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A Multi-Region Analysis of the Acquisition, Consolidation and Retention of Spatial Memory in the Morris Water Maze using Immediate Early Gene Imaging

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Summary

The Morris water maze has been extensively used in the study of spatial learning and memory, and a number of hippocampal, parahippocampal and neocortical brain regions have been identified as necessary for successful performance in this task. Immediate Early Genes (IEGs) have been implicated in learning and memory processes, and are used as markers of neural activity in a brain region in response to learning tasks. The use of adequate control conditions in these tasks has been identified as important, and we devised a novel control condition in an attempt to address these concerns. We examined the expression of IEGs over the course of spatial learning, finding that Zif268 expression was upregulated in a number of regions during early learning, and that c-Fos was upregulated during late learning. We investigated the role of IEGs in cellular consolidation at different time-points in the hours following learning, but we did not find support for multiple waves of IEG expression as previously reported in the literature. We also examined the course of systems consolidation by analysing IEG expression during recent and remote memory probe trials. The hippocampus was equivalently or increasingly activated over the course of time reflecting its continued involvement, while widespread increases in cortical activity were observed at remote time-points, consistent with systems consolidation theories. In addition, we showed that IEG expression was associated with error correction during learning and with superior performance during retention.

Chapter 1

Literature Review

1.1 Theories of Memory

Our understanding of the nature and classification of memory has been largely influenced by the study of cases where such processes are impaired. Research governing anterograde and retrograde amnesia, the inability to form new memories or retrieve older ones respectively, has revealed much about the different types of memory and how they are formed. In anterograde amnesia, the ability to hold novel information in memory for several minutes is spared, however the capacity to retain such information for longer periods of time is impaired. In their landmark study of the amnesic patient H.M., Scoville and Milner (1957) demonstrated that despite normal intelligence and a lack of impairment in perception, reasoning or motivation, H.M. could not retain recent events in memory or have any recollection of talking to someone moments before. Some forms of learning are preserved in amnesia, such as the ability to learn new motor skills, in the absence of any conscious recollection of such learning. Amnesic patients can learn the skill of reading new words which have been presented as a mirror reflection at an equivalent rate to non-amnesic controls, yet show marked forgetting of the words they have been presented with (Cohen & Squire, 1980). Amnesic patients also exhibit priming effects, such as the ability to recall previously shown words when given appropriate cues, but display an impairment in recognising the words themselves (Levy, Stark, & Squire, 2004).

The inability to retain information for a prolonged period of time in amnesia led to a distinction between short- and long-term memory (Squire, 1986). Furthermore, the preservation of certain types of learning in amnesia led to a classification of multiple types of memory. The declarative theory of memory proposed by Squire (2004), divides memory into two broad categories. Declarative, or explicit memory, refers to the learning and recollection of facts and events, of which we are consciously aware. Non-declarative,

or implicit memory, is an umbrella term which refers to types of learning which can occur in the absence of conscious recollection, as can be observed in amnesia. These include procedural memory, priming, classical conditioning and non-associative learning (Squire, 1986). The theory further subdivides declarative memory into episodic and semantic memory. Episodic memory refers to the recollection of events from a specific place and time, whereas semantic memory concerns the retention of general information and facts. While the declarative theory views this distinction as essentially descriptive, other theorists suggest that the ability to consciously re-experience past life events, that is the "where" and "when" of episodic memory, is a distinct entity from the "what" of semantic memory (Tulving, 2002). Tulving, Schacter, McLachlan, and Moscovitch (1988) presented the case of K.C., a 36 year old amnesic who retained a normal level of general knowledge about the world, and indeed many objective facts about his upbringing, but was unable to recount a single personal experience from any period in his life. The discovery of this type of flat retrograde amnesia, the inability to recall memories from any time prior to brain injury, calls into question a central tenet of the declarative theory. The theory does implicate the medial temporal lobe, and the hippocampus in particular, in the acquisition of new memories, and this assertion is not under dispute. However it suggests a time-limited role for the hippocampus in the consolidation and retrieval of memory. New memories are thought to depend on a neural trace between the hippocampus and participating areas in the neocortex. Over time, the hippocampus facilitates the strengthening of neural connections between these cortical areas, until the memory can be reactivated independently of the hippocampus (Squire, 1986). However, to account for the evidence that medial temporal lobe lesions can result in the loss of autobiographical knowledge extending up to a lifetime, Nadel, Samsonovich, Ryan, and Moscovitch (2000) proposed an alternative model known as

multiple trace theory. In this approach, the hippocampal complex rapidly stores a neural trace which binds neocortical and hippocampal neurons together, however consolidation is not a long slow process which eventually becomes independent of the hippocampus. Rather, every retrieval of a memory adds a new trace, rendering the memory less sensitive to disruption. Therefore the quality of remote episodic memories will always depend on the integrity of the hippocampus. General facts and knowledge about people and the world can be extracted from these episodes to form semantic memories (Moscovitch et al., 2005). Other theorists have sought to clarify a distinction in declarative memory between recollection, remembering based on the memory of a particular episode, and familiarity, the sense of knowing without the recall of its original context (M. W. Brown & Aggleton, 2001). This dual process theory maintains that recollection of an event is dependent on the integrity of the hippocampus, whereas the perirhinal cortex is responsible for recognising familiar items.

In attempting to encompass and build on existing theories of declarative memory and hippocampal functioning, Eichenbaum, Otto, and Cohen (1992) proposed that declarative memory is fundamentally relational. The relational account of memory formation posits that the hippocampal network processes sensory and behavioural inputs, creating a memory "space" where relationships between elements can be formed, and subsequently enabling the storage of this new information in neocortical sites. Episodic memory is constructed through associative representations, in other words an experience is encoded in terms of the relationships between items, people, locations and actions (Eichenbaum, 2004). Furthermore, these representations are sequentially organised to create a timeline for that experience. Finally, this theory maintains that declarative memories are stored in a relational network, where common elements overlap. This allows for the generation of semantic memories from episodic memories, where the

matching features in these memories become independent of context. Furthermore, these common elements help to link memories together and lead to a flexible declarative memory system. Eichenbaum (2004) proposed that while the details of memories are distributed throughout the neocortex, the hippocampus is responsible for the rapid encoding of associations between stimuli, organising them in sequence, and linking these episodes into a relational framework.

In an attempt to resolve the role of the hippocampus in the processing of different types of information, Ryan, Lin, Ketcham, and Nadel (2010) asked participants to make relational judgements on various stimuli and monitored hippocampal activation using functional magnetic resonance imaging (fMRI). Hippocampal activity increased during all relational tasks, in accordance with relational theory, was activated more by tasks taxing episodic than semantic memory, in accordance with multiple trace theory, and showed increased activity in spatial versus non-spatial relations as would be predicted by cognitive mapping theory, thus finding partial support for these three theories.

1.2 Cellular Consolidation

A common strand running through these theories is the concept that memories are linked together through increases in the strength of neural traces throughout the brain, and that the medial temporal lobe is critical in the facilitation of these connections. The nature of this strengthening was proposed by Donald Hebb (1949), where if the axon of one neuron is close enough to excite a second neuron persistently, this will lead to a growth or change in one or both cells, ultimately increasing the first neurons efficacy at exciting the second. Evidence for this phenomenon of synaptic plasticity was first provided by Bliss and Lomo (1973), who demonstrated that high frequency stimulation of the hippocampus in anaesthetised rabbits resulted in increased excitability to subsequent stimulation, an

effect which would last for hours, which was later termed Long-Term Potentiation (LTP). Evidence for the role of LTP in learning and memory came with the discovery that the blockage of N-Methyl-D-Aspartate (NMDA) receptors, which inhibits hippocampal LTP in rats, also led to severe impairment in the Morris water maze task (R. G. Morris, Anderson, Lynch, & Baudry, 1986). Furthermore, Whitlock, Heynen, Shuler, and Bear (2006) demonstrated enhanced LTP in the hippocampus following inhibitory avoidance training in rats. A second form of long-term plasticity that has been observed is that of Long-Term Depression (LTD), a prolonged reduction in neuronal excitability which can be induced by low frequency stimulation (Bear & Abraham, 1996). LTD has been shown to be important for learning and memory, with spatial memory in particular facilitating this form of plasticity (Goh & Manahan-Vaughan, 2013; Kemp, Tischmeyer, & Manahan-Vaughan, 2013).

In the hours following synaptic activation, a cascade of events occurs, involving second messenger systems, activation of transcription factors and the synthesis of new proteins in order to facilitate more permanent structural changes to the neuron (Frankland & Bontempi, 2005). Any attempt to disrupt this process, for example the administration of protein synthesis inhibitors during this period, will block the formation of memory (H. P. Davis & Squire, 1984). The synaptic tagging hypothesis (Frey & Morris, 1997) divides LTP into early and late stages, where a short-term increase in synaptic plasticity can be maintained for a few hours in the absence of protein synthesis, however these plasticity related proteins are required to facilitate late LTP. This theory proposes that synapses undergo a structural change during this early stage which serves as a "tag", allowing plasticity related proteins to stabilise the changes in synaptic strength at a later stage (Redondo & Morris, 2011). The time-limited process of cellular consolidation is regarded

as distinct from the prolonged consolidation and re-organisation of memories over months and years on a systems level in the brain (Frankland & Bontempi, 2005).

1.3 Spatial Memory

One domain of memory, which is also dependent upon the integrity of structures in the medial temporal lobe, is spatial memory, the capacity to retain knowledge about the spatial configuration of our environment and use this information to navigate successfully. Although declarative memory theorists do not draw any major distinction between spatial memory and other types of declarative memory, an influential theory dealing predominantly with spatial memory formation is that of the cognitive map, put forward by O'Keefe and Nadel (1978). Based on research with laboratory animals, this theory states that the brain has a "locale" system, whereby stimuli in the environment and their location in relation to each other are rapidly encoded into a mental framework. The brain also encodes the animal's precise location in relation to its environment. Both these sources of information can then be used to help an animal navigate from one location to another in the environment, using its current location and direction, manipulating this mental map of cues in the environment, and performing geometric calculations to aid successful movement through space. The information required for this "locale" system is proposed to be stored permanently in the hippocampus, as opposed to "taxon" spatial navigation, which involves simply heading towards cues in the environment, a process thought to be independent of the hippocampus.

Cognitive map theory was inspired by the discovery of "place cells" in the hippocampus, cells which were found to be active either solely or maximally when a rat was in a particular location in a testing environment, forming what is known as "place fields" (O'Keefe & Dostrovsky, 1971). The firing of these place cells has been found to

adapt in response to cue rotation, enlarge in response to upscaling of the environment, and be abolished once the shape of the environment has dramatically changed, implicating their role in spatial processing (Muller & Kubie, 1987). Place fields form within minutes of an introduction into a novel environment (Wilson & McNaughton, 1993), require new protein synthesis to stabilise (Agnihotri, Hawkins, Kandel, & Kentros, 2004), and can remain stable for up to 153 days (Thompson & Best, 1990). Hartley, Burgess, Lever, Cacucci, and O'Keefe (2000) put forward a "boundary vector" model to account for the rapid formation of new place fields upon introduction into a novel environment, suggesting that boundary vector cells which respond selectively to the distance and direction away from boundaries in the environment, provide a convergent input to place cells and influence their firing. Neurons with similar spatially sensitive firing properties have been found in other brain regions connected to the hippocampus. In the postsubiculum, "head direction" cells have been discovered, which fire when a rat is facing a particular direction in an environment, regardless of its location or trunk position (Taube, Muller, & Ranck, 1990a), and this preferred firing direction can be rotated in correspondence with the manipulation of available cues (Taube, Muller, & Ranck, 1990b). In the medial entorhinal cortex, "grid cells" have been reported to fire at regularly spaced locations in an environment, forming a grid-like pattern, which remain stable irrespective of the animals change in speed or direction (E. I. Moser & Moser, 2008).

While these neurobiological findings are put forward as support for the encoding of space as a neural map, they are not definitive evidence that the animal actually uses a viewer-independent representation of space. Hetherington and Shapiro (1997) found that place fields were distributed in a heterogeneous manner, tending to form close to walls and specifically to walls with salient cues. Furthermore, Gothard, Skaggs, Moore, and

McNaughton (1996) discovered that the majority of hippocampal place fields sampled (55%) fired in relation to local landmarks which defined the start and goal of a task, remaining tied to these continuously moved proximal cues regardless of their relationship to distal cues. Shapiro, Tanila, and Eichenbaum (1997), when rotating and reconfiguring local and distal stimuli, found that place fields would respond heterogeneously, often to subsets of available cues and in some cases, in response to a single distal cue. These findings suggest that place fields are mostly associated with particular cues, are malleable and heterogeneous, calling into question the notion of a stable, cohesive map. Furthermore, the demonstration of successful, albeit slow, spatial memory acquisition and normal retention in the absence of an intact hippocampus (R. G. Morris, Schenk, Tweedie, & Jarrard, 1990) casts doubt on the hippocampus as the exclusive site of permanent spatial memory storage.

Competing theories of spatial memory view a global representation of space as unnecessary for place learning, and offer simpler explanations. Configural association theory, proposed by Sutherland and Rudy (1989), asserts that the hippocampus resolves ambiguity in the environment by associating the configuration of stimuli with a particular outcome. A straightforward example would be the association of two different elemental stimuli, auditory tones A1 and A2, with food rewards F1 and F2 in light conditions (L), and the subsequent reversal of these associations under dark conditions (D), such that the reward is now dependent upon the configuration between the auditory stimulus and the lighting conditions. This theory was extended to place learning, in that an animal need only associate the configuration of cues with a particular location. In the water maze, for example, once an animal has stored a configural representation of cues with various local views of its environment, it learns to associate these with particular sequences of movements. In other words, when an animal is placed in the maze from two different

points, it has learned to associate a separate trajectory of movement with both of them, based on the differing local views of available cues, both of which are rewarded by successful escape (Sutherland & Rudy, 1989). The theories assertion that configural associations are stored in the hippocampus has been challenged by the finding that animals can perform configural association discriminations without an intact hippocampus (Davidson, McKernan, & Jarrard, 1993), prompting a revision of the theory. Rudy and Sutherland (1995), reformulated their original hypothesis, proposing that the hippocampal formation contributes to configural processing by enhancing the activation or salience of representations stored elsewhere in the cortex.

Evidence suggestive of associative learning in spatial learning was provided by Rodrigo, Chamizo, McLaren, and Mackintosh (1997), who demonstrated the phenomenon of "blocking" in the water maze. Whereas cognitive map theory maintains that new landmarks are automatically incorporated into a spatial representation, Rodrigo et al. (1997) showed that when rats are trained initially with three cues, and a fourth is added later, their memory performance is not above chance when using just two of the original cues plus the new cue, as opposed to a group trained with all four cues from the outset. In other words, learning of the original cue configuration "blocked" the learning of the novel cue. Similarly, "overshadowing" is a prediction of associative learning theory, where if two cues are presented together, one can be treated as more important in constructing representations. This was also demonstrated in the water maze by Sanchez-Moreno, Rodrigo, and Chamizo (1999), who again trained rats with four cues, but presented one with an auditory tone in the overshadowing group. The overshadowing group showed normal memory performance when tested with the other three cues alone, but were impaired relative to controls when required to use the fourth cue in the absence of this tone. This suggested the auditory stimulus overshadowed the visual cue, calling

into question the cognitive map assertion that animals encode a global representation of their environment which assigns equal importance to all cues.

Relational memory theory adopts a more inclusive approach, rejecting the cognitive map theory in favour of a more general model of hippocampal functioning and memory formation. Eichenbaum, Dudchenko, Wood, Shapiro, and Tanila (1999) proposed that hippocampal neurons encode regularities which are present across many experiences, and these can be spatial cues, non-spatial cues and behaviours. A place cell in this context, is regarded as a link between behavioural episodes that have occurred in the same location, as defined by the configuration of stimuli in the environment which define that place. Non-place cells can equally encode meaningful non-spatial stimuli such as odours, and actions, at any location in the environment, under the same principle. What sets the relational theory apart from cognitive map theory is that memories are organised as connected sequences in time, rather than spatial relations between objects in an environment. Spatial navigation is treated as an outcome of repeated behavioural episodes across time in the same environment. Individual cells are regarded as capable of encoding configurations of small sets of cues, viewed from a particular location in the environment. As more episodes are experienced and the environment viewed repeatedly from multiple locations, these spatial regularities which overlap and span across episodes become a range of spatial associations which allow the rat to take novel routes. The hippocampus can also, via the same mechanism, separate out discrete behavioural episodes which differ in their context. Wood, Dudchenko, Robitsek, and Eichenbaum (2000) trained rats in an alternation task in the T-maze, where the animals would choose a left or right turn in sequence in order to obtain a reward. The majority of hippocampal cells responded differentially while the animals approached the turn of the maze, firing

only during a left or right turn, suggesting that these cells assist in the encoding of discrete contexts rather than simply a location.

While approaches to spatial memory differ in their theoretical perspectives, there are a number of commonalities which have become evident. The hippocampus plays a substantial role in the encoding of spatial features of the environment and spatial navigation. However the processing of these features, and long-term representations, are calculated and stored elsewhere in the cortex.

1.3.1 Spatial memory tasks.

A number of tasks have been devised for use with animals in a laboratory setting, which, in combination with various neurobiological techniques, can be used to elucidate the precise role of particular brain regions to aspects of spatial navigation and memory. The Y-maze is a simple test of spatial memory, involving a maze containing three arms (Conrad, Galea, Kuroda, & McEwen, 1996). Animals are placed in the "start" arm, allowed to explore one of the remaining two arms, and following an interval, are placed back in the original arm, are allowed to explore the novel arm, and will choose it based on the availability of extra-maze spatial cues. The radial arm maze, devised by Olton and Samuelson (1976) consists of eight arms leading out from a central point. The ends of the arms are baited, and animals are allowed to choose arms sequentially to gain a reward. The procedure can be used to assess spatial working memory, where all arms are baited and the animal must remember which arms were visited on that session by using available extra-maze cues, and also reference memory, where only certain arms are baited every session, and the animal must remember only to choose those spatial locations. The Morris water maze (R. G. Morris, 1981) was devised as a more spatially demanding task. Consisting of a circular pool of opaque water and a platform submerged just below the

surface of the water, the goal of the task is to locate this hidden platform based on its relationship with distal cues surrounding the maze. Rats normally accomplish this task over a number of days, receiving multiple training trials per day. Aside from being a sensitive test of spatial learning, memory retention can be assessed during a probe trial where the platform is removed and time spent searching in the correct area is analysed (Vorhees & Williams, 2006). The water maze can be adapted to encourage the use of egocentric strategies, that is the use of internally generated information, by training in the dark (Moghaddam & Bures, 1996), taxic strategies, where the animal solves the task using proximal cues close to the platform to escape the maze, or spatial working memory, where the escape platform is relocated every day of training (Paul, Magda, & Abel, 2009).

1.4 Brain Regions Involved in Spatial Memory

A number of brain regions have been implicated in the formation and persistence of spatial memory. The area which has received most attention is the hippocampal formation, located in the medial temporal lobe. The hippocampal formation refers to the adjoining regions of the hippocampus, dentate gyrus, entorhinal cortex, subiculum, presubiculum and parasubiculum (Amaral & Lavenex, 2007). The hippocampus generally refers to the hippocampus proper and the dentate gyrus.

1.4.1 Hippocampus.

The hippocampus proper refers to the cytoarchitecturally defined fields Cornus Ammonis 1 (CA1), Cornus Ammonis 2 (CA2) and Cornus Ammonis 3 (CA3) (Amaral & Lavenex, 2007). The principal type of cell in the hippocampus is the pyramidal cell. CA2/3 receive projections from layer II of the entorhinal cortex while CA1 receives input from layer III

of the entorhinal cortex. Within the hippocampus itself, CA3 displays substantial interconnectivity, while also projecting to CA1 via the Schaffer collateral pathway. The dentate gyrus has a characteristic "V" shape containing two blades, one of which is located between the CA3 and CA1 field, known as the suprapyramidal blade, with the opposing side known as the infrapyramidal blade (Amaral & Lavenex, 2007). The principal type of cell in the dentate gyrus is the granule cell. The dentate gyrus receives input from the entorhinal cortex via a projection known as the perforant path. The dentate gyrus projects to area CA3 of the hippocampus via the mossy fibres, axons which originate from granule cells.

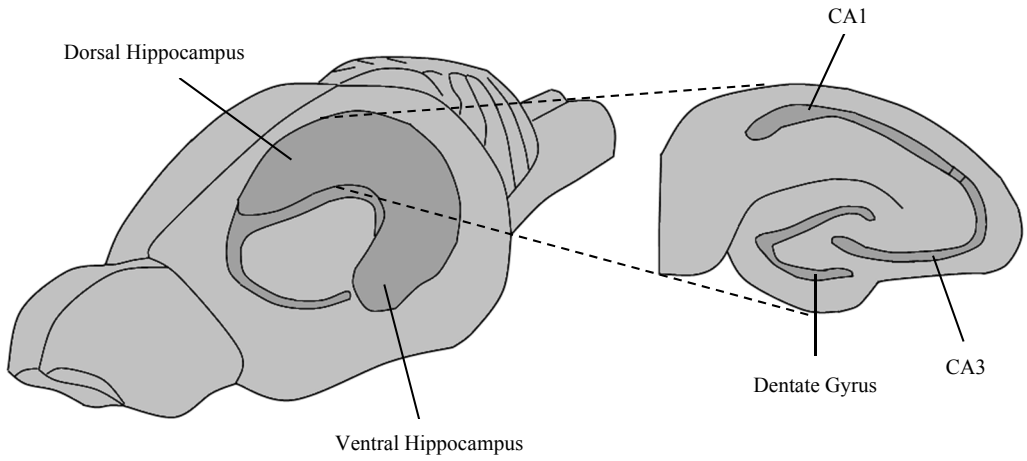

Figure 1.1: Diagram of the position of the hippocampus in the brain, alongside a magnification of a coronal slice of the dorsal hippocampus and its sub-regions. Adapted from Witter and Amaral (2004).

A plethora of human and animal research has identified the hippocampus as critical for successful spatial learning and memory. Maguire et al. (1998), when combining functional neuroimaging with navigation in a virtual environment, found increased navigational accuracy was associated with increased activity in the hippocampus. Using the same virtual reality environment with a patient with bilateral hippocampal damage,

Spiers, Burgess, Hartley, Vargha-Khadem, and O'Keefe (2001) demonstrated that navigation, scene recognition and map drawing were all severely impaired.

Since the discovery of "place cells" in the rat hippocampus, this region has been proposed as the neural basis of the representation of space (O'Keefe & Dostrovsky, 1971). When the rat hippocampus is lesioned (Moses, Cole, & Ryan, 2005; Wright et al., 2004), there is a resulting impairment in locating a hidden fixed platform in the water maze, yet performance is spared when a platform is visible (R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982), revealing this regions importance in building an allocentric spatial representation of the environment. This impairment can be observed across species, as a similar pattern has been observed in hippocampal-lesioned mice in the hidden and visible versions of the maze (Y. H. Cho, Friedman, & Silva, 1999). The usefulness of the Morris water maze in modelling spatial learning and memory and hippocampal functioning in humans has been demonstrated by Astur, Taylor, Mamelak, Philpott, and Sutherland (2002), who showed that humans with hippocampal damage were slower to find a hidden platform in a virtual Morris water maze and spent significantly less time searching in the correct quadrant during a probe trial.

The importance of the integrity of the hippocampus to spatial learning is underlined by the fact that lesions which encompass only 30-50% of the hippocampus are enough to impair spatial memory in the water maze (Broadbent, Squire, & Clark, 2004). Selective lesions of hippocampal subregions normally impair acquisition or retention of spatial tasks to some degree, but each subregion appears to play different roles in spatial information processing. Lesions of CA1 impair performance on the radial arm maze (Dillon, Qu, Marcus, & Dodart, 2008) and the water maze (Okada & Okaichi, 2009; Stubley-Weatherly, Harding, & Wright, 1996). Training in the Morris water maze also significantly increases spine density in area CA1, reflecting an increased number of

excitatory synapses following learning (M. B. Moser, Trommald, & Andersen, 1994). While lesions to CA1, CA3 and the dentate gyrus all affect sensitivity to metric changes in the environment, those based on distances, only CA1 lesions produce an alteration in the processing of topographical information, that is the arrangement of cues (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008).

Studies have found an impairment in the acquisition of the water maze when CA3 is lesioned (Stubley-Weatherly et al., 1996; Sutherland, Whishaw, & Kolb, 1983), as well as impaired retention (Brun et al., 2002; Steffenach, Sloviter, Moser, & Moser, 2002). Florian and Roullet (2004), in a thorough inactivation study, showed that when CA3 is inactivated prior to training in the Morris water maze, acquisition is disrupted, inactivation just after training affects consolidation and subsequent retention 24 hours later, but inactivation just before a probe trial has no effect on recall, suggesting this region is essential for the acquisition and consolidation of the task. The extensive interconnections of this hippocampal subregion have been proposed to create an autoassociative network which participates in pattern completion, the recreation of a representation based on partial information. Fellini, Florian, Courtey, and Roullet (2009) found that by blocking NMDA receptors in CA3, mice were impaired during acquisition and long-term retention of the water maze under partial, but not full cue conditions.

Selective lesions of the dentate gyrus impair acquisition and retention of the water maze (Jeltsch, Bertrand, Lazarus, & Cassel, 2001; Sutherland et al., 1983; Xavier, Oliveira-Filho, & Santos, 1999), and to a greater extent than CA1 or CA3 lesions (Okada & Okaichi, 2009). Performance in the radial arm maze is also affected (Jeltsch et al., 2001). The dentate gyrus appears to play a role in pattern separation, the ability to differentiate between similar cues, as lesions to this area disrupt performance in the radial

arm maze when goal arms are closer together (A. M. Morris, Churchwell, Kesner, & Gilbert, 2012).

The dorsal rather than the ventral hippocampus appears to be involved in spatial learning, as dorsal lesions disrupt performance on the radial arm maze, whereas ventral hippocampus-lesioned rats perform similarly to controls (Pothuizen, Zhang, Jongen-Relo, Feldon, & Yee, 2004; Potvin, Allen, Thibaudeau, Dore, & Goulet, 2006). Rats with ventral hippocampal lesions are also unimpaired on acquisition of the Morris water maze (W. N. Zhang, Pothuizen, Feldon, & Rawlins, 2004).

Some spatial navigation abilities are spared by hippocampal lesions, such as the ability to find a platform based on a fixed distance and direction from a landmark in the water maze, supporting the idea that the hippocampus builds a representation of space, while other regions are involved in navigation using vectors (Pearce, Roberts, & Good, 1998). Accordingly, path integration, the ability to find a novel route back to a starting point based on distance travelled and direction, is spared in rats with hippocampal lesions (Alyan & McNaughton, 1999). The hippocampus is involved in spatial working memory, although non-spatial working memory is spared in rats with hippocampal lesions (Aggleton, Hunt, & Rawlins, 1986).

1.4.2 Entorhinal cortex.

The entorhinal cortex is the main source of sensory information to the hippocampus, as well as being the main output back to the neocortex (Amaral & Lavenex, 2007). The entorhinal cortex can be divided into the lateral entorhinal area and the medial entorhinal area. It receives input from the temporal and frontal regions of the brain, as well as the parietal and retrosplenial cortices (Burwell & Amaral, 1998). It also projects to the prefrontal, perirhinal and retrosplenial cortices (Agster & Burwell, 2009).

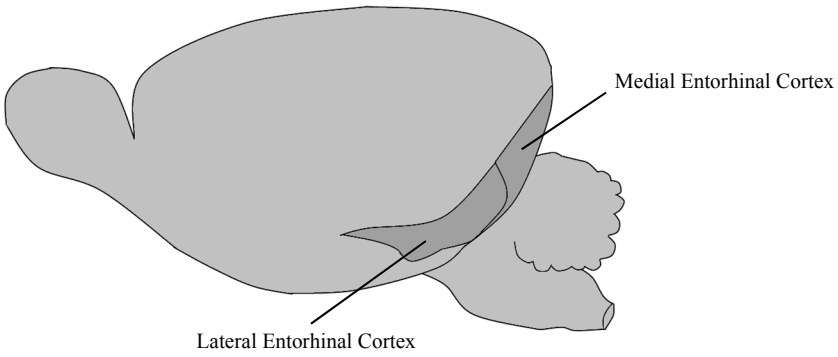

Figure 1.2: Lateral surface view of the brain showing the location of the lateral and medial entorhinal cortex. Adapted from Burwell and Amaral (1998).

The discovery of "grid cells" in the entorhinal cortex, regularly spaced place fields which predict a rats location in an environment as accurately as cells in the hippocampus, has presented a strong case for the neural encoding of space in this region (Fyhn, Molden, Witter, Moser, & Moser, 2004). The entorhinal cortex appears to be involved in the processing of spatial information, from arrangements of objects to complex spatial tasks. Parron and Save (2004) found that entorhinal cortex lesions result in a deficit in a reaction to a spatial change in the configuration of objects. Bilateral entorhinal cortex lesions also impair acquisition of a simple spatial alternation task in the Y-maze even after 12 weeks of training (Ramirez et al., 2007). In the Morris water maze, a more challenging task of spatial navigation and learning, bilateral lesions of the entorhinal cortex result in spatial learning impairments up to 70 days post-surgery (Hardman et al., 1997). The entorhinal cortex appears to be involved in the spatial aspect of the Morris water maze as lesions to this area impair the use of distal cues, but spare the use of proximal landmarks in this task (Parron, Poucet, & Save, 2004). The research is not unequivocal however, as Galani, Weiss, Cassel, and Kelche (1998) found that lesions of the entorhinal cortex impaired spatial working memory in the Morris water maze, but spared spatial reference memory.

1.4.3 Perirhinal cortex.

The perirhinal cortex, running along the rhinal sulcus, comprises of Brodmann's areas 35 and 36 and forms part of the parahippocampal region. The perirhinal cortex receives inputs from a range of sensory cortices. It inputs to and receives information from the entorhinal cortex and also connects directly and indirectly to the hippocampus and plays an important role in memory (Aggleton, Kyd, & Bilkey, 2004). It also projects to the anterior cingulate, prelimbic, infralimbic and parietal cortices (Agster & Burwell, 2009).

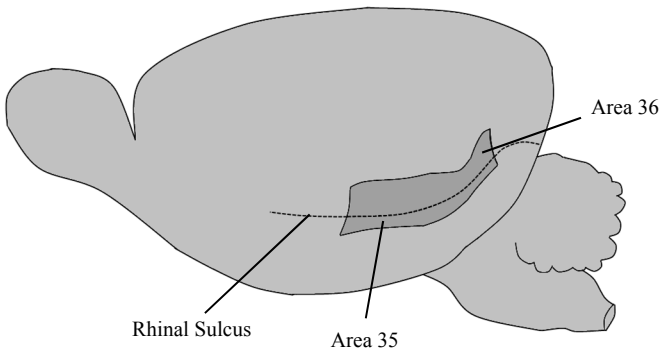

Figure 1.3: Lateral surface view of the brain showing the location of areas 35 and 36 of the perirhinal cortex. Adapted from Burwell and Amaral (1998).

However the role of the perirhinal cortex in spatial memory is controversial, with a considerable amount of research both supporting and refuting its involvement. Liu and Bilkey (1998) found perirhinal cortex lesions impaired both working and reference memory in the radial arm maze task, as well as a delay-dependent effect. However, Machin, Vann, Muir, and Aggleton (2002) failed to find any impairment in the radial arm maze following perirhinal cortex lesions even with a retention delay of 30 minutes. Similar inconsistencies have been found when assessing the effects of perirhinal cortex

lesions in the water maze. Moses et al. (2005) found rats with perirhinal cortex lesions unimpaired in all measures on the Morris water maze task. Furthermore, Futter, Davies, Bilkey, and Aggleton (2006) did not find any effect of perirhinal lesions on water maze performance, even when using two different rat strains. However, Wiig and Bilkey (1994) did find mild deficits in the Morris water maze with perirhinal cortex lesions, with rats taking longer to acquire the maze during earlier stages, swimming longer circuitous routes, showing consistent heading angle errors and less platform crossings during retention. Following a review of this conflicting literature, Aggleton et al. (2004) concluded that the effects of perirhinal cortex lesions on spatial memory are at best mild and transient, compensated for by an intact hippocampus, and may result from an inability to differentiate between available cues when their features overlap in some way.

1.4.4 Retrosplenial cortex.

The retrosplenial cortex can be subdivided into the granular (area 29) and dysgranular (area 30) subregions, with the granular area further divided into subarea granular a (Rga) and subarea granular b (Rgb). The retrosplenial cortex is densely interconnected with the hippocampal formation therefore implicating this region in spatial navigation and learning (Wyss & Van Groen, 1992). It is also connected to the prefrontal cortex (Hoover & Vertes, 2007; van Groen & Wyss, 2003) and the parietal cortex (Reep, Chandler, King, & Corwin, 1994).

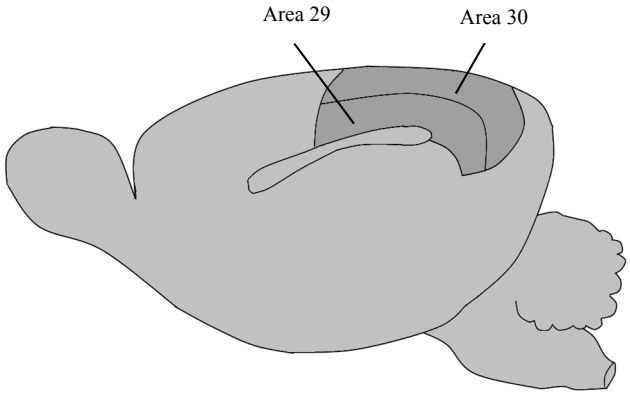

Figure 1.4: Medial surface view of the brain showing the location of areas 29 and 30 of the retrosplenial cortex. Adapted from Vann, Aggleton, and Maguire (2009).

Damage to this area in humans results in deficits in route learning and navigation (Maguire, 2001), and evidence from neuroimaging studies suggests that it increases in activity along with the hippocampus while learning about the spatial features of a virtual reality environment (Iaria, Chen, Guariglia, Ptito, & Petrides, 2007)

Insights from animal studies have implicated the retrosplenial cortex in the processing of spatial information. Parron et al. (2004) found that rats with retrosplenial cortex lesions display a deficit in reaction to a change in the spatial configuration of objects. It also appears to play a role in spatial navigation, as approximately 10% of cells in the retrosplenial cortex are head-direction cells, which fire when the animal is facing a particular direction (J. Cho & Sharp, 2001). Lesions of the retrosplenial cortex result in impairments in tasks which tax allocentric learning, such as the Morris water maze and the radial arm maze but spare performance on an egocentric task such as the cross maze, which simply requires rats to turn left or right in sequence (Vann & Aggleton, 2002). The disruption in performance of retrosplenial-lesioned rats following rotation of the radial arm maze mid-training suggests an over-reliance on idiothetic information and a deficit in the use of distal cues (Pothuizen, Aggleton, & Vann, 2008). Within the retrosplenial

cortex itself, granular lesions of the granular area b are more effective in disrupting spatial learning and memory in the water maze than granular area a (van Groen, Kadish, & Wyss, 2004). Lukoyanov, Lukoyanova, Andrade, and Paula-Barbosa (2005) also found that retrosplenial cortex lesions resulted in impaired learning of the Morris water maze, but this deficit could be somewhat retrieved by pretraining which familiarised rats with task demands, and performance towards the end of actual spatial training recovered to control levels. Therefore despite the obvious importance of its contribution to spatial learning and navigation, the retrosplenial cortex is not always essential to solving spatial tasks and other strategies and brain regions can be utilised instead (Aggleton, 2010).

1.4.5 Parietal cortex.

The parietal cortex receives input from the prefrontal cortex, the visual, auditory and somatosensory cortex, the perirhinal cortex, and the cerebellum and thalamus (Agster & Burwell, 2009; Save & Poucet, 2009). It projects to the hippocampus through the entorhinal cortex (Burwell & Amaral, 1998), implying its role in spatial information processing. It also projects to the anterior cingulate cortex (Kolb & Walkey, 1987) and the retrosplenial cortex (Reep et al., 1994).

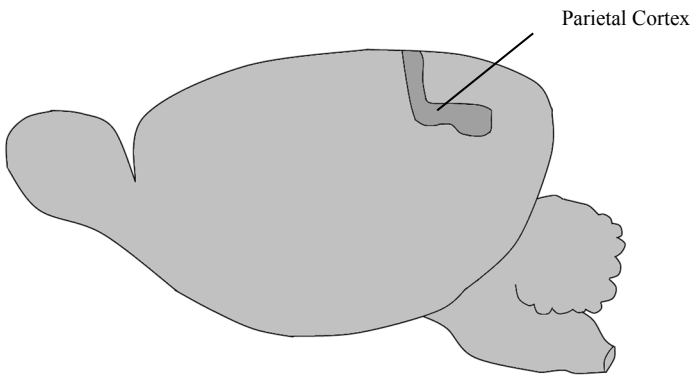

Figure 1.5: Medial surface view of the brain showing the location of the parietal cortex. Adapted from Burwell and Amaral (1998).

The parietal cortex appears to be involved in spatial information processing, in particular encoding the relationship of cues to each-other, as Goodrich-Hunsaker, Hunsaker, and Kesner (2005) showed a change in the topographical layout of objects went unexplored by parietal cortex-lesioned rats relative to controls. It also appears to contribute to water maze learning, as Kolb, Buhrmann, McDonald, and Sutherland (1994) found that rats with parietal cortex lesions were consistently slower to acquire the water maze than controls. In the cheese board task, an allocentric spatial task similar to the water maze, lesions of the parietal cortex also produce an impairment (Kesner, Farnsworth, & DiMattia, 1989). However, Save and Poucet (2000) found that rats with parietal lesions were unimpaired in a reference memory task requiring the use of distal cues, and were only impaired in a proximal cue task, where the platform was marked by a salient beacon, therefore it is unclear which aspects of the task the parietal cortex is engaged in. The parietal cortex does appear to contribute to egocentric strategies in the water maze, as bilateral lesions of this region, combined with disorientation, impair a rats ability to locate a platform in the dark, where no distal cues are available (Commins, Gemmell, Anderson, Gigg, & O'Mara, 1999). Impairments in path integration processes are also observed in rats with parietal cortex lesions (Save, Guazzelli, & Poucet, 2001).

1.4.6 Medial prefrontal cortex.

Located in the frontal lobes, the medial prefrontal cortex consists of the anterior cingulate, prelimbic and infralimbic cortices. The medial prefrontal cortex receives input from the lateral entorhinal, perirhinal, parietal and retrosplenial cortices (Agster & Burwell, 2009; B. F. Jones, Groenewegen, & Witter, 2005; Kolb & Walkey, 1987) as well as CA1 (Hoover & Vertes, 2007). The prelimbic and infralimbic cortices project to the lateral entorhinal cortex (Burwell & Amaral, 1998) and the anterior cingulate cortex

projects to the retrosplenial (B. F. Jones et al., 2005) and parietal cortices (Kolb & Walkey, 1987).

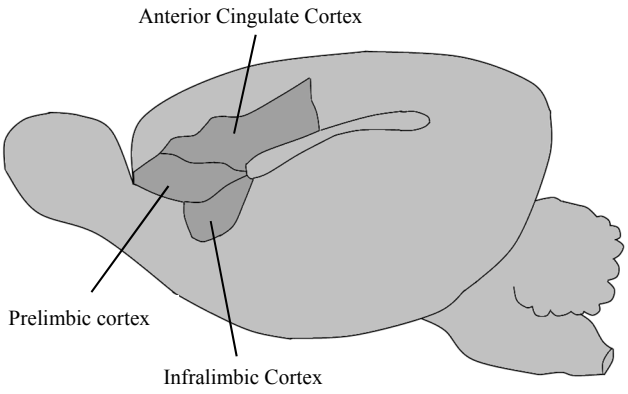

Figure 1.6: Medial surface view of the brain showing the location of the anterior cingulate cortex, the prelimbic cortex, and the infralimbic cortex. Adapted from Burwell and Amaral (1998).

The medial prefrontal cortex appears to encode a neural representation of space, similar to the hippocampus and entorhinal cortex. Hok, Save, Lenck-Santini, and Poucet (2005) found that place cells in the medial prefrontal cortex of the rat tended to fire in locations which were associated with the delivery of a food reward, rather than the location of the food itself, indicating they were encoding the motivational salience of locations. Accordingly, a number of studies have found that that medial prefrontal lesions produced an acquisition impairment in the Morris water maze (Kolb et al., 1994; Kolb, Sutherland, & Whishaw, 1983; Mogensen et al., 2004; Sutherland, Kolb, & Whishaw, 1982), as well as a retention deficit relative to sham controls (R. W. Brown, Gonzalez, & Kolb, 2000). In contrast, Ethier, Le Marec, Rompre, and Godbout (2001) found that rats with medial prefrontal cortex lesions performed similarly to controls in an allocentric water maze task consisting of a hidden fixed platform, but were markedly impaired in an egocentric task where they had to simply swim in a straight line to a variably placed platform. Similarly,

de Bruin, Sanchez-Santed, Heinsbroek, Donker, and Postmes (1994) found that rats with medial prefrontal cortex lesions learned and remembered the Morris water maze as well as controls, and only displayed a deficit when faced with spatial reversal training, where the platform was placed on the opposite side of the pool, or a visual platform to swim towards. While de Bruin et al. (1994) attribute the discrepancies between studies to a failure to place the animal on the platform after each trial to reinforce learning, R. W. Brown et al. (2000) followed this type of training protocol and still found an impairment in acquisition and retention. An acquisition and retrieval deficit following medial prefrontal cortex lesions has also been observed in the Hebbs-Williams maze, where rats have to calculate a route to an end goal through a maze for a food reward (Churchwell, Morris, Musso, & Kesner, 2010). Therefore the role of the medial prefrontal cortex in allocentric spatial learning is unclear.

A more consistent finding is that the medial prefrontal cortex is involved in behavioural flexibility or a change in strategy while completing spatial tasks. Compton, Griffith, McDaniel, Foster, and Davis (1997) demonstrated that both hippocampal and medial prefrontal cortex lesions impaired performance in the water maze relative to controls when the starting position was rotated. This effect was also demonstrated by Lacroix, White, and Feldon (2002), who found little impairment in the acquisition of the water maze following medial prefrontal cortex lesions, but slower performance when the platform location was reversed. This impaired flexibility was explored by Granon and Poucet (1995), who showed that rats with medial prefrontal lesions could learn two platform locations, and from two starting positions, but were severely impaired when the starting positions were increased to four. Jo et al. (2007) found that after rats were trained in full cue conditions, medial prefrontal cortex lesions disrupted memory retrieval tested in partial cue conditions, which would require a shift in strategy. The medial prefrontal

cortex also appears to be involved in remembering the temporal order of events in a spatial task such as the radial arm maze, rather than the spatial information itself (Hannesson, Vacca, Howland, & Phillips, 2004).

1.4.7 Connectivity between brain regions.

Supporting the idea that spatial learning is reliant on a network of brain regions working together to perform successfully on a task, the regions studied in this thesis display substantial interconnectivity. The efferent and afferent connections of all aforementioned brain regions are summarised in Figure 1.7.

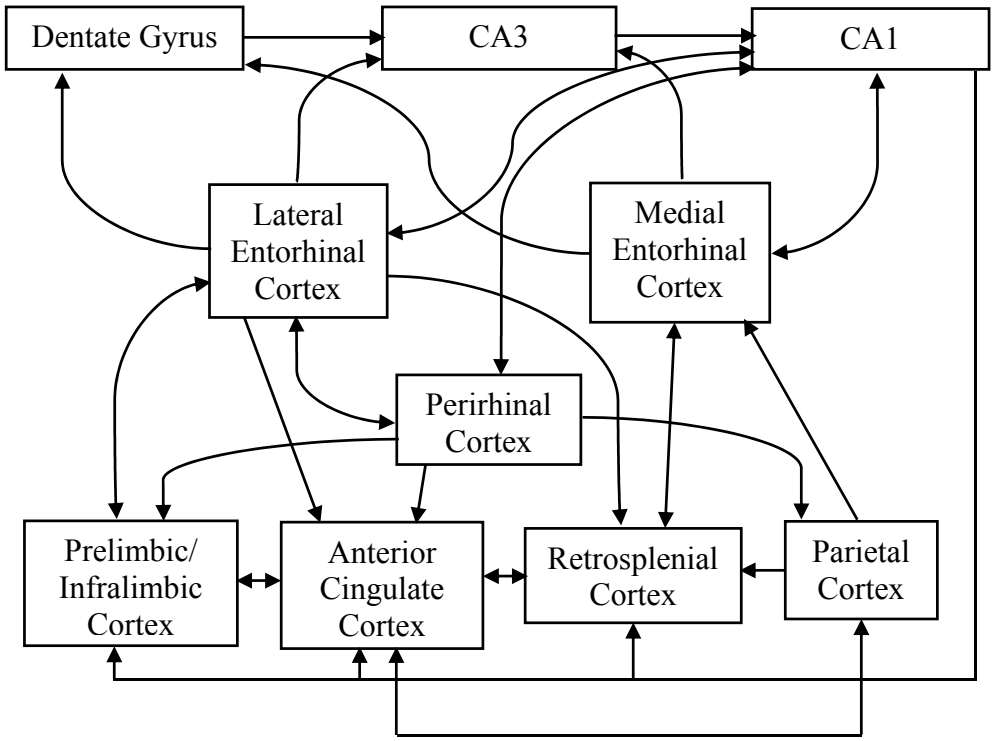

Figure 1.7: Interconnectivity of the brain regions studied in this thesis, summarised from Agster and Burwell (2009); Burwell (2000); Burwell and Amaral (1998); Hoover and Vertes (2007); B. F. Jones et al. (2005); Kealy & Commins, 2010; Kolb and Walkey (1987); Nelson, Sarter, and Bruno (2005); Witter et al. (2000).

1.5 Immediate Early Gene Imaging

While the aforementioned studies which have employed neuroscientific techniques such as lesioning and electrophysiological recording have yielded much information on the contribution of various regions to learning and spatial navigation, they are not without their drawbacks. Selective lesions of a brain region do not guarantee that nearby areas are functioning normally, as neural activity can be disrupted due to damage to input pathways, making it difficult to interpret observed impairments (R. G. Morris, 2007). Furthermore, electrophysiological studies have excellent temporal resolution but lack spatial resolution and fail to capture the activity of an entire region. Advances in IEG imaging have sought to address these issues. The analysis of IEG expression allows for the visualisation of complete patterns of neuronal activity during learning, while preserving neural circuits and neural functioning (Miyashita, Kubik, Lewandowski, & Guzowski, 2008).

The expression of IEG mRNA and proteins is low or undetectable in quiescent cells but is rapidly induced in response to trans-synaptic signalling between neurons (Sheng & Greenberg, 1990). The function of this expression is to facilitate long-term structural and functional changes to a neuron by encoding transcription factors, growth factors, metabolic enzymes, cytoskeletal proteins and proteins involved in signal transduction (Lanahan & Worley, 1998). There are two main categories of IEGs: regulatory transcription factor (RTF) IEGs regulate the expression of downstream genes, and effector IEGs directly influence cell functions. There are thought to be 30-40 IEGs involved in neuronal response to stimulation, of which 10-15 are classified as transcription factors (Lanahan & Worley, 1998). Once the protein products of RTF IEGs are translated in the cytoplasm, they enter the nucleus where they regulate the transcription of late-response target genes, which in turn directly affect cell structure and

function (Tischmeyer & Grimm, 1999). There are a small number of IEGs which have been well-characterised. Among these are Zif268, c-Fos and Arc.

1.5.1 Zif268.

Zif268 (also known as Egr-1, NGFI-A, Krox-24 and ZENK) is an RTF IEG. It encodes a zinc finger protein, and its expression is initiated in response to all subtypes of glutamatergic, adrenergic and dopaminergic receptors (S. Davis, Bozon, & Laroche, 2003). *Zif268* mRNA has been shown to increase in a linear fashion with a more prolonged LTP induction protocol underlining its close association with neuronal stimulation (Abraham et al., 1993). Furthermore, this study found *Zif268* mRNA to show a cumulative effect of expression over days, an effect which was not found for c-Fos or c-Jun. Basal expression of Zif268 is highest in layers II and IV of the cerebral cortex, and in CA1-3 in the hippocampus (Beckmann & Wilce, 1997). Zif268 has been implicated in learning and memory as its expression is tightly linked with NMDA receptors, a subtype of glutamate receptor which has been extensively studied for its role in synaptic plasticity. Administration of the NMDA receptor antagonist MK801 almost abolishes Zif268 expression in the neocortex (Gass, Herdegen, Bravo, & Kiessling, 1993). The excitatory amino acid glutamate appears to increase Zif268 expression through binding to both NMDA and α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptors (Vaccarino, Hayward, Nestler, Duman, & Tallman, 1992). Furthermore, showing a link between Zif268, LTP and NMDA receptor activation, Cole, Saffen, Baraban, and Worley (1989) demonstrated that the stimulus frequency and intensity required to increase Zif268 expression is similar to that required to induce LTP, and that both can be blocked by NMDA receptor antagonists.

The MAP kinase pathway appears to be particularly important for the transcription of Zif268. Within minutes of the induction of LTP, the MAP kinases ERK1 and ERK2 and two downstream transcription factors, Elk-1 and CREB are hyperphosphorylated, and begin transcription of Zif268 (S. Davis et al., 2003). Zif268 expression appears to be more important for late LTP than early LTP. Zif268 KO mice display normal induction of LTP but it decays back to baseline within 24 hours, indicating a role for this IEG in the stabilisation of memory (M. W. Jones et al., 2001). Two downstream target genes for Zif268 which have been identified are synapsin I and II, which are thought to link synaptic vesicles to the cytoskeleton, thereby playing a role in the exocytosis of synaptic vesicles and the control of neurotransmitter release (Petersohn, Schoch, Brinkmann, & Thiel, 1995; Thiel, Schoch, & Petersohn, 1994). Arc has also been shown to be a direct transcriptional target of Zif268 therefore it can indirectly modulate synaptic plasticity by regulating expression of this effector IEG (Li, Carter, Gao, Whitehead, & Tourtellotte, 2005).

1.5.2 c-Fos.

c-Fos is an RTF IEG which interacts with the product of c-Jun to form a heterodimeric transcription factor complex, binding with variable affinity to either Activator Protein 1 (AP1) consensus sites on DNA, or other transcription factor families (S. Davis et al., 2003). c-Fos is expressed at very low constitutive levels in the brain (Dragunow, Currie, Faull, Robertson, & Jansen, 1989), with particularly low levels in the rat hippocampus (Hughes, Lawlor, & Dragunow, 1992), although the cortex does show higher levels during the dark phase of the circadian rhythm, when the animal is more active (Grassi-Zucconi et al., 1993). This low basal expression makes c-Fos an ideal candidate for

studies involving stimulation, where there is an expected upregulation in the area of interest (Kaczmarek & Chaudhuri, 1997).

Similar to Zif268, c-Fos expression can be induced via the activation of NMDA and AMPA receptors (Vaccarino et al., 1992). c-Fos expression may also be induced following the opening of voltage sensitive calcium channels (Kaczmarek & Chaudhuri, 1997). Protocols which induce long-lasting LTP in the hippocampus also lead to increases in c-Fos expression in this region (Nikolaev, Tischmeyer, Krug, Matthies, & Kaczmarek, 1991), although this expression correlates poorly with the durability of LTP (Jeffery, Abraham, Dragunow, & Mason, 1990). Furthermore, electrophysiological stimulation of hippocampal slices from c-Fos KO mice reveal a deficit in the induction of LTP (Fleischmann et al., 2003). The late-response genes which c-Fos targets are yet to be fully identified, as although many genes have AP1 binding sites, only a few are targeted by c-Fos (Okuno, 2011). One of these target genes is brain-derived neurotrophic factor (BDNF) (J. Zhang et al., 2002), which has been strongly implicated in learning and memory.

The fact that c-Fos is not expressed under the conditions of normal neural activity, but rather is only induced by the changes in afferent inputs to a region, or by external stimuli make it a useful marker of activity in response to environmental changes (Kovacs, 2008). However, because its induction threshold appears to be higher than other IEGs, it tends to be used as a marker of neuronal activity in behavioural paradigms which have high cognitive demands or emotional burden (Okuno, 2011).

1.5.3 Arc.

Activity-regulated cytoskeletal-associated protein (Arc) is an effector IEG. It displays unique properties, in that its mRNA rapidly travels to dendrites and accumulates near

synapses that have been stimulated (Steward, Wallace, Lyford, & Worley, 1998), a process which requires activation of NMDA receptors (Steward & Worley, 2001). Here it is thought to facilitate the endocytosis of AMPA receptors resulting in depression of synaptic responses (Chowdhury et al., 2006; Rial Verde, Lee-Osbourne, Worley, Malinow, & Cline, 2006; Shepherd et al., 2006). Arc has also been shown to increase the size of dendritic spines and regulate their morphology (Peebles et al., 2010). The survival of newly born neurons in the dentate gyrus appears to be closely linked to whether or not they express Arc (Kuipers et al., 2009). The protocol used to induce LTP in the hippocampus is identical to that which induces Arc expression (Lyford et al., 1995), and its expression appears to be activated via signalling pathways that regulate LTP.

Arc appears to play a role in both early and late LTP. The application of Arc antisense oligodeoxynucleotides (AS-ODNs) which block the translation of mRNA into protein before neuronal stimulation, inhibit the induction of LTP, whereas the infusion of Arc AS-ODNs two hours after stimulation results in a rapid and permanent reversal of LTP (Messaoudi et al., 2007). Furthermore, although Arc KO mice display enhanced LTP initially, the late phase of LTP is absent, and LTD is also significantly impaired (Plath et al., 2006). The finding that AMPA receptor inhibition strongly increases Arc expression not only implicate this receptor in the regulation of Arc, but challenge the view that AMPA receptors are only involved in short-term plasticity (Rao et al., 2006). Group 1 metabotropic glutamate receptors (mGluRs) have been shown to trigger endocytosis of AMPA receptors via Arc transcription, which has been proposed as the likely mechanism by which Arc facilitates LTD (Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008). Arc also seems to play a role in homeostatic plasticity, where a neuron increases or decreases its activity to balance out extremes in activity induced by LTP and

LTD, which would lead to network instability, but how this is accomplished is not clear (Turrigiano, 2008).

1.5.4 Regulation of IEG expression.

The transcription of Zif268, c-Fos and Arc are regulated by multiple intracellular signalling pathways which interact with each other. The stimulation of NMDA, TrkB and group 1 metabotropic glutamate receptors (mGluRs), as well as the opening of voltage dependent calcium channels (VDCCs), promote Zif268, c-Fos and Arc transcription through the map kinase (MAPK) pathway, involving several downstream signalling kinases, including extracellular-signal-regulated kinase (ERK), which acts through a ternary complex factor ELK1 to activate serum response factor (SRF) and binds to a serum response element (SRE) to begin IEG transcription. The MAP kinase pathway also leads to the phosphorylation of CREB (Cahill, Janknecht, & Nordheim, 1996; Coulombe & Meloche, 2007; Hinoi, Balcar, Kuramoto, Nakamichi, & Yoneda, 2002; Knapska & Kaczmarek, 2004; Korb & Finkbeiner, 2011). Stimulation of NMDA receptors or opening of voltage gated calcium channels also leads to an influx of calcium, subsequent activation of calcium kinases and direct phosphorylation of CREB. mGlu receptors can also increase intracellular calcium and contribute to activation of calcium kinases via the IP3 pathway, and also result in the phosphorylation of PKC through the DAG pathway, which in turn phosphorylates RAS (S. Davis et al., 2003). Stimulation of NMDA receptors also initiates the cAMP/PKA pathway through calcium-calmodulin-dependent adenylyl cyclases (ACs) (Bloomer, VanDongen, & VanDongen, 2008). Both CREB and the Elk1/SRF complex begin transcription of c-Fos, Arc and Zif268 through binding to CRE and SRE sites on DNA (see Figure 1.8). Stimulation of AMPA receptors by glutamate appears to inhibit Arc production (Rao et al., 2006).

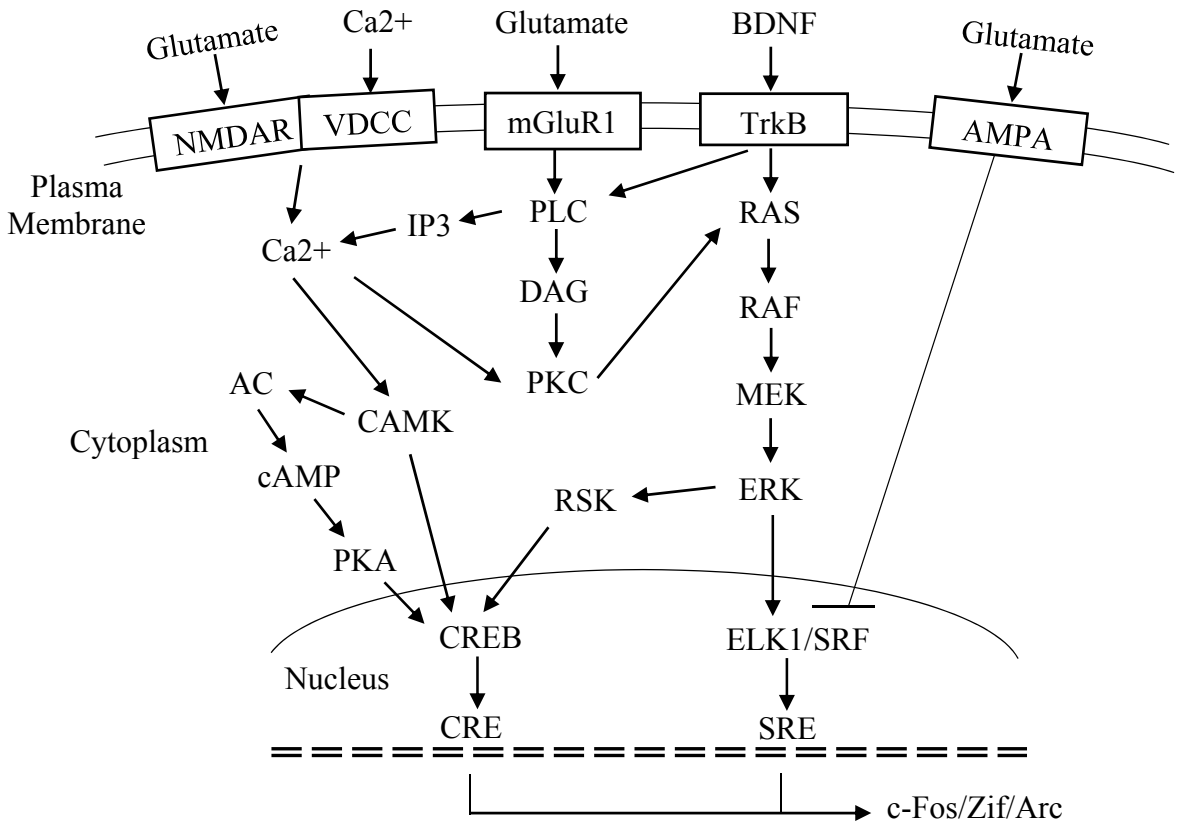

Figure 1.8: Schematic diagram showing the various intracellular signalling pathways by which stimulation of NMDA, TrkB AMPA, group 1 metabotropic glutamate receptors and voltage dependent calcium channels regulate the transcription of c-Fos, Zif268 and Arc. Abbreviations: AC, Adenylyl cyclase; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor; Ca2+, calcium; CAMK, calcium/calmodulin-dependent kinase; cAMP, cyclic adenosine monophosphate; CRE, cAMP responsive element; CREB, cAMP response element-binding protein; DAG, diacylglycerol; ELK1, Ets-like transcription factor; ERK, Extracellular Signal-Regulated Kinase; IP3, Inositol trisphosphate; MEK, MAPK/Erk kinase; mGLuR1, metabotropic glutamate receptor type 1; NMDAR, N-Methyl-D-Aspartate receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RSK, ribosomal protein S6 kinase; SRE, serum response element; SRF, serum response factor; TrkB, tropomyosin receptor kinase B.

1.6 IEGs in Learning and Memory

The characteristics of IEG expression and the conditions under which they are expressed make this method a useful tool for studying learning and memory. The close association between IEGs and LTP (Abraham et al., 1993; Nikolaev et al., 1991), combined with their transient expression in response to external stimulation and the ease at which their

mRNA and protein can be detected, makes them an ideal candidate for studying the activation of particular brain regions in response to a learning task (Kovacs, 2008).

IEG expression has been studied in a number of learning paradigms across a range of species. One such simple learning task is contextual fear conditioning, which involves reintroducing animals into an environment where they have previous received an aversive event, such as a foot shock. Re-exposure to the aversive environment results in an increase in *Zif268* (Malkani & Rosen, 2000; Rosen, Fanselow, Young, Sitcoske, & Maren, 1998) and *c-Fos* (Campeau et al., 1991) mRNA in the amygdala, while increases in *Arc* mRNA have also been found in the hippocampus (Pevzner, Miyashita, Schiffman, & Guzowski, 2012). Huff et al. (2006) demonstrated that observed increases in *Arc* and *c-Fos* mRNA in the hippocampus are due to the exposure of the context rather than the shock itself. While hippocampal IEG expression is highest during recent retention of this task (Hall, Thomas, & Everitt, 2001), Zif268 and c-Fos protein in the medial prefrontal cortex show increased expression during remote retention (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004).

Operant or instrumental conditioning tasks in animals normally involve pressing a lever in order to obtain a food reward. Kelly and Deadwyler (2002) trained rats in an operant conditioning task, and found increased *Arc* mRNA expression in the hippocampus, subiculum, prefrontal, perirhinal and entorhinal cortices. Furthermore, expression was highest during the early stages of training, and slow learners showed higher levels of Arc than fast learners. Rapanelli, Frick, and Zanutto (2009) also trained rats in an operant task, and found rats who had not yet mastered the task had higher levels of *Arc* and *c-Fos* mRNA in the hippocampus than fully trained animals. A similar effect for training was found by Svarnik, Alexandrov, Gavrilov, Grinchenko, and Anokhin

(2005), where the retrosplenial cortex displayed higher c-Fos protein expression in rats who were still learning the task.

IEG imaging techniques have also been used to differentiate the contribution of various brain regions to recognition memory. Wan, Aggleton, and Brown (1999) used a paired viewing procedure where rats were shown items or arrangements of items separately to each eye field of vision, allowing the activation of regions in different hemispheres of the same brain to be compared. The presentation of novel items resulted in higher c-Fos protein expression in the perirhinal cortex compared to the presentation of familiar items, supporting the role of this region in novel object recognition. However the presentation of novel spatial arrangements of the same items resulted in higher activation of CA1 in the hippocampus. This sensitivity of IEG expression to changes in spatial stimuli by relevant brain regions was further demonstrated by Vazdarjanova and Guzowski (2004), who analysed the co-localisation of *Arc* and *Homer 1a* mRNA expression in hippocampal neurons in response to environments after two exploration sessions where the second environment was either unchanged, slightly modified, or novel. As *Homer 1a* and *Arc* mRNA are expressed at different rates, they can be used to visualise activation in response to spatial exploration at two points in time. Animals exposed to the same environment twice exhibited the highest degree of overlap between activated neurons in the two sessions and animals exposed to two different environments exhibited a low degree of overlap. Where the environment was modified slightly, an intermediate degree of overlap was observed, however similarity scores between familiar and modified conditions were significantly higher in CA3 compared to CA1. This is consistent with the role of CA3 in pattern completion (Fellini et al., 2009).

The combined role of IEGs in learning and memory, and their activation in response to spatial information processing make this method an ideal candidate for

assessing the relative contribution of brain regions to spatial learning. While a number of studies have examined IEG expression following spatial tasks (Guzowski, Setlow, Wagner, & McGaugh, 2001; He, Yamada, & Nabeshima, 2002; Shires & Aggleton, 2008; Teather, Packard, Smith, Ellis-Behnke, & Bazan, 2005), they normally assess IEG activation at the end of training when the task is fully mastered, and at a particular time point when IEG expression is assumed to be maximal. Furthermore, studies investigating retention (Frankland et al., 2004; Gusev, Cui, Alkon, & Gubin, 2005), are generally restricted to two time-points, and analyse a limited number of regions.

1.7 Objectives of this Thesis

The overall objective of this thesis is to conduct an in-depth analysis of the contribution of a wide range of brain regions to the acquisition, consolidation and retrieval of spatial memory. The task used to measure spatial learning and memory will be the well-established Morris water maze task. A number of brain regions which have been implicated in spatial learning will be assessed, including the dorsal hippocampus and its subregions CA1, CA3 and the dentate gyrus, the lateral and medial entorhinal cortices, the retrosplenial, parietal and perirhinal cortices, and the medial prefrontal cortex and its subdivisions the anterior cingulate, prelimbic and infralimbic cortices. The method used to measure brain activation will be IEG imaging of Zif268, c-Fos and Arc protein, due to their established role in learning and memory.

Concerns have been raised regarding the appropriateness of control conditions used in studies investigating IEGs and spatial learning (Shires & Aggleton, 2008), therefore we will first address this issue by devising a new condition which will attempt to match the behaviour of spatially-trained animals without the learning component, and compare this with traditionally used controls. We hypothesise that although behaviour in

this novel control group will be matched to the spatial group, activity of the hippocampus as measured by IEG imaging, will be higher in the learning group.

Although some studies have assessed IEG expression following spatial learning in the water maze (Guzowski et al., 2001; Teather et al., 2005), they do not chart the change in activity of relevant structures throughout the course of learning. To achieve this aim, we will examine the change in expression of Zif268, c-Fos and Arc in 11 brain regions during early, middle and late training in the water maze. We hypothesise that IEG expression will be higher during early training, and will decrease as the task is mastered.

There is some evidence that the consolidation of spatial learning involves repeated waves of IEG expression in the hours following an experience (Ramirez-Amaya et al., 2005). We aim to investigate whether such delayed expression of Zif268, c-Fos and Arc can be observed in relevant brain regions at a number of time points following learning of the water maze task. We hypothesise that there will be two waves of IEG expression, one at 90 minutes and one at eight hours following training.

Finally, although it has been established that successful retention of the water maze task requires the recruitment of the medial prefrontal cortex at a remote time point (Frankland et al., 2004), it is not clear at what point in time the memory becomes dependent on this region for activation. Therefore we will assess the change in IEG activity of this and other cortical regions, as well as the hippocampus, at multiple retention time points between those traditionally used for recent and remote memory recall. We hypothesise that this reliance on the medial prefrontal cortex will emerge between seven and 14 days following acquisition of the Morris water maze task.

Chapter 2

General Methods

2.1 Subjects

Male Wistar rats, obtained from Charles River Laboratories, UK, were used as subjects throughout all experiments. Subjects were approximately three months old and weighed 200-300g at the beginning of experimentation. All animals were housed three per cage, in a temperature controlled environment (21±1°C), which was maintained on a fixed 12:12 hour light-dark cycle (07:00-19:00). All rats were given *ad libitum* access to food and water. Experimentation took place during the light phase and all subjects were well handled before experimentation began. The rats had no prior exposure to the maze and were experimentally naïve.

2.2 Morris Water Maze

The spatial navigation task used in all experiments was the Morris water maze. This apparatus has been used by our lab previously (Kealy et al., 2008). The water maze consisted of a black, circular fibreglass pool (diameter of 170cm, depth of 36cm), and was elevated on a table 70cm above the floor. The maze was filled with water to a depth of approximately 20cm and maintained at a temperature of 20±1°C. The escape platform was composed of black concrete and for the spatially-trained groups, always placed in the centre of the northeast quadrant of the pool. The platform was 18cm in height and 13.5cm in width. The platform was submerged 2cm below the surface of the water, ensuring the rats could not see the platform while navigating the maze.

The water maze area was enclosed by a black curtain which obscured the rest of the room from view, ensuring a uniform background and giving the experimenter more control over available spatial cues. For all experiments there were three cues available, which were located at fixed positions throughout each experiment. These consisted of two 25w bulbs which were suspended from the ceiling, at a distance of 75cm from the

edge of the pool and at an angle of 60°. One of these was located in the northeast and the other in the southeast (see Figure 2.1). The third cue was a rectangular piece of white card (55cm x 81cm) which was also suspended from the ceiling against the black background, on the west side of the maze. These cues remained constant for all water maze trained groups throughout the experiments in this thesis.

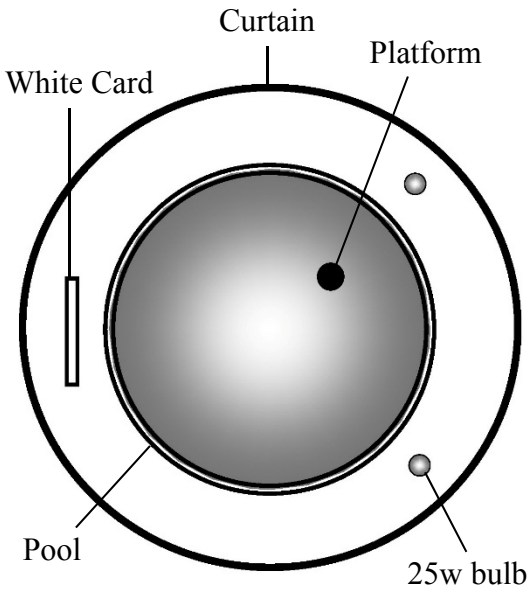

Figure 2.1: Layout of the Morris water maze used in all experiments in this thesis

A camera was positioned directly above the centre of the maze which recorded the animal's movements for each experimental trial. This information was collected by the digital tracking software EthoVision (Noldus Information Technologies, Wageningen, Netherlands), where an analysis of escape latencies, distance travelled, velocity and areas searched for each animal on every trial was calculated.

2.2.1 Acquisition.

Acquisition training followed protocols used previously by our lab (Kealy et al., 2008). The training procedure for water maze acquisition remained constant throughout all four

experiments for the spatially-trained groups. Animals were trained for up to five consecutive days in the water maze, with four trials per day. Animals were placed into the water maze facing the pool wall, from one of four pseudorandom starting positions, either north, south east or west, with each starting point being used just once per day. Animals were allowed 60 seconds to locate the escape platform in the northeast quadrant. If the animal had not located the platform after 60 seconds had elapsed, they would be guided to the platform by the experimenter using a ruler. Once the animal had mounted the platform, they were allowed to remain there for 15 seconds, following which they would be removed from the maze, placed in a container outside of the pool and allowed an inter-trial interval (ITI) of 10 seconds before being placed back into the maze from a different starting point. The available distal cues remained constant throughout the acquisition period. Successful acquisition of the maze was assessed by a statistically significant reduction in the time taken to escape the maze over the course of training.

2.2.2 Retention.

Where appropriate, water maze retention was assessed. The retention protocol has been used previously by our lab (McGauran et al., 2008). Following successful acquisition, animals were placed back into the water maze arena from the southwest, with the hidden platform removed from the maze. Animals were allowed 60 seconds to search the maze for an escape. Successful retention of the maze was assessed by analysis of time spent searching in the northeast quadrant, platform area, and platform crossings during the single retention probe trial.

2.3 Preservation of Tissue

Ninety minutes after the final acquisition or retention trial was chosen as the time point for sacrificing the animals as c-Fos and Zif268 are maximally expressed at this time-point (Zangenehpour & Chaudhuri, 2002). Rats were deeply anaesthetised with an intraperitoneal injection of sodium pentobarbital (100mg/kg, Euthatal), and subsequently perfused transcardially with ice cold 0.9% phosphate buffered saline (PBS, Ph7.4), followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, Ph7.4). Brains were then rapidly removed and post-fixed in 4% paraformaldehyde overnight, and then transferred to a 30% sucrose solution and stored at 4°C. Coronal sections were cut at 40μm using a freezing microtome, with every fourth section taken for subsequent immunohistochemical analysis.

2.4 Immunohistochemistry

Prior to the immunohistochemical procedure, free floating sections were stored in 0.1M PB containing 0.01% sodium azide at 4°C. In order to minimise variation due to the immunohistochemical procedure, brain sections representing a particular region from all subjects were processed in a single batch (unless otherwise stated). A standard immunohistochemical protocol was then followed (Coogan & Piggins, 2003). Sections were given two 10 minute washes in 0.1M PB, followed by a 10 minute wash in 0.1M PB containing 0.2% Triton-X-100 (PBX). A 20 minute wash in 0.1M PB containing 1.5% hydrogen peroxide was then carried out. This was followed by another two washes in 0.1M PB and one in PBX. Sections were then blocked for 60 minutes at room temperature in 5% normal goat serum (NGS) in 0.1M PBX. Sections were then incubated overnight in a primary antibody solution (2% NGS in 0.1M PBX). Labelling of Zif268, c-Fos and Arc were performed using the following primary antibodies: Zif268/Egr-1, rabbit

polyclonal antibody raised against the C terminus of human Egr-1 (dilution 1:3000; Santa Cruz Biotechnology); c-Fos, rabbit polyclonal antibody raised against the amino terminus of human c-Fos (dilution 1:2000; Santa Cruz Biotechnology); Arc, rabbit polyclonal antibody corresponding to amino acids 1-300 of Arc of human origin (dilution 1:800; Santa Cruz Biotechnology). After incubation with the primary antibody, sections were washed twice in 0.1M PB and once in PBX and then incubated with biotinylated secondary antibody (goat anti-rabbit, Jackson Laboratories, dilution 1:400) for 70 minutes. Sections were again washed twice in 0.1M PB and once in 0.1M PBX before incubation with avidin-biotin-peroxidase complex (0.4%; Vector Laboratories) for 90 minutes in lightproof conditions at room temperature. Sections were then given two 10 minute washes in PB followed by one 10 minute wash in 0.1M sodium acetate, pH6. This was followed by visualisation of the antigen using the nickel DAB method with glucose oxidase (Sigma, Poole, UK) as the catalyst. Sections were reacted for standardised lengths of time to ensure similar staining intensity across experimental groups. Sections were then mounted onto gelatine-coated slides, dried, dehydrated, cleared in Histoclear (National Diagnostics, Hull, UK), and coverslipped using Eukitt (Sigma, Poole, UK).

2.4.1 Verification of staining specificity.

To confirm specificity of staining, modified immunohistochemistry protocols were followed for Zif268, c-Fos and Arc. For Zif268, standard protocol was followed and staining can be observed in Figure 2.2A. Next, a standard protocol was followed with a Zif268 blocking peptide (dilution 1:300, Santa Cruz) during the primary incubation step. As can be seen in Figure 2.2B no immunoreactivity was observed. Standard protocols were also followed which excluded the primary antibody (Figure 2.2C) and secondary antibody (Figure 2.2D) and no immunoreactivity was observed.

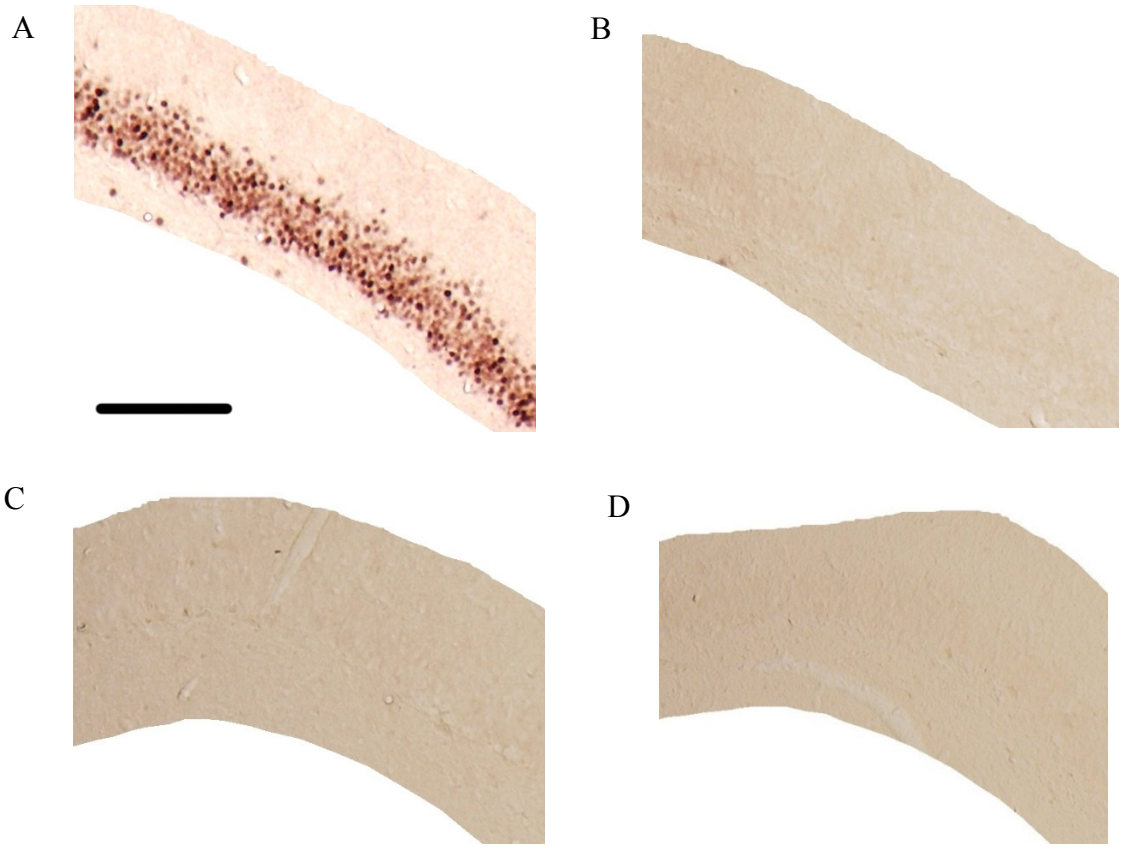

Figure 2.2: Sample sections of the dentate gyrus stained with Zif268 following a standard protocol (A), standard protocol with primary antibody incubated with a peptide block (B), standard protocol without primary antibody (C) and standard protocol without secondary antibody (D). Scale bar = 100 μm.

For c-Fos, a standard protocol was followed and c-Fos-positive nuclei can be observed in Figure 2.3A. A standard protocol was followed without the application of a primary antibody and no immunoreactivity was observed (see Figure 2.3B). A standard protocol was also followed without the application of a secondary antibody and no immunoreactivity was observed (see Figure 2.3C).

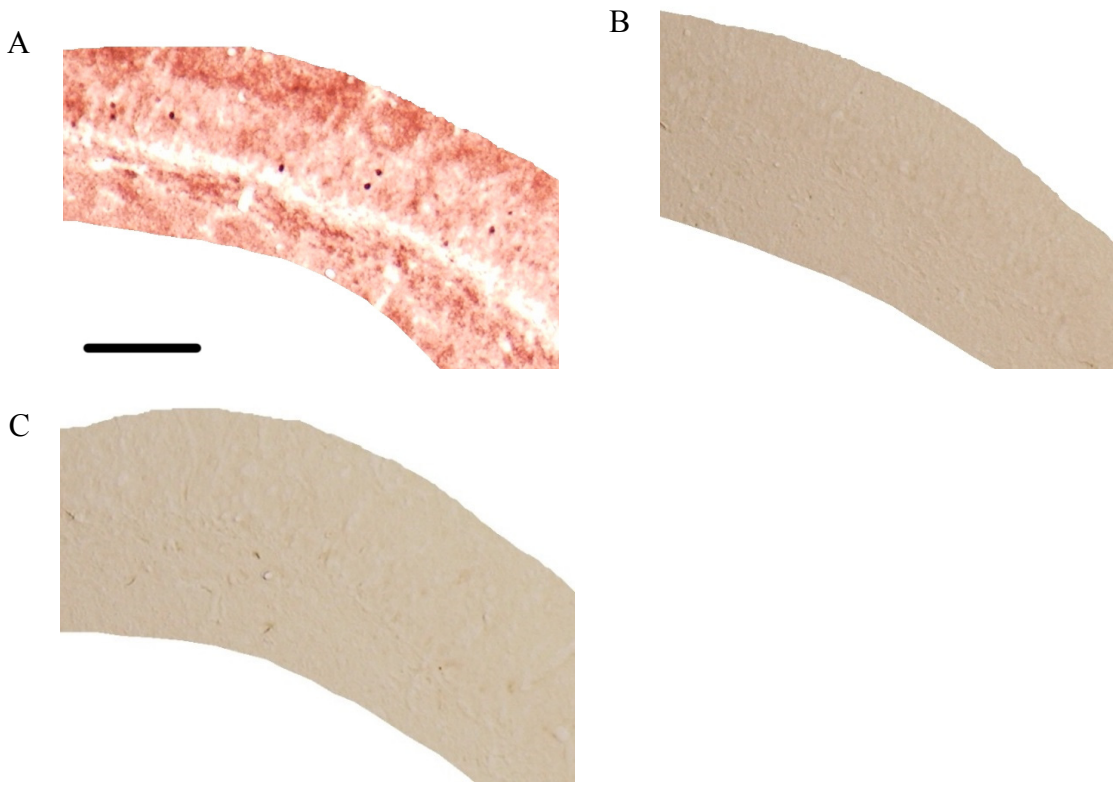

Figure 2.3: Sample sections of the dentate gyrus stained with c-Fos following a standard protocol (A), standard protocol without primary antibody (B), and standard protocol without secondary antibody (C). Scale bar = 100 μm.

For Arc, a standard protocol was followed and Arc-positive neurons can be observed in Figure 2.4A. A standard protocol was followed without the application of a primary antibody and no immunoreactivity was observed (see Figure 2.4B). A standard protocol was also followed without the application of a secondary antibody and no immunoreactivity was observed (see Figure 2.4C).

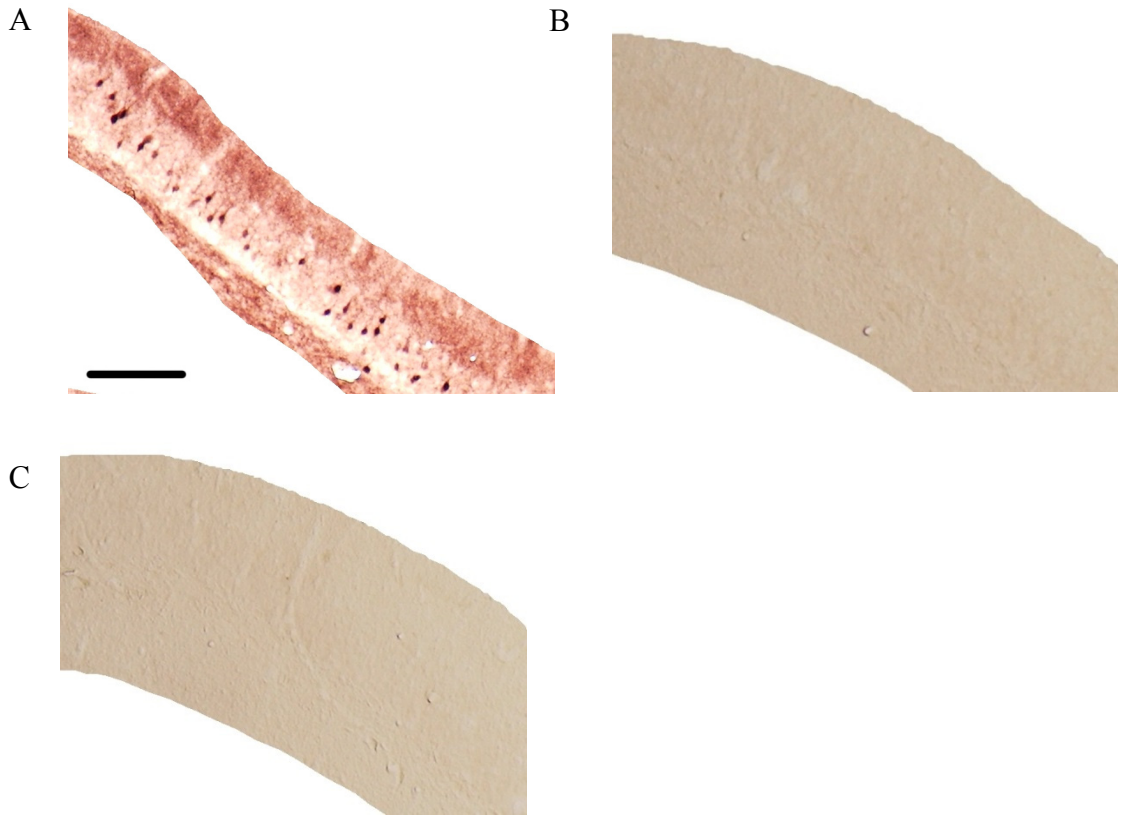

Figure 2.4: Sample sections of the dentate gyrus stained with Arc following a standard protocol (A), standard protocol without primary antibody (B), and standard protocol without secondary antibody (C). Scale bar = 100 μm.

2.5 Data Analysis

Estimates of IEG-activated cells, unless otherwise stated, were calculated using an automated cell counting procedure. The goal of this project was to compare relative counts to compare across conditions, rather than to obtain absolute cell counts, therefore stereological methods were not necessary. Images were taken of the 11 regions sampled using an Olympus DP12 digital camera, mounted on an Olympus BX51 microscope and captured using a 4x magnification. This level of magnification was sufficient to provide maximal coverage for areas of interest. However as this level of magnification usually sampled a larger area than that under investigation, a novel method to obscure adjacent brain regions during image acquisition was devised. Appropriately scaled images of the

coronal sections in question, adapted from Paxinos and Watson (2007), were printed onto clear acetate, with all regions except that of interest blacked out, and these were positioned over the section during image acquisition (see Figure 2.6 for sample masks). This procedure was carried out for all regions sampled except the hippocampus, where images were manually cropped following image acquisition. For all brain regions analysed, counts were obtained from four consecutive immunoreacted sections. These sections were 160 μm apart, as the tissue was taken in a one-in four (40 μm) manner.

Numbers of immunopositive cells were analysed using the public domain program ImageJ (National Institute of Health, USA). This programs allows for the automatic quantification of numbers of cells, which eliminates experimenter bias. Cell counts above a pre-defined brightness intensity threshold, and within a pre-defined particle area size were calculated. Counts from consecutive sections were averaged to produce a mean for each animal. Unless otherwise stated, raw values were used for statistical analyses as all animals were normally processed as a single batch during the immunohistochemical procedure.

2.6 Regions of Interest

The eleven regions selected for analyses, their coordinates, and the number of sections per region are displayed in Table 2.1. The dorsal hippocampus counts (CA1, CA3 and DG) were obtained from sections near AP level -3.24mm from Bregma. Adjacent regions included the retrosplenial cortex (AP -3.24), the perirhinal cortex (AP -3.24) and the parietal cortex (AP -3.24). The lateral entorhinal cortex (AP -5.76) and medial entorhinal cortex (AP -7.20) were also examined. Three frontal regions were also assessed, the anterior cingulate cortex (AP +3.72), prelimbic cortex (AP +3.72) and the infralimbic

cortex (AP +3.72). Figure 2.5 displays these regions on coronal sections, adapted from Paxinos and Watson (2007)

Table 2.1: Coordinates of regions selected and number of sections per region

Brain Region	Distance from Bregma		Sections
	Start	End	
CA1	-3.24 mm	-4.08 mm	6
CA3	-3.24 mm	-4.08 mm	6
Dentate Gyrus	-3.24 mm	-4.08 mm	6
Lateral Entorhinal Cortex	-5.76 mm	-6.36 mm	4
Medial Entorhinal Cortex	-7.20 mm	-7.80 mm	4
Retrosplenial Cortex	-3.24 mm	-4.08 mm	6
Perirhinal Cortex	-3.24 mm	-4.08 mm	6
Parietal Cortex	-3.24 mm	-4.08 mm	6
Anterior Cingulate Cortex	+3.72 mm	+2.76 mm	4
Prelimbic Cortex	+3.72 mm	+2.76 mm	4
Infralimbic Cortex	+3.72 mm	+2.76 mm	4

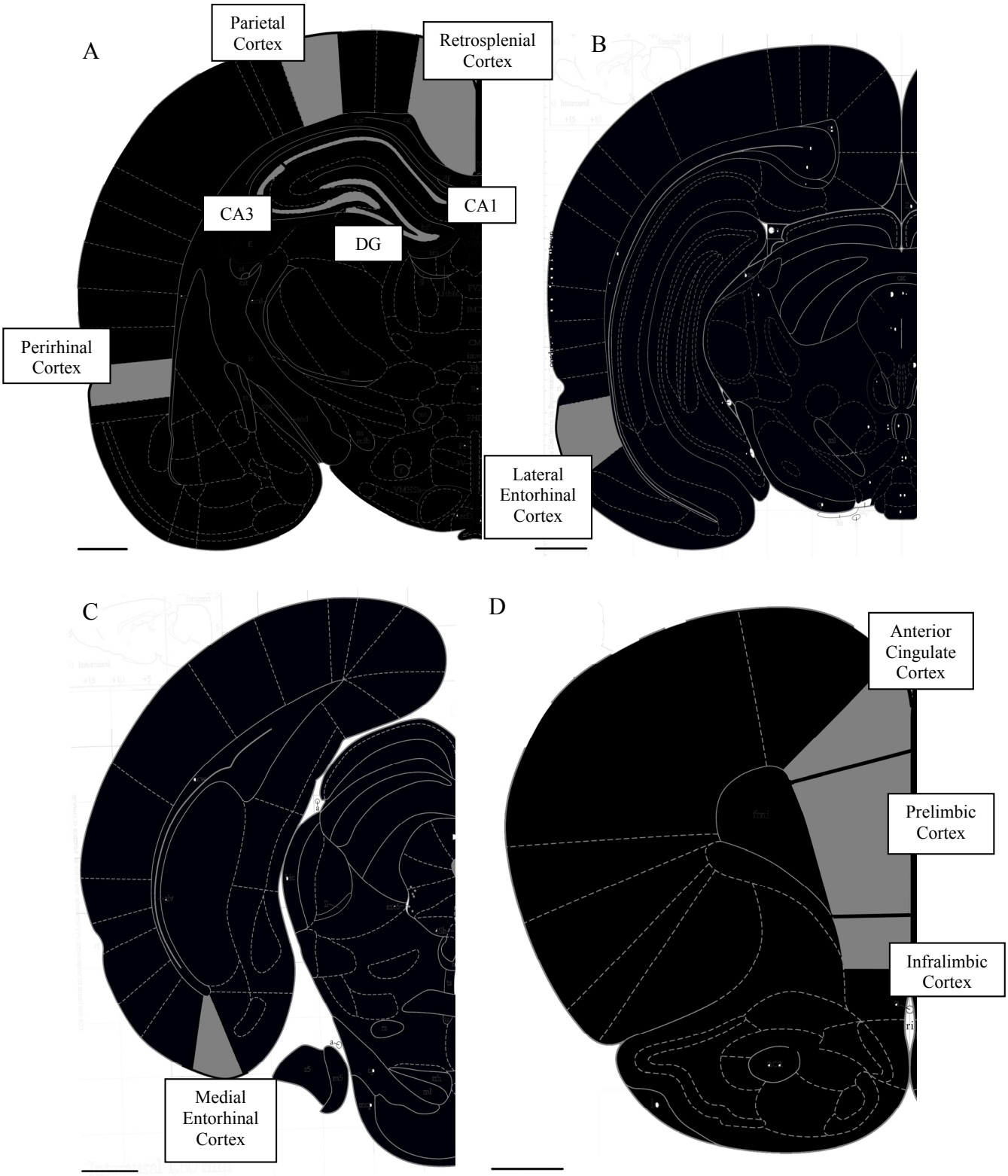

Figure 2.5: Coronal diagrams showing the regions of interest including CA1, CA3, the dentate gyrus, retrosplenial cortex, perirhinal cortex and parietal cortex (A, AP -3.24mm from Bregma), the lateral entorhinal cortex (B, AP -5.76mm from Bregma), the medial entorhinal cortex (C, AP -7.20mm from Bregma) and the anterior cingulate, prelimbic and infralimbic cortices (D, AP +3.72mm from Bregma). Adapted from Paxinos and Watson (2007). Scale bar = 1mm.

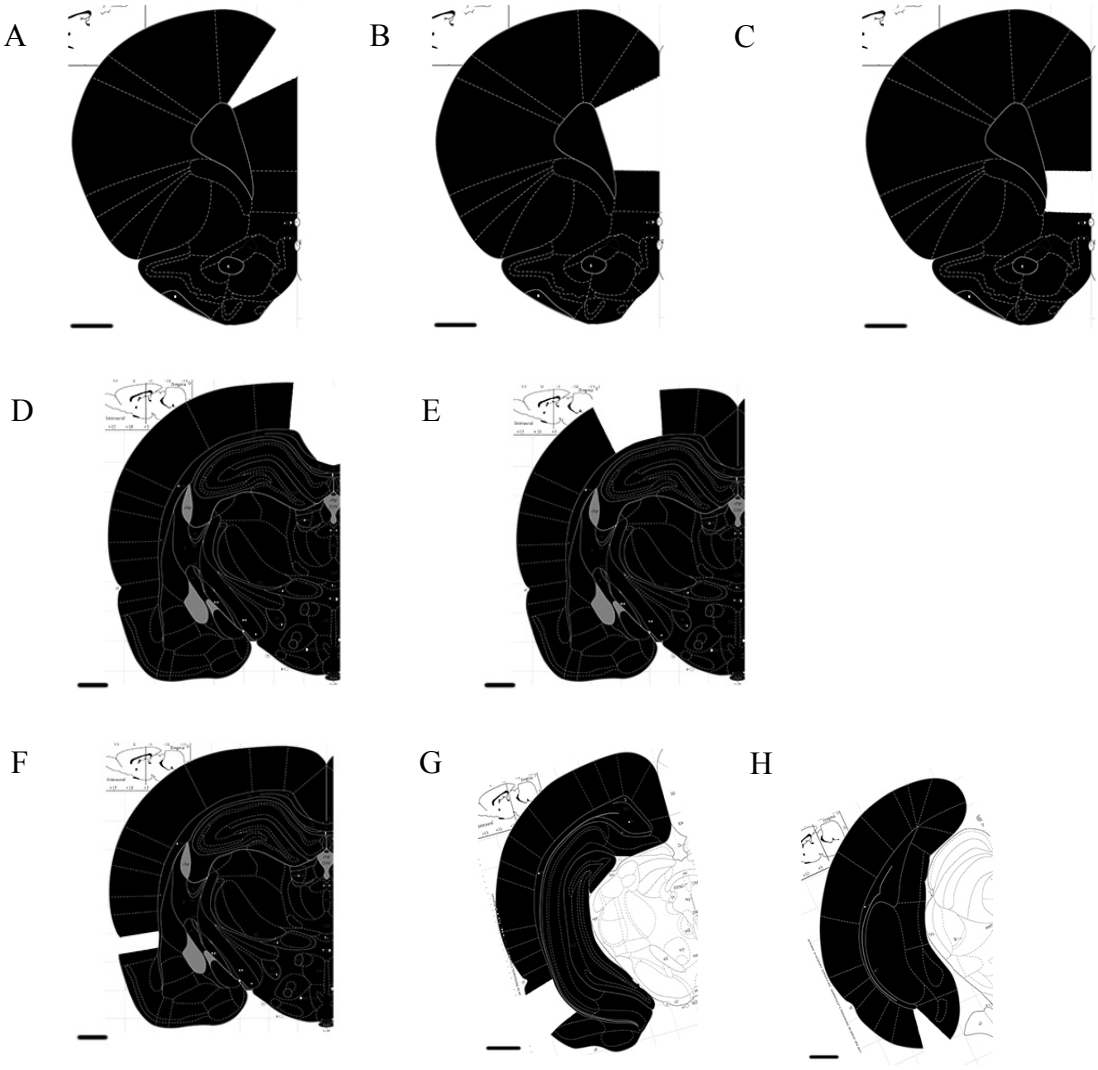

Figure 2.6: Sample masks used during image acquisition of the anterior cingulate cortex (A), prelimbic cortex (B), infralimbic cortex (C), retrosplenial cortex (D), parietal cortex (E), perirhinal cortex (F), lateral entorhinal cortex (G) and medial entorhinal cortex (I). Adapted from Paxinos and Watson (2007). Scale bar = 1mm.

2.7 Statistical Analysis

All statistical analyses were carried out using SPSS (Version 20). The significance of differences between groups was determined by using analysis of variance (ANOVA) with appropriate *post-hoc* test (Tukey at the 5% level of significance). The significance of differences within groups was determined by using repeated-measures analysis of

variance (ANOVA) with appropriate *post-hoc* test (Bonferroni at the 5% level of significance). Bivariate correlations were calculated using the Pearson product-moment correlation coefficient to examine the relationship between IEG activity and task performance. A star-rated system of representing significant results was used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.8 Ethical Considerations

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children (Ireland) guidelines under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

Chapter 3

Development of a Matched Control Condition for Spatial Learning in the Morris Water Maze

Part of this chapter has been previously published as Barry, D. N. and Commins, S. (2011). Imaging spatial learning in the brain using immediate early genes: insights, opportunities and limitations. *Reviews in the Neurosciences, 22*(2), 131-142.

Abstract

The importance of adequately matched control conditions in studies assessing IEG expression in response to learning tasks has been previously highlighted in the literature. Here we attempted to devise a novel control condition in the Morris water maze, which would simulate the experience of swimming in the maze and escaping the task, without requiring the formation of spatial memory. This was achieved through the use of increased numbers of randomly placed platforms over the course of training, which ensured control rats spent an equivalent amount of time in the maze but were not required to learn a platform location. This condition proved to be a superior control than free swimming rats, or a condition where a single hidden platform was randomly moved from trial to trial, on all behavioural measures analysed. Levels of c-Fos and Arc in the dentate gyrus were analysed, however there was no difference found in the level of IEG expression between spatially-trained and control groups. This reflects the findings of a previous attempt in the literature to create a matched control condition, and the equivalent hippocampal activation is attributed to the existence of incidental learning or the formation of navigational strategies to escape the maze in this control group.

3.1 Introduction

Experiments which use animals as subjects can often be influenced by a range of variables which are extraneous to those being manipulated (Johnson & Besselsen, 2002). Although IEGs such as Zif268, c-Fos and Arc have been strongly implicated in learning and synaptic plasticity (Knapska & Kaczmarek, 2004; Plath et al., 2006; Tischmeyer & Grimm, 1999), their induction can also be influenced by a range of variables and contexts which may be present in an experimental setting. Simply exposing an animal to novelty in their environment has been shown to affect the expression of IEGs. Placing a rat into a novel arena in the absence of task demands leads to an increase in c-Fos protein expression in CA1 and the lateral entorhinal cortex, regardless of environmental complexity (VanElzakker, Fevurly, Breindel, & Spencer, 2008). The exposure to novel stimuli as opposed to familiar ones leads to increased c-Fos protein expression in brain regions such as the perirhinal cortex and the anterior cingulate cortex in rats (Zhu, Brown, McCabe, & Aggleton, 1995). c-Fos and c-Jun immunoreactivity is maximally increased upon initial introduction to an exploratory apparatus such as the Làt maze, but this response declines with repeated exposures (Papa, Pellicano, Welzl, & Sadile, 1993).

This increase in IEG expression appears to be related to the exploratory potential of an experience rather than the stressfulness associated with novelty, as Pace et al. (2005) showed that hippocampal levels of c-Fos protein, *c-Fos* mRNA and *Zif268* mRNA were more closely associated with exploration rather than hypothalamic-pituitary-adrenal axis activity. Nonetheless, behavioural tasks can also involve a stress component, which has also been found to result in changes in the levels of IEG expression. Cullinan, Herman, Battaglia, Akil, and Watson (1995) analysed the alterations in levels of *c-Fos* and *Zif268* mRNA following restraint stress or swim stress in rats. *c-Fos* displayed the most widespread increase in expression across brain regions, with a similar response for

both swim and restraint stress. *Zif268* increases were also detected across similar brain regions. Duncan, Johnson, and Breese (1993) also demonstrated increases in c-Fos immunoreactivity in the medial prefrontal cortex in response to swim stress. *Arc* mRNA has also been shown to increase in the medial prefrontal cortex in response to free swimming stress and immobilisation stress, although this pattern was not observed in the hippocampus for either condition (Ons, Marti, & Armario, 2004). As is the case with novelty, the IEG response to stress appears to decline over time. Stamp and Herbert (1999) demonstrated that after 14 days of restraint stress, c-Fos immunoreactivity was reduced in the paraventricular nucleus and the amygdala. Nonetheless, the influence of novelty, exploration and stress on IEG induction are of concern when measuring neuronal activation in response to learning tasks.

The experiments in this thesis will use the IEG imaging method to elucidate the contribution of a number of brain regions to the acquisition, consolidation and retention of spatial memory. As such, there must be consideration of any extraneous variables associated with the chosen spatial learning task, how they may influence IEG expression, and how these variables can be controlled for. While the Morris water maze has proved a useful tool in the study of spatial memory, its greatest disadvantage is the employment of an aversive stimulus to motivate behaviour (Paul et al., 2009). Immersion in water introduces an element of stress into the task, which is of concern when combined with a measure sensitive to swim stress such as IEG imaging. The use of comparative controls (Johnson & Besselsen, 2002) to control for aspects of the task such as stress, swimming and novelty are required to make meaningful interpretations of results. Various control conditions have been utilised for the Morris water maze. As the maze tests place learning based on the relationship between distal cues and the escape platform, a "cued" condition, where the escape platform is visible and randomly placed during acquisition eliminates

the need to form a search strategy (R. G. Morris, 1981). This condition simply requires the use of a taxis strategy where the rat head directly towards a goal, but arguably still comprises a learning component and it is difficult to match swim times with spatially-trained rats. Alternatively, the "free swimming" control condition can be employed, where animals swim in the pool in the absence of an escape platform and are matched to the spatially-trained group for time (Mendez et al., 2008). However it is possible that this condition induces more stress than spatially-trained controls as animals have no control over the escape from the aversive stimulus of immersion. A third option is to compare spatially-trained animals to naïve controls, who are taken directly from their home cages and not exposed to the water maze. While useful as a measure of basal IEG expression, these controls are not exposed to the same stress, novelty and activity of the experimental groups. Therefore, there is a pressing need for an adequate control condition in the water maze task when using IEG imaging as a marker of neuronal activity.

In an attempt to address these concerns and provide a useful comparative control for spatial learning in the Morris water maze, Shires and Aggleton (2008) developed a novel condition which eliminated reliance on local or distal cues entirely, preserving the experience of exploring and escaping the water maze, without the stress of free-swimming. In attempting to match the behaviour of a spatial working memory group, a "procedural control" was devised, which was tasked with finding a submerged platform that was randomly located, but always at a fixed distance (either 5 or 13cm) from the pool wall. Rats learned to accomplish this task by swimming around the tank at the appropriate fixed distance from the wall until they reached the platform. Even when the platform remained static for the final session the animals did not deviate from this search strategy. However, although this group was matched for time with the spatial group, and displayed no evidence of place learning, no difference was found in hippocampal c-Fos

expression between the spatial working memory group and the two procedural groups. The spatial working memory group, however, did have higher counts than the 13cm procedural group in the lateral entorhinal cortex, and in the anterior cingulate, infralimbic and prelimbic cortices. Zif268 expression was actually higher in the procedural control groups compared to the spatial memory group, with the 5cm and 13cm procedural groups displaying higher Zif268 counts in CA1 and CA3, with the addition of the dentate gyrus in the 13cm group. Zif268 counts were also higher in the dorsal subiculum of both procedural groups, and in the medial entorhinal cortex and perirhinal cortex in the 13cm group. These results indicate that although some brain areas involved in working memory were not as engaged in the procedural control condition, the hippocampus and other structures involved in spatial navigation showed equivalent or higher activation in this group. Therefore the task performed by the control condition may have taxed spatial navigation to a similar or greater extent, and rats may have used available distal cues to continuously update their spatial location.

This raises the question as to whether or not it is possible to create a control condition which simulates the experience of swimming in the maze and escaping it in a similar time and manner to spatially-trained groups, without any additional stress or learning, or requiring the use of any navigational strategies. Previous attempts to create such a control condition have proved unsuccessful, therefore the aim of this experiment is to attempt to create such a condition and compare it to a spatially-trained group. This new control condition will involve a randomly placed hidden escape platform. The existence of a platform simulates the experience of escaping the maze, while the variable aspect of the task negates the use of platform-cue associations thus discouraging place learning. However, as escape latencies for spatially-trained groups decrease over the course of training, to match this time spent in the maze, the number of these hidden

platforms will be increased as training progresses, to gradually decrease the amount of time taken to escape the maze. Furthermore, in order to investigate whether the decrease in time spent in the maze by this novel control condition will be due to an increase in the number of available platforms, or more effective searching behaviour, a second control group where only one randomly placed platform will be available on each day will be employed. Finally, a widely used control condition, the free swimming control, will also be included, which will swim in the maze for the same amount of time as the spatially-trained group, in the absence of an escape platform. The expression of c-Fos and Arc protein in the hippocampus will also be analysed. We hypothesise that this novel control condition will match the spatially-trained group on a number of behavioural measures, including time spent in the maze, distance travelled, swimming speed, and thigmotactic behaviour. We also hypothesise that this novel control condition will prove to be superior to the other two control conditions, but will not display evidence of learning, and that this will be reflected in greater IEG expression in the hippocampus in the spatially-trained group.

3.2 Method

3.2.1 Subjects.

Male Wistar rats (n=28) obtained from Charles River Laboratories, UK, were used as subjects in the current study. The age and weight of subjects, housing conditions, handling, and time of experimentation were as described previously in Chapter 2.

3.2.2 Apparatus.

The Morris water maze was the behavioural task used in this study. Dimensions of the apparatus and cue configuration were as described previously in Chapter 2, however the availability and number of escape platforms varied depending on the experimental condition. Up to five escape platforms were utilised in this experiment.

3.2.3 Procedure.

Rats were randomly allocated to one of four experimental groups (n=7 per group). The spatially-trained group were given standard water maze training as described in Chapter 2, with a fixed hidden platform in the northeast quadrant of the pool (see Figure 3.1A). The multiple variable platform group were also trained for four trials per day for five days, but were tasked with locating an escape platform which could be in one of 20 possible locations (see Figure 3.1B). The number of available escape platforms in this condition increased by one per day, so that one randomly placed platform was available on day one for each trial, increasing to two platforms on day two, three on day three, four on day four, and five on the fifth day of training. A platform location was never repeated on the same day. The single variable platform group followed a similar training protocol but only had one randomly placed escape platform available for every trial over the five

days (See Figure 3.1C). The free swimming group swam in the maze in the absence of an escape platform (see Figure 3.1D).

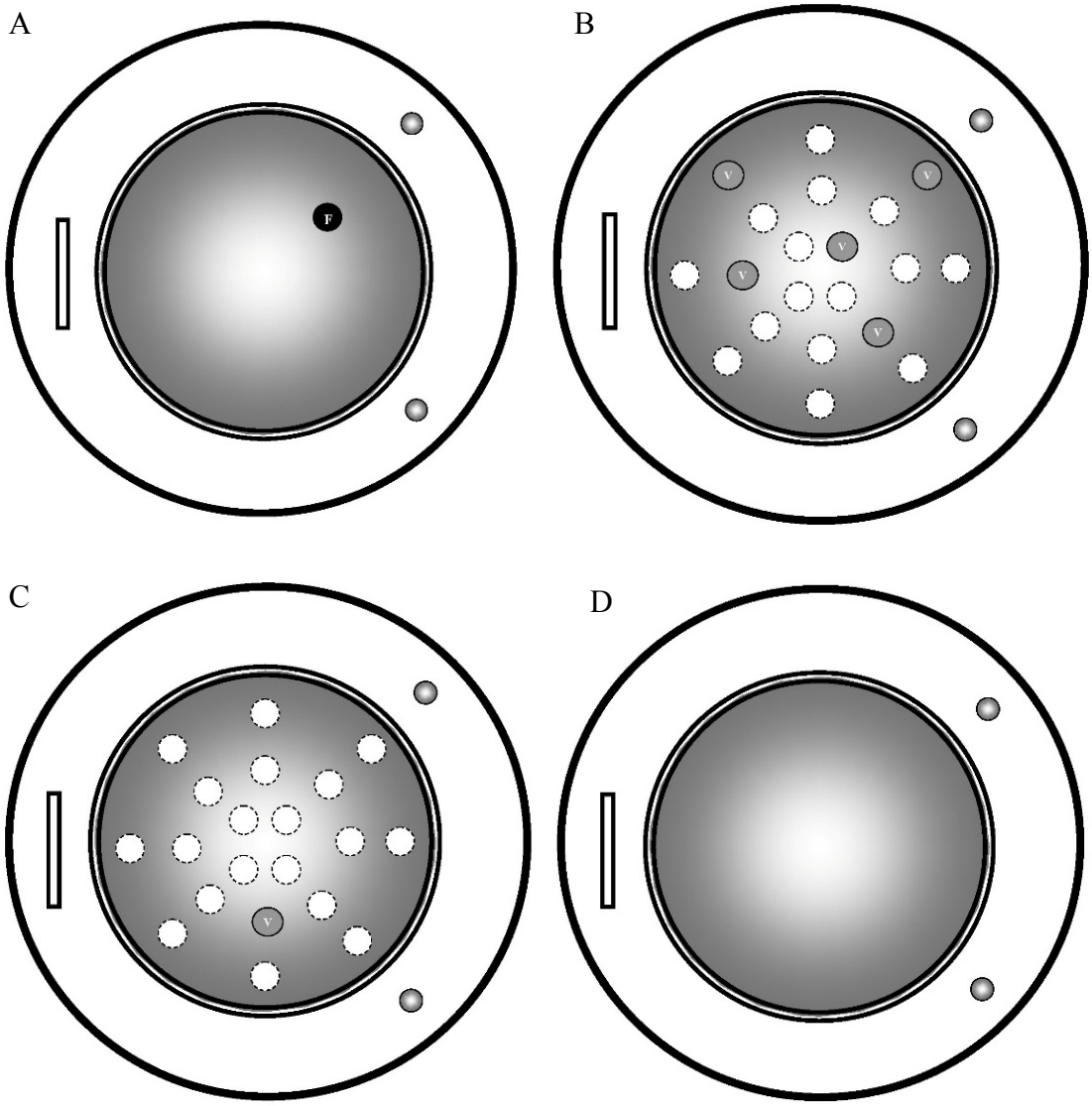

Figure 3.1: Morris water maze set-up and representative escape platform configurations for the spatially-trained group (A), the multiple variable platform group (B), the single variable platform group (C) and the free swimming group (D). F = fixed platform position. V = variable platform position. White circles represent possible locations for the variable platforms, whereas grey circles represent sample configurations of variable escape platforms.

3.2.4 Preservation of tissue.

Ninety minutes after the final trial on the fifth day of training rats were anaesthetised, transcardially perfused and their brains removed and post-fixed as described in Chapter 2. The hippocampal region was selected for analysis in this experiment. Forty μm coronal sections were cut on a freezing microtome from AP Bregma -2.64mm to AP Bregma -3.76mm (Paxinos & Watson, 2007) and every fourth section was taken for analysis, totalling seven sections for each IEG analysis.

3.2.5 Immunohistochemistry.

Immunohistochemical staining was performed on all four groups during one session, eliminating the need for subsequent normalisation of the data. Furthermore, staining was performed in cohorts of four, with one animal from each group processed side-by-side in the same well plate. Immunohistochemical protocol for the detection of c-Fos and Arc protein was followed as described previously in Chapter 2.

3.2.6 Data analysis.

3.2.6.1 Behavioural data.

To measure performance in the water maze, escape latencies, distance travelled and velocity for each trial were calculated, and averaged to produce a mean for every animal for each day. For the purposes of statistical analysis of swimming behaviour during acquisition trials, the swimming area of the water maze was divided into multiple sections. To measure preference for an overall area in the maze, it was divided into four quadrants, northeast, northwest, southeast and southwest (see Figure 3.2A), and percentage time spent in each quadrant for each trial was calculated. To quantify thigmotactic behaviour, percentage time spent swimming in corridor of 16cm width

around the circumference of the swimming area was also analysed (See Figure 3.2B). As a disproportionate amount of time searching in the location where a platform had been found on the previous trial would suggest some learning has taking place, to assess this an area around each previously located platform, comprising 5% of the total maze swimming area was also analysed, and percentage time of each trial spent in this area was computed (see Figure 3.2C).

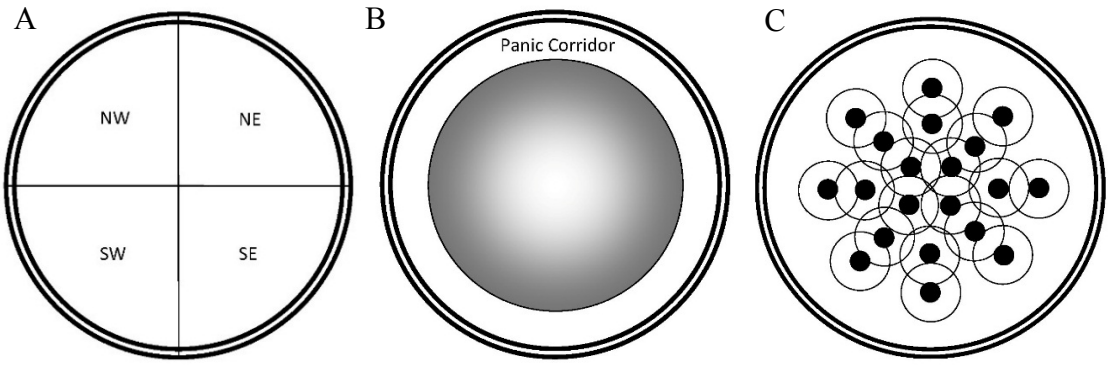

Figure 3.2: Division of the water maze area into quadrants (A), panic corridor (B), and platform areas (C) for the analysis of swimming behaviours during acquisition.

3.2.6.2 IEG data.

As there was only one region selected for analysis in this experiment, the use of automated cell counting procedures were not deemed necessary. Counts of c-Fos and Arc-positive neurons above a certain darkness intensity were instead made by visual inspection for this experiment, with the experimenter blind to the experimental condition. The dentate gyrus was selected for analysis, with both hemispheres included. Seven consecutive sections were analysed. Raw counts for all seven sections were then averaged to produce a mean for that region.

3.2.7 Statistical analysis.

To analyse escape latencies, distance travelled and velocity in the water maze, mixed between-within analyses of variance (ANOVA) were performed, with Bonferroni-corrected t-tests used to assess within group and Tukey *post-hoc* tests to assess between group differences. To compare time spent in water maze quadrants for each group, one-way repeated measures analyses of variance (ANOVA) were performed, with Bonferroni-corrected comparisons. To compare time spent in a previous platform location and time spent in the panic corridor across groups, one-way analyses of variance (ANOVA) were performed, with Tukey *post-hoc* tests. To compare levels of c-Fos and Arc across groups, one-way analyses of variance (ANOVA) were performed on mean numbers of counts. To assess relationships between levels of c-Fos and Arc and performance in the water maze, Pearson product-moment correlations were performed.

3.2.8 Ethical considerations.

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children (Ireland) guidelines under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

3.3 Results

3.3.1 Behavioural results.

3.3.1.1 Escape Latencies.

A number of measures were utilised to measure learning and performance in the water maze. The first measure was escape latency. As the spatial and free swimming groups were matched for time spent in the maze, the free swimming group are excluded from the escape latency analysis. A 3 x 5 mixed factorial ANOVA with group as the between group factor and day as the within group factor, confirmed there was an overall significant decrease in escape latency with a main effect for acquisition day, $F(4, 72) = 8.07$, $p < 0.001$, and Bonferroni *post-hoc* analyses revealed that escape latencies were significantly lower on days four (*M*: 22.29±1.72 sec, $p < 0.05$) and five (*M*: 18.18±1.76 sec, $p < 0.01$) than day one (*M*: 32.56±2.46 sec, see Figure 3.3).

Subsequent repeated measures ANOVAs with Bonferroni correction were performed on the escape latencies of the individual groups to further investigate how they changed over the course of training. A significant effect for day was found in the spatial group, $F(4, 24) = 7.42$, $p < 0.001$, with *post-hoc* analyses showing that escape latencies were significantly lower on day four (*M*: 14.40±2.33 sec, $p < 0.05$) and on day five (12.26±1.97 sec, $p < 0.01$) than day one (*M*: 33.04±4.18 sec). The multiple variable platform group also showed a significant effect for day, $F(4, 24) = 7.48$, $p < 0.001$, however *post-hoc* analyses revealed that the difference between escape latencies on day one (*M*: 33.54±5.66 sec) and day five (*M*: 9.72±1.74 sec) did not reach statistical significance ($p = 0.085$). In the single variable platform group a significant effect for day was not found, $F(4, 24) = 1.05$, $p > 0.05$.

There was an overall difference in escape latencies between the groups, $F(2, 18) = 14.63$, $p < 0.001$, with Tukey *post-hoc* analyses revealing escape latencies were higher

in the single variable platform group (*M*: 35.73±2.59 sec) than the spatial group (*M*: 21.17±2.04 sec, $p < 0.01$) and the multiple variable platform group (*M*: 19.80±2.27 sec, $p < 0.001$). A significant interaction effect was also revealed across days, $F(8, 72) = 3.53$, $p < 0.01$.

Subsequent one-way ANOVAS performed on days one and five showed there was no difference between the groups on day one, $F(2, 18) = 0.91$, $p > 0.05$), however a significant difference was found on day five, $F(2, 18) = 16.82$, $p < 0.001$), with Tukey *post-hoc* analyses revealing escape latencies were higher in the single variable platform group (*M*: 32.56±4.59 sec) than the spatial group (*M*: 12.26±1.97 sec, $p < 0.001$), and the multiple platform group (*M*: 9.72±1.74 sec, $p < 0.001$). These results suggest that the spatial group acquired the maze, the multiple variable platform group spent an equivalent amount of time in the maze compared to the spatial group, and that the single variable platform group spent longer in the maze than the other groups as training progressed, with escape latencies not decreasing in this group.

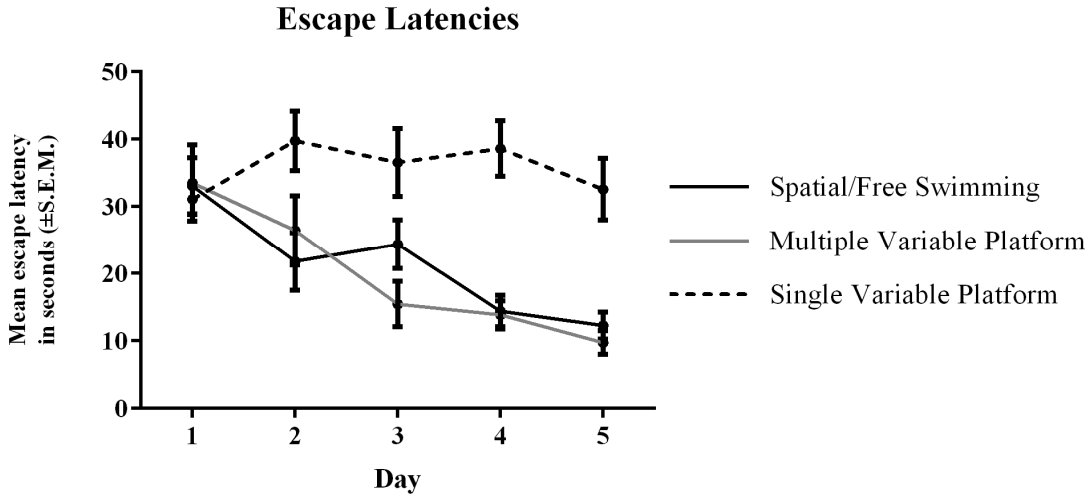

Figure 3.3: Mean escape latencies across the five days of training for the spatial/free swimming groups, the multiple variable platform group, and the single variable platform group.

3.3.1.2 Distance travelled

Mean distance travelled per day of training was also taken as a measure of water maze performance. All groups were included in the distance travelled analysis. A 4 x 5 mixed factorial ANOVA with group as the between groups factor and day as the within group factor, confirmed there was a main effect for acquisition day, $F(4, 96) = 20.34$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing distance travelled was significantly lower on days two (*M*: 512.99±42.83 cm, $p < 0.05$), three (*M*: 445.51±33.98 cm, $p < 0.01$), four (*M*: 361.40±30.92 cm, $p < 0.001$) and five (*M*: 300.92±31.01 cm, $p < 0.001$) than day one (*M*: 661.52±40.23 cm, see Figure 3.4).

Subsequent one-way repeated measures ANOVAs were performed on individual groups to further investigate how distance travelled changed over the course of training. A significant effect for day was found in the spatial group, $F(4, 24) = 10.43$, $p < 0.001$, with Bonferroni *post-hoc* analyses showing that distance travelled was significantly lower on day four (*M*: 253.39±53.69 cm, $p < 0.05$) and on day five (233.58±40.61 cm, $p < 0.001$) than day one (*M*: 710.77±84.39 cm). In the multiple variable platform group, a significant effect was also found for day, $F(4, 24) = 13.01$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing distance travelled was significantly lower on days three (*M*: 250.34±47.48 cm, $p <0.05$), four (*M*: 232.18±36.84 cm, $p = 0.05$), and five (*M*: 155.99±24.80 cm, $p < 0.05$) when compared to day one (*M*: 697.20±105.20 cm). A significant effect for day was not found in the single variable platform group, $F(4, 24) = 0.66$, $p > 0.05$. Significant differences were found however in the free swimming group, $F(4, 24) = 6.63$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing distance travelled was significantly lower on day five (*M*: 279.53±60.58 cm, $p < 0.01$ compared to day one (*M*: 633.75±74.58 cm).

Similar to escape latency, there was an overall difference found between the groups, $F(3, 24) = 5.86$, $p < 0.01$, with Tukey *post-hoc* analyses revealing distance travelled was higher in the single variable platform group (*M*: 618.20±51.58 cm) than the spatial group (*M*: 407.59±46.43 cm, $p < 0.05$), the multiple variable platform group (*M*: 363.28±36.05 cm, $p < 0.01$) and the free swimming group (*M*: 436.72±49.47 cm, $p < 0.05$). A significant interaction effect between day and group was also found, $F(4, 96) = 20.34$, $p < 0.01$.

Subsequent one-way ANOVAS showed there was no difference between the groups on day 1, $F(2, 24) = 0.39$, $p > 0.05$. However on day five, significant differences were found, $F(3,24) = 6.97$, $p < 0.01$, with Tukey *post-hoc* analyses revealing distance travelled was higher in the single variable platform group (*M*: 534.17±97.24 cm) than the spatial group (*M*: 233.58±40.61 cm, $p < 0.05$), the multiple variable platform group (*M*: 155.99±65.60 cm, $p < 0.01$), and the free swimming group (*M*: 279.53±60.58 cm, $p < 0.05$). These results suggest that the spatial group, the multiple platform group and the free swimming group travelled significantly less distance in the maze as training progressed, however the single variable platform group travelled further in the maze than the other three groups as training progressed.

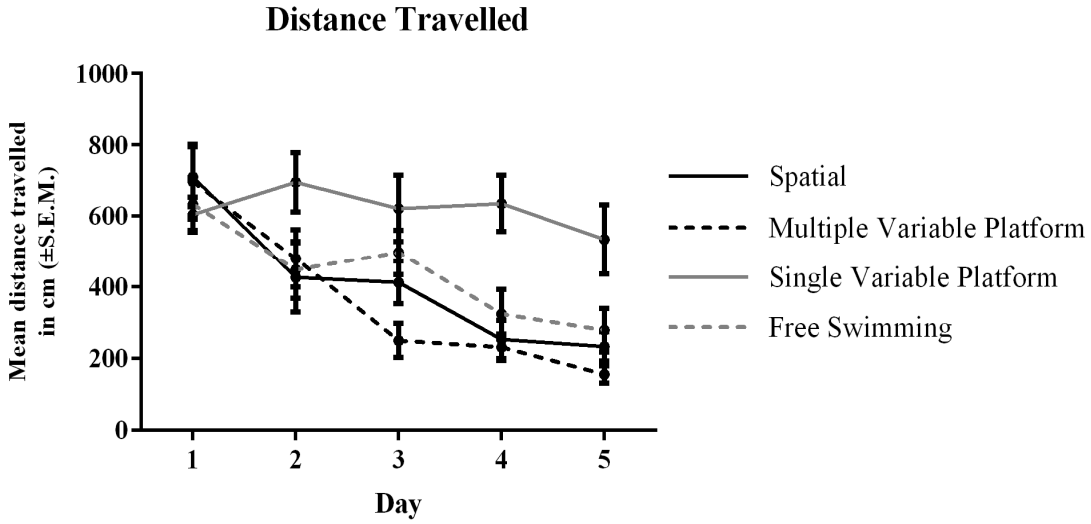

Figure 3.4: Mean distance travelled for all four groups over the five days of training in the water maze

3.3.1.3 Velocity

Velocity, measured in centimetres travelled per second, was also recorded. A 4 x 5 mixed factorial ANOVA with group as the between groups factor and day as the within group factor, did not find a main effect for day, $F(4, 96) = 1.70$, $p > 0.05$, or for group, $F(3, 24) = 2.32$, $p > 0.05$, however an interaction effect between day and group was found, $F(12, 96) = 3.71$, $p < 0.01$. Subsequent one-way ANOVAS showed there was no difference between the groups on day one, $F(3, 24) = 2.15$, $p > 0.05$. A significant difference between the groups was found on day five, $F(3, 24) = 3.46$, $p < 0.05$, with Tukey *post-hoc* analyses revealing the velocity of the free swimming group on day five (*M*: 24.16±3.18 cm/s, $p < 0.05$) was significantly higher than the multiple variable platform group (*M*: 16.17±1.47 cm/s). These results indicate that velocity remained largely constant for all four groups during acquisition although the free swimming group had a tendency to swim faster as training progressed (see Figure 3.5).

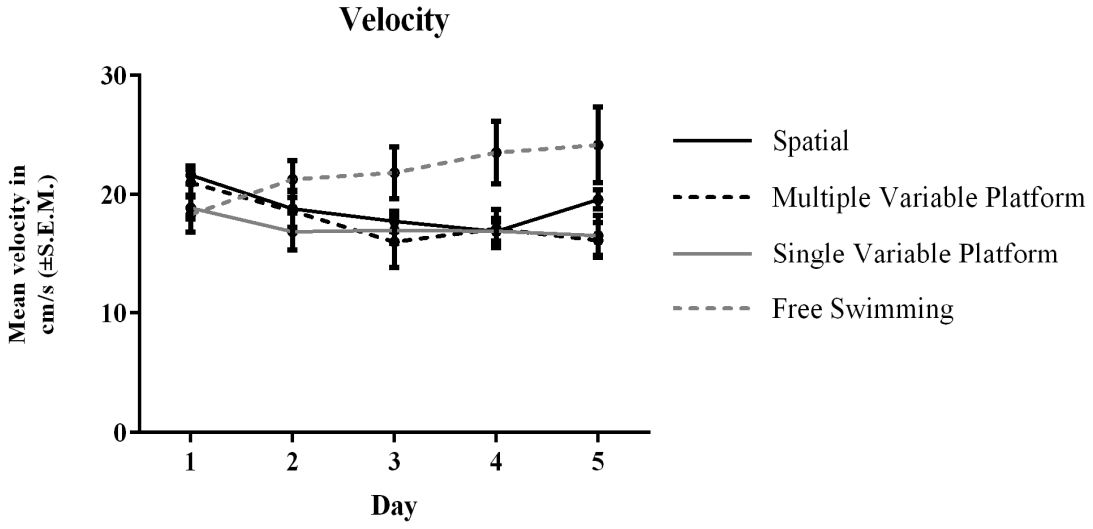

Figure 3.5: Mean velocity for all four groups over the five days of training in the water maze

3.3.1.4 Thigmotaxis

Thigmotaxis, or the tendency to stay close to the perimeter of an environment due to increased anxiety (Treit & Fundytus, 1988), was also measured. Time spent engaging in thigmotactic behaviour was assessed as percentage time spent swimming in a corridor of 16cm width around the circumference of the swimming area (McGauran, Harvey, Cunningham, Craig, & Commins, 2004) (see Figure 3.2B). A 4 x 5 mixed factorial ANOVA with group as the between groups factor and day as the within group factor, confirmed a main effect for acquisition day, $F(4, 96) = 4.80$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing thigmotaxis was significantly lower on day five (*M*: 40.12±3.29 %, $p < 0.01$) when compared to day one (*M*: 52.83±2.23 %, see Figure 3.6).

Subsequent repeated measures ANOVAs were performed on each group, revealing a significant effect for day in the spatial group, $F(4, 24) = 5.65$, $p < 0.01$, with Bonferroni *post-hoc* analyses showing that this group spent less time in the panic corridor on day five (24.20±3.50 %) when compared to day one (*M*: 48.09±5.03 %, $p < 0.01$). In the multiple variable platform group, a significant effect was also found for day, $F(4, 24)$

= 2.84, $p < 0.05$, however *post-hoc* analyses revealed that the difference between percentage time spent in the panic corridor on day one (*M*: 48.45±4.56 %) and day five (*M*: 27.10±8.26 %) did not reach statistical significance. In the single variable platform group, a significant effect for day was not found, $F(4, 24) = 1.51$, $p > 0.05$. The free swimming group did not differ significantly on this measure across days, $F(4, 24) = 0.70$, $p > 0.05$.

There was an overall difference found between the groups, $F(3, 24) = 15.82$, $p < 0.001$, with Tukey *post-hoc* analyses revealing percentage time spent in the panic corridor was lower in the spatial group (*M*: 33.69±3.36 %, $p < 0.001$), the multiple variable platform group (*M*: 34.19±3.94 %, $p < 0.001$), and the single variable platform group (*M*: 41.26±4.73 %, $p < 0.001$) than the free swimming group (*M*: 70.97±5.39 %). A significant interaction effect was also revealed across days, $F(12, 96) = 2.02$, $p < 0.05$.

Subsequent one-way ANOVAS performed on days one and five showed a significant difference between the groups on day one, $F(3, 24) = 3.83$, $p < 0.05$, with Tukey *post-hoc* analyses revealing time spent in the panic corridor was less in the spatial group (*M*: 48.10±5.04 %, $p < 0.05$) and the multiple platform group (*M*: 48.45±4.56 %, $p < 0.05$), than the free swimming group (*M*: 65.92±5.13 %). A difference between the groups was also found on day five, $F(3, 24) = 11.41$, $p < 0.001$), with Tukey *post-hoc* analyses revealing time spent in the panic corridor was lower in the spatial group (*M*: 24.20±3.50 %, $p < 0.001$), the multiple platform group (*M*: 27.10±8.26 %, $p < 0.001$), and the single variable platform group (36.74±6.94 %, $p < 0.01$) than the free swimming group (*M*: 72.44±6.65%). These results indicate that the spatial group spent less time engaging in thigmotactic behaviour over the course of training in the water maze, and that the free swimming group spent a significantly higher percentage of time in the panic

corridor compared to the other three groups, suggesting anxious behaviour is a limitation of this control group.

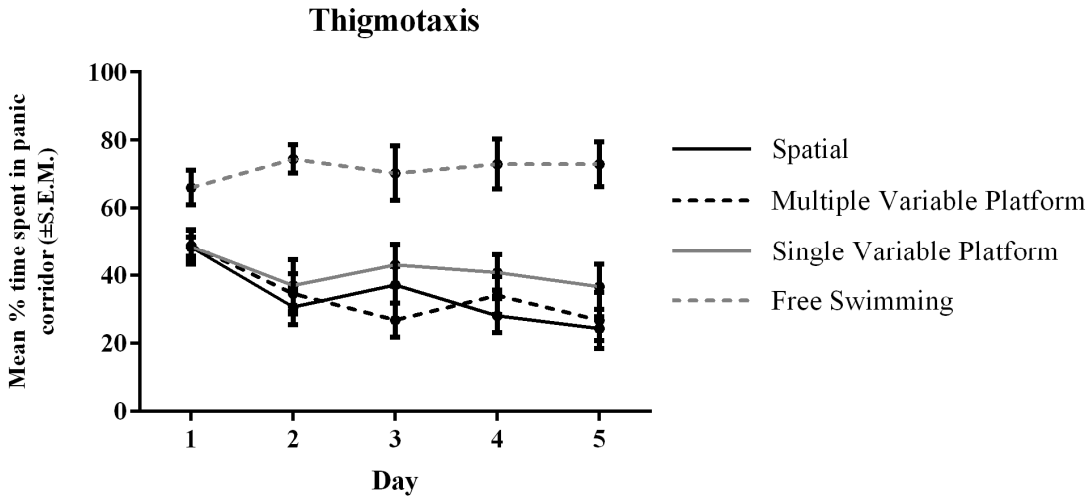

Figure 3.6: Mean percentage time spent swimming in the panic corridor over the five days of training in the water maze

3.3.1.5 Quadrant analysis

As the spatial group were trained to find a fixed platform in the northeast quadrant, it would be expected that this group display a significant preference for this quadrant at the end of training. It was not desirable for the control groups to display a preference for any particular quadrant, as this behaviour would suggest this quadrant was associated with escaping the maze. Therefore all groups were assessed on percentage time spent in each quadrant to ensure such learning behaviour was not evident in these control groups. The water maze arena was divided into four quadrants: northeast, northwest, southeast and southwest, and percentage time spent searching in each quadrant on day five was calculated.

A one-way repeated measures ANOVA found a significant effect for quadrant in the spatial group, $F(3, 18) = 14.64$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing more time was spent searching in the northeast quadrant (*M*: 47.27±3.80 %),

than the southeast (*M*: 14.38±3.49 %, $p < 0.01$) and southwest (14.15±3.11 %, $p < 0.01$, see Figure 3.7A). A significant effect for quadrant was not found in the multiple variable platform group, $F(3, 18) = 0.89$, $p > 0.05$ (see Figure 3.7B), the single variable platform group, $F(3, 18) = 0.29$, $p > 0.05$ (see Figure 3.7C), or the free swimming group, $F(3, 18) = 0.80$, $p > 0.05$ (see Figure 3.7D).

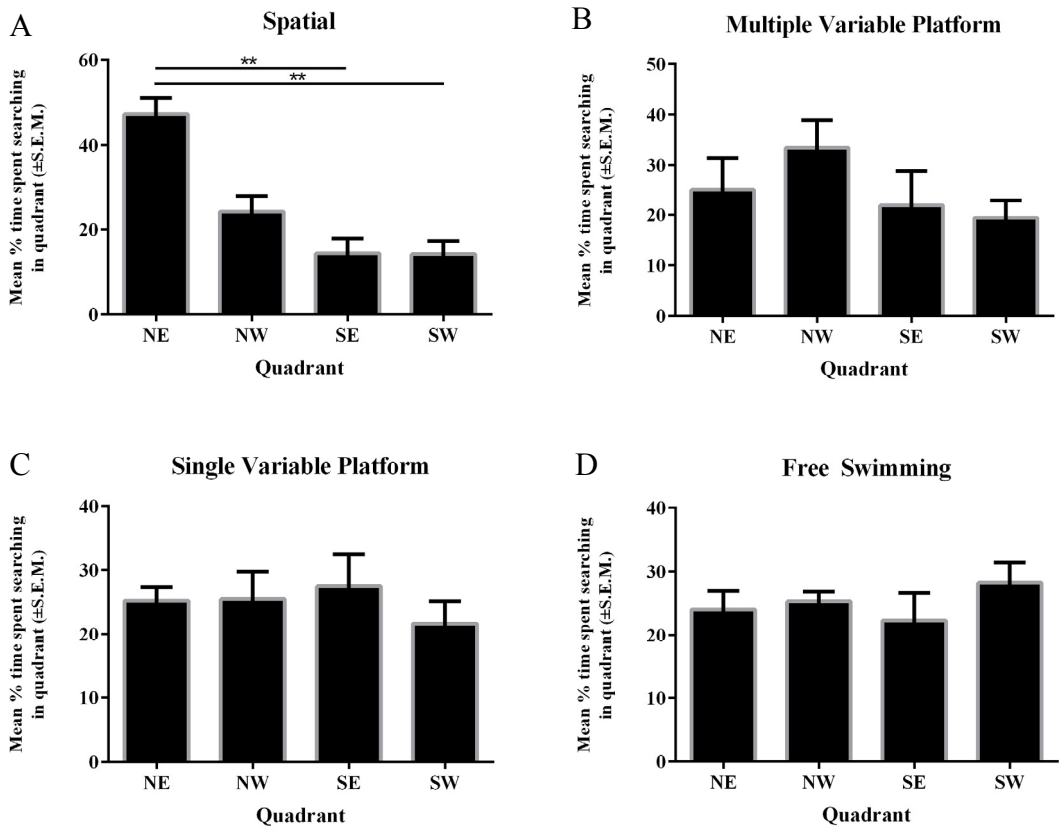

Figure 3.7: Mean percentage time spent searching in each quadrant of the water maze on the final day of training for all four groups

3.3.1.6 Searching in previous platform area

Although only the spatial group displayed a preference for a particular quadrant, this did not negate the possibility that the multiple variable or single variable platform groups returned to search the area where they had located a platform on a previous trial. Such

behaviour would be suggestive of spatial learning taking place. To rule out this possibility, percentage time spent searching in a circular area around where the previous platform had been located (comprising 5% of the area of the water maze) was calculated and groups were compared on this measure.

One-way ANOVAs to compare the spatial, multiple variable and single variable platform groups were performed on each day of training. A significant difference was found on day one, $F(2, 18), = 8.77), p < 0.01$, with Tukey *post-hoc* analyses showing the spatial group spent more time searching in the previous platform area (*M*: 10.87±1.85 %) than the multiple variable platform group (*M*: 4.34±1.42 %, $p < 0.05$), and the single variable platform group (*M*: 3.20±0.65 %, $p < 0.01$). A similar pattern was found on day two, $F(2, 18) = 8.64, p < 0.01$), with Tukey *post-hoc* analyses revealing the spatial group returned to the previous platform area (*M*: 18.58±4.09 %) more often than the multiple variable platform group (*M*: 6.21±1.70 %, $p < 0.01$) or the single variable platform group (*M*: 4.57±0.87 %, $p < 0.01$). A significant difference was again found on day three, $F(2, 18) = 3.92, p < 0.05$, however Tukey *post-hoc* analyses revealed the difference between the spatial group (13.88±3.60 %) and the multiple platform group (5.44±1.46 %, $p = 0.052$) and the single variable platform group (6.21±1.26 %,) did not reach statistical significance ($p = 0.08$). On day four, a significant difference between the groups was found, $F(2, 18) = 15.39, p < 0.001$, with Tukey *post-hoc* analyses revealing the spatial group again spent a significantly higher percentage of time searching in the previous platform area (*M*: 21.59±3.21 %) than the multiple variable platform group (*M*: 3.64±1.96 %, $p < 0.001$) and the single variable platform group (*M*: 8.34±1.64, $p < 0.01$). On the final day of training, a main effect was found for group, $F(2, 18) = 15.44, p < 0.001$), with Tukey *post-hoc* analyses revealing the spatial group spent more time in the previous platform area (*M*: 19.03±2.07) than the multiple platform group (*M*: 5.40±2.71,

$p < 0.01$), and the single variable platform group (M: 4.70±1.02, $p < 0.001$, see Figure 3.8).

These results show that the spatial group consistently returned to search the area surrounding the fixed northeast platform location, but the multiple variable and single variable platform groups did not return to where they had located an escape platform on a previous trial, only exploring this area at around chance level (5%). This suggests that the control groups did not display any evidence of spatial learning.

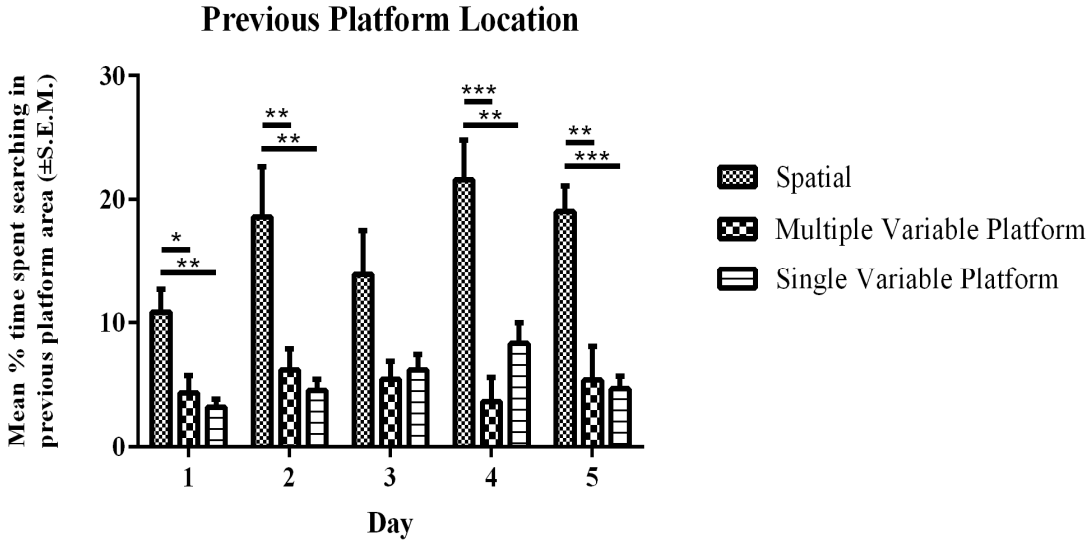

Figure 3.8: Mean percentage time spent searching in the area of the maze where an escape platform had been located on the previous trial

3.3.2 IEG results.

Levels of hippocampal c-Fos and Arc protein were compared across all groups 90 minutes post-training on day five. The dentate gyrus was selected for analysis. As no difference was found between the hemispheres the data was pooled together.

A one-way ANOVA found no difference between the four groups in the levels of c-Fos protein expressed in the dentate gyrus, $F(3, 24) = 1.99$, $p > 0.05$, see Figure 3.9A.

Similarly, no differences were found between the groups in the levels of Arc protein expressed in the dentate gyrus, $F(3, 24) = 2.52$, $p > 0.05$ (See Figure 3.9B).

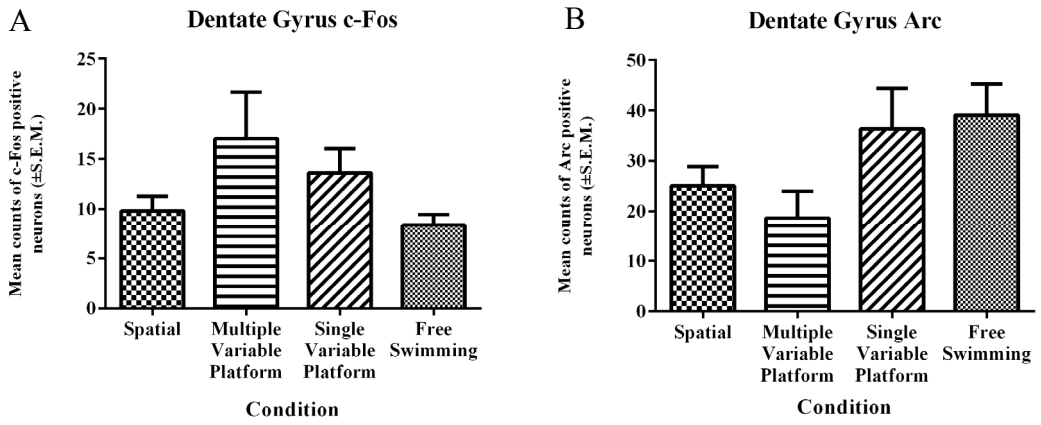

Figure 3.9: Mean counts of c-Fos (A) and Arc (B) positive neurons per section in the dentate gyrus for the spatial, multiple variable platform, single variable platform and free swimming groups

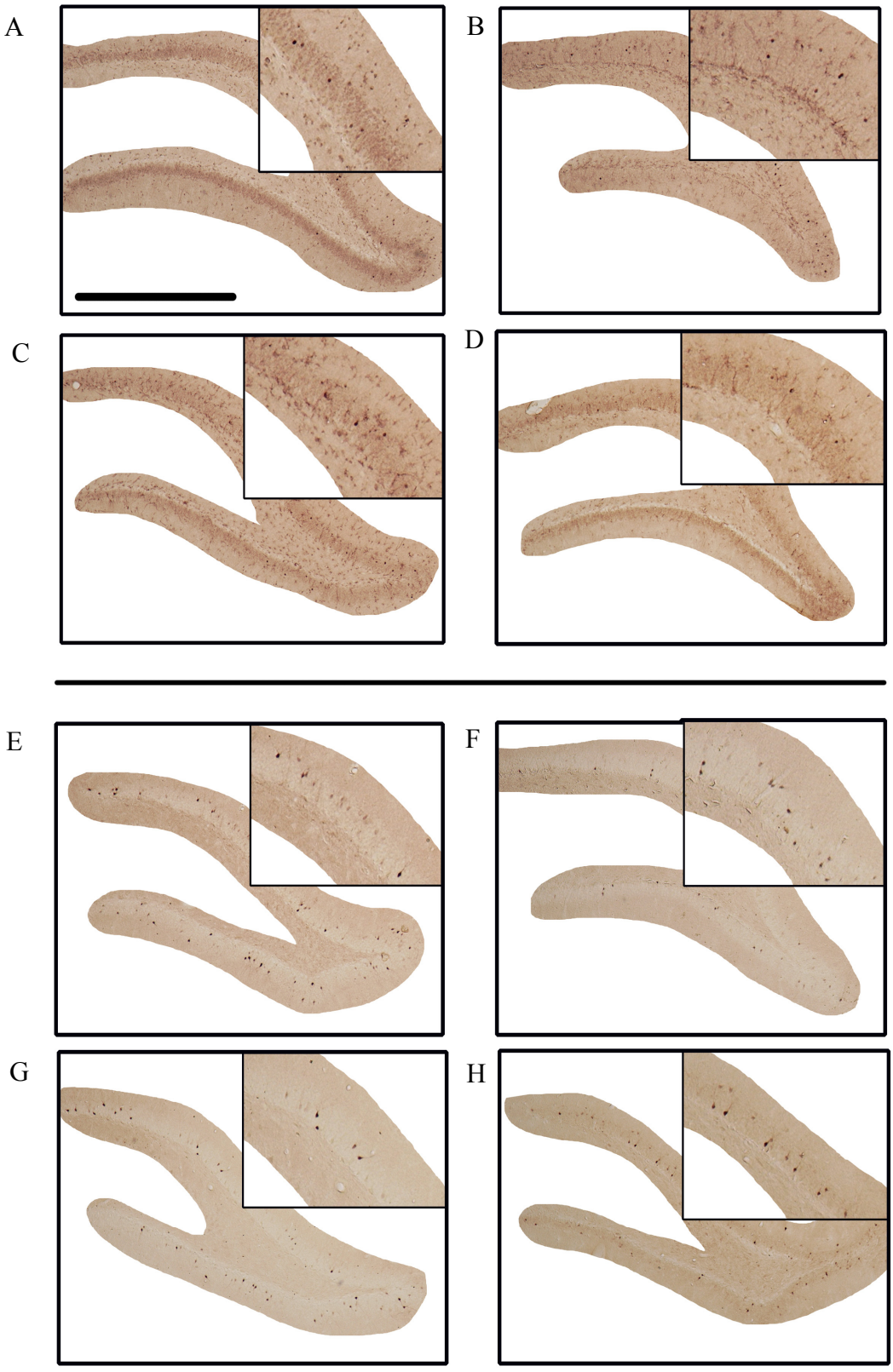

Figure 3.10: Representative images of c-Fos expression in the dentate gyrus for the spatial (A), multiple variable platform (B), single variable platform (C) and free swimming groups (D), and Arc expression in the spatial (E), multiple variable platform (F), single variable platform (G) and free swimming groups (H). Scale bar = 1mm.

3.3.2.1 Correlations with performance.

Finally, to assess the relationship between IEG expression and performance in the water maze, counts of Arc and c-Fos were correlated with mean escape latencies on the final day of training for each group. Significant correlations were not found (see Table 3.1).

Table 3.1: Correlations between IEG expression and escape latencies in the water maze

Region	Condition			
	Spatial	Multiple Variable Platform	Single Variable Platform	Free Swimming
Dentate Gyrus c-Fos	0.05	-0.52	-0.65	0.16
Dentate Gyrus Arc	0.19	-0.25	-0.54	-0.70

3.4 Discussion

The objective of this experiment was to create a novel control condition for the Morris water maze which matched spatially-trained groups on a number of behavioural measures, while not displaying any evidence of learning. By increasing the number of available randomly placed escape platforms by one per day, animals in the new multiple variable platform condition escaped the maze with daily trial times which were almost identical to the spatially-trained group. The distance travelled in the maze was similar to the spatial group for every day, as was the velocity for both groups. Furthermore, this control group did not show signs of additional stress, as measured by thigmotactic behaviour. Place learning did not appear to be evident, as they did not display a preference for a particular quadrant, nor did they spend a disproportionate amount of time returning to where they had located a platform on a previous trial. A particular navigational strategy was not encouraged to complete the task, as was the case with the procedural control devised by Shires and Aggleton (2008). Thus it would appear that this condition is an adequate matched control for learning in the water maze. Having just one variable platform in the pool every day was not sufficient to match the behaviour of spatially-trained rats. Although no evidence of spatial learning was evident in the single variable group either, they took a similar amount of time to locate a platform on every day of training, therefore escape latencies and distance travelled did not decrease significantly. The limitations of the free swimming group as a control was exposed through their much higher levels of thigmotactic behaviour, as they spent the majority of their time swimming around the edges of the pool, which is indicative of stress. Furthermore, their swimming velocity gradually increased as the week progressed.

Despite these clear distinctions in behaviour and learning performance between the experimental and control groups, there were no significant differences found across

groups in either the levels of c-Fos or Arc protein in the dentate gyrus. It is not surprising that differences were not found between the spatial and free swimming groups, as the stress associated with this control condition can increase IEG levels. Shires and Aggleton (2008) did not find any significant difference in hippocampal c-Fos expression between spatial working memory and free swimming groups, whereas levels of Zif268 were actually higher in the free swimming group in this region. c-Fos expression was also higher in the free swimming group in the lateral entorhinal cortex, the perirhinal cortex, and the dorsal subiculum. Furthermore, Teather et al. (2005) did not find statistically significant differences in c-Fos expression between spatially-trained rats in the water maze and yoked swimming controls in area CA1 of the hippocampus. Similarly, in the single variable platform group, with escape latencies almost three times higher than those of the spatial group on the final day of training, one might expect IEG expression to be elevated, and accordingly no differences were found between this group and the spatial group. However as the multiple variable platform group were matched for time with the spatial group, yet did not show any clear evidence of spatial learning, the lack of significant differences between these two groups in terms of hippocampal IEG expression is more difficult to account for.

Selective lesions of the dentate gyrus result in significant disruption to acquisition of the Morris water maze task (Xavier et al., 1999), indicating this region is critical for spatial learning. One possible explanation for the equivalent hippocampal activation in all groups is the existence of incidental learning, in that information about available spatial cues can be processed regardless of whether they are needed to solve a particular task (Ramos, 2010), and that information about one's environment is rapidly and automatically encoded by the hippocampus (R. G. Morris & Frey, 1997). The IEG response associated with exploration of an environment appears to be related to the

spatial features of the environment rather than the activity associated with exploration. Guzowski et al. (2006) used an approach called "cellular compartment analysis of temporal activity by fluorescence in-situ hybridization" (catFISH) to differentiate between nuclear and cytoplasmic *Arc* mRNA, allowing cells activated at two distinct points in time to be visualised during repeated exploration of the same environment. This study revealed 90% of the neurons activated during one exploration session were activated during a subsequent session. However, when this technique was used to differentiate between the explorations of two separate environments, three distinct populations of CA1 neurons displaying *Arc* mRNA emerged from the catFISH analysis. 22% of CA1 neurons contained only cytoplasmic mRNA from exposure to the first environment, 23% contained only intranuclear mRNA from the second exposure, and only 16% of cells contained both cytoplasmic and intranuclear mRNA from both experiences (Guzowski, McNaughton, Barnes, & Worley, 1999), presenting a strong case for the differential neural encoding of space through IEG expression in the absence of any task demands. Therefore the spatial environment may have been automatically encoded by all groups while in the maze. Furthermore, a key feature of the variable platform groups was that they were required to locate multiple escape locations every day, and while this information may not have been useful for subsequent trials, the possibility that these locations were encoded remains. If this is the case, the variable platform groups would have encoded up to 20 possible escape locations in the maze over the course of training.

However, if one is to make the reasonable assumption that hippocampal IEG expression should still be higher in a spatially-trained group than matched controls, as this task requires effortful rather than incidental spatial memory formation, then the use of some kind of spatial or navigational strategy in the variable platform groups cannot be

completely excluded. While successful escape in the spatially-trained group is contingent upon returning to search the same area where the platform was located on the previous trial, the most efficient search strategy for a variable platform group would be to navigate the maze in a systematic manner, avoiding areas which have already been searched on that trial. While such a search strategy could hypothetically be based upon egocentric information, the use of available distal cues to guide the animals search to unexplored areas is a possibility, and this process would be taxing spatial working memory and navigation. The lack of a preference for any particular quadrant or previous escape location, as well as levels of thigmotaxis and swimming velocity equivalent to the spatial group, and an unchanged escape latency across days in the single variable platform group suggest a random swimming pattern in the variable platform groups. However, this data also lends itself to the possibility of a purposeful, systematic and efficient search of the spatial environment which would in theory be easier to accomplish when available distal cues are used to guide it. Unfortunately, it is difficult to differentiate between a purely random search and a systematic one.

This combination of incidental learning and the emergence of spatial strategies to solve control condition tasks could account for both the findings of this study and that of Shires and Aggleton (2008). The noteworthy finding that hippocampal IEG expression was found to be higher in the procedural control groups in that study does suggest that the control task was even more taxing on the hippocampus. Although the pool wall was the most salient proximal cue to guide performance in the procedural control, the emergence of a strategy based on the use of distal cues which may significantly tax spatial navigational abilities cannot be ruled out, and appears to be the most parsimonious explanation for both sets of results. The potential stress of an unpredictable escape location in the variable groups when compared to the fixed location in the spatial group

could also be a factor, although one would assume that by the conclusion of training animals would have habituated to this aspect of the task.

In conclusion, the results of this experiment reflect those of Shires and Aggleton (2008), in that a novel control condition was devised which simulated the behaviour and environmental conditions of spatially-trained water maze rats, but that IEG expression in a key structure involved in the performance of this task did not appear to differentiate between this new control group and the spatially-trained group. This is most likely due to the similarities between experimental and control groups in terms of their experience and performance, rather than to imply the regions of interest are not involved, or that the IEG imaging method is an unreliable marker of neuronal activation. While it is important to use appropriately matched controls to make meaningful interpretations of neuronal activity, where a control condition is so closely matched to its experimental counterpart in terms of its behaviour, it is presumptive to conclude that the cognitive component of the task has not also been simulated in some way. Therefore the similar IEG expression observed between experimental and control groups may arise from similar cognitive processes, and lead to a failure to detect an effect where one is present.

Chapter 4

An Analysis of Immediate Early Gene Expression in Multiple Brain Regions over the Course of Learning in the Morris Water Maze

Abstract

A number of brain regions have been implicated in successful acquisition of the Morris water maze, a test of allocentric spatial memory. While some studies have assessed IEG expression in response to water maze training, a systematic examination of the changes which take place in relevant brain regions over the course of learning has not been carried out. We assessed the expression of Zif268, c-Fos and Arc in 11 brain regions implicated in spatial learning, including the hippocampus, the entorhinal cortex, the retrosplenial, parietal and perirhinal cortices and the medial prefrontal cortex, during early, middle and late training in the water maze. Zif268 was highest during early training in a number of regions, including CA1, the retrosplenial, parietal and perirhinal cortices, and the anterior cingulate and prelimbic cortices. In contrast c-Fos expression increased towards the end of training in the hippocampus, the entorhinal cortex, and the perirhinal and prelimbic cortices. This suggests there are different roles for these IEGs in learning and performance. A large increase in Zif268, c-Fos and Arc expression was observed in the dentate gyrus mid-training, implying its recruitment at this stage. Correlations with performance were observed in the hippocampus towards the end of training, where poorer learners had higher levels of IEGs, suggesting a role for error correction in this region. In summary, changes in IEG expression over the course of learning were observed in a number of brain regions, suggesting a network of structures co-operate in the learning and performance of this task.

4.1 Introduction

IEG imaging techniques have been utilised to study brain activity in a variety of learning paradigms, from one-trial learning such as contextual fear conditioning (Frankland et al., 2004; Hall et al., 2001; Malkani & Rosen, 2000) to multi-trial tasks such as operant conditioning (Kelly & Deadwyler, 2002; Rapanelli et al., 2009; Svarnik et al., 2005). IEGs are also a useful tool for visualising neural activity in response to spatial tasks, as there is evidence from genetic knockout studies that their expression is essential for spatial learning. Zif268 knockout (KO) mice take longer to learn a massed water maze protocol, and do not show significant bias for the target quadrant during retention testing, however this deficit can be overcome by extended training (M. W. Jones et al., 2001). Arc KO mice fail to show any improvement in the late stages of water maze acquisition, and although showing a preference for the platform location during retention, spend significantly less time in the correct location compared to WT mice, and are also slower to re-learn a new platform location (Plath et al., 2006). Mice lacking c-Fos in the entire brain display normal acquisition of the water maze, but spend an equal amount of time in each quadrant during retention, suggesting a consolidation or retrieval impairment (Fleischmann et al., 2003). These results appear to indicate that IEG expression is necessary for the consolidation of spatial learning in the water maze task, highlighting this method as a useful tool for studying neural activity in the experiments in this thesis.

A limited number of studies have used IEG imaging to investigate the contribution of a number of brain regions to spatial memory formation. Teather et al. (2005) found increased c-Fos expression in CA1, CA3 and the dentate gyrus after a single session of 10 trials in the reference memory version of the water maze. Feldman, Shapiro, and Nalbantoglu (2010) also utilised a mass training protocol of 15 session on a single day which resulted in rapid acquisition of the water maze task and robust increases in

Zif268 and *c-Fos* mRNA in the hippocampus when compared to controls. Guzowski et al. (2001) found that levels of *c-Fos*, *Zif268* and *Arc* mRNA were all significantly increased from basal levels in CA1, CA3 and the dentate gyrus of the dorsal hippocampus and the lateral entorhinal cortex in rats trained to locate a fixed hidden platform in the water maze over three days of training. Hippocampal IEG levels were highest after the first session and decreased by the final seventh session, suggesting IEG expression is critical during the early stages of learning. Changing the platform location in the final session increased Arc expression in the hippocampus and lateral entorhinal cortex, indicating the observed increases in hippocampal IEG expression were task-related. This expression appears to be specifically related to the spatial aspect of the task, as rats who do not display behaviourally-induced Arc expression in the hippocampus as a result of fornix lesions learn to approach a cued platform, but display marked impairment when the platform is removed and a spatial strategy is required (Fletcher, Baxter, Guzowski, Shapiro, & Rapp, 2007).

Although the evidence suggests that IEGs are required at particular stages of spatial learning in the water maze, a systematic examination of the changes in IEG expression that take place in relevant brain regions over time has not been carried out. Furthermore IEGs have been shown to be differentially expressed in response to water maze training (Shires & Aggleton, 2008), therefore a direct comparison of different IEGs is required. This systematic type of investigation has been carried out into the radial arm maze, a test of spatial discrimination memory, albeit only using c-Fos as a marker. He et al. (2002) analysed the expression of c-Fos in a number of brain regions on days one, three and five of training in the radial arm maze, and found that on day one of training in this task, the motor cortex and medial septal nucleus displayed increased c-Fos expression. On day three, CA3, the prelimbic, cingulate, somatosensory and motor

cortices showed increased expression, however after five days of training no brain region analysed displayed activation higher than controls. This study emphasises the importance of measuring IEG expression at multiple time-points, as given their role in plasticity, it would be reasonable to assume that their role in learning and consolidation is time-dependent. A similar spatial discrimination training protocol was followed by Poirier, Amin, and Aggleton (2008), where Zif268 expression in response to training in the radial arm maze was assessed during early (two day) or late (five day) training. Although no overall difference was found between spatially-trained and yoked control rats as regards the level of hippocampal IEG activation, during early or late training, the authors found a positive correlation between performance errors and Zif268 expression in the dentate gyrus during early learning, and in CA1 during late learning in the spatial group, suggesting these areas may be involved with error correction at different stages of learning. An opposite pattern was found in CA3, where IEG expression correlated with successful performance during late training. Structural equation modelling also revealed a loss of dentate gyrus efferents and uncoupling of CA3 and CA1 with additional training, whereas yoked controls showed no such pattern. The dynamic changes in activity of a number of brain regions over time observed in these studies suggests that similar changes may also occur over the course of learning in the Morris water maze.

An attempt to map out regional activation over the course of spatial learning in the water maze has been made using a method other than IEG imaging. Conejo, Gonzalez-Pardo, Gonzalez-Lima, and Arias (2010) used cytochrome oxidase histochemistry to chart the changes in metabolic activation of various brain regions on days one, three and five of training in the water maze. Increases from baseline controls were found on day one in the lateral septal nucleus, the anterodorsal and anteroventral thalamic nuclei, the lateral mammillary bodies and the basolateral amygdala. The lateral

septal nucleus and anterodorsal thalamic nucleus continued to show increased activity on days three and five, whereas the prelimbic and infralimbic cortices as well as CA3 showed increased activation on day five only. However the only regions which displayed a change in activity over time were the retrosplenial cortex and the lateral mammillary bodies, which decreased in activity between days one and three. Notably, metabolic activity in CA1, the dentate gyrus or the entorhinal cortex did not appear to be altered by training. These results suggest that measuring metabolic activity in brain regions may not be the optimal method for assessing the changes that take place in relevant brain regions in response to learning.

Immediate early gene imaging offers a significant advantage over a method such as cytochrome oxidase histochemistry, in that it is examining changes in neuronal plasticity rather than merely metabolic activity. While multiple brain regions have been implicated as important in spatial memory formation, at what point during the course of learning changes have been made to these structures has not been clearly defined. This experiment will seek to map out the changes that take place in a number of brain regions during early, middle and late learning in the Morris water maze using IEG expression as a marker of neuronal activity. Although the results of IEG genetic KO studies reveal that spatial performance deficits appear later in training, we hypothesise that IEG expression during early and mid-training is essential for the learning and consolidation of the task, therefore expression should be higher at these stages of learning. Rats will be trained for either one day, three days or five days in the water maze, and IEG expression will be examined at each time point. A range of brain regions implicated in solving the task will be stained for Zif268, c-Fos and Arc, including CA1, CA3, the dentate gyrus, the lateral and medial entorhinal cortex, the retrosplenial, parietal and perirhinal cortices, and the medial prefrontal cortex. The relationship between IEG expression and learning

performance will also be assessed, and it is hypothesised that levels of IEG expression will be positively correlated with time taken to escape the maze, indicating a learning-associated error correction process. Due to the difficulties in the interpretation of IEG levels of matched control conditions (see Chapter 3), this experiment will focus on the changes which occur in the spatially-trained groups over time, and will normalise to naïve controls at each time point to eliminate variations attributable to the staining procedure. Furthermore, to assess the relative contribution of different IEGs to the learning of this task, levels of Zif268, c-Fos and Arc will be compared in the same brain regions. As differential patterns of IEG expression have been observed following spatial training (Shires & Aggleton, 2008), we also hypothesise that all three IEGs analysed in this experiment will display unique patterns of activity.

4.2 Method

4.2.1 Subjects.

Male Wistar rats (n=36) obtained from Charles River Laboratories, UK, were used as subjects in the current study. The age and weight of subjects, housing conditions, handling, and time of experimentation were as described previously in Chapter 2.

4.2.2 Apparatus.

The Morris water maze was the behavioural task used in this study. Dimensions of the apparatus and cue configuration were as described previously in Chapter 2.

4.2.3 Procedure.

Rats were randomly allocated to one of three experimental groups (n=7 per group). All three groups underwent standard water maze training as described in Chapter 2, with a fixed hidden platform in the northeast quadrant of the pool, however the length of training differed for each group. The first group were given just one day of training in the water maze, the second group were given three days training, and the third group were given five days of training. Three naïve control groups (n=5 per group) were also employed at each time-point, to provide a measure of baseline IEG activity and to enable the process of normalisation due to staining variability.

4.2.4 Preservation of tissue.

Ninety minutes after the final trial on the relevant day of training for each spatially-trained group, rats were anaesthetised, transcardially perfused and their brains removed and post-fixed as previously described in Chapter 2. Eleven regions implicated in spatial processing were selected for analysis, as described in Chapter 2. Forty μm coronal

sections were cut on a freezing microtome and every fourth section was taken for analysis.

4.2.5 Immunohistochemistry.

All three water maze trained groups underwent immunohistochemical staining in different sessions. As a result, the use of a baseline control condition was deemed necessary for the purposes of normalisation to control for variations due to staining intensity. The one day, three-day and five-day water maze trained groups were each stained alongside a caged control group. Immunohistochemical protocol for the detection of Zif268, c-Fos and Arc protein was followed as described previously in Chapter 2.

4.2.6 Data analysis.

4.2.6.1 Behavioural data.

To measure learning performance in the water maze, escape latencies for each trial were calculated. For the one day group, escape latencies across four trials were analysed. For the three day and five day groups, escape latencies were averaged to produce a mean for every animal for each day, and these were analysed across the five days.

4.2.6.2 IEG data.

Images were taken of the 11 regions of interest using a digital camera (Olympus DP12) using the method outlined in Chapter 2. In the case of Arc protein, only the dentate gyrus was sampled in this experiment as staining was found to be sparse or absent in other regions. Computerised counts of Zif268, c-Fos and Arc positive neurons were analysed using the public domain program ImageJ (NIH) as previously described in Chapter 2. As each spatially-trained group and their corresponding caged control group were stained on

separate days, a normalisation procedure was followed. For each animal in the caged control groups a mean count for each region was calculated by dividing the total number of Zif/c-Fos/Arc positive neurons by the number of consecutive sections. This was then averaged to provide an overall group mean for the caged control group for that region. Next, for each animal in the spatially-trained groups, a mean count for each region was calculated by dividing the total number of Zif/c-Fos/Arc positive neurons by the number of consecutive sections. For each animal in the spatially-trained group this value was now expressed as a percentage increase or decrease from the mean of the caged control group, using the following formula:

$$\frac{\text{Spatially-trained individual mean}}{\text{Caged control group mean}} \times \frac{100}{1}$$

This procedure resulted in a percentage value for each rat for every region in all three spatially-trained conditions which could now be used to directly compare the three spatially-trained groups as it removed variations in the raw values attributable to staining intensity. (Shires & Aggleton, 2008).

4.2.7 Statistical analysis.

To analyse escape latencies, one-way repeated measures analyses of variance (ANOVA) were performed, with Bonferroni-corrected comparisons. To compare levels of Zif268, c-Fos and Arc across time-points, one-way analyses of variance (ANOVA) on normalised values were performed with Tukey *post-hoc* comparisons. To compare levels of Zif268, c-Fos and Arc between experimental and control groups at particular time points, independent samples t-tests were performed on raw values. To assess relationships between levels of Zif268, c-Fos and Arc and performance in the water maze, Pearson product-moment correlations were performed.

4.2.8 Ethical considerations.

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children (Ireland) guidelines under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

4.3 Results

4.3.1 Behavioural results.

When the trial escape latencies for animals trained for just one day were analysed, they decreased from 45.17±9.58 seconds on trial one to 17.86±3.84 seconds on trial four (see Figure 4.1A), however a one-way repeated measures ANOVA did not reveal any difference across the four trials, $F(3, 18) = 2.11$, $p > 0.05$. In the three day group, a significant effect was found across days, $F(2, 12) = 7.98$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing escape latencies decreased significantly from day one (*M*: 42.84±5.25) to day three (*M*: 22.57±3.04, $p < 0.05$, see Figure 4.1B). In the five day group, escape latencies decreased significantly over the course of training, $F(4, 24) = 3.95$, $p < 0.05$, with Bonferroni *post-hoc* analyses revealing escape latencies on day five (*M*: 15.52±1.30) were significantly lower than day one (*M*: 30.83±3.37, $p < 0.01$, see Figure 4.1C).

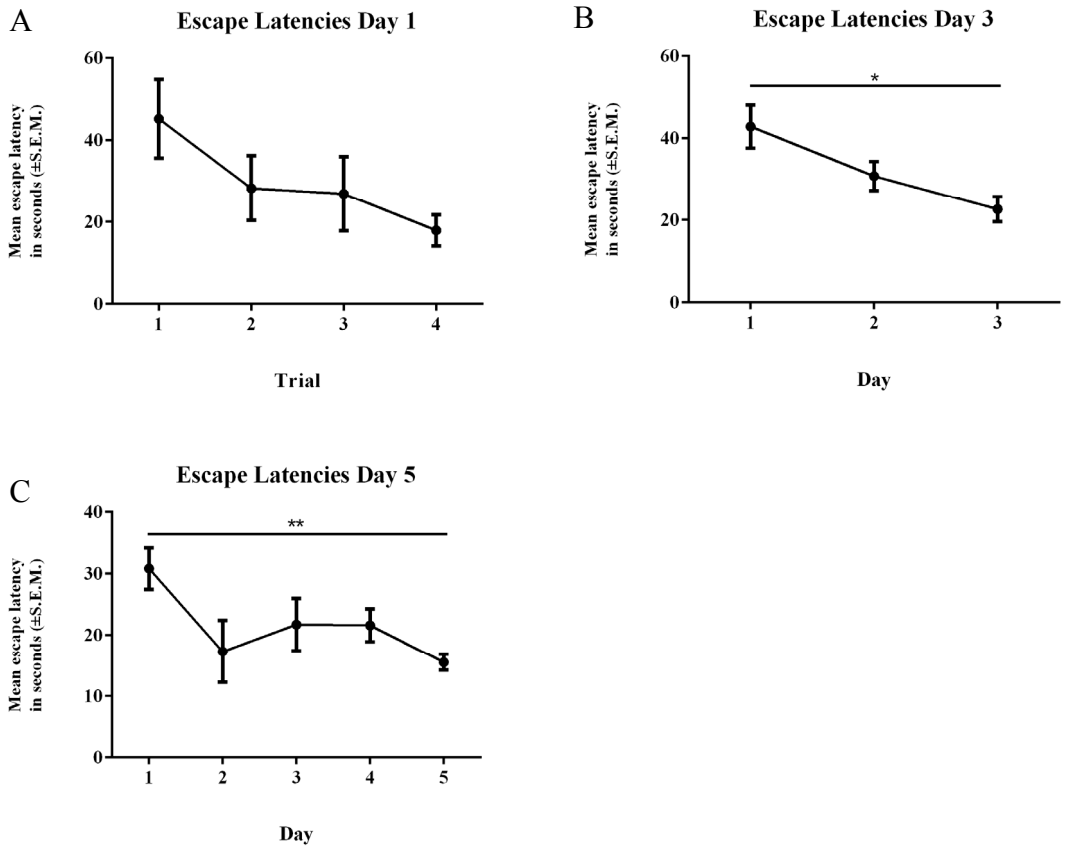

Figure 4.1: Trial escape latencies over one day of training in the one day group (A), mean daily escape latencies over three days of training in the three day group (B), and over five days of training in the five day group (C)

4.3.2 IEG results.

4.3.2.1 Zif268.

The expression of Zif268 was first examined across the three time-points, with a series of one-way ANOVAs with Tukey *post-hoc* tests performed on normalised values. Beginning with the hippocampal formation, a significant difference was found across groups in CA1, $F(2, 17) = 5.11$, $p < 0.05$, with Tukey *post-hoc* tests revealing Zif268 levels were significantly higher on day one (*M*: 955.41±271.80 %) than days three (*M*: 263.60±42.51 %, $p < 0.05$) and five (*M*: 250.85±92.51 %, $p < 0.05$, see Figure 4.2A). No difference across the three groups was found in CA3, $F(2, 15) = 0.58$, $p > 0.05$, see Figure 4.2B. A main effect for group was found in the dentate gyrus, $F(2, 16) = 75.10$, $p < 0.001$,

with Tukey *post-hoc* analyses showing Zif268 was significantly increased on day three (*M*: 447.12±34.79 %) compared to days one (*M*: 72.53±16.47 %, $p < 0.001$), and five (*M*: 58.88±16.69 %, $p < 0.001$, see Figure 4.2C and Figure 4.6ABC). A difference across groups was found in the lateral entorhinal cortex, $F(2, 17) = 11.75$, $p < 0.001$, with Tukey *post-hoc* analyses showing an increase in Zif268 expression from day one (*M*: 60.13±16.78 %) to days three (*M*: 230.57±25.95, $p < 0.001$) and five (*M*: 153.39±26.62 %, $p < 0.05$, see Figure 4.2D). A difference across training days was also found in the medial entorhinal cortex, $F(2, 16) = 4.74$, $p < 0.05$, with Tukey *post-hoc* analyses revealing levels of Zif268 increased from day three (*M*: 214.41±47.25 %) to day five (*M*: 477.14±21.80 %, $p < 0.05$, see Figure 4.2E).

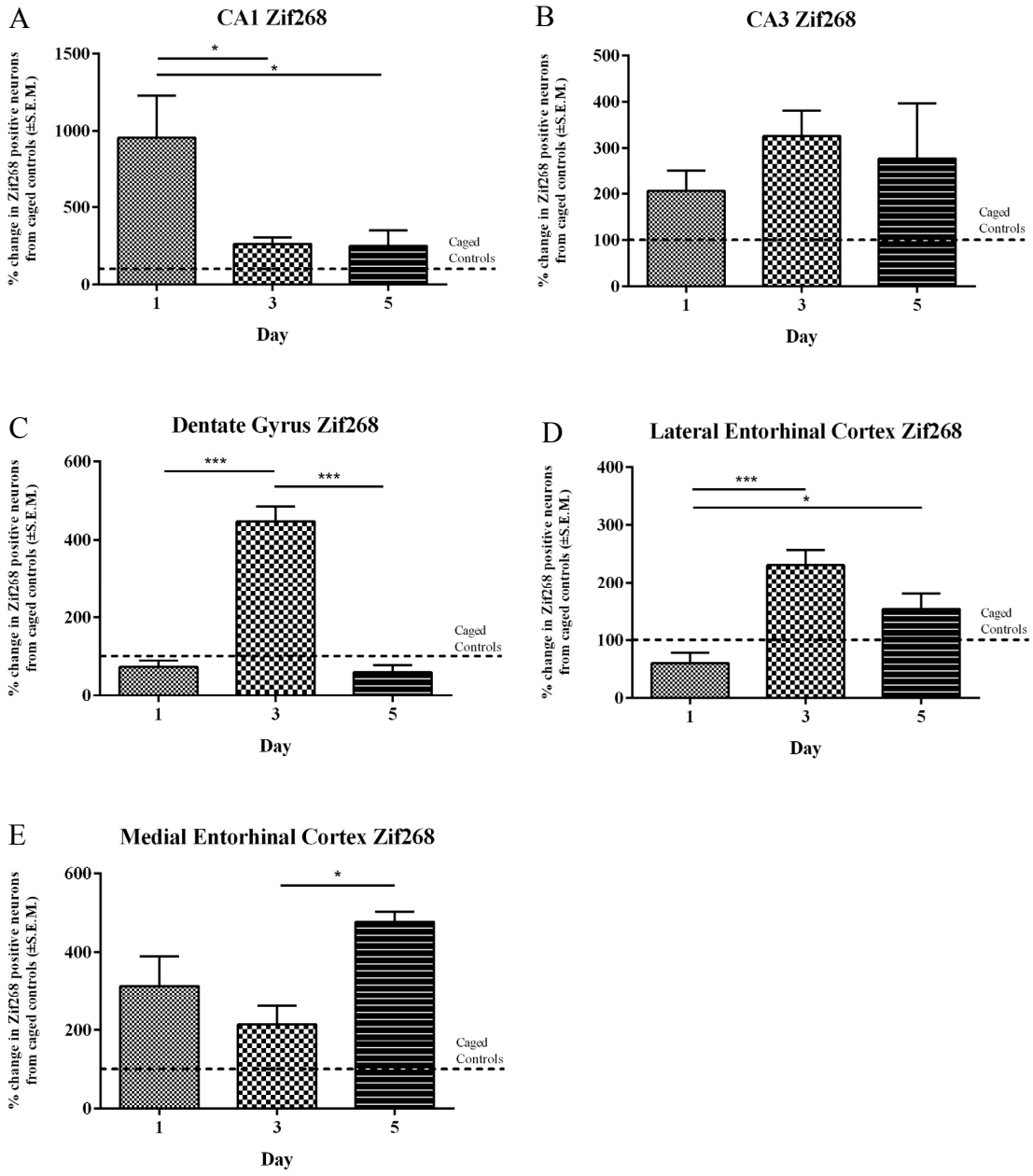

Figure 4.2: Changes in the level of Zif268 expression across one, three, and five days of training in the water maze in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E). Values are expressed as a percentage change from caged control levels at each time-point.

Changes in the level of Zif268 expression across days of training were also analysed in the retrosplenial, perirhinal and parietal cortices. A significant effect between groups was found in the retrosplenial cortex, $F(2, 17) = 20.23$, $p < 0.001$, with Tukey *post-hoc* analyses revealing levels of Zif268 were significantly higher on day one (*M*: 4791.05±959.93 %) than day three (*M*: 257.29± 22.24 %, $p < 0.001$) and day five (*M*: 306.22±73.42 %, $p < 0.001$, see Figure 4.3A). A significant effect across groups was also found in the perirhinal cortex, $F(2, 17) = 104.35$, $p < 0.001$, with Tukey *post-hoc* tests revealing a significant decrease from day one (*M*: 3640.85±308.29 %) to day three (*M*: 393.48±36.59 %, $p < 0.001$) and day five (*M*: 400.89±71.89 %, $p < 0.001$, see Figure 4.3B and Figure 4.6GHI). A difference across groups was also found in the parietal cortex, $F(2, 17) = 73.13$, $p < 0.001$, with Tukey *post-hoc* analyses revealing levels of Zif268 were significantly higher on day one (*M*: 5067±537.55 %) than day three (*M*: 239.29±28.29 %, $p < 0.001$) and day five (*M*: 249.05±62.64 %, $p < 0.001$, see Figure 4.3C).

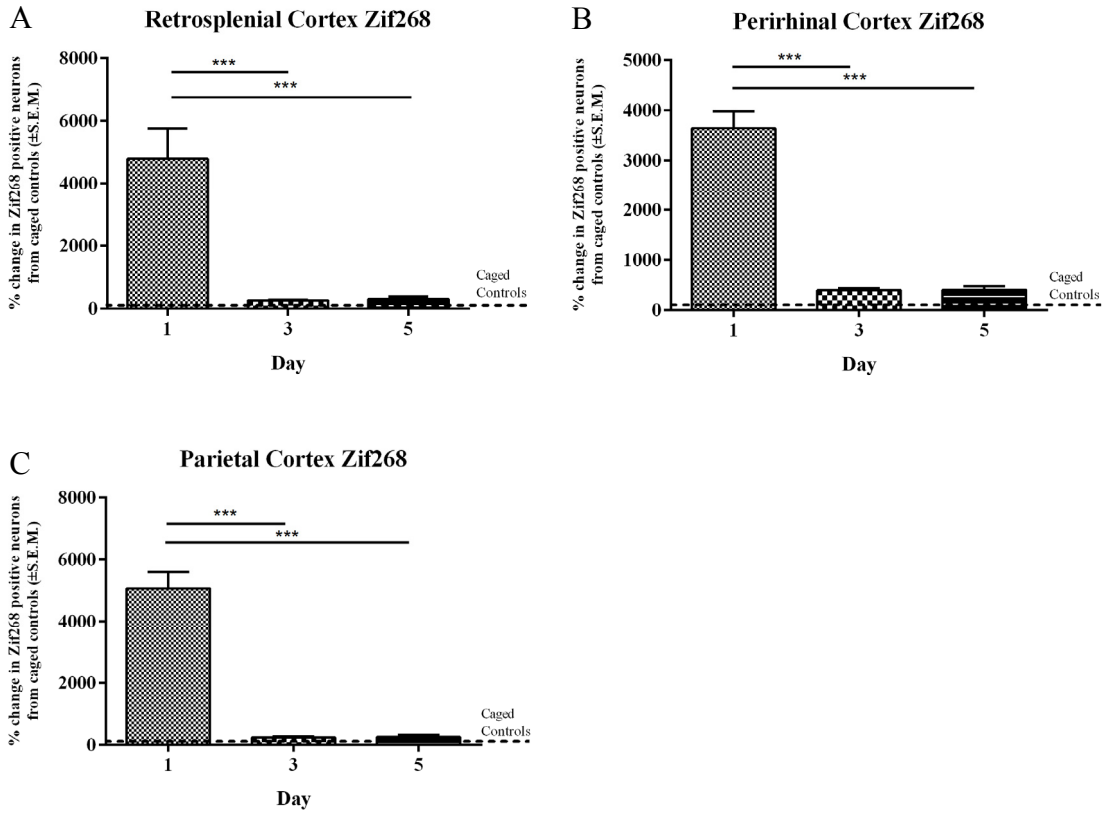

Figure 4.3: Changes in the level of Zif268 expression across one, three, and five days of training in the water maze in the retrosplenial cortex (A), perirhinal cortex (B) and parietal cortex (C). Values are expressed as a percentage change from caged control levels at each time-point.

The medial prefrontal cortex and its subregions were also analysed. A significant effect was found between groups in the anterior cingulate cortex, $F(2, 16) = 13.70$, $p < 0.001$, with levels of Zif268 significantly increased on day one (*M*: 1115.17±256.85 %) compared to day three (*M*: 293.51±22.52 %, $p < 0.01$) and day five (*M*: 112.64±26.79 %, $p < 0.001$, see Figure 4.4A and Figure 4.6MNO). A significant difference was also found across groups in the prelimbic cortex, $F(2, 16) = 11.09$, $p < 0.001$, with *post-hoc* Tukey tests revealing levels of Zif268 decreased from day one (*M*: 668.54±157.83 %) to day three (*M*: 298.70±16.74 %, $p < 0.05$) and day five (*M*: 74.96±24.07 %, $p < 0.001$, see Figure 4.4B). Levels of Zif268 also changed across days of training in the infralimbic cortex, $F(2, 16) = 4.72$, $p < 0.05$, with Tukey post-tests revealing levels of Zif268

increased from day 1 (*M*: 84.65±23.25 %) to day 3 (*M*: 194.67±24.79 %, $p < 0.05$, see Figure 4.4C).

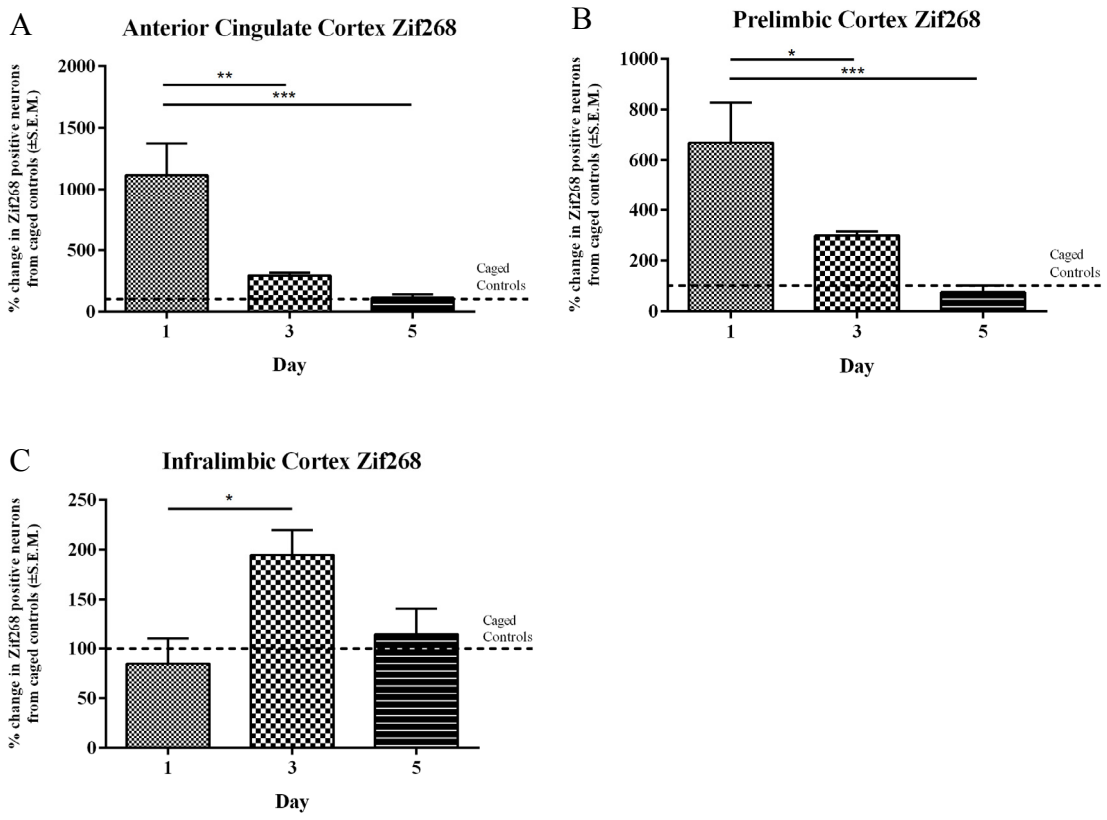

Figure 4.4: Changes in the level of Zif268 expression across one, three, and five days of training in the water maze in the anterior cingulate cortex (A), the prelimbic cortex (B) and the infralimbic cortex (C). Values are expressed as a percentage change from caged control levels at each time-point.

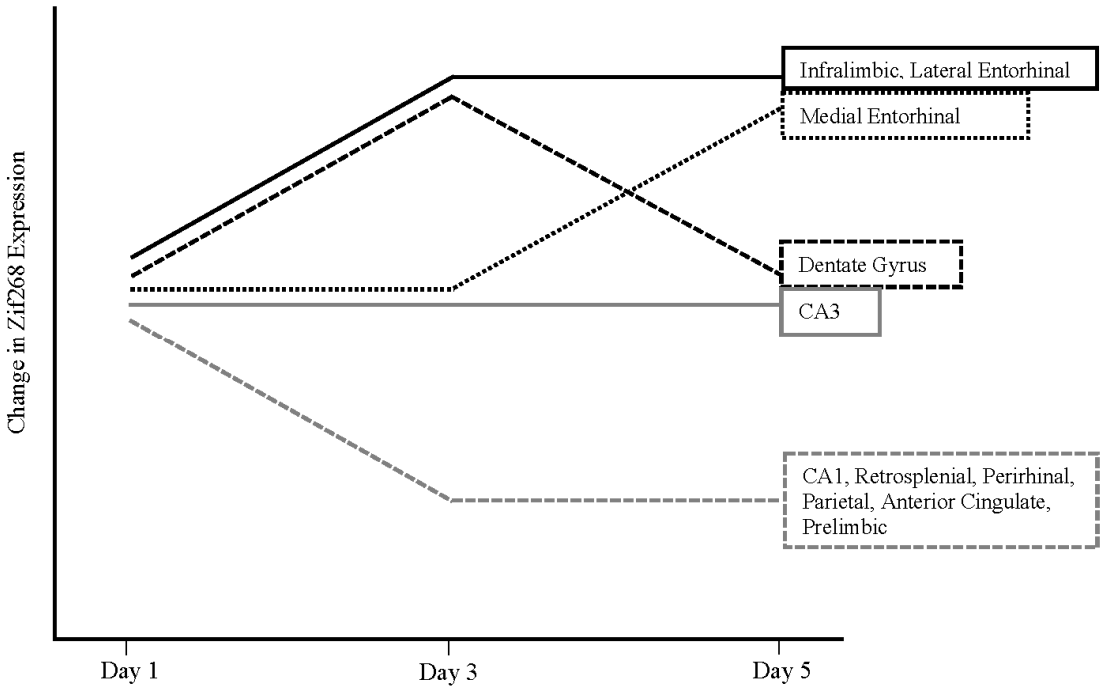

Figure 4.5: Schematic diagram summarising the changes in Zif268 expression in all brain regions analysed over days one, three and five of water maze training. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

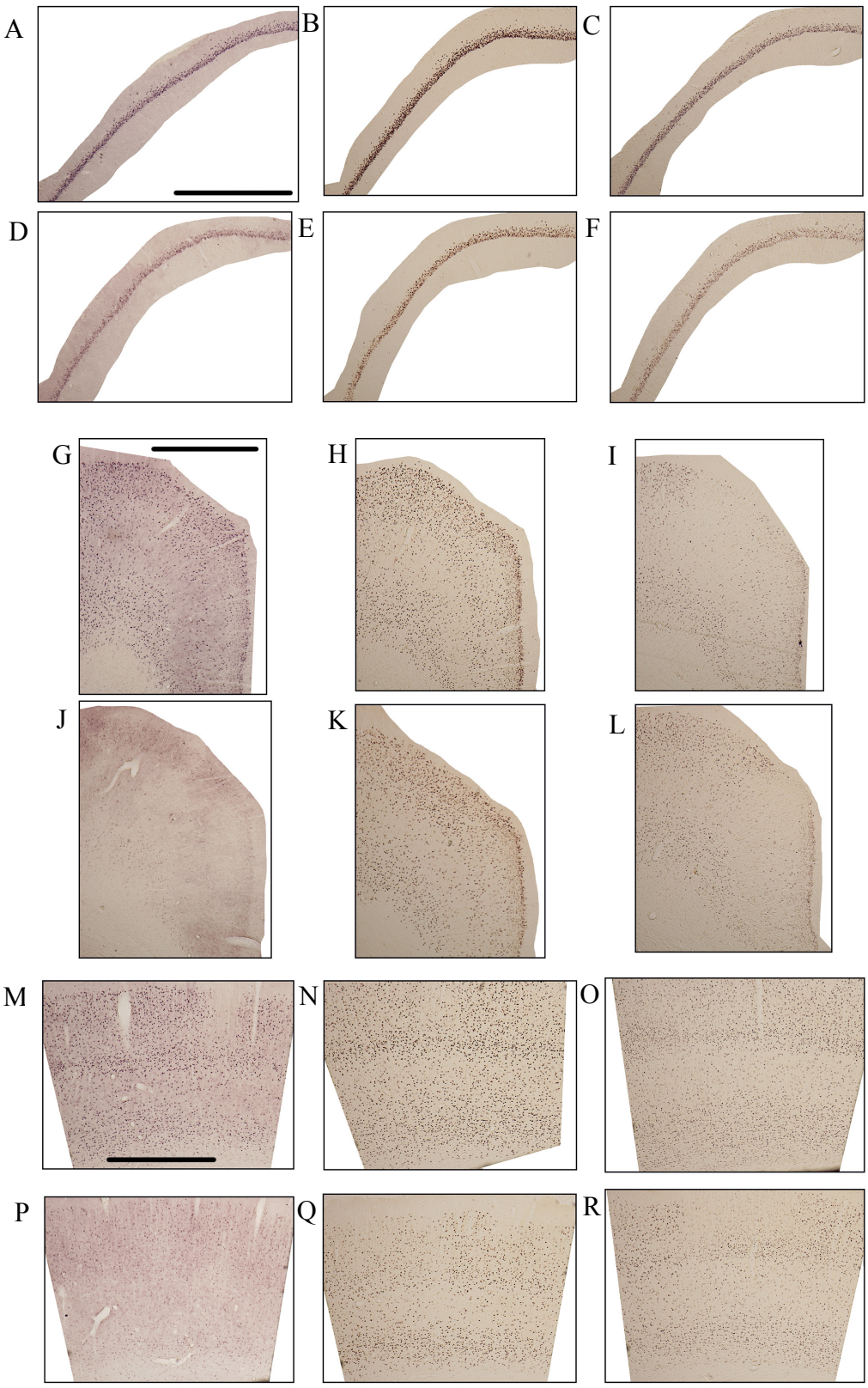

Figure 4.6: Representative images of Zif268 expression in selected regions: CA1 on days one, three and five (A-C) and respective caged controls (D-F), the retrosplenial cortex (G-I) and corresponding caged controls (J-L), and the parietal cortex (M-O), and caged controls (P-R). Scale bar = 1mm.

4.3.2.1.1 Correlations with performance.

To assess the relationship between levels of Zif268 and performance in the water maze, counts were correlated with mean escape latencies on the final day of training for each group. Significant positive correlations were found with performance in the five day group in area CA3 ($r = 0.85$, $p < 0.05$) and the dentate gyrus ($r = 0.82$, $p < 0.05$), as well as the prelimbic cortex ($r = 0.85$, $p < 0.05$, see Table 4.1).

Table 4.1: Correlations between Zif268 expression and escape latencies in the water maze

Region	Training duration		
	Day 1	Day 3	Day 5
CA1	-0.17	-0.57	0.20
CA3	-0.51	-0.60	0.85*
Dentate Gyrus	-0.58	0.25	0.82*
Lateral Entorhinal Cortex	0.11	-0.05	-0.53
Medial Entorhinal Cortex	0.74	-0.20	0.75
Retrosplenial Cortex	0.31	-0.18	-0.09
Perirhinal Cortex	0.50	0.05	-0.19
Parietal Cortex	0.20	0.28	0.36
Anterior Cingulate Cortex	-0.12	0.08	0.26
Prelimbic Cortex	0.09	0.23	0.85*
Infralimbic Cortex	0.16	-0.48	0.06

4.3.2.2 c-Fos.

A significant difference was found in the expression of c-Fos protein across groups in CA1, $F(2, 17) = 4.56$, $p < 0.05$, with Tukey *post-hoc* analyses revealing levels of c-Fos were higher on day five (*M*: 1635±727.91 %) than day one (*M*: 42.86±20.20 %, $p < 0.05$, see Figure 4.7A). A significant difference was also found in CA3, $F(2, 15) = 9.05$, $p <$

0.05, with Tukey *post-hoc* analyses revealing levels of c-Fos increased from day one (*M*: 41.21±14.57 %) to day three (*M*: 581.19±110 %, $p < 0.05$) and day five (*M*: 842.44±207.44 %, $p < 0.01$, see Figure 4.7B). A main effect for group was also found in the dentate gyrus, $F(2, 18) = 41.24$, $p < 0.001$, with Tukey *post-hoc* analyses revealing levels of c-Fos on day three (*M*: 356.73±33.46 %) were higher than day one (*M*: 13.15±2.95 %, $p < 0.001$) and day five (*M*: 177.56 %, $p < 0.001$), with the five day group also showing higher levels of c-Fos than the one day group ($p < 0.01$, see Figure 4.7C and Figure 4.11ABC). A difference between groups was also found in the lateral entorhinal cortex, $F(2, 16) = 23.77$, $p < 0.001$, with levels of c-Fos decreasing from day one (*M*: 1011.76±61.28 %) to day three (*M*: 347.74±39.33, $p < 0.001$) and day five (*M*: 627.12±83.48 %, $p < 0.01$) c-Fos expression also increased in this region from day three to day five ($p < 0.05$, see Figure 4.7D). There was also a difference found across groups in the medial entorhinal cortex, $F(2, 15) = 5.481$, $p < 0.05$, with Tukey *post-hoc* analyses revealing an increase in c-Fos expression from day one (*M*: 332.58±107.11 %) to day five (*M*:1094.37±248.17 %, $p < 0.01$, see Figure 4.7E and Figure 4.11GHI).

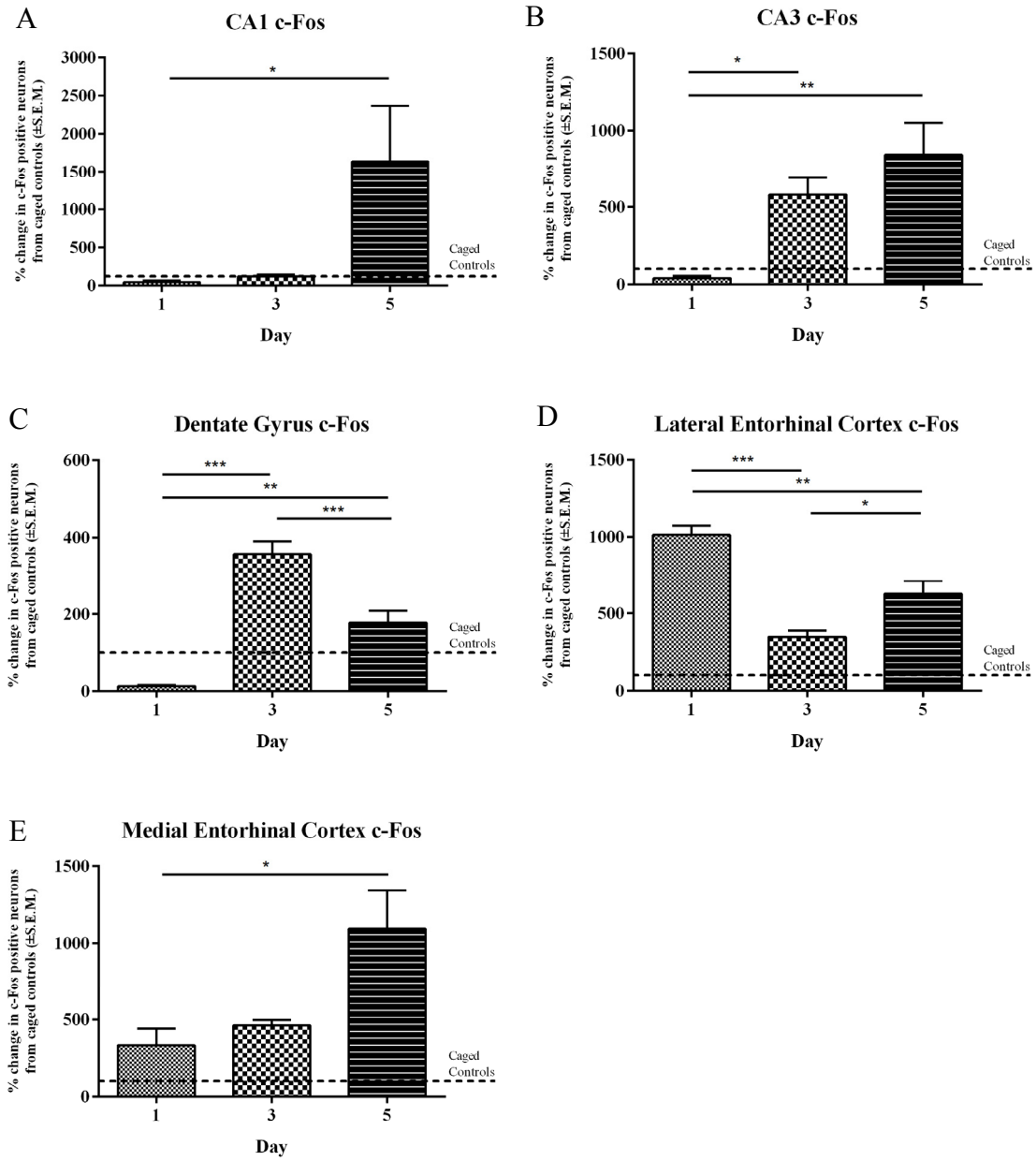

Figure 4.7: Changes in the level of c-Fos expression across one, three, and five days of training in the water maze in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E). Values are expressed as a percentage change from caged control levels at each time-point.

The three cortical regions of the retrosplenial, perirhinal and parietal cortices were then compared across groups. No difference was found between the groups in the retrosplenial cortex, $F(2, 15) = 1.60$, $p > 0.05$, see Figure 4.8A. A significant difference was found in the perirhinal cortex, $F(2, 15) = 6.48$, $p < 0.01$, with Tukey *post-hoc* analyses revealing that levels of c-Fos on day three (*M*: 472.91± 77.37 %) were lower than day one (*M*: 1000.61±123.01 %, $p < 0.05$) and day five (*M*: 997.72±150.22 %, $p < 0.05$, see Figure 4.8B). c-Fos expression in the parietal cortex did not change significantly across training, $F(2, 15) = 3.46$, $p < 0.05$, see Figure 4.8C)

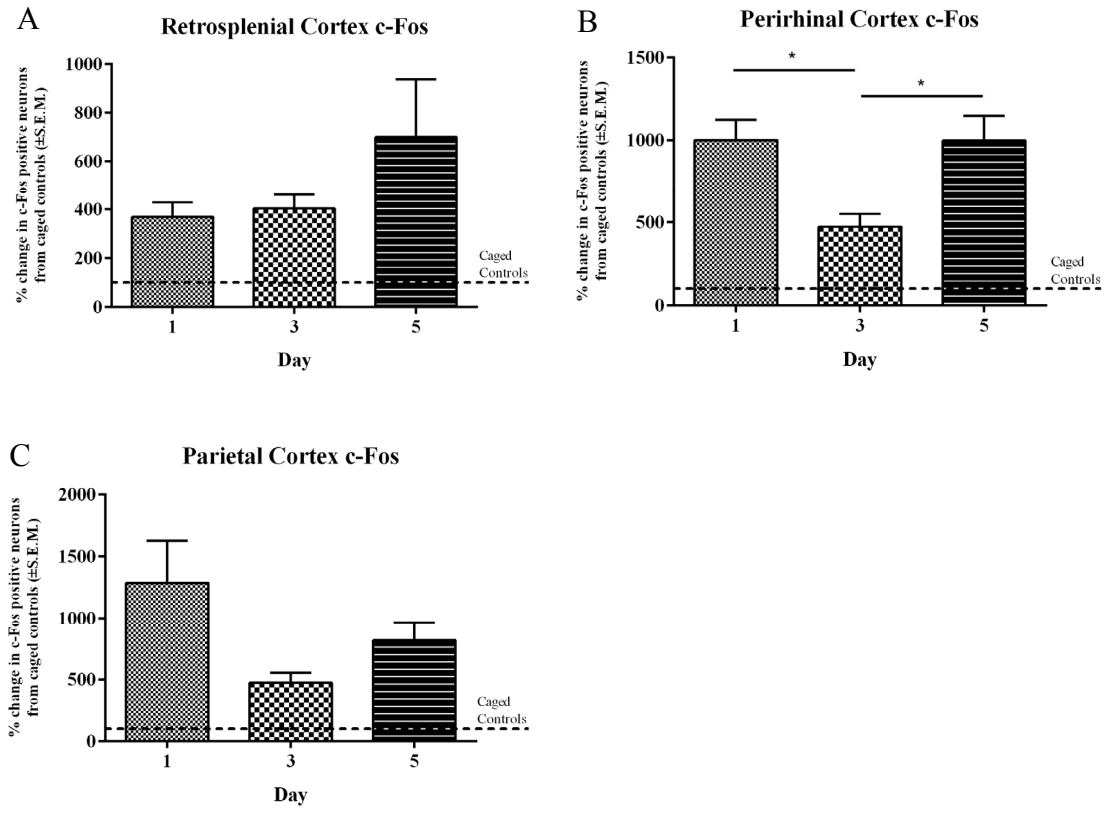

Figure 4.8: Changes in the level of c-Fos expression across one, three, and five days of training in the water maze in the retrosplenial cortex (A), the perirhinal cortex (B) and the parietal cortex (C). Values are expressed as a percentage change from caged control levels at each time-point.

Finally, the three medial prefrontal regions were analysed. A significant difference was not found in the levels of c-Fos in the anterior cingulate cortex between groups, $F(2, 15) = 0.65$, $p > 0.05$, see Figure 4.9A. c-Fos expression did change across days in the prelimbic cortex, $F(2, 15) = 7.77$, $p < 0.01$, with *post-hoc* Tukey analyses revealing levels of c-Fos expression were significantly higher on day five (*M*: 1586±431.04 than day one (*M*: 405.88±171.61 %, $p < 0.05$) and day three (*M*: 166.39±35.10 %, $p < 0.01$, see Figure 4.9B and Figure 4.11MNO). A main effect for group was also found in the infralimbic cortex, $F(2, 17) = 9.97$, $p < 0.001$, with Tukey *post-hoc* tests revealing levels of c-Fos expression were significantly higher on day five (*M*: 840.56±200.40 %) than day one (*M*: 145.69±41.59 %, $p < 0.01$) and day three (*M*: 182.58±27.62 %, $p < 0.01$, see Figure 4.9C).

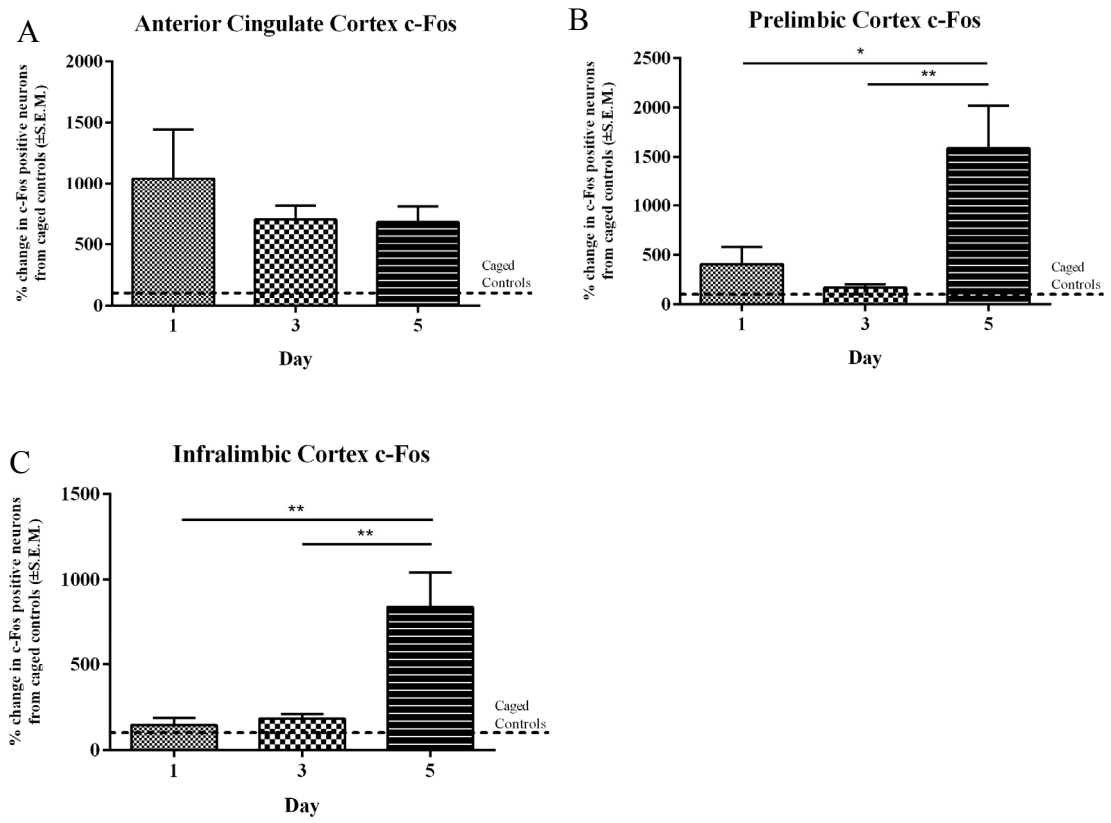

Figure 4.9: Changes in the level of c-Fos expression across one, three, and five days of training in the water maze in the anterior cingulate cortex (A), the prelimbic cortex (B) and the infralimbic cortex (C). Values are expressed as a percentage change from caged control levels at each time-point.

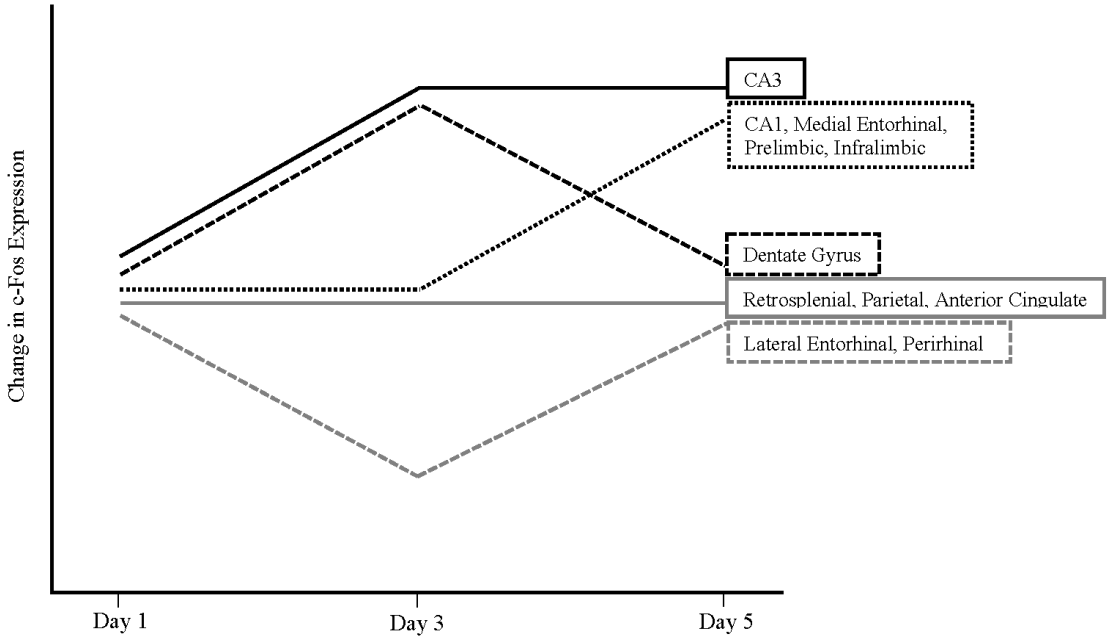

Figure 4.10: Schematic diagram summarising the changes in c-Fos expression in all brain regions analysed over days one, three and five of water maze training. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

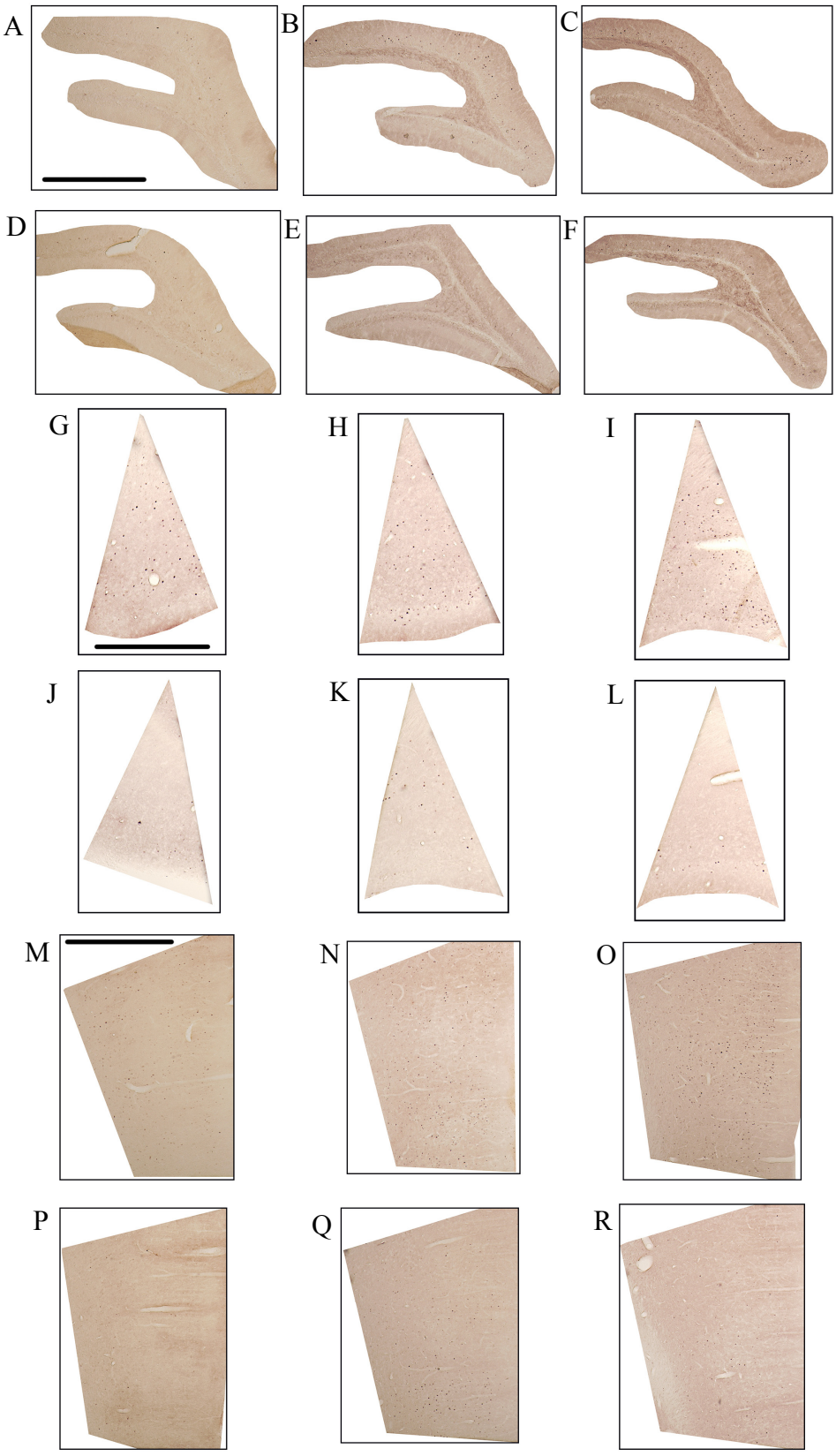

Figure 4.11: Representative images of c-Fos expression in selected regions: the dentate gyrus on days one, three and five (A-C) and respective caged controls (D-F), the medial entorhinal cortex (G-I) and corresponding caged controls (J-L), and the prelimbic cortex (M-O), and caged controls (P-R). Scale bar = 1mm.

4.3.2.2.1 Correlations with performance.

After one day of training, significant positive correlations were found between c-Fos expression and escape latency in the retrosplenial cortex ($r = 0.90$, $p < 0.05$) and the parietal cortex ($r = 0.91$, $p < 0.05$). In the three day group, a significant negative correlation was found between CA1 and escape latency ($r = -0.89$, $p = 0.01$). In the five day group, a significant positive correlation with escape latency was found in the dentate gyrus only ($r = 0.82$, $p < 0.05$, see Table 4.2).

Table 4.2: Correlations between c-Fos expression and escape latencies in the water maze

Region	Training duration		
	Day 1	Day 3	Day 5
CA1	0.36	-0.89**	-0.34
CA3	-0.15	-0.53	-0.22
Dentate Gyrus	-0.24	0.57	0.82*
Lateral Entorhinal Cortex	-0.35	0.49	-0.19
Medial Entorhinal Cortex	0.37	-0.82	-0.05
Retrosplenial Cortex	0.90*	-0.47	-0.27
Perirhinal Cortex	-0.28	0.75	-0.50
Parietal Cortex	0.91*	0.63	-0.62
Anterior Cingulate Cortex	0.75	-0.48	-0.31
Prelimbic Cortex	0.73	0.26	0.10
Infralimbic Cortex	0.56	-0.59	-0.28

4.3.2.3 Arc.

A significant difference across training days was found for levels of Arc expression in the dentate gyrus, $F(2, 18) = 8.90$, $p < 0.01$, with Tukey *post-hoc* analyses revealing Arc was more strongly expressed on day three (M: 356.73±33.46 %, $p < 0.01$) and day five (M: 177.56±31.93, $p < 0.01$) than day one (M: 13.15±2.95 %, see Figure 4.12)

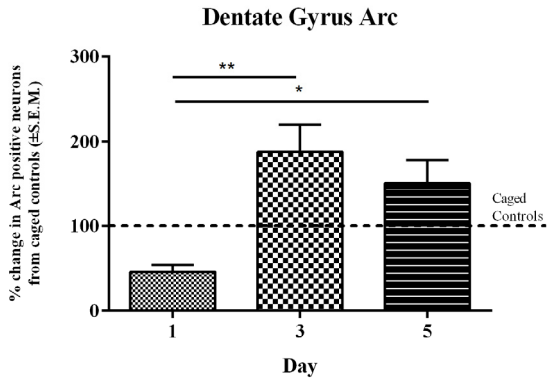

Figure 4.12: Changes in the level of Arc expression across one, three, and five days of training in the water maze in the dentate gyrus. Values are expressed as a percentage change from caged control levels at each time-point.

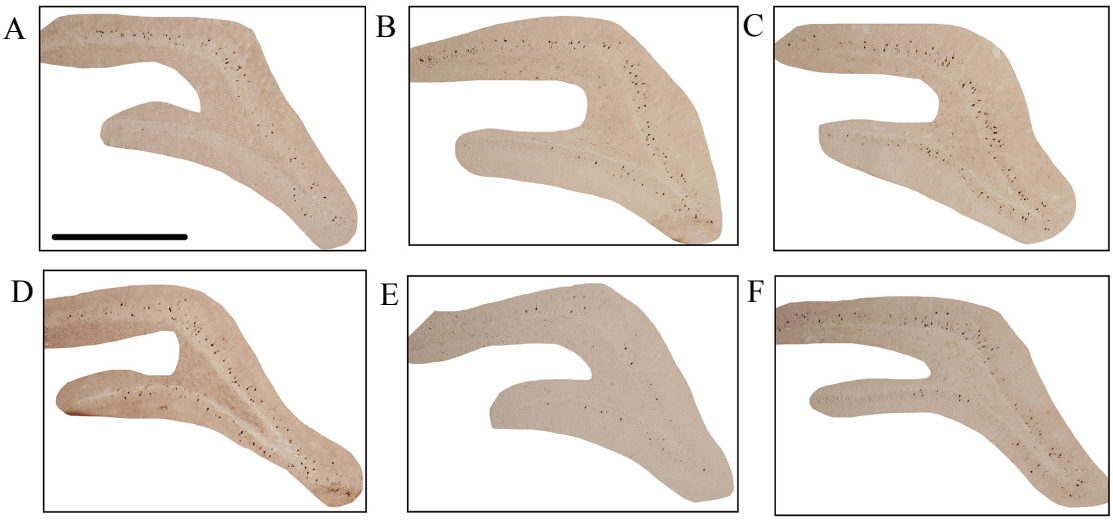

Figure 4.13: Selected representative images of Arc expression in the dentate gyrus on days one, three and five of training (A-C) and respective caged controls (D-F). Scale bar = 1mm.

4.3.2.3.1 Correlations with performance.

After five days of training, a significant positive correlation was found between levels of Arc expression in the dentate gyrus and escape latency ($r = 0.94$, $p < 0.01$, see Table 4.3).

Table 4.3: Correlations between Arc expression and escape latencies in the water maze

Region	Training duration		
	Day 1	Day 3	Day 5
Dentate Gyrus	-0.48	0.62	0.94**

4.4 Discussion

The objective of this experiment was to chart the changes in expression of IEGs in a number of brain regions in response to different stages of learning in the Morris water maze. All three groups displayed learning or improved performance over the course of training in the task. Changes in IEG expression were noted across a number of brain regions, and these appeared to differ across IEGs.

Beginning with Zif268, the general trend was that expression was highest during early learning, although this was not the case for all regions examined. Zif268 expression was highest in CA1 on day one of training, and this is consistent with the role of this protein in the stabilisation of place cells in this region. Renaudineau, Poucet, Laroche, Davis, and Save (2009) found that mice lacking Zif268 failed to reactivate the same place cells in CA1 24 hours following spatial exploration, when compared to wild-type controls. The retrosplenial, perirhinal and parietal cortices all displayed dramatic increases in Zif268 expression on day one of training when compared with day three and day five. The finding that retrosplenial lesions result in impairment of water maze acquisition (van Groen et al., 2004), combined with the observation that Zif268 expression is markedly decreased in this region following lesions to the hippocampus (Albasser, Poirier, Warburton, & Aggleton, 2007), suggest that Zif268 expression in this region is important during water maze acquisition, and it may play a particular important role at the beginning of spatial memory formation. The significant increase in Zif268 expression in this area during early training also reflect the findings of Conejo et al. (2010), where activity of the retrosplenial cortex decreased from day one to three of training in the water maze. Although the parietal cortex appears to be more engaged in the processing of proximal rather than distal landmarks (Save & Poucet, 2000), it has been proposed that this region is involved in the integration of spatial and motion

information (Save & Poucet, 2009), thus important for establishing a spatial representation of an environment, and this would presumably occur early in training. Lesions to the perirhinal cortex do not completely disrupt acquisition of the Morris water maze, but transient deficits can be seen during the initial one or two training sessions (Aggleton et al., 2004), suggesting it may be involved during early learning, consistent with the increase in Zif268 expression observed on day one. The anterior cingulate cortex and the prelimbic cortex also displayed maximal expression of Zif268 on day one, which subsequently declined over the course of training. This reflects the findings of Woolley et al. (2013), who showed that Zif268 expression was higher during early (three day) compared to late (30 day) learning in the medial prefrontal cortex of mice trained in the Morris water maze. In both cases IEG expression was higher than free swimming groups, suggesting this region plays a more important role in place learning than previously thought. Other regions in the current experiment displayed a delayed increase in Zif268, reaching their peak during mid-training. These regions included the dentate gyrus, the lateral entorhinal cortex and the infralimbic cortex. Given the known importance of the dentate gyrus (Xavier et al., 1999) and the entorhinal cortex (E. I. Moser & Moser, 2008) in place learning and spatial processing, this suggests that the formation of a spatial strategy emerges during mid-training. The similar pattern of expression in the infralimbic cortex during mid-training suggests it may also be involved in this process. The medial entorhinal cortex was the only area studied which revealed an increase during late training. However other regions showed a qualitative difference in expression towards the end of training, with CA3, the dentate gyrus, and the prelimbic cortex being positively correlated with escape latencies on the final day of training. In summary, the general decrease in Zif268 expression in key regions over the course of learning and mastery of the spatial task implicates this IEG in plasticity during early learning.

The analysis of c-Fos expression over the course of learning yielded an entirely different set of results for most regions studied. A number of regions displayed an increase rather than a decrease over the three time-points, culminating in maximum expression by the end of training. Area CA1, CA3, the dentate gyrus, the medial entorhinal cortex, the prelimbic cortex and the infralimbic cortex all showed significant increases at day five compared to day one. The marked increase in the hippocampal formation, compared to the lack of any significant between early and late training in areas such as the retrosplenial cortex, the perirhinal cortex and the parietal cortex, all areas which seem to play a minor role in spatial learning, suggest that towards the end of training c-Fos is being maximally expressed in areas which are related to the successful performance of the task. The most notable finding was the mid-training increase in c-Fos expression in the dentate gyrus, as this discovery was identical to the pattern observed with Zif268, further emphasising this regions role during this stage of learning. However c-Fos expression was still higher in this region on day five compared to day one, therefore the only region in the hippocampal formation in which IEG expression decreased from day one to day five was the lateral entorhinal cortex, although this region still displayed an increase from day three to day five. Taken together, these results indicate that c-Fos expression increased in critical brain regions as learning improved over time, suggesting it is a useful marker of successful performance. Correlations between c-Fos expression and performance were seen in mid to late training in CA1 and the dentate gyrus respectively, further emphasising the relationship between expression at these stages and learning performance. Although the amount of c-Fos expression did not change over the course of training in the retrosplenial and parietal cortex, there were positive correlations with escape latency at the beginning of training, suggesting the expression in these regions at this stage of learning is still task-related. Arc expression in the dentate gyrus

also reflected that of Zif268 and c-Fos, in that there was a mid-training increase, this being the only region to show similar activation across all markers of neuronal plasticity. Arc expression remained significantly higher on day five when compared to day one, and a significant correlation between Arc expression and escape latency in the water maze was found on this final day of training, reflecting a general trend for IEG expression to be more closely related to performance as the task was fully mastered.

The pattern of Zif268 expression in this study from early to late training does reflect that of Guzowski et al. (2001), in that expression was higher during initial sessions. The conflicting results with c-Fos and Arc between the two studies may be partially attributable to the length of training, as rats were only trained for three days in that study. However the increases in c-Fos and Arc expression over time observed here are somewhat consistent with the findings from genetic knockout studies, where Zif268 KO mice display much slower acquisition overall (M. W. Jones et al., 2001), yet Arc KO mice display impairment only in the later stages of learning (Plath et al., 2006), and c-Fos KO mice only begin to show impairment during retrieval (Fleischmann et al., 2003). Nikbakht et al. (2012) found that as the number of training trials in the 8-arm radial arm maze increased, with subsequent improvements in behavioural performance, so did *Arc* mRNA expression in the hippocampus increase in a linear fashion, therefore both c-Fos and Arc may be useful markers of successful performance in later training.

The correlations between IEG expression and performance at different stages of learning strongly implicate these proteins in the learning process. However the large numbers of areas under investigation presents a particular challenge when interpreting the results of this correlational analysis. When a large number of correlations between variables are performed together, the risk of a type I error increases, the probability of finding a statistically significant correlation where none exists. Conversely, when

Bonferroni correction for multiple comparisons of a large number of correlations is applied, the risk of making a type II error, the failure to detect a relationship where one exists, is also increased, particularly when applied to a small sample size in an experiment such as this. Two alternative solutions have been proposed (Curtin & Schulz, 1998), the first of which is focusing on the most relevant variables, however this is not a suitable approach for the experiments in this thesis as all brain regions are of equal interest. Secondly, the use of multivariate statistics has been advocated, however such an approach would not be appropriate in this instance. Thus, the multiple correlations observed between IEG expression and water maze performance in this experiment, and subsequent experiments in this thesis, should be interpreted with a degree of caution due to the increased risk of detecting a statistically significant difference where one may not be present.

In summary, the pattern of results suggest that changes are made to key structures at different times in training, as measured by different IEGs. This introduces an important caveat in the case of using just one IEG as a marker of neural activation, or just looking at IEG expression at a single stage during the learning process. The delayed increase in c-Fos, compared to the initial increase in Zif268, despite the fact that the expression of c-Fos has been shown to be more sensitive to stress than Zif268 (Cullinan et al., 1995), makes it unlikely that the changes in IEG expression observed in this experiment are due to alterations in stress levels over the course of training. The time-dependent role for each of the IEGs in spatial learning further implicates their role in cellular consolidation, which will be the focus of investigation for the next chapter.

Chapter 5

Charting the Course of Cellular Consolidation During the Hours Following Acquisition of the Morris Water Maze

Abstract

Cellular consolidation of newly acquired memories takes place in the hours following learning, leading to long-term structural and functional changes to neurons. *De novo* protein synthesis during this time has been shown to be critical for this process, and IEGs have been identified as important proteins in the stabilisation of a memory trace. Previous research has suggested there is more than one wave of expression of IEG protein in the hours following spatial learning. We analysed the expression of Zif268, c-Fos and Arc in a number of brain regions either 90 minutes, four hours or eight hours following spatial learning in the water maze task. A single wave of IEG expression was observed in most brain regions at 90 minutes, but a subsequent wave at eight hours was not found. Zif268 expression was slightly prolonged in a range of cortical regions at four hours, however these results are consistent with the role of IEGs during the earliest stages of cellular consolidation.

5.1 Introduction

The transition between short-term memory (seconds to hours) and long-term memory (hours to months) is known as consolidation (McGaugh, 2000). Cellular consolidation is initiated immediately following learning, where transcription factors are activated to modulate gene expression and protein synthesis, which results in remodelling of synapses and a more enduring and stable memory trace (Katche, Cammarota, & Medina, 2013). The observation that protein synthesis inhibitors administered in the hours following learning disrupt memory formation (H. P. Davis & Squire, 1984), implicates *de novo* protein synthesis as critical for cellular consolidation during this time frame. IEG expression represents the cells earliest genomic response to stimulation (Clayton, 2000) and IEGs are thought to activate late-response genes to facilitate the process of cellular consolidation, as well as directly affecting the structure and function of the cell to stabilise a memory. Zif268 KO mice show impaired late-LTP in the dentate gyrus, decaying to baseline at 24 hours, whereas induction of LTP in wild-type mice lasts at least 48 hours (M. W. Jones et al., 2001). Arc KO mice display impaired late-LTP and LTD in CA1 (Plath et al., 2006). Therefore IEGs appear to be critical to the formation and persistence of a stable memory, particularly in the hours following learning.

A number of studies have suggested that IEG expression facilitates the consolidation of spatial information. Arc for example, has been shown to be expressed exclusively in neurons following spatial exploration, and these neurons have also been found to be α-CaMKII positive (Vazdarjanova et al., 2006), which has been strongly implicated in the consolidation of memory (Irvine, von Hertzen, Plattner, & Giese, 2006). Where IEG expression is disrupted using various methods, consolidation of spatial memory is impaired. Zif268 KO mice fail to reactivate the same place cell ensemble in CA1 24 hours after exploration of a novel environment, suggesting consolidation of this

type of memory depends on Zif268 expression (Renaudineau et al., 2009). The use of antisense oligodeoxynucleotides (AS-ODNs) which block the translation of mRNA into protein, have demonstrated the necessity of IEG proteins in spatial memory formation. c-Fos antisense-ODNs infused into CA3 before training in the radial arm maze has been shown to increase both working and reference memory errors (He et al., 2002). Arc and c-Fos appear to have a time-dependent role in the consolidation of place learning in the water maze task. Guzowski (2002) showed that Arc AS-ODNs administered into the hippocampus before training had no effect on acquisition over two training sessions, but did affect retention 48 hours later, as did AS-ODNs administered immediately after training, whereas administration eight hours after training did not disrupt consolidation, suggesting there is a critical period of time following learning where this protein helps stabilise spatial memory. A similar effect was found for c-Fos AS-ODNs infused into to the hippocampus before training, where acquisition was unaffected but retention was impaired two days later (Guzowski et al., 2000). This suggests that IEG expression during and immediately after training is critically important for memory consolidation

While the expression of IEGs during spatial learning appears to facilitate the subsequent consolidation of memory, a number of studies have suggested their involvement in cellular consolidation may not be limited to the first few hours following learning. The pattern of basal IEG expression during resting periods appears to be altered based on behavioural experience. Marrone, Schaner, McNaughton, Worley, and Barnes (2008) found that while the amount of IEG-expressing neurons in CA1 of rats previously engaged in spatial exploration did not differ from naïve rats, the majority of neurons active during exploration were reactivated during rest. IEG-facilitated consolidation may also take place during sleep. Ribeiro et al. (2007) showed that four hours after spatial exploration, Arc and Zif268 expression were upregulated in the rat cortex during REM

sleep. Therefore subsequent reactivation of IEG expression may help to stabilise memories, and there is further evidence of multiple "waves" of consolidation, where neurons previously involved in processing spatial information reactivate after a period of time. Ramirez-Amaya et al. (2005) provided some insight into the time course of this reactivation, revealing a co-ordinated multi-phasic process. At two hours after spatial exploration, Arc expression in CA1 and CA3 were significantly correlated. At eight hours (but not four hours) after exploration, a second wave of IEG activity was observed in areas CA1, CA3 and the parietal cortex, albeit in only a proportion (40-55%) of the neurons initially activated. At 24 hours, Arc levels in CA3 and the parietal cortex were again significantly elevated, with correlations in IEG activity between CA1, CA3 and the parietal cortex. Demonstrating that the second and third wave of activity consisted of subpopulations of the original ensemble, double labelling of *Arc* mRNA and protein confirmed that 81% of neurons activated at 8 hours, and 82% of neurons activated at 24 hours belonged to the original ensemble. The dentate gyrus displayed a different pattern of activation, with sustained elevation of Arc expression in the upper blade from 30 minutes to eight hours, the lower blade showing in increase in Arc protein at six and eight hours, but not before. Arc expression in the dentate gyrus returned to baseline at 24 hours. These results indicate neural networks which participated in the initial spatial experience were reactivated and synchronised at later time points, and that IEG expression appeared to facilitate this process. This "multiple wave" of IEG expression has been artificially induced through electroconvulsive shock in mice, where a second wave of Arc expression has been observed in CA1 at eight hours, but not four or six hours following treatment, along with a sustained wave of expression in the dentate gyrus, in line with the aforementioned study (Penke, Chagneau, & Laroche, 2011). Interestingly, when the same treatment was applied to Zif268 KO mice in this study, the second wave of

expression in CA1 was abolished, whereas the first wave remained unaffected. This suggests that expression of Zif268 controls the second wave of Arc expression. However, when the authors attempted to replicate these findings under natural conditions of spatial exploration of a novel or familiar environment, as was carried out by Ramirez-Amaya et al. (2005), only a single wave of Arc expression was observed in CA1 and the dentate gyrus in both wild type and Zif268 KO mice. It is worthwhile noting Ramirez-Amaya et al. (2005) measured Arc protein at this time-point whereas Penke et al. (2011) analysed levels of *Arc* mRNA, therefore the time courses of mRNA and protein may account for these variations. However, a time-course study of *Arc*, *Zif268* and *c-Fos* mRNA expression in the hippocampus following place learning in the water maze has been carried out by Guzowski et al. (2001), finding that all three IEGs dropped to caged control levels or below at six hours, corresponding approximately to an eight hour time-point for IEG protein. Therefore the evidence for multiple waves of IEG expression over the course of consolidation of spatial memory remains inconclusive.

There is a pressing need to clarify the role of IEG expression in the hours following learning, whether or not their expression is confined to the first couple of hours, consistently elevated over subsequent hours, or reactivated at later time-points. This experiment will attempt to resolve the ambiguity in the literature, and to expand on the results which have been found. If the IEG expression observed in the previous acquisition experiment is involved in consolidating the task, one might expect a second wave of expression to be evident in these regions in the hours following training. Previous studies have assessed only a limited number of regions, therefore this experiment will assess the changes in IEG expression which take place in the same 11 regions previously analysed, the hippocampus, entorhinal cortex, medial prefrontal cortex, and retrosplenial, perirhinal and parietal cortices, at 90 minutes, four hours and eight hours following day

three of training in the water maze. As previous studies of consolidation have tended to focus on only one IEG in the hours following learning, we intend to examine the expression of Zif268, c-Fos and Arc. The 90 minute time-point was selected as IEGs are maximally expressed at this time-point following experience (Zangenehpour & Chaudhuri, 2002), and we hypothesise that all three IEGs are most strongly expressed at this time-point. We expect to observe a decline in IEG expression at four hours and a reactivation of IEG expression at the eight hour time-point. Three days of training was chosen as the learning stage for a number of reasons. Animals are still learning the water maze at this point but have not fully mastered the task yet, it is when the decline in Zif268 expression intersected with the increase in c-Fos expression in the previous chapter, and it is at this time-point where a coordinated expression of all three IEGs was observed in the critical region of the dentate gyrus. Based on the results of previous studies, we hypothesise that the dentate gyrus will display a sustained pattern of expression over the eight hour period. A repeated or sustained wave of expression of IEGs in any of the regions studied in the previous experiment would provide further support for these brain areas in the consolidation of the task.

5.2 Method

5.2.1 Subjects.

Male Wistar rats (n=26) obtained from Charles River Laboratories, UK, were used as subjects in the current study. The age and weight of subjects, housing conditions, handling, and time of experimentation were as described previously.

5.2.2 Apparatus.

The Morris water maze was the behavioural task used in this study. See Chapter 2 for details on dimensions of the apparatus and cue configuration.

5.2.3 Procedure.

Rats were randomly allocated to one of three experimental groups (n=7 per group). All three groups underwent standard water maze training as described before, with a fixed hidden platform in the northeast quadrant of the pool, however rats were only trained for three days. A fourth naïve control group (n=5) was also included, to provide a measure of baseline IEG activity.

5.2.4 Preservation of tissue.

The time of sacrifice differed for each spatially-trained group. The first group were sacrificed 90 minutes following the final trial, the second group were sacrificed four hours later, and the third group eight hours later. Naïve rats were taken directly from their home cages and sacrificed. Rats were anaesthetised, transcardially perfused and their brains removed and post-fixed as described in Chapter 2. Eleven regions implicated in spatial processing were selected for analysis, as described previously. 40μm coronal

sections were cut on a freezing microtome and every fourth section was taken for analysis.

5.2.5 Immunohistochemistry.

As the three spatially-trained groups and the caged control group were all stained in a single immunohistochemical session, normalisation procedures were not deemed necessary. Animals were processed in cohorts with one rat from each group stained side-by-side in a well plate. Immunohistochemical protocol for the detection of Zif268, c-Fos and Arc protein was followed as described previously in Chapter 2.

5.2.6 Data analysis.

5.2.6.1 Behavioural data.

To measure performance in the water maze, escape latencies for each trial were calculated, and averaged to produce a mean for every animal for each day.

5.2.6.2 IEG data.

Images were taken of the eleven regions of interest using a digital camera (Olympus DP12) using the method previously outlined. Computerised counts of Zif268, c-Fos and Arc positive neurons were analysed using the public domain program ImageJ (National Institute of Health, USA). Using a number of predefined parameters including a minimum and maximum size, darkness intensity and sphericity, the automated counting software delineated between neurons stained to a sufficient threshold, and non-specific staining. Raw counts from all sections for each region were averaged to produce a mean for each animal.

5.2.7 Statistical analysis.

To analyse escape latencies, one-way repeated measures analyses of variance (ANOVA) were performed, with Bonferroni-corrected comparisons. To compare levels of Zif268, c-Fos and Arc across groups, one-way analyses of variance (ANOVA) were performed with Tukey *post-hoc* tests. To assess relationships between levels of Zif268, c-Fos and Arc and performance in the water maze, Pearson product-moment correlations were performed.

5.2.8 Ethical considerations.

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children (Ireland) guidelines under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

5.3 Results

5.3.1 Behavioural results.

A 3 x 3 mixed factorial ANOVA with group as the between group factor and day as the within group factor, confirmed there was an overall significant decrease in escape latency with a main effect for acquisition day, $F(2, 36) = 3.80$, $p < 0.05$, and Bonferroni post-hoc analyses revealed that escape latencies were significantly lower on day three (*M*: 32.04±2.22 sec, $p < 0.05$) than day one (*M*: 40.86 ±2.40 sec, see Figure 5.1).

Subsequent repeated measures ANOVAs with Bonferroni correction were performed on the escape latencies of the individual groups to further investigate how they changed over the course of training. A significant effect for day was found in the 90 minute group, $F(2, 12) = 7.33$, $p < 0.01$, with post-hoc analyses showing that escape latencies were significantly lower on day three (*M*: 29.46±3.20 sec, $p < 0.05$) than on day one (*M*: 44.60±2.49 sec). Although the escape latencies for the four hour group decreased from day one (*M*: 39.26±4.63 sec) to day three (*M*: 32.67±3.56 sec), this did not reach statistical significance, $F(2, 12) = 0.64$, $p > 0.05$. A similar pattern was observed in the eight hour group, where escape latencies decreased from day one (*M*: 38.73±4.92 sec) to day three (*M*: 34.00±4.62 sec), but this difference did not reach statistical significance, $F(2, 12) = 0.70$, $p > 0.05$. However, a significant difference between the three groups was not found, $F(2, 18) = 0.29$, $p > 0.05$, nor was an interaction effect between day and group observed, $F(4, 36) = 0.88$, $p > 0.05$. Therefore despite the fact that the decrease in escape latencies did not reach statistical significance in the four hour and eight hour groups, the performance of the three groups over three days of training appeared to be comparable.

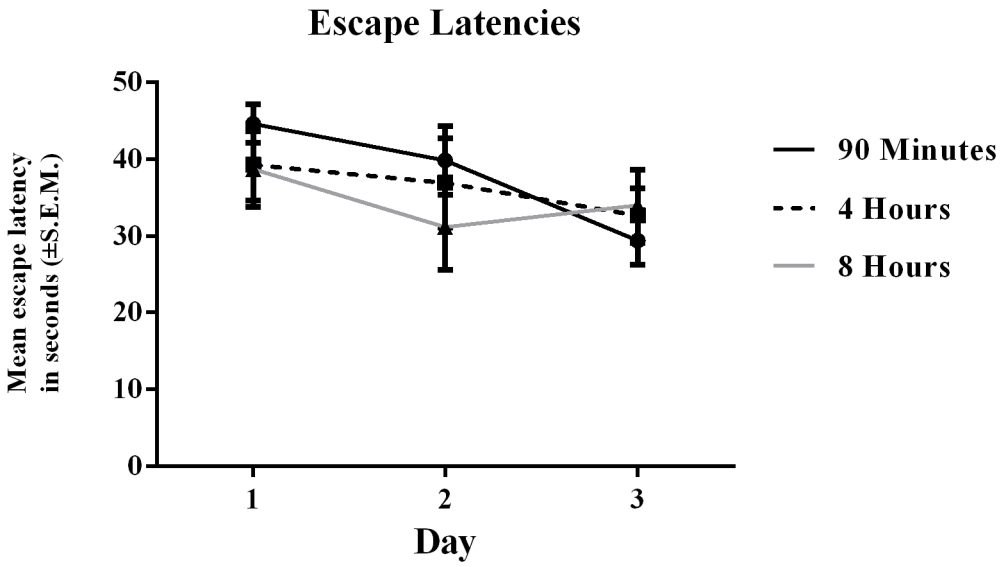

Figure 5.1: Mean daily escape latencies over three days of training for the 90 minutes, four hours and eight hours groups.

5.3.2 IEG results.

5.3.2.1 Zif268.

To determine the changes in IEG expression from caged control levels to 90 minutes, four hours and eight hours following water maze training, a series of one-way ANOVAs with Tukey *post-hoc* tests were performed. A significant difference was found across groups in CA1, $F(3, 22) = 11.07$, $p < 0.001$, with Zif268 counts significantly higher at 90 minutes (*M*: 308.43±14.22) than four hours (*M*: 204.16±27.05, $p < 0.05$) and eight hours post-acquisition (*M*: 112.84±25.14, $p < 0.001$, see Figure 5.2A). A significant difference across groups was also found in CA3, $F(3, 22) = 17.43$, $p < 0.001$, with *post-hoc* Tukey analyses revealing counts of Zif268 were again higher at 90 minutes (*M*: 79.14±11.38) than four hours (*M*: 39.39±4.43, $p < 0.01$) and eight hours following training (*M*: 12.38±4.24, $p < 0.001$), as well as caged controls (*M*: 20.79±4.50, $p < 0.001$, see Figure 5.2B). No effect for group was found in the dentate gyrus, $F(3, 22) = 2.44$, $p > 0.05$, see Figure 5.2C and Figure 5.6 Left. The lateral entorhinal cortex showed a difference across

groups, $F(3, 21) = 12.74$, $p < 0.001$, with Tukey *post-hoc* analyses showing Zif268 expression was significantly lower at eight hours after training (*M*: 72.57±19.84) than at 90 minutes (*M*: 477.18±53.64, $p < 0.001$) and four hours (*M*: 296.57±57.87, $p < 0.05$, see Figure 5.2). Counts were also significantly increased at 90 minutes post-training from caged controls (*M*: 163.65±52.06, $p < 0.01$, see Figure 5.2D). A difference across groups was also found in the medial entorhinal cortex, $F(3, 22) = 6.64$, $p < 0.01$, with Tukey *post-hoc* analyses revealing Zif268 counts were higher at 90 minutes following training (*M*: 90.86±19.92) than eight hours (*M*: 15.57±4.72, $p < 0.01$), as well as being significantly increased from caged control levels (*M*: 27.75±9.00, $p < 0.05$, see Figure 5.2E).

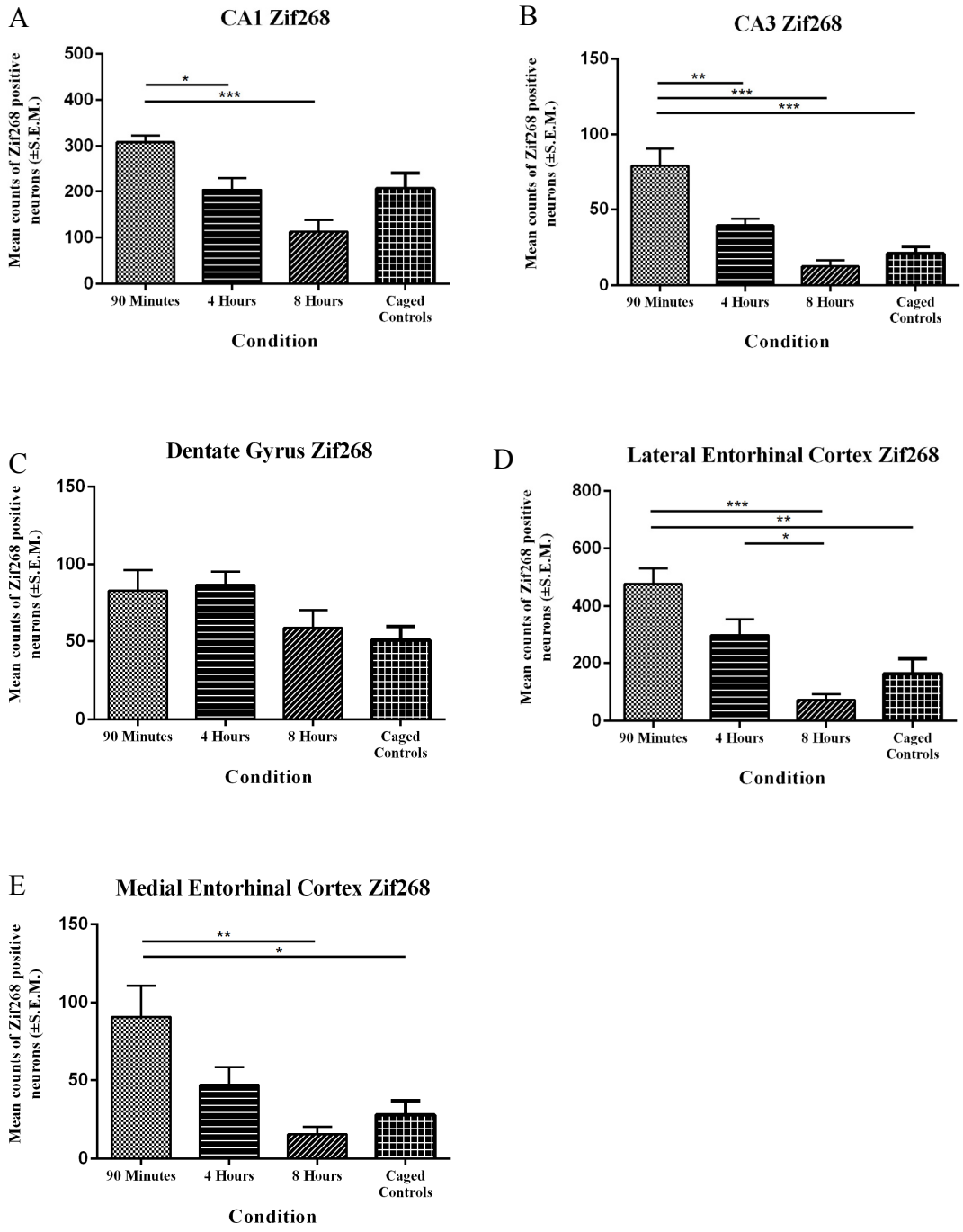

Figure 5.2: Mean Zif268 counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E).

Changes in the time-course of Zif268 expression in the retrosplenial, perirhinal and parietal cortices were also assessed. A significant effect between groups was found in the retrosplenial cortex, $F(3, 21) = 21.50$, $p < 0.001$, with Tukey *post-hoc* analyses revealing Zif268 counts were significantly higher at the 90 minute (*M*: 1397.79±100.68) than four hour (*M*: 502.39±105.27, $p < 0.001$) and eight hour time-points (*M*: 300.99±91.11, $p < 0.001$), as well as caged controls (*M*: 613.76±150.24, $p < 0.001$, see Figure 5.3A and Figure 5.6 Middle). A significant effect across groups was also found in the perirhinal cortex, $F(3, 22) = 30.36$, $p < 0.001$, with a significant decrease in Zif268 counts from 90 minutes (*M*: 288.40±25.44) to four hours (*M*: 147.01±19.14, $p < 0.001$) and eight hours following training (*M*: 34.00±7.24, $p < 0.001$), as well as being significantly increased from baseline (*M*: 116.47±24.22, $p < 0.001$). Counts at eight hours post-training were also significantly lower than four hours ($p < 0.01$) and caged controls ($p < 0.05$, see Figure 5.3B). A difference across groups was also found in the parietal cortex, $F(3, 22) = 19.52$, $p < 0.001$, with Tukey *post-hoc* analyses revealing Zif268 counts were significantly increased at 90 minutes following training (*M*: 2378.31±175.67) compared to four hours (*M*: 1278.29±240.05, $p < 0.01$), eight hours (*M*: 375.54±107.85, $p < 0.001$) and caged controls (*M*: 1072.04±244.86, $p < 0.001$). Counts were also significantly higher at four hours than eight hours ($p < 0.05$, see Figure 5.3C).

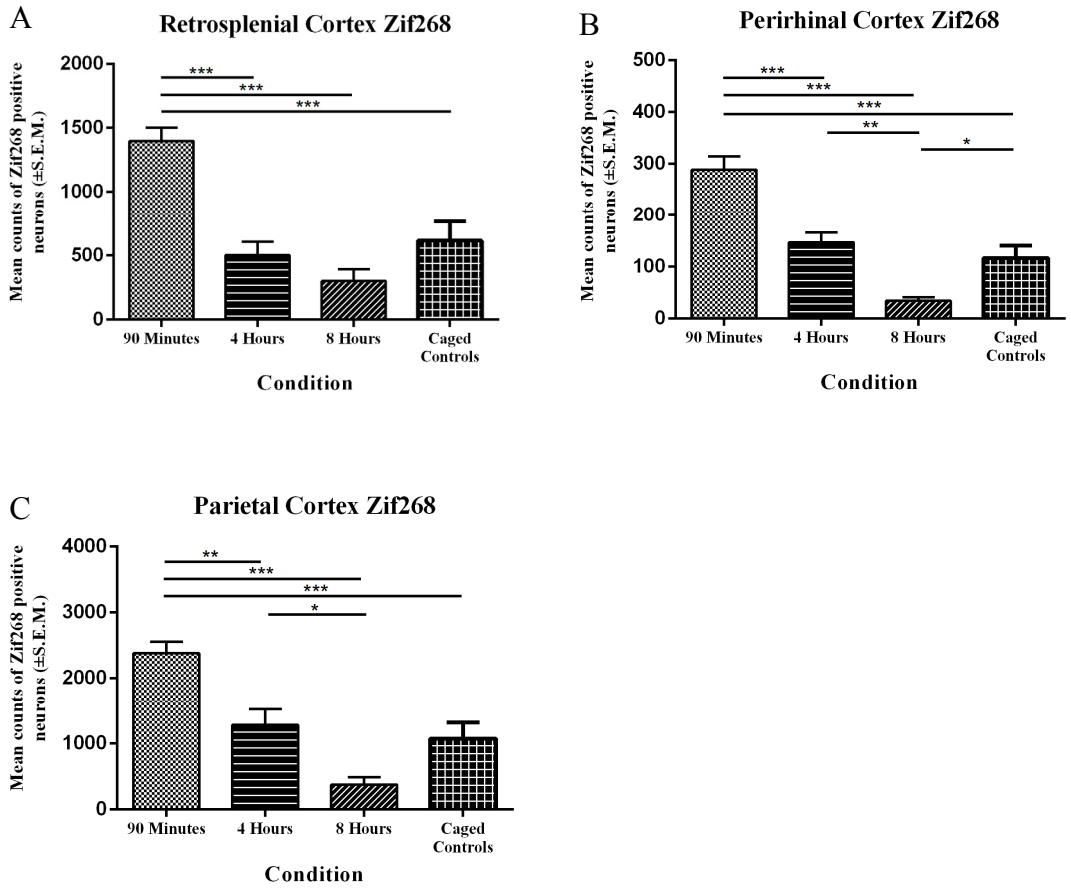

Figure 5.3: Mean Zif268 counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the retrosplenial cortex (A), perirhinal cortex (B) and parietal cortex (C).

The medial prefrontal cortex and its subregions were also analysed. A significant effect was found between groups in the anterior cingulate cortex, $F(3, 21) = 21.69$, $p < 0.001$, with counts of Zif268 significantly increased 90 minutes following training (*M*: 1292.07±82.52) compared to four hours (*M*: 764.76±83.79, $p < 0.01$), eight hours (*M*: 222.00±39.46, $p < 0.001$) and caged controls (*M*: 653.83±171.85, $p < 0.001$). Counts were also significantly lower at eight hours than four hours ($p < 0.01$) and lower than caged controls ($p < 0.05$, see Figure 5.4A and Figure 5.6 Right). A significant difference was also found for the prelimbic cortex, $F(3, 21) = 32.27$, $p < 0.001$, with *post-hoc* Tukey tests revealing counts of Zif268 decreased from 90 minutes post-training (*M*:

1557.64±101.34) to four hours (*M*: 672.78±16.24, $p < 0.001$), eight hours (*M*: 260.10±68.68, $p < 0.001$), and caged controls (*M*: 680.62±188.83, $p < 0.001$). Zif268 counts were also significantly lower in the eight hour group than the four hour ($p < 0.05$) and caged control groups ($p < 0.05$, see Figure 5.4B). Counts of Zif268 also differed across groups in the infralimbic cortex, $F(3, 22) = 25.05$, $p < 0.001$, with Tukey post-tests revealing levels of Zif268 were higher at 90 minutes post-acquisition (*M*: 262.25±28.35) than four hours (*M*: 77.58±14.66, $p < 0.001$), eight hours (*M*: 42.08±8.27, $p < 0.001$) and caged controls (*M*: 67.35±28.15, $p < 0.001$, see Figure 5.4C).

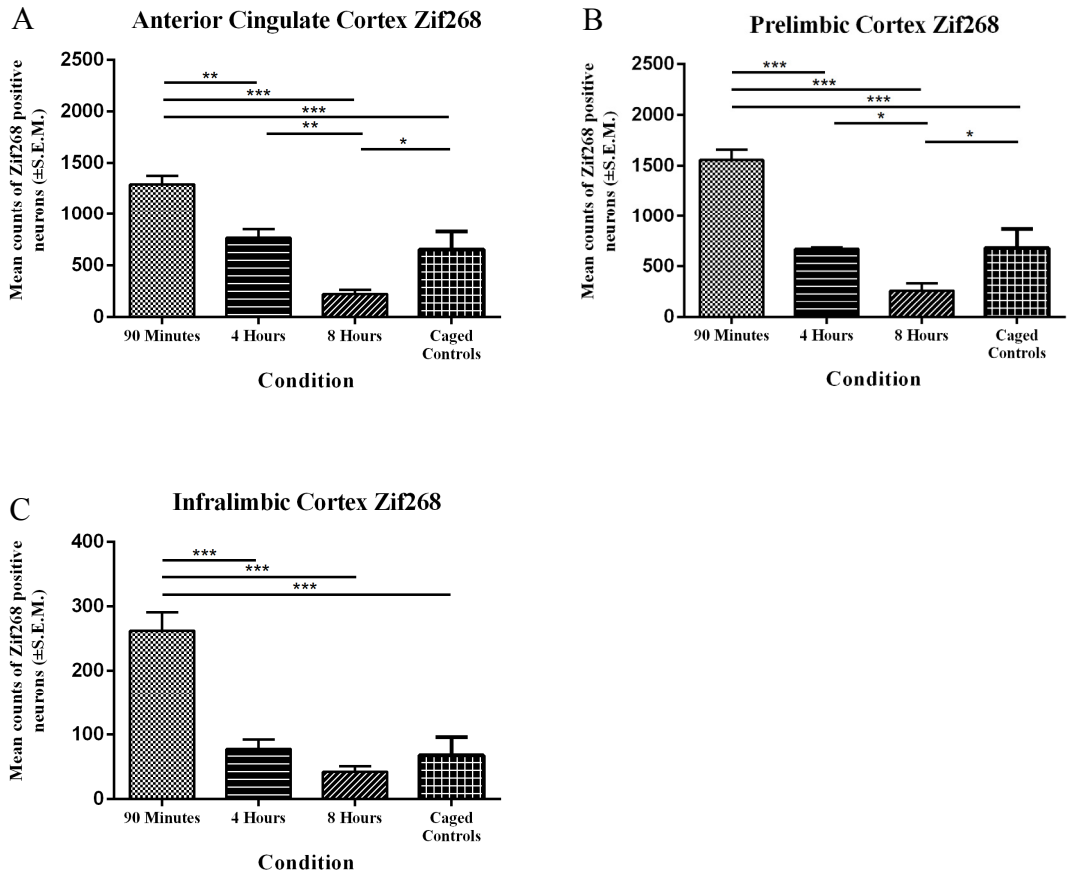

Figure 5.4: Mean Zif268 counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the anterior cingulate cortex (A), the prelimbic cortex (B) and the infralimbic cortex (C).

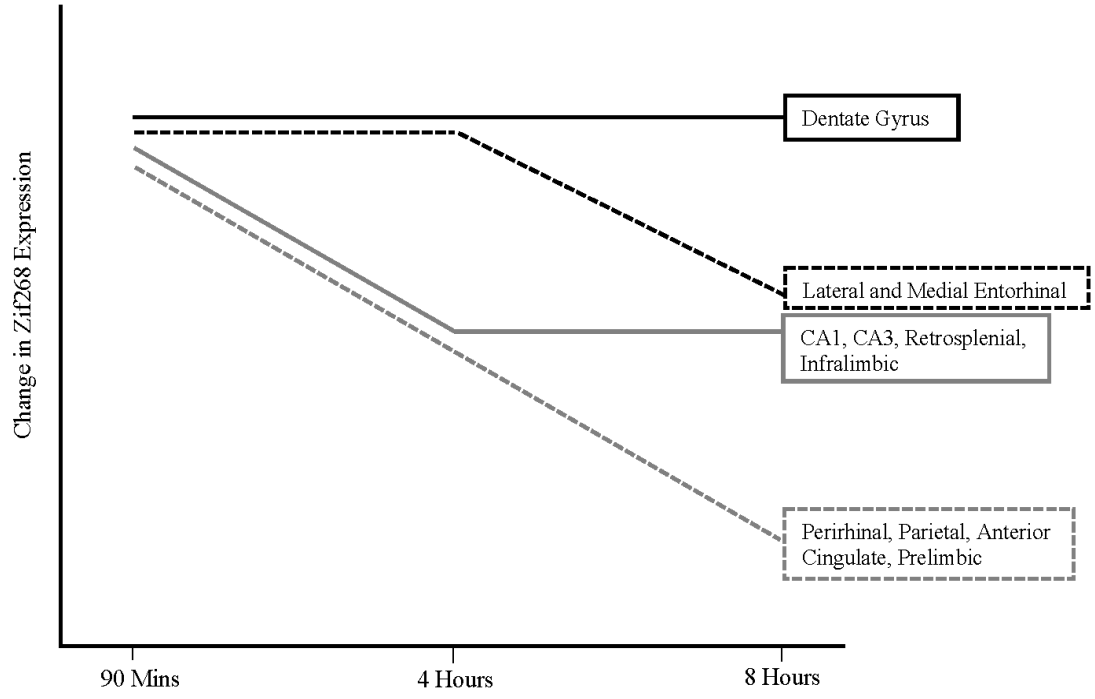

Figure 5.5: Schematic diagram summarising the changes in Zif268 expression in all brain regions analysed at 90 minutes, four hours, and eight hours following three days of water maze training. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

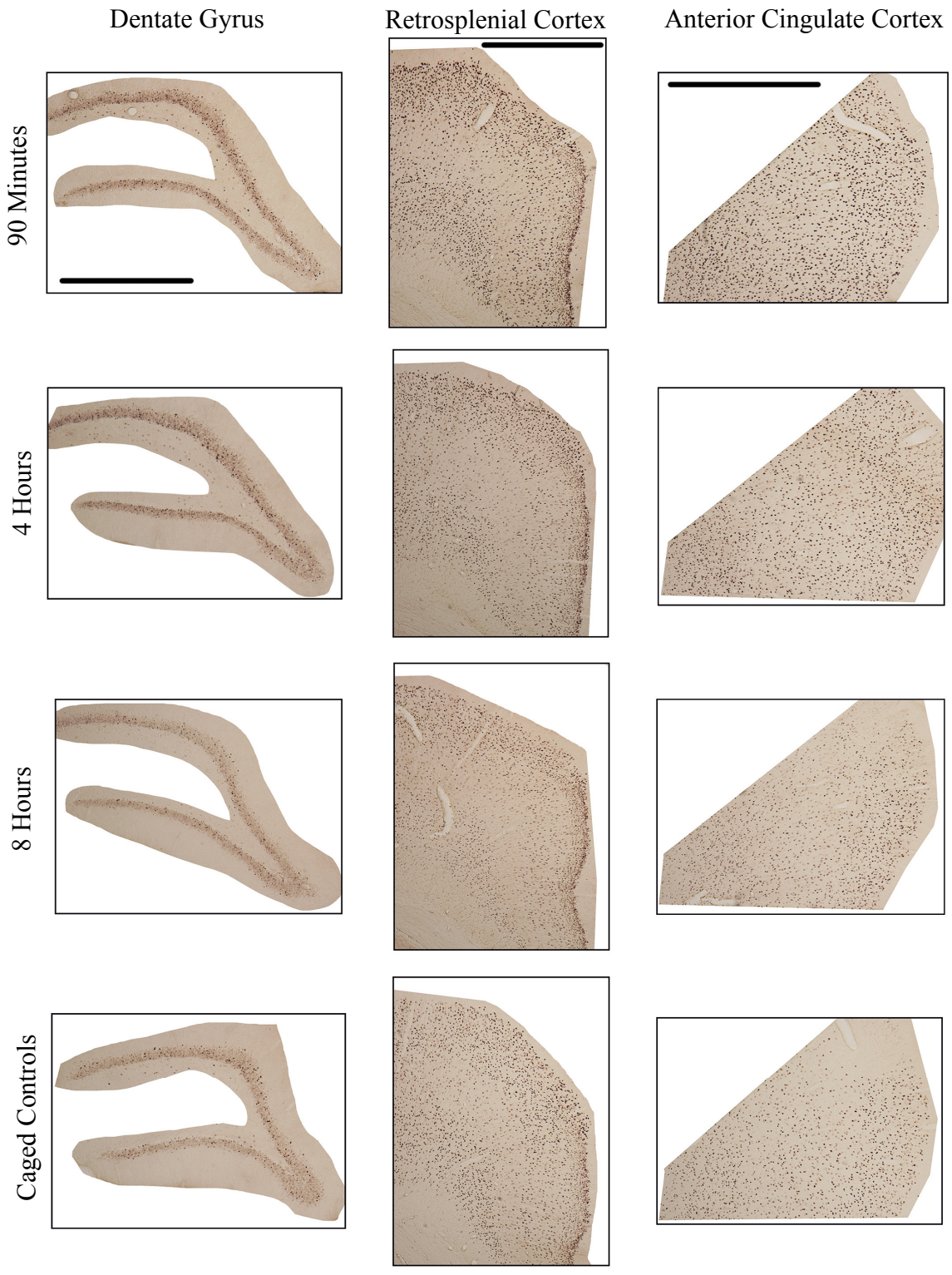

Figure 5.6: Representative photos of Zif268 expression in selected regions: the dentate gyrus, the retrosplenial cortex and the anterior cingulate cortex at 90 minutes, four hours, and eight hours post-acquisition, as well as caged controls. Scale bar = 1mm.

5.3.2.1.1 Correlations with performance.

No significant correlations were found between Zif268 expression and escape latencies in any of the water maze trained groups (see Table 5.1).

Table 5.1: Correlations between Zif268 expression and escape latencies in the water maze

Region	Time post-training		
	90 minutes	4 hours	8 Hours
CA1	0.37	-0.31	-0.34
CA3	0.08	-0.42	-0.43
Dentate Gyrus	-0.13	-0.36	-0.09
Lateral Entorhinal Cortex	0.26	0.07	-0.20
Medial Entorhinal Cortex	0.51	-0.42	-0.56
Retrosplenial Cortex	-0.26	-0.16	-0.34
Perirhinal Cortex	0.47	-0.17	-0.21
Parietal Cortex	0.29	-0.10	-0.48
Anterior Cingulate Cortex	-0.21	-0.23	-0.13
Prelimbic Cortex	-0.12	0.51	-0.45
Infralimbic Cortex	0.46	-0.67	0.12

5.3.2.2 c-Fos.

When c-Fos expression was examined, a significant difference was found across groups in CA1, $F(3, 21) = 4.36$, $p < 0.05$, with Tukey *post-hoc* tests revealing c-Fos counts were significantly higher at 90 minutes (*M*: 15.45±6.18) than eight hours post-training (*M*: 0.28±0.08, $p < 0.05$, see Figure 5.7A). A significant difference across the three groups was also found in CA3, $F(3, 21) = 4.21$, $p < 0.05$, with *post-hoc* analyses revealing c-Fos expression was higher 90 minutes following training (*M*: 17.27±5.96) than eight hours (*M*: 0.80±0.22, $p < 0.05$, see Figure 5.7B). A main effect for group was found in the dentate gyrus, $F(3, 20) = 4.21$, $p > 0.05$, with *post-hoc* Tukey analyses revealing c-Fos

counts were higher at 90 minutes (*M*: 25.63±3.02) than eight hours post-acquisition (*M*: 13.39±2.84, $p < 0.05$, see Figure 5.7C and Figure 5.11 Left). A difference across groups was also found in the lateral entorhinal cortex, $F(3, 22) = 8.78$, $p < 0.001$, with *post-hoc* analyses showing c-Fos expression was significantly higher 90 minutes following training (*M*: 91.31±20.46) than four hours (*M*: 30.87±8.72, $p < 0.01$), eight hours (*M*: 6.71±1.35, $p < 0.001$) and caged controls (*M*: 30.42±9.71, $p < 0.05$, see Figure 5.7D). A difference across training days was also found in the medial entorhinal cortex, $F(3, 21) = 6.24$, $p < 0.01$, with Tukey *post-hoc* analyses revealing c-Fos counts were higher at the 90 minute time-point (*M*: 37.43±8.95) than eight hours (*M*: 3.14±1.08, $p < 0.01$, see Figure 5.7E).

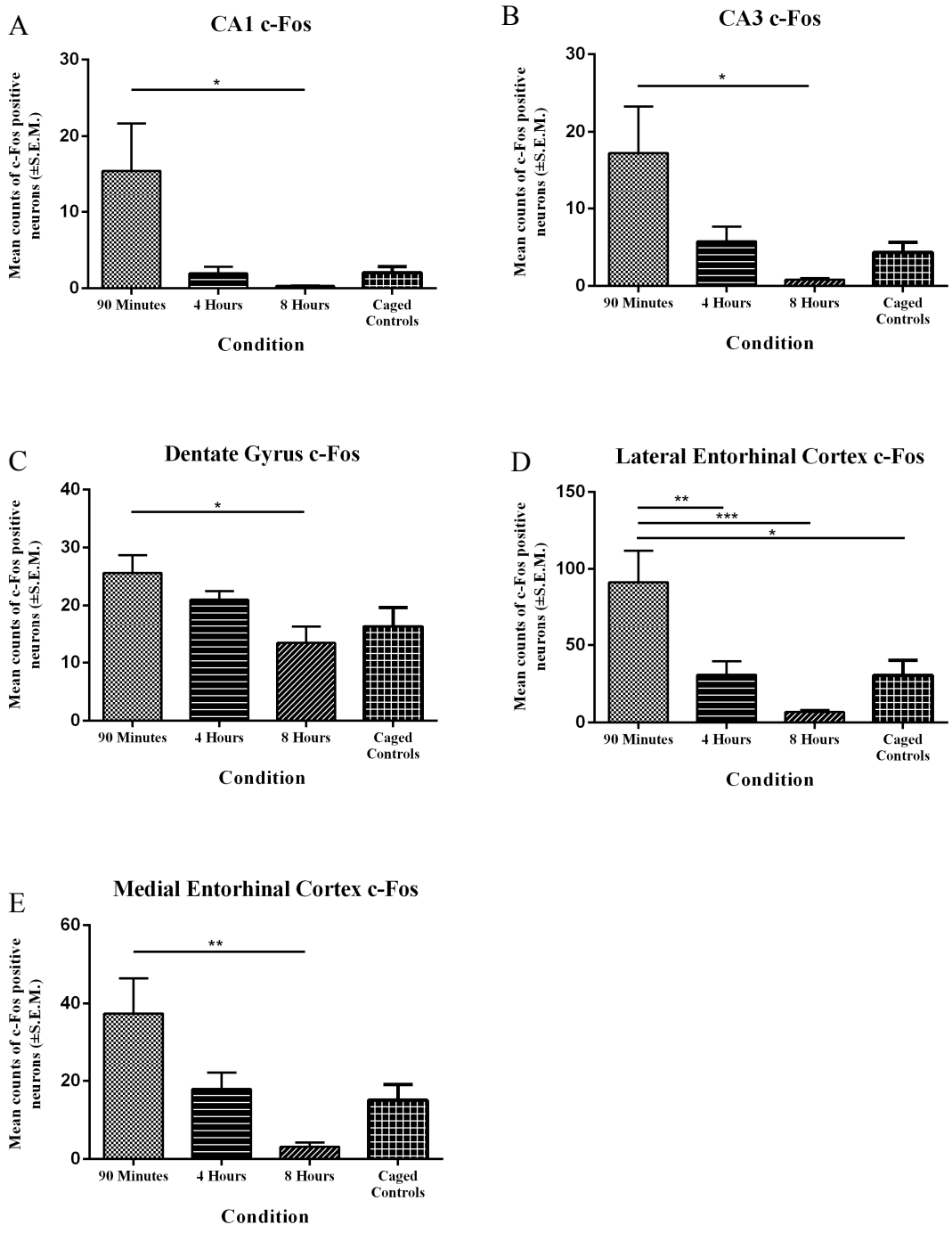

Figure 5.7: Mean c-Fos counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E).

A significant difference was found between the groups in the retrosplenial cortex, $F(3, 21) = 11.04$, $p > 0.001$, with Tukey *post-hoc* analyses revealing c-Fos expression was significantly higher at 90 minutes post-training (*M*: 163.96±39.25) than four hours (*M*: 8.47±2.25, $p < 0.001$), eight hours (*M*: 8.62±2.40, $p < 0.001$) and caged controls (*M*: 34.77±18.19, $p < 0.01$, see Figure 5.8A). Significant differences were found in the perirhinal cortex, $F(3, 21) = 14.55$, $p < 0.001$, with Tukey *post-hoc* analyses revealing c-Fos expression was significantly higher at 90 minutes (*M*: 68.70±13.47) than four (*M*: 14.56±3.88, $p < 0.001$) and eight hours post-acquisition (*M*: 2.85±0.39, $p < 0.001$) as well as caged controls (*M*: 14.58±5.59, $p < 0.01$, see Figure 5.8B and Figure 5.11 Middle). A difference across groups was also found in the parietal cortex, $F(3, 20) = 5.45$, $p < 0.01$), with *post-hoc* Tukey analyses revealing c-Fos expression was significantly higher at the 90 minute time-point (*M*: 128.68±51.45) than 4 hours (*M*: 5.96±1.80, $p < 0.05$) and eight hours (*M*: 5.65±1.30, $p < 0.05$, see Figure 5.8C and Figure 5.11 Right).

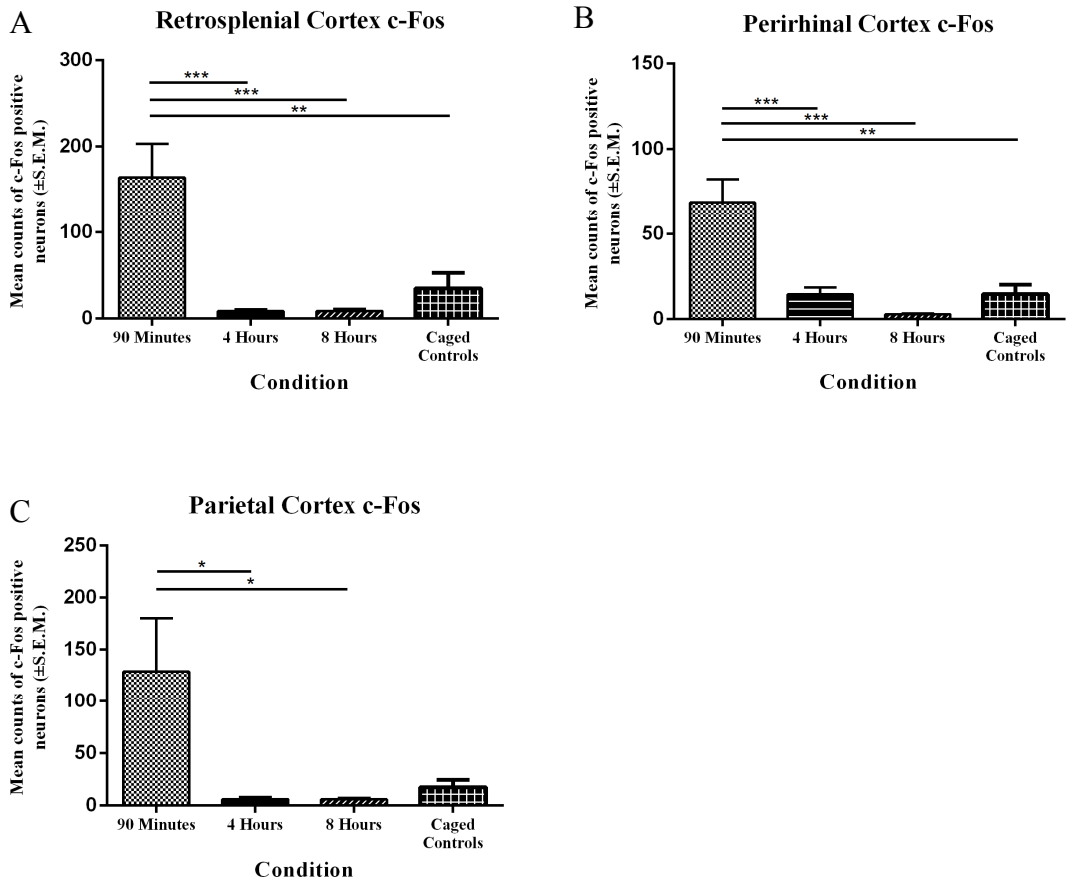

Figure 5.8: Mean c-Fos counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the retrosplenial cortex (A), the perirhinal cortex (B) and the parietal cortex (C).

A significant effect was found between groups in the anterior cingulate cortex, $F(3, 21) = 13.77$, $p < 0.001$, with counts of c-Fos significantly increased 90 minutes following training (M: 100.23±20.72) compared to four hours (M: 6.42.76±1.59, $p < 0.001$), eight hours (M: 5.27±1.02, $p < 0.001$) and caged controls (M: 25.30±11.95, $p < 0.01$, see Figure 5.9A). A significant difference was also found for the prelimbic cortex, $F(3, 20) = 12.62$, $p < 0.001$, with *post-hoc* Tukey tests revealing counts of c-Fos decreased from 90 minutes post-acquisition (M: 262.54±51.02) to four hours (M: 33.56±8.95, $p < 0.001$), eight hours (M: 12.24±3.35, $p < 0.001$), and caged controls (M: 79.53±35.24, $p < 0.01$, see Figure 5.9B). Counts of c-Fos also differed across groups in the infralimbic cortex, $F(3, 21) =$

12.62, $p < 0.01$, with Tukey post-tests revealing levels of c-Fos were higher at 90 minutes after training (M: 107.12±23.46) than four hours (M: 20.40±5.00, $p < 0.001$), eight hours (M: 7.32±1.04, $p < 0.001$) and caged controls (M: 28.53±5.88, $p < 0.01$, see Figure 5.9C).

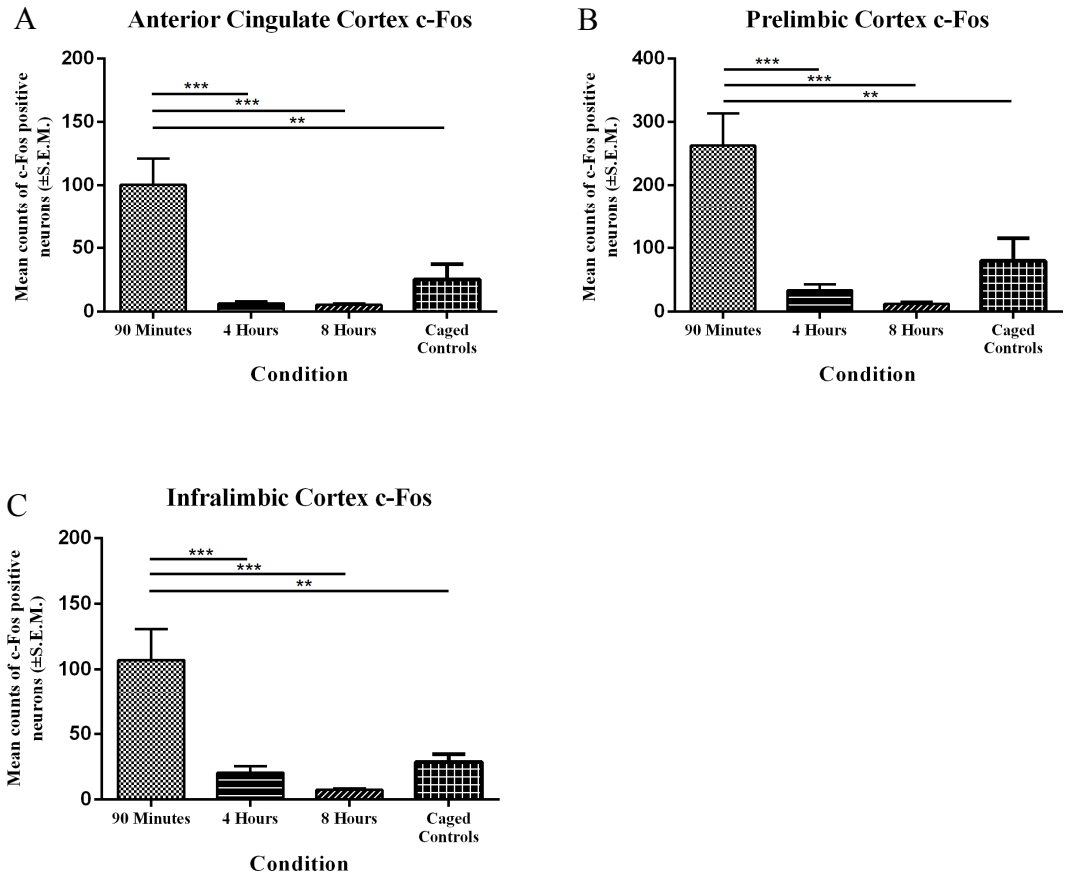

Figure 5.9: Mean c-Fos counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the anterior cingulate cortex (A), the prelimbic cortex (B) and the infralimbic cortex (C).

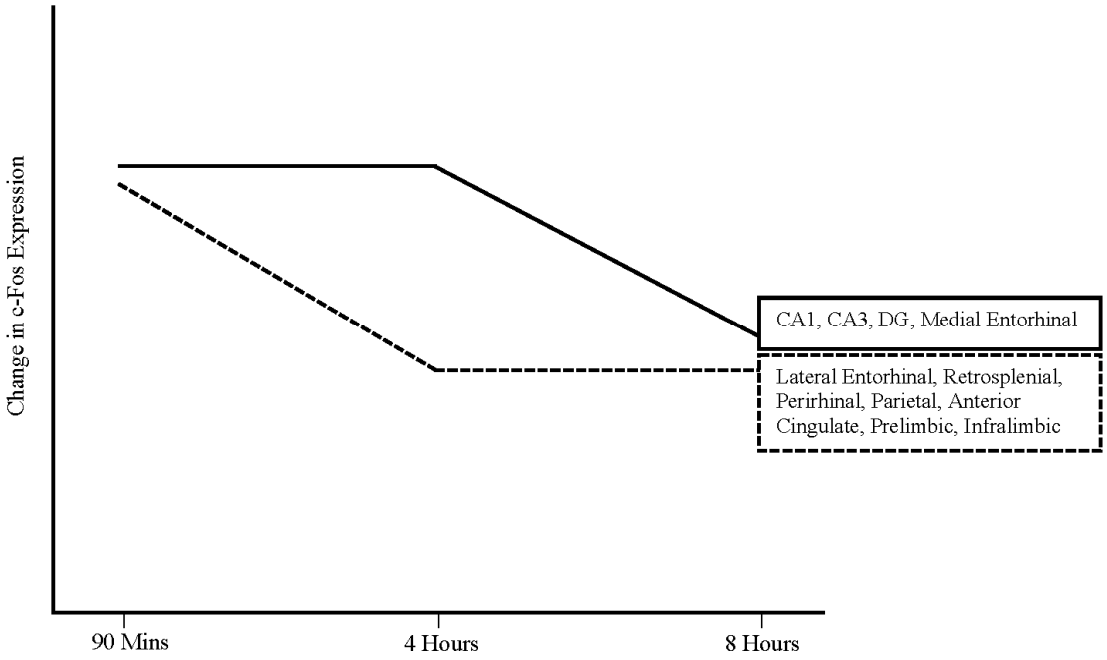

Figure 5.10: Schematic diagram summarising the changes in c-Fos expression in all brain regions analysed at 90 minutes, four hours, and eight hours following three days of water maze training. An ascending line represents a statistically significant increase from previous time points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

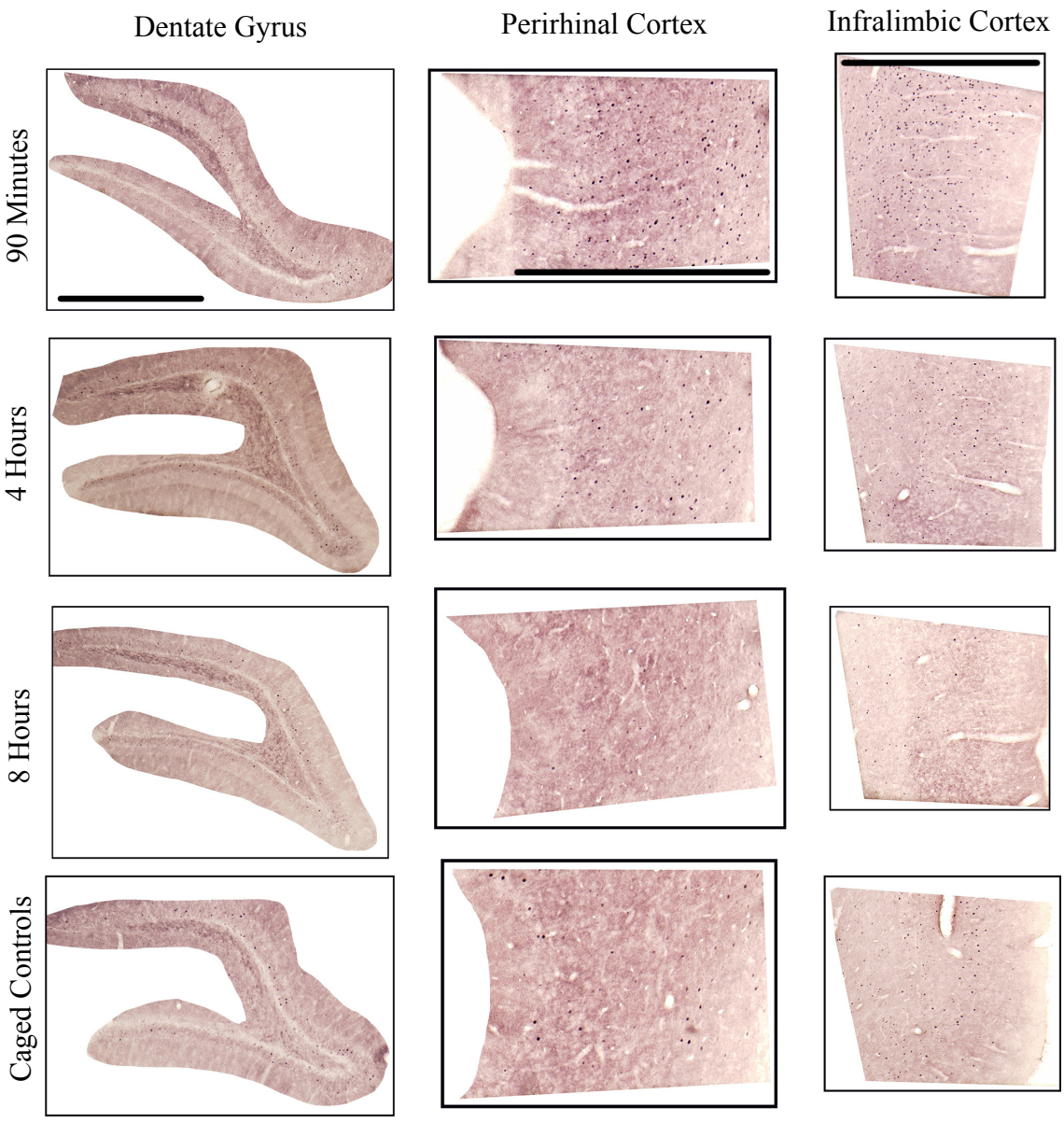

Figure 5.11: Representative photos of c-Fos expression in selected regions: the dentate gyrus, the perirhinal cortex and the infralimbic cortex at 90 minutes, four hours, and eight hours post-acquisition, as well as caged controls.

5.3.2.2.1 Correlations with performance.

Similarly to Zif268, no significant correlations between c-Fos expression and escape latency were found in any of the water maze trained groups (see Table 5.2).

Table 5.2: Correlations between c-Fos expression and escape latencies in the water maze

Region	Time post-training		
	90 minutes	4 hours	8 Hours
CA1	-0.14	-0.36	-0.56
CA3	-0.05	-0.21	0.35
Dentate Gyrus	-0.44	-0.32	-0.40
Lateral Entorhinal Cortex	0.08	-0.01	-0.45
Medial Entorhinal Cortex	0.37	-0.43	-0.32
Retrosplenial Cortex	0.16	-0.30	-0.06
Perirhinal Cortex	0.24	-0.27	-0.01
Parietal Cortex	-0.19	0.16	-0.28
Anterior Cingulate Cortex	-0.05	-0.05	-0.08
Prelimbic Cortex	0.05	0.19	-0.57
Infralimbic Cortex	0.22	-0.11	-0.54

5.3.2.3 Arc.

Finally, the expression of Arc was assessed across the three time points and caged controls. A significant difference in Arc expression was not found across groups in CA3, $F(3, 21) = 2.75$, $p > 0.05$ (see Figure 5.12A), or the dentate gyrus, $F(3, 22) = 1.60$, $p > 0.05$ (see Figure 5.12B and Figure 5.16 Left). The lateral entorhinal cortex showed a difference across groups, $F(3, 21) = 7.55$, $p < 0.01$, with Tukey *post-hoc* analyses showing Arc expression was significantly higher at 90 minutes post-training (*M*: 37.87±10.72) than four hours (*M*: 5.40±1.13, $p < 0.01$), eight hours (*M*: 1.52±0.34, $p < 0.01$) and caged controls (*M*: 8.80±4.30, $p < 0.05$, see Figure 5.12C). A difference across groups was also found in the medial entorhinal cortex, $F(3, 22) = 7.71$, $p < 0.01$, with Tukey *post-hoc* analyses revealing Arc counts were higher at 90 minutes following training (*M*: 5.49±1.39) than four hours (*M*: 1.02±0.23, $p < 0.01$), eight hours (*M*: 0.38±0.10, $p < 0.01$) and caged controls (*M*: 1.90±0.99, $p < 0.05$, see Figure 5.12D). Arc staining was absent in area CA1.

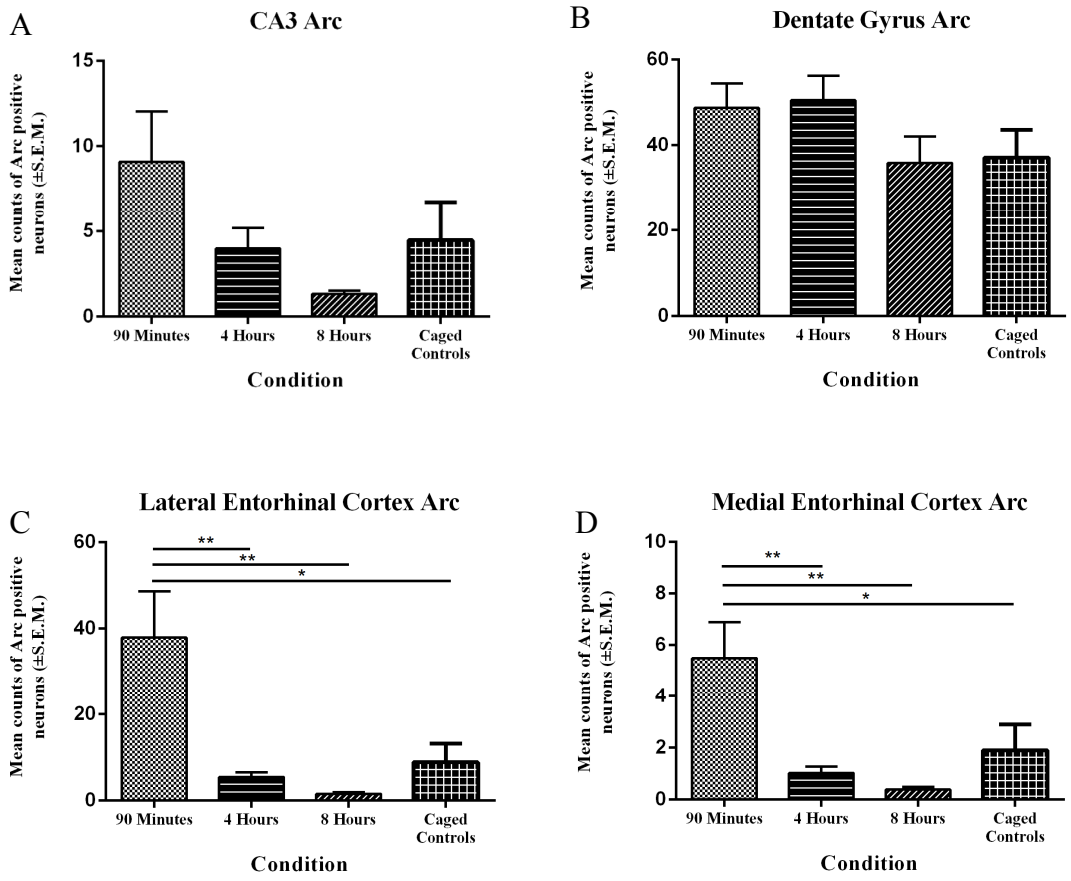

Figure 5.12: Mean Arc counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in CA3 (A), dentate gyrus (B), lateral entorhinal cortex (C) and medial entorhinal cortex (D).

A significant effect between groups was found in the retrosplenial cortex, $F(3, 21) = 5.82$, $p < 0.01$, with Tukey *post-hoc* analyses revealing Arc counts were significantly higher at the 90 minute time-point (*M*: 123.60±41.69) than four hours (*M*: 0.45±0.22, $p < 0.05$) and eight hours (*M*: 0.58±0.21, $p < 0.01$, see Figure 5.13A). A significant effect for group was also found in the perirhinal cortex, $F(3, 21) = 9.65$, $p < 0.001$, with Tukey *post-hoc* tests revealing a significant decrease in Arc counts from 90 minutes post-training (*M*: 24.56±6.97) to four hours (*M*: 4.45±1.56, $p < 0.01$) and eight hours (*M*: 0.75±0.19, $p < 0.001$), as well as being significantly increased from baseline (*M*: 3.56±1.28, $p < 0.01$, see Figure 5.13B). A difference across groups was also found in the parietal cortex, *F*(3,

22) = 5.80, $p < 0.01$, with Tukey *post-hoc* analyses revealing Arc counts were significantly increased at 90 minutes after training (*M*: 138.41±50.97) compared to four hours (*M*: 0.95±0.24, $p < 0.01$) and eight hours (*M*: 0.83±0.15, $p < 0.01$, see Figure 5.13C and Figure 5.16 Middle).

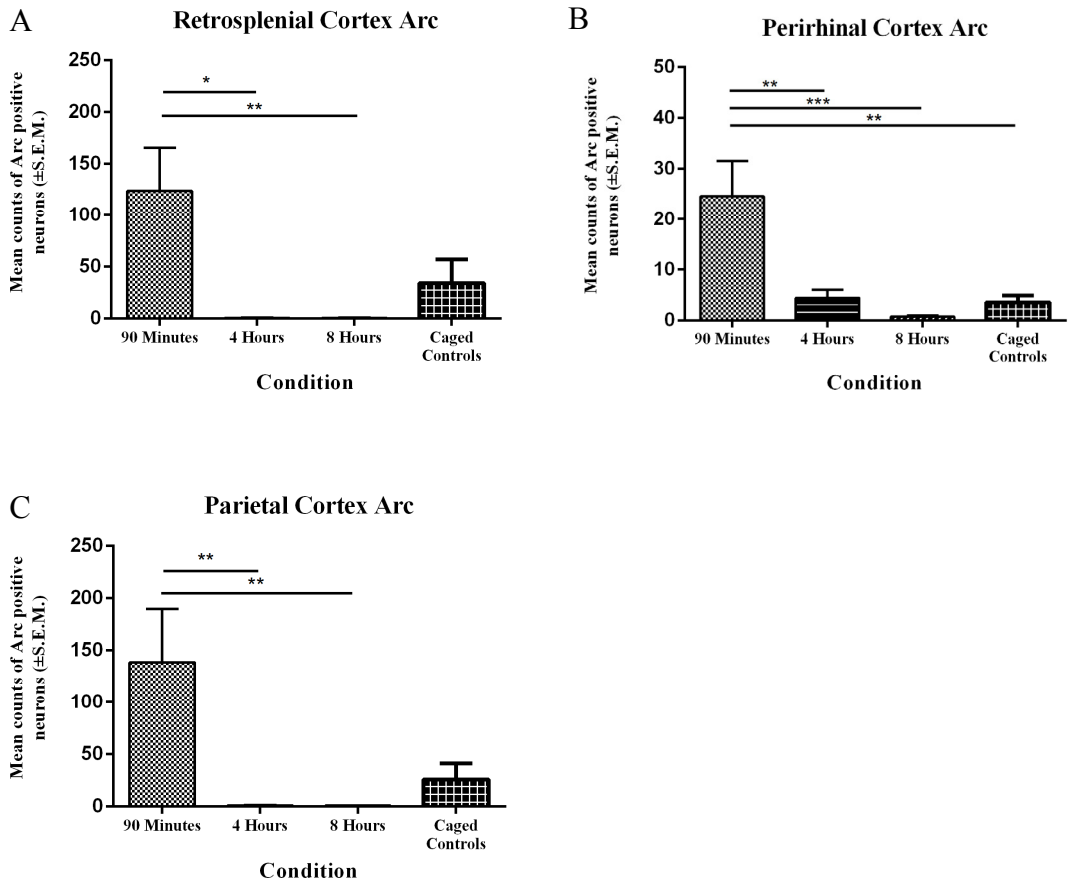

Figure 5.13: Mean Arc counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the retrosplenial cortex (A), perirhinal cortex (B) and parietal cortex (C).

A significant effect was found between groups in the anterior cingulate cortex, $F(3, 21) = 5.04$, $p < 0.01$, with counts of Arc significantly increased 90 minutes following training (*M*: 135.42±51.80) compared to four hours (*M*: 1.46±0.42, $p < 0.05$) and eight hours (*M*: 0.76±0.19, $p < 0.05$, see Figure 5.14A and Figure 5.16 Right). A significant difference

was also found for the prelimbic cortex, $F(3, 20) = 5.40$, $p < 0.01$, with *post-hoc* Tukey tests revealing counts of Arc decreased from 90 minutes (*M*: 163.18±61.00) to four hours (*M*: 0.92±0.29, $p < 0.05$) and eight hours (*M*: 0.39±0.15, $p < 0.05$, see Figure 5.14B), Counts of Arc also differed across groups in the infralimbic cortex, $F(3, 21) = 5.92$, $p < 0.01$, with Tukey post-tests revealing levels of Arc were higher at 90 minutes (*M*: 24.14±8.54) than four hours (*M*: 0.58±0.23, $p < 0.05$), eight hours (*M*: 0.43±0.14, $p < 0.01$) and caged controls (*M*: 3.98±1.62, $p < 0.05$, see Figure 5.14C).

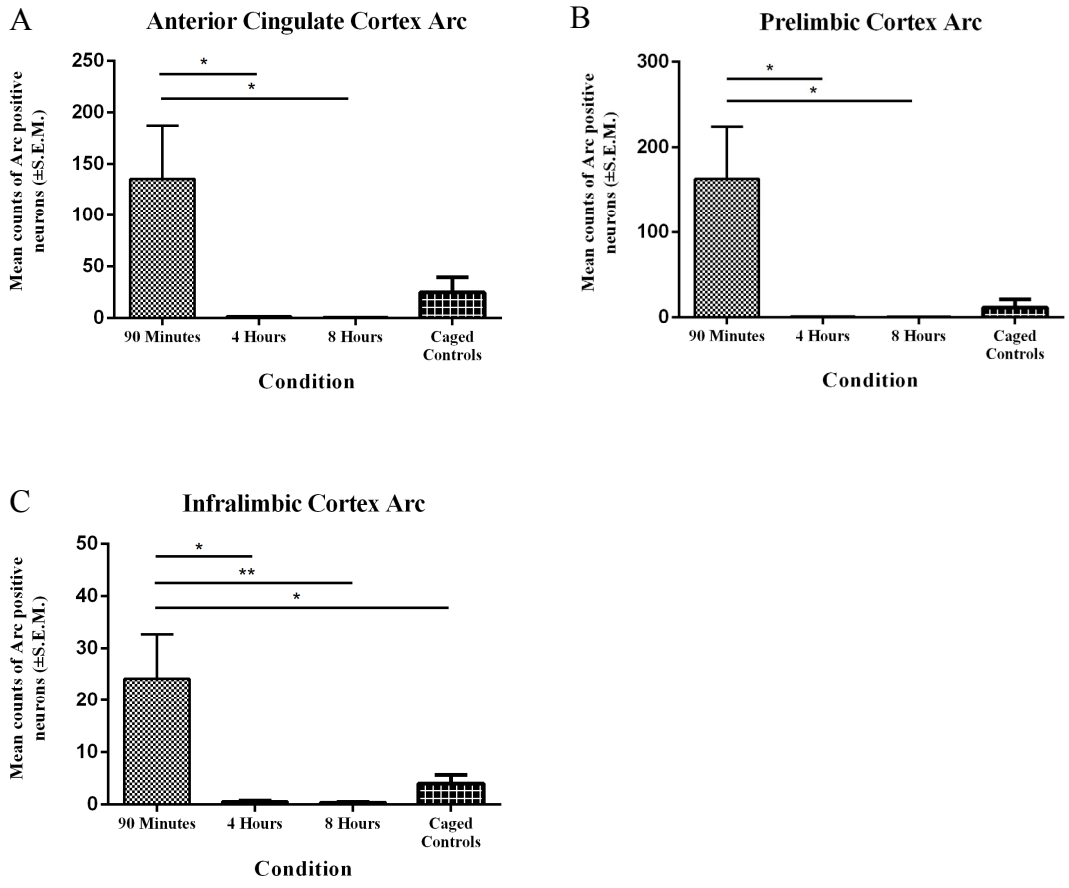

Figure 5.14: Mean Arc counts at 90 minutes, four hours, and eight hours after water maze training, as well as caged control levels, in the anterior cingulate cortex (A), the prelimbic cortex (B) and the infralimbic cortex (C).

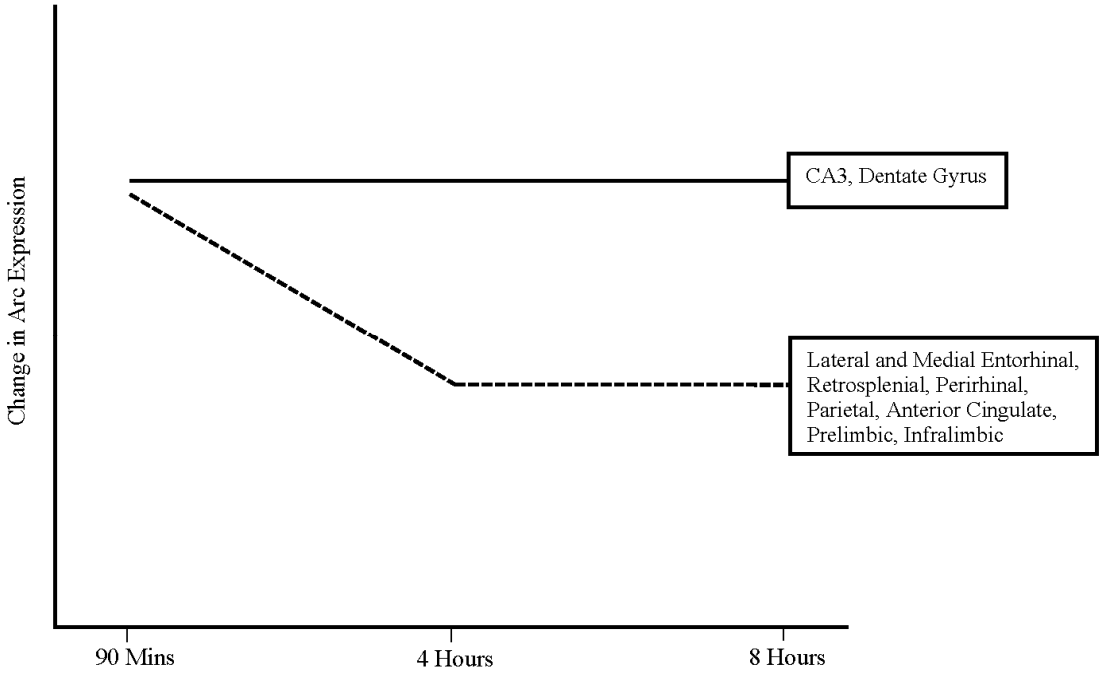

Figure 5.15: Schematic diagram summarising the changes in Arc expression in all brain regions analysed at 90 minutes, four hours, and eight hours following three days of water maze training. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

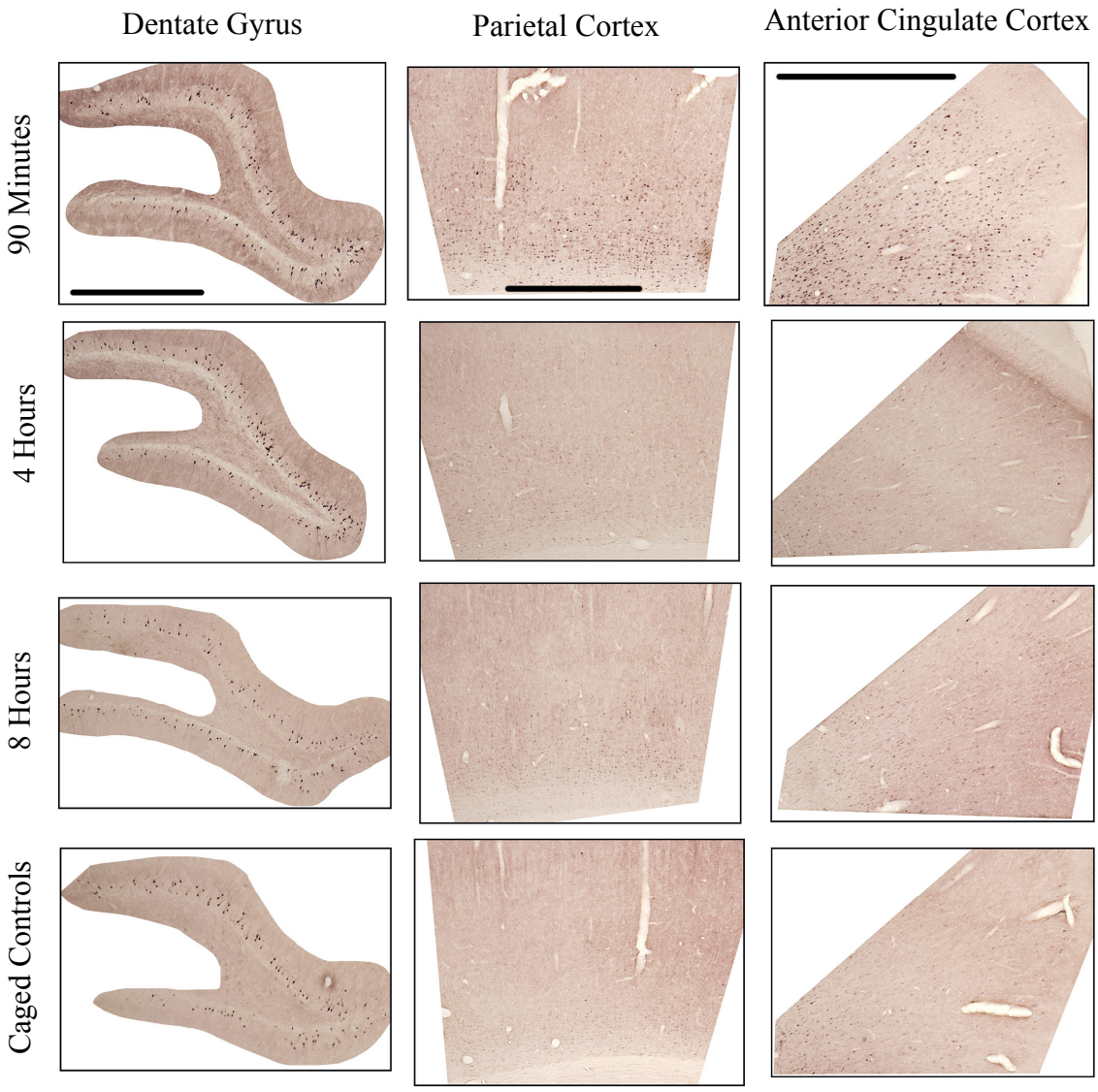

Figure 5.16: Representative photos of Arc expression in selected regions: the dentate gyrus, the parietal cortex, and the anterior cingulate cortex at 90 minutes, four hours, and eight hours post-acquisition, as well as caged controls. Scale bar = 1mm.

5.3.2.3.1 Correlations with performance.

Significant correlations between Arc expression and escape latency were not found in any of the water maze trained groups (see Table 5.3).

Table 5.3: Correlations between Arc expression and escape latencies in the water maze

Region	Time post-training		
	90 minutes	4 hours	8 Hours
CA3	-0.42	-0.41	0.58
Dentate Gyrus	0.01	-0.64	0.00
Lateral Entorhinal Cortex	0.12	-0.21	0.81
Medial Entorhinal Cortex	-0.14	-0.70	-0.72
Retrosplenial Cortex	0.38	0.44	0.11
Perirhinal Cortex	-0.70	-0.24	0.43
Parietal Cortex	0.01	0.36	0.70
Anterior Cingulate Cortex	0.19	0.29	-0.43
Prelimbic Cortex	0.22	0.38	-0.15
Infralimbic Cortex	0.16	-0.43	-0.29

5.4 Discussion

The objective of this experiment was to monitor the changes in IEG expression in 11 brain regions at multiple time-points post-training in the Morris water maze, and to resolve the inconsistency in the literature regarding multiple waves of IEG-facilitated consolidation in the hours following spatial learning. The most notable finding was the apparent absence of a second wave of expression for any IEG in any of the brain regions studied. However a prolonged expression was found in some areas at four hours following training.

Beginning with Zif268, as hypothesised, expression was higher at 90 minutes following training than at eight hours or caged controls in CA1, CA3, the lateral and medial entorhinal cortices, the retrosplenial, perirhinal and parietal cortices, and all three subregions of the medial prefrontal cortex. This is consistent with the time course of expression of this IEG, which is maximally expressed 90 minutes following experience (Zangenehpour & Chaudhuri, 2002), and has been shown to be upregulated at this time point in these areas following water maze training (Guzowski et al., 2001; Shires & Aggleton, 2008; Teather et al., 2005). However Zif268 expression in a number of regions had either not significantly decreased by four hours, or had yet to decrease from four hours to eight hours, indicating prolonged activity in these areas. These regions included the lateral and medial entorhinal cortices, the perirhinal and parietal cortices, and the anterior cingulate and prelimbic cortices. As no intermediate time-points between 90 minutes and four hours were assessed, it is not possible to elucidate whether this increased expression constituted a "second wave" of IEG expression, or was part of a prolonged initial period of expression. The finding that cortical areas, rather than the hippocampus showed prolonged activation at this time-point, is however, consistent with the observations of Ribeiro et al. (2007), where four hours following novel spatial

experience, Zif268 expression was increased in cortical areas, but not the hippocampus during sleep. Furthermore, the fact that overall levels of expression at this time-point were lower than that of 90 minutes, supports the idea that this would represent a "sub-set" of the original ensemble, as was shown by Ramirez-Amaya et al. (2005). However in contrast to the findings of Ramirez-Amaya et al. (2005), a "second wave" of IEG expression was not observed at eight hours post-training in any of the regions studied. This is surprising, as the spatial exploration of a novel environment in that study would not be nearly as taxing as the effortful learning required to consolidate the water maze task. In fact, Zif268 expression at the eight hour time-point was even lower than caged controls in the perirhinal, anterior cingulate and prelimbic cortices. This depletion of IEG protein below caged control levels is difficult to account for, but has been observed before by Guzowski et al. (2001), where *c-Fos* mRNA in the hippocampus dropped below caged control levels six hours following training in the water maze. When examining the time-course of Zif268 expression following fear conditioning, Lonergan, Gafford, Jarome, and Helmstetter (2010) observed hippocampal expression to drop below caged control levels from 1.5 to 24 hours. One finding of Ramirez-Amaya et al. (2005) which we have replicated here, is the prolonged IEG expression in the dentate gyrus from 90 minutes to eight hours. Whether this is the result of single or multiple waves of expression is unclear, and the failure to detect a significant increase from caged controls at any of the time-points makes this result difficult to interpret. Overall, there appears to be a prolonged expression of Zif268 in cortical areas over a four hour period, but no clear evidence of multiple waves of IEG expression, especially at eight hours.

The analysis of c-Fos expression over the course of hours following spatial training showed that there was a similar initial increase at the 90 minute time-point, with all regions displaying significantly higher levels of c-Fos at this stage when compared to

eight hours or caged controls. The only regions where expression at four hours did not differ significantly from 90 minutes, were CA1, CA3, the dentate gyrus and the medial entorhinal cortex. This differs markedly from the pattern of Zif268 results, in that it was the hippocampus rather than cortical areas which appeared to show prolonged expression of c-Fos. This somewhat reflects the results of the previous chapter, where c-Fos showed a marked increase in these structures as training progressed. Again however, similar to Zif268, a "second wave" of IEG expression was not observed with c-Fos, as a significant increase in expression did not become apparent at eight hours.

The pattern of Arc expression across the three time-points broadly reflected that of c-Fos. The lateral and medial entorhinal cortices, the retrosplenial, perirhinal and parietal cortices, and the anterior cingulate, prelimbic and infralimbic cortices all displayed dramatic decreases in Arc expression from 90 minutes to four hours, and did not display a second wave of activity at eight hours. The dentate gyrus once again showed prolonged expression for eight hours following training. These findings, for the most part, are at variance with Ramirez-Amaya et al. (2005) who used Arc as a marker of consolidation and found multiple waves of consolidation in CA3 and the parietal cortex, and support the findings of Penke et al. (2011), where only a single wave of expression was found following spatial exploration. IEG expression in the dentate gyrus appears to be largely unchanged over the eight hours following spatial training and may underline its importance at this stage of learning as shown in the previous chapter, although the non-significant increase from caged controls means these results should be interpreted with a degree of caution. No significant correlations were found with learning performance for any of the IEGs at any time-point, although this is consistent with the results of the previous acquisition experiment, which demonstrated that those relationships tend to form at the later stages of training when the task is fully acquired.

The findings of the current experiment are similar to the observations of Guzowski et al. (2001), who found a peak of *Arc*, *Zif268* and *c-Fos* mRNA expression in the hippocampus at 30 minutes following water maze training followed by a sharp drop at two hours, and returning to caged control levels or below at six hours, which approximately correspond to the 90 minute, four hour and eight hour time points for protein expression in this experiment. However neither set of results definitively rule out multiple waves of consolidation through IEG expression. The prolonged Zif268 expression seen at four hours in this experiment may be a second wave, furthermore Ramirez-Amaya et al. (2005) observed a third wave of IEG expression at 24 hours post-training, a time-point which was not assessed in this study. In fact there is evidence from other learning paradigms which point to a delayed expression of IEGs around this time. Katche, Goldin, Gonzalez, Bekinschtein, and Medina (2012) found a delayed increase of *Zif268* mRNA in the hippocampus at 12, 18 and 24 hours, but not nine or 30 hours following inhibitory avoidance training. They subsequently used AS-ODNs to specifically knock down Zif268 expression in the hippocampus either during or after training. Zif268 AS-ODNs administered 30 minutes before training impaired memory measured both one and seven days following training, whereas AS-ODNs infused into the hippocampus eight hours following training spared memory at one day but not seven days. The authors postulate that the second wave of Zif268 expression observed between 12 and 24 hours after training facilitated the persistence of long-term memory while not affecting its initial formation. Lonergan et al. (2010) charted the time-course of Arc expression following fear conditioning, and found a moderate increase at 24 hours, although it did not reach statistical significance. This raises the interesting possibility that the increased IEG expression observed towards the end of training in the previous experiment is the result of cumulative waves of expression from previous training days.

A further consideration is the pattern of basal IEG expression during resting periods that may be altered by behavioural experience, in that the same neurons activated during training are the ones expressing IEGs at rest (Marrone et al., 2008). Therefore the activity of which neurons, rather than how many neurons, may be a more successful measure of IEG-facilitated consolidation, however the method employed in the current study is not designed to detect these differences.

In summary, although multiple waves of IEG-facilitated consolidation were not clearly detected in this experiment, prolonged expression of Zif268 appears to take place in the cortex, with lasting expression of c-Fos and Arc in the hippocampus. Whether this is the result of single or multiple waves of IEG expression, it is likely to assist in the stabilisation of spatial memory in the regions analysed, ultimately facilitating the reorganisation of memory on a systems level in the brain, which will be the focus of the next chapter.

Chapter 6

Immediate Early Gene Imaging of Multiple Brain Regions During Retention of the Morris Water Maze From Recent to Remote Time-points

Abstract

Systems consolidation refers to the stabilisation of memory traces in the cortex over time, although the extent to which detailed memory traces simultaneously become independent of the hippocampus is under debate, particularly with regards to spatial memory. Remote memory appears to depend increasingly on the medial prefrontal cortex over time, yet the hippocampus appears to be essential for performance in the Morris water maze regardless of whether a memory is recent or remote. To assess the relative contributions of hippocampal and cortical regions to memory retention over time, we examined the expression of Zif268, Arc and c-Fos in relevant brain regions after a probe trial either one day, seven days, 14 days or 30 days following acquisition of the Morris water maze. Activity of the hippocampus actually increased over time, reaching a peak at seven to 14 days, whereas cortical areas also increased in activity from recent to remote retention, reaching a peak at 14 to 30 days. Successful retrieval was closely associated with Zif268 expression over the course of retention in a wide range of brain regions. These findings are consistent with multiple trace theory and cognitive mapping theory in that the hippocampus is required for the retrieval of detailed memory representations, spatial memory in particular. They also confirm a role for the medial prefrontal cortex in the long-term retrieval of spatial memory, revealing a gradual increase in activity over time.

6.1 Introduction

While cellular consolidation refers to the strengthening of connections between neurons in the hours following learning, the reorganisation of memory on a regional level in the brain is known as systems consolidation (Frankland & Bontempi, 2005). There remains an open debate on the nature of this consolidation and the extent to which newly formed memories become independent of the hippocampus over time and rely on the neocortex for successful retrieval. The declarative theory of memory (Squire, 2004) proposes a time-dependent role for the hippocampus in memory retrieval, the multiple trace theory (Moscovitch et al., 2005), maintains the quality of detailed contextual memory depends on the hippocampus indefinitely, while the cognitive map theory argues that the hippocampus is the permanent store of spatial memory (O'Keefe & Nadel, 1978). The discovery of "place cells" in the hippocampus (O'Keefe & Dostrovsky, 1971) would suggest that spatial memory is particularly dependent on the hippocampus for retrieval of spatial information. Spatial navigation experiments with hippocampal-damaged human subjects haven proven equivocal, but appear to suggest that at least detailed information for remote spatial memory is dependent upon the integrity of the hippocampus (Spiers & Maguire, 2007).

Studies using animals as subjects have investigated the relative contribution of hippocampal and cortical regions to remote spatial memory using a combination of lesion, temporary inactivation and IEG methods. Such a combined approach was adopted by Maviel, Durkin, Menzaghi, and Bontempi (2004), who compared the activity of the hippocampus and medial prefrontal areas at recent and remote retention in a test of spatial discrimination memory, using the radial arm maze. Rats were trained to locate a single baited arm in the radial arm maze based on surrounding cues, and were tested either one day or 30 days later to assess recent or remote spatial memory respectively. While

performance levels across the two sessions were comparable, Zif268 protein expression increased between recent and remote memory in the medial prefrontal and retrosplenial cortices. Increased c-Fos protein expression was also observed in the medial prefrontal cortex. The hippocampus exhibited an opposite pattern with Zif268 expression decreasing between recent and remote retention, when it was significantly lower than paired controls. Temporary inactivation of the hippocampus confirmed the IEG results, revealing a functional disengagement of this region. Infusions of lidocaine into the hippocampus impaired recent memory retrieval, but spared remote memory. Conversely, inactivation of the medial prefrontal cortices did not affect retrieval on day one, but impaired retrieval on day 30. Levels of Zif268 in the medial prefrontal cortex positively correlated with labelling of Growth Associated Protein 43 during remote retention. These results suggest a restructuring of cortical networks involved in remote recall of the reference memory radial arm maze task. When compared with a working memory version of the task, levels of Zif268 did not decrease in the hippocampus between day one and 30, further emphasising the role of the hippocampus in short-term rather than long-term spatial discrimination memory. While these findings appear conclusive, similar experiments investigating remote allocentric spatial memory in the water maze, which is the focus of this thesis, have yielded conflicting results.

Gusev et al. (2005) trained rats in the hidden version of the water maze and investigated Arc expression after recent (one day) and remote (30 day) retention. They found increased levels of *Arc* mRNA in CA1, CA3, the dentate gyrus and the subiculum during recent retention of the water maze. After one month, despite comparable retention performance, levels of *Arc* dropped dramatically in CA1, the dorsal segment of the dentate gyrus, and ventral segments of the subiculum. The magnitude and complexity of activation in the hippocampus and its circuitry was reduced, consistent with the findings

of Maviel et al. (2004) in the radial arm maze. However in contrast to these results, Teixeira, Pomedli, Maei, Kee, and Frankland (2006) found no difference in hippocampal IEG expression in mice trained in a fixed hidden version of the water maze during recent or remote retention. Mice acquired the task with the aid of a visible proximal cue, and the absence of this cue during retention enforced a change of strategy from cued to spatial, which could have resulted in a re-engagement of the hippocampus. However, inactivation of the hippocampus in this study impaired performance at both recent and remote retention irrespective of whether there was a visible or hidden platform during acquisition, a finding which has also been replicated with rats in the water maze (Broadbent, Squire, & Clark, 2006). Further inconsistencies in the literature exist with regards to the involvement of the anterior cingulate cortex during remote retention, as measured by IEG activation. When analysing IEG expression in cortical regions during retention of the water maze, Gusev and Gubin (2010) observed a reduction in expression of *Arc* mRNA in the parietal and cingulate areas from recent to remote retention. However, Teixeira et al. (2006) found that mice trained in the water maze displayed higher levels of Fos protein in the anterior cingulate cortex (ACC) at remote versus recent retention. Furthermore, inactivation of the ACC disrupted retention at remote testing but not recent, regardless of whether the platform was visible or hidden during training. These findings have been replicated recently by Lopez et al. (2012), who observed an increase in c-Fos expression in both the anterior cingulate cortex and the hippocampus between recent and remote retention in rats, with inactivation of the anterior cingulate cortex impairing remote memory, and inactivation of the hippocampus impairing both recent and remote memory. Therefore, although the literature is not equivocal, the general consensus appears to be that the anterior cingulate cortex becomes increasingly

involved in the retrieval of spatial memory in the water maze over time, whereas the hippocampus is involved in this type of memory regardless of its age.

Some key questions remain however. Given the fact that spatial memory is at least partially spared in hippocampal-lesioned patients (Spiers & Maguire, 2007), the relative contribution of other brain regions to the persistence of remote spatial memory remains unclear. Furthermore, although most studies demonstrate an increased reliance on medial prefrontal areas from one day to one month following learning, it is not clear when exactly during this time this shift occurs. Using methods which disrupt cellular consolidation can reveal the time-course of systems consolidation. Frankland, O'Brien, Ohno, Kirkwood, and Silva (2001) found that heterozygous α-CaMKII KO mice which have impaired cortical, but not hippocampal long-term potentiation display normal retention of the water maze up to 3 days, but are severely impaired from 10 to 17 days, suggesting a reliance on the cortex may emerge before 10 days have elapsed. Disrupting cellular functioning in the hippocampus during this specific period also seems to interfere with consolidation. Shimizu, Tang, Rampon, and Tsien (2000) devised a method where NMDA receptors in CA1 could be temporarily inactivated, finding that when NMDA receptors are switched off for seven days following water maze training, memory was impaired compared to wild-type controls at 15 days post-acquisition. Likewise, Riedel et al. (1999) temporarily inactivated the hippocampus using an AMPA receptor antagonist for seven days beginning one or five days after training, resulting in impaired retention at 16 days post-acquisition. Therefore systems consolidation may take place much sooner than 30 days, and IEG imaging would appear to be a useful method to assess the relative contribution of different brain regions to memory retention over the course of time. Analysing brain activity at a number of time points between one day and 30 days would likely yield more information about the course of systems consolidation.

One recent study has looked at IEG expression at multiple time-points over the retention period which has provided some insight into when a reliance on the cortex begins to develop. Bonaccorsi et al. (2013) analysed c-Fos expression in the hippocampus and medial prefrontal cortex following a probe trial at one day, 10 days, 20 days, 30 days, and 50 days post-acquisition of the water maze in mice. c-Fos expression in the dentate gyrus actually increased from one day to 20 and 30 days, and expression in CA1 on days 10, 20 and 30 were higher than day one. c-Fos expression in the anterior cingulate and infralimbic cortices only increased significantly from day one at day 30, although this process could be accelerated in the presence of an enriched housing environment. These results reflect those of Lopez et al. (2012), in that hippocampal involvement increased over time, and they show a delayed participation of the medial prefrontal cortex. No increase was found in the medial entorhinal or the parietal cortex over the course of retention under normal conditions in this study. Although this study only analysed the expression of one IEG, the results appear to be at odds with the standard theory of systems consolidation with regards to the role of the hippocampus, and the findings warrant further research.

The purpose of the current experiment is to gain a more complete understanding of the course of systems consolidation of spatial memory over the course of one month by using three markers of neural activity, and by assessing a wider range of brain regions. Rats will be given a probe test at one day, seven days, 14 days, or 30 days following acquisition of the water maze, and Zif268, c-Fos and Arc expression will be examined in CA1, CA3, the dentate gyrus, the lateral and medial entorhinal cortices, the retrosplenial, perirhinal and parietal cortices, and the anterior cingulate, prelimbic and infralimbic cortices. We hypothesise that the hippocampus will show equivalent or increased expression from day one to day 30, reflecting its continued involvement over time. We

also hypothesise that there will be an increase in activity in cortical areas as early as seven and 14 days following acquisition of the water maze. Furthermore, we expect to observe a positive correlation between IEG expression and performance during the probe trial as measured by searching accuracy, indicating a relationship between neural activity and successful retention.

6.2 Method

6.2.1 Subjects.

Male Wistar rats (n=33) obtained from Charles River Laboratories, UK, were used as subjects in the current study. The age and weight of subjects, housing conditions, handling, and time of experimentation were as described previously in Chapter 2.

6.2.2 Apparatus.

The Morris water maze was the behavioural task used in this study. Dimensions of the apparatus and cue configuration were as described previously in Chapter 2.

6.2.3 Procedure.

6.2.3.1 Acquisition.

Rats were randomly allocated to one of four experimental groups (n=7 per group). All four groups underwent standard water maze training, with a fixed hidden platform in the northeast quadrant of the pool, and all groups were trained for five days. A naïve control group (n=5) was also included, to provide a measure of baseline IEG activity.

6.2.3.2 Retention.

The four experimental groups were all given a retention probe trial, however the length of time between the final acquisition day and retention differed for each group. The first group were given a probe trial 24 hours following the fifth day of training. The second group were given a probe trial seven days after acquisition. The third group were tested at 14 days post-acquisition, and the fourth retention group were assessed at 30 days. The protocol for the retention probe trial was as previously described in Chapter 2.

6.2.4 Preservation of tissue.

All retention groups were sacrificed 90 minutes following their retention probe trial. Naïve rats were taken directly from their home cages and sacrificed. Rats were anaesthetised, transcardially perfused and their brains removed and post-fixed. Eleven regions were selected for analysis. Forty μm coronal sections were cut on a freezing microtome and every fourth section was taken for analysis.

6.2.5 Immunohistochemistry.

As the four retention probe trial groups and the caged control group were all stained in a single immunohistochemical session, normalisation procedures were not deemed necessary. Animals were processed in cohorts with one animal from each group stained side-by-side in a well plate. Immunohistochemical protocol for the detection of Zif268, c-Fos and Arc protein was followed as described previously in chapter 2.

6.2.6 Data analysis.

6.2.6.1 Behavioural data.

6.2.6.1.1 Acquisition.

To measure performance in the water maze, escape latencies for each trial were calculated, and averaged to produce a mean for every animal for each day.

6.2.6.1.2 Retention.

For the purposes of statistical analysis of swimming behaviour during the retention probe trial, the swimming area of the water maze was divided into multiple sections. To measure preference for an overall area in the maze, it was divided into four quadrants, northeast, northwest, southeast and southwest (see Figure 6.1A), and percentage time spent in each quadrant was calculated. For a more refined measure of time spent

searching in the correct area, percentage time spent in a circular area around the original platform location (comprising 13% of the area of the maze) was compared with equivalent areas in other quadrants of the pool (See Figure 6.1B). To measure the number of times the rats would have successfully mounted the platform if it were still in the pool, the number of crossings of the actual platform area were calculated (See Figure 6.1C).

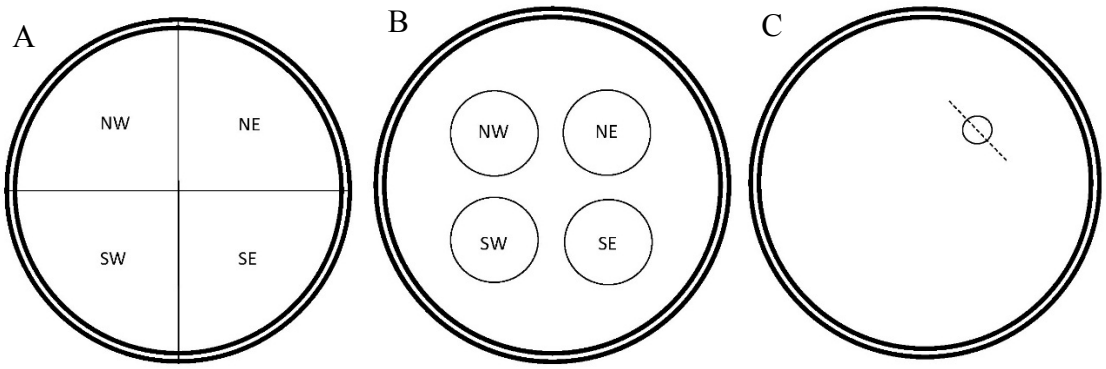

Figure 6.1: Pre-defined areas of the water maze for retention probe trials. Percentage time spent in quadrants (A), and platform areas (B) and number of platform crossings (C) were calculated.

6.2.6.2 IEG data.

Images were taken of the regions of interest using a digital camera (Olympus DP12). Computerised counts of Zif268, c-Fos and Arc positive neurons were analysed using the public domain program ImageJ (National Institute of Health, USA). Using a number of predefined parameters including a minimum and maximum size, darkness intensity and sphericity, the automated counting software delineated between neurons stained to a sufficient threshold, and other non-specific staining. Raw counts from all sections for each region were averaged to produce a mean for each animal.

6.2.7 Statistical analysis.

To analyse escape latencies during acquisition, one-way repeated measures analyses of variance (ANOVA) were performed, with Bonferroni-corrected comparisons. To compare percentage time spent in water maze quadrants and platform areas within groups during the retention probe trial, one-way repeated measures analyses of variance (ANOVA) were performed with Bonferroni-corrected comparisons. To compare platform crossings across groups during the retention trial, a one-way analysis of variance (ANOVA) was performed with Tukey *post-hoc* tests. To compare levels of Zif268, c-Fos and Arc across groups, one-way analyses of variance (ANOVA) were performed with Tukey *post-hoc* tests. To assess relationships between levels of Zif268, c-Fos and Arc and performance in the water maze during the retention trial, Pearson product-moment correlations were performed.

6.2.8 Ethical considerations.

Guidelines for the maintenance and experimentation of animals conformed to the Department of Health and Children (Ireland) guidelines under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU. The National University of Ireland, Maynooth ethics committee also approved all experimental work.

6.3 Results

6.3.1 Behavioural results.

6.3.1.1 Acquisition.

A 3 x 5 mixed factorial ANOVA with group as the between group factor and day as the within group factor, confirmed there was an overall significant decrease in escape latency with a main effect for acquisition day, $F(4, 96) = 36.22$, $p < 0.001$, and Bonferroni post-hoc analyses revealed that escape latencies were significantly lower on day five (*M*: 15.83±1.20 sec, $p < 0.001$) than day one (*M*: 39.28±1.27 sec, see Figure 6.2).

Subsequent repeated measures ANOVAs with Bonferroni correction were performed on the escape latencies of the individual groups to further investigate how they changed over the course of training. A significant effect for day was found in the one day group, $F(4, 24) = 5.85$, $p < 0.01$, with post-hoc analyses showing that escape latencies were significantly lower on day five (*M*: 18.76±3.26 sec, $p < 0.05$) than day one (*M*: 35.51±5.12 sec). The seven day retention group also showed a significant effect for day, $F(4, 24) = 11.67$, $p < 0.001$, with post-hoc analyses showing that escape latencies were significantly lower on day five (*M*: 17.46 ±2.33 sec, $p < 0.01$) than day one (*M*: 44.81±3.60 sec). A significant effect for day was found in the 14 day group, $F(4, 24) = 9.47$, $p < 0.001$, with post-hoc analyses showing that escape latencies were significantly lower on day five (*M*: 14.87±2.75 sec, $p < 0.05$) than day one (*M*: 40.76±3.61 sec). The 30 day retention group also showed a significant effect for day, $F(4, 24) = 11.84$, $p < 0.001$, with post-hoc analyses showing that escape latencies were significantly lower on day five (*M*: 12.23±1.49 sec, $p < 0.01$) than day one (*M*: 36.04±3.41 sec). There was no overall difference found in escape latencies between the groups, $F(3, 24) = 1.33$, $p > 0.05$, nor was an interaction effect between day and group observed, $F(12, 96) = 0.56$, *p*

> 0.05, see Figure 6.2). In summary, all groups acquired the maze, demonstrating comparable learning performance.

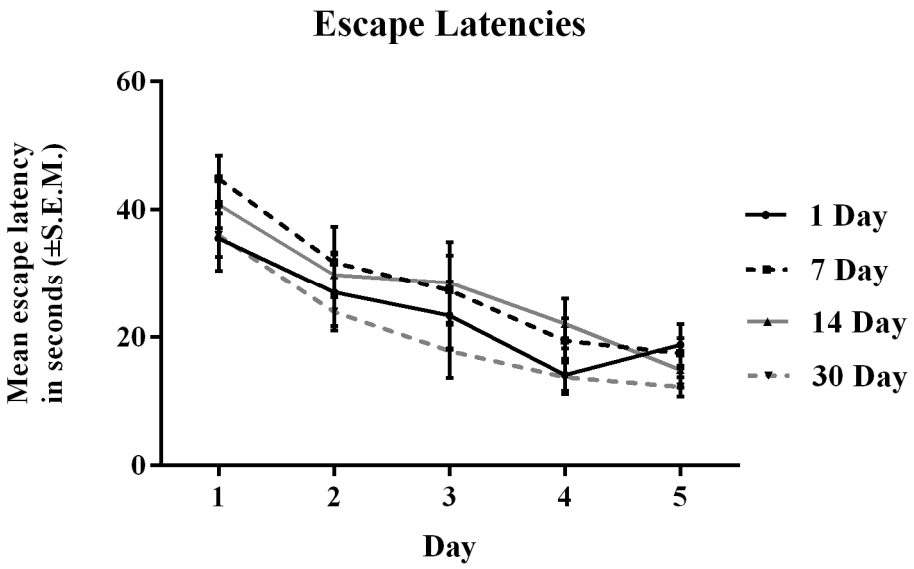

Figure 6.2: Mean daily escape latencies over five days of training for the one day, seven day, 14 day and 30 day retention groups.

6.3.1.2 Retention.

As a general measure of successful retention of the water maze, percentage time spent swimming in the target northeast quadrant of the maze was compared to other quadrants. One-way repeated measures ANOVAs were conducted on all four groups to compare time spent searching in each quadrant. In the one day retention group, a significant effect was found for quadrant, $F(3, 18) = 11.86$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing this group spent significantly more time searching in the northeast quadrant (*M*: 39.81±2.68 %) than the southeast (*M*: 18.96±2.32 %, $p < 0.05$) and the southwest quadrants (*M*: 19.10±1.98 %, $p < 0.01$, see Figure 6.3A). A significant effect for quadrant was also found in the seven day group, $F(3, 18) = 8.39$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing this group spent more time in the northeast quadrant (*M*: 36.95±1.24 %) than the northwest (*M*: 22.24±2.67 %, $p < 0.05$), southeast (*M*: 17.86±2.99

%, $p < 0.05$) and southwest quadrants (*M*: 22.95±2.63 %, $p < 0.01$, see Figure 6.3B). A significant effect for quadrant was not found in the 14 day, $F(3, 18) = 3.07$, $p > 0.05$ (see Figure 6.3C), or the 30 day group, $F(3, 18) = 3.05$, $p > 0.05$, see (Figure 6.3D).

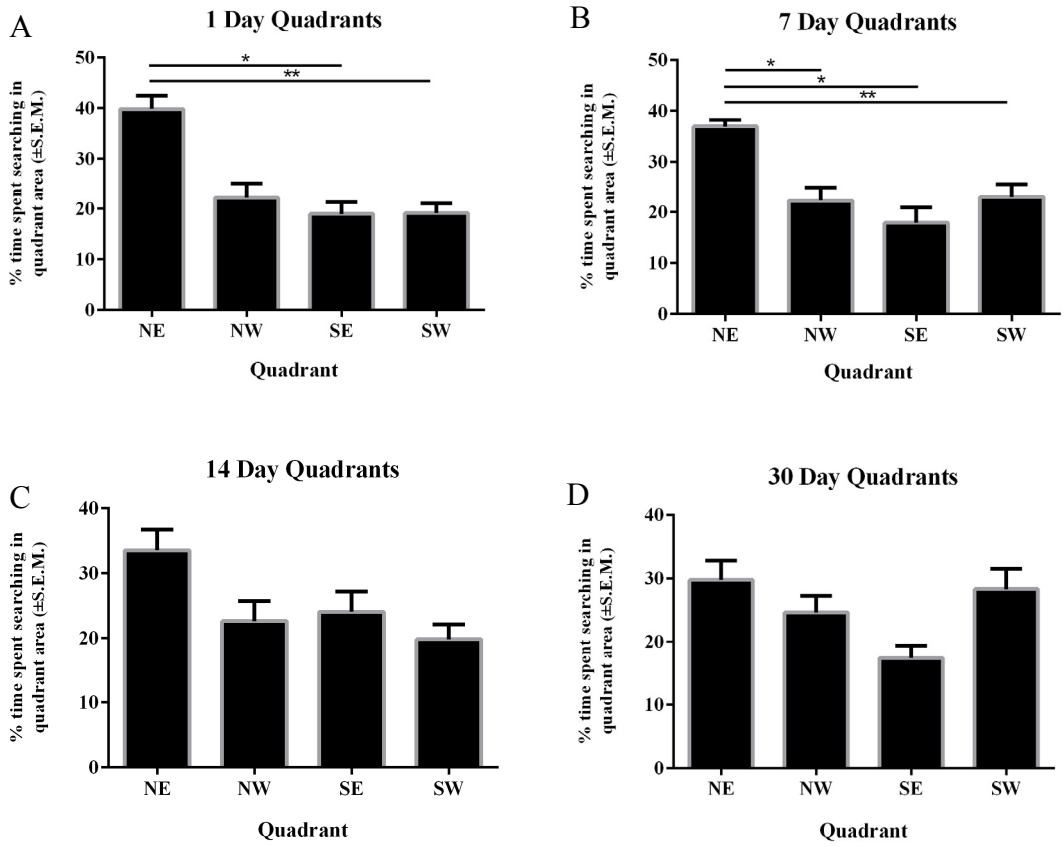

Figure 6.3: Percentage time spent searching in all four quadrants of the water maze during the probe trial for the one day (A), seven day (B), 14 day (C) and 30 day (D) retention groups.

To obtain a more refined measure of time spent searching in the correct area, a circular area around the target platform location, comprising 13% of total maze area, was compared with equivalent areas in the remaining three maze quadrants. One-way repeated measures ANOVAs were conducted on all four groups to compare time spent in the target northeast platform area to other equivalent areas. In the one day retention group, Mauchly's test indicated that the assumption of sphericity had been violated, $x^2(5)$

= 13.79, p < 0.05, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = 0.47). The results showed a significant effect for platform area, $F(1.42, 8.49) = 13.38$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing this group spent significantly more time searching in the northeast platform area (*M*: 14.00±2.15 %) than the southeast (*M*: 3.95±1.20 %, $p < 0.05$) and the southwest areas (*M*: 2.67±0.48 %, $p < 0.01$, see Figure 6.4A). A significant effect for platform area was also found in the seven day retention group, $F(3, 18) = 13.49$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing this group spent more time in the northeast platform area (*M*: 13.86±4.02 %) than the southeast (*M*: 4.33±3.52 %, $p < 0.05$) and southwest areas (*M*: 4.05±2.16 %, $p < 0.01$, see Figure 6.4B). A significant effect for platform area was also found in the 14 day retention group, $F(3, 18) = 9.31$, $p < 0.001$, with Bonferroni *post-hoc* analyses revealing this group spent more time in the northeast platform area (*M*: 13.71±1.88 %) than the southeast (*M*: 5.24±0.67 %, $p < 0.05$) and southwest areas (*M*: 4.76±0.93 %, $p < 0.05$, see Figure 6.4C). A significant effect for platform area was also found in the 30 day retention group, $F(3, 18) = 6.05$, $p < 0.01$, with Bonferroni *post-hoc* analyses revealing this group spent more time in the northeast platform area (*M*: 12.38±1.57 %) than the southeast area (*M*: 5.91±0.49 %, $p < 0.05$, see Figure 6.4D).

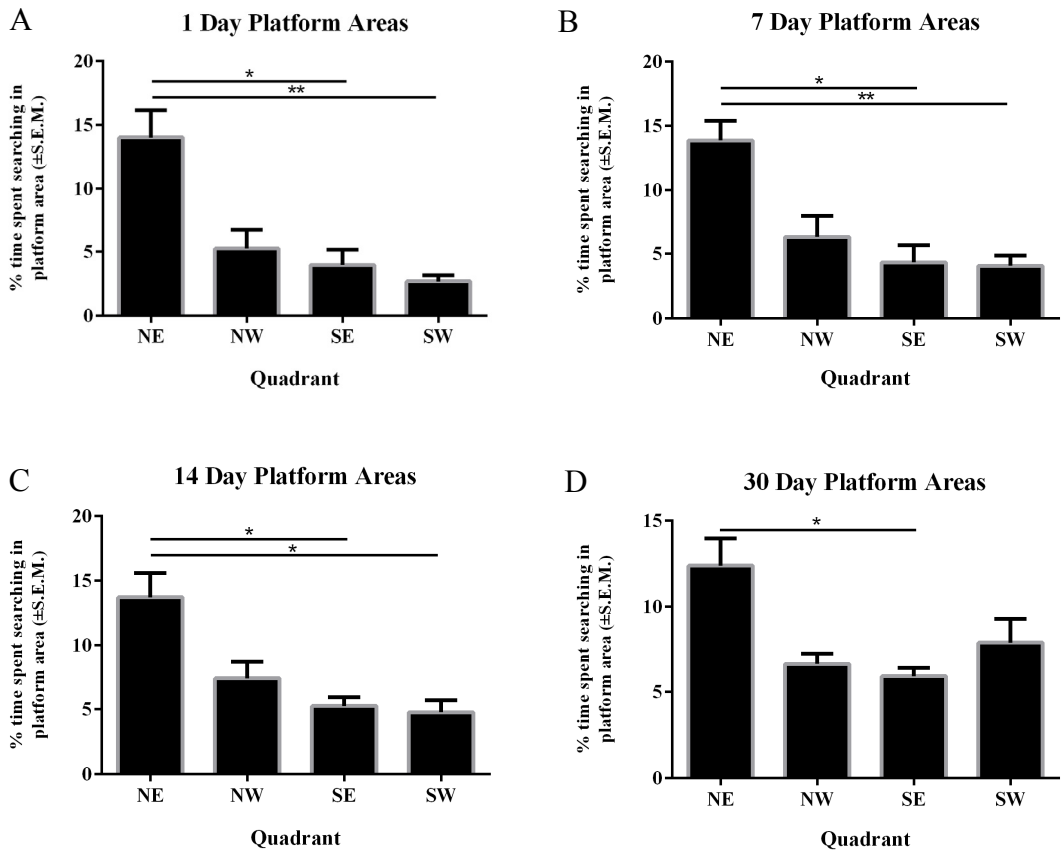

Figure 6.4: Percentage time spent searching in all four platform areas of the water maze during the probe trial for the one day (A), seven day (B), 14 day (C) and 30 day (D) retention groups.

A further measure of accurate searching was number of platform crossings, or the number of times the animal would have located the exact platform location over the course of the 60 second probe trial. In the one day retention group, the mean number of platform crossings was 3.14±0.37, with the 14 day retention group having an average of 3.86±0.51 crossings, the 14 day group crossing the platform an average of 3.29±0.68 times, and the 30 day group 1.86±0.26 times over the 60 second test (see Figure 6.5). A one-way ANOVA did not find a difference in the number of platform crossings across the four retention groups, $F(3, 24) = 2.49$, $p > 0.05$.

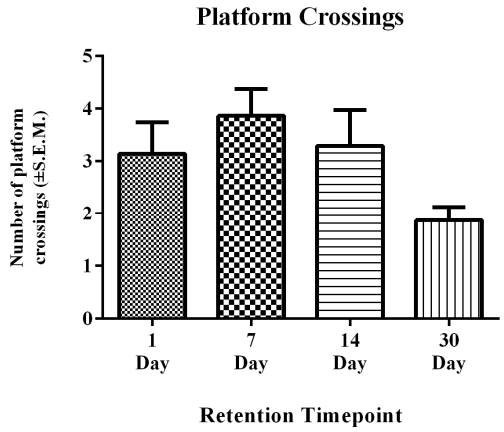

Figure 6.5: Number of platform crossings during the probe trial for the one day, seven day, 14 day and 30 day retention groups.

These findings suggest that although some memory degradation did occur from one day to 30 days following training, the remote group still displayed retention of the platform location.

6.3.2 IEG results.

6.3.2.1 Zif268.

To determine the changes in IEG expression from baseline to one day, seven days, 14 days and 30 days post-acquisition following a retention probe trial, a series of one-way ANOVAs with Tukey *post-hoc* analyses were performed. Beginning with the hippocampal formation, significant differences across groups were not found in CA1, $F(4, 26) = 2.65$, $p > 0.05$, CA3, $F(4, 27) = 1.85$, $p > 0.05$, the lateral entorhinal cortex, $F(4, 28) = 1.74$, $p > 0.05$, or the medial entorhinal cortex, $F(4, 26) = 0.89$, $p > 0.05$ (see Figure 6.6 and Figure 6.10 Left). A difference was found across groups in the dentate gyrus, $F(4, 28) = 3.18$, $p < 0.05$, with *post-hoc* Tukey tests revealing Zif268 expression was significantly lower in the one day retention group (*M*: 46.07±13.65) than the caged control group (*M*: 161.37±24.98, $p < 0.05$, see Figure 6.6C).

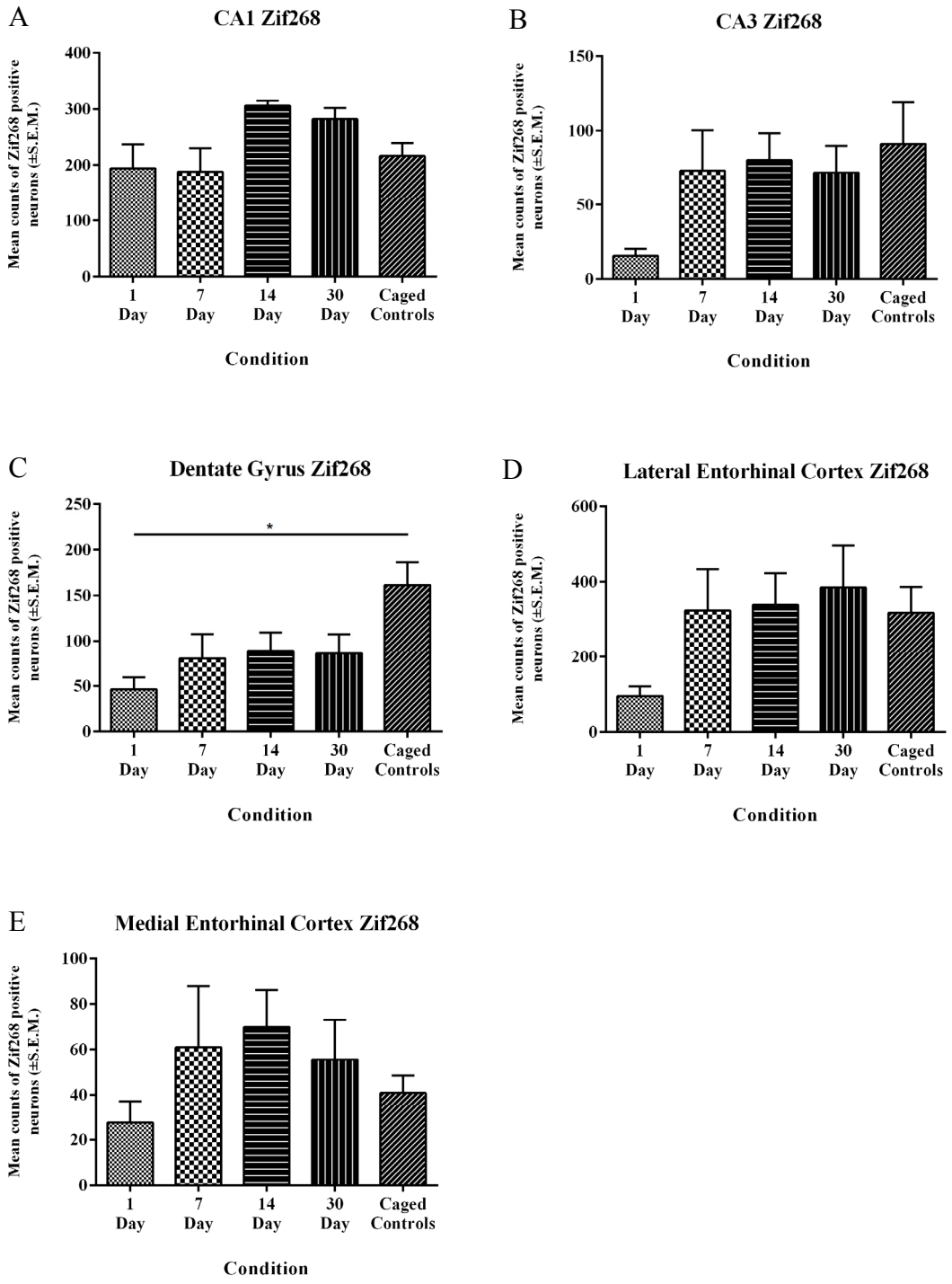

Figure 6.6: Changes in the level of Zif268 expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E).

No significant differences were found across groups in the retrosplenial cortex, $F(4, 28) = 0.47$, $p > 0.05$, or the parietal cortex, $F(4, 27) = 1.69$, $p > 0.05$, see Figure 6.7). A main effect for group was found in the perirhinal cortex, $F(4, 27) = 2.73$, $p < 0.05$, however Tukey *post-hoc* analyses did not reveal any significant differences between the groups (see Figure 6.7B).

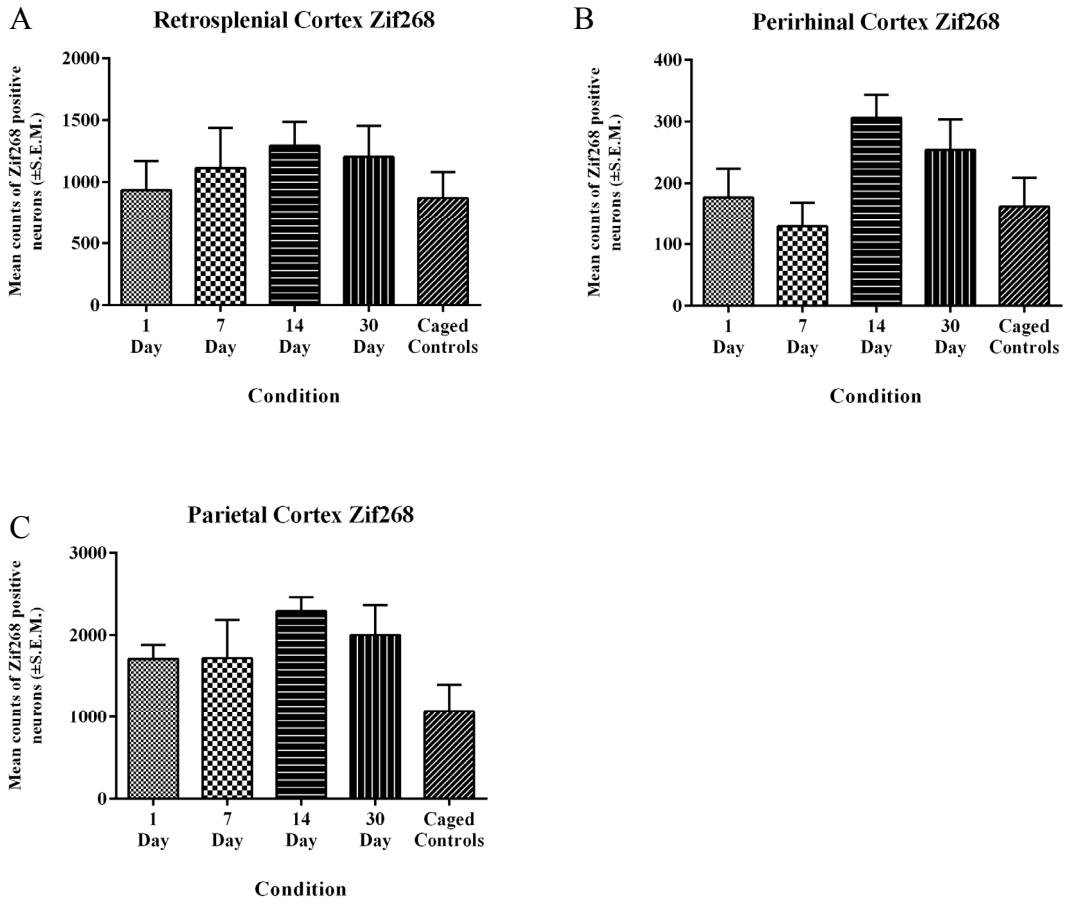

Figure 6.7: Changes in the level of Zif268 expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the retrosplenial (A), perirhinal (B), and parietal cortices (C).

A significant difference across groups was found in the anterior cingulate cortex $F(4, 26) = 2.97$, $p < 0.05$, with *post-hoc* Tukey analyses revealing levels of Zif268 were significantly higher at 30 day retention (*M*: 1362.46±19.92) than one day retention (*M*: 560.17±134.13, $p < 0.05$, see Figure 6.8A and Figure 6.10 Middle). A significant difference was also found in the prelimbic cortex, $F(4, 27) = 3.11$, $p < 0.05$, with Tukey *post-hoc* tests revealing Zif268 expression was significantly higher at 30 day retention (*M*: 1575.13±134.88) than one day retention (*M*: 557.25±182.45, $p < 0.05$, see Figure 6.8B). A significant difference was also found in the infralimbic cortex, $F(4, 25) = 8.31$, $p < 0.001$), with Tukey *post-hoc* tests revealing Zif268 counts increased significantly from one day retention (*M*: 23.04±5.05) to 14 day retention (*M*: 175.96±38.37, $p < 0.01$) and 30 day retention (*M*: 211.26±12.88, $p < 0.001$). There was also a significant increase in Zif268 levels from seven days, (*M*: 91.60±29.20) to 30 days ($p < 0.01$, see Figure 6.8C and Figure 6.10 Right).

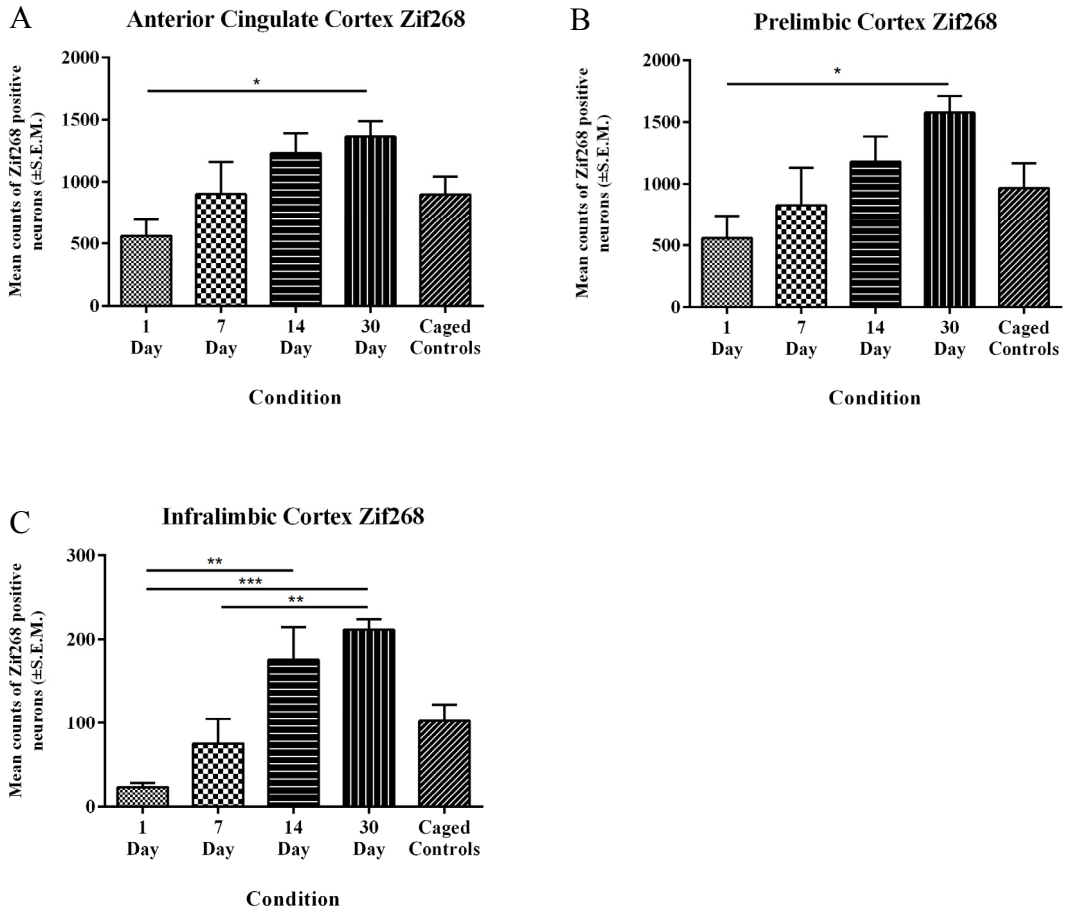

Figure 6.8: Changes in the level of Zif268 expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the anterior cingulate (A), prelimbic (B), and infralimbic cortices (C).

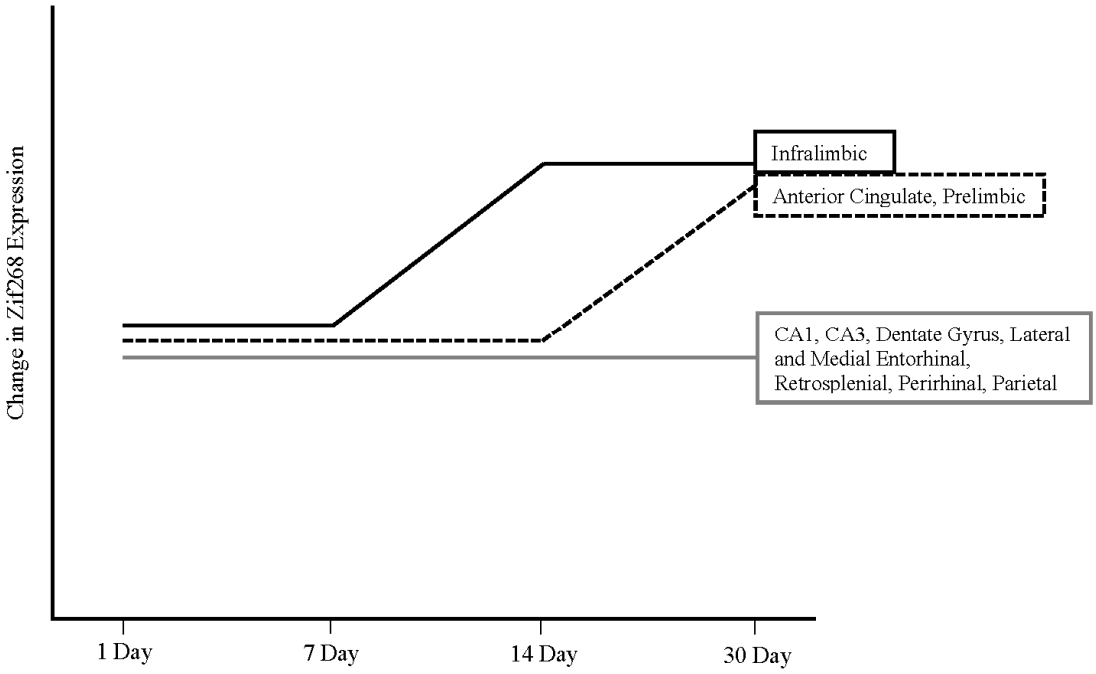

Figure 6.9: Schematic diagram summarising the changes in Zif268 expression in all brain regions analysed following a retention probe trial of the Morris water maze at one day, seven days, 14 days and 30 days. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

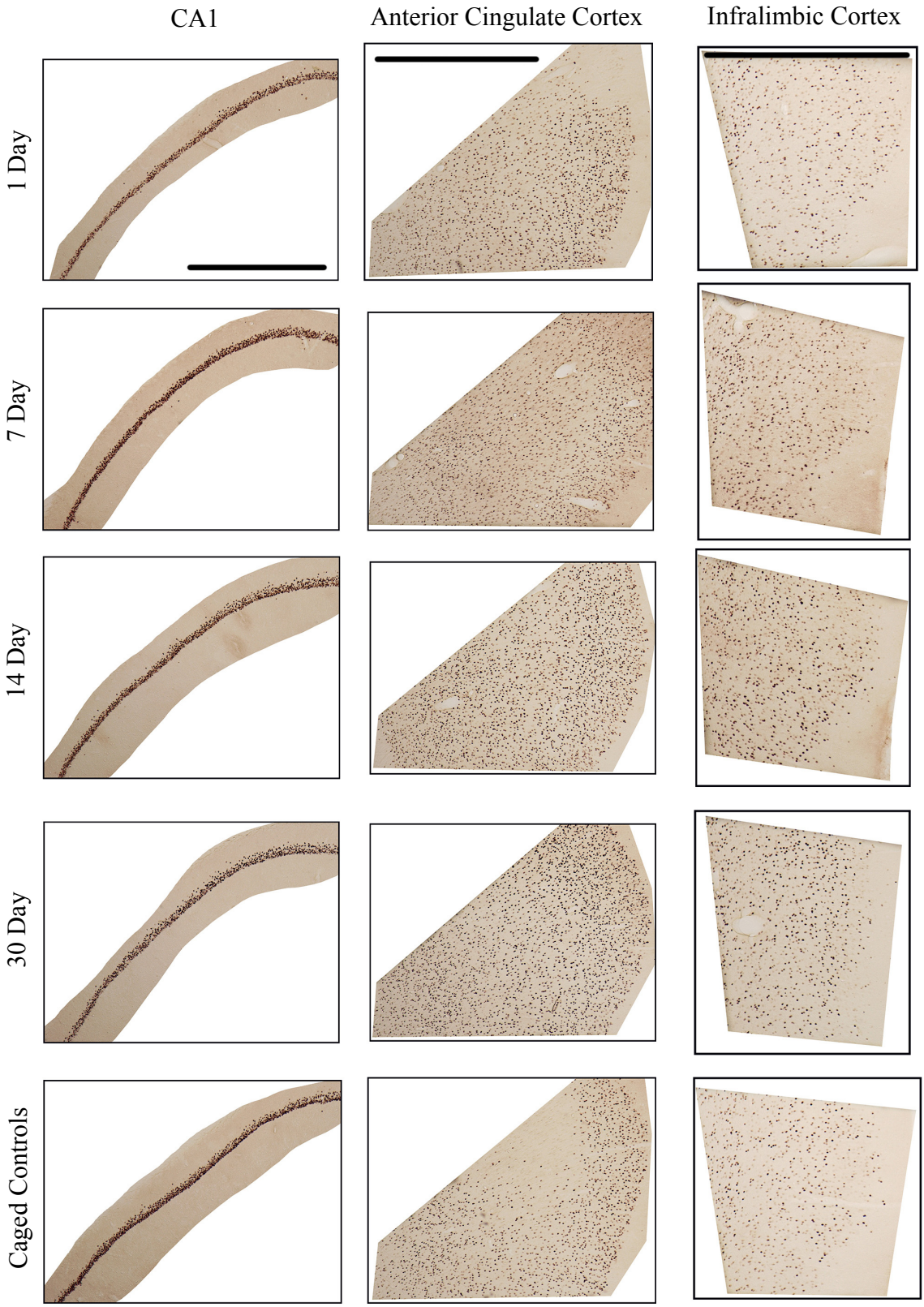

Figure 6.10: Representative photos of Zif268 expression in selected regions: CA1, the anterior cingulate cortex and the infralimbic cortex at one day, seven day, 14 day and 30 day retention, as well as caged controls. Scale bar = 1mm.

6.3.2.1.1 Correlations with performance.

To assess the relationship between Zif268 expression and performance during the retention probe trial, counts of Zif268 were correlated with two measures of searching accuracy, number of platform crossings and percentage time spent searching in the platform area. The relationship between levels of Zif268 and number of platform crossings are displayed in Table 6.1. Significant positive correlations were found between Zif268 counts and the number of platform crossings in the one day retention group for the dentate gyrus ($r = 0.80$, $p < 0.05$), the lateral entorhinal cortex ($r = 0.81$, $p < 0.05$), the retrosplenial cortex ($r = 0.92$, $p < 0.01$) the perirhinal cortex ($r = 0.91$, $p < 0.01$), and the prelimbic cortex ($r = 0.88$, $p < 0.01$).

Table 6.1: Correlations between Zif268 expression and number of northeast platform crossings

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA1	0.71	-0.11	-0.13	-0.32
CA3	0.25	0.18	0.13	-0.17
Dentate Gyrus	0.80*	-0.08	0.06	0.38
Lateral Entorhinal Cortex	0.81*	0.09	0.28	-0.01
Medial Entorhinal Cortex	0.75	0.07	-0.32	-0.19
Retrosplenial Cortex	0.92**	0.13	0.39	-0.17
Perirhinal Cortex	0.91**	-0.18	-0.36	0.11
Parietal Cortex	0.64	0.11	0.52	-0.08
Anterior Cingulate Cortex	0.21	0.12	-0.67	-0.06
Prelimbic Cortex	0.88**	-0.02	-0.54	-0.62
Infralimbic Cortex	0.05	0.00	-0.53	-0.81

Percentage time spent searching in the northeast platform area was also correlated with levels of Zif268 expression. Significant positive correlations were found in the seven day retention group in CA1 ($r = 0.83$, $p < 0.05$), CA3, ($r = 0.81$, $p < 0.05$), the dentate gyrus ($r = 0.81$, $p < 0.05$), lateral entorhinal cortex ($r = 0.92$, $p < 0.01$), retrosplenial cortex ($r = 0.94$, $p < 0.01$), parietal cortex ($r = 0.94$, $p < 0.01$), anterior cingulate cortex ($r = 0.89$, $p < 0.01$), prelimbic cortex ($r = 0.82$, $p < 0.05$) and infralimbic cortex ($r = 0.82$, $p < 0.05$).

Relationships between Zif268 expression and water maze performance were also found in the 14 day retention group in CA1 ($r = 0.82$, $p < 0.05$), CA3 ($r = 0.86$, $p < 0.05$), the dentate gyrus ($r = 0.82$, $p < 0.05$), medial entorhinal cortex ($r = 0.86$, $r = 0.86$) and the perirhinal cortex ($r = 0.79$, $p < 0.05$). In the 30 day retention group, a significant negative correlation was found between Zif268 expression and time spent searching in the target platform area ($r = -0.83$, $p < 0.05$, see Table 6.2).

Table 6.2: Correlations between Zif268 expression and percentage time spent searching in northeast platform area

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA1	0.48	0.83*	0.82*	-0.36
CA3	0.28	0.81*	0.86*	-0.83*
Dentate Gyrus	0.36	0.81*	0.82*	-0.69
Lateral Entorhinal Cortex	0.64	0.92**	0.61	-0.68
Medial Entorhinal Cortex	0.24	0.70	0.86*	-0.43
Retrosplenial Cortex	0.73	0.94**	0.34	-0.56
Perirhinal Cortex	0.67	0.79	0.79*	-0.68
Parietal Cortex	0.17	0.94**	0.40	-0.50
Anterior Cingulate Cortex	0.41	0.89**	-0.16	0.25
Prelimbic Cortex	0.65	0.82*	0.13	-0.17
Infralimbic Cortex	-0.04	0.82*	0.17	-0.58

6.3.2.2 c-Fos.

A significant difference in c-Fos expression across groups was found in CA1, $F(4, 26) = 4.02$, $p < 0.05$, with *post-hoc* Tukey analyses revealing counts increased significantly from one day retention (*M*: 2.26±1.15) to seven day retention (*M*: 22.25±7.39, $p < 0.05$, see Figure 6.11A). A significant difference across groups was found in CA3, $F(4, 26) = 5.03$, $p < 0.01$, with *post-hoc* Tukey analyses demonstrating c-Fos counts increased significantly from day one retention (*M*: 3.63±0.32) to seven day retention (*M*: 21.57±7.12, $p < 0.05$) and 14 day retention (*M*: 24.22±1.35, $p < 0.05$). Counts at 14 day retention were also higher than caged control levels (*M*: 3.47±1.22, $p < 0.05$, see Figure 6.11B and Figure 6.15 Left). A significant difference between groups was also found in the dentate gyrus, $F(4, 27) = 3.34$, $p < 0.05$, with Tukey *post-hoc* tests revealing c-Fos counts were higher at seven day retention (*M*: 41.72±9.84) than one day retention (16.80±2.83, $p < 0.05$, see Figure 6.11C). A main effect for group was also found in the lateral entorhinal cortex $F(4, 25) = 4.38$, $p < 0.01$, with Tukey *post-hoc* analyses revealing counts of c-Fos were significantly higher at seven day retention (*M*: 84.14±21.15) than one day retention (*M*: 19.15±4.03, $p < 0.05$) and caged controls (*M*: 15.23±6.30, $p < 0.05$, see Figure 6.11D). A significant difference was also found across groups in the medial entorhinal cortex, $F(4, 25) = 3.90$, $p < 0.05$, with *post-hoc* Tukey tests revealing levels of c-Fos were higher at 14 day retention (*M*: 34.13±5.02) than caged controls (*M*: 2.98±0.91, $p < 0.05$, see Figure 6.11E).

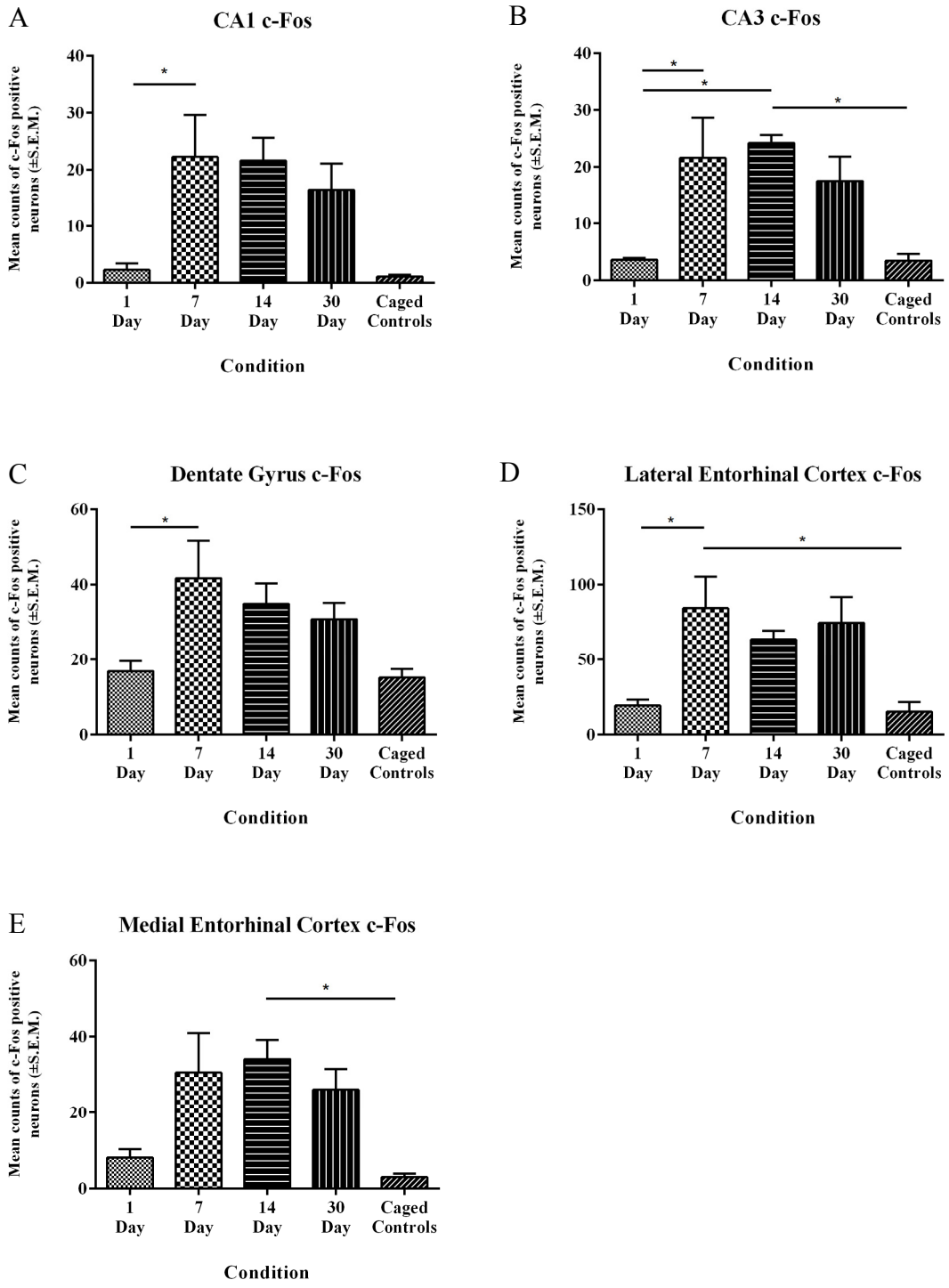

Figure 6.11: Changes in the level of c-Fos expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in CA1 (A), CA3 (B), dentate gyrus (C), lateral entorhinal cortex (D) and medial entorhinal cortex (E).

A significant difference was found across groups in the retrosplenial cortex, $F(4, 24) = 4.14$, $p > 0.05$, with Tukey *post-hoc* analyses revealing c-Fos counts were higher in the 30 day retention group (*M*: 145.67±37.49) than the caged control group (*M*: 19.19±8.77, $p < 0.05$, see Figure 6.12A). A main effect for group was also found in the perirhinal cortex, $F(4, 27) = 8.47$, $p < 0.001$, with Tukey *post hoc* analyses revealing c-Fos counts were higher at 14 day retention (*M*: 61.63±8.06) than one day retention (*M*: 19.82±4.79, $p < 0.01$) and caged controls (2.67±0.49, $p < 0.001$). c-Fos counts were also higher at 30 day retention (*M*: 56.40±8.27) than one day retention ($p < 0.05$) and caged controls ($p < 0.01$). c-Fos counts were also higher at seven day retention (M: 39.81±10.05) than caged controls, ($p < 0.05$, see Figure 6.12B and Figure 6.15 Middle). A significant difference was also found in the parietal cortex, $F(4, 27) = 4.07$, $p < 0.05$, with Tukey post hoc analyses revealing c-Fos counts were higher at 14 day retention (*M*: 221.36±52.69) than one day retention (*M*: 34.68±7.96, $p < 0.05$) and caged controls (8.40±3.50, $p < 0.05$, see Figure 6.12C).

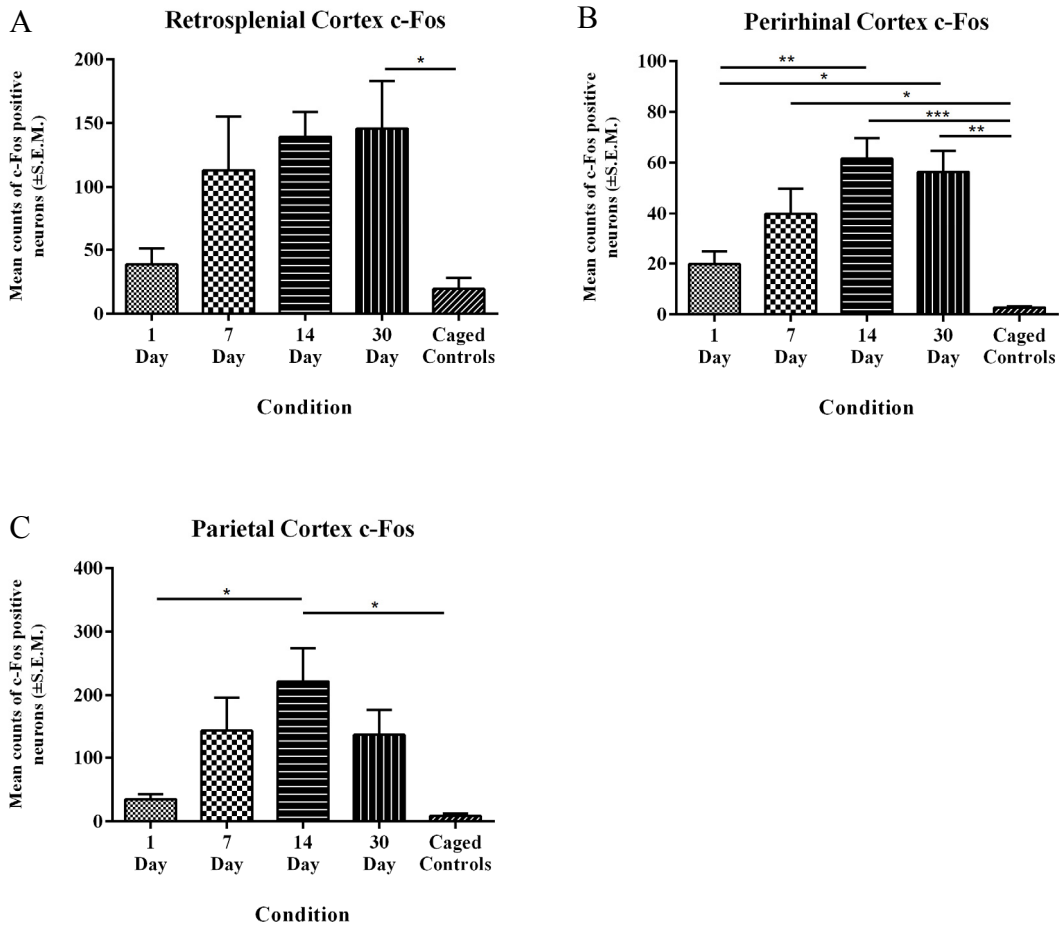

Figure 6.12: Changes in the level of c-Fos expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the retrosplenial (A), perirhinal (B), and parietal cortices (C).

A significant difference was found in the anterior cingulate cortex $F(4, 24) = 3.72$, $p < 0.05$, however *post-hoc* Tukey analyses did not reveal any differences between the groups (see Figure 6.13A). A significant difference was also found in the prelimbic cortex, $F(4, 25) = 8.49$, $p < 0.001$, with Tukey *post-hoc* tests revealing levels of c-Fos were significantly higher at 14 day retention (*M*: 198.97±11.36) than one day retention (M: 38.51±8.32, $p < 0.05$) and caged controls (*M*: 40.80±15.21, $p < 0.05$). Counts were also higher at 30 day retention (*M*: 270.79±55.34) than one day retention ($p < 0.001$), seven day retention (*M*: 129.54±39.26, $p < 0.05$), and caged controls ($p < 0.01$, see Figure 6.13B). A main effect for group was also found in the infralimbic cortex, $F(4, 26) =$

18.06, $p < 0.001$), with Tukey *post-hoc* tests revealing levels of c-Fos increased significantly from one day retention (*M*: 12.31±2.87) to 14 day (*M*: 70.74± 5.51, $p < 0.001$) and 30 day retention (*M*: 85.64±9.76, $p < 0.001$). There was also a significant increase in c-Fos levels from seven day retention (*M*: 36.22±11.51) to 14 day retention ($p < 0.05$) and 30 day retention ($p < 0.01$). Counts were also higher in the 14 day ($p < 0.001$) and 30 day ($p < 0.001$) retention groups than caged controls (*M*: 10.50±3.27, see Figure 6.13C and Figure 6.15 Right).

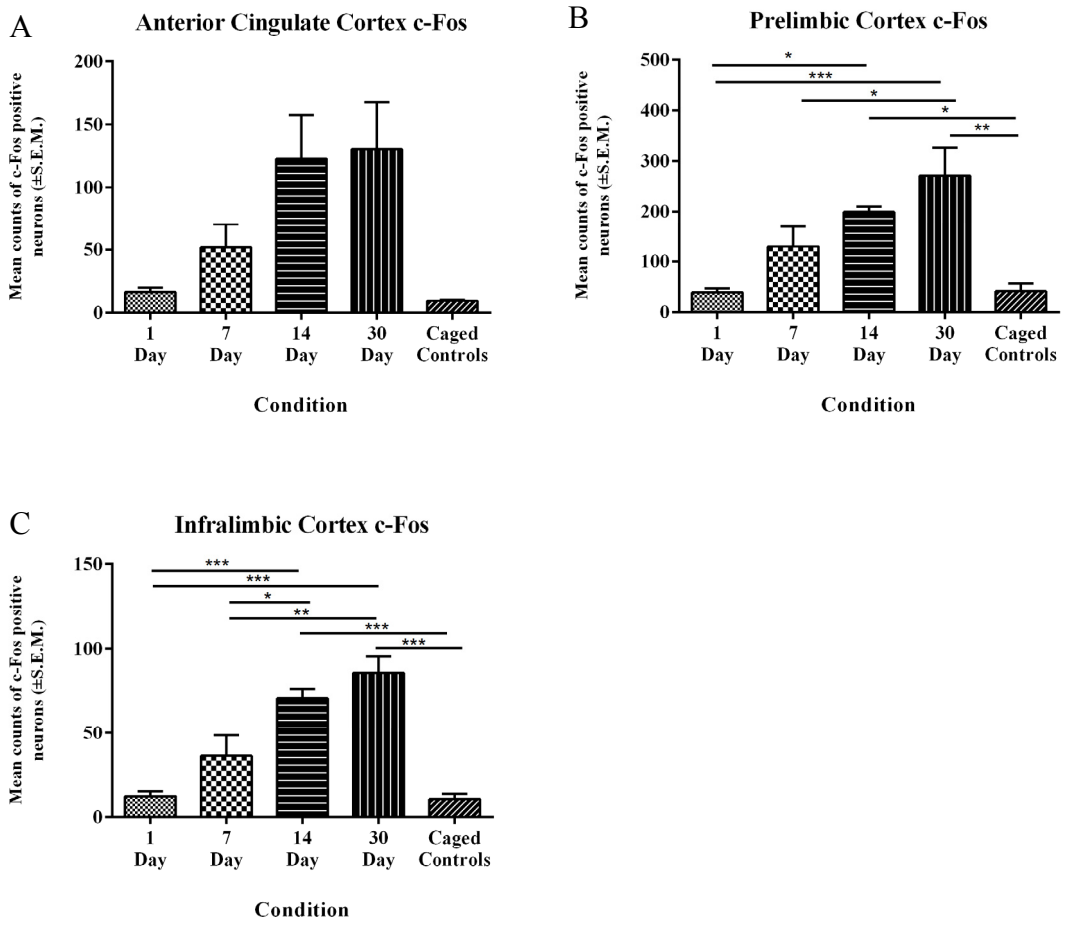

Figure 6.13: Changes in the level of c-Fos expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the anterior cingulate (A), prelimbic (B), and infralimbic cortices (C).

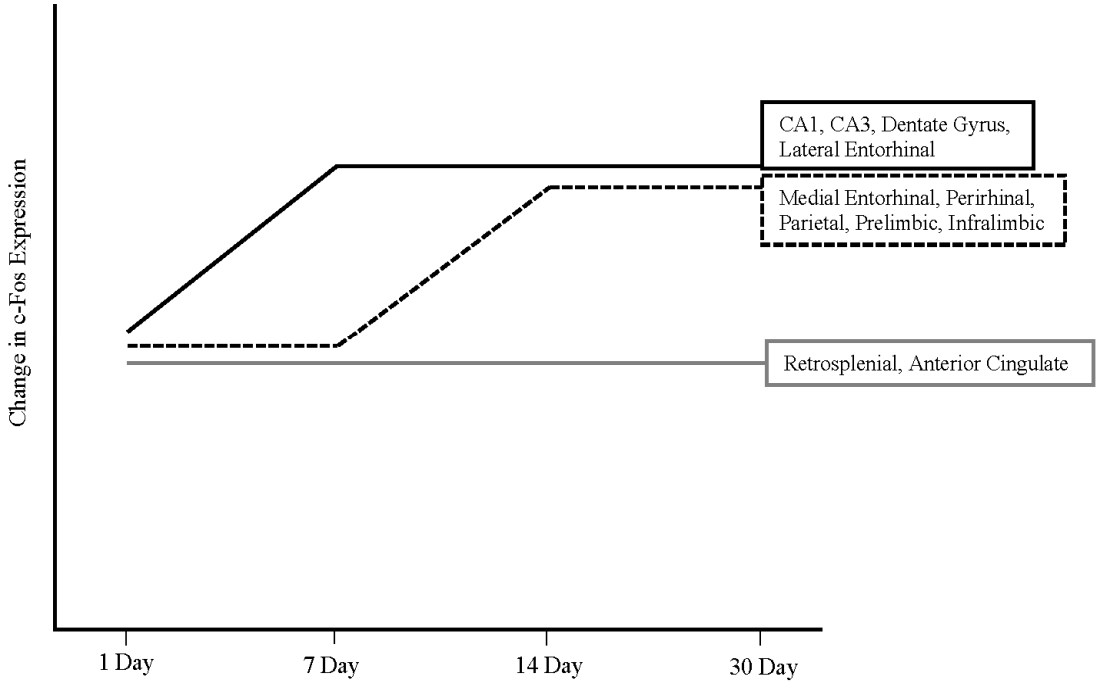

Figure 6.14: Schematic diagram summarising the changes in c-Fos expression in all brain regions analysed following a retention probe trial of the Morris water maze at one day, seven days, 14 days and 30 days. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

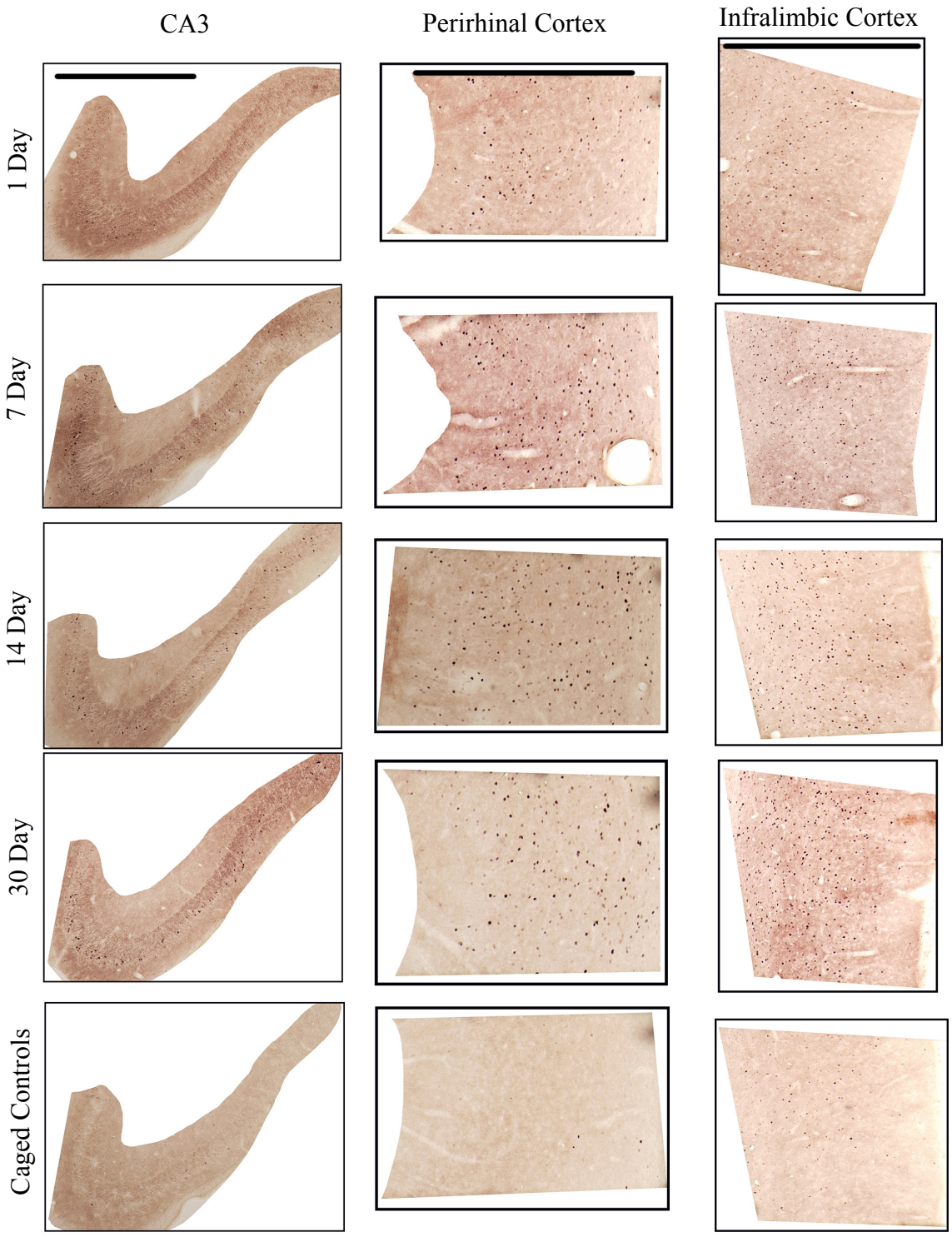

Figure 6.15: Representative photos of c-Fos expression in selected regions: CA3, the perirhinal cortex and the infralimbic cortex at one day, seven day, 14 day and 30 day retention, as well as caged controls. Scale bar = 1mm.

6.3.2.2.1 Correlations with performance.

The relationship between c-Fos expression and number of platform crossings are displayed in Table 6.3. In the one day retention group, a significant positive correlation was found between c-Fos counts and the number of platform crossings in CA1 ($r = 0.86$, $p < 0.05$).

Table 6.3: Correlations between c-Fos expression and number of northeast platform crossings

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA1	0.86*	0.14	0.10	-0.33
CA3	0.06	0.09	-0.75	-0.59
Dentate Gyrus	0.53	0.02	0.51	-0.31
Lateral Entorhinal Cortex	0.59	0.08	0.09	-0.48
Medial Entorhinal Cortex	0.78	-0.06	0.49	-0.21
Retrosplenial Cortex	0.53	-0.27	0.12	-0.39
Perirhinal Cortex	0.72	-0.12	0.71	-0.31
Parietal Cortex	-0.45	-0.09	-0.49	-0.17
Anterior Cingulate Cortex	0.41	-0.45	-0.25	-0.30
Prelimbic Cortex	0.80	-0.37	-0.10	-0.42
Infralimbic Cortex	0.57	-0.38	-0.19	0.00

Relationships between c-Fos expression and percentage time searching in the northeast platform area were also assessed. Significant negative correlations were found in the 30 day group in the parietal cortex ($r = -0.90$, $p < 0.01$) and the prelimbic cortex ($r = -0.81$, $p < 0.05$, see Table 6.4.

Table 6.4: Correlations between c-Fos expression and percentage time spent searching in northeast platform area

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA1	0.71	0.24	0.16	-0.67
CA3	0.31	0.56	-0.47	-0.46
Dentate Gyrus	0.11	0.64	0.68	-0.52
Lateral Entorhinal Cortex	0.76	0.36	0.53	-0.70
Medial Entorhinal Cortex	0.63	0.56	-0.05	-0.67
Retrosplenial Cortex	0.01	0.19	-0.45	-0.61
Perirhinal Cortex	0.37	0.46	0.64	-0.61
Parietal Cortex	-0.36	0.45	-0.06	-0.90**
Anterior Cingulate Cortex	0.18	0.42	-0.39	-0.69
Prelimbic Cortex	0.38	0.49	0.29	-0.81*
Infralimbic Cortex	0.16	0.21	0.50	-0.74

6.3.2.3 Arc.

A significant difference across groups was found in CA3, $F(4, 28) = 4.19$, $p < 0.01$, with *post-hoc* Tukey analyses revealing Arc counts were significantly higher at 14 day retention (*M*: 11.33±2.52) than one day retention (*M*: 0.98±0.31, $p < 0.05$), and caged controls (*M*: 1.27±0.53, $p < 0.05$, see Figure 6.16A). A significant difference was not found across groups in the dentate gyrus, $F(4, 27) = 1.46$, $p > 0.05$, see Figure 6.16B. There was a main effect for group found in the lateral entorhinal cortex, $F(4, 24) = 5.26$, $p < 0.01$, with Tukey *post-hoc* analyses revealing counts of Arc were significantly higher at 30 day retention (*M*: 14.04±3.66) than one day retention (*M*: 1.65±0.53, $p < 0.05$) and caged controls (*M*: 0.94±0.26, $p < 0.05$, see Figure 6.16C and Figure 6.20 Left). A significant difference was not found across groups in the medial entorhinal cortex, $F(4, 26) = 2.31$, $p > 0.05$, see Figure 6.16D).

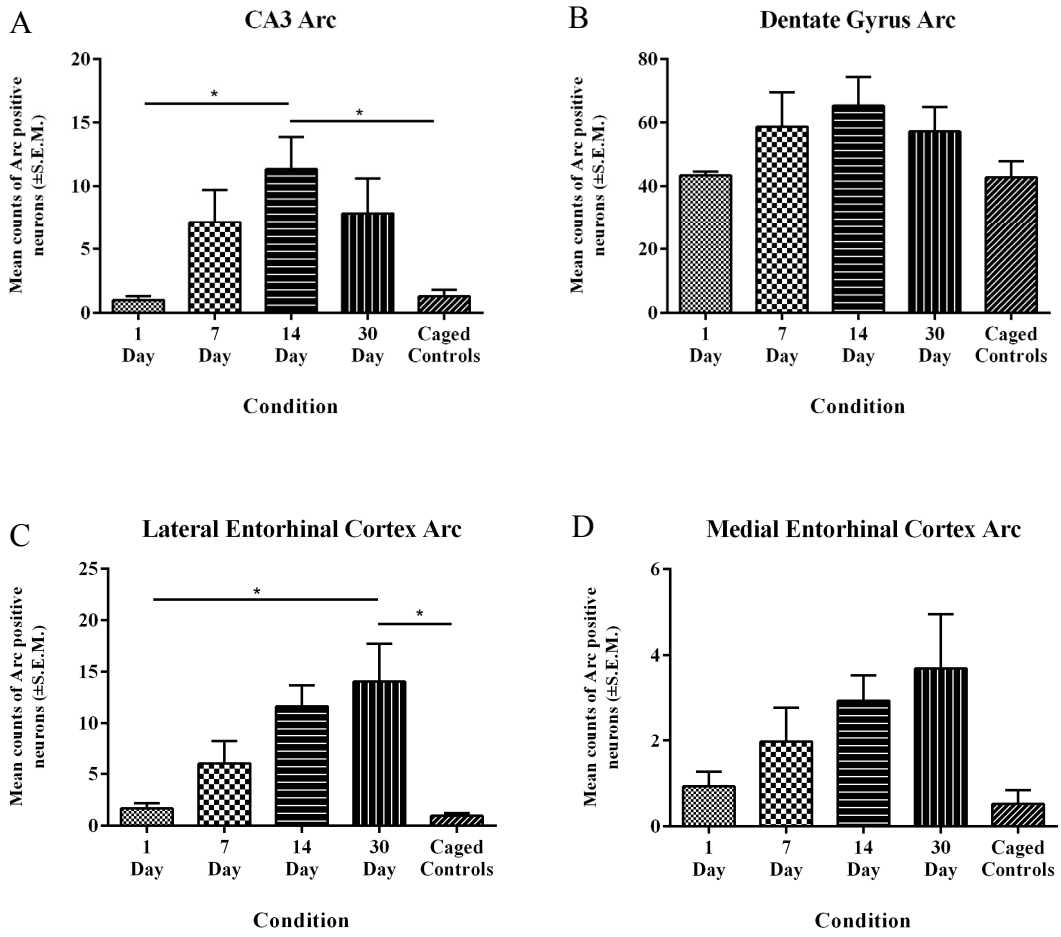

Figure 6.16: Changes in the level of Arc expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in CA3 (A), dentate gyrus (B), lateral entorhinal cortex (C) and medial entorhinal cortex (D).

A significant difference was found across groups in the retrosplenial cortex, $F(4, 26) = 3.63$, $p > 0.01$, however Tukey *post-hoc* analyses did not reveal any significant differences between the groups (see Figure 6.17A). A significant difference was also found in the perirhinal cortex, $F(4, 26) = 13.71$, $p < 0.001$, with Tukey post hoc analyses revealing Arc counts increased significantly from one day retention (*M*: 1.39±0.37) to 14 day retention (*M*: 24.85±1.91, $p < 0.001$) and 30 day retention (15.76±5.29, $p < 0.05$). Arc counts also increased from seven day retention (*M*: 4.15±0.77) to 14 day ($p < 0.001$) and 30 day retention ($p < 0.05$, see Figure 6.17B). A significant difference was also found in the parietal cortex, $F(4, 24) = 8.17$, $p < 0.001$, with Tukey post hoc analyses revealing

Arc counts were higher at 14 day retention (*M*: 202.21±52.14) than one day retention (*M*: 4.23±1.46, $p < 0.001$), seven day retention, (*M*: 23.75±7.85, $p < 0.01$) and caged controls (1.14±0.17, $p < 0.01$). Counts also significantly increased from seven day to 30 day retention (*M*: 75.06, $p < 0.05$, see Figure 6.17C).

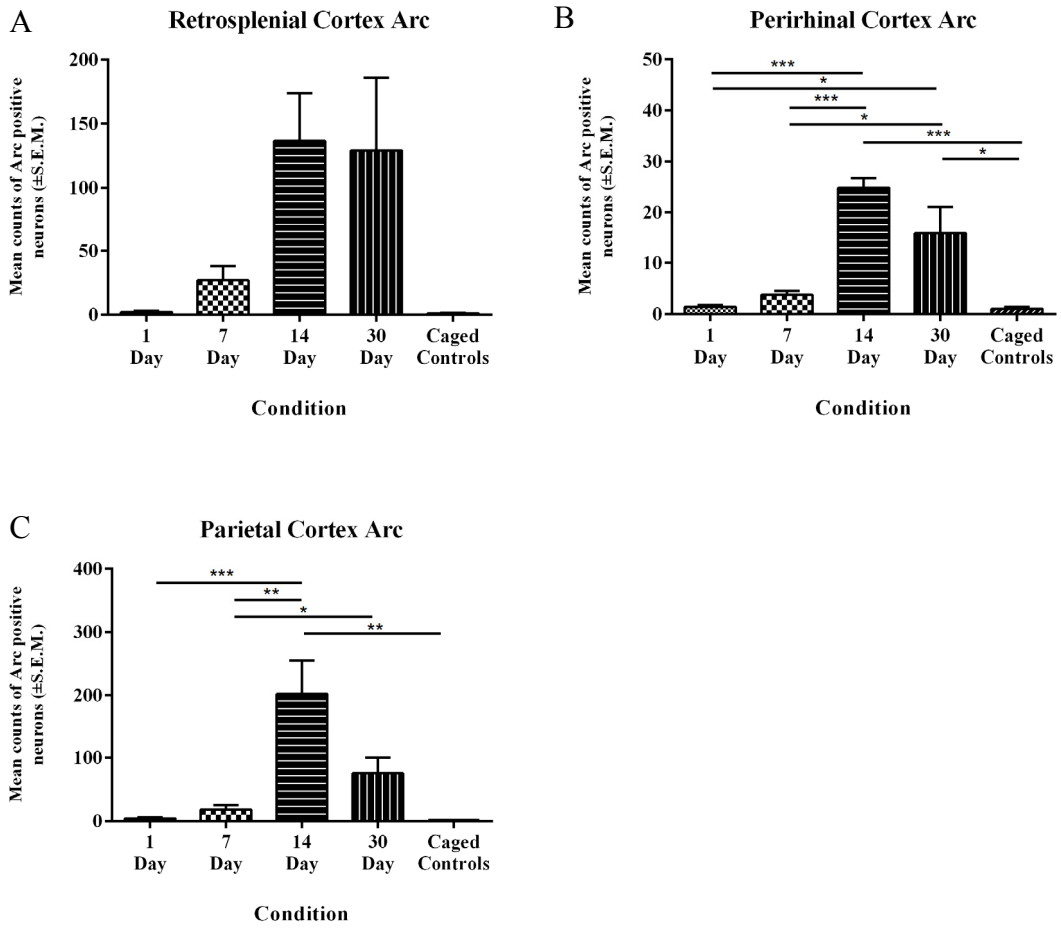

Figure 6.17: Changes in the level of Arc expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the retrosplenial (A), perirhinal (B), and parietal cortices (C).

A significant difference across groups was found in the anterior cingulate cortex $F(4, 25) = 5.37$, $p < 0.01$, with *post-hoc* Tukey analyses revealing levels of Arc were significantly higher at 30 day retention (*M*: 91.50±34.54) than one day retention (*M*: 1.46±0.24, $p < 0.05$, see Figure 6.18A and Figure 6.20 Middle). A significant difference was also found

in the prelimbic cortex, $F(4, 25) = 5.95$, $p < 0.01$, with Tukey *post-hoc* tests revealing levels of Arc increased significantly from one day retention (*M*: 1.50±0.43) to 14 day retention (*M*: 71.79±9.20, $p < 0.05$) and 30 day retention (*M*: 73.96±26.54, $p < 0.05$). Counts were also significantly higher at 14 day ($p < 0.05$) and 30 day ($p < 0.05$) retention than caged controls (*M*: 0.25±0.10, see Figure 6.18C). A significant difference was also found in the infralimbic cortex, $F(4, 26) = 4.30$, $p < 0.01$), with Tukey *post-hoc* tests revealing levels of Arc were significantly higher at 30 days (*M*: 85.64±9.76) than one day (*M*: 0.54±0.18, $p < 0.05$) and caged controls (*M*: 0.25±0.10, $p < 0.05$, see Figure 6.18C and Figure 6.20 Right).

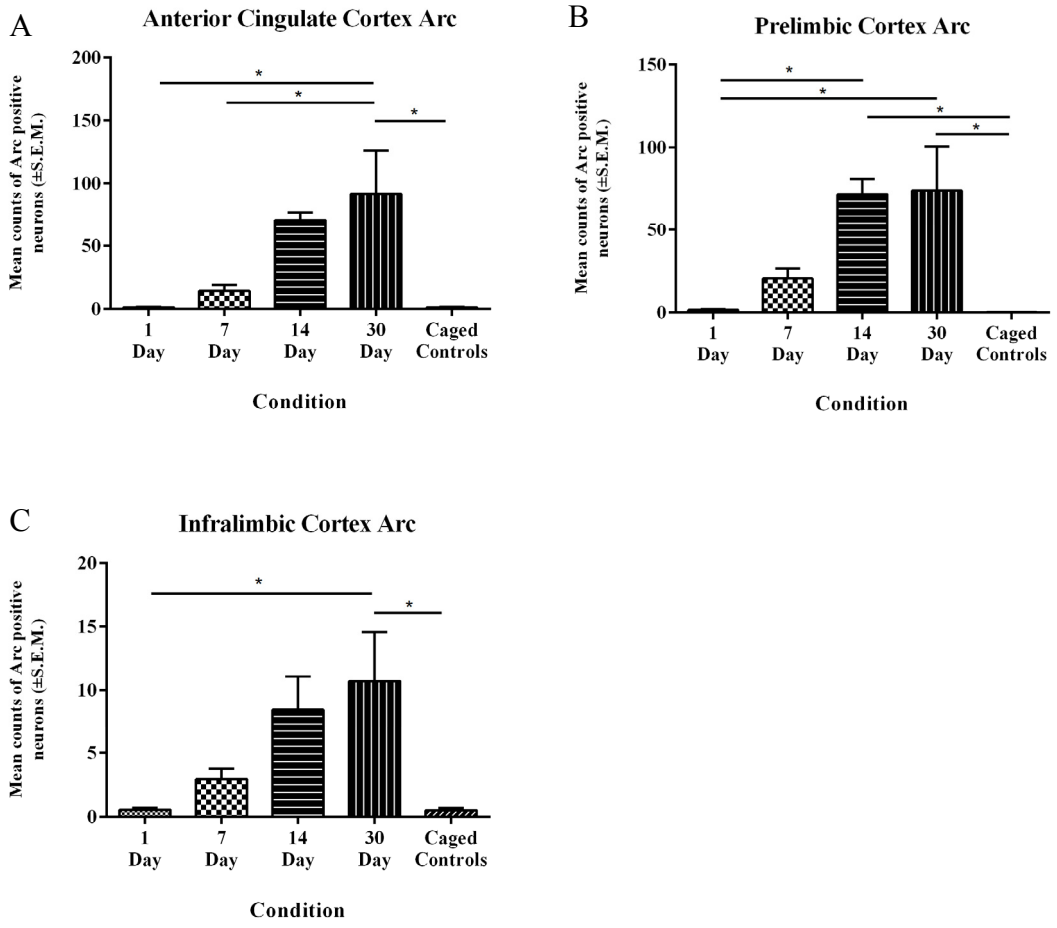

Figure 6.18: Changes in the level of Arc expression following a retention probe trial at one, seven, 14 and 30 days post-acquisition of the water maze in the anterior cingulate (A), prelimbic (B), and infralimbic cortices (C).

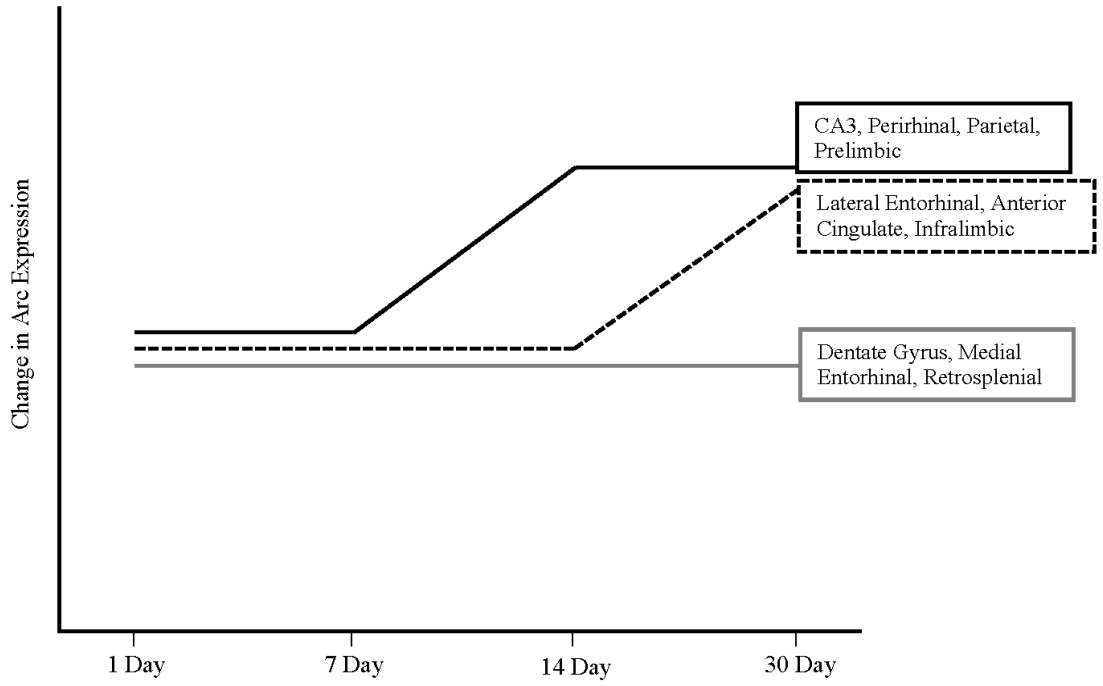

Figure 6.19: Schematic diagram summarising the changes in Arc expression in all brain regions analysed following a retention probe trial of the Morris water maze at one day, seven days, 14 days and 30 days. An ascending line represents a statistically significant increase from previous time-points, a descending line represents a decrease, and a horizontal line indicates no change. Brain regions displaying similar patterns are grouped together.

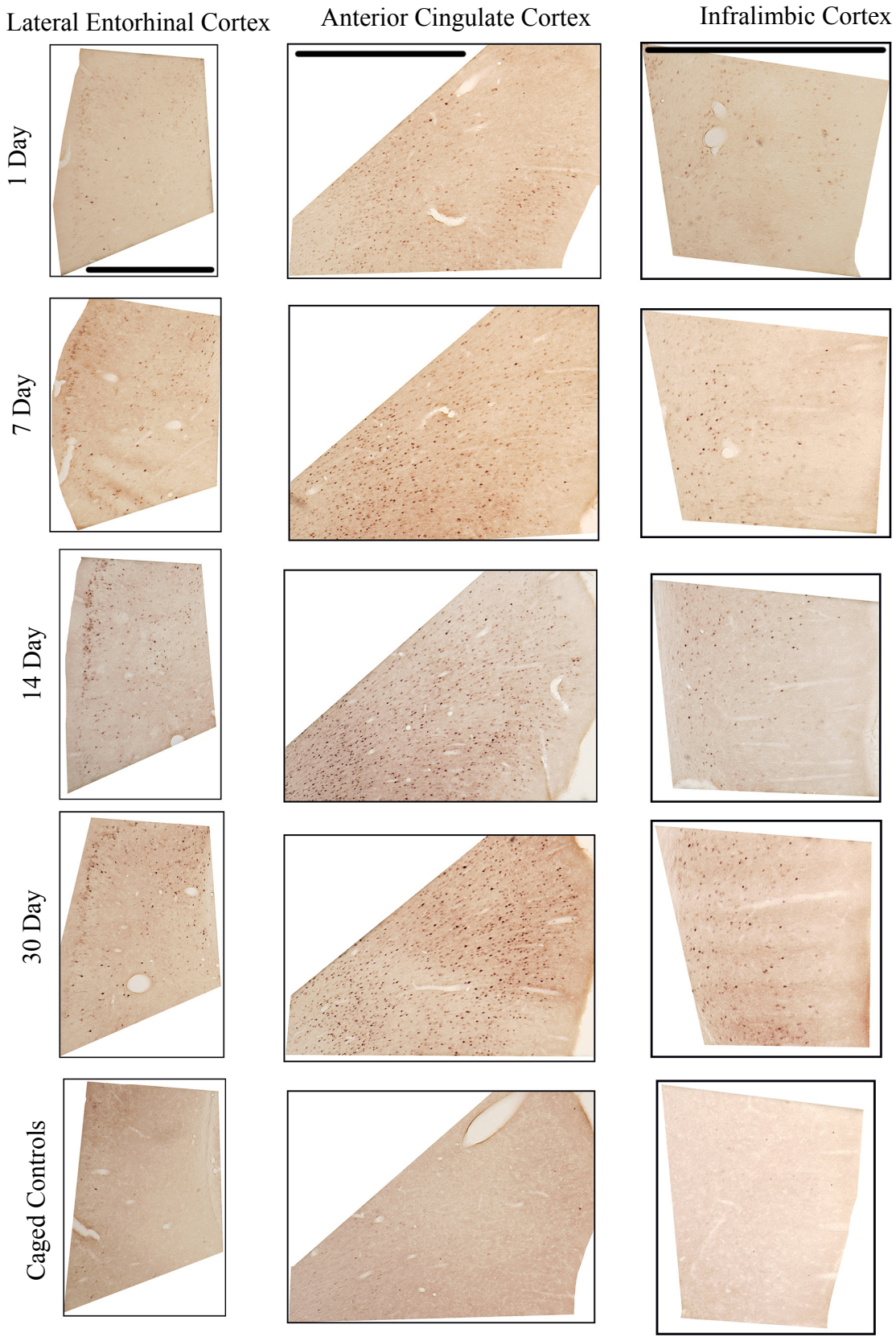

Figure 6.20: Representative photos of Arc expression in selected regions: the lateral entorhinal cortex, the anterior cingulate cortex and the infralimbic cortex at one day, seven day, 14 day and 30 day retention, as well as caged controls. Scale bar = 1mm.

6.3.2.3.1 Correlations with performance.

No significant correlations between levels of Arc expression and number of northeast platform crossings were found in any of the retention groups (see Table 6.5).

Table 6.5: Correlations between Arc expression and number of northeast platform crossings

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA3	0.43	-0.04	-0.61	-0.49
Dentate Gyrus	-0.14	-0.14	-0.21	-0.60
Lateral Entorhinal Cortex	0.23	-0.18	0.07	-0.52
Medial Entorhinal Cortex	0.03	-0.34	0.20	-0.31
Retrosplenial Cortex	0.72	-0.21	0.44	-0.26
Perirhinal Cortex	-0.18	0.61	-0.25	-0.33
Parietal Cortex	-0.17	-0.17	0.35	-0.55
Anterior Cingulate Cortex	-0.67	0.64	0.36	-0.34
Prelimbic Cortex	-0.11	0.60	0.32	-0.52
Infralimbic Cortex	0.39	0.41	0.54	-0.45

A relationship between Arc expression and percentage time spent searching in the northeast platform area was found in the 14 day retention group, with a significant positive correlation in the dentate gyrus ($r = 0.84$, $p < 0.05$), and a significant negative correlation in the anterior cingulate cortex ($r = -0.85$, $p < 0.05$, see Table 6.6).

Table 6.6: Correlations between Arc expression and percentage time spent searching in northeast platform area

Region	Retention time-point			
	1 day	7 days	14 days	30 days
CA3	0.26	0.74	0.63	-0.28
Dentate Gyrus	0.16	0.70	0.84*	-0.36
Lateral Entorhinal Cortex	0.55	0.61	0.46	-0.00
Medial Entorhinal Cortex	0.10	0.30	0.07	-0.34
Retrosplenial Cortex	0.48	0.62	-0.01	-0.35
Perirhinal Cortex	0.28	0.17	0.25	-0.40
Parietal Cortex	0.00	0.70	0.49	-0.48
Anterior Cingulate Cortex	-0.24	0.61	-0.85*	-0.44
Prelimbic Cortex	-0.01	0.47	-0.71	-0.27
Infralimbic Cortex	-0.24	0.08	-0.39	-0.25

6.4 Discussion

The objective of the current experiment was to chart the changes in a wide range of brain regions over four retention time-points using three IEGs as markers of neuronal activity. All animals acquired the maze over the course of five days of training. Although some memory degradation did take place over the course of 30 days as measured by quadrant analysis, rats at the remote time point still displayed a preference for the platform area, and the number of target platform crossings did not change significantly over the course of training.

As hypothesised, Zif268 expression increased in the anterior cingulate, prelimbic and infralimbic cortices from recent to remote time-points. The anterior cingulate cortex only became significantly higher from day one at day 30, corresponding to the c-Fos results of Bonaccorsi et al. (2013). The prelimbic cortex shared a similar pattern, although the infralimbic cortex had increased significantly by 14 days post-acquisition. Studies of memory retention have tended to focus primarily on the anterior cingulate cortex however the coordinated activity in all three subregions of the medial prefrontal cortex suggests this entire region becomes increasingly involved in retrieving remote spatial memory. Zif268 expression in the hippocampus did not change over the course of memory retention, suggesting it is equally involved at recent and remote retention, consistent with the findings of Teixeira et al. (2006). In fact no regions outside of the medial prefrontal cortex displayed a change in Zif268 expression over the four time points, nor however were they significantly higher than caged controls, which renders these results difficult to interpret. In fact, Zif268 levels were significantly lower than caged controls in the dentate gyrus at the one day retention time-point. While on the surface, the amount of Zif268 expression does not appear to mark a change in activity for most regions over the course of retention, a closer look at its relationship with

measures of accurate memory at each time-point revealed some striking results. At one day retention, Zif268 expression correlated with the most accurate measure of searching, number of platform crossings, in the dentate gyrus, the retrosplenial cortex, the perirhinal cortex and the prelimbic cortex. In other words, in rats who displayed the highest levels of Zif268 in these regions, memory performance was superior. At seven and 14 day retention, every region analysed displayed a significant positive correlation between Zif268 expression and time searching in the correct platform area at either one or both time points, with all three hippocampal subregions revealing associations with performance at both time points. Therefore the qualitative relationship with performance, rather than the mere amount of Zif268 expression in a region, may be more instructive of a regions involvement in a task, as was demonstrated by Poirier et al. (2008). The lack of a close association with performance at 30 day retention may be due to the slight degradation in memory observed at this time point. As previously discussed in Chapter 4, the number of regions inspected in the correlational analysis has resulted in a large number of correlations, therefore the risk of a Type I error is increased. Accordingly, the results should be interpreted with a degree of caution.

In contrast to Zif268, c-Fos expression did show quantitative changes from recent to remote time points in most regions. Beginning with the medial prefrontal cortex, a gradual increase was noted in all three subregions, although this did not reach significance in the anterior cingulate cortex. The prelimbic and infralimbic cortices increased significantly in activity from day one by 14 days, the latter finding consistent with those of Lopez et al. (2012). In area CA1, CA3, the dentate gyrus and the lateral entorhinal cortex, a peak of expression was observed at seven days compared to day one, with later increases at 14 days in CA3 and the medial entorhinal cortex. Delayed increases were also observed in the parietal and retrosplenial cortices at 14 and 30 days

respectively. The perirhinal cortex showed increased c-Fos expression at both 14 and 30 days.

Arc expression in the medial prefrontal cortex during retention broadly reflected that of Zif268 and c-Fos. An increase in expression was observed from recent to remote retention, reaching significance at 30 days in the anterior cingulate and infralimbic cortices, and earlier in the prelimbic cortices at 14 days. This increase over time was observed in most regions, peaking at 14 days in CA3, the perirhinal and the parietal cortex, and at 30 days in the lateral entorhinal cortex. Interestingly, the dentate gyrus appeared to remain unchanged over the four retention time-points, similar to Zif268.

The most consistent finding across all three IEGs was the significant increase in expression in the anterior cingulate, prelimbic and infralimbic cortices from recent to remote memory retention. This confirms the findings of a number of other similar studies investigating IEG expression in this area (Bonaccorsi et al., 2013; Lopez et al., 2012; Maviel et al., 2004; Teixeira et al., 2006). Although this increase tended to become significant at 14 or 30 days, the observed pattern was a linear increase in activity of these regions as more time between acquisition and retention elapsed. This suggests a progressive reliance on the medial prefrontal cortex for the integrity of remote memory rather than being fully recruited after a specific period of time. Inactivation of the anterior cingulate cortex disrupts remote memory retention, but what is the precise role of this region and the prelimbic and infralimbic cortices in the expression of spatial memory? While place cells have been found in this region which appear to selectively respond to locations which have motivational salience (Hok et al., 2005), this does not explain the increased reliance on this region over time. Rudy, Biedenkapp, and O'Reilly (2005) proposed than rather being a final storage site for memory, the medial prefrontal cortex becomes involved in the effortful recall of memory from storage sites elsewhere when

the memory has become weaker and difficult to retrieve over the course of time. This is consistent with the slight degradation of memory we observed over the four retention time-points.

IEG expression in the dentate gyrus remained largely unchanged over the course of retention, perhaps reflecting its continued importance to solving the task. The general trend for expression in other hippocampal regions however was to peak at either seven or 14 days. Lesions of the dorsal hippocampus in rats up to 100 days following acquisition impair retention of the water maze relative to controls, despite extended training, but it is unclear whether this is due to a retrieval deficit or performance impairment (Clark, Broadbent, & Squire, 2005). Lesions of the entorhinal cortex have been shown to impair both acquisition and retention of the Morris water maze (Hardman et al., 1997), possibly due to disruption of general location information (Hebert & Dash, 2004). Lesions of the entorhinal projection to CA1 impair retention of the water maze, but only up to three weeks following acquisition (Remondes & Schuman, 2004), indicating communication between these areas is necessary for systems consolidation, perhaps reflecting the similar pattern of expression observed between this region and the hippocampus in this experiment. Rats with retrosplenial cortex lesions show slight impairment during acquisition but no preference for the correct quadrant during a retention probe test (van Groen et al., 2004), and this appears to be reflected with the linear increase in activity of this area over the course of retention. Lesions of the perirhinal cortex do affect spatial memory retention, but only over longer time periods (Ramos & Vaquero, 2005), and its role appears to be specific to memory retrieval rather than consolidation (Ramos, 2008) therefore this may explain the sharp increase in perirhinal cortex activity during remote retention observed in this experiment. Limited research has been carried out on the role of the parietal cortex in retention of the water

maze, but the increase in IEG expression observed in the parietal cortex by Maviel et al. (2004) during remote retention of spatial discrimination memory is consistent with our findings.

The progressive increase in all medial prefrontal sub-regions for all IEGs over the four retention time-points is consistent with the theory of systems consolidation, in that memories become dependent on the neocortex over time for successful retrieval (Frankland & Bontempi, 2005). Other cortical regions also showed an increase in c-Fos and Arc expression over time, also supporting the idea that spatial memory becomes increasingly reliant on a distributed network of brain structures over time. Hippocampal expression remained either unchanged or peaked at intermediate retention time-points before declining again slightly. Therefore this area is still clearly involved over the different retention time-points, although it was not possible to separate out the roles of performance and memory retrieval in this experiment. The finding that IEG expression continues to climb in the medial prefrontal cortex and other cortical regions at the remote 30 day time-point despite slightly poorer memory performance in this group, adds some credence to the theory of Rudy et al. (2005), that the medial prefrontal cortex is attempting to reactivate and coordinate activity in other storage sites where the memory was first established. However it does not rule out a role for this site as a long-term storage site for spatial memory. The striking relationship between Zif268 expression and memory performance which was evident in all brain regions further supports the concept that successful memory retrieval is dependent on a range of brain regions working together. The finding that these associations occur during more recent time points suggests that where the quality of the memory is superior IEG expression can be a useful marker of a regions contribution to the performance of a task. As the memory becomes

more difficult to recall over time, a quantitative analysis of the overall amount of IEG activity in a region appears to implicate its involvement.

This experiment highlights the importance of using multiple markers of neural activity to gain a more informed understanding of regional activation, and also the use of multiple time-points, which highlights periods of heightened activity which may be masked when employing a more limited design. Of particular note was the relationship between Zif268 expression and memory performance, which proved to be more informative rather than simply assessing regional activation alone.

Chapter 7

General Discussion

7.1 Summary of the Findings of this Thesis

The main objectives of this thesis were to chart the changes in expression of three IEGs in a number of brain regions over the course of spatial learning, consolidation and retention using the Morris water maze task. While a number of studies have used IEG imaging to measure brain activity in response to allocentric spatial learning, the scope of the research has usually been limited to a single time-point or brain region, and a systematic investigation of how activity changes in a network of regions over time has not been carried out. We first attempted to create a control condition which would provide an adequate basis for comparison with spatially-trained rats when assessing IEG activation. Our control condition was designed to simulate the behavioural experience of swimming in the water maze, while also matching for time spent in the maze and finding an escape. The level of hippocampal IEG expression was compared across the experimental and control groups (Chapter 3). We then investigated neural activity during early, middle and late learning, by examining the expression of IEGs following one, three and five days of training in the water maze in 11 brain regions which have been implicated in spatial learning (Chapter 4). Following this, we investigated the role of IEGs in cellular consolidation by charting the time-course of IEG expression up to eight hours following spatial learning in the water maze, assessing the same 11 regions (Chapter 5). Finally, we used IEG imaging to examine the process of systems consolidation, by analysing hippocampal and cortical activity at a number of recent and remote time-points, from one day to 30 days post-acquisition (Chapter 6).

An attempt had previously been made to create an adequate matched control condition for the working memory version of the Morris water maze, although with limited success. Despite matching rats for time spent in the maze, and providing a reliable escape without any memory load, Shires and Aggleton (2008) found no difference

between spatially-trained rats and procedural controls in levels of c-Fos expression in the hippocampus, and actually found higher levels of Zif268 in controls in this region. Our findings reflected those of Shires and Aggleton (2008), in that our control condition matched the spatially-trained condition on a number of behavioural measures without the requirement to learn spatially, yet the groups did not differ significantly in terms of c-Fos or Arc expression in the hippocampus. The most likely explanation for these results is the existence of latent learning in this control condition (Ramos, 2010) and the spatial navigation demands of traversing the maze to find an escape.

While some studies have investigated IEG expression during acquisition of the Morris water maze (Feldman et al., 2010; Guzowski et al., 2001; Teather et al., 2005), they have tended to focus just on the hippocampus at one or two time points. We examined the expression of Zif268, c-Fos and Arc during early (one day), middle (three day) and late (five day) learning. We found that Zif268 displayed heightened expression during early training in CA1, the retrosplenial, perirhinal and parietal cortices, and in the infralimbic and prelimbic cortices. Mid-training increases were observed in the dentate gyrus, the infralimbic cortex and the lateral entorhinal cortex, with the medial entorhinal cortex increased during late training. c-Fos displayed a different pattern, with CA1, CA3, the medial entorhinal cortex and the prelimbic and infralimbic cortices displaying increased expression towards the end of training. c-Fos expression in the dentate gyrus was again increased mid-training, with a similar pattern also found for Arc in this region.

One study reported repeated waves of IEG consolidation at 8 hours following spatial exploration (Ramirez-Amaya et al., 2005), although this has not been successfully replicated (Penke et al., 2011). We aimed to clarify whether or not IEGs are expressed during the later stages of cellular consolidation in a wide range of brain regions implicated in spatial learning, by examining the expression of Zif268, c-Fos and Arc

protein at 90 minutes, four hours and eight hours following three days of training in the water maze. Our results did not support the hypothesis that there is a second wave of IEG expression at eight hours. Zif268 had decreased in all regions except the dentate gyrus by eight hours. Some regions displayed prolonged expression at four hours, including the lateral and medial entorhinal cortices, the perirhinal and parietal cortices, and the anterior cingulate and prelimbic cortices. The dentate gyrus displayed prolonged expression over eight hours, which was in accordance with the findings of Ramirez-Amaya et al. (2005). A decrease from 90 minutes to 8 hours was also observed with c-Fos for all regions analysed, with prolonged expression in the hippocampus and the medial entorhinal cortex at four hours. A similar pattern was also observed for Arc expression, with most regions displaying dramatic decreases from 90 minutes to four hours, without a second wave of activity at eight hours. The dentate gyrus once again showed prolonged Arc expression for eight hours following training. Therefore while a second wave of activity may occur at different time points to the ones investigated by us, a second wave of IEG expression was not observed at eight hours following spatial learning in the water maze in any relevant region studied.

Systems consolidation of spatial memory has been the focus of a number of IEG and lesioning studies (Frankland et al., 2001; Gusev et al., 2005; Lopez et al., 2012; Maviel et al., 2004; Teixeira et al., 2006), with the consensus being that for allocentric learning in the water maze, there is an increased reliance on the medial prefrontal cortex over time, whereas the hippocampus is always required for retrieval of the spatial memory regardless of the delay between learning and retention. However, studies have generally only focused on two time points, recent (one day) and remote (30 days), with only one study examining intervening time points (Bonaccorsi et al., 2013). We expanded on the findings of this study by examining four retention time points, using three different

markers of neural activity and looking at 11 brain regions. We found gradual increases in Zif268 expression in the medial prefrontal cortex which became significant between 14 and 30 days, however no other structures studied revealed changes in Zif268 expression over time. However significant correlations with searching accuracy were found at one day, seven days and 14 days in most regions analysed. c-Fos expression also increased in the medial prefrontal cortex, reaching significance at 14 days. The hippocampus and lateral entorhinal cortex displayed increased activity at seven days, with the medial entorhinal, parietal and perirhinal cortex increasing in activity at 14 days. c-Fos expression in the retrosplenial cortex was increased at 30 days. Arc expression was also increased in the medial prefrontal cortex, reaching significance at 30 days in the anterior cingulate and infralimbic cortices, and earlier in the prelimbic cortices at 14 days. Expression peaked in CA3, the perirhinal and parietal cortices at 14 days and at 30 days in the lateral entorhinal cortex. Similarly to Zif268, the expression of Arc in the dentate gyrus remained unchanged across the four retention time-points. These findings support the role of the medial prefrontal cortex in remote retention, suggesting a gradual increased reliance over time on this region. In addition, they suggest that other structures involved in spatial learning also display an increase in activity as the time between learning and retention increases.

7.2 Significance of Findings

7.2.1 Control conditions: Latent learning, navigation or stress?

Our observation that control conditions exhibit a similar magnitude of IEG expression in the hippocampus to spatially-trained rats is consistent with findings in the literature concerning acquisition of the water maze (Shires & Aggleton, 2008; Snyder, Radik, Wojtowicz, & Cameron, 2009), the radial arm maze (Poirier et al., 2008) and in the

acquisition of other learning paradigms (Bertaina & Destrade, 1995). What are the theoretical implications of this equivalent activation? One possible interpretation is that IEGs are not a reliable marker of learning, yet studies that show impaired learning under conditions where just one of these IEGs are not expressed (M. W. Jones et al., 2001; Plath et al., 2006) indicate that their expression is not only a useful marker of learning but is a prerequisite. Increased stress in a control group is also a possibility, particularly in the case of a free swimming rat, as this alone is used as a stress induction paradigm and increases levels of IEGs (Cullinan et al., 1995; Duncan et al., 1993). However, this should not be a confounding variable in conditions which have a reliable method of escape such as the multiple variable platform group in this study, or the procedural controls used by Shires and Aggleton (2008). Nor should increased stress be a factor in conditions where the platform is visible or indicated by a landmark, both conditions where IEG expression has been shown to be as high or higher than spatially-trained rats (Guzowski et al., 2001; Jenkins, Amin, Harold, Pearce, & Aggleton, 2003). Accordingly, Kavushansky, Vouimba, Cohen, and Richter-Levin (2006) investigated plasma levels of corticosterone in rats who were trained to swim to either a visible platform, an invisible platform, or forced to swim without a platform present, as well as naïve rats. Only the free swimming group displayed corticosterone levels higher than the other three groups, whereas there was no increase from baseline in the visible or invisible platform group, suggesting stress is not a factor when a reliable escape is present.

Incidental learning is a likely explanation. Mice trained to find a visible platform still display a spatial strategy and search in the correct location when it is removed (Teixeira et al., 2006), indicating that distal cues are still utilised. Varying the platform location in our control experiment does render the distal cues redundant, but that does not inhibit cue-platform associations being formed upon successful location of a

platform. R. G. Morris and Frey (1997) argued that the hippocampus is involved in rapid, automatic encoding which can take place in just one trial in the water maze, and is preferentially engaged by a mis-match in current versus previous experience of an environment, leading to an updating of spatial representations. This constant updating could be engaging the hippocampus in each trial of our control condition as the experience of a new platform location would conflict with previous experience. In other words, changes to the spatial environment would be as salient to a control group as the constant configuration to the spatial group.

Furthermore, these control groups are still engaged in a navigational task. In his original demonstration of place learning in the water maze, R. G. Morris (1981) included a random platform task akin to our single variable group, and noted semi-systematic searching of the maze arena to escape the task. Environmental cues would prove as useful to a group systematically searching an arena as a group using them to locate a particular area, thereby engaging the hippocampus and related regions. Given the remarkable sensitivity of IEG expression to spatial exploration, even in the absence of task demands (Vazdarjanova & Guzowski, 2004), it is likely similar information about the environment is being encoded or used in our control groups. Therefore their usefulness as a comparative control group at any particular time-point is questionable, and we felt justified in excluding them from subsequent experiments with a predominant focus on the changes that occur in IEG expression over the course of learning and memory, in line with their role in synaptic plasticity and neuronal activation.

7.2.2 IEGs: Markers of learning, performance or activity?

The particularly high expression of Zif268 observed during early learning in this study is consistent with its role in synaptic plasticity (Knapska & Kaczmarek, 2004), in that

key changes to relevant brain regions are made at this early stage when the task is first learned. It is also consistent with other studies which have found increased IEG expression in rats who are still learning a task compared to a group which have mastered it (Kelly & Deadwyler, 2002; Rapanelli et al., 2009; Svarnik et al., 2005). Although the overall magnitude of Zif268 expression declined as the task was mastered, it was associated with learning performance by day five, with hippocampal and medial prefrontal activity positively correlated with escape latency, suggesting it was involved in error correction, reflecting the findings of Poirier et al. (2008). This association with performance continued during retention, although there was a qualitative difference in this relationship. Rather than being higher in poorer performers, it was now associated with successful performance over three time-points in all regions, suggesting once a task has been consolidated, it is expressed in response to successful recreation of a spatial representation. The observed increase of this IEG in the medial prefrontal cortex during retention is likely to be attributable to increased involvement of this area. Therefore Zif268 appears to be a useful marker of learning, performance and activity, depending on the stage of learning and retention.

c-Fos expression showed a tendency to increase in most brain regions as escape latencies decreased towards the end of training, suggesting its expression was related to successful performance in the maze. Its expression in the hippocampus also correlated with time spent in the maze during mid to late training, and after one day retention, supporting this idea. However this correlation with performance did not continue throughout retention. Rather, the increases observed in most regions as retention time-points became more remote, implicates this IEG as a useful marker of general neural activity during remote recall.

While analysis of Arc expression was limited to the dentate gyrus during acquisition, its expression across learning and retention appears to be broadly similar to that of c-Fos. Arc expression increased sharply during mid to late training in the hippocampus, correlating with performance in the maze on day five of acquisition, but this IEG did not show any correlation with performance during retention at any time point. Rather it increased in most brain regions over the four retention time-points in a manner similar to c-Fos, suggesting both IEGs are useful as a marker of performance during acquisition and general neural activity during retention.

These findings are significant as they display the limitations of using one solitary IEG to assess brain activation during learning and memory tasks. Furthermore, they highlight the need for careful interpretation of IEG results, as they may be indicative of learning-related plasticity, behavioural performance or general activity depending on which IEG is used and at what stage of learning and memory.

7.2.3 A spatial network.

The results of this thesis suggest that rather than spatial memory being dependent on a small number of key regions, a wide range of structures contribute to the formation of spatial memory and its recollection.

The hippocampus has long been implicated in spatial learning (R. G. Morris et al., 1982). Consistent with this, area CA1 showed increased expression of Zif268 and c-Fos during early and late learning respectively, as well as being correlated with performance during recent retention as measured by c-Fos. The finding that place cells are formed within minutes of introduction into a new arena (Wilson & McNaughton, 1993), and are dependent on Zif268 expression in order for them to stabilise (Renaudineau et al., 2009) is the most likely explanation for the heightened expression

of Zif268 during early learning in this region. Area CA3 increased in activity during mid- to late-training as measure by c-Fos, displayed an association with performance as measured by Zif268, and increased in activity over the course of retention as measured by c-Fos and Arc, consistent with the role of this hippocampal subregion in spatial learning (Florian & Roullet, 2004; Stubley-Weatherly et al., 1996) and retention (Steffenach et al., 2002). The dentate gyrus appeared to play a key role during mid to late training as measured by all three IEGs, corroborating lesioning evidence that this is the most important hippocampal subregion for spatial learning in the water maze (Okada & Okaichi, 2009; Xavier et al., 1999). While an association with performance was found during recent retention, this region did not appear to display substantial quantitative changes over more remote retention time points. However, this is not to imply that it is not involved. The combined findings that blocking neurogenesis in the dentate gyrus results in impaired retention of the water maze two weeks following acquisition (Jessberger et al., 2009), along with the fact that these new neurons show increased Zif268 (Trouche, Bontempi, Roullet, & Rampon, 2009) and c-Fos (Snyder et al., 2009) expression at remote retention, suggest the expression in which neurons, rather than the amount of neurons may be a more accurate measure of the recruitment of this region.

While it is clear from these results that the hippocampus plays an important role in the acquisition of spatial memory, evidence suggests that it is not the only structure involved. Parron, Poucet, and Save (2001) demonstrated that in the absence of a functioning hippocampus, rats can learn the maze equally well as controls. After an initial acquisition protocol, the platform location was changed and rats were given hippocampal infusions of lidocaine before each trial, and were unimpaired at finding the new platform location, suggesting other structures were compensating during acquisition. Interestingly however, although their performance improved across days, it did not improve within a

trial block, suggesting the hippocampus was involved in offline processing while not performing the task. Nonetheless, other structures must be able to compensate for the lack of hippocampal involvement during spatial learning, and the other regions studied in this thesis are possible candidates.

The lateral and medial entorhinal cortex tended to increase in activity towards the end of acquisition as measured by Zif268 and c-Fos, and increased during retention, as measured by c-Fos and Arc. This could be attributable to the establishment and subsequent reactivation of grid cells in this region (Fyhn et al., 2004), enabling the animal to form a cohesive spatial representation during acquisition and navigate effectively during retention. The increase in activity of this region is also consistent with findings that it is necessary for solving the water maze task (Parron et al., 2004). The retrosplenial, perirhinal and parietal cortices all displayed dramatic increases in Zif268 expression during early learning, the retrosplenial and parietal cortices also showing strong correlations with c-Fos expression during this stage, suggesting they play a role in the initial formation of a spatial representation. The finding that pretraining can attenuate the impairments associated with retrosplenial lesions suggests this region is initially involved in the formation of a spatial strategy to solve the maze (Lukoyanov et al., 2005), although there is evidence to suggests it is involved in both spatial memory and strategies in the water maze (Cain, Humpartzoomian, & Boon, 2006). The finding that perirhinal cortex activity was extremely high during early learning is likely to be attributable to the novel aspects of the spatial environment (Liu & Bilkey, 2001). Rats with perirhinal lesions are also impaired at discriminating between objects with overlapping features (Eacott, Machin, & Gaffan, 2001), and since two of the cues were identical in this experiment, this region may have been involved in discriminating between them during early learning. The parietal cortex has been proposed to integrate spatial and motion

information to form a spatial strategy (Save & Poucet, 2009), which may explain its recruitment during early learning. The retrosplenial, perirhinal and parietal cortices all showed sharp increases in c-Fos and Arc expression over the course of remote retention, suggesting they are required to reactivate this representation. Lesions of the perirhinal (Ramos & Vaquero, 2005) and the retrosplenial cortex (van Groen et al., 2004) have been shown to disrupt long-term retention of the water maze, which is consistent with these findings.

All three medial prefrontal regions displayed increased expression of Zif268 during early to mid-training, with an increase in c-Fos expression in the prelimbic and infralimbic cortices during late learning, suggesting this region plays an important role in the acquisition as well as the retention of spatial memory. Their increase over the course of retention was the most consistent finding across the three IEGs. It is possible however, that the medial prefrontal cortex plays a different role in acquisition than retention. Its role in acquisition is likely to involve spatial working memory. Studies in humans have shown that the navigational impairments observed following medial prefrontal lesions are largely related to an inability to retain the goal destination in working memory (Ciaramelli, 2008), which may explain the increased activation in the medial prefrontal cortex during early training. The prelimbic cortex in particular appears to be involved in the working memory component of spatial memory (Ragozzino & Kesner, 1998). The medial prefrontal cortex appears to synchronise its activity with the hippocampus to facilitate this working memory. The firing of neurons in the medial prefrontal cortex are phase locked to hippocampal theta oscillations when rats are performing spatial tasks such as the radial arm maze and T-maze (Siapas, Lubenov, & Wilson, 2005). Accordingly, Lee and Kesner (2003) found that while the hippocampus and medial prefrontal cortex can compensate for each other in spatial working memory

tasks when one or the other is inactivated, the hippocampus become increasingly involved as the time required to hold information in memory increases. Therefore the medial prefrontal cortex may facilitate the transition from short-term spatial memory to long-term spatial memory, which involves the hippocampus. The medial prefrontal cortex may also be involved in early consolidation of the task. Leon, Bruno, Allard, Nader, and Cuello (2010) disrupted the MAPK pathway in the medial prefrontal cortex in rats after one day of training in the water maze, finding that retention was impaired 24 hours later. The role of the medial prefrontal cortex in memory retention will be discussed in more detail subsequently.

7.2.4 The role of IEGs in cellular consolidation.

Short-term memory, lasting minutes to hours, is thought to depend on the post-translational modification of post-synaptic proteins, and is thought to involve a process similar to early-LTP, whereas long term memory, lasting hours or longer, is dependent on intracellular signalling and transcription and translation of new proteins (Hernandez & Abel, 2008). The subsequent stabilisation of a memory trace is known as cellular consolidation (McGaugh, 2000), and this process can be disrupted by the application of protein synthesis inhibitors in a limited time-window of one to three hours following learning (H. P. Davis & Squire, 1984). IEGs and their downstream targets are thought to be the proteins which provide the structural components needed to stabilise memory during this time period. Consistent with this theory, we observed widespread expression of Zif268, c-Fos and Arc across all brain regions 90 minutes following spatial learning, followed by a marked decline to basal levels at four and eight hours, which corresponds with the critical time window thought to be involved with the production of plasticity related proteins. *De novo* protein synthesis and the establishment of late-LTP is necessary

for consolidation of the Morris water maze. Kelleher, Govindarajan, Jung, Kang, and Tonegawa (2004) found that in mutant mice with inhibited ERK activation, deficits in establishing late-LTP in hippocampal slices were observed. Furthermore, these mice spent significantly less time in the target quadrant than controls in a probe trial following water maze training, and made significantly less platform crossings. Likewise, disruption of CREB (Guzowski & McGaugh, 1997) or inhibition of the ERK signalling cascade (Blum, Moore, Adams, & Dash, 1999) in the dorsal hippocampus during water maze training preserves acquisition and short-term memory, but leads to impaired retention 24 or 48 hours later.

The expression of c-Fos (Guzowski, 2002) and Arc (Guzowski et al., 2000) immediately following training appears to be essential for consolidation of the task, as inhibition of this process impairs retention, identifying these proteins as likely candidates in the stabilisation of spatial memory. However, it has been suggested that multiple waves of expression of these proteins are involved in consolidation. The idea that there is more than one time window where protein synthesis inhibition can impair memory consolidation is not new. Grecksch and Matthies (1980) found that consolidation of a brightness discrimination reaction could be disrupted with hippocampal injections of anisomycin either four or six hours following training, although Meiri and Rosenblum (1998) could not replicate this result in the water maze, finding only PSIs administered around the time of training affected consolidation, whereas inhibition of protein synthesis up to 5.5 hours following training did not.

Previous findings suggest there is a second wave of IEG expression in the hippocampus and parietal cortex eight hours following a spatial experience (Ramirez-Amaya et al., 2005), however the results of this thesis largely refute this, as neither Zif268, c-Fos nor Arc were reactivated at this time-point in any brain regions studied. A

third wave of expression was observed at 24 hours in their study, but that time-point was not analysed in our experiments. In a different learning paradigm, Katche et al. (2010) showed that a delayed second wave of c-Fos expression was observed in the hippocampus 24 hours following inhibitory avoidance (IA) learning, and blocking protein synthesis at this time point, but not nine, 12, 18 or 36 hours disrupted memory retention seven days later. A similar second wave of Zif268 expression was found from 12 to 24 hours after IA training by Katche et al. (2012), and blocking its translation during this time period also resulted in a retention deficit. Another IEG, BDNF, displays a second wave of expression at 12 hours following IA training and its inhibition impairs retention seven days later (Bekinschtein et al., 2007). Therefore assessing IEG activation at these later time-points following spatial learning may yield positive results.

Zif268 expression appeared to be somewhat more prolonged over four hours compared to c-Fos and Arc, and this sustained expression took place in a number of cortical regions. This is consistent with the findings that Zif268 is upregulated in the cortex but not the hippocampus four hours following a novel spatial experience (Ribeiro et al., 2007). This could reflect the beginning of systems consolidation of the newly acquired spatial memory. IEG expression in the dentate gyrus was markedly sustained over eight hours for both Zif268 and Arc, consistent with the findings of Ramirez-Amaya et al. (2005), suggesting this region engages in continued consolidation of the task over this time period. A tendency for IEG expression to be lower than caged controls at the eight hour time point was observed, and this reached statistical significance in the anterior cingulate, prelimbic and perirhinal cortex, with Zif268 protein appearing to be depleted at this time-point relative to controls. This muted expression may be a protective mechanism which guards against further interference while consolidation is taking place, and emphasises the role of the medial prefrontal cortex in early consolidation of the water

maze task (Leon et al., 2010). Alternatively, this finding may add some credence to the controversial post-translational protein modification theory, where transcription and translation of novel proteins merely serves as a replenishment process after the necessary changes are made to synapses by pre-existing proteins (Routtenberg & Rekart, 2005). Thus it may take some time for IEG levels to be replenished to basal levels.

In summary, although the consolidation of memory is likely to involve post-training modifications in the hours or days following training (Katche et al., 2013), possibly involving reactivation of NMDA receptors during this time (Shimizu, 2000), it appears unlikely that this process involves IEG expression at eight hours following learning. While these results do not occlude the possibility of reactivation at a later time-point, they are consistent with the role of IEGs in the early phase of protein synthesis. The prolonged expression of Zif268 in cortical areas at four hours, and the unexpected depletion of Zif268 in the medial prefrontal cortex at eight hours may be indicative of systems consolidation in its earliest stages.

7.2.5 Systems consolidation of spatial memory.

As retention probe trials do not reinforce learning, the expression of IEGs during these trials were interpreted to reflect neural activity and behavioural performance. The observed changes in activity across a wide range of brain regions over the multiple retention time-points allowed us to examine the process of systems consolidation in detail.

There are numerous competing theories on the role of the hippocampus during recent retention of a learned task versus recall at a later time. Standard consolidation theory (Squire, 1986) proposes that over the course of time, memories become independent of the hippocampus. Multiple trace theory (Moscovitch et al., 2005) agrees

that while the neocortex is the permanent store of memory, recall of detailed memory representations will always rely on the recruitment of the hippocampus. Cognitive mapping theory (O'Keefe & Nadel, 1978) proposes that the hippocampus is the permanent store of spatial memory therefore it will always be involved with the successful recollection of this type of memory. The present set of results would provide support to the latter two theories, as the hippocampus appeared to be equivalently, if not increasingly activated at more remote time-points. These results are in accordance with IEG (Teixeira et al., 2006) and lesion studies (Broadbent et al., 2006; Clark et al., 2005) demonstrating that the hippocampus is required at both recent and remote retention time-points in the Morris water maze, and this impairment at remote time-points is not recovered by rewarded probe trials (Martin, de Hoz, & Morris, 2005). In fact, the increase in activity over time we observed in the hippocampus as measured by c-Fos and Arc mirrors that of Lopez et al. (2012) and Bonaccorsi et al. (2013), in that the hippocampus appeared to be increasingly recruited as the time between acquisition and retention increases. This is in stark contrast with observed temporally graded amnesia in other learning paradigms resulting from hippocampal lesions, such as contextual fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Kim & Fanselow, 1992) and socially-transmitted food preference (Clark, Broadbent, Zola, & Squire, 2002). The findings also conflict with the findings in a spatial discrimination learning paradigm, where IEG expression in the hippocampus was decreased at a remote time-point, and lesions to this area had no effect on recall (Maviel et al., 2004). Likewise, Bontempi, Laurent-Demir, Destrade, and Jaffard (1999) showed that metabolic activity in the hippocampus decreased from recent (five days) to remote (25 days) spatial discrimination memory retrieval. Gaskin, Tardif, and Mumby (2011) also showed that spatial memory can become independent of the hippocampus when it is dissociated from navigational

demands. Rats who had their hippocampus inactivated beginning three hours after exploration of objects did not notice when one was displaced in a retention test, whereas rats with their hippocampus deactivated beginning five days following object exploration noticed a change in the spatial configuration of objects. This raises the question as to whether the hippocampus is the permanent store of allocentric spatial memory in the water maze task, or performance deficits resulting from hippocampal inactivation at any time-point are a result of an impairment in the ability to navigate.

Riedel et al. (1999) found that inactivating the hippocampus of rats during acquisition of the water maze resulted in a random swimming pattern during retention, however rats who had their hippocampus inactivated during retention but not acquisition show a focused search strategy, albeit in the wrong location of the maze, suggesting they were unable to recall the precise location. However, other findings have suggested that hippocampal lesions disrupt more than just spatial information. Clark, Broadbent, and Squire (2007) trained rats in a novel water maze consisting of distal cues and local beacons, performing hippocampal lesions two months later and administering a probe trial. Lesioned rats not only failed to use the distal cues to search in the correct location, they did not use the proximal beacons either, suggesting they had amnesia for all aspects of the task. Thus, it is difficult to dissociate the "on-line" processing of spatial information by the hippocampus necessary for the performance of a task as complex as the water maze with the recall of an already consolidated spatial memory (Knowlton & Fanselow, 1998).

The precise role of the hippocampus in water maze retention may be informed by studies of allocentric learning where retention is at least partially spared by hippocampal lesions. Winocur, Moscovitch, Fogel, Rosenbaum, and Sekeres (2005) trained rats to find rewards over the course of three months in a complex "village", where distal cues were

used to help the rats navigate. They found that their spatial representation survived hippocampal lesions, indicating allocentric spatial memory could become independent of the hippocampus over time. However in a more recent study they showed that when a barrier was placed in the usual path of rats seeking out rewards in the village, control rats chose the most efficient alternate route, but hippocampal-lesioned rats took significantly longer to find a new route and made more errors (Winocur, Moscovitch, Rosenbaum, & Sekeres, 2010). This suggests that the hippocampus is needed for the flexible use of a spatial representation and the formation and access of a detailed cognitive map, which would explain the severe impairments observed following hippocampal inactivation in the water maze compared to other less demanding tasks. The increase in hippocampal activity observed in the present study over the course of retention, as measured by c-Fos and Arc, along with its close relationship with retention performance as measured by Zif268, suggests the hippocampus is involved in the performance and retention of this task regardless of the retention interval. It should be noted however that the increase in hippocampal activity from recent retention tended to peak at seven or 14 days, whereas significant increases cortical activation were slightly delayed in comparison, suggesting these regions play a more important role in remote recall.

Theories of systems consolidation agree on the neocortex as the permanent site of storage for memories, and the current findings support this view. An increase in c-Fos and Arc expression was observed in a wide range of cortical areas from recent to remote time-points, including the retrosplenial and parietal cortices, as well as the perirhinal and entorhinal cortices. Lesioning studies have demonstrated retrograde amnesia for allocentric spatial tasks in all of these brain regions. Retrosplenial cortex lesions result in impairment in a spatial discrimination task four weeks, but not one week after learning (Haijima & Ichitani, 2008). Lesions of the parietal cortex produce impairments in an

allocentric version of the Hebbs Williams maze (Rogers & Kesner, 2006). Ramos (2013) found that lesions to the perirhinal cortex immediately after acquisition of a spatial discrimination task produced a retrograde impairment when animals were retrained two weeks later. Y. H. Cho and Kesner (1996) found a temporally graded amnesia in entorhinal cortex-lesioned rats following spatial discrimination training in that memory was impaired up to four weeks following surgery but not six weeks, suggesting this region acts as a temporary store before consolidation in the neocortex. The increases in expression of c-Fos and Arc observed in these regions over the course of retention is likely to reflect their increased involvement in the recreation of the spatial representation.

Inactivation of the medial prefrontal cortex has been shown to disrupt remote memory in spatial tasks such as the water maze (Lopez et al., 2012; Teixeira et al., 2006), and the radial arm maze (Maviel et al., 2004), as well as other tasks such as contextual fear conditioning (Frankland et al., 2004), while leaving recent memory intact. These findings implicate the medial prefrontal cortex as a possible storage site for long-term memory, and spatial memory in particular. The simultaneous increase in Zif268, c-Fos and Arc expression in all three sub-regions of the medial prefrontal cortex over the four retention time-points reflect their increased involvement over time and are consistent with the theory of systems consolidation, which reflects the findings of similar studies (Bonaccorsi et al., 2013; Lopez et al., 2012).

There is, however, some debate on the precise role of the medial prefrontal cortex during memory retention, whether it is a site of storage or simply becomes more involved in the effortful retrieval of information from other brain regions as time goes on (Rudy et al., 2005). The extensive connections of the anterior cingulate cortex suggest it is in a position to function as an integrator of information, where it directs attention to stimuli on the basis of previous experience (Weible, 2013), while there is also evidence that it

displays the characteristics of a long-term storage site. Restivo, Vetere, Bontempi, and Ammassari-Teule (2009) found increased spine density in the anterior cingulate cortex during remote retention of contextual fear conditioning, which was essential for the expression of the memory (Vetere et al., 2011). However, even its role as an exclusively long-term storage site has come under scrutiny. There is accumulating evidence from a variety of learning paradigms that disruption of activity of the medial prefrontal cortex can affect both recent and remote memory. Leon et al. (2010) showed that disruption of the MAPK pathway in the medial prefrontal cortex of rats just before a recent (one day) memory probe trial in the water maze impaired retention. Tse et al. (2011) found increased expression of Zif268 and Arc in the prelimbic cortex in rats given paired associate training, and AMPA receptor inhibition in this region was sufficient to impair recently learned (one day) and remotely learned (six months) information. Einarsson and Nader (2012) found that infusion of a protein synthesis inhibitor into the anterior cingulate cortex immediately after training in contextual fear conditioning impaired retention one day later. These findings cast doubt over the traditional view that cellular consolidation occurs quickly and systems consolidation follows subsequently at a much slower rate, and suggest that they may occur in parallel. Accordingly, Lesburgueres et al. (2011) found that if the orbitofrontal cortex was inactivated in rats during acquisition of a socially transmitted food preference, retention was unimpaired at seven days but impaired at 15 and 30 days, suggesting an early "tagging" of cortical neurons was necessary for subsequent consolidation and recruitment. These results are consistent with our observed increases in IEG expression in the medial prefrontal cortex during early learning, and the subsequent increase in expression in this region over the course of retention, suggesting this region plays an increasingly important role in the expression of remote spatial memory over time. The pattern of IEG expression in cortical areas

suggested a gradual emergence of a reliance on the cortex over the course of retention, reaching a significant increase from recent retention at 14 or 30 days. This suggests that systems consolidation of spatial memory persists past the first week or 10 days following acquisition and cortical connections continue to strengthen over the course of time. These findings are however at variance with standard consolidation theory, and add credence to multiple trace and cognitive mapping theory, suggesting that detailed contextual and spatial memory does not become completely independent of the hippocampus.

7.3 Concluding Remarks.

The experiments in this thesis have provided an in-depth analysis of the contribution of a wide range of brain regions to the learning, consolidation and recall of spatial memory. We have highlighted the complications associated with the use of closely matched control conditions, and demonstrated the benefits of using detailed time-series designs. We discovered that IEG expression is often highest during early to mid-training in a spatial task, and that patterns of expression can differ across IEGs. We have shown that IEGs can display a close relationship with learning performance, with expression higher in poorer learners during acquisition and superior performers during retention. We have ruled out a second wave of IEG-facilitated consolidation at eight hours following spatial learning. We have demonstrated that hippocampal activity remains elevated during the expression of remote spatial memory, and that a number of cortical areas become increasingly involved in the recall of spatial memory as the time between acquisition and recall increases. Finally, we have demonstrated that a network of brain regions including the hippocampus contribute to the formation and persistence of allocentric spatial memory.

Chapter 8

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