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An Examination of Hippocampal and Prefrontal Contributions to Spatial Learning and Memory using Immediate Early Gene Imaging

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Table of Contents

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Summary

The hippocampus and medial prefrontal cortex are two brain regions which have repeatedly been linked to spatial learning and memory processing; however, the precise roles of individual sub-regions within these areas continue to be debated. The Morris water maze is a well-known behavioural task used to measure spatial memory. Despite its popularity, the type of spatial information animals encode and ultimately rely on for accurate navigation in this task remains unclear. Therefore, the primary objectives of this thesis were to conduct an in-depth investigation into the use of navigation strategies during memory encoding and retrieval in the water maze, and to characterise the specific contributions of the hippocampus and medial prefrontal cortex to these processes using Immediate Early Genes (IEG) imaging. In addition, we investigated the mechanisms underlying neuronal activation by inhibiting ionotropic glutamate receptors (NMDA and AMPA) during or after spatial learning. We found novel evidence that the salience (or noticeability) of environmental cues significantly impacted the type of learning strategy used (i.e. simple or complex), and that increased training led to more flexible responding (i.e. strategy switching). We also discovered that NMDA receptor-mediated activation in area CA1 (indexed by Zif268) was tightly linked to learning-related plasticity, and activation in CA3, prelimbic and anterior cingulate cortices was strongly associated with flexible spatial memory recall (i.e. pattern completion). Finally, we revealed that spatial memory deficits induced by NMDA receptor blockade could be partially prevented by extended environmental experience.

Chapter 1

Literature Review

1.1. Memory and navigation

Understanding how memories are instantiated in the brain remains one of the greatest challenges in the field of neuroscience. Current knowledge about memory processing has been informed by three broad strands of research: experimental analyses of learning and memory, studies of brain damage patients and the use of animal models (Nadel & Hardt, 2011). Collectively, these investigations have led to the classification of distinct memory systems, first according to length of storage (short-term or long-term), and subsequently by type (explicit or implicit) (Squire, 1986, 2004; Tulving, 1972). Explicit (or declarative) memory denotes the acquisition and recall of facts and events, later defined as semantic and episodic memory, respectively. Semantic information can be considered to represent the 'what' of memory, while episodic memory represents the 'where' and 'when' (Tulving, 2002). Implicit (or non-declarative) memory refers to learning in the absence of conscious awareness; for example, motor skill learning and priming (Squire, 2004). It is generally agreed that the medial temporal lobe of the brain, including the hippocampus, is crucial for recently acquired declarative memories, but not for non-declarative memories (see Good, 2002 for a review). One type of declarative memory which has been the subject of intense investigation and debate – particularly with regard to underlying brain mechanisms – is spatial memory (Eichenbaum & Cohen, 2014).

1.2. Theories of navigation

Spatial navigation is a fundamental behaviour shared by almost all animal species on our planet. The ability to navigate a complex environment requires constant coordination of sensory and proprioceptive information, learning and memory

processes, and planning (Chersi & Burgess, 2015; Penner & Mizumori, 2012). Human and non-human animals can avail of a variety of spatial strategies to navigate. These are broadly divided into two types: egocentric and allocentric (Burgess, 2008). Egocentric strategies generally involve learning the positions of objects or destinations in space relative to the navigator themselves. As such, no external cues are needed; instead, the animal uses stable self-motion cues, i.e. vestibular and kinaesthetic, to learn a fixed trajectory to the target (de Bruin, Moita, de Brabander, & Joosten, 2001; Tamara, Leffel, & Timberlake, 2010). This type of strategy is also termed path integration or dead reckoning (Cheung, 2014; Etienne & Jeffery, 2004). In addition, egocentric navigation can refer to procedural responding (termed 'taxon' learning). This entails learning to move towards a beacon cue which directly marks the goal location from a well-rehearsed start position (Chersi & Burgess, 2015; Liu, Turner, & Bures, 1994; O'Keefe & Nadel, 1978). Egocentric strategies are not considered to be strictly spatial in nature because the animal is not required to encode information about spatial relationship between the cue and the goal (or any other information about their environment); rather, it must only learn to associate movements towards the cue with reaching the target (Rodrigo, 2002).

In contrast, allocentric strategies involve learning spatial locations with reference to predictive environmental cues; accordingly, they are independent of the position of the navigator (Tamara et al., 2010). Using this kind of strategy, the animal navigates to a given destination by learning the spatial relationship between the available cues and the target (known as 'place' learning) (O'Keefe & Nadel, 1978; Rodrigo, 2002). Importantly, place learning is thought to culminate in the formation of internal representation or 'cognitive map' of the environment (Chersi & Burgess, 2015; O'Keefe & Nadel, 1978; Poucet, 1993; Tolman, 1948). Although the

precise definition continues to change, a cognitive map can broadly be defined as a global, unitary, mental representation of the spatial layout of an environment and all cues therein, which allows for flexible planning and navigating of novel routes (Ishikawa & Montello, 2006; Schinazi, Nardi, Newcombe, Shipley, & Epstein, 2013).

Support for the cognitive map proposal came from the discovery of 'place cells' in the hippocampus of rats (O'Keefe & Dostrovsky, 1971), and later in humans (Ekstrom et al., 2003). Place cells are a special class of cell that become active when an animal enters specific locations in the environment, which are known as a 'place fields' (O'Keefe & Dostrovsky, 1971). Place fields are formed within minutes of an animal being introduced into an environment and can be maintained robustly for up to 153 days (Thompson & Best, 1990; Wilson & McNaughton, 1993). In addition, modifying the environment (e.g. rotating or removing cues, or changing the borders or floor) has been shown to alter place fields (Cressant, Muller, & Poucet, 1999; Hetherington & Shapiro, 1997; Muller & Kubie, 1987). Importantly, place fields appear to be reliant on distal cues but not beacons; that is, rotating a distal cue results in a corresponding rotation of the place fields, while rotating proximal cues has no effect (Cressant et al., 1999). This suggests that the activation of place cells is directly related to complex, spatial processing. More recently, different types of spatial cells have been identified. These include boundary cells (which are most active when the animal is positioned at the edge of the environment; Hartley, Burgess, Lever, Cacucci, & O'Keefe, 2000), head direction cells (which respond to the animal's facing direction; Taube, Muller, & Ranck, 1990), and grid cells (which fire in multiple evenly spaced locations, forming a grid-like pattern; Moser & Moser, 2008). Similar to place cells, grid cell firing fields rotate in response to rotations of

distal cues (Hafting, Fyhn, Molden, Moser, & Moser, 2005). A number of additional spatial cell types have also been documented in the primate hippocampus and surrounding areas, including those which are responsive to specific views, goal locations and path directions (Ekstrom et al., 2003; Jacobs, Kahana, Ekstrom, Mollison & Fried, 2010).

Although there is strong physiological evidence that animals encode spatial information about the layout of the environment, the question of whether or not such representations are in fact 'global' (i.e. viewer-independent) continues to be debated (Benhamou, 1997; Shettleworth, 1999; Wang & Spelke, 2002). For example, Shapiro, Tanila and Eichenbaum (1997) showed that place fields could be significantly altered by rotating or removing a sub-set of available cues, or just a single cue. This finding argues against the idea of a global map, and suggests instead that spatial representations are linked to particular cues in the environment. Furthermore, it has been argued that map-like representations are unnecessary, and that successful navigation can be achieved using simpler processes. The most prominent opposing theory to the cognitive map hypothesis is associative learning (Pearce & Hall, 1980; Rescorla & Wagner, 1972; Rudy & Sutherland, 1995; Sutherland & Rudy, 1989). Based on the principles of Pavlovian conditioning (Pavlov, 1927), associative learning theory proposes that, over time, stored representations of elements in the environment (e.g. cues) become associated with specific actions or outcomes (e.g. sequences of movements towards a goal) (Hamilton, Driscoll, & Sutherland, 2002; Honey, Iordanova, & Good, 2014). These associations can be simple, whereby animals learn the spatial relationship between individual cues and the goal separately, or complex, which involve learning about a group of cues and their relationship to the goal (Sutherland & Rudy, 1989) (see

Chapter 3 for further description of the different types of associative learning theories).

Importantly, associative learning theory emphasises the formation of associations as required by the navigator (Leising & Blaisdell, 2009). Therefore, although both associative and cognitive mapping theories assume that allocentric spatial information is learned and represented in the brain, the two are not analogous (Mackintosh, 2002). Rather, the former are conceptualised as a collection of fragmented local views, or scenes, remembered from various locations in the environment, while the latter asserts that these scenes are combined to form a cohesive global representation (Ishikawa & Montello, 2006; Leonard & McNaughton, 1990; Rodrigo, 2002). Further, associative learning theory posits that spatial representations are stored in the cortex, and that the hippocampus contributes to spatial processing by enhancing activation of these representations (Rudy & Sutherland, 1995). One additional important distinction is that associative learning theory predicts 'cue competition' effects during learning. That is, cues which are more useful to the navigator (e.g. offer more reliable information about the location of the goal) will acquire greater control over behaviour than other, less useful cues (Diviney, Fey, & Commins, 2013; Redhead, Roberts, Good, & Pearce, 1997). From a cognitive map standpoint, these competitive effects should not emerge because all cues are thought to be incorporated spontaneously into the map, and thus, any combination of cues should allow for accurate navigation (Chamizo, 2002; Morris, 1981; Sánchez-Moreno, Rodrigo, Chamizo, & Mackintosh, 1999).

Contrary to cognitive mapping theory, two separate cue competition effects, known as 'blocking' and 'overshadowing', are well-documented in the literature (Chamizo, 2002). Blocking occurs where the presence of one cue during initial

learning delays or inhibits learning about a second cue presented subsequently (Kamin, 1969). This effect can also be reversed; that is, cues can be 'unblocked', if the location of the cues in relation to the goal is altered (Rodrigo, Arall, & Chamizo, 2005). Blocking has been demonstrated across species (Biegler & Morris, 1999; Cheng & Spetch, 2001; Hamilton & Sutherland, 1999; Miller & Escobar, 2002; Redhead et al., 1997). For example, Hamilton *et al.* (1999) demonstrated that participants initially trained to locate a hidden platform using four cues in a virtual navigation task failed to learn about four novel cues during a second training phase, and could not navigate to the goal location when only these novel cues were present. Stahlman and Blaisdell (2009) illustrated a comparable effect in rats, whereby pre-training with a beacon inhibited animals' ability to navigate using a second beacon introduced later.

Overshadowing is a similar phenomenon which denotes the inhibition of learning about one cue by a co-occurring cue, which is deemed more useful for finding the goal (Chamizo, Sterio, & Mackintosh, 1985). Overshadowing effects have also been observed in both human and non-human animals (Chamizo, Manteiga, Rodrigo, & Mackintosh, 2006; Chamizo & Rodrigo, 2004; Redhead, Hamilton, Parker, Chan, & Allison, 2013; Redhead et al., 1997; Sanchez-Moreno, Rodrigo, & Chamizo, 1999). For example, Chamizo and colleagues (2006) showed that rats navigating in the Morris water maze learned more about a cue positioned near to a hidden platform than similar cues located farther away, indicating that the near cue overshadowed the other cues. There a variety of factors which are known to influence which cues will overshadow others. These include the proximity between the cue and the target, with closer cues typically overshadowing farther cues (Redhead et al., 2013; Spetch, 1995), and the type of cues available, e.g. intra-maze

cues (textured flooring) or room cues surrounding the environment (Chamizo et al., 1985; March, Chamizo, & Mackintosh, 1992).

Importantly, it has been suggested that – instead of being reliant on one type of representation – spatial memory is supported by multiple representations in parallel, including both egocentric (self-motion and taxon learning) and allocentric (place learning via spatial representations) (Burgess, 2008). Concurrent use of strategies in rats has been observed in previous work from our laboratory via in-depth analyses of navigational behaviour (Harvey et al., 2008). Specifically, animals trained to find a hidden platform in the water maze were shown to rely on an egocentric strategy (i.e. movements towards particular cues) supported by allocentric learning (i.e. 'scanning' of the overall environmental layout). Over time, reliance on egocentric behaviours decreased, presumably as rats acquired a more stable spatial representation of the cue arrangement relative to the goal (Harvey et al., 2008). A similar effect was documented by Hamilton *et al.* (2004) using the egocentric (visible platform) version of the Morris water maze task. The authors demonstrated sequential use of strategies, whereby rats first employed an allocentric strategy by orienting relative to the available distal cues; once closer to the target, animals switched to an egocentric strategy using the platform itself as a beacon (Hamilton et al., 2004).

Much research has been carried out to investigate the conditions under which particular strategies become more dominant. One of the most robust findings is that egocentric strategies will be preferred when proximal cues (positioned close to the goal) are available (Carman & Mactutus, 2002; Cheng & Spetch, 1995; Harvey, Brant, & Commins, 2009). As the distance between the cue and the target increases, animals will typically decrease their dependence on procedural responding and

become more reliant on the surrounding configuration of cues (Tamara et al., 2010). In a systematic examination of cue proximity, Chamizo and Rodrigo (2004) revealed that rats can accurately locate a target using a single beacon positioned up to 110cm away from the goal. When the distance is greater than this, rats require additional information (e.g. the direction in which to travel) in order to navigate effectively using an allocentric strategy (Chamizo & Rodrigo, 2004; Mackintosh, 2002; Vorhees & Williams, 2014).

In addition, the reliability of the information provided by cues can have a significant influence on the strategy employed (Maaswinkel & Whishaw, 1999; Shettleworth & Sutton, 2005). That is, animals will use distal cues so long as they offer consistent spatial information about the goal location (Timberlake, Sinning, & Leffel, 2007). For example, Maaswinkel and Whishaw (1999) reported that forging rats preferentially used visual or olfactory cues when they were available, but could rely self-motion cues if necessary. However, animals may not always be able to switch between strategies; Kealy *et al.* (2008) found that rats trained with visual cues in the water maze for an extended period (12 days) were impaired when these cues were removed, despite both the start position and target remaining fixed. Accordingly, the precise factors governing the type of spatial information an animal will encode and utilise during navigation to a goal remain somewhat unclear.

1.3. The Morris water maze task

There are a variety of laboratory-based tasks that can be used to probe spatial processing in animals (Paul, Magda, & Abel, 2009). The most popular of these for examining rodent navigation is the Morris water maze task (Morellini, 2013). The water maze, originally developed by Richard Morris (1981), is an aversively

motivated task consisting of a circular pool of opaque water and a platform (the goal location) which is submerged just below the surface of the water in a fixed location, rendering it hidden to the navigating animal. The aim of the standard spatial reference version of task is for the animal to locate the hidden platform using a collection of distal cues which surround the maze. To acquire the task, the animal typically receives multiple training trials over a number of days, wherein they gradually learn the spatial relationship between the cues and the platform, eventually enabling them to find the goal. Spatial memory retrieval can subsequently be tested by removing the platform and examining where animals search during a probe trial.

The water maze has several advantages over other, land-based tasks such as the radial-arm maze (Olton & Samuelson, 1976) and the Y-maze (Conrad, Galea, Kuroda, & McEwen, 1996). For example, task acquisition does not require the animal to be food deprived, and the presence of water eliminates the potential use of confounding information such as olfactory or auditory cues (D'Hooge & De Deyn, 2001). Further advantages include fast and reliable learning, the absence of non-performers, and the elimination of any effects arising from differences in body weight (Vorhees & Williams, 2014). The main disadvantage of this task is its stressful nature, due to rodents' natural aversion to water (Vorhees & Williams, 2014). However, the stress induced under normal learning conditions is thought to be mild. Specifically, Kavushansky, Vouimba, Cohen and Richter-Levin (2006) measured levels of corticosterone in rats following training with a visible platform, a hidden platform, or no platform (forced swim test). The authors found that corticosterone was elevated in the group trained with no platform, i.e. where there was no escape from the water, indicating heightened stress in these animals only.

One final advantage of the water maze is that the ambiguity of this task – where effective navigation is not merely based on a single defined path – allows for an investigation of multiple navigational strategies (Kelly & Gibson, 2007; Penner & Mizumori, 2012). In addition to the standard version of the task which taxes allocentric spatial navigation, egocentric (or non-spatial) learning can also be assessed. This can involve training without cues (prompting the use of idiothetic information; Moghaddam & Bures, 1996), or training with a visual platform or a single beacon (via a taxon strategy; Morris, 1981; Roberts & Pearce, 1999). Working memory can also be examined by relocating the hidden platform to a new location on each day of training (Steele & Morris, 1999). In addition to navigational behaviour, the water maze can also be applied to the study of underlying brain mechanisms (Whishaw, 1985b). Since its development, the maze has been extensively used to examine the importance of specific brain areas for different types of spatial learning and memory (D'Hooge & De Deyn, 2001). Finally, the water maze has most recently been applied to human navigation using a virtual reality protocol, thereby demonstrated its cross-species relevance to the study of navigation (Driscoll, Hamilton, Yeo, Brooks, & Sutherland, 2005; Hamilton et al., 2002; Kelly & Gibson, 2007).

1.4. Brain regions involved in spatial learning and memory

A myriad of research over the past few decades has identified multiple brain regions which are thought to be important for representing space and enabling navigation. Chief among these is the hippocampal formation, which is widely accepted as a crucial structure for successful spatial memory processing (Burgess, Maguire, & O'Keefe, 2002; Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe & Nadel, 1978).

1.4.1. Hippocampal formation

1.4.1.1. Anatomy

The hippocampal formation is located in temporal lobe of the cerebral cortex. It comprises three distinct regions: the hippocampus proper, the dentate gyrus and the subiculum (Amaral & Lavenex, 2007). The hippocampus proper can, in turn, be divided into three separate fields based on the size and distribution of their cells; these include Cornus Ammonis 1 (CA1), Cornus Ammonis 2 (CA2) and Cornus Ammonis 3 (CA3) (Amaral & Witter, 1989). The term 'hippocampus' typically refers to the hippocampus proper and the dentate gyrus (see Figure 1.1).

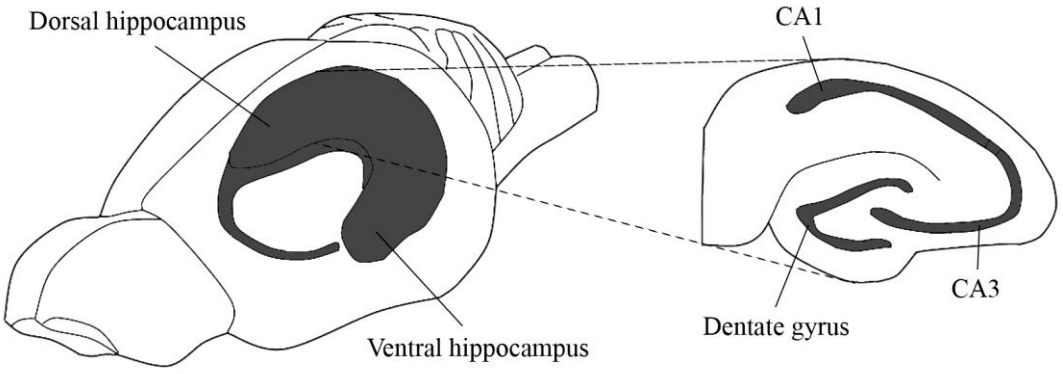

Figure 1.1: Left: schematic diagram of the position of the hippocampus in the brain including dorsal and ventral regions; Right: A coronal slice of the dorsal hippocampus and its sub-regions including CA1, CA3 and the dentate gyrus. Adapted from Barry (2013) and Witter and Amaral (2004).

The principal cell types of the hippocampus proper and dentate gyrus are the pyramidal cell and the granule cell, respectively (Amaral, Scharfman, & Lavenex, 2007; Lavenex & Amaral, 2000). The hippocampus receives input from the entorhinal cortex via the perforant path; projections to area CA1 and the subiculum originate mainly from cells in layer III and those to area CA2, CA3 and the dentate

gyrus originate from the cells in layer II (Amaral et al., 2007; Gigg, 2006). Area CA2 is the least well defined region and has been referred to as a transitionary zone between CA1 and CA3 cellular layers (Stubley-Weatherly, Harding, & Wright, 1996). The dentate gyrus is connected to the hippocampal formation via the mossy fibers (axons originating from the granule cells) which project to area CA3; projections from area CA3 pyramidal cells include the Schaffer collaterals which comprise the major projection to area CA1 (Amaral & Lavenex, 2007). Cells in area CA3 are also highly interconnected, forming a system of associational connections or 'recurrent collaterals' (Amaral & Lavenex, 2007). Cells in area CA1 project to the subiculum and to the deep layers of the entorhinal cortex (Naber, Lopes da Silva, & Witter, 2001). The unidirectional circuit from the entorhinal cortex through the hippocampus (dentate gyrus to CA3 to CA1) is known as the trisynaptic pathway (Amaral & Witter, 1989). Finally, area CA1 receives inputs from the perirhinal (Aggleton, Kyd, & Bilkey, 2004) and medial prefrontal cortices (Rajasethupathy et al., 2015).

1.4.1.2. Role in spatial learning and memory

Since the discovery of place cells over 40 years ago, the hippocampus has repeatedly been linked to spatial learning and memory. Collective evidence from lesion studies has revealed that rats tasked with navigating to a hidden platform in the water maze without a functioning hippocampus are impaired at both encoding and retrieval stages (Deacon & Rawlins, 2002; Dolleman-van der Weel, Morris, & Witter, 2009; Mogensen, Moustgaard, Khan, Wortwein, & Nielsen, 2005; Morris et al., 1982; Sutherland & Rodriguez, 1989). Moreover, performance deficits in lesioned animals have been shown to increase according to the spatial complexity of the task (Save &

Poucet, 2000). In contrast, hippocampal lesioned rats are unimpaired at navigating to a visible platform in the water maze (de Bruin et al., 2001; Morris et al., 1982; Save & Poucet, 2000). Additionally, Riedal and colleagues (1999) reported that temporary pharmacological inactivation of the hippocampus during water maze acquisition caused rats to swim randomly. The authors also showed that when the hippocampus was inactivated after training, rats continued to be impaired, exhibiting focused but inaccurate search patterns (Riedel et al., 1999). Together, these results indicate that an intact hippocampus is necessary for flexible place learning (i.e. when a representation of the environment is encoded), but not for taxon learning, in line with cognitive mapping theory (Poucet, 1993). However, they are also consistent with revised associative learning theory (Rudy & Sutherland, 1995), which posits that the hippocampus is involved in processing complex (but not simple) associative representations stored in the cortex.

Similar results have been documented in humans. Astur, Taylor, Mamelak, Philpott, and Sutherland (2002) tested patients with unilateral hippocampal damage in the hidden platform version of the virtual water maze and found that all patients displayed severe deficits in learning and remembering the goal location relative to a matched control group with no damage and a group of patients with extra-hippocampal lesions. Findings from neuroimaging studies also support the central role of the hippocampus in navigational processing. For example, Maguire and colleagues (2000) carried out a structural fMRI analysis of a group of expert navigators (London taxi drivers) and a control group (with no experience of driving taxis). The authors noted significantly enlarged posterior hippocampi in the experts, and a positive correlation between hippocampal size and time spent as a taxi driver (Maguire et al., 2000). In keeping with these results, a study by Schinazi, Nardi,

Newcombe, Shipley and Epstein (2013) showed that the size of the right posterior hippocampus predicted participants' proficiency at using recently acquired spatial knowledge about a real-world large-scale environment. More recently, work by Spiers and colleagues has provided specific evidence that the human hippocampus and entorhinal cortex are particularly engaged in processing direction and distance to the goal (Chadwick, Jolly, Amos, Hassabis, & Spiers, 2015; Howard et al., 2014).

Within the hippocampus, a functional distinction has been made between dorsal and ventral regions. Specifically, lesions to the dorsal hippocampus in rodents reliably produce severe deficits in spatial learning and memory, as well as spatial working memory, while ventral lesions have little or no effect on performance (Bannerman et al., 2002; Bannerman et al., 1999; Hock & Bunsey, 1998; Moser, Moser, & Andersen, 1993; Moser, Moser, Forrest, Andersen, & Morris, 1995; Potvin, Allen, Thibaudeau, Dore, & Goulet, 2006; Zhang, Pothuizen, Feldon, & Rawlins, 2004). On the other hand, ventral lesions lead to an attenuated anxiety response, indicating that this region is more involved in processing anxiogenic stimuli (Bannerman et al., 2003; McHugh, Deacon, Rawlins, & Bannerman, 2004). Of particular note, Moser and colleagues (1995) illustrated that spatial learning in the water maze could be achieved with only 26% of the hippocampus, provided that the remaining tissue was at the dorsal pole. The proportion of place cells in the ventral hippocampus is also lower than that of the dorsal hippocampus, and the place fields are less selective (Jung, Wiener, & McNaughton, 1994). Importantly, the posterior hippocampus in humans is considered to be the mammalian analogue of the dorsal region in rodents; as such, results from human studies (e.g. Maguire et al., 2000; Schinazi et al., 2013) lend further support to this functional segregation.

In addition, discrete sub-regions within the dorsal hippocampus also appear to play subtly different roles in spatial learning and memory (Kesner, Lee, & Gilbert, 2004). Focal lesions to area CA1 have been shown to impede spatial working memory performance in the Y-maze (Dillon, Qu, Marcus, & Dodart, 2008) and place learning in the water maze (Okada & Okaichi, 2009; Stubley-Weatherly et al., 1996). Further, Hunsaker, Fieldsted, Rosenberg and Kesner (2008) found that deficits in processing spatial locations were specific to lesions of dorsal CA1 region in rats. Importantly, rats with CA1 lesions resulting in 50% cellular loss have been shown to perform as poorly as those with 88% cellular damage to area CA3, suggesting that CA1 may be more engaged in encoding (Stubley-Weatherly et al., 1996). Moreover, while lesions to CA1, CA3 and dentate gyrus regions in rats all disrupt memory for metric information (i.e. learned distances between available distal cues), only CA1 lesions affect topographical memory (i.e. representations of the overall cue arrangement) (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008); again, this indicates that CA1 is particularly important for processing complex spatial representations. Bartsch and colleagues (2010) demonstrated a similar effect in patients with focal damage to area CA1. Specifically, participants were profoundly impaired at learning the location of the goal in a virtual water maze task, and performance deficits were positively correlated with lesion size (Bartsch et al., 2010). Finally, Rondi-Reig *et al.* (2006) tested CA1 knockout mice (lacking N-methyl-D-aspartate (NMDA) receptors) in a novel water star-maze task which taxed allocentric and egocentric strategy use. The authors found that while control mice could reach the platform using both strategies, knockout mice acquired neither, suggesting that CA1 also facilitates the use of multiple types of memory representations (Rondi-Reig et al., 2006).

In contrast to CA1, the role of CA3 in spatial encoding and retrieval is less clear, with lesion studies yielding equivocal results. For example, Stubley-Weatherly and colleagues (1996) showed that CA3 lesions impaired water maze performance in rats. Florian and Roullet (2004) found analogous deficits following temporary inactivation of CA3 before training. However, others have demonstrated accurate place learning in CA3 lesioned animals (Nakazawa et al., 2002; Okada & Okaichi, 2009; Steffenach, Sloviter, Moser, & Moser, 2002; Sutherland, Whishaw, & Kolb, 1983). Similarly, memory retrieval deficits have been reported in CA3 lesioned rats. Brun and colleagues (2002) found evidence for a functional segregation of areas CA1 and CA3. Using a lesion approach in rodents, the authors showed that removal of CA3 had no effect on place fields in CA1 or spatial recognition memory for goal locations (measured in the annular water maze). Conversely, recall of goal locations and routes towards them (tested in the standard water maze) depended on an intact CA3 sub-region. Together, these results suggest that CA1 supports spatial location encoding, while CA3 is required for memory recall (Brun et al., 2002). However, such effects have failed to be replicated following pre-testing pharmacological inactivation (Florian & Roullet, 2004) or genetic ablation of CA3 cells in mice (Nakazawa et al. 2002).

Area CA3 may be particularly important for rapid acquisition of novel spatial information (Rolls & Kesner, 2006). More specifically, deletion of NMDA receptors in CA3 prevents mice from learning a novel platform location in the water maze, despite normal performance when tested with previously learned locations (Nakazawa et al., 2003). Lee and Kesner (2002) found similar deficits in working memory with mice treated with selective CA3 injections of NMDA channel blocker APV. More specifically, mice were impaired on a previously acquired delayed-non-

matching-to-place task when tested on the same task in a novel environment (i.e. a different experimental room). Crucially, selective inactivation of CA1 or the dentate gyrus had no effect in the novel environment (Lee & Kesner, 2002). A further study by Lee, Rao and Knierim (2004) revealed that rotation of distal cues led to comparable shifts in CA3 place fields on first exposure to the novel environment (day 1), whereas place fields in CA1 were slower to change (day 2 onwards). Together, results strongly imply that CA3 facilitates the rapid formation of spatial representations.

During retrieval, CA3 is thought to mediate recall of stored information patterns when faced with partial inputs; a process known as pattern completion (Marr, 1971). A simple example of this would be training rats to find a hidden platform in the water maze using a distal cue arrangement, and subsequently testing them with only a sub-set of the original training cues. Area CA3 is considered to be particularly suited to this process due to its recurrent collaterals, which are said to enable reconstruction of an intact memory trace (Rolls & Kesner, 2006). Supporting evidence for this suggestion has been found in rodents (Fellini, Florian, Courtey, & Roullet, 2009; Jo et al., 2007; Nakazawa et al., 2002; Vazdarjanova & Guzowski, 2004) and humans (Deuker, Doeller, Fell, & Axmacher, 2014; Schapiro, Kustner, & Turk-Browne, 2012).

Interesting, Vazdarjanova and Guzowski (2004) found that – in addition to playing an important role in pattern completion – CA3 is also involved in pattern separation. Pattern separation refers to the process of separating spatially similar memories into distinct representations (Marr, 1971; Morris, Churchwell, Kesner, & Gilbert, 2012). Specifically, rats were exposed to two different environments with a 30 minute interval in between; in some cases the environments differed mildly from

one another (e.g. cues were moved), and in others the environments were markedly distinct (e.g. novel objects in a new testing room). Using a novel gene-based imaging approach (Arc/H1a catfish), the authors were able to monitor activation of neuronal ensembles in CA3 and CA1 as the animals explored. They found greater overlap of activated neurons in CA3 relative to CA1 when the environmental changes were small, indicative of pattern completion; in contrast, less overlap was seen in CA3 (compared to CA1) when the environments were drastically different, in line with pattern separation (Vazdarjanova & Guzowski, 2004).

Regarding the dentate gyrus, studies in rodents have shown that lesions to this sub-region lead to impaired acquisition and recall of both spatial reference and working memory tasks (Jeltsch, Bertrand, Lazarus, & Cassel, 2001; Nanry, Mundy, & Tilson, 1989; Okada & Okaichi, 2009; Sutherland et al., 1983; Walsh, Schulz, Tilson, & Schmechel, 1986; Xavier, Oliveira-Filho, & Santos, 1999). Okada and Okaichi (2009) and Sutherland and colleagues (1983) noted that dentate gyrus lesions caused greater navigation deficits than lesions to other sub-regions of the hippocampus. Further, Nanry *et al.* (1989) and Xavier *et al.* (1999) highlighted that rats with dentate gyrus lesions tested in the water maze exhibited comparable deficits to rats with complete hippocampal lesions. Together, these results indicate that the dentate gyrus is particularly important for spatial information processing. This could reflect its anatomical connectivity; specifically, because the dentate gyrus receives input from the entorhinal cortex and projects to CA3, it is in a position to control the flow of information within the hippocampus (Xavier et al., 1999).

Finally, the dentate gyrus has also been implicated in pattern separation (Kesner et al., 2004; Rolls, 2010). Studies have demonstrated that lesions to the dentate gyrus disrupt pattern separation in tasks of spatial working memory (Gilbert,

Kesner, & Lee, 2001; Goodrich-Hunsaker et al., 2008). For example, Goodrich-Hunsaker and colleagues (2008) demonstrated that rats with dentate gyrus lesions were unable to detect changes in distance between two objects, as evidenced by reduced exploration for displaced objects relative to control rats. Importantly, such deficits were not observed following lesions to areas CA1 or CA3, indicating that the dentate gyrus is specifically required for discriminating between similar spatial representations (Gilbert et al., 2001; Goodrich-Hunsaker et al., 2008). Recently, Morris *et al.* (2012) examined the role of the dentate gyrus in pattern separation for spatial reference memory using a place learning paradigm in the radial arm maze. Rats were trained to discriminate between a rewarded arm and a non-rewarded arm which were either next to each other (adjacent condition) or separated by two arms (separate condition). Results showed that in the separate condition, where the degree of overlap between spatial cues was low, lesion and control groups acquired the task at comparable rates. However, when the spatial overlap between cues was increased in the adjacent condition, lesioned rats took significantly longer to reach learning criterion relative to controls. These findings demonstrate that the dentate gyrus facilitates the formation of distinct memory representations when there is a high degree of spatial similarly (Morris et al., 2012).

Collectively, evidence to date strongly supports the integral role of the hippocampus in spatial processing. However, evidence of accurate navigation in the absence of this region has been reported (Pouzet, Zhang, Feldon, & Rawlins, 2002). In one study, Morris, Schenk, Tweedie and Jarrad (1990) found that hippocampal lesioned rats eventually learned to navigate via an allocentric strategy in the water maze, although they did not reach the same performance levels as control animals. Stubley-Weatherly and colleagues (1996) reported similar effects in CA1 and CA3

lesioned animals, i.e. acquisition improved over the course of training but rats remained impaired relative to controls. In addition, a retention study by Whishaw (1985a) revealed that fimbria-fornix lesioned rats retained some memory for a learned target location after prolonged training in the water maze (31 days), but not after standard five-day training. Taken together, these findings indicate that the hippocampus is not exclusively responsible for encoding and retrieval of spatial memories, and that its role in spatial processing may decrease with greater environmental experience.

1.4.2. Medial prefrontal cortex

The medial prefrontal cortex has also been implicated in spatial information processing (Simons & Spiers, 2003). Evidence strongly suggests that memories become increasingly dependent on the medial prefrontal region over time (Frankland & Bontempi, 2005). However, its importance for processing recently acquired spatial memories continues to be debated.

1.4.2.1. Anatomy

Current opinion remains divided as to whether or not rodents possess a prefrontal cortical region analogous to humans and other primates (Kesner, 2000; Uylings, Groenewegen, & Kolb, 2003). However, the prefrontal cortex of the rat can be separated anatomically into medial, orbital and lateral areas (Ongur & Price, 2000). Within the medial prefrontal cortex, there are four sub-regions; from dorsal to ventral, these are the medial agranular, anterior cingulate, prelimbic and infralimbic cortices (Hoover & Vertes, 2007; Ongur & Price, 2000) (see Figure 1.2). Each sub-region of the medial prefrontal cortex projects to the others, although the infralimbic

cortex receives comparatively fewer inputs from other medial prefrontal areas, with the prelimbic cortex being its primary source of afferent projections (Hoover & Vertes, 2007). The medial prefrontal cortex also receives projections from entorhinal, perirhinal, retrospenial and posterior parietal cortices, as well the hippocampus (Agster & Burwell, 2009; Hoover & Vertes, 2007; Kolb & Walkey, 1987; Valenti & Grace, 2009). Hippocampal inputs from area CA1 and the subiculum target prelimbic and infralimbic regions in particular (Hoover & Vertes, 2007; Jay & Witter, 1991; Laroche, Davis, & Jay, 2000). The infralimbic cortex also receives strong afferent projections from the amygdala (McDonald, Mascagni, & Guo, 1996; Vertes, 2004).

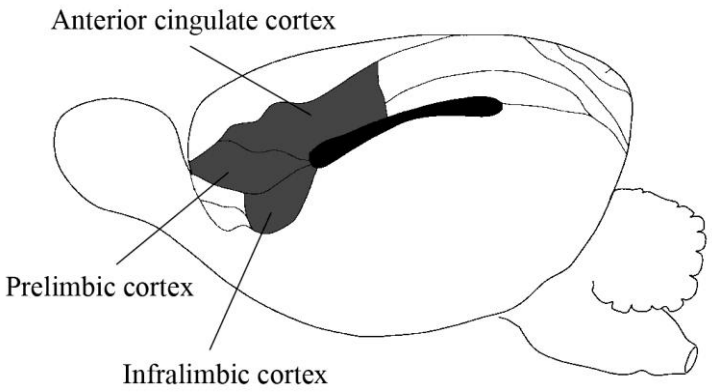

Figure 1.2: Medial view of the brain showing the location of the medial prefrontal cortex including anterior cingulate, prelimbic, and infralimbic sub-regions. Adapted from Burwell and Amaral (1998). Medial agranular cortex not shown.

1.4.2.2. Role in spatial learning and memory

In comparison to the hippocampus, the importance of the medial prefrontal cortex for spatial memory is less well characterised. Most of the research investigating its role in navigation has focused on spatial working memory. Lesions to the medial prefrontal cortex have been shown to impair this capacity on a range of delayed

response tasks in rodents (Granon & Poucet, 1995; Kesner, Hunt, Williams, & Long, 1996; Kolb, Buhrmann, McDonald, & Sutherland, 1994; Lee & Kesner, 2003; Wikmark, Divac, & Weiss, 1973). Lesions to the prelimbic/infralimbic cortices in particular, produce severe deficits (Delatour & Gisquet-Verrier, 2000; Ragozzino & Kesner, 1998), while anterior cingulate (Ragozzino, Adams, & Kesner, 1998) and agranular insular lesions have little effect (Ragozzino & Kesner, 1999). In an additional study, Granon and Poucet (1995) trained rats with medial prefrontal lesions in a modified version of the water maze task wherein the number of start positions was increased from one to four in consecutive stages. Results revealed that animals were impaired at locating the platform when tested from four distinct start positions, and poor performance was specific to the two most recently introduced platform locations. This was thought to be reflective of a working memory deficit which prevented rats from encoding a sufficient representation of all movements needed to reach the goal (Granon & Poucet, 1995). Similarly, prefrontal lesions have also been shown to impair working memory performance in humans, wherein the patient is required to maintain a goal destination as they navigate (Ciaramelli, 2008).

The role of the medial prefrontal cortex in place learning and spatial reference memory has received less attention. Initial electrophysiological recordings of prefrontal cells indicated that neuronal firing patterns in this region were not associated with animals' position or head direction (Jung, Qin, McNaughton, & Barnes, 1998; Poucet, 1997). However, a more recent study by Hok, Save, Lenck-Santini and Poucet (2005) showed that a sizeable proportion of cells in the prelimbic/infralimbic area (25% of cells analysed) had place fields. A smaller proportion of place cells were also found in the dorsal anterior cingulate region (4%). However, these place fields displayed less spatial coherence and were larger in size

compared to those of hippocampal cells. In addition, their distribution was not homologous; that is, place fields were mainly distributed at goal locations, indicating that cells in the prefrontal cortex encode spatial information about relevant places (or goals) in the environment (Hok et al., 2005).

The effects of lesions to the medial prefrontal cortex on spatial reference learning in the water maze are varied. For example, Lacroix, White and Feldon (2002) reported that medial prefrontal lesions had no impact on spatial acquisition of the water maze using an allocentric strategy. Similar results were also found by de Bruin and colleagues (de Bruin et al., 2001; de Bruin, Sanchez-Santed, Heinsbroek, Donker, & Postmes, 1994) and Compton, Griffith, McDaniel, Foster and Davis (1997). In contrast, a series of experiments by Mogensen *et al.* and Kolb *et al.* showed allocentric navigation was initially somewhat impaired in rats with medial prefrontal lesions, although animals eventually learned the task in some cases (Kolb, Sutherland, & Whishaw, 1983; Mogensen, Lauritsen, Elvertorp, Hasman, Moustgaard, & Wortwein, 2004; Mogensen, Pedersen, Holm, & Bang, 1995; Sutherland, Kolb, & Whishaw, 1982).

In support of these findings, recent research by Woolley and colleagues showed that prefrontal activation in both mice (measured by gene expression) and humans (using fMRI) was increased during initial encoding of the traditional and virtual water maze task, respectively (Woolley et al., 2013). Evidence for prefrontal involvement in egocentric navigation in the water maze has also been found (Ethier, Le Marec, Rompre, & Godbout, 2001; Mogensen et al., 2005). In addition, medial prefrontal lesions produce deficits in spatial reversal learning in the water maze, wherein the platform is moved to a new location in the middle of training (Kolb, Nonneman, & Singh, 1974). To accomplish this task, animals must inhibit their

original learning about the platform's location and encode the new target position. The failure of prefrontal lesioned rats to complete this task suggests a deficit in flexible responding, i.e. the ability to adopt a new strategy when the learned one becomes ineffective (Jones, Groenewegen, & Witter, 2005; Lacroix, White, & Feldon, 2002).

Several studies have reported deficits consistent with this idea. Ragozzino, Wilcox, Raso and Kesner (1999) inactivated prelimbic/infralimbic or dorsal anterior cingulate regions before training rats in spatial and cued versions of the cheeseboard task, which is similar a dry land version of the Morris water maze (the order of the tasks was counterbalanced). They found acquisition of both versions was unaffected by prelimbic/infralimbic or anterior cingulate inactivation; however, the former did impair rats' learning when they were required to switch between strategies, regardless of which version was presented second (Ragozzino, Wilcox, et al., 1999). These findings have been replicated with mice in the water maze (Latif-Hernandez et al., 2015) and rats in the cross maze (Ragozzino, Detrick, & Kesner, 1999). Interestingly, Floresco, Block and Tse (2008) demonstrated that, when the medial prefrontal cortex is inactivated, relative difficulty of tasks significantly impacts animals' ability to switch between them. Specifically, rats were trained on two discrimination tasks: visual-cue (i.e. always press the lever below a light cue) and response (i.e. always press the lever on the left; considered the more difficult task of the two). Results showed that inactivation of the medial prefrontal region impaired performance when rats were required to switch from the visual cue to the more demanding response task, but not vice versa (Floresco et al., 2008). Such behavioural flexibility is considered functionally similar to executive functioning in humans which is mediated by the dorsolateral prefrontal cortex (Granon & Poucet, 2000).

An additional study by Jo and colleagues (2007) investigated prefrontal involvement in behavioural flexibility and strategy switching. The authors tested rats with lesions to the medial prefrontal cortex (or to area CA3) in a hidden platform task under full and partial cue conditions. For the full cue condition, rats were tested with four distal training cues. For the partial cue condition, three of the cues were removed, leaving only one distant cue. Both prefrontal and CA3 lesion groups showed poor retrieval under partial, but not full, cue conditions. Temporary inactivation of the medial prefrontal cortex with infusions of muscimol administered before testing also produced impairments under partial cue conditions. Based on their results, Jo *et al.* (2007) proposed that the medial prefrontal cortex contributes to pattern completion processes during memory retrieval. More specifically, this region may be necessary to integrate the degraded memory provided by the hippocampus with additional inputs from the cortex, thereby producing a more complete representation which can be used to navigate to the target (Hok et al., 2005; Jo et al., 2007; Rudy, Biedenkapp, & O'Reilly, 2005).

Although studies aimed at investigating the specific roles of medial prefrontal sub-regions in spatial processing are limited, a functional distinction has been made between dorsal and ventral areas (Gisquet-Verrier, Winocur, & Delatour, 2000; Uylings et al., 2003). Dorsal areas (agranular insular and anterior cingulate cortices) are thought to be involved in motor behaviours (Dalley, Cardinal, & Robbins, 2004). For example, anterior cingulate lesions have been shown to cause impairments in temporal ordering of movements in space, i.e. when executing complex routes to a goal location (Eichenbaum, Clegg, & Feeley, 1983; Kesner, 2000; Kolb, 1984; Sutherland, Whishaw, & Kolb, 1988). In contrast, ventral regions (prelimbic and infralimbic cortices) have been implicated in a range of mnemonic processes, e.g.

task switching (Dalley et al., 2004). In keeping with the evidence outlined above, a recent study by Rich and Shapiro (2009) showed that place cell activity in prelimbic and infralimbic sub-regions was altered during spatial and non-spatial task switching in rats. The prelimbic cortex is thought to be particularly important for behavioural flexibility when task or attentional demands are high (Granon & Poucet, 2000). In comparison, the precise function of the infralimbic cortex is less clear. However, some evidence suggests that this area is important for emotional responding, particularly with regard to fear-related behaviours (Dalley et al., 2004; Hoover & Vertes, 2007; Uylings et al., 2003).

1.4.3. Connectivity between brain regions

The hippocampus and medial prefrontal cortex are highly interconnected (see Figure 1.3). Until recently, these connections were thought to be unidirectional, i.e. hippocampus to medial prefrontal cortex (Hoover & Vertes, 2007; Laroche et al., 2000). However, Rajasethupathy and colleagues (2015) discovered a direct return projection from the prefrontal cortex – primarily from the anterior cingulate area – to the CA3/CA1 region in mice.

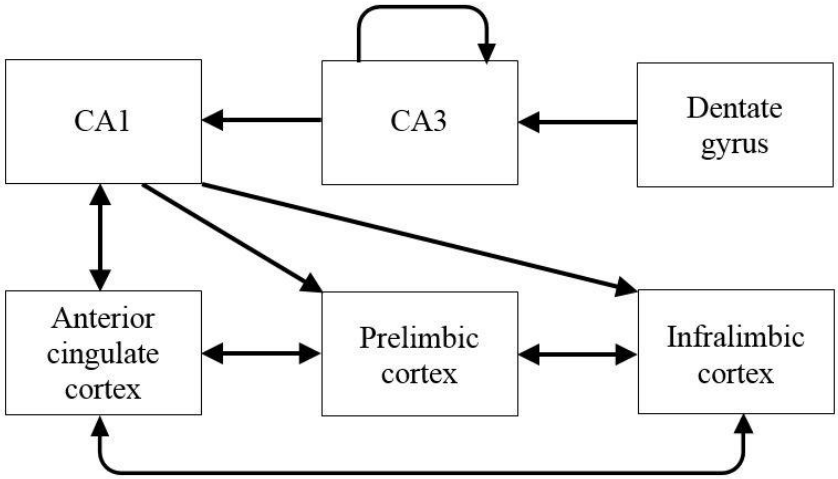

Figure 1.3: Diagram showing the direct interconnectivity of the hippocampal and medial prefrontal sub-regions examined in this thesis. Summarised from Agster and Burwell (2009), Amaral and Lavenex (2007), Burwell and Amaral (1998), Hoover and Vertes (2007), and Rajasethupathy *et al.* (2015).

Accordingly, it is reasonable to assume that hippocampal-prefrontal interactions are fundamental to spatial memory processing. In the last ten years, a number of studies have reported evidence in support of this suggestion. For example, Churchwell, Morris, Musso and Kesner (2010) demonstrated that disconnection of CA1 and the medial prefrontal cortex impaired encoding and retrieval of allocentric spatial memory in the Hebb-Williams maze, a task involving navigation to a food reward. Wang and Cai (2008) reported analogous effects in the water maze, whereby inactivation of the CA1-prelimbic circuit in rats resulted in significant performance deficits. This is consistent with an earlier finding by Kyd and Bilkey (2003), which showed that hippocampal place cell firing was altered by medial prefrontal lesions.

Together, results strongly suggest that functional interactions between the hippocampus and medial prefrontal cortex are essential for adaptive behaviour during encoding and retrieval of spatial representations (Churchwell et al., 2010). Finally, it should be noted that these regions represent only part of a wider brain

network thought to underlie navigational behaviour (Jenkins, Amin, Harold, Pearce, & Aggleton, 2003). More specifically, hippocampal and prefrontal regions are both connected (directly and/or indirectly) with the retrospenial, perirhinal and entorhinal cortices (Aggleton & Brown, 2005; Aggleton et al., 2004; Agster & Burwell, 2009; Hoover & Vertes, 2007; Valenti & Grace, 2009; Wyss & Van Groen, 1992), all of which likely contribute during one or more stages of spatial processing.

1.5. Mechanisms underlying memory formation

Synaptic plasticity is widely accepted as the physiological basis of memory storage in the brain (Collingridge, Isaac, & Wang, 2004). Synaptic plasticity refers to changes in the strength of the synapses between two cells. These changes can be positive or negative, resulting greater or less efficient information transfer. Persistent changes in synaptic strength are considered to underlie long-term memory (Lamprecht & LeDoux, 2004). The most prominent synaptic model of long-term memory formation is long-term potentiation (LTP), which has predominantly been studied in the hippocampus (Bliss & Collingridge, 1993). LTP was first described by Bliss and Lomo (1973) who noted a long-lasting increase in synaptic strength in the rabbit dentate gyrus following high-frequency stimulation. Since then, LTP has reliably been observed at synapses throughout the brain, including the prefrontal cortex (Zhuo, 2014), and has been shown to last from anywhere between one hour to one year (Abraham, 2003).

LTP is consistent with the physiological requirements of Hebb's theory of memory formation (1949) which states that when two neurons are repeatedly active at the same time, the connection between them will strengthen, such that subsequent activation of one neuron leads to activation of the other. According to the synaptic

tagging hypothesis, LTP can be divided into early and late phases (Frey & Morris, 1997). During early LTP, a short term increase in synaptic strength occurs in the absence of protein synthesis (lasting a few hours), causing structural changes to the synapses which act as a 'tag' for the later stage; in late LTP, these structural changes are stabilised via protein synthesis (Redondo & Morris, 2011). A second form of long-term synaptic plasticity has also been found. This is known as long-term depression (LTD), and refers to a prolonged reduction in neuronal excitability which can be induced by low frequency stimulation (Bear & Abraham, 1996; Lynch, Dunwiddie, & Gribkoff, 1977). LTD has also been shown to play an important role in spatial processing; Ge and colleagues (2010) found that an LTD-blocking glutamate antagonist impaired memory consolidation in the water maze.

Whether synapses show LTP or LTD is thought to depend on the absolute post-synaptic change in Ca^{2+}. That is, strong activation of NMDA receptors leads to large increases in Ca^{2+} (yielding a post-synaptic response above a critical threshold) which triggers LTP, whereas slower, more modest activation of NMDA receptors results in smaller increases in Ca^{2+} (less than the critical value) which leads to LTD (Dudek & Bear, 1992). According to the Bienenstock–Cooper–Munro (BCM) theory of bidirectional synaptic plasticity, the induction thresholds for LTP and LTD are dynamically adjusted to the level of previous post-synaptic activity; a history of low activity will lower the threshold for LTP and increase the threshold for LTD, while the opposite holds for a history of high synaptic activity (Bienenstock, Cooper, & Munro, 1982; Karabanov et al., 2015; Lüscher & Malenka, 2012).

The majority of synapses use glutamate to induce rapid neuronal excitation, making it the primary excitatory neurotransmitter in the central nervous system (Collingridge et al., 2004; Lamprecht & LeDoux, 2004). Glutamate regulates

synaptic transmission via activation of ionotropic (ion channel coupled) and metabotropic glutamate receptors (second messenger coupled) (Kew & Kemp, 2005), which are found in pre- and post-synaptic membranes (Pinheiro & Mulle, 2008). There are three families of ionotropic glutamate receptors which are named after the selective agonists NMDA (N-methyl-d-aspartic acid), AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic acid) and kainate (Granger, Gray, Lu, & Nicoll, 2011) (see Figure 1.4).

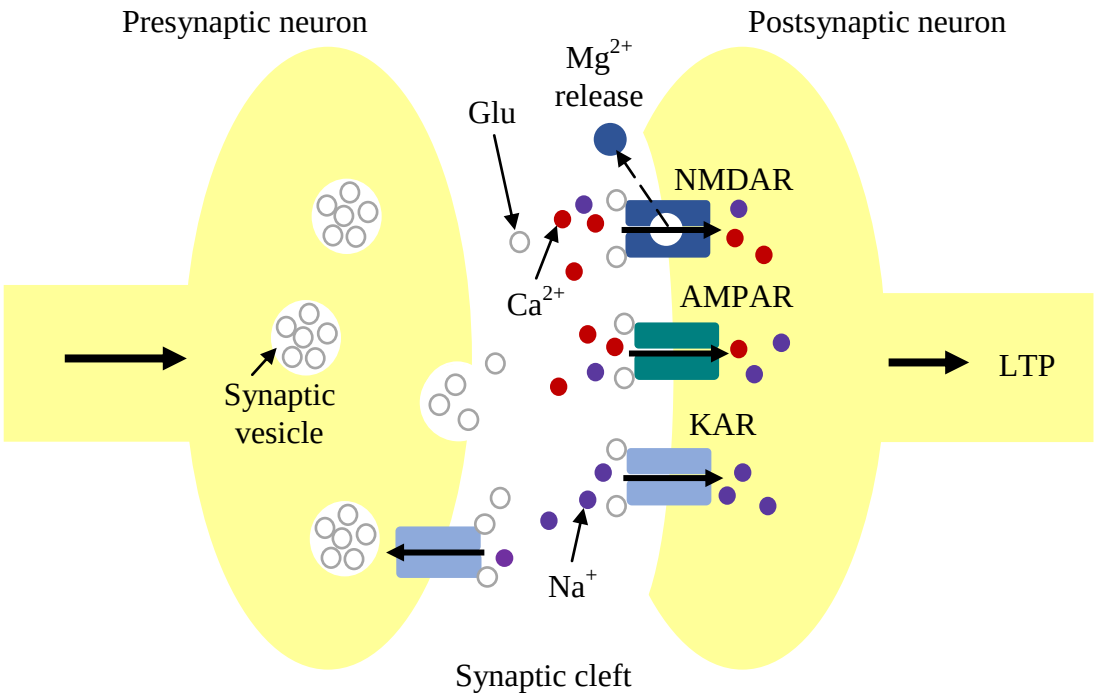

Figure 1.4: Schematic diagram of ionotropic glutamate receptor mediated synaptic plasticity. A series of impulses arrives at the presynaptic terminal which triggers the release of glutamate into the synaptic cleft (clear circles). Glutamate binds to receptors at the postsynaptic membrane. Activation of AMPA (AMPAR) and kainate receptors (KAR) causes an influx of sodium ions (Na^+; purple) which depolarises the membrane. Depolarisation leads to the release of magnesium ions (Mg^{2+}; red) blocking NMDA receptors (NMDAR). Once open, NMDA channels enable the influx of calcium ions (Ca^{2+}), which initiates long-term potentiation. Glutamate can also activate receptors located on the presynaptic terminal (autoreceptors), which modulate neurotransmitter release. Summarised from Voglis and Tavernarakis (2006), Lamprecht and LeDoux (2004), Pinheiro and Mulle (2008), and Engelman and MacDermott (2004).

Metabotropic glutamate receptors have also been classified into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR6-8), based on their pharmacology, sequence homology, G-protein coupling and specific associated second messenger systems (Conn & Pin, 1997; Pinheiro & Mulle, 2008). Broadly, ionotropic receptors mediate fast excitatory synaptic transmission, while metabotropic receptors modulate neuronal excitability (Pinheiro & Mulle, 2008; Schoepp, 2001).

1.5.1. NMDA receptors

NMDA receptors are composed of NR1, NR2 (NR2A-D) and, in some cases, NR3 subunits (NR3A and NR3B) (Madden, 2002). In order to become activated, NMDA receptors require two processes to occur. Firstly, glutamate must be released into the synapse from the presynaptic neuron and bind to the NMDA receptor; secondly, the postsynaptic neuron must be depolarised to remove the magnesium ion block in the NMDA receptor channel and allow for the influx of calcium (Malenka & Nicoll, 1999). This mode of action is unique to the NMDA receptor and reflects its role as a Hebbian 'coincidence detector', where neurons discriminate between correlated and uncorrelated synaptic inputs (Miyashita et al., 2012; Tsien, 2000).

NMDA receptors are considered to be the primary glutamatergic triggers for the induction of LTP and LTD (Bashir, Alford, Davies, Randall, & Collingridge, 1991; Christie & Abraham, 1992; Collingridge et al., 2004; Martin, Grimwood, & Morris, 2000; Peng et al., 2010; Thiels, Barrionuevo, & Berger, 1994), although NMDA receptor-independent LTP and LTD have been documented (Bortolotto et al., 1999; Johnston, Williams, Jaffe, & Gray, 1992; Wang, Rowan, & Anwyl, 1997). Importantly, the induction of LTP in the hippocampal-prefrontal pathway has been

shown to be NMDA receptor-dependent (Jay, Burette, & Laroche, 1995). NMDA receptor activation has also been directly implicated in learning and memory (see Levin, Buccafusco, & Rezvani, 2006; Gernot Riedel, Bettina Platt, & Jacques Micheau, 2003, for reviews).

A number of studies have shown that blockade of hippocampal NMDA receptor activation using selective antagonists (e.g. MK-801) leads to diminished LTP and impaired spatial learning and memory in rodents (Bannerman, Good, Butcher, Ramsay, & Morris, 1995; Lee & Kesner, 2002; Li, Matsumoto, Yamamoto, & Watanabe, 1997; Liang, Hon, Tyan, & Liao, 1994; Martin et al., 2000; Morris, Anderson, Lynch, & Baudry, 1986). MK-801 is a non-competitive antagonist which binds to the phencyclidine (PCP) binding site within activated NMDA receptor channels, thus preventing the flow of ions (Chahal, d'Souza, Barson & Slater, 1998; Foster & Wong, 1987). Evidence suggests that NMDA receptors may be particularly important for initial encoding. For example, Morris and colleagues (Morris, 1989; Morris, Davis, & Butcher, 1990) demonstrated that AP5 did not impair recall of a previously learned platform location in the allocentric water maze task. Interestingly, encoding deficits can be prevented by spatial pre-training prior to NMDA receptor blockade, despite the absence of LTP (Bannerman et al., 1995; Saucier & Cain, 1995).

In addition, it should be noted that antagonists such as MK-801 have a number of effects which are independent of synaptic plasticity. For example, acute injections of MK-801 into the rat prefrontal cortex decreases synchronization of action potential firing which is thought to result in disrupted information processing (Homayoun & Moghaddam, 2007; Molina, Skelin & Gruber, 2014). Further, administration of MK-801 (intraperitoneal and subcutaneous) increases basal gamma

band oscillations in rats, which is also considered to reflect cortical network dysfunction, e.g. in schizophrenia (Hiyoshi, Kambe, Karasawa, & Chaki, 2014; Pinault, 2008).

With regard to sub-regional NMDA receptor activation, Tsien, Huerta and Tonegawa (1996) showed LTP was absent in mice lacking the NR1 NMDA receptor sub-unit in CA1. These animals also exhibited poor acquisition of the allocentric water maze task, but were not impaired on a non-spatial version of the task. More specifically, CA1-KO mice successfully learned to find a submerged platform whose location was marked by a beacon, indicating that response memory was unaffected (Tsien et al., 1996). In contrast, Niewoehner *et al.* (2007) found that specific NR1 sub-unit deletion in the dentate gyrus had no effect on spatial performance in the water maze. Fellini *et al.* (2009) reported that selective inactivation of CA3 NMDA receptors in mice also had no effect on standard water maze performance, although animals were impaired on a pattern completion version of the task (i.e. when a sub-set of the distal cues were removed). In addition, Mei, Li, Gu, Cui and Tsien (2011) illustrated that knockout mice lacking NMDA receptors in CA1 or the entire hippocampus at the time of memory recall were not impaired in a spatial reference memory task under full or partial cue conditions. Collectively, these results indicate that NMDA receptors are crucial for encoding and/or consolidation of spatial memories, but not for retrieval (Martin et al., 2000; Mei et al., 2011; Nakazawa, McHugh, Wilson, & Tonegawa, 2004).

In relation to response memory, Mackes and Willner (2006) found that rats administered with the NMDA receptor antagonist MK-801 (subcutaneously) before water maze training were significantly less likely to use a place strategy during testing, instead relying on a response strategy. Similarly, Packard and Teather (1997;

1999) demonstrated that direct infusions of NMDA receptor antagonist AP5 into the caudate nucleus impaired memory in the visible platform water maze task, thus implicating this region in the use of response strategies. In a further study, Packard (1999) showed that post-training injections of glutamate could enhance place or response learning, depending on the site of injection (intrahippocampal or intracaudate). Specifically, rats that received intrahippocampal injections continued to rely on a place strategy after extended training, unlike saline-treated animals, who initially relied on a place strategy (day 8) but later switched to a response strategy (day 16). In contrast, rats administered with intracaudate injections displayed a response strategy after standard and extended training. Together, these results support the suggestion that the hippocampus and striatum are preferentially involved in place and response learning, respectively (Packard, 1999).

1.5.2. AMPA and kainate receptors

AMPA receptors consist of combinations of four sub-units (GluR1-4) (Hollmann & Heinemann, 1994). They exhibit extremely fast kinetics relative to NMDA receptors; that is, activation and deactivation occurs within milliseconds (Kleppe & Robinson, 1999). AMPA receptors require only glutamate binding to be activated and primarily conduct sodium and potassium (Gouaux, 2004). These receptors are thought to be responsible for fast excitatory synaptic signalling and modulation of synaptic strength (Nakazawa et al., 2004). Similar to NMDA receptors, AMPA and kainate receptors can also induce LTP (Castillo, Malenka, & Nicoll, 1997; Vignes & Collingridge, 1997; Yu, Wu, Liu, Ge, & Wang, 2008) and LTD (Chamberlain, Sadowski, Ruivo, Atherton, & Mellor, 2013; Holman, Feligioni, & Henley, 2007; Yu et al., 2008).

The GluR1 sub-unit has been shown to be particularly important for normal hippocampal LTP in rodents (Sanderson et al., 2008; Selcher, Xu, Hanson, Malenka, & Madison, 2012). In contrast, deletion of GluR4 has no effect on LTP in area CA1 (Sagata et al., 2010). Behaviourally, GluR1-deficient mice exhibit severe deficits in spatial working memory, while spatial reference memory is largely unaffected (Reisel et al., 2002; Sanderson et al., 2007; Schmitt et al., 2004; Schmitt, Deacon, Seeburg, Rawlins, & Bannerman, 2003; Zamanillo et al., 1999). Lee and colleagues (2003) did, however, report a reference memory deficit in GluR1 mice trained in the water maze. Specifically, mice successfully remembered the platform location when tested shortly after learning (2-4 hours), but were impaired at later time points (8 or 24 hours post-learning). Further, Bast, da Silva, and Morris (2005) found that hippocampal infusion of the AMPA receptor antagonist CNQX had no effect on acquisition of a one-trial allocentric place memory task, but resulted in poor retrieval. The authors found the opposite result in rats treated with NMDA receptor antagonist AP5. Therefore, it seems that both working and long-term term memory recall require fast excitatory transmission facilitated by AMPA receptors (Kessels & Malinow, 2009; Martin et al., 2000).

Like AMPA receptors, kainate receptors also mediate excitatory synaptic signals and are activated by glutamate binding (Nakazawa et al., 2004). Recently, pre-synaptic kainate receptors have been shown to play a role in modulating neurotransmitter release; specifically, these receptors act as ‘autoreceptors’ which can either facilitate or inhibit neurotransmission (Pinheiro & Mulle, 2008). Kainate receptors are composed of different combinations of five sub-units (KA1, KA2 and GluR5-7) (Wisden & Seeburg, 1993). GluR6 and GluR7 sub-units may be particularly important for the expression of LTP, as deletion of either sub-unit has

been shown to markedly impair its induction (Contractor, Swanson, & Heinemann, 2001; Lauri et al., 2001; Pinheiro et al., 2007). Although limited, existing evidence suggests that kainate receptors are involved in working memory processing (G. R. Barker et al., 2006), not unlike AMPA receptors.

1.6. Immediate Early Gene Imaging

The long-term structural cell changes which occur during late LTP are mediated, in part, by immediate early genes (IEGs) (Davis, Bozon, & Laroche, 2003). IEGs are rapidly and transiently expressed in response to neuronal activation and do not require protein synthesis to be induced (Sheng & Greenberg, 1990). The RNA transcripts of IEGs appear in the nucleus within minutes of neuronal activation and are subsequently transferred to the cytoplasm where – after 30-45 minutes – the protein products of these genes are translated (Guzowski et al., 1999; Murphy, MacKeigan, & Blenis, 2004). Their expression facilitates lasting cell modifications through encoding of transcription factors, cytoskeletal proteins, growth factors, metabolic enzymes and proteins involved in signal transduction (Lanahan & Worley, 1998). These long-term structural changes are, in turn, thought to underlie the maintenance of synaptic plasticity and the formation of long-term memories (Hughes & Dragunow, 1995; Lanahan & Worley, 1998; Tischmeyer & Grimm, 1999). IEGs are divided into two classes: regulatory transcription factor (RTF) IEGs, which encode proteins that increase or decrease downstream gene expression, or effector IEGs, which encode proteins that directly influence cell functions (Davis et al., 2003). There are approximately forty neuronal IEGs, of which 10-15 are classified as RTFs (Lanahan & Worley, 1998).

Because the expression of many IEGs is extremely low in quiescent cells, IEG imaging has become an increasingly popular method to investigate patterns of neuronal activation in response to a range of behavioural tasks (Aggleton, Brown, & Albasser, 2012; Barry & Commins, 2011; Kubik, Miyashita, & Guzowski, 2007). Using this technique, IEGs act as indirect markers of neuronal activation, where increased expression in particular brains areas is considered to reflect their involvement of in a given task (Aggleton & Brown, 2005). The primary advantage of this approach is that it allows for the visualisation of neuronal activity in multiple brain regions simultaneously, while preserving intact neural circuitry and functioning (Miyashita, Kubik, Lewandowski, & Guzowski, 2008). Accordingly, IEG imaging circumvents some of the problems faced by lesion studies. That is, lesions can impair functioning of nearby regions by disrupting input pathways, making any behavioural deficits difficult to interpret (Morris, 2007). In addition, IEG imaging provides excellent spatial resolution, i.e. down to an individual cell level. Further, because IEG imaging allows for the examination of multiple regions at the same time, patterns of coordinated activity across regions, reflective of wider brain networks, can be identified (Wheeler et al., 2013). Two of the most studied RTF IEGs are Zif268 and c-Fos, both of which have repeatedly been linked to LTP, LTD, learning and memory (Davis et al., 2003; Dragunow & Faull, 1989; Jones et al., 2001; Kovacs, 2008; Tischmeyer & Grimm, 1999; Worley & Shuler, 2014).

1.6.1. Zif268

Zif268 (also known as Egr-1, Krox-24, TZS8, NGFI-A and Zenk) is a member of the early growth response (Egr) family of genes (along with Egr-2, Egr-3 and Egr-4). Zif268 encodes a zinc finger protein and its expression is initiated by activation of

glutamatergic, dopaminergic, adrenergic and opiate receptors (Davis et al., 2003). Downstream target genes which are activated by Zif268 include Synapsin I and Synapsin II, which are thought to be important for controlling neurotransmitter release (Petersohn, Schoch, Brinkmann, & Thiel, 1995). Basal expression of Zif268 is highest in layers II and IV of the cerebral cortex, and in area CA1 of the hippocampus; expression is lower in areas CA2 and CA3, and negligible in the dentate gyrus (Schlingensiepen, Lüno, & Brysch, 1991). Zif268 is also found in the medial prefrontal, entorhinal, olfactory and cerebellar cortices, and in the striatum, amygdaloid nuclei and nucleus accumbens (Davis et al., 2003; Woolley et al., 2013).

Zif268 expression is tightly coupled with the induction of LTP. For example, Cole, Saffen, Baraban and Worley (1989) found that the frequency and intensity of neuronal stimulation needed to increase Zif268 mRNA levels were comparable to those required to induce LTP. Additionally, the authors demonstrated that both responses could be inhibited by administration of synaptic inhibitory inputs known to block LTP and by NMDA receptor antagonism. Gass, Herdegen, Bravo and Kiessling (1993) further emphasised the association between Zif268 and NMDA receptors, showing that MK-801 eradicates Zif268 expression in the cortex. Moreover, Jones *et al.* (2001) found that mutant mice lacking Zif268 failed to exhibit late phase LTP in the dentate gyrus (though the early phase was present). Together, these results indicate that Zif268 and LTP are regulated by similar synaptic mechanisms (Cole et al., 1989).

1.6.2. c-Fos

c-Fos is part of a group of transcription factors which comprises c-Fos, FosB and the Fos-related antigens 1 and 2 (Fra-1 and Fra-2) (Herdegen & Leah, 1998). Together

with the product of c-Jun, c-Fos forms a heterodimeric transcription factor complex which regulates gene expression by binding to the Activator Protein 1 (AP-1) recognition sequence found in various target genes (Fleischmann et al., 2003), although these genes have yet to be fully characterised. c-Fos is the most widely used marker of neuronal activation due to its low levels of basal expression throughout the brain (Dragunow, Currie, Faull, Robertson, & Jansen, 1989; Kovacs, 2008), especially in the rat hippocampus (Herdegen & Leah, 1998; Hughes, Lawlor, & Dragunow, 1992), which make it particularly suited to the detection of task-related neuronal activation. However, c-Fos also exhibits higher induction thresholds relative to other IEGs; therefore, it is thought to be a more useful marker of activation when task demands are high (Okuno, 2011). Like Zif268, c-Fos expression is induced by activation of glutamate receptors (Vaccarino, Hayward, Nestler, Duman, & Tallman, 1992), as well as hippocampal LTP (Nikolaev, Tischmeyer, Krug, Matthies, & Kaczmarek, 1991) and LTD (Kemp, Tischmeyer, & Manahan-Vaughan, 2013).

1.6.3. IEGs in learning and memory

In addition to their close association with LTP and LTD, Zif268 and c-Fos are highly expressed in response to a number of behavioural learning paradigms including odour discrimination (Hess, Lynch, & Gall, 1995; Magavi, Mitchell, Szentirmai, Carter, & Macklis, 2005), fear conditioning (Beck & Fibiger, 1995; Campeau et al., 1991; Hall, Thomas, & Everitt, 2001), object recognition (Albasser, Poirier, & Aggleton, 2010; Castilla-Ortega et al., 2012; Jones et al., 2001), paired associate learning (Tse et al., 2011) and long-term memory (Veyrac et al., 2015; Veyrac, Besnard, Caboche, Davis, & Laroche, 2014). In tests of spatial working memory,

elevated levels of Zif268 and c-Fos have been found in the hippocampus (particularly CA1 and CA3) and the medial prefrontal cortex (Mendez et al., 2008; Nagahara & Handa, 1995; Vann, Brown, Erichsen, & Aggleton, 2000). An additional study by He, Yamada and Nabeshima (2002) investigated c-Fos expression in rats at multiple time points during training (days one, three and five) in the radial arm maze. The authors found elevated expression in area CA3, the prelimbic and cingulate cortices on day three relative to control animals, but not on day five, suggesting that c-Fos may play a time-dependent role in memory encoding (He et al., 2002).

A number of studies have also analysed IEG expression during spatial reference memory (Gusev, Cui, Alkon, & Gubin, 2005; Guzowski, Setlow, Wagner, & McGaugh, 2001; Teather, Packard, Smith, Ellis-Behnke, & Bazan, 2005). Guzowski *et al.* (2001) reported that levels of Zif268 and c-Fos were significantly elevated from baseline in all regions of the dorsal hippocampus (and lateral entorhinal cortex) in rats trained in the allocentric water maze task for three days. Further, IEG expression was found to be highest early on in training, again indicating that learned-related increases in IEG activation are time-dependent. Teather and colleagues (2005) examined hippocampal c-Fos expression after a single day of training in the water maze, during which rats acquired either the hidden (spatial) or visible (cued) platform version. Results revealed increased c-Fos expression in area CA1 in the spatially trained group relative to rats trained in the cued task, as well as swim-yoked controls and naïve animals. These findings are in keeping with the idea that CA1 is particularly important for the formation of complex spatial representations (Teather et al., 2005).

In addition, genetic knockout studies have emphasised the functional role of IEGs in spatial learning and memory. Jones and colleagues (2001) demonstrated that mice lacking Zif268 displayed intact short-term memory but impaired long-term memory on both spatial and non-spatial tasks, indicating that Zif268 is crucial for memory consolidation. Subsequently, Bozon, Davis and Laroche (2002) illustrated that spatial learning is particularly sensitive to Zif268 activation. Specifically, they noted that a reduction in Zif268 mRNA levels (to approximately half that of wild-type mice) was sufficient to impede spatial learning; on the other hand, mutant mice were only somewhat impaired in a conditioned taste aversion task, and exhibited no deficits in a novel object recognition task (Bozon et al., 2002). Zif268 gene deletion has also been shown to weaken the long-term stability of newly formed hippocampal place fields, although they can be rescued by repeated exposure to the environment (Renaudineau, Poucet, Laroche, Davis, & Save, 2009). Together, these results indicate that Zif268 activation constitutes a crucial mechanism for the initial encoding of long-lasting spatial memories. Unlike Zif268, however, c-Fos deletion in mice has produced mixed results. For example, Zhang, McQuade, Vorhees and Xu (2002) found that mutant mice exhibited normal spatial learning in the water maze, whereas Fleishmann *et al.* (2003) reported deficits in spatial learning which correlated with a reduction in LTP in hippocampal CA3-CA1 synapses. Thus, the significance of c-Fos for spatial memory processing is less well defined at present.

1.7. Objectives of this thesis

The primary objectives of this thesis are to conduct an in-depth investigation into the use of allocentric navigation strategies during memory encoding and retrieval, and to characterise the specific contributions of the hippocampus and medial prefrontal

cortex to these processes. The popular Morris water maze will be employed as the behavioural task to measure spatial learning and memory. This task is particularly suitable due to its ambiguity, which facilitates the use of multiple types of strategies. A number of dorsal hippocampal and medial prefrontal sub-regions will be assessed, all of which have been implicated in spatial information processing to greater or lesser degrees. These will include CA1, CA3, dentate gyrus, prelimbic, anterior cingulate and infralimbic cortices. The mechanisms underlying neuronal activation in these areas will be examined by inhibiting different types of ionotropic glutamate receptors; namely, NMDA and AMPA receptors. Brain activity will be measured using IEG imaging of Zif268 and c-Fos protein, due to their known role in glutamate-dependent LTP and LTD, learning and memory.

Despite being one of the most widely used tasks of spatial learning and memory (Vorhees & Williams, 2014), the type of spatial information animals encode and ultimately rely on to navigate in the Morris water maze remains unclear. Therefore, we will first examine spatial strategy use in the maze, with a particular focus on two understudied, yet important, influencing factors. These are cue salience (i.e. what makes some cues more useful for finding the goal than others?) and environmental experience (i.e. does increased training lead to a change in the strategy used?). We hypothesise that when two cues are equally salient, animals will encode both into their navigation strategy; however, when one is markedly more useful than the other, rats will learn to rely on the more useful of the two. In addition, we predict that a proximal cue will acquire a higher salience than a distal cue, and a brighter cue will become more salient than a less luminous cue. Further, when one cue is closer to the target and the other is brighter, we expect the cues to compete for control over behaviour. Finally, we anticipate that increased training in the

environment will result in more flexible navigation, whereby animals can rely on any or all cues.

Following this behavioural examination, we will investigate the role of specific hippocampal and medial prefrontal sub-regions in allocentric spatial encoding in the water maze using Zif268 and c-Fos as markers of neuronal activation. We predict activation will be highest in area CA1 of the hippocampus, with heightened expression also in the anterior cingulate cortex relative to caged controls. In addition, we will assess the importance of NMDA and AMPA receptors for spatial learning, and for IEG expression in these brain regions. Importantly, few studies to date have examined the effects of glutamate receptor blockade on basal IEG expression, making any post-learning changes in IEG expression levels difficult to interpret. Thus, we will first measure dose-dependent effects of NMDA channel blocker MK-801 and AMPA receptor antagonist CNQX on baseline hippocampal and prefrontal IEG expression. We hypothesise that higher drug concentrations will lead to significant changes in IEG expression, while lower doses will have little or no effect. The impact of glutamate receptor inhibition on spatial learning will then be assessed. We expect NMDA receptor inhibition to impair task acquisition and attenuate IEG expression, and blockade of AMPA receptors to have little or no effect on learning or IEG activation.

Lastly, we will evaluate the role of hippocampal and medial prefrontal sub-regions during memory recall. Specifically, we will assess the use of spatial (distal cue) and non-spatial (beacon) strategies in the water maze and their associated brain regions following standard or extended training. We hypothesise that increased environmental experience will lead to better performance in spatially-trained rats, but have no impact on beacon-trained animals. Further, we expect greater

hippocampal IEG expression in the spatial group relative to the non-spatial group, and higher activation in CA3 and the prefrontal cortex in rats tested under partial cue conditions (i.e. when one of the two training cues is removed). Finally, we will delineate the importance of NMDA receptor activation for spatial and non-spatial memory retrieval via post-training injections of MK-801. Importantly, it has yet to be established if enhanced training can protect against the effects of NMDA receptor blockade. Rats will therefore be trained for standard or extended periods of time prior to MK-801 administration. We anticipate gross memory deficits and reduced IEG expression in all groups after standard training, but preserved memory following greater environmental experience.

Chapter 2

General Methods

2.1. Subjects

Male Wistar rats obtained from Charles River, UK, were used as subjects in this thesis. Animals were approximately three months old and weighed 250-300g at the beginning of all experiments. All rats were given a number with a non-toxic marker pen for identification purposes and housed three per cage in plastic-bottomed cages (56 x 38cm and 22cm high; NKP Cages, UK) with a 3cm layer of woodchip bedding, paper strip nesting material and cardboard tubes. All cages were cleaned out once a week. All rats had access *ad libitum* to water and food pellets and were maintained under a 12:12 hour light:dark cycle (lights on at 07:00h) at a fixed temperature of 21°C. All experimentation was conducted during the light phase. All rats were experimentally naïve and were well handled for one week prior to the onset of each experiment.

2.2. Morris water maze apparatus

The Morris water maze was employed as the spatial navigation task for all experiments. The Morris water maze task is widely known as a simple and effective measure of spatial learning and memory (Terry, 2009), and has been used previously in our laboratory (Harvey et al., 2008). The maze consisted of a black, circular fibreglass pool (170cm diameter, 35cm deep) resting 70cm above floor level on a metal support frame. The pool was filled with opaque water to a depth of 20cm and maintained at 21±1°C. A black concrete escape platform (13cm diameter, 13.5cm width) was placed in the centre of the northeast quadrant of the pool (25cm from the edge of the pool wall) for all training trials. The pool-to-platform area ratio was 171:1, and thus, was well-within the optimal range of task difficulty for rats (Vorhees & Williams, 2014). The platform rested 2cm below the water surface,

ensuring that rats could not see it when navigating in the maze. The maze was surrounded by a black curtain suspended from ceiling to floor at a distance of 60cm from the pool wall which provided a uniform background and prevented access to room cues.

Visual, distal cues located in fixed positions around the maze were used to guide the rats to the platform. Cues were fluorescent, inside-frosted, low energy Philips glass light bulbs which were suspended from the ceiling inside the curtain. The number, spatial position and brightness of the cues varied according to the experimental condition. Two cues, positioned northeast (NE; distance of 127cm, height angle of 42°; near cue) and northwest (NW; distance of 162cm, height angle of 25°; far cue) of the platform, or a single beacon positioned 50cm directly above the platform (Chamizo & Rodrigo, 2004), were used (see Figure 2.1). Cues were either 25 or 40 Watt brightness intensity. For all experiments, rats were trained and tested in complete darkness (i.e. the cues were the only light source) to ensure that they learned to navigate using these cues. To minimise distraction for the animals (e.g. noise), all trials were observed by the experimenter in an adjacent testing room via a video camera positioned directly above the centre of the maze. Behavioural data of the animals' movements were recorded using EthoVision© tracking system (Noldus Information Technologies, Wageningen, Netherlands).

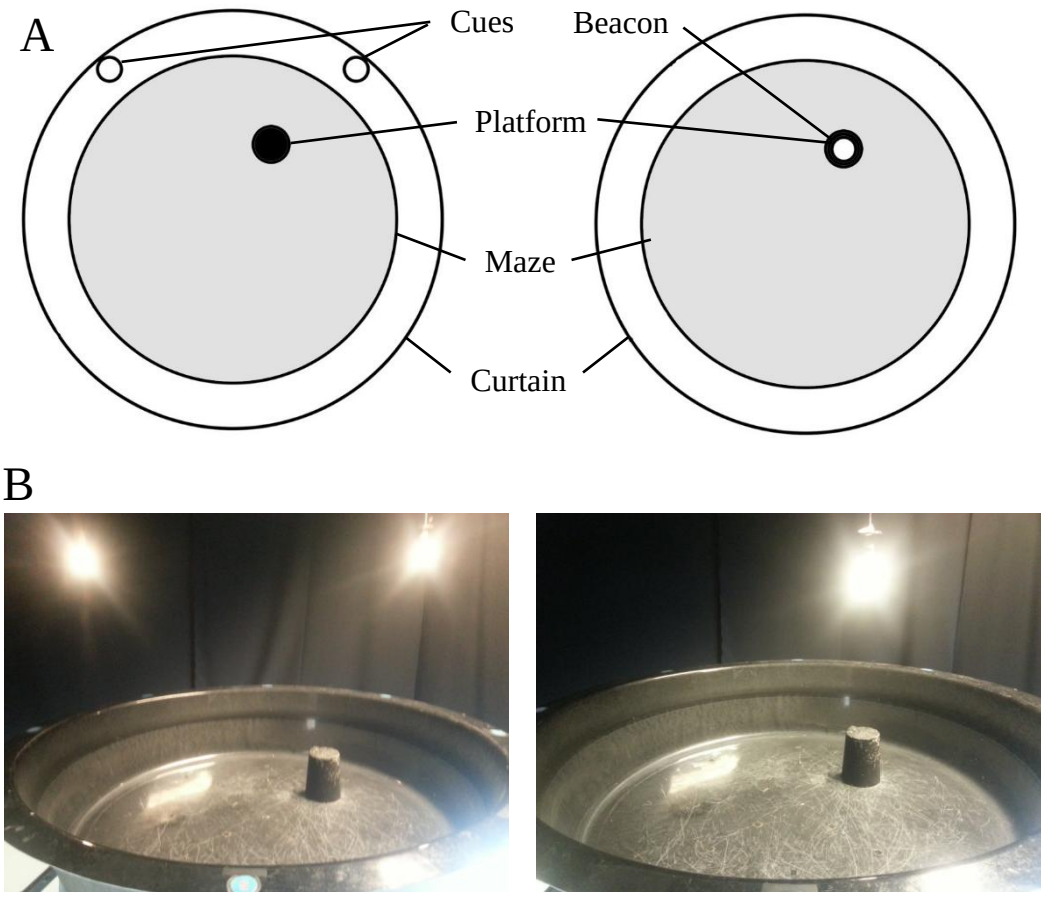

Figure 2.1: (A). Schematic diagram of a birds-eye view of the Morris water maze used in this thesis showing spatial positions of the distal cues and beacon. (B). Photographs of the maze with the platform in cue and beacon conditions.

2.3. Morris water maze procedure

2.3.1. Acquisition.

Acquisition training was based on previous procedures from our laboratory (Harvey et al., 2009). Rats were trained for up to ten days in the presence of two cues or a single beacon (depending on the experimental condition), which remained available throughout the training period. Training on each day consisted of four trials. For each trial, rats were placed into the pool near to and facing the pool wall from one of four pseudo-randomised directional starting positions (north, south, east or west). The time taken to reach the platform was recorded. Rats were allowed a maximum of

sixty seconds to find the platform (located in the NE quadrant). If they failed to locate the platform within this time, rats were guided there by the experimenter. Once on the platform, rats remained there for fifteen seconds after which they were removed from the maze and placed into an open-topped container for an inter-trial interval of at least ten seconds. Rats were placed back into the pool from a different starting position for the next trial. When all four trials had been completed, rats were returned to their home cage. Successful acquisition of the task was determined by a statistically significant decrease in time taken to escape the maze across training days.

2.3.2. Recall.

Where appropriate, water maze recall was examined. The procedure for assessing recall followed previously used protocols from our laboratory (McGauran, Harvey, Cunningham, Craig, & Commins, 2004). For all experiments, recall was assessed 24 hours after the final day of training. During recall, rats' memory for the platform location was tested in a single probe trial with the platform removed. Depending on the experimental condition, rats were tested in the presence of two distal cues, one distal cue or a single beacon. The position of the cues during testing also varied across conditions. For each probe trial, rats were placed into the pool near to and facing the pool wall from a novel start position and allowed to swim freely for sixty seconds. Start positions for each condition were chosen based on existing water maze protocols, where animals are released from the quadrant opposite to where the platform had been located (Steffenach, Witter, Moser, & Moser, 2005). After this time had elapsed, rats were removed from the maze and returned to their home cages. Successful recall was measured by examining the amount of time spent

swimming in the NE quadrant and platform area during the probe trial, relative to other quadrants and platform areas of the pool. As a visual representation of where animals' searched during the probe trial, heatmaps displaying swim distributions were generated using MATLAB (R2012b). Swim distributions for animals in each group were processed together, resulting in one heatmap per group.

2.4. Drug administration

Where applicable, animals were administered with glutamate receptor antagonists to examine their effects on spatial navigation and IEG expression. Rats were given intraperitoneal (i.p.) injections of the NMDA channel blocker MK-801 (0.05mg/kg or 0.1mg/kg body weight; Sigma-Aldrich) or the AMPA receptor antagonist CNQX (0.75mg/kg or 1.5mg/kg body weight; Tocris Bioscience). Sterile saline was used as the vehicle for all drugs (0.3ml total volume per injection). Depending on the experiment, injections were administered 20-30 minutes before training or testing, as per previous studies (de Lima, Laranja, Bromberg, Roesler, & Schroder, 2005). Selected doses were based on preceding research in our laboratory and in the wider literature where i.p. injections at the same concentrations led to a significant change in behaviour, but had no sensorimotor effects (Kealy & Commins, 2009; Murschall & Hauber, 2005; van der Staay, Rutten, Erb, & Blokland, 2011). Separate groups of animals were treated with physiological saline (0.1 ml/100 g body weight of 0.9 % NaCl; Sigma, Ireland) as a control, to ensure comparative stress levels across groups (related to receiving injections). All drugs were made up fresh for each experiment and dose-sized aliquots were frozen for daily use.

2.5. Tissue preservation

Animals were sacrificed ninety minutes after the final acquisition or recall trial, as IEGs are thought to be maximally expressed at this time point (Zangenehpour & Chaudhuri, 2002). Rats were terminally anaesthetised via i.p. injection with sodium pentobarbital (60mg/kg, Euthatal). Rats were then perfused transcardially with 0.9% phosphate buffered saline (PBS, 250ml, Ph 7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, 300ml, Ph 7.4). Brains were immediately removed and post-fixed in 4% paraformaldehyde overnight at 4°C before being cryoprotected in 30% sucrose solution. Brains were then frozen on dry ice and cut into 40-µm-thick coronal sections using a freezing stage sledge microtome (Brights Instruments, Huntingdon, UK). Free floating sections were stored in 0.1M PB containing 0.01% sodium azide (4°C).

2.6. Immunohistochemistry

Standard immunohistochemical staining methods were followed (Coogan & Piggins, 2003). Specificity of this staining procedure for Zif268 and c-Fos was confirmed previously in our laboratory (Barry, 2013). Sections were washed twice in 0.1M PB (ten minutes each), followed by a ten minute wash in 0.1M PB containing 0.2% Triton-X-100 (PBX). Sections were then washed in 0.1M PB with 1.5% hydrogen peroxide for twenty minutes. Two more ten minute washes in 0.1M PB and one in PBX followed. Subsequently, sections were blocked in 5% normal goat serum (NGS) in 0.1M PBX for sixty minutes at room temperature, and then incubated for 24 hours in a primary antibody solution (2% NGS in 0.1M PBX). Zif268 and c-Fos were labelled using the following antibodies: Zif268/Egr-1, rabbit polyclonal

antibody (dilution 1:3000; Santa Cruz Biotechnology), and c-Fos, rabbit polyclonal antibody (dilution 1:2000; Santa Cruz Biotechnology).

Post-incubation, sections were given two washes in 0.1M PB and one in PBX. Sections were then incubated with biotinylated secondary antibody (goat anti-rabbit, Jackson Laboratories, dilution 1:400) for seventy minutes. Two more washes in 0.1M PB and one in 0.1M PBX followed, after which sections were incubated with avidin-biotin-peroxidase complex (0.4%; Vector Laboratories) for ninety minutes in complete darkness at room temperature. Sections were again washed twice more in PB and once in 0.1M sodium acetate (Ph 6). The reaction product was visualised using the nickel-DAB technique with glucose oxidase (Sigma, Poole, UK) as the catalyst. The length of reaction time was standardised for all sections to ensure comparable staining intensity across sections. To further minimise variation in staining specificity, sections were stained in group cohorts where possible, with one animal from each group being processed side-by-side in the same well plate. Finally, sections were mounted onto gelatin-coated slides, dehydrated, cleared in Histoclear (National Diagnostics, Hull, UK), and coverslipped using Eukitt (Sigma, Poole, UK).

2.7. Regions of interest

Six areas were chosen for analysis including three regions from the dorsal hippocampus; CA1, CA3 and the dentate gyrus (DG), and three medial prefrontal regions; the prelimbic (PLC) anterior cingulate (ACC), and infralimbic cortices (ILC). All regions are illustrated on coronal sections in Figure 2.2 (adapted from Barry, 2013; Paxinos & Watson, 2007). These regions, their coordinates, and the number of sections sampled per IEG are displayed in Table 2.1. Dorsal hippocampal

sections were obtained as close as possible to AP level -3.24mm from Bregma. Medial prefrontal regions were attained as near to AP +3.72mm from Bregma as possible.

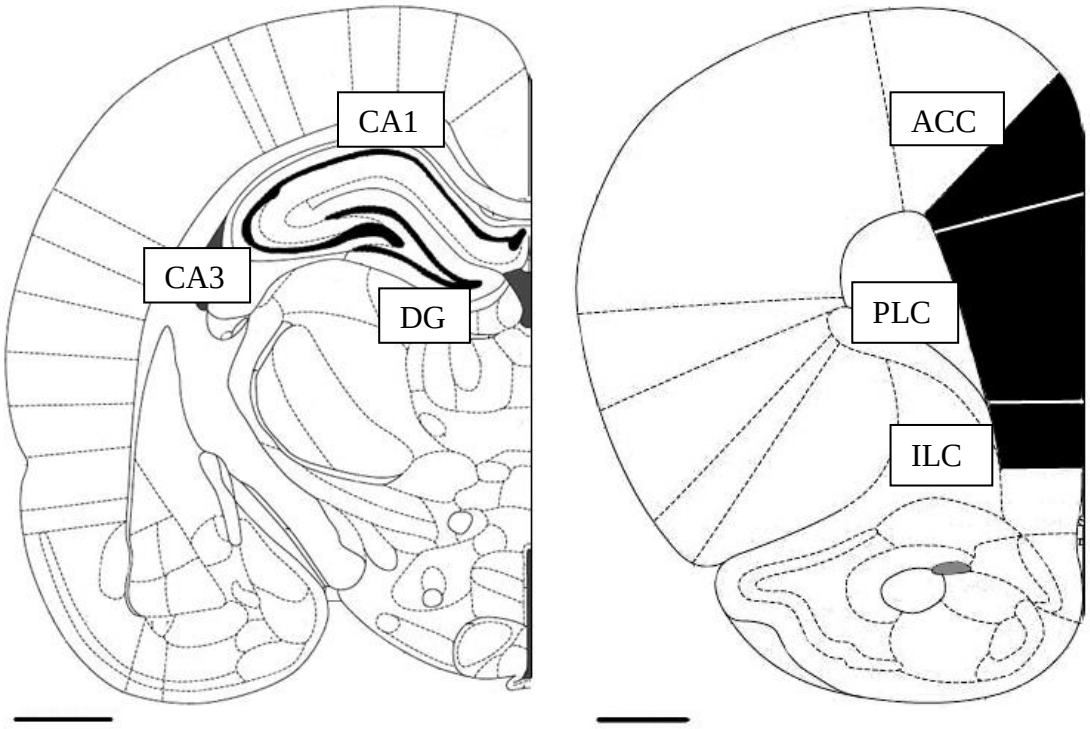

Figure 2.2: Coronal diagrams of the regions of interest in (A) CA1, CA3 and DG (the dentate gyrus), and (B) PLC (prelimbic cortex), ACC (anterior cingulate cortex), and ILC (infralimbic cortex). Modified from Barry (2013), and Paxinos and Watson (2007). Scale bar = 1mm.

Table 2.1: Coordinates of selected regions and numbers of sections for each region.

Brain region	Distance from Bregma		Number of sections
	Start	End	
CA1	-3.24mm	-4.08 mm	4
CA3	-3.24mm	-4.08 mm	4
DG	-3.24mm	-4.08 mm	4
PLC	+3.72mm	+2.76mm	4
ACC	+3.72mm	+2.76mm	4
ILC	+3.72mm	+2.76mm	4

2.8. IEG quantification

Images of the six regions were taken using an Olympus digital camera (Camedia C-2020-Z) mounted on an Olympus BX-50 microscope. To capture the maximum number of cells possible, all images were taken using a 4x magnification. For sub-regions of the medial prefrontal cortex, the sampled area was larger than the area under investigation; therefore, novel acetate coronal masks developed by Barry (2013) were placed over the section to obscure all adjacent regions during image acquisition (see Figure 2.3 for sample masks). For hippocampal regions, the images were manually cropped following acquisition. For all regions analysed, IEG cell counts were obtained from four sections per animal.

To eliminate experimenter bias in the cell counting process, counts were automatically calculated by ImageJ digitizing software (National Institute of Health, USA). In order for the software to distinguish active cells from inactive background tissue, a number of detection thresholds were used. These included brightness intensity (set between 70 and 100, depending on the experiment) and particle size

(20 to 200 pixel range). Detection thresholds for each IEG remained constant for all regions in a given experiment. Counts from each animal (from four sections) were averaged to produce a mean. Mean counts for individual rats in each group were then averaged to produce group means. Unless otherwise stated, raw counts were used for statistical analyses.

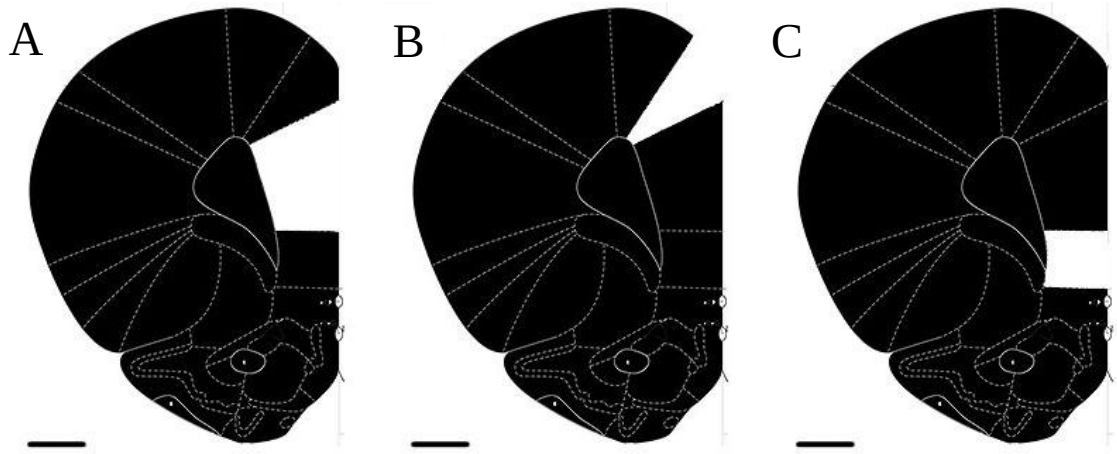

Figure 2.3: Coronal masks used to obscure surrounding tissue during image acquisition of the PLC, ACC and ILC (A-C). Reproduced from Barry (2013). Scale bar = 1mm.

2.9. Statistical analysis

All statistical analyses were carried out using SPSS (Version 22). Wherever possible, parametric inferential tests were conducted to test for differences between groups and conditions. One-way between groups, repeated measures or mixed factorial analysis of variance (ANOVA) were used, with appropriate Tukey and/or Bonferroni *post hoc* tests (at the 5% level of significance). Independent and dependent t-tests were also used to assess differences between conditions, where applicable. In cases of violations of normality, sphericity or homoscedasticity, non-parametric equivalents of the corresponding ANOVAs and *post hoc* tests were conducted.

Bivariate correlations were calculated using the Pearson product-moment correlation coefficient. Error bars on graphs depict standard error of the mean (S.E.M.). Statistical significance was indicated using an asterisk-based system representing p-values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) throughout.

2.10. Ethical considerations

All experimental work was approved by Maynooth University ethics committee and conformed to the Department of Health and Children (Ireland) and HPRA (Health Products Regulatory Authority) guidelines for the maintenance and experimentation of animals under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. Every effort was made to minimise the suffering of animals used in this thesis.

Chapter 3

An In-depth Behavioural Investigation of Cue Salience and Training Length in Allocentric Spatial Strategy Use

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Abstract

Most motile animals use environmental cues to aid navigation. Animals can learn to associate cues with a goal destination in one of two ways: individually (elemental learning) or in groups (configural learning). A number of factors are thought to influence the way in which these associations are formed. One such important factor is the salience (or noticeability) of the available cues. Despite its significance, however, few studies have examined the role of cue salience in allocentric strategy use to date. Here, we explored the influence of cue salience in the acquisition and recall of Morris water maze task. Experiments 1 and 2 investigated two salience factors: proximity to the goal and brightness. Results showed that a bright cue acquired more control over behaviour than a weaker cue, regardless of relative proximity to the platform and training length. Animals in Experiment 3 were trained with equally bright proximal and distal cues. Unexpectedly, probe tests revealed that rats tested with the farther cue outperformed those tested with the proximal cue – but only after extended training. Experiment 4 aimed to verify that animals were relying on the cues to navigate. Animals were trained with a bright (distal) cue and a proximal cue and tested with one or both cues in novel positions. Results demonstrated that rats did in fact modify their searching behaviour according to the spatial location of the cues. Overall, these findings point towards the use of an elemental learning strategy involving the more salient of two available cues, which emerges earlier when the relative saliences of the cues differ considerably.

3.1. Introduction

The importance of environmental cues for successful navigation is well-documented in many species (for reviews see Rodrigo, 2002; Tommasi, Chiandetti, Pecchia, Sovrano, & Vallortigara, 2012). According to associative theories of spatial learning, navigating animals form representations of cues from a collection of viewpoints, which then become associated with a goal destination (Hamilton et al., 2002; Honey et al., 2014; Leonard & McNaughton, 1990). These associations are thought to be created in one of two ways; elementally or configurally (Siegel & White, 1975; Sutherland & Rudy, 1989). Elemental learning strategies (e.g. Miller & Shettleworth, 2007; Rescorla & Wagner, 1972) occur where the animal forms direct associations between each cue and the destination separately (Pearce, 2002). When navigating to the goal, the animal must therefore identify the cues and remember their discrete spatial relationships to that location. Configural learning strategies (e.g. Rescorla, Durlach, & Grau, 1985; Rudy & Sutherland, 1995) involve the association of a group of cues with the destination, where a novel configural representation (independent of the individual cue components) is generated (Honey et al., 2014; Pearce, 2002). Here, the animal is required to remember the position of the goal relative to the complete configuration.

Research in various species has attempted to discriminate between configural and elemental strategies by altering the arrangement of cues between navigational training and testing phases. Using this approach, evidence for elemental strategy use has been found in children and non-human primates (MacDonald, Spetch, Kelly, & Cheng, 2004), gerbils (Collett, Cartwright, & Smith, 1986) and pigeons (Spetch, Cheng, & MacDonald, 1996). Specifically, results illustrated that, when trained to locate a goal in the centre of a fixed array of cues and tested with the distance

between these cues increased, animals tended to search for the goal at the absolute distance and direction from individual cues rather than at the relative midpoint of the configuration. Moreover, Collett and colleagues (1986) showed that when one of the two trained cues was removed, gerbils searched in two distinct locations which corresponded to the distances and directions from each cue to the target during training. On the other hand, adult humans (Spetch et al., 1996; Spetch et al., 1997) and honeybees (Cartwright & Collett, 1982) have been known to search in the same relative location during testing as in training; for example, if trained to navigate to the centre of a cue arrangement, they continue to search in the centre of the expanded array, suggesting a configural strategy. More interestingly, the use of both strategies has been documented in Clark's Nutcracker birds (Kamil & Jones, 1997, 2000), indicating that configural and elemental learning may not be mutually exclusive. Rather, the use of a particular strategy may be influenced by the nature of the cues available to the animal in a given scenario.

Cue salience arguably plays a vital role in determining the type of learning strategy an animal will use, although it has not yet been studied to any great extent in the spatial domain (Rodrigo, Gimeno, Ayguasanosa, & Chamizo, 2014). The term salience can be defined as the "significance or noticeability" of a cue (Chamizo, Rodrigo, Peris, & Grau, 2006, p. 340). There are a number of factors which can influence cue salience (Domjan, Grau, & Krause, 2010). One such well-established factor is the distance of a cue from the goal location, whereby proximal cues acquire more control over navigation (i.e. become more salient) than distal cues (Artigas, Aznar-Casanova, & Chamizo, 2005; Chamizo, 2002; Chamizo & Rodrigo, 2004; Cheng, Collett, Pickhard, & Wehner, 1987; Redhead & Hamilton, 2007; Spetch & Wilkie, 1994). For example, Chamizo and Rodrigo (2004) showed that rats

navigating in the water maze performed better when a single available cue was located near to the platform (on the same side of the pool) than when it was positioned far from the platform (on the opposite side of the pool). Further, performance was best when the cue was suspended directly above the platform, thus revealing the target location. This effect is thought to occur because proximal cues offer the most precise spatial information about the location of the goal (Spetch, 1995). That is, estimates of the distance and direction in which to travel are more variable for distant cues and, thus, more prone to error (Kamil & Cheng, 2001; Spetch, 1995). Specific features of a cue (e.g. size or luminance) have also been shown to effect salience (Chamizo, Rodrigo, Peris, et al., 2006; Chamizo, Rodriguez, Espinet, & Mackintosh, 2012; Young, Choleris, & Kirkland, 2006). Chamizo and colleagues (2006), for example, demonstrated that rats navigating in the Morris water maze with a bright distal cue performed as well as those navigating with a less luminous proximal cue.

Recently, Rodrigo and colleagues (2014) examined the effects of varying the salience of a cue configuration on the type of strategy employed by rats in the Morris water maze. Cues ranged from having approximately the same salience to having different saliences across conditions. Probe trials revealed that rats could adopt different spatial strategies, depending on the similarity of the cues' saliences (Rodrigo et al., 2014). Namely, when the salience was comparable, rats relied on the arrangement of cues (i.e. a configural strategy), and when salience was dissimilar, they used an elemental strategy involving the more salient of the two cues to reach the platform (Rodrigo et al., 2014). Notably, Rodrigo et al. (2014) suggest that the emergence of these distinct strategies may be somewhat dependent on a prolonged training period. Although this idea has not yet been thoroughly examined in a spatial

learning context, visual discrimination research in honeybees has demonstrated that extended training can in fact produce a change in the chosen strategy, from elemental to configural (Giurfa, Schubert, Reisenman, Gerber, & Lachnit, 2003). Giurfa and colleagues (2003) also showed that, at longer training lengths, perceptual similarity between cues promoted a configural learning approach.

This chapter aimed to expand on previous work in two ways; firstly, by further exploring the effects of altering cue salience on spatial learning strategies used in the Morris water maze, and secondly, by delineating the influence of training length on the type of strategy used. Four experiments were conducted. Experiment 1 examined two components of cue salience; distance from the goal and brightness. Rats were trained with a proximal (near) cue and a bright distal (far) cue for five or ten days, and subsequently tested with both or one of these cues. We hypothesised that if one cue acquired more salience than the other, rats would initially adopt an elemental strategy with the high salience cue; however, if both cues became equally salient, rats should readily incorporate both into a configural strategy after only five days of training.

Experiments 2 and 3 examined animals' learning behaviour in the presence of two cues with more distinct saliences. In Experiment 2, rats were trained with the original positions of the cues reversed. Here, as one cue was both brighter and closer to the goal, we expected rats to employ an elemental strategy with this cue. In Experiment 3, rats were trained with equally bright near and far cues. We predicted that rats would favour an elemental strategy involving the proximal cue to begin with, but after further training, may incorporate the farther cue into a configural strategy (similar to Giurfa et al., 2003). Finally, Experiment 4 aimed to verify that rats were in fact navigating via the distal cues, as opposed to unknown room cues.

Animals were trained with a near and bright cue, followed by testing with two cues or a single cue in a novel position. We hypothesised that rats would modify their searching behaviour according to the new locations of the cues.

3.2. Experiment 1

Experiment 1 had three goals: (1) to establish which component of cue salience (proximity or brightness), if any, acquired more control over navigation; (2) to identify the type of learning strategy rats were using (elemental or configural); and (3) to determine if increased training could lead to a change in strategy.

3.2.1. Method

3.2.1.1. Subjects.

Male Wistar rats (*n* = 39) obtained from Charles River, UK, were used as subjects. Rats' age and weight, housing conditions, handling, and time of experimentation were as described previously in Chapter 2.

3.2.1.2. Apparatus.

The water maze was used as the behavioural task in this experiment. Maze dimensions, position of the distal cues and platform location were as described in Chapter 2. For this experiment, two cues of unequal brightness were used; one cue was a 25 Watt light bulb (190 lumen light output; NE position; near cue) and the other was a 40 Watt light bulb (370 lumen light output; NW position; far cue).

3.2.1.3. Procedure.

Rats were randomly assigned to one of six experimental groups. Three of these groups (*n* = 21) were trained in the water maze for a total of five days (totalling 20 training trials) and the remaining three were trained for ten days (*n* = 18; 40 training trials). All training was carried out in the presence of both cues with a fixed hidden platform in the NE quadrant, followed by 24-hour recall without the platform, as

outlined in Chapter 2. Rats trained for five days were divided into a Control group, a Near group and a Bright group (n = 7 per group). Rats trained for ten days were also separated into Control, Near and Bright groups (n = 6 per group). The Control groups were tested with both the near (NE) and bright (NW) cues present, as per training (see Figure 3.1). The Near groups were tested with the near (NE) cue only, and the Bright groups were tested with the bright (NW) cue only (see Figure 3.1). For groups tested with a single cue, the alternate cue was removed from view by switching the light off and moving it outside of the curtain. During the probe trial, all rats were placed into the pool from the centre of the SW quadrant at the pool wall. The SW quadrant was chosen as a novel start position because it was the quadrant opposite to where the platform had been located, i.e. the NE quadrant.

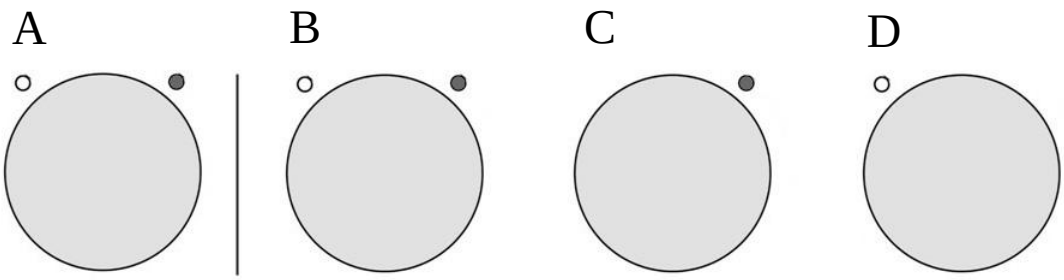

Figure 3.1: Representation of cue configuration during (A) training for all animals, and cue configuration during testing for (B) Control, (C) Near and (D) Bright groups during testing (A-C) with 25 Watt (closed circle) and 40 Watt bulbs (open circle).

3.2.1.4. Data analysis.

Task acquisition was quantified by escape latency (seconds) and distance travelled (centimetres): two measures which are widely used in the assessment of water maze learning (Terry, 2009). Values for each trial were calculated and averaged for each rat, which were then averaged to produce group means. To examine swimming behaviour during the recall trial, the maze was divided into a number of zones (see Figure 3.2). First, the maze was divided into four quadrants (NE, NW, SE and SW),

and mean percentage time (of sixty seconds) spent in each of these quadrants was recorded. Next, percentage time spent in four platform areas was assessed. Platform areas were defined as the circular areas surrounding the NE platform location and equivalent areas in the other three quadrants (NW, SE and SW; all 18cm diameter). These areas were included in the analyses as a more refined measure of rats' searching behaviour during the probe trial (Hoz, Martin, & Morris, 2004). Finally, thigmotactic behaviour – defined as a tendency to remain close to the perimeter of an environment (Treit & Fundytus, 1988) – was investigated as a general measure of anxiety by evaluating percentage time spent in an area defined to as the outer corridor (16cm width).

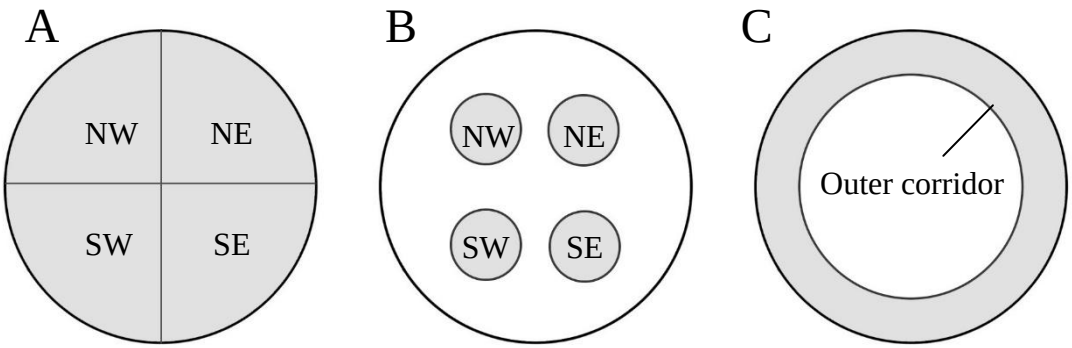

Figure 3.2: Zones of the maze used for recall including (A) quadrants, (B) platform areas and (C) the outer corridor.

3.2.1.5. Statistical analysis.

As all animals received identical training (with both cues), rats were first assessed a single group for acquisition analyses using one-way repeated-measures ANOVAs (within-groups factor: training day; days 1 to 5 or days 1 to 10, depending on the training length), with Bonferroni *post hoc* tests where appropriate. Rats were then assessed in their respective retention groups for acquisition analyses to ensure that they did not differ behaviourally at this stage using mixed factorial ANOVAs with

group as the between-groups factor (Control, Near and Bright) and training day as the within-groups factor (days 1 to 5 or days 1 to 10, depending on the training length). Significant differences were followed up by Tukey and Bonferroni post hoc tests and separate between- and within-groups ANOVAs, where appropriate. To establish whether rats showed a significant preference for any quadrant during the probe test, percentage time spent in each quadrant was compared to chance level (25%) for all groups using a series of one sample t-tests. As a more specific indicator of rats' searching behaviour, percentage time spent in all four platform areas was assessed using a 3 x 4 mixed factorial ANOVA with group as the between-groups factor and platform area as the within-groups factor (NE, NW, SE and SW). As an indicator of thigmotaxis, time spent in the outer corridor was examined using one-way between-groups ANOVAs with Tukey *post hoc* analyses.

3.2.2. Acquisition results

3.2.2.1. Escape latency.

One-way repeated-measures ANOVAs (for all animals as a single group) yielded significant main effects of training day after five days, $F_{4,80} = 27.79$, $P = 0.0001$, partial eta^2 = 0.58, and ten days, $F_{9,153} = 22.79$, $P = 0.0001$, partial eta^2 = 0.57. Bonferroni *post hoc* tests showed that escape latency on day 5 was significantly shorter than on day 1 ($P = 0.001$; see Figure 3.3A), and on day 10 compared to day 1 ($P = 0.001$; see Figure 3.3B). Mixed factorial ANOVAs (for animals separated into their recall groups) revealed significant main effects of day after five days, $F_{4,72} = 33.10$, $P = 0.0001$, partial eta^2 = 0.65, and ten days of training, $F_{9,135} = 23.84$, $P = 0.0001$, partial eta^2 = 0.61. Bonferroni *post hoc* tests confirmed that rats trained for five days were significantly faster at finding the platform on day 5 (14.36 ± 1.49s;

95% CI [11.22, 17.48]) compared to day 1 (38.49 ± 1.92s, CI [34.45, 42.52] P = 0.001). Similarly, rats trained for ten days escaped significantly faster on day 10 (10.48 ± 1.20s, CI [9.73, 13.03]) than on day 1 (38.03 ± 2.99, CI [31.66, 44.41], P = 0.001; see Figure 3.3B). No significant group x day interaction effects were found after five, $F_{8, 72}$ =1.25, P = 0.28, partial eta^2 = 0.12, or ten days, $F_{18,135}$ = 1.39, P = 0.21, partial eta^2 = 0.16.

The main effect of group was significant for five-day training groups, $F_{1,18}$ = 4.77, P = 0.05, partial eta^2 = 0.35. Tukey *post hoc* tests indicated an overall significant difference between the Control group and the Near group (P = 0.02). Between-groups ANOVAs were then carried out to determine on which days these groups differed. Although no main effects were found, the observed *post hoc* difference appeared to be driven by escape patterns on day 1, $F_{2,20}$ = 3.08, P = 0.07, and day 2 of training, $F_{2,20}$ = 3.09, P = 0.06. Main effects on day 3: $F_{2,20}$ = 1.03, P = 0.38, day 4: $F_{2,20}$ = 1.85, P = 0.19, and day 5: $F_{2,20}$ = 0.40, P = 0.67, were not significant. The main effect of group after ten days of training was not significant, $F_{1,15}$ = 0.72, P = 0.50, partial eta^2 = 0.09.

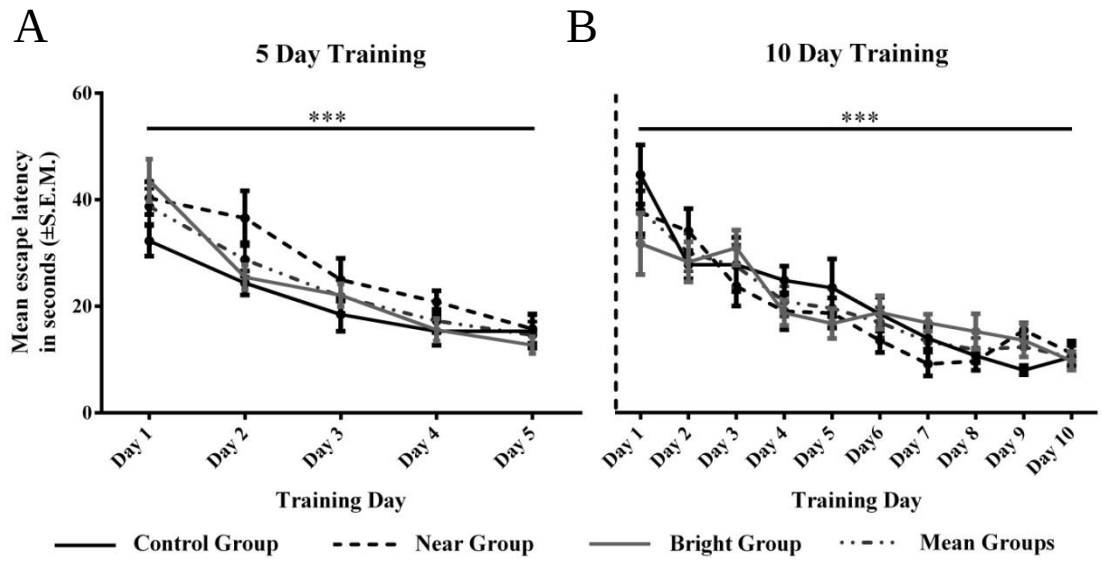

Figure 3.3: Mean escape latencies (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Near and Bright) across (A) five and (B) ten days of training.

3.2.2.2. Distance travelled.

One-way repeated-measures ANOVAs produced significant main effects of training day after five days, $F_{4,80} = 22.22$, $P = 0.0001$, partial eta$^2 = 0.53$, and ten days, $F_{9,153} = 19.75$, $P = 0.0001$, partial eta$^2 = 0.54$. Bonferroni *post hoc* tests indicated that distance travelled on day 5 was significantly less than on day 1 ($P = 0.001$; see Figure 3.4A) and on day 10 compared to day 1 ($P = 0.001$; see Figure 3.4B). Mixed factorial ANOVAs for distance travelled yielded similar results. Significant main effects of day after five-day training, $F_{4,72} = 22.74$, $P = 0.0001$, partial eta$^2 = 0.56$, and ten-day training, $F_{9,135} = 20.56$, $P = 0.0001$, partial eta$^2 = 0.58$. Pairwise comparisons (Bonferroni) showed that path lengths were significantly reduced on days 5 (371.30 ± 38.76cm; 95% CI [289.87, 452.72]) and 10 (231.92 ± 22.88cm; 95% CI [183.15, 280.69]) relative to initial training days (906.34 ± 62.09cm, CI [775.90, 1036.79], and 813.60 ± 66.26cm; 95% CI [672.37, 954.82], respectively; both $P = 0.001$). Main effects of group were not significant for either training length;

five day: $F_{1,18}$ = 0.79, P = 0.47, partial eta^2 = 0.08, ten day: $F_{1,15}$ = 1.80, P = 0.20, partial eta^2 = 0.19. Group x day interaction effects were also non-significant; five day: $F_{8,72}$ = 1.23, P = 0.30, partial eta^2 = 0.12, ten day: $F_{18,135}$ = 1.35, P = 0.23, partial eta^2 = 0.15. Acquisition results indicate equivalent learning for all groups by the end of the training period.

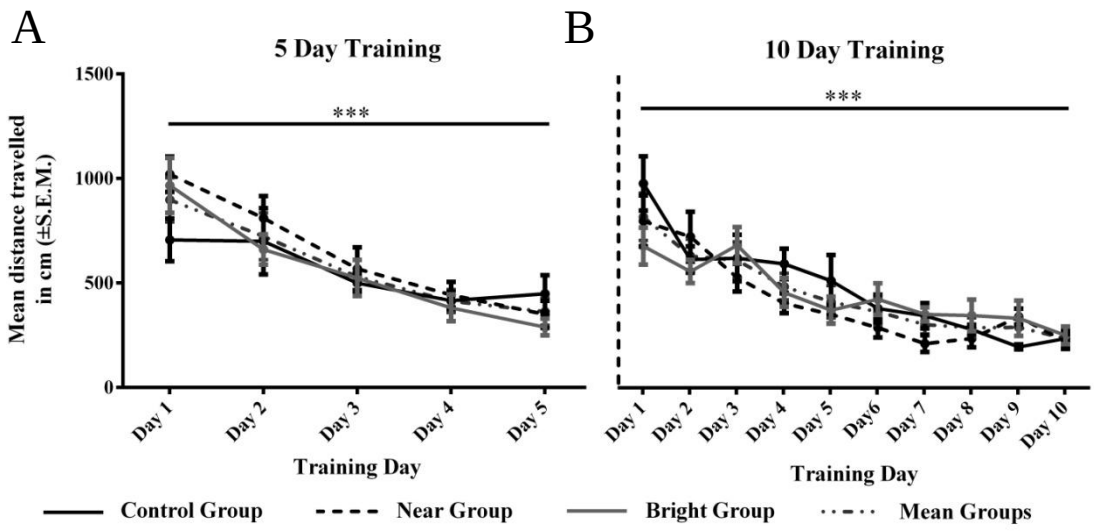

Figure 3.4: Mean distance travelled (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Near and Bright) across (A) five and (B) ten days of training.

3.2.3. Recall results

3.2.3.1. Quadrants.

Analyses of time spent in quadrants revealed Control and Bright groups trained for five days spent significantly more time in the target (NE) quadrant than expected by chance, t_{12} = 5.84, P = 0.001, and t_{12} = 2.70, P = 0.04, respectively (see Figure 3.5A). The Bright group also spent significantly longer in the SE quadrant compared to chance, t_{12} = 4.52, P = 0.01, while both groups spent significantly less time in the NW quadrant, t_{12} = 7.25, P = 0.001, and t_{12} = 4.37, P = 0.01, respectively. In contrast, time spent in the NE quadrant by the Near group was significantly below

chance, t_{12} = 6.79, P = 0.001. Control and Bright groups continued to favour the target quadrant after ten days of training, t_{10} = 2.75, P < 0.05, and t_{10} = 4.57, P < 0.05. The Near group showed no preference for any quadrant, with time spent in the NW quadrant being significant less than chance level, t_{10} = 5.30, P = 0.01 (see Figure 3.5B). No other significant differences were found.

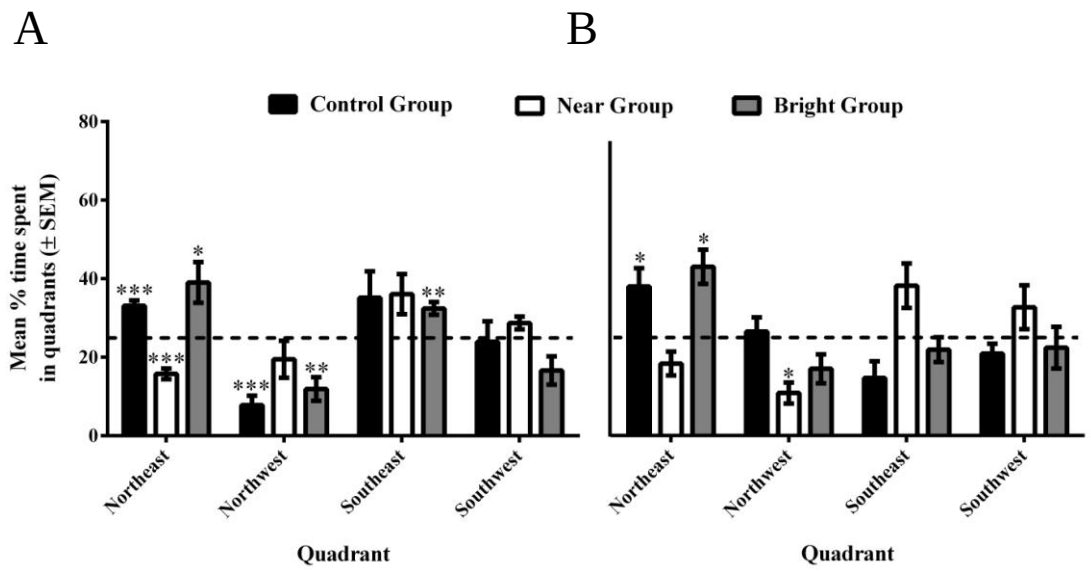

Figure 3.5: Mean percentage time (± SEM) in quadrants by five- and ten-day Control, Near and Bright groups (A-B). Dashed line indicates chance level (25%).

3.2.3.2. Platform areas.

Next, time spent in platform areas was assessed. Mixed factorial ANOVAs revealed a significant main effect of area ($F_{3,54}$ = 10.84, P = 0.001, partial eta^2 = 0.38) and area x group interaction effect ($F_{6,54}$ = 3.60, P = 0.02, partial eta^2 = 0.29) after five-day training. The main effect of group was not significant, $F_{1,18}$ = 0.96, P = 0.40, partial eta^2 = 0.10. *Post hoc* analyses showed that rats preferred the target platform area (8.16 ± 1.11s, CI [5.83, 10.49]) over the NW (2.33 ± 0.52s, CI [1.25, 3.42]; P = 0.01) and SW areas (3.41 ± 0.46s, CI [2.44, 4.39]; P = 0.04). When groups were examined separately with repeated measures ANOVAs, this preference was found to be driven by the Control group ($F_{3,18}$ = 10.32, P = 0.01, partial eta^2 = 0.63) who

favoured the target area compared to NW (P = 0.01) and SW areas (P = 0.04; see Figure 3.6A and 3.6C). The main effect of area was also significant for the Bright group ($F_{3,18}$ = 8.79, P = 0.02, partial eta^2 = 0.59) but no *post hoc* differences were discovered. Bonferroni corrected paired samples t-tests did, however, show that the Bright group spent longer in the target area compared to the NW (t_6 = 3.05, P = 0.04) and SW areas (t_6 = 3.09, P = 0.04). No main effect of area was noted for the Near group, $F_{3,18}$ = 1.07, P = 0.36, partial eta^2 = 0.15. To explore group differences within each area, between groups ANOVAs were then conducted. A significant main effect of area was discovered in the target region only ($F_{2,20}$ = 5.54, P = 0.02), with the Bright group spending more time here than the Near group (P = 0.02).

Platform area analyses after ten day training produced comparable results. The main effect of area ($F_{3,45}$ = 16.61, P = 0.0001, partial eta^2 = 0.53) and area x group interaction effect were significant ($F_{6,45}$ = 8.94, P = 0.0001, partial eta^2 = 0.54). However, the main effect of group was not significant, $F_{1,15}$ = 0.29, P = 0.75, partial eta^2 = 0.04. Bonferroni *post hoc* analyses showed that rats spent significantly longer in the target area (10.48 ± 1.01s, CI [8.32, 12.64]) compared to the three remaining areas (NW: 4.63 ± 0.58s, CI [3.39, 5.87], SE: 4.33 ± 0.64, CI [2.97, 5.70], SW: 4.07 ± 0.55s, CI [2.91, 5.24]; all P = 0.01). Repeated measures ANOVAs indicated that this result was mediated by the Control ($F_{3,15}$ = 22.80, P = 0.001, partial eta^2 = 0.82) and Bright groups ($F_{3,15}$ = 7.59, P = 0.02, partial eta^2 = 0.60). More specifically, the Control group spent significantly more time in the NE area than in the southern areas (both P = 0.01), and the Bright group spent more time in this area compared to the SE area (P = 0.04; see Figure 3.6B and 3.6C). Again, between-groups ANOVAs were used to assess group differences within areas. Analyses yielded main effects for the NE ($F_{2,17}$ = 14.04, P = 0.0001), NW ($F_{2,17}$ =

6.07, P = 0.02) and SE areas ($F_{2,17}$ = 5.31, P = 0.02). Tukey *post hoc* comparisons indicated that Control rats spent significantly longer in the NE and NW areas than the Near group (P = 0.01 and P = 0.02); the Bright group also outperformed the Near group in the NE area (P = 0.01), while the Near group spent more time in the SE area compared to the other two groups (P = 0.03 and P = 0.05).

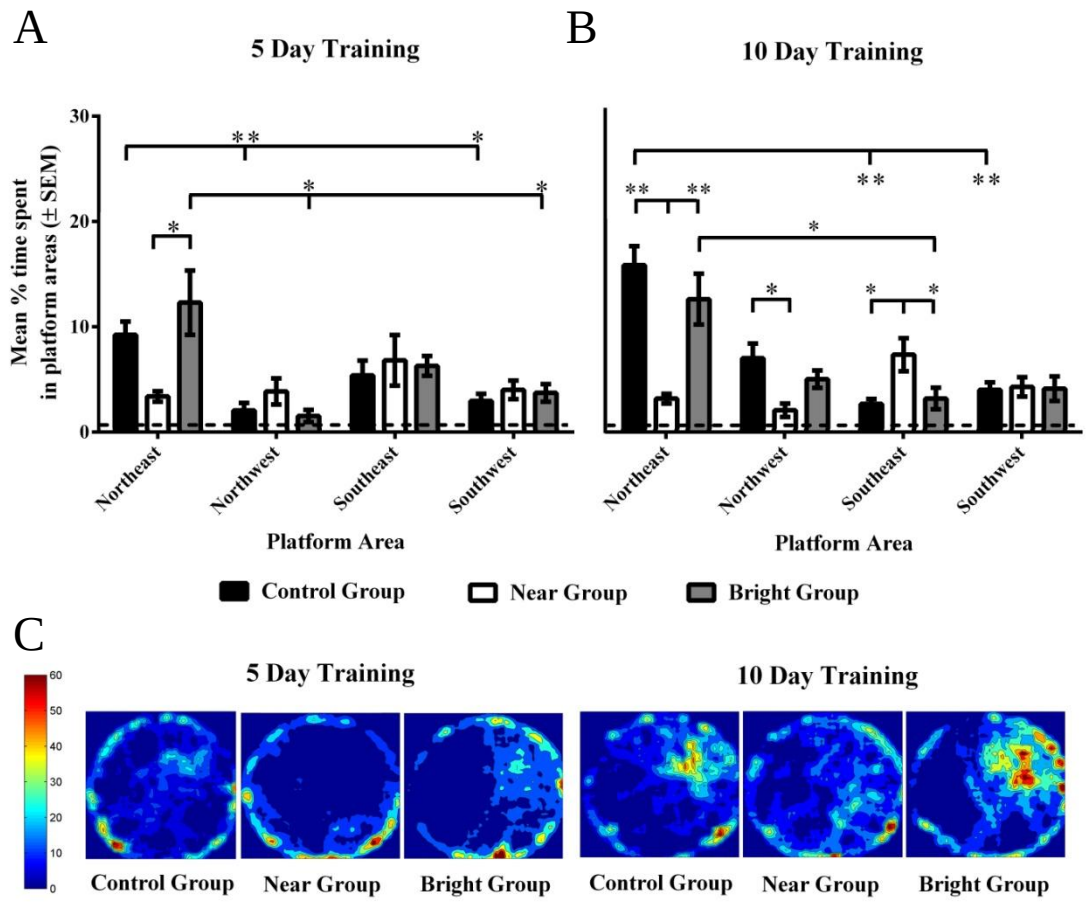

Figure 3.6: (A-B) Mean percentage time (± SEM) spent in platform areas by Control, Near and Bright groups trained for five and ten days. (C) Heat maps showing overall search distributions during the probe trial for five- and ten-day groups. Dashed line indicates chance level (0.6%).

3.2.3.3. Outer corridor.

One-way between-groups ANOVAs were carried out to explore groups differences in percentage time spent in the outer corridor of the maze after five and ten days of training. No significant main effects of group were found for either training length

(see Figure 3.7); five day: $F_{2,20}$ = 1.59, P = 0.23, ten day: $F_{2,17}$ = 1.88, P = 1.87, indicating that one cue groups were not more anxious relative to Controls.

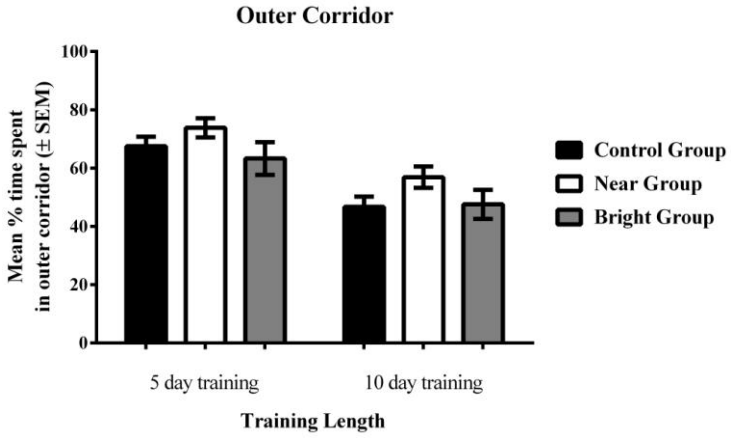

Figure 3.7: Mean percentage time (± SEM) spent in outer corridor of the maze by Control, Near and Bright groups trained for five and ten days.

3.2.4. Discussion

All groups acquired the task, as indicated by the decrease in escape latency and path length across five- and ten-day training periods. Furthermore, no group differences in either acquisition measure were found, signifying that groups showed equivalent learning. Analyses of time spent in quadrants and platform areas illustrated that, as expected, Control rats successfully located the correct region of the maze, with an increase in searching specificity observed from five- to ten-day training. Animals tested with the bright cue also showed a steady preference for the target quadrant and area across training lengths, indicating that rats correctly assigned the available cue to their representation of the bright cue in memory. In contrast, the Near groups had poor overall retention, spending the majority of their time in the southern regions of the maze. This behaviour is unlikely to be due to increased anxiety, since all groups showed comparable levels of thigmotaxis. Rather, the tendency to search in the SE area after ten days could indicate that the Near groups misidentified the near cue as

the bright cue, as searching in this region corresponds to the spatial relationship between the bright cue and the platform during training.

Taken together, results indicate that the cues did not acquire equal salience during training, but rather, the brighter distal cue rapidly became more salient than the proximal cue. This finding lends support to the idea that discrete features, in this case brightness, can influence the overall salience of a cue (Chamizo, Rodrigo, Peris, et al., 2006; Young et al., 2006). With regard to strategy use, the divergent performance of the one-cue groups seems to suggest that animals employed an elemental strategy involving the bright cue to find the platform, similar to rats in Rodrigo *et al.* (2014). Importantly, no evidence for a shift in learning strategy was observed, as rats continued to rely on the bright cue even after extended training.

3.3. Experiment 2

The aim of Experiment 2 was to demonstrate that rats could learn to navigate using a bright cue in the near position, thereby controlling for cue location. Since it was expected that the bright cue would acquire a higher salience than the near cue from the outset of learning (due to it being both brighter and closer to the platform), only one training length was employed.

3.3.1. Method

3.3.1.1. Subjects.

Twenty-one male Wistar rats (Charles River, UK) were used as subjects (see Chapter 2 for details regarding age, weight, housing and maintenance).

3.3.1.2. Apparatus and procedure.

All apparatus and procedures were identical to Experiment 1 with the exception of the cue locations. For this experiment, the location of the near and bright cues was reversed, such that the brighter cue was positioned closest to the platform (in the near NE position). All rats were trained for a total of ten days (four trials per day; 40 trials) in the presence of both cues, with probe trials being completed 24 hours later. Rats were assigned to a Control group, tested with both cues (NE and NW), Bright group, tested with the bright (NE) cue, or Far group, tested with the far (NW) cue (*n* = 7 per group; see Figure 3.8).

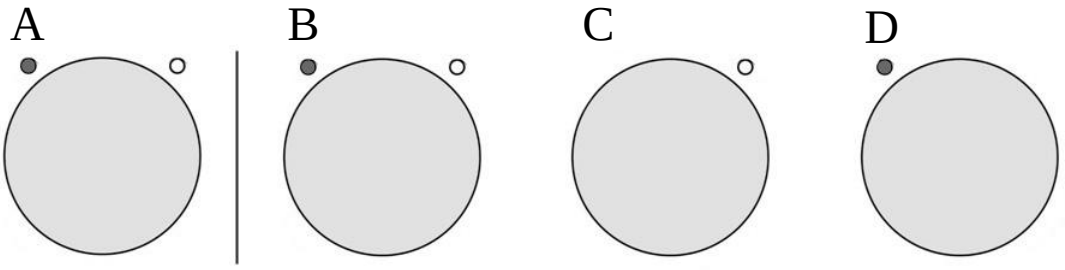

Figure 3.8: Representation of distal cue configuration during (A) training for all animals, and cue configuration during testing for (B) Control, (C) Bright and (D) Far groups. Closed circle indicates 25 Watt bulb and open circle indicates 40 Watt bulb.

3.2.1.4. Data and statistical analyses.

Water maze acquisition was measured by mean escape latencies and distances travelled and recall was assessed by average time spent in quadrants, platform areas and the outer corridor, as per Experiment 1. All statistical analyses carried out were the same as those used in Experiment 1.

3.3.2. Acquisition results

3.3.2.1. Escape latency.

A one-way repeated-measures ANOVA produced a significant main effect of training day, $F_{9,180} = 19.89$, $P = 0.0001$, partial eta$^2 = 0.50$, with Bonferroni *post hoc* tests showing that escape latency on day 10 was significantly faster than on day 1 ($P = 0.001$; see Figure 3.9A). Mean escape latencies for animals in their recall groups were examined using a 3 (Control, Bright and Far groups) x 10 (days 1 – 10) mixed factorial ANOVA, which revealed a significant main effect of day ($F_{9,162} = 29.20$, $P = 0.0001$, partial eta$^2 = 0.54$). *Post hoc* tests showed that mean escape latency decreased from 39.46 ± 2.91s (CI [33.33, 45.56]) on day 1 to 15.43 ± 1.10s (CI [13.12, 17.73]) on day 10 ($P = 0.001$). The main effect of group, $F_{1,18} = 0.27$, $P =$

0.77, partial eta^2 = 0.03, and day x group interaction effect, $F_{18,162}$ = 1.52, P = 0.14, partial eta^2 = 0.14, were not significant.

3.3.2.2. Distance travelled.

A one-way repeated-measures ANOVA produced a significant main effect of training day, $F_{9,180}$ = 15.91, P = 0.0001, partial eta^2 = 0.44, with Bonferroni *post hoc* tests showing that escape latency on day 10 was significantly faster than on day 1 (P = 0.001; see Figure 3.9B). When animals were grouped, a 3 x 10 mixed factorial ANOVA also yielded a main effect of day, ($F_{9,162}$ = 16.64, P = 0.0001, partial eta^2 = 0.48), with a marked reduction in distance travelled from 1047.26 ± 79.90cm (CI [879.39, 1215.12]) on day 1 to 399.96 ± 341.50cm (CI [1341.50, 458.41]) on day 10 (Bonferroni: P = 0.001). No significant main effect of group, $F_{1,18}$ = 0.28, P = 0.76, partial eta^2 = 0.03, or day x group interaction effect was found, $F_{18,162}$ = 1.46, P = 0.16, partial eta^2 = 0.14.

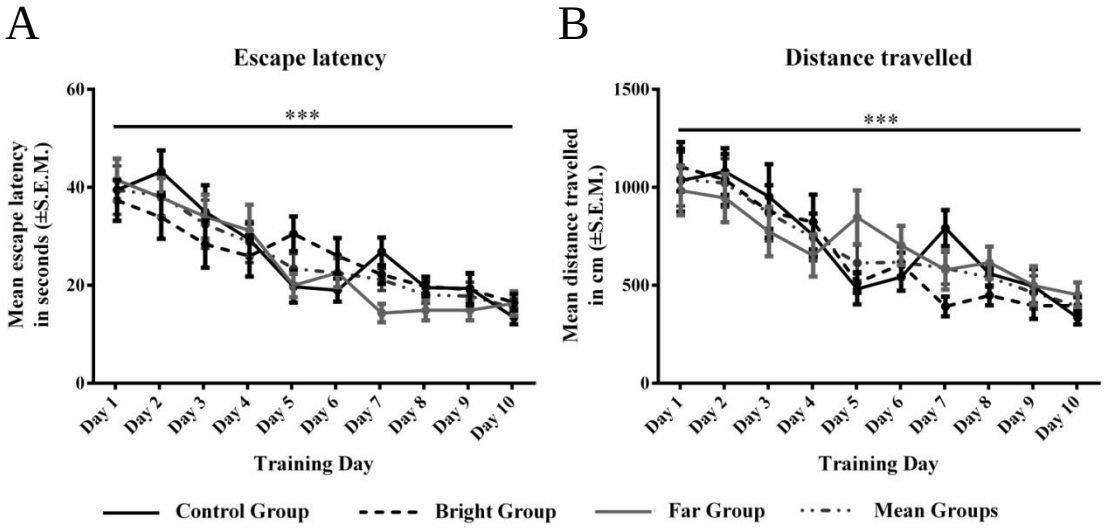

Figure 3.9: (A) Mean escape latencies (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Bright and Far). (B) Mean distances travelled (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Bright and Far).

3.3.3. Recall results

3.3.3.1. Quadrants.

During the probe trial, time spent in the target quadrant was significantly above chance for the Control (t_{12} = 3.34, P = 0.01) and Bright groups (t_{12} = 3.31, P = 0.01), but not for the Far group (see Figure 3.10A). No other significant differences were noted for the NW, SE or SW quadrants.

3.3.3.2. Platform areas.

Mixed factorial ANOVA results included significant main effects for area ($F_{3,54}$ = 18.75, P = 0.0001, partial eta^2 = 0.51) and group ($F_{1,18}$ = 15.17, P = 0.0001, partial eta^2 = 0.63), as well as a significant area x group interaction effect ($F_{6,54}$ = 4.89, P = 0.01, partial eta^2 = 0.31). Bonferroni *post hoc* tests revealed that rats spent more time in the NE area (7.49 ± 0.81s, CI [5.79, 9.19]) compared to the NW (2.75 ± 0.45s, CI [1.81, 3.68]), SE (3.49 ± 0.49s, CI [2.47, 4.52]) and SW areas (2.33 ± 0.33, CI [1.64, 3.03]; all P = 0.001).

When time spent in areas was examined for each group individually, significant main effects of area were found for the Control group ($F_{3,18}$ = 9.42, P = 0.01, partial eta^2 = 0.61) and the Bright group ($F_{3,18}$ = 14.17, P = 0.001, partial eta^2 = 0.70), but not for the Far group (see Figure 3.10B-C). Bonferroni corrected t-tests revealed that Control rats favoured the NE area over the NW (t_6 = 3.39, P = 0.045), SE (t_6 = 3.31, P = 0.048) and SW areas (t_6 = 3.71, P = 0.03). *Post hoc* tests were significant for the Bright group, which also preferred the target area over the three remaining areas: NW (P = 0.05), SE (P = 0.01) and SW (P = 0.02). In addition, Control and Bright groups spent significantly more time in the target area relative to

the Far group ($F_{2,18}$ = 12.19, P = 0.0001; both P = 0.01; see Figure 3.10B-C). Group differences in all other areas were non-significant.

3.3.3.2. Outer corridor.

A one-way between groups ANOVA revealed a significant main effect of group, $F_{2, 20}$ = 1314.39, P = 0.001. Tukey *post hoc* tests showed that the Far group spent significantly longer in the outer corridor (67.57 ± 3.72%, CI [58.48, 76.67]) compared to the Control (40.95 ± 4.82%, CI [29.17, 52.74]) and Bright groups (48.62 ± 4.26%, CI [38.18, 59.05]) during the probe trial (P = 0.001 and P = 0.02, respectively; see Figure 3.9D).

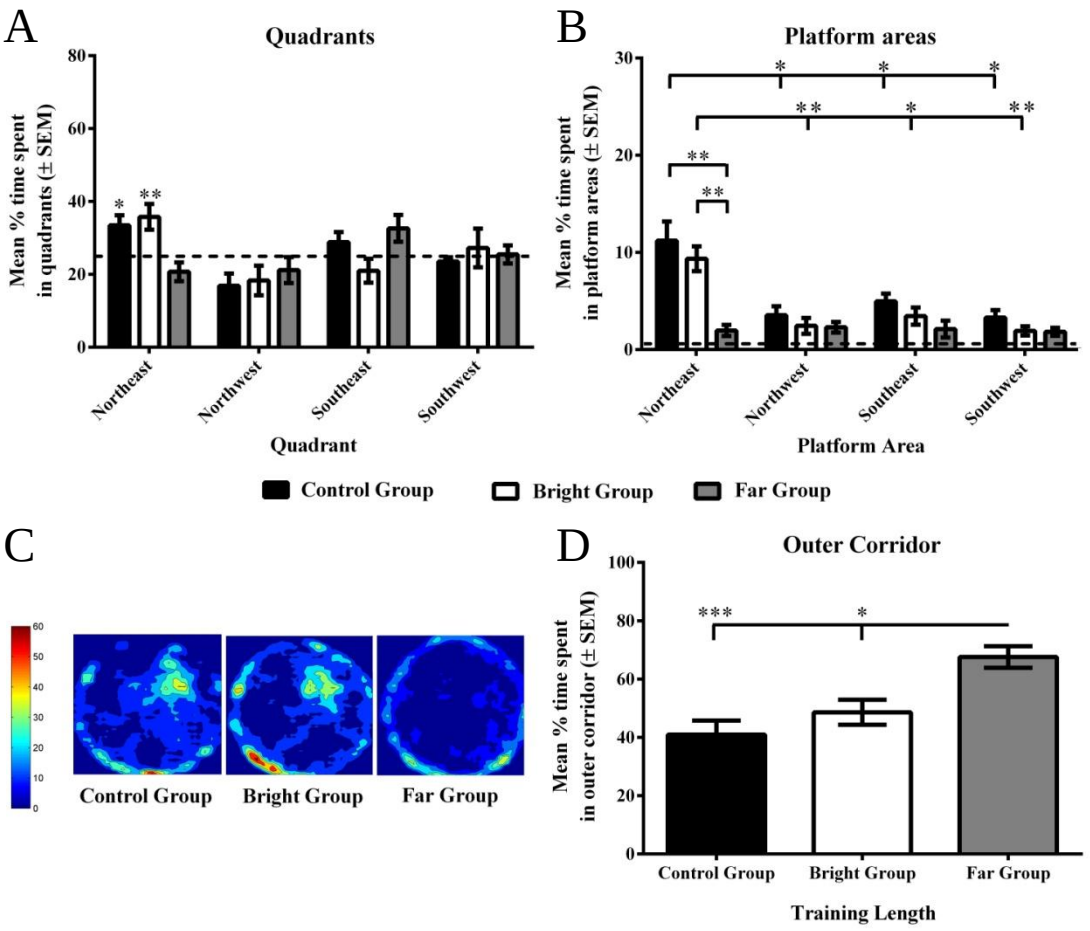

Figure 3.10: Mean percentage time (± SEM) spent by Control, Bright and Far groups in (A) quadrants and (B) platform areas during the probe trial. (C). Heat maps illustrating search patterns during the probe test for five- and ten-day groups. (D). Mean percentage time (± SEM) spent in outer corridor by Control, Bright and Far groups during recall. Dashed line indicates chance level (0.6%).

3.3.4. Discussion

As per Experiment 1, all groups learned to reach the platform equally well after ten days of training. During the recall test, the Control and Bright groups favoured the target quadrant and platform area, while rats navigating with the less luminous far cue were impaired. The Far group also demonstrated greater thigmotaxis relative to the other groups, which may indicate increased anxiety levels. Overall, results indicate that the bright cue again became the more salient of the two cues. Interestingly, the Far group did not appear to mistake the far cue for the bright cue here, as was noted in Experiment 1. Namely, this group showed no preference for

searching in the NW region of the pool. It is possible that because the bright cue was both brighter and closer to the platform relative to the far cue, this cue acquired a beacon-like control over navigation (Redhead et al., 1997), whereby animals paid little attention to the relationship between the far cue and the platform during training. Thus, rats were completely impaired with the near cue during the recall phase, resulting in greater thigmotactic behaviour. In line with Experiment 1, the discrepancy in performance level between the one-cue groups also implies the presence of an elemental over configural learning strategy.

3.4. Experiment 3

Findings from Experiments 1 and 2 showed that brightness can have a greater effect on cue salience than proximity to the goal. Therefore, the purpose of Experiment 3 was to more closely examine the role of proximity to the goal in determining cue salience and on the type of strategy learned. We also aimed to investigate if greater experience with a cue arrangement via increased training influenced the type of strategy used.

3.4.1. Method

3.4.1.1. Subjects.

Subjects were male Wistar rats (*n* = 38; Charles River, UK; see Chapter 2 for age, weight, housing and maintenance details).

3.4.1.2. Apparatus and procedure.

Apparatus and procedures were the same as Experiment 1 with the exception of the far cue, which was replaced with a 25 Watt light bulb, resulting in an environment with two equally bright distal cues. Rats were trained for five (*n* = 20; 20 trials) or ten days in total (*n* = 18; 40 trials), followed 24-hours later by a single probe trial. Before training, rats trained for five days were randomly allocated to one of three groups; Control group (*n* = 6), Near or Far (both *n* = 7). Rats trained for ten days were grouped in the same way (*n* = 6 per group). Control groups were tested with both cues (NE and NW), Near groups were tested with the near (NE) cue only and Far groups with the far (NW) cue only (see Figure 3.11).

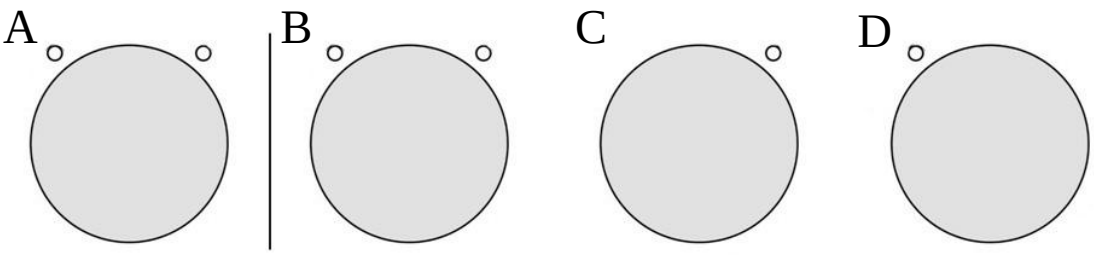

Figure 3.11: Diagram of (A) training cue configurations for all animals and testing cue configurations for (B) Control (C) Near and (D) Far groups 25 Watt bulbs (open circles).

3.2.1.4. Data and statistical analyses.

All behavioural data and statistical analyses were identical to those outlined in Experiment 1.

3.4.2. Acquisition results

3.4.2.1. Escape latency.

One-way repeated-measures ANOVAs (for animals as a single group) yielded significant main effects of training day after five days, $F_{4,76}$ = 31.84, P = 0.0001, partial eta^2 = 0.63, and ten days, $F_{9,153}$ = 26.25, P = 0.0001, partial eta^2 = 0.61. Bonferroni *post hoc* tests showed that escape latency on day 5 was significantly shorter than on day 1 (P = 0.001), and on day 10 compared to day 1 (P = 0.001; see Figure 3.12). Mixed factorial ANOVAs were then once again employed to investigate animals' escape latencies according to recall group. Main effects of day were found after five ($F_{4,68}$ = 33.86, P = 0.0001, partial eta^2 = 0.67) and ten days of training ($F_{9,135}$ = 25.88, P = 0.0001, partial eta^2 = 0.99). Bonferroni *post hoc* analyses showed that escape latencies after five days (11.97 ± 1.35s, CI [9.12, 14.82]) and ten days (10.39 ± 0.92s, CI [8.44, 12.34]) were significantly shorter compared to day 1 (31.23 ± 1.68s, CI [27.68, 34.79], P = 0.001, and 33.59 ± 1.92s, CI [29.54, 37.65], P = 0.001, respectively). No significant main effects of group (five day: $F_{1,17}$ = 2.21, P

= 0.14, partial eta^2 = 0.21, ten day: $F_{1,15}$ = 4.18, P = 0.36, partial eta^2 = 0.20) or day x group interaction effects were found (five day: $F_{8,68}$ = 1.66, P = 0.15, partial eta^2 = 0.16, ten day: $F_{18,135}$ = 0.88, P = 0.55, partial eta^2 = 0.11).

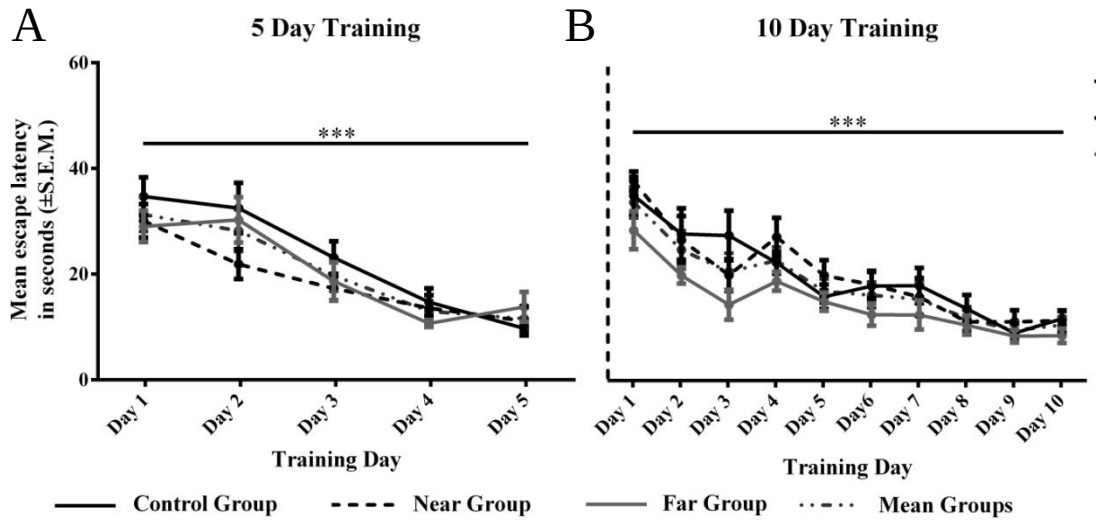

Figure 3.12: Mean escape latency (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Near and Far) across (A) five and (B) ten days of training.

3.4.2.2. Distance travelled.

One-way repeated-measures ANOVAs yielded significant main effects of training day after five days, $F_{4,76}$ = 30.78, P = 0.0001, partial eta^2 = 0.62, and ten days, $F_{9,153}$ = 22.35, P = 0.0001, partial eta^2 = 0.57. Bonferroni *post hoc* tests showed that path length on day 5 was significantly shorter than on day 1 (P = 0.001), and on day 10 compared to day 1 (P = 0.001; see Figure 3.12). Mixed factorial ANOVAs produced significant main effects of day after five- ($F_{4,68}$ = 31.39, P = 0.0001, partial eta^2 = 0.65) and ten-day training ($F_{9,135}$ = 21.37, P = 0.0001, partial eta^2 = 0.59; see Figure 3.13). Bonferroni *post hoc* tests illustrated that path lengths were significantly shorter on day 5 (219.05 ± 24.43cm, CI [167.52, 270.59]) and day 10 (280.76 ± 28.90cm, CI [219.15, 342.36]) in comparison to day 1 (644.52 ± 37.40cm, CI

[565.61, 723.43], and 758.70 ± 44.11cm, CI [664.67, 852.72], respectively; both P = 0.001). Main effects of group were not significant for either training length; five day: $F_{1,17}$ = 3.39, P = 0.06, partial eta^2 = 0.29, ten day: $F_{1,15}$ = 3.65, P = 0.06, partial eta^2 = 0.32. Day x group interaction effects also failed to reach significance; five day: $F_{8,68}$ = 1.24, P = 0.30, partial eta^2 = 0.13, ten day: $F_{18,135}$ = 0.62, P = 0.77, partial eta^2 = 0.08.

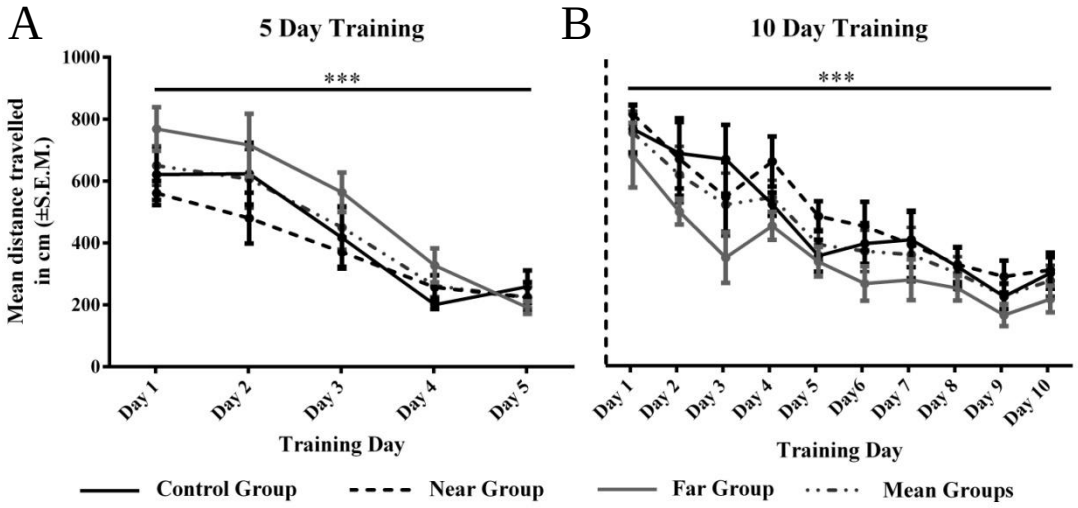

Figure 3.12: Mean distance travelled (± SEM) shown for all animals as a single group (Mean groups) and in their respective recall groups (Control, Near and Far) across (A) five and (B) ten days of training.

3.4.3. Recall results

3.4.3.1. Quadrants.

After five days of training, no group displayed a significant preference for any quadrant (see Figure 3.14A). One significant result was found for the Far group, which spent significantly less time in the SW quadrant compared to chance level, t_{12} = 2.48, P = 0.05. After ten days of training time spent in the NE quadrant was significantly greater than chance for the Control group (t_{10} = 6.93, P = 0.001) and the Far group (t_{10} = 6.63, P = 0.001), but not for the Near group (see Figure 3.14B). Percentage times were significantly below chance level in the NW quadrant for the

Near (t_{10} = 3.15, P = 0.03) and Far groups (t_{10} = 4.43, P = 0.01), and in the SW quadrant for the Control (t_{10} = 7.18, P = 0.001) and Far groups (t_{10} = 3.89, P = 0.02).

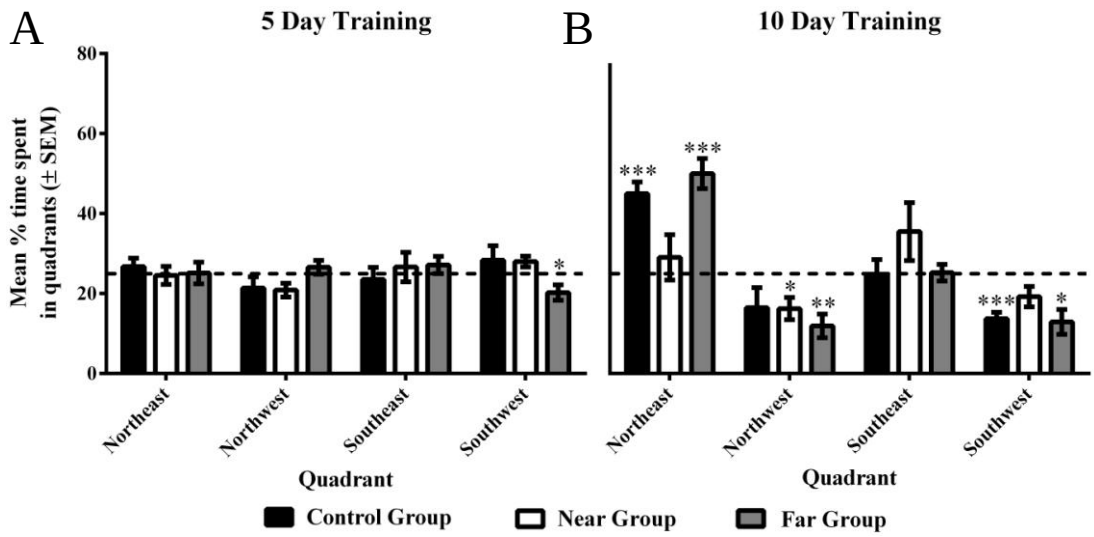

Figure 3.14: Mean percentage time spent in quadrants (± SEM) after (A) five-day and (B) ten-day training for Control, Near and Far groups.

3.4.3.2. Platform areas.

A comparison of time spent in platform areas after five days of training produced a significant main effect of area ($F_{3,51}$ = 3.13, P = 0.03, partial eta^2 = 0.16) and area x group interaction effect ($F_{6,51}$ = 5.08, P = 0.0001, partial eta^2 = 0.57), but no main effect of group, $F_{1,17}$ = 1.30, P = 0.89, partial eta^2 = 0.01. *Post hoc* tests were non-significant, however, repeated measures ANOVAs showed that, of the three groups, Control rats spent significantly more time in the target area compared to NW and SE areas ($F_{3,15}$ = 13.30, P = 0.001, partial eta^2 = 0.73; P = 0.01, and P = 0.04, respectively). Near and Far groups did not display a preference for any area. The Control group also spent significantly longer in the NE area compared to the Near group, $F_{2,15}$ = 13.30, P = 0.02 (P = 0.02; see Figure 3.15A and 3.15C).

A main effect of area ($F_{3,45}$ = 48.33, P = 0.001, partial eta^2 = 0.76) and area x group interaction ($F_{6,45}$ = 8.54, P = 0.001, partial eta^2 = 0.53) were also found after

10-day training. The main effect of group was not significant, $F_{1,15} = 0.15$, $P = 0.86$, partial eta^2 = 0.02. Bonferroni *post hoc* comparisons showed that rats spent significantly more time in the target area (9.57 ± 0.77s, CI [7.93, 11.22]) compared to all other areas (NW: 2.04 ± 0.43s, CI [1.13, 2.94]; SE: 4.35 ± 0.52s, CI [3.23, 5.47]; SW: 1.46 ± 0.31, CI [0.81, 2.21]; all $P = 0.001$). Individual main effects of area were also found for the Control ($F_{3,15} = 34.82$, $P = 0.001$, partial eta^2 = 0.87) and Far groups ($F_{3,15} = 37.13$, $P = 0.001$, partial eta^2 = 0.88). Both groups favoured the NE area over the NW ($P = 0.02$ and $P = 0.01$), SE ($P = 0.01$ and $P = 0.02$) and SW areas (both $P = 0.01$). Furthermore, between-groups ANOVA showed that the Control and Far groups spent significantly longer in the NE area compared to the Near group, $F_{2,17} = 11.84$, $P = 0.01$ (both $P = 0.01$; see Figure 3.15B-C). Time spent in all other areas did not differ across groups.

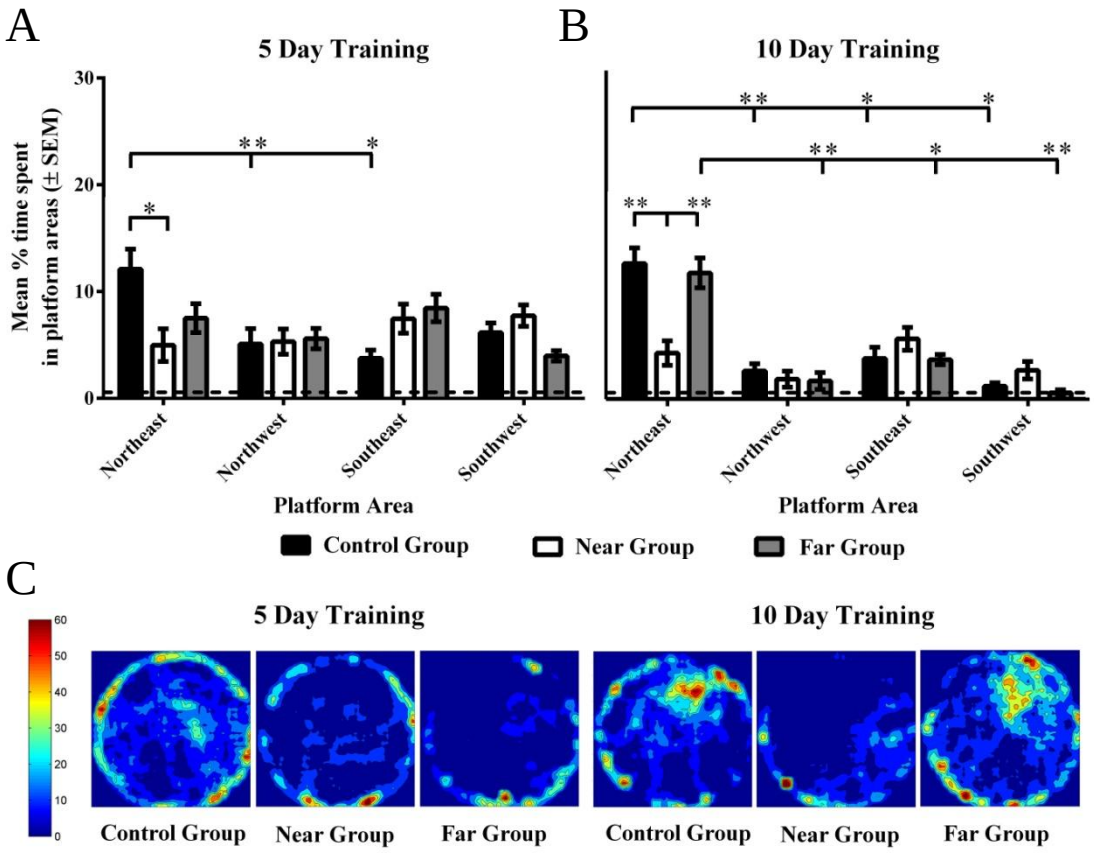

Figure 3.15: (A-B). Mean percentage time spent (± SEM) in platform areas by Control, Near and Far groups after five and ten days. (C). Heat maps showing search distributions during testing for five- and ten-day groups. Dashed line indicates chance level.

3.4.3.3. Outer corridor.

One-way between-groups ANOVAs failed to produce significant main effects of group after five- and ten-day training; $F_{2,19} = 1.49$, $P = 0.25$, and $F_{2,17} = 2.12$, $P = 0.16$, respectively (see Figure 3.16).

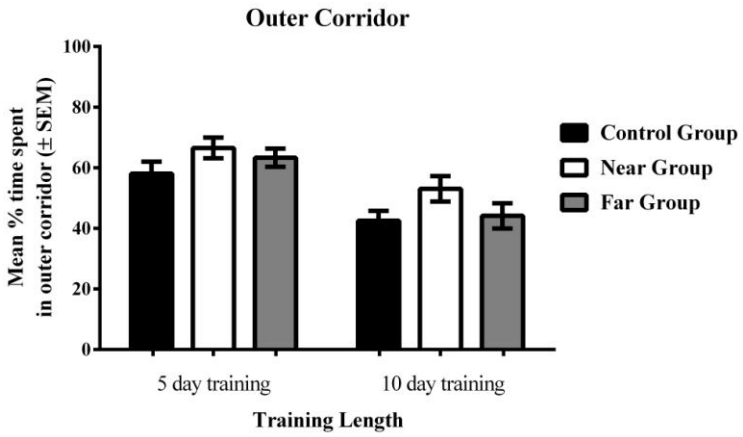

Figure 3.16: Mean percentage time (± SEM) spent in outer corridor by Control, Near and Far groups trained for five and ten days.

3.4.4. Discussion

All groups successfully acquired the task after standard and extended training, as before. After five days of training, no strong group preferences were observed for any quadrant of the maze. However, the Control group did search in the NE area (over the NW and SE regions, and compared to the Near group). When the number of training trials was doubled, the performance of the Control and Far groups improved significantly. Both groups favoured the NE quadrant and the NE area over all remaining areas and relative to the Near group. In contrast, rats navigating with the proximal cue failed to search in the target regions after ten days. Not entirely unlike Experiment 1, the Near group did exhibit a slight tendency to search in eastern over western regions of the maze – although these differences did not reach significance. This preference could potentially denote that the cues were confounded, and thus rats divided their time between searching in areas appropriate for each cue. Rats in the Far group showed no indication of a similar pattern of searching; time spent by this group in the NW quadrant was in fact significantly below chance level.

Overall, the results of Experiment 3 revealed that a distal cue can acquire greater salience than a proximal cue, contrary to our hypothesis and to some previous

literature (Artigas et al., 2005; Chamizo & Rodrigo, 2004; Redhead & Hamilton, 2007). Furthermore, findings do not support our prediction regarding strategy use. The poor navigation of the one-cue groups after five-day training indicates that rats did not adopt an elemental strategy from the beginning. Rather, it seems that animals initially engaged in configural learning with the entire cue arrangement, and the farther cue only became more salient after additional training. This could potentially have been due to the enhanced visual similarity of the cues (relative to Experiments 1 and 2) which may have promoted an initial configural learning approach, akin to the observations of Giurfa and colleagues (2003).

3.5. Experiment 4

As animals in the previous experiments were trained to navigate to a constant platform location (NE quadrant), it is possible that rats were relying other, unintentional room cues (e.g. noises, draughts or odours) to navigate. The goal of Experiment 4 was to exclude this possibility using a number of control conditions. A secondary aim of this experiment was to confirm that rats could discriminate cue brightness.

3.5.1. Method

3.5.1.1. Subjects.

Subjects were twenty-eight make Wistar rats (see Chapter 2 for description of age, weight, housing and maintenance).

3.5.1.2. Apparatus and procedure.

The apparatus and procedure were similar to Experiment 1. All animals were trained with two cues (one 25 Watt light bulb in the near position and one 40 Watt light bulb in the far position) for five days only (four trials per day; 20 trials). Rats were separated into five groups; a Control group (n = 6), a Swap group (n = 6), a Rotated Control group (n = 4), a Rotated Near group (n = 6) and a Rotated Bright group (n = 6) (see Figure 3.17). During recall, Control animals were tested with both cues, as per acquisition (from the SW start position). The Swap group was also tested in the presence of both cues (SW start position); however, their locations were reversed, such that the near cue was located in the NW position and the bright cue was located in the NE position. The Rotated Control group were tested with both cues rotated 180°, i.e. the bright cue was located in the SE position and the near cue was located

in the SW position. The Rotated Near and Rotated Bright groups were tested with a single cue only; the near cue and the bright cue, respectively, located in the SE position. All rotated groups were placed into the maze from the centre of the NW quadrant by the pool wall, i.e. opposite to the target quadrant as specified before (to ensure that start positions for all groups were equally distant from the cues).

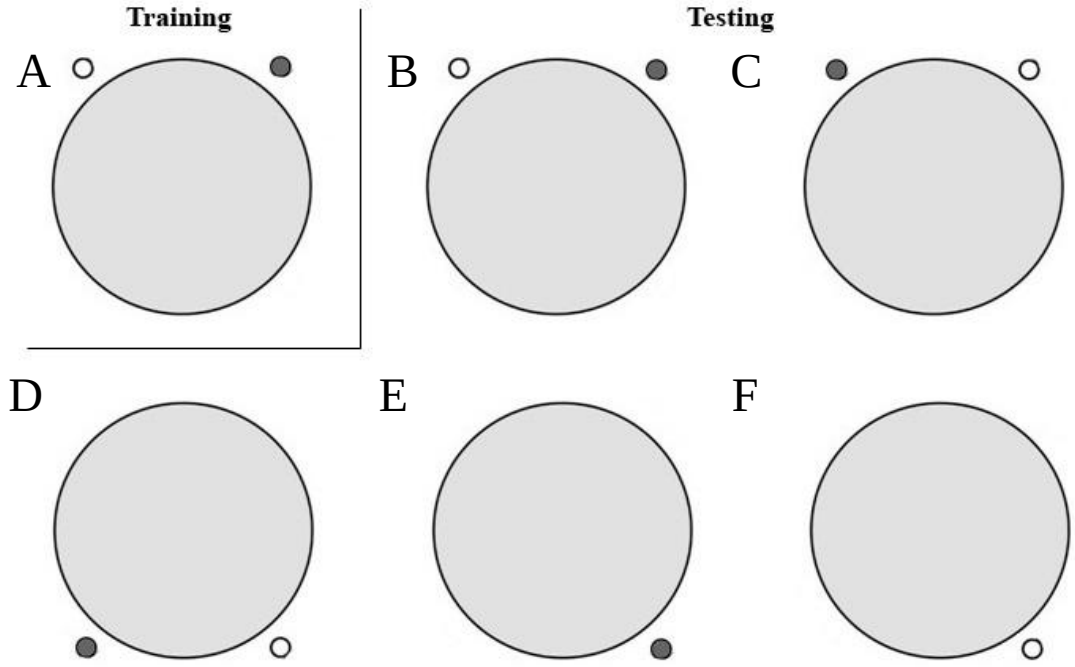

Figure 3.17: Cue configurations during (A) training and (B-F) testing for Control, Swap, Rotated Control, Rotated Near and Rotated Bright groups, with 25 Watt (closed circle) and 40 Watt bulbs (open circle).

3.2.1.4. Data and statistical analyses.

All analyses were the same as those outlined in Experiment 1.

3.5.2. Acquisition results

3.5.2.1. Escape latency.

A one-way repeated-measures ANOVA yielded a significant main effect of training day, $F_{4,108} = 27.43$, $P = 0.0001$, partial eta$^2 = 0.50$. Bonferroni *post hoc* tests showed

that escape latency on day 5 was significantly shorter than on day 1 (P = 0.001; see Figure 3.18A). A 5 x 5 mixed factorial ANOVA investigating escape latencies for animals according to recall group also produced a significant main effect of day, $F_{4,92}$ = 26.63, P = 0.0001, partial eta^2 = 0.54. Bonferroni pairwise comparisons showed that rats were significantly faster at escaping the maze on day 5 (16.20 ± 1.15s, CI [13.82, 18.58]) than on day 1 (33.81 ± 1.75s, CI [30.20, 37.43]; P = 0.0001). No main effect of group, $F_{1,23}$ = 1.01, P = 0.43, partial eta^2 = 0.16, or day x group interaction effect was noted, $F_{16,92}$ = 1.32, P = 0.24, partial eta^2 = 0.19.

3.5.2.2. Distance travelled.

A one-way repeated-measures ANOVA for path length yielded a significant main effect of training day, $F_{4,108}$ = 27.40, P = 0.0001, partial eta^2 = 0.50. Bonferroni *post hoc* tests indicated that path length on day 5 was significantly less than on day 1 (P = 0.001; see Figure 3.18B). A 5 x 5 mixed factorial ANOVA examining path lengths produced similar results. The main of day was significant, $F_{4,92}$ = 27.32, P = 0.0001, partial eta^2 = 0.54, and Bonferroni *post hoc* tests confirmed that mean distance travelled was significantly reduced on day 5 (390.47 ± 40.69cm, CI [306.30, 474.64]) compared to day 1 (834.64 ± 40.29cm, CI [751.30, 917.97]; P = 0.0001). The main effect of group, $F_{1,23}$ = 1.81, P = 0.16, partial eta^2 = 0.24, and day x group interaction effect, $F_{16,92}$ = 1.43, P = 0.14, partial eta^2 = 0.21, were non-significant.

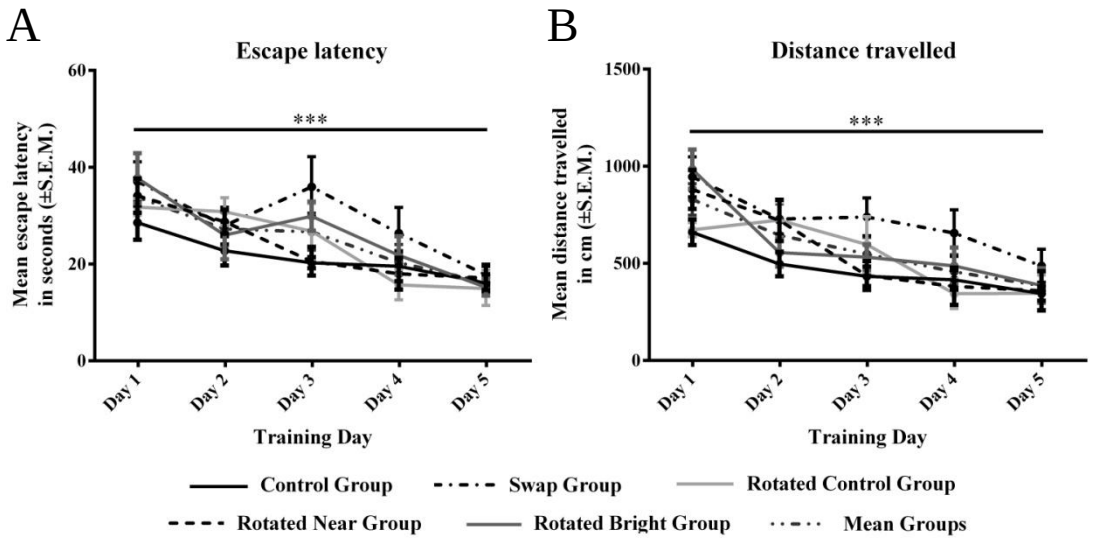

Figure 3.18: (A) Mean escape latencies and (B) distances travelled (± SEM) for all animals as a single group (Mean groups) and in their respective recall groups (Control, Swap, Rotated Control, Rotated Near and Rotated Bright).

3.5.3. Recall results

3.5.3.1. Quadrants.

Mean percentage time spent in each quadrant was compared to chance level for each group using a series of one sample t-tests. Only three significant results above chance were noted: the Control group spent significantly more time in the NE (t_{10} = 2.69, P = 0.05) and SE quadrants (t_{10} = 3.21, P = 0.04), and the Rotated Control group favoured the SW quadrant (t_8 = 2.70, P = 0.05; see Figure 3.19). A number of significant deviations below chance were found which included the Rotated Control group in the NE (t_8 = 4.79, P = 0.03) and SE quadrants (t_8 = 6.77, P = 0.02), and Control and Rotated Near groups in the NW quadrant (t_{10} = 3.65, P = 0.02, and t_{10} = 3.20, P = 0.02, respectively).

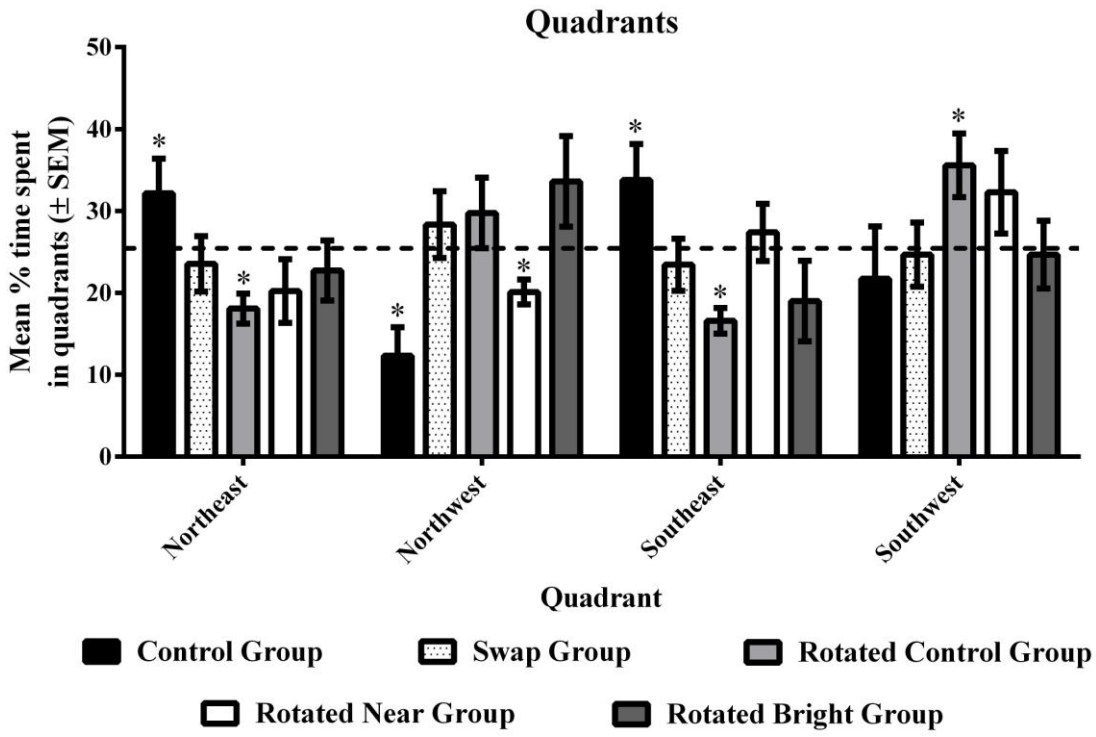

Figure 3.19: Mean percentage time (± SEM) spent in each quadrant by Control, Swap, Rotated Control, Rotated Near and Rotated Bright groups during the probe trial. Dashed line indicates chance level (25%).

3.5.3.2. Platform areas.

A 5 x 4 mixed factorial ANOVA was used to investigate time spent by groups in platform areas. Main effects of area, $F_{3,69} = 1.95$, $P = 0.15$, partial eta$^2 = 0.08$, and group, did not reach significance, $F_{1,23} = 1.30$, $P = 0.30$, partial eta$^2 = 0.19$. *Post hoc* tests were also non-significant. The area x group interaction effect was, however, significant, $F_{12,69} = 5.10$, $P = 0.0001$, partial eta$^2 = 0.47$. Group differences in individual areas were assessed using one-way between groups ANOVAs. Significant main effects were found in the NE ($F_{4,27} = 5.13$, $P = 0.01$), NW ($F_{4,27} = 10.08$, $P = 0.001$) and SW platform areas ($F_{4,27} = 3.66$, $P = 0.02$). No main effect was noted in the SE area, $F_{4,27} = 0.54$, $P = 0.71$.

Tukey multiple comparisons showed that Control group spent significantly longer in the NE platform area (10.28 ± 3.05%, CI [2.43, 18.13]) compared to the

three Rotated groups: Control (2.33 ± 0.81%, CI [0.23, 4.90]; $P = 0.03$), Near (2.00 ± 0.74%, CI [0.11, 3.90]; $P = 0.01$) and Bright (1.72 ± 0.66%, CI [0.02, 3.43]; $P = 0.01$) (see Figure 3.20). The Rotated Control group spent significant more time in the NW area (7.17 ± 2.91%, CI [2.53, 11.80]) than the Control (0.67 ± 0.23%, CI [0.08, 1.25]; $P = 0.001$), Rotated Near (1.83 ± 0.52%, CI [0.51, 3.16]; $P = 0.001$) and Swap groups (2.72 ± 0.53%, CI [1.35, 4.10]; $P = 0.01$) (see Figure 3.20). Despite a significant main effect in the SW area, Tukey *post hoc* tests were non-significant.

However, t-tests corrected for multiple comparisons indicated that Control animals spent less time in this region (2.06 ± 0.86%, CI [0.15, 4.26]) than the Rotated Control (7.25 ± 0.19%, CI [2.80, 11.70]; $t_8 = 3.37$, $P = 0.01$) and Rotated Bright groups (6.06 ± 1.46%, CI [2.30, 9.81]; $t_{10} = 2.36$, $P = 0.04$). The Swap group also spent less time here (1.83 ± 0.46%, CI [1.35, 2.32]) than Rotated Control ($t_8 = 4.80$, $P = 0.03$) and Bright groups ($t_{10} = 2.87$, $P = 0.02$). Although the Rotated Near group also preferred the SW area compared to Control and Swap groups, these differences did not reach statistical significance ($t_8 = 2.13$, $P = 0.07$, and $t_{10} = 2.38$, $P = 0.06$, respectively).

Time spent in platform areas was then assessed for each group individually using within-groups ANOVAs. Significant main effects were found for Control ($F_{3,15} = 6.26$, $P = 0.01$, partial eta$^2 = 0.56$), Swap ($F_{3,15} = 5.28$, $P = 0.02$, partial eta$^2 = 0.51$) and Rotated Control groups ($F_{3,9} = 7.71$, $P = 0.01$, partial eta$^2 = 0.72$). No main effect was found for the Rotated Near ($F_{3,15} = 3.66$, $P = 0.04$, partial eta$^2 = 0.42$) or Rotated Bright groups ($F_{3,15} = 1.92$, $P = 0.17$, partial eta$^2 = 0.28$). Bonferroni *post hoc* comparisons were non-significant for all groups. Accordingly, repeated measures t-tests were employed to determine if Control, Swap and Rotated

Control groups favoured specific areas over others, however, no significant differences were found for any group after Bonferroni correction.

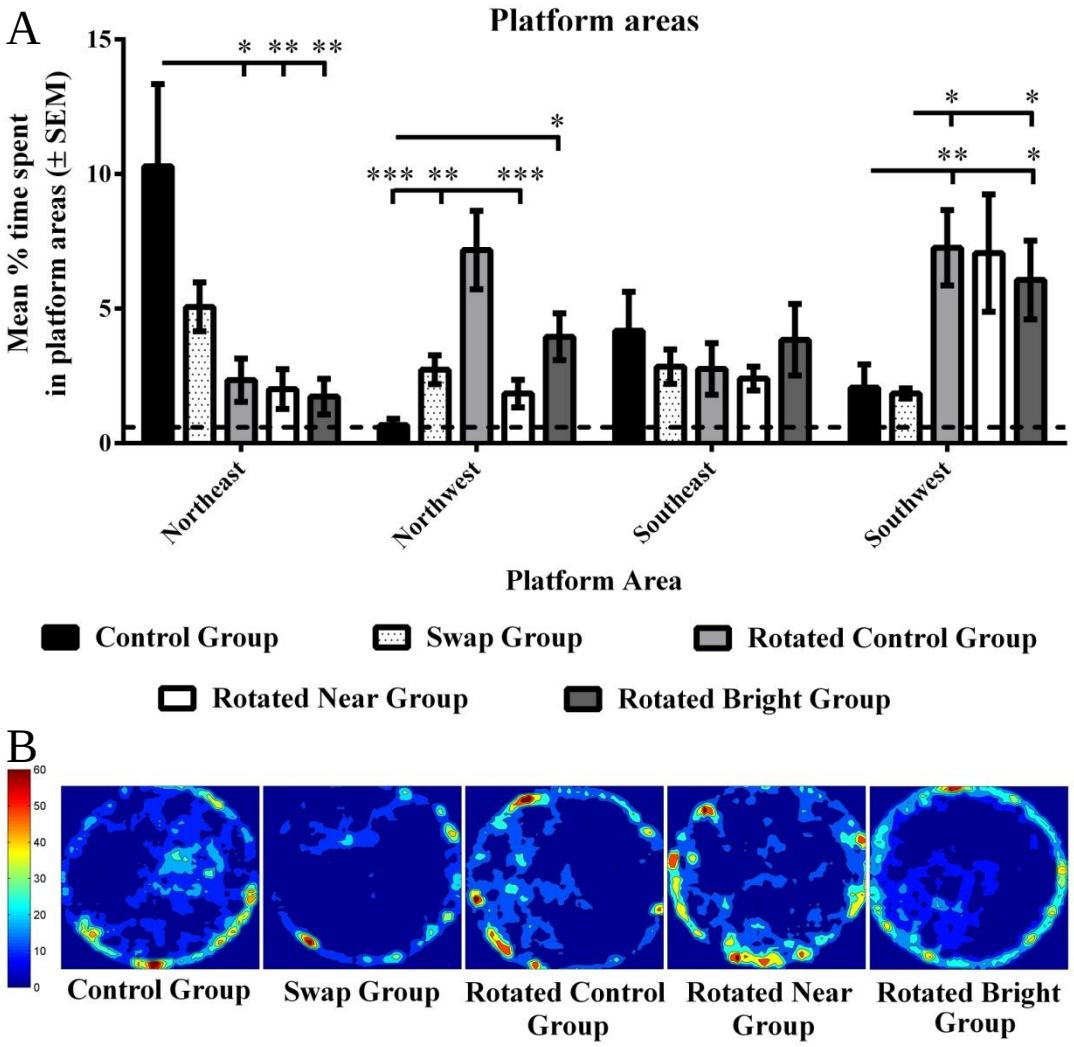

Figure 3.20: (A). Mean percentage time (± SEM) spent in platform areas by Control, Swap, Rotated Control, Rotated Near and Rotated Bright groups during the probe trial. (B). Heat maps illustrating overall searching behaviour during testing by each group. Dashed line indicates chance level (0.6%).

3.5.3.3. Outer corridor.

A one-way between groups ANOVA failed to yield a significant main effect of group, $F_{4,27} = 0.40$, $P = 0.81$ (see Figure 3.21).

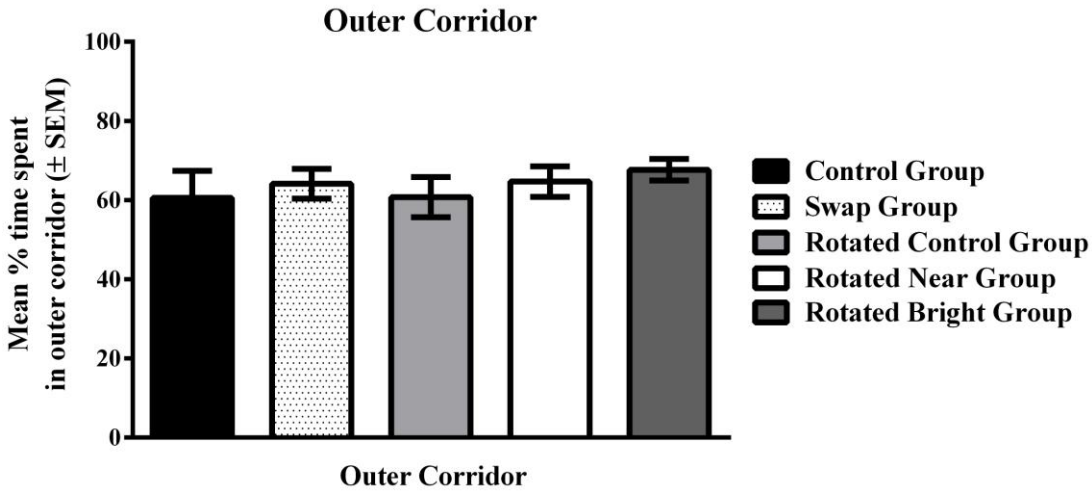

Figure 3.21: Mean percentage time (± SEM) spent in the outer corridor by Control, Swap, Rotated Control, Rotated Near and Rotated Bright groups during testing.

3.5.4. Discussion

All rats acquired the task, as illustrated by the significant reduction in escape latencies and path lengths across training. Regarding recall, the observed difference between Control and Swap groups (in the NE quadrant) showed that the latter group identified the change in cue configuration; thus suggesting that rats could in fact distinguish between the cues based on their brightness. This finding is important because it confirms that rats were not merely relying on the overall cue configuration (regardless of individual cue characteristics), but rather, the cues were acquiring individual saliences, as suggested by Experiments 1-3. The lack of searching specificity displayed by the Swap group can likely be explained by the altered cue configuration, i.e. this condition was the only one in which the spatial relationship of the cues was different from that of training. As expected, when both cues were rotated 180°, rats' searching behaviour adjusted accordingly. More specifically, the Rotated Control group displayed a preference for the SW quadrant and platform area, indicating that the spatial relationship between the platform and the cues was preserved. By comparison, rats tested with a single cue rotated appeared to be

somewhat impaired, although they did appear to modify their searching behaviour in response to the novel testing environment (i.e. neither showed a preference for the original NE target zones). In addition, the searching behaviour of the Rotated Bright group (in the SW platform area) is indicative that this group were relying on the learned relationship between the bright cue and the platform, as observed in previous experiments. Interestingly, the Rotated Near group did not favour the SE region of the pool – as would be expected if rats had navigated via the near cue. Instead, they exhibited similar searching patterns to the Rotated Bright group, although these differences were not significant. Taken together, these results suggest that rats tested with a rotated near cue may have misidentified it as the bright cue (akin to Experiment 1). Overall, findings support the suggestion that rats learned the location of the platform with reference to the distal cues, as opposed to other unknown environmental cues, and that the brighter of the two cues acquired more salience during training.

3.6. General discussion

The goal of this chapter was to examine the influence of cue salience and training on the type of allocentric spatial learning strategy used in the Morris water maze. Experiment 1 revealed that individual cue characteristics – in this case brightness – can become more salient, and thus acquire more control over animals' searching behaviour, than relative proximity to the goal. Experiment 2 showed that reliance on the more salient (brighter) cue can be increased if this cue is also closer to the goal. This finding supports the idea that multiple components of cue salience can have an additive effect on behaviour (Bennett, 1996; Chamizo, Rodrigo, & Mackintosh, 2006). Together, results also suggest that the presence of a brighter cue may have interfered with the amount of spatial information rats learned about the alternate cue throughout training (near cue in Experiment 1 and far cue in Experiment 2), i.e. an interference by salience effect (Crespo, Rodriguez, & Chamizo, 2012; Rodrigo et al., 2014). Furthermore, we observed that greater disparity between the saliences of cues can lead to more interference. More specifically, rats navigating with a less luminous near cue misdirected their searching to regions (i.e. SE quadrant and area) appropriate for the bright cue (Experiment 1), whereas those navigating with a cue that was both dimmer and farther from the platform appeared to be completely impaired (Experiment 2). This disproportionate reliance on one cue relative to the other is indicative of an elemental strategy which was acquired quickly, i.e. after only twenty training trials in Experiment 1, which was less than half of the number administered by Rodrigo and colleagues (2014).

When brightness was removed as a component of cue salience in Experiment 3, a different pattern of results emerged. Rats trained with two equally bright cues for five days failed to find the correct platform area using either cue in isolation,

whereas after forty training trials, rats navigating with the far cue (in addition to Controls) displayed good recall (NE quadrant and area). The perceived change in navigational ability suggests that the cues initially acquired similar saliences, but that the far cue became more salient than the near cue over time. This may point towards the use of a configural strategy with the intact cue arrangement at first, followed by a shift towards elemental processing involving the far cue. Here, the interference by salience effect appears to have been dependent on additional training (Crespo et al., 2012; Rodrigo et al., 2014). As mentioned, the delayed emergence of an elemental strategy in Experiment 3 compared with Experiments 1 and 2 could be due to the fact that cues were visually indistinguishable in this experiment. It is probable that the enhanced perceptual similarity of the cues made it difficult for rats to differentiate between them during the early stages of training, causing them to rely on both cues to orient towards the platform. This result is in line with Rodrigo and colleagues (2014), who showed that rats trained in the water maze with two cues of equivalent saliences navigated via a configural strategy.

Unexpectedly, the far cue acquired greater behavioural control after extended training, despite offering no obvious advantage over the near cue. One simple explanation for this is that rats made use of incidental room cues unknown to the experimenter. Indeed, it is difficult to determine the features of an environment that will be considered most salient to a rat (Young et al., 2006). However, unintentional visual cues were obscured from view by the addition of the surrounding curtain and by the administration of all training and testing in complete darkness. Further, it is doubtful that animals were relying on static auditory or olfactory cues (e.g. air conditioning) as, if this were the case, we would have expected groups to navigate equally well regardless of which cue was removed. Moreover, the results of

Experiment 4 – whereby rats altered their behaviour when the cue positions were rotated (i.e. increased searching in the SW quadrant and area) – suggest that animals were using the distal cues to navigate. One further straightforward suggestion is that rats were using an inertial sense of direction via their vestibular system to guide navigation, as previously shown by Cheng (1986). However, the use of multiple start positions during training as well as novel start positions during recall makes this, or the use of habitual or procedural responding (Packard & McGaugh, 1992), an unlikely explanation.

Nevertheless, rats evidently learned to distinguish between the cues on some non-salient physical feature, which resulted in the far cue acquiring more salience. We propose that rats discriminated between the cues based on their spatial position relative to the platform. Furthermore, we suggest that the positioning of the distal cue allowed for a more reliable estimation of the platform location than the proximal cue, causing the former to become more salient. Research has shown that errors in estimating distance tend to increase more rapidly than directional errors as a cue gets farther from the goal (Kamil & Cheng, 2001; Kamil & Jones, 1997, 2000; Kelly, Kamil, & Cheng, 2010). Therefore, we can reasonably assume that the far cue in the current experiments was a better indicator of directional (rather than distance) information. Previous work with rats in the water maze has also demonstrated that a loss of directional information affects performance more negatively than a comparable loss of distance information, suggesting that the former is weighted more heavily (Diviney et al., 2013; see Forloines, Bodily, & Sturz, 2015; Kamil & Jones, 2000, for similar evidence in humans and birds, respectively).

In addition, research in desert ants has highlighted the importance of cue elevation for navigation, whereby cues of a lower elevation allow for a more precise

estimation of direction (Müller & Wehner, 2007). Crucially, the elevation of the farther cue (positioned 162cm from the platform) was lower than that of the closer cue (127cm from the platform) in the present set of experiments. Moreover, the elevation of the near cue would have increased as animals approached and mounted the platform (making it more difficult to gauge directional information), whereas the elevation of the far cue would have remained relatively stable. Therefore, taking the elevations of the cues into account, in combination with the importance of directional information, it seems reasonable that rats would regard the distal cue as more useful; however, future work exploring cue elevations systematically in the water maze is needed to confirm this suggestion.

If rats had established the far cue as a primary source of directional information, the question of how they navigated without a second cue to provide distance information still remains. To account for this, we suggest that the perimeter of the maze played an important role in the estimation of distance, and ultimately in establishing the far cue's higher salience. The use of the pool wall as an aid in locating the platform is well-documented (Austen, Kosaki, & McGregor, 2013; Hamilton, Akers, Weisend, & Sutherland, 2007; Harvey et al., 2009). Specifically, rats have been shown to swim in circles around the maze at a set distance from the pool wall in search of the platform, indicating that they can easily estimate distance information from the wall (Alvarado & Rudy, 1995; Artigas et al., 2005; Chamizo, Manteiga, et al., 2006; Maurer & Derivaz, 2000). Importantly, animals would be unable to obtain directional information from the shape of the maze in the current set of experiments, as has been illustrated previously (Pearce, 2009), due to its circular shape. We posit that in these experiments, the near cue could have been replaced by

the pool wall relatively easily as a result of its position close to and in the same quadrant as the platform.

Although our results are indicative of an elemental learning strategy involving the more salient of two cues, we cannot definitively rule out the use of a configural strategy. As Rodrigo and colleagues (2014) state, the separation of elemental and configural learning strategies is not easily achievable. According to configural accounts, elemental representations are retained in memory, although they do not become directly associated with the goal (Pearce, 1987; 1994). Thus, once established, a configural representation can be proportionately activated by any of its original elements (Rodrigo et al., 2014; Sutherland & Rudy, 1989). As such, it is possible that rats established a configural representation with both cues (and the pool wall) which was then generated during testing with a single cue (Rodrigo et al., 2014). However, if this were the case, we would have expected animals to find the correct platform location using either cue in isolation, i.e. with the near cue in Experiments 1, 3 and 4 and the far cue in Experiment 2. That is, the remaining cue should have triggered a representation of the overall configuration including the absent cue, allowing rats to navigate accurately (Rodrigo et al., 2014).

In sum, findings from the current chapter lend support to the idea of an enhanced flexibility of spatial behaviour (Sturz & Katz, 2009). Rather than being mutually exclusive, it seems more likely that searching behaviour can come under the control of whichever strategy (elemental or configural) is most beneficial for navigating a particular environment (Biegler & Morris, 1999; Kamil & Jones, 1997, 2000; Rodrigo et al., 2014). Our results provide novel evidence that the utility of a strategy is at least partially determined by the relative saliences of the cues and the length of training. When one cue is notably more salient than the other, rats quickly

learn to rely on the spatial information offered by this cue instead of the entire arrangement, which may be suggestive of a learning efficiency. Furthermore, rats' ability to navigate using this strategy develops more slowly when the available cues are of similar saliences.

Having investigated some of the key behavioural features associated with allocentric spatial learning in the water maze, we next explored the neurochemical and anatomical underpinnings of such behaviour. Specifically we investigated the role of NMDA and AMPA receptors in two key brain regions – the hippocampus and medial prefrontal cortex – during water maze acquisition. We also used IEG imaging to probe for evidence of plasticity in these areas.

Chapter 4

Examining the Effects of Glutamate Receptor Blockade on Spatial Learning and Immediate Early Gene Expression in the Hippocampus and Prefrontal Cortex

Parts of this Chapter have been published as Farina, F. R., & Commins, S. (2016). Differential expression of immediate early genes Zif268 and c-Fos in the hippocampus and prefrontal cortex following spatial learning and glutamate receptor antagonism. *Behavioural Brain Research, 307*, 194-198.

Abstract

The hippocampus is critical for spatial memory encoding, and is facilitated by NMDA receptor activation. The medial prefrontal cortex is also involved in spatial processing; however, its specific role during early stages of memory formation remains unclear, as preceding research has yielded mixed results. Over two experiments, we investigated the contribution of glutamate receptor activation to spatial memory acquisition and IEG expression in the hippocampus and medial prefrontal cortex. In Experiment 1, the effects of glutamate antagonism on basal expression of two IEGs, Zif268 and c-Fos, in hippocampal and prefrontal sub-regions were examined. Experimentally naïve rats received injections of NMDA channel blocker MK-801, AMPA receptor antagonist CNQX or saline (all i.p.) over five days. Results failed to show any significant differences between drug and saline groups, indicating that glutamate receptor blockade had no impact on baseline gene expression. In Experiment 2, rats received MK-801, CNQX or saline i.p. injections before water maze training each day for five days. Levels of Zif268 and c-Fos expression were quantified after training on day 5. Behaviourally, Saline and CNQX groups acquired the water maze task while MK-801-treated animals were impaired, as evidenced by significantly slower escape latencies on day 5. IEG imaging revealed different patterns for Zif268 and c-Fos across brain regions, with Zif268 levels being more closely related to learning-related activation in the hippocampus and prefrontal cortex.

4.1. Introduction

The dorsal hippocampus is widely accepted to be a crucial brain region for spatial memory encoding. Over the last 40 years, converging evidence from lesion, genetic, electrophysiological and neuroimaging studies has supported its central role in spatial processing (Burgess, Maguire, & O'Keefe, 2002; Moser, Moser, & Andersen, 1993; Nakazawa, McHugh, Wilson, & Tonegawa, 2004; Silva, Giese, Fedorov, Frankland, & Kogan, 1998). Hippocampal involvement in spatial memory acquisition is mediated by NMDA receptors (Bliss & Collingridge, 1993; Martin et al., 2000). Research has shown that antagonism of these receptors within the hippocampus reliably impairs performance in the water maze (Bast et al., 2005; Davis, Butcher, & Morris, 1992; Morris, Halliwell, & Bowery, 1989; Pitkänen et al., 1995; Whishaw & Auer, 1989). Encoding deficits have also been observed following hippocampal AMPA/kainate receptor antagonism (Cain, Saucier, Hall, Hargreaves, & Boon, 1996; Filliat, Pernot-Marino, Baubichon, & Lallement, 1998; Liang et al., 1994; Riedel et al., 1999). However, because AMPA/kainate receptor blockade can reduce NMDA receptor activation, it is possible that these effects were NMDA receptor-related (Riedel, Platt, & Micheau, 2003).

The importance of the medial prefrontal cortex for successful way-finding is also well-established (Simons & Spiers, 2003). This region is thought to be involved in motivational aspects of spatial performance such as route planning and flexible responding (Hok et al., 2005; Rich & Shapiro, 2009). Recently, place cells have been identified within the prefrontal cortex (Hok et al., 2005). In addition, prefrontal cells which respond to hippocampal stimulation are activated by both NMDA and AMPA receptor agonists (Jay, Thierry, Wiklund, & Glowinski, 1992), and can be blocked by antagonists (Gigg, Tan, & Finch, 1994; Jay et al., 1992). These findings confirm

that neurotransmission between the hippocampus and medial prefrontal cortex is glutamate receptor-dependent (Laroche et al., 2000).

Despite much research into the functioning of the medial prefrontal cortex, its significance – and that of its specific sub-regions – during the early stages of memory formation remains unclear (Wang & Cai, 2008). Lesion studies investigating prefrontal involvement in allocentric spatial learning have yielded mixed results. Some authors have reported no learning deficits in rats with whole or partial (prelimbic) lesions in water maze tasks (Compton et al., 1997; de Bruin et al., 2001; de Bruin et al., 1994; Lacroix et al., 2002; Maaswinkel, Baars, Gispen, & Spruijt, 1996). In contrast, others have found mild or marked effects on spatial task acquisition in rats with lesions to the entire medial prefrontal cortex or to the anterior cingulate sub-region (Mogensen, Lauritsen, Elvertorp, Hasman, Moustgaard, & Wörtwein, 2004; Sutherland et al., 1988; Warburton, Aggleton, & Muir, 1998). While these contrasting findings might be explained by subtle procedural differences as Hok and colleagues (2005) suggest, it seems likely that they also reflect limitations of the lesion approach. Specifically, lesion site specificity is difficult to achieve; thus, variations in behavioural performance could be due to differences in the extent or location of cortical damage (Aggleton & Brown, 2005; Poirier, Amin, & Aggleton, 2008).

Immediate early gene (IEG) imaging circumvents this problem, allowing for the non-invasive visualisation of neuronal activation patterns across multiple intact brain regions simultaneously (Sauvage, Nakamura, & Beer, 2013). As expected, considerable increases in Zif268 and c-Fos expression have been found in CA1, CA3 and dentate gyrus areas of the dorsal hippocampus following short- and long-term water maze training (Feldman, Shapiro, & Nalbantoglu, 2010; Guzowski et al.,

2001;Jenkins et al., 2003; Teather, Packard, Smith, Ellis-Behnke, & Bazan, 2005; Teather et al., 2005); although, this effect appears to be time-dependent. That is, while the abovementioned studies revealed heightened IEG expression up to ninety-minutes post-training, Richter-Levin, Thomas, Hunt and Bliss (1998) failed to find any differences in Zif268 expression between trained and naïve rats in the dentate gyrus after three hours.

To date, two studies have also examined IEG expression levels in the medial prefrontal cortex during spatial learning in the water maze (Jenkins et al., 2003; Woolley et al., 2013). Jenkins and colleagues (2003) trained two groups of rats in the water maze over twelve days; a landmark group and a place group. The landmark group were trained to locate a hidden platform using a beacon, which was rotated (along with the platform) for each trial. For the place group, matching-to-place training was used whereby rats learned to navigate to the platform using room cues, with the platform location changing on each training day. c-Fos expression for both groups was measured ninety-minutes post-training on day 12. Interestingly, the authors found greater levels of c-Fos expression in the anterior cingulate cortex, but not in the prelimbic cortex, for the landmark group compared to the place group (Jenkins et al., 2003). This increase could be explained by a key procedural difference between the groups; namely, the landmark group encoded twice as many platform positions as the place group (24 versus 12) during training. Therefore, these expression patterns could reflect an increased demand on goal representation. More recently, Woolley *et al.* (2013) examined Zif268 expression in the medial prefrontal cortex for mice trained in the water maze for three or thirty days. Zif268 levels quantified forty-five minutes after the final training session were significantly higher for the early (3-day) compared to the late (30-day) learning group, further indicating

the involvement of the prefrontal cortex in initial memory formation (Woolley et al., 2013).

Collectively, the abovementioned data demonstrate that both glutamate receptor activation and IEG expression are strongly associated with spatial memory encoding. However, the way in which these processes interact during the formation of new spatial memories has yet to be investigated. Furthermore, the importance of these interactions within specific brain regions is unknown. Recently, Czerniawski and colleagues (2011) provided the first description of glutamate-IEG interdependency in hippocampal-dependent fear conditioning. Rats received bilateral dorsal hippocampus infusions of NMDA receptor antagonist APV before a single session of fear conditioning. Activation of the IEG Arc was examined one hour later. Results illustrated a significant attenuation of Arc expression in APV rats compared to cage controls, signifying that NMDA receptor activation and Arc expression are functionally coupled during memory for fear conditioning (Czerniawski et al., 2011).

The overarching aim of this chapter was to characterise the contributions of NMDA and AMPA receptors to spatial memory encoding and IEG expression in the hippocampus and medial prefrontal cortex. Existing evidence regarding the prefrontal cortex is largely mixed, and studies to date have provided IEG data as a single structure only (Woolley et al., 2013) or have failed to include all sub-regions (Jenkins et al., 2003). Here, we analysed Zif268 and c-Fos expression in all hippocampal and prefrontal sub-regions. This enabled us to quantify the relative contributions of individual areas for memory acquisition. Moreover, including all areas allowed us to directly compare expression across hippocampal and prefrontal regions. Two experiments were conducted. Experiment 1 examined the effects of NMDA and AMPA blockade on basal expression levels of Zif268 and c-Fos, relative

to saline-treated animals. We hypothesised that higher drug concentrations would lead to significant changes in IEG expression, and that lower doses would have no effect. Following on from Experiment 1, we compared levels of IEG activation in hippocampal and medial prefrontal areas following water maze training in saline and drug treatment groups. It was predicted that NMDA channel blockade would significantly impair rats' ability to acquire the water maze task and cause a reduction in IEG expression, but that AMPA receptor antagonism would have little or no effect on either.

4.2. Experiment 1

The goal of Experiment 1 was to establish the effects of NMDA channel blocker MK-801 and AMPA receptor antagonist CNQX on basal expression levels of Zif268 and c-Fos in the hippocampus and medial prefrontal cortex. Previous research has shown that administration of MK-801 at high concentrations (between 0.3 and 3mg i.p.) results in a down regulation of basal hippocampal Zif268 expression (Gass et al., 1993), but also induces significant locomotor deficits (Hargreaves & Cain, 1992; Whishaw & Auer, 1989). Similar effects have been reported for high doses of CNQX (between 10 and 30µg i.c.v. infusions) (Cain et al., 1996). Increases in medial prefrontal Zif268 and c-Fos expression have been documented using a lower dose of MK-801 (0.1mg) one hour post-injection (as well as three hours post-injection for Zif268) (Gao, Hashimoto, & Tamminga, 1998). These effects were amplified with a stronger dose (1mg); however, no differences in hippocampal expression were found between MK-801 and control animals (Gao et al., 1998). Accordingly, a secondary aim of this experiment was to determine if NMDA or AMPA receptor antagonism influenced expression of Zif268 of c-Fos in a dose-dependent manner.

4.2.1. Method.

4.2.1.1. Subjects.

Male Wistar rats (*n* = 25; Charles River, UK) were used as subjects (see Chapter 2 for age and weight specifications). All were managed and housed in similar conditions as described previously.

4.2.1.2. Procedure.

All animals were placed in the testing room for two hours prior to injections. Rats were randomly divided into five different groups (*n* = 5 per group). Four experimental groups were used; two MK-801 groups and two CNQX groups. The MK-801 groups were administered with MK-801 at one of two doses (Low: 0.05 mg/kg or High: 0.1 mg/kg). The CNQX groups received injections of CNQX at a dose of 0.75 mg/kg (Low) or 1.5 mg/kg (High). Two concentrations of each drug were used to investigate potential dose dependent differences in IEG expression. Sterile saline was used as the vehicle for all drugs (0.3ml total volume per injection). As a control, a fifth group of animals were injected with a saline solution (0.1 ml/100 g body weight of 0.9% NaCl). Each rat received one i.p. injection per day for a total of five days (matched to spatial learning conditions; see Section 4.3.1.3). All injections were administered in a separate experimental room, in order to minimise animals' stress. After drug administration, animals were returned to their home cages and periodically monitored for drug-induced locomotive behaviours.

4.2.1.3. Tissue preservation.

Ninety minutes post-injection on day five, rats were terminally anaesthetised, perfused transcardially, and their brains were removed, post-fixed and cryoprotected as outlined in Chapter 2. Brains were sliced on a freezing microtome from -3.24mm to -4.08mm Bregma into 40-μm-thick coronal sections (four sections per IEG per region). The hippocampus and medial prefrontal cortex were included as regions of interest.

4.2.1.4. Immunohistochemistry.

Immunohistochemical staining was performed for all groups in a single session, thus negating the need for subsequent normalisation of the cell counts. Staining was carried out in cohorts of five, with one animal from each group being processed simultaneously in the same well plate. Immunohistochemical protocol was completed as described previously in Chapter 2.

4.2.1.5. Data analysis.

Zif268 and c-Fos immunopositive cell counts in hippocampal and medial prefrontal sub-regions were automatically calculated using ImageJ software with pre-defined brightness intensity and particle size thresholds (see Chapter 2 for details). Raw counts from each section were averaged to produce a mean for each animal. Mean counts for each animal were then averaged to yield group means.

4.2.1.6. Statistical Analysis.

To compare levels of Zif268 and c-Fos expression across groups, a series of one-way between-groups ANOVAs were carried out on mean cell counts in each region. Tukey *post hoc* tests were employed where appropriate.

4.2.2. Results

4.2.2.1. Zif268 expression.

One-way between-groups ANOVAs failed to yield any significant main effects of group for any region of the hippocampus or medial prefrontal cortex; CA1: $F_{4,24}$ = 1.63, P = 0.21, CA3: $F_{4,24}$ = 1.96, P = 0.14, dentate gyrus (DG): $F_{4,24}$ = 0.20, P =

0.93, prelimbic cortex (PLC): $F_{4,24}$ = 1.21, P = 0.34, anterior cingulate cortex (ACC): $F_{4,24}$ = 0.98, P = 0.44, and infralimbic cortex (ILC): $F_{4,24}$ = 0.32, P = 0.86 (see Figure 4.1). Sample sections of Zif268 expression in hippocampal and medial prefrontal sub-regions are shown in Figures 4.2 and 4.3, respectively.

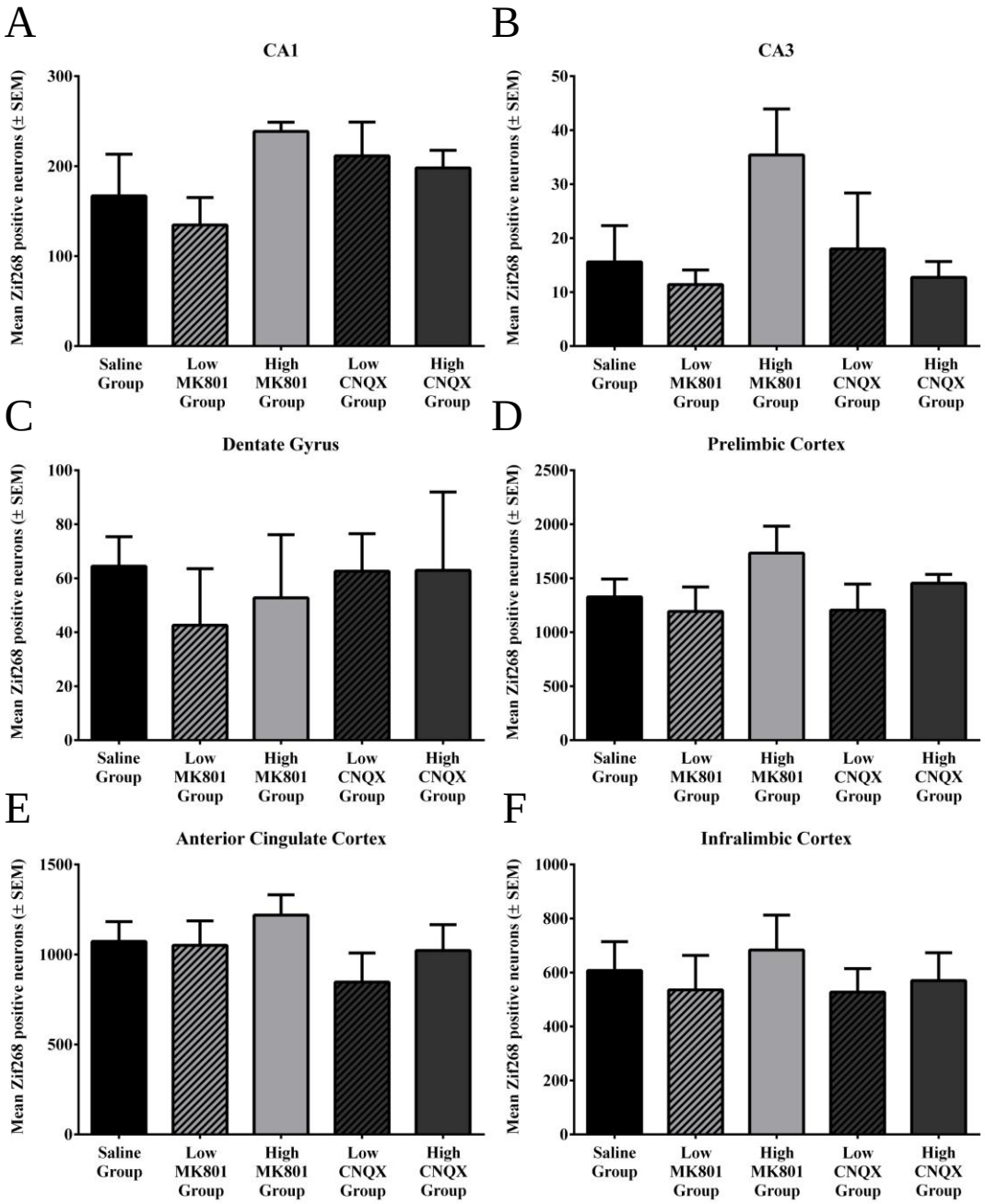

Figure 4.1: Mean cell counts of Zif268 positive neurons for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

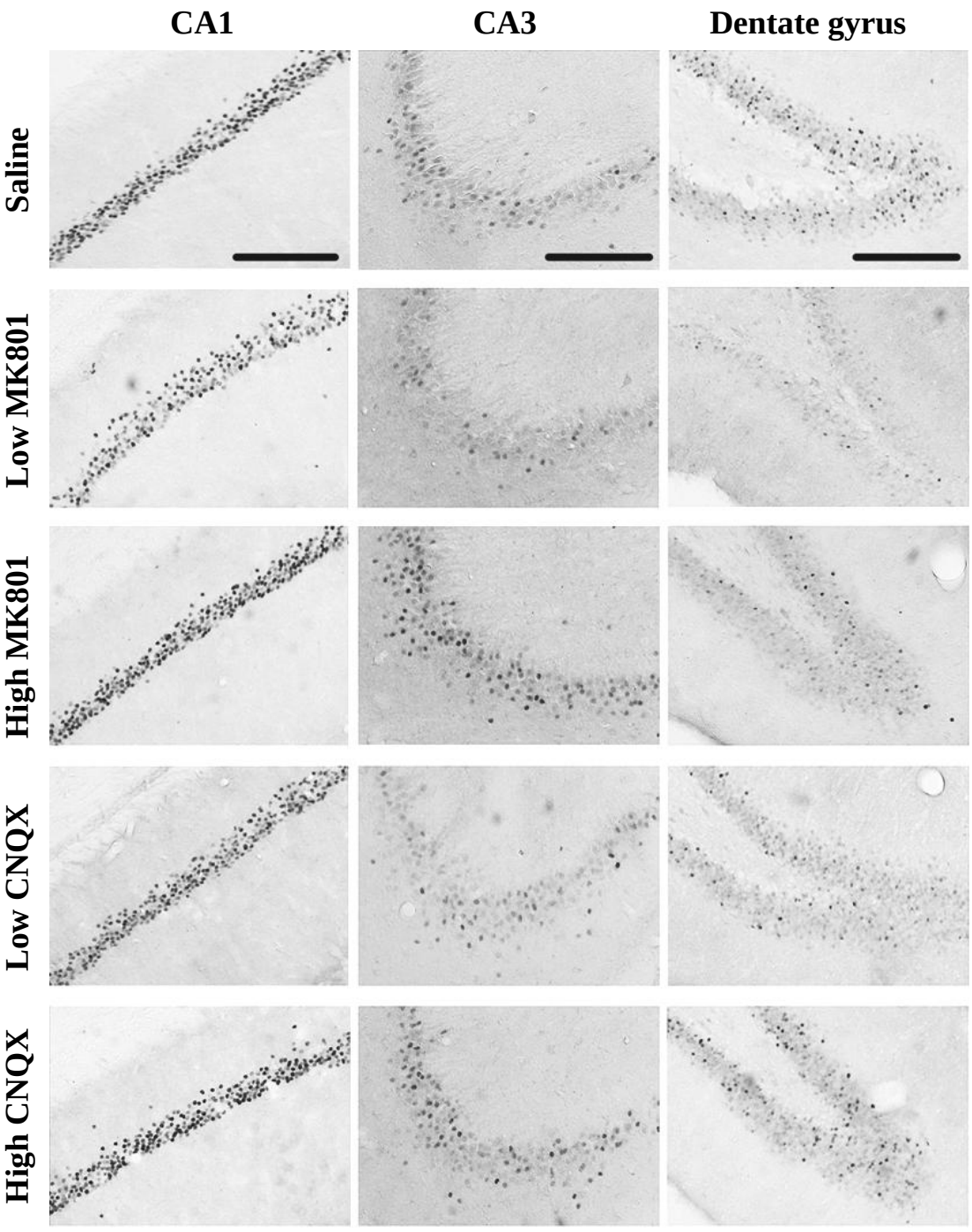

Figure 4.2: Representative images of Zif268 expression for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in CA1, CA3 and the dentate gyrus. Scale bar = 100μm. For ease of viewing, all representative images have been cropped from the overall cell area analysed.

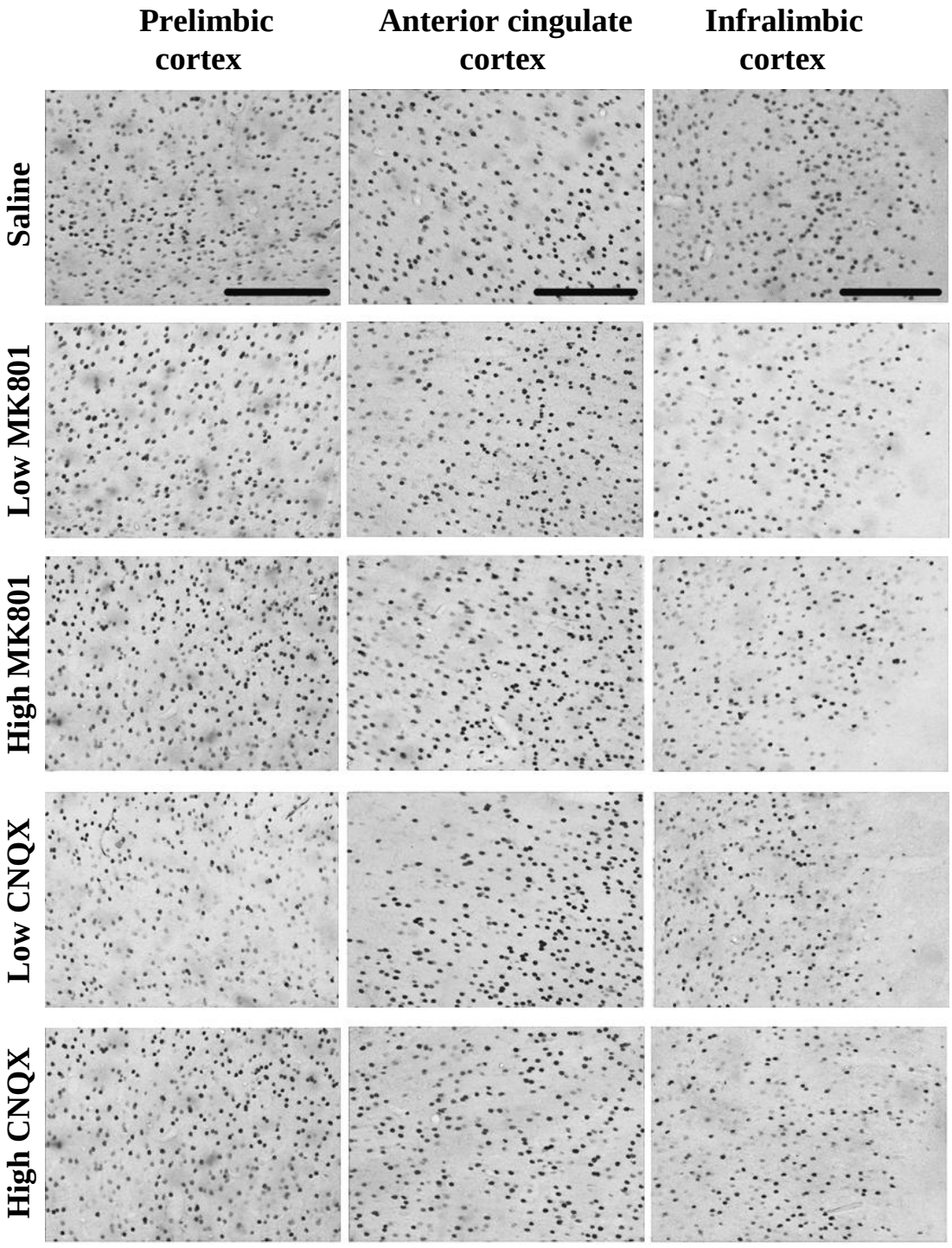

Figure 4.3: Representative images of Zif268 expression for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

4.2.2.2. c-Fos expression.

For c-Fos expression, no significant group differences were found in the hippocampus; CA1: $F_{4,24} = 0.99$, $P = 0.44$, CA3: $F_{4,24} = 0.22$, $P = 0.92$, DG: $F_{4,24} = 1.91$, $P = 0.15$ (see Figure 4.4A-C). In the medial prefrontal cortex, main effects of group were not significant: PLC ($F_{4,24} = 2.65$, $P = 0.06$), ACC ($F_{4,24} = 2.82$, $P = 0.05$) and ILC ($F_{4,24} = 1.31$, $P = 0.30$; see Figure 4.4D-F. Representative sections of c-Fos expression in sub-regions of the hippocampus and medial prefrontal cortex are illustrated in Figures 4.5 and 4.6, respectively.

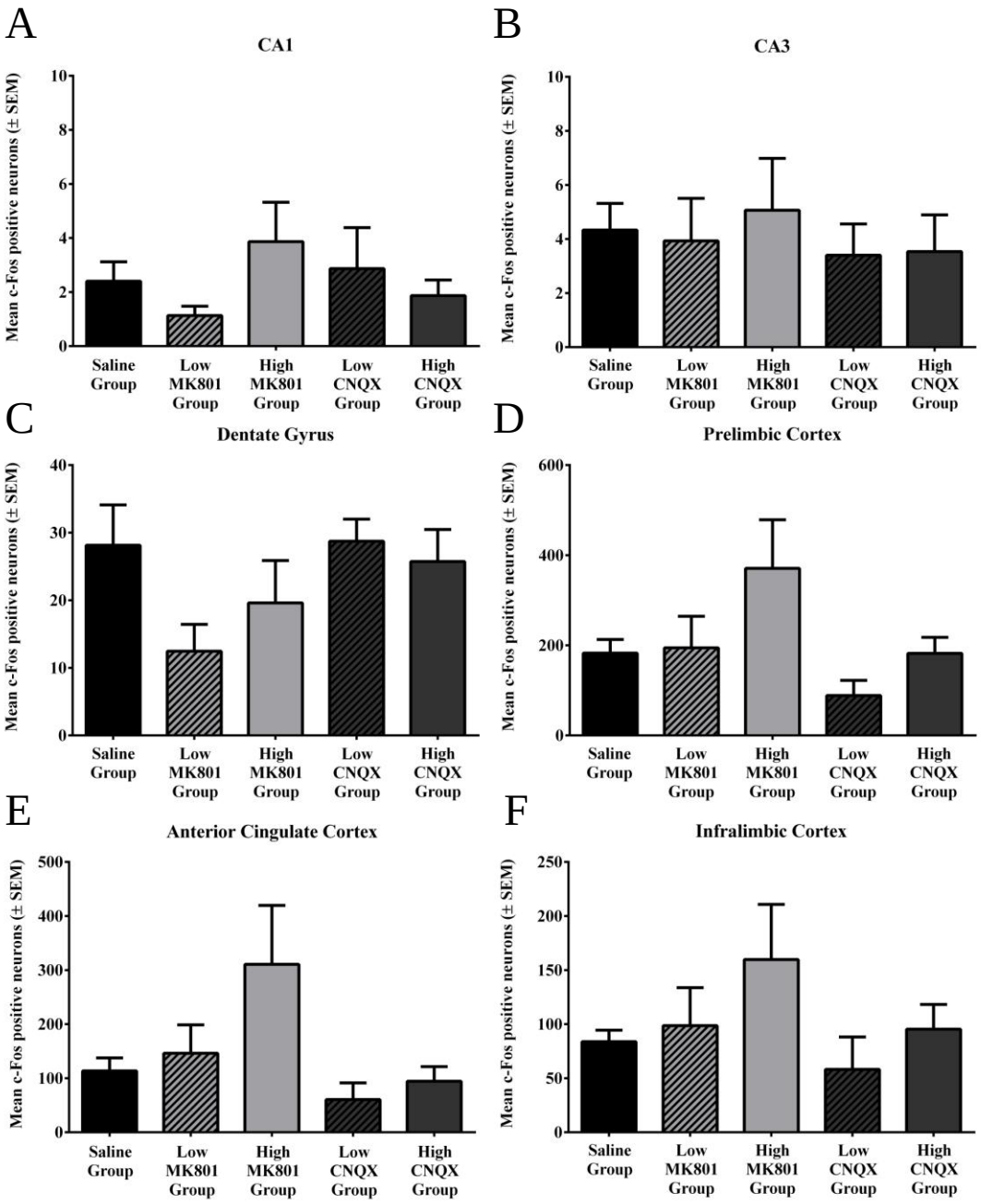

Figure 4.4: Mean cell counts of c-Fos positive neurons for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

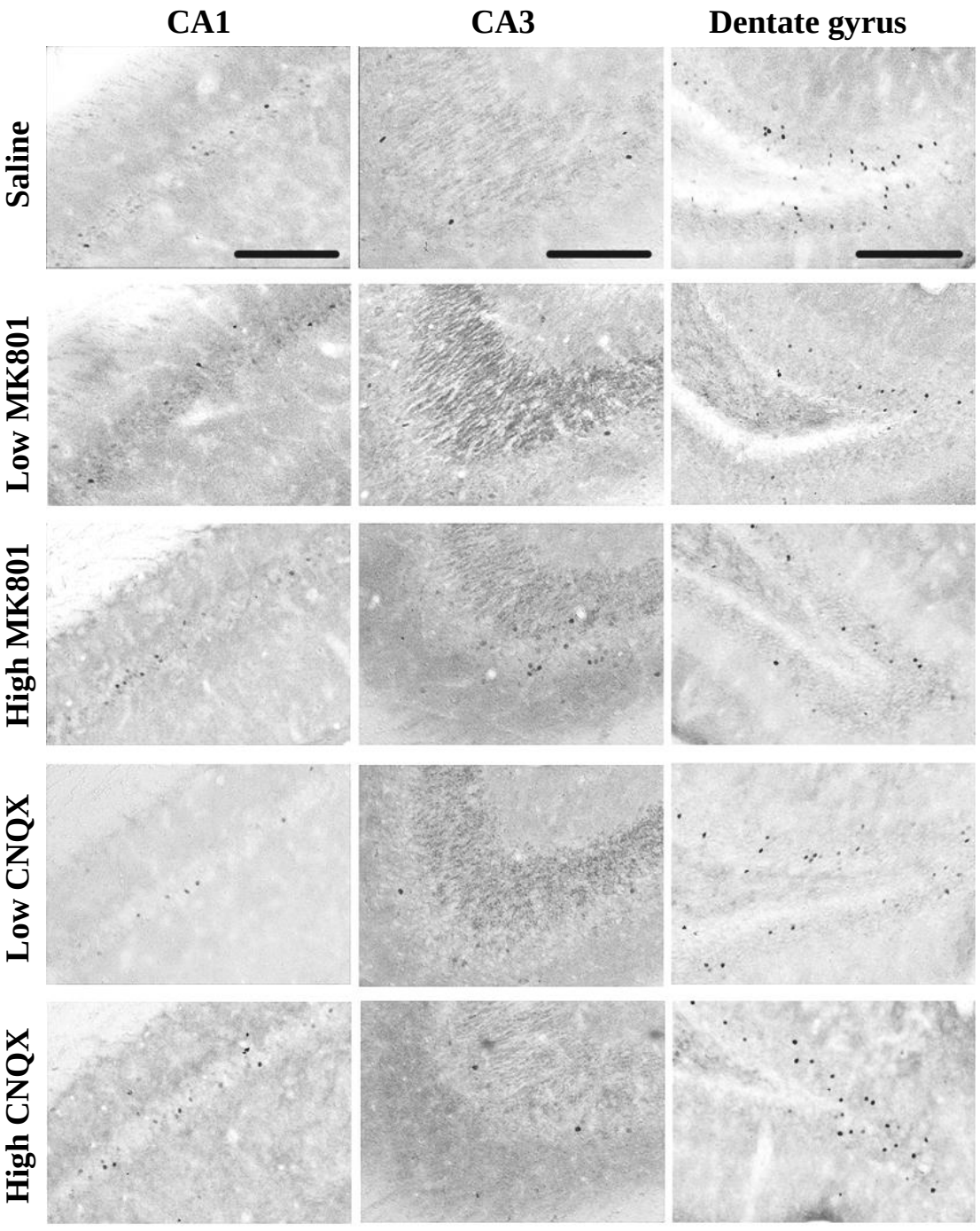

Figure 4.5: Representative images of c-Fos expression for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in CA1, CA3 and the dentate gyrus. Scale bar = 100μm.

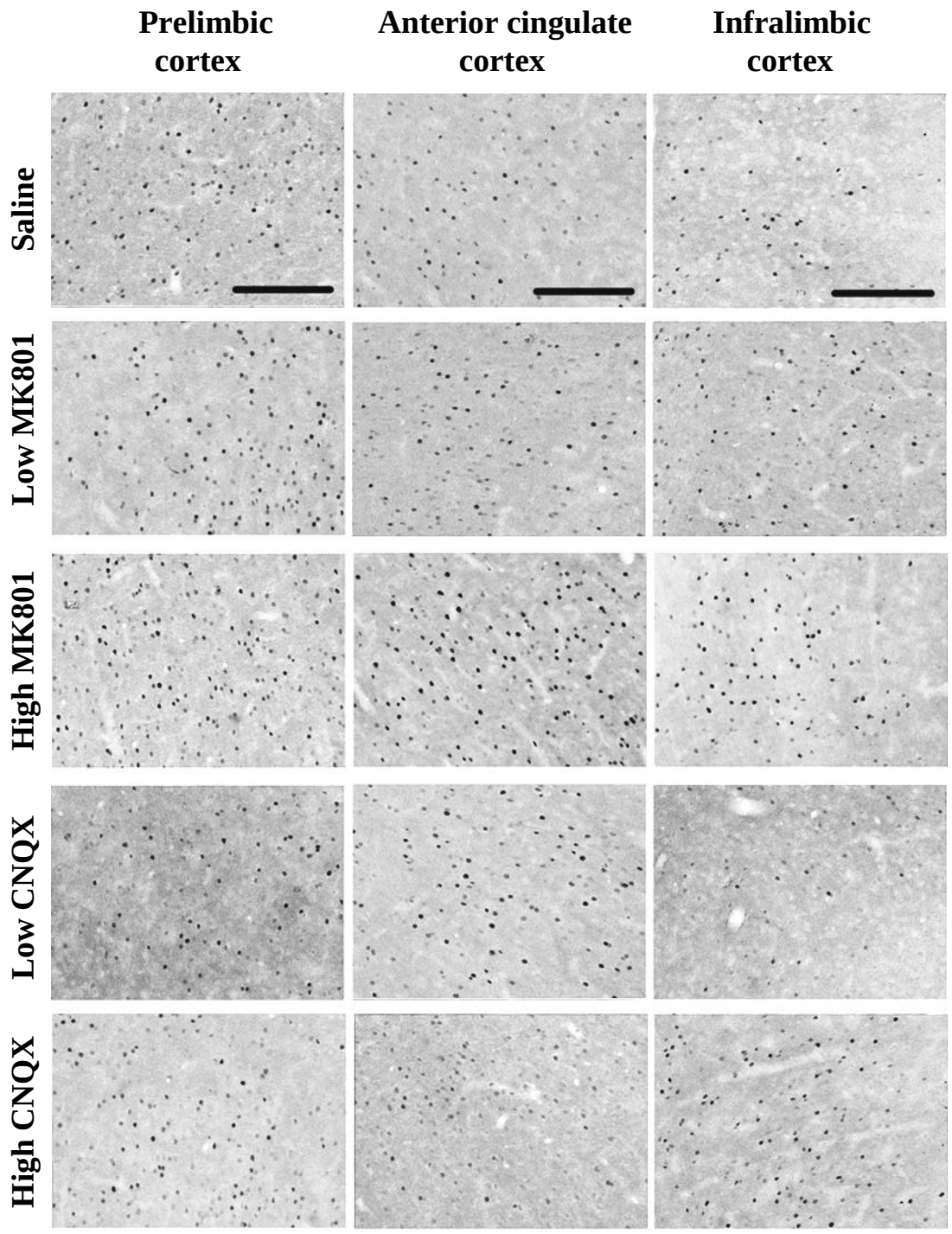

Figure 4.6: Representative images of c-Fos expression for Saline, Low MK-801, High MK-801, Low CNQX and High CNQX groups in the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

4.2.3. Discussion

No significant deviations from Saline animals' Zif268 expression levels were found for any drug-treatment group. This result suggests that administration of MK-801 or CNQX had no effect on basal Zif268 activation in any sub-region of the hippocampus or medial prefrontal cortex at the doses used. Additionally, no differences in expression were observed between low and high doses of MK-801 or CNQX, indicating that these drugs did not influence expression of Zif268 in a dose-dependent manner. Similarly that administration of MK-801 or CNQX failed to influence hippocampal or prefrontal c-Fos expression (relative to saline-treated rats), and no dose dependent changes were found. In contrast to preceding research (Gao et al., 1998), findings from this experiment support the hypothesis that NMDA and AMPA receptor antagonism has no effect on baseline IEG expression. The absence of drug effects here could be explained by the concentrations used, which were comparatively low (relative to Cain et al., 1996; Gass et al., 1993), as well as differences in the time at which IEG expression was measured, i.e. Gao and colleagues (1998) quantified activation one or three hours post-injection, while we used a ninety-minute protocol.

4.3. Experiment 2

The purpose of Experiment 2 was to investigate the influence of NMDA and AMPA receptor blockade on spatial learning and post-learning expression of the IEGs Zif268 and c-Fos in the hippocampus and medial prefrontal cortex. Two control groups were employed for this experiment: a Cage Control group and a Saline group, to control for varying aspects of task acquisition such as stress and swimming, which may obscure the interpretation of results (Johnson & Besselsen, 2002; Shires & Aggleton, 2008). The Cage Control group were included to provide a direct comparison between IEG expression in trained and untrained animals. The Saline group acted as a trained comparison group, allowing us to contrast IEG activation between treatment conditions.

4.3.1. Method.

4.3.1.1. Subjects.

Thirty-two male Wistar rats (Charles River, UK) were used as subjects in this experiment. Animals' age and weight, housing conditions, handling and maintenance were as outlined in Chapter 2.

4.3.1.2. Apparatus.

The Morris water maze was used to spatially train animals. Dimensions of the maze and experimental set up were as described in Chapter 2, with a fixed hidden platform in the centre of the NE quadrant. Two 25 Watt Philips glass light bulbs were used as visual, distal cues, which were suspended from the ceiling in NE and NW positions, respectively.

4.3.1.3. Procedure.

Animals were randomly divided into three experimental training groups; Saline, MK-801 and CNQX, and one Cage Control group (n = 8 per group). The experimental groups were trained in the water maze for five days (four trials per day), as per the training protocol described in Chapter 2. Rats in these groups received an i.p. injection of saline (0.1 ml/100g body weight of 0.9% NaCl), MK-801 (0.1mg/kg body weight) or CNQX (1.5mg/kg body weight), 20-30 minutes before training each day (de Lima et al., 2005). Given that no evidence for dose dependent effects was found in Experiment 1, we chose to use the higher doses of MK-801 and CNQX for this experiment, to maximise the chances of observing any behavioural or cellular effects. The Cage Control group, included as a baseline IEG level comparison group, was not trained in the water maze. These animals were administered with i.p. saline injections (0.1 ml/100g body weight of 0.9% NaCl) once a day for each of the five training days.

4.3.1.4. Tissue preservation.

Ninety minutes post-injection on day five, all rats were terminally anaesthetised, perfused transcardially and their brains removed, post-fixed and sliced as described in Chapter 2. Hippocampal and medial prefrontal regions were, again, examined as regions of interest (four sections per region).

4.2.1.5. Immunohistochemistry.

Staining for all groups was carried out in a single session as documented in Chapter 2, therefore normalisation of the data was not necessary. In this experiment, staining was performed in cohorts of four (one animal from each group).

4.3.1.6. Data analysis.

Behavioural data was examined using four measures of water maze acquisition: escape latency (seconds), distance travelled (centimetres), velocity (centimetres per second), and percentage time spent in the outer corridor. Values from four trials were obtained and averaged for each animal on each training day to produce individual means. Mean values were then averaged according to group to yield group means. For the MK-801 group, values for all trials were also analysed to examine performance on a trial-by-trial basis. For IEG data, numbers of Zif268 and c-Fos immunopositive cells in the hippocampus and medial prefrontal cortex were automatically counted using ImageJ software as outlined before. Raw counts in each section were averaged to produce means for each animal. Group means for each region were then attained by averaging individual means.

4.3.1.7. Statistical analysis.

Escape latencies, distances travelled, velocities and percentage time spent in the outer corridor were examined using 3 x 5 mixed factorial ANOVAs, with group as the between-groups factor (Saline, MK-801 and CNQX group) and training day as the within-groups factor (days 1 to 5). A separate repeated measures ANOVA was also carried out for the MK-801 group, with trial as the within-groups factor (trials 1-20). The Cage Control group were not included in behavioural analyses as they did not receive any behavioural training. Tukey and Bonferroni *post hoc* tests were included where appropriate. Post-training levels of Zif268 and c-Fos expression in each region were investigated using a series of one-way between-groups ANOVAs, with Tukey *post hoc* comparisons. Pearson product-moment correlations were also employed to examine the relationship between behavioural performance and IEG

expression levels. For correlational analyses, only those mean counts which were above a pre-defined value (minimum ten immunopositive cells) were included to minimise statistical artefacts, i.e. significant correlations for sub-regions with extremely low IEG expression levels. Limiting the number of correlations in this way also served to reduce the risk of type I errors (finding a significant correlation where none exists) which can occur when multiple correlations are performed together.

4.3.2. Behavioural results.

4.3.2.1. Escape latency.

A 3 x 5 mixed factorial ANOVA revealed significant main effects of training day, $F_{4,84}$ = 15.55, P = 0.001, partial eta^2 = 0.43, and group, $F_{1,21}$ = 50.42, P = 0.001, partial eta^2 = 0.83, but no day x group interaction effect, $F_{8,84}$ = 0.58, P = 0.80, partial eta^2 = 0.06. Bonferroni *post hoc* comparisons indicated that overall mean escape latency on day 5 was significantly faster than on day 1 (P = 0.001). Tukey *post hoc* tests showed that the MK-801 group was significantly slower at escaping the maze compared to both Saline and CNQX groups (both P = 0.001; see Figure 4.7).

One-way repeated measures ANOVAs were then conducted to examine individual group performance across training days. A main effect of day was found for the Saline group, $F_{4,28}$ = 17.02, P = 0.001, partial eta^2 = 0.71, with mean escape latency decreasing significantly from 35.48 ± 4.74s (CI [24.28, 46.64]) on day 1 to 12.04 ± 1.89s (CI [7.57, 16.50]) on day 5 (Bonferroni; P = 0.02). The main effect of day was also significant for the CNQX group, $F_{4,28}$ = 6.22, P = 0.001, partial eta^2 = 0.47. Although this group were faster at escaping the maze on day 5 (15.29 ± 1.1s,

CI [12.63, 17.96]) compared to day 1 (32.81 ± 4.83s, CI [21.39, 44.22]), this difference was not significant (P = 0.11). In contrast, no main effect of day was observed for the MK-801 group, $F_{4,28}$ = 2.35, P = 0.08, partial eta^2 = 0.25. On average, MK-801-treated rats took 33.28 ± 14.24s (CI [21.37, 45.18]) to reach the platform on day 5, compared to 54.06 ± 8.31s (CI [47.12, 61.01]) on day 1.

Next, one-way between-groups ANOVAs were used to compare mean group escape latencies on each day of training. Analyses produced significant main effects for all days; day 1: $F_{2,23}$ = 7.40, P = 0.01, day 2: $F_{2,23}$ = 9.02, P = 0.01, day 3: $F_{2,23}$ = 22.57, P = 0.001, day 4: $F_{2,23}$ = 14.51, P = 0.001, and day 5: $F_{2,23}$ = 13.00, P = 0.001. Tukey *post hoc* tests revealed that the MK-801 group were significantly slower at finding the platform relative to both Saline and CNQX groups on all training days (all P = 0.02).

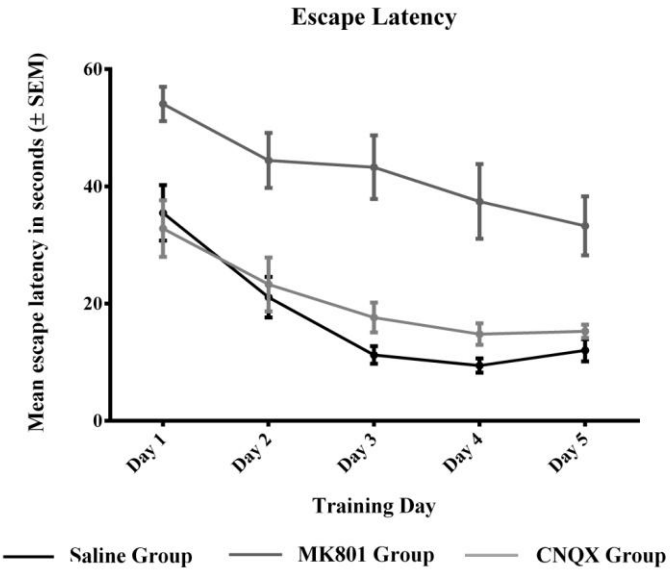

Figure 4.7: Mean escape latencies (± SEM) for Control, MK-801 and CNQX groups on days 1 to 5 of training.

4.3.2.2. Distance travelled.

Analyses of distances travelled yielded comparable results. Main effects of training day, $F_{4,84} = 9.85$, $P = 0.001$, partial eta$^2 = 0.32$, and group, $F_{1,21} = 162.92$, $P = 0.001$, partial eta$^2 = 0.94$, were significant. However, the day x group interaction effect was not significant, $F_{8,84} = 2.17$, $P = 0.06$, partial eta$^2 = 0.17$. Bonferroni pairwise comparisons showed that mean escape latency for all animals on day 5 was significantly faster than on days 1 ($P = 0.001$) and 2 ($P = 0.01$). In addition, Tukey *post hoc* tests illustrated that MK-801-treated animals travelled a significantly greater distance than Saline- and CNQX-treated rats (both $P = 0.001$; see Figure 4.8).

Groups were assessed individually with one-way repeated measures ANOVAs. A significant main effect of day was found for the Saline group, $F_{4,28} = 20.06$, $P = 0.001$, partial eta$^2 = 0.74$. Bonferroni *post hoc* tests indicated that this group travelled a significantly shorter distance on day 5 (232.34 ± 48.59, CI [117.45, 347.24]) relative to day 1 (706.85 ± 83.14, CI [510.25, 903.45]; $P = 0.02$). A significant main effect of day was also noted for the CNQX group, $F_{4,28} = 4.75$, $P = 0.01$, partial eta$^2 = 0.40$. Mean distance travelled decreased from 675.06 ± 97.79cm (CI [431.81, 906.30]) on day 1 to 341.23 ± 27.09cm (CI [277.17, 405.28]) on day 5; however, this difference was non-significant ($P = 0.13$). For the MK-801 group, the main effect of day was non-significant, $F_{4,28} = 2.25$, $P = 0.13$, partial eta$^2 = 0.24$. For these animals, mean distance travelled on day 5 was 723.19 ± 81.62cm (CI [530.19, 916.19]), compared to 1096.67 ± 81.90cm (CI [903.00, 1290.33]) on day 1.

To compare distances travelled by groups across training days, one-way between-groups ANOVAs were conducted. Significant main effects were found for all days; day 1: $F_{2,23} = 7.13$, $P = 0.01$, day 2: $F_{2,23} = 25.27$, $P = 0.001$, day 3: $F_{2,23} =$

32.25, $P = 0.001$, day 4: $F_{2,23} = 22.78$, $P = 0.001$, and day 5: $F_{2,23} = 20.43$, $P = 0.001$. Tukey multiple comparisons confirmed that the average distance travelled by the MK-801 group was significantly longer than both Saline and CNQX groups on day 1 (both $P = 0.01$), day 2, day 3, day 4 and day 5 (all $P = 0.001$).

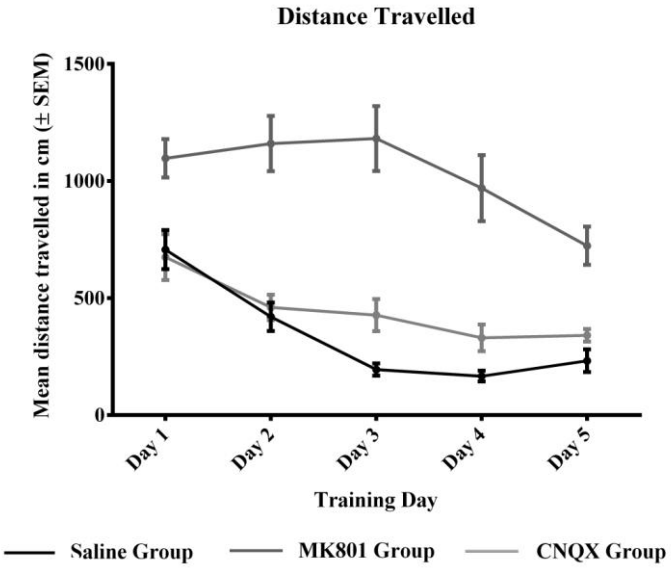

Figure 4.8: Mean distances travelled (± SEM) for Control, MK-801 and CNQX groups for each training day.

4.3.2.3. Velocity.

Due to the inclusion of different drug groups in this experiment, mean velocities (i.e. swim speeds) were analysed as a measure of sensorimotor performance during task acquisition (Vorhees & Williams, 2006). A 3 x 5 mixed factorial ANOVA yielded a significant main effect of group, $F_{1,21} = 47.44$, $P = 0.001$, partial eta$^2 = 0.82$, and day x group interaction effect, $F_{8,84} = 3.17$, $P = 0.01$, partial eta$^2 = 0.23$, but no main effect of day, $F_{4,84} = 2.00$, $P = 0.10$, partial eta$^2 = 0.09$. Tukey *post hoc* tests showed that the Saline group's average swim speed was significantly slower than MK-801- and CNQX-treated animals (both $P = 0.001$; see Figure 4.9). The CNQX group also

had a significantly slower mean swim speed than the MK-801 group (P = 0.01). Bonferroni pairwise comparisons were non-significant.

Mean velocity across days was then examined for each group individually. No main effect of day was found for the Saline group, $F_{4,28}$ = 1.90, P = 0.14, partial eta^2 = 0.21, or the CNQX group, $F_{4,28}$ = 1.05, P = 0.40, partial eta^2 = 0.13. In contrast, the main effect of day was significant for the MK-801 group, $F_{4,28}$ = 5.46, P = 0.03, partial eta^2 = 0.44. Bonferroni pairwise comparisons revealed that mean velocity for this group increased significantly on day 3 (27.01 ± 1.60, CI [23.23, 30.79]) relative to day 1 (19.84 ± 1.02, CI [17.43, 22.25]) (P = 0.01). No other differences were noted.

To explore group differences on each day further, one-way between-groups ANOVAs were carried out. The main effect of group was not significant on day 1, $F_{2,23}$ = 0.22, P = 0.81. Significant main effects of group were discovered for days 2 ($F_{2,23}$ = 6.98, P = 0.01), 3 ($F_{2,23}$ = 22.09, P = 0.001) and 4 ($F_{2,23}$ = 9.82, P = 0.001). The main effect of group on day 5 was not significant, $F_{2,23}$ = 3.31, P = 0.06. Tukey *post hoc* tests revealed that the MK-801 group swam significantly faster on average compared to the Saline group on days 1 (P = 0.01), 2 and 3 (both P = 0.001). The CNQX group also swam significantly faster than the Saline group on day 3 (P = 0.001) and significantly slower compared to the MK-801 group on day 4 (P = 0.05).

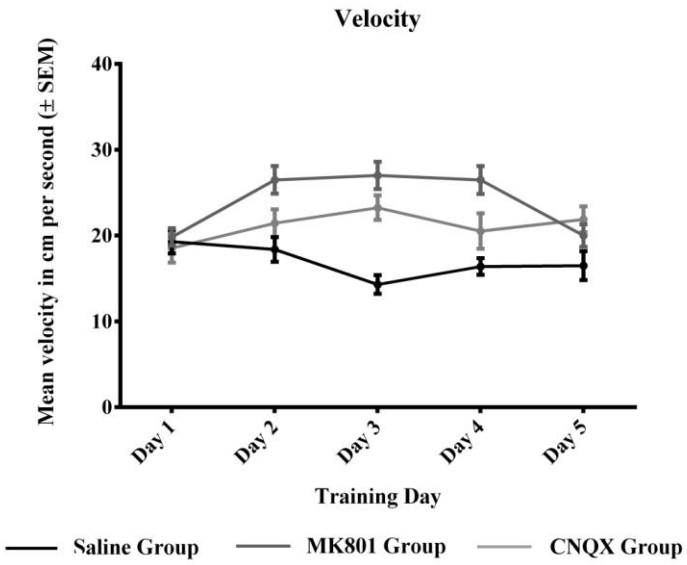

Figure 4.9: Mean velocity (± SEM) for Control, MK-801 and CNQX groups across training days.

4.3.2.4. Outer corridor.

Finally, percentage time spent in the outer corridor of the maze was investigated as a general measure of stress during acquisition (Treit & Fundytus, 1988). A mixed factorial ANOVA produced significant main effects of day, $F_{4,84} = 6.43$, $P = 0.001$, partial eta$^2 = 0.23$, and group, $F_{1,21} = 77.02$, $P = 0.001$, partial eta$^2 = 0.88$. The day x group interaction effect was also significant, $F_{8,84} = 2.09$, $P = 0.04$, partial eta$^2 = 0.17$. Bonferroni *post hoc* tests illustrated that rats spent significantly less time in the outer corridor on day 5 compared to day 1 ($P = 0.001$). Additionally, Tukey *post hoc* comparisons showed that the Saline group spent less time in this zone than both drug groups (Tukey: both $P = 0.001$), and the CNQX group spent less time here than the MK-801 group ($P = 0.001$) (see Figure 4.10).

One-way repeated-measures ANOVAs were carried out to examine groups individually across days. A significant main effect of day was found for the Saline group, $F_{4,28} = 8.48$, $P = 0.001$, partial eta$^2 = 0.55$, whose time spent in the outer corridor was significantly reduced on day 5 (9.11 ± 2.54, CI [3.10, 15.13]) compared

to days 1 (30.51 ± 4.05, CI [20.95, 40.08]; $P = 0.01$) and 2 (30.61 ± 5.06, CI [18.65, 42.57]; $P = 0.03$). Main effects were also noted for the CNQX group, $F_{4,28} = 2.75$, $P = 0.40$, partial eta$^2 = 0.28$, and the MK-801 group, $F_{4,28} = 2.88$, $P = 0.04$, partial eta$^2 = 0.29$. Subsequent planned comparisons (Bonferroni-corrected) showed that time spent in the outer corridor decreased significantly from day 1 to day 5 for the CNQX group (58.87 ± 7.37, CI [41.46, 76.29] versus 37.92 ± 3.69, CI [29.20, 46.63]; $t_7 = 2.97$, $P = 0.02$), but not for the MK-801 group (64.59 ± 5.73, CI [51.06, 78.13] versus 45.41 ± 6.24, CI [30.65, 60.16]; $t_7 = 2.32$, $P = 0.06$).

One-way between-groups ANOVAs were then conducted for each day separately. Main effects of group were found on all days; day 1 ($F_{2,23} = 9.67$, $P = 0.001$), day 2 ($F_{2,23} = 19.11$, $P = 0.001$), day 3 ($F_{2,23} = 31.16$, $P = 0.001$), day 4 ($F_{2,23} = 11.60$, $P = 0.001$) and day 5 ($F_{2,23} = 18.67$, $P = 0.001$). Tukey *post hoc* analyses yielded a number of significant differences. On day 1, the Saline group spent less time in the outer corridor than the MK-801 and CNQX groups (both $P = 0.01$). On day 2, Saline- and CNQX-treated groups spent less time in this area than the MK-801 group (both $P = 0.001$). On day 3, time in the outer corridor was significantly reduced for the Saline group relative to the CNQX ($P = 0.01$) and MK-801 groups ($P = 0.001$), and for the CNQX group compared to the MK-801 group ($P = 0.001$). On day 4, the MK-801 group spent longer in this corridor than the Saline ($P = 0.001$) and CNQX groups ($P = 0.05$). Lastly, on day 5, the Saline group, again, spent less time swimming in the outer corridor relative to the other groups ($P = 0.001$).

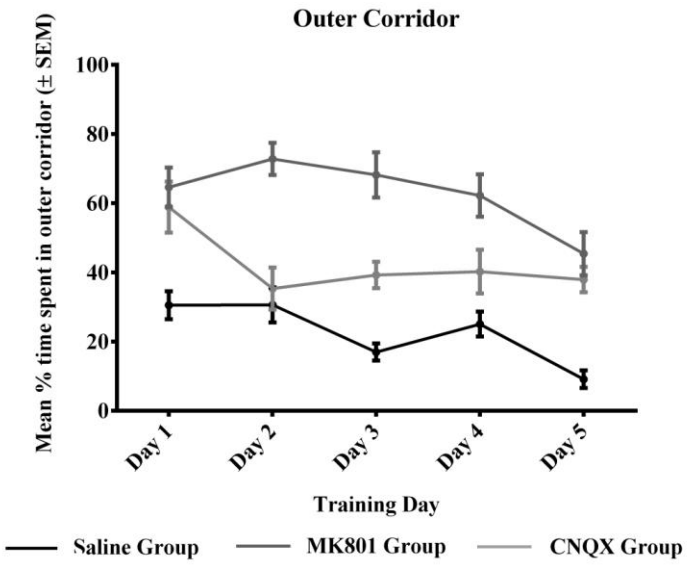

Figure 4.10: Mean percentage time (± SEM) spent in the outer corridor by Control, MK-801 and CNQX groups for each day of training.

4.3.2.5. Trial-by-trial analysis

Due to the increased variance of the MK-801 group relative to the other groups, an additional trial-by-trial analysis was carried out for these animals to further investigate their behaviour during training. Repeated measures ANOVAs (with trial as the within groups factor) failed to yield a significant main effect of trial for escape latency, $F_{19, 133} = 1.41$, $P = 0.14$, partial eta$^2 = 0.17$, distance travelled, $F_{19, 133} = 1.43$, $P = 0.13$, partial eta$^2 = 0.17$, and time spent in the outer corridor, $F_{19, 133} = 1.99$, $P = 0.11$, partial eta$^2 = 0.22$ (see Figure 4.11A, B and D). The main effect of trial was significant for velocity, $F_{19, 133} = 3.30$, $P = 0.03$, partial eta$^2 = 0.32$. Bonferroni *post hoc* tests indicated that rats swam significantly faster on trial 13 compared to trials 3 ($P = 0.05$) and 4 ($P = 0.04$; see Figure 4.11C).

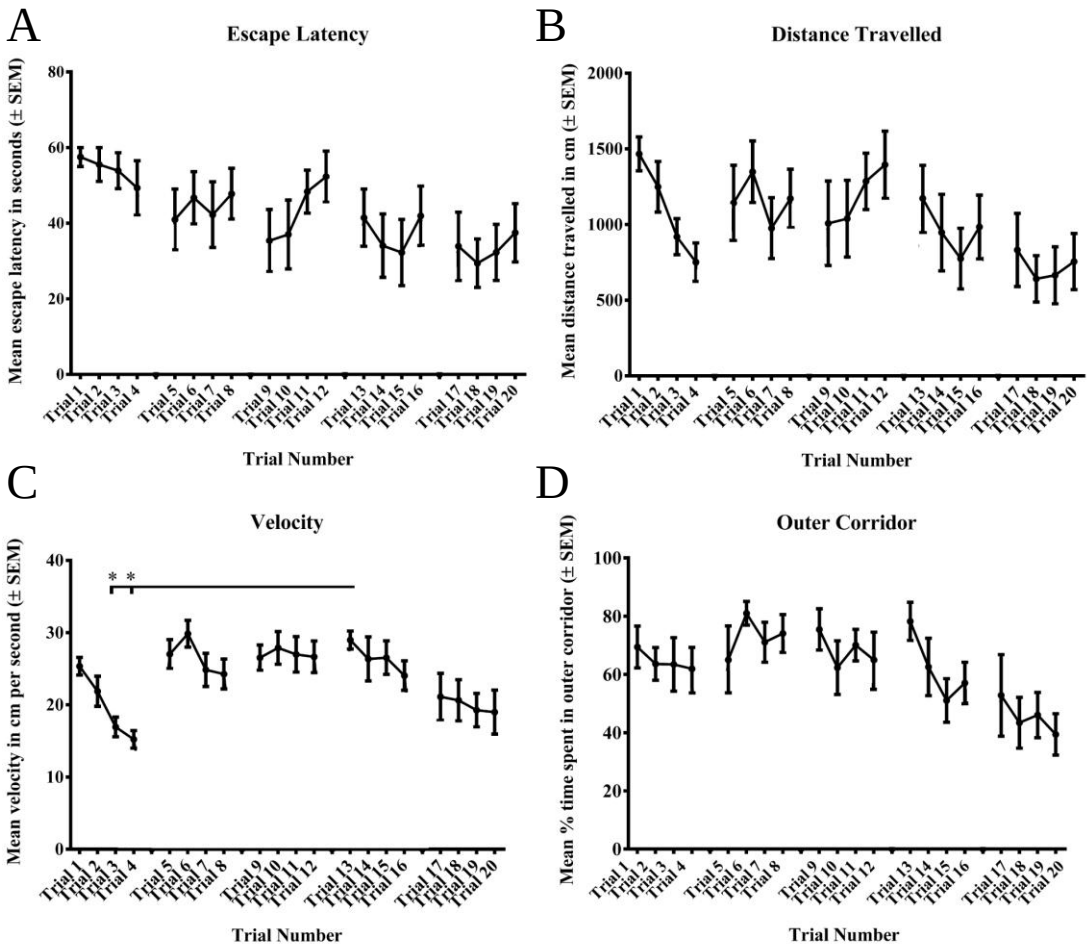

Figure 4.11: Mean (A) escape latency, (B) distance travelled, (C) velocity and (D) percentage time spent in the outer corridor (± SEM) by the MK-801 group for each training trial.

4.3.3. IEG results.

4.3.3.1. Zif268 expression.

One-way between-groups ANOVAs were carried out to compare Zif268 expression across groups. In the hippocampus, a significant main effect of group was noted in area CA1, $F_{3,31}$ = 9.71, P = 0.001, with Tukey *post hoc* tests showing that levels of Zif268 expression for the Saline (40.51 ± 12.74, CI [10.39, 70.63]) and CNQX groups (56.04 ± 9.48, CI [32.84, 79.24]) were significantly higher compared to the Cage Control (4.86 ± 1.98, CI [0.18, 9.54]) (P = 0.02 and P = 0.001, respectively) and MK-801 groups (7.58 ± 1.72, CI [3.51, 11.65]) (P = 0.03 and P = 0.001,

respectively) (see Figure 4.12A). No significant main effects of group were found in area CA3, $F_{3,31} = 1.53$, $P = 0.23$, or the DG, $F_{3,31} = 1.55$, $P = 0.23$ (Figure 4.12B-C). In the medial prefrontal cortex, the main effect of group was significant in the PLC, $F_{3,31} = 5.44$, $P = 0.01$. Here, significantly more Zif268 positive cells were present for the CNQX group (66.79 ± 19.13, CI [21.55, 112.03]) relative to the Cage Control (16.21 ± 5.76, CI [2.59, 29.83]; $P = 0.02$), Saline (15.29 ± 7.36, CI [2.72, 33.30]; $P = 0.02$) and MK-801 groups (14.00 ± 5.29, CI [1.48, 26.52]; $P = 0.02$) (see Figure 4.12D). No significant group main effects were discovered in the ACC, $F_{3,31} = 2.01$, $P = 0.13$, or ILC, $F_{3,31} = 0.38$, $P = 0.77$ (Figure 4.12E-F). Sample sections of hippocampal and medial prefrontal Zif268 expression are shown in Figures 4.13 and 4.14.

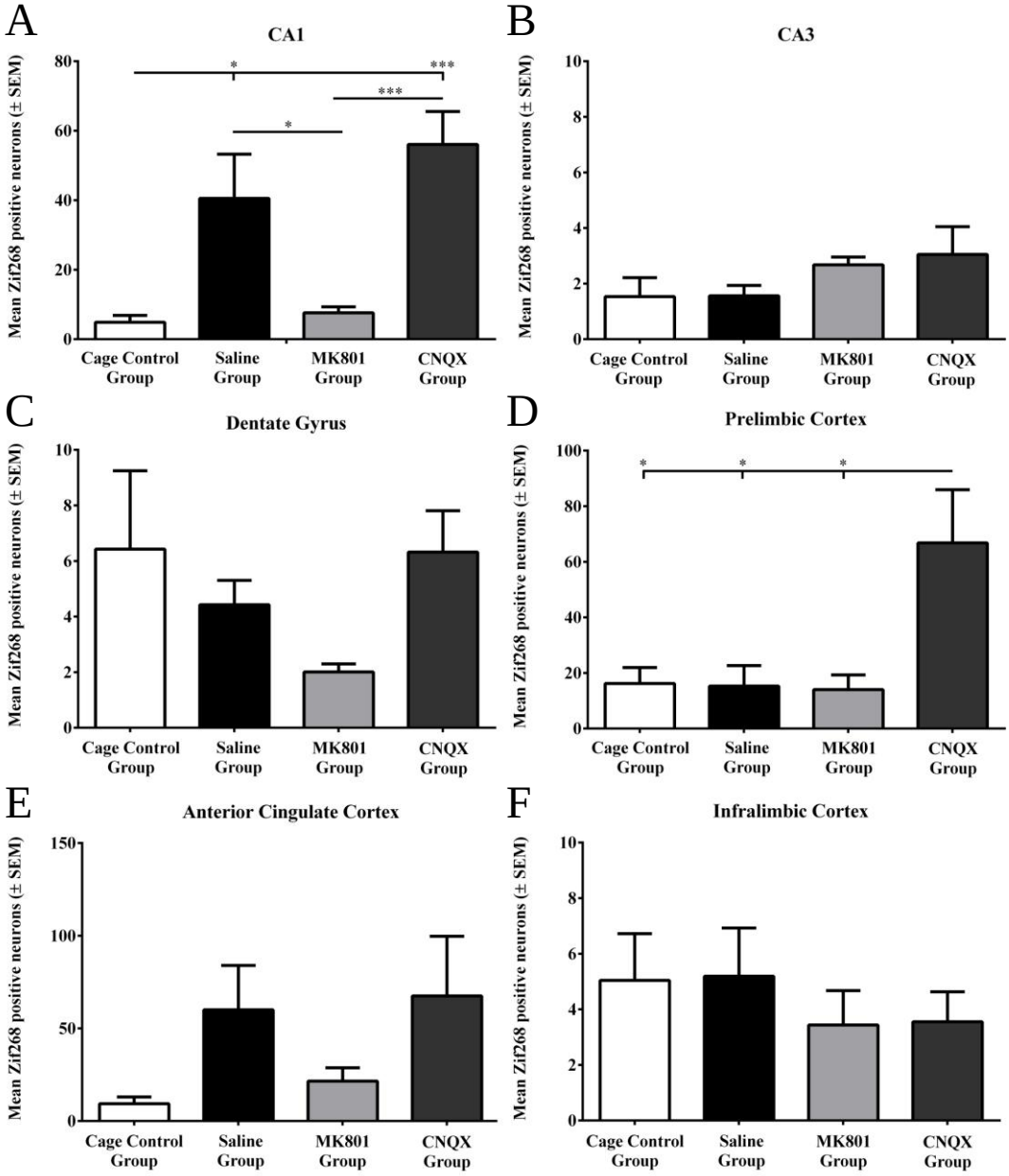

Figure 4.12: Mean cell counts of Zif268 positive neurons for Saline, MK-801 and CNQX groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

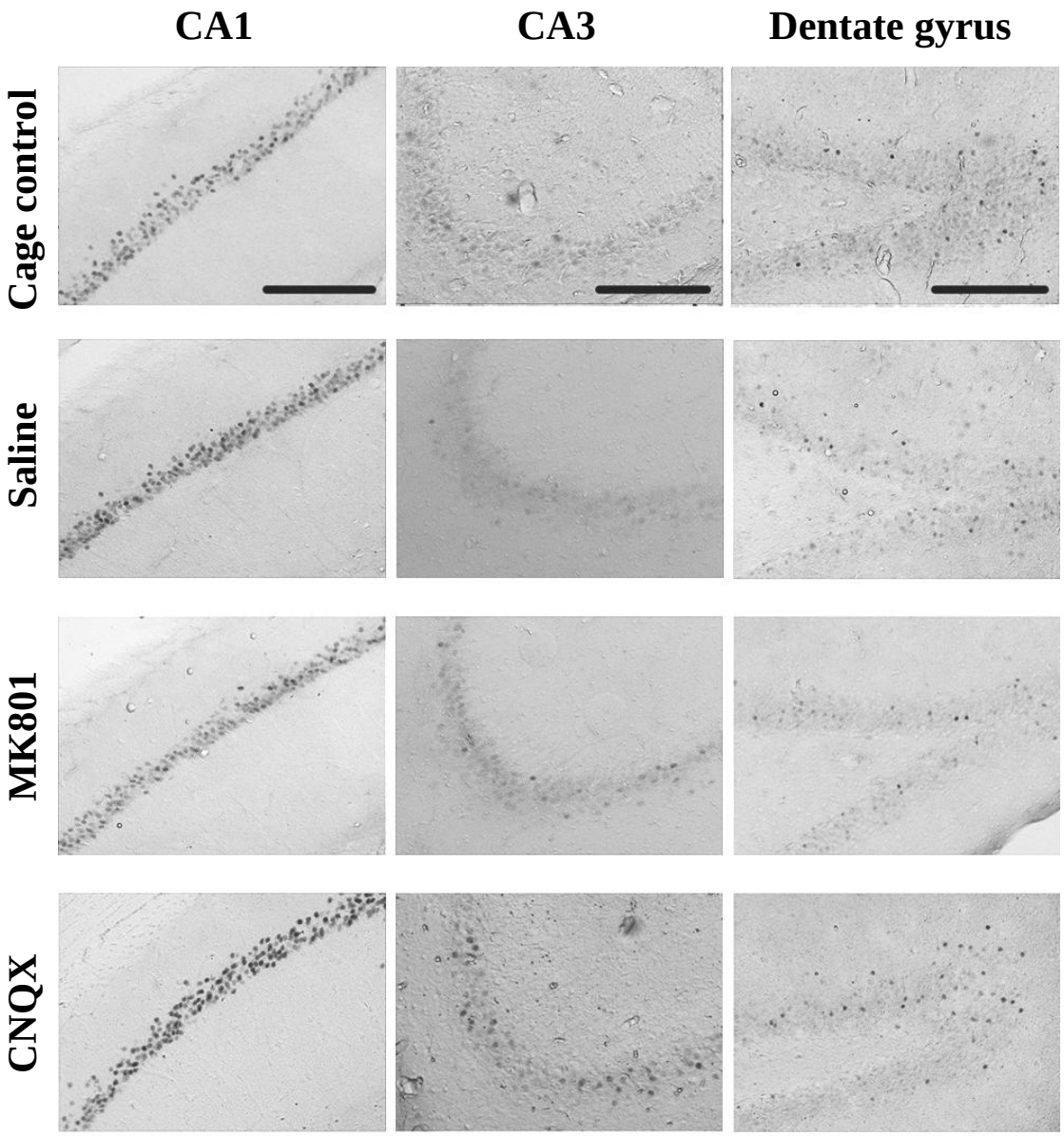

Figure 4.13: Sample images of Zif268 expression for Cage Control, Saline, MK-801 and CNQX groups in area CA1, area CA3 and the dentate gyrus. Scale bar = 100μm.

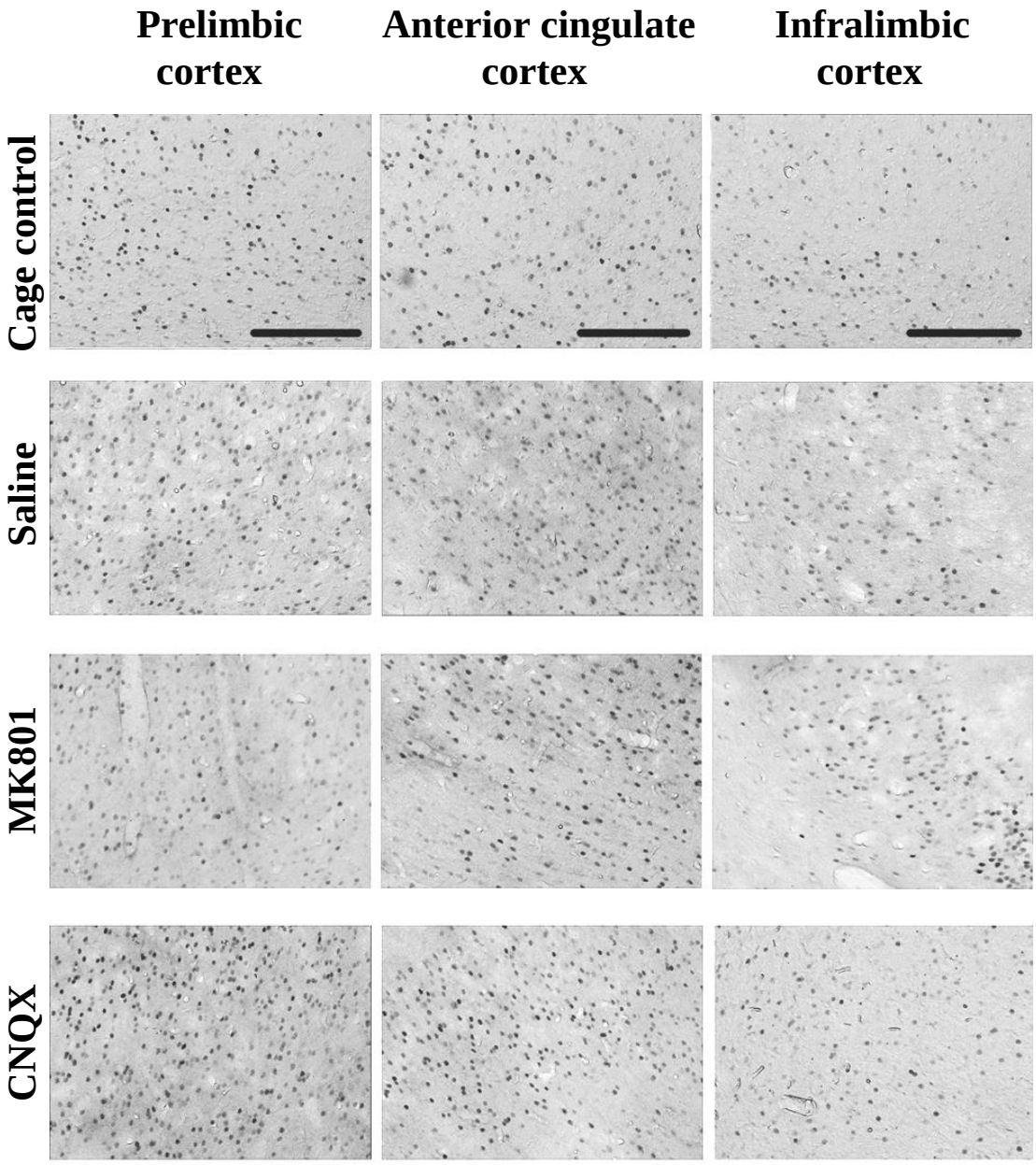

Figure 4.14: Sample images of Zif268 expression for Cage Control, Saline, MK-801 and CNQX groups in the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

4.3.3.2. c-Fos expression.

One-way between-groups ANOVAs investigating c-Fos expression levels produced a different pattern of results. No main effect of group was found in area CA1, $F_{3,31}$ = 1.90, P = 0.15, or the DG of the hippocampus, $F_{3,31}$ = 0.64, P = 0.60. Despite a low number of overall cell counts, a significant main effect was discovered in area CA3,

$F_{3,31}$ = 4.14, P = 0.02, where the mean number of c-Fos positive cells was greater for the CNQX group (3.06 ± 1.12, CI [0.42, 5.70]) compared to the Saline group (0.15 ± 0.07, CI [0.01, 0.32]; P = 0.02) (see Figure 4.15A-C). Within the medial prefrontal cortex, the main effect of group was not significant for the PLC, $F_{3,31}$ = 2.45, P = 0.08, and significant for the ACC, $F_{3,31}$ = 3.15, P = 0.04, and ILC, $F_{3,31}$ = 3.94, P = 0.02 (see Figure 4.15D-F). Tukey *post hoc* comparisons illustrated that mean c-Fos counts in the ACC were significantly higher for MK-801-treated animals (57.63 ± 17.23, CI [16.87, 98.38]) than the Cage Control group (9.63 ± 1.84, CI [5.27, 13.98]; P = 0.04). Mean counts were also greater in the ILC for the MK-801 group (38.88 ± 8.47, CI [18.84, 58.91]) compared to Cage Controls (10.29 ± 2.70, CI [3.92, 16.67]; P = 0.02). Figures 4.16 and 4.17 illustrate sample sections of c-Fos expression levels in the hippocampus and medial prefrontal cortex.

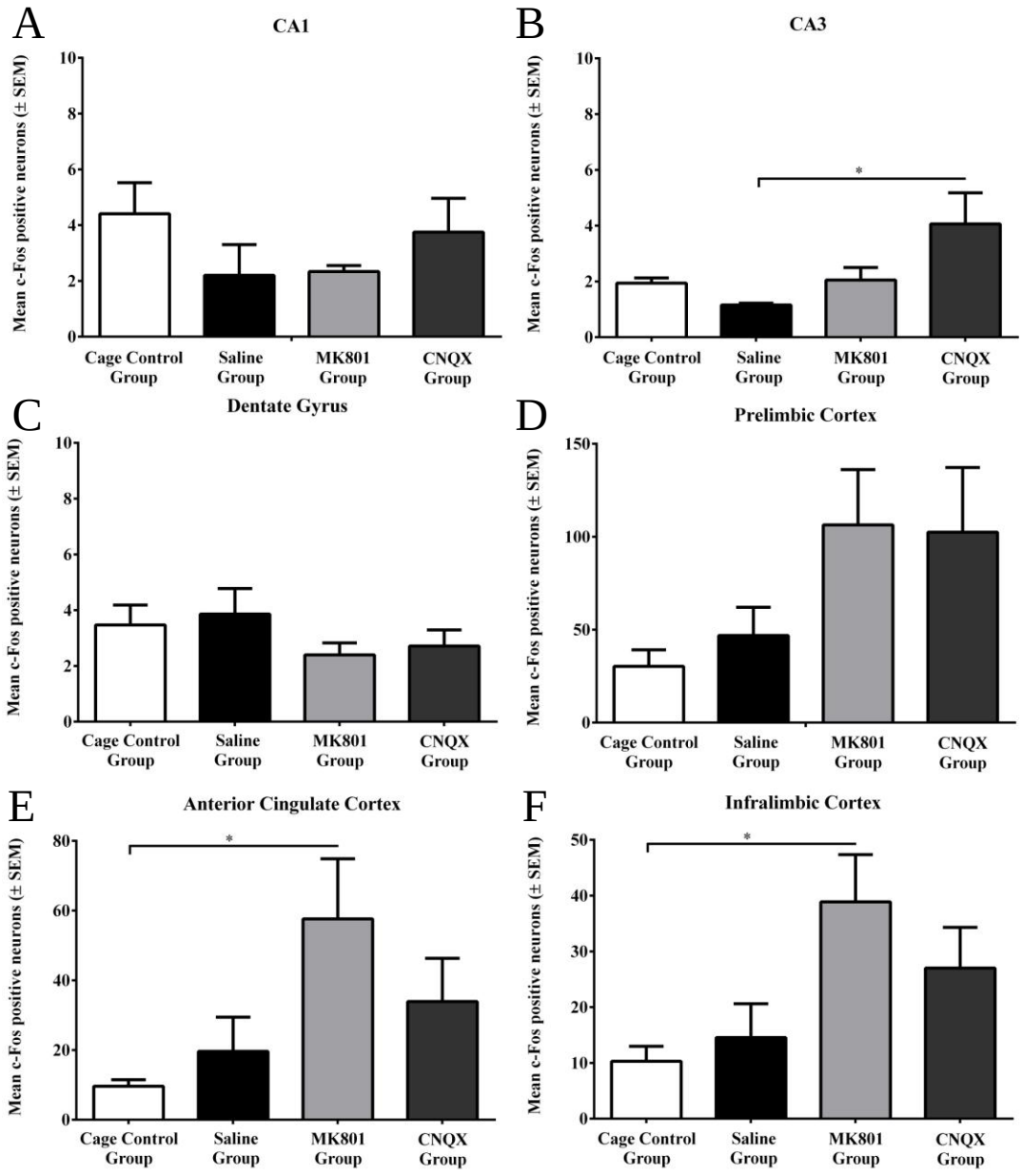

Figure 4.15: Mean cell counts of c-Fos positive neurons for Saline, MK-801 and CNQX groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

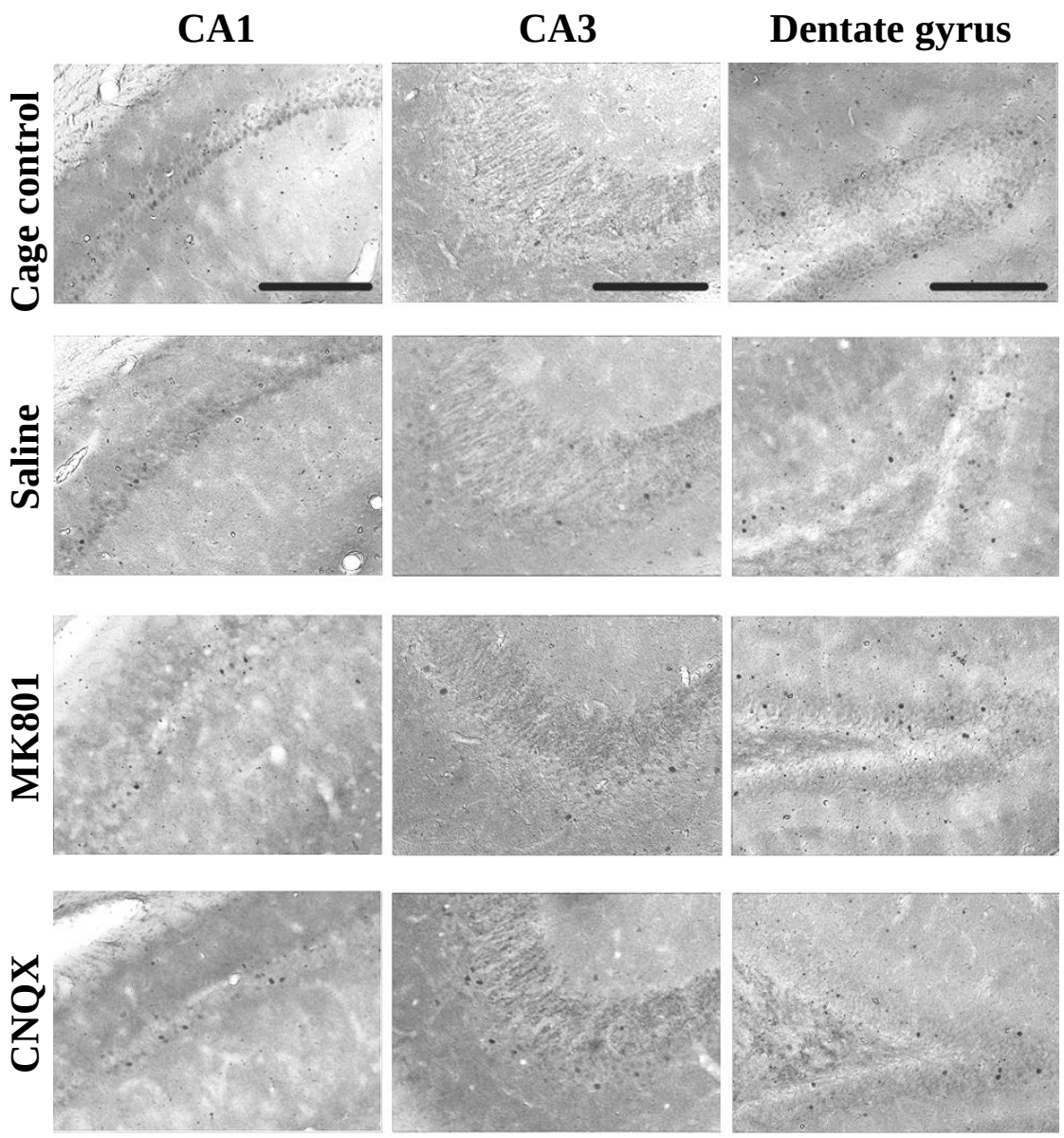

Figure 4.16: Sample images of c-Fos expression for Cage Control, Saline, MK-801 and CNQX groups in CA1, CA3 and the dentate gyrus. Scale bar = 100μm.

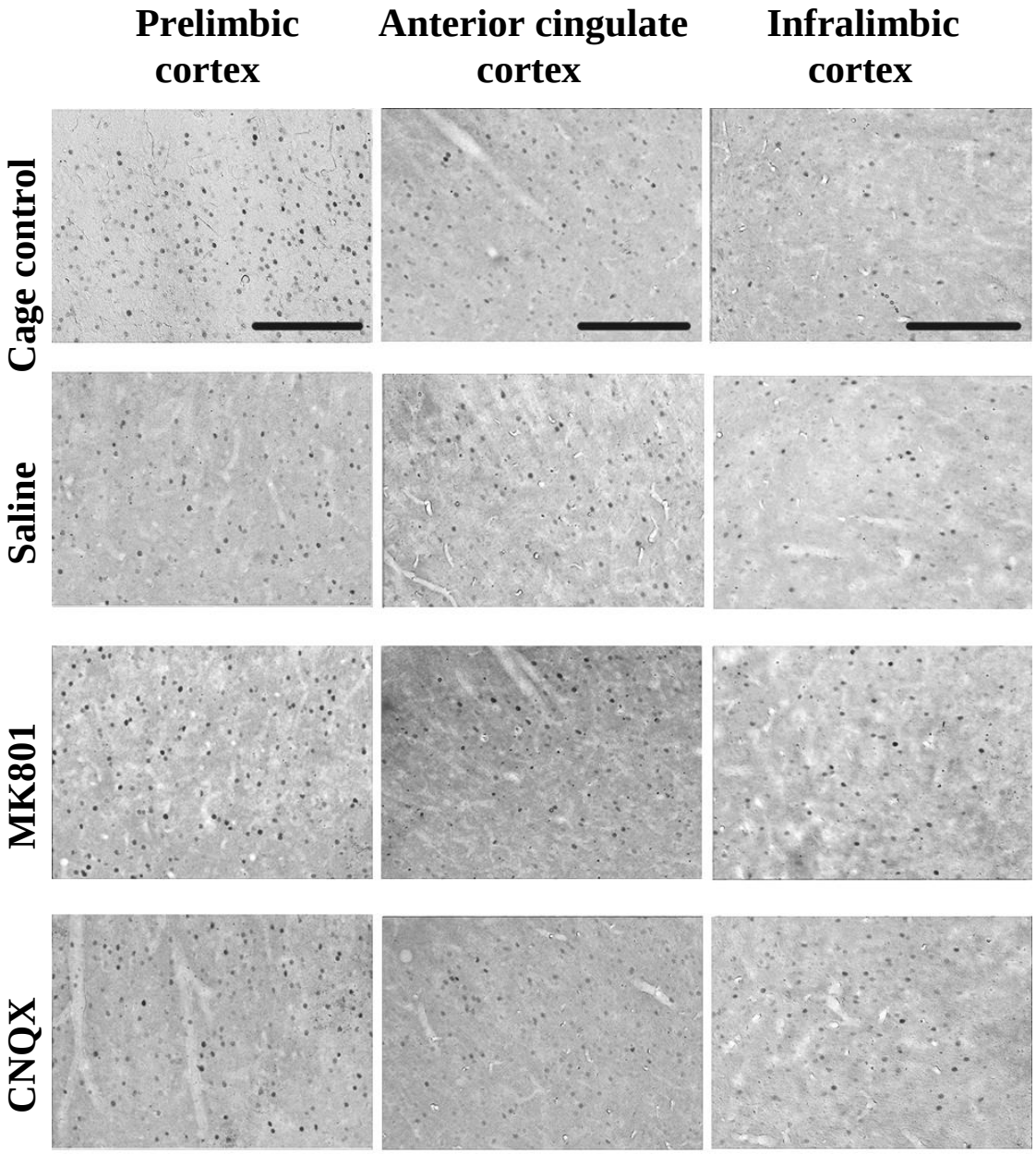

Figure 4.17: Sample images of c-Fos expression for Cage Control, Saline, MK-801 and CNQX groups in the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

4.3.3.3 Correlations with behaviour

Finally, mean counts of Zif268 and c-Fos in selected sub-regions for each group were correlated with each of the four behavioural measures on the final day of training (escape latency, distance travelled, velocity and time spent in the outer corridor) to determine the relationship between IEG expression and water maze performance (as per previous research, e.g. Guzowski et al., 2001). As mentioned above, certain regions were excluded from correlational analyses due to very small numbers of cell counts. A number of significant correlations were found between Zif268 expression and behaviour. For the Saline group, a significant negative correlation was found with time spent in the outer corridor in area CA1 (r = 0.75, P = 0.03; see Table 4.1 Top). In contrast, data from the MK-801 group produced a positive correlation with distance travelled in the ACC (r = 0.72, P = 0.04; see Table 4.1 Middle). For the CNQX group, a significant negative correlation was noted with escape latency in the ACC (r = 0.82, P = 0.03; see Table 4.1 Bottom). Pearson product-moment correlations between c-Fos expression and behaviour failed to yield any significant results for the Saline group (see Table 4.2 Top) or CNQX group (Table 4.2 Bottom). For the MK-801 group, a significant positive correlation was found with time spent in the outer corridor (r = 0.75, P = 0.04; see Table 4.2 Middle).

Table 4.1: Correlations between Zif268 expression and acquisition measures in the water maze for the Saline, MK-801 and CNQX groups.

Group	Brain region	Behavioural Measure			
Saline		Escape latency	Distance travelled	Velocity	Outer corridor
	CA1	-0.28	-0.13	0.12	**-0.75***
	ACC	-0.57	-0.53	-0.21	-0.74
MK-801					
	ACC	0.38	**0.72***	0.55	0.68
CNQX					
	CA1	0.16	0.13	-0.22	-0.32
	PLC	0.46	0.50	0.23	-0.08
	ACC	**-0.82***	-0.27	0.37	0.04

Table 4.2: Correlations between c-Fos expression and acquisition measures in the water maze for the Saline, MK-801 and CNQX groups.

Group	Brain region	Behavioural Measure			
Saline		Escape latency	Distance travelled	Velocity	Outer corridor
	PLC	0.30	0.41	0.34	-0.40
	ACC	0.61	0.70	0.35	-0.07
	ILC	0.35	0.46	0.47	-0.29
MK-801					
	PLC	0.63	0.55	0.05	0.66
	ACC	0.65	0.63	0.13	**0.73***
	ILC	0.46	0.45	0.06	0.39
CNQX					
	PLC	0.58	0.55	0.23	0.31
	ACC	0.36	0.33	0.17	0.35
	ILC	0.36	0.35	0.23	0.40

4.3.5. Discussion

Results from this experiment revealed a number of important group differences on both behavioural and cellular levels. With regard to behaviour, findings demonstrate that Saline- and CNQX-treated rats successfully acquired the water maze task – as indicated by considerable decreases in mean time taken to reach the platform and path lengths. In contrast, the MK-801 group showed no change in escape latency or distance travelled across training; furthermore, these animals were slower to find the platform and took longer routes on all training days, relative to the other groups. In addition, locomotor effects (indexed by velocity) were most pronounced in the MK-801 group, although no group differences were found on the final day of training.

Finally, results for the outer corridor indicate that administration of MK-801 (and to a lesser extend CNQX) influenced searching behaviour relative to Saline. Similar to previous work (Pitkänen et al., 1995), these findings support the suggestion that MK-801 channel blockade impairs spatial memory acquisition, while AMPA receptor antagonism does not (at the doses used here).

Regarding IEG expression, different patterns emerged for Zif268 and c-Fos. Zif268 levels were upregulated in Saline and CNQX groups compared to Cage Control and MK-801 groups in area CA1. These group differences may be indicative of a learning-related increase in expression, not unlike previous findings (Feldman et al., 2010). The significant negative relationship between mean CA1 Zif268 counts and time spent in the outer corridor by saline-treated rats supports this suggestion. Higher Zif268 counts were also noted for the CNQX group in the PLC relative to the other groups, and ACC Zif268 counts for these animals were correlated with faster escape times. Conversely, ACC counts were positively correlated with distance travelled for the MK-801 group, and no significant results were observed for Saline-treated animals. Thus, findings seem to suggest that AMPA and NMDA receptor blockade had differential task-related effects on Zif268 expression in the prefrontal cortex. In contrast to Zif268, c-Fos cell counts were consistently higher for impaired animals in prefrontal sub-regions (ACC and ILC) relative to Cage Controls, and increased expression was related to greater time in the outer corridor. Accordingly, it appears that prefrontal c-Fos expression may have been mediated by poor performance, or associated swim stress.

4.4. General discussion

The goal of this chapter was to investigate the role of NMDA and AMPA receptors in spatial memory encoding and IEG expression in the hippocampus and prefrontal cortex. Previous research has highlighted that glutamate receptor antagonism can influence basal expression of IEGs, making it difficult to interpret results from behavioural training studies (Knapska & Kaczmarek, 2004; Tischmeyer & Grimm, 1999). However, results from Experiment 1 failed to indicate any such significant effects following treatment with low or high doses of MK-801 or CNQX. Crucially, these findings showed that glutamate antagonism at the selected doses was not modulating gene expression, and thus, any cellular changes detected following water maze training were not merely caused by the drugs themselves.

Pharmacological blockade of glutamate receptors, even at very low concentrations (e.g. MK-801 at 0.05mg), has also been shown to produce mild behavioural modifications such as hyper-activity (increased movement speed) and hyper-reactivity (vocalisation when handled) (Hargreaves & Cain, 1992). Although no formal assessment of locomotive behaviour was carried out here, visual inspection of all animals revealed some evidence of hyper-activity for the MK-801 group in Experiment 2, who displayed faster swim speeds compared to the other groups. However, no effects on swimming ability were observed for these (or any) rats; thus, it is unlikely that their water maze performance or IEG expression levels were attributable to motor-dysfunctions. Instead, our results support the proposal that NMDA receptor activation is critical for efficient spatial memory encoding (Bast et al., 2005; Davis et al., 1992; Morris et al., 1989; Pitkänen et al., 1995; Whishaw & Auer, 1989). We found no evidence of a similar role for AMPA receptors, in contrast to earlier work (Cain et al., 1996; Filliat et al., 1998; Liang et al., 1994; Riedel et al.,

1999). Rather, findings are consistent with more recent research suggesting that AMPA receptors are necessary for spatial memory retrieval but not encoding (Bast et al., 2005).

Interestingly, post-training IEG imaging highlighted very different patterns of expression for Zif268 and c-Fos. Zif268 expression in selective hippocampal and prefrontal sub-regions (CA1 and PLC) was associated with groups that acquired the task, i.e. Saline- and CNQX-treated rats. In particular, group differences in area CA1 strongly support the importance of this region for spatial learning, and are in line with previous work which has shown that inactivation or deletion of CA1 NMDA receptors in mice attenuates LTP and impedes water maze acquisition (Shimizu, Tang, Rampon, & Tsien, 2000; Tsien, Huerta, & Tonegawa, 1996). Taken together, results indicate that Zif268 expression is tightly linked to CA1 NMDA receptor activation, LTP and spatial encoding. Conversely, Zif268 expression in CA3 and the DG did not appear to be mediated by water maze learning. Zif268 results also imply some interaction between hippocampal and prefrontal structures during encoding (i.e. high counts in CA1, PLC and ACC areas). This would not be entirely unexpected, given the known anatomical connections from CA1 to the medial prefrontal cortex (particularly to the PLC and ILC) (Hoover & Vertes, 2007); however, lack of significant differences in the ACC limits the conclusions which can be drawn here.

Unlike Zif268 expression, levels of c-Fos in the hippocampus generally did not differ between learning- and non-learning groups, and expression in the medial prefrontal cortex (PLC and ACC) was highest for rats that failed to acquire the task. Moreover, the hippocampus and prefrontal cortex displayed markedly different patterns of c-Fos expression (low versus high); suggesting that expression across

regions was not synchronised. It is important to note that c-Fos counts were significantly higher for the MK-801 group compared to Cage Controls, and thus, increased expression was specifically related to task performance. One explanation for this result is that MK-801-treated animals experienced greater stress (relative to the other groups) as a result of prolonged swimming in the maze. Indeed, Duncan and colleagues (1993), demonstrated a similar effect on c-Fos expression in the medial prefrontal cortex (but not in the hippocampus) during the forced swim test. In addition, numerous studies have highlighted the importance of the prefrontal region for controlling the hypothalamo-pituitary-adrenal (HPA) stress response (Figueiredo, Bruestle, Bodie, Dolgas, & Herman, 2003; Spencer, Buller, & Day, 2005). Most recently, Radley, Arias and Sawchenko (2006) localised this inhibitory process to the dorsal (PLC) region, showing that lesions to this area resulted in a greater increase in stress-related c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN) compared to ventral (ILC) lesions. However, it is also possible that the increase in prefrontal c-Fos expression was the result of diminished learning, or indeed a combination of impaired learning and stress.

More generally, there are a number of reasons which might explain the differences in Zif268 and c-Fos expression seen in Experiment 2. Firstly, c-Fos is known to be expressed at lower levels in the rat hippocampus (Hughes et al., 1992) than Zif268, particularly in area CA1 (Davis et al., 2003). c-Fos also has a higher induction threshold (Wisden et al., 1990; Worley et al., 1993). Thus, it is likely that the water maze acquisition task employed here (wherein the cue and platform positions remained constant throughout training) was not sufficiently complex to provoke high levels of c-Fos expression in the learning groups. This would explain the differences between our results and those of Jenkins and colleagues (2003),

whose procedure included multiple cue and platform rotations throughout training. In addition, c-Fos is thought to be more sensitive to stress than Zif268. More specifically, Cullinan, Herman, Battaglia, Akil, and Watson (1995) examined changes in c-Fos and Zif268 expression in rats following swim or restraint stress. The authors found that c-Fos expression was greatly elevated across multiple brain regions following both types of stress, whereas the effects for Zif268 were less pronounced.

Another factor which may have influenced our results is the time point at which IEGs were quantified. For example, Abraham *et al.* (1993) showed that Zif268 – but not c-Fos – continued to be expressed in the dentate gyrus for up to five days in response to stimulus-induced neuronal plasticity. It is therefore possible that c-Fos levels peaked earlier during our water maze training procedure, and were returning to baseline by day five. Indeed, previous studies demonstrating heightened c-Fos expression in the hippocampus employed much shorter water maze acquisition protocols, such as one day training, lasting 10 or 15 minutes in total, respectively (Feldman et al., 2010; Teather et al., 2005), or three day training (Guzowski et al., 2001). On the other hand, Zif268 knockout mice have been shown to exhibit slower water maze acquisition relative to yoked controls (Jones et al., 2001), while c-Fos knockout mice only begin to show impairments during memory recall (Fleischmann et al., 2003). Therefore, it may be that the significance of c-Fos expression is more closely coupled with later stages of memory processing (i.e. retrieval).

Taken together, IEG imaging results from this chapter reveal that Zif268 and c-Fos are differentially expressed within hippocampal and prefrontal sub-regions during spatial memory encoding. This observation is particularly important given that these IEGs are often used interchangeably as markers of neuronal activation

throughout the literature, and are also thought to be somewhat coordinated during spatial learning (Guszowski et al., 2001); though it should also be noted that Guzowski *et al.* performed correlations on all animals as a single group (regardless of their experimental condition), which is likely to have affected their results.

In summary, findings from this chapter show that NMDA and AMPA receptor blockade has no impact on baseline expression of Zif268 or c-Fos in hippocampal and prefrontal sub-regions at the selected doses (Experiment 1), contrary to previous work, thus highlighting the significance of drug concentrations used. Results from Experiment 2 highlight the importance of CA1 for spatial memory encoding and support the role of NMDA receptors in this process; however, they demonstrate little evidence for AMPA receptor involvement. Finally, trends from IEG imaging analyses indicate that Zif268 may represent a more appropriate index of spatial learning in the Morris water maze task. In the next chapter, we continue to explore the roles of hippocampal and prefrontal sub-regions during spatial navigation, this time by investigating neuronal changes associated with the retrieval phase of the task.

Chapter 5

Investigating the Neural Correlates of Spatial and Non-Spatial Memory Retrieval over Time Using Immediate Early Gene Imaging

Abstract

Animals use a range of allocentric strategies for memory recall. These include simple (non-spatial) stimulus-response strategies and more complex (spatial) place strategies, which are thought to have distinct neural substrates. The hippocampus is considered to be crucial for place, but not response strategies, while the opposite has been shown for the caudate nucleus. The medial prefrontal cortex has also been implicated in memory retrieval; however, evidence concerning its specific role is equivocal. Recent research suggests that both hippocampal and prefrontal regions are critical for flexible behavioural responding, e.g. when task demands change. The aim of this chapter was to further investigate the use of spatial and non-spatial strategies in the Morris water maze and their associated brain areas using IEG imaging of Zif268 and c-Fos. In Experiment 1, we charted hippocampal and prefrontal involvement during retrieval of spatial and non-spatial memories after standard (5 day) and extended training (10 day). Behavioural flexibility was examined using intact and partial cue arrangements. Results indicated that specific sub-regions of the hippocampus (CA3) and prefrontal cortex (PLC and ACC) were preferentially engaged in spatial memory recall. In Experiment 2, the importance of NMDA receptor activation for memory retrieval, behavioural flexibility and IEG expression was examined. Results demonstrated that spatial and non-spatial memories were initially dependent of NMDA receptor activation; however, with increased training, spatial memory could be preserved under full cue conditions. Finally, results suggest that Zif268 is a more useful indicator of regional brain activation relating to memory retrieval than c-Fos.

5.1. Introduction

Animals can employ a range of allocentric navigational strategies to reach a goal; from simple stimulus-response associations – such as approaching a prominent beacon – to the use of more complex spatial representations, which can be acquired through overt or latent learning (Rodrigo, 2002; Tolman, 1948; Whitlock, Sutherland, Witter, Moser, & Moser, 2008). Learning via a beacon strategy occurs rapidly, as the animal only needs to remember whether or not to move towards the cue. In his original study, Morris (1981) showed that rats navigating to a visible platform in the water maze could reach asymptotic performance after just three trials in both fixed and variable platform conditions. By comparison, animals navigating to a hidden platform using distal room cues (i.e. place learning) are thought to construct a 'map' of the overall environment, and therefore, take considerably longer to acquire the task (Morris, 1981; Tolman, 1948). Subsequent experiments utilising visible platforms (Carman & Mactutus, 2002; Sutherland & Dyck, 1984) and beacons (Harvey et al., 2009; Redhead et al., 1997; Roberts & Pearce, 1999) have reported similar findings, thus confirming the differing behavioural complexity of these strategies.

In addition to behavioural differences, evidence from the existing literature strongly indicates that response and place strategies are supported by distinct neural substrates. Specifically, the hippocampus is considered to be essential for the retrieval of newly acquired place memories, but not for beacon navigation (Broadbent, Squire, & Clark, 2006; de Bruin et al., 2001; McDonald & White, 1994; Morris et al., 1982; Save & Poucet, 2000; Simon, Stevens, Curtis, & Ramus, 2011; Sutherland & Rodriguez, 1989). For example, Save and Poucet (2000) established that dorsal hippocampal lesions administered pre-training impaired water maze recall

in rats using distal (room) and proximal (intramaze) cues; in contrast, lesions had no effect on navigation with a beacon (attached to the platform). Sutherland and colleagues (1989) showed that post-training lesions to the fornix also lead to poor recall in rats navigating to a hidden platform, but not to a visible platform. Comparable results were recently reported by Simon et al. (2011), who trained rats with fornix or sham lesions on a water maze task in which they had to discriminate between two visually distinct beacons; one which indicated the platform location and the other which acted as a foil. Rats' memory was then tested in a probe trial without the platform. The authors found that both groups performed equally well during acquisition (i.e. correctly discriminating between the beacons to reach the platform) and recall (i.e. favouring the quadrant with the correct beacon) (Simon et al., 2011).

With regard to regions that are involved in response strategies, the dorsal striatum has been highlighted as an important area (Devan, McDonald, & White, 1999; McDonald & White, 1994; Packard & McGaugh, 1996). McDonald and White (1994), for example, found that lesions to the caudate nucleus significantly impaired rats' ability to navigate to a visual platform (but not to a hidden platform), indicative of a deficit in simple associative responding. The medial prefrontal cortex has also been implicated in response strategies. In a series of experiments de Bruin and colleagues (de Bruin et al., 2001; 1994), discovered that rats with medial prefrontal lesions were impaired at navigating to a visible platform in the water maze, but displayed normal recall on a hidden platform version of the task, suggesting that this area is involved in non-spatial strategies only. However, because all animals in these experiments performed the spatial task first and the non-spatial task second, the observed results may have reflected a failure to adapt their strategy in keeping with task demands, as opposed to a deficit in beacon navigation *per se* (de Bruin et al.,

1994). Recent findings from Jo and colleagues (2007) support this suggestion. The authors found that rats with lesions to the medial prefrontal cortex, or to area CA3, were impaired at finding a hidden platform under partial, but not full, cue conditions (i.e. when some of the training cues were removed). Further, temporary inactivation of the medial prefrontal cortex with infusions of muscimol administered before testing produced similar impairments. Jo *et al.* (2007) also measured expression of the IEG c-Fos after recall and found that navigation with an incomplete cue arrangement elevated the number of immunopositive c-Fos cells in prefrontal and CA3 regions (but not in CA1 or the dentate gyrus).

Together with earlier findings, these results are consistent with the suggestion that both the hippocampus and prefrontal cortex are crucial for the flexible use of stored representations (Compton et al., 1997; Jo et al., 2007). Although limited, existing evidence from the spatial domain indicates that these processes are mediated by NMDA receptor activation (Kubik et al., 2007). Studies by Nakazawa and colleagues (2002) and Fellini, Florian, Courtney and Roullet (2009) found that mutant mice with specific ablation of NMDA receptors in area CA3 successfully acquired and retrieved spatial memories in the water maze task using distal cues, but were unable to navigate when presented with a sub-set of the original cue configuration. Gold and Kesner (2005) demonstrated an analogous effect in rats with lesions to area CA3 in a dry land version of water maze.

Zif268 and c-Fos have also been implicated in long-term memory recall, both in a functional capacity and as neuronal markers of regional activation (Fleischmann et al., 2003; Guzowski, 2002; Jones et al., 2001; Kubik et al., 2007; Lanahan & Worley, 1998; Renaudineau et al., 2009). Jones and colleagues (2001), for example, noted impaired memory in Zif268 knockout mice on spatial and non-spatial water

maze tasks. These memory deficits could, however, be rescued through extended and distributed training over ten days, suggesting that Zif268 plays a time-dependent functional role in memory retrieval (Jones et al., 2001). As markers of neuronal activity, changes in Zif268 and c-Fos expression levels have been reported under a variety of behavioural conditions (see Chapter 1; and also Kubik et al., 2007). However, research comparing IEG expression patterns associated with long-term spatial and non-spatial memory retrieval is limited. One study carried out by Guzowksi and colleagues (2001) examined place and response memory using hidden and visible platform water maze tasks, respectively. The authors measured hippocampal expression of Zif268, c-Fos and Arc in these groups and in a group of untrained rats. Interestingly, they found equivalent increases in hippocampal expression of all IEGs in spatial and non-spatial groups relative to caged controls. These results appear to indicate that rats processed spatial information about their surroundings even when it was not necessary for completion of the task (Clark, Broadbent, & Squire, 2007). However, rats in this study were trained for a relatively short period of time (three days), thus, it is possible that different patterns of expression would have emerged with longer training.

Importantly, IEG expression outside of the hippocampus has yet to be investigated with regard to spatial and non-spatial strategy use. In particular, patterns of expression in the medial prefrontal region during strategy switching are currently unknown. The main goal of this chapter is to further investigate the use of such strategies in the Morris water maze and their associated brain areas. Two experiments will be carried out. In Experiment 1, we aim to delineate specific sub-regions of the hippocampus and medial prefrontal cortex implicated in the recall of place and response memories using IEG imaging. Extending on previous research

(Jo et al., 2007), we will characterise the expression of two IEGs, Zif268 and c-Fos, in all sub-regions of the dorsal hippocampus and medial prefrontal cortex. The behavioural flexibility of place memory will also be examined by testing rats under intact and partial cue conditions. Expanding on findings from Chapter 3, a final aim of this experiment is to characterise the effects of extended experience with the environment on the nature of these memories and their neural substrates. In Experiment 2, we aim to examine the importance of NMDA receptor activation for spatial and non-spatial memory retrieval and behavioural flexibility, and to determine how inactivation of these receptors influences the regional patterns of Zif268 and c-Fos expression documented in Experiment 1.

5.2. Experiment 1

The purpose of this experiment was to investigate how spatial and non-spatial strategies utilise hippocampal and prefrontal brain regions over time via IEG imaging. Results from Chapter 3 of this thesis showed rats trained for an extended period (10 days) could rely on a partial cue arrangement (with the more salient cue); therefore, we predicted that longer training here would lead to similar behavioural effect for the spatial groups trained with distal cues, but not for rats navigating via a non-spatial beacon strategy. With regard to the brain regions involved, we predict an increase in hippocampal IEG expression for spatially trained rats after extended training, reflecting successful memory recall under both full and partial cue conditions. In addition, we hypothesise that IEG expression will be increased in CA3 and in the medial prefrontal cortex for animals navigating under partial cue conditions relative to the other groups (Jo et al., 2007). For beacon-trained animals, we expect no changes in regional activation from five- to ten-day training conditions.

5.2.1. Method

5.2.1.1. Subjects.

Subjects were 42 male Wistar rats obtained from Charles River, UK. Animals' age and weight, housing conditions, handling procedures, and time of experimentation were as outlined in Chapter 2.

5.2.1.2. Apparatus.

The apparatus for this experiment was the water maze. Maze dimensions, position of the cues or beacon and platform location were as described previously in Chapter 2. Rats were trained with two cues of equal brightness; two 25 Watt light bulbs (NE

position; near cue and NW position; far cue), or a single beacon (directly above the platform). Cues of equal brightness were chosen based on the results of Chapter 3, where changes in learning strategies were observed across training lengths using these cues.

5.2.1.3. Procedure.

Rats were assigned to one of six experimental groups randomly; three groups were trained in the maze for five days (n = 21) and three groups were trained for ten days (n = 21). In the five-day training condition, two groups were trained to find the fixed, hidden platform (NE quadrant) using both cues (Control and One Cue groups), and the third group was trained with the beacon (Beacon group) (n = 7 per group). Animals in the ten-day training condition were divided into identical groups (n = 7 per group). All groups were trained with four trials per day as described in Chapter 2. Rats trained with the distal cues acted as spatial strategy groups, i.e. animals were required to learn the spatial relationships between the cue configuration and the goal in order to navigate effectively (Rodrigo, 2002). Conversely, rats trained with the beacon served as the non-spatial strategy groups, i.e. animals needed only to learn to associate movement towards the beacon with reaching the goal location (Chamizo, 2002).

Memory recall was assessed 24 hours after the final day of training (day 6 or day 11) with one probe trial lasting sixty seconds. Twenty minutes before testing, all rats were administered with an i.p. injection of saline solution (0.1 ml/100g body weight of 0.9% NaCl), in order to match the experimental conditions of Experiment 2 (see section 5.3.1.3). Control groups were tested with both near (NE) and far (NW) cues (full cue condition), One Cue groups were tested with the far cue only (partial

cue condition), and Beacon groups were tested with the beacon (non-spatial condition; see Figure 5.1). The far cue was chosen based on the results of Chapter 3, wherein a considerable performance difference was observed with this cue across five and ten day training lengths. All rats were placed into the maze near to and facing the wall from the centre of the SW quadrant.

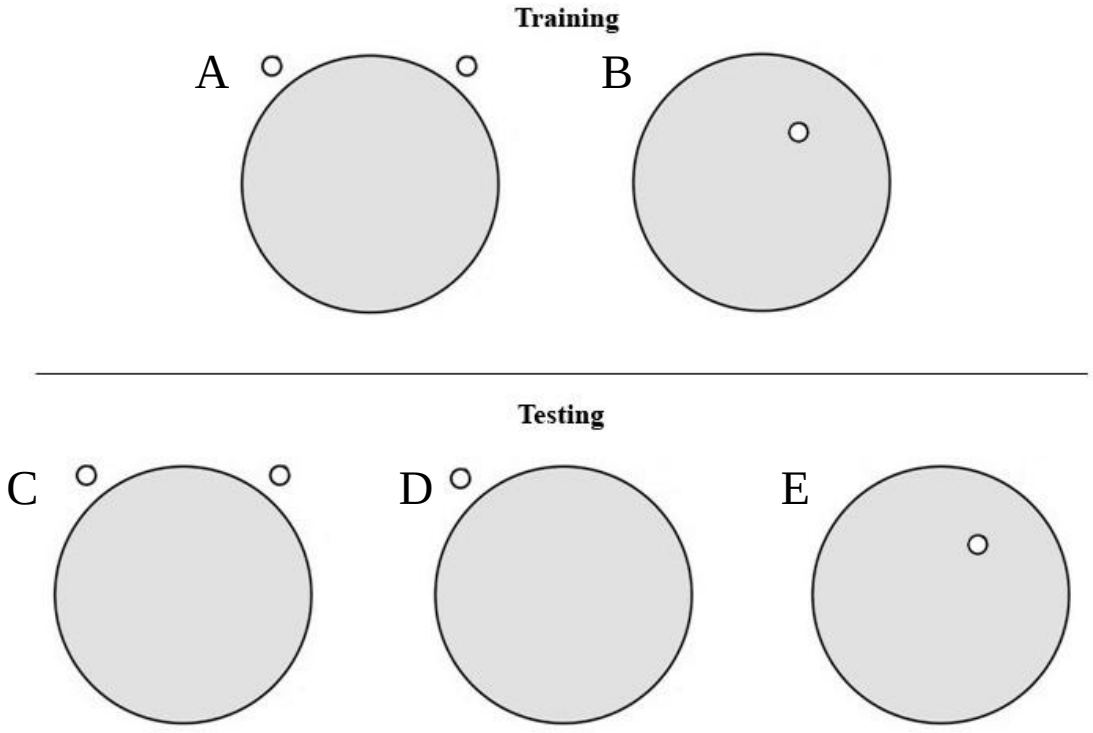

Figure 5.1: Top panel: representation of cue configuration during five- and ten-day training for (A) Control and One Cue groups, and (B) Beacon groups. Bottom panel: representation of cue configuration during testing for (C) Control, (D) One Cue, and (E) Beacon groups. Open circles outside maze denote 25 Watt bulbs, respectively. Open circle inside maze denotes beacon.

5.2.1.4. Tissue preservation.

Rats were terminally anaesthetised ninety minutes post-testing on the final day of training; they were perfused transcardially and their brains were removed, post-fixed and sliced as outlined in Chapter 2. Sub-regions of the hippocampus (CA1, CA3 and

DG) and medial prefrontal cortex (PLC, ACC and ILC) were included in IEG imaging analyses (four sections per region).

5.2.1.5. Immunohistochemistry.

Staining procedures were carried out as documented in Chapter 2 (in cohorts of three; one animal from each group).

5.2.1.6. Data analysis.

Acquisition of the water maze task was measured by escape latency (seconds) and distance travelled (centimetres). Mean trial values for each rat were averaged to produce group means. Recall was examined as percentage time spent in quadrants, platform areas and the outer corridor of the pool for each group. Numbers of Zif268 and c-Fos immunopositive cells in hippocampal and medial prefrontal sub-regions were automatically counted using ImageJ software and group means were obtained (see Chapter 2). Mean raw counts were then normalised. Because all immunohistochemistry could not be performed as a single batch (due to the difference in training lengths across groups), normalisation of the IEG data was required to control for any variability in staining specificity (see Jenkins et al., 2003, for similar procedures). Normalisation was carried out as follows. First, mean raw counts for each staining cohort of three were summed (one Control, one Bright and one Beacon rat). Counts for each individual rat were then divided by this total and expressed as a percentage; thus, all sets of normalised values summed to 100. Normalised values for each rat were then averaged, producing normalised group means.

5.2.1.7. Statistical analysis.

Group differences in escape latencies and distances travelled in each training condition were analysed using mixed factorial ANOVAs, with group as the between-groups factor (Control, One Cue and Beacon group) and training day as the within-groups factor (days 1 to 5 and days 1 to 10, respectively). One sample t-tests were used to compare percentage time spent in quadrants to chance level; time spent in platform areas was assessed using 3 x 4 mixed factorial ANOVAs, and the outer corridor was examined with one-way between-groups ANOVAs. Tukey and Bonferroni *post hoc* tests were included in these analyses where appropriate. Zif268 and c-Fos expression in the different regions were examined with a number of one-way between-groups ANOVAs, with Tukey *post hoc* comparisons (carried out on normalised data).

Five and ten day conditions were directly compared in terms of behaviour (i.e. recall performance) and IEG expression to determine any changes across training using independent-samples t-tests. To assess IEG expression in the five and ten day training conditions, difference scores were computed using the normalised mean counts. Difference scores were calculated for each sub-region by subtracting the mean score for each group on day ten from the corresponding score on day five (time 2 – time 1); thus, difference scores represent a percentage increase or decrease from IEG expression on day five. Pearson product-moment correlations were also carried out to explore the relationship between memory performance and IEG expression (see Chapter 4; raw IEG counts used). Correlations were conducted for all brain areas to allow for comparisons across training lengths.

5.2.2. Behavioural results

5.2.2.1. Acquisition.

5.2.2.1.1. Escape latency. Mixed factorial ANOVAs yielded a significant main effects of training day after five days, $F_{4,72}$ = 11.30, P = 0.001, partial eta^2 = 0.40, and ten days, $F_{9,162}$ = 39.29, P = 0.001, partial eta^2 = 0.69 (see Figure 5.2). The main effect of group was also significant after five days, $F_{1,18}$ = 18.95, P = 0.001, partial eta^2 = 0.68, but not after ten days, $F_{1,18}$ = 1.31, P = 0.30, partial eta^2 = 0.13. The day x group interaction effects were not significant; five day: $F_{8,72}$ = 1.26, P = 0.30, partial eta^2 = 0.12, ten day: $F_{18,162}$ = 0.60, P = 0.90, partial eta^2 = 0.62. For five-day trained animals, *post hoc* comparisons showed that mean escape latency on day 5 was significantly faster than on day 1 (Bonferroni: P = 0.001), and that the Beacon group was significantly faster at escaping the maze compared to the Control and One Cue groups (Tukey: both P = 0.001). Regarding rats trained for ten days, Bonferroni *post hoc* analyses showed that escape latency on day 10 was significantly shorter than on day 1 (P = 0.001).

One-way repeated measures ANOVAs conducted for each group separately produced a number of significant effects. A main effect of day was found for five- and ten-day trained Control groups; $F_{4,24}$ = 7.15, P = 0.001, partial eta^2 = 0.54, and $F_{9,54}$ = 15.10, P = 0.001, partial eta^2 = 0.72. Mean escape latency for the five-day group decreased from 42.54 ± 4.86s (CI [30.65, 54.42]) on day 1 to 19.91 ± 2.59s (CI [13.58, 26.25]) on day 5; however, this difference failed to reach statistical significance (P = 0.10). *Post hoc* tests for the ten-day group did produce a significant difference between day 1 (36.49 ± 3.56s, CI [27.79, 45.19]) and day 10 (10.12 ± 1.66s, CI [6.06, 14.19]) (P = 0.03). The main effect of day was also significant for the One Cue groups; five day: $F_{4,24}$ = 3.81, P = 0.02, partial eta^2 = 0.39, ten day: $F_{9,54}$

= 13.71, $P = 0.001$, partial eta^2 = 0.70. Again, Bonferroni *post hoc* analyses indicated that rats were not significantly faster at escaping the maze on day 5 (21.19 ± 2.26s, CI [15.67, 26.71]) relative to day 1 (39.99 ± 3.62s, CI [31.14, 48.83]) ($P = 0.14$), but were significantly faster on day 10 (11.79 ± 2.28s, CI [6.22, 17.36]) compared to day 1 (46.19 ± 5.18s, CI [33.51, 58.86]) ($P = 0.02$). For beacon groups, the main effect of day was not significant after five days, $F_{4,24} = 2.27$, $P = 0.09$, partial eta^2 = 0.27, but was significant after ten days, $F_{9,54} = 12.21$, $P = 0.001$, partial eta^2 = 0.67. For the five-day trained group, time taken to escape the maze on day 5 (17.31 ± 3.08s, CI [9.76, 24.85]) was similar to day 1 (25.24 ± 1.81s, CI [20.80, 29.67]). In contrast, escape latency for the ten-day group on the final day of training (11.62 ± 1.40s, CI [8.21, 15.03]) was significantly shorter than on day 1 (41.31 ± 3.56s, CI [32.63, 50.00]) ($P = 0.02$).

One-way between-groups ANOVAs examining group differences on each day produced significant main effects for five-day trained animals. Specifically, main effects were found on day 1: $F_{2,20} = 6.55$, $P = 0.01$, day 2: $F_{2,20} = 10.36$, $P = 0.001$, and day 4: $F_{2,20} = 5.08$, $P = 0.02$. *Post hoc* tests showed that the Beacon group were significantly faster at finding the platform than cue-trained groups on each of these days: day 1 (Control: $P = 0.01$; One Cue: $P = 0.05$), day 2 (both $P = 0.01$) and day 4 (both $P = 0.05$). No significant main effects were noted for ten-day groups (all $P > 0.05$). Importantly, all groups reached similar mean escape latencies by the final day of training.

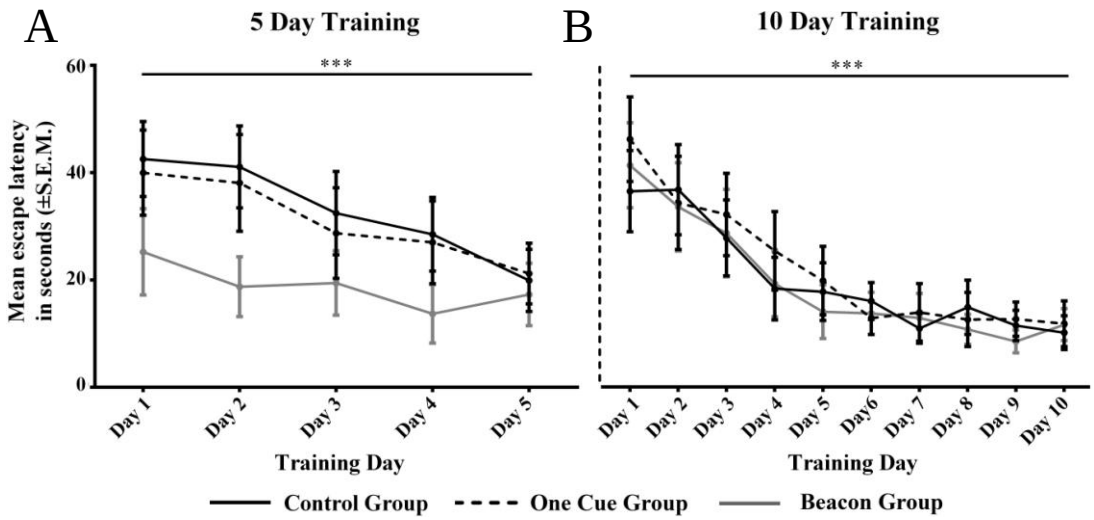

Figure 5.2: Mean escape latencies (± SEM) for Control, One Cue and Beacon groups trained for (A) five and (B) ten days.

5.2.2.1.2. Distance travelled. Similar results were found for distance travelled. Mixed factorial ANOVAs produced significant main effects of day for five-day, $F_{4,72}$ = 8.50, P = 0.001, partial eta^2 = 0.32, and ten-day groups, $F_{9,162}$ = 26.15, P = 0.001, partial eta^2 = 0.59 (see Figure 5.3). Significant main effects of group were also found after five days, $F_{1,18}$ = 7.76, P = 0.01, partial eta^2 = 0.46, and ten days, $F_{1,18}$ = 5.86, P = 0.01, partial eta^2 = 0.39. Day x group interaction effects were not significant; five day: $F_{8,72}$ = 0.54, P = 0.78, partial eta^2 = 0.06, ten day: $F_{18,162}$ = 1.20, P = 0.27, partial eta^2 = 0.12. Bonferroni *post hoc* tests revealed that mean path length on day 5 was significantly shorter than on day 1 (P = 0.01) and Tukey *post hoc* comparisons showed that the Beacon group travelled significantly shorter paths compared to the Control and One Cue groups (P = 0.01 and P = 0.02, respectively). Identical *post hoc* effects were found in the ten-day condition, i.e. distance travelled on day 10 was significantly less than on day 1 (P = 0.001) and that the mean path length of the Beacon group was shorter than cue-trained groups (P = 0.02 and P = 0.04, respectively).

A one-way repeated measures ANOVA yielded a main effect of day for both Control groups; $F_{4,24}$ = 6.73, P = 0.001, partial eta^2 = 0.53, and $F_{9,54}$ = 14.02, P = 0.001, partial eta^2 = 0.70. Similar to escape latency results, path lengths on the first (1011.35 ± 119.93cm, CI [717.90, 1304.80]) and last day of training (504.73 ± 55.05cm, CI [370.02, 639.44]) did not differ significantly in the five-day condition (P = 0.18). A significant *post hoc* difference was found for the ten-day Control group, with shorter distances travelled on day 10 (215.97 ± 33.91cm, CI [132.99, 298.95]) relative to day 1 (963.03 ± 117.72cm, CI [674.99, 1251.07]) (P = 0.04). Main effects of day were noted for the One Cue groups; five day: $F_{4,24}$ = 2.77, P = 0.04, partial eta^2 = 0.32, ten day: $F_{9,54}$ = 14.51, P = 0.001, partial eta^2 = 0.71. Again, *post hoc* tests did not indicate any significant differences between path lengths on day 1 (1025.64 ± 96.94s, CI [788.42, 1262.85]) and day 5 (586.41 ± 75.41cm, CI [401.89, 770.93]) (P = 0.32).

Distance travelled was, however, significantly shorter on day 10 (285.40 ± 63.80cm, CI [129.29, 441.50]) compared to day 1 (1287.76 ± 161.44cm, CI [892.74, 1682.78]) (P = 0.03). The main effect of day for the five-day Beacon group was not significant, $F_{4,24}$ = 2.40, P = 0.08, partial eta^2 = 0.29; mean distance travelled decreased from 887.92 ± 207.25cm (CI [380.80, 1395.04]) on day 1 to 464.19 ± 81.99cm (CI [263.56, 664.81]) day 5 (P = 0.88). A significant main effect was found after ten days, $F_{9,54}$ = 7.08, P = 0.001, partial eta^2 = 0.54, where path length on day 9 (256.90 ± 44.59cm, CI [147.78, 366.02]) was shorter than on day 1 (1518.42 ± 190.05cm, CI [1053.40, 1983.44]) (P = 0.01). One-way ANOVAs investigating between-groups effects yielded no significant differences between groups on the final days of training (all $P > 0.05$).

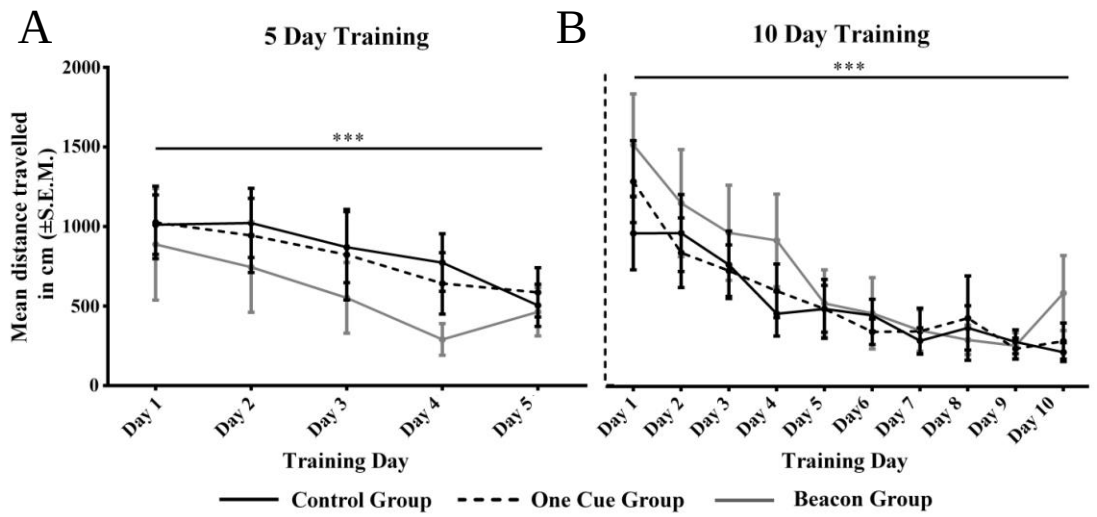

Figure 5.3: Mean path lengths (± SEM) for Control, One Cue and Beacon groups trained for (A) five and (B) ten days.

5.2.2.2. Recall.

5.2.2.2.1. Quadrants. Analyses of time spent in quadrants for five-day groups showed that – compared to chance level – the Beacon group spent significantly more time in the NE quadrant, $t_{12} = 4.19$, $P = 0.01$, and significantly less time in the NW, $t_{12} = 2.78$, $P = 0.03$, and SE quadrants, $t_{12} = 5.42$, $P = 0.01$ (see Figure 5.4A). No other significant deviations from chance were noted. After ten days of training, the Control and Beacon groups displayed a significant preference for the NE quadrant relative to chance, $t_{12} = 3.04$, $P = 0.02$, and $t_{12} = 4.68$, $P = 0.01$ (see Figure 5.4B). Time spent in the NE quadrant for the One Cue group was also significant ($P = 0.05$). In addition, all three ten-day groups spent significantly less time in the SE quadrant compared to chance; Control: $t_{12} = 4.67$, $P = 0.01$, One Cue: $t_{12} = 4.98$, $P = 0.01$, Beacon: $t_{12} = 2.67$, $P = 0.05$ (see Figure 5.4B).

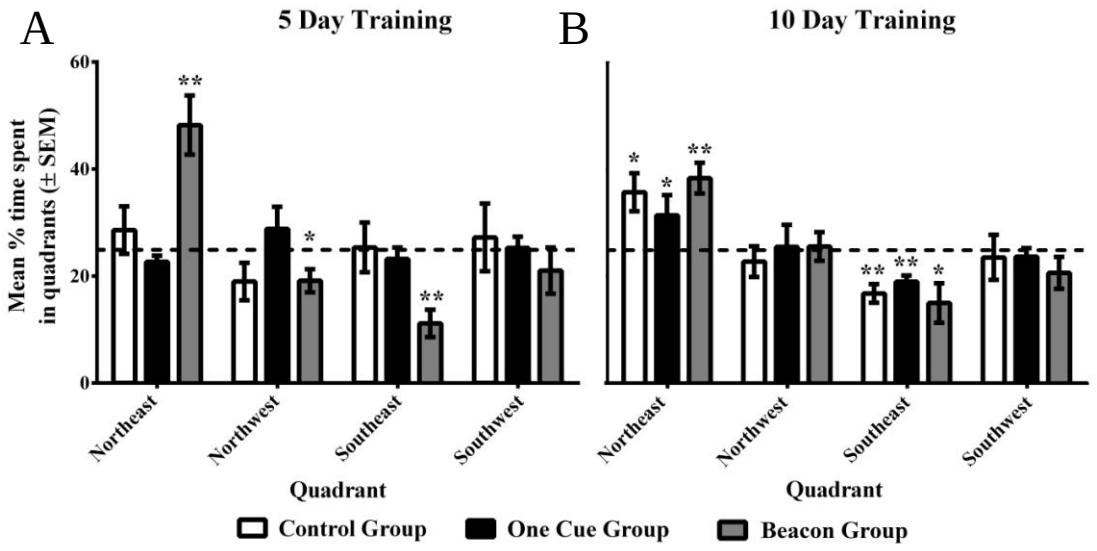

Figure 5.4: Mean percentage time (± SEM) spent in quadrants of the maze for Control, One Cue and Beacon groups after (A) five- and (B) ten-day training. Dashed line indicates chance level.

5.2.2.2.2. Platform areas. 3 x 4 mixed factorial ANOVAs examining time spent in platforms after five and ten days of training yielded identical results. More specifically, significant main effects of area, $F_{3,54}$ = 12.43, P = 0.001, partial eta^2 = 0.41, and $F_{3,54}$ = 29.48, P = 0.001, partial eta^2 = 0.62, as well as group x area interaction effects, $F_{6,54}$ = 8.16, P = 0.001, partial eta^2 = 0.48, and $F_{6,54}$ = 3.50, P = 0.02, partial eta^2 = 0.28, were found for five and ten days of training, respectively. Both main effects of group were not significant, $F_{1,18}$ = 3.50, P = 0.06, partial eta^2 = 0.28, and $F_{1,18}$ = 2.15, P = 0.15, partial eta^2 = 0.19. Bonferroni *post hoc* tests revealed that five and ten day groups spent longer in the NE area than in the NW (P = 0.01 and P = 0.001), SE (P = 0.01 and P = 0.001) and SW area (both P = 0.001).

Next, one-way repeated-measures ANOVAs were carried out to investigate within-groups differences. In the five-day training condition, main effects of area were noted for the Control group, $F_{3,18}$ = 5.77, P = 0.01, partial eta^2 = 0.49, and Beacon group, $F_{3,18}$ = 19.99, P = 0.001, partial eta^2 = 0.77, but not for the One Cue group, $F_{3,18}$ = 1.21, P = 0.33, partial eta^2 = 0.17 (see Figure 5.5A). Bonferroni *post*

hoc comparisons failed to indicate any significant differences between areas for the Control group, however, the Beacon group spent more time in the NE area (10.62 ± 1.33%, CI [7.36, 13.88]) compared to the SE (2.29 ± 0.71%, CI [0.56, 4.02]) and SW areas (2.38 ± 0.67%, CI [0.76, 4.01]) (both P = 0.01). In the ten-day training condition, main effects of area were, again, documented for the Control, $F_{3,18}$ = 26.07, P = 0.001, partial eta^2 = 0.81, and Beacon groups, $F_{3,18}$ = 8.31 P = 0.03, partial eta^2 = 0.58 (see Figure 5.5B). No main effect was found for the One Cue group, $F_{3,18}$ = 2.71, P = 0.08, partial eta^2 = 0.31. Bonferroni *post hoc* tests showed that the Control group favoured the NE area (15.24 ± 2.27%, CI [9.70, 20.78]) over the NW (4.67 ± 0.82%, CI [2.66, 6.78]; P = 0.05), SE (4.05 ± 0.81%, CI [2.08, 6.02]; P = 0.05) and SW areas (2.19 ± 0.51%, CI [0.94, 3.45]; P = 0.01).

Lastly, one-way between-groups ANOVAs were used to compare groups in each area. After five days of training, the main effect of area was significant for the NE region only, $F_{2,20}$ = 12.97, P = 0.001. The One Cue group (1.43 ± 0.76%, CI [0.43, 3.29]) spent less time here compared to the Control (6.76 ± 1.60%, CI [2.84, 10.69]) and Beacon groups (P = 0.05 and P = 0.001, respectively) (see Figure 5.5A). After ten days, no main effect was found in the NE area, $F_{2,20}$ = 3.25, P = 0.06, however, a main effect was noted in the SW area, $F_{2,20}$ = 4.28, P = 0.03. *Post hoc* analyses indicated that the One Cue group spent more time in this area than the Control group (5.14 ± 1.19%, CI [2.24, 8.04] versus 2.19 ± 0.51%, CI [0.93, 3.45]) (P = 0.03; see Figure 5.5B).

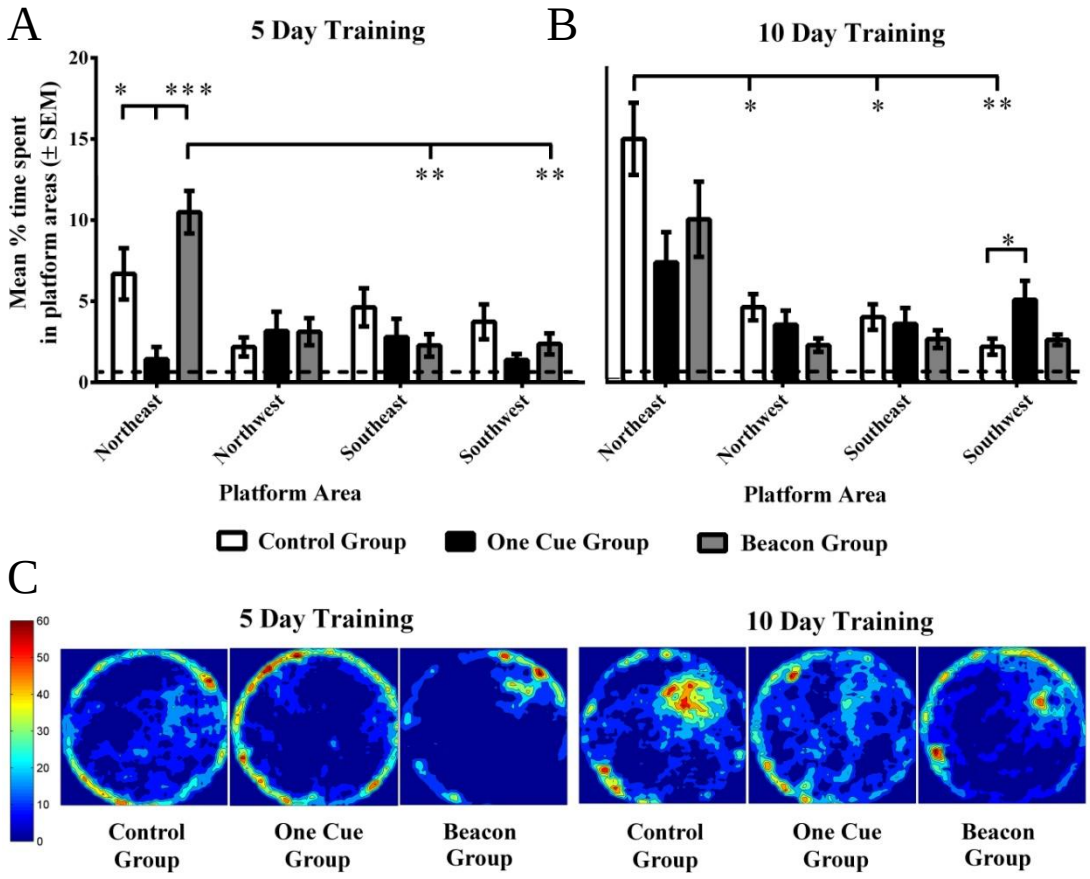

Figure 5.5: (A-B) Mean percentage time (± SEM) spent in platform areas by Control, One Cue and Beacon groups trained for five and ten days. (C) Heat maps showing overall search distributions during the probe trial for five- and ten-day groups. Dashed lines indicate chance level.

5.2.2.2.3. Outer corridor. One-way ANOVAs comparing time spent by groups in the outer corridor yielded no main effect after five, $F_{2,20} = 2.27$, $P = 0.07$, or ten days, $F_{2,20} = 2.48$, $P = 0.06$ (see Figure 5.6).

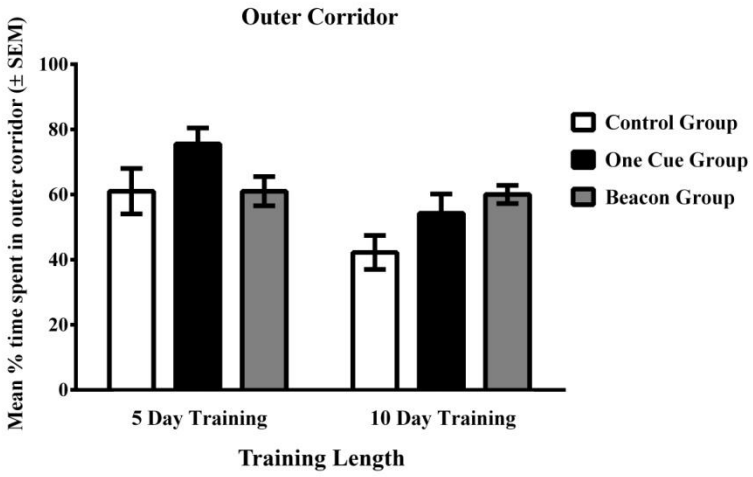

Figure 5.6: Mean percentage time (± SEM) spent in the outer corridor Control, One Cue and Beacon groups after five and ten days of training.

5.2.2.3. Comparison between five and ten day training.

Mean percentage time spent in the NE platform area by groups after five and ten days of training was compared using independent samples t-tests. Significant differences were found for the Control group, $t_{12} = 3.06$, $P = 0.01$, and the One Cue group, $t_{12} = 5.99$, $P = 0.001$, both of which spent more time in the NE area after ten days of training. No significant difference was noted for the Beacon group, $t_{12} = 0.91$, $P = 0.39$ (see Figure 5.5). No other differences were found in any other platform area. Time spent in the outer corridor also decreased significantly from five to ten days for the Control, $t_{12} = 2.16$, $P = 0.05$, and One Cue groups, $t_{12} = 2.75$, $P = 0.02$, but not for the beacon group, $t_{12} = 0.19$, $P = 0.85$ (see Figure 5.6).

5.2.3. IEG results.

5.2.3.1. Zif268.

One-way between-groups ANOVAs were carried out to compare Zif268 expression across groups for each training condition. In the five-day training condition, significant main effects of group were found in all sub-regions. In area CA1 ($F_{2,20} =$

30.51, P = 0.001), Tukey *post hoc* tests yielded significant differences between all groups. Specifically, the mean count for the Beacon group (57.49 ± 5.73, CI [43.47, 71.50]) was significantly greater than those of the Control (8.69 ± 3.80, CI [0.60, 17.98]; P = 0.001) and One Cue groups (33.83 ± 3.37, CI [25.59, 42.07]; P = 0.01), and the One Cue group had a higher mean count than the Control group (P = 0.01) (see Figure 5.7A). In area CA3 ($F_{2,20}$ = 8.76, P = 0.01), the Beacon group (61.43 ± 12.46, CI [30.95, 91.91]) had a significantly higher mean normalised count compared to the Control group (5.37 ± 3.22, CI [2.51, 13.25]; P = 0.01) (see Figure 5.7B). In the DG ($F_{2,20}$ = 9.44, P = 0.002), mean counts for Beacon (41.12 ± 10.79, CI [14.72, 67.52]; P = 0.001]) and One Cue groups (56.57 ± 11.34, CI [28.86, 84.28]; P = 0.02) were significantly higher than that of the Control group (2.32 ± 1.87, CI [-2.25, 6.88]) (see Figure 5.7C).

In the PLC ($F_{2,20}$ = 11.11, P = 0.001), the mean count for the Beacon group (61.70 ± 11.13, CI [34.45, 88.94]) was significantly higher than the Control group mean (5.48 ± 2.54, CI [0.74, 11.71]; P = 0.001) (see Figure 5.7D). In the ACC ($F_{2,20}$ = 9.18, P = 0.01), the Beacon group (61.16 ± 11.72, CI [31.49, 88.84]), again, had a higher mean Zif268 count compared to the Control group (4.41 ± 1.97, CI [0.41, 9.22]; P = 0.01) (see Figure 5.7E). Finally, the same pattern emerged in the ILC ($F_{2,20}$ = 9.54, P = 0.001), with a lower count for the Control group (7.50 ± 5.21, CI [-5.23, 20.24]) relative to Beacon group (58.35 ± 10.52, CI [32.60, 84.10]; P = 0.01) (see Figure 5.7F). Sample sections of hippocampal and medial prefrontal Zif268 expression are shown in Figure 5.8. For comparison, scatterplots depicting raw scores in each sub-region for all animals are shown in Figure 5.9.

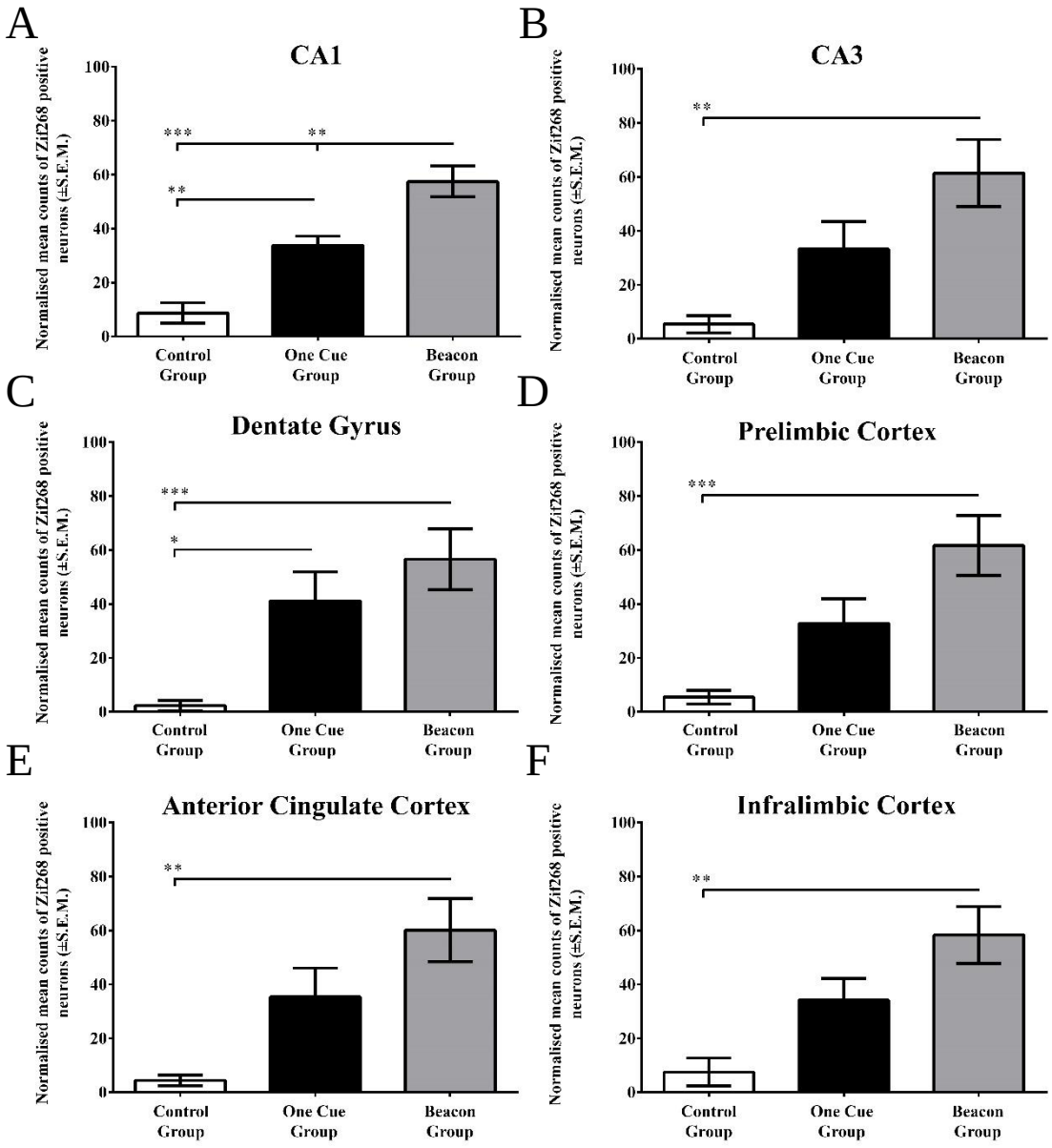

Figure 5.7: Mean normalised cell counts of Zif268 positive neurons for five-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

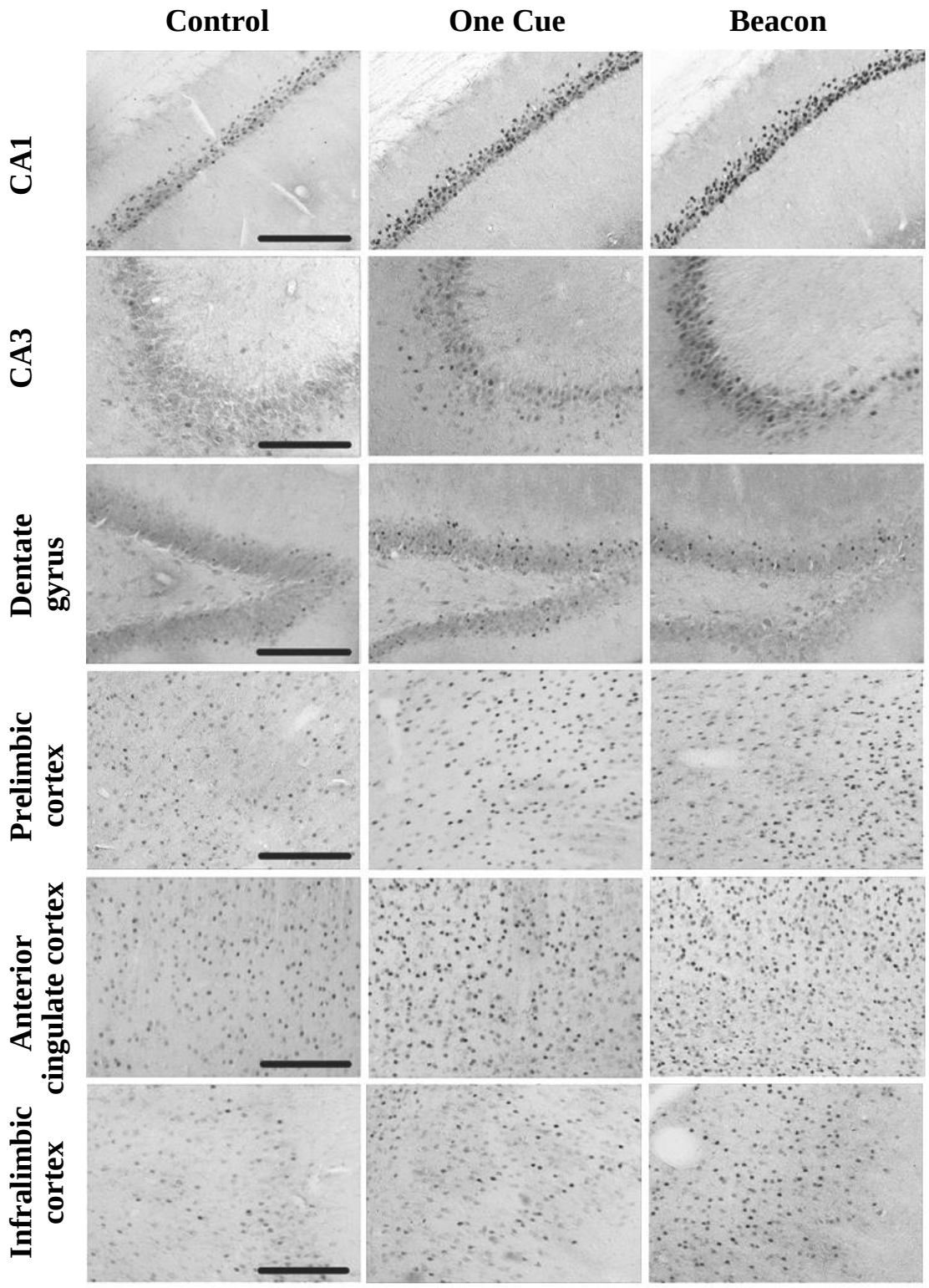

Figure 5.8: Representative images of Zif268 expression for five-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

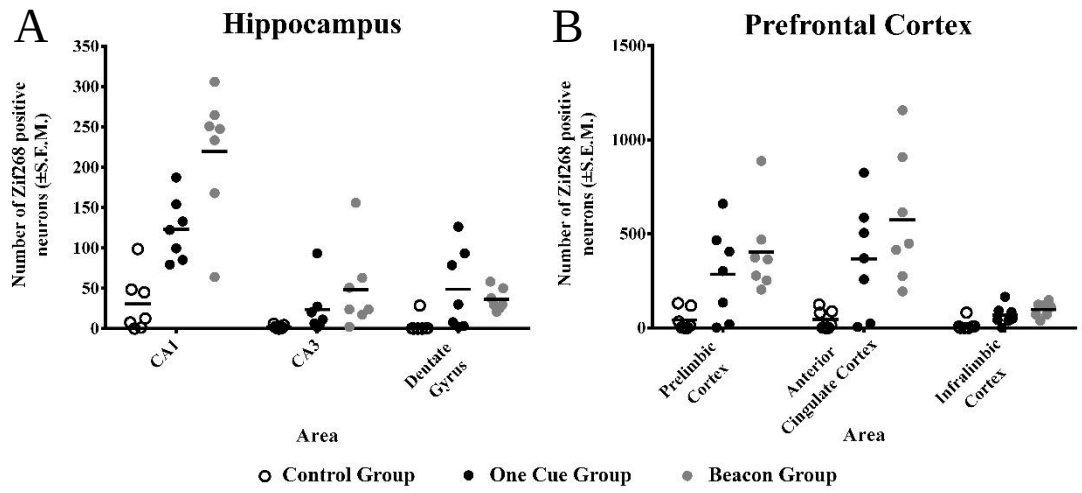

Figure 5.9: Scatterplots showing individual raw Zif268 counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after five days. Horizontal lines represent group means.

In the ten-day training condition, one extreme outlier (defined by SPSS) in the ACC sub-region was removed from the Beacon group. Significant main effects of group were found in area CA3 ($F_{2,20}$ = 10.65, P = 0.001), the PLC, ($F_{2,20}$ = 4.28, P = 0.03), and the ACC ($F_{2,19}$ = 13.73, P = 0.001). Main effects were not significant in area CA1 ($F_{2,20}$ = 3.00, P = 0.08), the DG, ($F_{2,20}$ = 2.12, P = 0.15), or the ILC, ($F_{2,20}$ = 1.76, P = 0.20) (see Figure 5.10). Tukey *post hoc* analyses showed that in area CA3, normalised mean counts for the Control (44.03 ± 3.42, CI [35.76, 52.40]) and One Cue groups (36.25 ± 4.26, CI [25.82, 45.68]) were significantly higher than that of the Beacon group (19.71 ± 3.69, CI [10.68, 28.74]) (P = 0.001 and P = 0.02, respectively) (see Figure 5.10B). In the PLC, the count for the Beacon group (13.80 ± 6.45, CI [2.00, 29.59]) was also lower than that of the Control group (45.28 ± 8.84, CI [23.63, 66.92]) (P = 0.04) (see Figure 5.10D). In the ACC, the count for the Beacon group (6.51 ± 2.47, CI [0.16, 12.86]) was, again, lower than those of the Control (55.73 ± 8.14, CI [33.82, 75.74]) and One Cue groups (38.70 ± 6.86, CI [21.91, 55.48]) (P = 0.001 and P = 0.01, respectively) (see Figure 5.10E). Sample

sections of hippocampal and medial prefrontal Zif268 expression are shown in Figure 5.11. For comparison, scatterplots depicting raw scores in each sub-region for all animals after ten-day training can be seen in Figure 5.12.

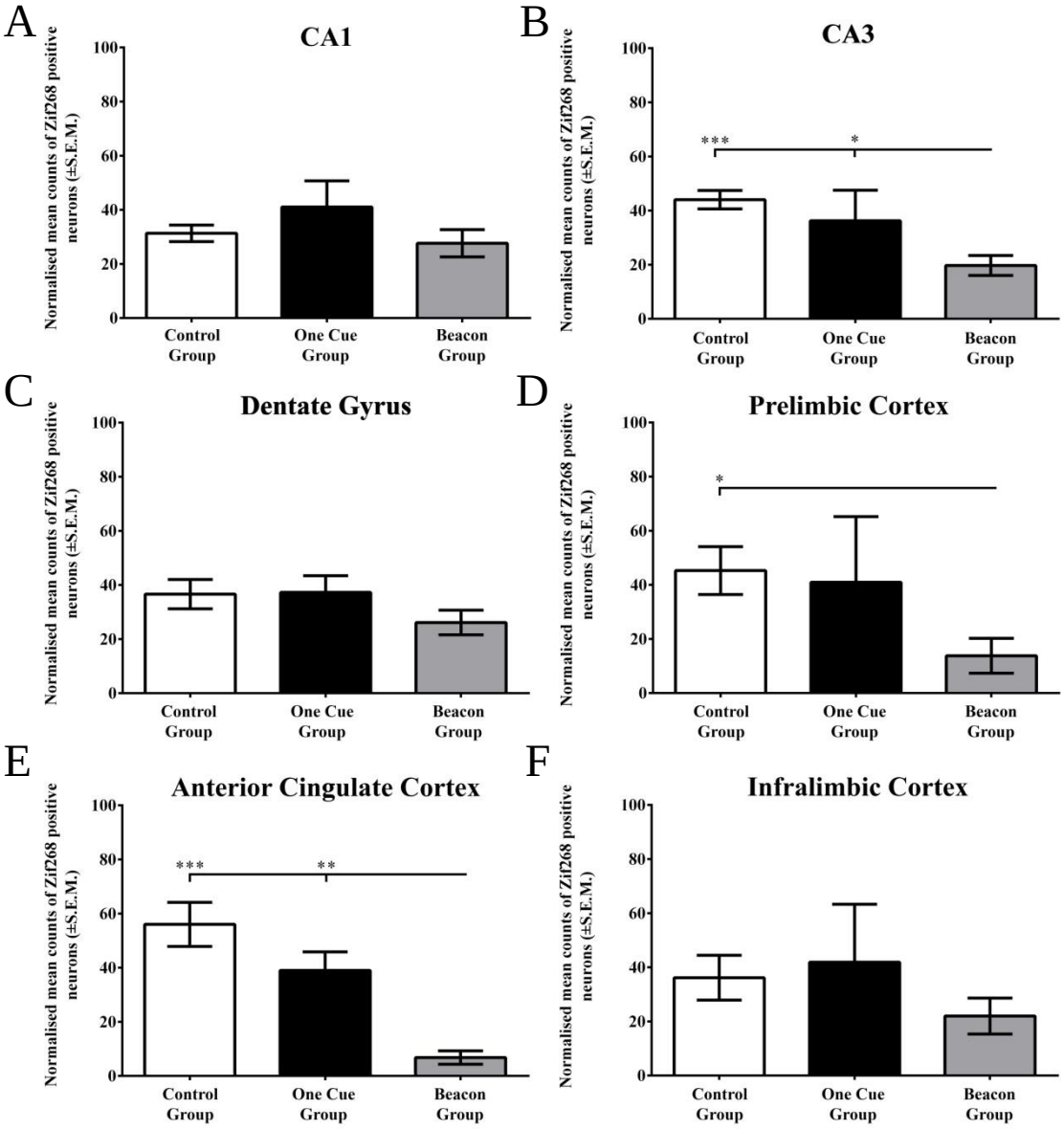

Figure 5.10: Mean normalised cell counts of Zif268 positive neurons for ten-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

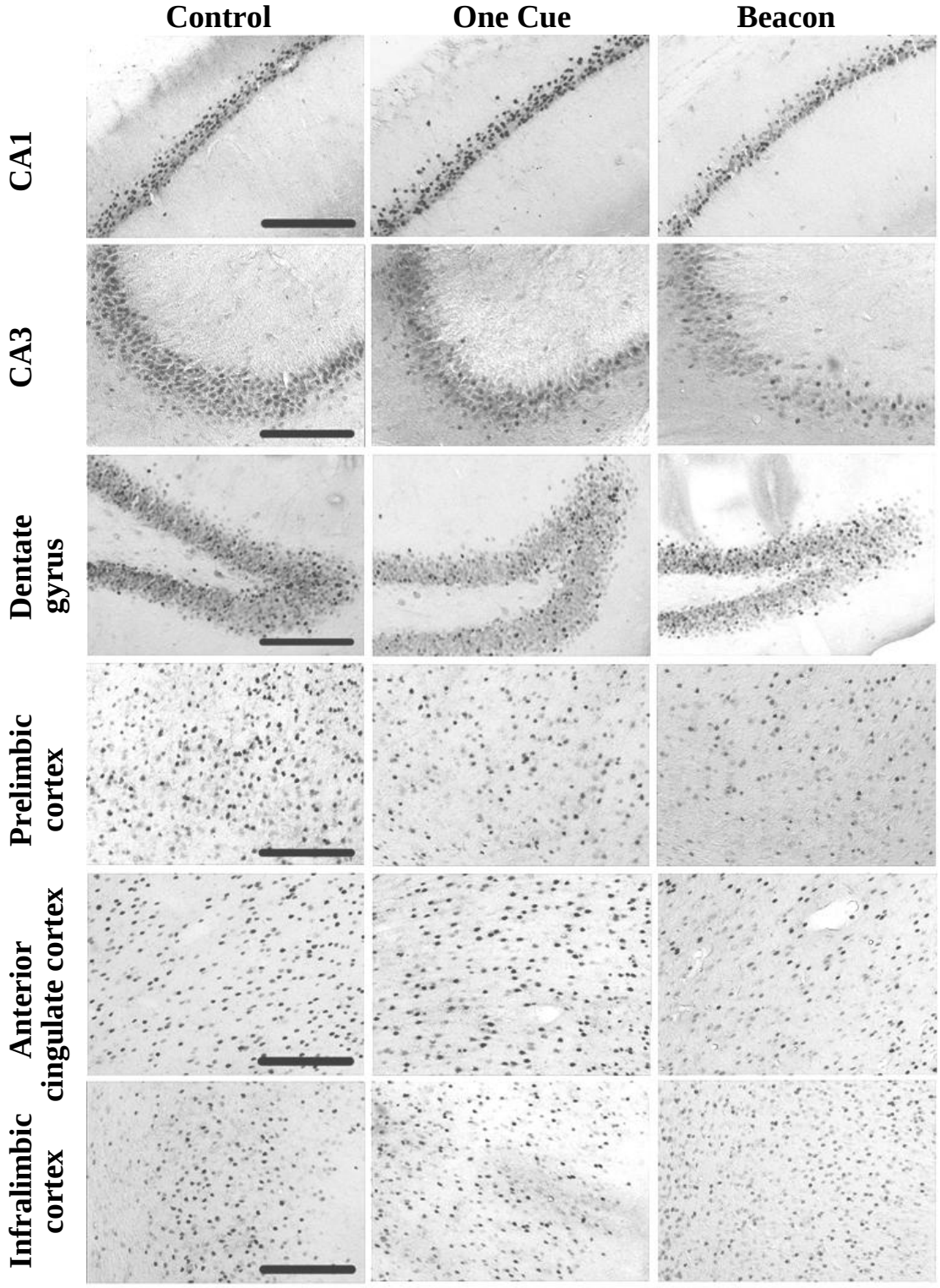

Figure 5.11: Representative images of Zif268 expression for ten-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

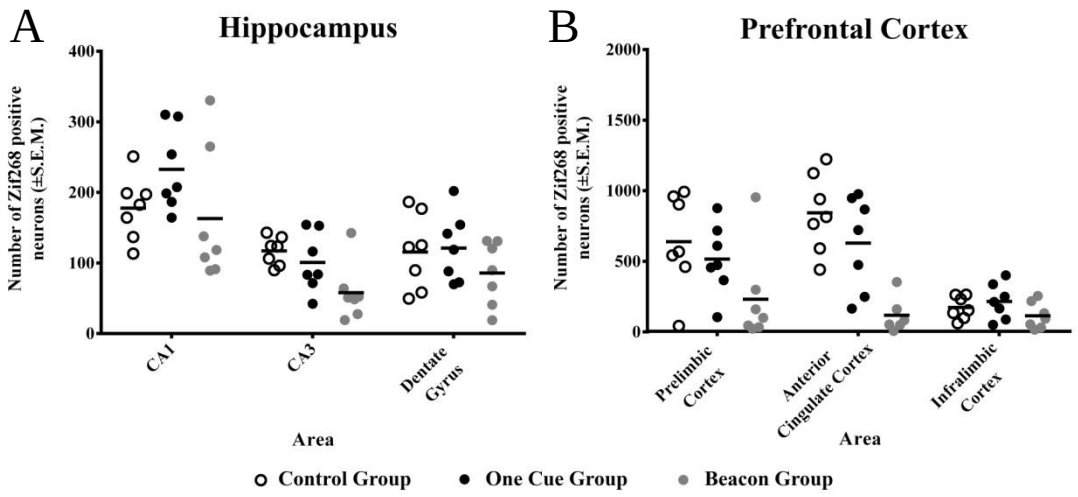

Figure 5.12: Scatterplots showing individual raw Zif268 counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after ten days. Horizontal lines represent group means.

5.2.3.2. c-Fos

One-way between-groups ANOVAs were conducted to compare c-Fos expression across groups after five and ten days of training. In the five-day condition, no significant main effects of group were found; CA1: $F_{2,20} = 0.10$, $P = 0.90$, CA3: $F_{2,20} = 0.20$, $P = 0.82$, DG: $F_{2,20} = 0.03$, $P = 0.97$, PLC: $F_{2,20} = 0.52$, $P = 0.60$, ACC: $F_{2,20} = 0.17$, $P = 0.85$, and ILC: $F_{2,20} = 0.34$, $P = 0.72$ (see Figure 5.13). After ten days of training, no significant main effects of group were noted in any area; CA1: $F_{2,20} = 0.61$, $P = 0.56$, CA3: $F_{2,20} = 0.05$, $P = 0.95$, DG: $F_{2,20} = 0.15$, $P = 0.86$, PLC: $F_{2,20} = 0.06$, $P = 0.94$, ACC: $F_{2,20} = 0.38$, $P = 0.69$, and ILC: $F_{2,20} = 0.62$, $P = 0.55$ (see Figure 5.14). Sample sections of hippocampal and prefrontal c-Fos expression are shown in Figures 5.15 and 5.16. Figures 5.17 and 5.18 depict scatterplots with individual raw scores in each sub-region.

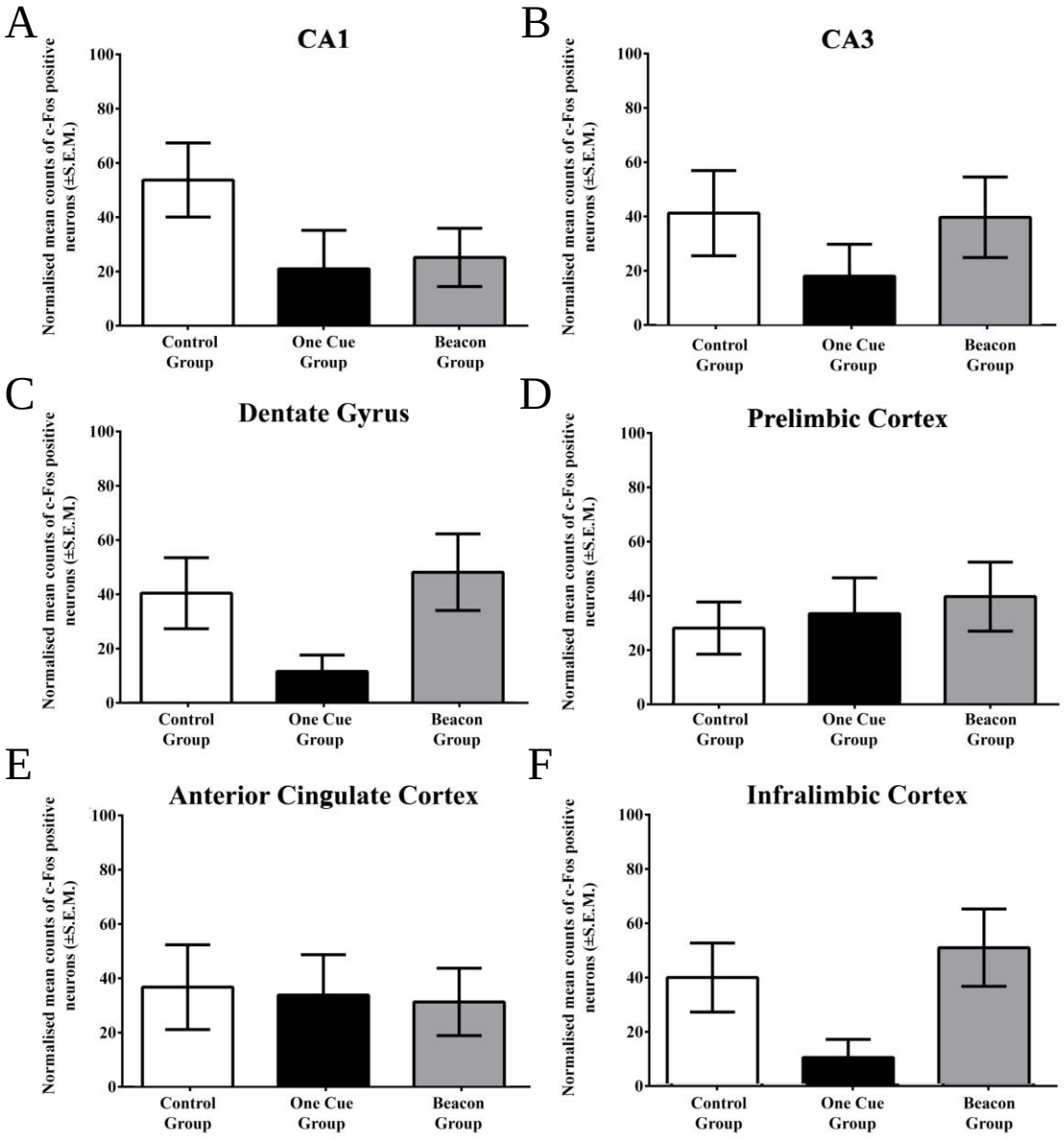

Figure 5.13: Mean normalised cell counts of c-Fos positive neurons for five-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

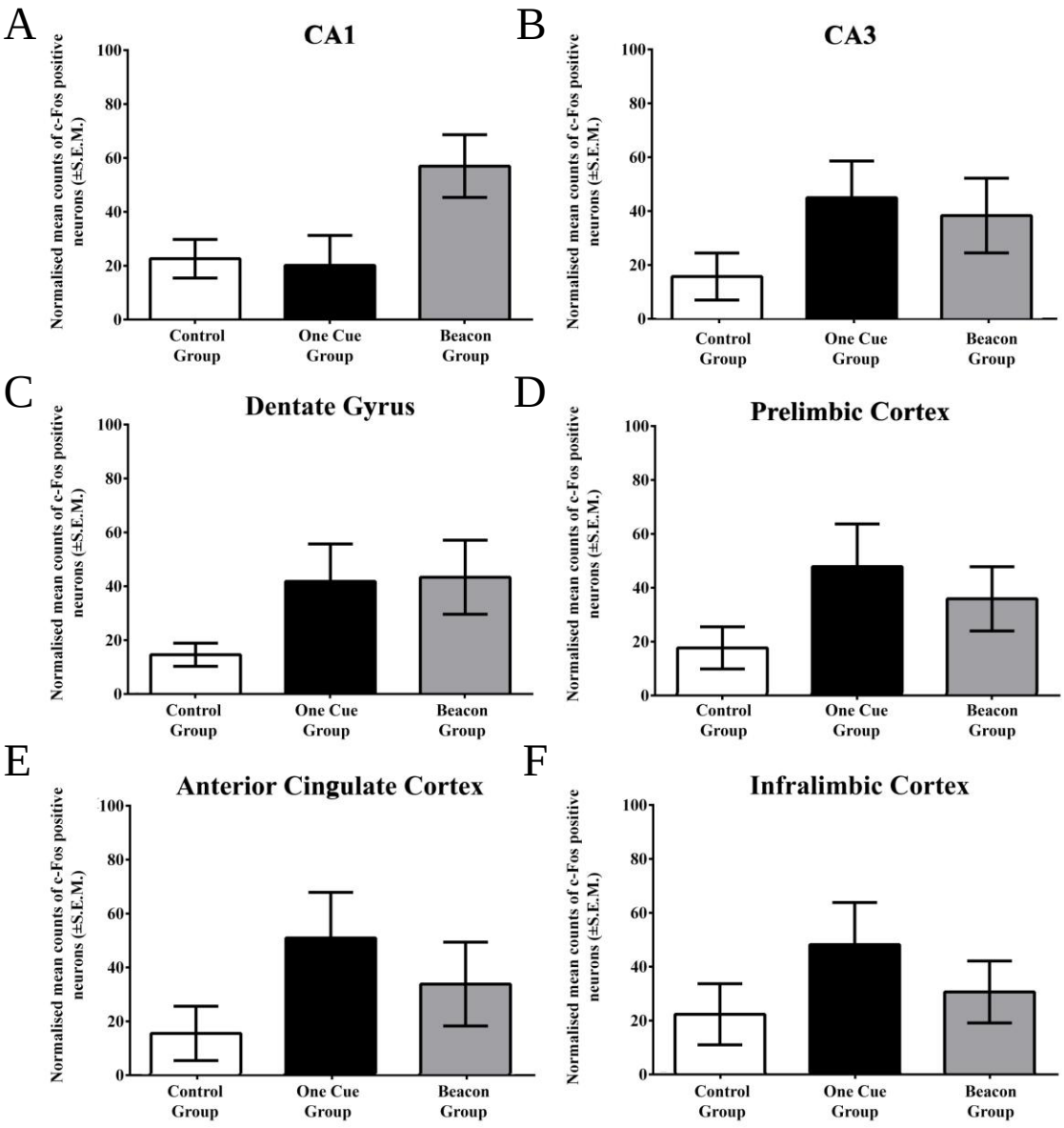

Figure 5.14: Mean normalised cell counts of c-Fos positive neurons for ten-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

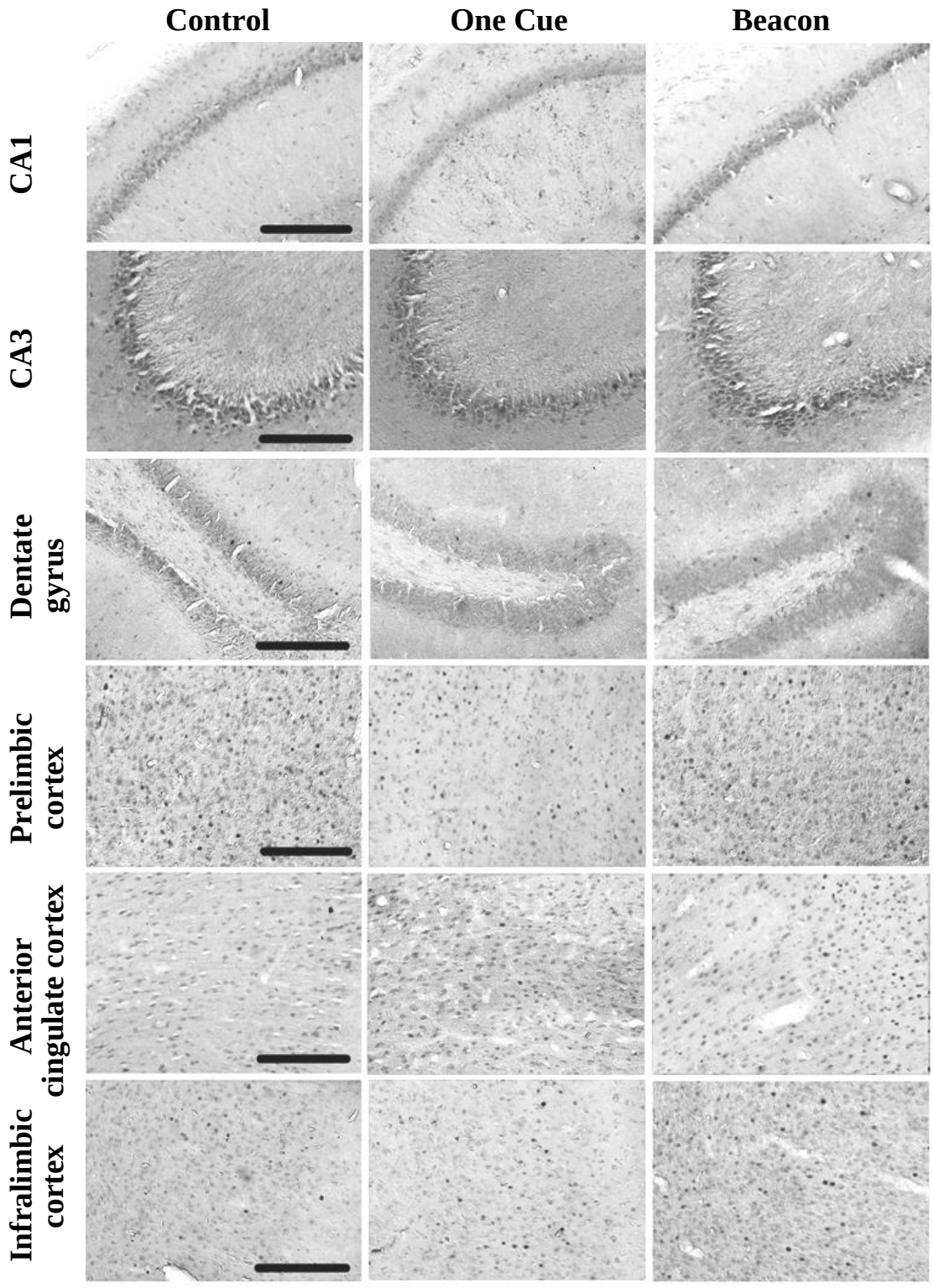

Figure 5.15: Representative images of c-Fos expression for five-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, and the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

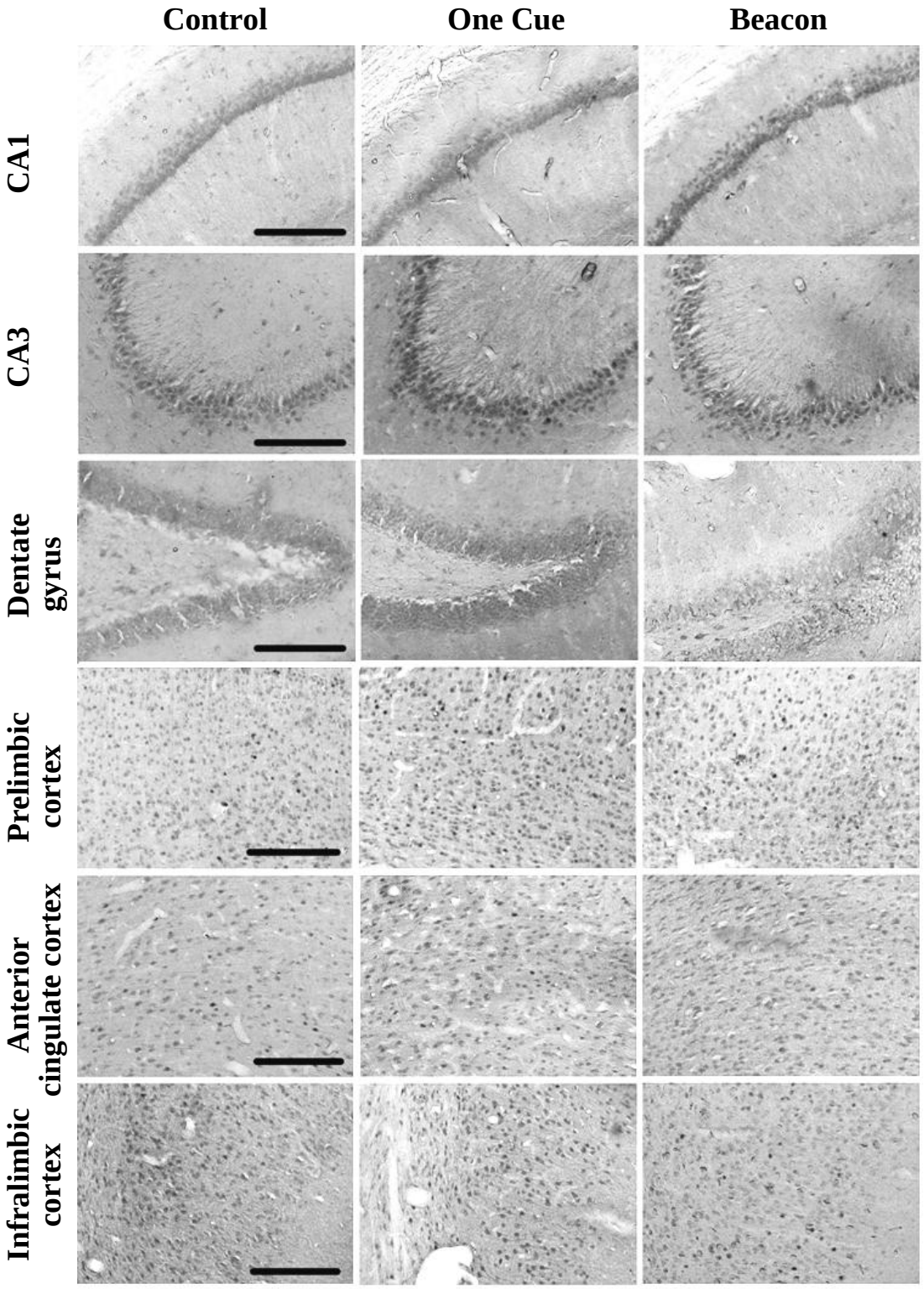

Figure 5.16: Representative images of c-Fos expression for ten-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

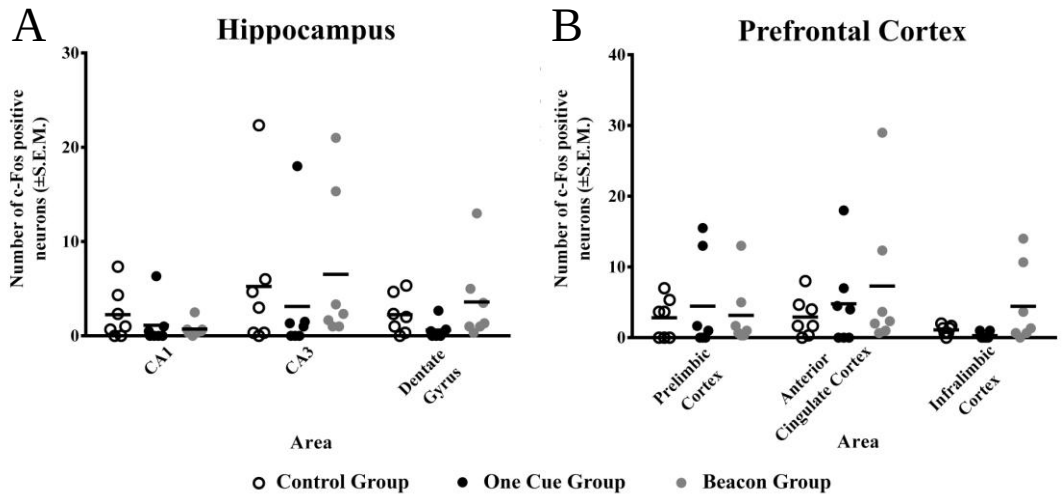

Figure 5.17: Scatterplots showing individual raw c-Fos counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after five days. Horizontal lines represent group means.

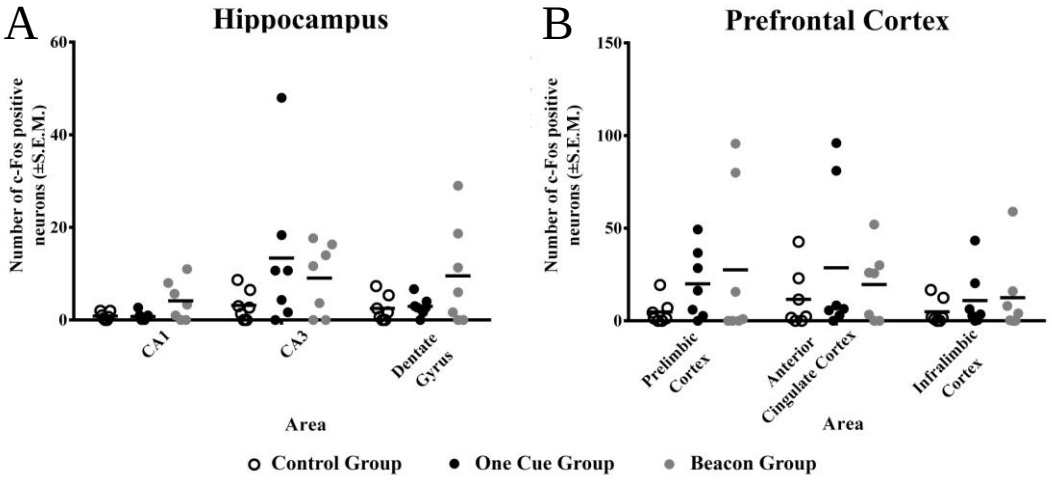

Figure 5.18: Scatterplots showing individual raw c-Fos counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after ten days. Horizontal lines represent group means.

5.2.3.3. Comparison between five and ten day training.

Independent-samples t-tests revealed a number of significant differences in Zif268 expression for the Control and Beacon groups (see Figure 5.19A). For the Control group, Zif268 expression increased significantly in all sub-regions across training conditions; CA1: $t_{12} = 3.85$, $P = 0.01$, CA3: $t_{12} = 7.24$, $P = 0.001$, DG: $t_{12} = 7.83$, $P =$

0.001, PLC: t_{12} = 4.11, P = 0.01, ACC: t_{12} = 5.76, P = 0.001, and ILC: t_{12} = 2.88, P = 0.03. In contrast, significant decreases were observed for the Beacon group in all sub-regions; CA1: t_{12} = 2.94, P = 0.03, CA3: t_{12} = 3.16, P = 0.02, DG: t_{12} = 2.55, P = 0.04, PLC: t_{12} = 3.45, P = 0.02, ACC: t_{12} = 4.88, P = 0.01, and ILC: t_{12} = 2.89, P = 0.03. No significant differences were noted for the One Cue group. For c-Fos expression, no significant differences were found for any group (see Figure 5.19B).

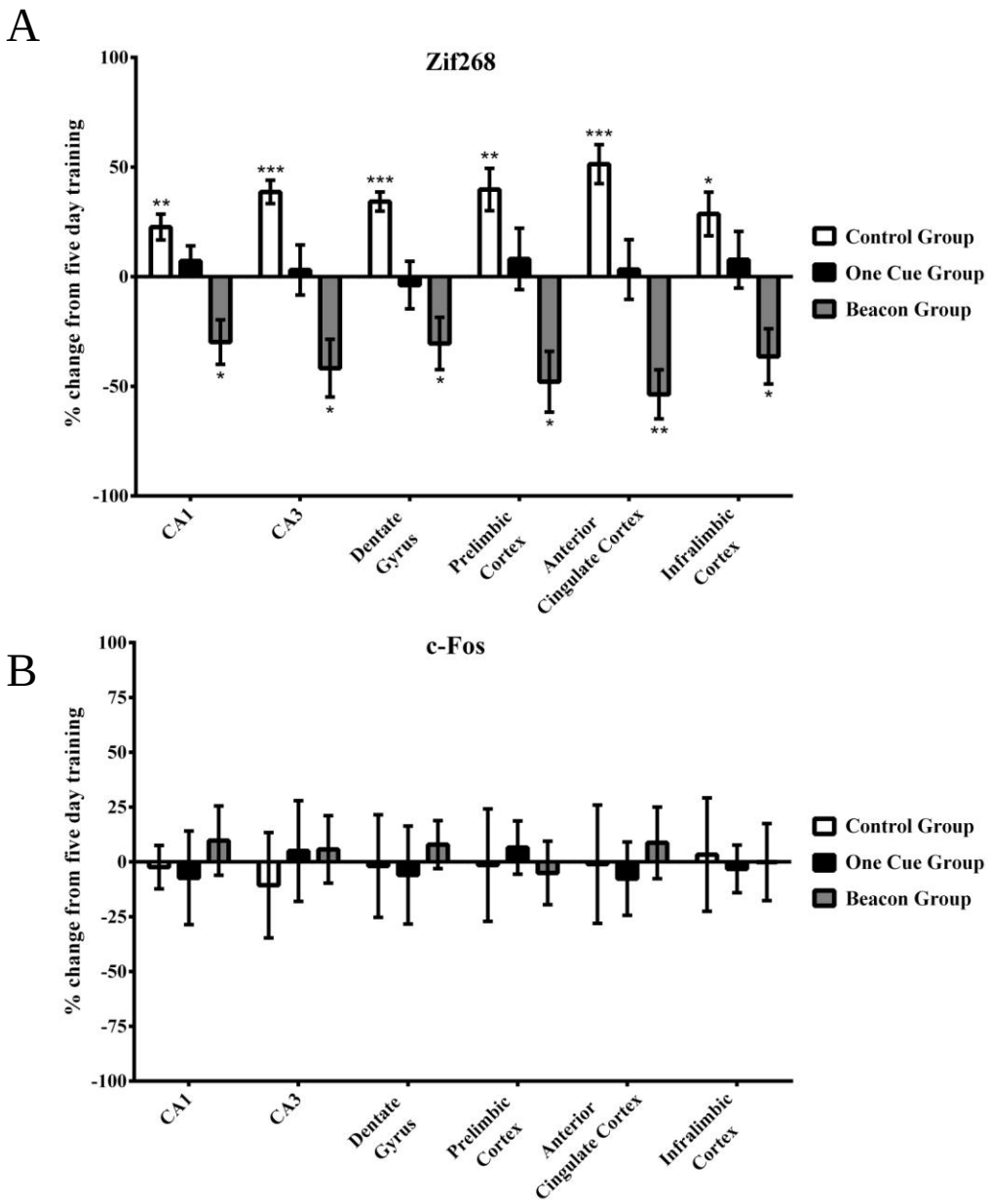

Figure 5.19: mean percentage increase or decrease in (A) Zif268 and (B) c-Fos expression from five- to ten-day training conditions for Control, One Cue and Beacon groups in all sub-regions.

5.2.4. Correlations with behaviour.

Finally, mean counts of regional Zif268 and c-Fos for each group were correlated with percentage time spent in the target (NE) platform area to determine the relationship between IEG expression and water maze recall after five and ten days. All regions were analysed. Only one significant correlation was found; this result was for the Beacon group, for which a significant positive correlation between Zif268 expression in CA1 and time spent in the NE area after five days was identified ($r = 0.81$, $P = 0.03$; see Table 5.1 Bottom). All other correlations between Zif268 and c-Fos expression after five and ten days of training were not significant (see Tables 5.1 and 5.2 and Figures 5.20-5.23).

Table 5.1: Correlations between Zif268 expression and percentage time spent in the NE platform area for five- and ten-day Control, One Cue and Beacon groups.

Group	Brain region	Training condition	
Control		Five days	Ten days
	CA1	0.05	-0.44
	CA3	-0.46	0.31
	DG	-0.07	0.50
	PLC	-0.09	-0.08
	ACC	0.23	-0.36
	ILC	-0.05	-0.34
One Cue		Five days	Ten days
	CA1	0.30	0.18
	CA3	-0.31	0.43
	DG	0.22	-0.60
	PLC	0.75	-0.59
	ACC	0.56	-0.02
	ILC	0.32	0.02
Beacon		Five days	Ten days
	CA1	**0.81***	-0.31
	CA3	0.52	-0.06
	DG	0.62	-0.34
	PLC	0.11	-0.04
	ACC	0.67	0.11
	ILC	-0.09	-0.13

Table 5.2: Correlations between c-Fos expression and percentage time spent in the NE platform area for five- and ten-day Control, One Cue and Beacon groups.

Group	Brain region	Training condition	
Control		Five days	Ten days
	CA1	-0.29	0.21
	CA3	-0.70	0.22
	DG	-0.05	-0.13
	PLC	0.19	-0.27
	ACC	-0.26	0.64
	ILC	-0.03	-0.17
One Cue		Five days	Ten days
	CA1	-0.25	-0.18
	CA3	-0.30	0.58
	DG	-0.32	0.65
	PLC	0.26	-0.01
	ACC	0.12	-0.33
	ILC	0.68	-0.08
Beacon		Five days	Ten days
	CA1	0.55	0.70
	CA3	0.14	0.53
	DG	0.21	0.68
	PLC	0.63	0.73
	ACC	0.47	0.70
	ILC	0.68	0.77

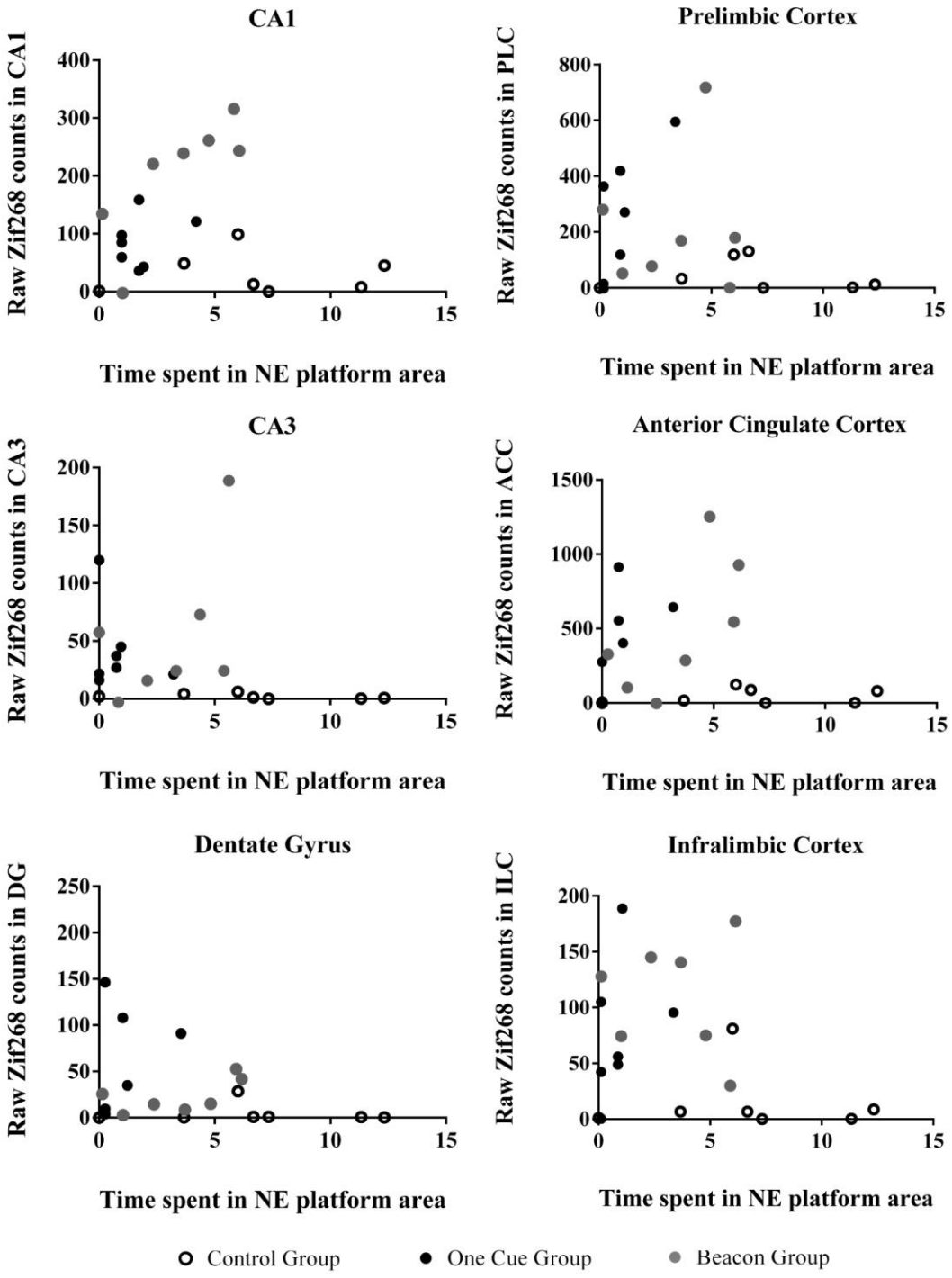

Figure 5.20: Scatterplots showing regional Zif268 counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after five days of training.

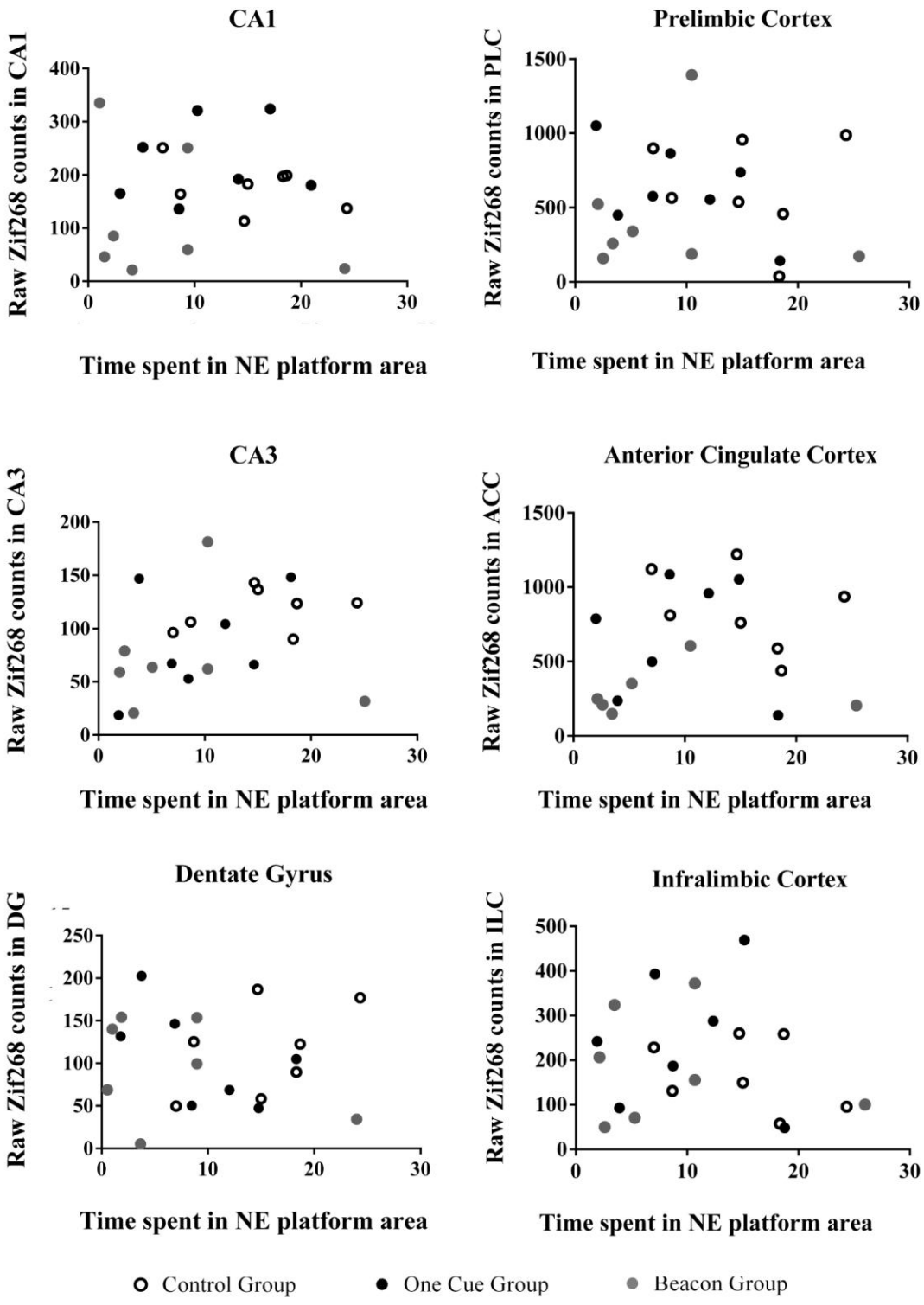

Figure 5.21: Scatterplots showing regional Zif268 counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after ten days of training.

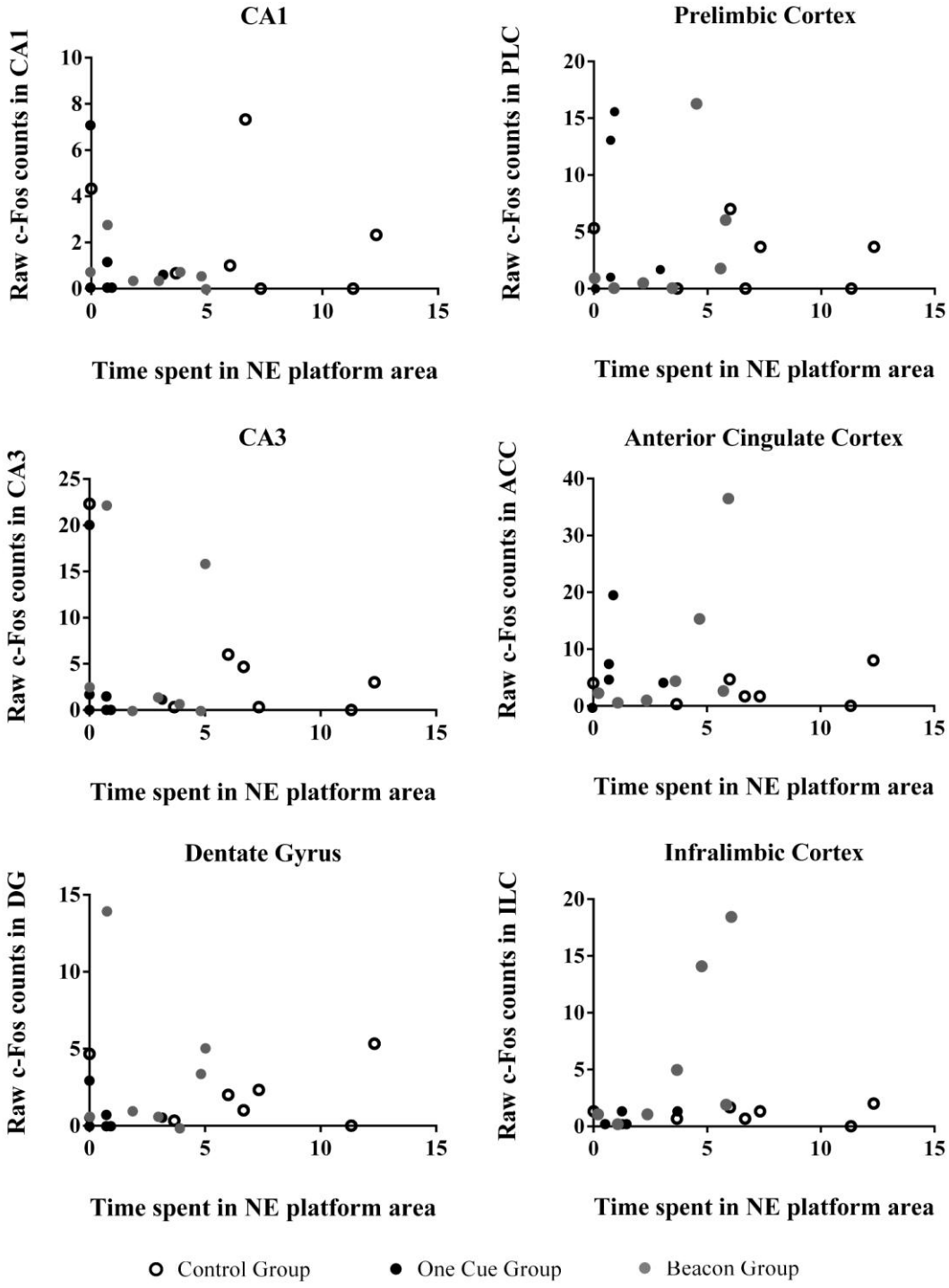

Figure 5.22: Scatterplots showing regional c-Fos counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after five days of training.

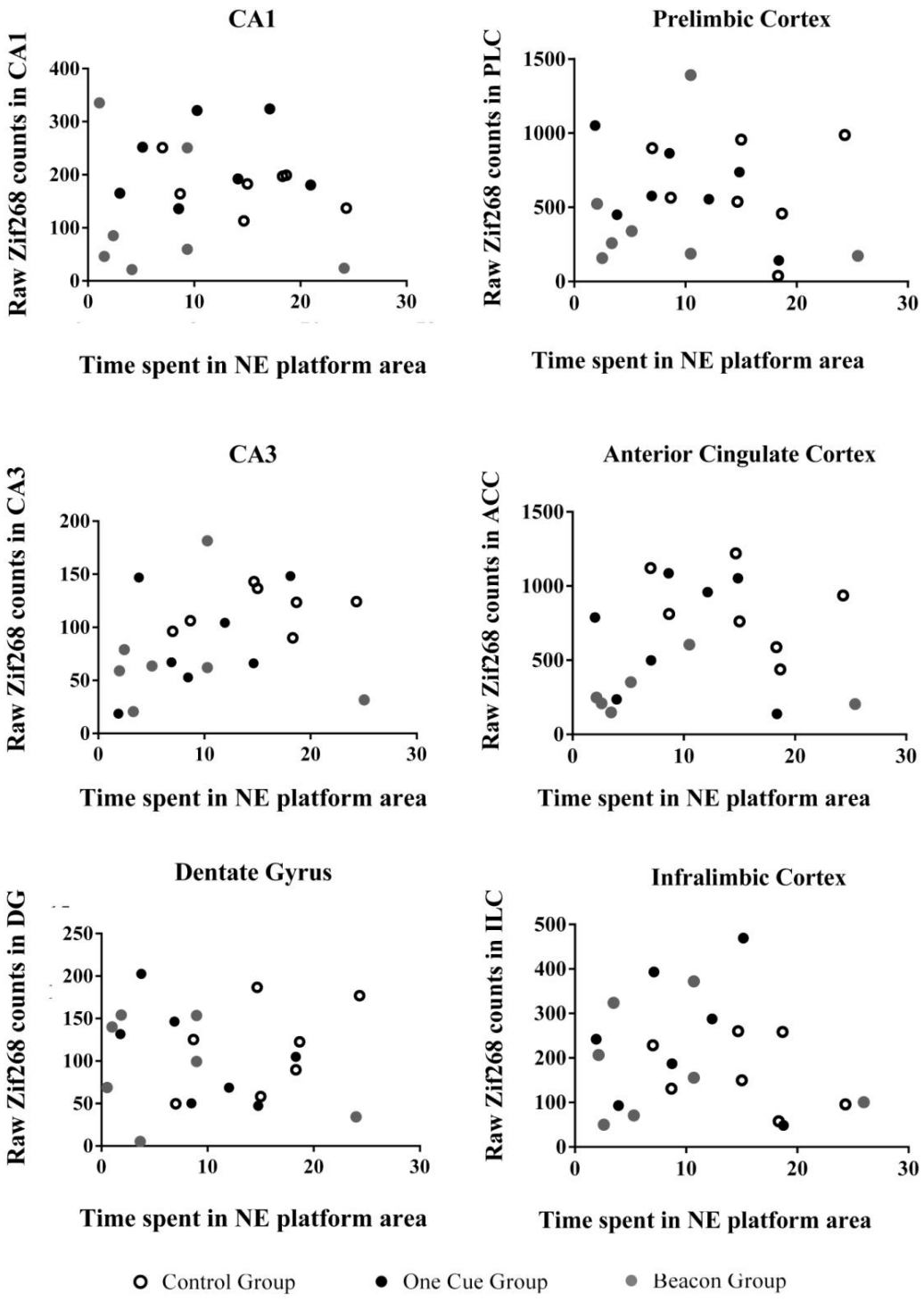

Figure 5.23: Scatterplots showing regional c-Fos counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after ten days of training.

5.2.5. Discussion

All animals learned to locate the hidden platform after five and ten days of training. Importantly, groups did not differ in average time taken to escape the maze or distance travelled on final days of training, signifying equivalent learning. Both Control and One Cue groups (trained with two cues) took less time and used shorter paths to reach the platform as training progressed, with performance (i.e. memory for the platform location) being strongest after ten days of training. Results for the Beacon groups (trained with a single cue) were less clear; that is, while the five-day group displayed a shallow learning curve – indicative of traditional beacon-type learning (Morris, 1981) – the ten-day group showed a similar pattern of learning to the Control and One Cue groups. Thus, it is difficult to determine from the acquisition results what type of learning strategy these animals were using.

During the probe test, only the Beacon group favoured the target quadrant after five days, while the Control and One Cue groups failed to show any preferences. After ten days of training, all groups favoured the correct quadrant, thereby demonstrating that more training improved memory recall for the Control and One Cue groups, but not for Beacon group. Results from the platform area and outer corridor analyses support this suggestion; specifically, performance of the Control and One Cue groups was significantly better after ten days (i.e. more time in the NE area and less time in the outer corridor), while the performance of Beacon group remained constant. Together, findings suggest that animals trained with the beacon were relying on a response-type strategy. In addition, time spent in the platform areas revealed that the One Cue group was considerably impaired relative to the other groups at five- but not at ten-day recall. These results are line with those of Chapter 3 (Experiment 3), whereby extended training can lead to increased

behavioural flexibility which, in turn, enables memory recall under diminished cue conditions (Jo et al., 2007; Rodrigo et al., 2014).

On a cellular level, Zif268 cell counts were significantly higher for the Beacon group in all sub-regions relative to the other groups when tested after five days of training, with expression in area CA1 positively correlated with percentage time in the target platform area. This is consistent with behavioural findings, i.e. this was the only group to display accurate memory recall after five days. The One Cue group also exhibited greater Zif268 expression in the hippocampus (CA1 and DG) compared to the Control group, who had the lowest overall counts. Interestingly, the opposite pattern was seen at ten-day recall, with the Beacon group yielding the lowest levels of Zif268 expression across regions. Specifically, Zif268 expression in CA3 and ACC regions was increased for the both spatial groups.

These patterns were reflected in the difference scores; percentage Zif268 expression in all sub-regions increased from five to ten days for the Control group, decreased for the Beacon group and remained the same for the One Cue group. Consequently, it would seem that changes in Zif268 expression reflect accurate memory recall by the spatially trained groups after extended training (which resulted in a comparative decrease for the beacon group). In stark contrast to Zif268, no group differences were found for c-Fos expression in any sub-region after five or ten days of training, and levels of expression did not appear change as a function of training length (unlike Jo et al., 2007). Together, findings imply that expression of Zif268 in the regions analysed is more sensitive to memory type (spatial or non-spatial) than c-Fos.

5.3. Experiment 2

The purpose of Experiment 2 was to investigate the effects of NMDA receptor blockade (via MK-801) on spatial and non-spatial memory processing over time, and on corresponding IEG expression in the hippocampus and medial prefrontal cortex. Due to the lack of effects observed following AMPA receptor antagonism in Chapter 4, which may be explained by poor penetration of CNQX across the blood-brain barrier (Rogawski, 2011), a CNQX group was not included for this experiment. As MK-801 was administered i.p. (thus inactivating NMDA receptors throughout the brain), we anticipate gross memory deficits in all groups after five days of training. In addition, given the known association between NMDA receptor activation and IEG expression, we also expect widespread decreases in Zif268 and c-Fos (Chapter 4; and also Gass et al., 1993; Vaccarino et al., 1992). Crucially, however, whether or not increased experience with the environment prior to spatial or non-spatial testing can protect against the effects of NMDA receptor blockade is unknown. If this is the case, we predict that spatially-trained rats in the full cue condition will show good memory recall and increased IEG expression, while the partial cue group will continue to be impaired (similar to Fellini et al., 2009; Nakazawa et al., 2004; Nakazawa et al., 2002). Based on the results of Experiment 1, we hypothesise that increased training will have no effect on performance of the non-spatial group; that is, if rats are impaired at five-day recall, these deficits will persist after ten days.

5.3.1. Method

5.3.1.1. Subjects.

Forty-two male Wistar rats (Charles River, UK) were used as subjects. Rats' age and weight, housing conditions, handling procedures, and time of experimentation were the identical to those described previously.

5.3.1.2. Apparatus.

The apparatus, position of the platform, cues and beacon were identical to Experiment 1. Animals were trained with two cues or a single beacon.

5.3.1.3. Procedure.

Rats were, again, divided into six experimental groups (n = 7 per group); two Control and two One Cue groups trained with both cues for five or ten days (spatial strategy groups), and two Beacon groups trained with the beacon for five or ten days (non-spatial strategy groups) (all four trials per day). Sixty second probe trials were conducted on day 6 or day 11. Rats received an i.p. injection of NMDA receptor antagonist MK-801 (0.1mg/kg body weight) twenty minutes prior to testing. Sterile saline was used as the vehicle (0.3ml total volume per injection). As per Experiment 1, Control groups were tested with both cues, One Cue groups were tested with the far cue only, and Beacon groups were tested with the beacon only (see Figure 5.1).

5.3.1.4. Tissue preservation and immunohistochemistry.

As before, ninety minutes post-testing, rats were terminally anaesthetised and perfused, their brains were removed, post-fixed and sliced (see Chapter 2). Regions

of interest included CA1, CA3, DG, PLC, ACC and ILC. Staining procedures were followed as described in Chapter 2 and data were normalised as previously outlined.

5.3.1.5. Data and statistical analyses.

All data and statistical analyses were identical to those used in Experiment 1.

5.3.2. Behavioural results

5.3.2.1. Acquisition.

5.3.2.1.1. Escape latency. For the five day condition, a 3 x 5 mixed factorial ANOVA produced a significant main effect of day, $F_{4,72} = 11.39$, $P = 0.001$, partial eta$^2 = 0.39$, with rats escaping the maze significantly faster on day 5 than on day 1 (Bonferroni: $P = 0.001$) (see Figure 5.24A). No significant main effect of group, $F_{1,18} = 0.25$, $P = 0.79$, partial eta$^2 = 0.03$, or day x group interaction effect was found, $F_{8,72} = 0.90$, $P = 0.52$, partial eta$^2 = 0.09$. A 3 x 10 mixed factorial ANOVA for the ten day condition also yielded a main effect of day, $F_{9,162} = 27.27$, $P = 0.001$, partial eta$^2 = 0.60$ (see Figure 5.24B), and day x group interaction effect, $F_{18,162} = 2.68$, $P = 0.01$, partial eta$^2 = 0.23$. Bonferroni *post hoc* tests showed that escape latency on day 10 was significantly shorter compared to day 1 ($P = 0.001$). The main effect of group was not significant, $F_{1,18} = 1.22$, $P = 0.32$, partial eta$^2 = 0.12$.

Separate repeated-measures ANOVAs were then carried out for each group. No main effects were found for the five-day Control group, $F_{4,24} = 2.26$, $P = 0.09$, partial eta$^2 = 0.27$, or One Cue group, $F_{4,24} = 3.85$, $P = 0.07$, partial eta$^2 = 0.39$. The main effect of day was significant for the five-day Beacon group, $F_{4,24} = 8.94$, $P = 0.01$, partial eta$^2 = 0.60$; escape latency for this group was significantly shorter on day 5 than on day 1 (Bonferroni: $P = 0.03$). After ten days of training, main effects

were significant for all groups; Control: $F_{9,54}$ = 8.02, P = 0.01, partial eta^2 = 0.57, One Cue: $F_{9,54}$ = 20.43, P = 0.001, partial eta^2 = 0.77, Beacon: $F_{9,54}$ = 6.98, P = 0.01, partial eta^2 = 0.54. However, significant *post hoc* differences were noted for the One Cue and beacon groups only. Time taken to find the platform for the One Cue group was significantly reduced on day 10 (12.74 ± 1.75s, CI [8.46, 17.01]) than on day 1 (45.59 ± 3.63s, CI [36.72, 54.47]). Similarly, escape latency for the beacon group was shorter on day 10 (10.94 ± 1.30s, CI [7.76, 14.11]) compared to day 1 (31.68 ± 2.40s, CI [25.80, 37.55]).

One-way between-groups ANOVAs were then carried out to investigate group differences on each day. No main effects of group were discovered in the five-day training condition; day 1: $F_{2,20}$ = 0.96, P = 0.40, day 2: $F_{2,20}$ = 0.10, P = 0.90, day 3: $F_{2,20}$ = 0.52, P = 0.60, day 4: $F_{2,20}$ = 1.14, P = 0.34, day 5: $F_{2,20}$ = 0.13, P = 0.88. For the ten-day trained groups, significant main effect were noted on day 1, $F_{2,20}$ = 4.71, P = 0.02, day 6, $F_{2,20}$ = 5.60, P = 0.01, and day 7, $F_{2,20}$ = 17.12, P = 0.001. Tukey *post hoc* tests highlighted a number of significant differences; on day 1, the One Cue group were significantly slower at locating the platform than the Control group (P = 0.03), on day 6, escape latency for the Control group was longer than the Beacon group (P = 0.01), and on day 7, both One Cue and Beacon groups escaped the maze faster than the Control group (both P = 0.001). Importantly, no significant differences were noted between groups on the final day of training.

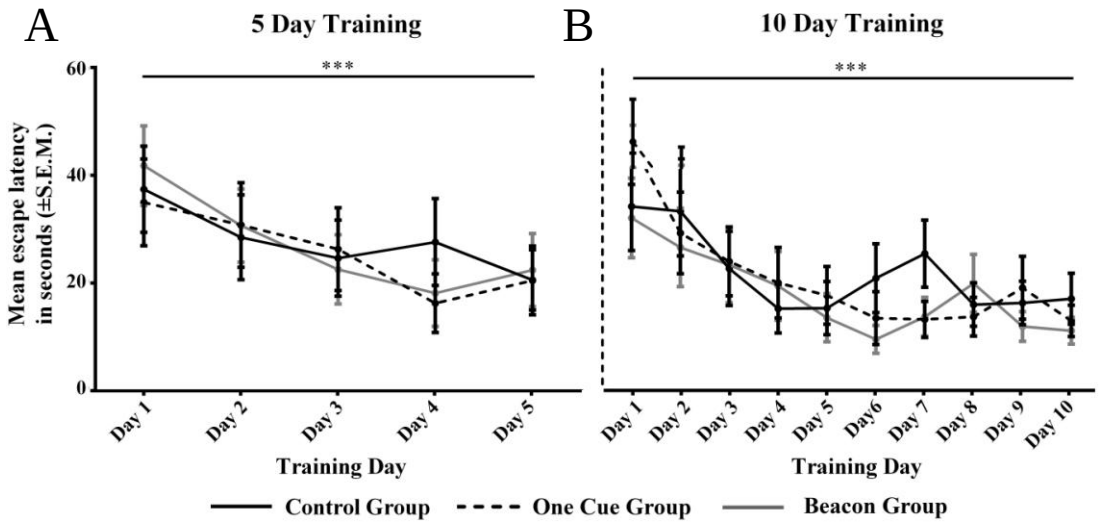

Figure 5.24: Mean escape latencies (± SEM) for Control, One Cue and Beacon groups over (A) five and (B) ten days of training.

5.3.2.1.2. Distance travelled. A 3 x 5 mixed factorial ANOVA yielded a significant main effect of day, $F_{4,72} = 24.38$, $P = 0.001$, partial eta$^2 = 0.58$, and day x group interaction effect, $F_{8,72} = 4.68$, $P = 0.001$, partial eta$^2 = 0.34$ (see Figure 5.25A). The main of group was not significant, $F_{1,18} = 1.62$, $P = 0.23$, partial eta$^2 = 0.15$. Bonferroni *post hoc* tests showed that distance travelled on day 1 was significantly slower than on day 5 ($P = 0.001$). Results from a 3 x 10 mixed factorial ANOVA were similar; the main of day, $F_{9,162} = 25.50$, $P = 0.001$, partial eta$^2 = 0.59$, and day x group interaction effect were significant, $F_{18,162} = 6.22$, $P = 0.001$, partial eta$^2 = 0.41$, but the main effect of group was not, $F_{1,18} = 2.35$, $P = 0.12$, partial eta$^2 = 0.21$ (see Figure 5.25B). Again, *post hoc* analyses highlighted a significant difference between day 1 and day 10 ($P = 0.001$).

Individual main effects of day were found for both Control groups; five day: $F_{4,24} = 4.40$, $P = 0.01$, partial eta$^2 = 0.42$, and ten day: $F_{9,54} = 7.30$, $P = 0.001$, partial eta$^2 = 0.55$. *Post hoc* comparisons were not significant after five days of training; however, a significant difference between path lengths on day 1 (816.11 ± 72.82cm, CI [637.93, 994.30]) and day 10 (480.56 ± 74.98cm, CI [297.10, 644.01]) was found

after ten days (P = 0.04). Main effects of day were significant for both One Cue groups; $F_{4,24}$ = 7.10, P = 0.001, partial eta^2 = 0.54, and $F_{9,54}$ = 20.87, P = 0.001, partial eta^2 = 0.78, for five and ten days, respectively. No significant *post hoc* differences were noted for the five-day group, but distance travelled was significantly shorter on day 10 (332.77 ± 38.03, CI [239.71, 425,83]) compared to day 1 (1532.01 ± 178.15, CI [1096.12, 1967.91]) (P = 0.02). Lastly, main effects were also significant for the Beacon groups; $F_{4,24}$ = 18.50, P = 0.001, partial eta^2 = 0.76, and $F_{9,54}$ = 7.11, P = 0.001, partial eta^2 = 0.54, for five and ten days, respectively; however, *post hoc* tests between the first and last days of training for both groups failed to reach significance (five day: P = 0.06, ten day: P = 0.07).

With regard to between-groups differences, main effects of group for five-day groups were noted on day 1, $F_{2,20}$ = 7.39, P = 0.01, and day 4, $F_{2,20}$ = 3.88, P = 0.04. Tukey *post hoc* revealed that the Beacon group travelled longer routes to the platform compared to the cue-trained groups on day 1 (P = 0.01 and P = 0.02, respectively), and these animals took shorter routes than the Control group on day 4 (P = 0.04). For the ten-day condition, main effects of group were significant for day 1, $F_{2,20}$ = 13.74, P = 0.001, day 6, $F_{2,20}$ = 7.71, P = 0.01, and day 7, $F_{2,20}$ = 25.31, P = 0.001. On day 1, mean distance travelled by the One Cue group was longer relative to the Control and Beacon groups (both P = 0.001). On day 6 and day 7, the Control group travelled larger distances than the One Cue group (P = 0.05 and P = 0.001, respectively) and the Beacon group (P = 0.01 and P = 0.001, respectively).

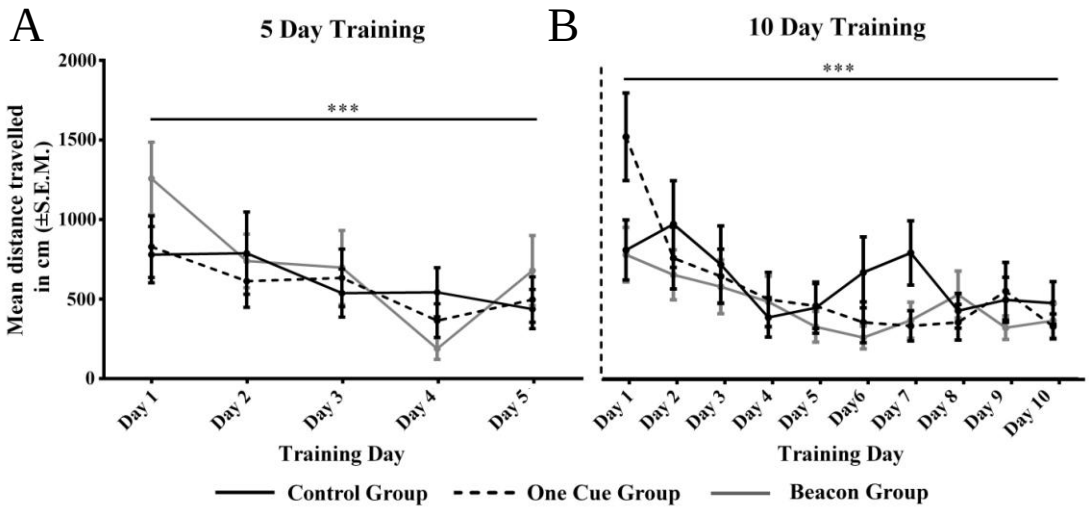

Figure 5.25: Mean distance travelled (± SEM) for Control, One Cue and Beacon groups over (A) five and (B) ten days of training.

5.3.2.2. Recall.

5.3.2.2.1. Quadrants. Twenty minutes pre-recall, animals in both conditions were administered with a single dose of MK-801 (0.01mg/kg, i.p.). After five days of training, the only significant result found was for the Beacon group, which spent less time in the SE quadrant than would be expected by chance level, $t_{12} = 3.14$, $P = 0.02$ (see Figure 5.26A). After ten-day training, the Control group were shown to have spent significantly more time in the NE quadrant compared to chance, $t_{12} = 3.22$, $P = 0.02$, whereas time spent in this region was significantly below chance for the One Cue group, $t_{12} = 2.47$, $P = 0.04$. Instead, the One Cue group appeared to favour the SW quadrant, $t_{12} = 3.37$, $P = 0.02$ (see Figure 5.26B). No other significant differences were noted.

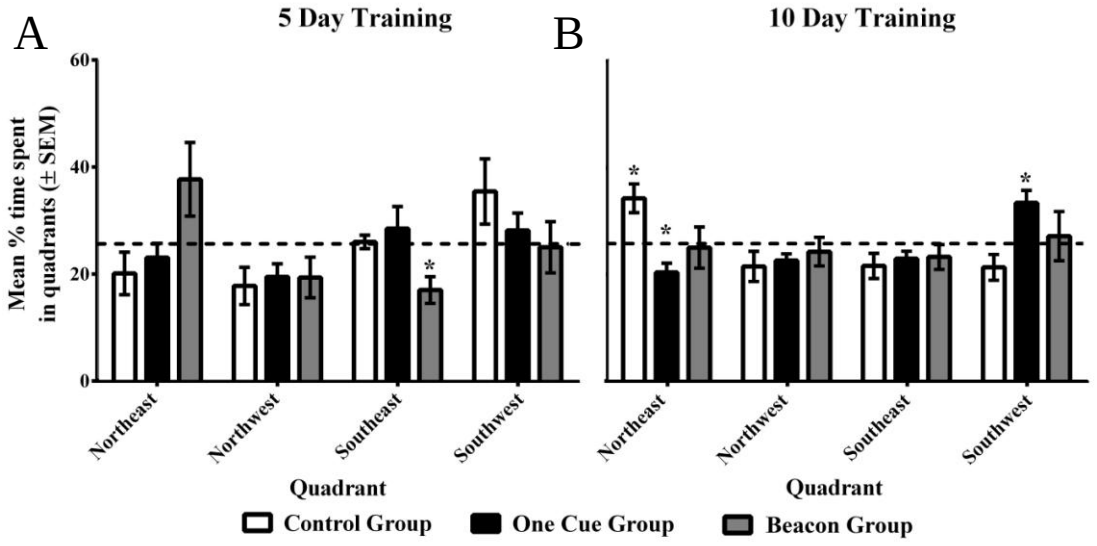

Figure 5.26: Mean percentage time (± SEM) spent in quadrants of the maze for Control, One Cue and Beacon groups after (A) five and (B) ten days of training. All animals were treated with MK-801 (0.01mg/kg, i.p.) approximately twenty minutes before recall. Dashed line signifies chance level.

5.3.2.2.2. Platform areas. A 3 x 4 mixed factorial ANOVA generated for the five-day condition did not produce any main of area, $F_{3,54} = 2.75$, $P = 0.10$, partial eta^2 = 0.13, main effect of group, $F_{1,18} = 3.01$, $P = 0.08$, partial eta^2 = 0.25, or area x group interaction effect, $F_{6,54} = 2.43$, $P = 0.09$, partial eta^2 = 0.21 (see Figure 5.27A). After ten days, main effects of area, $F_{3,54} = 3.99$, $P = 0.04$, partial eta^2 = 0.18, and group, $F_{1,18} = 4.38$, $P = 0.03$, partial eta^2 = 0.33, were significant. The area x group interaction effect was not significant, $F_{6,54} = 2.01$, $P = 0.13$, partial eta^2 = 0.18. Bonferroni *post hoc* tests failed to indicate any differences across areas; however, Tukey *post hoc* comparisons did highlight a significant difference between the One Cue and Beacon groups ($P = 0.04$).

Repeated measures ANOVAs were carried out for the ten-day groups to investigate differences in time spent across areas; however, no main effects of area were found; Control group, $F_{3,18} = 4.99$, $P = 0.06$, partial eta^2 = 0.45, One Cue group, $F_{3,18} = 2.71$, $P = 0.22$, partial eta^2 = 0.22, Beacon group, $F_{3,18} = 0.51$, P =

0.68, partial eta^2 = 0.08 (see Figure 5.27B). Similarly, between-groups ANOVAs for each area did not yield any significant main effects: NE: $F_{2,20}$ = 2.87, P = 0.08, NW: $F_{2,20}$ = 3.32, P = 0.06, SE: $F_{2,20}$ = 2.94, P = 0.08, SW: $F_{2,20}$ = 1.23, P = 0.32 (see Figure 5.27B).

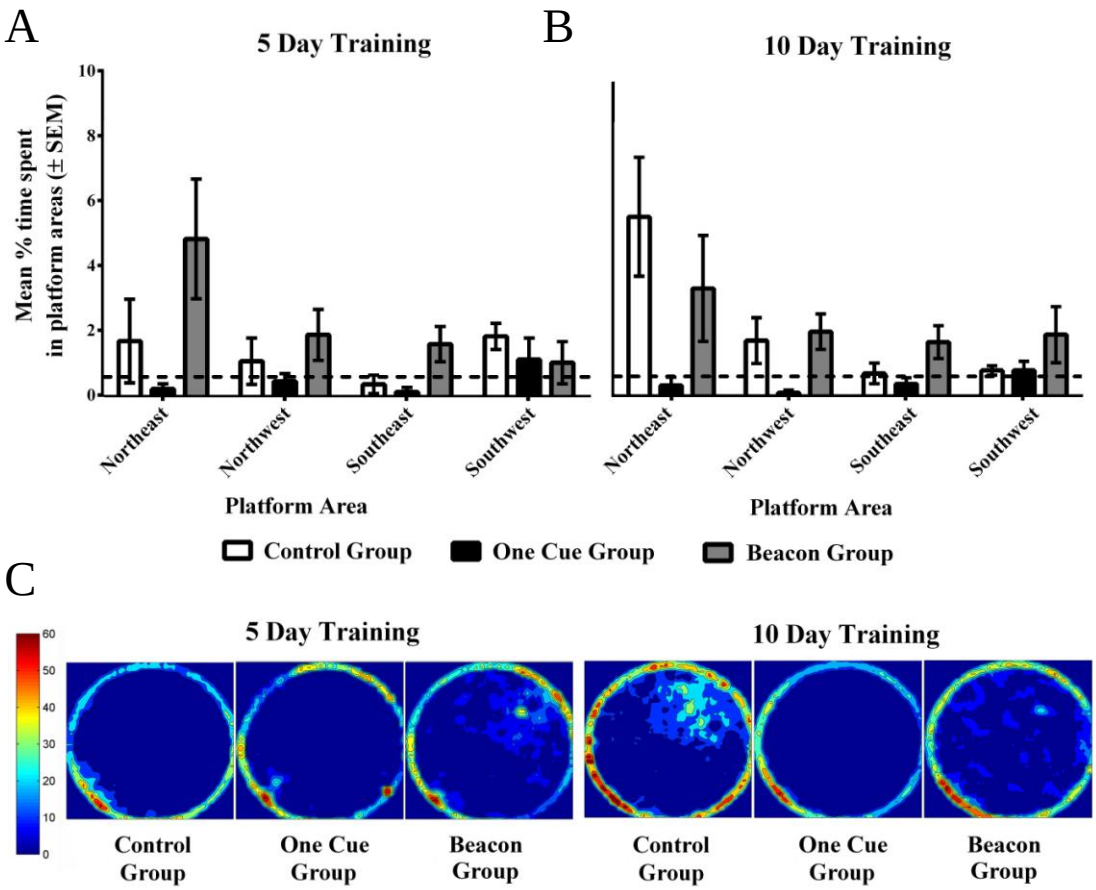

Figure 5.27: (A-B) Mean percentage time (± SEM) spent in platform areas by Control, One Cue and Beacon groups trained for five and ten days. (C) Heat maps displaying overall search distributions during the probe trial for five- and ten-day groups. All animals were treated with MK-801 (0.01mg/kg; i.p.) approximately twenty minutes before recall. Dashed line signifies chance level (0.6%). Note difference in scale from Figure 5.5.

5.3.2.2.3. Outer corridor. A one-way between-groups ANOVA comparing time spent in the outer corridor did not yield a significant main effect after five days of training, $F_{2,20}$ = 2.40, P = 0.12. However, a main effect was found after ten days, $F_{2,20}$ = 3.77, P = 0.04, with Tukey *post hoc* tests demonstrating that the One Cue

group (91.43 ± 3.25, CI [83.49, 99.37]) spent significantly more time in the perimeter of pool than the Control group (66.57 ± 9.73, CI [42.77, 90.37]) (P = 0.04; see Figure 5.28).

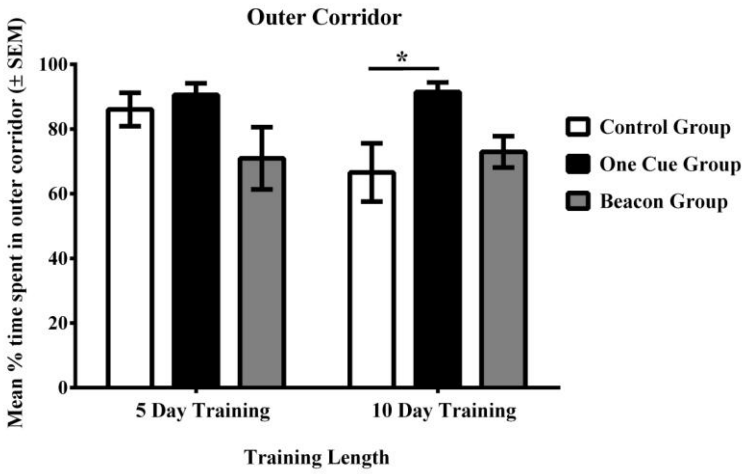

Figure 5.28: Mean percentage time (± SEM) spent in the outer corridor Control, One Cue and Beacon groups after five- and ten-day training. All animals were treated with MK-801 (0.01mg/kg; i.p.) approximately twenty minutes before recall.

5.3.2.3. Comparison between five and ten day training.

No significant differences between time spent in the NE platform area after five and ten days of training were found for any group; Control: t_{12} = 1.65, P = 0.13, One Cue, t_{12} = 0.29, P = 0.78, and Beacon, t_{12} = 0.55, P = 0.59 (see Figure 5.27). Similarly, time spent in the outer corridor did not decrease significantly from five- to ten-day training for any group; Control: t_{12} = 1.77, P = 0.11, One Cue, t_{12} = 0.18, P = 0.86, and Beacon, t_{12} = 0.18, P = 0.86 (see Figure 5.28).

5.3.3. IEG results

5.3.3.1. Zif268.

In the five-day training condition, one extreme outlier was removed from the Control group analyses in each of the CA1, PLC, ACC and ILC sub-regions. One-way between-groups ANOVAs produced significant main effects for all sub-regions of

the hippocampus; CA1: $F_{2,19}$ = 15.89, P = 0.001, CA3: $F_{2,20}$ = 7.13, P = 0.005, and DG: $F_{2,20}$ = 6.51, P = 0.01 (see Figure 5.29A-C), with a number of significant Tukey *post hoc* differences. In CA1, the mean counts for the Control (32.38 ± 9.04%, CI [9.12, 55.64]) and Beacon groups (7.49 ± 3.36%, CI [0.74, 49.29]) were significantly smaller than the One Cue group (64.76 ± 23.25%, CI [28.64, 81.84]; P = 0.01 and P = 0.001, respectively). In CA3, the mean count for the Beacon group (6.06 ± 1.60%, CI [2.16, 9.97]) was significantly smaller than those of the Control (41.77 ± 10.82%, CI [15.29, 66.26]; P = 0.03) and One Cue groups (52.17 ± 11.24%, CI [24.66, 79.67]; P = 0.01). In the DG, the mean count for the One Cue group (55.43 ± 12.09%, CI [25.84, 85.02]) was, again, higher than the mean count for the Beacon group (6.39 ± 5.82%, CI [-7.85, 20.63]; P = 0.01).

No significant main effect of group was noted in the PLC: $F_{2,19}$ = 2.98, P = 0.08 (see Figure 5.29D). Significant main effects were found in the ACC, $F_{2,19}$ = 6.10, P = 0.01, and ILC: $F_{2,19}$ = 4.32, P = 0.03 (see Figure 5.29E-F). *Post hoc* tests showed that in the ACC, the mean counts for the Control (27.23 ± 7.72%, CI [7.39, 47.07]) and Beacon groups (20.40 ± 5.31%, CI [7.40, 33.40]) were significantly lower than the One Cue group (56.26 ± 9.79%, CI [32.32, 80.21]; P = 0.05 and P = 0.01, respectively). In the ILC, the mean count for the Control group (19.80 ± 4.87%, CI [7.28, 32.32]) was significantly lower than the One Cue group (53.12 ± 10.33%, CI [27.84, 78.39]; P = 0.03). Sample sections of Zif268 expression in the hippocampus and prefrontal cortex are shown in Figure 5.30, and scatterplots depicting raw scores in all regions are shown in Figure 5.31.

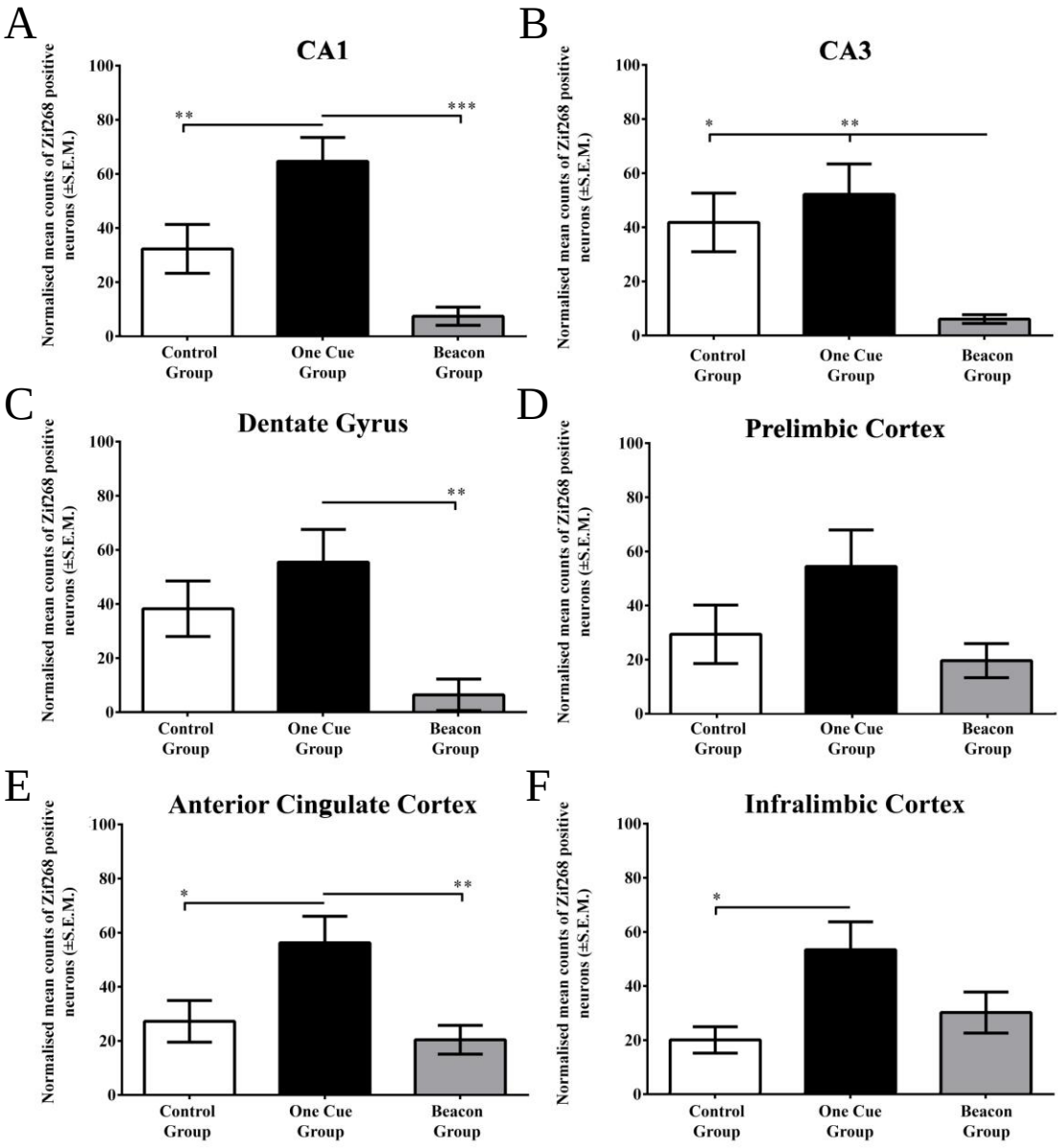

Figure 5.29: Mean normalised cell counts of Zif268 positive neurons for five-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

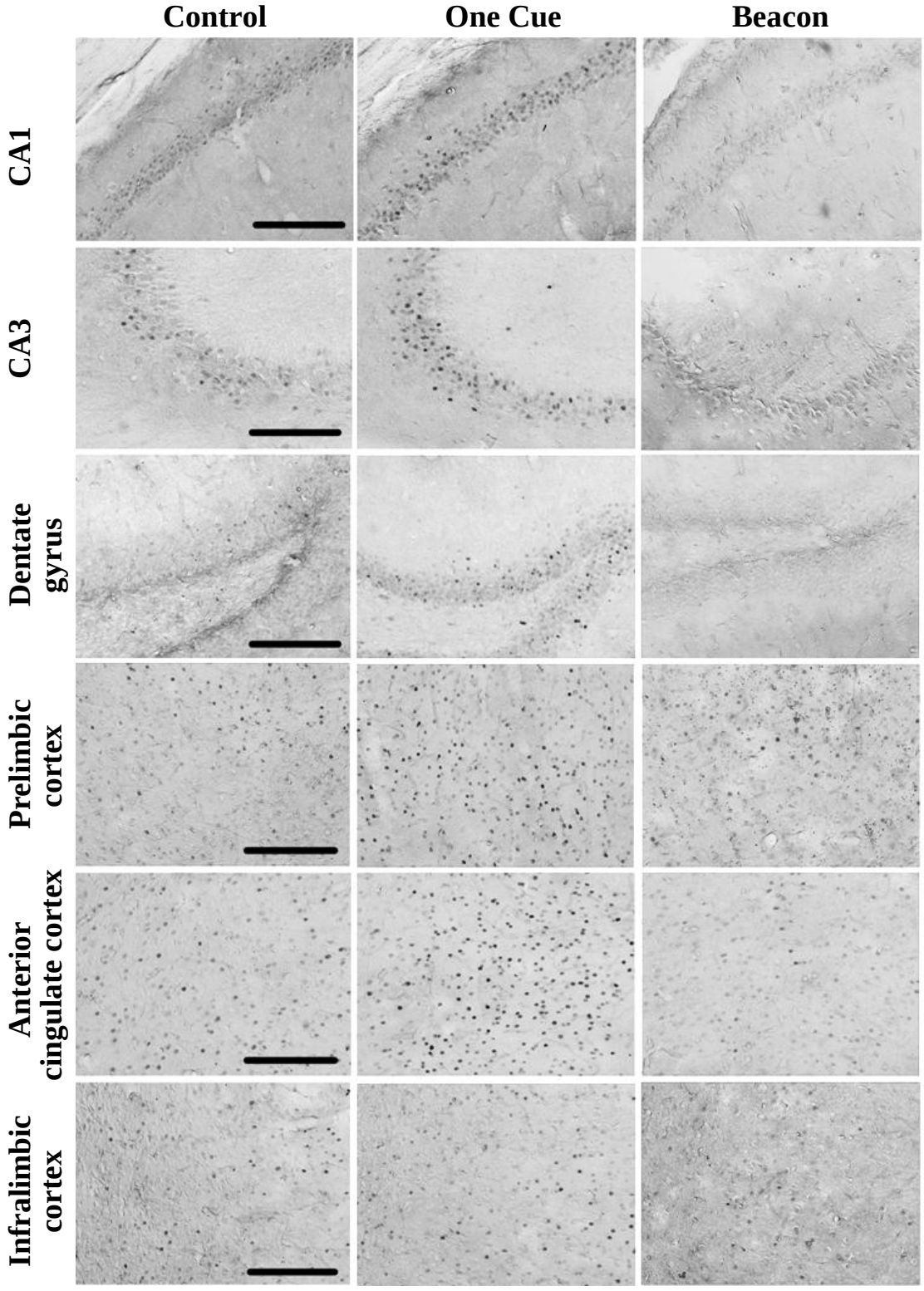

Figure 5.30: Representative images of Zif268 expression for five-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

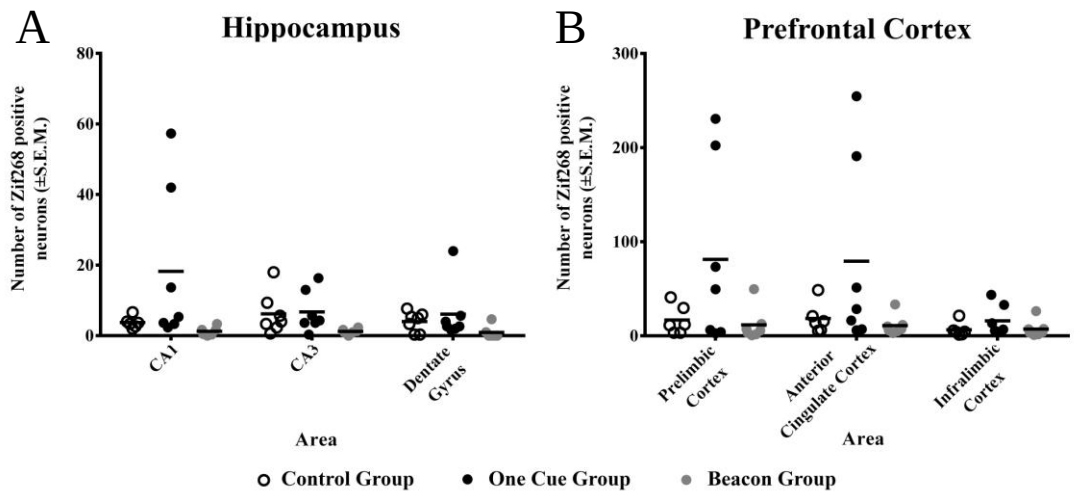

Figure 5.31: Scatterplots showing individual raw Zif268 counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after five days. Horizontal lines represent group means.

One extreme outlier in the Beacon group for area CA1 was removed prior to analyses for the ten-day training condition. ANOVAs failed to yield any significant main effects of group for any sub-region sampled; CA1: $F_{2,19} = 0.61$, $P = 0.56$, CA3: $F_{2,20} = 1.65$, $P = 0.24$, DG: $F_{2,20} = 0.45$, $P = 0.64$, PLC: $F_{2,20} = 1.29$, $P = 0.30$, ACC: $F_{2,20} = 3.26$, $P = 0.06$, and ILC: $F_{2,20} = 1.70$, $P = 0.21$ (see Figure 5.32). Sample sections of Zif268 expression in the hippocampus and prefrontal cortex are shown in Figure 5.33, and scatterplots depicting raw scores in all regions are shown in Figure 5.34.

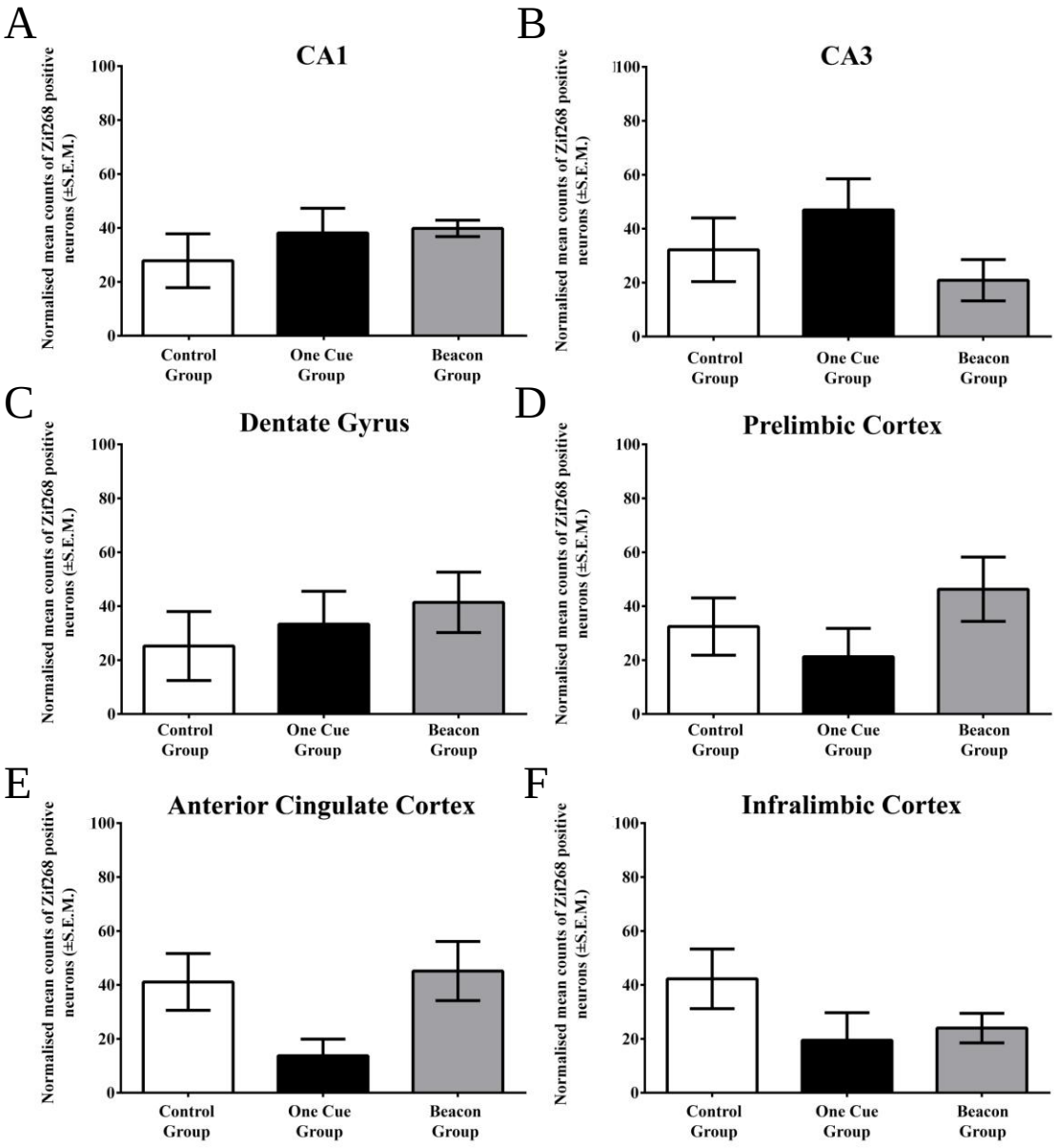

Figure 5.32: Mean normalised cell counts of Zif268 positive neurons for ten-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

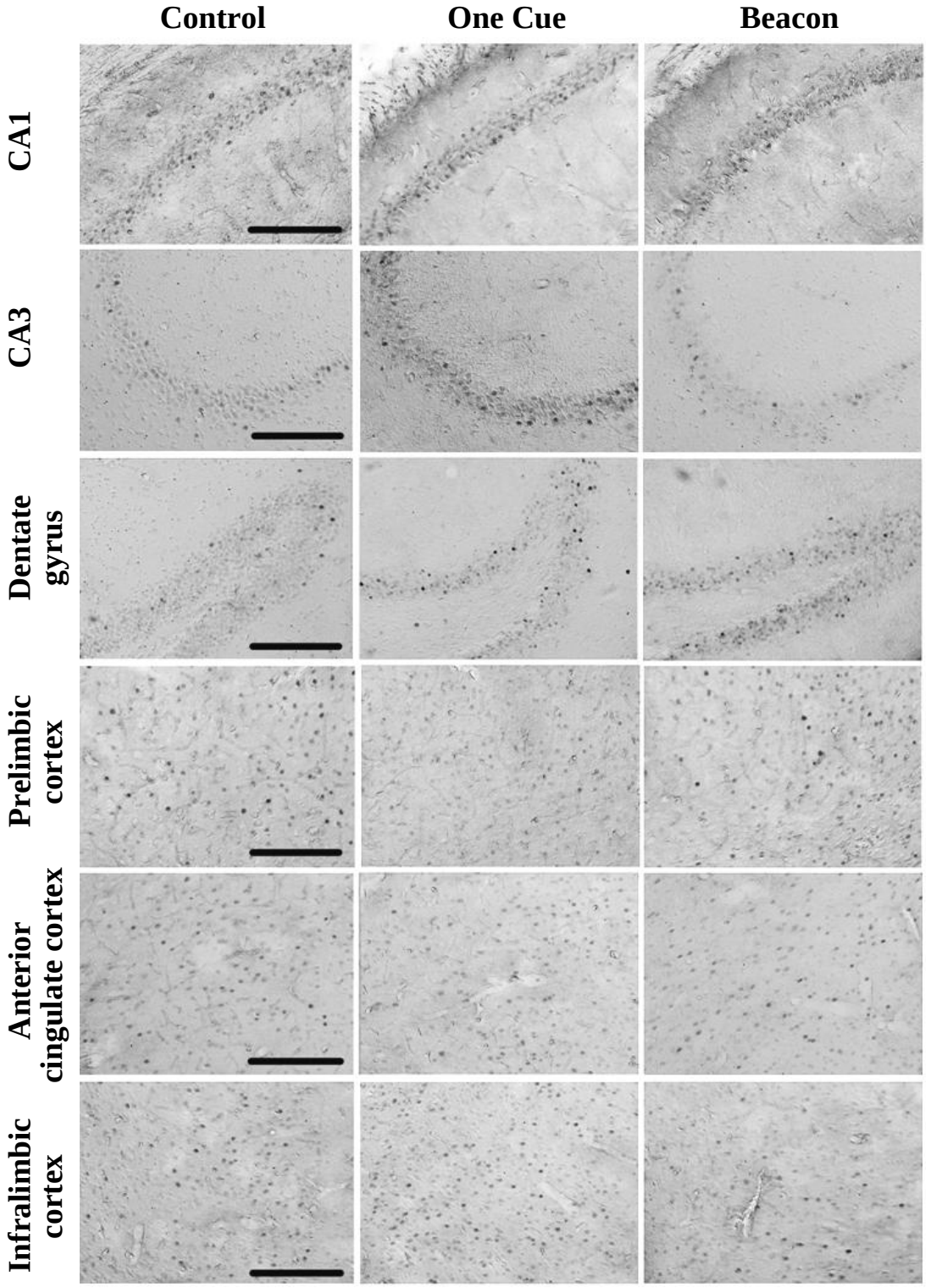

Figure 5.33: Representative images of Zif268 expression for ten-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

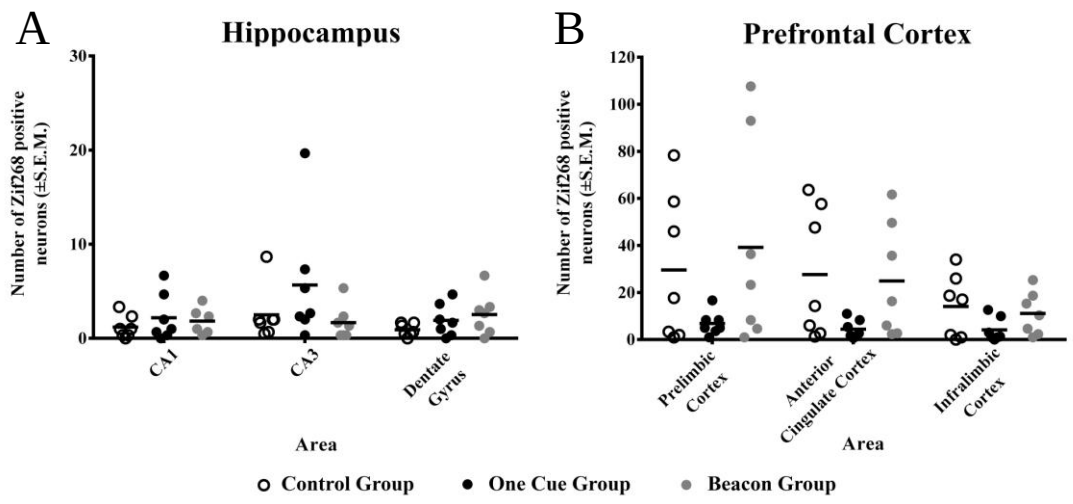

Figure 5.34: Scatterplots showing individual raw Zif268 counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after ten days. Horizontal lines represent group means.

5.3.3.2. c-Fos.

One extreme outlier was removed from area CA3 in the ten day condition (Beacon group). One-way between-groups ANOVAs comparing c-Fos expression in each sub-region after five and ten days of training failed to yield any significant main effects of group; five-day condition: CA1: $F2,20 = 0.79$, $P = 0.47$, CA3: $F2,20 = 1.12$, $P = 0.35$, DG: $F2,20 = 1.30$, $P = 0.30$, PLC: $F2,20 = 1.41$, $P = 0.27$, ACC: $F2,20 = 3.01$, $P = 0.07$, and ILC: $F2,20 = 1.28$, $P = 0.30$ (see Figure 5.35); ten-day condition: CA1: $F2,20 = 0.78$, $P = 0.47$, CA3: $F2,19 = 1.34$, $P = 0.29$, DG: $F2,20 = 0.22$, $P = 0.80$, PLC: $F2,20 = 0.25$ $P = 0.78$, ACC: $F2,20 = 0.61$, $P = 0.56$, and ILC: $F2,20 = 0.02$, $P = 0.99$ (see Figure 5.36). Sample sections of c-Fos expression in the hippocampus and prefrontal cortex after five and ten days are included in Figures 5.37 and 5.38, respectively, and scatterplots depicting raw scores are shown in Figures 5.39 and 5.40, respectively.

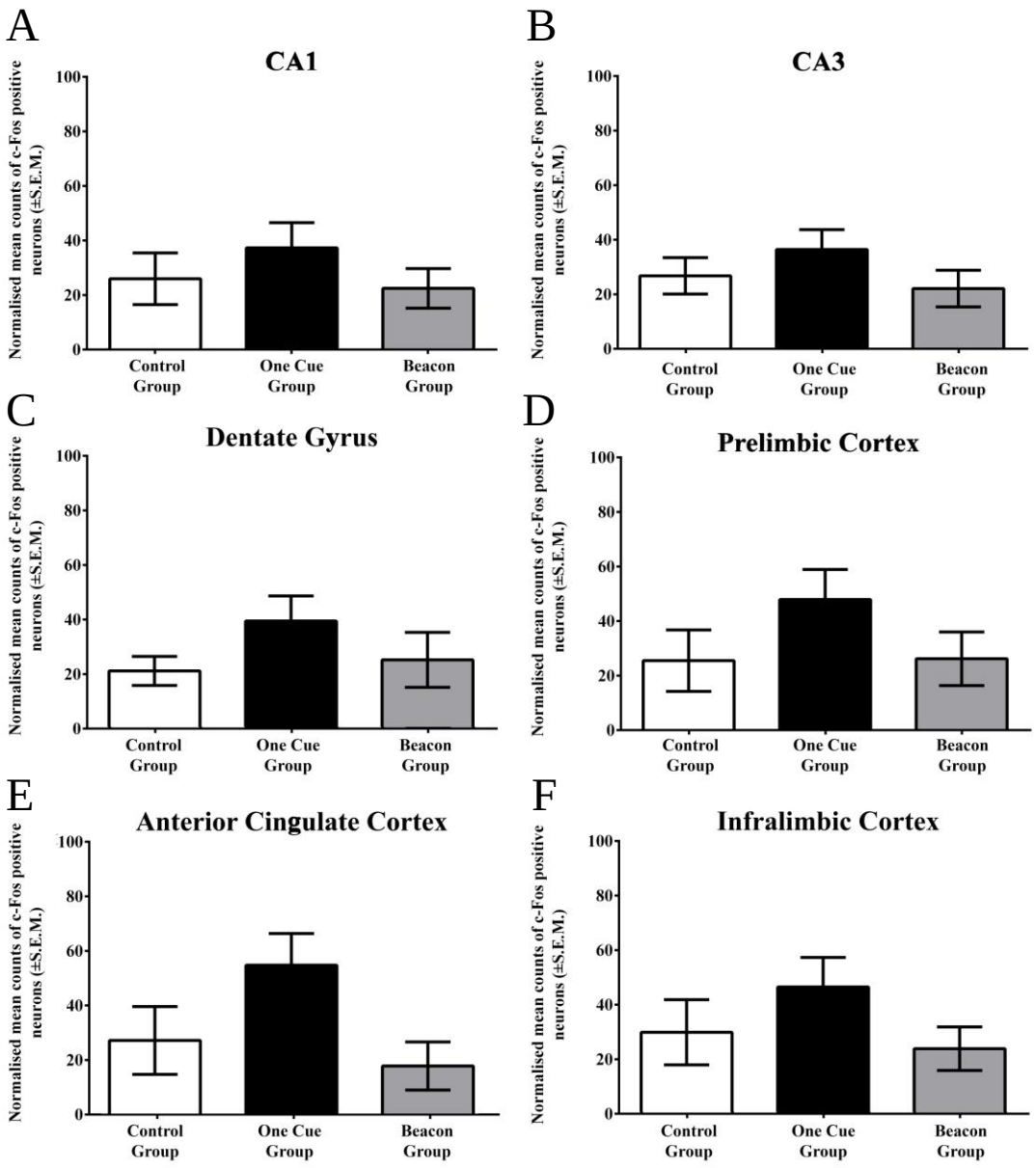

Figure 5.35: Mean normalised cell counts of c-Fos positive neurons for five-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

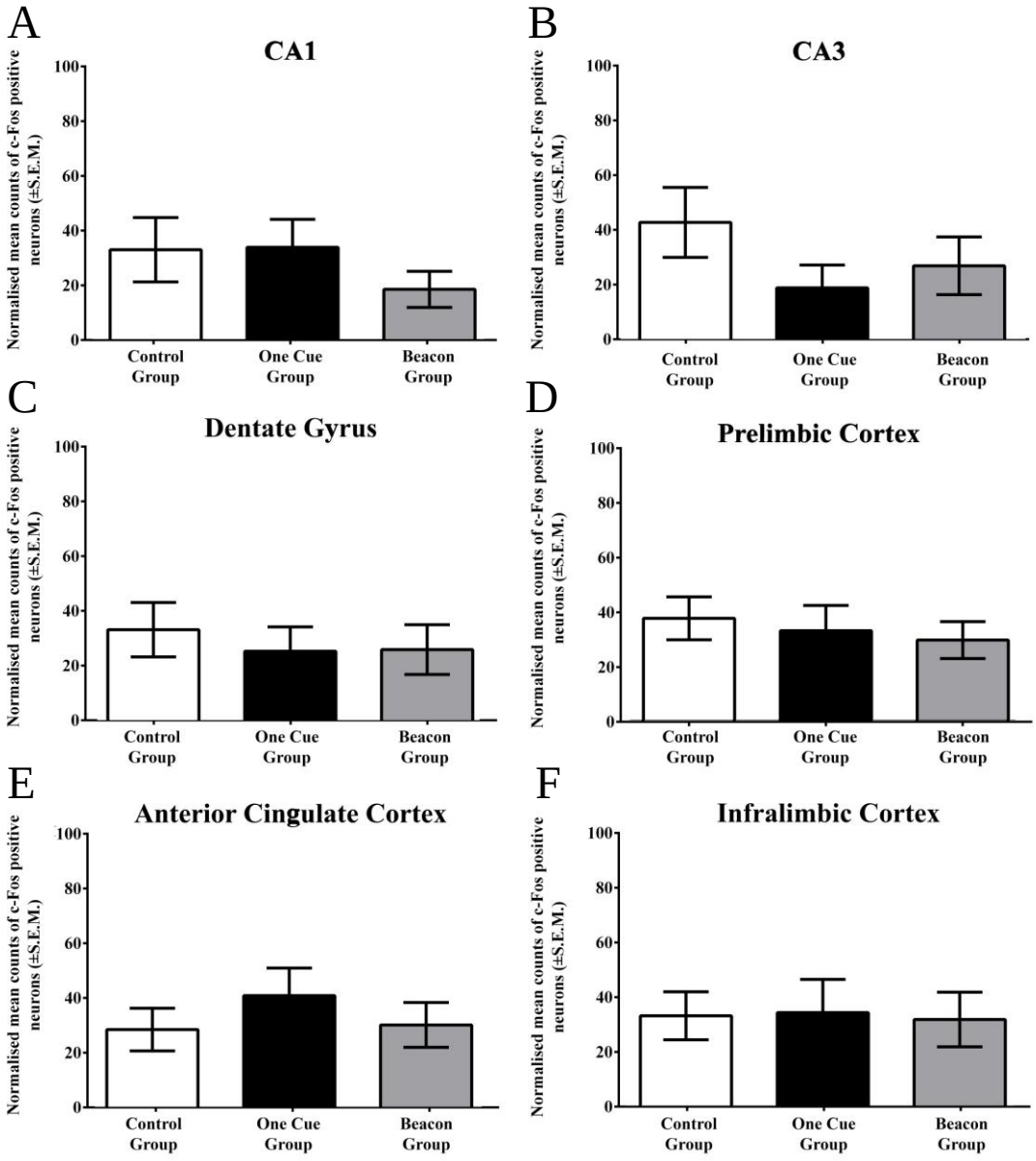

Figure 5.36: Mean normalised cell counts of c-Fos positive neurons for ten-day Control, One Cue and Beacon groups in (A) CA1, (B) CA3, (C) dentate gyrus, (D) prelimbic cortex (E) anterior cingulate cortex and (F) infralimbic cortex.

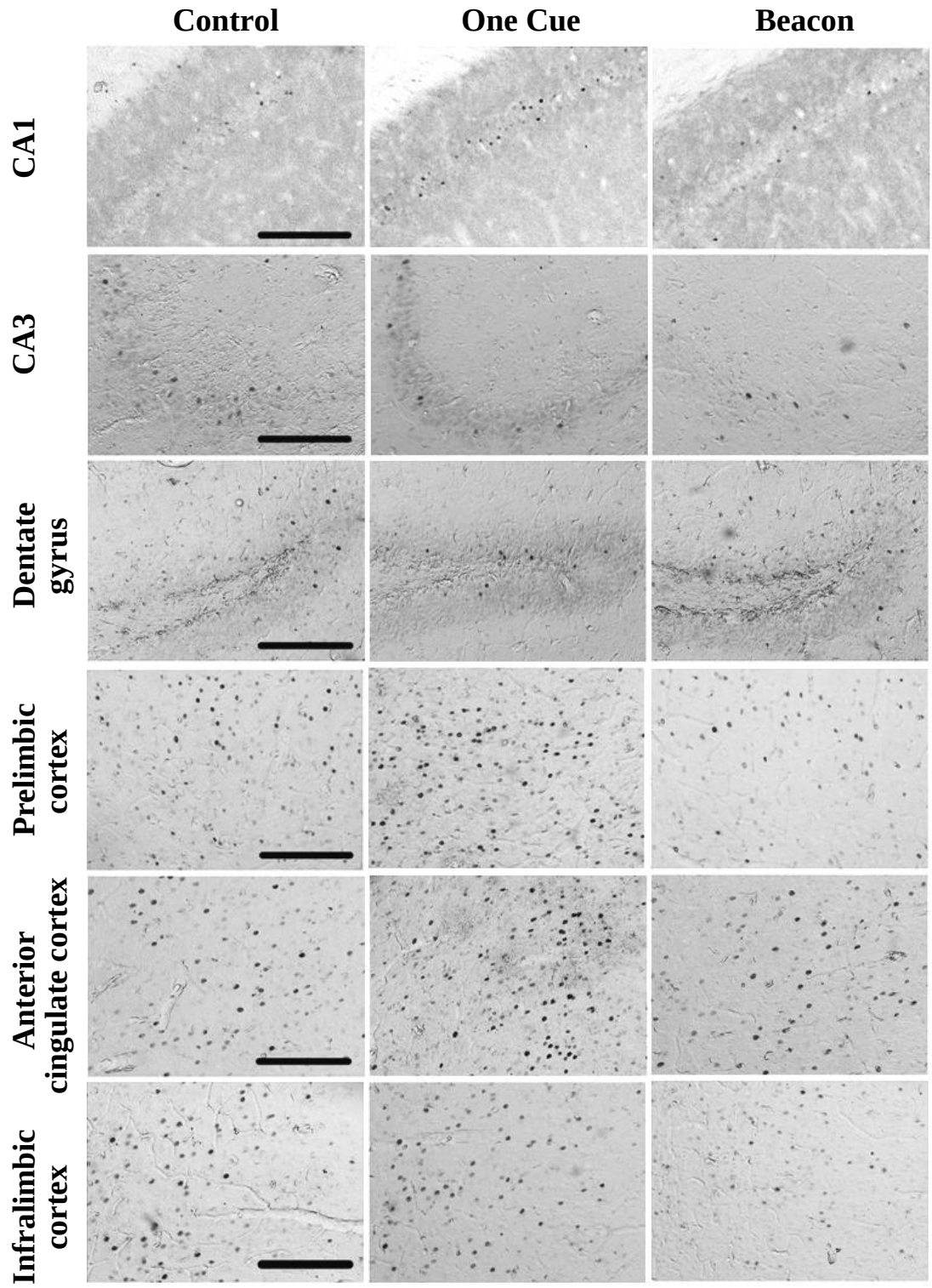

Figure 5.37: Representative images of c-Fos expression for five-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

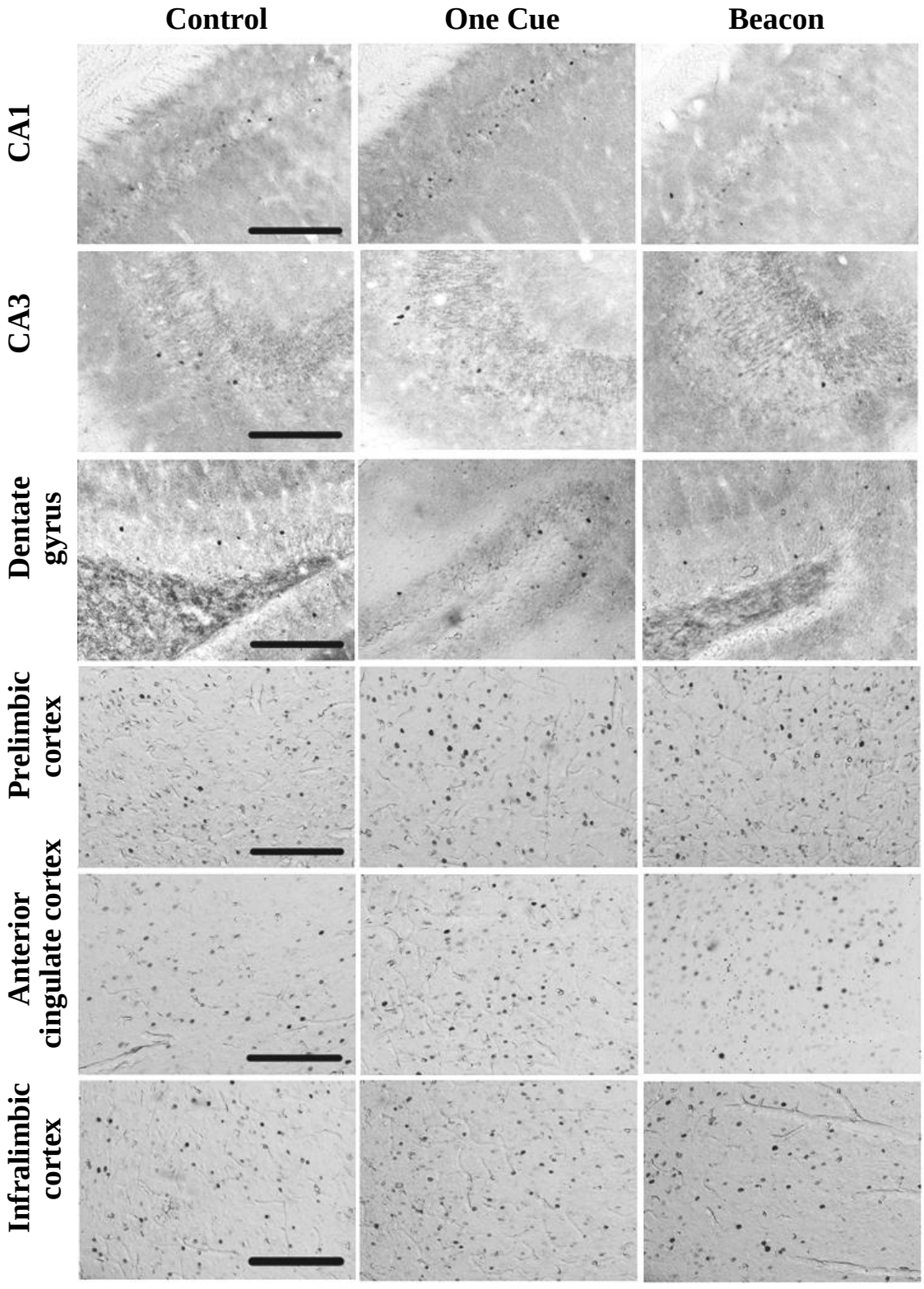

Figure 5.38: Representative images of c-Fos expression for ten-day Control, One Cue and Beacon groups in CA1, CA3, the dentate gyrus, the prelimbic, anterior cingulate and infralimbic cortices. Scale bar = 100μm.

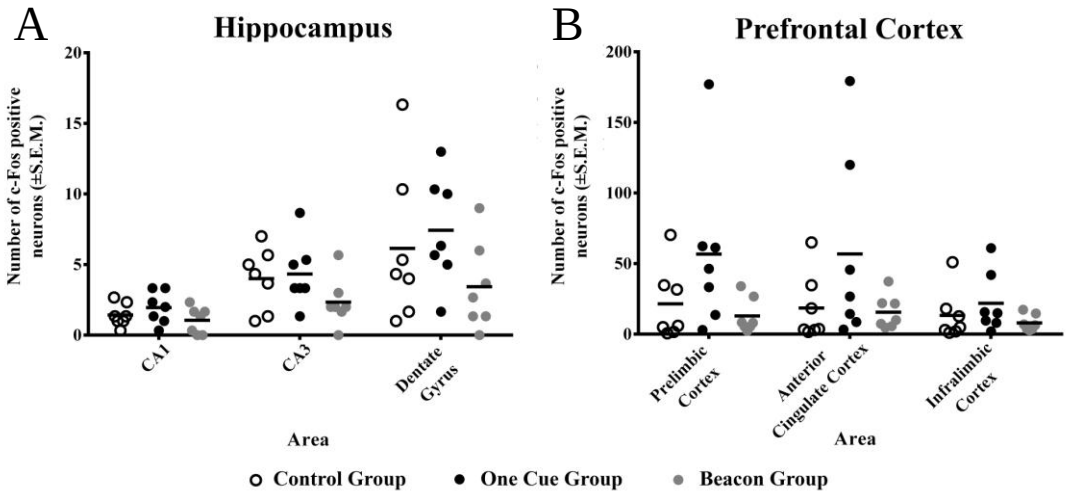

Figure 5.39: Scatterplots showing individual raw c-Fos counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after five days. Horizontal lines represent group means.

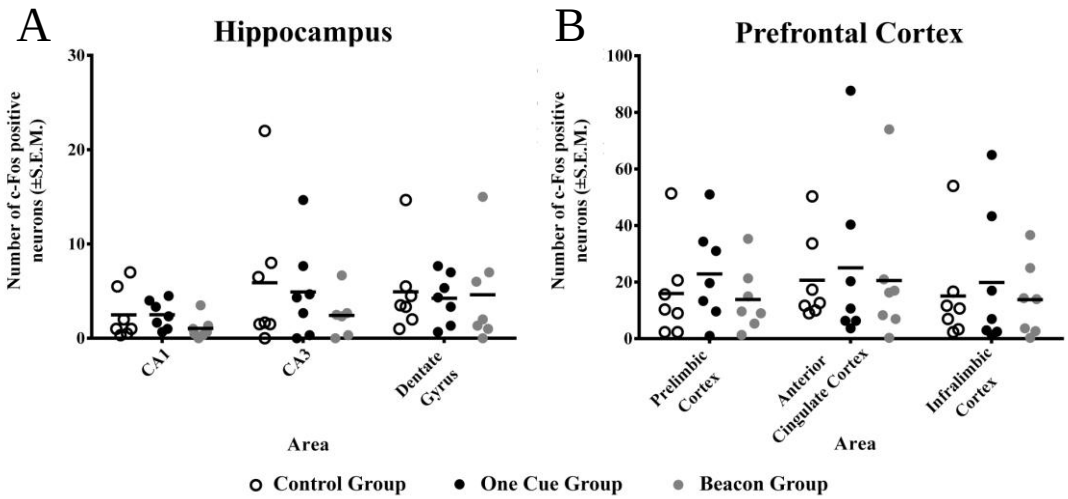

Figure 5.40: Scatterplots showing individual raw c-Fos counts for all animals in (A) CA1, CA3, dentate gyrus, and (B) prelimbic cortex, anterior cingulate cortex and infralimbic cortex after ten days. Horizontal lines represent group means.

5.3.3.3. Comparison between five and ten day training.

For Zif268 expression, significant decreases from five to ten days were found for the One Cue group in CA1, $t_{12} = 4.08$, $P = 0.01$, and ACC, $t_{12} = 2.78$, $P = 0.03$, while significant increases were found for the Beacon group in CA1, $t_{12} = 4.82$, $P = 0.01$, and DG sub-regions, $t_{12} = 3.83$, $P = 0.01$ (see Figure 5.41A). No differences were

noted for the Control group. Again, no significant differences in c-Fos expression were found for any group in any area (see Figure 5.41B).

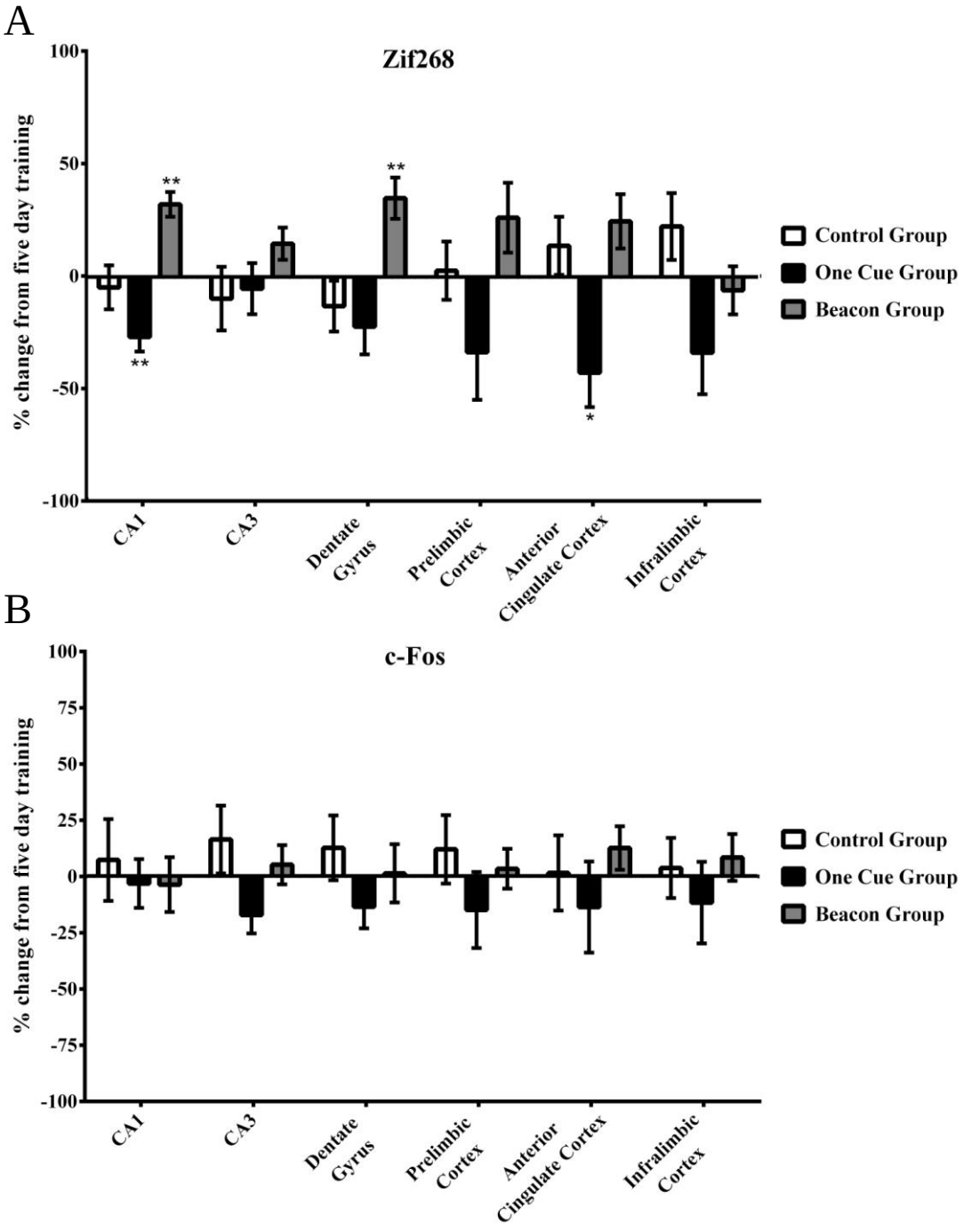

Figure 5.41: mean percentage increase or decrease in (A) Zif268 and (B) c-Fos expression from five- to ten-day training conditions for Control, One Cue and Beacon groups in all sub-regions.

5.3.4. Correlations with behaviour

Correlations between Zif268 expression for five- and ten-day groups and percentage time spent in the NE platform area were yielded a number of significant results. For both Control group, Zif268 expression in CA1 was positively correlated with time spent in the NE area (r = 0.95, P = 0.001, and r = 0.81, P = 0.02; see Table 3.3 Top and Figure 5.42). Further, for the five-day Control group, significant positive correlations were noted between mean Zif268 counts in CA3 (r = 0.87, P = 0.01), PLC (r = 0.93, P = 0.002), ACC (r = 0.94, P = 0.002) and ILC (r = 0.94, P = 0.002) (see Table 3.3 Top and Figure 5.42). A significant positive correlation was also found for the One Cue group after ten days, with increased Zif268 expression in the PLC associated with more time spent in the NE area (r = 0.84, P = 0.02; see Table 5.3 Middle and Figure 5.43). No significant correlations were documented between mean group c-Fos counts in any sub-region and time in the NE area after five or ten days of training (see Table 5.4 and Figures 5.44 and 5.45).

Table 5.3: Correlations between Zif268 expression and percentage time spent in the NE platform area for five- and ten-day Control, One Cue and Beacon groups.

Group	Brain region	Training condition	
Control		Five days	Ten days
	CA1	**0.95****	**0.81***
	CA3	**0.87***	-0.27
	DG	0.12	-0.13
	PLC	**0.93****	-0.45
	ACC	**0.94****	0.25
	ILC	**0.94****	0.07
One Cue		Five days	Ten days
	CA1	-0.26	-0.39
	CA3	-0.58	-0.41
	DG	0.36	-0.54
	PLC	-0.24	**0.84***
	ACC	-0.30	0.41
	ILC	-0.22	0.71
Beacon		Five days	Ten days
	CA1	-0.01	-0.23
	CA3	-0.32	-0.07
	DG	-0.13	0.02
	PLC	-0.16	-0.53
	ACC	-0.27	0.60
	ILC	-0.43	-0.06

Table 5.4: Correlations between c-Fos expression and percentage time spent in the NE platform area for five- and ten-day Control, One Cue and Beacon groups.

Group	Brain region	Training condition	
Control		Five days	Ten days
	CA1	-0.31	0.03
	CA3	-0.11	-0.49
	DG	-0.12	-0.41
	PLC	-0.37	-0.52
	ACC	-0.36	-0.39
	ILC	0.31	0.44
One Cue		Five days	Ten days
	CA1	0.45	-0.51
	CA3	0.33	-0.26
	DG	-0.51	-0.50
	PLC	0.56	-0.12
	ACC	0.72	-0.52
	ILC	0.64	-0.20
Beacon		Five days	Ten days
	CA1	0.10	-0.23
	CA3	-0.43	-0.13
	DG	-0.50	0.73
	PLC	-0.34	-0.11
	ACC	-0.30	-0.13
	ILC	-0.44	0.04

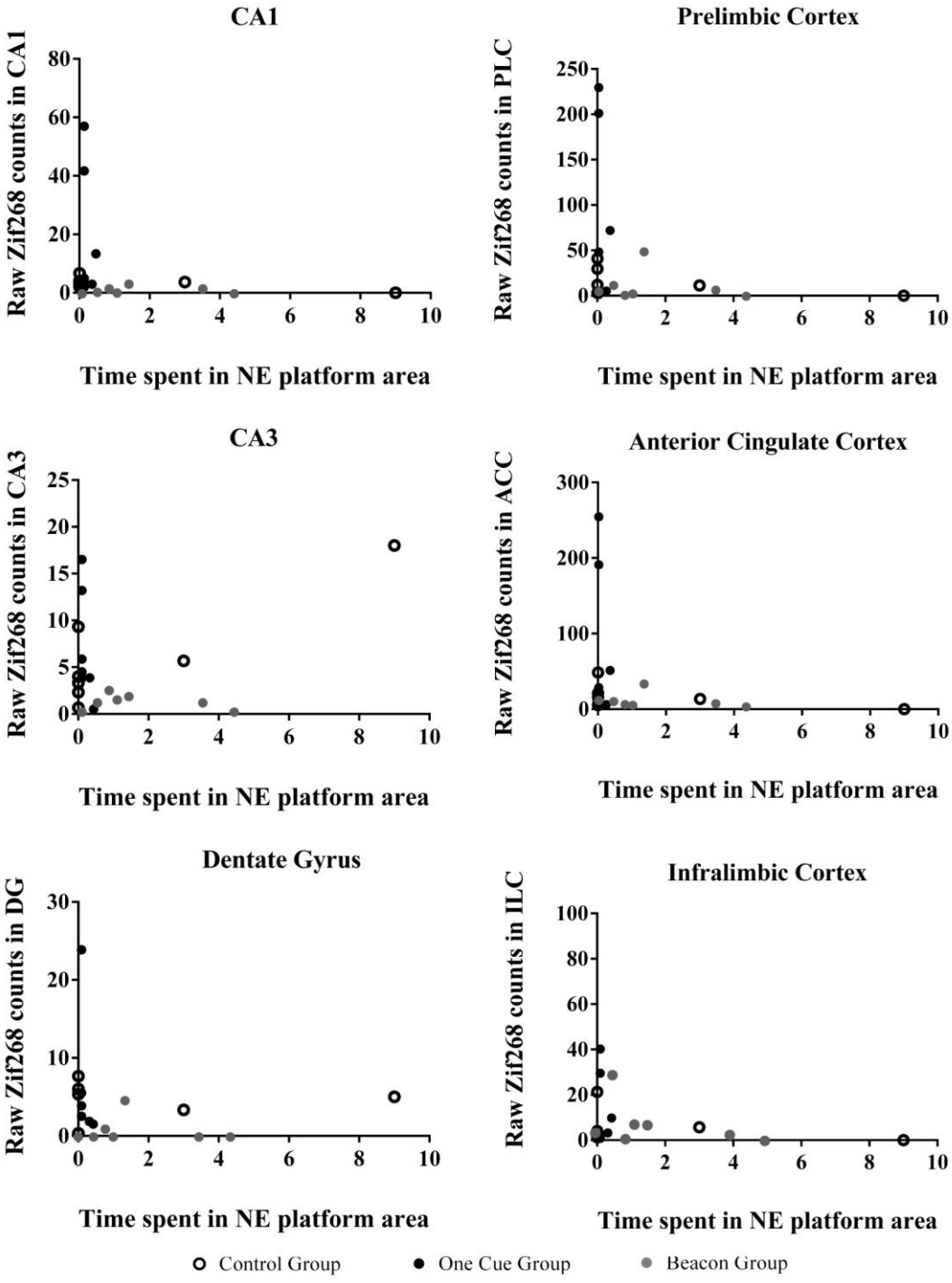

Figure 5.42: Scatterplots showing regional Zif268 counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after five days of training. All animals were treated with MK-801 prior to testing.

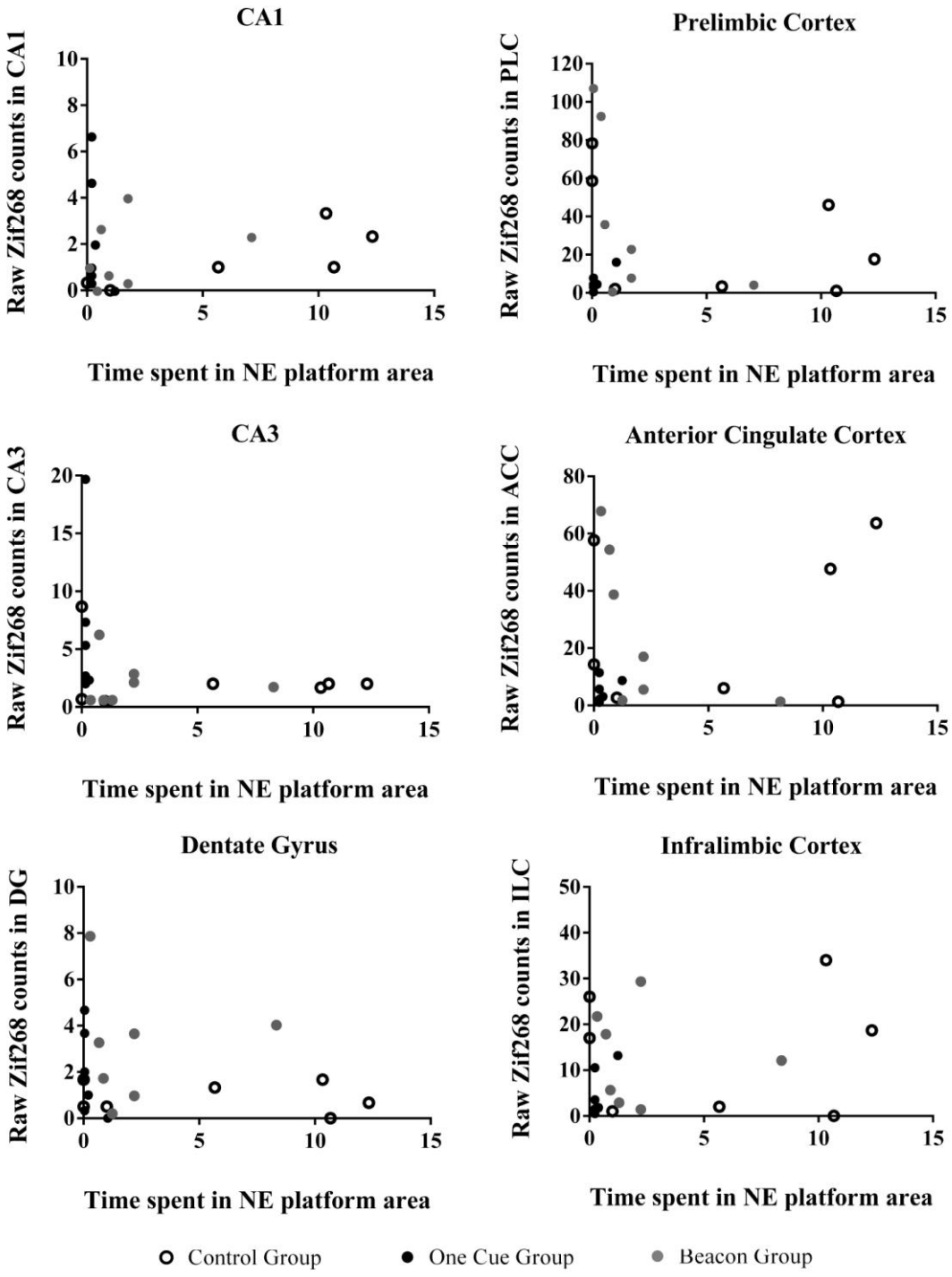

Figure 5.43: Scatterplots showing regional Zif268 counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after ten days of training. All animals were treated with MK-801 prior to testing.

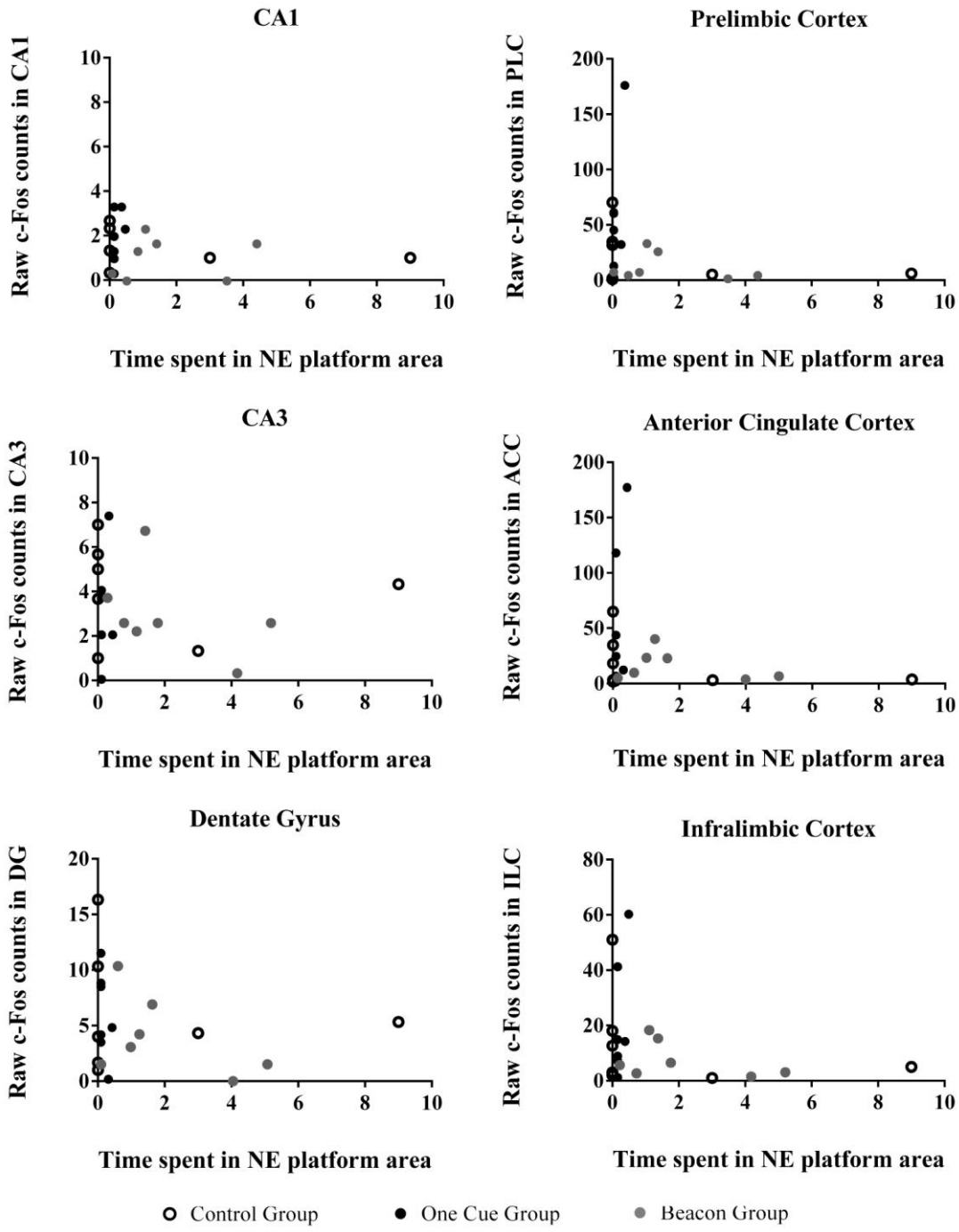

Figure 5.44: Scatterplots showing regional c-Fos counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after five days of training. All animals were treated with MK-801 prior to testing.

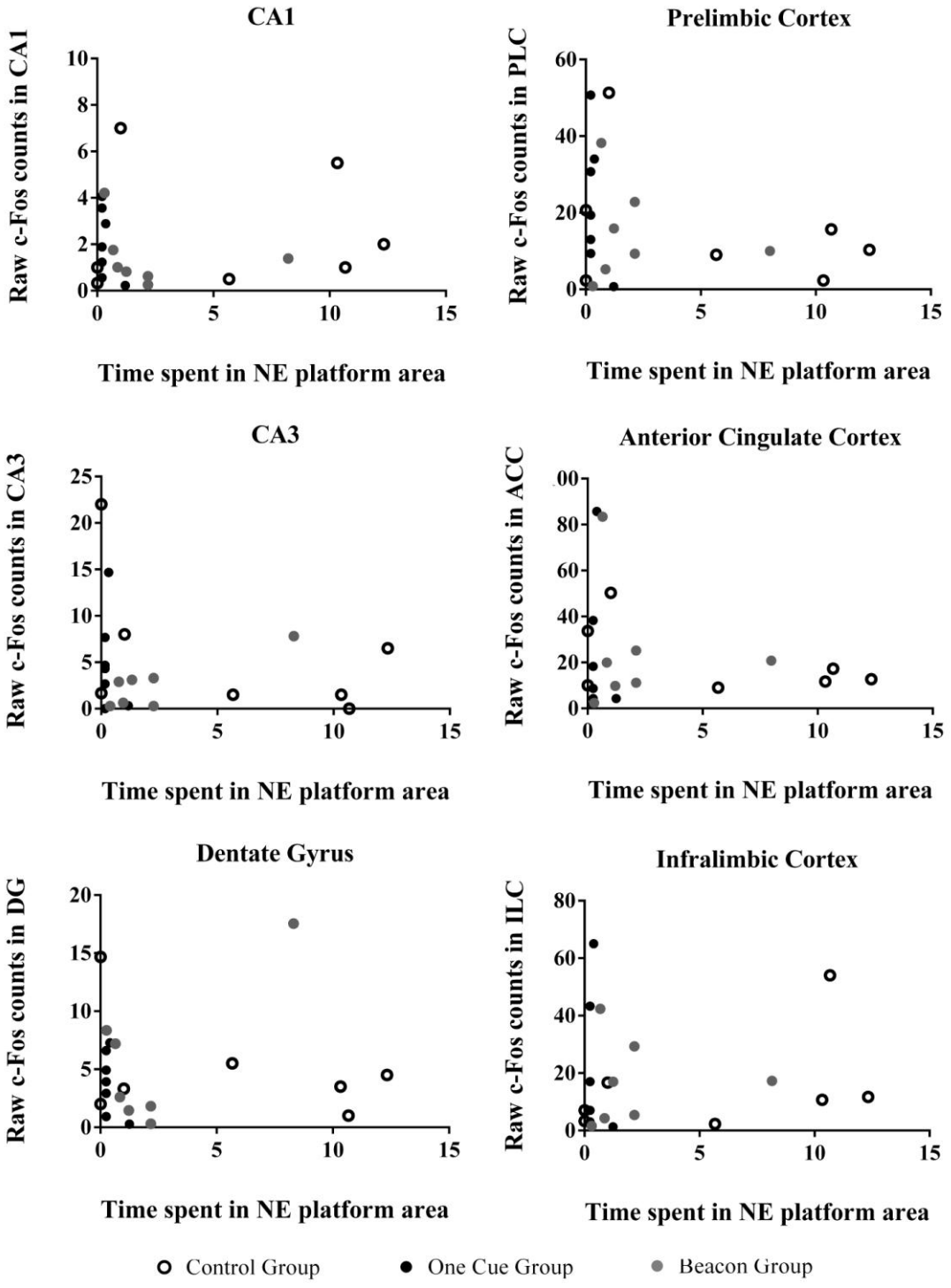

Figure 5.45: Scatterplots showing regional c-Fos counts (Y axis) and percentage time spent in the NE platform area (X axis) for Control, One Cue and Beacon groups after ten days of training. All animals were treated with MK-801 prior to testing.

5.3.5. Discussion

Task acquisition was similar to Experiment 1, with equivalent performance between five and ten day conditions, i.e. escape latencies of ~20 seconds or lower. Again, no group differences were found on the final days of training (signifying analogous learning) and acquisition for all groups improved with increased training. As expected, none of the five-day groups showed a significant preference for the target NE quadrant after MK-801 administration, demonstrating that spatial and non-spatial memory recall were equally impaired. After ten days of training, only the Control group favoured the correct quadrant, indicating that spatial memory for the platform location was preserved under full cue conditions. However, analyses of time spent in platform areas highlighted that memory for the exact position of the goal was poor. Overall, results show that increased experience with the environment can partially protect against the effects of NMDA receptor channel blockade in rats trained and tested with an intact cue configuration, indicating that NMDA receptors may only be necessary early in training (Mei et al., 2011; Nakazawa et al., 2004). In contrast, extended training did not facilitate spatial memory recall with a single cue or beacon.

Additionally, although no differences were noted between groups with regard to time spent in platform areas, memory recall under partial cue conditions appeared to be the most affected by MK-801, consistent with our original hypothesis. More specifically, the ten-day One Cue group spent significantly less time in the target (NE) quadrant and more time in the starting (SW) quadrant compared to chance level, and spent more time at the edge of the pool relative to the Control group. Results therefore demonstrate that blockade of NMDA receptors negatively affected flexible use of stored spatial representations. Non-spatial memory was also impaired

after extended training. This result is in line with those of Experiment 1, where level of experience navigating the environment had no effect on performance.

Crucially, the patterns of Zif268 expression seen in Experiment 1 were largely erased following MK-801 administration, in line with previous studies (Gass et al., 1993). Here, the One Cue group exhibited significantly higher Zif268 expression across multiple brain regions relative to the Control (CA1, ACC and ILC) and Beacon groups (CA1, CA3, DG and ACC) tested after five days. The Control group also exhibited greater expression in CA3 compared to the Beacon group. Moreover, Zif268 expression in the hippocampus decreased for the One Cue group (CA1) and increased for the Beacon group (CA1 and DG) across training conditions, and no group differences were found at the ten-day recall time point. Although it is difficult to interpret why activation was initially higher in the partial cue condition following NMDA receptor blockade, there appears to be an overall pattern of enhanced Zif268 expression during the use of spatial, as opposed to a non-spatial, strategies.

One further point of note is that, although the Control group failed to indicate successful recall after five days, mean Zif268 counts for this group in all regions examined (except for the dentate gyrus) were positively correlated with percentage time in the NE area; this relationship was maintained in area CA1 after ten days. Importantly, these findings highlight qualitatively different patterns of regional engagement across groups (akin to Poirier et al., 2008), which may otherwise have been overlooked. However, as mentioned in Chapter 4, results from the large number of correlations performed here must be interpreted with caution due to an increased risk of Type I error. Lastly, as per Experiment 1, no group differences were found for

c-Fos in any sub-region after five or ten days of training, and levels of expression did not change from five to ten days.

5.4. General discussion

This chapter had four aims: (1) to delineate the involvement of hippocampal and medial prefrontal sub-regions during the retrieval of spatial and non-spatial memories using IEG imaging; (2) to examine how extended experience in an environment affects memory recall, behavioural flexibility, and activation in associated brain areas; (3) to establish the relative importance of NMDA receptors for spatial and non-spatial strategy use; and (4) to explore how NMDA receptor blockade during retrieval influences expression of Zif268 and c-Fos in the hippocampus and prefrontal cortex. To address these aims, we trained rats to use spatial (place) and non-spatial (response) strategies in the Morris water maze task. For the spatial strategy, rats learned to navigate to a hidden platform via a configuration of two distal cues (near and far). For the non-spatial strategy, rats learned to swim towards a beacon placed directly above the platform. Spatial and non-spatial memory recall was then tested in a probe trial without the platform.

Firstly, it is essential to establish whether or not these two distinct strategies did in fact emerge from our protocol. Overall acquisition results indicated that all groups learned at similar rates (with the exception of the five-day Beacon group in Experiment 1). Previous research using visible platforms has shown considerably faster learning in non-spatial compared to spatial groups (Carman & Mactutus, 2002; Morris, 1981; Sutherland & Dyck, 1984). However, studies that used hanging or standing cues as their beacon found that rats learned gradually across days (Chamizo & Rodrigo, 2004; Clark et al., 2007; Timberlake et al., 2007), consistent with our results. Collectively, these findings suggest that learning is less efficient with a beacon cue than with a visible platform; however, both can be used to establish a response strategy.

An alternate possibility is that rats in the Beacon groups were employing a spatial strategy to find the platform, having learned the spatial relationship between the beacon, platform and the edge of the maze (Harvey et al., 2009), in the same way as the One Cue group (Experiment 1). A number of studies have shown that rats can learn and remember the location of a hidden platform using a single cue only (see Chapter 3; and also Chamizo & Rodrigo, 2004; Harvey et al., 2009; Vorhees & Williams, 2014). However, analyses of the probe trial data indicate that this is unlikely. Specifically, the Beacon group displayed accurate recall after five days of training, whereas both Control and One Cue groups were not as accurate. Further, while increased training lead to better memory performance in the groups trained with two cues, the Beacon group showed no improvements. These distinctions strongly suggest that animals navigating with the beacon were using a different strategy to the other groups. More broadly, these results imply that increased experience aids spatial – but not non-spatial – memory recall, as originally hypothesised. Moreover, extended training appears to allow for more flexible use of spatial representations, i.e. where animals can switch between configural or elemental spatial strategies involving whole or partial cue arrangements, depending on task demands (see Chapter 3; Farina et al., 2015).

An additional aim of this experiment was to identify the specific hippocampal and prefrontal sub-regions implicated in the retrieval of these memories. Previous research has shown that the hippocampus is essential for spatial memory recall (Morris et al., 1982; Save & Poucet, 2000) and flexible responding (Jo et al., 2007) but not for non-spatial memory (Packard & McGaugh, 1992). Therefore, we expected to see overall increases in hippocampal IEG expression for both spatial groups relative to the Beacon group, and higher expression in area CA3

for the One Cue group compared to the other groups (Jo et al., 2007). The medial prefrontal cortex has also been implicated in behavioural flexibility, particularly with regard to strategy switching (de Bruin et al., 1994), although the role of specific sub-regions in such processes remains unclear (Kubik et al., 2007). Accordingly, we predicted that the highest prefrontal IEG expression would also be found in the One Cue group.

Analyses of Zif268 expression revealed markedly different patterns of activation across groups, in line with the theory that animals were using distinct strategies. These results were generally well-matched with behavioural findings. The Beacon group (who demonstrated intact recall after five days) had higher Zif268 expression in all sub-regions examined relative to the spatial groups (which showed poorer recall after five days). Expression in CA1 and DG was also higher for the One Cue group compared to the Control group. One possible explanation for this may have been the increased ambiguity of the environment in the partial cue condition. Bannerman and colleagues (2012) recently reported that mice lacking NMDA receptor sub-unit GluN1 selectively in CA1 and DG cells were unable to distinguish between two visually similar beacons in the water maze, implying that CA1 and DG facilitate visual discrimination. Thus, it is possible that the selection choice encountered by the One Cue group here (i.e. which cue is present?) resulted in increased engagement of these areas relative to the full cue condition, where no such choice was necessary.

Overall patterns of Zif268 expression were reversed following extended training (greater mean counts in the Control and One Cue groups relative to the Beacon group), likely reflecting the successful memory recall displayed by the spatial groups under both full and partial cue conditions. However, no differences

were found between spatial and non-spatial groups in CA1 or DG. This is somewhat surprising, as lesion studies have established that non-spatial memory recall can be accomplished in the absence of a functional hippocampus (McDonald & White, 1994). One explanation for our finding could be that, although the hippocampus is not necessarily required for beacon responding, it will continue to be engaged in navigation tasks if intact (Jenkins et al., 2003; Simon et al., 2011). Importantly, a significant difference was noted between spatial and non-spatial groups in area CA3, consistent with its suggested role in flexible responding (Jo et al., 2007). Differences were also observed between these groups in the PLC and ACC. Given that few studies have investigated prefrontal IEG expression during spatial and non-spatial memory recall to date, results could signify that PLC and ACC sub-regions are particularly important for more complex, spatial responding.

In Experiment 2, we explored the effects of NMDA receptor blockade on spatial and non-spatial memory retrieval. As predicted, i.p. administration of MK-801 before recall caused memory impairments (and greater performance variability) for all groups after standard five-day training. Together with results from Chapter 4 of this thesis and previous research (Holahan et al., 2005), our findings demonstrate that NMDA receptor activation is critical for encoding and retrieval stages of memory processing. Importantly, we found that prolonged experience in the environment prior to NMDA receptor blockade protected against these memory deficits, but only under certain conditions. Specifically, memory was preserved in rats tested with the intact arrangement of training cues only. These results are consistent with reports from Shapiro and colleagues (Shapiro & Caramanos, 1990; Shapiro & O'Connor, 1992), who found intact spatial reference memory in well-trained rats treated with MK-801 (i.p.; 0.06–0.1mg/kg) before testing in the radial

arm maze. Accordingly, we propose that NMDA receptor activation is predominantly engaged in memory recall in less familiar environments.

The deficits observed in the One Cue group indicate that NMDA receptors are also required for the flexible use of spatial representations, regardless of the animals' experience. Previous research (Fellini et al., 2009; Nakazawa et al., 2002) has shown that deletion or inactivation of NMDA receptors in CA3 alone can impair recall under partial cue conditions; therefore, it is not surprising that blockade of NMDA throughout the brain resulted in comparable effects here. Equally, NMDA receptors appear crucial for non-spatial memory use, as evidenced by the poor performance of the five- and ten-day Beacon groups. Taken together, results highlight the significant role played by NMDA receptors in multiple types of navigation (Vorhees & Williams, 2014).

With regard to IEG expression, Zif268 activation patterns were drastically altered from Experiment 1. The One Cue group exhibited significantly higher levels of Zif268 in all hippocampal sub-regions and in the ACC and ILC. This could reflect a novelty response (Hall et al., 2001; Renaudineau et al., 2009). More specifically, animals navigating in the partial cue condition were the only group to experience an environmental change between training and testing, which may explain the increase in Zif268 expression. However, although these group differences were found, visual inspection of the raw data (Figures 5.9 and 5.12) indicated that Zif268 expression was considerably attenuated following drug administration, akin to previous research (Gass et al., 1993). Taken together, results therefore emphasise the complex relationship between memory, NMDA receptors and IEG expression (Veyrac et al., 2014).

In complete contrast to Zif268, no group differences in c-Fos expression were found in any sub-region after standard or prolonged training (similar to findings from Chapter 4). This result is in line with those of Guzowski and colleagues (2001), who also failed to find differences in hippocampal c-Fos expression between spatial and non-spatial groups. However, they are inconsistent with the results of Jo et al. (2007), which revealed higher c-Fos expression in CA3 and the prefrontal cortex in a partial cue condition. These divergent findings can most likely be accounted for by variations in the experimental procedures used, such as recall intervals (ranging between 30 minutes and 24 hours) or the number of cues present during initial training (between one cue and four cues). One further explanation is the small sample size used; for example, a power analysis on these data (G*Power Analysis Tool) indicated that a sample size of 60 (n = 20 per group) would be required to detect any significant effects. On the whole, however, it appears that although c-Fos is necessary for normal long-term memory retrieval (Fleischmann et al., 2003), its expression was not sensitive to differences underlying spatial and non-spatial strategies in the context of this study.

Additionally, because no changes in c-Fos were observed in either Experiment, the effects of NMDA receptor inactivation on c-Fos expression are unclear. That said, visual inspection of raw counts (Figures 5.27 and 5.30) in Experiments 1 and 2 revealed a similar overall reduction in c-Fos expression to that seen for Zif268. Therefore, it is reasonable to assume that NMDA receptor blockade leads to a general decrease in IEG activity (Vaccarino et al., 1992). Collectively, IEG results indicate that expression of Zif268 was a more useful indicator of regional activation during memory retrieval, and support the suggestion that Zif268 plays a functional role in the recall of long-term memories (Jones et al., 2001). Again, these

divergent patterns of IEG activation also highlight the importance of using multiple markers of neural activity in order to obtain a more informed understanding of regional activation.

In summary, findings from this Chapter indicate that, once established, spatial memories preferentially recruit specific sub-regions of the hippocampus (CA3) and medial prefrontal cortex (PLC and ACC), when compared with non-spatial strategies (Experiment 1). These areas likely work in tandem to mediate flexible use of spatial representations between cues in their environment (Churchwell et al., 2010). Further, findings illustrate that both spatial and non-spatial memory retrieval is largely NMDA receptor dependent (Experiment 2); however, given sufficient experience with an environment, rats can recall spatial memory for a learned goal destination.

Chapter 6

General Discussion

6.1. Summary of the findings from this thesis

The main objectives of this thesis were to differentiate the use of discrete allocentric navigation strategies over the course of learning and recall in the Morris water maze, and to characterise the specific roles of hippocampal and medial prefrontal sub-regions in these processes using IEG imaging. We first examined spatial strategy use during acquisition of the maze (Chapter 1). Although the Morris water maze is one of the most widely used tasks of spatial learning and memory (Vorhees & Williams, 2014), precisely how animals encode information about their environment during this task remains unclear. Accordingly, we investigated two influencing factors which have yet to been studied in detail. These were cue salience and length of training.

Results revealed that the determinants of cue salience are more complex than previously thought (Chamizo, 2002; Chamizo & Rodrigo, 2004). That is, proximal cues will not always become more salient than distal cues. Instead, we found that salience is at least partially dependent on the type of spatial information cues convey, i.e. a cue offering stable directional information (in this case the far cue) can be more useful than a cue positioned closer to the goal (near cue). This is in keeping with previous findings from our laboratory which showed that knowing the direction in which to travel is more important for accurate navigation than knowing the distance to the goal (Diviney et al., 2013). In addition, we found novel evidence that cue elevation – which has not yet been explored in rodent navigation – plays an important role in determining cue salience (Collett, 2010; Muller & Wehner, 2007). More specifically, our results indicate that cues whose elevation appears lower when viewed from the goal location allow for more precise estimations of distance and direction than those at a higher elevation. Finally, we demonstrated that relative cue

salience has a significant effect on whether animals learn to rely on individual cues (elemental learning) or groups of cues (configural learning) to navigate, but that increased experience in the environment can facilitate more flexible responding, i.e. strategy switching (in line with Rodrigo et al., 2014). Together, findings are largely consistent with associative learning theories of navigation and oppose cognitive mapping theory (discussed further in Section 6.2).

Next, we investigated the neuronal underpinnings of spatial learning in the water maze (Chapter 4). Specifically, we aimed to determine the role of distinct classes of ionotropic glutamate receptors in spatial learning, and their effects on associated IEG expression in the hippocampus and medial prefrontal cortex. Successful encoding of spatial information is widely accepted to be hippocampal-dependent, with activation of NMDA receptors being crucial to this process (Bast et al., 2005; Pitkänen et al., 1995). However, the involvement of AMPA/kainate receptors in spatial processing is less well understood (Riedel et al., 1999; Riedel et al., 2003). Moreover, existing evidence for the involvement of the prefrontal cortex in spatial learning is equivocal (Wang & Cai, 2008), and the specific contributions of its sub-regions during this initial stage are largely unknown.

Extending on previous IEG imaging studies (Feldman et al., 2010; Guzowski et al., 2001; Teather et al., 2005), we charted the expression of two IEGs, Zif268 and c-Fos, in sub-regions of hippocampus and prefrontal cortex following spatial learning. IEG imaging confers the advantage of being able to observe patterns of activation in functional brain regions in parallel, thereby revealing functional connectivity between regions during a task (Aggleton et al., 2012). Finally, as little is known about how glutamate receptors and IEGs interact to influence spatial

behaviour and learning (or, more broadly, memory), our study was one of the first to explicitly investigate this.

Results were consistent with previous reports that NMDA receptor activation is necessary for spatial learning (Bast et al., 2005), as evidenced by the poor performance of animals treated with MK-801. Conversely, AMPA/kainate receptor inactivation (via CNQX) had no effect on spatial encoding, contrary to previous work (Liang et al., 1994). This disparity may be attributable to differences in the administration routes used in the two experiments (hippocampal infusions versus i.p. administration). CNQX has been shown to penetrate the blood-brain barrier relatively poorly (Rogawski, 2011); therefore any effects may have been substantially diluted in our animals. Further, evidence suggests that AMPA receptor activation may be more critical to spatial memory retrieval, rather than encoding (Bast et al., 2005); this may also account for the lack of CNQX effect observed here.

We found that expression of Zif268 was associated with the successful encoding, where animals that learned the task displayed elevated levels of Zif268 relative to those who did not. This was particularly evident in area CA1 of the hippocampus, which is consistent with previous research implicating this region as a critical area for spatial processing (Bartsch et al., 2010; Shimizu et al., 2000; Tsien, Huerta, & Tonegawa, 1996). In contrast, area CA3 and the dentate gyrus were not associated with task acquisition, while the prelimbic cortex only showed some evidence of involvement in learning. Overall, the data suggest minimal involvement of the prefrontal cortex in spatial learning, which may be expected given the relative ease of the task in the absence of any major environmental changes. Importantly, the observed patterns of Zif268 expression were reliably dissociable from the pharmacological effects of blocking NMDA and AMPA/kainate receptors – that is,

any increases in Zif268 expression cannot be attributed to drug effects – as drug administration at baseline had no observable effect on expression. In complete contrast to Zif268, c-Fos expression was associated with animals that performed poorly on the task (i.e. elevated levels in the MK-801 group), suggesting that c-Fos may be more closely linked to other, non-navigational aspects of the task such as stress induced by prolonged swimming (Duncan et al., 1993) or error-correction responses (Poirier et al., 2008).

In Chapter 5, we investigated memory recall in the water maze. Specifically, we explored the potential benefits of extended training on retrieval of spatial and non-spatial memories (via distal cues and a beacon, respectively), and its effect on related hippocampal and medial prefrontal activation (i.e. does regional involvement change with increased experience in the environment?). Not unlike memory encoding, how and when discrete sub-regions within these structures contribute to memory recall are unclear at present, particularly with regard to the medial prefrontal cortex (Kubik et al., 2007). A final aim of this chapter was to establish whether or not NMDA receptor activation is equally important for spatial and non-spatial strategy use, and how memory impairments are reflected in regional IEG expression. Again, this study represents one of the first investigations of this kind in the spatial domain.

Results revealed a clear behavioural distinction between non-spatial memories (which were accurately recalled following standard exposure to the environment) and spatial memories (which necessitated further training). Further, although both types of memory were initially impaired following NMDA receptor blockade, spatial memories could be preserved with sufficient training (in keeping with Shapiro & O'Connor, 1992). Similar to acquisition, Zif268 expression was

tightly coupled with successful performance; that is, expression was higher for the non-spatial group initially, but increased for the spatial groups once they remembered the goal location. Patterns of Zif268 expression indicated that CA1 and the dentate gyrus were involved in both spatial and non-spatial memory, likely reflecting the overall engagement of the hippocampus in navigation even when no spatial representations are required (Simon et al., 2011; Teixeira, Pomedli, Maei, Kee, & Frankland, 2006). Interestingly, however, we found that the prelimbic and anterior cingulate cortices were preferentially engaged in spatial processing, signifying differing roles of the prefrontal sub-regions during this task. After NMDA receptor blockade, Zif268 levels were highest in spatially-trained rats tested in diminished cue conditions, which may have been indicative of a novelty response (Renaudineau et al., 2009). Lastly, c-Fos levels were similar for all groups in all sub-regions regardless of NMDA receptor functioning, which suggest that this marker is less sensitive to the type of memory being recalled.

6.2. Significance of findings

6.2.1. Navigation strategies: Cognitive map or associative learning?

A central question explored in this thesis was: how do animals form allocentric representations of their environment? At present, there are a number of theories explaining how this might be achieved, the most prominent of which are cognitive mapping theory (O'Keefe & Nadel, 1978) and associative learning theory (Pearce & Hall, 1980). Cognitive mapping theory defines two types of navigation strategies: taxon, wherein the animal learns to directly approach a cue located at or near to the goal, and locale learning, which involves the formation of a cognitive map of the environment incorporating the spatial relationships between cues and the goal

(O'Keefe & Nadel, 1978). This representation is thought to automatically update as the animal navigates, incorporating any environmental changes in an all-or-nothing manner (Schinazi et al., 2013). Accordingly, cognitive maps are flexible by nature (i.e. allowing the animal to plan and execute novel routes). Associative learning theory also posits that cue representations become associated with the goal through repeated exposure during navigation (Honey et al., 2014; Pearce & Hall, 1980). However, such representations are thought to lie somewhere in between taxon and locale strategies, in that they necessitate more than a simple approach strategy but do not require a global representation.

Overall, results from this thesis (in particular those from Chapters 3 and 5) are not well accounted for by locale learning as defined by cognitive map theory. We found that animals do not appear to encode information about all cues equally; rather, certain cues proved more important for locating the goal than others. This is not predicted by cognitive map theory, which assumes that accurate navigation should be possible with any training cue (Morris, 1981; Sanchez-Moreno et al., 1999). These data are more in line with associative learning accounts – that is, some cues acquired a higher salience than others, and remained essential for successful way-finding. Moreover, we observed effects consistent with cue competition, i.e. overshadowing (Chamizo, Rodrigo, & Mackintosh, 2006). Specifically, highly salient cues appeared to inhibit learning about other cues. We also found additive effects of cue salience (Crespo et al., 2012), whereby animals relied more on an already salient cue when its prominence was enhanced, i.e. using a cue which was both brighter and closer to the platform. These effects are hallmarks of associative learning, and as such our findings provide strong support for this theory.

Equally, results could also be interpreted as supportive of the vector model of learning (Collett et al., 1986; Kubie & Fenton, 2009). This theory states that the navigating animal uses the cues as vectors to compute distance and direction to the goal. The vector model is similar to associative learning theory in that cues are imbued with weighted salience, and again assumes less elaborate representations, i.e. the animal does not need to know its position relative to the overall layout of the environment, rather it only needs to update its progress along the vector (Kubie & Fenton, 2009). One further model which could potentially explain our results is view-based navigation theory, derived largely from studies of insect navigation (Cheung, Stürzl, Zeil, & Cheng, 2008; Collett, 2010). According to this theory, the animal navigates by searching for a location at which the current retinal image matches the remembered view (or 'snapshot') at the target position. While plausible, this would not have been the most efficient strategy for animals relying on distal cues in the present studies. More specifically, because the cues did not unambiguously define the goal location, retinal matching one its own would not have been sufficient to find the platform.

In Chapter 5, we also explored navigation using a beacon cue, which is classified by cognitive mapping theory as taxon learning. This type of strategy has also been defined as non-spatial or egocentric (Brown, 1992; de Bruin et al., 2001), as the animal encodes little information about their surroundings. Our results support the distinction between spatial and non-spatial strategies with regard to behavioural complexity (i.e. beacon-trained rats demonstrated rapid acquisition and recall of the task, with extra training offering no benefit). In line with previous work, we suggest that rats learn to relate movements towards the beacon with escaping the maze, which in turn reinforces this behaviour (Sheynikhovich, Chavarriaga, Strosslin,

Arleo, & Gerstner, 2009). With regard to navigational theories, findings are consistent with most models (e.g. cognitive mapping, associative learning and vector models), all of which predict a simple stimulus-response association between the beacon and the platform. Equally, these results could also be explained by the view-based model of navigation; that is, matching their retinal image to the remembered image would, in this case, lead animals directly to the goal location. However, this model would be difficult to directly test in the water maze given the limited size of the environment.

Crucially, it is probable that many of the models discussed here (e.g. associative, vector and view-based models) are not mutually exclusive. Indeed, Kubie and Fenton (2009) have highlighted the difficulty in distinguishing between strategies based on performance alone, as there may be a large degree of overlap between the characteristics of these models. For example, our results strongly suggest that vector information (i.e. distance and direction) is incorporated into spatial representations. Further, we demonstrated that rats could adopt different strategies (i.e. using single or multiple elements) depending on the cues available and on their level of experience with the environment (Biegler & Morris, 1999; Harvey et al., 2008; Kamil & Jones, 1997; and in humans, Redhead & Hamilton, 2007). In particular, training length had a significant effect on rats' navigational abilities, i.e. ten-day training lead to considerably improved performance relative to five-day training. This is an important point given that standard water maze procedure is five or six days (Vorhees & Williams, 2006). Thus, we suggest that similar studies in the future should employ longer training protocols to ensure that animals' have acquired a robust spatial memory.

Finally, while our results appear to indicate increased behavioural flexibility (Jo et al., 2007; Sturz & Katz, 2009), exactly what this entails is difficult to determine as the term itself is generally not well defined. For example, greater efficiency may not necessarily lead to flexibility. Rather, animals may become increasingly reliant on the most useful cue at the expense of others, which, in turn, would imply that the representations employed are less elaborate. If this were case, these patterns would argue against the idea that configural cue representations can be activated by any single element (associative learning theory; Rodrigo et al., 2014) or that animals acquire a global representation of the environment (cognitive map theory; O'Keefe & Nadel, 1978).

6.2.2. Brain regions involved in navigation

A second goal of this thesis was to delineate the involvement of the hippocampus and medial prefrontal cortex in facilitating the different types of strategies outlined above, i.e. spatial and non-spatial. Although both structures are strongly implicated in navigation (de Bruin et al., 2001; Simon et al., 2011), how and when specific sub-regions contribute to these processes remains unclear (Aggleton, Vann, Oswald, & Good, 2000). Below, we review our findings with regard to the wider neuroscientific literature.

6.2.2.1. Hippocampus

We found that activity in area CA1 (indexed by Zif268) was strongly associated with spatial encoding, and as well as being negatively correlated with thigmotaxis, i.e. swimming around the edges of the pool (a measure of anxiety or stress; Treit & Fundytus, 1988). In contrast, no differences were noted between animals that learned

the task and those who did not in CA3 or dentate gyrus regions. These results indicate that, within the hippocampus, CA1 is a key region for establishing an accurate spatial representation of the environment, consistent with the majority of human and non-human research (Bartsch et al., 2010; Goodrich-Hunsaker, Hunsaker, & Kesner, 2008; Okada & Okaichi, 2010). During spatial memory retrieval, both CA1 and the dentate gyrus were preferentially engaged when rats were required to make a visual discrimination (i.e. following removal of one of two similar cues), but only when exposure to the environment was limited. Given extended training, during which animals had sufficient time to establish a more robust memory representation, no differences were documented between rats navigating with intact or partial cue arrangements, or via a non-spatial beacon strategy.

Interestingly, similar patterns were seen when NMDA receptor functioning (and consequently memory recall) was inhibited; expression of Zif268 in CA1 and the dentate gyrus was initially highest in the partial cue condition, but these differences attenuated following further training. Results support the suggestion that CA1 and the dentate gyrus are critical for successful recall of newly formed spatial and non-spatial memories. Further, with regard to non-spatial memory, CA1 may be more important than the dentate gyrus, as Zif268 expression here was positively correlated with time spent in the target quadrant for the beacon-trained animals. With regard to CA3, activation of this region during recall was specific to spatial memory (regardless of cue condition or NMDA receptor blockade). This suggests that CA3 plays a more substantial role in the retrieval of complex spatial representations as opposed to mediating stimulus-response type behaviours.

Together, findings illustrate that CA1 is crucial for acquisition and recall stages of spatial and non-spatial navigation. This is consistent with previous research showing that lesions to this area result in impaired performance (Okada & Okaichi, 2009; Stubley-Weatherly et al., 1996). More specifically, results support the suggestion that CA1 facilitates a range of behaviours including response selection (Bannerman et al., 2012) and adapting to task demands (Dillon et al., 2008). Area CA3 appears to be engaged in spatial strategies only, as is essential for flexible responding involving more complex representations (Jo et al., 2007). This is in line with preceding work which has also implicated CA3 in spatial learning (Florian & Roullet, 2004; Stubley-Weatherly et al., 1996) and memory (Steffenach, Witter, Moser, & Moser, 2005).

In particular, the results of Florian and Roullet (2004) add weight to our data; the authors demonstrated that temporary inactivation of CA3 impaired place learning in the water maze (using distal cues), but had no effect on beacon learning. In comparison, the role of dentate gyrus is less clear. Our results implicated this region in the retrieval but not the acquisition stage of memory processing. However, a previous study by Okada and Okaichi (2009) showed that both encoding and retrieval were impaired following dentate gyrus lesions, while Xavier, Oliveira-Filho and Santos (1999) found task acquisition was somewhat preserved in lesioned rats. One possible explanation for the discrepancy in findings is that dentate gyrus lesions could have led to disrupted functional connectivity with other regions (e.g. CA3), resulting in performance deficits (Jerman, Kesner, & Hunsaker, 2006); this would account for the absence of similar effects here.

More broadly, findings from this thesis indicate that an intact, functional hippocampus will be engaged by any task which includes a navigational element,

regardless of behavioural complexity. However, a hippocampal contribution is less important, and may not be necessary for simpler forms of non-spatial memory (Broadbent et al., 2006; McDonald & White, 1994; Morris et al., 1982; Packard & McGaugh, 1996; Save & Poucet, 2000). These memories are likely mediated by other cortical areas including the caudate nucleus and striatum (Devan et al., 1999). One simple explanation for the continued involvement of the hippocampus in the various navigation strategies examined here is the additional memory processing required during recall (Martin, de Hoz, & Morris, 2005; Teixeira et al., 2006). More specifically, remembering the goal location in the water maze when the platform is absent requires the animal to continually monitor and update their position relative to their environment, thus reengaging the hippocampus.

Studies have also shown that the hippocampus continues to be crucial for accurate performance in the water maze at a remote time point (30 days post-learning) (Broadbent et al., 2006). In addition, while Winocur, Moscovitch, Fogel, Rosenbaum and Sekeres (2005) originally demonstrated that rats with hippocampal lesions could navigate a complex 'village' environment following extensive (three month) training, a follow up study revealed that when the optimal route was blocked, animals took significantly longer to reach the target relative to controls (Winocur, Moscovitch, Rosenbaum, & Sekeres, 2010). Therefore, it appears that 'online' processing in the hippocampus continues to be required for flexible use of spatial representations, in line with the role of place cells in this area (Muller & Kubie, 1987; O'Keefe & Dostrovsky, 1971).

6.2.2.2. Medial prefrontal cortex

In comparison to the hippocampus, the role of the medial prefrontal cortex in navigation is less well defined. During task acquisition, the prelimbic cortex was the only prefrontal sub-region that appeared to be associated with memory encoding (as evidenced by increased Zif268 expression). In contrast, levels of Zif268 in the anterior cingulate and infralimbic cortices were equivalent for animals that learned the task, those who were impaired and untrained controls, suggesting that these regions were not engaged in the formation of spatial representations. At the recall stage, higher activation in both prelimbic and anterior cingulate regions was linked to spatial strategy use (with a single cue or multiple cues), thereby demonstrating their importance for spatial (as opposed to non-spatial) responding. Not unlike the patterns observed in the hippocampus, memory disruption (via MK-801) resulted in greater activation for rats navigating under partial cue conditions. This was specific to anterior cingulate and infralimbic sub-regions, which may imply that these areas are sensitive to environmental changes. In support of this suggestion, a study by Granon, Save, Buhot and Poucet (1996), reported that prelimbic lesions did not impair rats ability to detect spatial changes in an environment, even when their surroundings were complex.

Together, results from this thesis illustrate that the medial prefrontal cortex is more involved in memory recall than in acquisition. Although some research has demonstrated that lesions to the entire medial prefrontal area prevent rats from learning the location of a hidden platform in the water maze (Kolb, Pittman, Sutherland, & Whishaw, 1982; Mogensen, Lauritsen, Elvertorp, Hasman, Moustgaard, & Wortwein, 2004), others have failed to find such deficits (de Bruin et al., 1994; Lacroix et al., 2002). Rather than reflecting a role in place learning *per se*,

there is a growing body of literature which suggests that the prefrontal cortex is involved in more global processing such as monitoring behaviour (Miller, 2000), flexible use of strategies (Churchwell et al., 2010) and inhibitory control (i.e. inhibiting the use an ineffective strategy or response behaviour; Caetano et al., 2013). In keeping with this, our results indicate that prefrontal regions were particularly implicated in the processing of complex spatial representations (akin to Churchwell et al., 2010; Jo et al., 2007). This would also explain previously documented deficits during reversal tasks, wherein the platform is moved to a new location, signifying a shift in task demands (de Bruin et al., 1994). Similarly, it has also been shown that rats with prefrontal lesions are unable to switch from using a spatial strategy to a non-spatial one (de Bruin, Swinkels, & de Brabander, 1997; Mogensen et al., 2005).

Based on our results, we propose that the prelimbic and anterior cingulate regions are particularly important for behavioural monitoring and flexibility (i.e. when one of the training cues is removed), with the infralimbic cortex playing a limited role. This is in keeping with, but also extends, the suggestion by Jo *et al.* (2007), that the medial prefrontal cortex is crucial for successful navigation in a modified environment. Additionally, the prelimbic cortex in particular may also facilitate goal-directed behaviours. More specifically, Hok and colleagues (2005) found that place cells in the prelimbic/infralimbic region (and to a lesser extent the anterior cingulate cortex) were primarily distributed at goal locations and around cues, suggesting that these cells encode information about salient features of the environment.

More generally, the anterior cingulate cortex has been implicated as a 'hub-like' region which supports the integration of information from multiple sources

(Frankland & Bontempi, 2006; Frankland, Teixeira, & Wang, 2007). This would not be surprising given the variety of afferent connections this region receives (i.e. prelimbic cortex, CA1, entorhinal cortex, perirhinal cortex and subiculum; Hoover & Vertes, 2007), and consequently supports our finding that the anterior cingulate region was consistently engaged during recall. Recent findings by Rajasethupathy and colleagues (2015), which showed that optogenetic stimulation of a newly discovered anterior cingulate to CA1/CA3 projection in mice elicited memory retrieval, also add considerable weight to this suggestion. Finally, we noted limited involvement of the infralimbic cortex, indicating that this area may not be as important for spatial navigation as prelimbic and anterior cingulate regions. This region may be more involved in visceromotor functions (Wang & Cai, 2008) and anxiety-related behaviours (consistent with its heavy projections to amygdala; Jinks & McGregor, 1997; Vertes, 2004).

6.2.2.3. Hippocampal-prefrontal interactions

As outlined in Chapter 1, the hippocampus and medial prefrontal cortex are anatomically connected through direct (CA1 to prelimbic and anterior cingulate, and anterior cingulate to CA1/CA3) and indirect projections (via entorhinal cortex) (Hoover & Vertes, 2007; Rajasethupathy et al., 2015). Further, cell firing in the medial prefrontal cortex is phase locked to hippocampal theta oscillations during spatial tasks (Siapas, Lubenov, & Wilson, 2005), indicating functional connectivity. Therefore, it is reasonable to assume a high degree of interaction during the experiments employed in this thesis. In line with this assumption, we saw evidence of hippocampal-prefrontal interactions during both stages of memory processing. For acquisition, we observed similarly high expression of Zif268 (associated with

successful learning of the task) in CA1, prelimbic and anterior cingulate areas. During recall, we also saw coordinated activity between structures; specifically CA3, prelimbic and anterior cingulate regions (higher activation for spatial relative to non-spatial strategy use). These patterns of activation are consistent with previous lesion and IEG studies showing hippocampal-prefrontal interactions support flexible encoding and retrieval of complex spatial memories (Churchwell et al., 2010; Jo et al., 2007; Lee & Kesner, 2003). Furthermore, they support the current consensus within the field that the hippocampus and prefrontal cortex comprise part of a wide network of brain regions subserving navigation (i.e. entorhinal, perirhinal, postrhinal and retrosplenial cortices) (Aggleton, Vann, Oswald, & Good, 2000; Kubie & Fenton, 2009; Spiers & Barry, 2015). In the future, it will be important to characterise the functions of all areas within this network for different types of navigation strategies using IEG imaging, and further, how regional patterns of activation change over time (e.g. with increasing experience). The application of structural equation modelling (SEM) to these data would also be particularly informative here, as it would allow for the characterisation of functional interactions between regions in the network (Aggleton & Brown, 2005; Kinnavane, Albasser, & Aggleton, 2015).

6.2.3. Glutamate receptors and memory

Synaptic plasticity (e.g. LTP) is widely accepted to be the physiological mechanism by which memories are encoded and stored in the brain (Martin et al., 2000). Glutamatergic signalling (including both NMDA and AMPA/kainate receptors) is implicated in LTP (Bannerman et al., 1995; Castillo et al., 1997; Collingridge, Herron, & Lester, 1988; Wozny, Maier, Schmitz, & Behr, 2008; Wu, Rush, Rowan,

& Anwyl, 2001; Yu et al., 2008). We therefore investigated the effects of glutamate receptor inactivation on the encoding and retrieval of spatial (and non-spatial) memories. Our results strongly support the view that NMDA receptors underpin learning and memory, and that NMDA receptor plasticity is crucial to these processes (Barker, Bird, Alexander, & Warburton, 2007; Barker & Warburton, 2008; Liang et al., 1994; Morris et al., 1986).

Findings are consistent with previous research indicating that NMDA receptors in areas CA1 and CA3 of the hippocampus are particularly important for encoding and retrieval (Adams et al., 2001; Bannerman et al., 2012). For example, inactivation or genetic deletion of these receptors in CA1 has been shown to result in unstable place fields in mice (Kentros et al., 1998; McHugh, Blum, Tsien, Tonegawa, & Wilson, 1996). Our results are also in line with the more recent suggestion that NMDA receptors in these sub-regions facilitate flexible behavioural responding (i.e. choosing between competing response options) through synaptic modification (Bannerman et al., 2014; Jo et al., 2007; Taylor et al., 2014). In contrast, NMDA receptors in the dentate gyrus may not be required for memory recall, as demonstrated by Niewoehner and colleagues (2007). Interestingly, the acquisition deficits observed following NMDA receptor inhibition can be eliminated by non-spatial pre-training, although rats remain impaired during reversal testing (when the platform is moved to a new location) (Bannerman et al., 1995; Vorhees & Williams, 2014). Thus, the influence of NMDA receptor activation on spatial learning – but not flexible memory retrieval – appears to be dependent on experience with the environment.

On the other hand, we failed to find evidence that AMPA receptors were required for encoding in the water maze, similar to Filliat et al. (1998) but in contrast

to Liang and colleagues (1994). Although this may have been due to drug administration routes, as discussed, an alternate explanation is that AMPA receptors (in the hippocampus and prefrontal cortex) are more tightly coupled to the retrieval stage of memory processing (Barker et al., 2006; Bast et al., 2005; Teixeira et al., 2006; Tse et al., 2011). Importantly, Teixeira *et al.* (2006) found that AMPA inhibition in the anterior cingulate region caused impaired navigation to a hidden platform in the water maze at a remote time point (one month); thus, these receptors may become increasingly import as memories age. Lastly, it should be noted that although our findings indicate differing roles for distinct sub-regions, the global administration of drugs used somewhat limits the conclusions which can be drawn here. Therefore, further studies which utilise direct infusion or region-specific genetic knockout methods are needed to confirm the precise functions of these regions for the acquisition and recall of spatial and non-spatial memories.

6.2.4. IEGs as markers of neuronal activity

IEG imaging is a popular approach for measuring brain activity in response to behavioural experience (Aggleton et al., 2000; Dragunow & Faull, 1989; Kubik et al., 2007; Tischmeyer & Grimm, 1999). Although they are often used interchangeably within the literature, different IEGs may be associated with distinct processes (e.g. learning-related plasticity, general neuronal activity or physiological stress). Therefore, a key aim in this thesis was to chart the expression of two widely used IEGS – Zif268 and c-Fos – in the hippocampus and prefrontal cortex during memory encoding and retrieval. Overall results demonstrated that these two IEGs were in fact related to task performance in different ways. During encoding, increased Zif268 expression in CA1 was associated with successful task acquisition,

indicating that Zif268 was a useful marker of learning-related plasticity (Feldman et al., 2010). In contrast, c-Fos counts in the hippocampus and prefrontal cortex were elevated in poorer learners. This suggests that c-Fos expression was associated with encoding under conditions where the task demands were high (i.e. when MK-801-treated rats were prevented from learning) (Okuno, 2011), when stress levels were consistently elevated (as a result of prolonged swimming) (Cullinan et al., 1995), or both.

During recall, Zif268 expression proved to be a valuable indicator of successful performance, as illustrated previously (Hall et al., 2001; Knapska & Kaczmarek, 2004). Zif268 levels in the hippocampus and prefrontal cortex were also highly sensitive to the type of memory being retrieved (i.e. spatial or non-spatial) and to spatial changes in the environment (i.e. modifications to the cue arrangement). This is in line with previous findings from Ribeiro and colleagues (2007), which showed that Zif268 was upregulated following a novel spatial experience. On the other hand, expression of c-Fos was not associated with memory-related neuronal activity; that is, counts did not differ between groups that remembered the task and those who did not. Further, stress as a result of impaired performance did not appear to be a factor in c-Fos expression at the recall stage (i.e. expression was not higher in rats with poor memory). Thus, it seems that c-Fos may not be a suitable marker of post-learning neuronal activation at the retention interval examined here. Rather, we suggest that the expression patterns of c-Fos occur more rapidly, i.e. early in training and shortly after recall. This would account for the discrepancies between our results and those of previous researchers who employed shorter training protocols (one day training; Feldman et al., 2010; Teather et al., 2005) and retention intervals (thirty minutes post-training; Jo et al., 2007).

On the whole, our results demonstrate that Zif268 is a superior marker of neuronal activation relating to allocentric navigation in the water maze, relative to c-Fos. Based on previous research, we propose that this is largely due to the differing functions of these IEGs (Davis, Bozon, & Laroche, 2003; Knapska & Kaczmarek, 2004). A number of studies have demonstrated that Zif268 activation is tightly linked to neuronal plasticity underlying learning and memory. More specifically, Zif268 knockout mice demonstrate reliably diminished LTP, and impaired acquisition and retention of the water maze (Bozon et al., 2002; Jones et al., 2001), and exhibit unstable place fields (Renaudineau et al., 2009). However, these deficits can be overcome with extended and distributed training (Jones et al., 2001), indicating that Zif268 plays a time-dependent role in memory processing. This is supported by our findings, i.e. that group differences in Zif268 expression disappeared after ten days of training (Chapter 5; Experiment 2). Conversely, evidence of a role for c-Fos in these processes is currently equivocal. For example, Zhang, McQuade, Vorhees and Xu (2002) showed that deletion of c-Fos from the hippocampus had no effect on water maze acquisition, while Fleishmann and colleagues (2003) found that mice lacking c-Fos from the entire nervous system were impaired at spatial learning. Thus, it seems that c-Fos activation outside of the hippocampus (and medial prefrontal cortex, according to our findings) may be necessary for spatial processing.

Importantly, results from this thesis highlight the importance of careful interpretation with regard to IEG imaging data, as IEG expression can reflect a number of processes, e.g. learning-related plasticity, task performance or stress. Crucially, the patterns observed will likely depend on the IEG, task and stage of memory processing investigated. The level of experience will also have a significant

influence on IEG expression (similar to NMDA receptor activation); thus, future studies should aim to investigate a number of IEGs at multiple time points throughout training, ranging from a single trial to one month of training. This would allow for a more complete characterisation of IEG expression patterns associated with navigation.

6.2.5. Long-term memory

With regard to more general long-term memory processing, our results contribute to the limited existing literature on pattern separation and completion in navigation, and the role of the hippocampus and medial prefrontal cortex in these phenomena (Bannerman et al., 2014; Jo et al., 2007). Pattern separation – the ability to differentiate between overlapping memories – is thought to rely mainly on the dentate gyrus, and activation of NMDA receptors therein (Bannerman et al., 2012; Bannerman et al., 2014; Gilbert et al., 2001; Marr, 1971; McHugh et al., 1996). Here, we demonstrated this effect in a spatial context with rats navigating via two visually identical cues, whereby animals were initially unable to distinguish between the cues (likely due to the overlap in spatial information provided by them) and exhibited elevated Zif268 expression in the dentate gyrus. Thus, our findings support existing theory that the dentate gyrus plays a fundamental role in separating overlapping inputs to create distinct memory traces (Leutgeb, Leutgeb, Moser, & Moser, 2007; Yassa & Reagh, 2013).

Pattern completion – defined as the reactivation of a stored representation when presented with degraded information – is mediated by NMDA receptor-dependent activity in area CA3 (Gold & Kesner, 2005; Jo et al., 2007; Marr, 1971; Nakazawa et al., 2002; Yassa & Reagh, 2013). Our findings showed that CA3 and

anterior cingulate regions were preferentially engaged in spatial memory retrieval (full and partial cue conditions); that is, expression of Zif268 was not higher in rats navigating with an incomplete cue arrangement than those using the full arrangement, in contrast to previous work. One possible explanation for our finding is the reduced number of cues utilised here (two cues) compared to earlier studies (four cues) (Gold & Kesner, 2005; Jo et al., 2007; Nakazawa et al., 2002). Specifically, because the degree of environmental change was smaller in our experiments, it is reasonable to assume that retrieval of the intact representation was easier for these animals, which in turn required less synaptic modification of recurrent connections in area CA3 (Marr, 1971; Nakazawa et al., 2002).

An alternate possibility is that animals had learned to rely completely on a single (far) cue by day ten, and thus, did not need to recall the overall cue arrangement to navigate, i.e. no pattern completion was required. One way to test this idea would be to carry out a probe trial with the other (near) cue only. More specifically, because this cue was less salient, pattern completion would be required for accurate navigation; thus, we would expect to see higher activation in CA3 for these animals. In terms of the anterior cingulate cortex, this result is in keeping with the suggestion by Jo *et al.* (2007) that the prefrontal cortex mediates complex memory retrieval by integrating information from other cortical areas with that of the hippocampus, which is facilitated by direct projections from the anterior cingulate cortex to areas CA1/CA3 (Rajasethupathy et al., 2015).

6.3. Concluding remarks

A final emergent conclusion from this thesis is that overall experience in an environment has a significant impact on navigational behaviour and its neural

underpinnings. Specifically, the amount of training will influence: how animals learn about cues in their environment (i.e. forming individual or group cue associations); the specific brain regions involved in representing and recalling spatial (and non-spatial) memories; and the neural mechanisms that mediate these processes (i.e. NMDA receptor synaptic transmission). Therefore, we propose that future research following on from this thesis should focus on a systematic investigation of environmental experience.

In sum, the experiments in this thesis have provided an in-depth analysis of allocentric navigation in the Morris water maze, and have investigated the relative contributions of hippocampal and medial prefrontal regions during the acquisition and recall of spatial and non-spatial memories. We have provided novel evidence that cue salience plays a crucial role in determining the type of type of strategy an animal will use to locate a goal, and that increased experience in the environment allows for greater flexibility of responding. These findings strongly support associative learning and vector model theories of learning, and refute cognitive mapping theory. We have shown that ionotropic glutamate receptors contribute differently to spatial learning, whereby NMDA receptor activation is necessary for successful encoding but AMPA receptors are not.

We have also demonstrated that Zif268 expression in the hippocampus (area CA1) is tightly coupled to learning-related plasticity, while c-Fos in the prefrontal cortex (anterior cingulate and infralimbic cortices) was associated with poorer performance and/or physiological stress. In addition, we have shown that NMDA receptor activation is also required for successful memory recall – but that extended training can partially protect against spatial memory deficits caused by NMDA receptor inhibition. Finally, we have revealed that elevated activity in the

hippocampus (CA3) and prefrontal cortex (prelimbic and anterior cingulate cortices) is strongly associated with flexible spatial memory retrieval.

Chapter 7

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