaw, 1998a).

Specifically, it has been suggested that the deficits seen in spatial tasks reside in lesioned animals' inability to adapt to procedural features of the task, such as understanding that escape cannot be attained by scrabbling at the wall, and subsequently learning to move away from the edge of the maze into the centre of the pool (Whishaw, 1998a; Wright et al., 2004). Whishaw and colleagues (1985a, b; 1995)

provided evidence for this in a series of MWM experiments, where through adapted training procedures that were aimed at reducing the use of maladaptive behaviours, they showed that hippocampal lesioned animals could successfully locate a hidden goal when only distal cues were present to guide their search (see also Chapter 1). Following training in the adapted protocol, the same animals also displayed accurate place finding when later trained in a conventional place version of the MWM, indicating that their prior exposure to the procedural aspects of the task resulted in improved performance. From this examination of the behavioural component of the task, others have further suggested that hippocampal lesions lead to a failure in altering searching behaviour in relation to environmental information, resulting in perseveration in ineffective behaviours, rather than simply an inability to learn the location of place (Day et al., 1999; Eichenbaum et al., 1990).

Proceeding from the abovementioned, we intend here to conduct a detailed examination of the swimming patterns and platform behaviour of both sham and dorsal hippocampal lesioned animals trained in the MWM with distal cues in the near position. We propose that an in-depth analysis of their movements in the maze will enable a clear assessment of spatial and behavioural processing throughout training. This would be particularly informative as to date, there has been no direct analysis of how exactly the hippocampus contributes to spatial navigation and the use of cues. Based on our previous findings, we hypothesise that the dorsal lesioned animals will display similar cue-directed search patterns as sham controls, as the near position of cues allows for more view-dependent, egocentric-based processing. However, as an allocentric inferring component still remains in this task, we may expect the lesioned

animals to have some observable impairment in view-independent, allocentric behaviours, in comparison to controls.

4.2 Method

4.2.1 Subjects

Male Wistar rats (n=19) served as subjects in the current study. Subjects were approximately three months old and weighing 200-300g at the beginning of experimentation. All were well handled prior to experimentation and housed as previously described (see Chapter 2).

4.2.2 Apparatus

The hidden platform version of the MWM task was again used in this experiment. The three distal cues and submerged escape platform remained in a fixed position throughout training in the Near cue position. Gross acquisition measures, platform and swimming behaviours of the animals were digitally recorded, as previously described, and later used for in-depth analysis (Chapter 3a for details).

4.2.3 Surgery

Subjects were, initially, randomly assigned to one of two groups: a sham control group (Near sham; n=8), or a dorsal hippocampal lesion group (Near DH; n=11). Rats were anaesthetised with isoflurane gas (1.8-3.0% isolflurane delivered in O_2 at 1 l/min). The animal's head was shaved and was then placed in a Kopf stereotaxic frame and the incisor bar was adjusted so that bregma was level with lambda. Surgical anaesthesia was monitored by a lack of responsiveness to tail or foot pinch, respiratory rate and a lack of responsiveness to surgical stimulus, when present. The head was cleansed with betadine and alcohol. A 1-2 cm long incision was made along the midline of the scalp and the skin and muscles were retracted and infusion site coordinates marked. For DH

lesioned animals, a small burr hole was made in the skull, with a small hand-held drill, at each marked coordinate. N-methyl-D-aspartate dissolved in 0.1 M sterile PBS, pH 7.4 (NMDA; Sigma-Aldrich, 10mg/ml) was injected bilaterally along the longitudinal axis of the hippocampus at the coordinates and volumes listed in Table 4.1 (following Bardgett et al., 2006 and Paxinos & Watson, 2005). Solutions were infused with a 5µl Hamilton syringe over approximately 30-60 seconds. The needle was left in place for one minute after each infusion. The burr holes were closed using bone-wax (Johnson & Johnson Ltd). The incision was closed using 4-5 sutures (Size 3-0, Ethicon, Johnson & Johnson Ltd.) and an antiseptic powder was applied to the wound. Buprenorphine (0.3 mg/kg, s.c; Temgesic) was given as an analgesic, prior to the cessation of anaesthesia. Sham-operated rats were anaesthetised in the same manner as above, had their skin and muscles cut and had two small holes burred in the skull. They were then sutured and administered buprenorphine (0.3 mg/kg, s.c.; Temgesic); they received no damage to the cortex. Following surgery, animals were placed in an individual recovery cage until they regained mobility. Animals were housed in individual cages for the duration of the experiment. All animals were allowed to recover for 7 days before behavioural testing began.

Table 4.1: Stereotaxic coordinates for dorsal hippocampal lesions (Bardgett et al., 2006).

Anterior-Posterior	Medial-Lateral	Dorsal-Ventral	Infusion Amount (µl)
-2.0	± 1.2	-3.7	**0.15**
-3.0	± 1.6	-3.7	**0.08**
-3.0	± 3.0	-3.6	**0.15**
-3.8	± 2.0	-3.7	**0.08**
-3.8	± 3.6	-3.6	**0.15**
-4.6	± 2.9	-3.7	**0.08**
-4.6	± 4.0	-3.9	**0.15**
-5.5	**± 5.0**	**-5.0**	**0.15**

4.2.4 Procedure

Following recovery, sham lesioned and DH lesioned animals were trained in the MWM with distal cues in the Near position, receiving 4 trials/day for 5 days (see Chapter 2 for detailed training protocols and cue location). Briefly, each trial consisted of transporting animals from a transport container and placing them into the water facing the pool wall, from one of four pseudorandom starting positions (N, S, E, W). Once the animal reached the platform they remained there for 15 seconds before being placed in a holding cage for an inter-trial interval of 10 seconds. All animals received a retention trial which was assessed 7 days post-acquisition by allowing animals a single 60 second trial with the platform removed from the maze. All animals were placed in the pool at the NW position for the single retention trial (see Chapter 6 for retention analysis).

4.2.5 Assessment of the platform interval

Digital footage of animal movements during the platform interval (15 sec) was recorded and saved on a connecting computer using a video capture software package (VirtualDubMod 1.5 10.2). Analysis of platform behaviour of each group of animals was carried out by observing video footage along with recorded behavioural tracks, as described in Chapter 3a. In total for the current study, there were 4800 digital stills (i.e. 16 animals x 4 trials x 15 seconds x 5 days) assessed for animal head direction analysis.

4.2.6 Searching strategies used during in trial locomotion

Swimming trials for each animal were digitally recorded throughout the acquisition phase. Exploratory behaviours, as outlined in Chapter 3a were examined for both groups. For the detailed examination of swimming behaviours, EthoVision (Noldus Information Technologies, Wageningen, Netherlands) provided *x, y* coordinates (0.2s increments apart) for the animal's position throughout each trial and these tracks were then examined alongside digital recordings.

4.2.7 Histological Analysis

At the completion of behavioural testing, animals were administered a lethal overdose of sodium pentobarbital (100mg/kg i.p.; Euthatal). The brains were then removed and stored in 4% paraformaldehyde dissolved in 0.1M phosphate buffer (PFA) and later transferred to a 30% sucrose solution and stored at 4°C. Coronal 40µm thick sections were then cut on a freezing microtome (Leica SM2010R, Leica Microsystems, Germany). Every fourth section was mounted on gelatin-coated slides and stained with cresyl violet (Sigma-Aldrich). Images of the stained slices were taken and then transferred to a PC where they were analysed using a specifically designed Matlab R2008a programme. Six sections rostrocaudally, which included 2 rostral sections at bregma -2.16, 2 mid sections at bregma -3.12 and 2 caudal sections at bregma -4.08, were examined for each animal. The area of total dorsal hippocampus along with the area of damaged dorsal hippocampal tissue was measured from each of the 6 sections. The total area and damaged area from the 6 sections were then summed and damage presented as a percentage of the total area. Lesions were reconstructed using Paxinos and Watson (2005).

4.2.8 Statistics

All linear statistical analysis was carried out using SPSS (version 17). Circular statistical analysis was carried out using Oriana (Version 2.0, Kovach Computing Services, UK). Statistics used included analysis of variance with appropriate Bonferroni pairwise comparisons and independent, dependent and one-sample t-tests, where appropriate. Rayleigh Uniformity tests ($p < 0.05$) and Watson-William F-tests were also employed to assess circular data. The symbol ± was employed throughout to indicate standard mean error. Error bars, where present, show standard error of the mean, which is in turn denoted by S.E.M. A star-based system for significance representing p-values of *<0.05, **<0.01, ***<0.001, respectively, was used throughout.

4.2.9 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

4.3 Results

4.3.1 Histology

Three animals were removed from the study following histology which indicated less than 40% damage to the dorsal portion of the hippocampus resulting in an n=8 for the DH group. All other animals included in analyses sustained damage >40% to the dorsal hippocampus and also displayed behavioural impairments. A one-sample t-test was used to compare the percentage area of damage to a representative sample (no damage represented as 0%). Near DH lesioned animals had a mean area damaged of 65.43±6.57% and this was found to be a significantly higher percentage damage when compared to the representative sample (t(7) = 9.96, p<0.001). There was only slight damage to the overlying corpus callosum and somatosensory cortex at the sites of cannula penetration. It is important to note that all animals displayed normal motor and coordinated swimming movements and that damage to these cortical areas, adjacent to the hippocampus, have not been correlated with spatial acquisition deficits previously seen in the water maze (Horne et al., 2010; Wright et al., 2004). There was some damage to the habenula and limited damage to the laterodorsal nuclei of the thalamus in 3 out of 8 animals. The ventral hippocampus was left intact and there was also no damage to the entorhinal cortex or amygdala in any animal. See Figure 4.1 for representative examples of Near DH photomicrographs.

a) **Bregma – 2.04**

b) **Bregma – 3.12**

c) **Bregma – 4.08**

Coronal drawings

Dorsal hippocampal photomicrographs

Magnified dorsal hippocampal photomicrographs
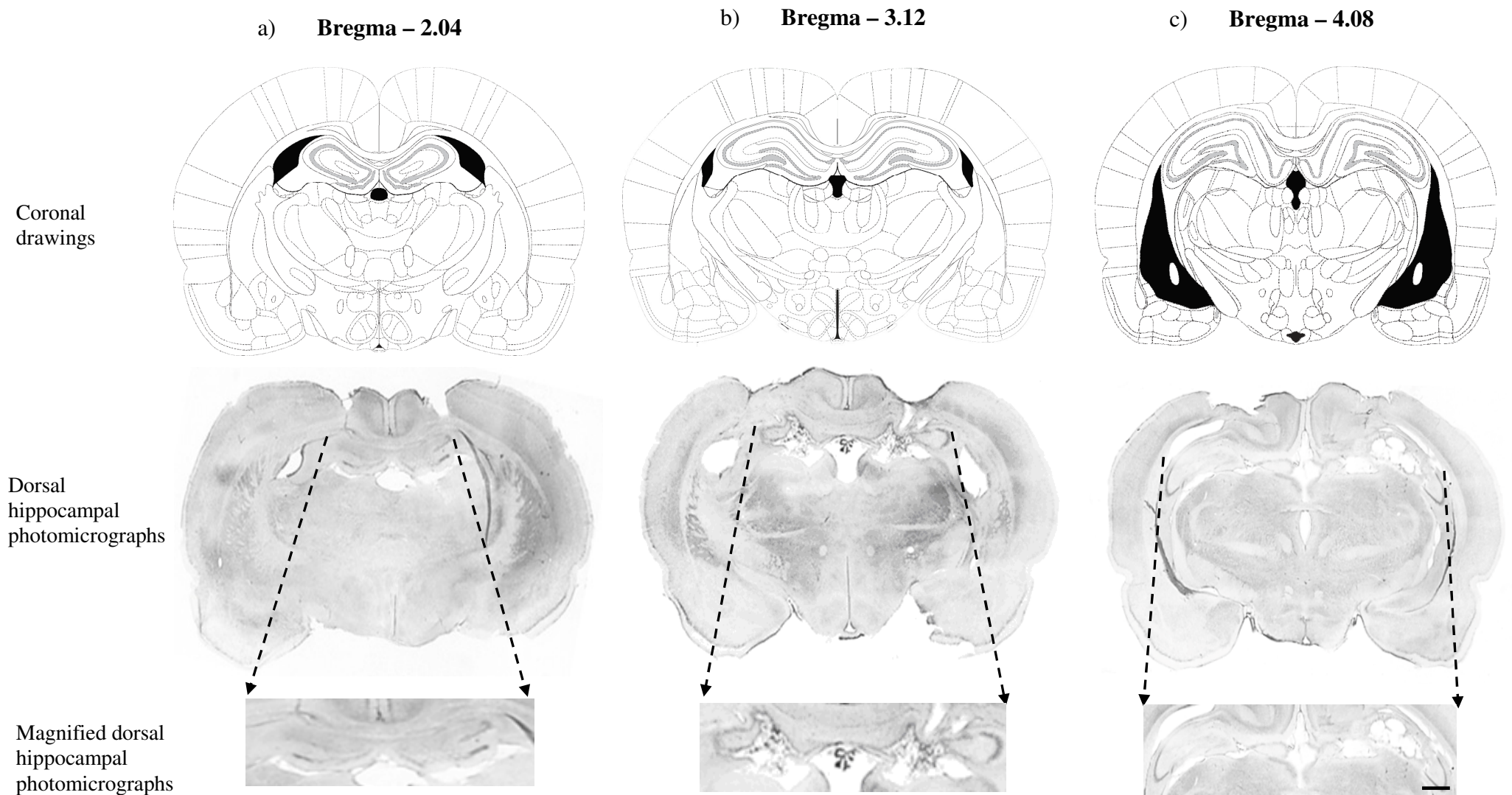
Figure 4.1: Schematic drawings of normal coronal sections of intact brain regions* (top row), representative photomicrographs of dorsal hippocampal damage (middle row) and photomicrographs of magnified dorsal hippocampal damage (bottom row), at rostrocaudal levels from bregma; -2.04 (a), -3.12 (b), and -4.08 (c). Scale bar = 500µm.
*Adapted from Paxinos & Watson (2005).

4.3.2 Basic Measures of Acquisition

Near sham lesioned animals successfully acquired the MWM over 5 days of training with escape latencies (EL) decreasing from a mean of 41.86±4.53 sec on Day 1, to a mean on Day 5 of 10.96±1.76 sec. Near DH animals' ELs decreased from a mean on Day 1 of 46.43±4.27 sec to 30.89±7.15 sec on Day 5 (see Figure 4.2). A 2 x 5 mixed factorial ANOVA revealed a significant main effect for day [$F(4, 56) = 10.27$, $p<0.001$] indicating an overall decrease in EL throughout training. Bonferroni-corrected t-tests revealed a significantly shorter EL on Days 4 and 5 when compared to Day 1 ($p<0.01$) as would be expected as the task is learned. In addition to this, a main effect for group was also found [$F(1, 14) = 12.59$, $p<0.01$], with animals with hippocampal damage performing significantly slower in the task (M: 38.60±6.06 sec) when compared to the Near sham group (M: 21.40±3.40 sec). Finally, there was no interaction effect found between day and group [$F(4, 56) = 2.01$, $p>0.05$].
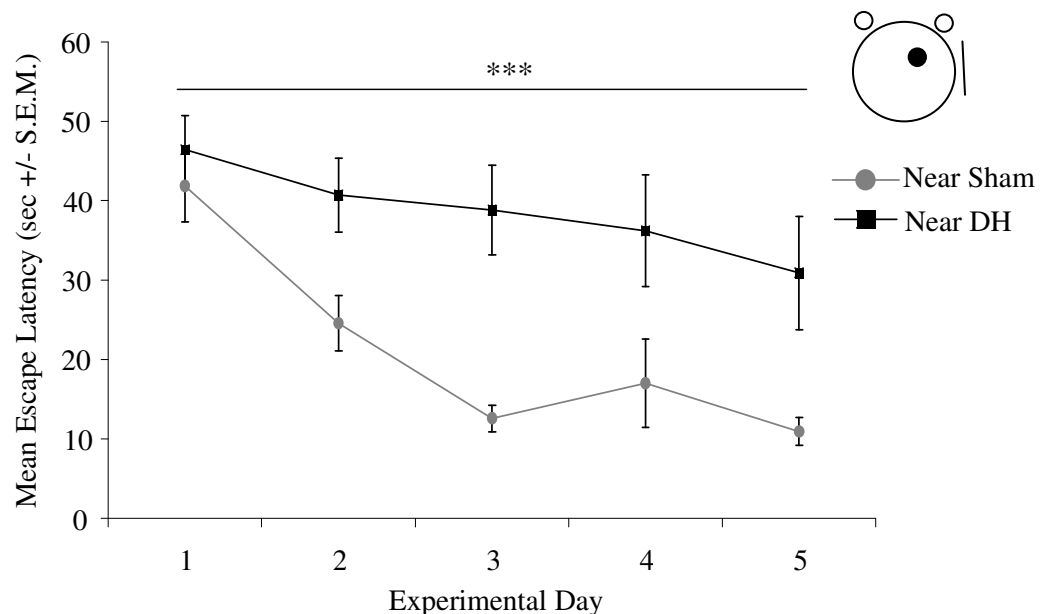
Figure 4.2: Mean escape latencies (sec ± S.E.M.) of Near sham and Near DH lesioned animals during water maze training. Insert; Near cue configuration.

In addition, a 2 x 5 mixed factorial ANOVA examining the total distance moved, revealed an overall effect for day [$F_{(4, 56)} = 8.47$, $p<0.001$]. Subsequent Bonferroni-adjusted t-tests revealed that the distance travelled on Day 5 (M: 548.37±107.22 cm) was significantly shorter than Day 1 (M: 1095.22±78.95 cm; $p<0.01$), suggesting more accurate platform finding as training progressed overall. A significant effect for group was also found [$F_{(1, 14)} = 10.29$, $p<0.01$], with the Near DH animals travelling longer distances (M: 1009.46±164.47 cm) throughout training overall when compared to the Near sham group (M: 560.72±78.26 cm). Additionally, no interaction effect between day and group was noted [$F_{(4, 56)} = 1.68$, $p>0.05$; see Figure 4.3].
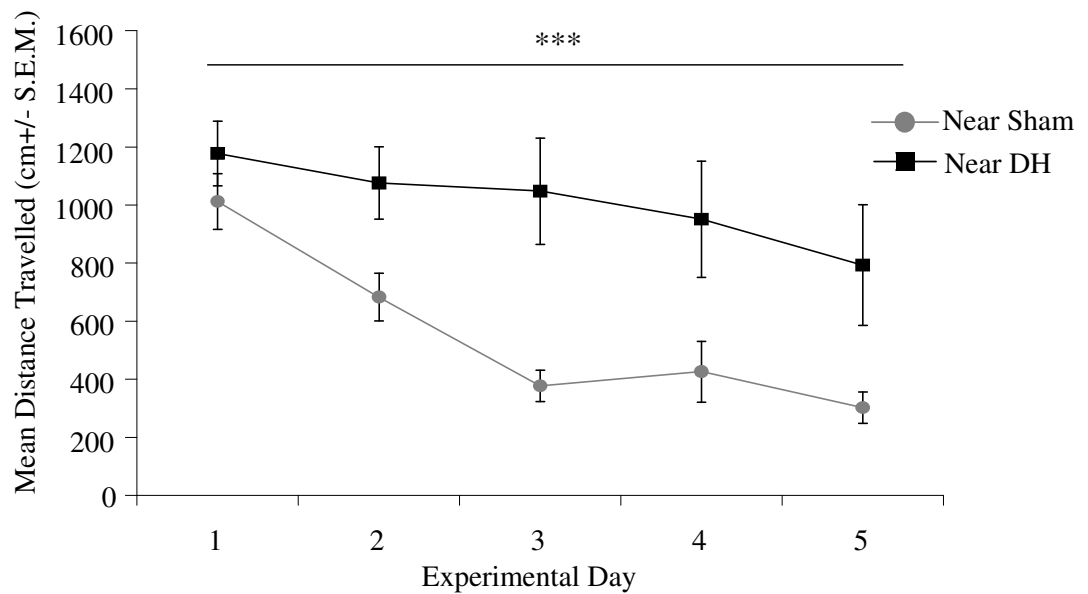
Figure 4.3: Mean distance travelled (cm ± S.E.M.) for the Near sham and Near DH lesioned animals throughout training days.

When the mean swimming velocity during training was assessed, no significant main effect for day was found [$F_{(4, 56)} = 2.71$, $p>0.05$]. There was also no effect for group [$F_{(1, 14)} = 2.39$, $p>0.05$] and no interaction effect between day and group [$F_{(4, 56)} = 1.41$, $p>0.05$]. Escape latency and distance travelled, standard measures of

acquisition, would suggest there were differences in the acquisition of the task between the Near sham and Near DH groups, with the Near DH having a significantly poorer performance in the task overall.

4.3.3 Behavioural Analysis; Platform Behaviour

4.3.3.1 Range of Head Movement

Analysis of head direction and the range of head movements, while on the platform, were conducted by observing digital stills of the 15 sec platform interval for each animal on all training trials (Figure 4.4). A 2 x 5 x 20 mixed factorial ANOVA initially revealed that there was no change in head direction movements across days [$F(4, 56) = 0.60$, $p>0.05$]. There were also no differences between the Near sham and Near DH groups in the range of movement while on the platform [$F(1, 14) = 0.24$, $p>0.05$] or interaction effect found between day and group [$F(4, 56) = 0.95$, $p>0.05$]. However, when assessed across trials, an overall decrease in the range of movements was found, with a significant main effect for trial [$F(3, 42) = 17.77$, $p<0.001$]. While there was no interaction effect found between trial and group [$F(3, 42) = 0.78$, $p>0.05$], there was, however, a significant interaction effect between trial and day [$F(12, 168) = 1.88$, $p<0.05$]. A series of repeated measures ANOVAs with Bonferroni-corrected t-tests revealed trial differences on Day 1 [$F(3, 45) = 8.63$, $p<0.001$], with the greatest range in movement on trial 1 (M: 74.72 ± 4.94^{o}) compared to trials 2 (M: 49.72 ± 7.32^{o}, $p<0.05$), 3 (M: 46.13 ± 6.27^{o}, $p<0.05$), and 4 (M: 35.46 ± 5.27^{o}, $p=0.001$). A similar finding was revealed on Day 2 [$F(3, 45) = 8.21$, $p<0.001$], with differences between trial 1 (M: 65.06 ± 5.79^{o}), and trials 2 (M: 39.83 ± 5.87^{o}, $p<0.01$) and 3 (M: 36.09 ± 4.02^{o}, $p<0.01$). No differences on Day 3 were found [$F(3, 45) = 2.37$ $p>0.05$], however trial 1

(M: 63.72±9.64°) and trial 2 (M: 38.76±6.99°, p<0.05) differed significantly on Day 4 [F(3, 45) = 3.62, p<0.05], suggesting increased movement that coincides with the animals' reintroduction to the maze on a new training day and increased curiosity of the environment. However, no differences were noted on Day 5 where animals are most familiar with the environment [F(3, 45) = 1.98, p>0.05].
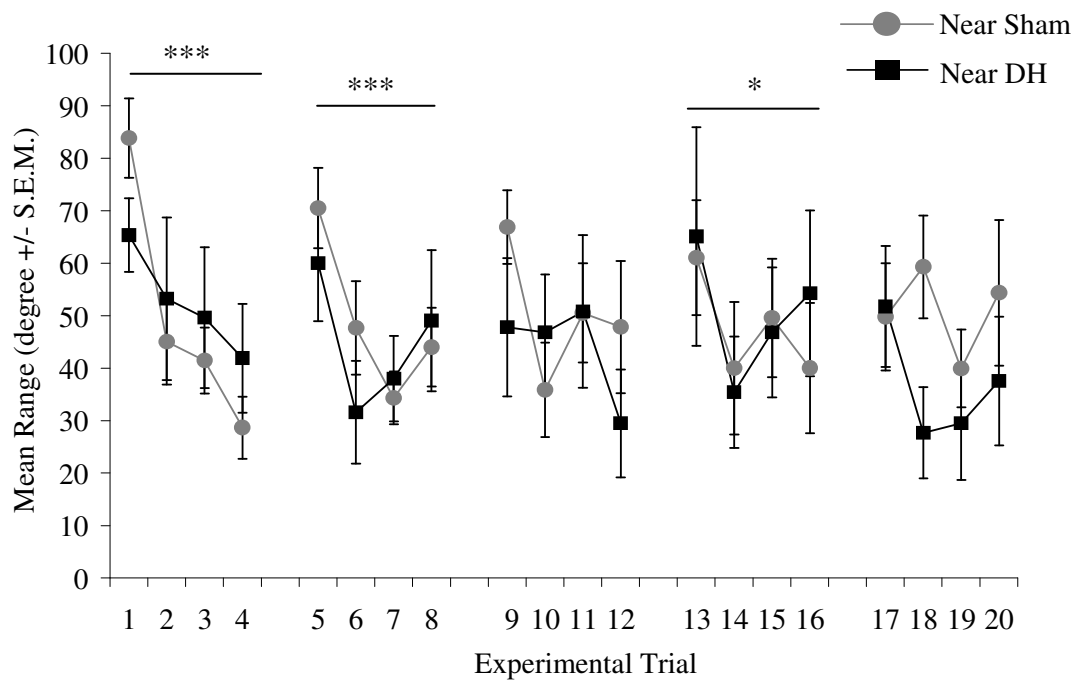
Figure 4.4: Mean range of head movement (degree ± S.E.M.) for the Near sham and Near DH groups across training trials.

4.3.3.2 Mean head direction

To investigate this further, the mean head directions of the animals during the platform interval were also examined. Overall it was found that the Near sham animals appeared to have a dispersal of viewpoints across days. However, these remained somewhat within the range of the distal cues (see Table 4.2). When examined in depth, Rayleigh Uniformity tests revealed that the only significantly preferred orientation for the Sham group was to the NW on Day 1 of training (Z = 2.94, p<0.05). Near sham animals

exhibited no significantly preferred head orientations on any other training day. When assessing platform behaviour of the Near DH animals, it was found that they displayed a mean head direction towards the SE of the pool on Day 1. There was a slight shift in the mean head orientation on Day 2 to the S of the pool with the mean head direction on Day 3 to the NW, Day 4 to the S and Day 5 shifted towards the NW of the pool (see Table 4.2). Near DH animals' head orientations, overall, did not appear to focus on the distal cues, with mean viewing direction concentrated to the south of the pool. When statistical examination of the head directions were carried out, no statistically preferred angle of orientation on any day of training was revealed. Thus, results indicate that neither animals in the Near sham nor the Near DH group spent the majority of their time looking towards the cues at the north of the pool during the platform interval but rather both groups tended to look around the entire arena during the platform interval.

Table 4.2: Mean head direction (± S.E.M.) during training for the Near sham and Near DH groups (* denotes significant orientation).

	Near Sham Lesioned			Near Dorsal Hippocampal Lesioned		
Day	Mean±S.E.M.	Rayleigh Uniformity	Compass Location	Mean±S.E.M.	Rayleigh Uniformity	Compass Location
Day1	276.11±22.9°	Z=2.94,p<0.05*	W	56.89±23.24°	Z= 2.77, p>0.05	SE
Day2	287.06±32.9°	Z= 1.74, p> 0.05	NW	8.48±39.96°	Z= 0.06, p>0.05	S
Day3	115.0±62.9°	Z= 0.84, p>0.05	NE	276.85±31.8°	Z= 0.42, p>0.05	W
Day4	275.81±76.6°	Z= 0.72, p>0.05	W	340.85±65.6°	Z= 0.46, p>0.05	S
Day5	67.83±35.6°	Z= 0.35, p>0.05	E	230.64±51.3°	Z= 1.02, p>0.05	NW

4.3.4 Behavioural Analysis; Swimming Behaviours

4.3.4.1 Thigmotaxis

Swimming behaviours were analysed in detail as in Chapter 3a. The mean percentage time, of total time in the pool, spent in thigmotaxis was first examined (Figure 4.5a). From initial inspection, there appeared to be an overall decrease in the time animals spent in thigmotaxis as training progressed, with a mean on Day 1 of $56.49\pm4.20\%$ reduced to $38.54\pm5.21\%$ by Day 5. A 2 x 5 mixed factorial ANOVA revealed no differences in time spent in thigmotaxis between groups $[F(1, 14) = 1.91, p>0.05]$, there was also no interaction effect between day and group $[F(4, 56) = 2.12, p>0.05]$. However, an overall effect for day was found $[F(4, 56) = 4.61, p<0.01]$, with the percentage time on Day 1 significantly higher than Days 4 $(p<0.01)$, and 5 $(p<0.05;$ Bonferroni-adjusted t-tests). Further assessment revealed that only the Near shams had a significant decrease in this behaviour across training $[F(4, 28) = 7.19, p<0.001]$, unlike the Near DH animals who did not significantly reduce their time spent in thigmotaxis despite continued training, with performance, instead, remaining relatively stable across days $[F(4, 28) = 0.89, p>0.05]$.

The mean percentage time spent by animals in parallel and vertical thigmotaxis was also assessed (Figure 4.5b and c). A 2 x 5 mixed factorial ANOVA investigating parallel thigmotaxis during acquisition revealed no significant main effect for day $[F(4, 56) = 0.58, p>0.05]$ or group $[F(1, 14) = 1.0, p>0.05]$. There was also no interaction effect between day and group $[F(4, 56) = 0.43, p>0.05]$ indicating a similar level of performance by both groups in parallel thigmotaxis. Following this, the mean percentage time spent by animals in vertical thigmotaxis was investigated. A 2 x 5 mixed factorial ANOVA revealed a main effect for day $[F(4, 56) = 12.34, p<0.001]$

with Bonferroni-corrected t-tests revealing that the time spent in vertical thigmotaxis on Day 1 (M: $34.38\pm4.15\%$) was significantly higher than Days 2 (M: $18.80\pm1.95\%$, $p<0.01$), 3 (M: $13.81\pm2.46\%$, $p<0.05$), 4 (M: $12.87\pm3.16\%$, $p<0.05$), and 5 (M: $9.46\pm2.01\%$, $p<0.01$), suggesting an overall reduction in the behaviour as animals became more familiar with the training environment. There was, however, no main effect for group [$F_{(1, 14)} = 1.76$, $p>0.05$] nor interaction effect between day and group [$F_{(4, 56)} = 1.87$, $p>0.05$]. Further assessment revealed that the Near sham groups' time spent in vertical thigmotaxis decreased with continued training [$F_{(4, 28)} = 16.99$, $p<0.001$]. The Near DH group, on the other hand, did not reduce their performance of this behaviour as training progressed [$F_{(4, 28)} = 1.99$, $p>0.05$].
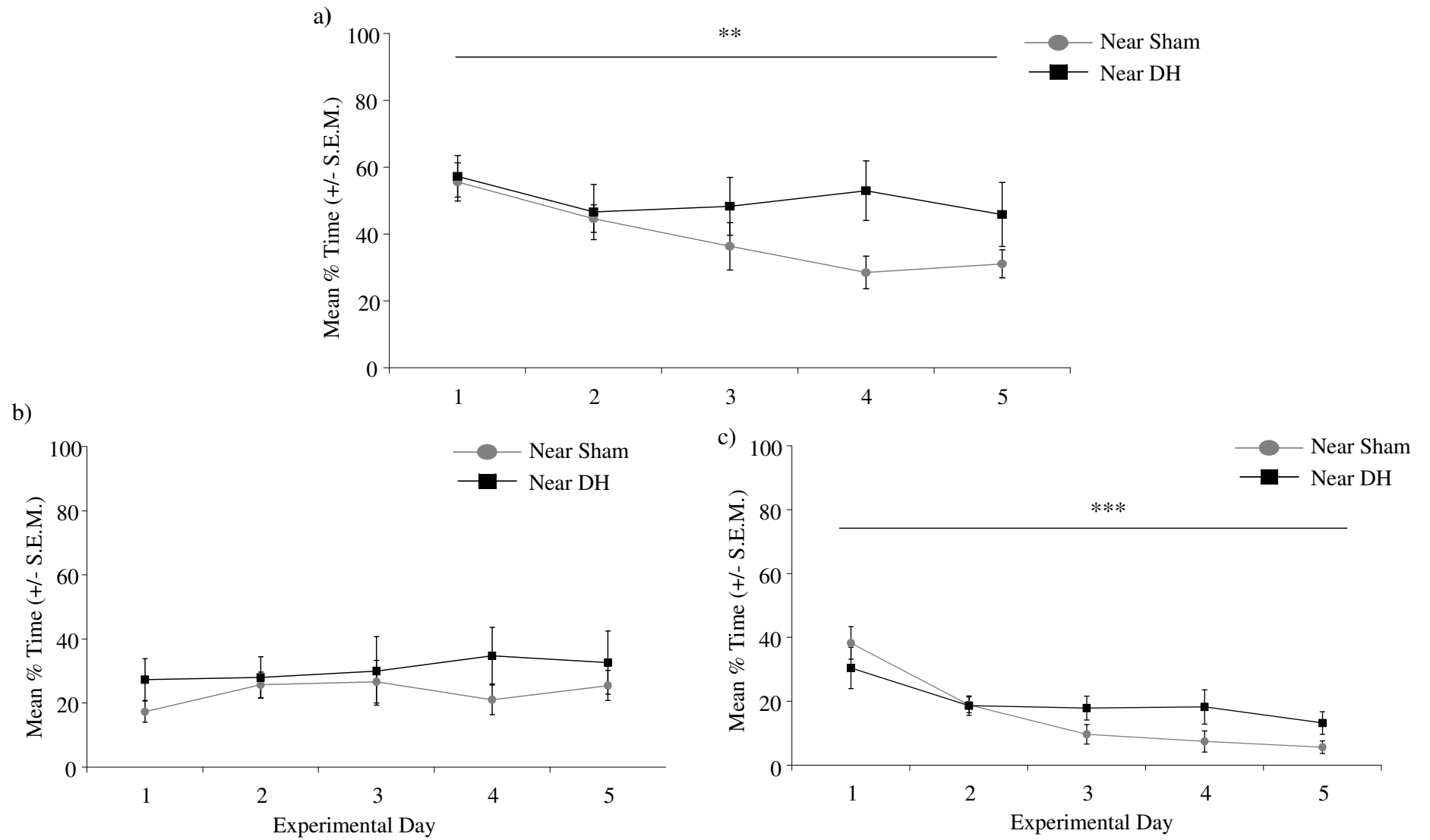
Figure 4.5: Mean percentage time spent by the Near sham and Near DH animals in a) total thigmotaxis, b) parallel thigmotaxis and c) vertical thigmotaxis throughout training.

The location at which animals performed parallel and vertical thigmotaxis in the pool was also investigated. Assessment of parallel thigmotaxis (data not shown) revealed a peak in performance on Day 1 at 90-100° for the Near sham group, which is at the approximate location of the white card cue. Further to this, however, there are few peaks at any particular location for the Near sham and DH groups on any of the remaining days, with performance equal across degree points around the maze.

Interestingly, however, vertical thigmotaxis revealed a number of differences between the groups (see Figure 4.6). Animals in the Near sham group, on Day 1 and 2 in particular, had a peak in performance between 180-220°, falling between the location of the two light cues. A similar finding was also noted for Day 2. However, as training continued, the number of occurrences of this behaviour decreased, with few distinguishable peaks visible, corresponding with the overall reduction in time spent performing this behaviour (this was a similar pattern to that observed in the previous Chapter). On the other hand, the Near DH group showed a different pattern of performance. On Day 1 there was some evidence of animals' focus towards the cues with a slight peak at 140°, close to the light cue. Day 2, however, showed a more sporadic performance, with the mean number of performances generally ranging equally across all degree locations. On Day 3, a peak in vertical thigmotaxis was noted at the cues between 170-230°. Additionally, peaks within range of the cues appeared for the Near DH group on Days 4 and 5, respectively (170-190°; 190-200°). An additional, subtle difference between groups is the peak that appears at approximately 350-20°, in the Near DH group on Days 3 to 5. This peak falls outside the range of the distal cues and is not seen in the Near sham animals' behaviour.
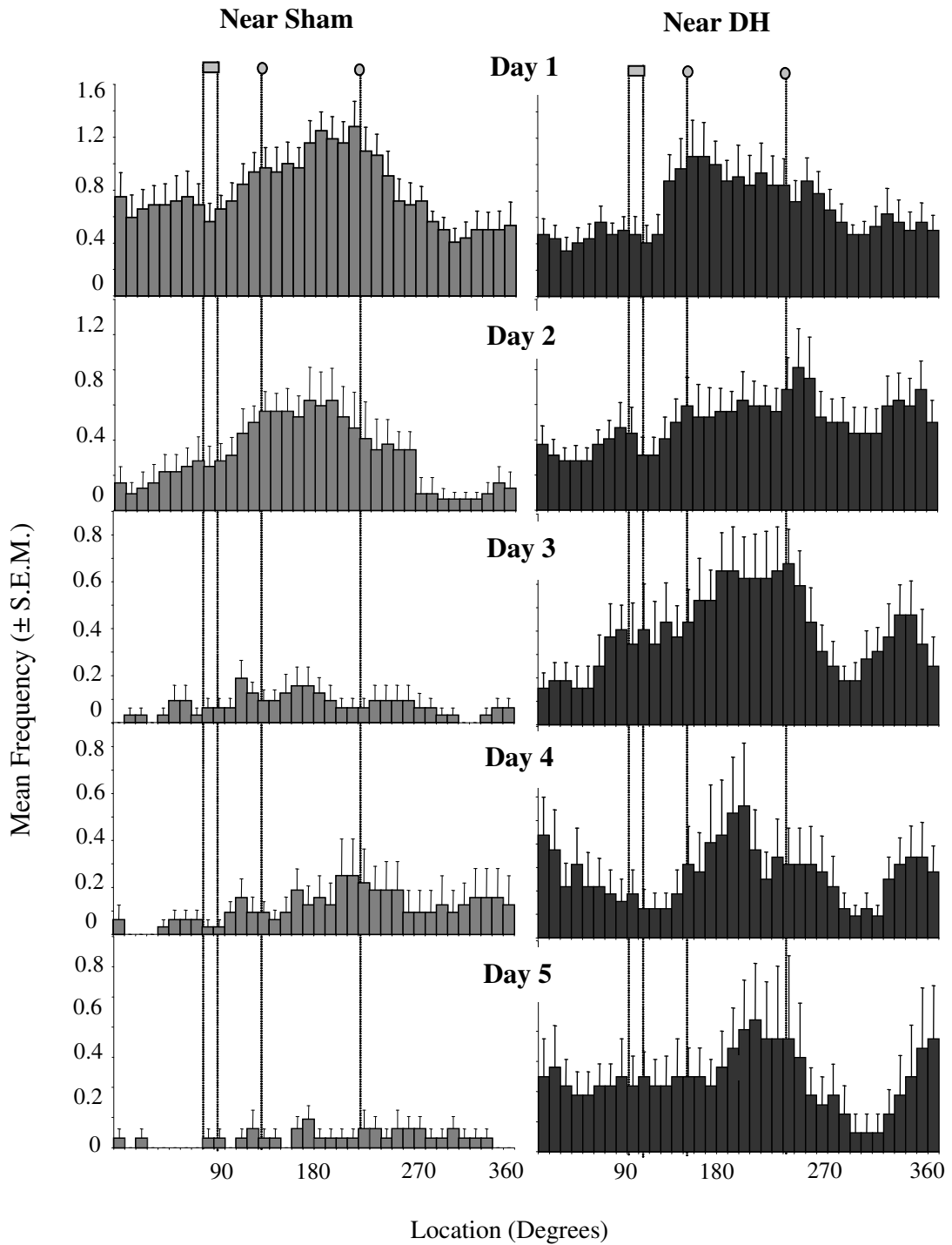
Figure 4.6: Mean number of occurrences of thigmotaxis vertical at each degree location (0-360°) around the Morris water maze across training days for Near shams (grey) and Near DH (dark grey) animals. Included are the locations of the distal cues (insert).

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4.3.4.2 Direct

The next behaviour investigated was direct behaviour (i.e. movement in a definite direction for at least 1 second), where the mean percentage time and location of direct swims were analysed for both Near sham and Near DH lesioned animals. Overall, the mean percentage time spent by Near sham animals in direct behaviour appeared to increase with continued training (Day 1, M: 14.79±1.17%; Day 5, M: 31.99±4.72%). Conversely, the mean percentage time spent by the Near DH group, in this behaviour, appeared to decrease across days (Day 1, M: 16.94±3.10%; Day 5, M: 14.34±3.65%). A 2 x 5 mixed factorial ANOVA was carried out on data from both groups and from this it was noted that there was no overall effect for day [$F_{(4, 56)} = 1.78$, $p > 0.05$]. However, an overall effect for group was found [$F_{(1, 14)} = 1.82$, $p < 0.05$], with Near sham animals (M: 22.86±3.09%) spending significantly more time in direct behaviour than the Near DH lesioned animals (M: 16.17±1.62%; see Figure 4.7). An interaction effect between day and group [$F_{(4, 56)} = 5.8$, $p < 0.001$] was also noted with further independent t-tests revealing differences between the groups on Days 3 ($t_{(14)} = 3.20$, $p < 0.01$) and 5 of training ($t_{(14)} = 3.17$, $p = 0.01$). A repeated measures ANOVA revealed that the Near sham group spent an increasing amount of time performing direct behaviour as training continued [$F_{(4, 28)} = 5.39$, $p < 0.01$], however, it was found that the Near DH animals did not alter their performance of direct swims, displaying no significant increase or decrease in the time spent in this behaviour with continued training [$F_{(4, 28)} = 1.82$, $p > 0.05$].

Following this, the location towards which the groups headed when in direct behaviour was also investigated. For this, locations were, once again, segmented as described in Chapter 3a. Briefly, to examine the time spent by the animals heading

towards the cues we grouped a segment of the pool containing cues which ranged from $60\text{-}240^{\circ}$ and compared this area to the region of the maze where no cues were present (i.e. $240\text{-}60^{\circ}$). Dependent t-tests revealed a significant increase in heading towards the cued segments compared to the non-cued regions for the Near sham group on Day 1 ($t(7) = 3.56$, $p<0.01$), Day 3 ($t(7) = 3.86$, $p<0.01$), Day 4 ($t(7) = 3.48$, $p<0.01$) and Day 5 ($t(7) = 7.17$, $p<0.001$; Figure 4.8). The Near sham animals displayed no significantly preferred heading on Day 2 only ($t(7) = 1.75$, $p>0.05$). The Near DH animals' direct behaviours were also examined in a similar manner. Unlike the Near shams, however, the Near DH group only headed towards the cues on Day 3 ($t(7) = 2.54$, $p<0.05$). All other days had no statistically significant preferred location for direct behaviour; [Day 1: $t(7) = 0.33$, $p>0.05$; Day 2: $t(7) = 0.76$, $p>0.05$; Day4: $t(7) = 0.84$, $p>0.05$; Day5: $t(7) = 1.12$, $p>0.05$]. Overall, this would suggest that there was no obvious preferred location for direct behaviour for the Near DH animals (Figure 4.8).
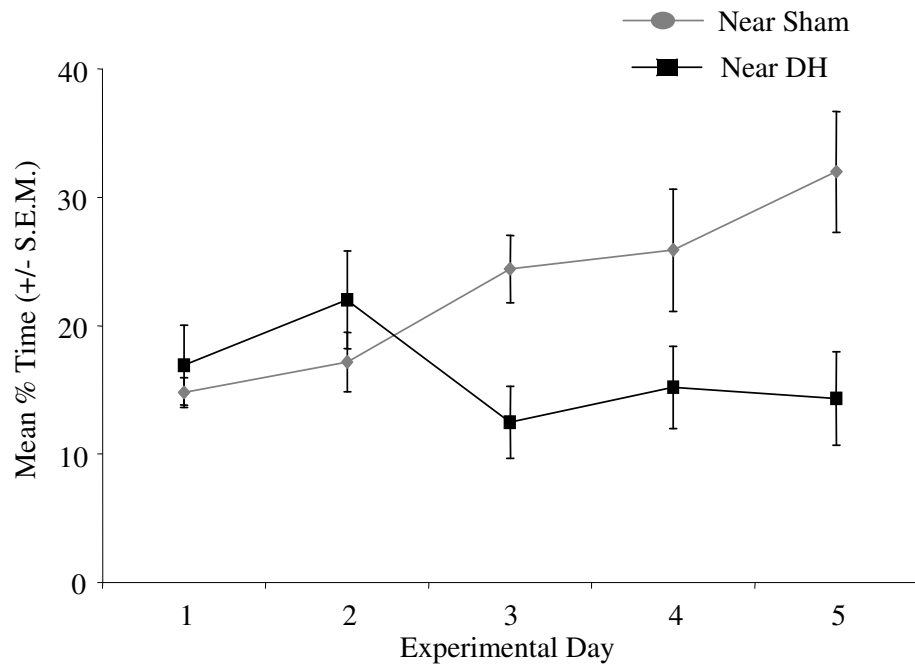
Figure 4.7: Mean percentage time (± S.E.M.), of total time in the maze, spent in direct behaviour, on all training days, of the Near sham and Near DH groups.
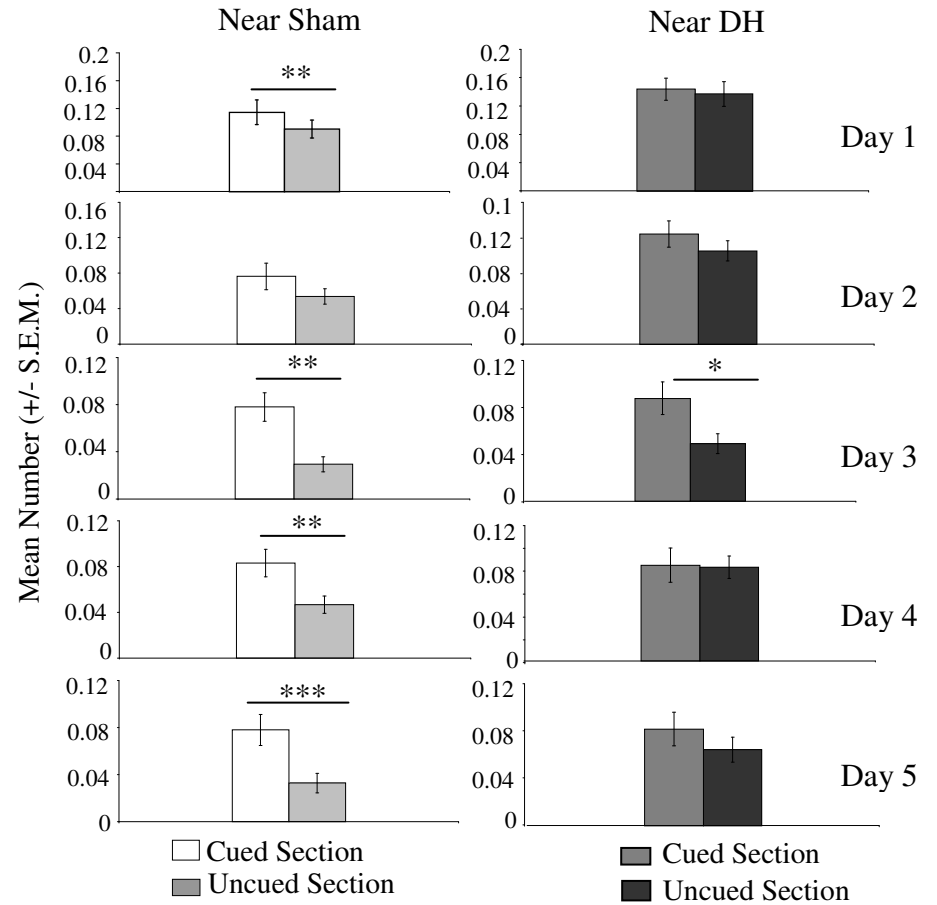
Figure 4.8: Mean number of occurrences of direct behaviour (± S.E.M.) in cued (encompassing Near cues from 60-240°) and uncued (no distal cues at 240-60°) sections in the maze for the Near sham and Near DH lesioned groups across training days.

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4.3.4.3 Turning Behaviour

The mean overall number of turns (including both turns towards and turns-away from the cues) made by each group of animals was initially examined (see Figure 4.9). A 2 x 5 mixed factorial ANOVA revealed an overall significant difference in the mean number of turns made throughout acquisition [$F(4, 56) = 2.59$, $p<0.05$], with subsequent Bonferroni-adjusted t-tests revealing the highest number of turns were performed on Day 1 (M: 24.56 ± 2.36) when compared to Day 5 (M: 15.75 ± 2.09, $p<0.05$). There was also no main effect for group [$F(1, 14) = 3.1$, $p>0.05$] nor interaction effect between day and group [$F(4, 56) = 1.00$, $p>0.05$], indicating that both groups performed a similar number of turns for the entire training period.
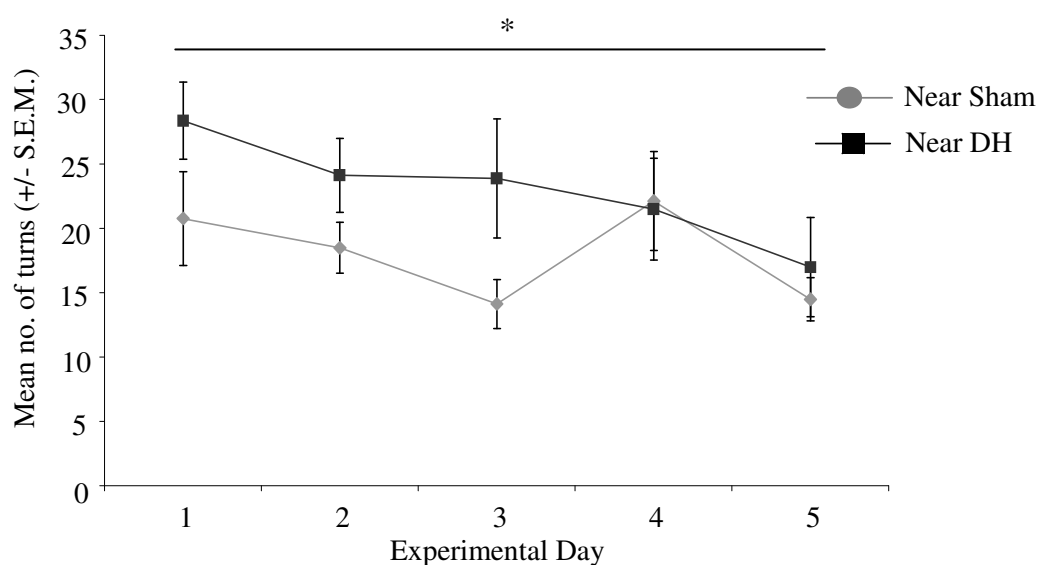
Figure 4.9: Mean overall number of turns made (±S.E.M.) by the Near sham and Near DH group across training.

Further examination revealed that the mean number of turns made *towards* the cues by Near sham animals across days did not differ greatly, for example, the mean for Day 1 was 12.75 ± 1.83, Day 3 was 10.25 ± 1.39 and Day 5 was 11.25 ± 0.73. The

mean number of turns towards the cues for the Near DH group, however, decreased as training progressed; the mean on Day 1 was 18.01±2.67, Day 3 was 14.63±2.90 and Day 5 was 10.63±2.51 (Figure 4.10a and b). A 2 x 5 mixed factorial ANOVA, however, revealed no significant main effect for day [$F_{(4, 56)}$ = 1.58, $p > 0.05$] or group [$F_{(1, 14)}$ = 0.52, $p > 0.05$], with no interaction effect between day and group being noted [$F_{(4, 56)}$ = 1.91, $p > 0.05$].

The spatial position of turns made towards cues was subsequently assessed. When evaluated, it was revealed that the turn positions for each respective cue were within refined locations of the pool and not randomly dispersed throughout the maze for the Near sham group (See Figure 4.11a). In fact, significantly preferred locations were identified for turns towards each cue on Days 1, 3 and 4 using Rayleigh Uniformity tests (see Table 4.3). Significantly preferred positions were not seen for turns towards the blue cue on Days 2 and 5 only. Watson William F-tests were also used to examine any change in turn locations across days. For the Near sham group, turns towards the blue and red cue remained stable throughout training with no significant differences in turn location across days (all $p > 0.05$), suggesting relatively steady cue use between training days. When the mean location of turns for the green cue were examined, however, there appeared to be some change in the location at which animals performed their turns with significant differences found between Day 1 and Days 4 [$F_{(1, 73)}$ = 7.87, $p < 0.01$] and 5 [$F_{(1, 62)}$ = 8.42, $p < 0.01$], and between Day 2 and Day 3 [$F_{(1, 45)}$ = 4.44, $p < 0.05$], and between Day 3 and Days 4 [$F_{(1, 63)}$ = 10.17, $p < 0.01$], and 5 [$F_{(1, 52)}$ = 11.85, $p < 0.001$]. This fluctuation in turn positions may reflect the larger surface area occupied by this cue (i.e. the cue card).

Interestingly, the Near DH group also showed significantly preferred turn positions towards the distal cues on all days (Figure 4.11b and Table 4.4). In addition, Watson William F-tests revealed that only Day 2 turns towards the blue cue were significantly different to the other training days, with the exception of Day 3 [Days 1: $F(1, 96) = 6.16$, $p<0.05$; Day 4: $F(1, 86) = 6.34$, $p<0.05$, Day 5: $F(1, 71) = 8.68$, $p<0.01$]. Similarly, the location of turns for the red cue on Day 1 was revealed to be significantly different to Day 2 [$F(1, 93) = 8.35$, $p<0.01$], Day 3 [$F(1, 86) = 4.91$, $p<0.05$], and Day 4 [$F(1, 75) = 13.13$, $p<0.001$]. Day 4 was also found to be significantly different to Day 5 [$F(1, 58) = 4.54$, $p<0.05$]. When the mean location of turns for the green cue were examined significant differences were found between Day 1 and Days 2 [$F(1, 61) = 4.29$, $p<0.05$], 3 [$F(1, 71) = 10.02$, $p<0.01$], and 4 [$F(1, 61) = 5.66$, $p<0.05$].

Watson-William F tests were also used to assess any differences in turn locations between groups. Significant differences between the Near sham and Near DH group were only apparent for the location of turns towards the red and green cue on Day 1 only [Red: $F(1, 76) = 5.17$, $p<0.05$; Green: $F(1, 66) = 10.22$, $p<0.01$; see Table 4.5], indicating that both groups focused their attention towards the cues in a similar manner when performing turning behaviours.
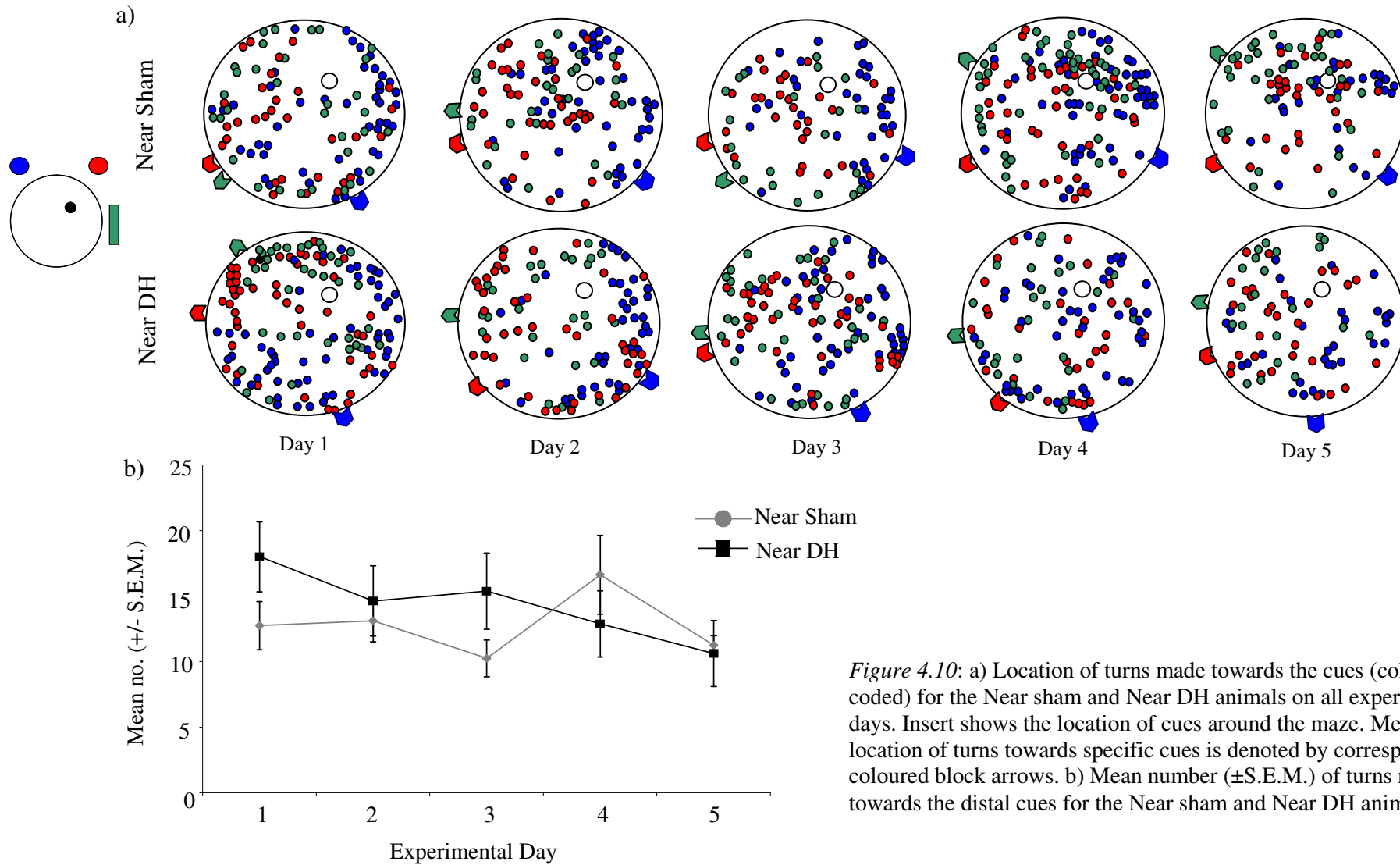
Figure 4.10: a) Location of turns made towards the cues (colour coded) for the Near sham and Near DH animals on all experimental days. Insert shows the location of cues around the maze. Mean location of turns towards specific cues is denoted by corresponding coloured block arrows. b) Mean number (±S.E.M.) of turns made towards the distal cues for the Near sham and Near DH animals.

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a)
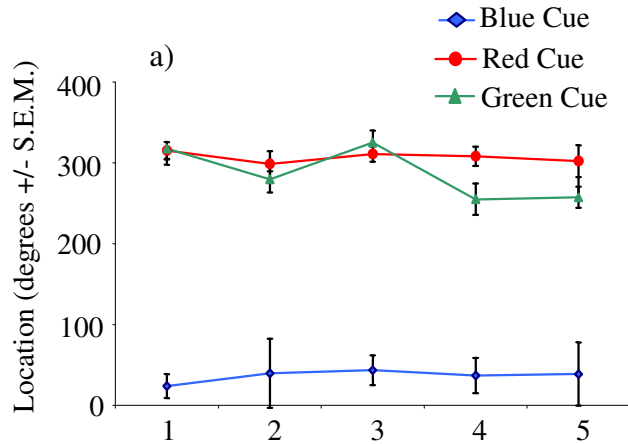
Table 4.3: Rayleigh Uniformity test results of the mean position of turns towards each cue, for the Near sham group, over 5 days.

Near Sham Lesioned Group

Cue	Day 1			Day 2			Day 3			Day 4			Day 5		
	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green
Mean	23.9	315.4	317.5	39.7	298.8	279.9	43.6	310.9	325.02	37.1	308.2	254.9	38.8	302.3	257.5
Z Score	6.79	11.68	3.93	0.89	15.15	5.35	4.58	12.93	6.07	3.36	9.91	4.19	1.05	3.97	8.42
p	***	***	*	-	***	**	**	***	**	*	***	*	-	*	***

b)
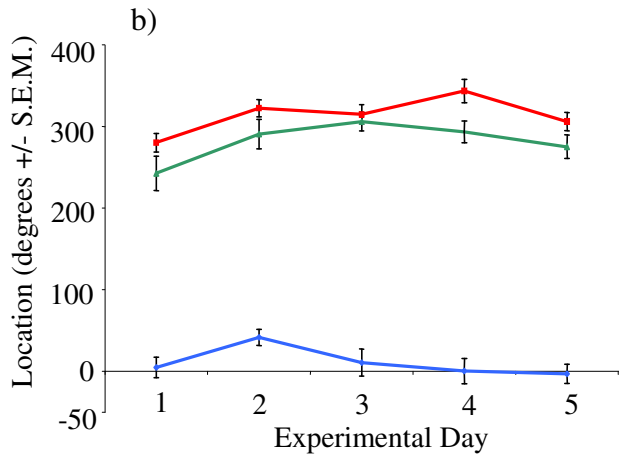
Table 4.4: Rayleigh Uniformity test results of the mean position of turns towards each cue, for the Near DH group, over 5 days.

Near Dorsal Hippocampal Lesioned Group

Cue	Day 1			Day 2			Day 3			Day 4			Day 5		
	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green	Blue	Red	Green
Mean	4.8	280.4	242.7	41.7	322.76	290.8	10.7	315.04	306.2	0.5	343.7	293.6	357	306.3	275.4
Z Score	9.63	10.98	3.19	13.83	12.94	4.48	5.62	9.82	10.38	6.31	6.95	7.69	9.77	10.51	6.48
p	***	***	*	***	***	**	**	***	***	**	***	***	***	***	***

Figure 4.11: a) Near sham group and b) Near DH group, mean location (± S.E.M) of turns made towards each respective cue across training days.

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Table 4.5: Mean ± S.E.M. and Watson-William F tests results comparing differences in the location of turns towards each cue for the Near sham and Near DH groups. Denotations for p-values: **-** non-significant, ***** p<0.05, ****** p<0.01.

	Blue Cue			Red Cue			Green Cue		
Day	Sham	DH	p	Sham	DH	p	Sham	DH	p
D1	23.9±14.9	4.8±12.5	-	315.4±10.4	280.4±11.4	*	317.5±19.8	242.7±21.2	**
D2	39.7±42.7	41.7±9.8	-	298.8±9.0	322.7±10.4	-	279.9±16.5	290.8±18.3	-
D3	43.6±18.3	10.7±16.5	-	310.9±9.5	315.0±12.0	-	325.0±15.1	306.2±11.5	-
D4	37.1±21.7	0.5±15.5	-	308.2±11.9	343.7±14.4	-	254.9±19.3	293.6±13.4	-
D5	38.8±39.1	357±11.7	-	302.3±19.6	306.3±11.2	-	257.5±12.9	275.4±14.5	-

4.3.4.4 Turns-Away

Following the examination of the turns made towards the distal cues, turns animals made away from the external cues were investigated in the same manner. As above, the mean number of turns-away was initially examined to determine if this behaviour changed as training progressed. Overall, Near sham animals' mean number of turns-away appeared to decrease with training, with a mean on Day 1 of 8.01±2.09 and Day 5 of 3.25±0.99. There was also somewhat of a decrease for the Near DH group across days with a mean of 10.38±1.06 on Day 1 and 6.38±1.46 on Day 5 (Figure 4.12b). Further to this, a 2 x 5 mixed factorial ANOVA revealed a significant main effect for day [$F_{(4, 56)} = 3.02$, $p < 0.05$]. Subsequent Bonferroni-corrected t-tests, however, revealed no differences between any of the training days ($p > 0.05$). Furthermore, a main effect for group was found [$F_{(1, 14)} = 10.05$, $p < 0.01$] where the Near DH animals made more turns-away overall (M: 8.67±0.66) compared to the Near sham group (M: 5.2±0.82), perhaps suggesting that the DH animals needed to perform more turns in attempts to reorient in the maze. Finally, there was no interaction effect between day and group [$F_{(4, 56)} = 0.23$, $p > 0.05$].
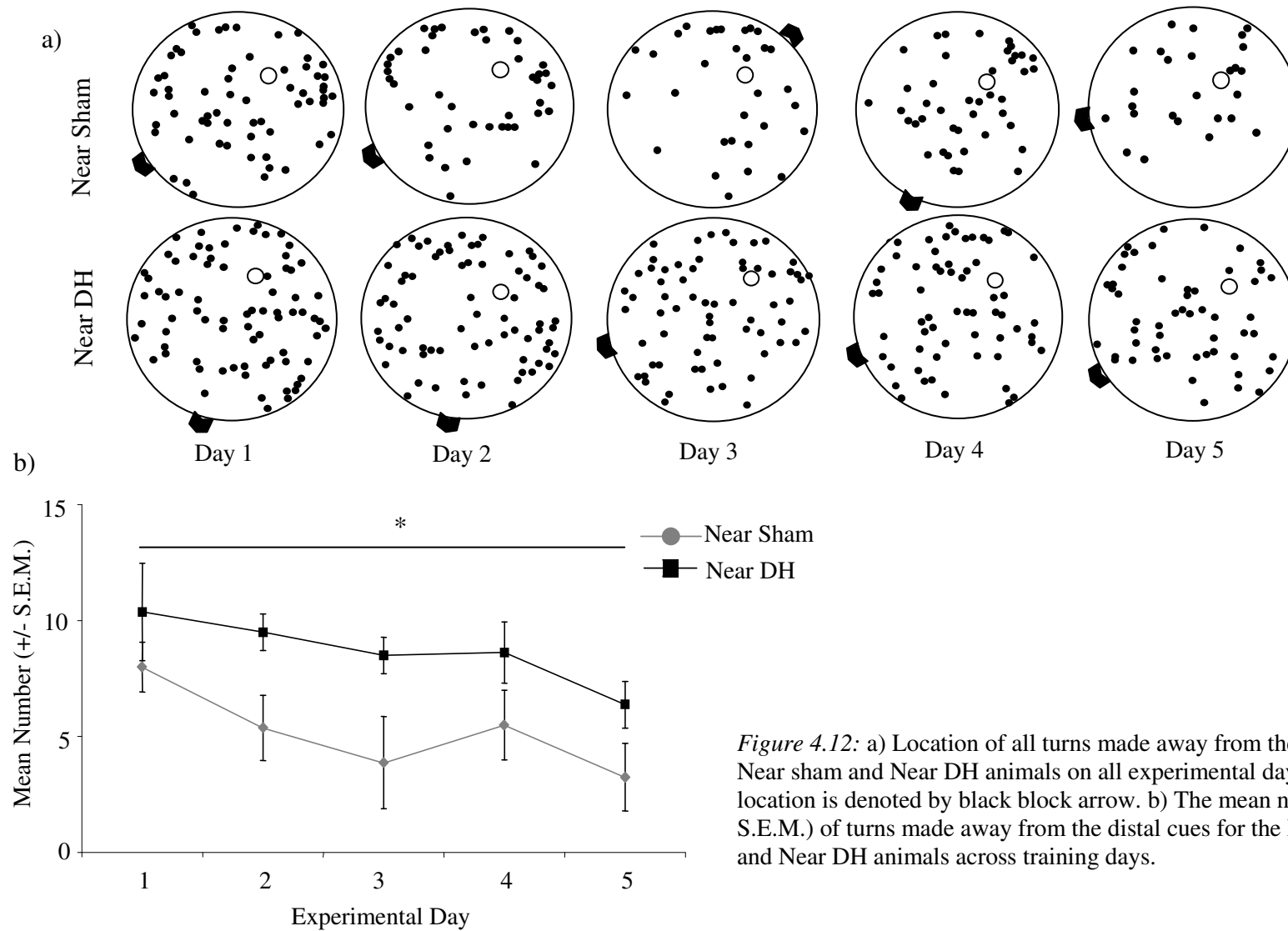
Figure 4.12: a) Location of all turns made away from the cues for the Near sham and Near DH animals on all experimental days. Mean location is denoted by black block arrow. b) The mean number (± S.E.M.) of turns made away from the distal cues for the Near sham and Near DH animals across training days.

4.3.4.5 Turns in Zones

As subtle differences between the groups were seen when turn locations were examined, we subsequently investigated the distance at which the turns were made in relation to the platform. To examine this, the pool was divided into three zones (near, middle and far) according to distance from the platform (see Chapter 3a for details). A 3 x 5 repeated measures ANOVA was initially conducted to assess the mean number of turns made in each zone, throughout training, for Near sham animals (Figure 4.13a). No overall day effect was found [$F(4, 28) = 2.92$, $p>0.05$]. However, a main effect for zone was revealed [$F(2, 14) = 50.19$, $p<0.001$] with subsequent Bonferroni corrections illustrating the highest mean number of turns occurred in the far zone (M: 9.07±1.24), which was significantly different to both the near (M: 3.33±0.54; $p<0.001$) and middle zones (M: 5.57±0.96; $p<0.01$), overall. There was also an interaction effect between day and zone [$F(8, 56) = 5.01$, $p<0.001$]. When studied in more detail using daily one-way repeated measures ANOVAs, a significant difference between the zones on Day 1 [$F(2, 14) = 24.31$, $p<0.001$], 2 [$F(2, 14) = 9.87$, $p<0.01$], 3 [$F(2, 14) = 23.83$, $p<0.001$] and 4 [$F(2, 14) = 6.57$, $p<0.05$] was revealed. Significant differences between the near and far zone ($p<0.01$) and the middle and far zones ($p<0.05$) were noted on Day 1, with highest turns presented in the far zone. A similar finding was revealed on Day 2, 3 and 4 with most turns occurring, once again, in the far zone ($p<0.05$; Figure 4.13a). There were no differences in the mean number of turns made in each zone on Day 5 [$F(2, 14) = 1.28$, $p>0.05$]. Results suggest that the location in which Near shams perform turns shifts throughout training, with turns being performed closer to the platform by Day 5.

The mean number of turns in each zone was also assessed for the Near DH group. A 3 x 5 repeated measures ANOVA revealed no day effect [$F_{(4, 28)}$ = 2.37, $p > 0.05$]. However, a main effect for zone [$F_{(2, 14)}$ = 29.04, $p < 0.001$] with an interaction effect between day and zone was found [$F_{(8, 56)}$ = 3.26, $p < 0.01$]. Subsequent Bonferroni-corrected t-tests revealed that the highest mean number of turns were in the far zone (M: 12.90±1.91), which was significantly different to both the near (M: 2.71±0.67; $p < 0.001$) and middle zones (M: 6.92±1.62; $p < 0.05$) overall. Further comparisons using a one-way repeated measures ANOVA revealed differences between zones on Day 1 [$F_{(2, 14)}$ = 26.86, $p < 0.001$], 2 [$F_{(2, 14)}$ = 42.35, $p < 0.001$], 3 [$F_{(2, 14)}$ = 8.26, $p < 0.01$], 4 [$F_{(2, 14)}$ = 11.31, $p = 0.001$], and Day 5 [$F_{(2, 14)}$ = 8.77, $p < 0.01$]. Subsequent Bonferroni comparisons revealed differences between all zones on Days 1 and 2 ($p < 0.05$), and differences between the near and far zones on Days 3 ($p < 0.01$), 4 ($p < 0.05$), and 5 ($p < 0.05$), suggesting that the Near DH group did not perform their turns closer to the platform despite continued training in the maze (see Figure 4.13b).
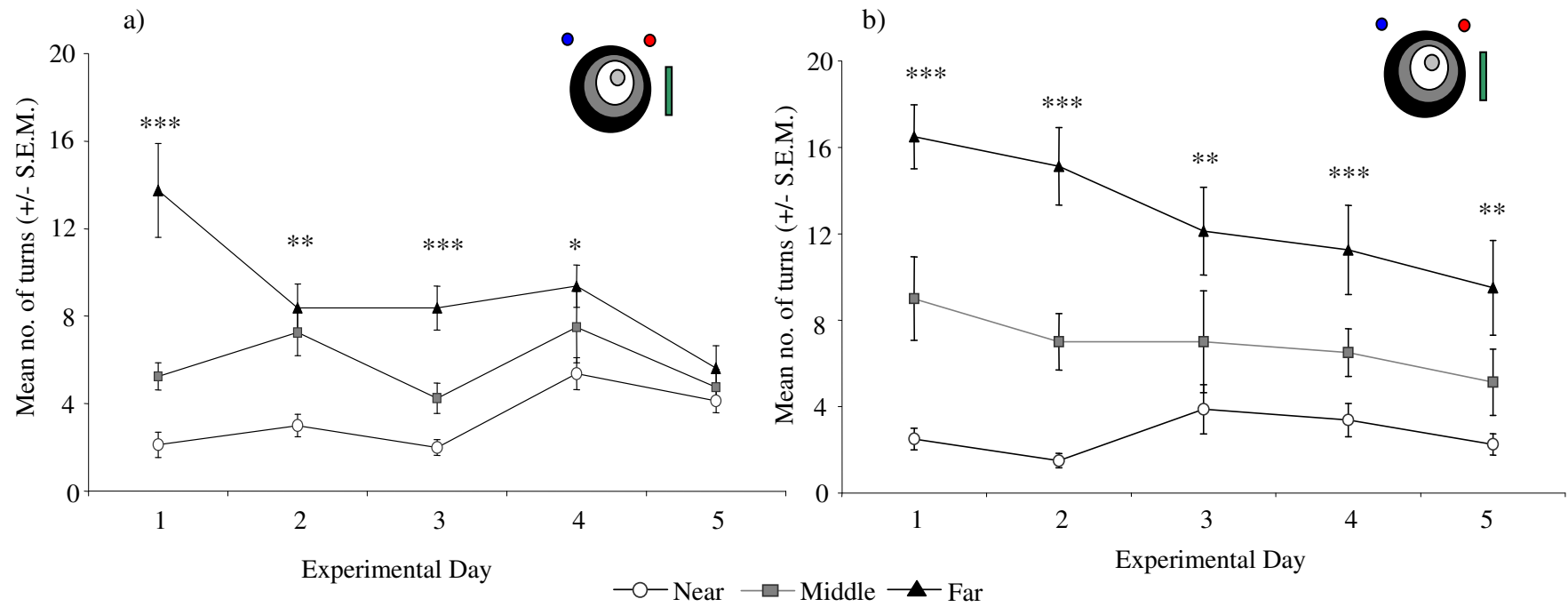
Figure 4.13: Mean number (± S.E.M.) of turns in the near, middle and far zones for a) the Near Sham and b) Near DH groups over 5 days of training. Inset; Schematic representation of the three zones used for analysis

4.4 Summary

Analysis of the data indicates that, while the Near dorsal hippocampal lesioned animals are impaired in learning the task, both the sham and DH groups use similar types of swimming behaviours in combination to solve the task. However, subtle differences between the groups' exploratory behaviours, revealed through in-depth analysis, highlighted impairments in the Near DH group in solving the task. Specifically, the DH group appear to be unable to effectively inhibit maladaptive behaviours from early in training. For example, while both groups initially avail of thigmotactic behaviour, particularly at the location of the cues, the Near DH animals' are impaired in their ability to reduce their overall level of performance of thigmotaxis throughout the training period. Specifically, while the Near shams' level of thigmotaxis decreased early in training, the Near DH animals did not reduce their time spent performing the behaviour as efficiently, or to the same extent, suggesting some perseveration of behaviour. The location at which thigmotaxis was performed also highlights an impairment in the lesioned animals ability to alter their behaviour. Specifically, sham animals' vertical thigmotaxis was performed at peaks located at the external cues from as early as Day 1 of training; however, similar peaks were not seen until Day 3 for the Near DH animals. Despite the inability to effectively adapt their swimming behaviour, the Near DH animals still recognised the importance of the external cues, as they eventually altered the location at which vertical thigmotaxis was performed, and concentrated this behaviour towards the environmental stimuli. Similarly, when turns towards the cues were assessed, both the Near sham and Near DH animals performed turns at the same, cue-appropriate locations in the maze.

However, this recognition of the value of the external cues alone did not enable accurate performance in the maze by the Near DH animals. Rather, further assessment of exploratory movements indicates that animals display significant navigational difficulties following hippocampal ablation. Specifically, while lesioned animals retain the ability to perform direct swims they are unable to maintain this performance, spending less time overall in the behaviour than the Near sham animals. Furthermore, they also often head in inappropriate directions, with the location at which the direct swims were performed being less focused in the Near DH group; only becoming directed towards the cues on latter training days. In addition, lesioned animals also displayed impaired processing of distance, with the majority of their turns being located at the periphery of the pool rather than moving closer to the platform. Alongside this, the DH animals also perform more turns-away from the cues than the shams, in attempts to reorient in the maze. Therefore, overall, it appears that a delay in altering behaviours initially, combined with navigational difficulties such as less direct behaviour and more turns-away from the cues, leads to the Near DH animals being less successful in solving the task. In-depth discussion of the implications of these findings will be conducted later (see Chapter 8).

Chapter 5

The effect of dorsal hippocampal lesions on exploratory behaviour under Far cues Morris water maze training conditions.

Abstract

In-depth behavioural examination of animals trained with distal cues in either a near or far position, revealed that cue location is an important factor in determining the strategies employed by animals in solving the MWM (Chapter 3a). In addition, results from Chapter 4 revealed that animals with dorsal hippocampal lesions, trained in the Near cue MWM, have altered behaviour when compared to sham controls, manifesting in perseveration and delay in altering their navigational strategies. We proposed that this may be due to hippocampal damage, leading to disruption in behavioural rather than spatial responses. However, the relative contribution of the hippocampus to spatial and behavioural components of the task remains ambiguous. Here, we trained animals with sham (Far sham; n=8) and dorsal hippocampal lesions (Far DH; n=7) to locate a hidden platform, using distal cues that are in a position further away from the goal (i.e. a more cognitively demanding task), to determine hippocampal function in the MWM. A detailed behavioural analysis of the animals' swimming movements during training trials and head directions while on the platform was performed. Results indicate that Far DH lesioned animals are significantly impaired in the MWM task generally. They also demonstrate subtle differences in their pattern of behaviour during acquisition when compared to sham animals, such as delays in altering their behaviours to more efficient search strategies, as well as performing cue-directed behaviours at inaccurate locations in the pool.

5.1 Introduction

Chapter 4 findings revealed differences between the Near sham and Near DH lesioned animals in overall measures of acquisition. In addition to this, Near DH animals also displayed behavioural deficits, with evidence of perseveration of behaviour and an inability to efficiently reorient in the pool using the cues. From this, we proposed that hippocampal lesions may lead to navigational impairments rather than purely, spatial memory deficits in the water maze. However, as the cues were in a position close to the goal, it is difficult to definitively say this, as there is less inference and putatively less hippocampal involvement needed during the Near cues version of the task (as shown in Chapter 3a and 3b).

As previously discussed, when the hippocampus is damaged, lesioned animals can still successfully learn tasks that require less spatial processing, or that are based on egocentric guidance (Mogensen et al., 2005; Rasmussen et al., 1989; Schenk & Morris, 1985). However, as spatial demands increase in a task, for example, when the position of a goal must be inferred using information gained from external cues (i.e. using allocentric information), hippocampal lesioned animals performance declines (Jarrard, 1993; Kim & Lee, 2011; Potvin et al., 2009). In the water maze, this deficit is seen repeatedly (Cassel et al., 1998; DiMattia & Kesner, 1988; Morris et al., 1982; Sutherland et al., 1983) and tends to remain even when protocols that move away from the traditional place training regime are employed. For example, lesions of the hippocampus lead to impairments in an annular water maze, where the position of the goal had to be inferred from distal information (Hollup et al., 2001). Similar deficits were also seen under conditions where multiple beacon cues were available and the

spatial location of the correct beacon marking the goal was defined only by a configuration of distal cues (Clark et al., 2007).

Alongside lesion data, information from hippocampal place cells has also indicated a role for the hippocampus in the organisation of distal information. Cressant et al. (1997) recorded place cell firing under both proximal and distal cue conditions to assess the relative importance of cue positioning on hippocampal activity. Interestingly, it was found that when objects that were in close proximity to the animal were rotated, the place cell firing field became unstable, suggesting that proximal cues could not control the angular position of the fields. However, when the objects were moved further away to a more distal position, they gained more accurate control over the place fields of hippocampal cells and displayed stable firing patterns in line with the rotation of the distal cues. These results suggest that the position of objects in the environment may be critical in determining the extent of hippocampal involvement in the processing of spatial information.

Therefore, as the abovementioned and our previous findings (Chapter 3a and 3b) highlight the importance of considering cue location when examining performance in the water maze, we now aim to examine the effect of cue positioning, further away from the hidden platform, on hippocampal lesioned animals' ability to solve the MWM. While there is ample evidence for the increased importance of the hippocampus in the processing of information from distal cues to infer a goal position, there is some ambiguity surrounding the underlying processes that prevent successful navigation in such tasks. Furthermore, lesioned animals' exploratory behaviours have been shown to be significantly altered during cognitively demanding spatial tasks, leading to suggestions that the hippocampus may be involved in monitoring

movements rather than purely spatial processes (Eichenbaum et al., 1990; Wallace et al., 2002b; Whishaw et al., 1994; Whishaw, 1998a). Therefore, in-depth behavioural analysis will be used here to determine the extent of differentiation in swimming patterns between Far sham and Far dorsal hippocampal (DH) lesioned animals. We hypothesise that the Far DH group will be impaired in the MWM, generally, and that this impairment will manifest as a deficit in view-independent search patterns, which are critical for successful performance in the Far training condition in the maze (see FT group in Chapter 3a).

5.2 Method

5.2.1 Subjects

Male Wistar rats (n=15), obtained from Charles River Laboratories, UK, were subjects in the current study. Animals were approximately three months old and weighing 200-300g at the beginning of experimentation. All subjects were handled as described in previous chapters.

5.2.2 Apparatus

The hidden platform version of the MWM task was used in this experiment with three distal cues in the far cue position (see Chapter 3a), and the submerged escape platform remaining in a fixed position throughout training, in the NE quadrant. As in Chapter 3a the platform and swimming behaviour of the animals was recorded and later used for in-depth behavioural analysis.

5.2.3 Surgery

Subjects were randomly assigned to one of two surgery groups: a sham control group (Far sham; n=8) or a dorsal hippocampal lesion group (Far DH; n=7). All surgical procedures were as described in Chapter 4.

5.2.4 Procedure

Following a recovery period from surgery, all animals were trained with cues in the far location in the MWM, as previously described (see Chapter 3a). Retention was assessed 7 days post-acquisition by allowing animals a single 60 sec trial with the platform removed from the water maze (see Chapter 6 for retention analysis).

5.2.5 Assessment of the platform interval

Animals' head movements while on the platform were digitally recorded and analysed as previously detailed (see Chapter 3a). For the current study, there were 4500 digital stills (i.e. 15 animals x 4 trials x 15 seconds x 5 days) of animal head directions.

5.2.6 Searching strategies used during in trial locomotion

Swimming behaviours, as outlined in Chapter 3a and 4 were also examined for Far sham and Far DH groups. EthoVision (Noldus Information Technologies, Wageningen, Netherlands) provided *x, y* coordinates (0.2s increments apart) for the animal's position throughout each trial and these tracks were subsequently examined in depth alongside digitally recorded video footage.

5.2.7 Histology

All animals were terminally anaesthetised at the completion of behavioural testing. The brains were extracted and preserved in 4% PFA and later transferred to 30% sucrose and stored at $4^{o}C$. 40µm thick sections were cut, mounted onto gelatin-coated slides and stained with cresyl violet for later analysis.

5.2.8 Statistics

As described in Chapter 3a the statistical packages used were SPSS (version 17) and Oriana (Version 2.0, Kovach Computing Services, UK). A series of mixed factorial and repeated measures ANOVAs were conducted, along appropriate Bonferroni-corrected t-tests. Independent, dependent and one-sample t-tests were also used where appropriate. Circular statistics and appropriate Watson-William's F tests and Rayleigh

tests of uniformity were also used. The symbol **±** was employed throughout to indicate standard error of the mean. Error bars, where present, show standard error of the mean, which is in turn denoted by S.E.M. A star-based system for significance representing p-values of *<0.05, **<0.01, ***<0.001, respectively, was used throughout.

5.2.9 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

5.3 Results

5.3.1 Histology

All animals included in analyses sustained damage >40% to the dorsal hippocampus and also displayed behavioural impairments. A one-sample t-test was used to compare the percentage area of damage to a representative sample (no damage represented as 0%). Far DH lesioned animals had a mean area damaged of 79.09±5.80% and this was found to be a significantly higher percentage damage when compared to the representative sample ($t(6) = 13.62$, $p<0.001$). There was some damage to the overlying corpus callosum, the primary somatosensory cortex and parietal region of the posterior association cortex at the sites of cannula penetration. It is important to note that all animals displayed normal motor and coordinated swimming movements and that damage to these cortical areas, adjacent to the hippocampus, have not been correlated with spatial learning deficits previously seen in the water maze (Horne et al., 2010; Wright et al., 2004). There was some damage to the habenula and to the laterodorsal nuclei of the thalamus in 3 out of 7 animals. There was no damage to the entorhinal cortex or amygdala in any animal. See Figure 5.1 for Far DH photomicrographs.
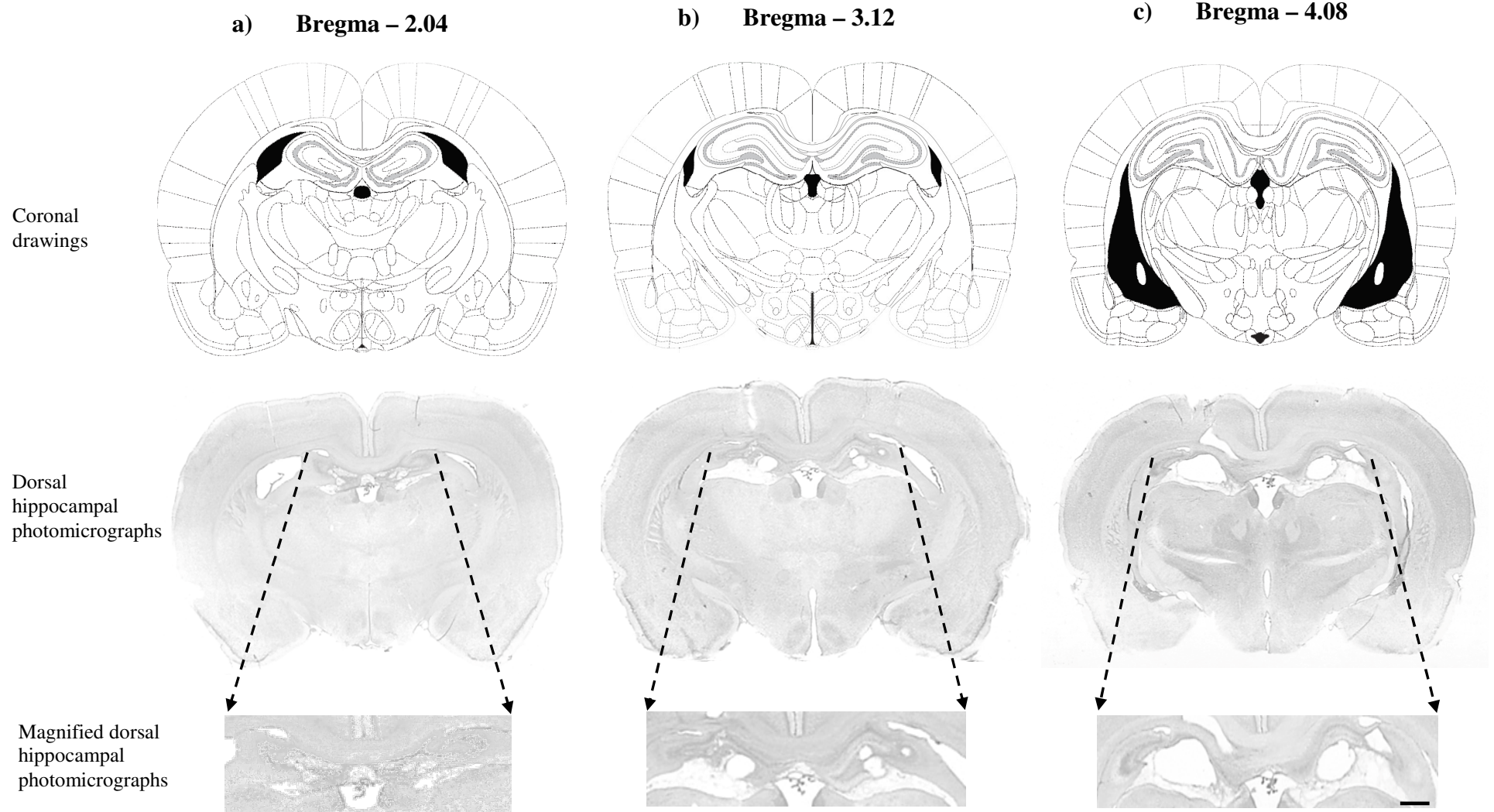
Figure 5.1: Schematic drawings of normal coronal sections of intact brain regions* (top row), representative photomicrographs of dorsal hippocampal damage (middle row) and magnified photomicrographs of dorsal hippocampal damage (bottom row), at rostrocaudal levels from bregma; -2.04 (a), -3.12 (b) and -4.08 (c). Scale bar = 500µm.

*Adapted from Paxinos & Watson (2005).

5.3.2 Basic Measures of Acquisition

Overall acquisition of the MWM was assessed, initially, by examining both groups escape latencies in the maze. Far sham lesioned animals successfully learned the MWM, with a reduction in mean escape latencies (EL) throughout training [Day 1, M: 38.53±4.14 sec; Day 5, M: 15.94±2.81 sec]. Far DH animals also showed some reduction in EL as training progressed, albeit to a lesser degree [Day1, M: 47.51±3.68 sec; Day5, M: 35.88±5.16 sec; see Figure 5.2). A 2 x 5 mixed factorial ANOVA comparing Far sham and Far DH animals ELs in the maze, revealed a significant main effect for day [$F_{(4, 52)} = 15.39$, $p<0.001$]. Bonferroni-adjusted t-tests revealed significantly shorter escape latencies towards the end of training with ELs on Day 5 significantly faster when compared to Day 1 ($p<0.001$), Day 2 ($p<0.01$) and Day 3 ($p=0.001$; see Figure 5.2). There was also a significant main effect for group [$F_{(1, 13)} = 17.92$, $p=0.001$], with the Far shams (M: 26.53±4.05 sec) performing significantly better than the Far DH animals (M: 46.29±3.81 sec). A significant interaction effect between day and group was also revealed [$F_{(4, 52)} = 3.13$, $p<0.05$]. Further assessment with independent t-tests indicated that there was no difference between groups on Day 1 ($t(13) = 1.59$, $p>0.05$). However, significant differences were recorded on all other training days (all $p<0.01$).

The distance covered in the pool during training was also assessed. A 2 x 5 mixed factorial ANOVA revealed a significant main effect for day [$F_{(4, 52)} = 9.06$, $p<0.001$], indicating a decrease in distance travelled from Day 1 (M: 981.09±69.55 cm) to Day 5 (M: 738.11±92.07 cm). Bonferroni-corrected t-tests further revealed significantly shorter distances travelled on Day 5 than Day 1 ($p<0.05$), Day 2 ($p<0.01$) and Day 3 ($p<0.01$). A significant main effect for group was also found [$F_{(1, 13)} =$

24.8, p<0.001] with the Far DH group (M: 1354.04±159.01 cm) travelling significantly longer distances than the Far sham animals (M: 576.32±93.32 cm; see Figure 5.3) when attempting to locate the submerged platform. A significant interaction effect between day and group was also revealed [$F(4, 52) = 7.39$, p<0.001] with further independent t-tests showing significant differences between the groups on Days 2, 3, 4 and 5 (all p<0.01).
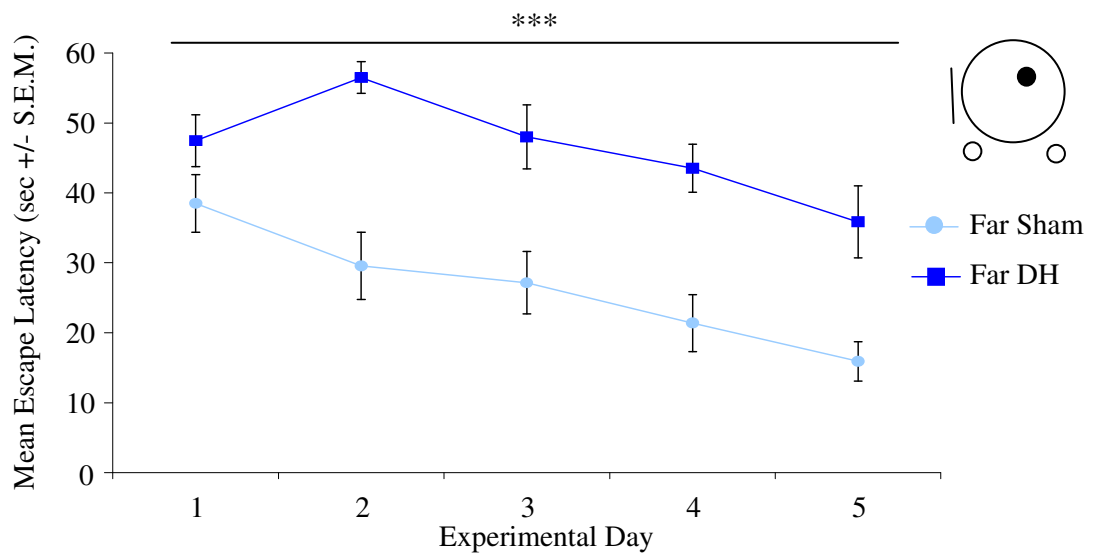
Figure 5.2: Mean escape latencies (sec ±S.E.M.) of Far sham and Far DH animals during MWM training. Insert; Far cue configuration.
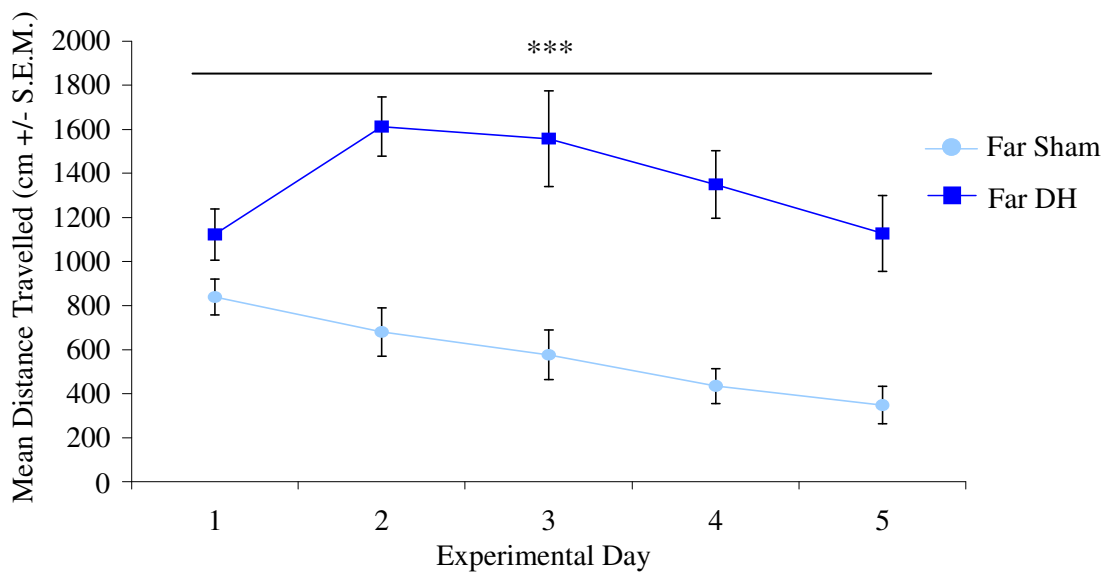
Figure 5.3: Mean distance travelled (cm ±S.E.M.) for the Far sham and Far DH lesioned animals during MWM training.

Mean swimming velocity was also assessed for both groups of animals. A 2 x 5 mixed factorial ANOVA revealed a significant main effect for day [$F_{(4, 52)} = 4.97$, $p<0.01$] with animals swimming speed increasing throughout training. Bonferroni-corrected comparisons further highlighted significantly quicker swim velocities on the last day of training when compared to Day 1 ($p<0.01$). A significant main effect for group was also noted [$F_{(1, 13)} = 32.48$, $p<0.001$], with the Far DH group (M: 28.48±1.49 cm/sec) swimming significantly faster than the Far sham animals overall (M: 21.43±0.89 cm/sec). A significant interaction effect between day and group was also revealed [$F_{(4, 52)} = 8.22$, $p<0.001$]. Further independent t-tests showed no difference between the groups mean velocity on Day 1 alone ($t_{(13)} = 1.65$, $p>0.05$). However, significant differences were found on all other days of training ($p<0.05$).

5.3.3 Behavioural Analysis; Platform Behaviour

5.3.3.1 Range of Head Movement

The head direction and range of head movement during the 15sec platform interval was assessed by observing digital stills of all animals while sitting on the platform. A 2 x 5 x 20 mixed factorial ANOVA revealed no change in head movements across days [$F_{(4, 52)} = 2.13$, $p>0.05$]. There was also no significant difference between the Far sham and Far DH groups overall [$F_{(1, 13)} = 0.54$, $p>0.05$], and no significant interaction between day and group [$F_{(4, 52)} = 0.87$, $p>0.05$]. Furthermore, there was no interaction effect between day and trial [$F_{(12, 156)} = 1.04$, $p>0.05$] or trial and group [$F_{(3, 39)} = 1.69$, $p>0.05$]. Overall, it appears that both groups observe the same range during the platform interval (see Figure 5.4). However, a decrease in the range of head movements across trials was found [$F_{(3, 39)} = 6.28$, $p=0.001$]. A series of

repeated measures ANOVAs with Bonferroni-corrected t-tests, however, revealed trial differences on Day 1 [$F_{(3, 42)} = 3.92$, $p<0.05$], with the greatest range in movement on earlier trials when the animal is first introduced to the environment; significant differences for example were noted between trial 1 (M: 86.73 ± 4.01^{o}) and trial 4 (M 55.86 ± 6.89^{o}, $p<0.05$) on Day 1. There were no differences found on Day 2 [$F_{(3, 42)} = 1.25$, $p>0.05$], however a significant effect for trial was noted on Day 3 [$F_{(3, 42)} = 3.11$, $p<0.05$]. Subsequent analysis with Bonferroni-corrections, however, revealed no differences between trials on Day 3. Furthermore, no differences between trials were noted on Day 4 [$F_{(3, 42)} = 1.57$, $p>0.05$] or 5 [$F_{(3, 42)} = 0.29$, $p>0.05$].
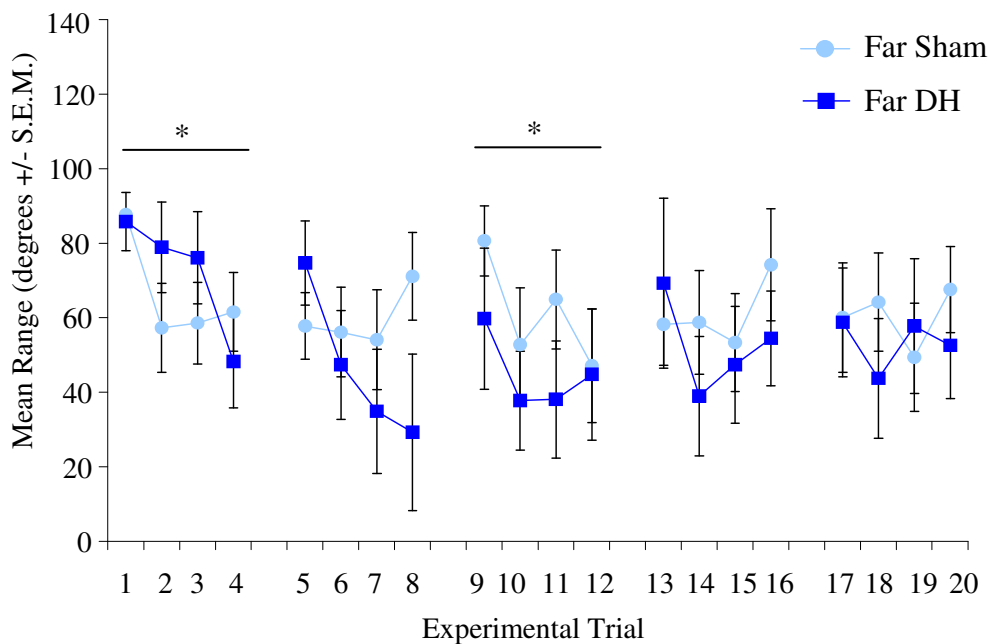
Figure 5.4: Mean range of head movement (±S.E.M.) made by Far sham and Far DH animals during the platform interval on each trial across 5 training days.

5.3.3.2 Mean Head Direction

The mean head direction of all animals during the platform interval was also assessed. The Far sham animals had a wide distribution of viewpoints across training days

ranging from the N on Day 1, to the NW on Day 3 and to the E on Day 5 (see Table

5.1). Rayleigh Uniformity tests revealed that the Far sham animals only had a

significantly preferred head orientation on Day 1 of training (Z = 4.92, p<0.01), with

no significantly preferred head direction on any other day. The Far DH animals also

displayed a wide range of head movements during the platform interval throughout

acquisition, with no significantly preferred direction on any day being revealed

(Rayleigh Uniformity test; p>0.05; Table 5.1). The data suggests neither the Far sham

nor Far DH group directed their attention to the Far cue arrangement to the south of the

pool while on the platform.

Table 5.1: Mean head direction (± S.E.M.) during training for the Far sham and Far DH groups
(* denotes significant orientation).

	Far Sham Lesioned			Far Dorsal Hippocampal Lesioned		
Day	Mean±S.E.M.	Rayleigh Uniformity	Compass Location	Mean±S.E.M.	Rayleigh Uniformity	Compass Location
Day1	174.95 ± 17.1^{o}	Z= 4.92, p<0.01*	NE	274.69 ± 24.7^{o}	Z= 2.47, p>0.05	W
Day2	60.39 ± 39.35^{o}	Z= 0.18, p>0.05	SE	43.80 ± 43.38^{o}	Z= 0.12, p>0.05	SE
Day3	242.43 ± 34.7^{o}	Z= 1.62, p>0.05	NW	275.76 ± 56.2^{o}	Z= 0.92, p>0.05	W
Day4	302.27 ± 95.6^{o}	Z= 0.63, p>0.05	W	214.17 ± 22.3^{o}	Z= 2.82, p>0.05	NW
Day5	81.95 ± 38.84^{o}	Z= 0.20, p>0.05	E	88.16 ± 44.04^{o}	Z= 0.11, p>0.05	E

5.3.4 Behavioural Analysis; Swimming Behaviours

5.3.4.1 Thigmotaxis

The swimming movements of both the Far sham and Far DH animals were assessed by

observing recorded video footage alongside detailed EthoVision tracks (Noldus,

Wageninen, Netherlands). The first behaviour analysed was thigmotaxis which

included analysis of percentage time spent in total, vertical and parallel thigmotaxis. The location at which animals performed these behaviours was also examined.

Initial investigation of the mean percentage time spent in total thigmotaxis indicates that the Far sham animals reduced the amount of time performing this behaviour throughout training, with a mean on Day 1 of 53.78±4.60% and Day 5 of 26.26±7.78%. Far DH animals, however, do not appear to reduce the time spent in this behaviour, with a recorded Day 1 mean of 68.66±4.95% and Day 5 of 57.87±8.30%. A 2 x 5 mixed factorial ANOVA confirmed that there was a significant effect for day $[F(4, 52) = 8.57, p<0.001]$, with Bonferroni-corrected t-tests revealing that a significantly higher percentage of time was spent in thigmotaxis on Day 1 when compared to Days 4 ($p<0.01$) and 5 ($p=0.001$). A significant main effect for group was also found $[F(1, 13) = 14.93, p<0.01]$, whereby the Far DH group (M: 66.82±7.05%) spent a significantly higher percentage of time performing thigmotaxis than the Far sham animals (M: 39.66±6.27%; Figure 5.5a). However, there was no significant interaction effect between day and group $[F(4, 52) = 1.8, p>0.05]$.

The mean percentage time spent in parallel and vertical thigmotaxis was assessed in a similar manner. A 2 x 5 mixed factorial ANOVA assessing time spent by both the Far sham and Far DH animals in thigmotaxis parallel revealed no significant effect for day $[F(4, 52) = 0.74, p>0.05]$. However, a significant effect for group was found $[F(1, 13) = 14.68, p<0.01]$, with the Far DH group (M: 39.65±5.31%) spending a higher percentage of time in parallel thigmotaxis when compared to the Far sham group overall (M: 11.78±4.97%). A significant interaction effect between day and group was also revealed $[F(4, 52) = 4.25, p<0.01]$. Independent t-tests confirmed that Far DH animals spend a significantly higher percentage of time in parallel thigmotaxis

than the Far sham animals on Day 2 $(t(13) = 2.55, p<0.05)$, Day 3 $(t(13) = 3.71, p<0.05)$, Day 4 $(t(13) = 2.85, p<0.05)$ and Day 5 $(t(13) = 6.26, p<0.001)$, but not on Day 1 $(t(13) = 1.75, p>0.05$; Figure 5.5b). Further assessment, with repeated measures ANOVAs, revealed that the Far sham reduced the time spent in parallel thigmotaxis throughout training $[F(4, 28) = 4.80, p<0.01]$. The Far DH animals, on the other hand, did not alter their performance of this behaviour $[F(4, 24) = 1.69, p>0.05]$.

The mean percentage time spent in vertical thigmotaxis, a behaviour that has previously highlighted search pattern differences, was also assessed (Figure 5.5c). A 2 x 5 mixed factorial ANOVA revealed a significant reduction in this behaviour across training days $[F(4, 52) = 8.98, p<0.001]$, with Bonferroni-corrected t-tests indicating a significant difference between Day 1 and Days 4 $(p<0.01)$ and 5 $(p<0.01)$, and between Day 2 and Day 4 $(p<0.05)$. No significant effect for group was found $[F(1, 13) = 0.01, p>0.05]$, and there was no interaction effect between day and group $[F(4, 52) = 0.73, p>0.05$; Figure 5.5c). Furthermore, a repeated measures ANOVA revealed that the Far sham groups' time spent in vertical thigmotaxis decreased with continued training $[F(4, 28) = 2.94, p<0.05]$. Similarly, the Far DH group also spent a decreasing amount of time performing vertical thigmotaxis $[F(4, 24) = 6.54, p=0.001]$.
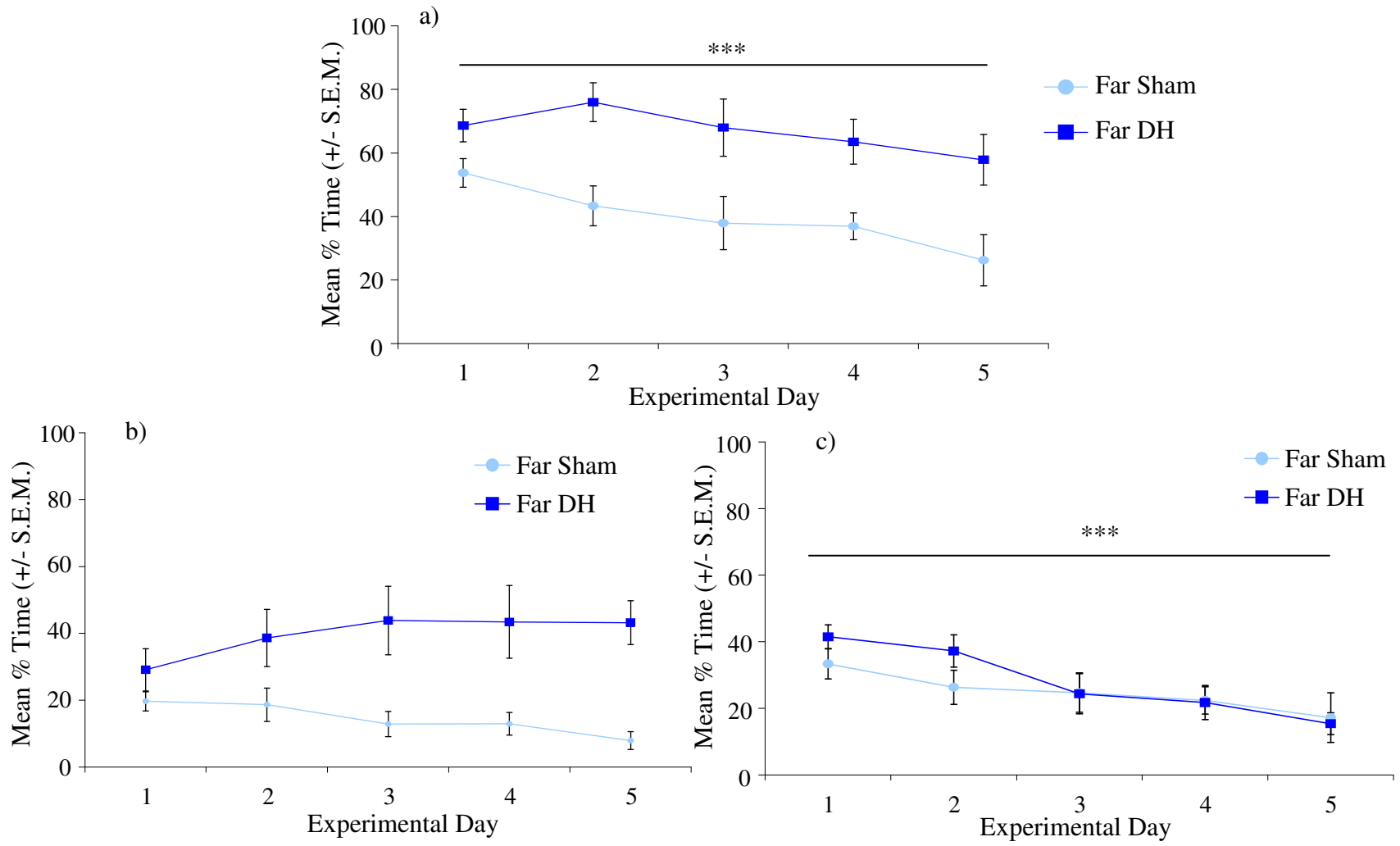
Figure 5.5: Mean percentage time (± S.E.M.) spent by the Far sham and Far DH animals in a) total thigmotaxis, b) parallel thigmotaxis and c) vertical thigmotaxis.

The location at which animals performed parallel and vertical thigmotaxis was also examined. This was carried out by measuring the mean number of times the behaviour was executed at a particular location around the pool. The results of this analysis are presented in Figure 5.6. From visual inspection of parallel thigmotaxis, there appears to be a peak in performance from 80-260° on Day 1 of training for the Far sham group, which is towards the north of the pool. Peaks were visible from 120-200° and from 230-250° on Day 2. There was a reduction in performance with continued training, with a relatively flat pattern seen on the remaining days of training (see Figure 5.6a). Similar analysis was carried out for the Far DH group. A small peak in performance at 240-250° was noted on Day 1, however the remaining days revealed relatively flat patterns of thigmotaxis parallel for this group.

Analysis of vertical thigmotaxis revealed peaks in the Far sham pattern of performance ranging from 20-90° and 260-350° on Days 1- 4 (specifically D1: 20-60° and 270-350°; D2: 20-90° and 290-330°; D3: 40-60° and 280-350°; D4: 10-60° and 260-290°) with performance encompassing the location at which all three cues are positioned around the maze. As there was a reduced amount of time spent in this behaviour as training progressed, analysis of Day 5 performance did not reveal any prominent peaks in location of thigmotactic performance around the maze (see Figure 5.6b). Far DH animals, displayed a similar pattern of performance, with peaks at the position of the cues throughout training. Some peaks were visible on Day 1 at 10-50°, 210-230° and 320-340°, however these peaks became more prominent and cue focused as training progressed; D2: 10-30° and 330-360°; D3: 10-30° and 290-350°; D4: 20-60° and 270-350°. Day 5, however, revealed a prominent peak at 190-230°, which falls outside the range of the distal cues. Peaks were, however, also observed at 10-30° and

280-330° at distal cue locations (see Figure 5.6b). Thus, examination of initial exploratory behaviour indicates few differences between the groups. However, further examination of more complex navigating behaviours may highlight differences in the groups' acquisition of the task.
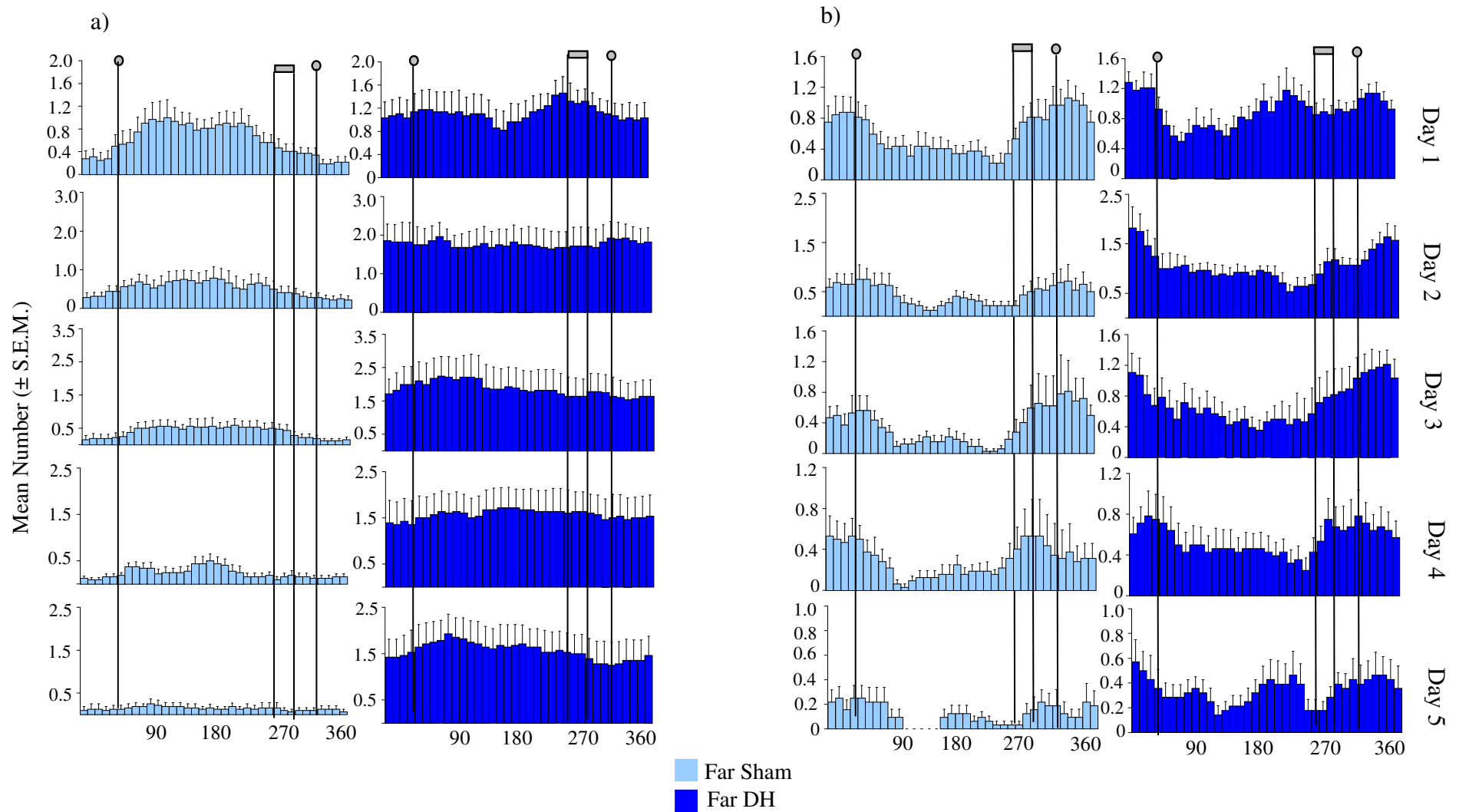
Figure 5.6: Mean number of occurrences of **a**) parallel thigmotaxis and **b**) vertical thigmotaxis at each degree location (0-360°) around the Morris water maze across training days for Far shams (grey) and Far DH (dark grey) animals. Included are the locations of the distal cues (insert).

5.3.4.2 .Direct

The percentage time spent in direct behaviour was also assessed for both the Far sham and Far DH animals on all training days. A 2 x 5 mixed factorial ANOVA revealed a small significant effect for day [$F(4, 52) = 3.39$, $p<0.05$], with a slight increase in performance of the behaviour from Day 1 (M: 14.52±1.20) to Day 5 (M: 18.76±1.61; see Figure 5.7). Subsequent analysis with Bonferroni-corrections, however, revealed no differences between days. There was also a significant difference between the groups [$F(1, 13) = 15.80$, $p<0.01$], with the Far shams spending, on average, 19.97±2.13% of time in this behaviour and Far DH animals spending 11.71±1.69% of their time performing direct behaviour overall. There was no interaction effect found between day and group [$F(4, 52) = 0.71$, $p>0.05$].

The direction towards which animals swam in the maze was also examined. For this, the pool was segmented as described in Chapter 3a. Briefly, to examine the time spent by the animals heading towards the cues we grouped a segment of the pool which contained the distal cues which ranged from 240-60° and compared this area to the region of the pool where no cues were present (i.e. 60-240°). Dependent t-tests revealed differences between segments in the Far sham group on Day 2 ($t(7) = 4.58$, $p<0.01$) and Day 5 ($t(7) = 3.27$, $p<0.05$) with a higher number of direct behaviours towards grouped segments 1, 5, 6, where no cues were present (D2, M: 0.08±0.01; D5, M: 0.08±0.01). No differences were found on any other day [Day 1: $t(7) = 1.37$, $p>0.05$; Day 3 $t(7) = 1.55$, $p>0.05$; Day 4 $t(7) = 0.43$, $p>0.05$]. The Far DH animals' direct behaviours were also examined in a similar manner. A significant difference between segments for the Far DH group was found on Day 2 ($t(6) = 2.59$, $p<0.05$) and Day 3 ($t(6) = 3.39$, $p<0.05$), the preferred direction on both days was, however,

towards the north of the pool, encompassing the area where no cues were present (Day 2, M: 0.13±0.02; Day 3, M: 0.11±0.01; see Figure 5.8). There was no statistically preferred location for direct behaviour on any other day [D1, t(6) = 0.11, p>0.05; D4, t(6) = 1.8, p>0.05; D5, t(6) = 2.17, p>0.05].
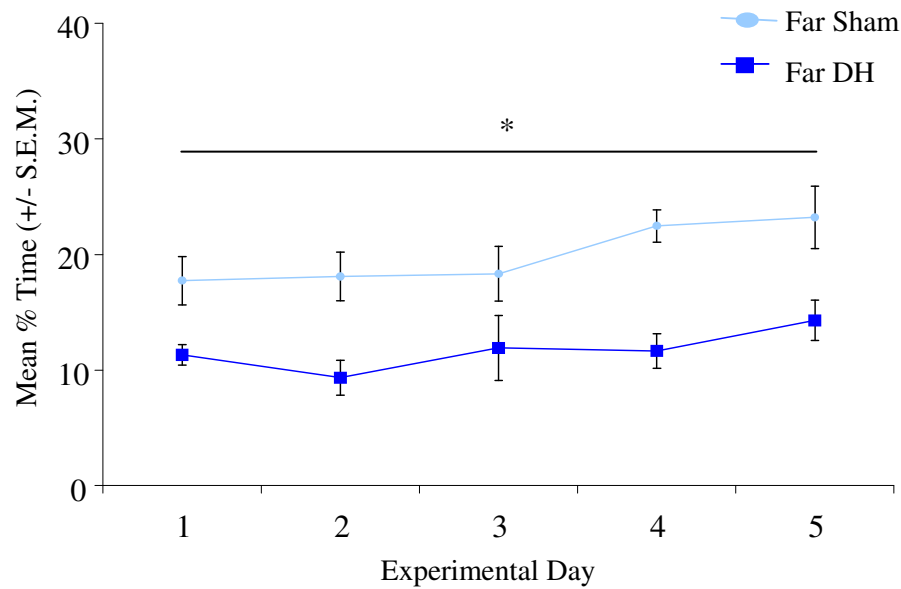
Figure 5.7: Mean percentage time (± S.E.M.), of total time in the maze, spent in direct behaviour, on all training days, of the Far sham and Far DH groups.
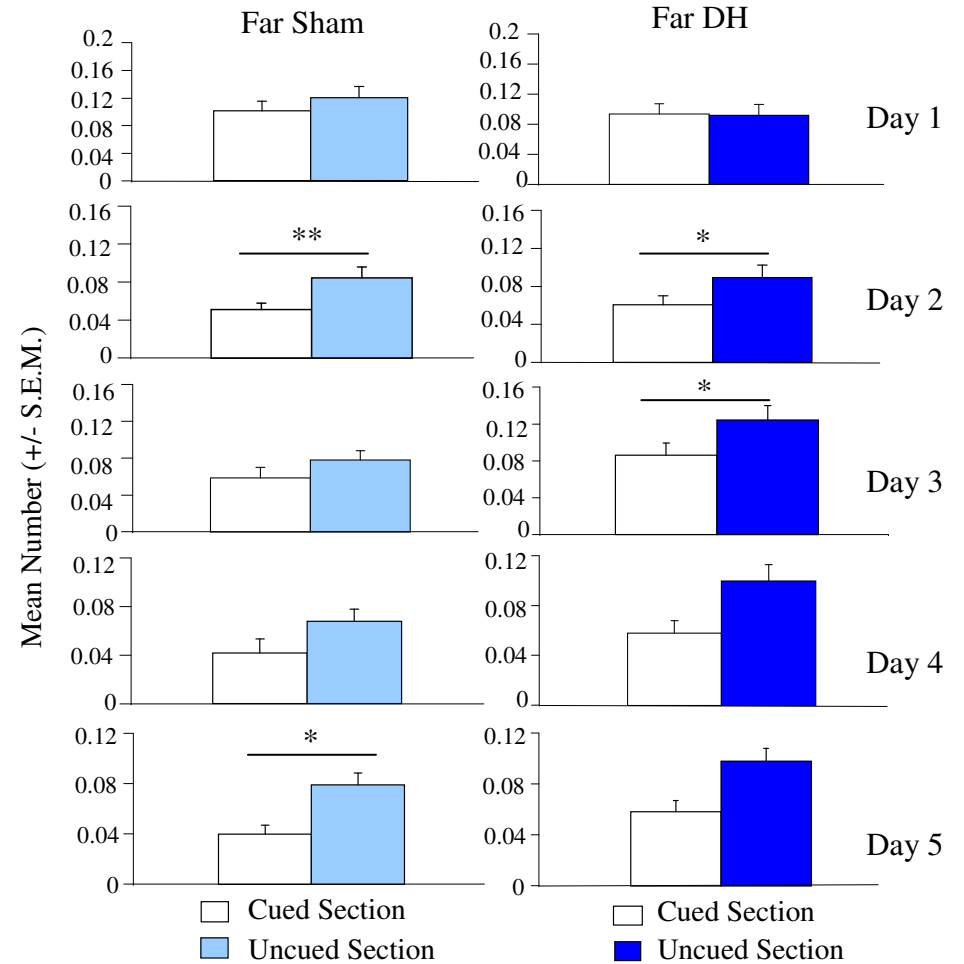
Figure 5.8: Mean number of occurrences of direct behaviour (± S.E.M.) in cued (Far cues from 60-240°) and uncued (no distal cues at 240-60°) sections in the maze for the Far sham and Far DH lesioned groups across training days.

5.3.4.3 Turning Behaviour

The more navigationally complex turning behaviour, including the mean number and spatial distribution of turns, was also examined. The mean number of turns overall (including turns towards and away from the cues) was assessed. A 2 x 5 mixed factorial ANOVA revealed no significant effect for day [$F_{(4, 52)}$ = 0.41, p>0.05] or group [$F_{(1, 13)}$ = 1.1, p>0.05], with Far shams having a mean number of turns of 23.65±2.47 and the Far DH group having a mean of 19.86±2.64 overall. There was also no significant interaction between day and group [$F_{(4, 52)}$ = 0.16, p>0.05; see Figure 5.9].
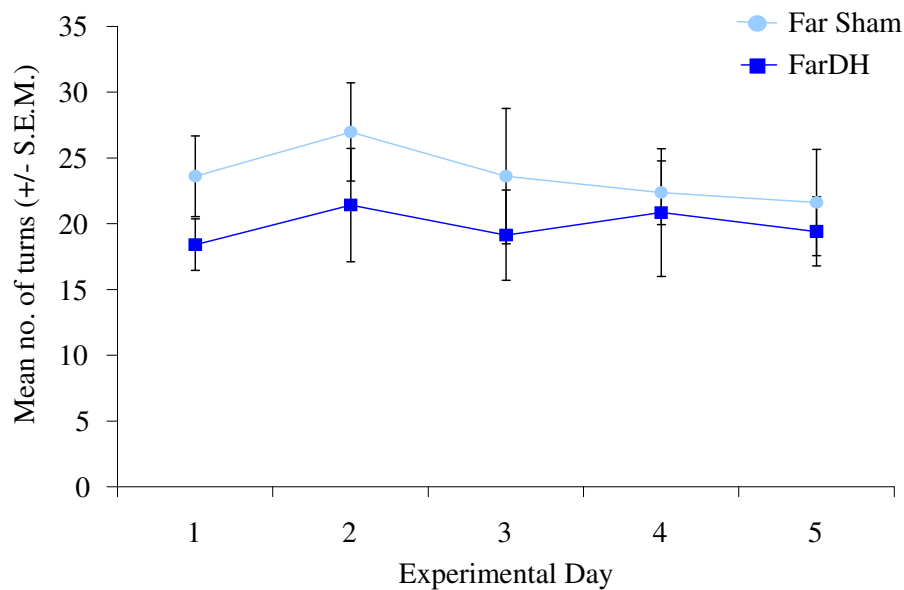
Figure 5.9: Mean overall number of turns made (±S.E.M.) in the Far sham and Far DH group throughout training.

The mean number of turns made *towards* the cues on each day in the MWM did not differ greatly throughout training for either the Far sham (D1, M: 10.63±1.8; D5, M: 11.75±2.6) or the Far DH groups (D1, M: 10.15±1.87; D5, M: 9.14±2.82; see also Figure 5.10). A 2 x 5 mixed factorial ANOVA confirmed that there was no significant

day [F(4, 52) = 0.23, p>0.05] or group effect [F(1, 13) = 0.73, p>0.05]. There was also no interaction effect between day and group noted [F(4, 52) = 0.10, p>0.05], indicating a similar level of turns towards the cues for both groups of animals.

The spatial distribution of turn positions for the Far sham group were also assessed and are represented in Figure 5.10a and 5.11a (see also Chapter 3a for detailed description of colour-coded cues). Table 5.2 reports all Rayleigh Uniformity data on the location of turns towards each cue throughout training. Briefly, the mean daily direction of turns towards the blue cue were found to be in statistically preferred directions on Days 1, 2, 4, and 5 (all p<0.05). Turns towards the red cue, however, were not performed at any preferred location throughout training (all p>0.05). Finally, turns towards the green cue were generally in statistically preferred locations, with the only non-significantly preferred positions found on Day 5 (see Table 5.2). Watson-Williams F-tests were used to examine any change in turn location throughout training for each cue. For the Far sham group, the location of turns towards the blue cue did not differ between days (all p>0.05). When the mean location of turns towards the red cue were assessed, however, significant differences were found between Day 1 and Days 2 [F(1, 68) = 17.86, p<0.001], 4 [F(1, 62) = 13.49, p<0.001] and 5 [F(1, 62) = 22.1, p<0.001], between Day 2 and Days 3 [F(1, 61) = 14.92, p<0.001] and 4 [F(1, 68) = 7.21, p<0.01], and also between Day 3 and Day 5 [F(1, 55) = 16.1, p<0.001]. Analysis of turn position for the green cue throughout training revealed a significant difference between Day 2 and Day 5 only [F(1, 47) = 4.09, p<0.05]. All other days had similar and stable turn positions towards the green cue (all p>0.05).

The spatial position of turns towards each of the distal cues was also assessed for the Far DH group (see Figure 5.10a and Figure 5.11b). Rayleigh Uniformity tests,

revealed statistically preferred locations for turns towards the blue cue on all acquisition days. Analysis of turns towards the red cue revealed statistically preferred locations on the first three days of training only ($p<0.05$). When turns towards the green cue were assessed, statistically preferred turn positions were noted on Days 1, 2, 3 and 4, but not Day 5 (see Table 5.3). Watson-William F-tests revealed stable turns positions for turns towards the blue, red and green cues between all acquisition days (all $p>0.05$).

Watson-Williams F-tests were also used to assess any differences in turn locations between groups on each day. Significant differences between the Far sham and Far DH group were apparent for the location of turns towards only the red cue on Day 2 [$F(1, 66) = 17.52$, $p<0.001$], Day 4 [$F(1, 56) = 6.01$, $p<0.05$] and Day 5 [$F(1, 51) = 19.99$, $p<0.001$; see Table 5.4] indicating that the animals' level of focused attention towards the red cue differed between the groups.
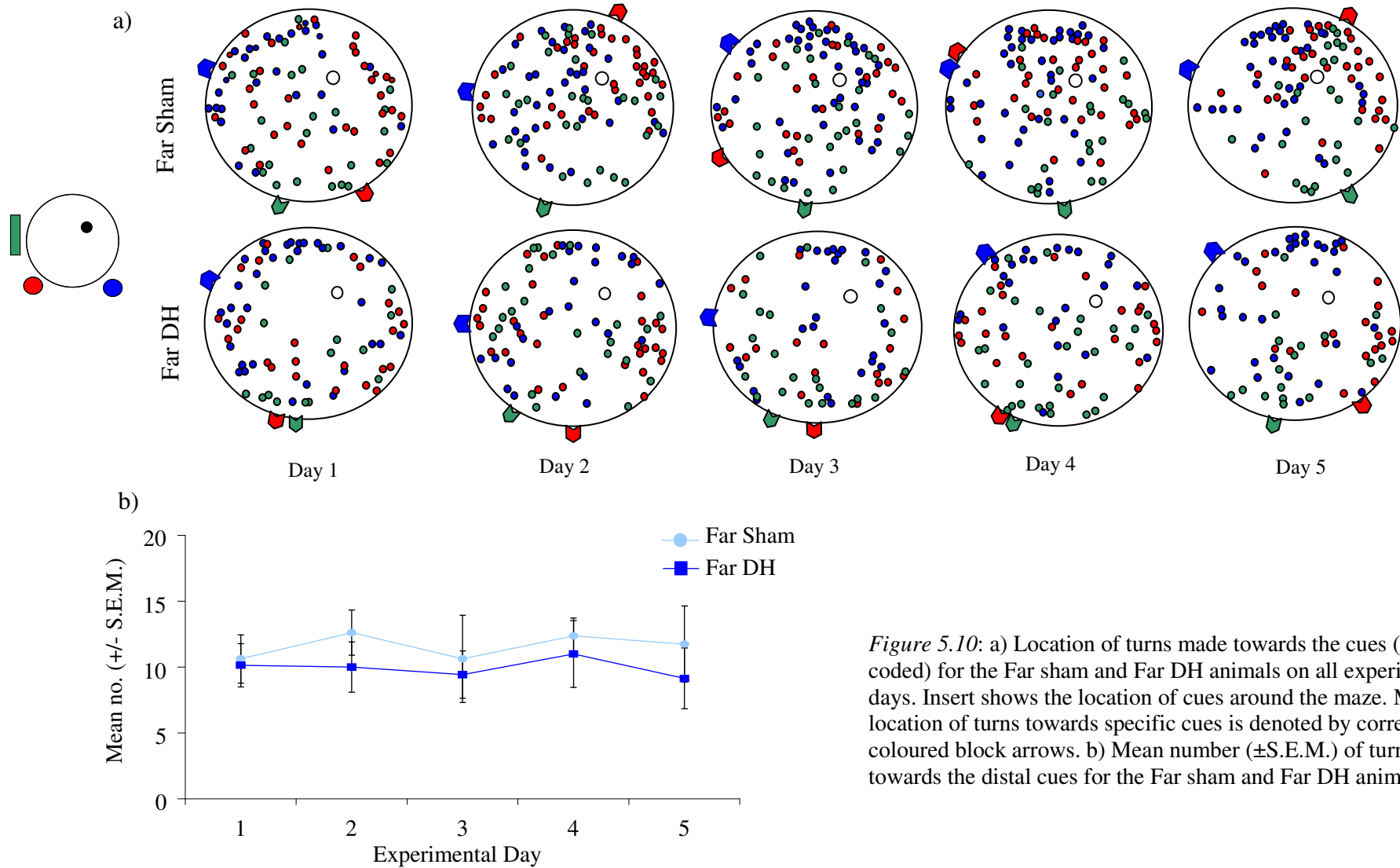
Figure 5.10: a) Location of turns made towards the cues (colour coded) for the Far sham and Far DH animals on all experimental days. Insert shows the location of cues around the maze. Mean location of turns towards specific cues is denoted by corresponding coloured block arrows. b) Mean number (±S.E.M.) of turns made towards the distal cues for the Far sham and Far DH animals.

a)

Table 5.2: Rayleigh Uniformity test results of the mean position of turns towards each cue, for the Far sham group, over 5 days.

Far Sham Lesioned Group

Cue	Day 1 Blue	Red	Green	Day 2 Blue	Red	Green	Day 3 Blue	Red	Green	Day 4 Blue	Red	Green	Day 5 Blue	Red	Green
Mean	260.6	7.75	330.1	278.4	162.6	322.7	243.9	307.3	341.4	265.1	252.9	355.5	260.1	152.5	18.9
Z Score	12.1	1.44	7.47	11.3	0.40	3.19	1.19	0.57	4.45	9.49	1.07	6.8	9.11	1.59	2.13
p	***	-	***	***	-	*	-	-	**	***	-	***	***	-	-

b)

Table 5.3: Rayleigh Uniformity test results of the mean position of turns towards each cue, for the Far DH group, over 5 days.

Far Dorsal Hippocampal Lesioned Group

Cue	Day 1 Blue	Red	Green	Day 2 Blue	Red	Green	Day 3 Blue	Red	Green	Day 4 Blue	Red	Green	Day 5 Blue	Red	Green
Mean	262.1	338.9	349.1	290.1	337.7	314.4	283.4	342.5	322.4	249.3	326.3	328.8	250.3	17.1	335.2
Z Score	5.26	4.87	4.83	3.76	4.95	6.31	0.84	3.97	8.87	6.8	2.54	14.83	6.29	2.73	2.73
p	**	**	**	*	**	***	*	*	***	***	-	***	***	-	-

Figure 5.11:a) Far sham group and b) Far DH group, mean location (±S.E.M) of turns made towards each respective cue across training days.

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Table 5.4: Mean ± S.E.M. and Watson-William F tests results comparing differences in the location of turns towards each cue (degrees) for the Far sham and Far DH groups. Denotations for p-values: **-** non-significant, ***** p<0.05, ****** p<0.01, ***p<0.001.

	Blue Cue			Red Cue			Green Cue		
Day	Sham	DH	p	Sham	DH	p	Sham	DH	p
D1	260.6±10.1	262.1±16.9	-	367.7±33.3	338.9±17.4	-	330.1±13.4	349.1±21.2	-
D2	278.4±10.9	290.1±20.1	-	162.6±63.7	337.7±17.4	***	322.8±21.9	314.4±14.9	-
D3	243.9±36.7	283.4±43.6	-	307.3±53.2	342.5±19.2	-	341.5±18.4	322.4±12.1	-
D4	265.1±12.3	249.3±14.4	-	252.9±38.8	326.3±24.8	*	355.6±14.2	328.8±8.5	-
D5	260.1±12.5	250.3±15.3	-	152.5±31.7	377.1±23.6	***	378.9±27.1	335.2±10.5	-

5.3.4.4 Turns-Away

The turns animals made away from the cues were also assessed and are presented in Figure 5.12a. There were few changes in the level of turning away behaviour throughout training with the mean number of turns-away from the cues remaining relatively consistent throughout the training period for both the Far sham (D1, M: 13.0±1.31; D5, M: 9.8±1.4) and Far DH animals (D1, M: 8.29±1.08, D5, M: 10.29±1.48). A 2 x 5 mixed factorial ANOVA confirmed this, revealing no significant effect for day [$F(4, 52) = 1.47$, $p>0.05$] or group [$F(1, 13) = 1.35$, $p>0.05$]. There was also no significant interaction between day and group [$F(4, 52) = 1.19$, $p>0.05$; see Figure 5.12b).
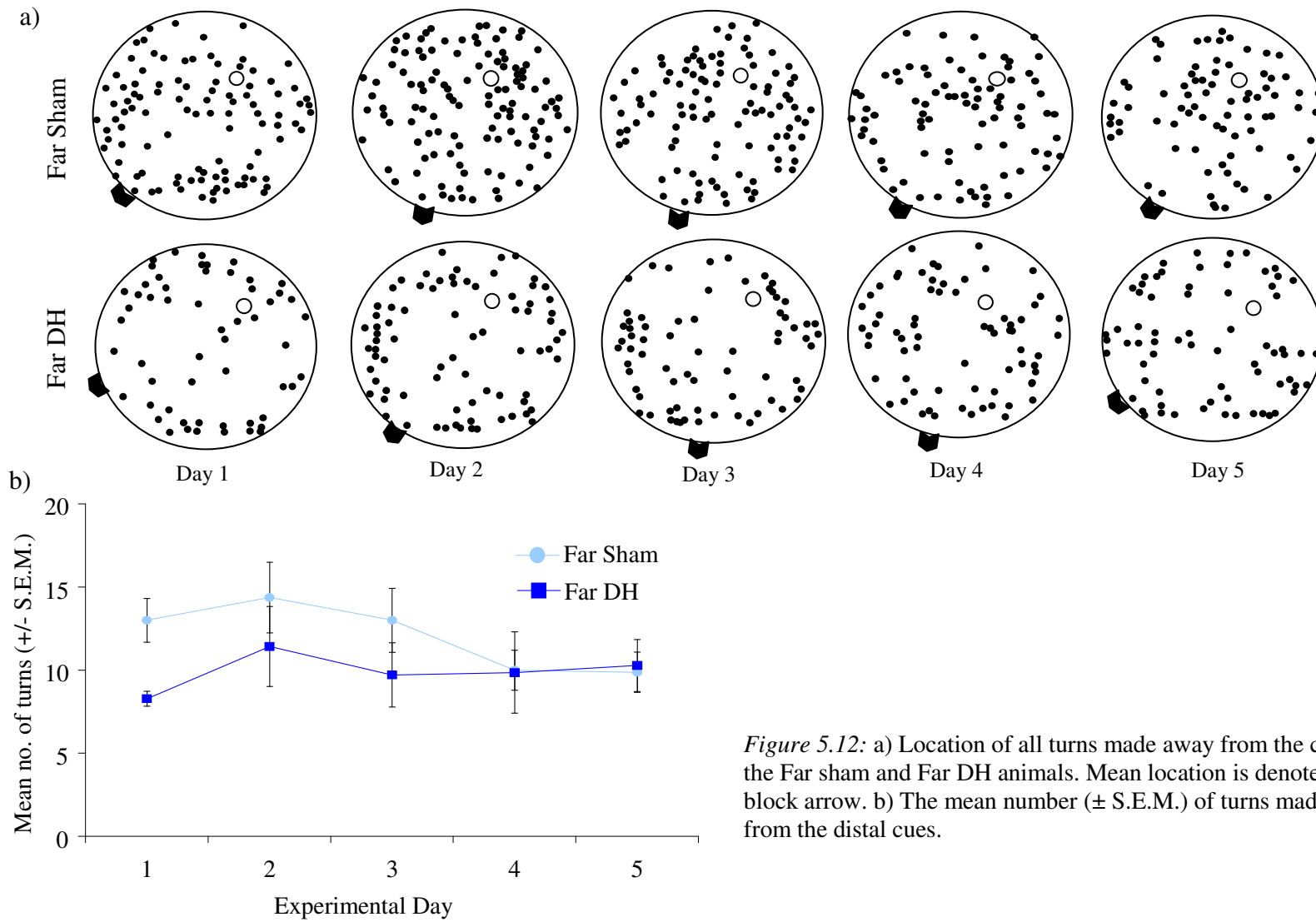
Figure 5.12: a) Location of all turns made away from the cues for the Far sham and Far DH animals. Mean location is denoted by black block arrow. b) The mean number (± S.E.M.) of turns made away from the distal cues.

5.3.4.5 Turns in Zones

Following assessment of turn locations, we subsequently examined the distance of the turns from the platform as training progressed. To examine this, the pool was divided into three zones (near, middle and far) according to distance from the platform (see Chapter 3a for details). The Far sham animals' turns in the pool were first assessed and a 5 x 3 repeated measures ANOVA revealed no significant effect for day [$F_{(4, 28)}$ = 0.85, $p>0.05$]. However, a significant difference between zones was found [$F_{(2, 14)}$ = 40.14, $p<0.001$] with subsequent Bonferroni-adjusted t-tests revealed the highest number of turns were performed in the far zone (M: 11.95±1.81) than the near (M: 4.3±0.98, $p<0.001$) and middle zones (M: 7.10±1.58, $p<0.05$). A significant interaction interaction effect between day and zone was also noted [$F_{(8, 56)}$ = 3.25, $p<0.001$]. Further examination of the data was conducted using daily one-way repeated measures ANOVAs to determine if these differences occurred across training days. Significant differences were revealed between zones on Day 1 [$F_{(2, 14)}$ = 54.34, $p<0.001$], 2 [$F_{(2, 14)}$ = 11.68, $p=0.001$], 3 [$F_{(2, 14)}$ = 12.78, $p<0.001$], 4 [$F_{(2, 14)}$ = 4.35, $p<0.05$] and Day 5 [$F_{(2, 14)}$ = 5.19, $p<0.05$]. Further Bonferroni corrected t-tests revealed differences on Day 1 between the near and far ($p<0.001$) and middle and far zones ($p<0.001$). Differences between the near and far ($p<0.05$) and the middle and far zones ($p<0.05$) on Day 2 were also noted. Significant differences between the near and far zone only ($p<0.01$) was revealed on Day 3 only. However, following Bonferroni corrections, no differences between zones were noted on Day 4, with differences only between the near and middle zones ($p<0.05$) on Day 5. Results suggest that the location in which Far shams perform turns shifts, somewhat, as training progresses, with turns being performed closer to the platform by Day 4, with only small

differences noted between zones on Day 5, indicating increased understanding of the goal location (see Figure 5.13a).

The mean number of turns made in each zone for the Far DH group was also assessed using a 5 x 3 repeated measures ANOVA. Results revealed no significant effect for day [$F_{(4, 24)} = 0.38$, $p > 0.05$]. However, a significant effect for zone was found [$F_{(2, 12)} = 32.79$, $p < 0.001$], where the highest number of turns were performed in the far zone (M: 14.20 ± 2.86) than the near (M: 1.31 ± 0.47, Bonferroni-corrected, $p < 0.01$) and middle zones (M: 4.2 ± 0.96, Bonferroni-corrected $p < 0.01$). However, no interaction effect between day and zone was noted [$F_{(8, 48)} = 0.23$, $p > 0.05$]. Further daily one-way repeated measures ANOVAs revealed significant differences between zones on Day 1 [$F_{(2, 12)} = 32.97$, $p < 0.001$], 2 [$F_{(2, 12)} = 11.42$, $p < 0.01$], 3 [$F_{(2, 12)} = 20.73$, $p < 0.001$], 4 [$F_{(2, 12)} = 10.11$, $p < 0.01$] and 5 [$F_{(2, 12)} = 30.33$, $p < 0.001$]. Further Bonferroni corrected t-tests revealed significant differences between the near and far ($p < 0.01$) and middle and far zones ($p < 0.05$) on all days of training, with the highest number of turns being performed in the far zone (see Figure 5.13b). This would suggest that the Far DH animals' turn locations, in the maze, did not move towards the platform with continued training.
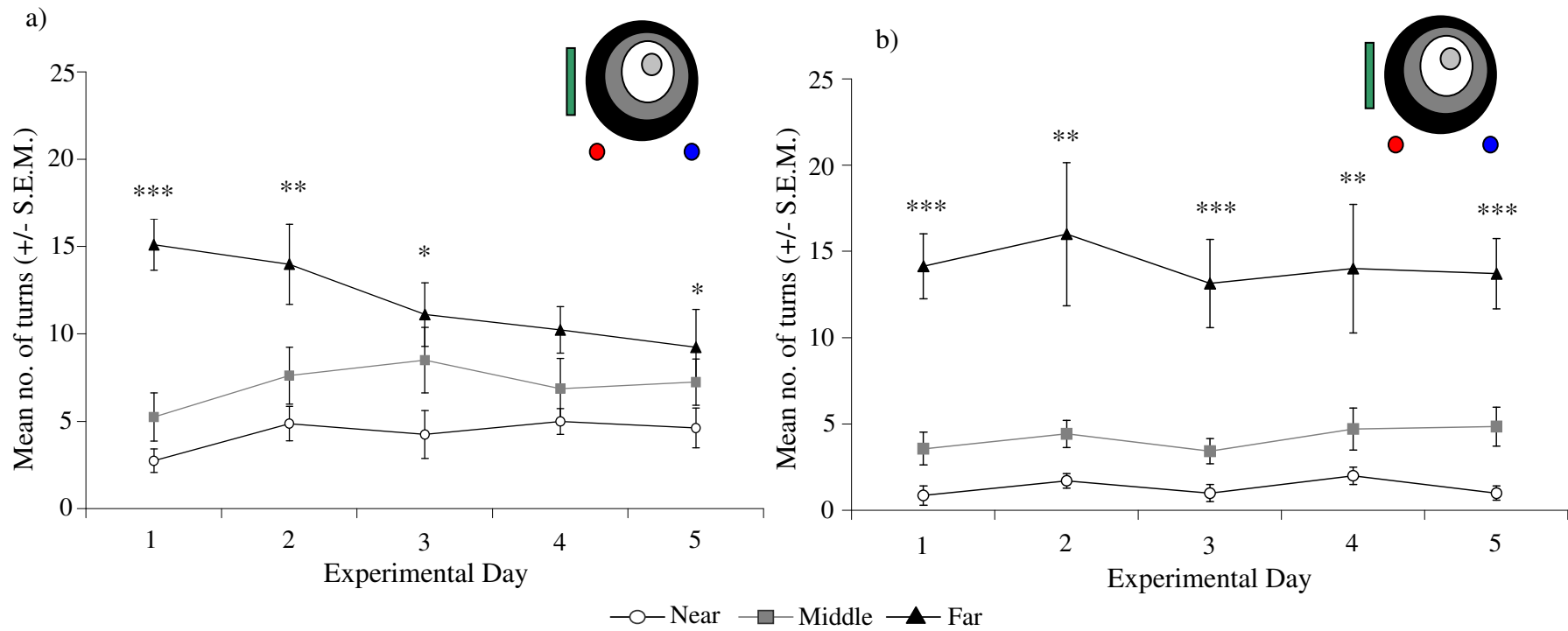
Figure 5.13: Mean number (± S.E.M.) of turns in the near, middle and far zones for **a**) the Far sham and **b**) Far DH groups over 5 days of training. Inset; Schematic representation of the three zones used for analysis.

5.4 Summary

Analysis of overall measures of acquisition indicated that the Far DH animals were significantly impaired in learning the MWM task when compared to the Far sham animals. Furthermore, assessment of exploratory behaviours also showed that the Far DH animals had a higher level of performance of overall thigmotaxis when compared to sham animals. In addition to this, the lesioned animals did not reduce their time spent in parallel thigmotaxis as training progressed, indicating a perseveration of behaviour throughout acquisition. Interestingly, this perseveration was not seen in vertical thigmotaxis, with the Far DH animals learning to reduce their time spent in this behaviour with continued exposure to the task. The Far DH group also appear to perform vertical thigmotaxis at a similar location as the Far shams; however the peaks in performance at the cues were less prominent than in the sham group. In addition to this, closer analysis on a number of days (specifically Day 1 and Day 5), revealed the location of Far DH performance was, somewhat, less accurate than the shams, and not always carried out at the location of the cues indicating further subtle differences in behaviour between the groups.

Direct behaviour also revealed interesting results with the Far sham group performing significantly more direct swims than the Far DH animals, who displayed a consistently lower level of performance throughout training. While the lesioned animals could perform direct swims, indicating a retained ability to execute appropriate behaviours in the maze, the reduced number overall indicate an inability to maintain the performance throughout acquisition. Similarly, the location at which they were performed also suggests problems in the direction of their heading while in the maze. However, as the cues were located in a position further away from the goal, the

accuracy of this behaviour was also reduced in the Far sham animals (a finding also observed in the FT group in Chapter 3a).

In addition, turning behaviour highlighted performance differences between the groups. Specifically, the location at which turns towards the SW light cue were made differed between the Far sham and Far DH group on a number of days, which may indicate that while the DH group are displaying some attention towards the cues in the environment, they are not incorporating the information effectively in their search strategy. Further credence to this lies in the examination of the location of the turns, which suggests an inability of the lesioned animals to alter their movements in relation to the cues, during acquisition. Specifically, assessment of the zones in which turns were performed by the animals, revealed that the Far DH group, while displaying similar numbers of turns, did not move their positions closer to the platform, instead remaining at the periphery of the pool throughout the entire training period.

While some behaviours performed by the Far sham and Far DH groups are similar, the subtle differences in exploration between the groups, such as initial perseveration and inaccurate positioning of behaviours, led to longer escape latencies and poorer performance overall in the Far DH group. The implications of these findings will be further discussed in the General Discussion Chapter, alongside evidence from Chapters 4 and 6.

Chapter 6

Direct comparative assessment of hippocampal lesioned animals during acquisition and retention in the Morris water maze.

Abstract

Lesioned animals in both Near and Far cue conditions appear to show significantly different patterns of searching in the maze when compared to their control counterparts, as evidenced through in-depth behavioural analysis (Chapter 4 and 5). Furthermore, earlier Chapters elucidated that cue position affects intact animals' performance in the task, with Near trained animals, in particular, availing of distal cues more directly than those trained with cues in a Far position, who instead must infer more to locate their goal (Chapter 3a). The Far cue condition also appears to be a more hippocampal-dependent task, with higher dorsal hippocampal BDNF expression evident following training in this condition (Chapter 3b). Therefore, here we directly compare the findings from Chapter 4 and 5, and assess the Near and Far dorsal hippocampal lesioned animals' performance, on basic and behavioural acquisition measures, to determine whether dorsal hippocampal lesions exacerbate performance under the higher spatially demanding Far training condition. In addition to the acquisition period we also compare the performance of the Near and Far lesioned animals during a 7-day post-acquisition, retention trial in the MWM, as the hippocampus has also been implicated in the storage and retrieval of spatial memories. Overall, the results suggest subtle differences between the groups in both acquisition and retention measures. However, overall it appears that the key role played by the hippocampus in spatial tasks is in the organisation and monitoring of exploratory behaviours in the maze over spatial processing.

6.1 Introduction

Results from Chapter 4 and 5 suggest that both the Near and Far dorsal hippocampal lesioned animals were impaired in locating the hidden goal in comparison to their control counterparts. Specifically, general measures of acquisition, such as escape latency and distance travelled, provided initial evidence for impairments in the task with further detailed assessment of the animals' exploratory behaviours, during the acquisition period, also offering interesting results with respect to navigational difficulties in both lesion groups.

In addition to the acquisition period however, retention or probe trials are also often used to assess animals' performance and ability to retain and recall information that has been previously encountered in the MWM (Hannesson & Skelton, 1998; Morris et al., 1990). It has been well documented that retrieval of spatial information shares similar mechanisms as the acquisition period, whereby an intact animal avails of the same strategy to retrieve a memory as the one that they used to learn it (Abel & Lattal, 2001; Kealy et al., 2008; McGauran et al., 2004). Moreover, evidence from a substantial number of studies, assessing the effect of hippocampal lesions on performance in the water maze, have shown that re-expression of learned information at a later time also requires a functioning hippocampus (Broadbent et al., 2010; R.E. Clark et al., 2005; Martin et al., 2005; Morris et al., 1982; Mumby et al., 1999; Sutherland et al., 2001). Interestingly, the retention probe trial has also been used to further assess the controversy surrounding the exact role of the hippocampus in spatial learning, in particular its contribution to the relative spatial and navigational aspects involved in acquiring the water maze (Clark et al., 2007; Morris et al. 1990; Ramos, 2010; Whishaw & Jarrard, 1996). Whishaw and Jarrard (1996), for example,

successfully trained hippocampal lesioned animals in the MWM, when initial spatial demands were reduced by including a visible platform to encourage more efficient performance behaviours. Following training in the modified task, the lesioned animals were then assessed in a retention probe trial where the platform was removed and the animal required to use distal information to guide their search. Interestingly, the retention trial further confirmed the lesioned animals' intact ability to locate place, with animals displaying accurate searching in the correct goal location, and performing behavioural pauses and turns at appropriate locations. However, the lesioned animals could not subsequently learn a new place location, leading authors to the conclusion that the retained ability to reach place, during retention, was due to encouraged behavioural performance during the initial training period (Whishaw & Jarrard, 1996).

In addition to the general deficits seen during acquisition and retention of spatial tasks following hippocampal lesions, a number of studies have noted that differences in performance may emerge as a result of the location and size of the lesion made (Aznar et al., 1998; de Hoz et al., 2005; Martin et al., 2005). Moser et al. (1995) for example, found that as the extent of damage to the hippocampus increased so too did the resulting impairment observed in tasks examining spatial learning (see also Bannerman et al., 1999). In addition to this, the extent of lesion size to specific subregions of the hippocampus has also revealed interesting results. Hernandez-Rabaza et al. (2007), for example, assessed the performance of animals with either large dentate gyrus (DG) lesions or small DG lesions, and found that larger lesions resulted in greater impairments in a spatial task, evidenced in the animals' inabilities to execute goal-directed actions in a flexible manner. Beyond lesion size, examination of damage (irrespective of extent) to the specific hippocampal subregions has also

highlighted differential roles of the DG, CA3 and CA1 in spatial tasks, ranging from encoding, to intermediate and long-term retrieval of memories (Dillon et al., 2008; Lee & Kesner, 2004; Okada & Okaichi, 2009; see Chapter 1 for a more detailed review).

Therefore, in this Chapter, we initially examine the size of dorsal hippocampal lesions and extent of subregion damage on all measures of acquisition including both standard criteria and exploratory behaviours, as well as investigating retained performance in a retention probe trial. From this analysis, we aim to ascertain individual subregional function and also determine if these relate to specific behavioural criteria as measured through in-depth analysis. Furthermore, to determine if cue positioning, in particular the far cues, augments the negative effects of dorsal hippocampal lesion damage on performance in the MWM as spatial demands increase, we also compared the Near DH and Far DH groups on a number of acquisition, exploratory behaviour and retention measures.

6.2 Method

6.2.1 Subjects

Data from sham and dorsal hippocampal lesioned, male, Wistar rats (n=31), that served as subjects in Chapter 4 and 5, was further assessed in this Chapter. In addition, retention data, which was not examined previously, was also assessed.

6.2.2 Procedure

Following surgery to the dorsal portion of the hippocampus, Near dorsal hippocampal lesioned animals (n=8) and Far dorsal hippocampal lesioned animals (n=7) were trained in the MWM with cues in either the near position or far position, as detailed in Chapters 4 and 5. Briefly, both the Near DH and Far DH animals were trained in the MWM for 5 days with 4 trials per day from four pseudorandom start positions. Following the training period, animals had a 7 day break, and then received a single 60 second retention trial with the platform removed from the pool. For this trial, all animals were placed in the pool from the NW position.

6.2.3 Lesion size and Performance Correlate Analysis

The extent of damage to the hippocampus, regardless of training condition, was assessed to determine the effect of lesion size on both basic acquisition measures and on the animals' swimming behaviours in the maze. In addition to this, we also assessed the effect of damage to the specific subregions of the hippocampus (DG, CA3 and CA1) on the same measures. For this, the size of the lesion to each of the subregions within the hippocampus was calculated. Images of stained coronal slices were analysed using a specifically designed Matlab R2008a programme. Six sections rostrocaudally,

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which included 2 rostral sections at bregma -2.16, 2 mid sections at bregma -3.12 and 2 caudal sections at bregma -4.08, were examined for each animal. The size of the area of the intact regions (DG, CA1 and CA3) was calculated at each level for each animal in both of the sham groups (i.e. Near sham and Far sham) and the area of damage was, similarly, calculated at each level for each animal in both of the DH groups (i.e. Near DH and Far DH). The extent of subregional damage in each of the DH animals was then presented as a percentage of the intact area for each of the animals in the sham group.

6.2.4 Comparative Analyses of Basic Measures of Acquisition

Initially, Near DH and Far DH animals were compared on basic measures of acquisition including escape latency, distance travelled and swimming velocity. Following this, assessment of the 7 day post-acquisition retention trial, including the percentage time spent swimming in each of the quadrants of the pool, the platform areas, platform corridors and the outer corridors, as defined in Chapter 2, were carried out for the Near and Far DH groups. To assess the expected pattern of behaviour in intact animals during a retention trial, data from the Near and Far sham animals was also examined.

6.2.5 Comparative Analyses of Behaviours

The recorded data from the Near DH and Far DH animals during training in the MWM was compared to determine any similarities or differences between the groups' acquisition in the maze. For this, we assessed percentage time spent in total, vertical and parallel thigmotaxis. The percentage time spent in direct behaviour and the number

of turns-towards the cues was also compared between groups. Turns-away were not analysed as we found in Chapter 3a that Far trained animals (without lesions to the hippocampus) generally performed more of these behaviours in comparison to Near trained animals. Therefore, comparison of turns-away between the Far DH and the Near DH would not provide an accurate assessment of how the task is solved by the respective group.

6.2.6 Statistics

All linear statistical analysis was carried out using SPSS (version 17). Statistics used included repeated-measures analysis of variance with appropriate Bonferroni-corrected t-tests and independent t-tests where required and Pearson product-moment correlations. The symbol ± was employed throughout to indicate standard mean error. Error bars, where present, show standard error of the mean, which is in turn denoted by S.E.M. A star-based system for significance representing p-values of $*<0.05$, $**<0.01$, $***<0.001$, respectively, was used throughout.

6.3 Results

6.3.1 Histology

An independent samples t-test comparing the extent of damage to the dorsal hippocampus between the Near DH and Far DH groups revealed no significant difference in the size of the lesioned area between groups ($t(13) = 1.54$, $p>0.05$; see Figure 6.1). The Near DH group had a mean of $65.43\pm6.56\%$ damage to the dorsal portion of the hippocampus and the Far DH group had $79.09\pm5.81\%$ damage to the dorsal hippocampus (see Chapter 4 and 5 for lesion photomicrographs) indicating that any subsequent differences observed between groups should be as a direct result of task demands and performance, rather than the extent of hippocampal damage, *per se*.
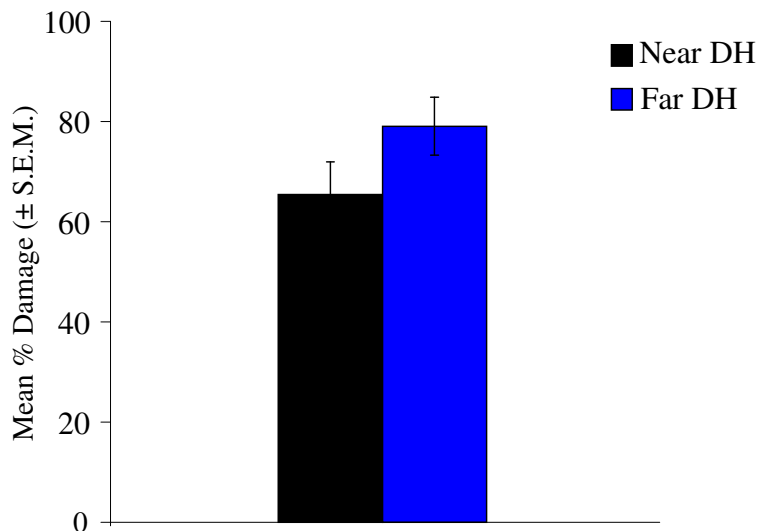
Figure 6.1: Mean percent (± S.E.M.) of dorsal hippocampal damage for the Near DH and Far DH groups

6.3.2 Lesion Size and Performance Correlations

We initially examined the relative impact of lesion size on acquisition and retention in general in the maze. For this, we correlated the total percentage of dorsal hippocampal

damage for each animal (irrespective of group) with an overall mean escape latency (EL) throughout training for each animal (see also Hernandez-Rabaza et al., 2007). Pearson product-moment correlations revealed a significant positive correlation, with larger lesion damage leading to increased ELs during acquisition ($r = 0.54$, $p<0.05$; see Figure 6.2). A similar finding was also revealed when distance travelled was correlated with the extent of hippocampal damage ($r = 0.57$, $p<0.05$). However, swimming velocity did not appear to be affected by the level of damage to the hippocampus ($r = 0.23$, $p>0.05$).
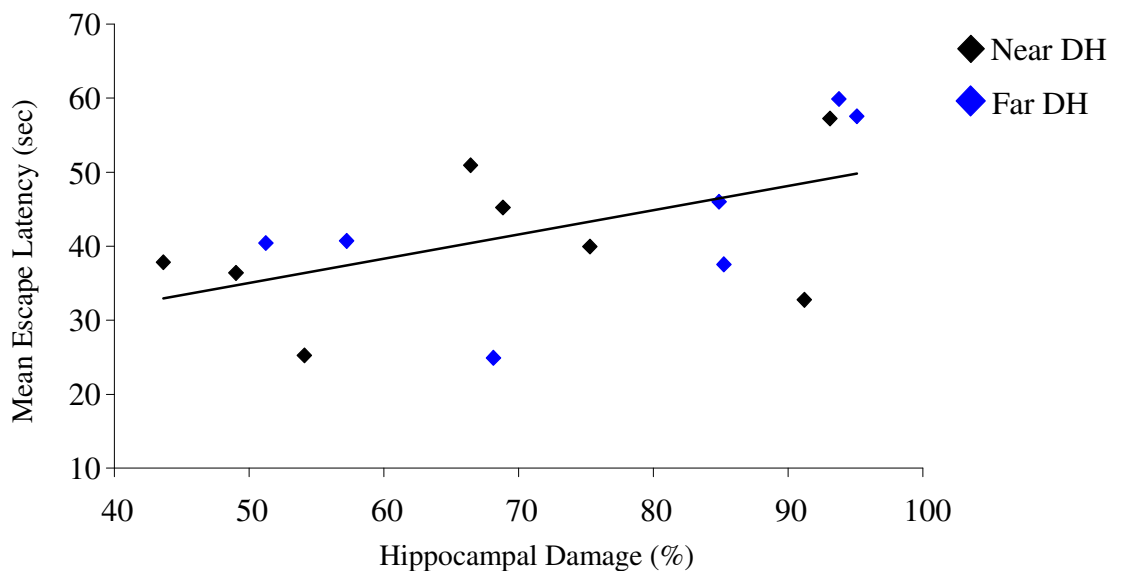
Figure 6.2: Correlations between mean escape latency across the 5 days of training and percentage of hippocampal damage for all dorsal hippocampal lesioned animals.

As we have previously seen, exploratory behaviours of the intact and hippocampal damaged navigating animals has revealed subtle differences in performance. Therefore, we also applied lesion analysis to individual observed behaviours. Specifically, total hippocampal damage was correlated with the mean percentage time spent by animals in total thigmotaxis, parallel thigmotaxis (PT) and vertical

thigmotaxis (VT) throughout the training period. From this assessment, we found a significant positive correlation between hippocampal damage and time spent in total thigmotaxis (r = 0.62, p<0.05; see Figure 6.3) and PT (r = 0.55, p<0.05) with a larger lesion resulting in a higher performance of thigmotactic behaviour overall. However, a significant correlation was not found between lesion damage and VT (r = 0.26, p>0.05) or indeed with any other exploratory behaviour (direct; r = -0.392, p>0.05; turning behaviour; r = -0.49, p>0.05), suggesting that lesion size does not have an impact on behavioural performance beyond initial thigmotaxis.
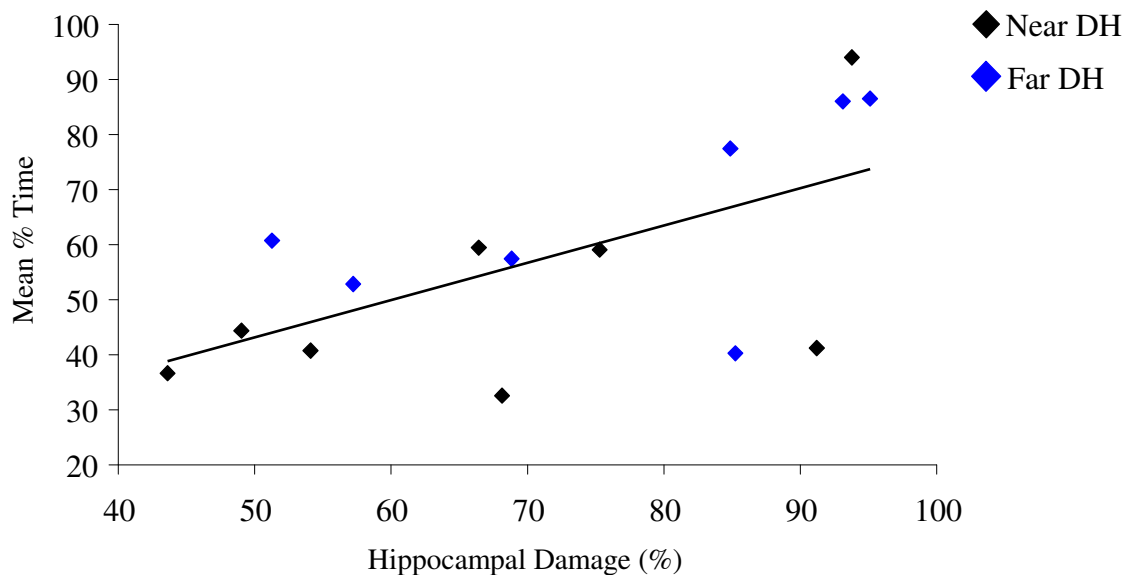
Figure 6.3: Correlations between mean percentage time spent in total thigmotaxis throughout training and percentage of hippocampal damage for all dorsal hippocampal lesioned animals.

6.3.3 Subregion Lesion Size and Performance Correlations

6.3.3.1 Basic Measures of Acquisition

Following from overall lesion size investigation, Pearson product-moment correlations were subsequently conducted to assess the relationship between the three hippocampal subregions; DG, CA3 and CA1, and general acquisition measures. A significant

positive correlation was noted between the extent of damage to the DG and escape latency (EL) (r = 0.65, p<0.01) and also with distance travelled (r = 0.69, p<0.01). However, there was no correlation between the extent of DG damage and velocity (r = 0.39, p>0.05). Damage to area CA3 was assessed in a similar manner and it was revealed that the extent of damage positively correlated with distance travelled by the animal (r = 0.57, p<0.05) and also with swimming velocity (r = 0.55, p<0.05), but unlike DG, there was no significant correlation between CA3 and EL (r = 0.44, p>0.05). Interestingly, there was no correlation between the size of CA1 damage and any of the basic acquisition measures; EL (r = 0.48, p>0.05), distance travelled (r = 0.57, p>0.05), and velocity (r = -0.09, p>0.05).

6.3.3.2 Exploratory Behaviours

In a further attempt to dissociate subregion function, correlations were made between the extent of subregion damage and individual exploratory behaviours in the task. From this, we found that the greater the damage to the DG, the longer the animals spent in total thigmotaxis (r = 0.66, p<0.01) and PT (r = 0.59, p<0.05). However, there was no significant correlation between the extent of DG damage and percentage time spent in VT (r = 0.27, p>0.05), direct (r = -0.39, p>0.05) and turns towards the cues (r = -0.39, p>0.05). Area CA1 correlations returned similar findings with significant correlations between the extent of CA1 damage and time spent in total thigmotaxis (r = 0.55, p<0.05). However, beyond this there was no significant correlation with any other behaviour noted; PT (r = 0.38, p>0.05), VT (r = 0.49, p>0.05), direct (r = -0.36, p>0.05) and turning behaviour (r = -0.50, p>0.05). CA3 correlations revealed a similar result with the extent of damage positively correlating with the time spent in total

thigmotaxis (r = 0.64, p=0.01) and PT (r = 0.72, p<0.01). In addition, when the same correlation analysis was carried out on the mean number of turns towards the cues, it was found that the greater the damage to CA3, the less turns towards cues made (r = -0.55, p<0.05). There was also no significant correlation between CA3 damage and VT (r = -0.06, p>0.05) or direct behaviour (r = -0.42, p>0.05).

6.3.3.3 Retention

The retention trial was also subjected to similar, full and subregional damage, correlational analyses. For this investigation, the NE quadrant was focused on, particularly the NE *platform area* as this was the expected goal location that animals were trained towards. Pearson product-moment correlations revealed no significant relationships between total dorsal hippocampus damage (r = -0.39, p>0.05), DG damage (r = -0.51, p>0.05) or CA1 damage (r = -0.11, p>0.05) and the time spent in the NE platform area. However, the extent of CA3 damage was related to the time spent in the NE platform area, where greater damage was associated with poorer searching and less time spent in the target area (r = -0.54, p<0.05; Figure 6.4).
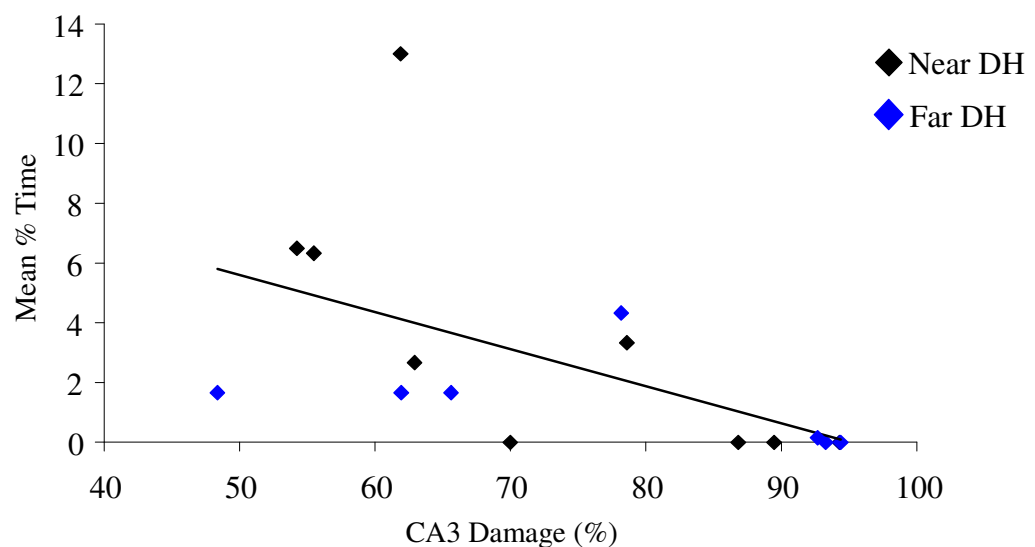
Figure 6.4: Correlations between mean percentage time spent in the NE quadrant during the retention trial and percentage of CA3 damage for all lesioned animals.

6.3.4 Comparative Analysis; Acquisition

As there were no differences in the extent of damage to the dorsal hippocampus between the Near DH and Far DH groups, we subsequently assessed any direct differences between the groups in their performance in the maze to determine if the higher spatially demanding far cue task augmented the impairment caused by lesions.

6.3.4.1 Basic Measures

Initial examination of the basic acquisition measures revealed that the Near DH group had an overall mean EL of 38.61±5.75 sec with the Far DH group having a mean EL of 46.29±3.81 sec. Further, a 2 x 5 mixed factorial ANOVA revealed an overall effect for day [$F(4, 52) = 4.91$, $p<0.01$] with Bonferroni-corrected pairwise comparisons revealing significantly shorter ELs on Day 5 (M: 33.39±4.53 sec) than Day 1 (M: 46.96±2.86 sec; $p<0.05$) suggesting that as training progressed some improvement in task performance occurred. However, there was no significant difference seen between the groups [$F(1, 13) = 2.03$, $p>0.05$] or interaction effect between day and group [$F(4, 52) = 0.99$, $p>0.05$; see Figure 6.5], suggesting that both groups performed at a similar level, irrespective of cue location.
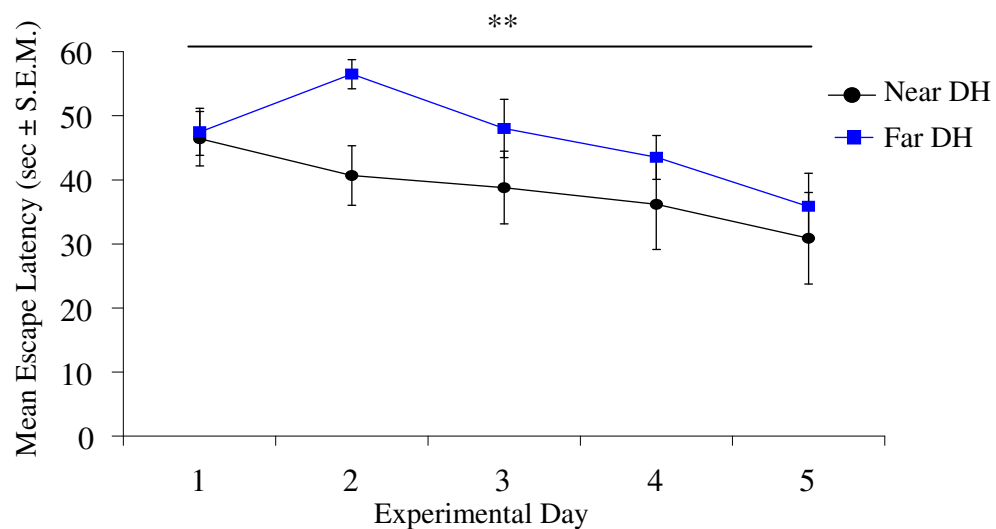
Figure 6.5: Mean escape latency (sec ± S.E.M.) for the Near DH and Far DH lesioned animals throughout training.

A similar assessment of distance travelled revealed an overall effect for day $[F_{(4, 52)} = 3.64, p<0.05$; see Figure 6.6] with longer distances travelled on Day 1 (M: 1150.42±80.74 cm) than Day 5 (M: 960.44±137.16 cm). However, following Bonferroni-corrections no significant differences between days were found. Furthermore, no differences between the Near DH or Far DH group $[F_{(1, 13)} = 3.29, p>0.05]$ or interaction effect between day and group was noted $[F_{(4, 52)} = 2.24, p>0.05]$.
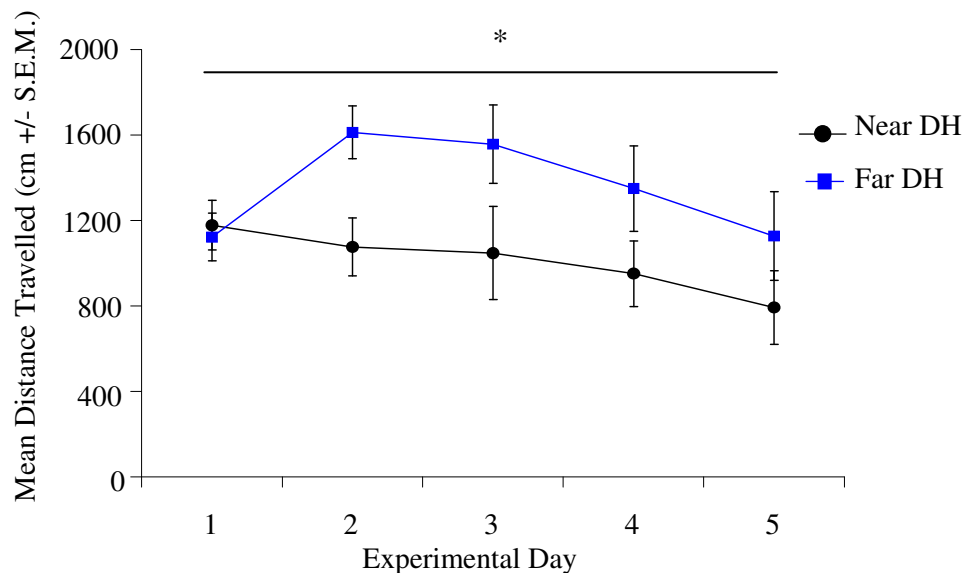
Figure 6.6: Mean distance travelled (cm ± S.E.M.) for the Near DH and Far DH lesioned animals throughout training.

A 2 x 5 mixed factorial ANOVA assessing the mean swimming velocity for the groups also revealed a significant effect for day $[F_{(4, 52)} = 3.59, p<0.05]$, with Bonferroni-corrected pairwise comparisons revealing a significant difference in velocity on Day 1 (M: 24.19±0.96) compared to Day 2 (M: 27.36±1.22, p<0.05). However, there was no significant difference between groups $[F_{(1, 13)} = 2.01, p>0.05]$ or interaction effect between day and group $[F_{(4, 52)} = 2.27, p>0.05]$.

6.3.4.2 Swimming Behaviours

Taken together, the standard measures of acquisition suggest that there were no differences between the two lesion groups despite their cues being positioned in different locations. However, as previously reported, these measures do not always reveal existing underlying differences (D. Harvey et al., 2009). Therefore, we further explored differences in the Near DH and Far DH swimming behaviours to assess any subtle differences in their performance during training. The mean percentage time of total time in the pool, spent in thigmotaxis, was initially examined. A 2 x 5 mixed factorial ANOVA revealed no effect for day [$F_{(4, 52)} = 1.77$, $p>0.05$], no overall differences between the groups [$F_{(1, 13)} = 2.97$, $p>0.05$] and no interaction effect between day and group [$F_{(4, 52)} = 1.58$, $p>0.05$]. Examination of percentage time spent in PT throughout training also revealed no day effect [$F_{(4, 52)} = 1.24$, $p>0.05$]. Similarly, no differences between the groups in time spent in PT was noted [$F_{(1, 13)} = 0.84$, $p>0.05$] nor interaction effect between day and group [$F_{(4, 52)} = 0.32$, $p>0.05$]. However, examination of VT, a behaviour that has previously highlighted critical differences in search patterns between training groups (Chapter 3a, 4 and 5), revealed a significant effect for day [$F_{(4, 52)} = 6.98$, $p<0.001$], with Bonferroni-corrected t-tests revealing a significant differences between Day 1 (M: 35.98±3.87) and Day 5 (M: 14.33±2.42; $p<0.01$) and Day 2 (M: 27.96±2.79) and Day 5 ($p<0.05$), indicating a reduction in performance of the behaviour as training progressed. In addition, a significant difference between the Near DH and Far DH group was also revealed [$F_{(1, 13)} = 5.72$, $p<0.05$], with the Far DH group (M: 28.06±2.55%) spending significantly more time performing this behaviour than the Near DH animals (M: 19.71±2.83%).

There was no further interaction effect between day and group noted [F(4, 52) = 1.12, p>0.05].

Subsequently, assessment of the more navigationally complex behaviours was also carried out. Analysis of percentage time spent in direct behaviour initially revealed no overall effect for day [F(4, 52) = 0.75, p>0.05] and no significant differences in the performance between the groups overall [F(1, 13) = 2.3, p>0.05]. However, a significant interaction effect between day and group was found [F(4, 52) = 2.85, p<0.05], with further independent t-tests highlighting a significant difference between the groups, with the Near DH group (M: 22.02±10.70%) spending significantly more time performing direct movements than the Far DH group (M: 9.35±3.99%) on Day 2 of training (t(13) = 2.94, p<0.05). In addition, similar analysis of the mean number of turns towards the cues revealed no differences between the groups [F(1, 13) = 2.89, p>0.05]. There was also no day [F(4, 52) = 1.23, p>0.05] or day x group interaction effect noted [F(4, 52) = 1.01, p>0.05], suggesting that both lesion group performed this behaviour to a similar level.

6.3.5 Comparative Analysis; Retention

Retention trials have also been used in a number of studies to assess the accuracy of performance and retained information learned throughout the acquisition period and have been particularly useful in the examination of the role of the hippocampus in the MWM (e.g. Morris et al., 1990). As a result, a retention trial carried out 7 days post-acquisition was assessed to determine the search patterns of both sham and lesioned animals trained under either near or far cue conditions. To assess retention the pool was divided into a number of zones including quadrant, platform area, inner corridor

and outer corridor and percentage time spent in these areas was examined. Maze maps (adapted from McGauran et al., 2004), were used initially to determine where animals in each group spent the majority of their time searching during the 60 second probe trial. Figure 6.7 illustrates the percentage time spent by a) the sham and b) the DH animals in each zone of the maze
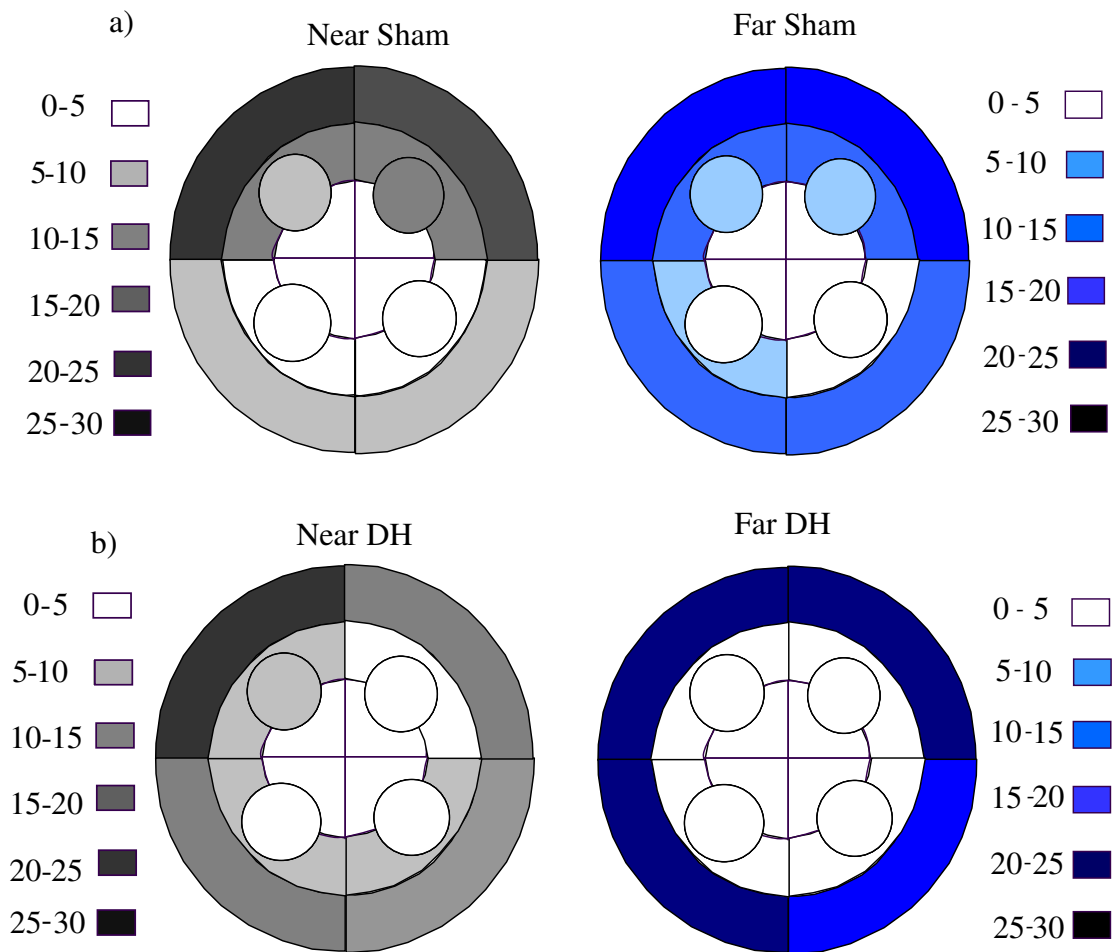
Figure 6.7: Maze maps illustrating the mean percentage time spent by a) the Near and Far sham and b) the Near and Far DH groups in the different zones of the maze during their respective retention trials.

The time spent by the Near sham and Far sham groups in each platform area of the maze was initially assessed to highlight the accurate and successful performance and search patterns of intact animals during retention. A 2 x 4 mixed factorial ANOVA revealed a significant difference in the mean percentage time spent in each of the four platform areas during the retention trial [$F_{(3, 42)} = 13.86$, $p<0.001$; Figure 6.8], with Bonferroni-adjusted t-tests highlighting the highest percentage time was spent in the NE platform area (M: 10.34±1.16%) when compared to the SW (M: 3.91±0.92%; $p<0.001$) and SE areas (M: 4.22±1.20%; $p<0.001$). Both groups also spent the majority of their time searching in this target area with no significant differences found between the groups [$F_{(1, 14)} = 0.01$, $p>0.05$] or interaction effect between group and area [$F_{(3, 42)} = 0.85$, $p>0.05$].
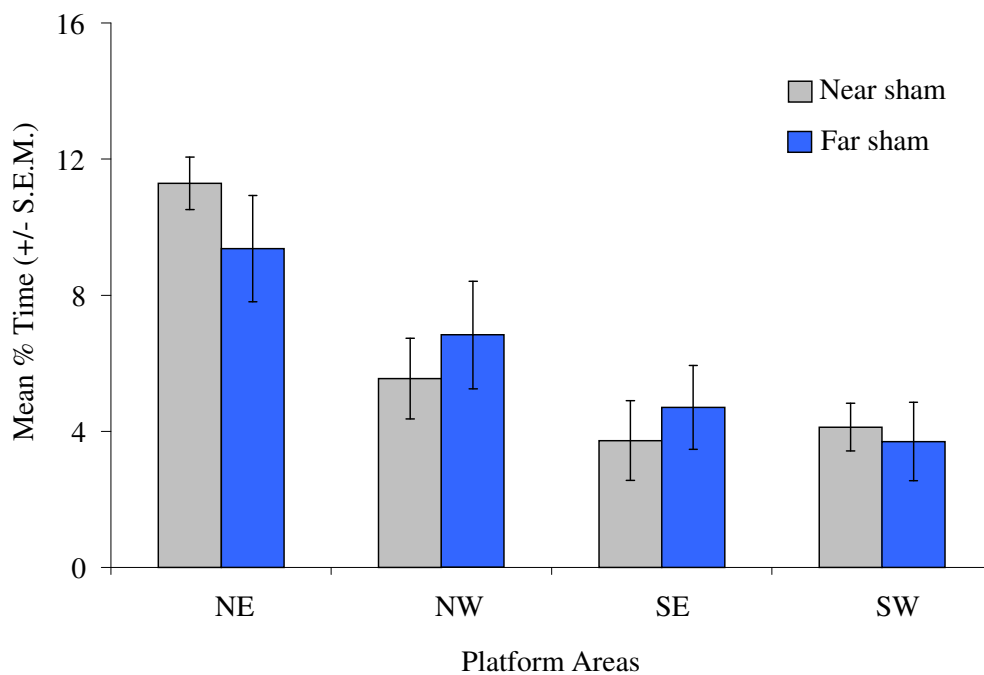
Figure 6.8: The mean percentage time (± S.E.M.) spent by the Near sham and Far sham animals in each of the four platform areas during the retention trial.

Following initial illustration of a successful retention trial, subsequent assessment of the time spent by both the Near DH and Far DH groups in each platform area of the maze was carried out. A 2 x 4 mixed factorial ANOVA revealed no significant difference in the mean percentage time spent in each of the four respective platform areas during the retention trial [$F_{(3, 39)} = 0.47$, $p > 0.05$], with lesioned animals showing no preference for the expected platform area (NE) or any other platform position. Furthermore, there was no significant differences found between the groups [$F_{(1, 13)} = 4.37$, $p > 0.05$] or interaction effect between group and quadrant [$F_{(3, 39)} = 0.27$, $p > 0.05$; see Figure 6.9] suggesting equal searching throughout all areas of the maze, by both groups.
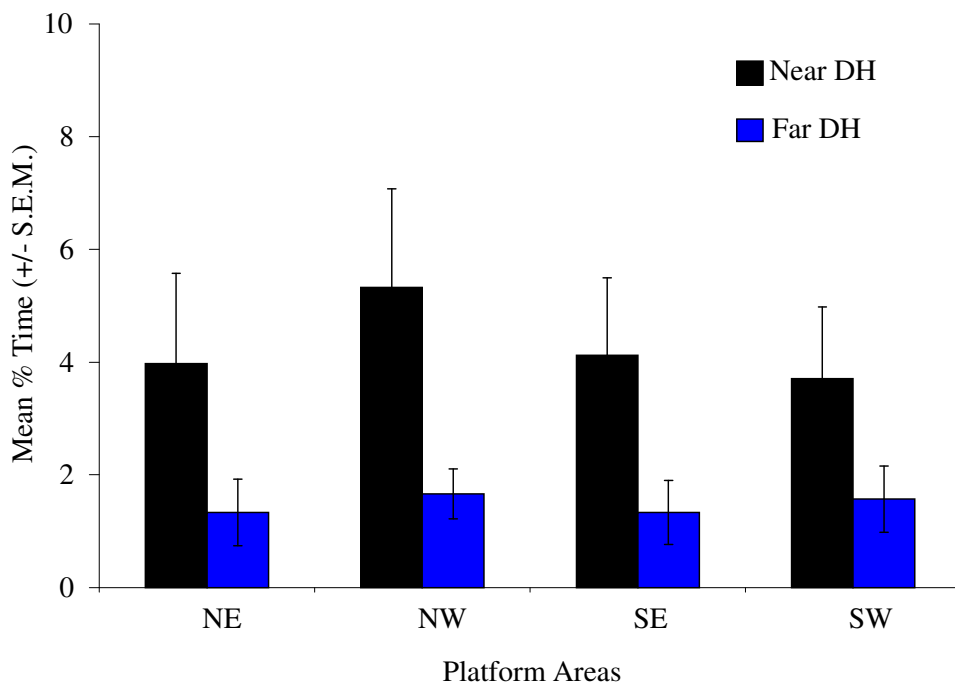
Figure 6.9: The mean percentage time (± S.E.M.) spent by the Near DH and Far DH animals in each of the four platform areas during the retention trial.

Further detailed examination of subsections of the expected NE goal quadrant was also carried out. An independent samples t-test revealed no significant differences between lesioned groups in the time spent searching in the NE quadrant as a whole ($t(13) = 1.77$, $p>0.05$). Similarly, assessment of the NE platform corridor, which is a corridor encompassing the expected platform location, revealed no significant differences between the groups ($t(13) = 0.81$, $p>0.05$) nor did assessment of percentage time in the precise platform area ($t(13) = 1.46$, $p>0.05$). However, there was a significant difference between the groups in the time spent in the NE outer corridor ($t(13) = 2.49$, $p<0.05$), with the Far DH animals (M: 22.62±1.13%) spending significantly more time in the outer area of the NW quadrant than the Near DH group (M: 15.58±2.44%; Figure 6.10).
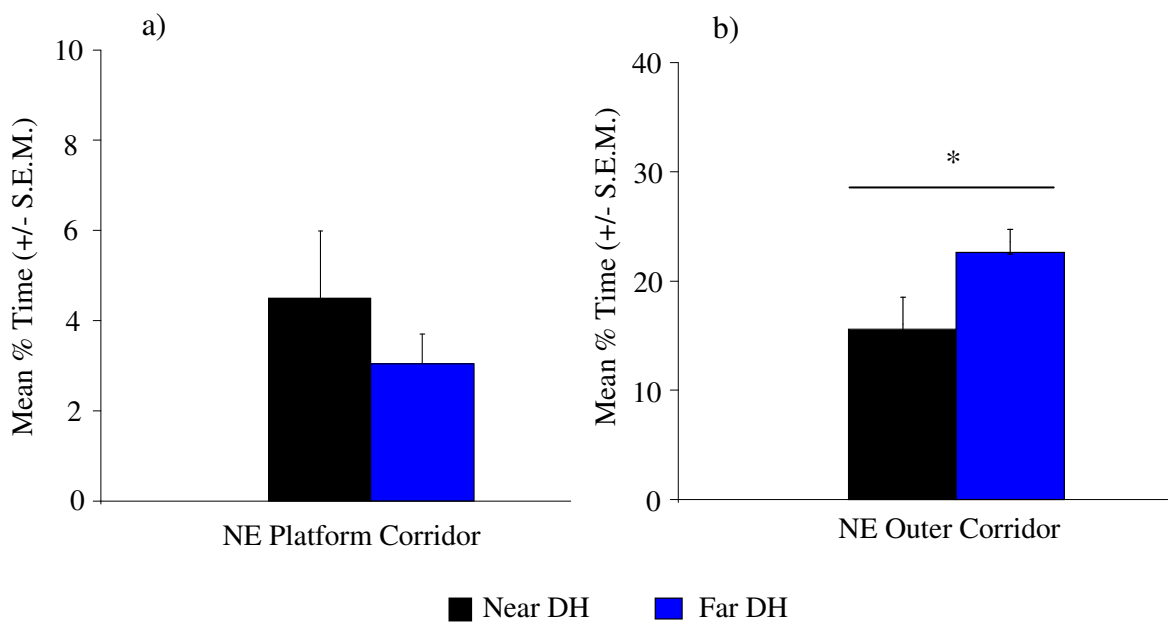
Figure 6.10: Mean percentage time spent by the Near DH and Far DH groups in the a) NE platform corridor and b) NE outer corridor during the retention trial.

6.4 Summary

The effect of lesion size on animals' performance in the maze was first assessed to determine if the extent and also the location of damage to the hippocampus affected basic performance and exploratory movements in the task. Overall assessment of lesion size from both groups combined, revealed that the larger the lesion the poorer the animals' performance in the maze, as evidenced by escape latencies and distance travelled. In addition, assessment of subregional damage on animals' performance in the maze revealed that damage to the DG resulted in similar impairments to that seen following complete dorsal lesions, with increased DG damage leading to an increase in escape latencies and distance travelled. Damage to area CA3 had a slightly different effect, with greater lesions resulting in an increase in the distance travelled, and also in the speed at which animals swum in the maze. Interestingly, damage to area CA1 had no impact on animals' general performance in the maze.

In addition to this, it was also found that when damage to the total dorsal hippocampus was large, the animals reverted to performing more thigmotactic behaviour, with the extent of the lesion having no effect on direct or turning behaviours. When analysis was extended to assess the effect of subregional lesions on the exploratory movements of the animals in the maze, it was found that the larger the damage to all areas, the longer the animals spent in parallel and total thigmotaxis, suggesting that all hippocampal subregions are involved in monitoring basic exploratory behaviours and indeed, may be required for reducing animals' time spent in these initial behaviours. In addition, an increase in the area of damage in the DG and CA1 did not have a significant impact on the more complex navigational behaviours such as direct or turning behaviour. However, as the damage to area CA3 increased,

the animals' performance in the more navigationally complex behaviours reduced, with CA3 perhaps being required for initiating and maintaining these essential exploratory movements. In addition, analysis of the retention trial also suggests that CA3 may be important in the retrieval of spatial information and in the ability to accurately search for a goal during a retention trial.

Furthermore, a key aim of this Chapter was to assess the effect of dorsal hippocampal lesions on performance in the Near and Far cue conditions in the MWM. Initial assessment of the extent of the lesion revealed no significant differences between the Near DH and Far DH groups, allowing for direct comparison of the lesion groups. In addition, histological analysis revealed both groups had similar patterns of damage external to the hippocampus including damage to the overlying corpus callosum and somatosensory cortex at the sites of cannula penetration. While these may have impacted on the behavioural results, it is important to note that all animals displayed normal motor and coordinated swimming movements and that damage to these cortical areas have, previously, been shown not to significantly impact on spatial acquisition in the water maze (Horne et al., 2010; Wright et al., 2004). There was some damage to the habenula which has been previously shown to lead to impairments in learning a place MWM (LeCourtier et al., 2004). However, these patterns of damage external to the hippocampus are not unusual when large infusions of neurotoxin are used to produce a lesion. Furthermore, while the habenula has been shown to disrupt learning in the water maze and is also thought to play an important role in attention processes (LeCourtier et al., 2004), LeCourtier and Kelly (2004) illustrated that habenula damage did not result in significant behavioural impairments, including no perseverative responding, in a choice serial reaction time task. However, the exact role

of these external areas in MWM acquisition and navigational behaviour remains unclear, and as with all lesion studies (see Morris, 2007), results should be interpreted with this in mind.

Following initial histological assessment, animals in the Near and Far DH groups were compared on both basic and behavioural measures in the MWM. We predicted that performance of dorsal hippocampal lesioned animals would be worse in the Far cue condition, as evidence from Chapter 3a and 3b suggested a higher hippocampal involvement when the cues were in a position far away from the goal. Contrary to this, initial analysis of basic measures of acquisition indicated that there were no differences between the Near DH and Far DH animals' performance in the task. The only difference that emerged between the groups was in the mean percentage time spent in thigmotactic behaviour, with the Far DH group spending a significantly longer time in vertical thigmotaxis. While there was no overall difference between the groups in the time spent in direct behaviour, a slight difference emerged on Day 2, with the Near DH performing more of this behaviour than the Far DH animals. Assessments of the retention probe trial also revealed no significant differences between the groups' searching patterns, with neither group displaying a significant preference for any particular quadrant, unlike shams, who quickly showed accurate knowledge of the goal location. The Far DH group, however, did spend significantly more time in the NE outer corridor than the Near DH group, highlighting a reduced time spent in the inner areas of the maze, with animals, instead, remaining at the periphery of the pool. However, there were no differences between the percentage time spent by each group in either the inner corridor or platform areas suggesting both groups searching did not differ during the retention trial.

Overall assessment of swimming behaviours suggests that the Far DH animals are only slightly more impaired in the task than the Near DH animals. Furthermore, these findings suggest that the hippocampus does not play a strong and distinct role in inference, rather the evidence emerging from this Chapter (and the previous two Chapters) would suggest that the hippocampus is differentially more involved in the monitoring of exploratory behaviours over spatial processing. However, these findings, in conjunction with summaries of Chapters 4 and 5, will be further explored in relation to current theory in the concluding Chapter 8.

Chapter 7

Immediate early gene activation in subregions of the dorsal hippocampus; the effect of cue location during Morris water maze training.

Abstract

Immediate early genes (IEG) have been suggested as good markers for neuronal activity and are readily expressed soon after learning. They also enable anatomical examination of the activation of a structure and the subregions within it, following experimental manipulation. Increased IEG expression in the hippocampus, in particular, has been observed following training in a number of spatial tasks (Guzowski et al., 2001; Mayer et al., 2010). While Chapter 3b initially demonstrated that the hippocampus is involved in inference components of the task, this was not subsequently observed in the lesion data (Chapter 4-5). Despite this, however, there was evidence of a differentiation of function within the subregions of the structure seen in Chapter 6. However, this analysis did not reveal, firstly, the full extent to which the specific subregions of the hippocampus may be involved in an intact brain and secondly, whether cue positioning will have a noticeable impact on activation within the functioning hippocampus. Therefore, in the current experiment, animals were trained as described in previous Chapters in a Near Trained (n=7) or Far Trained (n=7) condition. Two yoked-control groups; Near control (n=7) and Far control (n=7), matched to escape latency for the spatially trained groups, were also used and following training, expression of the IEG c-Fos was analysed in all hippocampal subregions for each group. Overall, the DG expressed the highest number of active cells when compared to CA3 or CA1, for all groups. Further analysis revealed higher c-Fos expression in area CA1 only, for the Far trained group when compared to the Near trained group. This increase, however, was not observed between the matched-control groups. These results suggest that as cognitive demands increase, activation within hippocampal area CA1 is simultaneously augmented.

7.1 Introduction

Lesion, electrophysiological and molecular studies have all demonstrated the importance of the hippocampus in the acquisition of spatial tasks (Cain et al., 2006; C.D. Harvey et al., 2009; Harvey et al., 2008; Morris et al., 1982). In addition, even within the hippocampus, specific subregions may play different roles in spatial processing in the MWM, as seen in Chapter 6 (see also Chapter 1). It has previously been proposed that this reported differentiation of function between hippocampal regions could be due to the presence of parallel projections from the EC to the DG, CA3 and area CA1 in the trisynaptic (Amaral & Witter, 1989), disynaptic (Tamamaki & Nojyo, 1993) and monosynaptic circuits (Steward & Scoville, 1976; see Chapter 1 for additional detail). However, debate remains regarding the exact contribution made by each of the subfields in hippocampal-dependent tasks. Attempts at clarifying the subregional contribution to spatial learning has generally taken the form of lesion studies (Hunsaker & Kesner, 2008; Jeltsch et al., 2001; Maglakelidze et al., 2010; Okada & Okaichi, 2009), however these have led to a number of functions being attributed to each region. Okada and Okaichi (2009), for example, found differential involvement for the DG in the acquisition of the MWM over CA1 and CA3. Whereas, others have implicated CA1 in both the encoding (Rondi-Reig et al., 2006) and retrieval of spatial information (Hunasker & Kesner, 2008) with CA3 thought to be more involved in the recall of spatial memories (Nakazawa et al., 2003).

While both complete and targeted lesion types are informative to our understanding of different brain regions and their contributions to the processing of spatial learning and memory (Churchwell et al., 2010; Hunsker & Kesner, 2008; Mogensen et al., 2005), lesion studies have inherent problems associated with them;

interpretation and analyses is conducted on a damaged brain and information gained, while very useful, only reveals information on a specific brain region necessary for a particular function. As such, extrapolating and applying findings to a functioning brain can raise some difficulties (Amin et al., 2006; Poirier et al., 2008). Therefore, other methods of analyses in conjunction could be more informative, such as IEG expression which provides an anatomical view of a functioning brain (Albasser et al., 2007; He et al., 2002; Pothuizen et al., 2009; Vann et al., 2000a).

IEG mapping is particularly useful in examining activation of a functioning brain. Specifically, the mnemonic capacity of an animal is thought to depend on neural networks whose synaptic plasticity is dependent on specific patterns of protein synthesis (Okuno, 2011). LTP, a cellular model of learning, has been shown to be prevented when mRNA synthesis is blocked immediately after learning, suggesting the critical importance of protein and gene expression very soon after an event to be memorised (Lanahan & Worley, 1998). IEG mapping, therefore, is a useful method to visualise active neurons related to learning and memory as IEG levels are typically low in resting animals but are dramatically increased following neural activity associated with the induction of synaptic plasticity (Cole et al., 1989). c-Fos in particular is often examined following cognitively demanding spatial tasks (Herrera & Robertson, 1996; Kubik et al., 2007; Santin et al., 2003). It is particularly suitable for identifying neurons acutely involved in hippocampal functioning as basal levels are generally low and not highly expressed in this region (Cullinan et al., 1995; Sheng & Greenberg, 1990); allowing for clear detection of hippocampal subregional activation following various behavioural paradigms (Guzowski et al., 2006; Kubik et al., 2007). Evidence for a role of c-Fos during memory formation has come from a number of areas of

investigation, including genetic manipulation, where knock out mice for c-Fos were shown to be impaired in hippocampal-dependent LTP formation (Fleischmann et al., 2003; Paylor et al., 1994) and also behavioural studies, where increased c-Fos expression was observed following a number of spatial tasks including the water maze and radial-arm maze (Countryman et al., 2005; Guzowski et al., 2001; He et al., 2002; Koponen et al., 2004; Mayer et al., 2010; Teather et al, 2005; Tischmeyer & Grimm, 1999).

While allowing clear anatomical visualization of activity within hippocampal subregions, some ambiguity of function has been observed using IEGs, with Guzowksi et al. (2001) reporting no differences in activity between hippocampal regions following spatial learning, whereas others have recorded clear differences in expression between subfields. French et al. (2001), for example, found increased IEG activity only in the DG following induction of LTP in the perforant path but not in area CA1 following induction of LTP by stimulation of commissural projections to the pyramidal cells. Teather et al. (2005), similarly, reported higher expression of c-Fos in area CA1, but not CA3, following spatial training in the water maze. Further, clearer differentiation of IEG activity between regions, however, was found to occur when assessed under specific conditions such as when the cognitive demands of a task increased in a training paradigm. Vann et al. (2000a), for example, found a greater rise in CA1 activation relative to area CA3 when animals were exposed to a novel version of the radial-arm maze. Moreover, Satvat et al. (2011) reported distinct patterns of Egr-1 expression in the DG only, when animals were trained in a more difficult place over a response plus-maze. These findings would suggest that detecting changes in the pattern of IEG activation across hippocampal subregions, under various learning

conditions, provides a good alternative method and level of analysis, for identifying changes in neural plasticity associated with learning.

While we demonstrated that hippocampal BDNF expression was higher in intact animals in the Far trained group over the Near trained group (Chapter 3b), suggesting a role for the hippocampus in inference components of the water maze task, this was not subsequently shown in our lesion data (Chapters 4, 5, and 6). However, as IEG imaging enables the visualisation of distinct regions within the hippocampus, we assess activation of the IEG c-Fos under NT and FT conditions in order to further clarify this issue, and to determine any subtle differences that may arise within the intact hippocampus under differing task demands. However, it is not only spatial learning and memory that contributes to increased IEG activation; physical movement without the occurrence of learning can also influence IEG expression (Kavushansky et al., 2006; Shires & Aggleton, 2008). Therefore, we have included a yoked-control group, similar to that in Chapter 3b to differentiate potential learning-dependent alterations in IEG expression from those caused by non-mnemonic confounding factors. Similarly, as the hippocampus is vulnerable to stress-induced damage (Cullinan et al., 1995; Maroun & Richter-Levin, 2003; Vedhara et al., 2000; Woolley et al., 1990), a caged control group was also used to evaluate basal levels of IEG expression in the different hippocampal subregions. However, we did not use the caged controls as a direct comparison of our learning group as the conditions to which this group was exposed were completely different to the training environment, thereby making any comparison difficult to interpret and reconcile.

7.2 Method

7.2.1 Subjects

Male Wistar rats (n=32) served as subjects in the current study. All were managed and housed in similar conditions as described previously.

7.2.2 Apparatus

The standard MWM paradigm was, again, used in this experiment (see Chapter 3a for details), with the hidden escape platform located in the centre of the NE quadrant. All settings and experimental protocols were identical to those in the previous chapters.

7.2.3 Procedure

All animals (n=14) received identical training to that outlined in Chapter 3a; 4 trials for 5 consecutive days in the MWM, commencing from one of four pseudo-random start positions (N, S, E, W). Animals in the Near trained group (NT; n=7) and Far trained (FT; n=7) groups had their distal cue configurations in the same locations as described in Chapter 3a. Two sets of motor control groups; Near control (NC) and Far control (FC; n=7 per group) were also used for comparative purposes with the spatial learning groups. These animals were placed in the pool for the same length of time as their learning counterparts without a platform present. The length of time each group spent in the pool was determined by their spatial equivalent group's mean time spent swimming on each respective training day. As there was no spatial learning involved, these animals were therefore "yoked-controls". A further sedentary caged control group (n=4) was also used. These animals did not receive any training or exercise in

the MWM. This group was included to obtain a representation of basal levels of IEG expression in various subregions of the hippocampus.

7.2.4 Immunohistochemistry

Ninety minutes (Albasser et al., 2007; Amin et al., 2006; Zangenehpour & Chaudhuri, 2002) after completion of the last training trial in the MWM or the final matched yoked trial, rats were deeply anaesthetised with sodium pentobarbital (100mg/kg i.p.; Euthatal) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M PB (PFA). The brains were then removed and postfixed in 4% PFA for approximately 12 hours and then transferred to a 30% sucrose solution at 4°C overnight. Coronal sections were cut at 40μm on a freezing microtome and every fourth section was taken for analysis.

To minimize variability in staining attributable to the histological procedure rather than to the behaviour, brain sections of representatives for all conditions were processed in a single batch. Free floating sections were stored in 0.1M PB with 0.01% sodium azide at 4°C. Slices were washed (10 min) twice in 0.1M PB followed by a single 10 minute wash in 0.1M PB with 0.03% triton-x-100 (PBX). A further 20 minute PBX wash containing 1.5% hydrogen peroxidase was carried out. This was followed by two 10 minute PB washes and a final 10 minute PBX wash. The slices were then blocked for 60 minutes in 5% normal goat serum (NGS; for rabbit polyclonals) in 0.1M PBX. Sections were then incubated in primary antibody solution (2% NGS in 0.1M PBX overnight). The primary antibody used was c-Fos, rabbit polyclonal IgG (1:8000; Santa Cruz Biotechnology). After incubation with primary antisera, sections were washed at room temperature (two 10 min PB, one 10 min

PBX). They were then incubated with the appropriate biotinylated secondary antibody (1:400 goat anti-mouse) for 70 minutes in 2% NGS in PBX. Sections were subsequently washed and incubated in avidin biotin complex (ABC, 0.4%) for 90 minutes at room temperature. Sections were then washed twice for 10 minutes in PB and once, for 10 minutes, in 0.1M sodium acetate. Immunoreactivity was then visualised using the nickel-diaminobenzidine (DAB) technique. Sections were reacted for standardised lengths of time for each group to minimize variability. Finally, the sections were washed twice for 10 minutes each in PB. Sections were then mounted onto gelatin coated slides, air dried overnight, dehydrated and delipified, cleared and cover-slipped.

7.2.5 Image Analysis and c-Fos cell Counts

Digital images of sections were taken with an Olympus Camedia C2020-Z camera mounted on a BX-50 microscope (Olympus). Numbers of c-Fos immunoreactive cells were counted by visual inspection. Both right and left hemispheres at each rostrocaudal level from bregma -1.80 to -4.92 mm in each animal were examined for c-Fos activated cells. The region of interest for analysis was the dorsal hippocampus. This was examined by looking at IEG activity in the structure's three cytoarchitectonic subfields: the dentate gyrus (DG), area CA1 and area CA3.

To reduce the impact of staining variability between sets of sections stained at different times, all counts were normalised. For statistical analyses, counts were normalised according to matched sets of animals for the spatially trained (one animal from the NT and FT each) and the yoked-control groups (one animal from each of the NC and FC groups). For this, the mean number of activated neurons for each animal, at

a given site, was divided by the combined mean of the pair in each cohort, and was then expressed as a percentage (Shire & Aggleton, 2008; Albasser et al., 2007; Vann et al., 2000b). For initial statistical analysis, normalised c-Fos counts were used. In addition to this, when assessing the level of expression between areas, within a group, the mean number of cells/mm^2 were calculated to account for the variation in size of each of the hippocampal subregions. For this, the size of each of the three regions at each rostrocaudal level from bregma -1.80 to -4.92mm was calculated and the number of activated cells in each region counted and expressed as cells/mm^2.

7.2.6 Statistics

All statistical analyses of collated data were carried out using SPSS statistical package (Version 17). The significance of differences was determined by using analysis of variance (ANOVA) with Bonferroni-corrected t-tests. Independent t-tests were also used, where appropriate. The symbol ± was employed throughout to indicate standard error of the mean (S.E.M.) A star-based system for significance representing p-values of *<0.05, **<0.01, ***<0.001, respectively, was used throughout.

7.2.7 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children under the Cruelty to Animals Act 1876 guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

7.3 Results

7.3.1 Basic Measures of Acquisition

Overall learning in the maze was initially assessed using standard measures including escape latency (EL), total distance travelled and mean swimming velocity. The mean EL for both groups decreased across training with the NT group's mean EL across 5 training days recorded as 34.92±5.23 sec, 26.83±2.82 sec, 14.87±2.81 sec, 7.55±1.10 sec and 9.38±1.78 sec, respectively. However, the FT group appeared to have consistently slower mean ELs throughout training when compared to the NT group; D1: 37.37±3.90 sec, D2: 29.96±3.60 sec, D3: 17.77±2.86 sec, D4: 13.25±1.08 sec and D5: 12.7±1.20 sec (see Figure 7.1). To further examine this, a 2 x 5 mixed factorial ANOVA was conducted. Results revealed that there was an overall effect for day [$F(4, 48) = 39.29$, $p<0.001$] with Bonferroni-corrected t-tests revealing that Day 5 was significantly faster than Days 1 ($p<0.001$) and 2 ($p<0.001$). However, there were no significant differences between the NT and FT animals [$F(1, 12) = 1.84$, $p>0.05$] and no day x group interaction effect [$F(4, 48) = 0.11$, $p<0.05$].
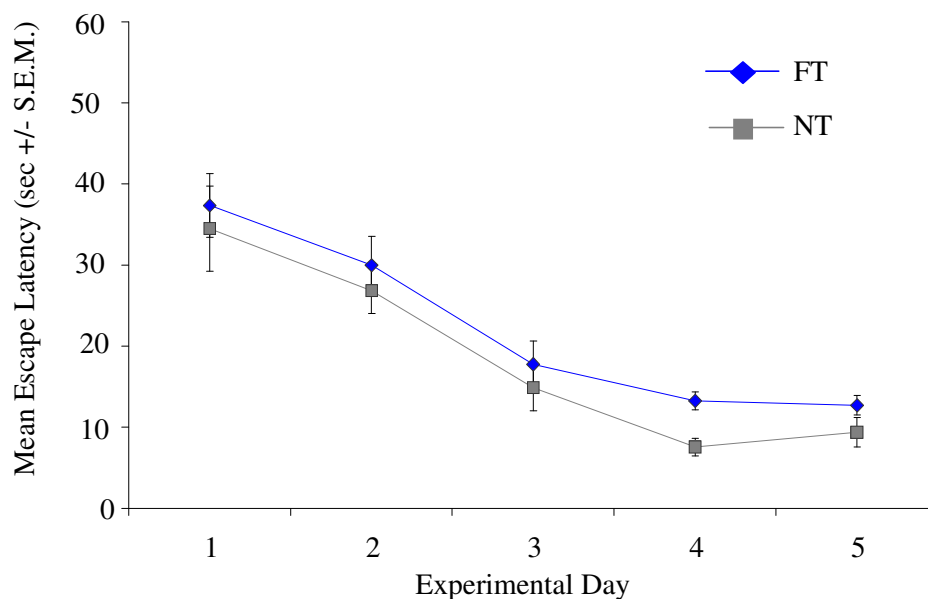
Figure 7.1: Mean escape latency (sec ± S.E.M.) for the NT and FT groups.

Initial assessment of the mean distance travelled over training indicated a decrease in path length for the NT group from 728.02±90.10 cm on Day 1 to 243.23±42.38 cm on Day 5. The FT group also displayed a similar decrease across training (D1, M: 692.15±55.64 cm and D5, M: 235.01±20.10 cm; see Figure 7.2). Similarly, a 2 x 5 mixed factorial ANOVA revealed an overall effect for day [$F_{(4, 48)}$ = 37.35, $p<0.001$] with Day 5 distances significantly shorter than Days 1 ($p<0.001$) and 2 ($p<0.001$). There was, again, no effect for group [$F_{(1, 12)}$ = 0.30, $p>0.05$] or day x group interaction effect [$F_{(4, 48)}$ = 0.63, $p>0.05$].
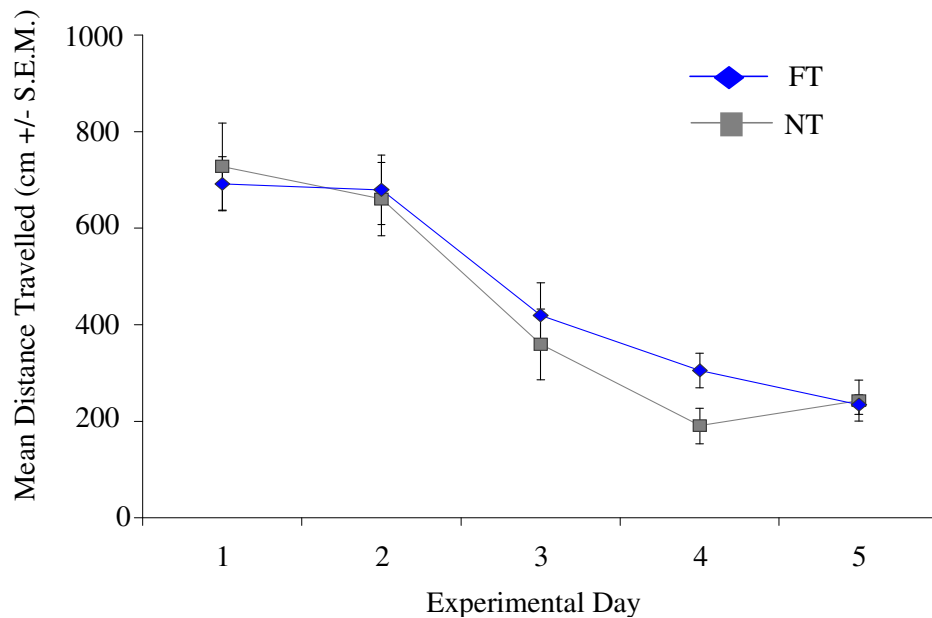
Figure 7.2: Mean distance travelled (cm ± S.E.M.) for the NT and FT groups.

Similar assessment of the mean velocity travelled revealed no effect for day [$F_{(4, 48)}$ = 2.29, $p>0.05$]. However, an overall effect for group was found [$F_{(1, 12)}$ = 12.75, $p<0.01$], with the NT group (M: 24.80±1.40 cm/sec) swimming significantly faster than the FT group (M: 20.91±0.87 cm/sec). There was also a day x group interaction effect [$F_{(4, 48)}$ = 3.42, $p<0.05$] with further independent t-tests revealing differences between groups on Day 1 ($t_{(12)}$ = 2.7, $p<0.05$) and Day 5 ($t_{(12)}$ = 3.67, $p<0.01$).

7.3.2 Baseline hippocampal IEG expression

Before comparing the level of IEG expression between groups, we first measured basal expression of c-Fos within the hippocampus in a group of caged control animals, to determine resting levels of activity within the structure. For this, we measured the mean number of activated cells per mm^2 in the DG, CA3, and CA1 from the dorsal portion of the hippocampus in 4 caged control animals. The expression of c-Fos remained very low with a mean number of activated cells of 1.60±0.78, 0.33±0.05, and 0.21±0.09, in the DG, CA3 and CA1, respectively (see Figure 7.3). A repeated measures ANOVA revealed no significant difference between the regions at basal levels [$F(2, 6) = 2.86$, $p>0.05$].
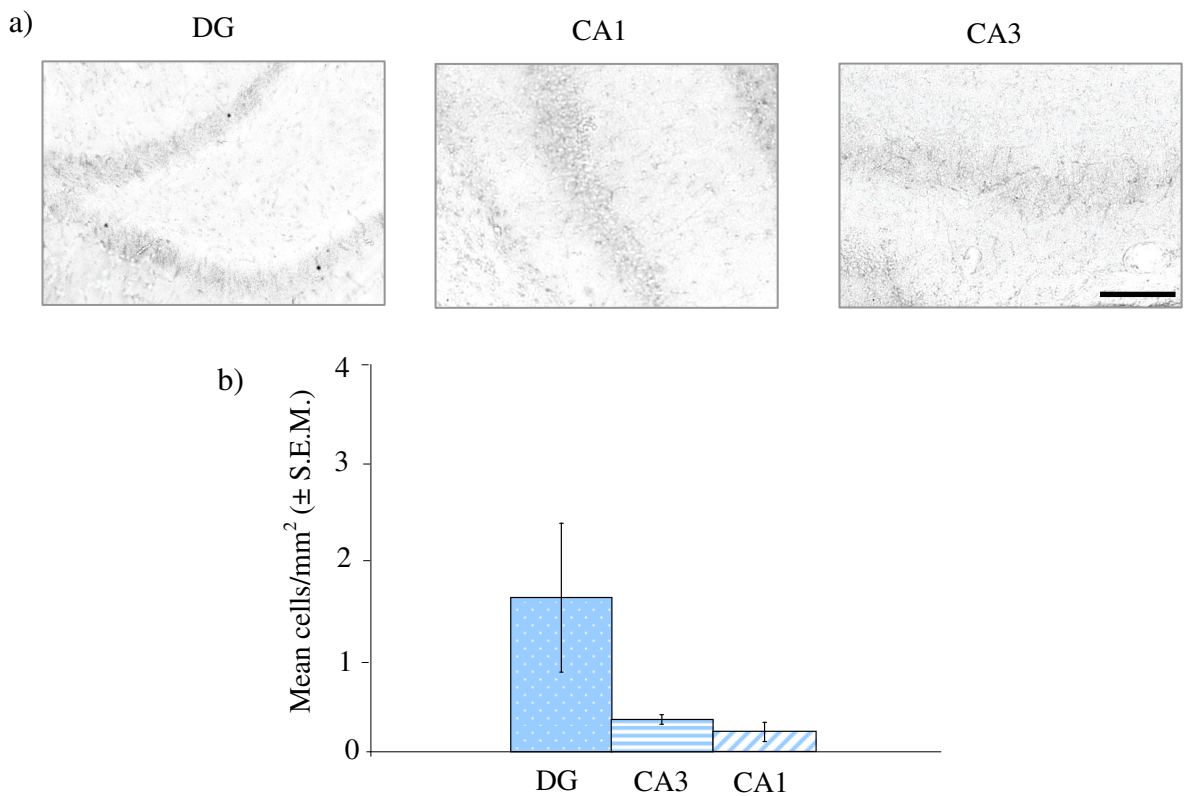
Figure 7.3: a) Photomicrographs illustrating c-Fos expression in the DG, CA1 and CA3 for caged control animals. b) Basal levels of hippocampal IEG expression in the DG, CA3 and CA1 per mm^2 for the caged control group. Scale bar = 100µm

7.3.3 Water maze activation of hippocampal IEG expression

Following this, as we had found differences in regional effects from our lesion data (Chapter 6), we wanted to assess if any one region of the hippocampus was more highly activated while navigating in the pool than the other subregions. For this, we again examined the mean number of activated cells/mm^2 in each of the groups. From this, we noted significant differences in the number of activated cells across the three hippocampal subregions in each of the groups, with overall subregional differences noted in the NT [$F_{(2, 12)} = 33.98$, $p<0.001$], FT [$F_{(2, 12)} = 39.51$, $p<0.001$], NC [$F_{(2, 12)} = 18.74$, $p<0.001$] and FC groups [$F_{(2, 12)} = 45.81$, $p<0.001$]. Bonferroni-corrected pairwise comparisons revealed that activation within the DG was significantly higher than area CA3 ($p<0.01$) and CA1 ($p<0.01$) for all groups. In addition, expression in area CA3 was found to be significantly higher than CA1 for all groups ($p<0.05$; Figure 7.4 and Figure 7.5).
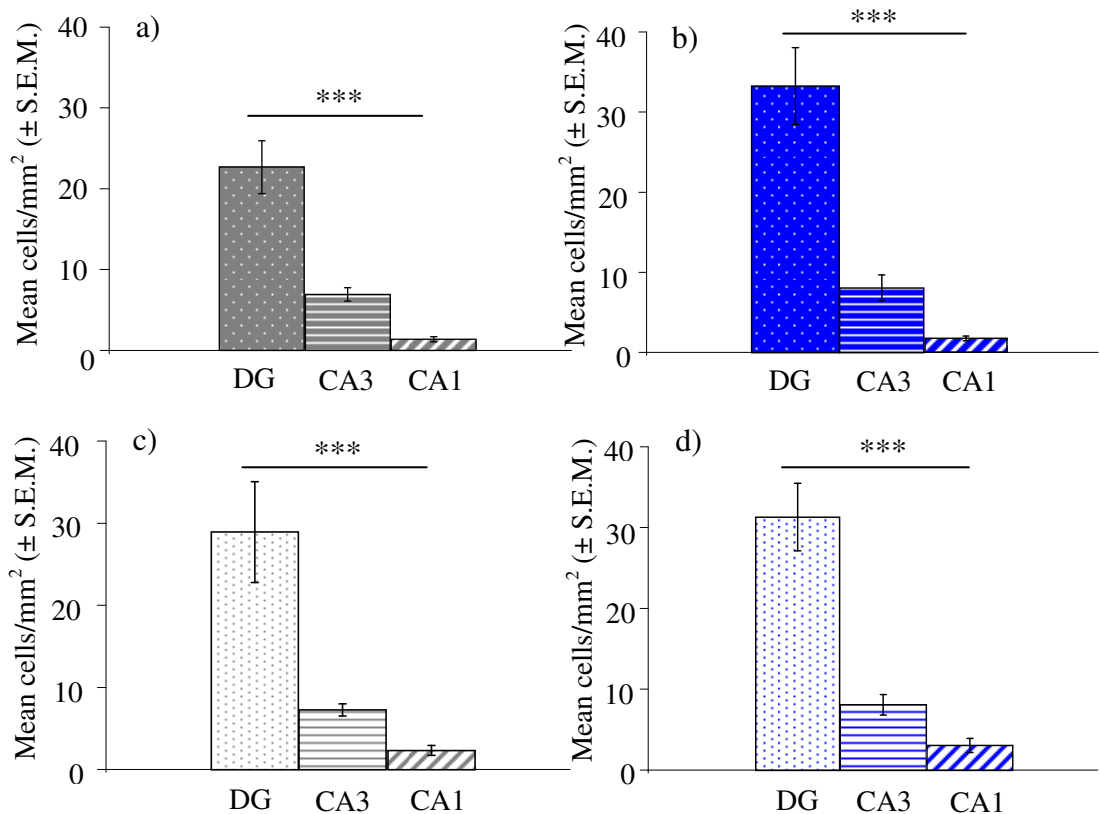
Figure 7.4: Hippocampal IEG expression in the DG, CA3 and CA1 for the a) NT, b) FT, c) NC, and d) FC groups.
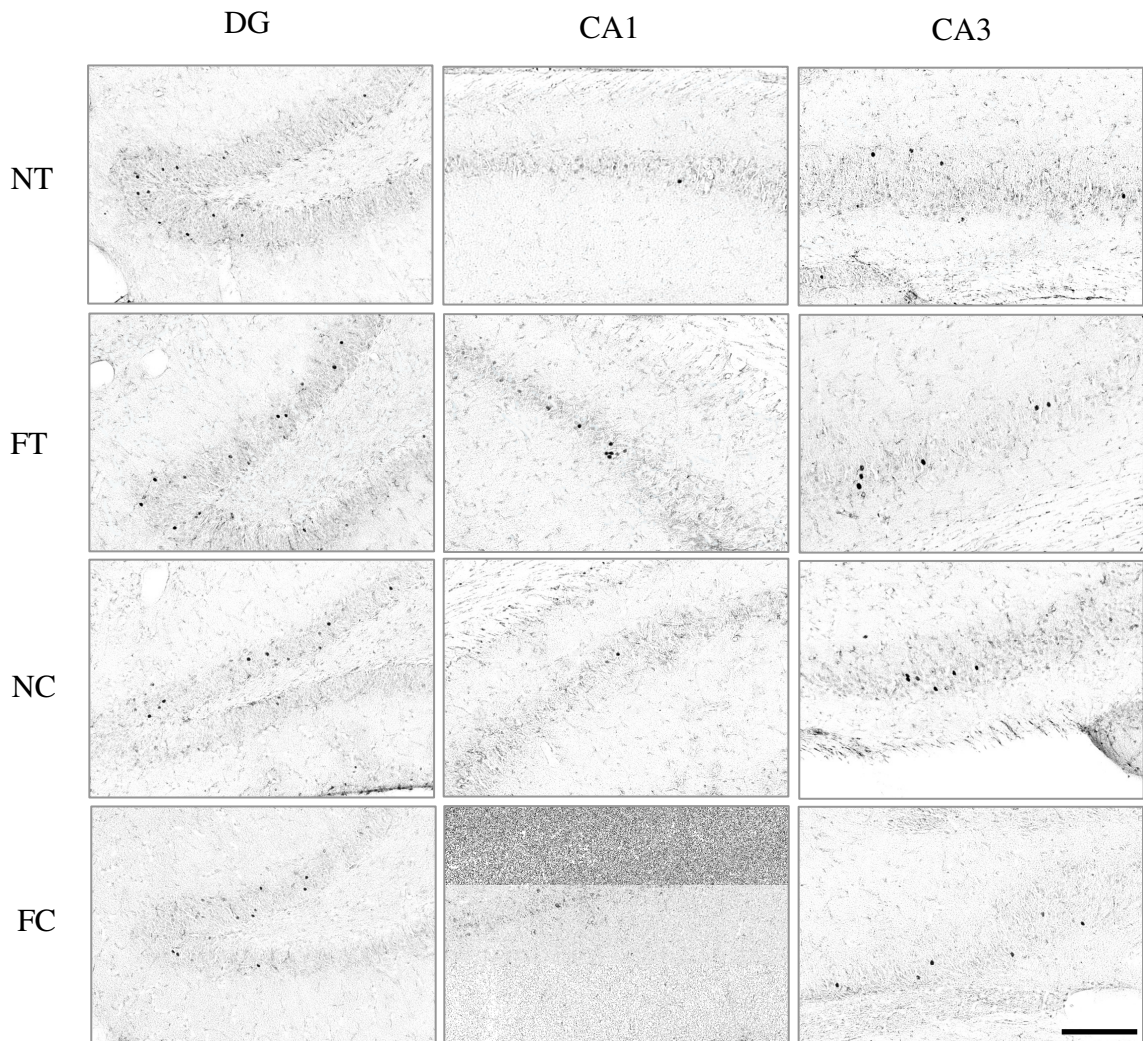
Figure 7.5: Representative photomicrographs showing c-Fos activation following exposure to the MWM in the DG, CA1 and CA3 for the NT, FT, NC and FC groups. Scale bar = 100μm.

7.3.4 Between-condition comparison of hippocampal IEG expression

Examination of differences in c-Fos expression between groups was also assessed to determine if cue condition had any effect on IEG expression within the hippocampus. Independent t-tests comparing the normalised c-Fos counts for the NT and FT groups revealed no differences in the DG ($t(12) = 1.88$, $p>0.05$), with the NT group having a mean normalised expression of $42.9\pm5.33\%$ and the FT group having a mean

expression of 57.12±5.33%. However, further assessment of the individual subregions revealed significant differences between the NT (M: 37.64±5.89%) and FT (M: 62.4±5.88%) groups in area CA1 (t(12) = 2.97, p<0.05; Figure 7.5). No significant differences, however, were found between groups in area CA3 (t(12) = 1.41, p>0.05). Furthermore, independent t-tests examining differences in levels of c-Fos activation in the NC and FC groups revealed no differences in any area of the hippocampus including the DG (t(12) = 1.23, p>0.05), CA1 (t(12) = 0.58, p>0.05) and CA3 (t(12) = 0.95, p>0.05; see Figure 7.6), suggesting that length of time in the pool alone does not account for the IEG difference seen in the training groups.
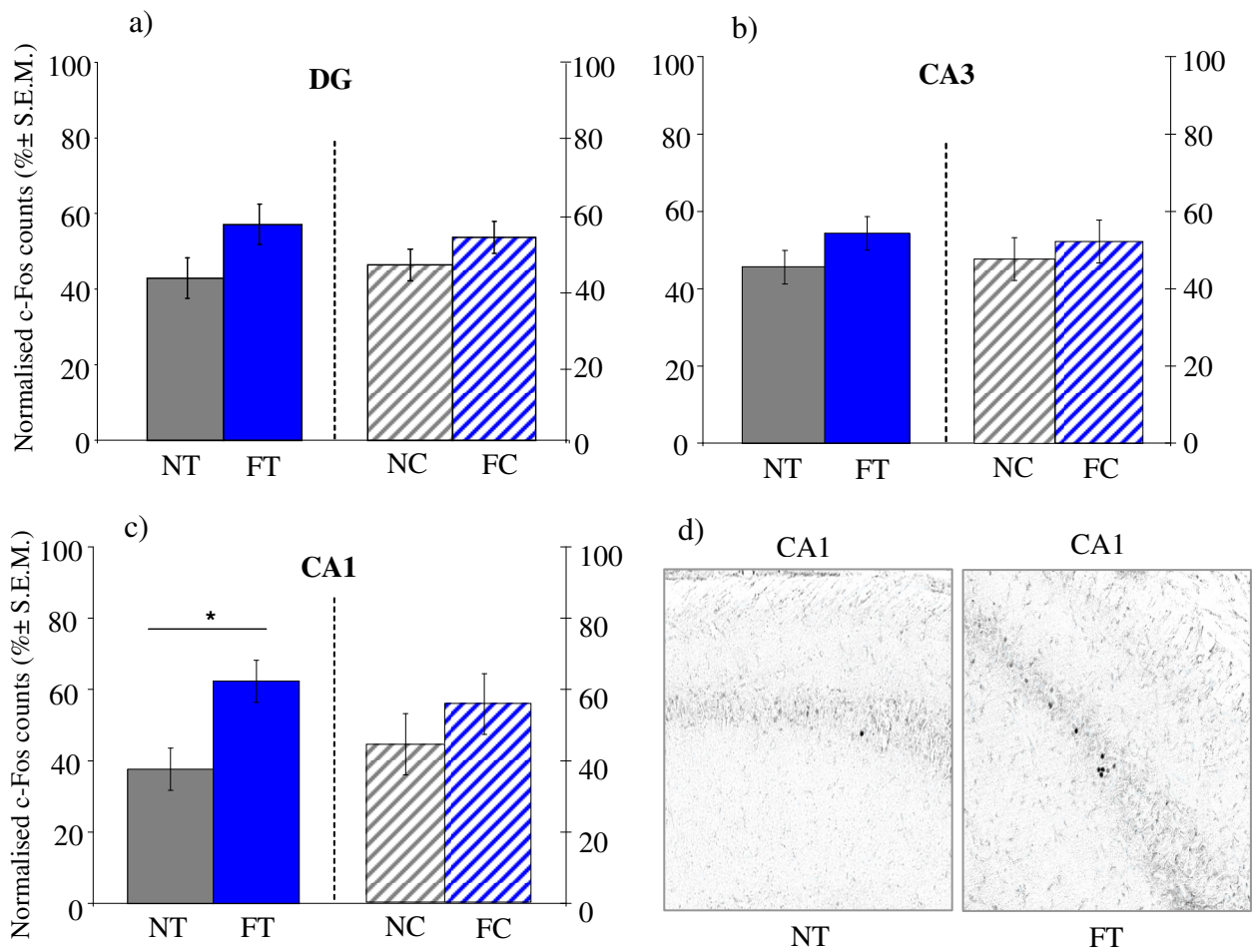
Figure 7.6: c-Fos levels in all groups in a) the DG b) CA3 and c) CA1 following water maze exposure Normalised c-Fos counts are expressed as mean % (± S.E.M.). d) Photomicrographs illustrating the level of c-Fos expressed in CA1 for the NT and FT groups.

7.3.5 Summary

Overall, there appeared to be no differences in the Near and Far trained groups when basic acquisition measures were examined. Examination of c-Fos expression in the hippocampal region revealed that in all groups there is an increased level of immunoreactive cells in the DG, indicating its higher importance in MWM navigation over other hippocampal subregions. Further comparison of c-Fos expression between conditions revealed a greater level of activation only in area CA1 in the FT group than in the NT group. Similar differences were not observed for the Near and Far control groups.

7.4 Discussion

Our research, thus far, has shown that the hippocampus is critically important for solving the MWM task (Chapter 4 and 5), however the exact nature of this involvement is somewhat unclear (Chapter 3b and Chapter 6). In addition, hippocampal subregional assessment indicated involvement of the DG in acquisition and a possible role for CA3 in the retrieval of spatial memories, however, with only correlational evidence available, we were unable to fully specify the extent of subregional involvement (Chapter 6). Therefore, in the current Chapter we aimed to further develop the insights gained from our previous behavioural results and examine cellular activation within specific subregions of the hippocampus under different environmental conditions.

Our initial results demonstrated that the largest activation of c-Fos was in the DG over other regions. This compliments our previous findings and available lesion data. Okada and Okaichi (2009), for example, found that damage to the DG results in similar levels of impairment as that of full hippocampal lesions, in the MWM, suggesting that the DG appears to be differentially more involved than the other subregions in tasks of spatial learning. BDNF molecular data has also supported the higher involvement of the DG over other hippocampal regions in learning and LTP (Messaoudi et al., 2002; O'Callaghan et al., 2007; Silhol et al., 2007). Furthermore, a number of IEG studies have also distinctively reported activity-dependent IEG activation within the DG following spatial tasks (Countryman et al., 2005; Vann et al., 2000a), again supporting the current findings and also reflecting the importance of the structure in spatial learning. Furthermore, while others have demonstrated that CA1 lesions may also lead to impairments in animals in the acquisition of a place over a

cued version of the MWM (Kesner et al., 2004; Maglakelidze et al., 2010; Nunn et al., 1998), the DG has been suggested as being differentially more involved, likely due to its positioning within the trisynaptic circuit (Witter & Amaral, 2004). Damage to CA3 has also been shown to lead to only minor or no differences in performance when compared with shams in the place MWM (Brun et al., 2002; Nakazawa et al., 2002), supporting the lower level of c-Fos activation in the CA3 region, compared to the DG, seen in the current study.

Examination of performance between the NT and FT groups in the task also revealed some interesting results. Although basic acquisition measures (e.g. EL and distance) suggested that there were no differences in how the NT and FT animals solved the task, there remained a pattern of slower escape latencies in the FT group across training; a finding that reached significance in previous Chapters and was reflected in the use of different strategies when solving the task (Chapter 3a). We did, however, find that FT animals displayed an increase in activated c-Fos cells in area CA1 when compared to the NT group. This finding compliments previous work like Vann et al. (2000a) and Poirier et al. (2008), who demonstrated that the more difficult a task or the more demanding a task becomes (e.g. moving from egocentric to allocentric learning), the higher the levels of c-Fos activity within the hippocampus. Vann et al. (2000a) specifically reported an increase in the CA1 region, over other regions, in line with spatial task demands. Similarly, Teather et al. (2005) reported that relative to a cued water maze task, training in a more cognitively demanding spatial task produced a marked increase in area CA1 only, with similar increases not seen in the DG or CA3.

While these findings may indicate that c-Fos expression is upregulated in a task-specific manner shortly after acquisition of hippocampal-dependent spatial learning, it is important to note that this difference was only observed in CA1, where expression of c-Fos was low, and not in any other region of the hippocampus. This suggests the entire dorsal hippocampus may not be differentially activated when task demands increase; this is inline with the behavioural results observed following damage to all regions of the dorsal hippocampus in Chapter 4 and 5. Instead, perhaps the increased activity seen only in area CA1, but not in CA3 or the DG, during Far training, may be due to different patterns of cortical afferents and efferents of the hippocampus (Witter et al., 2000). For example, although it was previously thought that CA1 and CA3 function interdependently, as CA3 projects directly to CA1, in the trisynaptic circuit (Kesner et al., 2004), the entorhinal cortex has also been shown to project to each of these areas separately; supporting the idea that these regions may be differentially involved in independent memory functions (Poirier et al., 2008; Witter et al, 2000; see also Chapter 1). This, however, poses the question that other regions outside the hippocampus may influence activation within the structure, particularly under greater cognitive demands; this will be explored further in Chapter 8 (see also Aggleton et al., 2000).

Moreover, as the DG has been shown to be critically involved in spatial learning, it would be expected that differences between groups may also emerge in this region, particularly as the DG demonstrated a high level of c-Fos activation. However, perhaps some areas may become less influential with additional training. It has been suggested that the level of learning that occurs at different points throughout training, may affect the amount of expression observed, with animals that were sacrificed late in

learning (having usually fully acquired the task) displaying lower levels of IEG (Guzowski et al., 2001; Mayer et al., 2010; Teather et al., 2005). Therefore, there is the possibility that more pronounced differences may occur early in training that later diminish as animals become familiar with a task.

This time-course and learning-dependent activation may go towards explaining the discrepancy between the low levels of c-Fos seen here and higher levels of BDNF expression seen in the full dorsal hippocampus in Chapter 3b, when sampled on the last day of training. Specifically, BDNF expression has been shown to gradually increase over time as a task is learned. For example, Harvey et al. (2008) found highest BDNF expression on Day 5 of MWM training when compared to earlier BDNF expression assessed on Days 1 and 3. Similarly, Kesslak et al. (19998) showed higher levels of BDNF mRNA that significantly correlated with lower escape latencies on Day 3 of training in the water maze, as animals began to reach asymptotic performance. Some have suggested that this is due to BDNF being particularly involved in late-LTP (Lu, 2003) which has been implicated in longer-term memory and consolidation of learned information (Abel et al. 1997; Miller et al. 2002), which may be occurring on latter training days. In contrast, c-Fos expression has been shown to peak earlier in training, with Guzowski et al. (2001) noting lower c-Fos levels during later trials where animals' performance in a task had stabilised compared to earlier trials where performance and learning was increasing. Thus, while BDNF and c-Fos have been implicated in similar signalling cascades, including mitogen-activated protein kinase (MAPK)/ERK (Ha & Redmond, 2008; Marsh et al., 1993) and phosphatidylinositol 3-kinase (PI3K; Ip et al., 1993; Roback et al., 1995), these results

may indicate that different pathways are involved at different time points during learning.

In addition, to further ensure the changes seen between the FT and NT groups were not due to exercise or simply traversing the training environment (Shires & Aggleton, 2008), we assessed IEG activation in a number of control groups. The most commonly used controls are caged-control animals, as they provide a baseline for IEG activity (Guzowski et al., 2001; Santin et al., 2003) and while assessment of c-Fos revealed very low levels of activation in all animals in the present study, their naïvety to the experimental environment makes it difficult to fully compare them to an experimental group. Therefore, we also examined IEG activation in two yoked-control groups (see also Teather et al., 2005; Shires & Aggleton, 2008). From this we found no differences in the level of immunoreactive cells between these groups, suggesting that the increase in c-Fos in CA1 seen in the FT over the NT group is an accurate result relating to the cognitive demands of the task.

However, there appeared to be higher IEG expression in the yoked-control groups in comparison to the caged controls. This has been seen in a number of studies (Bertaina-Anglade et al., 2000; Beiko et al., 2004; Duncan et al., 1993; Ons et al., 2004) and has been suggested as resulting from stress induced activation (Cullinan et al., 1995; Shires & Aggleton, 2008), which may be the case as free-swimming is considered highly stressful for rats (Kavushansky et al., 2006). However, an alternative explanation could be that IEG activation in the swim yoked-controls occurs as a result of ongoing hippocampal processing of swim movements and navigational components of the task (Guzowski et al., 2001; Teather et al., 2005); a suggestion that fits with the behavioural findings from our lesioned animals (Chapter 4 and 5).

While our study is not the first to look at IEG activity in a spatial task (Albasser et al., 2007; Guzowski et al., 2001; Maviel et al, 2004; Poirier et al., 2008; Vann et al., 2000a; 2000b), it allowed for a detailed examination of subregional activation within the hippocampus under differing water maze task demands. This was of particular importance as there remained some discrepancy in findings between our molecular (Chapter 3b) and lesion data (Chapters 4, 5, and 6). Overall, the findings from this Chapter highlight the presence of subtle differences in subregional hippocampal activation between NT and FT animals, which we suggest may be due to input from extrahippocampal structures; the implications of which will be further discussed in Chapter 8.

Chapter 8

General Discussion
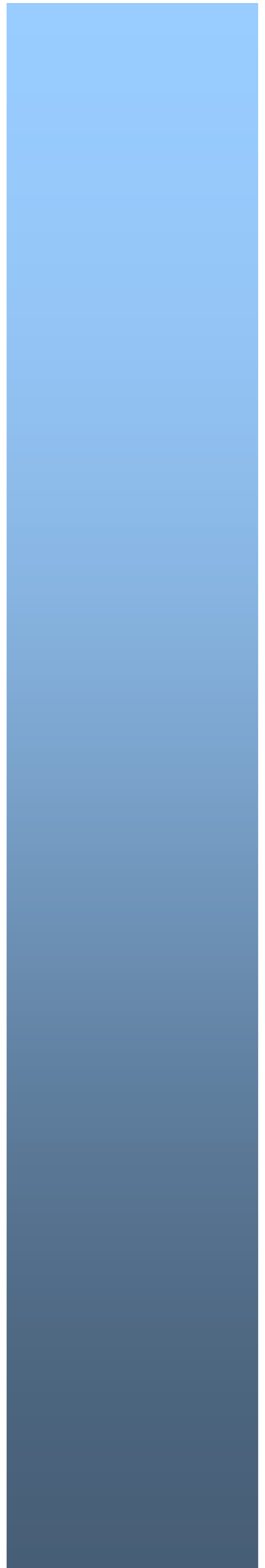
8.1 Summary of the findings from this thesis

This thesis set out to examine, in detail, the behavioural and neural differences between two groups of animals in the MWM trained under different cue conditions. While countless investigators have used the water maze in attempts at understanding the strategies and mechanisms used when learning and remembering the task (Kealy et al., 2008; Moghaddam & Bures, 1996; Morris, 1984), few have taken steps towards quantifying individual animal behaviours in the maze; a method that has proven invaluable in differentiating subtle differences in learning the task (D. Harvey et al., 2008; 2009). In addition, the MWM has traditionally been deemed as a task that requires navigation relying on the formation of a cognitive map (Morris et al., 1982; O'Keefe & Nadel, 1978). However, a number of studies have questioned this idea, suggesting that associative learning may account for acquisition of the task, and that the navigating animal need not build up spatial representations of every feature in the environment (Benhamou, 1996; Chamizo et al., 2004; 2006; Rodrigo et al., 1997; Sanchez-Moreno et al., 1999).

In the first two experimental chapters of this thesis, therefore, we aimed at providing a clearer account of water maze acquisition under two cue conditions; a Near cue condition and a Far cue condition; a task thought to be incrementally more difficult, corresponding with the distance between the cue and the goal. Initial investigation in Chapter 2 revealed that both groups of animals successfully learned the task as measured by escape latency, distance and velocity. Also, by displacing the cues during the retention phase of the task, we verified animals will typically follow them, displaying concurrent rotation in their searching of the pool. This primarily indicated to us that the overarching strategy in use in both conditions in the maze was

an allocentric one. Consequently, we suggested that the use of the relations between the distal cues and the platform position appear critical in knowing the location of the goal.

However, it has been previously reported that general acquisition measures can miss important variations in task acquisition under only slightly altered conditions in the MWM (D. Harvey et al., 2009). Therefore, Chapter 3a was designed to further enlighten the strategies used in acquiring the Near and Far cue version of the task. At a general level, we found that the Far trained animals were significantly slower in solving the task than the Near trained animals. However, to determine where the differences lay, we used a more detailed behavioural analysis (Graziano et al., 2003; Harvey et al., 2008; Leggio et al., 2003). This proved to be a highly complex task, attesting to the dearth of literature that fully document animal behaviour in detail. However, using accurate x, y coordinate output from EthoVision, alongside digitally recorded footage, we examined the moment-to-moment movement of the animals while in the pool and throughout the platform interval. From this detailed analysis, we noted a number of behaviours ranging from spatially simple to more complex, inferring movements. From this range of behaviours, we determined that the Near trained group remained reliant on more egocentric, view-dependent movements throughout acquisition. Specifically, the Near animals performed more cue-focused behaviours such as direct-swims and turns towards the cues, both of which reflect the animals' reliance and retained dependence on the external environmental stimuli, despite continued training. The Far trained group, on the other hand, displayed more view-independent strategies, with less focused behaviour towards the cues. This was apparent the assessment of the location at which they performed cue-directed

behaviours, which were often in a direction away from the cues. However, escape latencies indicated that they still learned enough information from the cues to successfully locate the goal. Analysis revealed that it was through the use of more inferring behaviours, such as turns away from distal cues that enabled them to locate place. In addition, assessment of dorsal hippocampal BDNF corresponded with our behavioural findings with the Far trained group having significantly higher levels of expression than the Near trained group (Chapter 3a).

As the behavioural and molecular evidence supported the idea that Far training was a more cognitively demanding task, Chapter 4 and 5 were designed to examine the impact of removal of the hippocampus on Near and Far acquisition. The assessment of the behaviours of both Near and Far trained dorsal hippocampal lesioned animals indicated that they had difficulties in the accurate execution of exploratory behaviours in the maze, with both groups displaying perseveration in initial basic movements and inaccuracies in the locations at which they performed these behaviours. The comparison of performance between the two lesion groups in the task was central to the thesis and the analysis in Chapter 6 aimed to determine a differentiation in relative spatial or navigational difficulties in the hippocampal lesioned animals. However, there was little difference between the two groups, with only slightly increased accuracy and less perseveration noted in the Near group than the Far group (as suggested by Chapter 4 and 5). While evidence from this Chapter leaves some ambiguity surrounding the function of the hippocampus, further subregional analysis conducted in Chapter 6, along with immunohistochemical assessment of c-Fos expression in each subregion of the structure in Chapter 7, highlighted interesting functional properties of each region. Lesion and IEG findings both led to the

distinction of the dentate gyrus, in particular, as critical in the acquisition of the water maze. The evidence relating to CA3 suggested a role in retention of the task, a finding that was supported by the lack of increased expression of c-Fos following acquisition in Chapter 7. The function of CA1, however, remained somewhat unclear, with the lesion data indicating no clear role for the structure in acquisition or retention of the maze. However, higher c-Fos expression in area CA1 following Far training suggested it may be required in learning the task, perhaps when cognitive demands increase.

8.2 Varying forms of distal cue use; Theoretical implications.

Here we will review all of our findings together to show how they contribute to the wider neuroscience field in appreciating how spatial memories are acquired. Specifically, the critical question when examining spatial learning and memory in the MWM, and one which we have attempted to address is how does the navigating animal learn to locate the hidden platform? This debate primarily focuses on the idea that animals acquire knowledge of a cognitive map (Tolman, 1948), or that animals' learning consists of the formation of associative stimulus–response (S-R) habits (Hull, 1943).

8.2.1 Cognitive Mapping Theory

Cognitive mapping, a central proponent in the theoretical debate of spatial learning and memory, appears initially to be a suitable model to explain our findings. Within this, it is suggested that animals can learn both a taxon and locale representation of their environment (O'Keefe & Nadel, 1978). Critically, however, according to this theory, these occur separately and are governed by different neural structures. Specifically, taxon learning is recognised as a form of guidance where cues or landmarks are

approached directly when navigating. Locale learning, on the other hand, would involve the animal building up a cognitive map of their environment using the spatial relationships between cues. These maps are thought to be developed in an all-or-none manner unlike associative mechanisms which are learned elementally (O'Keefe & Nadel, 1978). Our findings may initially appear to fit with this model as both groups learned to use only distal cues to get to their goal (Chapter 2) and they both displayed a level of allocentric inferring by moving away from their cues, a strategy thought to be central to the locale system of cognitive mapping (Chapter 3a). This performance in the water maze is also similar to that initially reported by Morris (1981), who provided the first account for 'cognitive mapping' in the water maze, where swimming rats were capable of getting to a hidden goal from multiple start points using only distal cues and without using a response strategy (i.e. turn-right). However, if a map was developed in an all-or-none manner in our experiments, cue location should have no visible impact on performance; which it does with the Far group being slower at getting to their goal (Chapter 3a). Our behavioural findings also do not display clear evidence for just an allocentric mechanism being used, as all animals (both Near and Far) display cue-directed behaviour throughout the entire training period; a finding not expected if the animal was no longer reliant on its movements towards a cue. Specifically, Cognitive mapping theory has also been described as preserving the spatial relationships between environmental features with no one feature being necessary to maintain all remaining relationships (O'Keefe & Conway, 1978). If this is true for water maze learning, then why do our recorded behaviours indicate the persistent and increased use of individual cues? In particular, our findings indicate that the cues are being used in a very

specialised manner, as elucidated by the stable positions at which turns were performed towards the cues (Chapter 3a).

8.2.2 Associative Learning Theory

Alternatively, the initial findings that the Near group were significantly quicker in reaching their goal, mirrors findings by Chamizo et al. (2006) who found that when a cue was located nearer to a goal, within a configuration of cues, it gained more accurate control of task completion than when the same cue within the configuration was placed in a Far position. Chamizo et al. (2006) attributed this distinction to a mechanism of associative learning, a finding they further supported when subsequent tests revealed evidence of overshadowing by the near cue; a phenomenon that occurs from the development of S-R associations (see also Chamizo & Rodrigo, 2004; Lechelt & Spetch, 1997; Sanchez-Moreno et al., 1999). This conclusion was also made on the basis of research by Cheng et al. (1986; 1995) and Spetch (1995), both of whom report the occurrence of overshadowing when proximal cues are presented alongside distal cues. These findings and the importance of stable distal cues suggest the possible use of an associative form of learning in the MWM (Biegler & Morris, 1996; Rescorla & Wagner, 1972; Sutton & Barto, 1998).

Our behavioural analysis also lends further support to this theoretical view of spatial learning. In particular, when the cues were closer to the platform, the animals performed more cue-directed behaviours, providing the first indication that they may learn to associate movements towards the cues with subsequent reward through goal finding. Sheynikhovich et al. (2009) developed a computer model of spatial learning in a MWM which reflected similar findings. Specifically, they suggested that a simulated

rat in the MWM will use 'snap-shots' (i.e. view-dependent images) of the environment and simply learn to associate these views with rewarded motor action. Our intact animals performance in the maze also reflects this, as they continuously approach and avail of the cues; evident in thigmotaxis vertical, direct behaviour and turns towards the cues. Collett (2010), similarly, noted that ants, using stored views, can remember and recall correct guidance movements and head directly towards a landmark in order to get to their target. However, with cues in the Far position, while animals performed cue-directed behaviour, they needed to perform more view-independent behaviours to get to their goal, highlighted in more turns away from the distal cues, which are indicative of inferring (Harvey et al., 2008).

While the differences in performance under the two training conditions initially indicates similarities to associative mechanisms of learning, i.e. nearer cues gain greater control than far cues, the behavioural analysis indicates some anomalies that cannot be accounted for by associative mechanisms alone. Specifically, both groups visualise the cues and then must infer where to go by performing turns away from their cues; albeit more prominent in the Far group. Collett (2010), similarly, noted that ants travelling on a curved path cannot use individual learned heading directions to get to their goal; rather the ant must change their guidance commands to fit the task demands. Therefore, as the cues in our task did not directly mark the goal, their direct association with behaviour would be insufficient to get to the platform; rather some information about distance would also be needed to accurately reach the hidden target in every trial (Collett et al., 1986; Kubie & Fenton, 2009; Sheynikhovich et al., 2009). As a result, we cannot definitively state that associative learning solely accounted for acquisition in the task.

8.2.3 Vector-Modelling

As mentioned, cognitive mapping theory has been unable to fully incorporate the behavioural findings seen in the first half of this thesis (Chapter 2 and 3). Alongside this, individual associative mechanisms in the sense of S-R actions cannot fully account for our behavioural evidence, particularly as the navigator will be unable to accurately compute distance information from merely approaching cues. Rather, extending associative mechanisms of learning, a number of studies, including those done on insects (Nicholson et al., 1999) and more recently rats (Cheung et al., 2008), have recognised that perhaps associative learning is, in part, representative of taxon learning that has been outlined as a feature of cognitive mapping (O'Keefe & Nadel, 1978). Taking this view, Collett et al. (1986) further developed the idea of associative learning between single cues, and instead suggested that animals can use multiple cues in the form of vectors to guide them to a goal. A vector, in this instance, incorporates both distance and direction information of the location of a landmark to a goal and will enable shortcuts to be made once vectors are established (Collett et al., 1986).

The emergence of direct use of the cues by movement both towards and away, for the Near and Far trained groups (Chapter 3a), appears to be indicative of vector use based on the direct use of landmarks (Collett, 1986). Specifically, when the animal is using more than one cue, which was seen on all training days in turning towards the cues, it is speculated that a number of heading-vectors will be established, providing distance and direction information from each of the respective cues to the goal (Collett, 2010; McGregor et al., 2004). Once the animal has this information it can plan path trajectories from multiple start points and also enable the animal to take short-cuts to a goal. This is done by using a current perception of the environment in the form of a

'seen' vector, where the animal's position is defined with respect to a landmark as it moves, and a 'stored' or remembered vector, which provides the position of the goal with respect to the landmark as it was previously encountered. The animal can then compute a direct path (i.e. short-cut) by taking the difference between the two, which will enable the navigator to plan an intended path trajectory to a goal (Cheng, 1986; Collett et al., 1986). This increased understanding of accurate distance to the goal was also witnessed in the change in location at which turns were performed within the maze as training progressed, corresponding with the development of the association between the cues, behaviour and goal location. This method of navigation also accounts for the animal's ability to plan their path and head directly towards a goal on later training days, seen in the increased number of direct behaviours in both the Near and Far trained groups.

The idea of multiple heading-vectors also provides a more comprehensive account of learning in the MWM, as it takes into consideration the fact that navigating animals are likely going to attend to some landmarks on some trials and others on other trials (McGregor et al., 2004), a finding we have noted in the disparity of heading directions between days. Kubie and Fenton's (2009) simulated heading-vector model replicated a similar pattern of behaviour as our Near and Far trained animals. Specifically, they reported that when establishing a heading-vector, animal movement along wall edges and between prominent landmarks is reliably seen. They also added that this behaviour is not conducive to creating a map of continuous space, such as a cognitive map. This was also reflected in both the Near and Far trained animals searching at the wall in thigmotactic behaviour between the available distal cues, particularly evident on early training days. Adding to this, the vector-model also

accounts for the differences seen between the Near and Far trained groups. Specifically, when multiple spatial cues are available during learning, the vector-model provides rules by which various landmarks are weighted by the subject, similar to the idea of overshadowing seen in associative learning theory (Collett et al., 1986; Lechelt & Spetch, 1997; Spetch, 1995).

Our behavioural analysis has expanded on what was previously taken for granted in studies using the water maze and we suggest that the presence of distal cues to locate a hidden goal does not automatically result in 'truly spatial', allocentric learning (Morris, 1981; O'Keefe & Nadel, 1978). The vector-model, while appearing to have some similarities to a cognitive map, has been interpreted as a simpler representation, as it is developed through associative processes instead of an all-or-none manner (Collett et al., 1986; Esber et al., 2005; Leising & Blaisdell, 2009; Pearce et al., 2004) and appears to account well for our behavioural findings.

8.3 The nature of spatial navigation dependent on the hippocampus.

Adding to this theoretical debate is the contribution of different brain structures to spatial learning and memory (Morris et al., 1982; Packard & McGaugh, 1996; White & McDonald, 2002). In particular, the hippocampus has been implicated for decades in spatial navigation and has been the centre of theoretical debate in the area. The most significant division between the various accounts of hippocampal function are between non-spatial (Eichenbaum et al., 1992; Olton & Werz, 1978; Rudy & Sutherland, 1995) and spatial theories (O'Keefe & Nadel, 1978). Specifically, it has been implicated in behavioural flexibility, habituation including the control of behavioural responses such as perseveration, and also involvement in path integration (Eichenbaum et al., 1990;

Whishaw et al., 1995). On the other hand, it has also been solely implicated in spatial aspects of the task which have posited that the hippocampus is required for the formation of allocentric relations between cues in an environment and the location of a goal in the form of a cognitive map (O'Keefe & Nadel, 1978).

Critically, therefore, the behavioural assessment conducted on hippocampal lesioned animals in this thesis occurred in tasks that were spatial in nature. This enabled a clear account of the navigation processes that occur when an animal is required to learn place. From this we found that hippocampal lesioned animals displayed significant navigation impairments. This initially manifested in the form of perseveration and was particularly evident in the prolonged performance of thigmotactic behaviour and the hippocampectomised animals' inability to efficiently alter the location at which they performed this behaviour. They also displayed delays in focusing their direct behaviour towards the cues (particularly the Near DH group). We further recognised this as having an inability to inhibit maladaptive behaviours when it was required, such as learning to move away from the edge and perform accurate searches in the centre of the maze. This idea of hippocampal function was supported by findings from Day et al. (1999) who found that when maladaptive behaviours are discouraged from the beginning of training and strategy choice is removed, lesioned animals can actually perform well in a place task. Kim and Frank (2009) also noted that hippocampal lesioned rats displayed difficulty in adapting behaviour to suit the task demands in a 'W' maze (see also Leggio et al., 2006; Morris et al., 1982). Our hippocampectomised rats' inability to refine their initial performance of exploratory movements in the task is also inline with findings that, under standard training procedures, lesioned animals generally fail to habituate to their environment,

displaying only early exploratory behaviours, resulting in poor performances in the maze (Wright et al., 2004). This type of perseverative behaviour has also been noted in tasks that are not necessarily spatial in nature, with a number of lever pressing (Steward & Blampied, 1975; Ellen & Wilson, 1963), spontaneous alternation (Dalland, 1970) and open environment exploration tasks (Mitchell et al., 1993) showing prolonged and rigid performance in inappropriate behaviours (e.g. extended time spent lever-holding; Ellen & Wilson, 1963). While this suggests that perseverative behaviour does not necessarily directly relate to an alteration in spatial strategies, it does further highlight a behavioural impairment in these animals that is often interpreted as an inability to develop spatial representations of an environment (O'Keefe & Nadel, 1978; Morris et al., 1982). Furthermore, perseveration is also thought to represent an animal's inability to learn and apply new rules to a task (Kim & Frank, 2009), which becomes particularly evident as demands in a task alter. Therefore, while perseveration is not necessarily a 'spatial' behaviour, it is critical in the effective acquisition of spatial tasks with an intact hippocampus appearing essential in the control of this behaviour.

Following this, we also noted lesioned animals' difficulty in coherently integrating their behaviours to move towards their goal. They often displayed poor judgement of distance, seen in the inability to alter turn locations, along with performance of behaviours at inappropriate locations, for example inaccurate positioning of direct behaviour (Chapter 4 and 5). This is in keeping with a number of accounts of hippocampal dysfunction that have been elucidated from studies applying adapted protocols in the MWM (Allen et al., 2007; Eichenbaum et al., 1990; Whishaw et al., 1995). It has also been reported that, while lesioned animals can learn to locate a

goal with extended training, they have significant difficulty in re-calculating their directional position in the maze if they miss their target on approach (Whishaw et al., 1995). This is in accordance with our findings which crucially, found that while lesioned animals were capable of performing similar movements as shams, such as direct behaviour, they displayed increased numbers of turns-away, suggesting that they may be unable to efficiently and accurately re-orient their position in space. In addition, we also noted a significant correlation between the extent of CA3 damage and the animals' reduced performance in the more navigationally complex behaviours (Chapter 6). This is in keeping with suggestions that area CA3 is a likely hippocampal candidate in the monitoring of path-integration, due to its recurrent collaterals (Rolls & Kesner, 2006).

Together our findings and evidence from adapted MWM paradigms (Day & Schallert, 1996; Whishaw et al., 1995) indicate a significant navigation impairment following hippocampal ablation. Moreover, it appears that lesioned animals still recognise the value of the cues (i.e. both lesion groups perform vertical thigmotaxis at their cues), thus, further indicating that the difficulty observed is due to their inability to accurately calculate movement information gained from the cues, such as distance and direction. This is a finding also supported by Gaffan et al. (2000) who found that when the need to encode navigation movements was removed, lesioned animals could accurately process information acquired from external stimuli to locate a target. Furthermore, additional assessment of the platform interval revealed that both lesioned groups retained the ability to effectively attend to the cues when not required to actively navigate (Chapter 4 and 5). This ability for lesioned rats to remain capable of observing an environment when they are not required to move has also been reflected

in place cell activity where firing does not occur when the animal is aware that its orientation in space is not changing; i.e. when a cue that elicited spatial firing is moved in the presence of the animal (unlike conditions where the cue is moved when the animal is absent; Rotenberg & Muller, 1997).

8.3.1 Theoretical Implications

These findings lend themselves to the extension of what has been previously suggested, that the hippocampus is involved in more than just developing the spatial relations between environmental features and forming a map from them (Day et al., 1999; Eichenbaum et al., 1999; Whishaw & Jarrard, 1996). In particular, while the hippocampus has been the centre of Cognitive mapping theory, our behavioural findings do not fit with the proposal that when the hippocampus is destroyed all exploratory behaviours should disappear (D'Hooge & De Deyn, 2001; Morris, 1984; O'Keefe & Nadel, 1978). Indeed, the debate surrounding the function of the hippocampus, implicating it in the monitoring of navigation behaviour, fits with our suggestion of the use of a heading-vector in the MWM. Specifically, as vectors, by definition, are used to calculate distance and direction to a goal from landmarks (Collett et al., 1986), the presence of a retained understanding that the cues hold value, alongside the inaccurate heading directions and the inability to accurately judge distance, seen in our lesioned animals, supports the idea that the hippocampus is required for navigation using heading-vectors.

Adding further weight to this, Collett and Graham (2004) and Kubie and Fenton (2009) extended Collett et al.'s (1986) account of heading-vectors and suggested that path integration (PI) is initially used to construct a vector. They suggest

that a navigating animal initially uses path integration (i.e. the continuous organising and planning of movements based on self-generated motion cues; Gallistel, 1990) to update their sense of position between known locations. From this, as the animal becomes familiar with available cues and the environment, they can apply their learned positional information to developing heading-vectors (Collett & Graham, 2004). Our hippocampectomised rats' inability to accurately integrate their movements to effectively reach their goal, such as an inability to effectively switch early swimming strategies from basic, thigmotactic behaviours to more efficient direct and turning behaviours to enable them to move towards their goal, further highlights the possible role of the hippocampus in the development of heading-vectors. Lesion studies have also implicated the hippocampus in PI processes. Allen et al. (2007), for example, noted that lesioned animals were impaired when only PI could be relied upon to guide them to a goal in the dark. Hippocampal place cells have also been seen to fire as rats move in the dark, appearing to process idiothetic, PI information (O'Keefe & Speakman, 1987; Quirk, Muller & Kubie, 1990).

However, the recording of these place cells in the hippocampus originally, have been taken as clear evidence for the hippocampus as the centre of a cognitive map as they are thought to represent the animals location in space, in particular in relation to distal cues (Muller & Kubie, 1987; O'Keefe & Conway, 1978; O'Keefe & Dostrovsky, 1971). It has been noted, however, that place cells may not fully account for, or represent an entire environment. Instead, they have been shown to fire and encode previous or anticipated locations (Ainge et al., 2007a, b; Bower et al., 2005; Ferbinteanu & Shapiro, 2003; Lipton et al., 2007). Specifically, Wood et al. (2000) found that different place cells fired according to a prospective left or right turn that

the animals intended to make in a T-maze. This may highlight the need for the hippocampus in the planning of future situations, which also corresponds with Collett et al.'s (1986) idea that animals, when navigating, plan their path trajectory from a start point by using heading-vectors. The current data is also in accordance with the idea that the animal can learn to associate its current position with its next choice of goal-directed behaviour. For example, once an intact animal encounters the cues in a particular part of the environment where they know their relation to the platform's position, the goal can be found; a strategy that the lesioned animals are clearly unable to effectively use.

Finally, it can also be seen that hippocampal place cells react to the same sources of spatial information that animals use when solving the MWM using heading-vectors, i.e. reacting to cue alterations (Rosenzweig et al., 2003; Rotenberg & Muller, 1997) and also path integration inputs (Gothard et al., 2001; Hargreaves et al., 2007). Hippocampal place cells are also susceptible to associative processes, with firing in relation to cues shown to be effected by overshadowing (Fenton et al., 2000a, b) and blocking (Barry & Muller, 2006). Together, our behavioural and lesion evidence suggest that the hippocampus is required for the development of heading-vectors, with evidence from hippocampal place cell studies also highlighting how activation of the hippocampus is consistent with the components of vector navigation.

8.3.2 The hippocampus as an integrator of sensory inputs

The detail of hippocampal cell organisation is also of interest in examining spatial learning in that neurons in many of the structures attached to the hippocampus also encode spatial information (see below). So while our lesion evidence suggests that the

hippocampus is key to navigating using heading-vectors, it is likely that the structure is part of a larger cortical and subcortical spatial information processing circuit that underlies the ability for an animal to recognise its location, direction and locomotor movements in an environment (Aggleton et al., 2000; Kubie & Fenton, 2009; Touretzky & Redish, 1996).

The proposition that multiple systems interact in learning to navigate may be apparent in our molecular data from unlesioned animals (Chapter 3b and 7), where we saw increased levels of hippocampal BDNF and c-Fos activation in the Far trained group, a finding that was not subsequently reflected in our lesion evidence (Chapter 6). We posit that perhaps the interactions between the hippocampus and other structures play an important role in navigating and that under certain circumstances higher input from other areas will lead to increased activation within the intact hippocampus. The higher activation of these other cortical areas, however, may not be sufficient to compensate once the hippocampus is removed (as seen in Chapter 6; also Aggleton et al., 2000). Specifically, Aggleton et al. (2000), in a comprehensive review, highlight that lesions to some of these areas do not result in the expected level of impairment seen following hippocampal lesions, suggesting they may not play as an important role in navigation. However, assessment of the intact brain using immediate-early gene analysis has highlighted the normal importance and standard activation of these cortical structures in spatial tasks (Aggleton et al., 1997; Albasser et al., 2007; Bussey et al., 1999), which may go towards explaining the retained presence of increased hippocampal activity in the Far trained groups.

Kubie and Fenton (2009) highlight some prominent candidate areas that could be involved in navigation using heading-vectors including head-direction cells in the

retrosplenial cortex (Cho & Sharp, 2001), thalamus (Taube, 1995), and entorhinal cortex (Sargolini et al., 2006) and place cells in the hippocampus (O'Keefe & Dostrovsky, 1971). The entorhinal cortex (EC) in particular may be critical as it has direct inputs to the hippocampus (Burwell & Amaral, 1998; Myers et al., 1995; Naber et al., 1997) and has also been associated with animals' self-monitoring of movement, in particular distance and direction (Etienne & Jeffery, 2004; Fiete et al., 2008; Hafting et al., 2005). In addition, high levels of the TrkB receptor and BDNF have also been reported in the EC (Conner et al., 1997; Tokuyama et al., 1998), with Nagahara et al. (2009), crucially, showing that injections of a lentiviral vector, expressing BDNF, directly into the EC leads to a subsequent increase of BDNF in the hippocampus. This lends itself to the idea that if the EC is more highly activated during spatial tasks where behaviour must be monitored (i.e. in the Far condition), it may also lead to increased BDNF expression in the hippocampus with further downstream activation of c-Fos possibly via the mitogen-activated protein kinase (MAPK) signal transduction pathway (Ip et al., 1993; Marsh et al., 1993; Roback et al., 1995; as seen in Chapter 3b and 7). In support of this, Vann et al. (2000a), measuring c-Fos expression, found that when activation of the hippocampus increased, EC activation correspondingly augmented when task demands were higher in a spatial reference memory paradigm. In line with this, we also reported higher IEG activation in the DG over other hippocampal subregions overall (Chapter 7) and strong correlations between the extent of DG damage and performance in the maze; a finding not prominent in the other regions (Chapter 6). This is worth noting as the DG receives greatest input from the EC and related sensory cortices and is, therefore, the first hippocampal region to process this information (Witter & Amaral, 2004). This places it as a key area in the proposed

hippocampal-cortical circuit for the processing of vector-navigation, which may explain its marked role in MWM acquisition that has been reported in a number of lesion and IEG studies (Jeltsch et al., 2001; Lee & Kesner, 2004; Okada & Okaichi, 2009; Xavier et al., 1999).

Other sensory regions including the visual inputs from the occipital cortex (Burwell & Amaral, 1998), and the prefrontal cortex (PFC; Burwell & Amaral, 1998) could also be involved. The PFC, in particular, has been recognised as being critical in goal-directed action (Corbit & Balleine, 2003; Hasselmo, 2005; Vertes, 2006) and the monitoring and flexible adaptation of future movements (Compton et al., 1997; Seamans et al., 2008). Burgess (2008) also suggested that the PFC may supply a motor efference copy to enable accurate planning of movements; with Miller (2000) reporting that activity in the PFC is dependent on the associations between environmental cues with future responses. These findings lend well to our current data where the animal directly uses landmarks to plan movement to a goal via heading-vectors. In addition, anatomical data indicates that hippocampal area CA1 sends inputs to the medial PFC (mPFC), with further physiological evidence suggesting that LTP can be induced in the mPFC by stimulating CA1 (Jay et al., 1996). This cortical interaction with the hippocampus, in particular, may account for the higher c-Fos expression in area CA1 seen in the Far trained group (Chapter 7). We suggest it is higher in the Far group than the Near group as they are required to infer their position by moving away from the cues and thus must rely on their ability to keep record of their movements and plan extended path trajectories without the direct use of the cues. So, rather than relying solely on the hippocampus to navigate, with cues in a position further away, other cortical areas may begin to play a more prominent role.

The D$_1$ dopamine receptor in the PFC may play a role in modulating this transfer of spatial information from the hippocampus to the PFC and importantly, has been thought to be particularly necessary under conditions where a prospective series of movements must be organized, which we suggest is pertinent to the Far group (Goto & Grace, 2008; Gurden et al., 2000; Seamans et al., 1998). Furthermore, the importance of the D$_1$ receptor for activity dependent IEG expression was noted by Granado et al. (2008), who found impaired IEG expression following LTP induction, specifically in area CA1, in mice lacking the receptor. Our increased levels of c-Fos in area CA1 in the Far trained group is in agreement with this PFC-CA1 dopamine association. Interestingly, the EC also sends projections to the PFC (Uylings et al., 2003; Valenti & Grace, 2009). The PFC, while not having direct connections to the hippocampus, sends indirect projections via the EC (Heidbreder & Groenewegen, 2003; Vertes, 2006). This may indicate an important role for the EC, PFC and hippocampus in heading-vector navigation.

A number of lesion studies have also been conducted in the examination of the importance of the PFC and EC in MWM acquisition with some conflicting results. Kolb et al. (1982), for example, showed that mPFC lesions result in an inability to learn the location of a hidden platform in the MWM. Others, however, have shown that lesions to the mPFC leave learning of the place MWM intact, and instead only cause disruption to an animal's ability to learn the position of a new hidden platform during a reversal paradigm (de Bruin et al., 1994; LaCroix et al., 2002). It has been suggested that this impairment is as a result of an inability to flexibly alter appropriate behaviours in order to locate a new goal. Similarly, Compton et al. (1997) also noted impairment in mPFC lesioned animals' ability to flexibly adapt their behaviour when a new start

position was used in the MWM. Others have further suggested that the mPFC is required for accurate goal localisation that is dependent on the monitoring of ongoing, appropriate behaviours and not necessarily learning the goal position based on cues alone (de Bruin et al., 2001).

A number of studies have also assessed the effect of lesions to the EC on spatial learning in the MWM. Parron et al. (2004), for example, found that animals with lesions to the EC displayed significant impairments in acquiring the standard, place MWM. Specifically, they noted that the position of cues in the environment is a critical factor affecting the impact of EC lesions on learning the task, with EC animals capable of learning the MWM when proximal cues were available but not when only distal cues could be used to locate a hidden goal (Parron et al., 2004). Furthermore, Oswald et al. (2003) suggested that in tasks where animals are required to switch attention to different features of the environment (Prados et al., 1998), damage to the EC results in significant impairment, for example when lesioned animals are required to switch between using proximal to distal cues. Interestingly, Bannerman et al. (2001) found that while hippocampal lesioned animals were significantly impaired in the MWM, EC lesioned animals displayed no impairments in acquiring the task. They were, however, impaired in the alternating T-maze, indicating that an intact EC is not necessary for normal hippocampal function in the MWM, yet remains critical for some aspects of spatial processing that may require more online, attention processing. While a number of studies have assessed the effect of lesions to the EC and mPFC, it remains unclear the exact nature of the involvement of these regions in learning the spatial position of a hidden goal in the MWM (Aggleton et al., 2000). Therefore, it may be worthwhile to further examine the impact of lesions on these proposed areas and to use

the detailed behavioural analysis adopted in this thesis to determine the presence of any subtle variations in behaviour that may emerge between animals trained with cues in differing positions and with different spatial demands; for example, we would expect the Far group to be more impaired than the Near group based on findings from Chapters 3b and 7.

Aggleton et al. (2000) further suggested that to differentiate the components of spatial navigation and the cortical contributions, very defined tasks should be used, as under normal MWM procedures differences often do not emerge following damage to these structures (Galani et al., 1998; Pouzet et al., 1999). By employing the in-depth behavioural analysis used throughout this thesis along with normal and modified navigational tasks, differentiation of function would become evident. In particular, the utility of this analysis can be seen even in tasks in intact animals where groups appear to learn the task very similarly, however assessment of their movements reveal otherwise (D. Harvey et al., 2009; also Chapter 3a). In addition, it would also be interesting to examine the effect of lesioning the hippocampus and examining, anatomically, the changes in IEG expression in the EC and PFC. A study conducted by Albasser et al. (2007) successfully used this technique to examine the impact of lesioning on the functional properties of adjacent structures. Such an experiment, would, initially, highlight whether damage to the hippocampus results in dysfunction of other areas and so further clarify the disparity between our lesion and molecular findings under altering cue conditions. This combined form of assessment would also clarify whether the areas we propose (i.e. EC, PFC and hippocampus) work conjointly to support navigation in the water maze, and determine if the three areas have interdependent functions when tasks demands change.

8.3.3. Implications to human spatial navigation

Our findings may also contribute to the understanding of the neural structures underlying human spatial navigation. Specifically, as in the animal data, there is also conflict regarding the exact nature of hippocampal involvement in navigation tasks in intact human participants. Hartley et al. (2003), for example, examined activity associated with navigating in a large-scale virtual environment either by way-finding (allocentric) or by route-following (egocentric). They noted higher activity in the medial temporal lobe (MTL), specifically in the parahippocampal area during the way-finding task when compared to the route-following task. More interestingly, accuracy in the task was associated with greater hippocampal activation, with the assessment of individual participants revealing greater MTL activity in the better navigators. Hartley et al. (2003) took this as evidence for the hippocampus as being critical for the development of a cognitive map. However, McNamara and Shelton (2003) suggest that the increased hippocampal activation in the better navigators may also reflect the view that the hippocampus processes memory in a way that allows for its efficient and flexible use in guiding behaviour (e.g. Eichenbaum, 2000). Interestingly, increases in activation were also seen outside the hippocampus, with more accurate performance in the way-finding task strongly correlating with activity in the perirhinal cortex; further illustrating the involvement of other regions in spatial navigation. So while there is evidence in humans for hippocampal activation in allocentric-type tasks, there also remains conflict surrounding the exact nature of this involvement and the involvement of other regions. The behavioural analysis and findings from this thesis further enlighten the role of the hippocampus beyond a simple cognitive map, highlighting its involvement in navigational aspects of the task. Alongside this, our suggestion of the

hippocampus as possibly being involved in navigation based on heading vectors, may lead to a novel area of assessment in humans. In addition, developing detailed understanding of spatial navigation processes in intact humans may also enable clearer appreciation of what is occurring when spatial difficulties present as a result of brain damage or neurodegeneration.

Specifically, it has been shown in Alzheimer's disease (AD) patients, and to some extent adults with Mild Cognitive Impairments (MCI; deIpolyi et al., 2007), that early symptoms can manifest as difficulties in spatial navigation. Specifically, some key difficulties with AD patients often manifest as way-finding (allocentric) disorientation (Kalova et al., 2005; Pai & Jacobs, 2004), problems in recognising new landmarks, along with an inability to flexibly adapt to spatial demands in an environment (Burgess et al., 2006). The neural structures and circuits underlying these different pathologies have not been fully elucidated, however the brain areas that undergo the earliest damage in the disease are the same as those, which, in rodents, contain head direction and place cells. For example, damage to the hippocampus, entorhinal cortex and anterodorsal thalamic nuclei (Braak & Braak, 1991). Therefore, the extension of these areas in the rodent data, such as through the use of more thorough analytical techniques, as used in this thesis, may further elucidate what goes wrong when spatial difficulties arise in clinical populations, such as Alzheimer's disease.

8.4 Concluding remarks

Overall the results of this thesis expand upon explanations on how animals search in and learn the MWM. The in-depth analysis employed allowed us to provide an exact

outline of how the animals interact with the testing environment with the use of varying cue locations enabling us to separate the strategies used to reach a goal. We noted an emerging focus on the distal visual cues throughout the training period for both groups of animals, with further assessment of individual behaviours leading us to the suggestion that the MWM may be solved using heading-vectors. These findings provide a marked contribution to the field of spatial navigation in suggesting that the MWM is not solved simply using an allocentric, cognitive map, as is widely reported. Adding to this, detailed behavioural assessment of hippocampectomised animals indicated marked navigational impairments in the maze once the hippocampus is removed, contrary to the widely reported involvement of the hippocampus in purely spatial processes (Morris et al., 1982; Yamada et al., 2002). This, we suggest, further supports the use of heading-vectors in solving the MWM with the deficits seen following lesions as a result of their inability to correctly judge distance and direction movement to develop an accurate vector. When the hippocampus is left intact we also suggest that other connecting regions may influence the level of hippocampal activity; particularly when cognitive demands are increased such as when the cues are placed further away from their target area. This adds to the growing body of literature highlighting the importance of looking at multiple structures when assessing spatial learning and memory (Aggleton et al., 2000; Albasser et al., 2007; Poldrack & Packard, 2003). Overall, the evidence provided in this thesis strongly contributes to the current theoretical debate on spatial learning and memory, and may go towards the development of a model of cortical and hippocampal-dependent navigation which is initially based on the processing of individual components of spatial information, the synthesis of which supports later successful, goal-directed behaviour.

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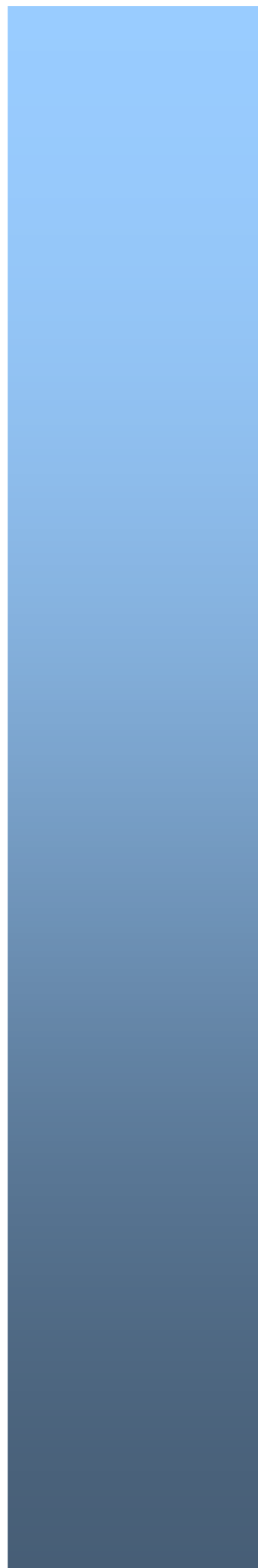
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Conference presentations

Refereed Abstracts
Diviney, M. & Commins, S. (2011). Dorsal hippocampal lesions, erformed in the anaesthetised rat, lead to impairments in exploration in the Morris water maze (in press). Abstract from Neuroscience Ireland, September 2011.

Diviney, M. & Commins, S. (2010). Effect of distal cue configuration location on Morris water maze performance: An in-depth behavioural and molecular analysis. *The Irish Journal of Medical Science, 179 (Supp 3):* S116.

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Conference Presentations
Diviney M., & Commins S. (2011). Dorsal hippocampal lesions, erformed in the anaesthetised rat, lead to impairments in exploration in the Morris water maze. Oral presentation at the Neuroscience Ireland Conference, Maynooth, Co Kildare.

Diviney M., & Commins S. (2009). Dorsal hippocampal lesions alter exploratory behavioural strategies in the Morris water maze . Poster presented at the European Brain and Behaviour Society (EBBS), Rhodes, Greece.

Diviney M., & Commins S. (2009). Effect of distal cue configuration location on Morris water maze performance: An in-depth behavioural and molecular analysis. Poster presented at the Neuroscience Ireland Conference, Dublin, Ireland.

Diviney M., & Commins S. (2008). The effects of overtraining in the Morris water maze on allocentric and egocentric learning strategies in rats. Poster presented at the Federation of European Neurosciences (*FENS*), Geneva, Switzerland.

Diviney M., & Commins S. (2008) The effect of hippocampal lesions, in the anaesthestised rat, on cue-platform associations in the Morris watermaze task. Poster presented at the Neuroscience Ireland Conference, Galway, Ireland.