Using IEG's to uncover pathways for spatial learning in the rat

A thesis submitted for the degree of doctor of philosophy

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i

Abstract

This thesis concentrates on the induction of the immediate early genes *zif268* and *c-fos* in a functional and dysfunctional brain network. Initial studies focused on the creation of a task to allow the study of immediate early gene activation after working memory. Previous studies using such a paradigm have compared the animals performing the experimental task with poorly matched control groups. Experiments in this thesis attempted to rectify this. A group of experiments using the water maze found decreases in Zif268 activation in the experimental group as compared to control groups, mainly in the hippocampus and some parahippocampal areas. This was believed to have arisen because of a streamlining of the brain network in the Working Memory group. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to controls in prefrontal regions. Structural equation modelling analysis was performed, which allows immediate early gene counts to be used to analyse networks of brain regions. In the Working Memory group connections were seen between the parahippocampal regions and the subiculum that progressed via the hippocampus, indicating that the hippocampus was still engaged by the task. In the control group analysed no such hippocampal pathway was found.

This water maze task was then used to study *zif268*-EGFP activation in a novel transgenic rodent model, where the Zif268 promoter drives the expression of a fluorescent protein EGFP. Activation of both EGFP and Zif268 immunoreactivity was seen in the CA1 region of the animals performing the control task. No EGFP activation was seen in this region in the Working Memory group even though EGFP expression was seen in other regions. The GFP protein was also able to be seen under direct visualisation in the CA1 and dentate gyrus region of control animals.

Concerning the dysfunctional brain, gene expression was analysed in the retrosplenial cortex after NMDA lesions of the anterior thalamic nuclei. Previous research has shown that lesions produce a dramatic hypoactivity in the protein products of the immediate early genes *c-fos* and *zif268*. Microarray analysis of retrosplenial tissue revealed that as well as decreases in expression of genes related to repair and cell adhesion/neurogenesis, an increase in *c-fos* mRNA was seen in the lesion hemisphere of the brain. This pattern of expression is opposite to that of the protein. Possible reasons for this are discussed.

Acknowledgements

Publications

Shires, K.L. and Aggleton, J.P. (2005) Decreases in Immediate Early Gene Activation Following Spatial Learning in the Watermaze by Rats: Use of a Novel Control. (European Brain and Behaviour Society Abstract).

Poirier,G; Shires, K.L.; Carter, D.A. and Aggleton, J.P. (2004) Transcriptome Analysis of Granular Retrosplenial Tissue Following Anterior Thalamic Excitotoxic Lesions in the Rat Reveal a Distal Effect (Society for Neuroscience Abstract)

Shires, K.L. and Aggleton, J. P. (2004) Immediate Early Gene Expression Following Learning in the Water Maze: Comparison with a Novel Control (Society for Neuroscience Abstract)

Abbreviations

Neuroanatomy

Aca	anterior cingulate cortex
AD	anterodorsal nucleus
AM	anteromedial nucleus
Atn	anterior thalamic nuclei
AV	anteroventral nucleus
LD	lateral dorsal nucleus
Bfd	barrel fields
DG	dentate gyrus
ENTl	lateral entorhinal cortex
ENTm	medial entorhinal cortex
IL	infralimbic cortex
Mop	motor cortex
MTL	medial temporal lobe
Peri	perirhinal cortex
PL	prelimbic cortex
Por/Post	postrhinal cortex
Rga	retrosplenial granular a
Rgb	retrosplenial granular b
rRSP	rostral retrosplenial cortex
Ssp	somatosensory cortex
Subd	dorsal subiculum

Genes

AP-1	activating protein 1
Chgb	Chromogranin B
CREB	cyclic AMP response element binding protein
EGFP	enhanced green fluorescent protein
ERE	Egr response element
EST	expressed sequence tag
Fra1	Fos related antigen 1
Fra2	Fos related antigen 2
GFP	green fluorescent protein
Hsd11beta1	Hydroxysteroid dehydrogenase 11 beta type 1
IEG	immediate early gene
Klf5	kruppel-like factor 5
Lsamp	Limbic system associated membrane protein
MAPK	mitogen-activated protein kinase
Mmp9	matrix metalloproteinase 9
Nrxn1b	non-processed neurexin 1-alpha
Olfm3	Olfactomedin 3
PKA	protein kinase A
RTF	regulatory transcription factor
SCN1B	voltage-gated sodium channel subunit beta1-A
Usp3	Ubiquitin specific protease 3
YFP	yellow fluorescent protein
ZFP91	zinc finger protein 91

Pharmacology and chemistry

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AS-ODN	antisense oligodeoxynucleotides
DAB	diaminobenzidine
DEPC	Diethylpyrocarbonate
GABA	gamma-aminobutyric acid
LTD	long term depression
LTP	long term potentiation
NMDA	N-methyl-D-aspartic acid
PBS	phosphate buffered saline
PBST	PBS with 0.2% Triton-X
PCR	polymerase chain reaction
PFA	paraformadehyde
QPCR	quantitative polymerase chain reaction

Others

AGFI	adjusted goodness of fit index
EEG	electroencephalogram
fMRI	functional magnetic resonance imaging
GFI	goodness of fit index
ICM	implied covariance matrix
MCI	mild cognitive impairment
OCM	observed covariance matrix
PET	positron emission tomography
RAM	radial arm maze
ROS	reactive oxygen species
SEM	structural equation modelling

Contents

Chapter One

General Introduction

During learning the brain must undergo changes that reflect this process. These changes can be measured at a variety of levels (structural, molecular) and take place in multiple brain sites. An intrinsic problem in understanding the neurobiology of learning and memory is how to select approaches that combine i) high temporal resolution ii) high anatomical resolution, and iii) the ability to make simultaneous comparisons across multiple brain sites. For example, electrophysiological techniques provide high temporal resolution, but because of sampling (e.g. single unit) or localisation (e.g. EEG) limitations provide poorer anatomical coverage or resolution. Although functional imaging techniques (e.g. fMRI, PET) can provide greater anatomical coverage, these techniques have poorer temporal resolution and the anatomical resolution is relatively crude. At present there simply is no single technique that will suffice, and our understanding of the where and what of learning mechanisms depends on bringing together data from these various techniques. A huge advance would be to map a marker within the brain that signifies plastic changes that could be detected at high temporal and high anatomical resolution. In this way, different networks for different types of learning would emerge. Furthermore, by not relying on the outcome of abnormal brain states (e.g. as in lesion studies) one would derive a far better insight into normal brain function. It is this set of issues that has driven the aims of this thesis. The goal of the studies described in this thesis has been to map changes in proteins that are markers linked to learning. The approach adopted was selected as it can provide two of the three desired component features (high anatomical specificity, mapping of multiple brain sites simultaneously). The main focus has been on measuring changes in the expression of a gene *zif268* following learning. This gene was selected as it is an example of an 'immediate early gene'.

1.1 What are Immediate Early Genes?

For memories to be laid down, permanent changes within the cell must be brought about. Initially this process is believed to begin with a group of genes known as 'immediate early genes (IEGs)', that are the first in a cascade that lead to permanent changes within a cell. By definition IEGs are a "class of genes that are rapidly and transiently activated in response to neuronal activation, require no protein

synthesis to be activated and, upon activation, can in turn activate downstream targets" (see Davis et al., 2003). IEGs can be split into two categories: Regulatory transcription factors (RTFs) and 'effector' IEGs that effect functional changes. Effector IEGs perform a range of functions throughout the nervous system including cellular growth, with such genes as *BDNF* and *narp*, intracellular signalling (*homer 1a*), synaptic modification (*arc*) and metabolism (*cox-2*) (Guzowski, 2002). There are believed to be about 30 to 40 IEGs and of these 10 to 15 are RTFs, the rest being effector IEGs (Lanahan and Worley, 1998). RTFs can regulate many other different genes more 'downstream' than the RTF itself (Herdegen and Leah, 1998; O'Donovan et al., 1999; Tischmeyer and Grimm, 1999). In other words, they have no direct effect on the cell themselves but rather begin a chain of reactions that does (Clayton, 2000). The *fos* and *krox* families are RTFs that include the two genes of interest here, *c-fos* and *zif268* (also known as *egr-1*, *krox 24*, *NGFI-A*, *zenk*, etc).

This review and thesis experiments will focus on two IEG's, *zif268* and *c-fos*. This is mainly because they are the two genes that are most well characterised out of all IEG's with respect to neuronal function. There is a large body of evidence as discussed below that link their expression to a number of different forms of stimulation. *Zif268* is of most interest because although there is much evidence relating *c-fos* to spatial learning and cognition there is less known about *zif268* in this sphere of research.

1.2 What do Zif268 and c-Fos do?

c-Fos is a nuclear protein (Curran et al., 1987) with DNA binding and transcriptional regulatory properties (Sambucetti and Curran, 1986; Setoyama et al., 1986). The *c-fos* gene is located in the (E-D) region of chromosome 12 and the c-Fos protein is 380 amino acids long in the rat (Herdegen and Leah, 1998). To be fully functional c-Fos must dimerize with proteins of the Jun family, producing the transcription factor *AP-1* (Activating protein 1) (See Chaudhuri et al., 2000). The level of c-Fos in each cell after induction is roughly 0.001% to 0.01% of the total protein in the cell (Kovary and Bravo, 1991), which means it has a very low basal activity level (Herrera and Robertson, 1996) compared to other IEG proteins. This is believed to be the case because *c-fos* has negative feedback regulation (See Chaudhuri et al., 2000). It is also not expressed in every brain region (Chaudhuri, 1997). *c-fos* mRNA is found at basal level in the visual cortices of the rat, cat, monkey and human

(Dragunow and Robertson, 1988; Sagar et al., 1988; Zhang et al., 1994) and at lower levels in other cortical regions (Zhang et al., 1994). Higher levels of mRNA expression are found in the anterior olfactory nucleus, and moderate levels in the piriform and entorhinal cortices, the dorsal endopiriform nucleus, lateral septum and subiculum (Kasof, Mahanty et al., 1995; Kasof, Mandelzys et al., 1995). Kasof et al. (Kasof, Mahanty et al., 1995; Kasof, Mandelzys et al., 1995) also found weak expression in the frontal, agranular, orbital and cingulate cortices, the CA1 and CA3 and the amygdala and tegmentum. There is some conflict over expression in the hippocampus with Kasof et al. (Kasof, Manhanty et al., 1995; Kasof, Mandelzys et al., 1995) finding weak expression in CA1 and 3 but others have found no expression of the mRNA or protein (Wisden et al., 1990; Gass et al., 1992; Hughes et al., 1992). Target genes that *c-fos* is thought to induce include those encoding nerve growth factor, proenkephalin, prodynorphin, endorphin, neurotensin, tyrosine hydroxylase and neuropeptide Y (Kaczmarek, 2002).

Zif268 has a DNA binding site that consists of three zinc finger motifs (O'Donovan et al., 1999). The protein is 508 amino acids long in the rat and 553 in the human (Herdegen and Leah,1998). Within the human cell it is found on chromosome 5 in band 5q23-31 (Sukhatme et al., 1988). *Zif268* has a much higher basal level than *c-fos* in all areas of the brain (Milbrandt, 1987; Lemaire et al., 1988; Sukhatme et al., 1988; Mack et al., 1990; Waters et al., 1990; Chaudhuri et al., 2000). Both the mRNA and protein are found in the cortex of the rat, cat and monkey (Chaudhuri et al., 1995) and particularly in the visual cortex (Worley et al., 1991; Zhang et al., 1994). There are differential distributions of the mRNA and protein amongst the hippocampal regions (Schlingensiepen et al., 1991; Gass et al., 1992; Hughes et al., 1992; Herdegen et al., 1993; Herdegen et al., 1995). These studies all generally show that expression is at its highest in CA1, of medium intensity in CA3 and very low within the dentate gyrus. Target genes for Zif268 include phenylethanolamine N-methyl transferase, neurofilament, synapsin I, synaptophysin I and II, apolipoprotein A1, platelet-derived growth factor B and *nur 77* (Leah and Wilce, 2002). It also is believed to repress adenosine deaminase and because the Zif268 promoter contains an ERE (Egr response element) it induces *egr1/zif 268* (Leah and Wilce, 2002).

The time courses of Zif268 and c-Fos protein and mRNA differ considerably. Zangenehpour and Chaudhuri (2002) used light-dark transitions to investigate the temporal expression characteristics of *zif268* and *c-fos* (Fig. 1).
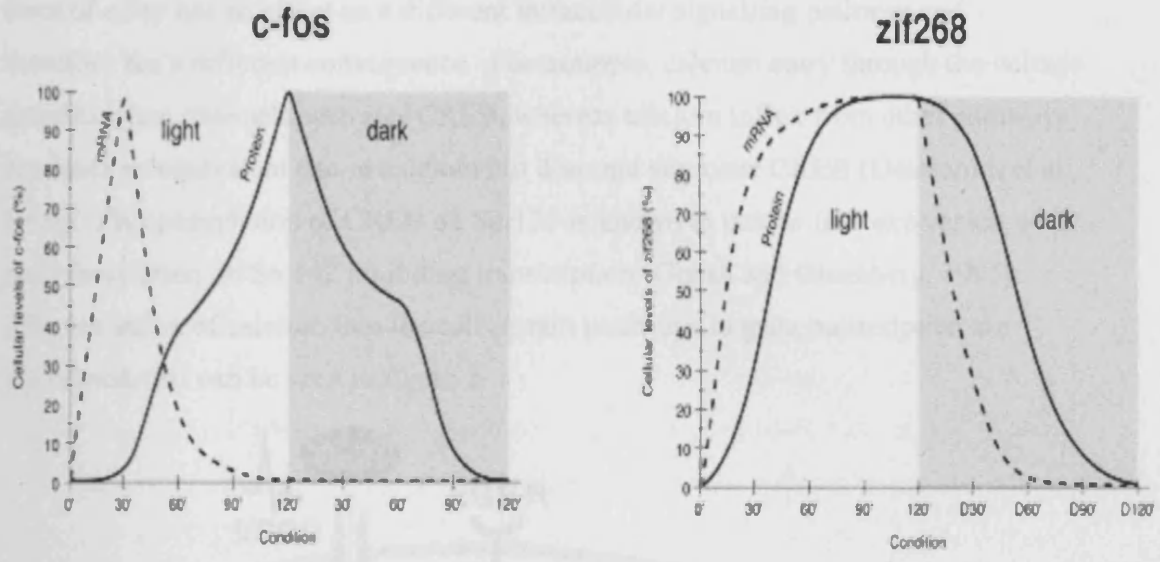
Figure 1.
Figure depicting the time course of *zif268* and *c-fos* mRNA and protein in the primary visual cortex (Taken from Zangenehpour and Chaudhuri, 2002)

For *c-fos*, within 30 minutes of the light phase onset the mRNA level had reached a maximum and after 120 minutes had returned to base level. Protein detection began 30 minutes into the light cycle coinciding with the mRNA peak and reached maximum expression at 120 minutes. With the beginning of the dark phase the protein level dropped, reaching base line 120 minutes after the dark phase commenced. At the beginning of the light phase both *zif268* mRNA and protein is detected. The mRNA level reaches a plateau at 60 minutes and drops rapidly after 120 minutes at the beginning of the dark cycle. Protein levels reach a peak at 80 minutes and remain there until 20 minutes into the dark phase when they begin to decline. By 60 minutes into the dark phase the mRNA levels reach zero and so does the protein levels by 120 minutes.

1.3 Regulation of IEG expression

IEG induction in neurons relies on the propagation of the signals from the plasma membrane to the nucleus. For some IEG's this signal involves phosphorylation of a transcription factor called CREB (Cyclic AMP Response Element Binding protein). CREB phosphorylation occurs with the entry of calcium

into the cytoplasm. Calcium can enter into the cytoplasm in one of four ways: either through the NMDA or AMPA (Jonas and Burnasher, 1995) glutamate receptors, through voltage-gated channels or from intra cellular stores (Berridge, 1998). Each form of entry has an effect on a different intracellular signalling pathway and therefore has a different consequence. For example, calcium entry through the voltage gated calcium channels activates CREB, whereas calcium influx from other pathways produces an equivalent rise in calcium but does not stimulate CREB (Deisseroth et al., 1998). Phosphorylation of CREB on Ser133 is known to induce IEG expression with phosphorylation on Ser142 inhibiting transcription (Ghosh and Greenberg, 1995). After an influx of calcium into the cell certain pathways to gene transcription are stimulated, this can be seen in figure 2.
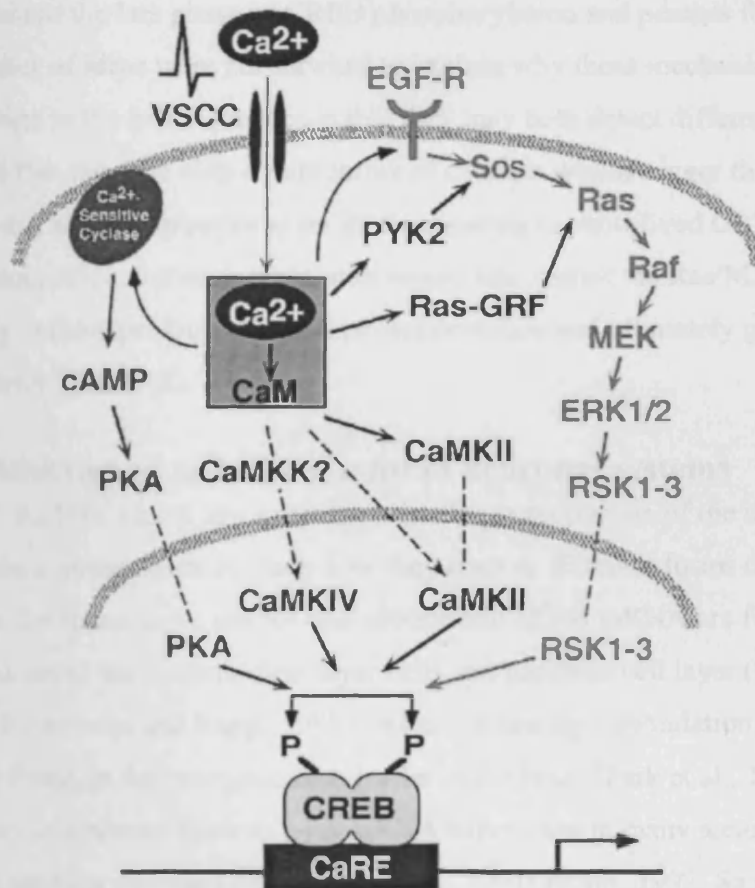
Figure 2.
Three pathways to CREB phosphorylation: PKA, Calcium/Calmodulin and Ras/MAPK.
(Shaywitz and Greenberg, 1999)

Three main pathways have been discovered; the Calcium/Calmodulin dependent kinase pathway, the Ras/MAPK pathway and the PKA pathway. They are thought to stimulate CREB in different ways which leads to different expression of IEG's, however more research is needed to indicate specifically what occurs. It is known that both the Calcium/Calmodulin and Ras/MAPK pathways phosphorylate CREB at Ser133 (Matthews et al., 1994; Rosen et al., 1994; Bito et al., 1996; Xing et al., 1996) but which predominates when it comes to IEG expression is unknown. Wu et al. (2001) tried to answer this question. Inhibitors were used to block both pathways independently. It was discovered that the Calcium/Calmodulin pathway was very fast acting and effected the initial phase of CREB phosphorylation but decayed quickly. The Ras/MAPK pathway on the other hand was slow acting and can be linked to the late phase of CREB phosphorylation and persists for a longer period. A number of ideas were put forward to explain why these mechanisms may be of an advantage to the cell. One idea is that they may both detect different strength stimuli. Stimuli that produce only a mild influx of calcium would trigger the Calcium/Calmodulin pathway on its own leading to short lived CREB phosphorylation, but stronger stimuli would also recruit the Ras/MAPK pathway leading to more prolonged CREB phosphorylation and ultimately greater IEG expression (Wu et al., 2001).

1.4 Induction of *zif268* and *c-fos* in neuronal systems

As IEG's have low expression in a large proportion of the unstimulated brain it is a natural progression to study how they react to different forms of stimulation. Within the retina, *c-fos* mRNA and protein and *zif268* mRNA are found after light stimulation in the inner nuclear layer cells and ganglion cell layer (Gudehithlu et al., 1993; Koistinaho and Sagar, 1995). After intense light stimulation activation of c-Fos is also found in the intergeniculate leaflet of the brain (Park et al., 1993). Binaural auditory stimulation leads to *c-fos* mRNA expression in many areas including the dorsal cochlear nucleus (Ehret and Fischer, 1991; Friauf, 1992; Sato et al., 1992) and the superior olivary complex, lateral lemniscus and inferior colloclus (Ehret and Fischer, 1991; Friauf, 1992; Rouiller et al., 1992; Brown and Liu ,1995; Friauf, 1995; Keilmann and Herdegen, 1997). It has also been found that the number of c-Fos positive cells increases with more intense sounds (Brown and Liu, 1995). Herdegen

and Leah (1998) postulated that stronger IEG expression is seen when the stimulation is of greater significance to the animal.

Noxious stimulation of the hind paw skin, hind leg muscle or tail leads to activation of c-fos in many brain regions including sensory, nociceptive, motor, autonomic, hypothalamic and thalamic areas and some amygdala nuclei (Bullitt, 1989; Bullitt, 1990; McKitrick et al., 1992; Bellavance and Beitz, 1996; Dong et al., 1997; Hermanson and Blomqvist, 1997). This activation occurs with mechanical, chemical and thermal stimulation. Hypoxia-ischemia produces activation of c-fos mRNA and protein in the forebrain of the rat and gerbil (Onodera et al., 1989; Gunn et al., 1990; Popovici et al., 1990; Uemura et al., 1991). Ten to fifteen minutes after a seizure strong c-fos and zif268 activation is seen in the dentate gyrus (Lanaud et al., 1993). Activation is then seen in the CA1, CA3 and cortex 30 minutes later (White and Gall, 1987; Cole et al., 1989), returning to basal level in the dentate gyrus and CA1/3 within one to two and two to four hours respectively. This follows the path the seizure is believed to take from the dentate gyrus through CA1 to CA3 and CA4 (Herdegen and Leah, 1998). Zif268 expression has also been found in the hippocampus, cortex and the amygdala after kainate-induced seizures (Honkaniemi and Sharp, 1999).

Changes in IEG expression are also seen with exposure and dependency to drugs. Acute cocaine and amphetamine administration produces c-fos and zif268 activation in the nucleus accumbens and striatum (Persico et al., 1993) returning to basal level after several hours. Activation is minimised if the drug is administered repeatedly (Couceyro et al., 1994; Klitenick et al., 1995). c-Fos protein is increased in many brain areas following acute amphetamine and methamphetamine administration including the cortex, amygdala, thalamus, hypothalamus, nucleus accumbens and the striatum (Umino et al., 1995). Withdrawing from chronic amphetamine administration causes a decrease in c-fos and zif268 mRNA in the prefrontal cortex (Persico et al., 1995). Morphine administration leads to a decrease in c-fos in the locus coeruleus of naive animals but in dependent animals an increase of c-fos and zif268 mRNA and protein is seen in the locus coeruleus, cerebral cortex, hippocampus, thalamus, cerebellum, brain stem and spinal cord (Hayward et al., 1990; Beckmann et al., 1995). Withdrawal from ethanol vapours leads to an increase in c-fos, c-jun and zif268 mRNA in the hippocampus and the cortex (Matsumoto et al., 1993).

A factor to consider when studying IEG expression is the effect of age. Lee et al. (1998) found a significant difference in basal c-Fos expression in old (20-29 months) and young (4-6 months) rats. A decrease in active c-Fos nuclei was seen in the aged rats in the hippocampus with the biggest difference in the dentate gyrus. There was also a significant decrease in the piriform and temporal cortices compared to the young rats. Desjardins et al. (1997) studied the differences found with age in a water maze task. In the initial phase the platform was hidden in a fixed location in one of the four quadrants of the pool. In phase two the platform was visible above the water. The number of c-Fos positive nuclei in the hippocampus was not significantly different between the groups. For Zif268 however there were fewer nuclei in the CA1 region in the aged group compared to the young animals.

1.5 IEGs and learning

With such a broad range of simple stimuli initiating IEG induction it is now pertinent to consider whether more advanced stimuli could produce similar effects. A particular area of interest is complex forms of learning such as recognition, recall, novelty and spatial working memory. Being able to use IEG's to study areas of brain activation in these paradigms is important because of the problems encountered using lesions which has up to now been one of the main techniques employed. Here, I will discuss studies that use IEG's in a number of different learning paradigms to discover more about brain function in this context.

Beginning with a very simple form of learning, Zhu et al. (1995) used c-Fos expression to map brain activation induced by novel and familiar stimuli. Rats were shown objects in a light box three times a day, a familiar set was shown either twice (on days two, four and five) or three times (on days one and three). On days two and five the rats were also shown a set of novel objects and on day four the rats had trials where the box was illuminated with no object present. On the test day (day six) rats were shown the familiar objects twice. Rats were then split into three groups, where one group were shown the familiar set, one the novel set and one received light only trials. Higher c-Fos counts were found in rats shown the novel objects compared to familiar in the occipital cortex, area TE, perirhinal cortex and the anterior cingulate gyrus. In an expansion of this study (Zhu et al., 1997), c-Fos activation was compared after rats were exposed to a novel or familiar environment. In the perirhinal cortex, the number of c-Fos stained nuclei was non-significantly lower in the rats

8

experiencing the environment for the first time compared to the habituated animals. In the hippocampus, however, the number of stained nuclei was lower in the habituated rats compared to those exposed to the novel environment.

c-Fos expression induced by avoidance and escape has also been studied using the elevated T maze (Silveira et al., 2001). The apparatus was constructed such that one arm was enclosed and the other two were not. The rats were split into three groups; avoidance, escape and control. The avoidance group were placed at the end of the enclosed arm and the time taken for the rat to leave the enclosed arm was recorded. In the escape group the rat was placed at the end of one of the open arms. Again the time taken for the rat to leave this arm was recorded. The control group performed the same task as the avoidance group but in an enclosed T-maze. More c-Fos stained nuclei were discovered in the avoidance group versus control in the medial amygdaloid nucleus, the paraventricular nucleus of the thalamus, the anterior and dorsomedial hypothalamic nuclei and in the median raphe nucleus. The avoidance group also had significantly more staining in the medial amygdala nucleus and dorsomedial hypothalamic nucleus compared to the escape group. For the escape task more c-Fos was found in the dorsomedial hypothalamic nucleus, in the basolateral amygdala nucleus, in the paraventricular nucleus of the thalamus and the dorsal periaqueductal gray matter of the mesencephalon compared to the control group. Only the periaqueductal grey had increased activation in the escape group compared to the avoidance group.

Bertania and Destrade (1995) investigated differential c-fos mRNA expression within the hippocampus after acquisition and recognition in mice. Mice were split into two groups; trained and sham conditioned. Animals in the experimental group had to bar press to receive food and then navigate around a partition to get to the food well. The trained group received 15 rewarded trails. In the sham conditioned group the bar was not connected to the cage controls so pressing the bar had no effect on the food reward. These animals were yoked with the trained group and were rewarded depending on the trained groups performance. Half of the animals were sacrificed 30, 90 and 180 minutes after testing (acquisition group) and the other half received 5 more trails and were killed 30, 90 and 180 minutes later (recognition group). For the acquisition group more c-fos mRNA was seen in the trained animals sacrificed after 90 and 180 minutes compared to the sham-conditioned in the hippocampus and posterior cingulate cortex. After 30 minutes more expression was seen in the sham

animals in CA3 and the dentate gyrus. In the recognition group there was significantly more expression in CA1, CA3, dentate gyrus and the posterior cingulate cortex in trained animals after 90 minutes. There was also an increase in CA3 and dentate gyrus in the trained animals at 180 minutes. Comparing the two groups it was shown that at 180 minutes there was significantly more *c-fos* expression in CA3 and dentate gyrus of recognition animals compared to the acquisition animals. This study shows that not only does *c-fos* mRNA produced different time courses of activation depending on the neuronal activation produced by the distinct task demands but also it has differential activation within the discrete hippocampal subfields.

Less research has been carried out using *zif268* as a mapping tool for learning. *Zif268* is ultimately a better tool than *c-fos* because of its wider range of expression within the brain and its more flexible time course of expression i.e. maximum expression is present for a longer period compared to *c-fos* mRNA and protein (see 1.2). Rosen et al. (1998) used *zif268* in conjunction with *c-fos* to study contextual fear conditioning in the amygdala. Four test groups were used; context-no-footshock, where rats were placed in the chamber for five minutes and then removed; immediate-footshock, with rats receiving a shock as soon as they were placed in the chamber; delayed-footshock, where rats received a shock after being in the chamber for a minute; the fourth group acted as a control and were handled every day for five minutes. Half of the rats were killed 15 minutes after performing the task the other half performed a retention test 24 hours later. Behaviourally the rats in the delayed footshock condition showed a fear conditioning response but the other groups did not. In the delayed footshock condition significantly more *zif268* nuclei were seen in the lateral nucleus of the amygdala as compared with the context-no-footshock animals. At retention no significant differences in *zif268* activation was found between the groups. These results indicate that *zif268* activation in the amygdala may be associated with the fear response.

Zif268 has also been used in mapping a visual memory task in monkeys (Tokuyama et al., 2002). Surgery was performed on the animals prior to testing transecting the corpus callosum so that the two halves of the brain could be studied independently. The monkey performed two tasks; a visual paired-association and a visual discrimination that acted as a control. Each task was performed in a different hemisphere. In the paired-association task, a cue stimulus was presented on the screen and then two choice stimuli were presented one of which was a paired associate of the

cued stimulus. After this, the monkey had to touch a choice of two targets in the stimulus' previous locations and had to select the one that was originally paired with the cue. For the visual discrimination task, the same procedure was followed in the opposite hemisphere but the cue stimulus was not presented. The monkey had to select the one choice stimulus that was rewarded. In each case the monkey had been previously trained on other visual sets to learn the rules for the tasks. It was discovered that there were more *zif268* positive nuclei in area 36 (analogous to the perirhinal cortex in the rat) in the pair associated hemisphere as compared to the visual discrimination side. This confirms reports from lesion work that found that paired associations could not be performed after perirhinal lesions (discussed in Tokuyama et al., 2002).

Because changes in IEG expression have been found in many forms of learning, the next logical step would be to look at one of the most complex and highly researched areas, spatial learning. Vann et al. (2000) looked at c-Fos expression using the radial arm maze. In their first experiment, one group of rats (8arm-1) performed a standard working memory task. All eight arms of the maze were food baited and the animal had free choice of all arms, having to visit all eight to finish the trial. Therefore, animals had to use surrounding visual cues to remember which arms it had previously visited. A second group (1arm) had access to only one arm and received a reward depending on when group 8arm-1 made a correct choice. In experiment two, group 8arm-2 carried out the same task as group 8arm-1 i.e. a standard working memory task. Group 8arm-novel performed the same working memory task but in a room containing different spatial landmarks than 8arm-2. For the final session, group 8arm-2 did the same task as previously. Group 8arm-novel however was tested in the same room as group 8arm-2 and were therefore working with the landmarks present for the first time. c-Fos expression was found to be higher for group 8arm-1 and group 8arm-novel in both the dorsal and ventral hippocampus compared to their respective control groups. The authors suggested that this arose because both of these tasks were more spatially demanding than the control tasks. Also, for group 8arm-1 c-Fos expression was higher than group 1arm in the dentate gyrus, CA3, CA1 and dorsal, ventral and caudal subiculum. This was also true for group 8arm-novel as compared to group 8arm-2 but not in the ventral subiculum. This was again due to these tasks being more spatially demanding. A further study (Vann et al., 2000) using the same protocol found the an increase in expression in the 8arm-1 group in all three

anterior thalamic nuclei (anterodorsal, anteroventral and anteromedial) which have strong links with the hippocampus. With few studies focussing on the IEG *zif268* in relation to forms of learning and spatial learning in particular this appears to be the perfect area for further research.

As discussed above there is evidence to show that IEG activation correlates with different forms of learning. Another aspect of plasticity within the cell is the induction of long term potentiation (LTP). Long-term potentiation (LTP) is "A long term increase in the excitability of a neuron to a particular synaptic input caused by repeated high frequency activity of that input"p.428 (See Carlson, 2001). LTP has also been shown to occur within the hippocampus (Bliss and Collingridge, 1993) and is a strong correlate with many different forms of learning (Martin et al., 2000). With IEG induction occurring within the hippocampus and numerous sub fields (Vann et al., 2000) and in response to different behavioural paradigms (Hess et al., 1995; Seeds et al., 1995; Vann et al., 2000), it is of interest to see whether LTP is related to IEG induction. LTP has three stages dependent on the time it takes for the prolonged activation to cease. 'LTP 1' decays after about two hours, 'LTP 2' after 4 days and 'LTP 3' lasts up to 23 days (Racine et al., 1983). It has been found that LTP 2 and 3 require new protein synthesis to occur but LTP 1 does not (Krug et al., 1984). Abraham et al. (1991) refined this by suggesting that altered gene expression is required for LTP 3 but not for LTP 2.

1.6 Long Term Potentiation and IEGs

IEG induction has been shown to correlate both in vivo and in vitro with LTP. *Zif268* induction occurred with the same stimulation that produced LTP in the hippocampus (Cole et al., 1989). Wisden et al. (1990) also showed that *zif268* was up-regulated by LTP with blockade of NMDA receptors preventing LTP and IEG induction. This did not occur for *c-fos*, leading them to conclude that *c-fos* was not involved in LTP. Others have, however, shown that *c-fos* activation does occur with LTP like stimulation (Cole et al., 1989). Further studies are required to try and resolve this discrepancy.

There is conflicting evidence as concerns the role of IEGs with respect to the time course of LTP. *Zif268* mRNA has been found to be up regulated between ten minutes and two hours following LTP induction (Cole et al., 1989; Wisden et al., 1990; Richardson et al., 1992). Jones et al. (2001) suggested that this was an

indication that IEGs are probably involved with the transition from early to late LTP and not with any particular stage. However, IEG induction has also been linked with the late phase of LTP dependent on protein synthesis and with the maintenance of this state (Abraham et al., 1991; Richardson et al., 1992; Abraham et al., 1993; Worley et al., 1993; Dragunow, 1996). Roberts et al. (1996) put forward evidence that IEGs are not directly involved with the activation of LTP but more with its maintenance. Within 1 hour of LTP induction, there is an increase in *c-fos* and *zif268* mRNA levels (Cole et al., 1989; Wisden et al., 1990; Richardson et al., 1992), with *zif268* mRNA levels returning to basal level after three hours (Chinestra et al., 1994). With *de novo* protein synthesis being required to maintain LTP for longer than three hours (Krug et al., 1984; Otani et al., 1989; Fazeli et al., 1993) this suggests that Zif268 is required to stimulate the movement of LTP 1 to LTP 2 and to maintain LTP up to three hours but not beyond this point. The induction of IEGs could therefore stimulate an activation cascade that begins the later phase of LTP without being actively involved per se (Davis et al., 2003).

1.7 What happens without the presence of IEGs?

Although many studies have shown a correlation between IEG's and learning this in itself is not conclusive evidence that the two are linked. Research using methods that prevent IEG expression can further confirm these results. Jones et al. (2001) carried out a series of experiments using *zif268* mutant mice to assess what effect a complete absence of *zif268* would have on spatial learning. Three different genotypes of mice were generated: *Zif268+/+* mice with normal *zif268* mRNA levels, *zif268+/-* mice with 50% of normal levels and *zif268-/-* mice which had no *zif268* expression. Mice were initially tested in a T-maze on a spontaneous alternation task. All animals were able to perform as normal with both a 30 second and ten minute delay between the sample and test trials. This led to the conclusion that *zif268* was not required for this type of simple spatial learning. Mice were then tested in the water maze on a massed training protocol with all genotypes taking roughly the same amount of time to find the platform on the first trial. Escape latencies decreased as training continued for all genotypes, however *zif268+/-* and *zif268-/-* mice took longer than the *zif268+/+* mice to find the platform. At probe, *zif268+/+* mice showed a preference for the target quadrant whereas the *zif268-/-* and *zif268+/-* mice did not. However, with extended training the *zif268-/-* and *zif268+/-* mice were able to perform

the spatial task (Jones et al., 2001). This result indicates that *zif268* is not required for short-term memory tasks such as the T-maze and initial training in the water maze. However, this data does suggest that *zif268* may be needed for retention of spatial information. Bozon et al. (2002) also showed that *zif268* may be required for retention using a modified object recognition task. On the first day *zif268* knock out mice explored an open field containing three novel objects. On the second day one of the objects was moved to a new location. On the third day mice were given three new objects to explore. Twenty four hours later they were tested with one of the objects being replaced with a novel one. For the spatial aspect of the experiment, the *zif268+/+* mice spent more time exploring the moved object, with *zif268-/-* mice spending the same amount of time exploring all objects equally. *Zif268+/-* mice also spent no extra time exploring the object in the new spatial location, showing that *zif268* may in fact be implicated in long term memory.

A *c-fos* knockout mouse was also shown to be impaired in spatial and cued tasks in the water maze (Paylor et al., 1994). However, these mice have been revealed to have developmental abnormalities (Johnson et al., 1992), which makes it difficult to draw conclusions from these results. Zhang, McQuade et al. (2002) however have produced a *c-fos* mutant mouse that is lacking in expression within the hippocampus and not the brain as a whole. *c-fos* mRNA loss was 97% within the CA1, CA2 and CA3 and reduced by 70% within the dentate gyrus compared to wild type controls (Zhang, Zhang et al., 2002). Water maze testing consisted of three phases – the spatial learning phase, the random platform phase and the visible platform phase. In the spatial learning phase mice had to find a hidden platform placed in the south east quadrant for four trials a day. In the probe trials the platform was removed and the number of platform site crossings was recorded. The random platform phase consisted of two days of trials. On day one mice had two trials with the platform in the south east quadrant and two with it in the north east and north west quadrants. On the second day mice had one trial with the platform in the original location and three more with the platform located in the north west, south west and north east quadrants. The visible platform phase was identical to the hidden platform phase but the platform was visible above the water and a curtain was drawn around the maze so only proximal cues were available.

Both groups of mice were able to perform the hidden and random platform tasks, with escape latencies being similar in both groups (Zhang, McQuade et al.,

2002). This finding also occurred in the visible platform phase which does not require the integrity of the hippocampus for normal performance. These mice were also tested using a Barnes maze which is less physically demanding than the water maze. Mice had to find an escape tunnel when an aversive stimulus was turned on. For each trial the escape tunnel was under the same hole. During testing the mice were placed in the centre of the maze and had to find the escape tunnel. A cued version of the test was also performed where a curtain was drawn around the maze so only proximal cues were available. For the spatial and cued versions of the task both groups had similar escape latencies (Zhang, McQuade et al., 2002). This lack of deficit on spatial tasks led the authors to claim that c-fos within the hippocampus was not required for spatial learning. Although this knockout is more viable than those previously constructed because a select brain region is targeted and not the whole brain it still has fundamental problems. In particular, mice were born with this knockout and therefore compensatory mechanisms may have developed.

A more precise approach to studying learning in the absence of IEG's is to use antisense oligodeoxynucleotides (AS-ODN) which inhibit the synthesis of a specific protein as required. This technique has a number of advantages, in particular it can be very specific, a certain brain area can be targeted and it can be carried out in adults which reduce compensation responses. Guzowski (2002) used antisense to study the effect of c-Fos loss on a water maze task. Rats were injected into the dorsal hippocampus either with c-fos AS-ODN or a controlled scrambled ODN. This was carried out two and half hours before training. The first training session consisted of six trials where rats had to find a submerged platform; the second session was carried out two hours after the first and also consisted of six trials. Forty eight hours later retention was tested, with platforms in the training position. No difference was found between the two groups in the two training sessions but the c-fos AS-ODN rats were impaired during retention compared to the scrambled controls. These results very much mirror what has been found with the zif268 mutant mice and lends further support to the role of IEGs in retention of spatial information. He, Yamada and Nabeshima (2002) also found learning impairments using c-fos antisense on a radial arm maze task. The animals were placed in the centre of the maze in which four arms were baited and four arms were not. A training trial continued until all food had been eaten. Working and reference memory errors were recorded. Before training rats received injections of either antisense or saline into the dorsal hippocampus or the

15

cingulate and motor cortex. Rats received 20 trials twelve hours after the injections. The animals that received the antisense injections into the hippocampus made significantly more working and reference memory errors compared to the saline injected animals. No difference was found for the cingulate and motor cortices. The same results were found when testing was carried out ten hours after the injections. These results were confirmed by immunohistochemistry where the numbers of c-Fos positive nuclei were reduced in the hippocampus and cingulate and motor cortices of the antisense injected animals compared to saline controls.

1.8 Induction of IEG's in a dysfunctional network

As described above IEG's are activated by many different stimuli in a functioning brain network. However changes in IEG induction are also seen in dysfunctional networks, under injury, disease and lesion. There is a great amount of evidence to show that IEG's are activated after ischemia in the brain. Ischemia is the prevention of blood flow into one of the main arteries of the brain and models human stroke, the amount of time this is carried out for can vary but blood flow can be restored at the end (reperfusion). Butler and Pennypacker (2004) studied c-Fos activation after an ischemic injury that lasted for 60 minutes. Activation was seen to begin in the CA1 and CA3 region of the hippocampus 6 hours following reperfusion. An increase of *zif268* expression after ischemia has also been seen (Honkaniemi et al. 1997) with the gene showing prolonged up-regulation in the thalamus and the CA1 region up to 72 hours after the ischemic episode. The authors suggest that *zif268* expression could be related to delayed cell death.

Popovici et al. (1990) looked at c-Fos expression after kainic acid induced seizures and ischemia. Ninety minutes after kainic acid injection an increase in c-Fos immunoreactivity was seen in the hippocampus and more diffusely in subicular cortices and the entorhinal cortex. At 3 hours other areas showed c-Fos activation including the retrosplenial and cingulate cortices. At 12 hours the area with the greatest density of staining was the frontal cortex with all previous areas that had strong staining beginning to decrease. At 24 hours a few labelled neurons were still present in the hippocampus and by 48 hours all immunolabelling had disappeared. After ischemia a slight increase in immunoreactivity was seen at 3 hours in the hippocampus with no other differences from controls at any other time point.

Changes in IEG expression are also seen in tissue from patients suffering from neurodegenerative disorders. Marcus et al. (1998) found an increase in c-Fos and c-Jun in the post mortem brains of Alzheimer's patients. This was seen in all hippocampal areas apart from for c-Fos in the CA1 and in all cases there was significantly higher levels than normal age matched controls. This is believed to have occurred because c-Fos has been shown to be up-regulated by abeta and could be related to amyloid neurotoxicity (Gillardon et al., 1996). Both *c-fos* and *c-jun* have also been shown to make a contribution to apoptosis and the death of cells (Smeyne et al., 1993; Ham et al., 1995).

Within rodent models changes in IEG expression are observed after lesion. Glenn et al. (2005) looked at c-Fos levels after different lesion types of the perirhinal cortex. An hour after completion of the surgery animals were killed and immunohistochemistry performed. In the dentate gyrus, temporal cortex, frontal cortex, parietal cortex and retrosplenial cortex NMDA perirhinal lesion produced greater c-Fos immunoreactivity than sham controls. In the entorhinal cortex, temporal cortex, frontal cortex and parietal cortex electrolytic lesions produced greater density of c-Fos positive neurons compared to sham controls. No effect on IEG induction was seen after aspiration lesions. This was believed to occur because electrolytic and excitotoxic lesions work in similar ways killing the cells by hyperexcitation, which could trigger similar activation of cells in connected brain regions, this however, does not occur with aspiration lesions as tissue is completely removed in the region without producing this hyperexcitation.

Further more, unilateral lesions in the anterior thalamic nuclei produced significantly decreased c-Fos expression in five regions within the hippocampus and the retrosplenial cortex (Jenkins et al., 2002). After receiving anterior thalamic nuclei or sham lesion animals performed a standard working memory task in the radial arm maze. Decreases of c-Fos immunoreactivity were seen in the dorsal and ventral hippocampus, retrosplenial cortex, and prefrontal regions. These regions have been associated with learning and memory which led the authors to suggest that this hypoactivity could be related to amnesia.

1.9 The aims of this thesis

The aims of this thesis are to study immediate early gene activation in a functional and dysfunctional brain network. This will be achieved in a number of

17

ways. Firstly, the activation of both c-Fos and Zif268 will be studied in two different spatial working memory paradigms. This will be an attempt to improve on tasks that have been used to study immediate early gene activation. This will include testing in both the radial arm maze and the water maze. Structural equation modelling will also be used to derive a more dynamic representation of the brain systems that are engaged during a working memory task. The working memory task will then be used to test the viability of a novel transgenic rodent model, where *zif268* drives EGFP expression, for use in spatial memory tasks. Finally a dysfunctioning brain network will be studied using microarray technology to examine changes in immediate early gene mRNA activation in the retrosplenial cortex after lesions of the anterior thalamic nuclei.

Chapter two

Expression of c-Fos in a working memory task in the radial arm maze

2.1 Introduction

As detailed in the introduction, IEG induction has been investigated in a number of different spatial learning paradigms. This thesis will focus on working memory in a number of different forms. This is a type of task that has been shown to induce immediate early gene activation (Vann et al. 2000). Working Memory will be focused on because it allows us to demonstrate that the animal is learning within a test session unlike reference memory where a ceiling effect in behaviour is often reached. Working memory will also be discussed in relation to lesion studies.

2.1.1. Working memory in the rodent

In animals working memory is generally defined as information that animals need to remember for a testing session but is no longer needed once the session has ended (Honig, 1978; Dudchenko 2004). It differs from working memory as defined in humans as " a limited capacity system, which temporarily maintains and stores information, supports human thought processes by providing an interface between perception, long term memory and action" (Baddeley, 2003 p.829). A further issue is that tests of working memory in rodents very often do not demand an active maintenance or manipulation of information i.e. it could simply reflect a store with attentional properties that degrade. Uylings et al. (2003) suggested that rodents have a 'scratch pad' memory system that is a short term erasable form of memory and is analogous to working memory. This information is used to direct behaviour in relation to presented stimuli or context. This is controlled by a master or executive control system that directs the relevant behaviour. However, it has been proposed that working memory in the rodent is just in fact short term memory and it differs from standard short term memory in the way that the animal uses it and because of its nature could be seen as a form of forgetting as unneeded information is discarded (Dudchenko, 2004). For the purpose of this thesis working memory will be operationally defined as suggested by Dudchenko (2004) as information that animals

need to remember for a testing session but is no longer needed once the session has ended.

2.1.2 Tests of Spatial Working Memory in the rat

There are a number of different spatial working memory paradigms in the rodent that utilise diverse apparatus. These include tests in the T-maze, 8 or 12 arm radial maze and the water maze. Discussion here will focus on the T-maze and radial arm maze (RAM); working memory in the water maze will be reviewed elsewhere. Both the T-maze and RAM are appetitive forms of spatial learning requiring animals to perform the task for a food reward. In the T maze animals can be tested either on a forced choice alternation (non matching to place) or matching to place task. A session consists of two trials; on the first trial animals are forced to enter one arm of the T shaped maze. On the second trial animals are given free choice of both arms and will either be rewarded for entering the arm they had not previously visited (non-matching to place), or in the case of matching-to-place, receive a food reward for entering the arm presented in the sample phase.

Standard working memory in the radial arm maze consists of baiting every arm with food (Olton and Samulson, 1976). Animals are then allowed free choice of all arms and have to enter only the ones they have not previously visited to receive a food reward. The trial continues until all arms have been visited and all food rewards retrieved. A number of different permutations of testing working memory in the radial arm maze have been used and will be described in more detail as required.

It important to note, that it is possible to solve working memory tasks in the T-maze and radial arm maze using other strategies (Dudchenko, 2004; Baird et al., 2004; Futter and Aggleton, 2006). For example, within the radial arm maze it is possible to solve it by making consecutive arm entries in a clockwise or anticlockwise direction. In the T-maze it is possible to perform a response strategy based on remembering the turn direction previously made and then making the same or opposite response on subsequent trials as required.

2.1.3 Hippocampal lesions and spatial working memory

The hippocampus has been implicated in many different forms of learning, but particularly spatial learning (O'Keefe and Nadel, 1978). The main body of research has focused on lesion studies with hippocampal lesions producing impairments in

20

many different tasks (Holscher, 2003). A large body of evidence has shown that the hippocampus has been implicated in working memory. Olton et al. (1982) looked at the effect of disconnecting the hippocampus on spatial working memory in the radial arm maze. Lesions that severed both hippocampal commissures; thereby disconnecting the hippocampus from other parahippocampal regions produced deficits on the task. Lesions that kept an ipsilateral or contralateral connection through the hippocampus did not impair behaviour.

Pothuizen et al. (2004) employed a standard working memory task in the radial arm maze to elucidate ventral and dorsal hippocampal function. Animals with either dorsal, ventral or complete hippocampal lesions were exposed to one trial a day for 25 days with four out of eight arms baited. Animals with complete and dorsal lesions made more 'correct' errors (re-entries into baited arms) and 'incorrect' errors (entering a un baited arm). This shows the dorsal hippocampus' role in working memory and the lack of deficit seen with ventral lesions.

Bannerman et al. (2002) have also shown deficits with dorsal hippocampal lesions using a standard working memory task in the T-maze using a delay of a few seconds between the sample and choice runs. Subsequent to that a further 24 trials were run with a delay between the sample and choice run of either 30 or 600 seconds. Complete and dorsal lesioned animals performed just above chance at all delays with the ventral and sham lesioned animals performing well making 81.9% and 81.5% correct choices respectively.

Recent research has started to draw out the function of the different subfields of the hippocampus. Lee and Kesner (2003) utilised selective lesions to study the function of the different hippocampal subfields in working memory. Rats were tested on delayed non matching to place in the radial arm maze with short and intermediate delays between trials and in a familiar and a novel room. Lesions of the dentate gyrus and CA3 but not CA1 affected performance at short delays in the familiar room and also at both delays in the novel environment. At the intermediate delay performance was impaired by lesions of all subregions in the familiar environment. Lee and Kesner (2003) suggested that this difference between the subregions could occur because the dentate gyrus and CA3 have 'recurrent networks', a neural network that keep a representation active whilst it is required, a type of short term memory, and CA1 does not.

Jeltsch et al. (2001) focused on the function of the dentate gyrus in forced choice alternation in the T-maze and standard working memory in the RAM. Animals were tested on 2 trials a day for 5 days in the T-maze and for 1 trial a day for 32 days in the RAM using only 4 baited arms out a choice of 8. More errors were made by animals that had received dentate gyrus lesions on both tasks.

2.1.4 Other lesion studies and working memory

Although a functioning hippocampus is widely regarded as necessary for spatial working memory a number of other brain regions have also been implicated. There is a large body of evidence to suggest a role of the prefrontal cortical regions. Dias and Aggleton (2000) used separate lesions of the prelimbic-infralimbic (PL-IL) and anterior cingulate cortices and a combined lesion to study working memory using both matching and non matching to place in the T-maze. In the non-matching to place procedure a significant deficit was found in animals with combined lesions (PL-IL and anterior cingulate) but not for the separate lesions. For the matching to place task animals were tested to criterion of 5 out of 6 correct choices on 5 consecutive sessions (stage 1). After learning this task the rule was reversed and animals had to now non-match to place (stage 2).

Animals with combined lesions and PL-IL lesions showed impairment at the first stage. The animal's performance was analysed by splitting it into two different phases 'perseveration', where performance was below chance and 'learning', where animals were above chance. Animals with PL-IL and anterior cingulate lesions showed the greatest amount of perseveration. In the learning phase animals with complete lesions and lesions of PL-IL showed a greater number of errors. At reversal all animals showed a certain amount of perseveration but animals with PL-IL lesions showing the greatest amount. Lesions of the prelimbic cortex could be seen to produce an inability to shift responding as they tended to show a greater use of response strategies and therefore learnt slower. Lesions of the anterior cingulate cortex on the other hand seemed to leave response learning intact but may disrupt stimulus-reward learning.

Lesions of the caudal retrosplenial cortex have also been shown to impede working memory. Vann et al. (2003) used a two stage task in the RAM with stage 1 being a standard working memory version of the task and stage 2 involving a rotation of the maze. This rotation involved animals performing the first 4 trials as normal but

then being removed from the maze and it being rotated 45° with the food pellets being placed in the same allocentric location. For stage 1 no effect of group was seen. At stage 2 a significant effect of group was found with the lesion group needing a greater number of arm entries to retrieve the last 4 food pellets. The authors suggest this could occur because the rotation puts a greater demand on the animal's capacity to reorient.

There is also evidence that the anterior thalamic nuclei lesions produce deficits in spatial working memory tasks. Aggleton et al. (1995) showed that on spatial forced choice alternation in the T maze animals with anterior thalamic nuclei, mamillary bodies and fornix lesion were found to be impaired. Also lesions of the mamillary body and mamillothalmic tract lesions impaired performance on the radial arm maze and T maze but only on initial trials (Vann and Aggleton, 2003). An improvement was seen over trials but further rotation trials showed that animals failed to use extra maze cues effectively so therefore were able to solve the task using other strategies.

A brain region that has caused some controversy in its relation to working memory is the perirhinal cortex. Two studies by Liu and Bilkey (1998, 2001) have found deficits in working memory after perirhinal lesions. Training in the RAM demonstrated that lesioned animals made more errors than the sham group. The animals in the lesion group improved their performance over trials but increased their tendency to make adjacent arm entries suggesting they had found another strategy to be successful at the task other than using spatial cues.

This result was replicated with a comparison between perirhinal and hippocampally lesioned animals (Liu and Bilkey, 2001). Hippocampal lesioned animals performed poorly as compared to perirhinal lesioned and sham animals. Perirhinal lesioned animals were impaired but their performance improved over training. Again this could be because they tended to make more adjacent arm entries than the sham animals which suggests performance improved because animals were using another strategy than utilising allocentric cues.

However a study by Machin et al. (2002) found no evidence of a deficit after perirhinal lesions. They used a 4 stage procedure in the RAM to test working memory. In stage 1(days1-14) animals were tested on a standard working memory task with one trial a day. In stage 2 (days 15-20) the maze was rotated after 4 choices had been made and the further food pellets moved to the same allocentric positions. In stage 3 (days 21-25) animals were tested on 2 trials a day and in stage 4 (days 24-

25) animals were tested for one trial in the same maze but in a novel room. At no stage did the perirhinal lesioned group show any deficit as compared to the sham group. Bussey et al. (1999) also found a similar lack of effect of combined perirhinal and postrhinal lesions in the RAM.

2.1.5 IEG's and the study of working memory

Using lesion studies to study the functionality of brain regions is difficult because of the complicated connections of the hippocampus and parahippocampal subfields. This means one is looking at an abnormally functioning brain. Removal of a brain area can lead to other regions compensated or other pathways between regions being utilised. Also some lesions can destroy fibres of passage disconnecting other brain regions unintentionally. There is also the issue of whether a brain region is necessary or sufficient for the task. Damaging a brain structure may be sufficient to produce a deficit (e.g. working memory is impaired with hippocampal lesions) but damage to this region might not be necessary for that impairment to occur (e.g. working memory deficits are also seen with lesions of other brain structures). Also, distal lesion effects can be seen whereby a lesion to one area causes damage to another (Jenkins et al., 2002). The use of techniques such as Immediate Early Gene (IEG) activation which has shown to indirectly correlate with neuronal activation and learning and memory (Herdegen and Leah, 1998) may be a better way of studying brain activation in working memory tasks.

Vann et al. (2000) employed the IEG *c-fos* to study hippocampal and parahippocampal activation in a working memory task in the radial arm maze. In their first experiment activation in one group (8arm-1) which performed a standard working memory task was compared with group 1arm who were forced to repeatedly enter only one arm and received a reward depending on group 8arm-1's performance. In their second experiment groups 8arm-2 and 8arm novel executed a standard working memory task during training. On the final session, group 8arm-2 performed the task as previously whereas group 8arm-novel was tested in the same task in a novel room. c-Fos expression was found to be higher for group 8arm-1 and group 8arm-novel in both the dorsal and ventral hippocampus. The authors suggest that this was because group 8arm-1 and 8arm novel were seen to be performing a task that was more spatially demanding than the other tasks and therefore led to more activation. No disassociation was seen within the hippocampal subfields with group 8arm-1 c-Fos

24

expression being higher than group 1arm in the dentate gyrus, CA3, CA1. This was also true for group 8arm-novel as compared to group 8arm-2. Comparisons in the hippocampus for both experiments showed a greater increase in c-Fos positive neurons in the dorsal hippocampus in experiment 2 as compared to the ventral, whereas in experiment one both were similarly activated. This is in line with findings from lesion studies comparing the dorsal and ventral hippocampus, with the dorsal section needed for spatial tasks but not the ventral (Pothuizen et al., 2004; Moser et al., 1998).

c-Fos activation was also studied using a variation of the standard working memory task in the radial arm maze (Jenkins et al., 2004). Animals were tested on consecutive trials for 30 minutes every day for 16 sessions. Towards the end of the experiment animals would be performing on average 5 trials during the 30 minutes time period. Two groups of animals were tested; group 'novel' and group 'familiar'. Both groups were exposed to an array of the same cues placed on a curtain around the maze. Group familiar encountered the same arrangement of cues on every session. Group novel were exposed to a different arrangement of the cues than group familiar until the final test day when they were exposed to the arrangement that group familiar always encountered. c-Fos immunoreactivity was highest in group novel in the hippocampal subfields, the postrhinal cortex and the parietal cortex.

'Control' groups used in previous studies in the radial arm maze only allowed the rat to enter one arm of the maze repeatedly so they do not experience the whole array of visual cues available to them. The current experiment will try to rectify this problem by replicating Vann et al. (2000) but with another 'control' group added. Animals will be trained by restricting entry to all but one of the arms of the maze. These animals will be yoked to the behaviour of the working memory group and will enter each arm the experimental group does but without having to learn its spatial location. This allows the animals to enter every arm within a trial in the same manner as the experimental group without having to use working memory to remember which arms they have visited previously. This comparison will allow us a closer contrast between the animals performance than seen previously. It would be expected that if animals are automatically learning there would be no difference between the working memory group and the 'control' group.

2.2 Experiment One

2.2.1 Materials and methods

Subjects

Subjects were 15 male albino rats (Sprague Dawley, Charles River) weighing between 350 and 550g at the time of perfusion. The rats were food deprived to 85% of their free feeding weight in the days preceding the experimental procedure and this was maintained throughout testing. Water was made available *ad libitum*. Animals were caged in pairs and were given additional handling before the experiment began. All procedures were carried out in accordance with the UK animals (scientific procedures) act 1986.

Apparatus

The experiment was carried out in an eight arm radial maze, which consisted of a central platform (34cm diameter) and eight equally spaced radial arms (87cm long, 10cm wide). The base of the arms and platform was made of wood surrounded by Perspex walls 24cm high. At the end of each arm was a food well 2cm in diameter and 0.5cm deep. The central platform and each arm were separated by a clear Perspex guillotine door 12cm high. The doors were connected to a pulley system which allowed the experimenter to open and close them at will. The maze was contained within a rectangular room (295 x 295 x 260cm), surrounded by visual cues. The experimenter was visibly located in the same location throughout testing and acted as a further cue.

Behavioural training

Animals were divided into three conditions, group Working Memory (group WM), group Passive (group P) and group 1-arm (group 1-arm). Group Working Memory followed the standard working memory procedure (Olton et al., 1978). Each arm of the maze was baited with two sucrose pellets (Noyes, 45mg). Animals were placed in the centre of the maze with all doors closed for 10 seconds. Then all doors were raised and the animal allowed a free choice. Once an arm choice was made the doors were closed again. When the pellets had been consumed the door for that arm was

raised allowing the rat to return to the centre of the maze. Upon returning to the centre of the maze all the doors were closed and the rat remained there for 10 seconds. Once this time delay had elapsed the doors were raised again and rats were given free choice of the arms. The trial continued until all of the eight arms had been visited or 30 minutes had elapsed. When this had been achieved the rat was placed into its carrying box (28x45x21cm) which was covered with a towel for 2 minutes while the maze was rebaited. Trials continued until 30 minutes had elapsed. Training continued until the rats were able to retrieve all 16 pellets making as few errors as possible i.e. not going down an arm they had already visited, performing five trials or over in the 30 minutes. This took on average 18 days.

Group Passive was a stimulus yoked control group. Animals matched the arm choices made by group Working Memory. At the beginning of a trial animals were placed in the centre of the maze. Only one arm was made available for the rat to enter. Again once the rat had entered the arm the door was lowered and only raised again when the food pellets had been consumed. Animals visited all eight arms but their presentation was dictated by group Working Memory's performance. Group 1-arm only entered one arm of the maze which remained the same over all trials. Animals were rewarded according to the choices made by group Working Memory. If the animal in group Working Memory made a correct choice, the animal in group 1-arm received a reward upon entering the arm. If the animal in group Working Memory was incorrect no reward was received.

Before each animal was tested it was placed within a cage in a dark and quiet room for 30 minutes. When transporting the animal from the dark room to the experimental room the cage was covered with a towel to keep the animal in the dark. After the experiment had finished the animals were placed back into the dark, quiet room for 90 minutes. This cut down on any extraneous activation of IEGs by other unwanted stimuli.

Immunohistochemistry

Ninety minutes after the final session animals were anaesthetized with sodium pentobarbital (60mg/kg) and perfused cardially with 0.1m PBS (Phosphate Buffered Saline) followed by 4% paraformadehyde (PFA) in 0.1M PBS. The 90 minute time point was chosen because there is evidence to suggest this is when IEG protein activation peaks (see 1.2). After removal the brain was placed in PFA for 4 hours and

then in 30% sucrose solution overnight. Sections were cut coronally at 40μm on a freezing microtome and one in four sections were collected in PBS with 0.2% TritonX-100 (PBST). The sections were then blocked using 0.3% hydrogen peroxidase in PBST for 10 minutes. After four 10 minute washes in PBST sections were incubated in the primary antibody anti *c-fos* (1:5000, C-19,) for 48 hours at 4°C. Sections were again washed four times in PBST then incubated in the secondary antibody with goat serum (1:2000 in PBST, vector laboratories) for two hours at room temperature. After washing with PBST, sections were incubated in avidin-biotinylated horseradish peroxidase complex diluted in PBST (Elite kit, Vector laboratories) for one hour at room temperature. Sections were washed again in PBST and then in 0.05M tris buffer. The sections were then stained using diaminobenzidine (DAB kit, Vector laboratories). The reaction was stopped by washing in cold PBS. Sections were then mounted on gelatine-covered slides, dehydrated using a series of increasing alcohol concentrations and then cover slipped. A separate one-in-four series of sections was directly mounted after cutting and stained using cresyl violet for histological purposes. Animals were processed for immunohistochemistry in the triplets they were tested in.

Image Analysis

Sections were scanned onto a PC (IBM) computer using a Leica DMRB microscope and an Olympus DP70 camera. Counts of the stained nuclei were carried out using the program analySIS (Soft Imaging Systems). The threshold was set at the same level for all sections and counts were made in a frame area of 0.84 x 0.63 mm using a 10x magnification. For hippocampal counts the entire area of interest was captured in different frames using a 5x magnification using the analysis software to montage the images. Counts were taken from 3 consecutive sections from both hemispheres for all regions of interest.

Counts were normalized to reduce variation between animals and variations in the immunostaining. Mean counts for each animal at a given site were divided by the overall mean for that site for all animals in the triplet. This produced a percentage ratio for each triplet of animals (Fig. 3). These normalized counts were analysed using a 2 factor analysis of variance (group and region). Regions were chosen as detailed below and areas were grouped as shown. Tables of raw cells counts can be seen in Appendix A Figure 1.

WM: Number of cells in WM subject
   Number of cells in WM subject + Number of cells in P subject +Number of cells in 1-arm subject

P: Number of cells in P subject
   Number of cells in WM subject + Number of cells in P subject +Number of cells in 1-arm subject

1-arm: Number of cells in 1-arm subject
   Number of cells in WM subject + Number of cells in P subject +Number of cells in 1-arm subject

Figure 3
Equations for normalization procedure for Working Memory and 'control' groups

Regions of Interest

Cell counts were taken from regions as detailed in figure 4. These regions were chosen because of their relation to learning and memory processes in the rodent brain. Also included were regions that should show no change over the conditions and this choice was driven by previous findings in IEG spatial learning experimentation (Vann et al., 2000). As detailed previously (2.1.3 and 2.1.4) there are a number of brain regions that have been implicated in working memory tasks in the radial arm maze. In this experiment we will focus on the hippocampus and its subfields, prefrontal areas (prelimbic, infralimbic and anterior cingulate cortex), parahippocampal areas (entorhinal, perirhinal and postrhinal cortex). Also included were cingulate regions (retrosplenial cortex) and the subicular region. The subicular region is the principal junction of the hippocampal pathways to the mamillary bodies and anterior thalamic nuclei, two structures also required for working memory. The sensorimotor regions chosen were the motor and somatosensory cortex.
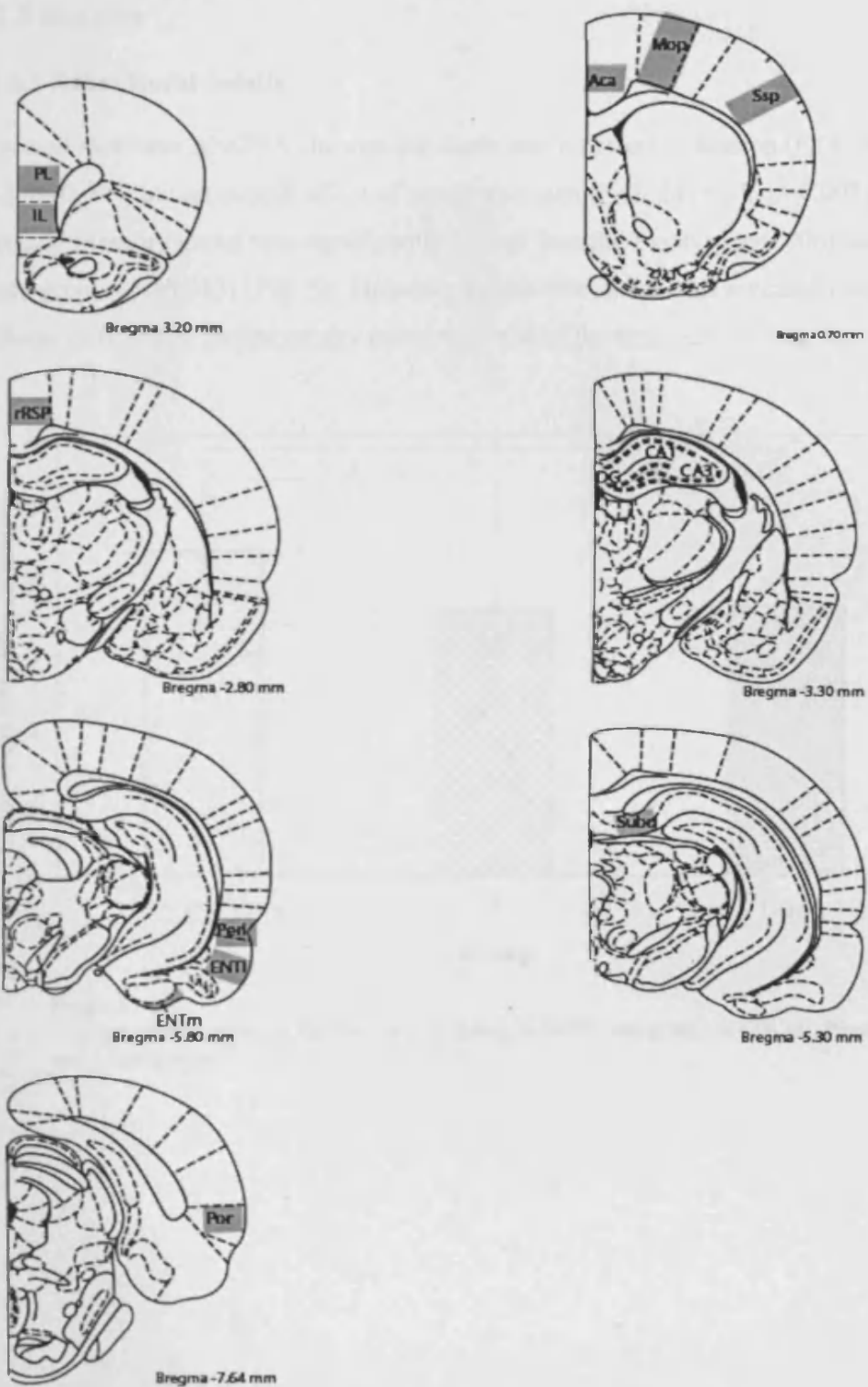
Figure 4
Diagrams showing coronal sections of regions of interest and distance from bregma (mm)
(Paxinos and Watson, 1998). (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior
cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial
cortex, DG-dentate gyrus, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-
medial entorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

2.2.2 Results

2.2.2.1 Behavioural results

Repeated measures ANOVA showed that there was no effect of session (F (4, 56) =0.2, p=0.944) but an overall effect of group was seen (F (2, 14) =8.8, p=0.003). The Working Memory group was significantly slower then the Passive (p=0.006) and the 1-arm groups (p=0.013) (Fig. 5). However simple effects analysis revealed no differences between groups on any individual trial of the final session (Fig. 6).
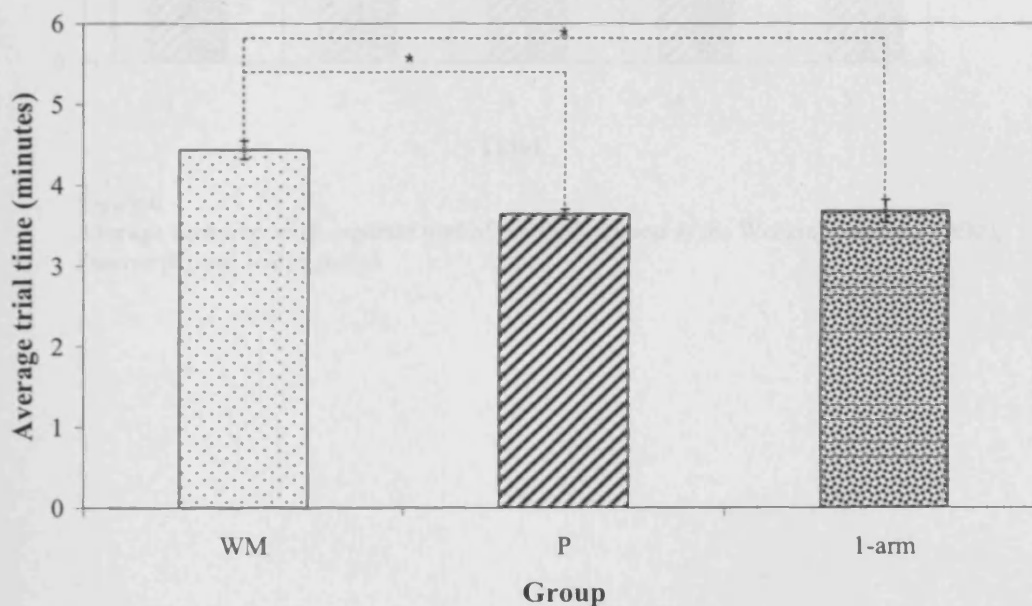
Figure 5
Average session times on the final day of testing in the Working Memory (WM), Passive (P) and 1-arm groups.

Passing time during measurement over five trials of the final session can be seen in the Working Memory, Passive control and 1-arm groups. These can be seen in Figure 6.
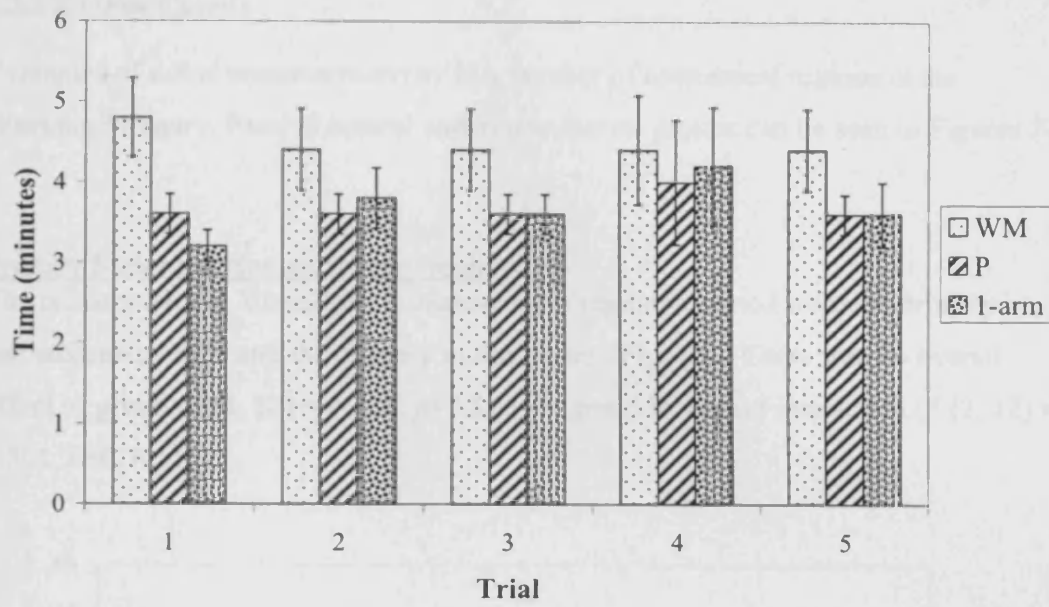
Figure 6
Average time over each separate trial of the final session in the Working Memory (WM),
Passive (P) and 1-arm groups

2.2.2.2 c-Fos Counts

Examples of c-Fos immunoreactivity in a number of anatomical regions in the
Working Memory, Passive control and 1-arm control groups can be seen in Figures 7-
9.

<u>Primary Motor and Somatosensory regions</u>
The primary Motor, Visual and Somatosensory regions counted were the primary
somatosensory area and the primary motor cortex (Fig. 10). There was no overall
effect of group (F $(2, 12) = 0.134$, $p=0.876$) or group by region interaction (F $(2, 12) =$
0.301, $p=0.745$).
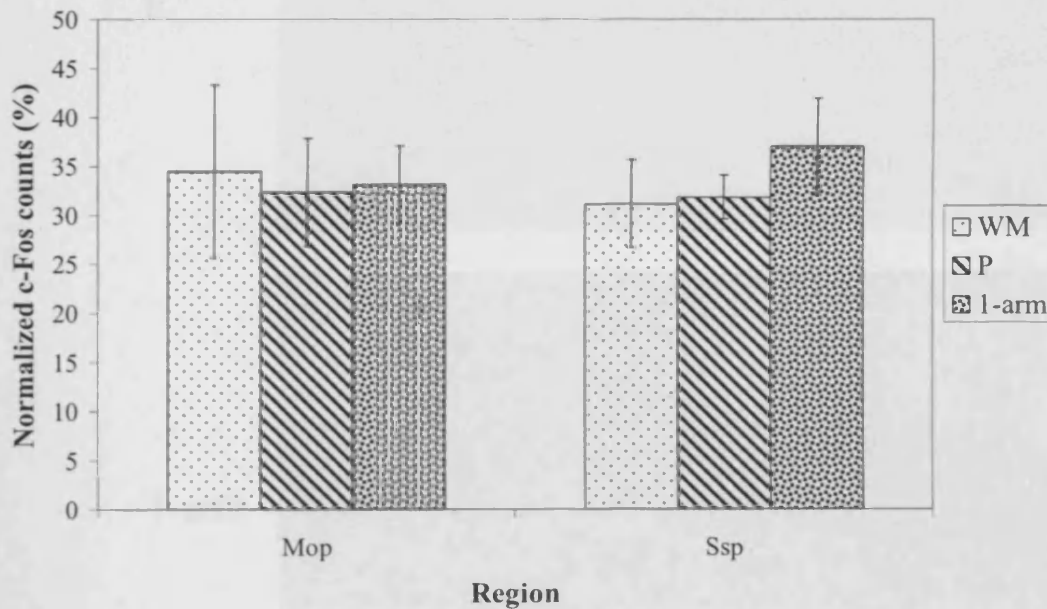
Figure 10
Normalized c-Fos counts in the primary motor (Mop) and somatosensory regions (Ssp)

WM
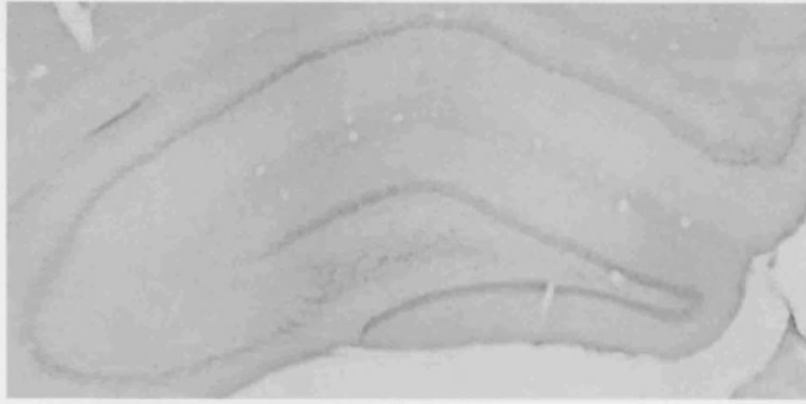
P

1-arm
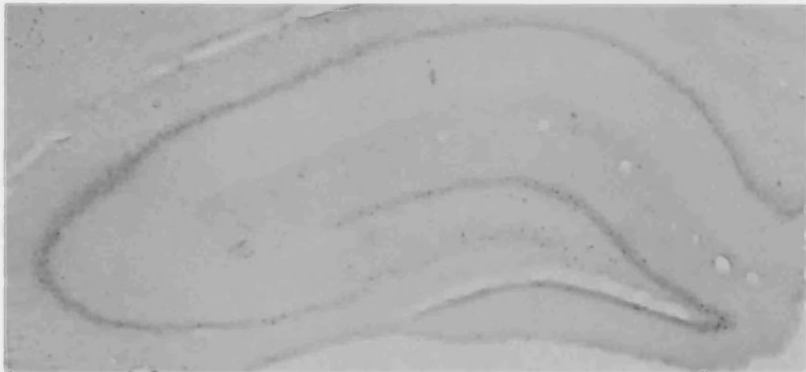
Figure 7
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Passive
Control (P) and 1-arm control (1-arm) groups in the hippocampus.

WM
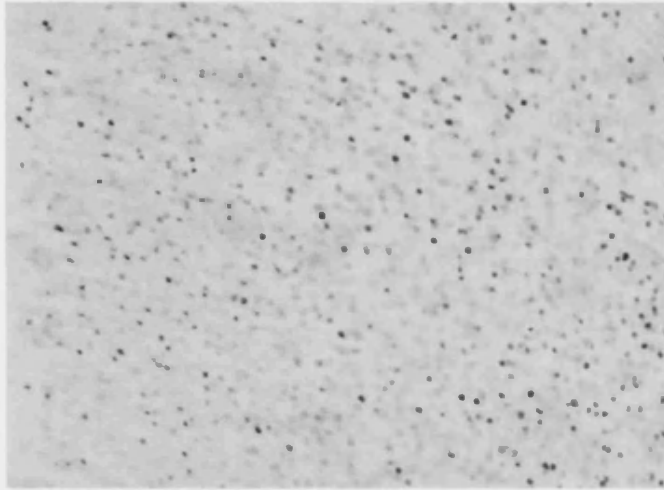
P
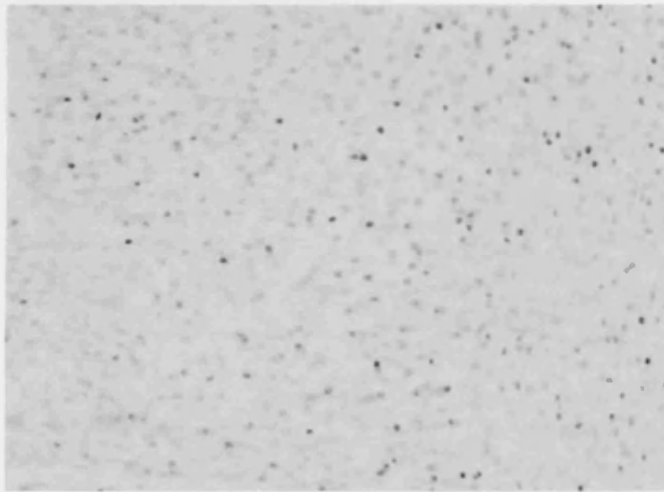
1-arm
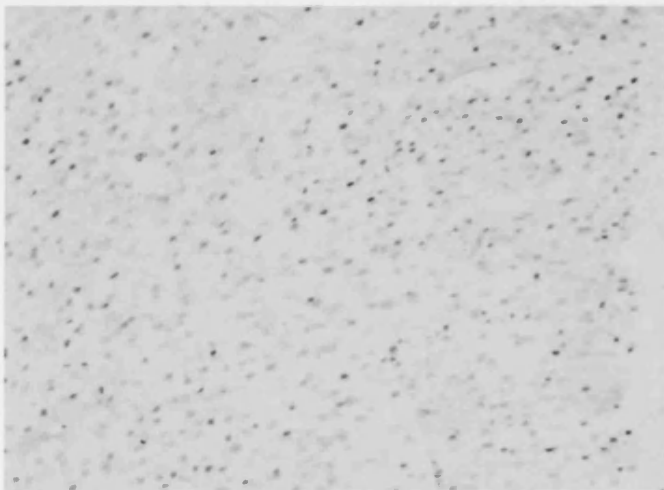
Figure 8
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Passive Control (P) and 1-arm control (1-arm) groups in the prelimbic cortex.

35

WM
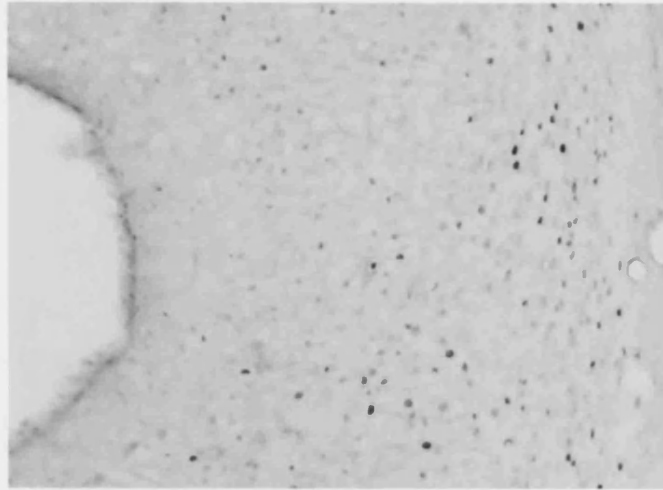
P
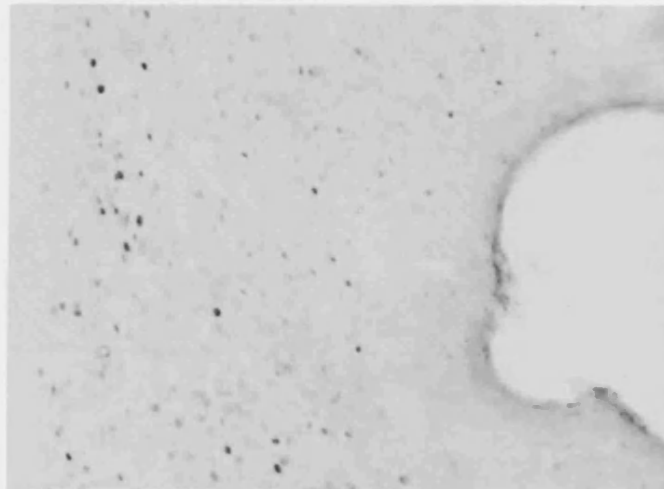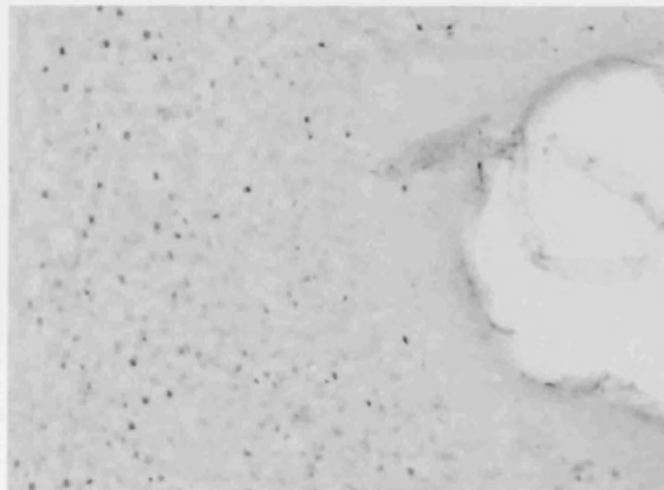
1-arm

Figure 9
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Passive Control
(P) and 1-arm control (1-arm) groups in the perirhinal cortex.

36

Prefrontal and Cingulate regions

The prefrontal and cingulate regions counted were the rostral retrosplenial cortex, anterior cingulate cortex and prelimbic and infralimbic cortices (Fig. 11). No differences were seen between any of the group ($F_{(2, 12)} = 0.976$, $p=0.405$) neither was a group by region interaction ($F_{(6, 36)} = 1.728$, $p=0.143$).
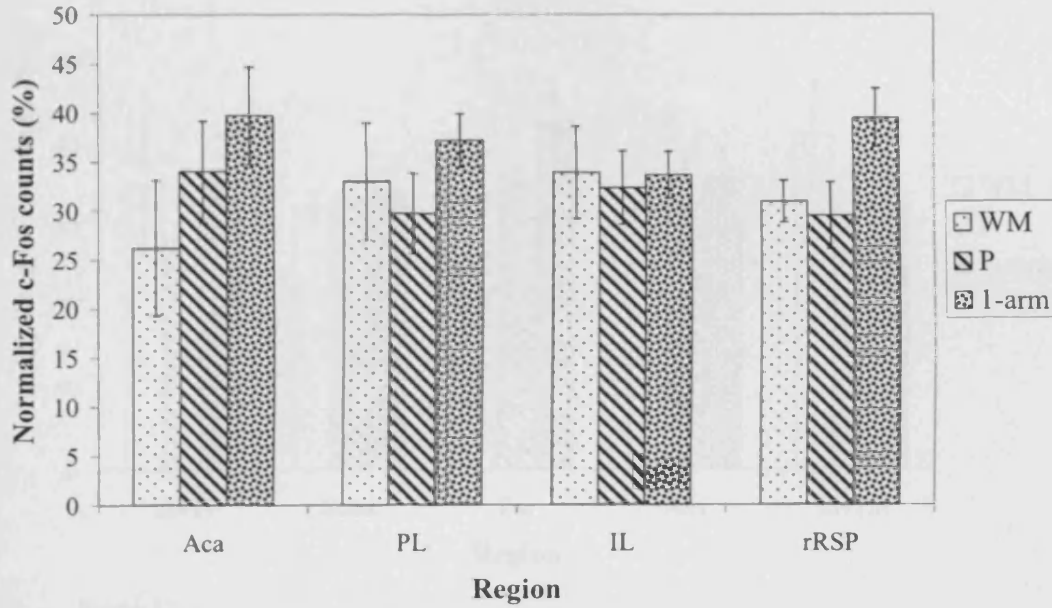
Figure 11
Normalized c-Fos counts in the prefrontal and cingulate regions (Aca-anterior cingulate cortex, PL-prelimbic cortex, IL-infralimbic cortex, rRSP-rostral reterosplenial cortex)

Parahippocampal cortices and adjacent regions

The parahippocampal cortices and adjacent regions counted were the medial and lateral entorhinal cortices, perirhinal cortex, postrhinal cortex and dorsal subiculum (Fig. 12). No overall effect of group was seen (F $(2, 12)$ =0.456, p=0.644) neither was a group by region interaction (F $(8, 36)$ =2.062, p=0.066).
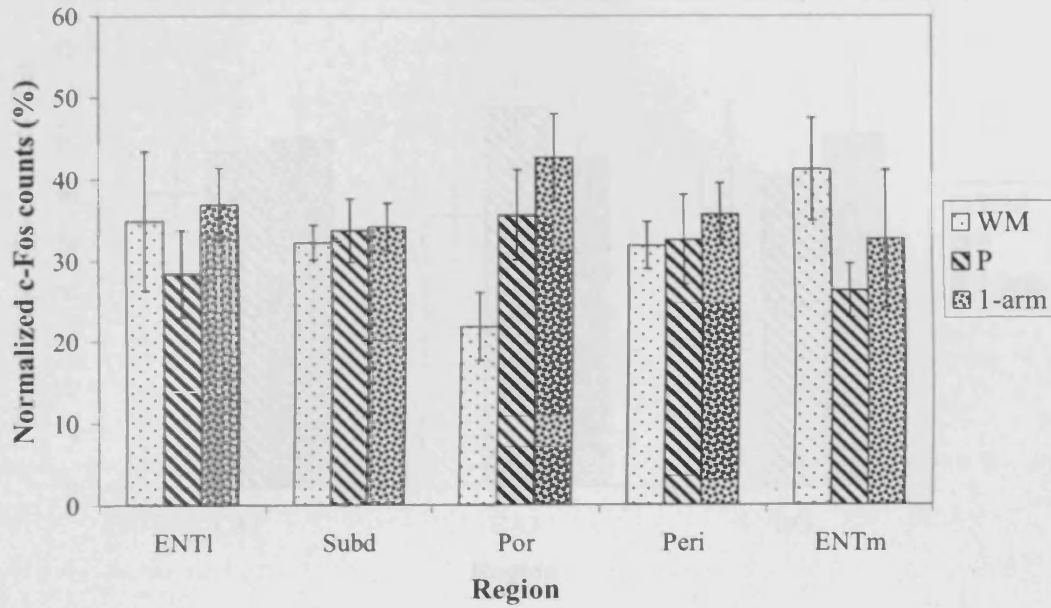
Figure 12
Normalized c-Fos counts in the parahippocampal regions (ENTl-lateral entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex, Peri-perirhinal cortex, ENTm-medial entorhinal cortex)

<u>Hippocampal subfields</u>
The hippocampal subfields counted were CA1, CA3 and dentate gyrus (Fig. 13). No overall effect of group was seen (F (2, 9) =0.547, p=0.597) nor was a group by region interaction (F (4, 42) =0.577, p=0.682).
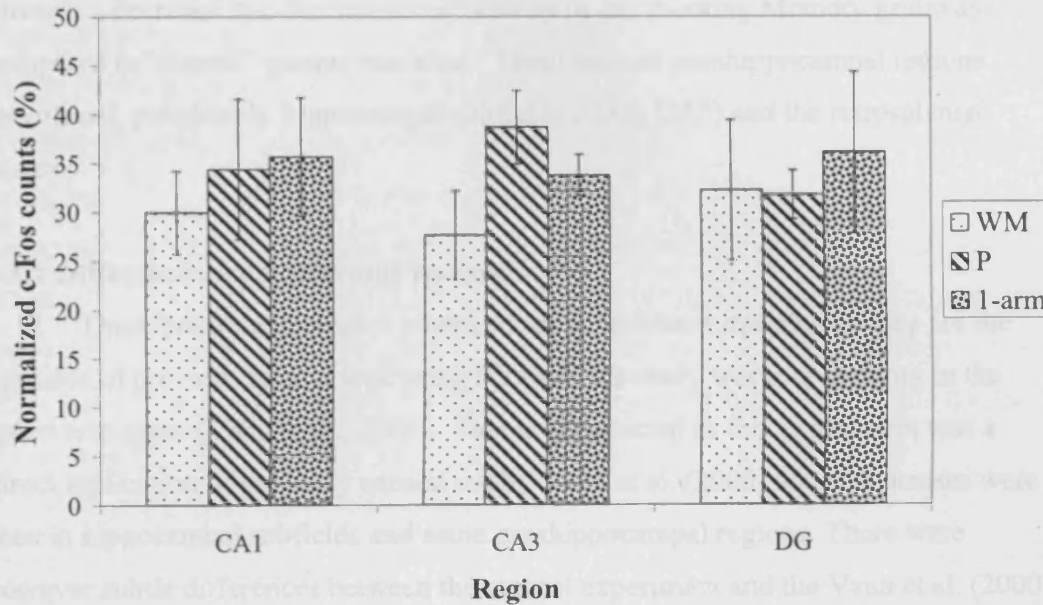
Figure 13
Normalized c-Fos counts in the hippocampal subfields (DG-dentate gyrus)

2.3 Discussion

This study looked at *c-fos* activation in a working memory paradigm comparing this with two different 'control' groups. No significant differences were seen in any brain region between the groups. However in a number of regions a trend towards a decrease in c-Fos immunoreactivity in the Working Memory group as compared to 'control' groups was seen. These include parahippocampal regions (perirhinal, postrhinal), hippocampal subfields (CA1, CA3) and the retrosplenial cortex.

2.3.1 Differences from previous research

These possible decreases produced are somewhat surprising as they are the opposite of previous results seen using IEG's in the study working memory in the radial arm maze (Vann et al., 2000). This is unexpected as this experiment was a direct replication of the study carried out by Vann et al. (2000) where increases were seen in hippocampal subfields and some parahippocampal regions. There were however subtle differences between the current experiment and the Vann et al. (2000) study which may account for the changes seen. In the Vann et al. (2000) experiment the different groups of animals were well matched on the amount of time spent in the maze. This was not the case in the current experiment where although no differences in amount of time spent in the maze between the groups were seen if each trial on the final day is compared individually overall the Working Memory group spent more time in the maze than the other groups. However this difference in the amount of time spent in the maze between the Working Memory and the Passive group could reflect a difference in responding and the differences in the demands of the task. This could suggest that animals in the Working Memory group are in fact taking longer because they are having to make a choice of which arm to enter. The other groups are faster because they have learnt to just enter an arm once the door has been raised, in other words have learnt a response strategy. In the Vann et al. (2000) study as in this current experiment animals were trained to criterion; however Vann et al. (2000) trained their animals until they were successfully able to perform 7 trials in the 30 minute training time whereas this experiment trained animals to perform only 5 trials.

2.3.2 Decreases seen in the Working Memory group

It could be argued that the possible decrease in the Working Memory group could in fact not be a decrease but reflect an increase in activation in the 'control' groups. The animals in the 'control' groups have no control over when they receive a reward unlike the Working Memory group and so could constantly be under stress. Stress stimuli have been shown to increase activation of immediate early genes using a number of paradigms (Senba, 1997). However, this seems unlikely as a very specific subset of regions were activated and a more global increase across the brain might be expected with stress stimulation. Another possibility is that the animals in the 'control' groups could some how been more alert because they never know when a reward is going to be received whereas the animals in the Working Memory group are less attentive as to a certain extent they know what stimuli predict a rewarded outcome. This is exemplified by the Pearce- Hall theory (1980) which states that more attention is paid to stimuli that have a surprising or unpredictable outcome.

There is some indication of this occurring in a study by Bertania and Destrade (1995). They studied c-fos mRNA expression within the hippocampus in acquisition and recognition testing in mice. Mice were split into two groups; trained and sham conditioned. Animals in the trained group had to lever press to receive food and then navigate around a partition to get to the food well. In the sham conditioned group the lever was not connected to the cage controls so pressing the lever had no effect. These animals were yoked with the trained group and were rewarded depending on their performance. Animals in the acquisition group were sacrificed 30, 90 and 180 minutes after testing whilst those in the recall group received 5 more trials and were killed 30, 90 and 180 minutes later.

As described previously (see 1.5) higher c-fos activation was generally seen in the experimental group as compared to the conditioned group. However, Bertania and Destrade (1995) also found higher c-fos mRNA in CA1, CA3 and dentate gyrus in their sham conditioned group as compared to their experimental group at acquisition at the 30 minute post test time interval. The authors stated that this may have occurred because of the stress of having no control over when a reward is received. However, this seems unlikely as this pattern was not seen in the other region tested (retrosplenial cortex). It was also stated that it could have occurred because c-Fos expression is repressed by the stimulation. It does however seem plausible that as

stated above the animals in the sham conditioned group may have been more alert to their surroundings because of the unpredictability of their reward.

2.3.3 Differences between rat strains

Another issue related to the differences seen between the current experiment and the Vann et al. (2000) study is the strain of rats used in each experiment. Vann et al. (2000) used the Dark Agouti strain whereas the current experiment used Sprague Dawley rats. There is some evidence to suggest that differences are seen between different rat strains on different behavioural tasks. Harker and Whishaw (2002) compared a number of different rat strains on tests of memory in the water maze. Animals were trained on both a reference (platform remained stable over trials and sessions) and working memory (platform remained stable over trials but moved session to session) task. The Long Evans rat strain performed in a superior manner to all other rat strains apart from wild rats with which they had comparable performance. All other strains including Sprague Dawley and Dark Agouti showed no difference on both tasks as compared to each other. This does suggest that, as far as testing in the water maze is concerned; there is no difference between the Dark Agouti and Sprague Dawley strains. There is however some evidence that Sprague Dawleys are slightly better at water maze tasks than other rat strains (not including Dark Agouti) (Andrews et al. , 1995; Andrews, 1996). Sprague Dawley rats trained to criterion to lever press for food were slower to acquire the task than other strains (Long Evans, Wistar) and slightly better than S3 rats (Andrews et al., 1995). Although there is evidence to suggest that differences between strains are seen on behavioural tasks it seems improbable that strain differences would lead to a completely opposite pattern of results.

2.3.4 Problems with current experiment

The most likely explanation for the changes seen in the current experiment is the low number of subjects that were used. This experiment had a low number of subjects due to operational difficulties. The strain of rats used, Sprague Dawley, although commonly used in behavioural experimentation had difficulty in performing the task. They seemed unmotivated to perform for a reward so although they did eventually learn the task it was a long and arduous process. This could have been because of the strains poor visual acuity, common in albino strains. Prusky et al.

(2002) looked at the performance of different rat strains on a forced choice visual discrimination task in a water tank. Animals were trained to distinguish a sine wave pattern cue from a plain grey cue. Incremental changes in the spatial frequency of the sine wave gratings were made until animals performance dropped below 70%. Albino rats (Fisher 344, Sprague Dawley, Wistar) showed the poorest visual acuity as compared to pigmented strains (Dark Agouti, Fisher Norway, Long Evans, Wild). If the rats could not see the extramaze cues it may have made them unmotivated to perform the task. This is a possibility as rats seemed to habituate to the maze as normal and were readily eating the reward pellets when no choice was needed to be made based on the use of visual cues. There is however no evidence that these animals used any other method to solve the task other than using the visual cues (e.g. sequential arm choices); which may have occurred if the visual cues were not as salient.

Another possibility is that the task was too difficult for the animals so they gave up. This is not the case because this lack of motivation was seen in not just the Working Memory group but in the other groups where no learning and remembering was required. It seems likely that the subjects were unmotivated for some unknown reason which made this experiment impossible to complete to a satisfactory level. This is somewhat unusual as many studies have used Sprague Dawley animals in such a task.

This evidence taken together suggests that Sprague Dawley rats maybe better equipped for aversive rather than appetitive tasks. It may also be of use to employ more distinct visual cues when testing Sprague Dawley rats on spatial tasks.

Chapter three

Induction of Zif268 and c-Fos in a working memory task in the water maze

3.1 Introduction

As discussed earlier there is a large body of lesion evidence that implicates a number of interlinked brain areas in spatial working memory and these lesion studies have been complemented with immediate early gene findings. Another arena in which to test spatial working memory is the water maze, and again numerous findings appear to demonstrate a large network of brain regions that are required to perform the task. The water maze allows the animal to locate the platform using allocentric cues just like the radial arm maze but it removes the rats ability to learn the task using other strategies (see 2.1.2). For example, in the radial arm maze it is possible to learn the task by a response strategy (e.g. always making a sequential arm choice) which is not possible in the water maze. Also as problems were encountered studying working memory in the radial arm maze, in that the animals were difficult to train and lacked motivation to find the food rewards (2.3.4), using an aversive task will remove these difficulties whilst controlling the way the animals are able to solve the task .

3.1.1 Working Memory in the Water Maze

In the standard water maze working memory paradigm the submerged hidden platform is kept fixed across trials in a session but moves from session to session. This means that the platform location on the first trial of a session is in a novel spatial location and the animal has to maintain this information to be able to locate the platform on subsequent trials. However the animal then must forget the location as it will differ in further sessions. A learning curve should be seen over a session with escape latencies getting progressively faster as the animal finds it easier to orient to the platforms location. In all studies discussed below this is the standard testing procedure only the number of trials per session and number of sessions differ.

3.1.2 Hippocampal lesions and working memory in the water maze

Lesions of the hippocampus have been shown to impair spatial learning (O'keefe and Nadel, 1978) and more specifically, as will be discussed below, working memory in the water maze. Morris et al. (1982) studied such a task where hippocampal lesioned and sham operated rats had 5 days of training to find a submerged platform in a fixed position on a given day with 4 trials a day on the first two days of training, 8 trials on the two subsequent days and then 4 trials a day on the final day. Hippocampally lesioned rats were shown to be significantly impaired as compared to sham operated animals. Cassel et al. (1998) also looked at the effect of complete hippocampal lesions on working memory in the water maze. There was a significant group effect with hippocampal lesioned rats having slower escape latencies than the sham rats. Both groups showed a decrease in latencies over trials.

It has been demonstrated that this deficit seen with hippocampal lesions is restricted to the dorsal hippocampus and that lesions of the ventral hippocampus produce no such effect. Bannerman et al. (2002) studied the effect of dorsal, ventral and complete hippocampal lesions on working memory in the water maze. Animals with complete and dorsal lesions showed a deficit in this task by increased escape latencies with animals with ventral and sham lesions performing the task adequately. This again indicates that the dorsal but not the ventral hippocampus is required for spatial working memory.

Pouzet et al. (2002) also looked at the effect of dorsal hippocampal lesions on working memory in the water maze. Sham rats reduced their latencies to find the platform over days where as the hippocampally lesioned animals did not. Analysing the days where the platform was located in the central part of the maze and those where it was located nearer the edge a differing pattern of results were seen. When the platform was centrally located lesioned animals were impaired in locating the platform. However when the platform was located near the periphery no such deficits were seen. This was because the animals used a looping strategy to find the platform in both cases but it only benefited the animals whilst the platform was in less central position. This shows that animals with hippocampal lesions are able to learn a working memory task in the water maze to a certain extent using non spatial strategies.

Lesions of the dorsal hippocampus can lead to both working memory and reference memory deficits (Galani et al., 1998; Cassel et al., 1998). When this occurs

it is difficult to elucidate the differences between the two tasks particular if using the water maze and the conclusion could be drawn that the animals are not both impaired at working and reference memory but may have a more general spatial learning deficit. In the radial arm it is feasible to separate working memory and reference memory errors so it is achievable to make sure that both reference and working memory are being tested. This is somewhat harder in the water maze if standard working and reference memory procedures are used. However if a delay is placed after the sample phase (i.e. the first trial) the working memory load is increased and it is easier to be sure that that is what is being tested. Also testing animals on other spatial cognition paradigms could indicate whether a general spatial learning deficit is present or whether animals are impaired simply on both working and reference memory.

3.1.3 Other lesion studies and working memory in the water maze

Other parahippocampal and cingulate regions have also been implicated in working memory tasks in the water maze. Galani et al. (1998) examined the effect of lesions of the hippocampus, entorhinal cortex and the subiculum. A significant effect of trial was seen with animals in the sham group having faster escape latencies than those in the lesion groups with no difference between the lesion groups. Only the sham group showed a significant trial by trial improvement. The authors suggest that these structures may interact in spatial learning that requires flexibility like working memory. Vann et al. (2003) tested animals with caudal retrosplenial lesions for 14 days with 4 trials a day. A borderline group effect was seen with animals in the lesion group taking longer to locate the platform on trial 2. As no differences were seen between the further trials it suggests that with repeated training removes any deficits seen with caudal retrosplenial lesions.

Deficits have also been seen with animals with entorhinal cortex lesions. Nagahara et al. (1995) tested animals with entorhinal lesions with some perirhinal damage on a single trial procedure. Animals had an acquisition trial to find the platform and then after a delay of either 30 seconds or 5 minutes the animals were reintroduced to the pool and had to relocate the platform. No difference between the groups was seen at the 30 second delay but at the 5 minutes delay lesioned animals were impaired at locating the platform.

46

Lesions of the lateral mamillary nuclei have been shown to impair working memory in the water maze. Vann (2005) found that lesioned animals were significantly impaired compared to controls but their performance improved over trials. This deficit could have occurred because of the presence of head direction cells in this area and their loss led to the deficits seen in spatial learning. However this would only be true if animals relied on head direction cells at the beginning of acquisition.

As in other working memory paradigms there is a certain amount of conflict as regards the role of the perirhinal cortex in working memory in the water maze. Liu and Bilkey (2001) studied the effect of hippocampal and perirhinal lesions. Each session consisted of 2 trials a sample phase and a test phase ("single sample trials"). They also tested animal for two days with 4 trials ("repeated sample trials"). In both cases the animals received a delay of either 30 seconds or 180 seconds between the sample and test phase. For the single sample trials the hippocampal lesioned rats were significantly impaired at both delays on the test phase. The perirhinal lesioned animals performed well at the 30 second delay but were impaired as compared to sham at the 180 second delay. For the repeated sample trials the hippocampal lesioned animals were impaired on the sample phase as compared to the other groups. This suggests a non-spatial deficit produced by these lesions and the authors state that this may have occurred because of damage to the overlying cortex. No differences were seen in the test phase. The delay dependent deficits seen in the perirhinal cortex led the authors to suggest that the perirhinal cortex may play role in a spatial paradigm whilst the test or environment is novel. However, Machin et al. (2002) tested rats with perirhinal lesions for 12 days with 4 trials a day with further testing on days 13-16 with an increase in the delay between trial 1 and 2 to 30 minutes. For both the short and long delay no significant effect of group was seen showing that lesions of the perirhinal cortex do not impair working memory in the water maze.

Deficits have also been seen with lesions of the postrhinal cortex. Liu and Bilkey (2002) tested animals with postrhinal lesions for 4 days with 2 sessions a day with each session consisting of a sample phase and a test phase. Two different delays between the sample and test phase were used 30 seconds and 300 seconds. No difference between groups was seen on the sample phase. During the test phase escape latencies in the lesion group were significantly longer than the sham animals at both time delays.

3.1.4 IEG's and water maze paradigms

These lesion studies have been complemented to a certain extent by studies of immediate early gene activation studies. Guzowski et al. (2001) found higher *zif268*, *c-fos* and *arc* activation in the dorsal hippocampus of rats performing a standard working memory task in the water maze as compared to cage control animals. Guzowski et al. (2001) also looked at the correlation between behavioural performance and *arc* activation. They found that there was a negative correlation between the amount of activation and the mean escape latencies. In other words, the best learners had the most *arc*. This is in conflict with results found by Kelly and Deadwyler (2003) using a free lever pressing for food task (see 3.3.3.3 for further discussion).

Another study which looked at working memory in the water maze (Santin et al., 2003) found higher *c-fos* activation in the medial mamillary nucleus of the mamillary bodies again with a standard working memory task. The rats had to locate a submerged platform and were given 2 trials a day; an acquisition and a retention trial. As a control group comparison animals were allowed to freely swim for 30 seconds each day without a platform being present.

Teather et al. (2005) used both types of control groups as described in the above studies, cage controls and free swimming animals. The animals were trained in one session to a criterion on a spatial (fixed submerged platform) and cued (visible moving platform) task in the water maze. The yoked free swimming animals swam without a platform present for the same amount of time as an animal in the experimental group. Increased c-Fos immunoreactivity was seen in all hippocampal subfields in all groups that experienced the water maze as compared to cage controls and that activation was higher in the spatial than the cued task in CA1. c-Jun activation was also increased in CA3 in groups that had experienced the water maze as compared to naive controls and that higher activation was seen in CA1 and CA3 in spatial animals compared to cued animals.

Jenkins et al. (2003) looked at c-Fos activation comparing a landmark and place task in the water maze. Rats had to locate a hidden platform which in the place task was in the same location trial to trial but moved session to session (i.e. working memory), and in the landmark task moved trial to trial but was always indicated by a beacon. Higher c-Fos activation was seen in the landmark group in many different areas including the hippocampal subfields, the retrosplenial and anterior cingulate

cortices, and the visual and motor areas. It was argued that these results occurred because the landmark group visited eight spatial locations in one session whereas the place group only visited one.

3.1.5 'Knockout' of IEG's in water maze paradigms

Work has also been carried out using methods of 'knocking-out' IEG activation and looking at its effects on learning and memory.

One such method is antisense and it has been used to study the effect of c-fos loss on a water maze task (Guzowski, 2002). Rats were injected into the dorsal hippocampus either with c-fos AS-ODN or a controlled scrambled ODN. The first training session consisted of six trials where rats had to find a submerged platform; the second session was carried out two hours after the first and also consisted of six trials. Forty eight hours later retention was tested, with platforms in the training position. No difference was found between the two groups in the two training sessions but the c-fos AS-ODN rats were impaired during retention compared to the scrambled controls.

However, Zhang, McQuade et al. (2002) have shown that c-fos activation within the hippocampus and the dentate gyrus was not necessary for learning and memory in the water maze. They produced c-fos mutant mice with a loss of 97% within the CA1, CA2 and CA3 and reduced by 70% within the dentate gyrus compared to wild type controls (Zhang, Zhang et al., 2002) but with no change in any other region. Water maze testing consisted of three phases – the spatial learning phase, the random platform phase and the visible platform phase. In the spatial learning phase mice had to find a hidden platform placed in the south east quadrant for four trials a day. In the probe trials the platform was removed and the number of platform site crossings was recorded. The random platform phase consisted of two days of trials. On day one mice had two trials with the platform in the south east quadrant and two with it in the north east and north west quadrants. On the second day mice had one trial with the platform in the original location and three more with the platform located in the north west, south west and north east quadrants. The visible platform phase was identical to the hidden platform phase but with a curtain around the maze so only proximal cues were available. Both groups of mice were able to perform the hidden and random platform tasks, with escape latencies being similar in both groups. This finding also occurred in the visible platform phase which

does not require the hippocampus to be performed. This could have occurred because the animals had this decrease in *c-fos* in the hippocampus from birth and a compensatory mechanism may have taken place. Although spatial learning in the water maze has been shown to be hippocampally dependent other brain regions have also been implicated. Testing the transgenic animals on a very specific hippocampally dependent task may therefore produce deficits.

Jones et al. (2001) utilized *zif268* mutant mice to investigate the effect of the absence of the gene on a massed training protocol in the water maze a task for which the hippocampus is required (Holscher, 2003). Mice had to find a submerged platform in a fixed position. Latencies reduced with training for all groups but *zif268+/-* and *zif268 -/-* mice took significantly longer to find the platform than *zif268* +/+ mice. A probe trial was performed 48 hours after training with *zif268+/+* mice showing a significant preference for the training quadrant with *zif268* +/- and *zif268 -/-* mice showing no preference. This indicates that although knocking out *zif268* does not completely abolish the ability to perform the water maze task it does inhibit it and also removes the ability to retain information about the task with a delay.

3.1.6 Problems with previous IEG research in the water maze

Previous research using IEG's to study learning in the water maze has a certain number of pitfalls that often make interpretation of the results somewhat ambiguous. This is principally due to comparisons of the experimental groups with inadequate control groups. One of the most common controls is a cage control, but it is impossible to be aware of what the animal is exposed to and their experience is very unlike that of the experimental group. The cage control itself is of use, however, as a base line measure alongside other better controls. However because of the baseline nature of the cage control often any comparison with an experimental group will produce higher activation in that group. This higher activation can not be attributed to any learning experience because the groups differ on many other factors and up regulation of the IEG could be ascribed to any of these.

Animals that perform a free swimming task have also been used as a control. This is problematic as forced swimming with no escape platform is regarded as a stressor which has been shown to increase *c-fos* expression in many brain areas (Cullinan et al., 1995). Evidence has shown that animals that swim in a water maze with no platform present have higher levels of the stress hormone corticosterone than

baseline controls and animals that do have a platform present (Kavushansky et al., 2006). Also because no platform is present even though the animals do experience the water maze their experience is not the same as the experimental group. They do not sit on the platform for anytime so they do not experience a controlled spatial position.

A different strategy is to compare activation between two different tasks. This was used by Jenkins et al. (2003) as they compared a working memory with a reference memory task. This is problematic because, as was seen in this experiment, different patterns of activation were seen in each task that could be attributed to the difference in the way the animals were trained. For example, global increases including 'control' regions where no changes would be expected, were seen in the reference memory group which the authors attributed it to this set of animals having had experienced eight platform positions on a given day with the comparable working memory group having experienced one.

The goal of the next series of experiments is to try and rectify the above faults. A novel 'control' group was used that allowed animals to be time matched without the confound of them having to perform another learning task. The control group and experimental group will not only be matched on the amount of time that is spent in the water maze, they will also both locate a platform that is closely matched on spatial position. The control group will have all these features but unlike the experimental group will display no learning across trials in a session.

The 'control' or Procedural Task group will be encouraged to perform a response strategy by keeping the platform a fixed distance from the water maze walls and moving it from trial to trial. Animals will be released from the centre of the maze and will learn to adopt thigamotaxic swimming to locate the platform. IEG activation in these animals will be compared with animals performing a standard working memory task. From previous results (Vann et al., 2000; Jones et al., 2001) it is expected that higher activation will be seen in the hippocampal subfields and connected cortical areas in the Working Memory group as compared to the Procedural Task group.

3.2 Experiment 2

3.2.1 Method

Subjects

Subjects were 16 male Dark Agouti rats (Harlan, UK) weighing between 260g and 300g at end of testing. Rats were housed in pairs and kept on a 14/10 dark/light cycle. Food and water was available *ad libitum* through out the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Apparatus

The water maze used was made of white fibreglass 1m in depth and 2m in diameter and mounted on a platform 0.6m from the floor. The maze was filled to a depth of 60cm with water and 1L of opacifier (Roehm and Haas, Dewsbury, UK). Water temperature was maintained between 22 and 24°C. The pool was located in the centre of a square room 3m x 4m. Three of the walls were painted white with extra maze cues in the form of posters present; the fourth wall contained a door covered in stainless steel. This door was central to the wall and was due north of the water maze. A 1.4m high blue opaque curtain was suspended from the ceiling and runners allowed it to be drawn around the pool 25cm below it's edge. When the curtain was not required it could be drawn into the north east corner of the room. The animal's movements were recorded using a closed circuit video camera mounted into a false ceiling 1.75m above the pool. Images were recorded on a video camera connected to a computer (PC-6266 Professional II, RM) in an adjacent room. The platform was made of clear Perspex and was 10 cm in diameter. The top of the platform was always 3cm below the surface of the water. A stick was used to indicate platform location in some conditions and this was attached 0.5cm from the edge of the platform. The stick protruded 23cm above the water and was grey in colour; it had a thick disc on the top which was 2.5cm thick and 3 cm in diameter. Another irrelevant stick cue was also used and was identical to the one attached to the platform but it was free standing in the pool on a heavy metal base. The pool was illuminated by eight 45w spotlights mounted in the ceiling above the arena and the cues were lit by halogen lights mounted in the corners of the room. The rat's movement was tracked using water maze software on the computer (Morris and Spooner, 1990).

Procedure

Animals were split into 2 groups; group Working Memory and group Procedural Task. All animals performed 8 consecutive trials which lasted no more than 90 seconds each with an inter trial interval of 30 seconds in each session for 12 sessions. Upon reaching the platform animals remained on it for 10 seconds. Animals were placed into a quiet and dark room for twenty minutes before each session began and for an hour and half afterwards. This was to cut down on any extraneous stimulation the animals may have received. When animals were moved from room to room they were placed in a covered carry box measuring 28x45x21cm to maintain the dark conditions.

Pretraining

Animals were pretrained for 2 days before the experiment began. The curtain was drawn around the maze to block all extra maze cues and the platform position was changed for each trial on that day. Each group performed 8 pretraining trials a day. For the Working Memory group the platform was placed in random positions throughout the pool. For the Procedural Task group the platform was placed around the edge of the pool. On the first pre training day if the animals had not found the platform in 120 seconds they were guided to it and allowed to remain on it for 10 seconds. On the second pretraining day the animals timed out after 90 seconds.

Working Memory group

For the Working Memory group the platform position remained constant from trial to trial but moved session to session (Fig. 14). The platform could be placed in a possible 12 different positions in the maze; at different compass points both near the periphery of the pool and nearer the centre. These positions were presented in a pseudorandom order. Animals were released from the edge of the pool and the start points differed on each trial. From day 8 of training onwards a stick was placed on the platform for the first trial of each session to help animals find the platform more easily. Latencies to find the platform were recorded as were the length of the swim paths. On the final test day (day 12) the Working Memory group performed the task as normal.
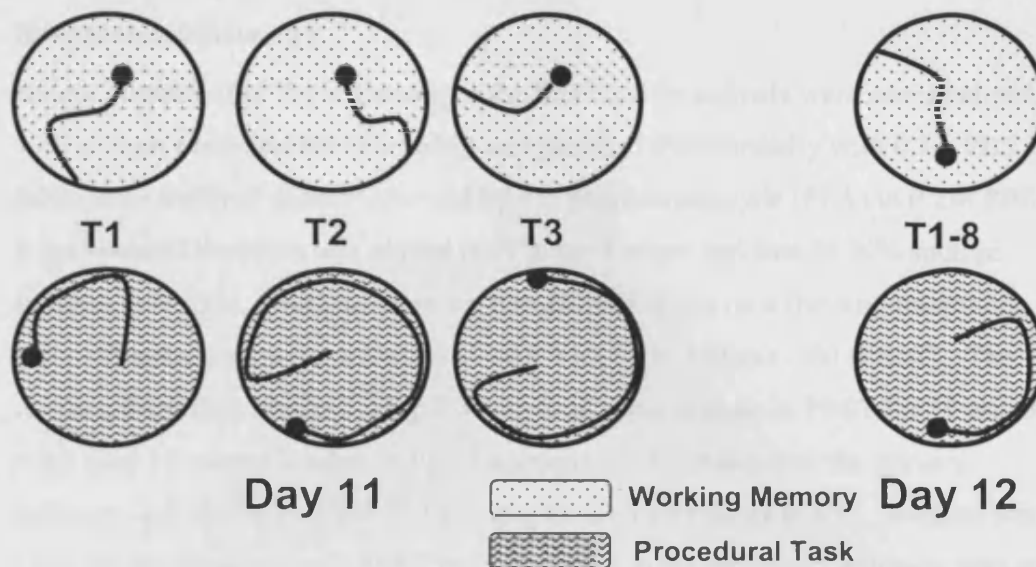
Figure 14
Figure to show a representation of swim paths for group Working Memory and group
Procedural Task on a training day (day 11) and the final test day (day 12).

Procedural Task group

For the Procedural Task group the platform position was always around the perimeter
of the water maze to encourage thigamotaxic behaviour (Fig. 14). The platform could
be in a possible 8 different positions. As the platform position altered trial to trial it
was in all of the positions on any given day. The animals were released from the
centre of the maze. From day 8 onwards a stick was introduced into the pool for the
first trial of each session as in the Working Memory group. The stick was placed
away from the periphery of the pool and the platform and had no significance to the
animals. On the final test day the platform position remained in the same place on
each trial and was in the equivalent position as for the Working Memory group. The
decision to stop moving the platform on the final test was not only based on the desire
to equate platform position with the Working Memory group but also because of the
results found by Jenkins et al. (2003). Jenkins et al. (2003) compared a working
memory group (group place) with a reference memory group (group landmark) in
which the platform moved every trial (including the final test day) and was demarked
with a landmark. They found higher activation in group landmark as compared to
group place and equated this to the fact the platform was moving on every trial in one
group and not in the other.

Post hoc, animals were paired on total time spent in the pool and processed for
immunohistochemistry together.

54

Immunohistochemistry

Ninety minutes after the beginning of the final session animals were anaesthetized with sodium pentobarbital (60mg/kg) and perfused transcardially with 0.1m PBS (phosphate buffered saline) followed by 4% paraformadehyde (PFA) in 0.1M PBS. After removal the brain was placed in PFA for 4 hours and then in 30% sucrose solution overnight. Sections were cut coronally at 40μm on a freezing microtome and one in four sections were collected in PBS with 0.2% TritonX-100 (PBST). The sections were then blocked using 0.3% hydrogen peroxidase in PBST for 10 minutes. After four 10 minute washes in PBST sections were incubated in the primary antibody, anti *zif268* (1:3000, C-19, Santa Cruz) for 48 hours at 4°C. Sections were again washed four times in PBST then incubated in the secondary antibody with goat serum (1:2000 in PBST, vector laboratories) for two hours at room temperature. After washing with PBST, sections were incubated in avidin-biotinylated horseradish peroxidase complex diluted in PBST (Elite kit, Vector laboratories) for one hour at room temperature. Sections were washed again in PBST and then in 0.05M tris buffer. The sections were then stained using diaminobenzidine (DAB kit, Vector laboratories). The reaction was stopped by washing in cold PBS. Sections were then mounted on gelatine-covered slides, dehydrated using a series of increasing alcohol concentrations and then cover slipped. A separate one in four series of sections was directly mounted after cutting and stained using cresyl violet for histological purposes.

Image Analysis

Sections were scanned onto a PC (IBM) computer using a Leica DMRB microscope and an Olympus DP70 camera. Counts of the stained nuclei were carried out using the program analySIS (Soft Imaging Systems). The threshold was set at the same level for all sections and counts were made in a frame area of 0.84 x 0.63 mm using a 10x magnification. For hippocampal counts the entire area of interest was captured in different frames using a 5x magnification and montaged together. Counts were taken from 3 consecutive sections from both hemispheres for all regions of interest (Fig.13).

Counts were normalized to reduce variation between animals produced by differences in immunohistochemical staining. For this, mean counts for each animal at a given site were divided by the overall mean for both animals in the pair. This produced a percentage ratio with each pair of animals adding up to 100 (Fig. 15).

These normalized counts were analysed using a 2 factor analysis of variance (group and region). Areas were grouped as shown below to reduce type one errors. One pair of animals was removed from the analysis due to poor immunohistochemistry. Tables of raw cells counts can be seen in Appendix A Figure 2.

WM:
$$\frac{\text{Number of cells in WM subject}}{\text{Number of cells in WM subject} + \text{Number of cells in PT subject}}$$

PT:
$$\frac{\text{Number of cells in PT subject}}{\text{Number of cells in WM subject} + \text{Number of cells in PT subject}}$$

Figure 15
Equations for normalization procedure for Working Memory and Procedural Task groups

Regions of interest

Regions were chosen as described previously (2.2.1). However counts were also taken from the barrel fields. This area was chosen because the barrel fields become activated when the vibrasse are stimulated (Filipkowski, 2000). This could be of importance because the animals in the Procedural Task group swim very close to the edge of the maze and the vibrasse sometimes do brush against the walls (Fig. 16).
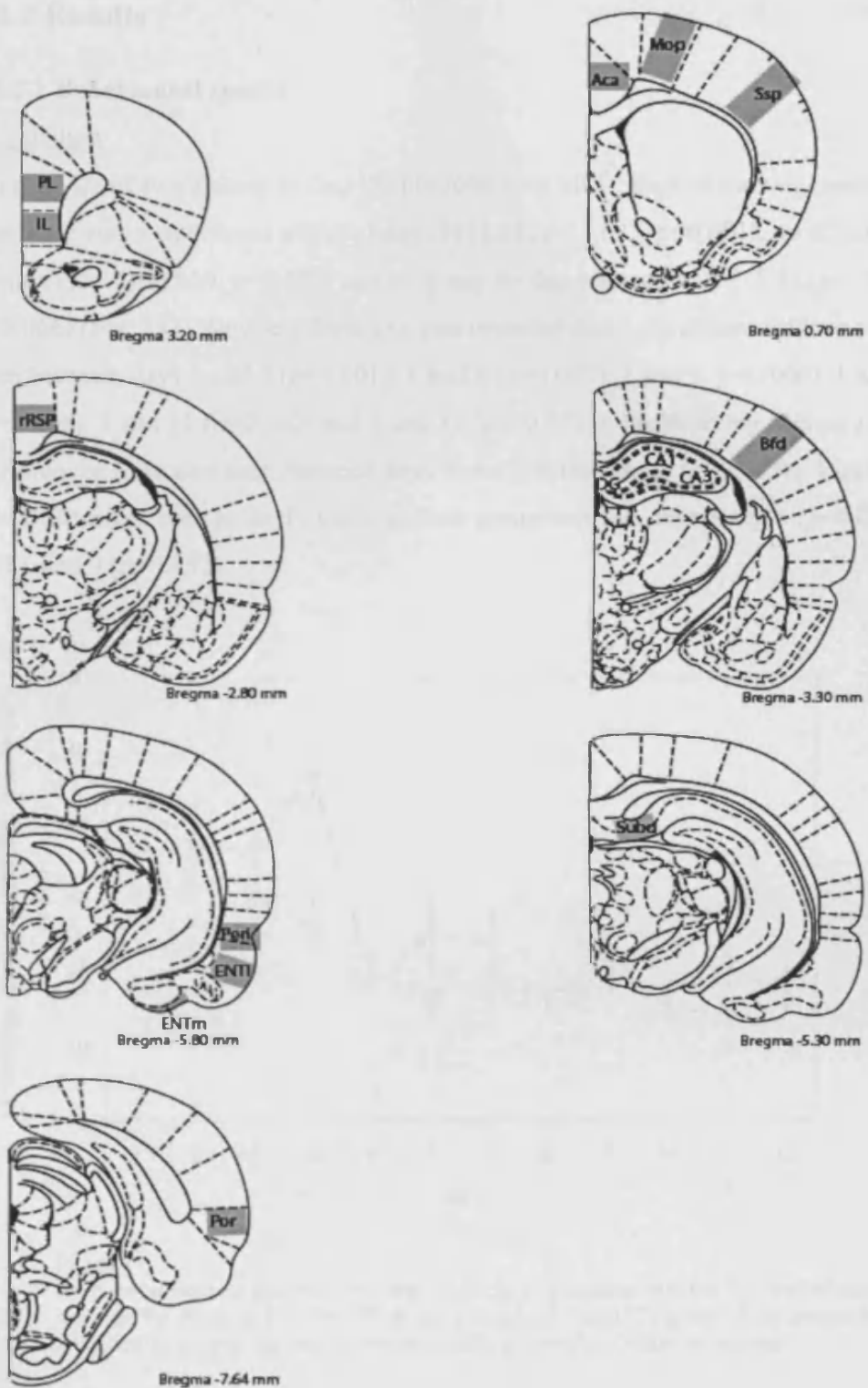
Figure 16
Diagrams showing coronal sections of regions of interest and distance from bregma (mm)
(Paxinos and Watson, 1998). (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior
cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial
cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal
cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.2.2 Results

3.2.2.1 Behavioural results

Acquisition

An analysis of swim times to find the platform over all 12 days of training revealed that there was a significant effect of day $(F(11,132)=11.625, p<0.001)$, no effect of group $(F(1,12)=0.659, p=0.433)$ and no group by day interaction $(F(11,132)=1.769, p=0.066)$ (Fig. 17). Simple effects analysis revealed that a significant difference was seen between days 1 and 5 $(p=0.001)$, 1 and 8 $(p=0.003)$, 1 and 9 $(p=0.006)$, 1 and 10 $(p=0.005)$, 1 and 11 $(p=0.002)$ and 1 and 12 $(p=0.037)$ in the Working Memory group. Differences were also seen between days 4 and 5 in this group $(p=0.012)$. There were also differences seen in the Procedural Task group between days 1 and 5 $(p=0.028)$ and 1 and 9 $(p=0.032)$.
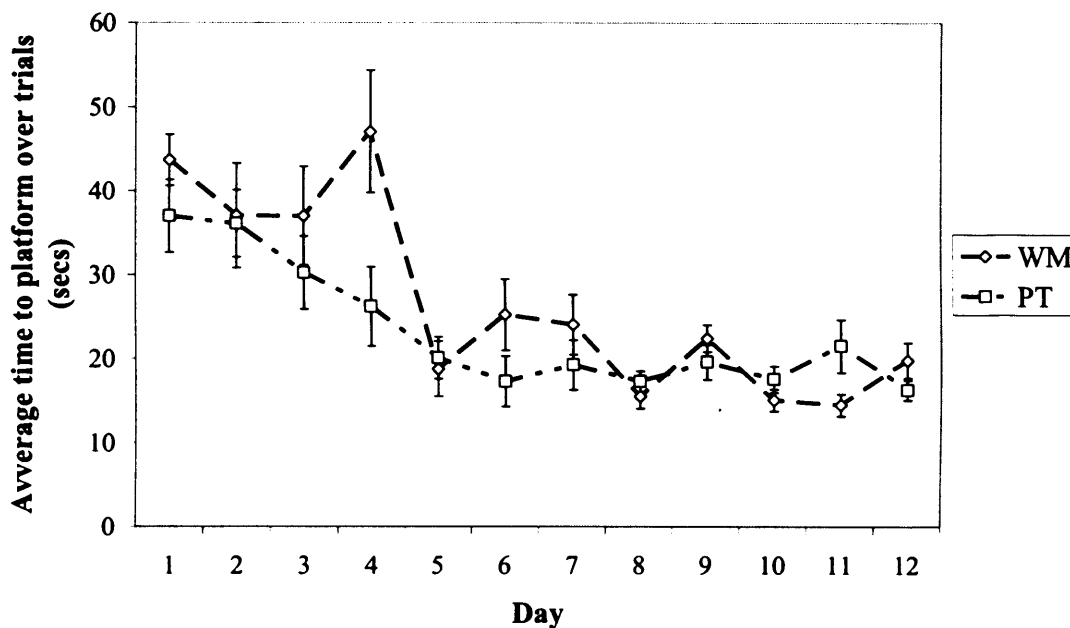
Figure 17
Average latencies to find platform over all 12 days of training with the first trial of each day removed for Working Memory (WM) and Procedural Task (PT) group. Both groups display a reduction in the average time to find the platform over the 12 days of training.

Final session

On the final day of testing, half the animals performed a standard version of the working memory task in the water maze, whilst the others performed a procedural task. A one way ANOVA collapsed over trials on the final day showed there was no

effect of group (F (1, 12) =0.1, p=0.991) (Fig. 18). For all further analyses the data from the first trial was discounted because of the presence of the stick. No effect of group was seen (F (1, 12) =1.229, p=0.289) showing that overall latencies between the groups did not differ (Fig. 19). A group versus trial interaction was also found (F (6, 72) = 4.283, p =0.001). An overall effect of trial was found (F (6, 72) = 5.396, p <0.001) with latencies in the Working Memory group reducing over the trials. Post hoc analysis (Bonferonni) revealed that the differences were significant in the Working Memory group between trials 2 and 6 (p=0.026), 2 and 7 (p=0.009) and 2 and 8 (p=0.009) with no significant differences between any trials in the Procedural Task group.
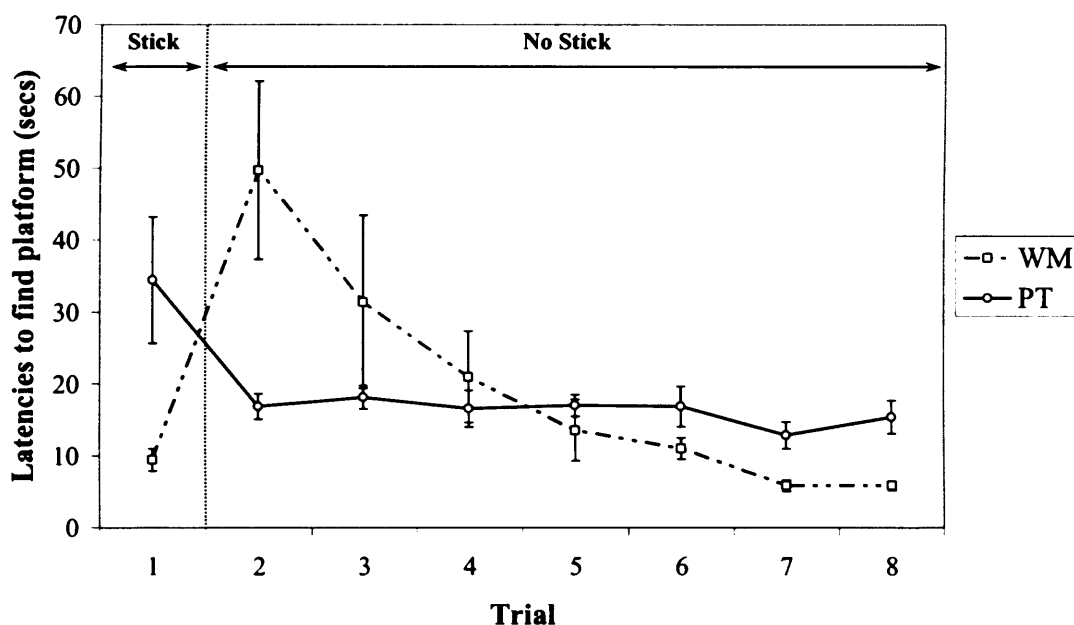
Figure 18
Latencies to find platform over all 8 trials on final test day for Working Memory (WM) and Procedural Task (PT) groups. The Working Memory group shows a decrease in latencies to find the platform over the 8 trials whilst the Procedural task group does not.
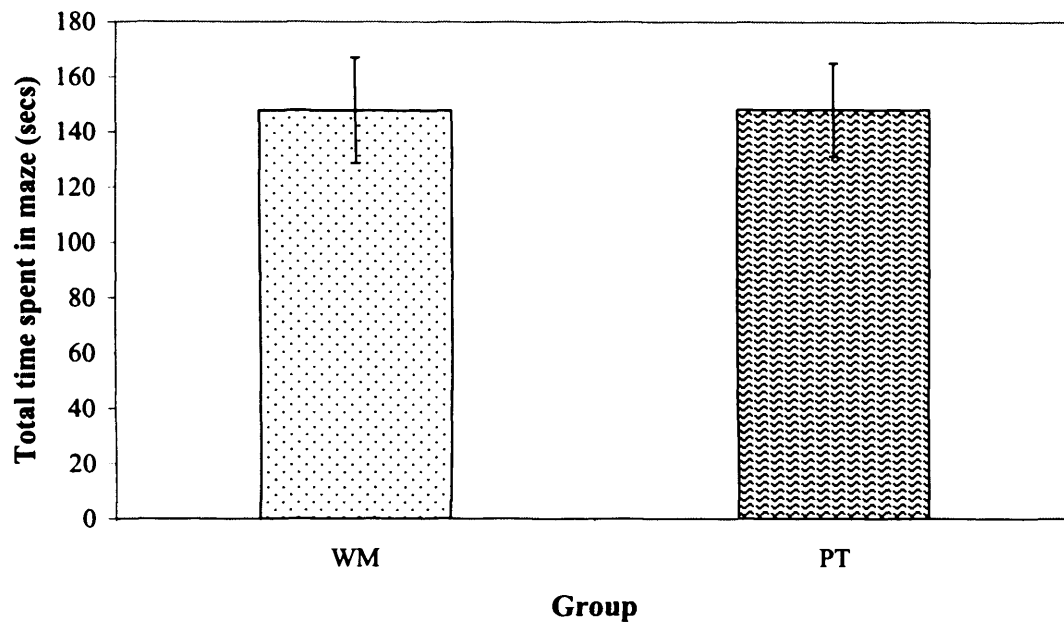
Figure 19
Total amount of time spent in the maze over all 8 trials on final test day for Working Memory (WM) and Procedural Task (PT) groups. No difference between the total time spent in the maze was seen between the two groups.

3.2.2.2 Zif268 Counts

Examples of Zif268 immunoreactivity in a number of anatomical regions in the Working Memory and Procedural Task groups can be seen in Figures 20 and 21.

Primary Motor, Visual and Somatosensory regions

The regions counted were the primary somatosensory area, the primary motor area and the barrel cortex (Fig. 22). No overall effect of group was seen (F (1, 12) =2.441, p=0.144).
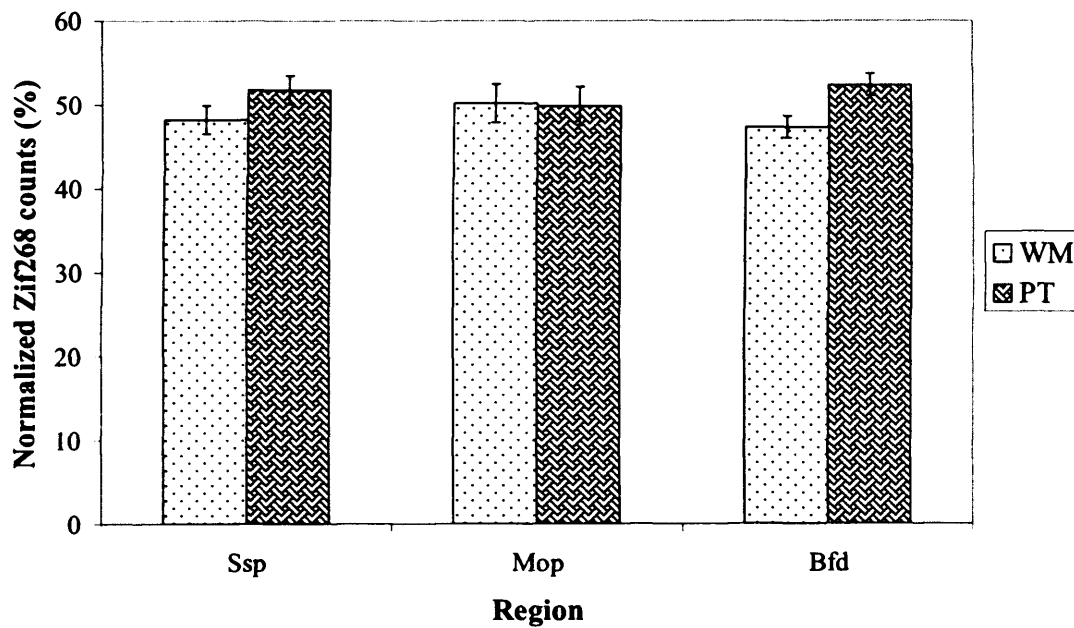
Figure 22
Normalized Zif268 counts for the primary motor and somatosensory regions (Ssp-somatosensory cortex, Mop-motor cortex, Bfd-barrel cortex)

Aca WM

Aca PT

Mop WM

Mop PT

rRSP WM

rRSP PT
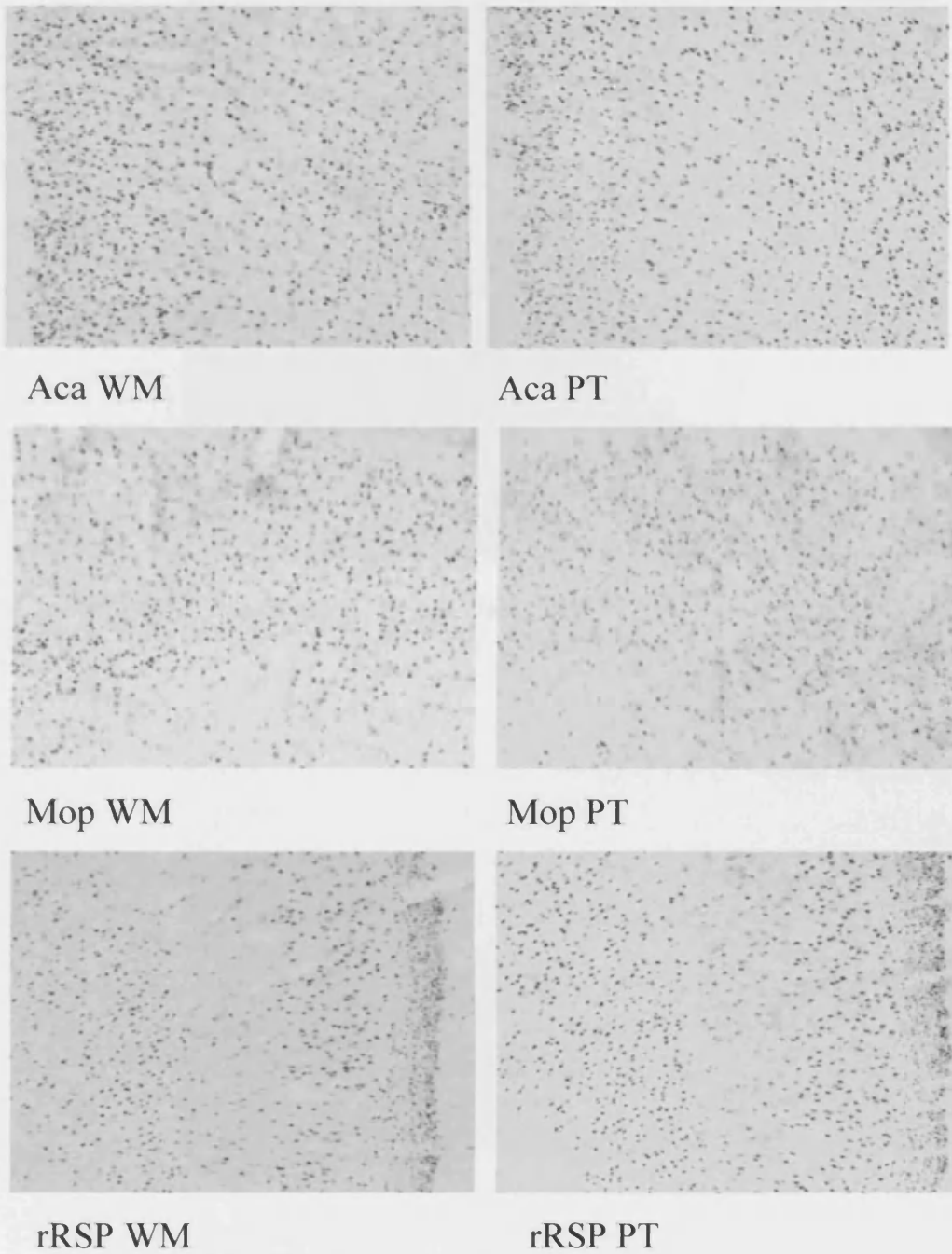
Figure 20
Figure depicting Zif268 immunoreactivity in the Working Memory (WM) and
Procedural Task (PT) groups in a number of anatomical regions. (Aca-anterior
cingulate cortex, Mop-motor cortex, rRSP-rostral reterosplenial cortex).

WM
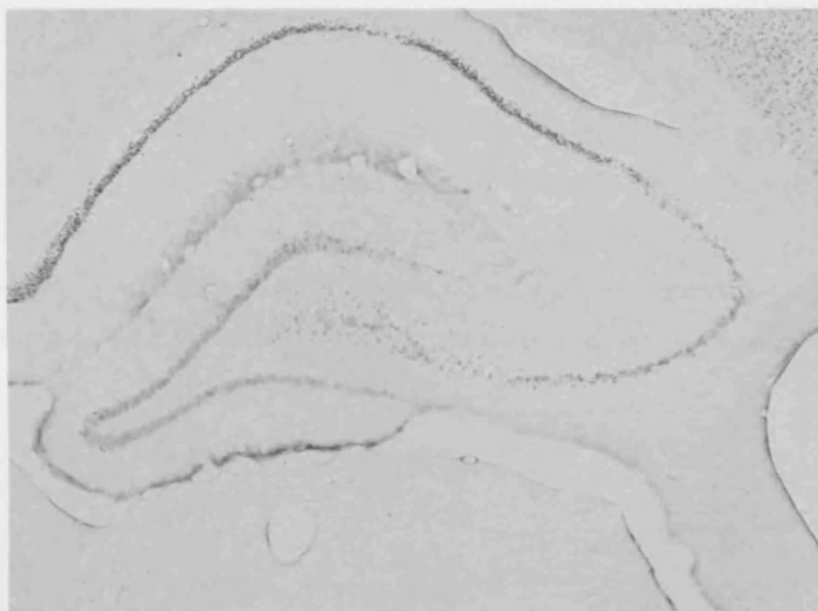
PT
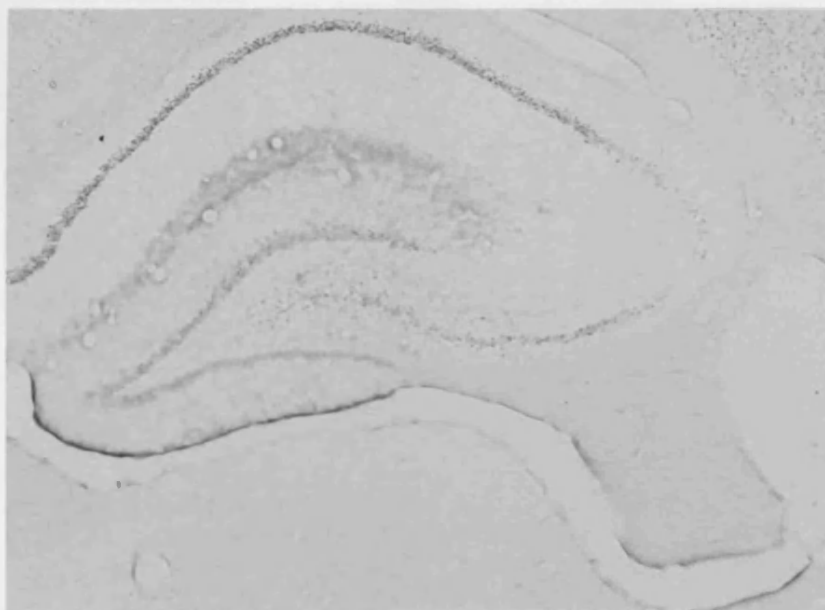
Figure 21
Figure depicting Zif268 immunoreactivity in the Working Memory (WM) and
Procedural Task (PT) groups in the hippocampus.

63

Prefrontal and Cingulate regions

The prefrontal and cingulate regions counted were the granular rostral retrosplenial cortex, anterior cingulate cortex and prelimbic and infralimbic cortices (Fig. 23). An overall group difference was seen (F (1, 12) =10.581, p=0.007). Simple effects analysis revealed that the Working Memory group had significantly lower counts than the Procedural Task group in the prelimbic (p<0.001) and anterior cingulate regions (p=0.037).
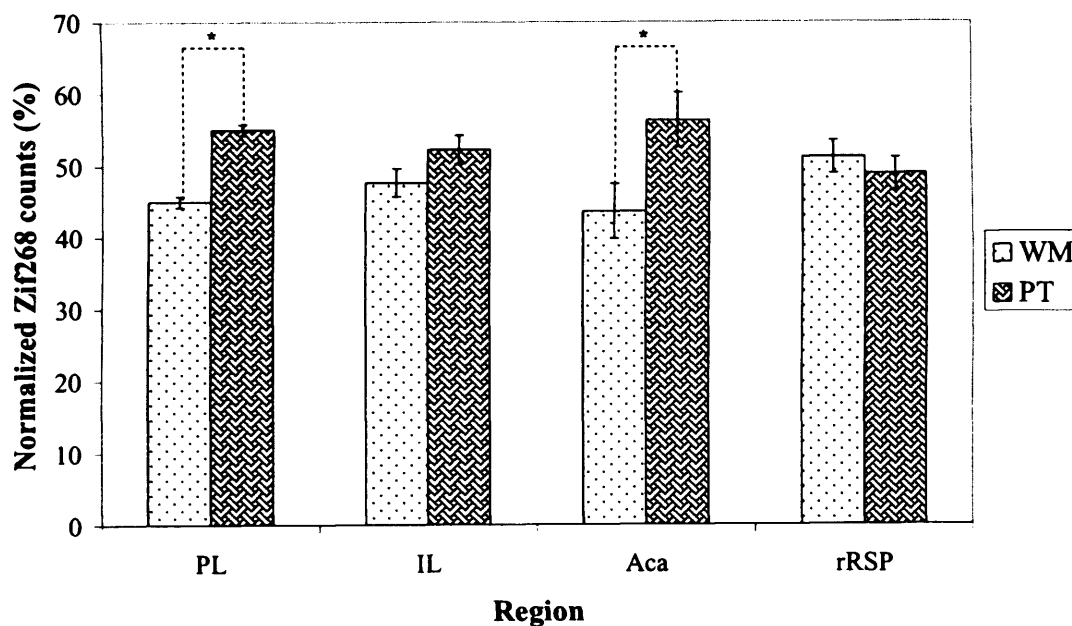
Figure 23
Normalized Zif268 counts for prefrontal and cingulate regions. A decrease in Zif268 immunoreactivity was seen in the prelimbic cortex and anterior cingulate cortex in the Working Memory group as compared to the Procedural Task group. (PL-prelimbic cortex, IL-infralimbic, Aca-anterior cingulate cortex, rRSP-rostral reterosplenial cortex)

Parahippocampal cortices and adjacent regions

The parahippocampal regions counted were the medial and lateral entorhinal cortices, dorsal subiculum, perirhinal and postrhinal cortices. (Fig. 24). An overall effect of group was found (F (1, 12) = 26.039, p <0.001) with protein positive cell counts in the Working Memory group being lower than the Procedural Task group. Subsequent analysis showed the difference to be significant in the lateral entorhinal cortex (p = 0.001) the postrhinal cortex (p=0.029) and the dorsal subiculum (p=0.022).
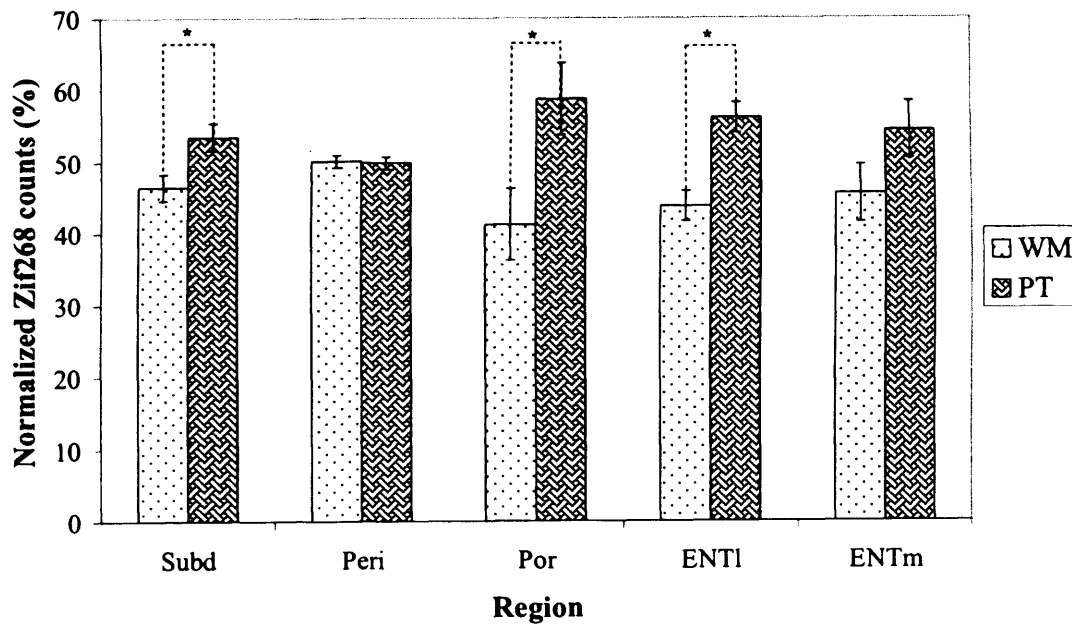
Figure 24
Normalized Zif268 counts for the parahippocampal regions. A decrease in Zif268 immunoreactivity was seen in the dorsal subiculum, postrhinal cortex and the lateral entorhinal cortex in the Working Memory group as compared to the Procedural Task group. (Subd-dorsal subiculum, Peri-perirhinal cortex, Por-postrhinal cortex, ENTl-lateral entrorhinal cortex, ENTm-medial entorhinal cortex)

Hippocampal subfields

The hippocampal subfields counted were CA1, CA3 and dentate gyrus (Fig. 25). An overall effect of group was found (F (1, 12) = 55.070, p < 0.001) with counts again being lower in the Working Memory group than the Procedural Task group. Simple effects analysis revealed significant differences in all sites (CA1, p =0.016; CA3, p <0.001; DG, p <0.001).
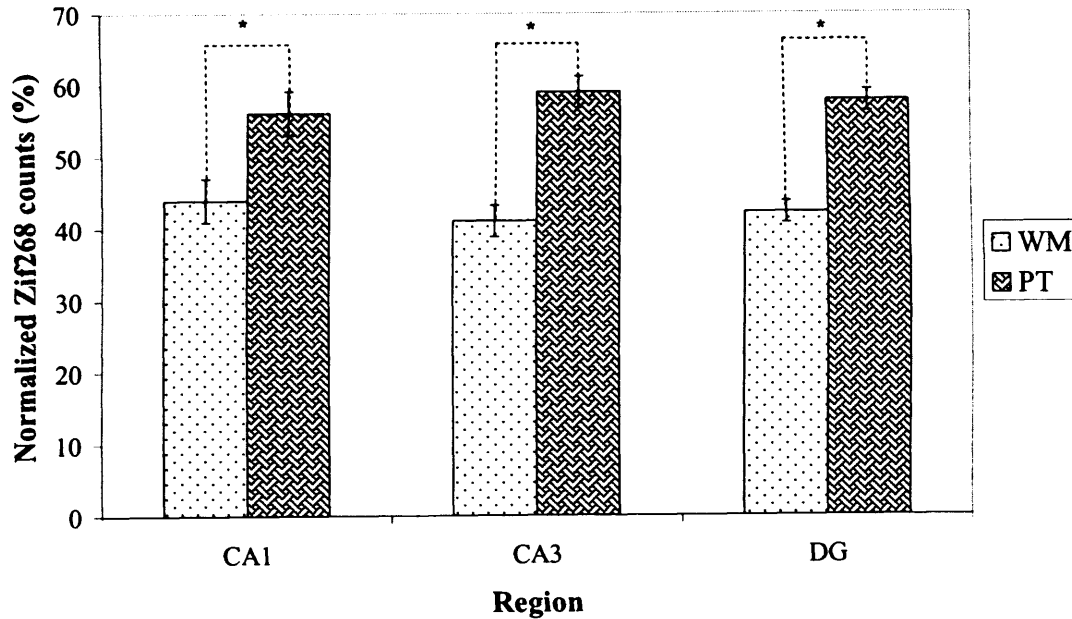
Figure 25
Normalized Zif268 counts for hippocampal subfields. A decrease in Zif268 immunoreactivity was seen in all hippocampal subfileds the Working Memory group as compared to the Procedural Task group. (DG-dentate gyrus)

3.2.3 Discussion

Animals tested on a working memory version of the water maze showed decreases in Zif268 activation in a number of brain areas as compared to a novel control group. The implications of this will be discussed elsewhere (3.6). Although this experiment is an improvement on past research, in that animals are matched on swim times and spatial location of the platform, there are still a number of faults with it. The platform positions are not identical for the final session in the Working Memory and Procedural Task groups and the platform in the Procedural Task group is very close to the edge of the pool. Also the presence of the stick on the first trial of each session which although may have aided learning in the Working Memory group produced longer latencies in the Procedural Task group for that trial. In the next group of experiments these faults were remedied. Two new control groups were tested; one where the platform was still close to the edge of the pool (5cm from the edge) and another where the platform was 13cms from the edge of the pool. The 13cm Procedural Task group allows the platform to be placed in exactly the same spatial location as the Working Memory group for the final session. Also included was a Cage Control and Free Swim comparison. As well as looking at counts of the immediate early gene Zif268 activation of c-Fos was also studied.

3.3 Experiment 3a

3.3.1 Introduction

To rectify faults with the previous experiment (see 3.2.3) two further Procedural Task groups were tested and IEG activation was again compared to animals performing a standard working memory task. Both Procedural Task groups were tested in the same way as previously (3.2.1.) but group Procedural Task 5cm had the platform located 5cm from the edge of the pool wall and group Procedural Task 13cm had the platform located 13cm from the wall. Also the number of days of training was extended so the presence of the stick on the platform to aid learning of it's position for the first trial of a session in the Working Memory group was not required.

3.3.2 Method

Subjects

Subjects were 27 male Dark Agouti rats (Harlan) weighing between 260g and 300g at end of testing. Rats were housed in pairs and kept on a 14/10 dark/light cycle. Food and water was available *ad libitum* throughout the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Apparatus

The apparatus was the same as in Experiment one, except that a different platform was substituted for some groups. This new platform was made out of Perspex and was 15cm in diameter.

Procedure

Animals were split into 3 groups with nine animals in each group; group Working Memory, group 5cm Procedural Task (5cmPT) and group 13cm Procedural Task (13cmPT). All animals performed 8 consecutive trials, lasting for a maximum of 90 seconds for each, with an inter trial interval of 30 seconds. Upon reaching the platform animals remained on it for 10 seconds. All rats received 20 sessions. In all cases swim paths were recorded. Animals were placed into a quiet and dark room for

twenty minutes before the experiment began and for an hour and half afterwards. This was to reduce any extraneous stimulation the animals may have received. When animals were moved from room to room they were placed in a covered carry box measuring 28x45x21cm to maintain the dark conditions.

Pretraining

Animals in all groups were pretrained for 2 days before the experiment began in the same manner as Experiment two.

Working Memory group

For the Working Memory group the platform position remained constant from trial to trial but moved from session to session (Fig. 26). The platform could be placed in a possible 12 different positions in the maze; both near the periphery of the pool and nearer the centre. These positions were balanced throughout the sessions. Animals were released from the edge of the pool and the start points differed on each trial. Latencies to find the platform were recorded as were the swim paths taken. On day 20, the final test day, the Working Memory group performed the task as previously but the platform was 13cms from the edge of the pool. This distance was not novel as rats had been exposed to it on a previous session but the platform position had never been experienced before.
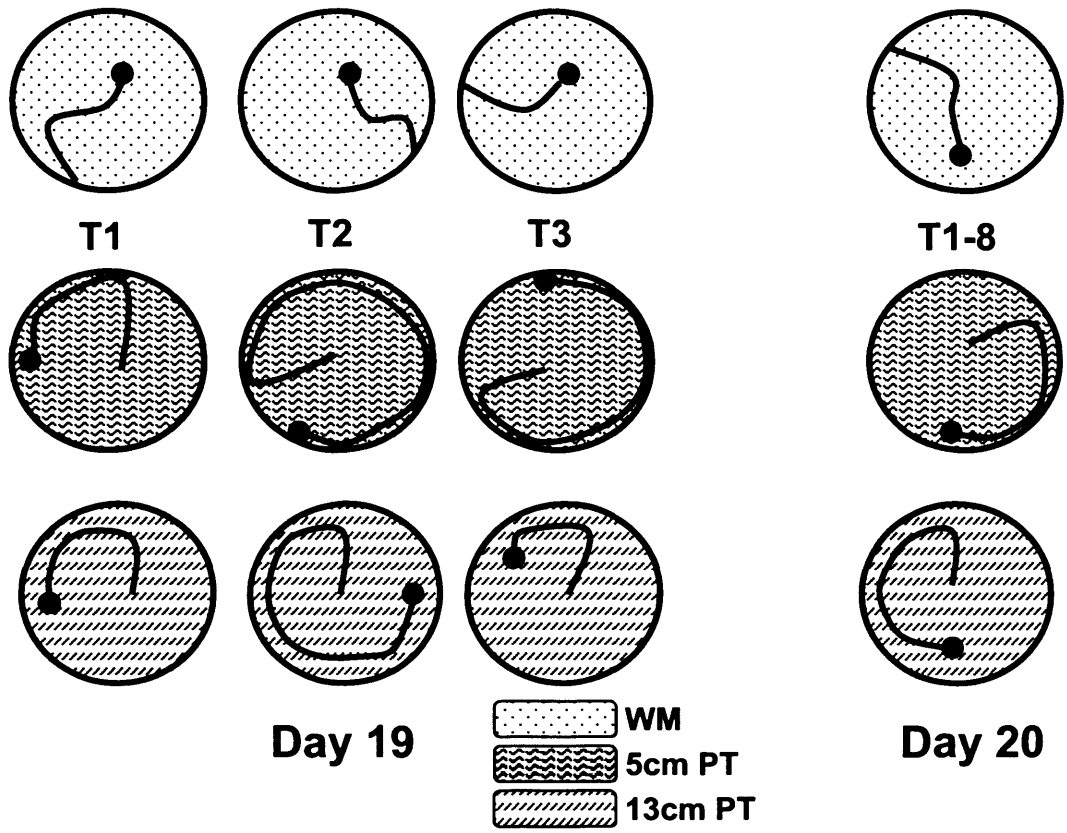
Figure 26
Diagram to show a representation of swim paths for group Working Memory, group 5cmPT and group 13cmPT on a training day (day 19) and the final test day (day 20).

5cm Procedural Task group

For the 5cmPT group the platform position was always around the perimeter of the water maze 5cm from the edge to encourage thigmotaxic behaviour (Fig. 26). The platform could be in a possible 8 different positions. As the platform position altered trial to trial it was in all of the positions on any given day but the order of presentation differed from day to day in a pseudorandom manner. The animals were released from the centre of the maze. On the final test day the platform position remained in the same place on each trial and was adjacent to the working memory platform location (i.e. in the south east position) but close to the side walls.

13cm Procedural Task group

For the 13cmPT group the platform position was always around the perimeter of the water maze 13cms from the mazes edge (Fig. 26). The platform used was slightly larger than for the other groups to make the task easier for the rat and so equate the swim times. The platform could be in a possible 8 different positions. As the

70

platform position altered from trial to trial it was in all of the positions on any given day but the order of presentation differed from day to day in a pseudorandom manner. The animals were released from the centre of the maze. On the final test day the platform position remained in the same place on each trial and was in the same position as for the Working Memory group.

Post hoc animals in the Working Memory group and the 2 Procedural Task groups (5cmPT, 13cmPT) were matched on total time spent in the pool and processed for immunohistochemistry together.

Immunohistochemistry

Ninety minutes after the beginning of the final session animals were anaesthetized with sodium pentobarbital (60mg/kg) and perfused intracardially with 0.1m PBS followed by 4% PFA in 0.1M PBS. After removal the brain was placed in PFA for 4 hours and then in 30% sucrose solution overnight. Sections were cut coronally at 40μm on a freezing microtome and one in four sections were collected in PBS with 0.2% TritonX-100 (PBST). The sections were then blocked using 0.3% hydrogen peroxidase in PBST for 10 minutes. After four 10 minute washes in PBST sections were incubated in the primary antibody, anti *zif268* (1:3000, C-19, Santa Cruz) and anti *c-fos* (1:5000, Ab-5, Oncongene) respectively, for 48 hours at 4°C. Sections were again washed four times in PBST then incubated in the secondary antibody with goat serum (1:2000 in PBST, vector laboratories) for two hours at room temperature. After washing with PBST, sections were incubated in avidin-biotinylated horseradish peroxidase complex diluted in PBST (Elite kit, Vector laboratories) for one hour at room temperature. Sections were washed again in PBST and then in 0.05M tris buffer. The sections were then stained using diaminobenzidine (DAB kit, Vector laboratories). The reaction was stopped by washing in cold PBS. Sections were then mounted on gelatine-covered slides, dehydrated using a series of increasing alcohol concentrations and then cover slipped. A separate one in four series of sections was directly mounted after cutting and stained using cresyl violet for histological purposes.

Image Analysis

Sections were scanned onto a PC (IBM) computer using a Leica DMRB microscope and an Olympus DP70 camera. Counts of the stained nuclei were carried out using the program analySIS (Soft Imaging Systems). The threshold was set at the same

level for all sections and counts were made in a frame area of 0.84 x 0.63 mm using a

10x magnification. For hippocampal counts the entire area of interest was captured in

different frames using a 5x magnification. Then a montage was created using the

analySIS software. Counts were taken from 3 consecutive sections from both

hemispheres for all regions of interest. Counts were normalized to reduce variation

between animals. Mean counts for each animal at a given site were divided by the

overall mean for all animals in the comparison. This produced a percentage ratio with

each group of animals adding up to 100 (Fig. 27). These normalized counts were

analysed using a 2 factor analysis of variance (group and region). Tables of raw cells

counts can be seen in Appendix A Figure 3 for Zif268 and Figure 4 for c-Fos.

WM: $$\frac{\text{Number of cells in WM subject}}{\text{Number of cells in WM subject} + \text{Number of cells in 5cmPT subject} + \text{Number of cells in 13cmPT subject}}$$

5cmPT: $$\frac{\text{Number of cells in 5cmPT subject}}{\text{Number of cells in WM subject} + \text{Number of cells in 5cmPT subject} + \text{Number of cells in 13cmPT subject}}$$

13cmPT: $$\frac{\text{Number of cells in 13cmPT subject}}{\text{Number of cells in WM subject} + \text{Number of cells in 5cmPT subject} + \text{Number of cells in 13cmPT subject}}$$

Figure 27
Equations for normalization procedure for Working Memory and Procedural Task groups

Regions of interest

Regions of interest were the same as in Experiment two.

3.3.3 Results

3.3.3.1 Behavioural results

<u>Acquisition</u>

Comparing the average latencies for each group over the 20 sessions of training an overall effect of session (F (19,456) =11.134, p<0.001) was seen with no effect of group (F (2, 24) =2.575, p=0.097) and no group by session interaction (F (38,456) =1.273, p=0.133) as all groups showed decreases in latencies over sessions (Fig. 28).
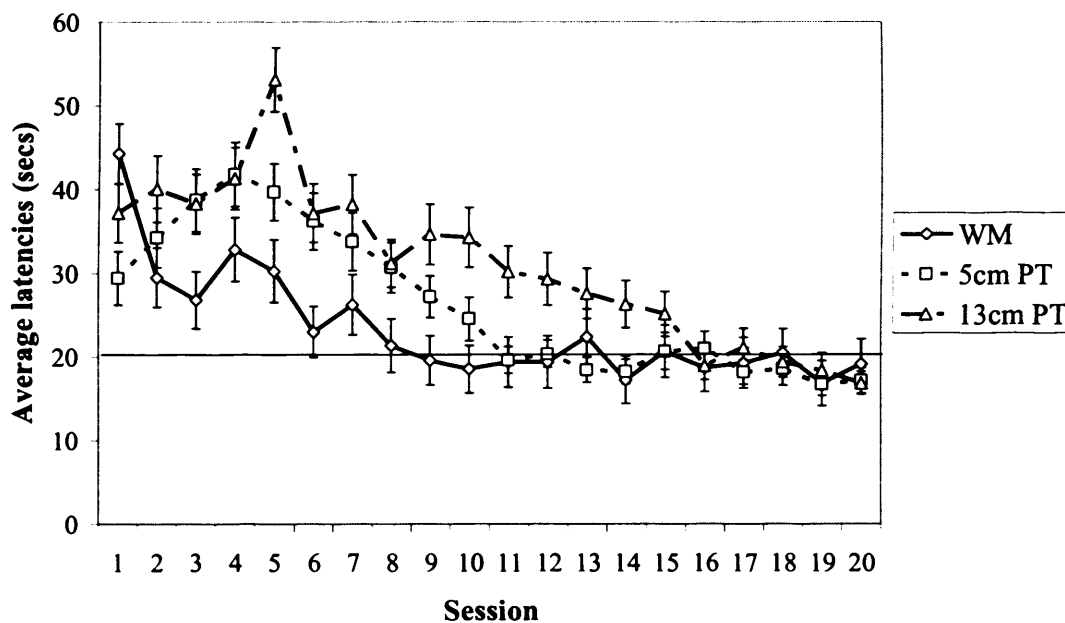
Figure 28
Average latencies to find platform across all trials over all days of training for the Working Memory, 5cm PT and 13cm PT groups. All groups show a decrease in average latencies across the 20 sessions.

<u>Final session</u>

On the final day of testing, a group of animals performed a standard version of the working memory task in the water maze, whilst the other two groups performed two different procedural tasks (Fig. 29). For swim latencies there was no effect of group (p=0.709) (Fig. 30) while the expected group by trial interaction was found (F (14, 168) = 4.377, p =0.004). This interaction reflects the improvement in performance by the Working Memory group, contrasting with the flat latency properties of the other two Procedural Task groups. An overall effect of trial was found (F (7, 168) = 7.059, p <0.001) with latencies in the working memory group reducing over the trials. Simple effect analysis revealed that there was a significant effect of trial in the

73

Working Memory group ($p<0.001$) but no effect in either Procedural Task group (5cmPT, $p=0.159$; 13cmPT, $p=0.634$).
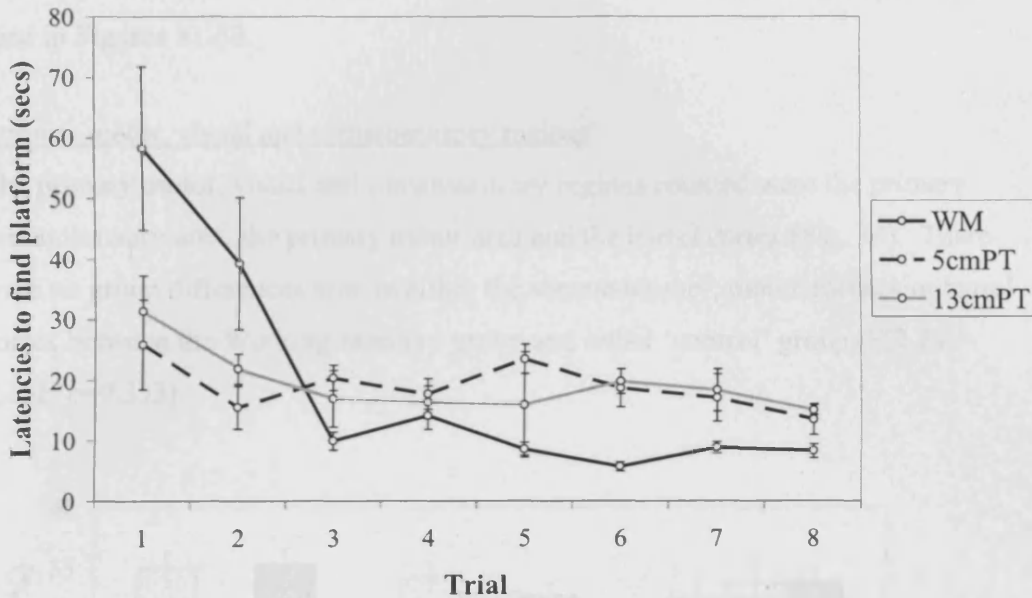
Figure 29
Latencies to find platform over all 8 trials on final test day for Working Memory, 5cm PT and 13cmPT groups. The Working Memory group showed a decrease in latencies to find the platform across the 8 trials whilst the 5cmPT and 13cmPT groups did not.
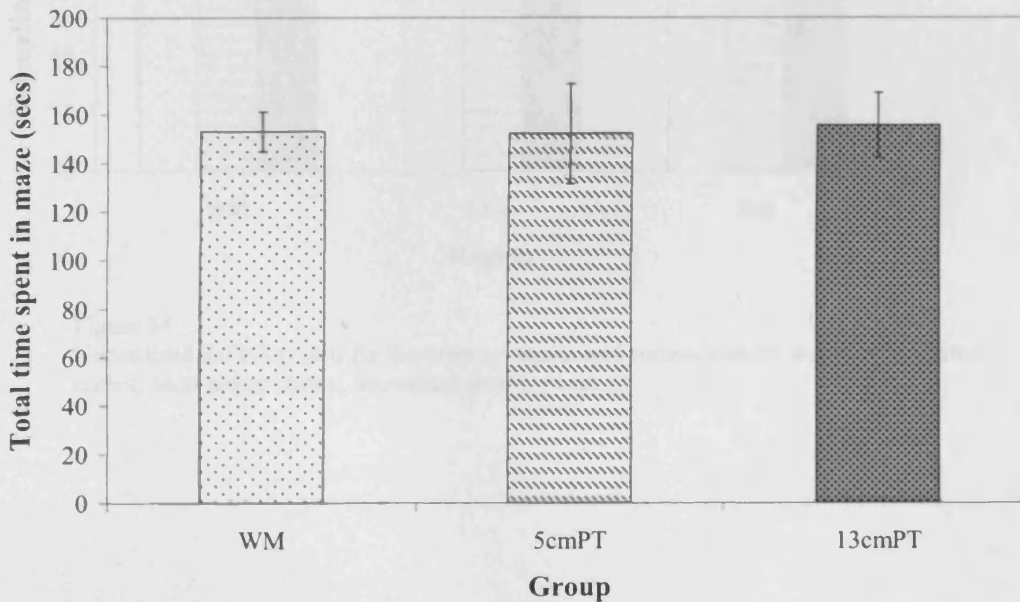
Figure 30
Total amount of time spent in the maze over all 8 trials on final test day for Working Memory, 5cm PT and 13cm PT groups.

74

3.3.3.2 Zif268 Counts

Examples of Zif268 immunoreactivity in a number of anatomical regions in the
Working Memory 5cm Procedural Task and 13cm Procedural Task groups can be
seen in Figures 31-33.

<u>Primary motor, visual and somatosensory regions</u>
The primary motor, visual and somatosensory regions counted were the primary
somatosensory area, the primary motor area and the barrel cortex (Fig. 34). There
were no group differences seen in either the somatosensory, motor cortices or barrel
cortex between the Working Memory group and either 'control' group ($F_{(2,24)}$=
1.152, p=0.333).
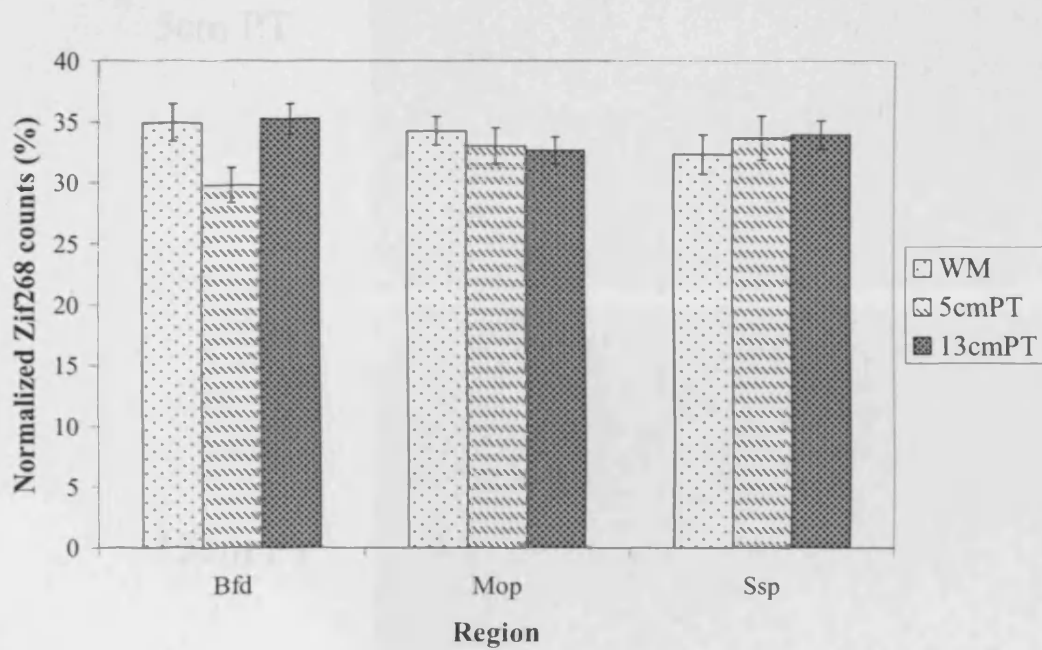
Figure 34
Normalized Zif268 counts for the primary, motor and somatosensory regions (Bfd-barrel
cortex, Mop-motor cortex, Ssp-somatosensory cortex)

WM
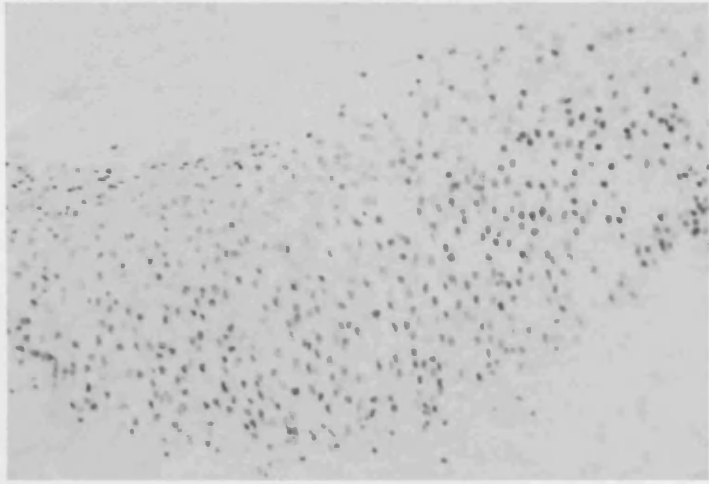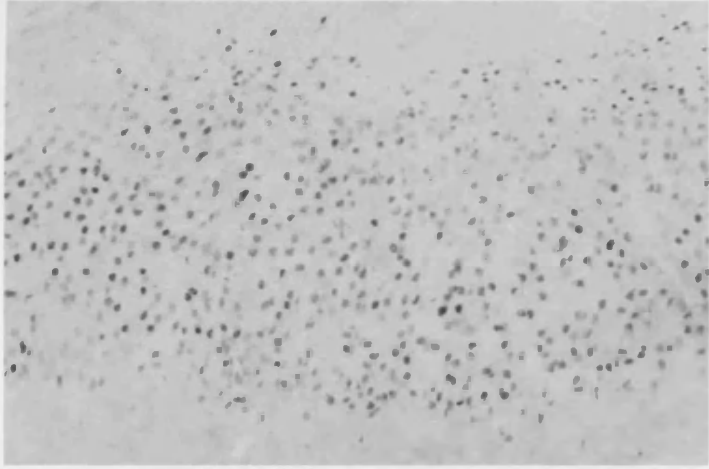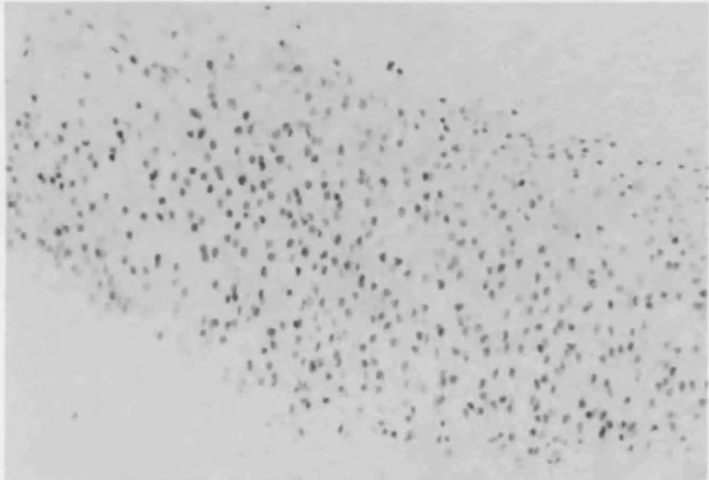
5cm PT

13cm PT

Figure 31
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the dorsal
subiculum

WM
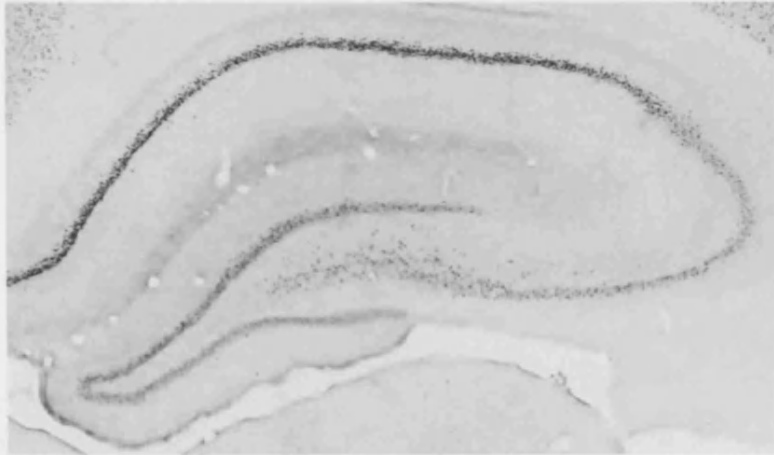
5cm PT
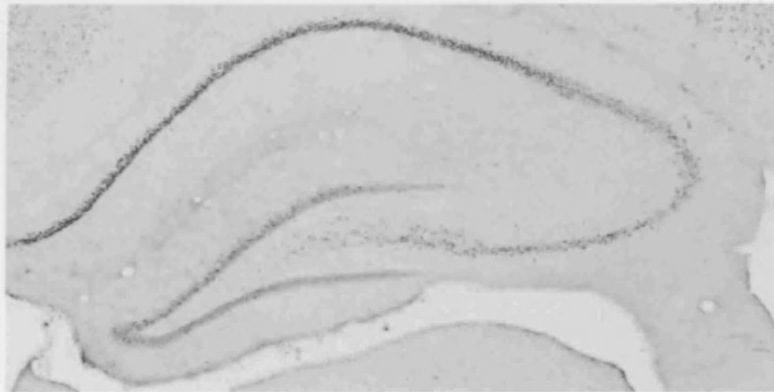
13cm PT

Figure 32
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the
hippocampus.

WM

5cm PT

13cm PT

Figure 33
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the barrel
cortex.

<u>Prefrontal and Cingulate regions</u>

The prefrontal and cingulate regions counted were the rostral granular retrosplenial cortex, anterior cingulate cortex and prelimbic and infralimbic cortices (Fig. 35). No group differences were seen between the Working Memory and procedural groups for all areas (F $(2, 24)$ = 3.136, p=0.062).
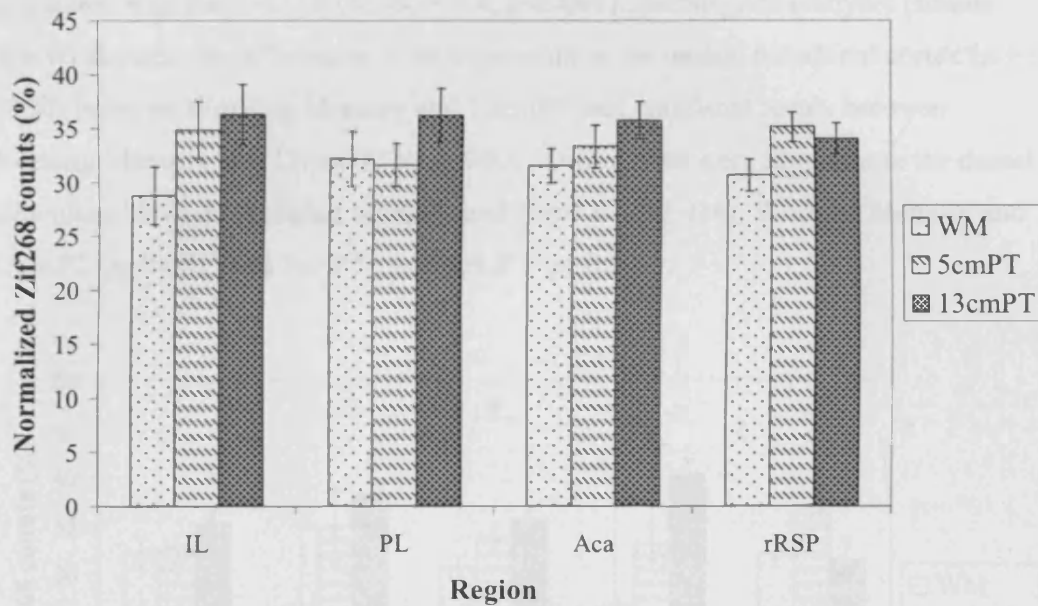
Figure 35
Normalized Zif268 counts for prefrontal and cingulate regions. (IL-infralimbic cortex, PL-prelimbic cortex, Aca-anterior cingulate cortex, rRSP-rostral retrosplenial cortex)

Parahippocampal cortices and adjacent regions

The parahippocampal and adjacent regions counted were the medial and lateral entorhinal cortices, perirhinal cortex, postrhinal cortex and dorsal subiculum (Fig. 36). An overall effect of group was found (F (2, 24) = 5.8, p =0.009) with counts in the Working Memory group being lower than the procedural groups. A area by group interaction was also seen (F (8, 96) = 4.4, p<0.001). Subsequent analyses (simple effects) showed the differences to be significant in the medial entorhinal cortex (p = 0.050) between Working Memory and 13cmPT and perirhinal cortex between Working Memory and 13cmPT (p = 0.040). Differences were also seen in the dorsal subiculum between Working Memory and 5cmPT (p=0.014), Working Memory and 13cmPT (p=0.001) and 5cmPT and 13cmPT (p=0.007).
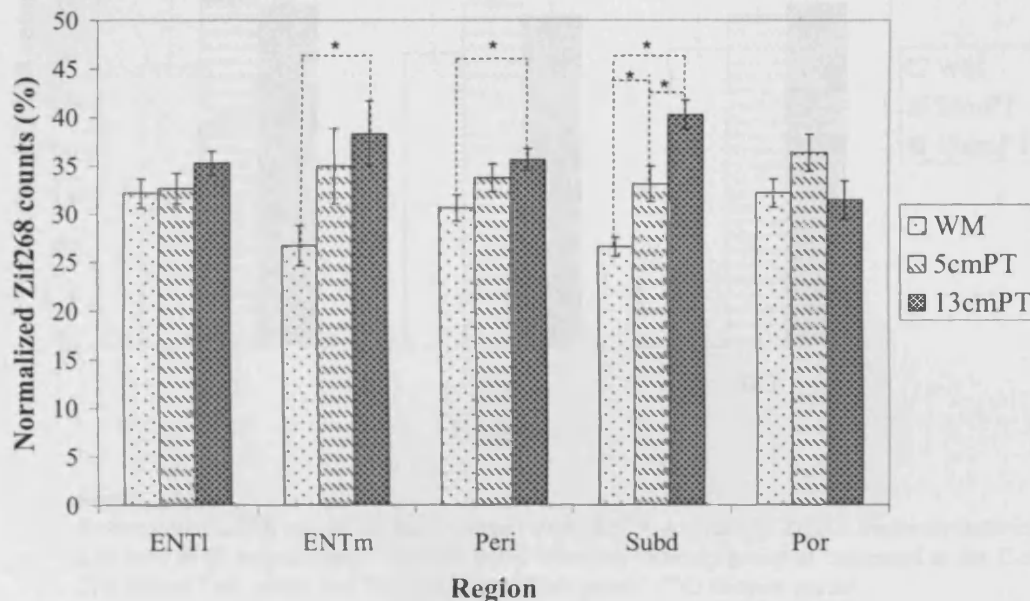
Figure 36
Normalized Zif268 counts for the parahippocampal regions. A decrease in Zif268 immunoreactivity was seen in the medial entorhinal cortex, perirhinal cortex and dorsal subiculum in the Working Memory group as compared to the 13cm Procedural Task group. (ENTl-lateral entorhinal cortex, ENTm-medial entorhinal cortex, Peri-perirhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

<u>Hippocampal subfields</u>

The hippocampal subfields counted were CA1, CA3 and dentate gyrus (Fig. 37). An overall effect of group was found (F (2, 24) = 17.1, p <0.001) with counts again being lowest in the Working Memory group than the procedural groups. Simple effects analysis revealed significant differences in all sites. In CA1 Working Memory was significantly lower than 5cmPT (p<0.001) and 13cmPT (p<0.001). In CA3 differences were seen between Working Memory and 5cmPT (p=0.006) and between Working Memory and 13cmPT (p=0.011).
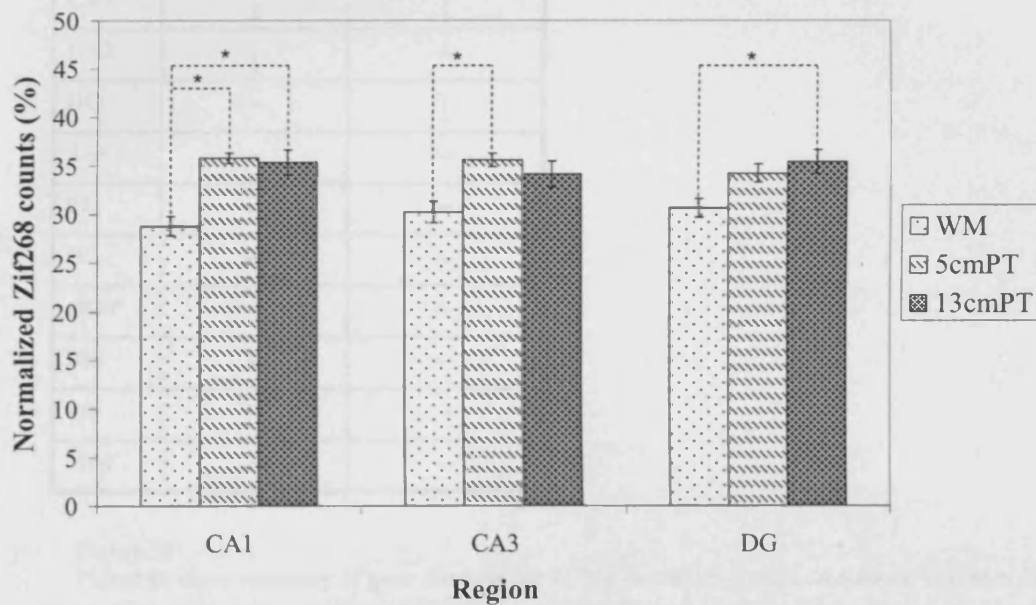
Figure 37
Normalized Zif268 counts for hippocampal subfields. A decrease in Zif268 immunoreactivity was seen in all hippocampal subfields in the Working Memory group as compared to the 13cm Procedural Task group and 5cmProcedural Task group. (DG-dentate gyrus)

	WMvs5cm PT	WMvs13cm PT
ENTl	−	−
ENTm	−	↓
Subd	↓	↓
Peri	−	↓
Por	−	−
CA1	↓	↓
CA3	↓	−
DG	−	↓
Aca	−	−
PL	−	−
IL	−	−
rRSP	−	−
Bfd	−	−
Ssp	−	−
Mop	−	−

Figure 38

Figure to show summary of gene changes for Zif268 across all groups. An arrow indicates the direction of the effect and a − indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.3.3.3 Correlations

Correlations between behavioural performance and Zif268 counts were carried out. Correlations were conducted on data collapsed across the first 3 trials on the final day (before animals had reached asymptote) for the Working Memory group and across the last 5 trials. This was done to try and work out the conflict seen between the findings of Guzowski et al. (2001) and Kelly and Deadwyler (2003). Both looked at how IEGs correlated with behaviour and found contradictory results. Guzowski et al. (2001) found that there was a negative correlation between *arc* expression and mean escape latencies, the best learners had the most *arc*. Kelly and Deadwyler (2003) however found that slower learners had the most *arc*. These authors stated however that there was a difference between the way the results were analysed. They looked at the number of freely made level presses for food when the animals were still learning the task. Guzowski et al. (2001) however looked at the last 3 trials on the final day of testing when animals had reached asymptotic performance. Kelly and Deadwyler (2003) stated that Guzowski et al. (2001) may have found similar results as them if they had looked at the first 3 trials instead.

Results show that in the lateral entorhinal cortex and DG using the 1st 3 trials as the behavioural measure (before animals had reached asymptote) there is a significant positive correlation (Fig. 39), the longer the animals took to found the platform the greater amount of Zif268. In fact all areas display a positive correlation but only lateral entorhinal and DG reached significance. This is inline with the results Kelly and Deadwyler (2003) found in their experiment. Using the last 5 trials as the behavioural measure (when the animals had reached asymptote) the anterior cingulate cortex and CA1 showed a significant negative correlation (Fig. 39), the longer the animals took to find the platform the less Zif268 there was. Overall most areas showed a negative correlation. This is a replication of Guzowski et al. (2001) results. This proves Kelly and Deadwyler (2003) point that if different stages of learning are studied opposite results are seen.

Area	1st 3 trials	Last 5 trials
Prelimbic Cortex	.584	-.429
Perirhinal Cortex	.445	.008
Lateral Entorhinal Cortex	**.698**	-.654
Dentate Gyrus	**.832**	-.137
CA3	.205	-.216
CA1	.313	**-.711**
Dorsal Subiculum	.432	-.396
Rostral retrosplenial Cortex	.182	.129
Medial Entorhinal Cortex	.134	-.023
Postrhinal Cortex	.370	.532
Anterior Cingulate Cortex	.495	**-.677**
Infralimbic Cortex	.503	-.606

Figure 39
Correlation between Zif268 counts and latencies to find the platform on the 1st 3 and last 5 trials of the final test day. Numbers in bold signify a significant correlation.

3.3.3.4 c-Fos counts

Examples of c-Fos immunoreactivity in a number of anatomical regions in the Working Memory 5cm Procedural Task and 13cm Procedural Task groups can be seen in Figures 40-42.

<u>Primary motor, visual and somatosensory regions</u>
The primary motor, visual and somatosensory regions counted were the primary somatosensory area, the primary motor area and the barrel cortex (Fig. 43). An overall effect of group was seen (F $(2, 24)$ =4.708, p=0.019) with simple effects analysis shows that the Working Memory and the 5cmPT group were significant different in the motor cortex (p=0.003).

Figure 43
Normalized c-Fos counts for the primary, motor and somatosensory regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the 5cmProcedural Task group in the motor cortex. (Bfd-barrel cortex, Mop-motor cortex, Ssp-somatosensory cortex)
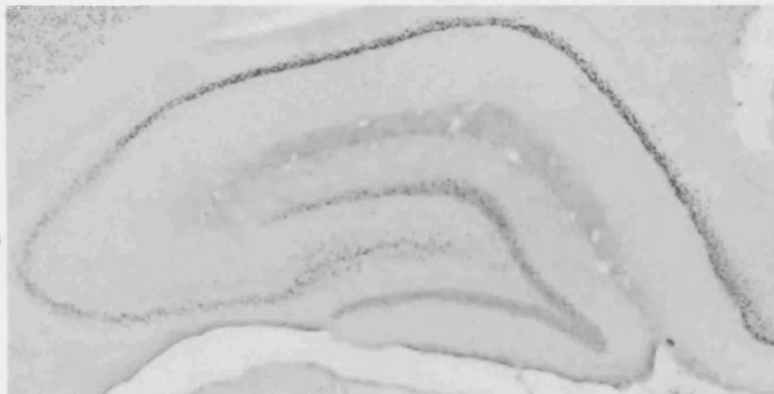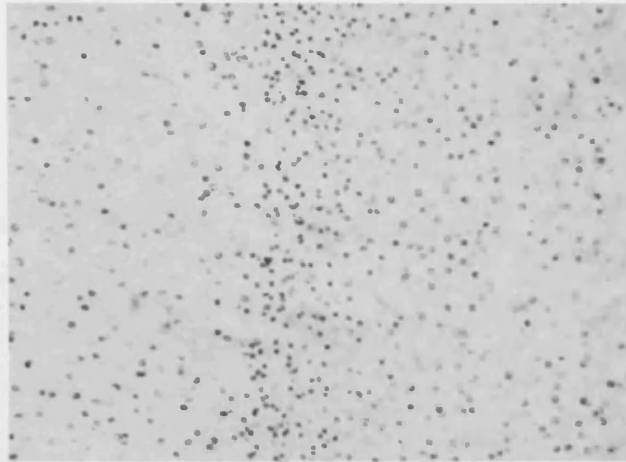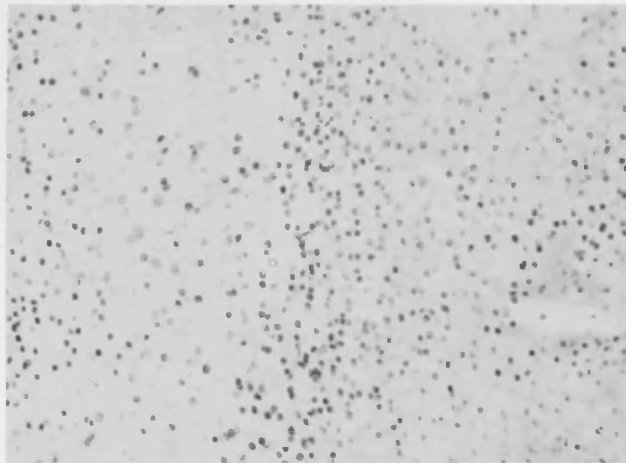
WM
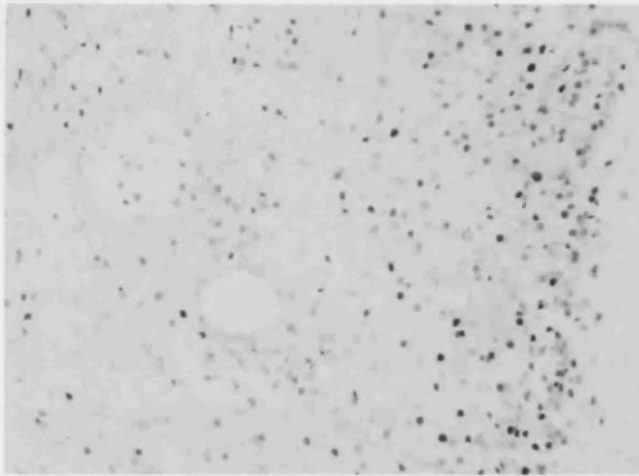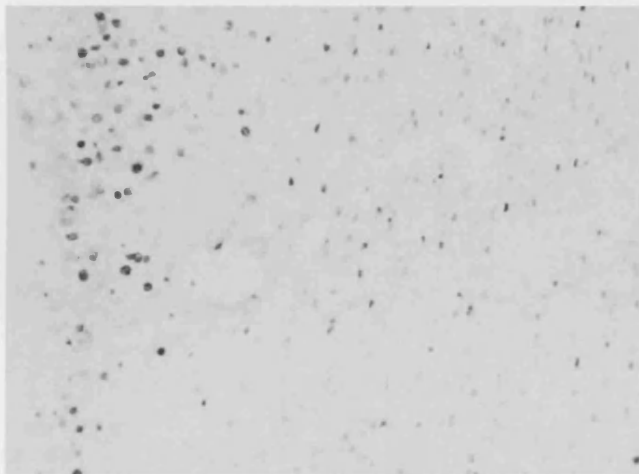
5cm PT

13cm PT

Figure 40
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the lateral
entorhinal cortex.

WM
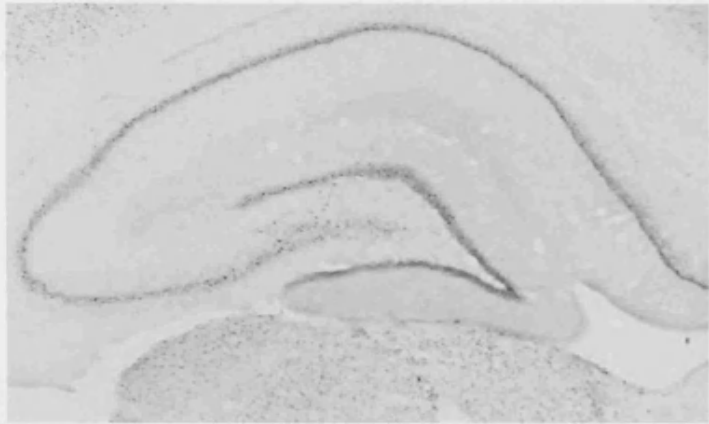
5cm PT
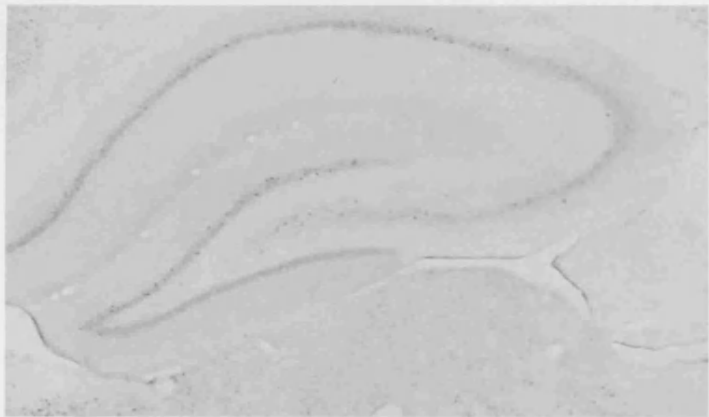
13cm PT
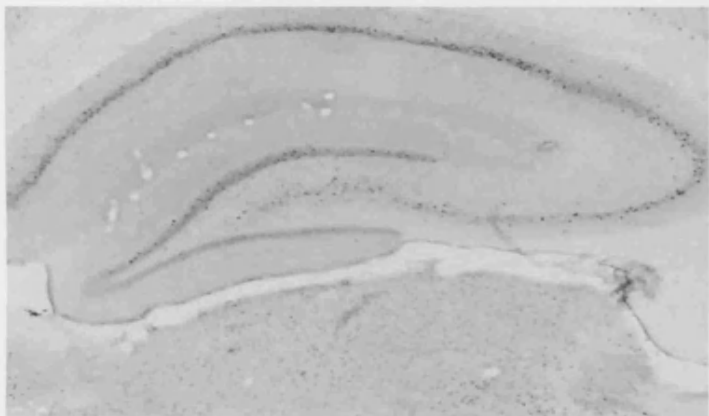
Figure 41
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the
hippocampus.

WM
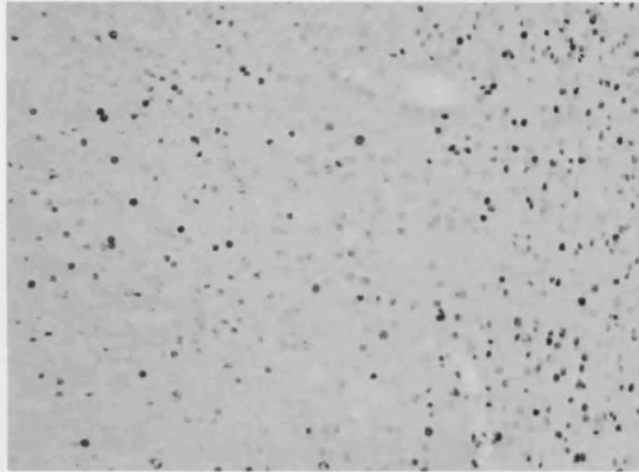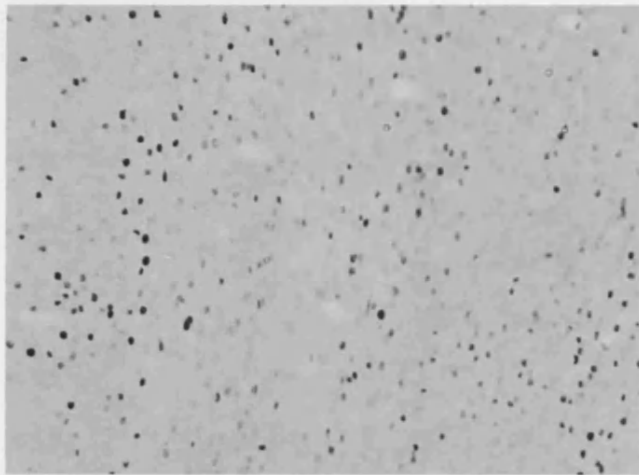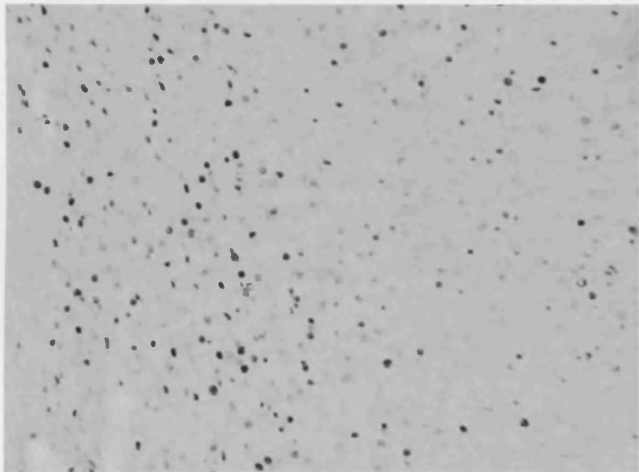
5cm PT

13cm PT

Figure 42
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), 5cm
Procedural Task (5cm PT) and 13cm Procedural Task (13cm PT) groups in the
postrhinal cortex.

Parahippocampal cortices

The parahippocampal regions counted were the medial and lateral entorhinal cortices, perirhinal cortex, postrhinal cortex and dorsal subiculum (Fig. 45). An overall effect of group was found (F (2, 24) = 3.943, p =0.033) with counts in the Working Memory group being higher than the Procedural Task groups. Subsequent analysis showed the only significant difference to be in the lateral entorhinal cortex (p = 0.002) between Working Memory and 13cmPT.
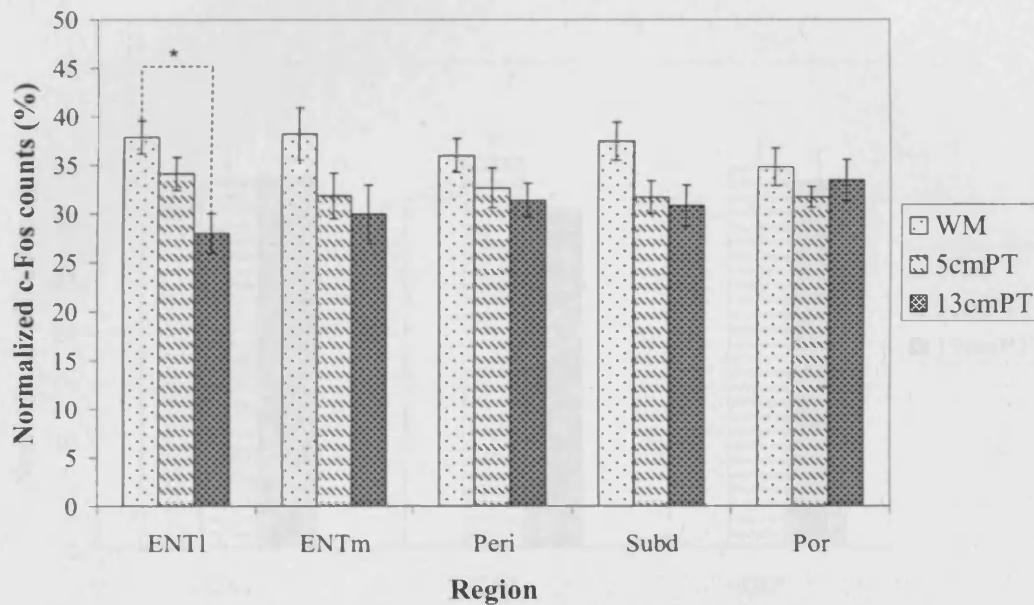
Figure 45
Normalized c-Fos counts for the parahippocampal regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the 13cmProcedural Task group in the lateral entorhinal cortex. (ENTl-lateral entorhinal cortex, ENTm-medial entorhinal cortex, Peri-perirhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

Hippocampal subfields

The hippocampal subfields counted were CA1, CA3 and dentate gyrus (Fig. 46).
Overall the effect of group was approaching significance (F (2, 24) = 3.346, p =
0.052). In accordance with Howell (1997, p351) due to the marginal nature of the F
statistic's significance simple effect analysis was performed. The simple effects show
that over all areas the Working Memory group was significantly lower than the
5cmPT group (p=0.041).
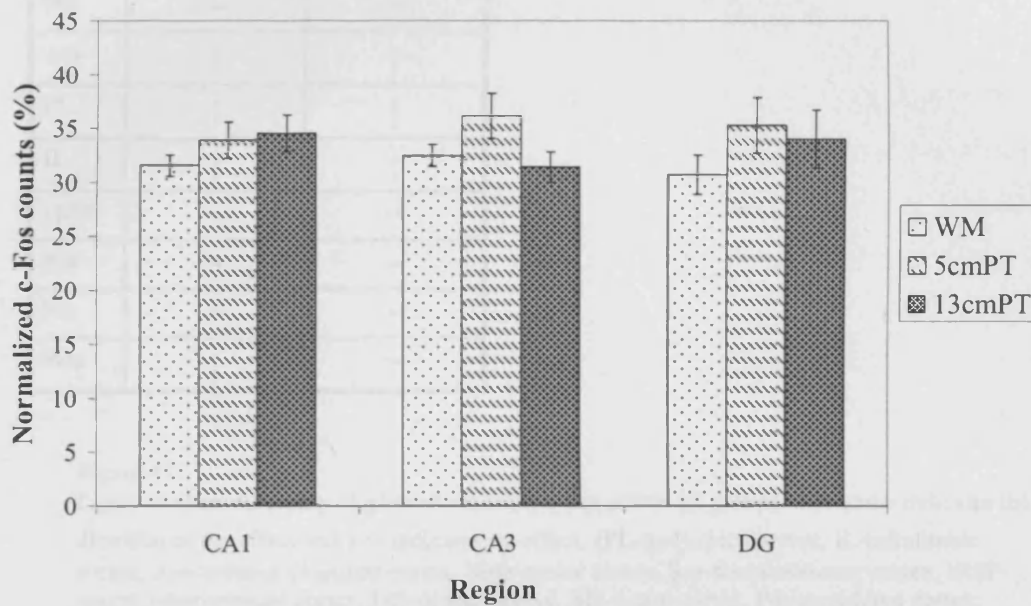
Figure 46
Normalized c-Fos counts for hippocampal subfields. (DG-dentate gyrus)

	WMvs5cm PT	WMvs13cm PT
ENTl	–	↑
ENTm	–	–
Subd	–	–
Peri	–	–
Por	–	–
CA1	–	–
CA3	–	–
DG	–	–
Aca	↑	↑
PL	↑	↑
IL	↑	↑
rRSP	–	–
Bfd	–	–
Ssp	–	–
Mop	↑	–

Figure 47

Figure to show summary of gene changes for c-Fos across all groups. An arrow indicates the direction of the effect and a — indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.3.4 Discussion

As in Experiment two decreases in Zif268 immunoreactivity was seen in a number of brain regions in the Working Memory group as compared to Procedural Task groups (5cmPT, 13cmPT). Again the implications of this are discussed below. However an increase in the number of c-Fos positive cells was seen in the prefrontal regions in the Working Memory group which is more in line with previous research (Vann et al., 2000).

3.4 Experiment 3b

3.4.1 Introduction

Analysis of cage control animals was also performed to see if the assumption that, due to the low level of activation of IEGs in such animals, any comparison with an experimental group will produce higher activation in such group is true.

3.4.2 Method

Subjects

Subjects were 12 male Dark Agouti rats (Harlan) weighing between 260g and 300g at end of testing. Rats were housed in pairs and kept on a 14/10 dark/light cycle. Food and water was available *ad libitum* throughout the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Apparatus

Apparatus were the same as Experiment two.

Procedure

Animals were split into 2 groups with six animals in each group, group Working Memory and group Cage Control.

Pretraining

Animals in Working Memory group were pretrained as Experiment 3a.

Working Memory group

The Working Memory group was trained as in Experiment 3a.

Cage Control

Cage Control animals were handled for 5 minutes every day during the 20 days the other animals were tested. On the final test day (day 20) animals were removed from their home cages and killed. Cage Control animals were housed together and were paired with an animal from the Working Memory group in a random manner.

Immunohistochemistry

Immunohistochemistry was performed as in Experiment 3a.

Image Analysis

Image analysis was the same as Experiment 3a. Tables of raw cells counts can be seen in Appendix A Figure 5 for Zif268 and Figure 6 for c-Fos.

Regions of interest

Regions of interest were the sane as Experiment 3a.

3.4.3 Results

3.4.3.1 Behavioural results

On the final day of testing, half of the animals performed a standard version of the working memory task in the water maze, whilst the others were taken from their home cages. (Fig. 48). An overall effect of trial was found (F (7, 35) = 16.7, p <0.001) with latencies in the Working Memory group reducing over the trials.
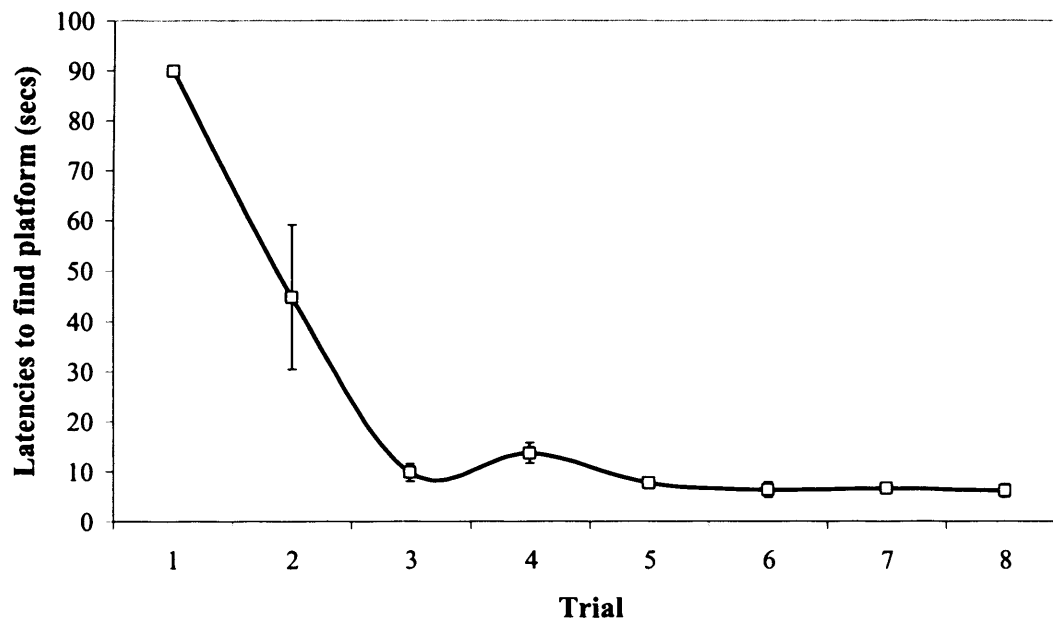
Figure 48
Latencies to find platform over all 8 trials on final test day for Working Memory group used for Cage Control comparisons. The Working Memory group show a decrease in latencies to find the platform over the 8 trials.

3.4.3.2 Zif268 Counts

<u>Primary motor, visual and somatosensory regions</u>

For the primary motor, visual and somatosensory regions counted an overall effect of group was seen (F (1, 10) =75.6, $p<0.001$) (Fig. 49). Simple effects analysis showed that for all areas Working Memory group cell counts were significantly higher than the Cage Control group (Ssp: $p=0.0002$; Mop: $p<0.001$; Bfd: $p<0.001$).
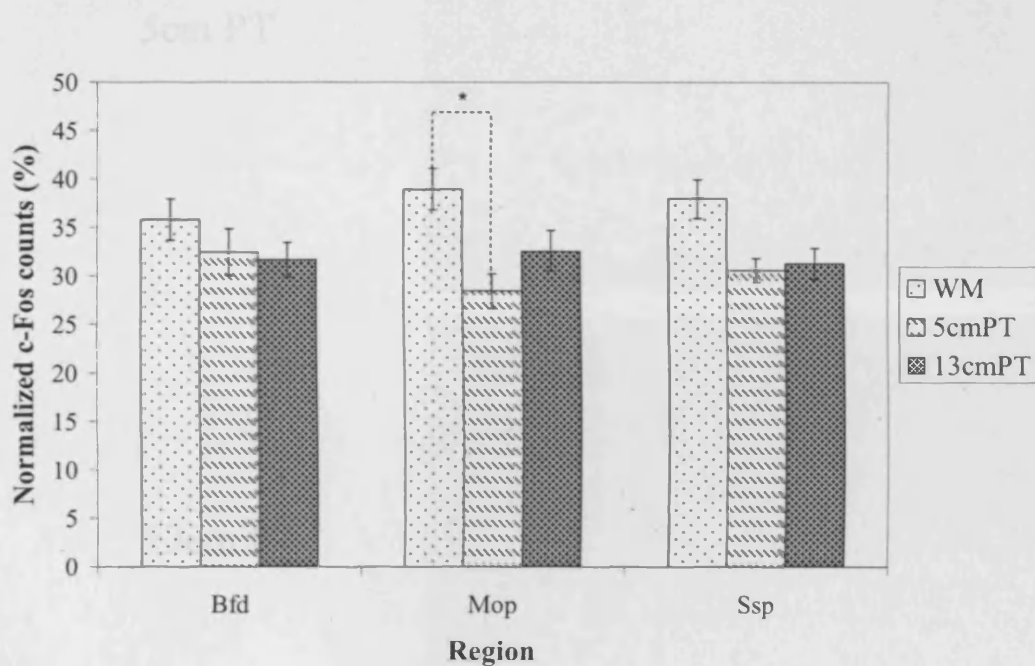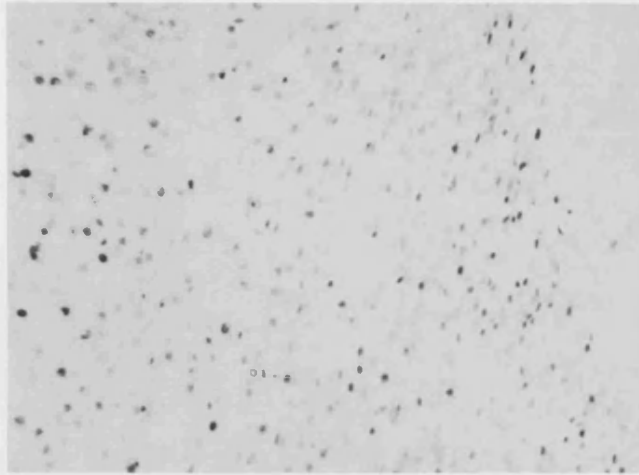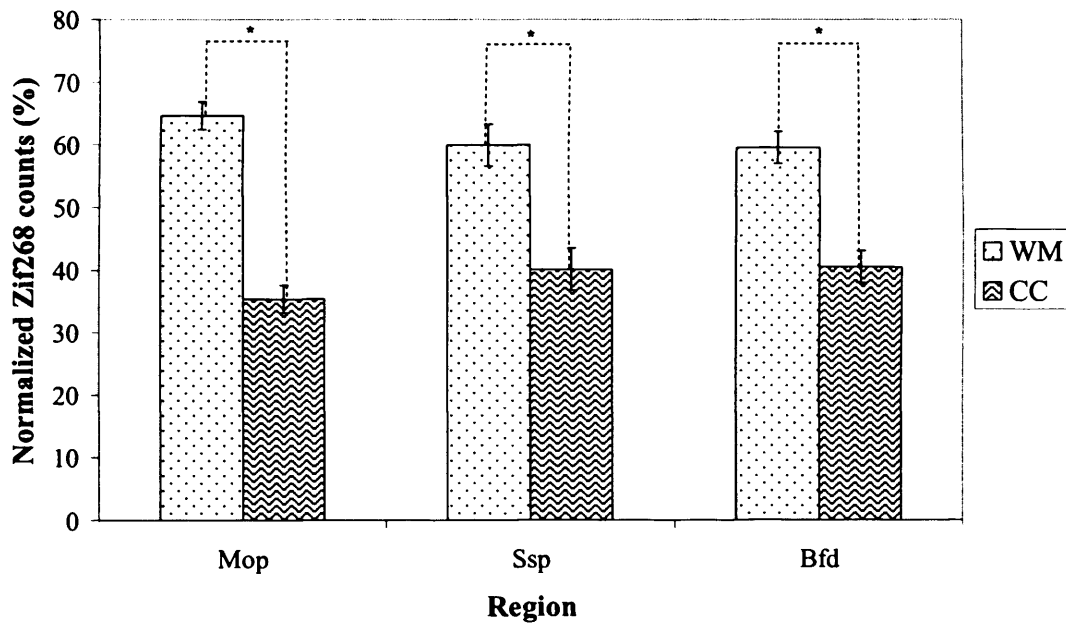

Figure 49

Normalized Zif268 counts for the primary, motor and somatosensory regions. An increase in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in all control regions. (Mop-motor cortex, Ssp-somatosensory cortex, Bfd-barrel cortex)

Prefrontal and Cingulate regions

In the prefrontal and cingulate regions an overall effect of group was found (F (1, 10) =68.7, $p<0.001$) (Fig. 50). For all areas apart from the retrosplenial cortex the Cage Control group had significantly lower numbers of c-Fos positive cells than the Working Memory group (Aca $p=0.002$; PL $p=0.004$; IL= $p<0.001$).
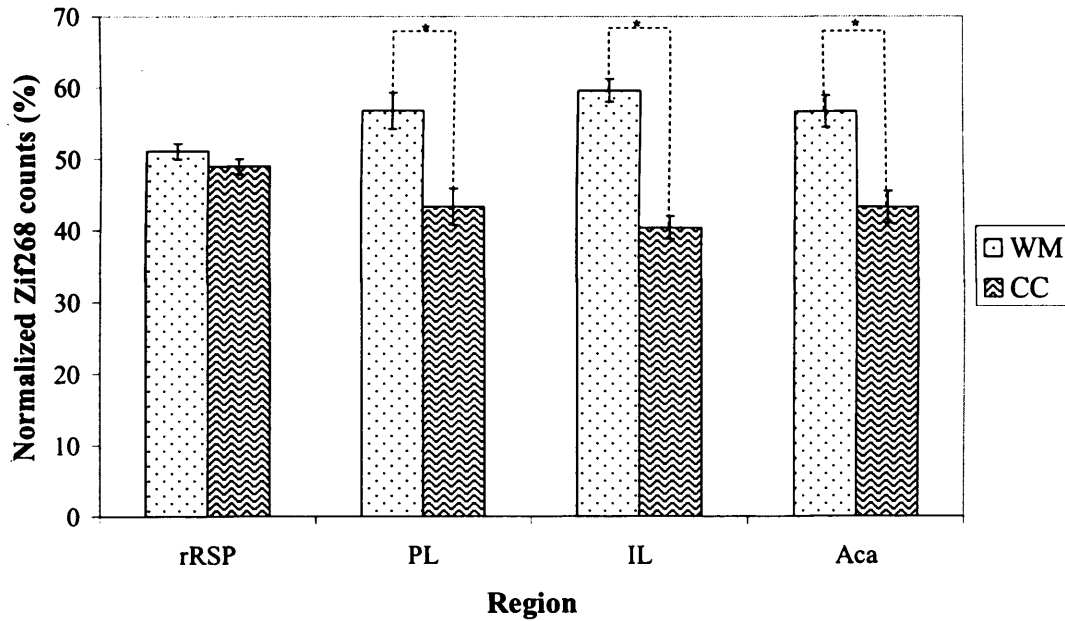
Figure 50
Normalized Zif268 counts for prefrontal and cingulate regions. An increase in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the prelimbic, infralimbic and anterior cingulate cortices. (rRSP-rostral reterosplenial cortex, PL-prelimbic cortex, IL-infralimbic cortex, Aca-anterior cingulate cortex)

Parahippocampal cortices

For the parahippocampal cortices there was no overall effect of group (F (1, 10) =3.8, p=0.080) but a group by area interaction was seen (F (4, 40) =10.1, p<0.001) (Fig. 51). Subsequent analysis revealed differences in the postrhinal cortex where the Working Memory group was higher than the Cage Control group (p=0.001) and in the perirhinal cortex where the Cage Control was higher than the Working Memory group (p=0.049).
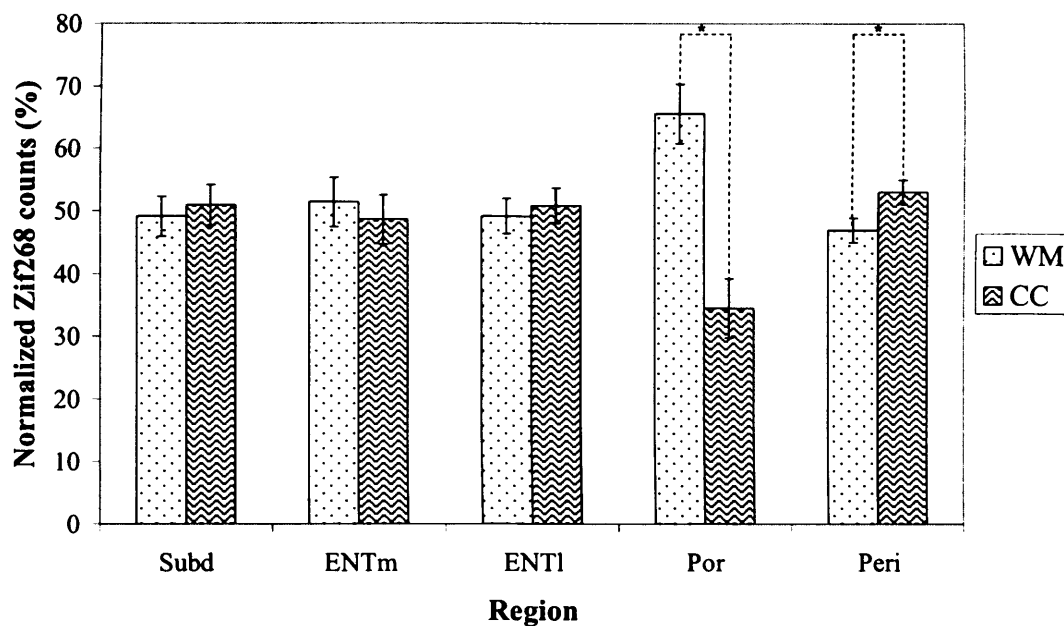
Figure 51
Normalized Zif268 counts for the parahippocampal regions. An increase in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the postrhinal cortex, a decrease in the Working Memory group was seen in the perirhinal cortex. (Subd-dorsal subiculum, ENTm-medail entrorhinal cortex, ENTl-lateral entrorhinal cortex, Por-postrhinal cortex, Peri-perirhinal cortex)

Hippocampal subfields

In the hippocampal subfields an overall effect of group was seen (F (1, 10) =8.04, p=0.018) (Fig. 52). The only significant differences were seen in CA3 where the Working Memory group was lower than the Cage Control group (p=0.009).
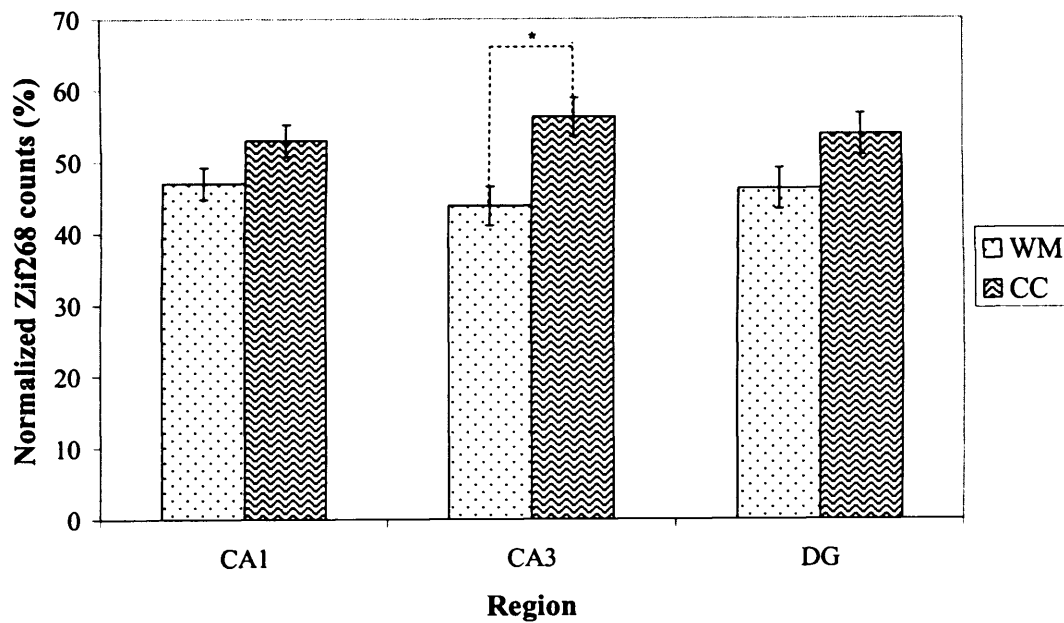
Figure 52
Normalized Zif268 counts for hippocampal subfields. A decrease in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the CA3 region. (DG-dentate gyrus)

	WM vs CC
ENTl	–
ENTm	–
Subd	–
Peri	↓
Por	↑
CA1	–
CA3	↓
DG	–
Aca	↑
PL	↑
IL	↑
rRSP	–
Bfd	↑
Ssp	↑
Mop	↑

Figure 53

Figure to show summary of gene changes for Zif268 between the Working Memory and Cage Control groups. An arrow indicates the direction of the effect and a — indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.4.3.3 c-Fos Counts

<u>Primary Motor, Visual and Somatosensory regions</u>

For the primary motor, visual somatosensory regions counted an overall effect of group was seen (F (1, 10) =849.7, $p<0.001$) as was a group by area interaction (F (2, 20) =26.5, $p<0.001$) (Fig. 54). Simple effects analysis showed that for all areas the Working Memory group was significantly higher than the Cage Control group (Ssp: $p<0.001$; Mop: $p<0.001$; Bfd: $p<0.001$).
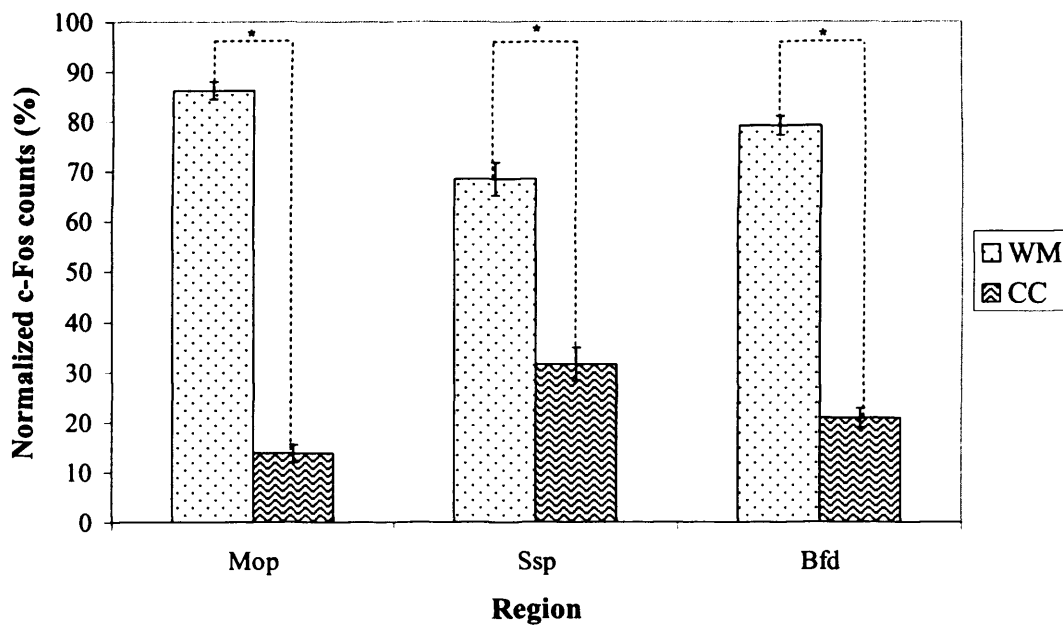
Figure 54

Normalized c-Fos counts for the primary, motor and somatosensory regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in all control regions. (Mop-motor cortex, Ssp-somatosensory cortex, Bfd-barrel cortex)

Prefrontal and Cingulate regions

In the prefrontal and cingulate regions an overall effect of group was found (F (1, 10) =297.1, $p<0.001$) as was a group by area interaction (F (3, 30) =24.4, $p<0.001$) (Fig. 55). For all areas the Cage Control group was significantly lower than the Working Memory group (rRSP $p<0.001$; Aca $p<0.001$; PL $p<0.001$; IL $p<0.001$).

Figure 55
Normalized c-Fos counts for prefrontal and cingulate regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in all prefrontal and cingulate regions. (rRSP-rostral reterosplenial cortex, PL-prelimbic cortex, IL-infralimbic cortex, Aca-anterior cingulate cortex)

Parahippocampal cortices

For the parahippocampal cortices there was an overall effect of group $(F\ (1,\ 10)$ $=211.5, p<0.001)$ and a group by area interaction was also seen $(F\ (4,\ 40) =29.2$, $p<0.001)$ (Fig. 56). For all areas apart from the dorsal subiculum the Cage Control group was significantly lower than the Working Memory group (ENTl $p<0.001$; ENTm $p<0.001$; Por $p<0.001$; Peri $p<0.001$).

Figure 56
Normalized c-Fos counts for the parahippocampal regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in all parahippocampal regions apart from the dorsal subiculum where no difference was seen. (Subd-dorsal subiculum, ENTm-medial entrorhinal cortex, ENTl-lateral entrorhinal cortex, Por-postrhinal cortex, Peri-perirhinal cortex)

Hippocampal subfields

In the hippocampal subfields an overall effect of group was seen (F (1, 10) =35.0, p<0.001) as was a group by area interaction (F (2, 20) =12.4, p<0.001) (Fig. 57). For the CA1 and dentate gyrus the Working Memory group was significantly higher than the Cage Control (CA1 p<0.001; DG p=0.004).
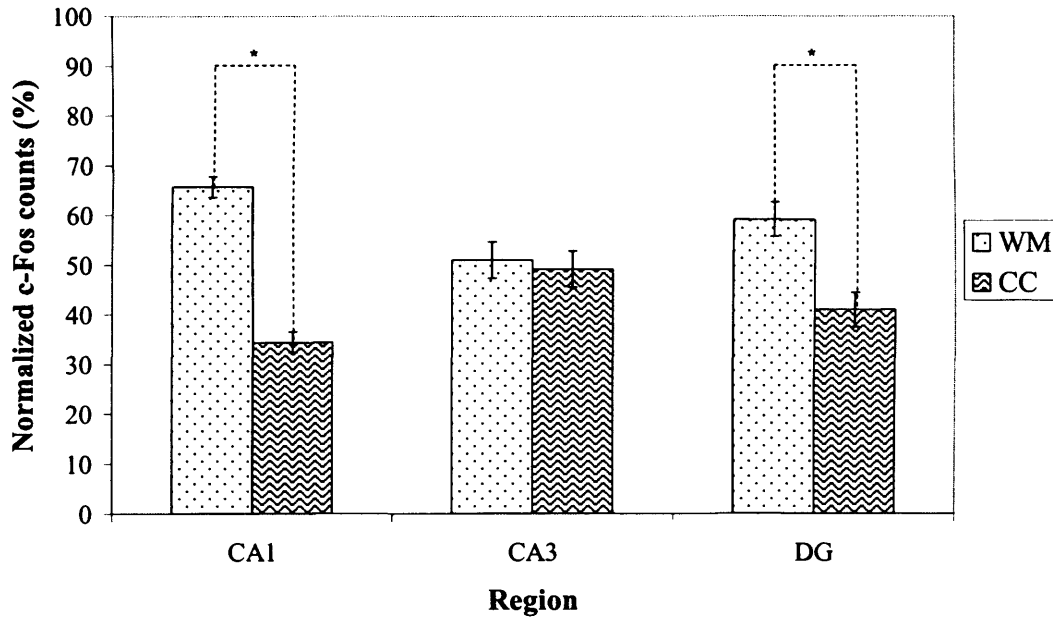
Figure 57
Normalized c-Fos counts for hippocampal subfields. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the CA1 region and the dentate gyrus. (DG-dentate gyrus)

	WM vs CC
ENTl	↑
ENTm	↑
Subd	—
Peri	↑
Por	↑
CA1	↑
CA3	—
DG	↑
Aca	↑
PL	↑
IL	↑
rRSP	↑
Bfd	↑
Ssp	↑
Mop	↑

Figure 58

- Figure to show summary of gene changes for c-Fos between the Working Memory and Cage Control groups. An arrow indicates the direction of the effect and a — indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.4.4 Discussion

As illustrated in nearly all regions for Zif268 and c-Fos comparison of the Working Memory group with a Cage Control group produces higher immunoreactive activation in the Working Memory group. This includes the sensorimotor areas where no differences would be expected to be seen if the 'control' group comparison was a good one. There was however decreases seen in the Working Memory group compared to the Cage Control in the CA3 region using Zif268 as a marker. This could have occurred because of a ceiling effect and that because of such a high basal level of Zif268 in this region saturation may have occurred. These results do show that as stated earlier although, in the case of Zif268, decreases in immunoreactive cells are seen in the Working memory group when compared to a 'tight' control this group do show increases in activation if compared with a Cage Control.

3.5 Experiment 3c

3.5.1 Introduction

An analysis of IEG immunoreactivity in a Free Swimming animal as compared to a Working Memory animal was also carried out. This comparison was carried out to see if an increased pattern of activation would be seen in the Free Swimming group due to stress factors as previously predicted.

3.5.2 Method

Subjects

Subjects were 12 male Dark Agouti rats (Harlan) weighing between 260g and 300g at end of testing. Rats were housed in pairs and kept on a 14/10 dark/light cycle. Food and water was available *ad libitum* throughout the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Apparatus

Apparatus was the same as Experiment two.

Procedure

Animals were split into 2 groups with six animals in group Working Memory (WM) and six in group Free Swim (FS).

Pretraining

Animals in the Working Memory group were pretrained as Experiment 3a.

Working Memory group

The Working Memory group was trained as in Experiment 3a.

Free swim

The Free Swim group was yoked to the performance of the Working Memory group. Animals were released from the same position around the pool and swam for the same

amount of time as the Working Memory group. No platform was present so the animal was removed from the pool by the experimenter when the trial had ended.

Immunohistochemistry

Immunohistochemistry was performed as in Experiment 3a.

Image Analysis

Image analysis was the same as Experiment 3a. Tables of raw cells counts can be seen in Appendix A Figure 5 for Zif268 and Figure 6 for c-Fos.

Regions of interest

Regions of interest were the same as Experiment 3a.

3.5.3 Results

3.5.3.1 Behavioural results

On the final day of testing, half of the animals performed a standard version of the working memory task in the water maze, whilst the others performed a free swim task. (Fig. 59). An overall effect of trial was found (F $(7, 70)$ = 61.0, p <0.001) with no overall effect of group (F $(1, 10)$ = 0.003, p=0.959).
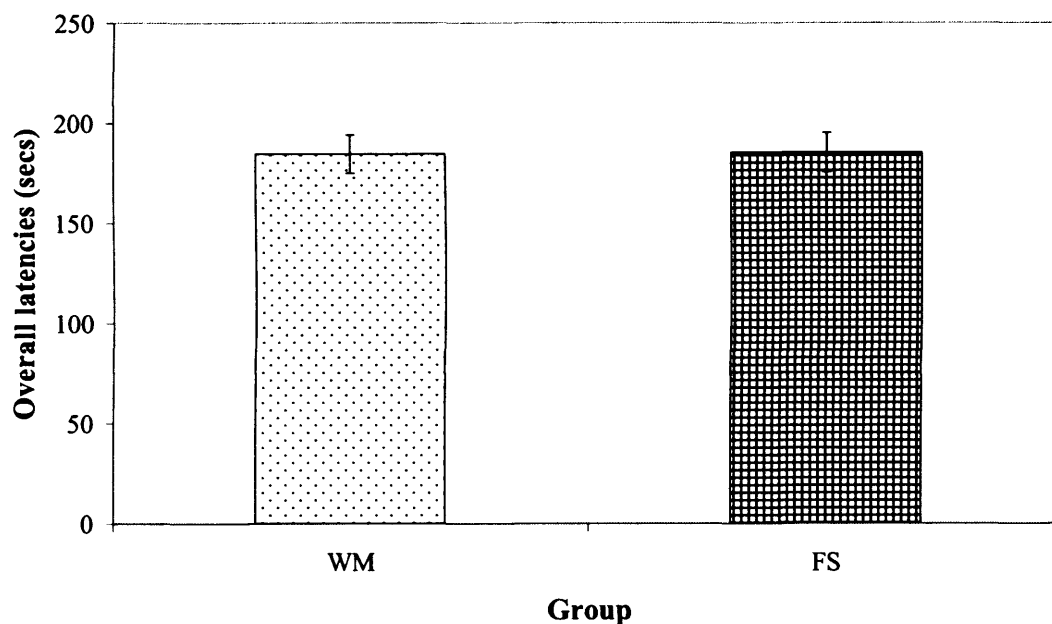
Figure 59

Latencies to find platform over all 8 trials on final test day for Working Memory (WM) and Free Swimming (FS) groups. No differences were seen between the two groups in the overall latencies to find the platform.

Analysis of the speed and distance the two groups travelled in the water maze was performed. Differences in the distance travelled over days was seen (F $(1,200)$ =26.554, p<0.001) with the Working Memory group travelling further than the Free Swimming group (Fig. 60). There was also an effect of day (F $(19,200)$ = 13.640, p<0.001) with no day by group interaction (F $(19,200)$ =0.565, p=0.927). A group effect was also seen in the speed the animals travelled (F $(1,200)$ =136.739, p<0.001) with the Free Swimming animals travelling slower than the Working Memory group (Fig. 61). An effect of day was also seen (F $(19,200)$ = 3.345, p<0.001) with no group by day interaction (F $(19,200)$ =1.143, p=0.313).
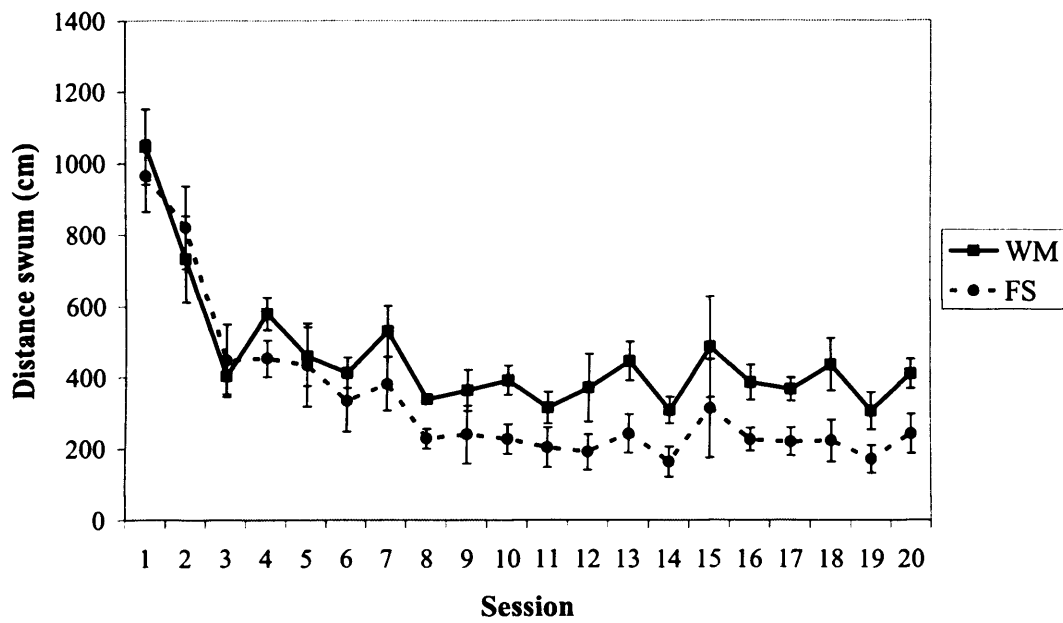
Figure 60
Distance swum over all sessions of training for the Working Memory (WM) and Free Swimming (FS) group. Animals in the Working Memory group swam a further distance as compared to those in the Free Swimming group towards the end of the training sessions.
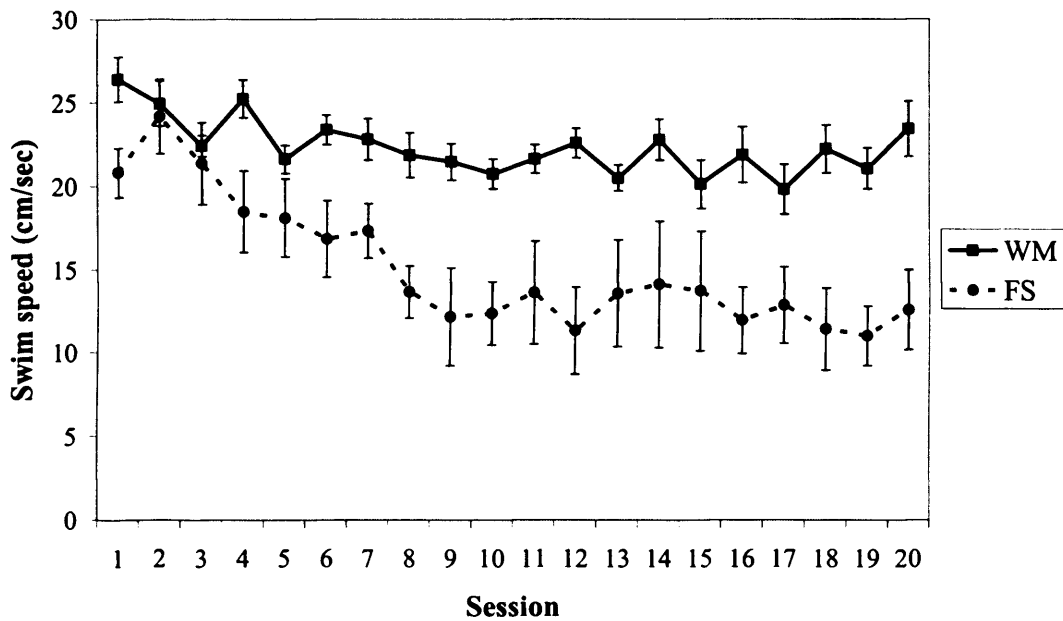
Figure 61
Animals speed over all sessions of training for the Working Memory (WM) and Free Swimming (FS) group. Animals in the Working Memory group swam at a faster speed as compared to those in the Free Swimming group towards the end of the training sessions.

3.5.3.2 Zif268 Counts

Examples of Zif268 immunoreactivity in a number of anatomical regions in the Working Memory, Cage Control and Free Swimming groups can be seen in Figures 62 and 64.

Primary motor, visual and somatosensory regions

For the primary motor, visual and somatosensory cortical regions counted no overall group effect was seen (F $(1, 10)$ =0.002, p=0.970) (Fig. 65).
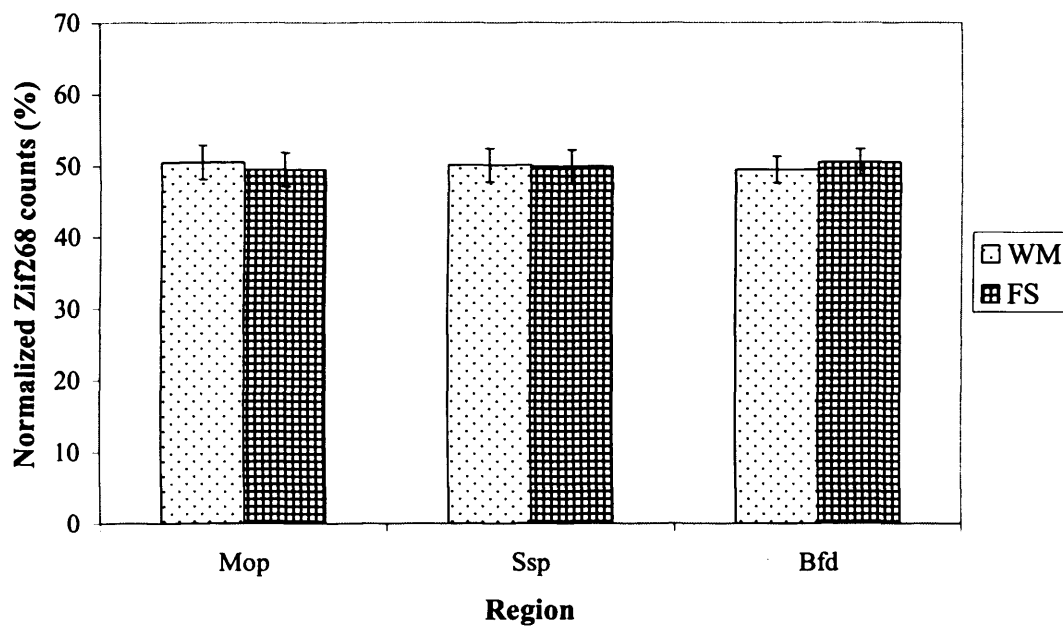
Figure 65
Normalized Zif268 counts for the primary, motor and somatosensory regions. (Mop-motor cortex, Ssp-somatosensory cortex, Bfd-barrel cortex).

WM
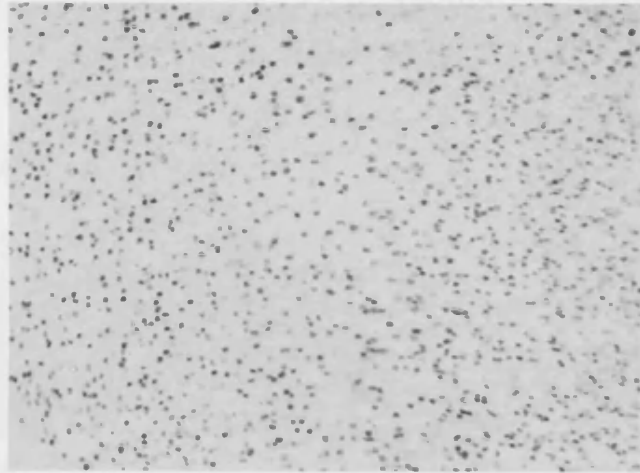
CC
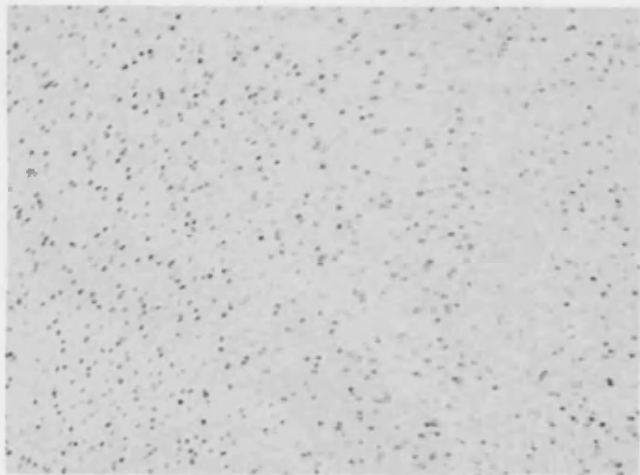
FS
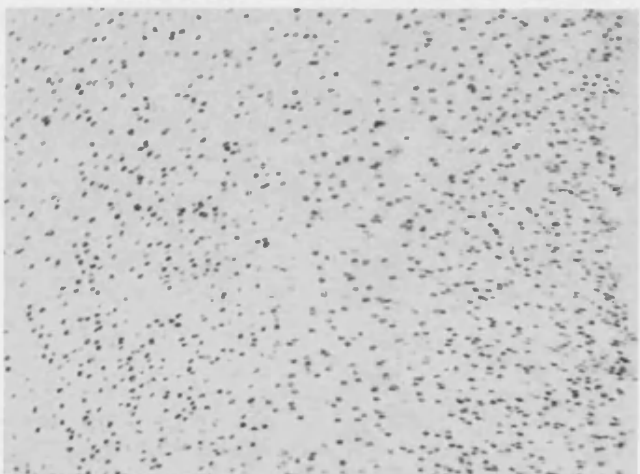
Figure 62
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), Cage
Control (CC) and Free Swimming (FS) groups in the anterior cingulate cortex.

WM
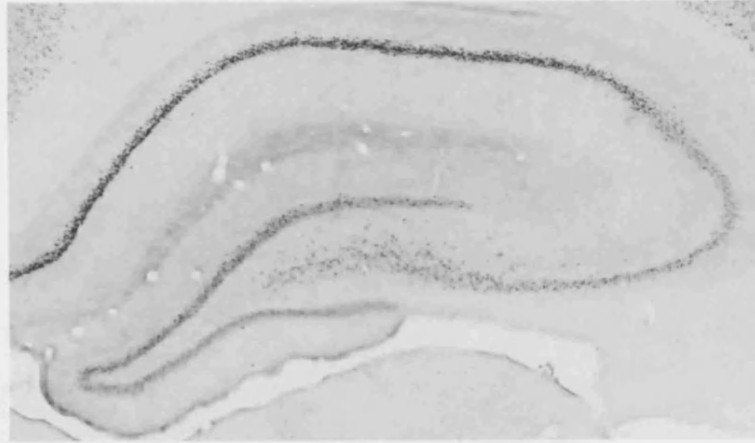
CC
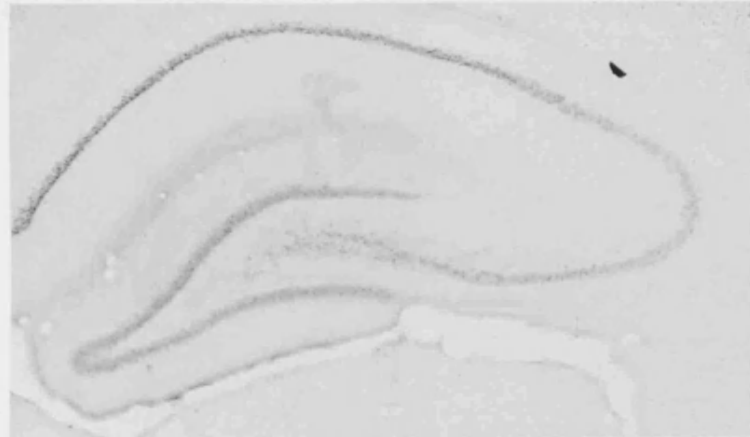
FS
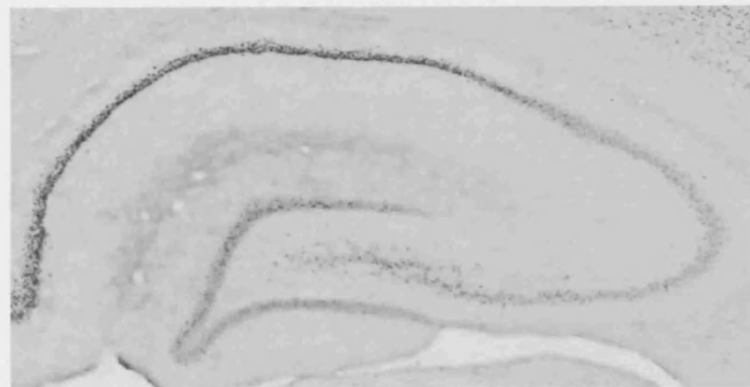
Figure 63
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), Cage Control (CC) and Free Swimming (FS) groups in the hippocampus.

112

WM
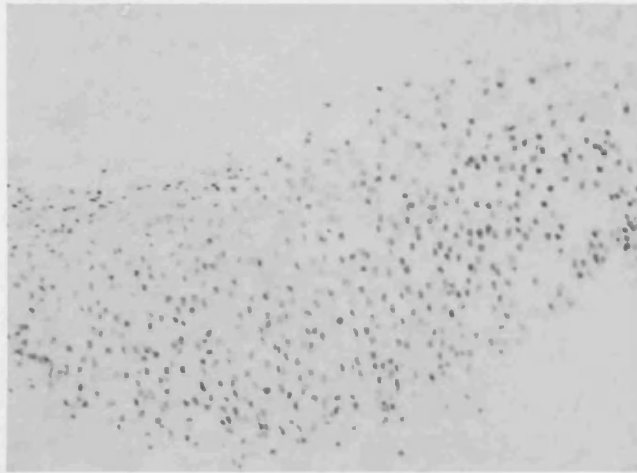
CC
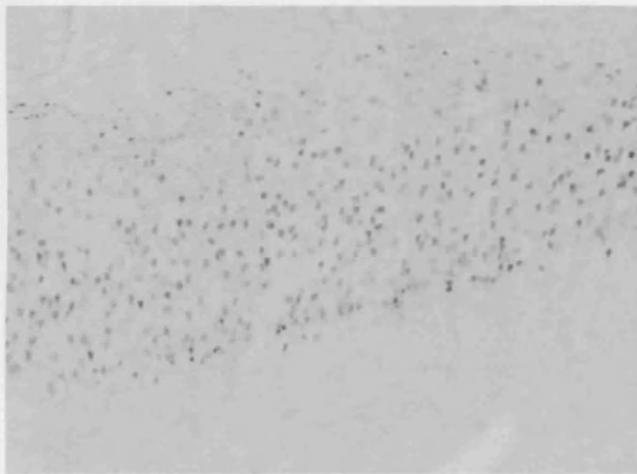
FS
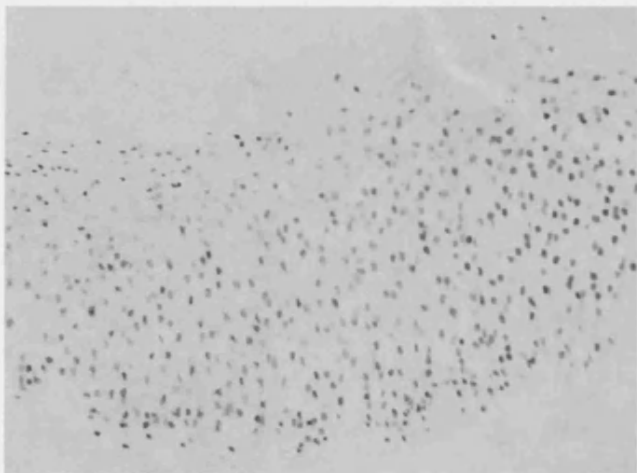
Figure 64
Figure depicting Zif268 immunoreactivity in the Working Memory (WM), Cage
Control (CC) and Free Swimming (FS) groups in the dorsal subiculum.

Prefrontal and Cingulate regions

In the prefrontal and cingulate regions an overall effect of group was found (F (1, 10) =78.2, $p<0.001$) and a group by area interaction was also seen (F (3, 30) = 4.9, $p=0.007$) (Fig. 66). For the retrosplenial cortex and prelimbic cortex the Zif268 counts were lower in the Working Memory group (rRSP $p<0.001$; PL $p=0.001$).
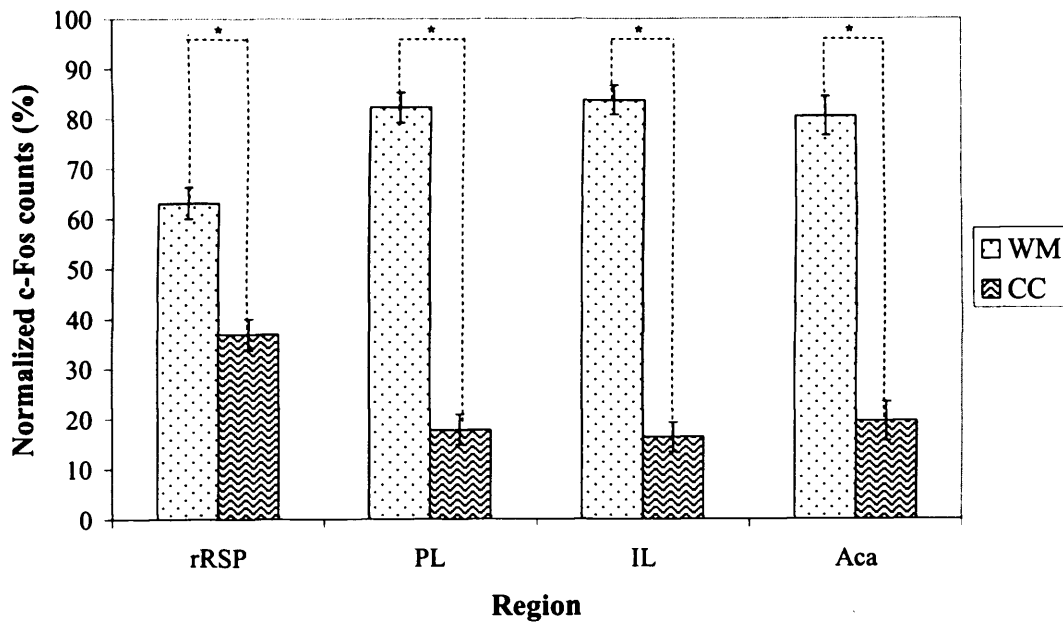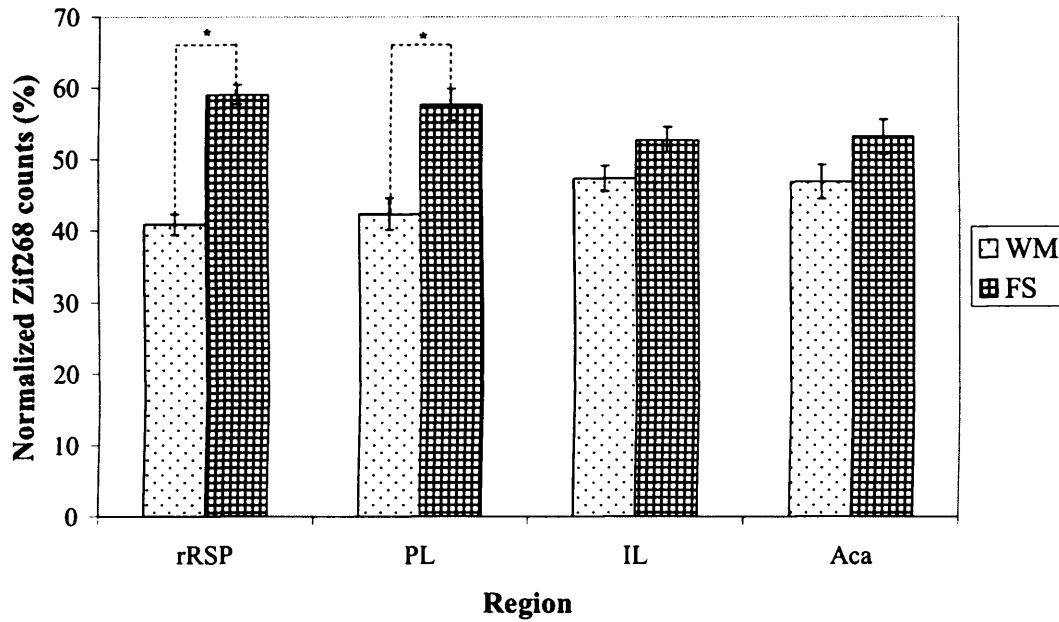

Figure 66
Normalized Zif268 counts for prefrontal and cingulate regions. A decrease in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Free Swimming group in the reterosplenial cortex and the prelimbic cortex. (rRSP-rostral reterosplenial cortex, PL-prelimbic cortex, IL-infralimbic cortex, Aca-anterior cingulate cortex).

Parahippocampal cortices

For the parahippocampal cortices there was an overall effect of group (F (1, 10) =53.5, $p<0.001$) and a group by area interaction was also seen (F (4, 40) = 3.6, $p=0.013$) (Fig. 67). For all areas apart from postrhinal cortex the Working Memory group was lower than the Free Swim group (ENTl $p=0.003$; ENTm $p=0.021$; Subd $p<0.001$, Peri $p=0.029$).
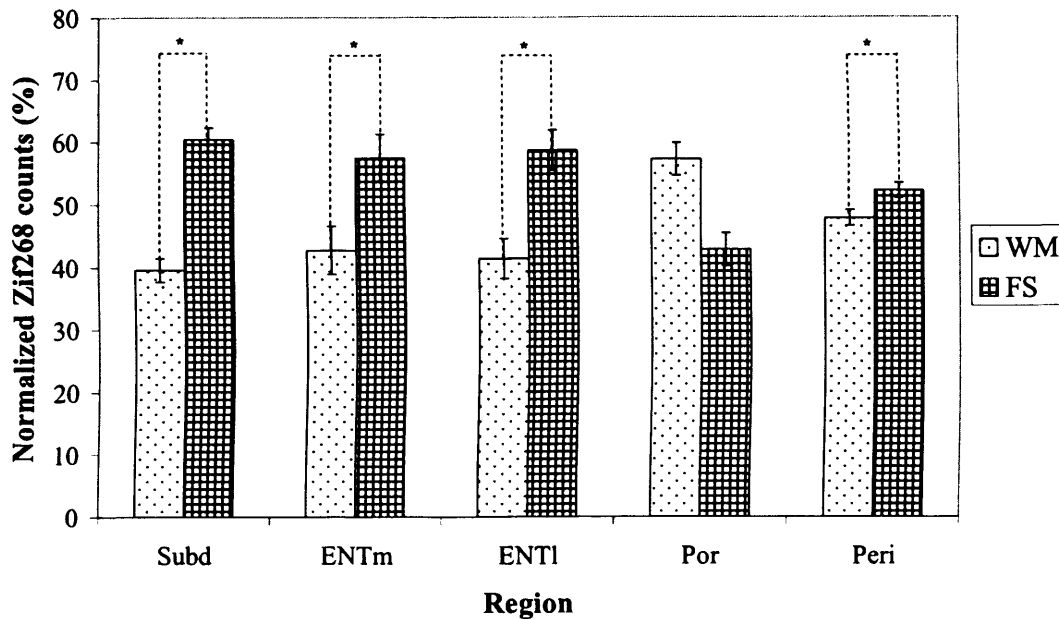
Figure 67
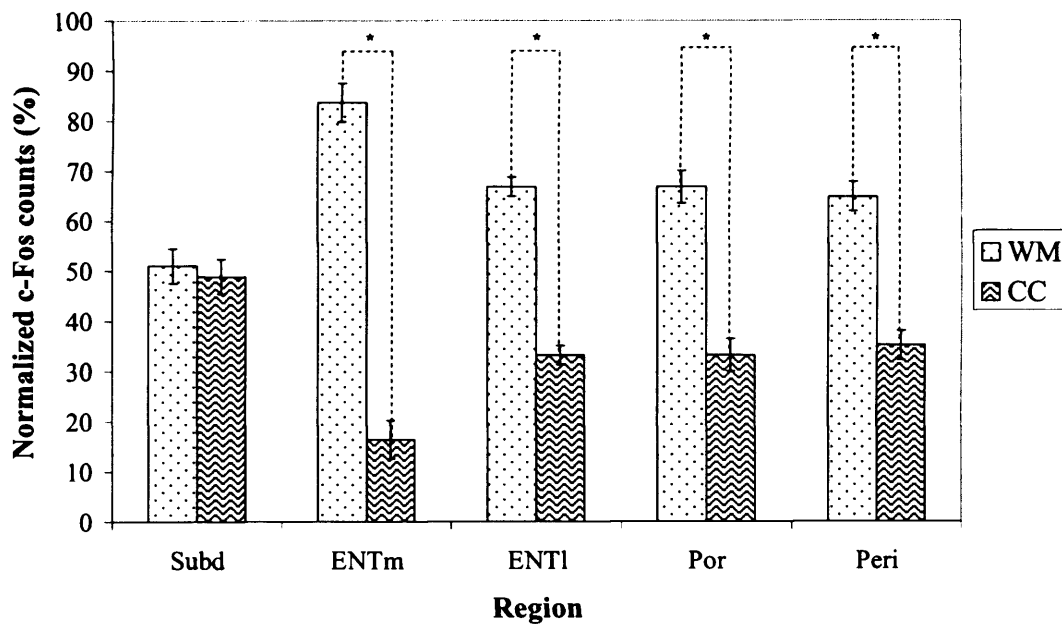Normalized Zif268 counts for the parahippocampal region. A decrease in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Free Swimming group in the dorsal subiculum, lateral and medial entrorhinal cortices and the perirhinal cortex. (Subd-dorsal subiculum, ENTm-medial entorhinal cortex, ENTl-lateral entorhinal cortex, Por-postrhinal cortex, Peri-perirhinal cortex).

Hippocampal subfields

In the hippocampal subfields an overall effect of group was seen (F (1, 10) =31.7, $p<0.001$) and a group by area interaction was also seen (F (2, 20) = 4.4, $p=0.027$) (Fig. 68). The cell counts of the Working Memory group were significantly lower than the Free Swim group in the CA1 and CA3 regions (CA1 $p=0.003$; CA3 $p<0.001$).
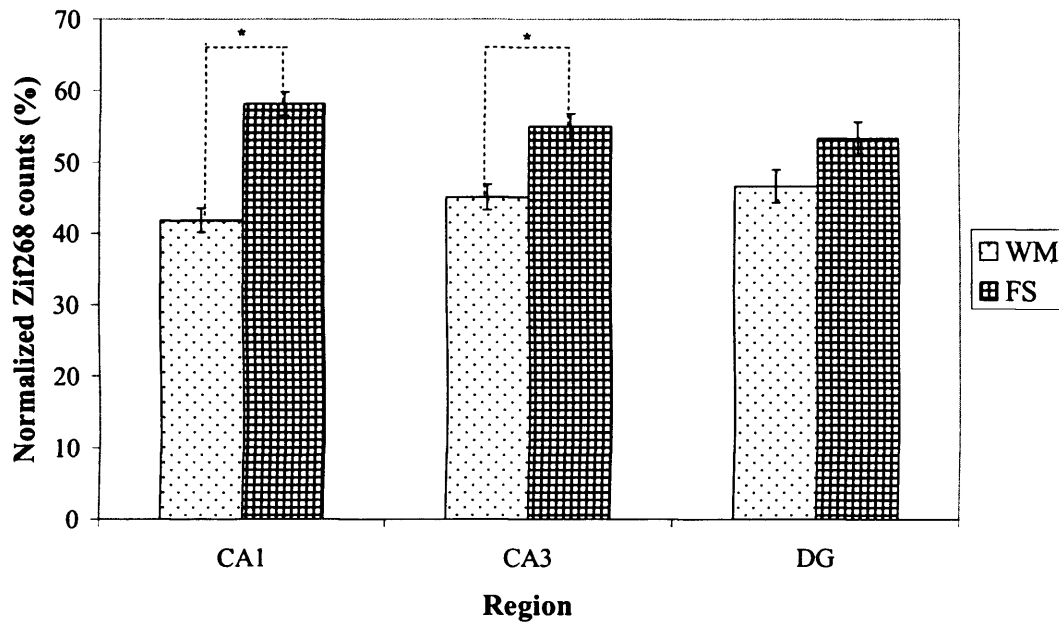
Figure 68
Normalized Zif268 counts for hippocampal subfields. A decrease in Zif268 immunoreactivity was seen in the Working Memory group as compared to the Free Swimming group in the CA1 and CA3 regions. (DG-dentate gyrus).

116

	WM vs FS
ENTl	↓
ENTm	↓
Subd	↓
Peri	↓
Por	—
CA1	↓
CA3	↓
DG	—
Aca	—
PL	↓
IL	—
rRSP	↓
Bfd	—
Ssp	—
Mop	—

Figure 69

Figure to show summary of gene changes for Zif268 between the Working Memory and Free Swim groups. An arrow indicates the direction of the effect ($p<0.05$) and a — indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.5.3.3 c-Fos counts

Examples of c-Fos immunoreactivity in a number of anatomical regions in the
Working Memory, Cage Control and Free Swimming groups can be seen in Figures
70 and 72.

<u>Primary motor, visual and somatosensory regions</u>

For the primary motor, visual and somatosensory regions counted no overall effect of
group was seen (F $(1, 10)$ =0.47, p=0.833) but there was a group by area interaction (F
$(2, 20)$ =7.7, p=0.003) (Fig. 73). Subsequent analysis revealed that differences were
seen in the motor cortex, where the cell counts of the Working Memory group were
higher than the Free Swim (p=0.018), and in the barrel fields where the Free Swim
group was higher than the Working Memory group (p=0.041).
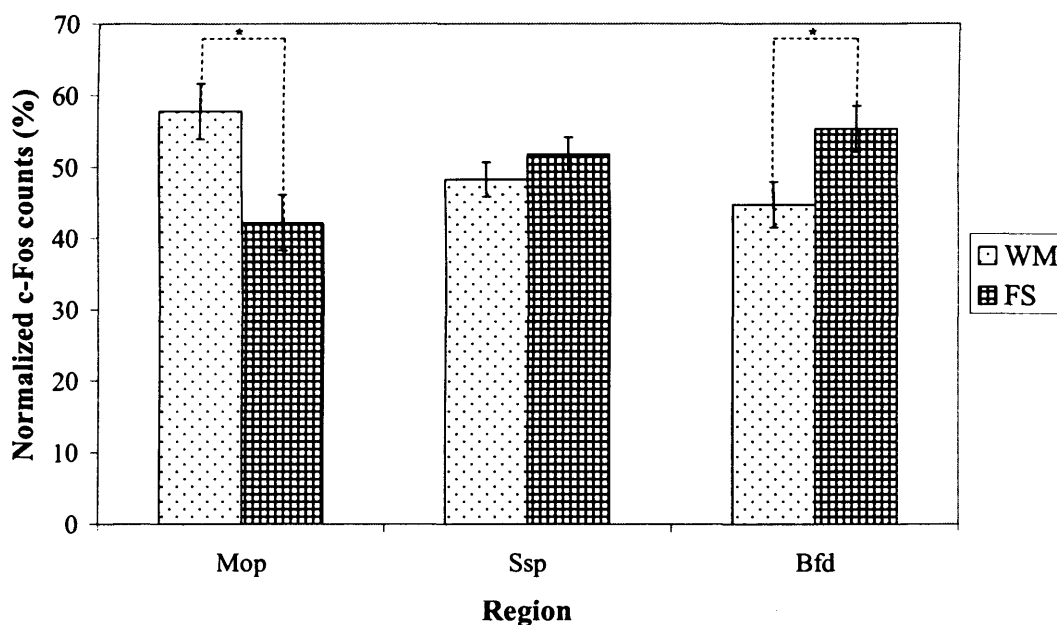
Figure 73
Normalized c-Fos counts for the primary, motor and somatosensory regions. An increase in c-
Fos immunoreactivity was seen in the Working Memory group as compared to the Cage
Control group in the motor and barrel cortices. (Mop-motor cortex, Ssp-somatosensory cortex,
Bfd-barrel cortex)

WM
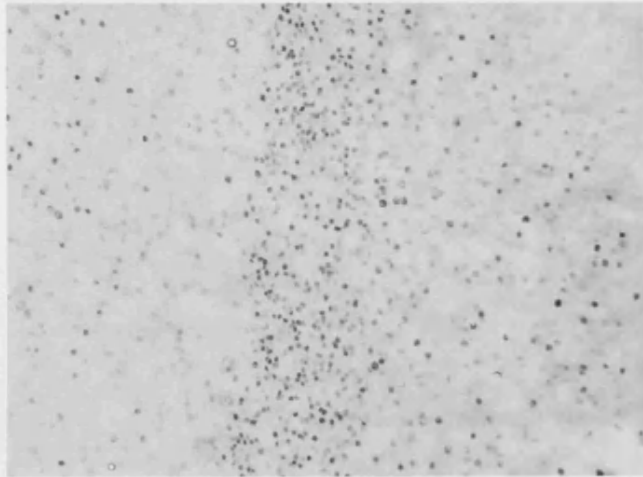
CC
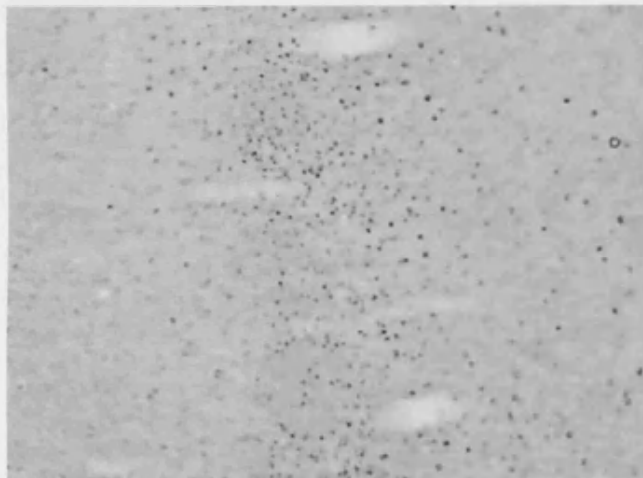
FS
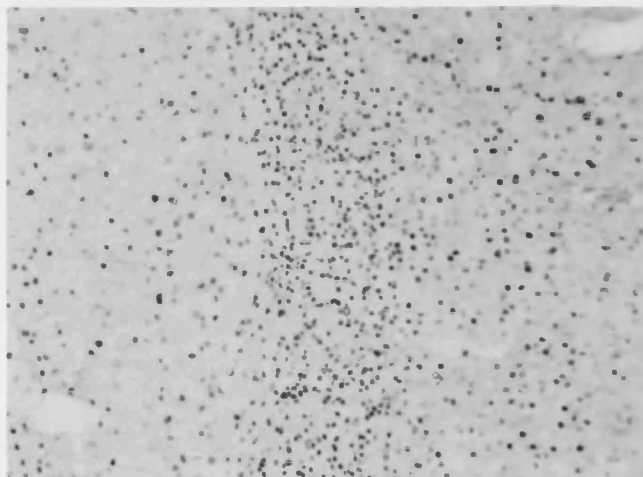
Figure 70
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Cage Control
(CC) and Free Swimming (FS) groups in the somatosensory cortex.

119

WM
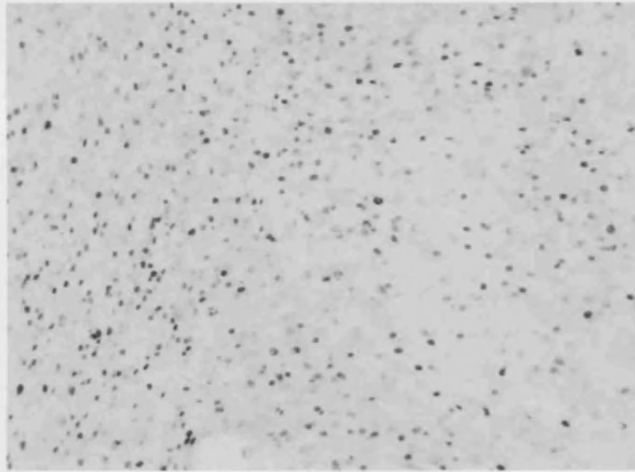
CC
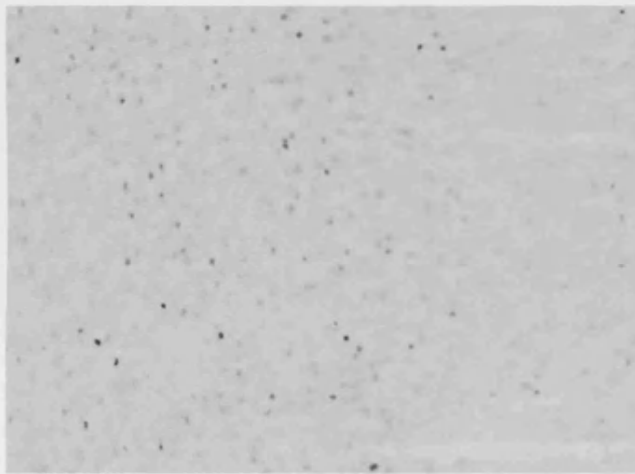
FS
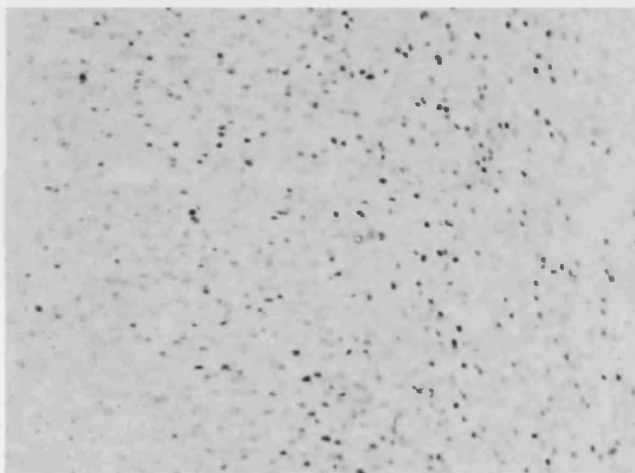
Figure 71
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Cage Control
(CC) and Free Swimming (FS) groups in the infralimbic cortex.

WM
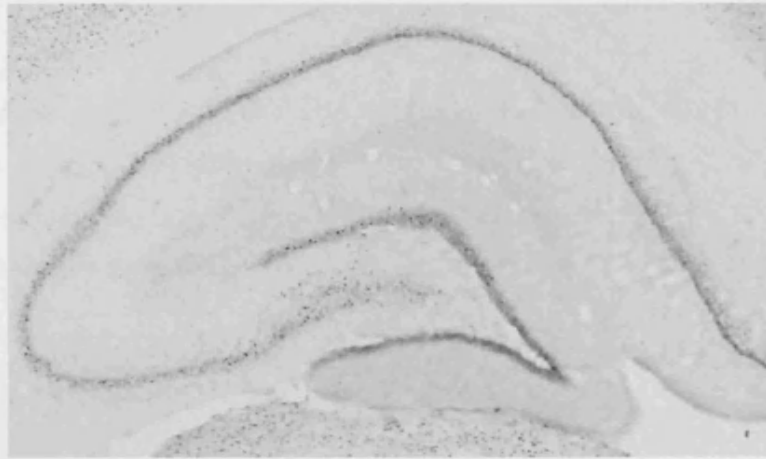
CC
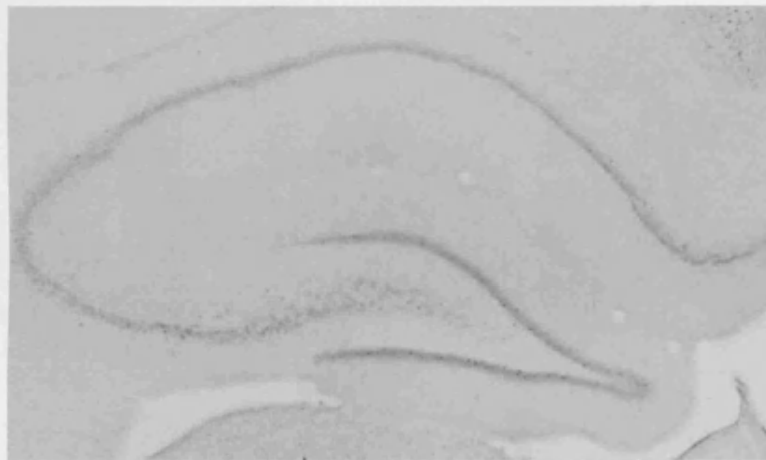
FS
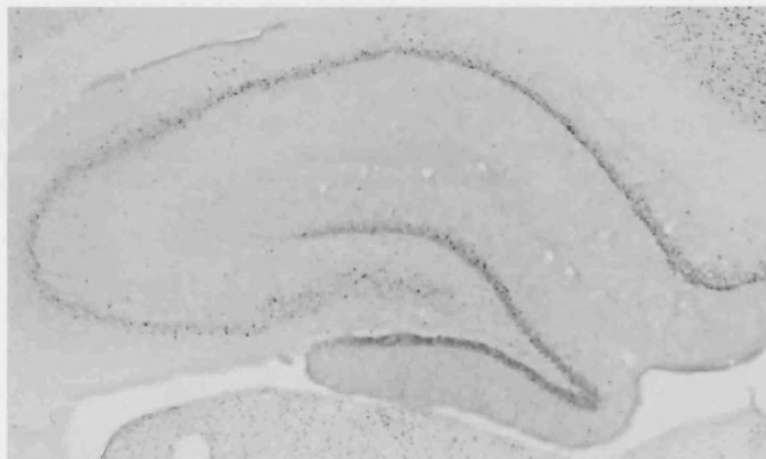
Figure 72
Figure depicting c-Fos immunoreactivity in the Working Memory (WM), Cage Control (CC) and Free Swimming (FS) groups in the hippocampus.

Prefrontal and Cingulate regions

In the prefrontal and cingulate regions an overall effect of group was found (F (1, 10) =45.0, $p<0.001$) as was a group by area interaction (F3, 30) =19.1, $p<0.001$) (Fig. 74). For the infralimbic and prelimbic cortices the Free Swim group was significantly lower than the Working Memory group (IL $p<0.001$; PL $p<0.001$).
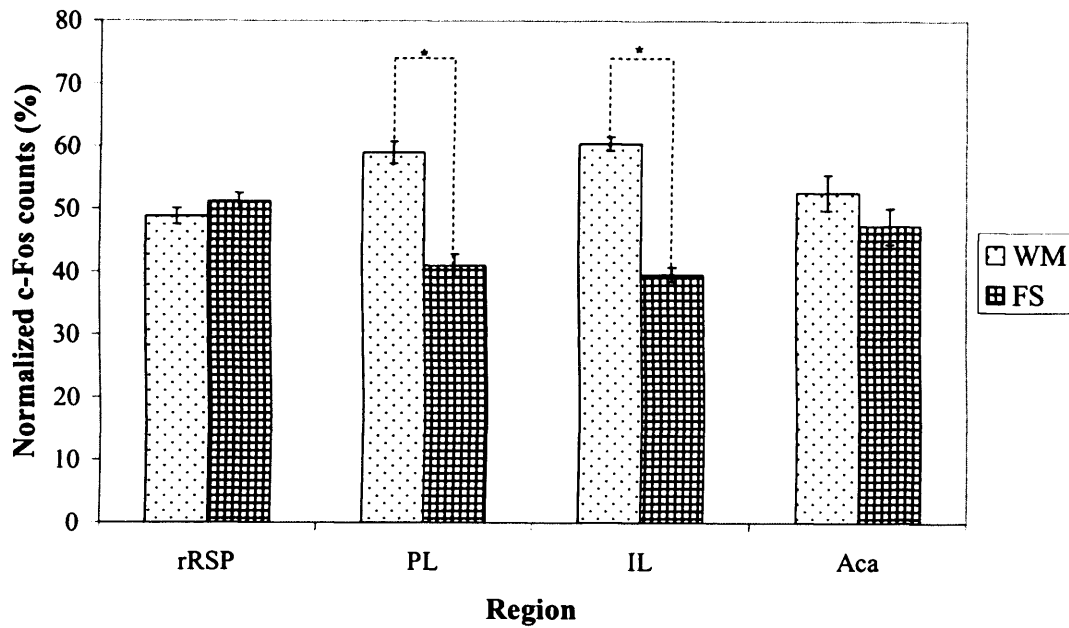
Figure 74
Normalized c-Fos counts for prefrontal and cingulate regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the prelimbic and infralimbic cortices. (rRSP-rostral reterosplenial cortex, PL-prelimbic cortex, IL-infralimbic cortex, Aca-anterior cingulate cortex)

Parahippocampal cortices

For the parahippocampal cortices there was an overall effect of group (F (1, 10) =18.4, p=0.002) as well as a group by area interaction (F (4, 40) =11.6, p<0.001) (Fig. 75). For the dorsal subiculum, lateral entorhinal cortex and perirhinal cortex the counts in Working Memory group were significantly lower than the Free Swim group (Subd p=0.001; ENTl p=0.001; Peri p=0.001). For the medial entorhinal cortex the Working Memory was higher than the Free Swim group (p=0.011).
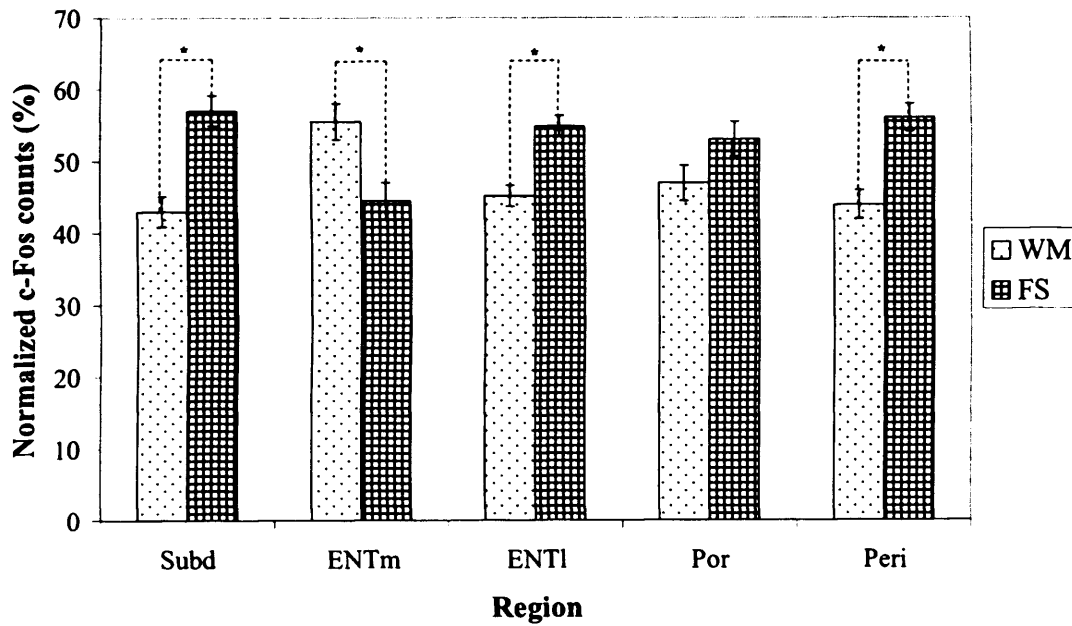
Figure 75
Normalized c-Fos counts for the parahippocampal regions. An increase in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the medial entorhinal cortex. A decrease in activity in the Working Memory group was seen in the dorsal subiculum, lateral entorhinal and perirhinal cortices. (Subd-dorsal subiculum, ENTm-medial entorhinal cortex, ENTl-lateral entorhinal cortex, Por-postrhinal cortex, Peri-perirhinal cortex)

Hippocampal subfields

In the hippocampal subfields no overall effect of group was seen (F $(1, 10)$ =0.46, p=0.835) but an area by group interaction was found (F $(2, 20)$ = 145.608, p=0.036) (Fig. 76). Subsequent analysis revealed a significant increase in c-Fos cells in the Free Swim group in the dentate gyrus (p=0.035).
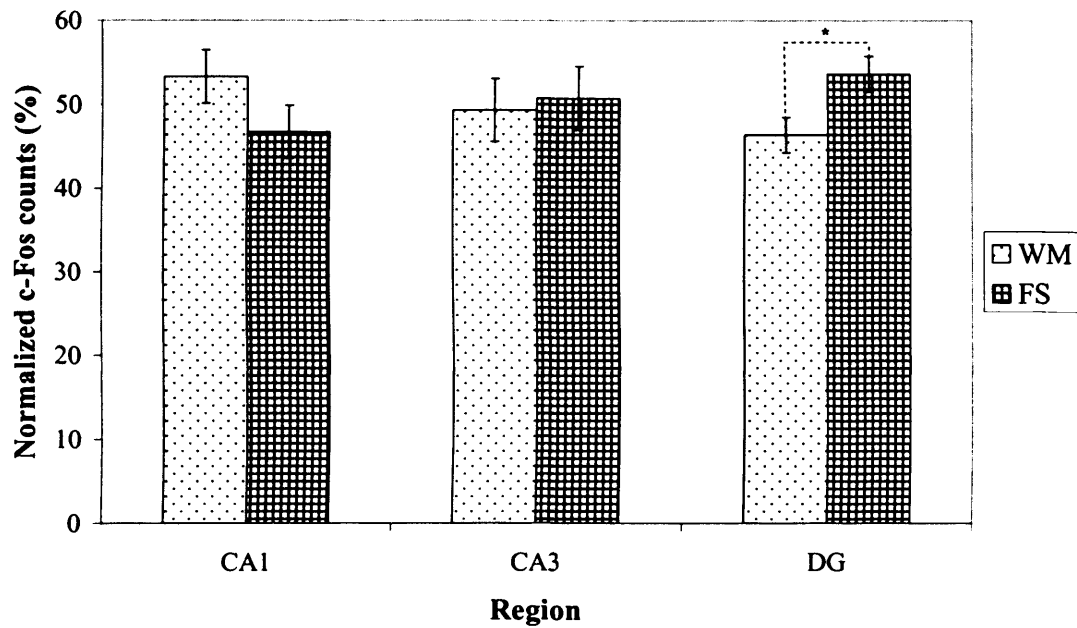
Figure 76
Normalized c-Fos counts for hippocampal subfields. An decrease in c-Fos immunoreactivity was seen in the Working Memory group as compared to the Cage Control group in the dentate gyrus. (DG-dentate gyrus)

124

	WM vs FS
ENTl	↓
ENTm	↑
Subd	↓
Peri	↓
Por	–
CA1	–
CA3	–
DG	↓
Aca	–
PL	↑
IL	↑
rRSP	↓
Bfd	↓
Ssp	–
Mop	↑

Figure 77

Figure to show summary of gene changes for c-Fos between the Working Memory and Free Swim groups. An arrow indicates the direction of the effect (p<0.05) and a — indicates no effect. (PL-prelimbic coretex, IL-infralimbic cortex, Aca-anterior cingulate cortex, Mop-motor cortex, Ssp-somatosensory cortex, rRSP-rostral reterosplenial cortex, DG-dentate gyrus, Bfd-barrel cortex, Peri-perirhinal cortex, ENTl-lateral entorhinal cortex, ENTm-medial entrorhinal cortex, Subd-dorsal subiculum, Por-postrhinal cortex)

3.5.4 Discussion

For Zif268 Free Swimming animals do show a greater amount of activation in many brain regions as compared to the Working Memory group. In fact apart from the postrhinal cortex and the 'control' regions every other region shows an increase in activation in the Free Swimming most of which are statistically significant. For c-Fos the results differ somewhat with some regions showing an increase in c-Fos positive cells in the Working Memory group as compared to Free Swim (pre and infra limbic and medial entorhinal cortex). Other regions showed a decrease in immunoreactive cells in the Working Memory group (lateral entorhinal cortex, dorsal subiculum, perirhinal cortex and rostral retrosplenial cortex). Although these results reflect somewhat those seen in experiment two and three a, as regards decreases in Zif268

protein in the Working Memory group, because of the nature of the Free Swimming task it is difficult to attribute these changes to the learning aspects of the task. The Free Swim task has been shown to be stressful and that stress can increase immediate early gene levels (see 3.1.6). A further experiment would be required to check stress hormone levels to determine if differences are seen between the Working Memory and Free Swimming groups.

3.6 Discussion

Comparisons between animals performing a working memory task and two 'control' tasks in the water maze produced interesting results. Results for c-Fos immunoreactivity were similar to previous research. Increases were seen in the Working Memory group in prefrontal areas (anterior cingulate, infralimbic and prelimbic). However Zif268 immunoreactivity produced some unusual results. Decreases were seen in the Working Memory group in a number of areas including the hippocampal subfields. Although seeing a reduction in IEGs in the experimental group is unusual there is an emerging body of evidence that show similar results.

3.6.1 Decreases in Zif268 expression in the Working Memory condition

3.6.1.1 Experiment 2

In Experiment two a number of brain regions showed decreases in Zif268 immunoreactivity in the Working Memory group as compared to the Procedural Task group. These included prefrontal regions (prelimbic, anterior cingulate cortex), all hippocampal subfields and some parahippocampal regions (subiculum, postrhinal cortex, lateral entorhinal cortex). These are all regions that have previously shown increases in immediate early gene studies of spatial working memory or areas that have been shown to be implicated using lesion studies. As was expected no differences were seen in the primary motor and somatosensory regions.

3.6.1.2 Experiment 3a

Again in Experiment three a as in Experiment two decreased Zif268 counts in the Working Memory group was seen as compared to the two Procedural Task groups. In all regions bar one (CA3), the decreases were seen in the comparisons between the 13cmPT and Working memory groups or between both Procedural Task groups and the Working Memory group. Significant Zif268 count decreases were seen in the hippocampus (CA1, dentate gyrus) and the parahippocampal regions (medial entorhinal cortex, perirhinal cortex, subiculum). No differences were seen in the prefrontal and cingulate regions.

127

3.6.2 Increases in c-Fos expression in the Working Memory condition

In experiment 3a c-Fos expression was also studied. Increases were seen in a number of regions, which is consistent with past research (Vann et al., 2000). These increases were mainly confined to the prefrontal areas (infralimbic, prelimbic, anterior cingulate cortex). Increases were also seen in lateral entorhinal cortex and in the motor cortex. Interestingly the hippocampal subfields showed a trend towards an overall decrease in the Working Memory group which approached statistical significance. This replicates the results found with Zif268.

3.6.3 Use of Cage Control and Free Swimming animals as controls

Comparisons of the Working Memory group with Cage Control animals produced a huge number of areas that show increases in activation of both Zif268 and c-Fos. These encompassed all different subdivision of brain areas. This shows as previously suggested that almost any experimental group compared with a cage control group may show IEG increases. This is an especially pertinent point because comparison of the Working Memory group with a very stringent 'control' does in fact show decreases in the case of Zif268 activation.

Comparisons with a Free Swimming group produced decreases of Zif268 immunoreactivity in the Working Memory group in many brain regions. Changes in c-Fos were also seen but with some regions showing increases in protein positive cells in the Working Memory group and some showing decreases. However, the nature of the Free Swimming task dictates that to accept that the decreases seen in the Working Memory group are not a function of increases in immunoreactivity in the Free Swimming group, which could be attributable to stress factors as previously suggested, (see 3.1.6) further research needs to be carried out.

The differences seen in the Cage Control group as compared to the Working Memory task has been shown previously. Guzowski et al. (2001) showed higher activation in the dorsal hippocampus after a water maze training as compared to cage control. Teather et al. (2005) showed the same result after spatial training in the water maze with increased c-Fos activation in the dorsal hippocampus. These authors also compared animals performing a spatial task with those free swimming and again saw increases in protein in the dorsal hippocampus after the experimental task. A comparison with free swimming animals was also tested by Santin et al. (2003) who found increases in the medial mamillary nucleus after a working memory task.

Although the results of the current experiment as regards cage control comparisons does reflect what has been shown previously the results obtained from free swimming comparisons are opposite to past studies. In both past experiments where free swimming animals have been used the animals were tested over a much shorter time frame than that used in the current experiment. One group were trained over one session of eight trials (Teather et al., 2005) and the other for two trials a day for eight sessions (Santin et al., 2003). In the current experiment animals were tested for eight trials a day for twenty sessions. Also the described studies only focused on a small number of brain regions it is possible that if a larger number were sampled the patterns of expression seen in the current experiment would occur.

3.6.4 Decreases in IEG expression seen in previous studies

Although it was predicted on past research that increases in immediate early gene activation would be seen in the Working Memory group compared to the Procedural Task groups, decreases were seen in a number of regions including the hippocampus. There is some evidence, as discussed below, that decreases in immediate early gene activation can be seen in the experimental group in some circumstances.

<u>3.6.4.1 Decreases in spatial learning paradigms</u>

He, Yamada, Nakajima et al. (2002) utilized different tasks within the radial arm maze to study *c-fos* activation, with both intra maze and extra maze cues present. Group FPRT (Fixed position of reward task) has the same 4 arms baited for each trial with the 4 other arms never being baited. In group VPRT (Variable position of reward task) again 4 arms were baited but they were baited in a pseudo- random fashion for each trial – with this task seen as a control for the working memory task. Each group was also tested with the maze rotated 180° and extra maze cues moved so the baited arms were in different spatial locations. Animals were processed for immunohistochemistry on days 1, 3 and 5 of training. For the FPRT group removal of the intra maze cues had no effect but changing the location of the extra maze cues inhibited performance. For the VPRT group rearrangement of either cues had no effect. This indicates that the VPRT group are not using spatial cues to find the baited arms and led He, Yamada, Nakajima et al. (2002) to state that the VPRT group would not be using spatial localization but may be depending on body orientation or another

stereotypic response. Increased activation was seen in the VPRT group compared to the FPRT group on day 3 in CA2/3 and the dentate gyrus. He, Yamada, Nakajima et al. (2002) explained this result by saying that the different subfields were used for the different tasks.

3.6.4.2 Decreases in recent and remote memory

Decreases have also been seen in experiments that study recent and remote memories. Maviel et al. (2004) looked at retention in a spatial task in mice using a five arm maze. Retention was tested 2 or 30 days after training and animals were compared with controls that were exposed to the same stimuli but were not confronted with arm choices during testing. An increase in Zif268 activation was seen in cortical areas between recent and remote memories. In the hippocampal and parahippocampal areas a decrease in Zif268 immunoreactivity was seen between recent and remote memory. This was thought to occur because the hippocampus was becoming disengaged as information was sent for long term storage in the neocortex. To illustrate this Maviel et al. (2004) also looked at GAP-43 which is a protein that controls axon growth. They found an increase at 30 days compared to 2 days in anterior cingulate and prefrontal cortex which correlated with Zif268 activation. This suggests that the Zif268 changes could be attributed to synaptogenesis and indeed that the Zif268 increase in the neocortex is because of rearrangement to store new information.

3.6.4.3 Decreases with extended training

A number of studies suggest that IEGs are activated upon the first learning experience and that declines with extended training. Guzowski et al. (2001) compared IEG mRNA activation on day 1 of spatial training in the water maze with day 7. They found decreases in zif268, c-fos and arc on day 7, this was attributed to the rats on the 7th session having learnt the task to a greater degree and therefore having more direct swim paths and being less attentive to the surrounding cues. Kelly & Deadwyler (2003) also displayed decreases in IEG expression between animals which were newly trained and those who had experienced extended training using a paradigm where animals had to lever press for water reward. Arc mRNA activation was compared in animals trained to asymptote with those newly trained, with higher activation in the newly trained animals seen in infra/prelimbic, somatosensory, perirhinal, entorhinal,

subiculum, CA1 and CA3 regions. In the newly trained group the number of lever presses showed a negative correlation with *arc* levels in the anterior cingulate, limbic cortex, CA1/3, subiculum, entorhinal and perirhinal cortices. It was also found that animals that were slower to acquire the task on the day of acquisition showed higher *arc* mRNA. This led the authors to state that *arc* is important in the plastic processes related to new behaviours. However, Guzowski et al. (2001) also correlated IEG activation with behaviour and found the opposite result. There was a significant negative correlation between *arc* levels and mean escape latencies, the best spatial learners tended to have the highest levels of *arc*. This issue was addressed by Kelly and Deadwyler (2002) where they suggested that if Guzowski et al. (2001) had looked at early training trials rather than after the animal's behaviour had stabilized their results may have been replicated. This indeed is the case as analysis from experiment three a show positive correlations between behaviour and Zif268 levels before the animals reach asymptote and negative correlations after that point.

Passino et al. (2002) looked at the comparison between place and response learning by using different strains of mice that favour each behavioural response. C57 mice are more accomplished at hippocampally dependent spatial tasks and are seen as "place learners". DBA mice are better at tasks that require them to form a simple S-R response and are "response learners". Using a plus maze, animals were trained to move from a start point in the south arm to retrieve food from the east arm. At all times the north arm was blocked. Animals were trained for 8 or 17 days, with probe trials given on the 9th or 18th day respectively. On a probe trial animals were released from the north arm, with south arm blocked, and were observed to see if they displayed a place (east arm) or response (west arm) strategy. A control group of animals was included which continued to be released from the south arm on probe trials. For the C57 mice c-Fos expression at the 1st probe was significantly higher in the experimental group as compared to control in all hippocampal subfields. At the 2nd probe in DG and CA3 this difference no longer existed. For the DBA mice no difference was seen between the 1st and 2nd probe tests. The authors suggest that the decrease seen in the hippocampus at the 2nd probe occurs because the animals are mastering the task and therefore the hippocampus becomes less engaged.

This decrease with over training has also been seen in chicks (Anokhin and Rose, 1991), with higher forebrain activation of *c-jun* in the group exposed to food

discrimination for 1 day compared to 2. The authors concluded that IEG activation was required for the learning of a new experience.

Avian studies have also shown a decline in IEG activation after repeated exposure to a stimulus. Mello et al. (1995) inspected Zenk activation (the avian equivalent of Zif268) in songbirds after exposure to a novel or familiar song. First they repeatedly exposed a bird to a song and it was noted that initial exposure to this song induced Zenk activation in the caudo-medial neostriatum. This activation peaked at 30 minutes of exposure but slowly declined reaching baseline activation after 180 minutes of exposure. Interestingly, introduction of a novel song to birds that had received prolonged exposure of a familiar song re-induced Zenk activation back to similar levels that had been seen at the previous peak. The authors state that this decrease in activation with familiarity and reactivation with novelty could suggest that neurons were able to change their responding to a certain stimulus without effecting their response to other stimuli.

Declines in IEG activation has also been studied along side single unit recording (Svarnik et al., 2005). Animals were trained to lever press for food reward and were sacrificed either after the first lever pressing session (acquisition group) or after the fifth session (performance group). IEG activation and electrophysiological recordings were studied in two animal cohorts in the retrosplenial cortex with the motor cortex used as a control area. During single unit recording neurons were classified into 2 groups; *Specific Element cells* if they fired during a specific part of the lever pressing behaviour in all cases (pressing lever, approaching hopper etc.), this included cells that fired at particular spatial points. *Non specific cells* were those which fired when an operation was performed that did not relate to the behavioural task (turning left/right, raising head etc.). In the retrosplenial cortex more immunoreactive cells were seen in the acquisition group as compared to the performance group. There were also a greater number of specific element-neurons compared to non specific element-neurons in the retrosplenial cortex. In the motor cortex there was no difference in the amount of immunoreactivity in all groups. There were a greater number of non specific element-neurons compared to specific element-neurons in the motor cortex. The decrease in c-Fos activation seen between the acquisition group and the performance group was attributed to the acquisition of a new experience. Interestingly, the single unit recording data showed that this decline

was observed in the same brain region where a greater number of specific element neurons were seen.

This idea of differences seen in IEG activation with extended training is interesting because it does seem like a likely explanation for the decreases seen in this study. Studying the animals behaviour over days of training (Fig. 22) indicates that the Working Memory group appear to have reached asymptote (the point at which no improvement in behaviour is seen even if training continues) on about day 8 of training with the 5cm Procedural Task group maybe reaching it on day 11 and the 13cm Procedural Task group reaching it on day 16 or maybe not at all. The animals in the Working Memory group could be said to have had extended training as it is has been shown that animals can learn a working memory task in the water maze with only a few days of training (Galani et al., 1998). This extended training was necessary for the animals in the Procedural Task groups to be performing the strategy required of them.

3.6.4.4. Extended training and synaptogenesis

Research has also shown that an increase in synaptogenesis has been seen with extended training which could suggest that the decreases in immunoreactivity seen could be attributed to a more streamlined brain system at work. Ramirez-Amaya et al. (1999) looked at synaptic density using the Timm's stain after extended training in the water maze. Rats were trained for 1 (WM1), 2 (WM2) or 3 (WM3) days with a fixed submerged platform over 10 trials. Also included was a swim control that experienced the water maze but with no platform present this was to cut out the likelihood that any changes had occurred because of stress. An increase in staining was seen in the mossy fibres of the CA3 region in the WM3 group as compared to all other groups and there was greater staining in the WM2 group compared to WM1. It was suggested that this reflected a change in synaptic efficacy (Geinisman et al., 1996) and was a mechanism for long term memory. This mechanism may also make animals more efficient at any spatial task. Moser et al. (1994) found that exposure to a complex environment made animals quicker to acquire a water maze task carried out at a later point and increased dendrite branching in CA1.

Although the above studies do suggest that increases in synaptogenesis are seen with extended training and this may be the reason why a decrease in IEGs is seen there is no direct evidence of this. A more direct assessment of this hypothesis was

133

carried out by Kleim et al. (1996) who looked at synaptogenesis and c-Fos expression in animals that had undergone motor skills learning. Animals in the Acrobatic condition (AC) had to traverse an obstacle course, the motor control condition (MC) were yoked to an AC animal and walked across a flat Plexiglas surface the same length as the obstacle course. Inactive control (IC) animals were taken from their home cage. Animals were killed after 1, 2, 5, 10 and 20 days of training. AC animals had significantly more synapses per neuron than the animals in the other groups and this was greater in the maintenance phase than in acquisition and that this increased over training. The AC group also had significantly more c-Fos positive cells than the other groups. Linear trend analysis showed that the c-Fos activation decreased over the 5 time points in the AC group but not in the MC group. This seems to indicate that as the number of synapses increases the amount of c-Fos decreases, therefore IEG's may be involved in the early stages of synapse formation for storage of long term memories.

3.6.5 Differences between Zif268 and c-Fos

The current studies allowed a direct comparison of the immediate early genes Zif268 and c-Fos. A differing pattern of results was seen between the two, with Zif268 showing decreases in the Working Memory group and c-Fos showing increases. Previous studies have found that these genes produce similar pattern of activation after water maze training (Guzowski et al., 2001) and brightness discrimination training (Grimm and Tischmeyer, 1997). Zif268 immunoreactivity decreases were seen in the hippocampus proper and a number of parahippocampal regions. c-Fos on the other hand showed increases very distinctly in the prefrontal regions. This suggests that the two genes may be mapping two distinct aspects of learning. As detailed in the introduction (1.2) c-Fos and Zif268 do have different downstream targets so therefore could conceivably model different things. The increases seen in the Working Memory group in the prefrontal cortex could reflect the "executive component" of the task. There is debate over what the function of the prefrontal cortex is. It is a common conclusion that it is involved in working memory which is an over simplification. Lesions of the prefrontal cortex do indeed impair performance in working memory tasks (see 2.1.5) but is often seen as inability to shift responding (Dias and Aggleton, 2000; Lacroix et al., 2002; De Bruin et al., 1994; Delatour and Gisquet-Verrier, 1996). There is also evidence as shown by Maviel et

al.(2004) that decreases in immediate early gene activation in the hippocampus are twinned with increases in cortical regions and more specifically the prefrontal regions. The pattern of results seen for c-Fos does somewhat suggest this, with increases seen in the prefrontal regions and a marginal decrease in activity seen in the hippocampus.

This differential pattern of c-Fos and Zif268 expression could also be due to the nature of the stimuli that activate the different genes. It is well documented in the literature that Zif268 is regulated by stress stimuli (Cullinan et al., 1995; Fujisaki et al., 2004; Honkaniemi et al., 2000) and c-Fos is regulated by motor activity (Timofeeva et al., 2003; Puntschart et al., 1998; Ruigrok et al., 1996) which in this experiment could have led to the differential expression seen. However it seems unlikely that the Procedural Task groups could have experienced a greater amount of stress as compared to the Working Memory animals as both groups swam for the same amount of time within the water maze and both found a submerged platform. There is also evidence to suggest that c-Fos is also activated by stress stimuli (Cullinan et al., 1995) which if the interpretation that the pattern of Zif268 expression in this experiment is because of stress does not tally with what is found here. Again it seems unlikely that the motor activity is responsible for the expression patterns seen with c-Fos as both experimental and 'control' group experienced the same motor demands related to the task. However a significant increase in c-Fos immunoreactivity was seen in the motor cortex in the Working Memory group as compared to the 5cm Procedural Task group in this experiment. Further research is needed to discover why these genes are differentially activated in the same behavioural paradigm.

3.6.6 What these results tell us

Two parallel processes could be at work in the different groups in the current experiment. For the Working Memory group repetition of the same task has led to the brain being very focused at what it does. Only neurons that need to be activated are activated, the amount of "noise" is reduced. As Kleim et al. (1996) show, as the amount of c-fos decreases in a well learned task the number of synapses per neuron increases. This could be consolidation of behaviourally relevant information. The Procedural Task groups will have formed a spatial representation of the water maze just as the Working Memory group has done. Note that although the groups' platform is much closer to the edge of the pool compared to the Working Memory group recent work has shown that the spatial representation is built up as the animals swim around

not when they are on the platform (McGauran et al., 2005). However unlike the Working Memory group this spatial information will be completely irrelevant to the rats. As the platform moves every single trial their brains could be permanently "on-line" because they are having to adapt to an ever changing task and environment and are therefore paying more attention to their surroundings. This is exemplified by the Pearce-Hall model (1980) that states that animals pay more attention to stimuli that have no predictable outcome than those that do. Therefore if the immediate early gene activation was studied at an earlier point in training one would expect to see no difference between the groups if IEG expression decreases with habituation, which research suggests it does. It is also possible that the "increase" seen in the Procedural Task group could be due to the fact that the platform is remaining stable for the first time. Although the animals don't display the use of the extra-maze cues they may be preparing to do so and so if the number of trials were increased they may begin to use the spatial cues and therefore start to pay more attention to them. However, because the Working Memory group have performed the task many times their attention to the cues would be less.

It could also be proposed that the Working Memory group are undergoing an active form of forgetting. Initially in training animals would retain the information of the platforms location over sessions, however, as training progressed and it became evident that the platform location information would not be required for the next session, it would be quickly discarded on completion of that days session as suggested by Dudchenko (2004).

Three novel 'control' groups were studied in this group of experiments. They were matched on amount of time spent in the maze and showed no learning over trials in the final session. Also in the case of the 13cm PT group, the platform was in the same spatial location as the Working Memory group. Although they are not perfect, for example the fact that the platform in the Procedural Task group changes to remain stable on the final test day, they are far better than 'control' groups used in previous research (see 3.1.6). They also produced an interesting array of results that show that not only can increases of IEG activation correlate with the learning experience but also decreases.

Chapter Four

Structural equation modelling analysis of a working memory task

4.1 Introduction

Generally in research one looks at how each brain area changes across conditions independently of any other factors. However this is a not biologically valid approach because the brain is a network of different regions which all exert influence on each other. A procedure that would allow us to look at the brain as a complete network would be an advantage. Structural equation modelling is a statistical technique that allows us to use cell counts of immediate early gene activation to do this.

4.1.1 What is SEM?

Structural Equation Modelling is a statistical technique most commonly used in social psychology experimentation. It allows one to look at the connections between variables and how a change in one variable affects another. This means that when SEM is applied to imaging data it can test whether a change in activation in one brain area affects another. It is a confirmatory technique that tests an *a priori* theoretical model. SEM is data driven and the theoretical model is respecified according to the data. This is an exploratory method of data analysis and is most validly used for developing theories for further experimental testing. A commonly used programme to implement SEM is LISREL which will be discussed in relation to SEM.

4.1.2 How is it done?

There are a number of different steps and issues related to structural equation modelling which will be discussed below.

i) Model specification

SEM begins by specifying a model to be tested. This model must be based on theory or previous research. Any model could be created that will fit the data

but may have no theoretical grounding. The model must represent only unidirectional linear relationships between variables. SEM tries to explain the pattern of covariances seen between the different variables. Between the variables there is a group of structural relations and they can be represented as a set of structural equations. These structural equations can be solved to recreate the original correlation matrix derived from the data. If this occurs the model is a perfect fit for the data. However this rarely happens, in other words there is not often one solution to the model.

ii) Estimation

SEM entails estimating unknown parameters, in the case of network analysis the path coefficients between brain areas, based on the correlation matrix produced from the data. LISREL tries to solve the structural equations to recreate the original data driven correlation matrix. The program does this by iterative estimation. It commences by making a 'guess' at the unknown parameter values. It will then solve the structural equations and calculate an 'implied' covariance matrix (ICM). This is the matrix that would result from the set of model parameters that LISREL 'guessed'. The ICM is then compared with the observed covariance matrix (OCM), taken from the actual data, to see if they are similar. If they are well matched the process stops, if not LISREL will make another guess. LISREL uses a fitting criterion as specified by the user to know when a 'guess' is good or not. In most cases the fitting criterion used is a maximum likelihood estimation although others do exist. When the two different covariance matrices are the same the maximum likelihood estimate would be zero. Therefore LISREL tries to minimise the fitting function.

iii) Assessing Model fit

As stated above there is rarely one solution to a model so the model that produces the 'best fit' for the data needs to be found. There are a number of different indices of fit that are available but three will be focused on here. The first index of fit is the chi squared statistic. A non significant chi squared means that there is no difference between the implied and data driven

covariance matrices. The Goodness of Fit Index (GFI) is based on the "ratio of the sum of the squared discrepancies to the observed variance" (Kelloway, 1998 p.27). The GFI ranges from 0 to 1 with values closes to 1 representing a good fit. The Adjusted Goodness of Fit (AGFI) is the same as the GFI but adjusts for the models degrees of freedom. Again an AGFI closer to 1 is seen as a good fit.

iv) Model respecification

If the model originally tested is not a good fit to the data it can be modified till the best fit of the data can be found. This can be done by using the modification indices calculated by LISREL. For each parameter set to zero i.e. each parameter the user has asked LISREL not to predict, the program will calculate how the chi squared statistic would decrease if that parameter was predicted. A rule of thumb as concerns modification indices is that a value above 5.0 is worth predicting.

v) Path coefficients

LISREL will calculate standardized parameter estimates which in essence are path coefficients. They are the direct influence of one brain region on another if all other regions are left unchanged. In other words it is the way one brain region changes as the brain region influencing it changes. The statistical significance of these coefficients are shown by the t statistic produced from the maximum likelihood estimate. T statistics over 1.96 are seen to be significant at the 0.05 level.

4.1.3 Use of SEM in human neuroimaging

Although SEM is not commonly used in neuroscience research a number of studies are starting to emerge that have used this technique. Human neuroimaging studies have, for example, used it to research episodic memory in different tasks.

Nyberg et al. (1996) employed episodic memory retrieval to study networks of brain regions that are activated in PET scans. Subjects were scanned in four conditions, one baseline and 3 episodic retrieval conditions. In the 3 episodic conditions subjects had to recognize words that were visually presented from two lists

that had been previously presented audibly. Subjects had to press a left hand button if they recognized the word and the right hand button if they did not. The conditions differed in the type of words presented; words studied with respect to meaning, words studied in respect to the speakers voice and non studied words. The baseline condition consisted of studying words without recall and randomly pressing each button. Areas that showed decreases during recognition included left middle frontal gyrus and right medial frontal cortex, bilateral anterior and posterior temporal cortex and posterior cingulate. Regions that showed increases included right prefrontal cortex, left anterior cingulate, left occipital cortex and cerebellum. SEM network analysis revealed that in the recognition condition the decreases seen in regional blood flow occurred because of an inhibitory influence from the regions that showed increases. This was shown by the fact that the influence from regions with increased activation was stronger and more negative on regions showing decreased activation.

Another PET scan study that utilized SEM to study network connections during episodic memory retrieval was carried out by Kohler et al. (1998). At an initial scan participants performed an object based and a spatially based perceptual matching task. This involved subjects comparing a pair of displays and having to decide whether they were the same or different. The pairs differed on two dimensions either in respect to the location of one of the objects (spatial matching) or in respect to the objects identity (object matching). After this scan participants had to memorize 22 displays from the perceptual matching task as no scan was taken. A memory test for these items was then performed whilst subjects were scanned. They performed spatial retrieval and object retrieval. In the spatial retrieval condition target displays were presented with test displays where the location of the one of the objects was altered. In the object retrieval condition the test display was altered by changing the identity of one of the objects. In both cases participants had to decide if the test display was altered in relation to what they had seen previously.

Brain regions for SEM analysis were chosen based on changes seen between the conditions and anatomical connections. The dorsal lateral middle frontal gyrus and the parieto-occiptal sulcus showed higher activation during retrieval than perceptual matching. The middle temporal gyrus showed higher activation during perceptual matching. The medial uncus and fusiform gyrus showed higher activation during processing of object rather than spatial information whilst the supramarginal gyrus showed the opposite. Also selected was the right medial-temporal region which

140

showed no change over conditions. Interactions between the medial temporal lobe (MTL) and dorsally located posterior region were positive in the spatial retrieval task and negative in the object retrieval task. The opposite was true of the ventrally located posterior regions. During perceptual matching all connections between MTL and all posterior regions was negative apart from the connection from BA 21. These results overall show that connections between MTL and dorsally located regions were positive in the spatial retrieval task and negative in object retrieval. The opposite was true of the connections involving ventrally located regions.

A further neuroimaging experiment that utilized SEM was carried out by Krause et al. (1999). Participants were visually presented with pairs of words that were semantically unrelated. Two PET scans were performed. The first, the storage scan, was executed when subjects were presented with the words and had to read them out loud. At the second scan, the retrieval scan, the participants were presented with the first word of the previously presented pair and had to recall its partner word. There was a common pathway of connections in both encoding and retrieval networks from the primary visual cortex to extrastriate areas and then to the parahippocampal region in the right hemisphere. Connections were also seen in both groups between extrastriate areas and parahippocampal regions and precuneus and posterior cingulate both in the left hemisphere. The main differences seen were that during encoding there were strong connections between anterior neocortical regions (anterior cingulate cortex, prefrontal cortex) which did not occur during retrieval. During retrieval strong connections were seen in more posterior regions (parahippocampal cortex, posterior cingulate gyrus, extrastriate cortex). There was also an obvious bihemispheric asymmetry with stronger connections being seen in the left hemisphere during encoding and the opposite during retrieval.

4.1.4 Use of SEM in animal testing

Very little work has been carried out in animal research using SEM as a network analysis tool. Jenkins et al. (2003) used SEM to study the differential activation of a working memory and reference memory task in the water maze. Animals were trained for 12 days with 4 trials for the 1st 6 sessions and 8 trials for the last 6. Animals were split between 2 different conditions, place (working memory) and landmark (reference memory) conditions. In the place condition the platform remained fixed over trials in a session but moved from session to session i.e. a

standard working memory task. In the landmark condition the platform moved from trial to trial. In the landmark condition a beacon was always 25cm due north of the platform. The place group also had a beacon present in the same spatial location as the landmark group but it had no significance to the animal. No difference was seen between the amounts of time the animals spent in the maze. Animals in the place condition showed a decrease in latencies over trials on the final test day with the landmark group showing no such decrease. Animals were processed for c-Fos immunoreactivity.

A global decrease in c-Fos immunoreactivity was seen in the place condition in all brain regions sampled (Jenkins et al., 2003). SEM modelling was performed on the data using an anatomically derived model (Fig. 78). In the place condition models a significant pathway was seen between the parahippocampal regions and the hippocampus but this did not progress through to the subiculum (Fig. 79). In the landmark condition there were connections within the parahippocampal regions and connections within the hippocampus that progressed to the subiculum (Fig. 79). However, the parahippocampal and hippocampal regions were disconnected because of a lack of connection between the entorhinal cortex and the dentate gyrus. A significant pathway between the parahippocampal region and the subiculum was seen but this bypassed the hippocampus. The authors suggest that this pattern fits with the idea of the entorhinal cortex as a key region in sensory information reaching the hippocampus. This also suggests that that the "place" or working memory task requires the integrity of the hippocampus to be performed but the "landmark" task does not.

Perirhinal ⟶ **Entorhinal Cortex**
Cortex   **Lateral**
           ↘   **(CA3)**
            DG ⟶ **CA1** ⟶ **Subiculum**
           ↗
Postrhinal ⟶ **Entorhinal Cortex**
Cortex   **Medial**
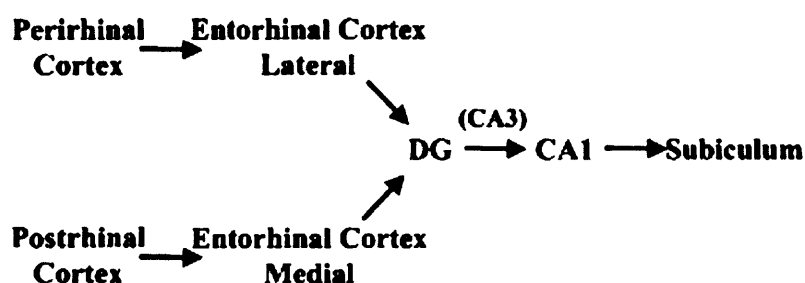
Figure 78
Pathway diagrams showing the models tested by Jenkins at el. (2003).

Group 'Place'

$R^2=.68$ $R^2=.42$ $R^2=.56$ $R^2=.34$

Peri $\xrightarrow{.83^{**}}$ EntL $\xrightarrow{.65^{**}}$ DG $\xrightarrow{.75^{**}}$ CA1 $\xrightarrow{.03^{NS}}$ Sub

$.57^{NS}$

$\chi^2_s=3.90, df=5$
GFI=0.82
p=0.56

$R^2=.62$ $R^2=.42$ $R^2=.56$ $R^2=.18$

Post $\xrightarrow{.79^{**}}$ EntM $\xrightarrow{.65^{**}}$ DG $\xrightarrow{.75^{**}}$ CA1 $\xrightarrow{.28^{NS}}$ Sub

$.22^{NS}$

$\chi^2_s=5.92, df=5$
GFI=0.75
p=0.31

Group 'Landmark'

$R^2=.44$ $R^2=.06$ $R^2=.48$ $R^2=.75$

Peri $\xrightarrow{.66^{**}}$ EntL $\xrightarrow{.24^{NS}}$ DG $\xrightarrow{.69^{**}}$ CA1 $\xrightarrow{.48^{**}}$ Sub

$.64^{*}$

$\chi^2_s=1.10, df=5$
GFI=0.94
p=0.95

$R^2=.38$ $R^2=.03$ $R^2=.48$ $R^2=.85$

Post $\xrightarrow{.62^{**}}$ EntM $\xrightarrow{.17^{NS}}$ DG $\xrightarrow{.69^{**}}$ CA1 $\xrightarrow{.51^{**}}$ Sub

$.71^{**}$

$\chi^2_s=6.16, df=5$
GFI=0.74
p=0.29
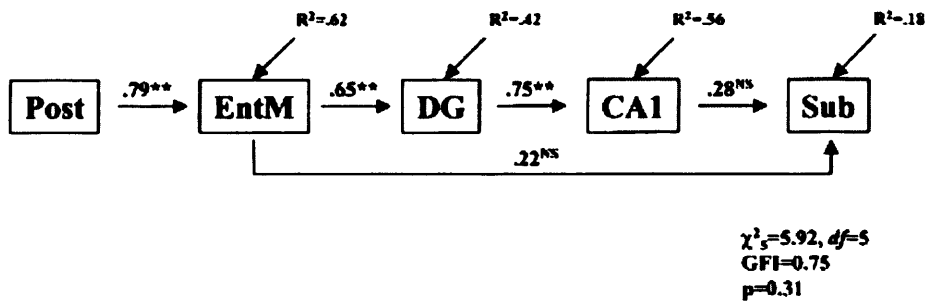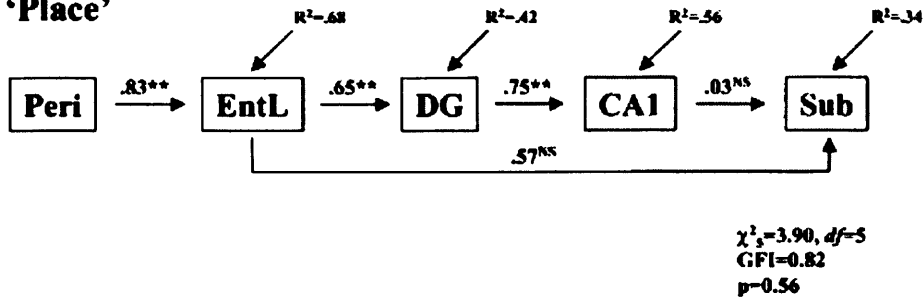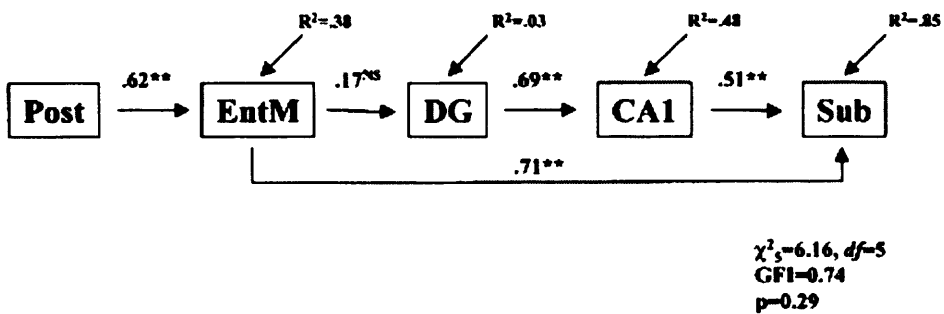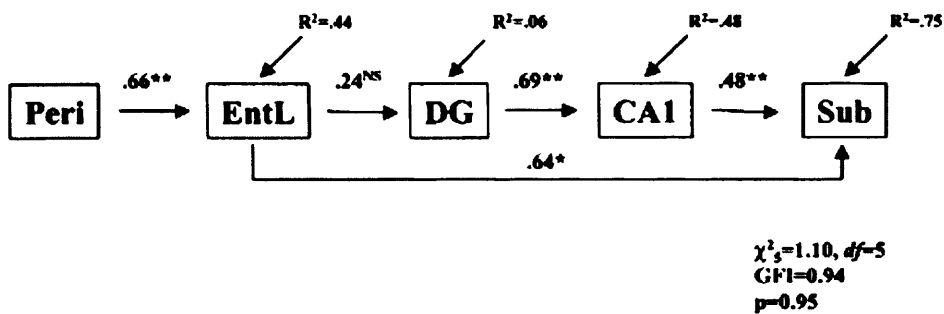
Figure 79
Pathway diagrams showing significant and non significant path coefficients from the model tested in the Jenkins at el. (2003) study. These diagrams show that significant pathways in the 'place' group progress through the hippocampus whilst this structure is sub served in group 'landmark'.

4.1.5 Brain connections
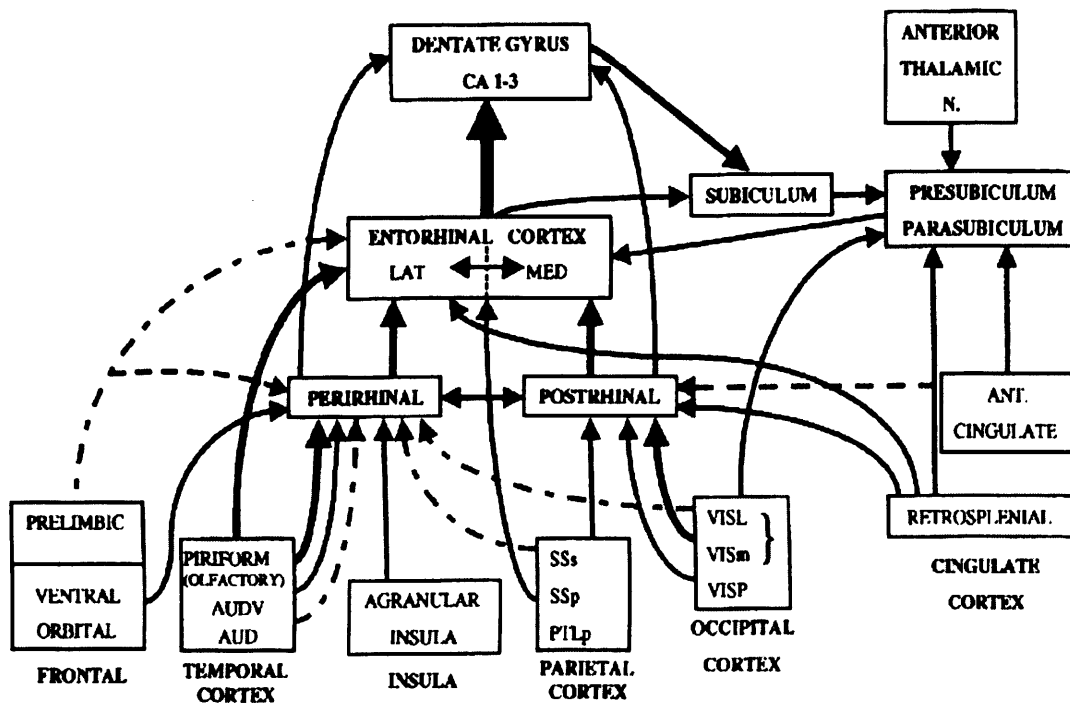
Figure 80
Diagram taken from Aggleton et al. (2000) representing the main connections between cortical, hippocampal and parahippocampal regions.

Above is a simplified representation of the connections in the rat brain that potentially provide sensory inputs to the hippocampal and parahippocampal regions. Information received from sensory inputs is transferred from the occipital and parietal and temporal cortices to distinct areas of the brain. Visual and somatosensory information is primarily passed to the postrhinal cortex and auditory information is primarily passed to the perirhinal cortex. There is interplay between these two regions with both connecting to the entorhinal cortex: the postrhinal cortex mainly projecting to the medial part of the cortex and the perirhinal cortex to the lateral entorhinal cortex. There are reciprocal connections between both parts of the entorhinal cortex, which both provide dense projections to the hippocampus proper. The CA1 field then projects to the subiculum and onwards to the pre and para subiculum, which have dense forward projections with other brain areas. There is also a direct project from the entrorhinal cortex to the subiculum bypassing the hippocampus.

144

As shown in the experiments described above, SEM can be used to supplement research in many ways. This experiment will use SEM to deal with two issues. First, using a similar model to that described in Jenkins et al. (2003) it will try and elucidate the network connections seen in a working memory task in the water maze as compared to a novel 'control' group. The goal is to derive a more dynamic representation of the brain systems that are engaged during these differing memory tasks. This will be done by extending the model tested by Jenkins et al. (2003) using the immediate early gene *zif268* as the neuronal marker. Second, it will perform a direct comparison of Jenkins et al. (2003) model making use of the immediate early gene *c-fos* but comparing the working memory model with one obtained using a much tighter control group (see chap 3).

4.2 Experiment Four

4.2.1 Materials and methods

Due to the nature of SEM, groups were combined from the previous experiments (exp2 and 3a). In SEM analysis the number of subjects must exceed the number of pathways the analysis predicts. Therefore the greater number of subjects the greater number of pathways LISREL can predict and therefore produce a better model.

Subjects

Data was obtained from two previously run experiments. Subjects were 32 male Dark Agouti rats (Harlan, UK) weighing between 260g and 300g at time of testing. Rats were housed in pairs and kept on a 14/10 dark/light cycle. Food and water was available *ad libitum* through out the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Apparatus

Apparatus was the same as in Experiment 2a.

Procedure

Animals were tested as described previously in Experiment 2 and 3a.

Working Memory group

To produce the Working Memory group animals from the Working Memory groups from Experiment 2 and 3a were combined. They differed on the number of days of training they received, 12 session in Experiment 2 and 20 sessions in Experiment 3a. Also in Experiment 2 from day 8 onwards the animals had a stick present on the platform on the first trial of every session which animals in Experiment 3a did not. Analysis reveals however that there was no significant difference in the amount of time both groups spent in the maze on the final test day (F $(1, 15)$ =0.071, p=0.794).

Procedural Task group

To produce the Procedural Task group animals from the Procedural Task groups from Experiment 2 and 3a were combined (PT and 5cmPT). They differed on the number of days of training they received, 12 session in Experiment 2 and 20 sessions in

Experiment 3a. Also in Experiment 2 from day 8 onwards the animals had a stick present on the platform on the first trial of every session which animals in Experiment 3a did not. Also for the Procedural Task group from Experiment 2 the platform was right against the maze walls whereas for the 5cmPT group from Experiment 3a the platform was 5cm from the edge of the walls. Analysis reveals however that there was no significant difference in the amount of time both groups spent in the maze on the final test day (F $(1, 15)$ =0.020, p=0.888).

Immunohistochemistry

Immunohistochemistry was performed as previously (3.3.2)

Image Analysis

Image analysis was carried out as previously (3.3.2).

Counts were normalized to reduce variation between animals. This was carried out because groups were combined across experiments. Raw data was normalised to counts from one of the sensorimotor regions for each animal to produce good reliability as demonstrated by a Cronbach alpha which is essential for SEM analysis. Cronbach Alpha is a test of reliability with high alpha's indicating that the data reliably measures a latent construct. It is calculated using the formula below:

$$\alpha = \frac{N - \bar{r}}{1 + (N - 1) - \bar{r}}$$

with N indicating the number of items and r-bar the average inter-item correlation.

Model specification

A model was produced based on one tested previously (Jenkins et al, 2003). Due to a higher number of subjects used than in the previous experiment more connections could be added to the current model. The retrosplenial cortex was chosen as the end point of the model due to its involvement with working memory paradigms (Fig. 82a). Also the dentate gyrus was removed and the CA3 region added due to the low correlations between other brain regions in the former and strong correlations with other brain regions in the latter (see Fig. 82). However the retrosplenial cortex has some back projections within the model which is assumed to be unidirectional, so an alternative model was also tested that removed the retrosplenial cortex and terminated in the dorsal subiculum which has dense forward projections to many areas (Fig. 82b)

and has previously been used as a model end point in other research (Jenkins et al. 2003). A model for c-Fos was also tested which differed from the one tested for Zif268 as the study had less subjects (Fig. 81). This model was the same as specified by Jenkins et al. (2003) and so provided a direct comparison with that study.

$$\text{POST} \longrightarrow \text{ENTM} \longrightarrow \text{DG} \overset{\text{(CA3)}}{\longrightarrow} \text{CA1} \longrightarrow \text{SUBD}$$

$$\text{PERI} \longrightarrow \text{ENTL} \longrightarrow \text{DG} \overset{\text{(CA3)}}{\longrightarrow} \text{CA1} \longrightarrow \text{SUBD}$$
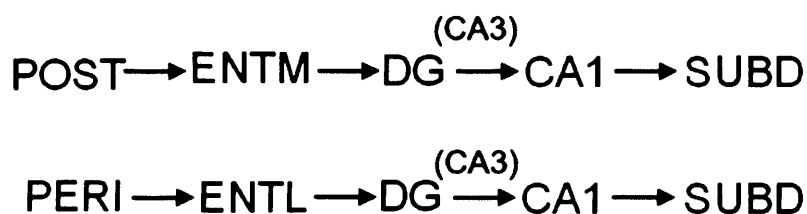
Figure 81
Representation of the two c-Fos models to be tested

a) $$\text{POST} \longrightarrow \text{ENTM} \overset{\text{(DG)}}{\longrightarrow} \text{CA3} \longrightarrow \text{CA1} \longrightarrow \text{SUBD} \longrightarrow \text{rRSP}$$

$$\text{PERI} \longrightarrow \text{ENTL} \overset{\text{(DG)}}{\longrightarrow} \text{CA3} \longrightarrow \text{CA1} \longrightarrow \text{SUBD} \longrightarrow \text{rRSP}$$

b) $$\text{POST} \longrightarrow \text{ENTM} \overset{\text{(DG)}}{\longrightarrow} \text{CA3} \longrightarrow \text{CA1} \longrightarrow \text{SUBD}$$

$$\text{PERI} \longrightarrow \text{ENTL} \overset{\text{(DG)}}{\longrightarrow} \text{CA3} \longrightarrow \text{CA1} \longrightarrow \text{SUBD}$$
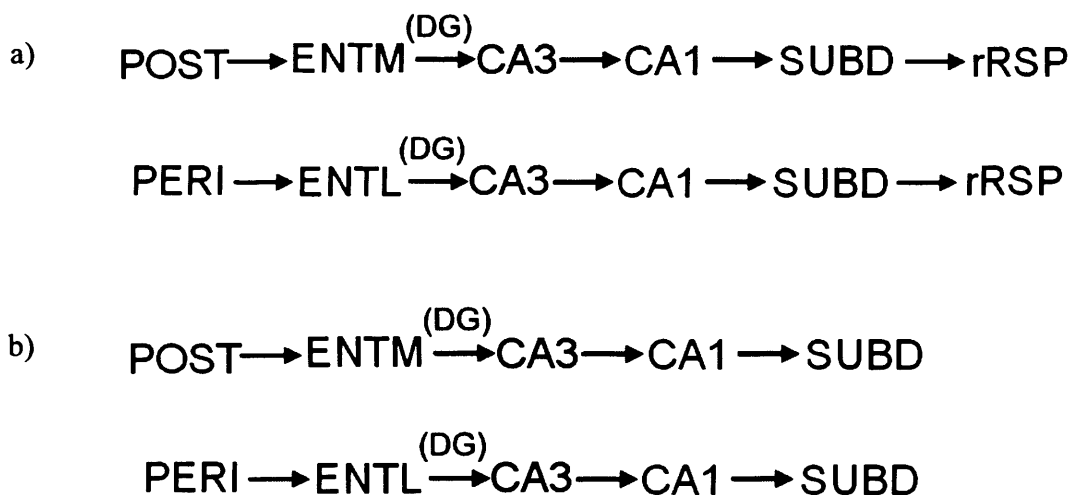
Figure 82
Representation of the four Zif268 models to be tested; two with the retrosplenial cortex (a) two without (b).

SEM analysis

Correlations for the Working Memory group and Procedural Task group were produced separately. These were calculated using data from all brain regions counted apart from the sensorimotor regions. Correlations were inputted into the SEM programme LISREL. Two models were tested for each group one encompassing the connections between the perirhinal cortex and the lateral entorhinal cortex the other the connections between the postrhinal cortex and the medial entorhinal cortex (Figs

81 and 82). The simplex model was run for each condition. This model consists of comparing a linear forward moving connection between the brain regions. The model was then modified as dictated by modification indices produced by LISREL. This continued until the best fitting model for the data was produced. This decision was based on the indices of best fit produced by the programme and by the plausibility of the anatomical connections.

4.2.2 Results

4.2.2.1 Correlations

Figure 83 shows the correlations between brain regions for Zif268 and figure 84 shows the correlations for c-Fos. Numbers in bold signify significant correlations. These are the start points for all SEM models.

Region	Peri	ENTl	CA3	CA1	Subd	rRSP	ENTm	Por
Peri		**.810**	**.874**	**.848**	**.926**	**.927**	**.629**	-.276
ENTl	**.737**		**.794**	**.946**	**.865**	**.790**	**.602**	-.157
CA3	**.759**	**.676**		**.880**	**.856**	**.877**	**.635**	-.415
CA1	**.651**	**.814**	.827		**.935**	**.843**	**.524**	-.181
Subd	**.605**	**.834**	**.658**	.787		**.894**	.490	-.162
rRSP	**.845**	**.919**	**.833**	**.872**	**.843**		**.592**	-.228
ENTm	.301	.341	.140	.170	**.507**	.280		**-.661**
Por	.137	-.112	.052	-.172	-.261	-.080	-.362	

Figure 83
Correlations for the Zif268 counts. Counts from the Working Memory group are above the diagonal and counts below are from the Procedural Task group

Region	Peri	ENTl	DG	CA1	Subd	ENTm	Por
Peri		**.898**	**.751**	.251	.381	**.823**	**.917**
ENTl	.579		.605	.081	**.906**	**.876**	**.906**
DG	**.853**	.197			.470	**.732**	**.850**
CA1	.319	.272	.233		**.860**	.194	.263
Subd	**.960**	.479	**.807**	.468		.255	.308
ENTm	**.723**	.664	.571	.553	**.683**		**.962**
Por	**.831**	**.767**	.518	.309	**.805**	**.833**	

Figure 84
Correlations for the c-Fos counts. Counts from the Working Memory group are above the diagonal and counts below are from the Procedural Task group

4.2.2.2 Zif268 Postrhinal model

Model terminating in rostral retrosplenial cortex

For the postrhinal-rostral retrosplenial cortex model both Working Memory (WM) and Procedural Task (PT) groups have a main significant pathway from postrhinal cortex to CA3 and from CA3 to rostral retrosplenial (Fig. 85). Both models also include the postrhinal to CA3 direct pathway and the indirect pathway from CA3 to rostral retrosplenial cortex via CA1 and dorsal subiculum. The Procedural Task group also has a significant pathway from postrhinal to CA1 and from CA1 to rostral retrosplenial cortex via dorsal subiculum. The Working Memory group had less significant pathways than the Procedural Task group.
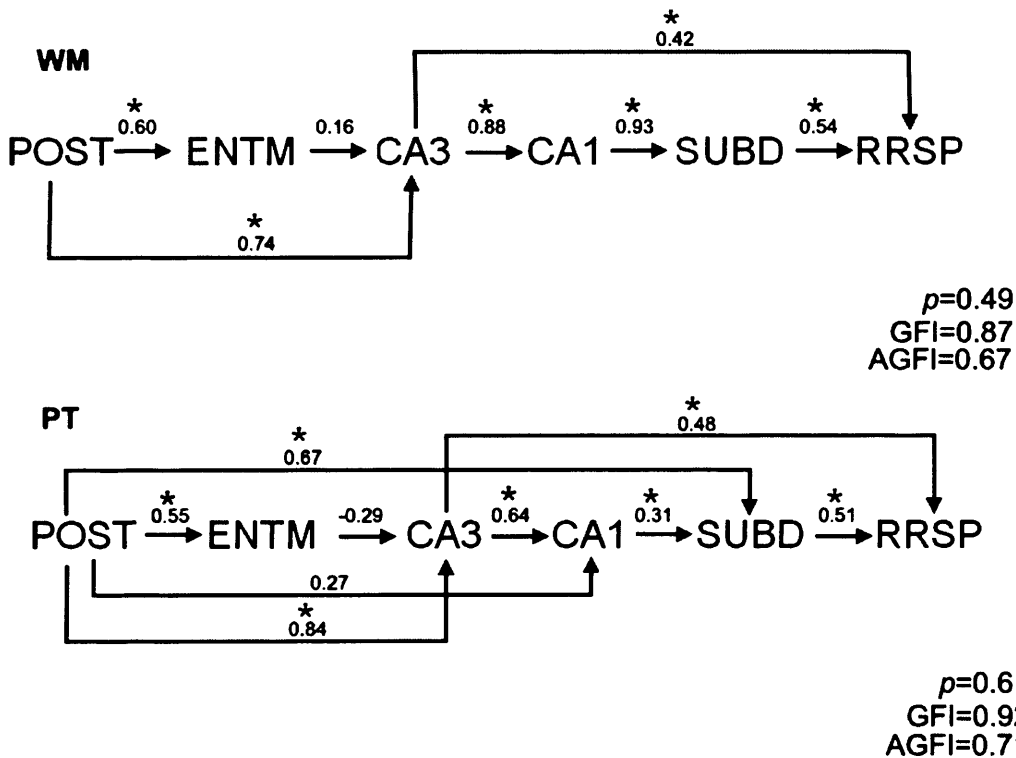
Figure 85

Postrhinal to retrosplenial cortex model for Zif268 including significant path coefficients and indicies of fit ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (POST-postrhinal cortex, ENTM-medial entorhinal cortex, SUBD-dorsal subiculum, RRSP-rostral reterosplenial cortex)

Model terminating in the dorsal subicular region

For the postrhinal-dorsal subiculum models again the Working Memory group (WM) had less significant pathways as compared to the Procedural Task group (PT), in fact only one which progressed from the postrhinal cortex to CA3 and on through CA1 to the dorsal subiculum (Fig. 86). Again the Procedural Task group did have a significant pathway through the hippocampus but had others that evaded this structure.

Figure 86
Postrhinal to dorsal subiculum model for Zif268 including significant path coefficients and indices of fit ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (POST-postrhinal cortex, ENTM-medial entorhinal cortex, SUBD-dorsal subiculum)

4.2.2.3 Zif268 Perirhinal model

Model terminating in rostral retrosplenial cortex

In the Perirhinal –rostral retrosplenial cortex models both groups have a direct perirhinal to rostral retrosplenial cortex connection which is the only significant connection for the Working Memory group (WM) (Fig. 87). The Procedural Task group (PT) also has pathways from perirhinal to lateral entorhinal cortex and then either directly from lateral entorhinal to rostral retrosplenial cortex or from lateral entorhinal to rostral retrosplenial cortex via the dorsal subiculum.
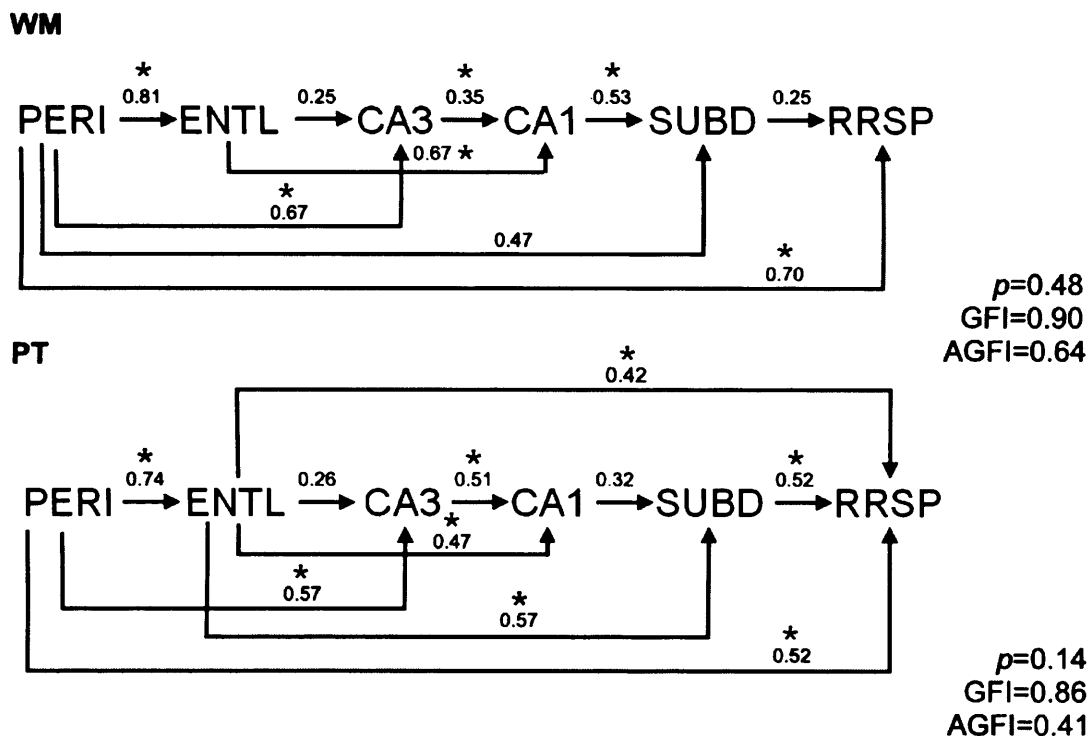
Figure 87
Perirhinal to retrosplenial cortex model for Zif268 including significant path coefficients and indices of fit ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, SUBD-dorsal subiculum, RRSP-rostral reterosplenial cortex)

Model terminating in the dorsal subicular region

In the case of the perirhinal-rostral retrosplenial cortex models the significant pathways evade the hippocampus proper (Fig. 88). Within the perirhinal cortex to dorsal subiculum model a certain amount of change was seen. The Working Memory group (WM) now has more significant pathways than the Procedural Task group (PT), which only has one which sub serves the hippocampal subfields.

WM

```
        *           *        *
     0.81        0.35     0.53
PERI ──►ENTL ──►CA3 ──►CA1 ──►SUBD
        0.25      ▲0.67 *    ▲
      │         │   *
      │    *    │  0.67
      │   0.67  │        *
      │         │       0.47
      └─────────┴──────────────┘
```

p=0.28
GFI=0.92
AGFI=0.59

PT

```
                      *
                    0.57
        *                    *
     0.74                  0.51
PERI ──►ENTL ──►CA3 ──►CA1 ──►SUBD
        0.26      ▲    *  0.32  ▼
      │         │  0.47
      │    *    │
      │   0.57  │
      └─────────┘
```

p=0.61
GFI=0.96
AGFI=0.78
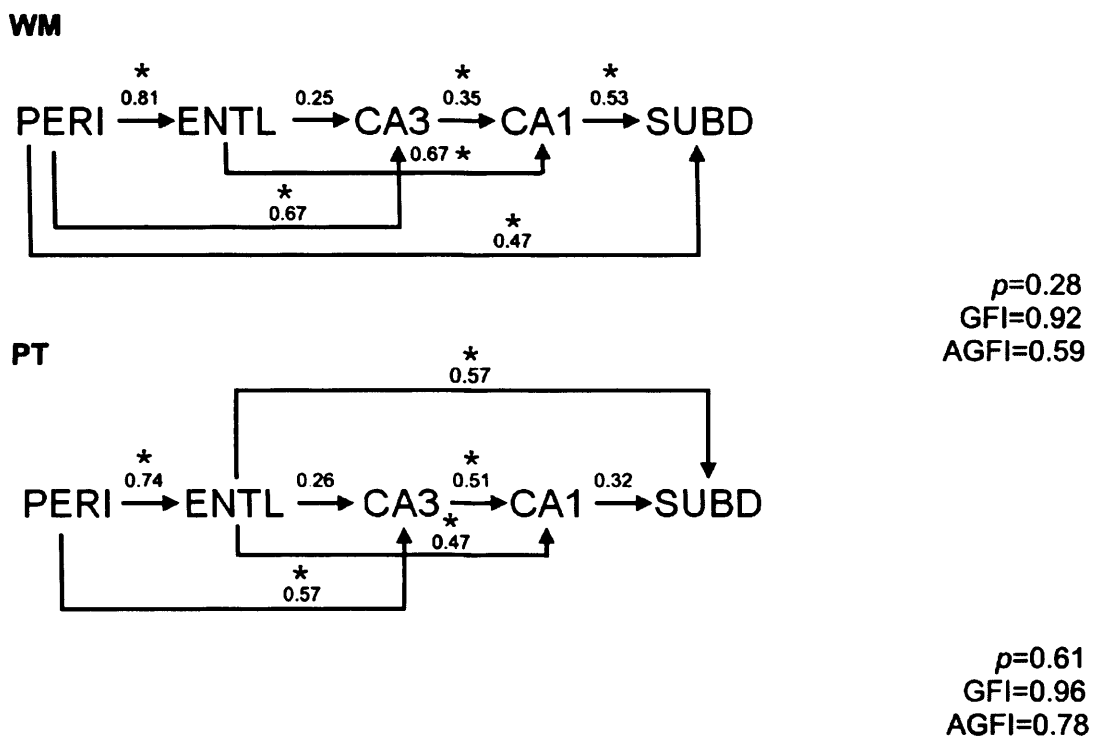
Figure 88
Perirhinal to dorsal subiculum model for Zif268 including significant path coefficients and indices of fit ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, SUBD-dorsal subiculum)

154

4.2.2.4 Cross- validation of Zif268 models

It is usual practice in SEM research to test your model on one group of subject and then to cross validate this model by testing on another group. In the current experiment this is problematic because of such a small number of subjects. However cross validation was attempted on two models. The group of 16 animals was randomly split to produce two groups of 8. The first model tested was the postrhinal-medial entorhinal to dorsal subiculum model in the Working Memory group. However the constraints of SEM modelling dictated only one further pathway than the simplex model could be tested. In the case a model was tested that removed the non-significant CA3 to dorsal subiculum connection (Fig. 89). This model produced a good fit for both sets of data and produced the same pattern of path coefficients with a significant pathway travelling from the postrhinal cortex to CA3 and then through CA1 to the dorsal subiculum mirroring what was seen in the originally tested model.

Rep 1

$$
\text{POST} \xrightarrow[\underset{0.66}{*}]{} \text{ENTM} \xrightarrow{0.01} \text{CA3} \xrightarrow[\underset{0.86}{*}]{} \text{CA1} \xrightarrow[\underset{0.88}{*}]{} \text{SUBD}
$$

$$
\underset{0.92}{\overset{*}{}}
$$
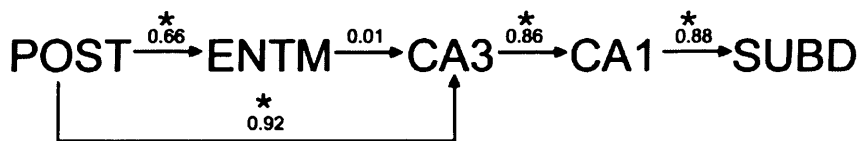
p=0.24
GFI=0.79
AGFI=0.37

Rep 2

$$
\text{POST} \xrightarrow[\underset{0.65}{*}]{} \text{ENTM} \xrightarrow{0.19} \text{CA3} \xrightarrow[\underset{0.91}{*}]{} \text{CA1} \xrightarrow[\underset{0.99}{*}]{} \text{SUBD}
$$

$$
\underset{0.76}{\overset{*}{}}
$$
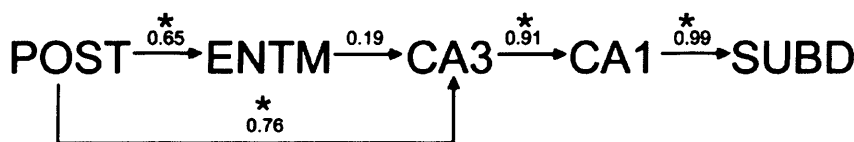
p=0.28
GFI=0.80
AGFI=0.40

Figure 89
Figure depicting the cross validation of the postrhinal-medial entorhinal to dorsal subiculum model in the Working Memory group. (p=, GFI and AGFI). p= represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (POST-postrhinal cortex, ENTM-medial entorhinal cortex, SUBD-dorsal subiculum).

The other model that was chosen to be cross-validated was the perirhinal-lateral entrorhinal to dorsal subiculum model in the Procedural Task group. As this had three significant pathways beyond the simplex model each pathway was cross-validated

separately (Figs 90, 91 and 92). All models tested fitted both groups of data

somewhat however different patterns of path coefficients were produced. However in

all but one case no significant pathway was seen from the parahippocampal cortex

through the hippocampus to the dorsal subiculum which replicates what is seen in the

original model. This slightly messy pattern of results and the inability to completely

cross-validate the model in this case may be due to they way the models were tested.

It was not possible to test the complete model due to lack of subjects and if it was

possible to test the complete model the model may be able to be satisfactorily cross

validated.

Rep 1

PERI $\xrightarrow{*}{0.68}$ ENTL $\xrightarrow{0.50}$ CA3 $\xrightarrow{*}{0.83}$ CA1 $\xrightarrow{*}{0.69}$ SUBD

0.13
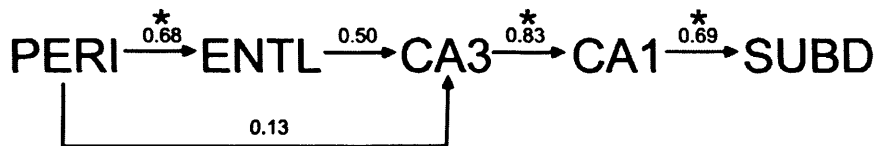
p=0.35
GFI=0.82
AGFI=0.47

Rep 2

PERI $\xrightarrow{*}{0.80}$ ENTL $\xrightarrow{0.04}$ CA3 $\xrightarrow{*}{0.83}$ CA1 $\xrightarrow{*}{0.86}$ SUBD

*
0.91
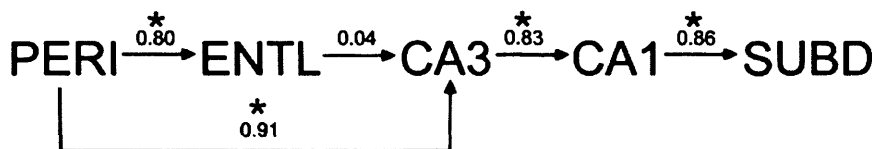
p=0.063
GFI=0.76
AGFI=0.27

Figure 90
Figure depicting the cross validation of the perirhinal-lateral entorhinal to dorsal subiculum
model in the Procedural Task group. (p=, GFI and AGFI). p= represents the significance of
chi-squared testing with non-significant values indicating good fit. Values close to one
represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit
indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, SUBD-dorsal subiculum).

Rep 1

$$PERI \xrightarrow{0.42} ENTL \xrightarrow{0.41} CA3 \xrightarrow[\;*\;]{0.47} CA1 \xrightarrow[\;*\;]{0.69} SUBD$$

$$\xrightarrow[\;*\;]{0.52}$$

Rep 2

p=0.052
GFI=0.75
AGFI=0.24

$$PERI \xrightarrow[\;*\;]{0.70} ENTL \xrightarrow{0.36} CA3 \xrightarrow[\;*\;]{0.79} CA1 \xrightarrow{-0.02} SUBD$$

$$\xrightarrow{-0.11}$$
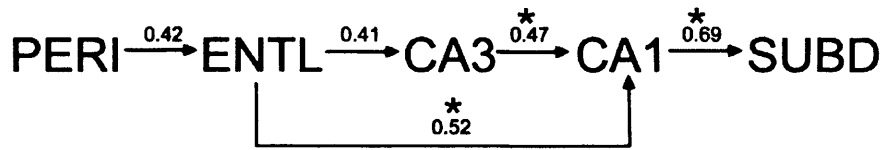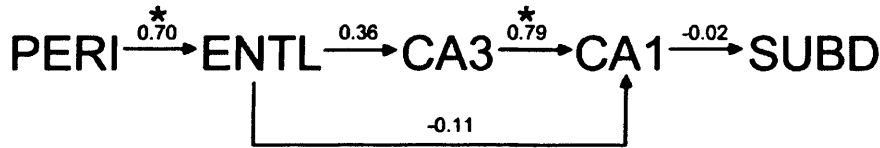
p=0.065
GFI=0.76
AGFI=0.27

Figure 91
Figure depicting the cross validation of the perirhinal-lateral entorhinal to dorsal subiculum model in the Procedural Task group. ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, SUBD-dorsal subiculum).

Rep 1

$$PERI \xrightarrow{0.42} ENTL \xrightarrow{0.41} CA3 \xrightarrow[\;*\;]{0.68} CA1 \xrightarrow{0.22} SUBD$$

$$\xrightarrow[\;*\;]{0.74}$$
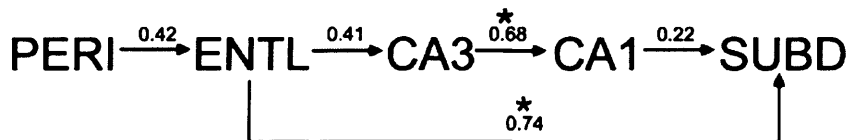
p=0.064
GFI=0.73
AGFI=0.18

Rep 2

$$PERI \xrightarrow[\;*\;]{0.70} ENTL \xrightarrow{0.36} CA3 \xrightarrow[\;*\;]{0.75} CA1 \xrightarrow{-0.03} SUBD$$

$$\xrightarrow{0.09}$$
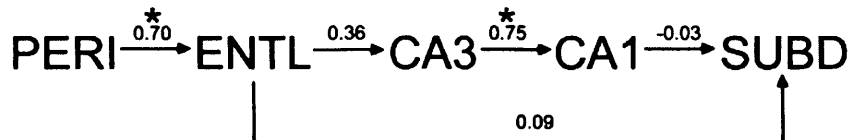
p=0.062
GFI=0.75
AGFI=0.25

Figure 92
Figure depicting the cross validation of the perirhinal-lateral entorhinal to dorsal subiculum model in the Procedural Task group. ($p=$, GFI and AGFI). $p=$ represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, SUBD-dorsal subiculum).

4.2.2.5 c-Fos Postrhinal model

The postrhinal to dorsal subiculum model for both the Working Memory (WM) and

Procedural Task groups (PT) had only one significant pathway terminating in the

subiculum, but the Working Memory group's pathway passes through the

hippocampus whereas the Procedural Task pathway is direct from postrhinal to

subiculum (Fig. 93).

WM

$$POST \xrightarrow[0.96]{*} ENTM \xrightarrow[0.73]{*} DG \xrightarrow[0.93]{*} CA1 \xrightarrow[0.88]{*} SUBD$$

0.54

p=0.15
GFI=0.79
AGFI=0.37

PT

$$POST \xrightarrow[0.89]{*} ENTM \xrightarrow[0.77]{*} DG \xrightarrow[0.83]{*} CA1 \xrightarrow{-0.14} SUBD$$

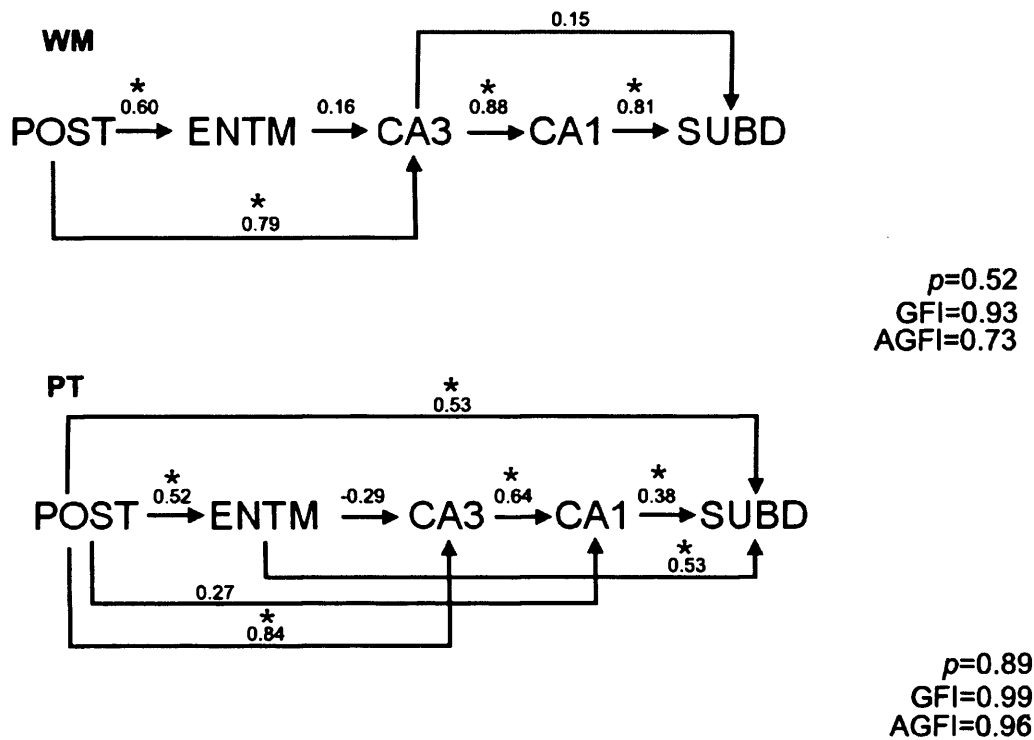$\overset{*}{0.82}$

p=0.47
GFI=0.83
AGFI=0.46
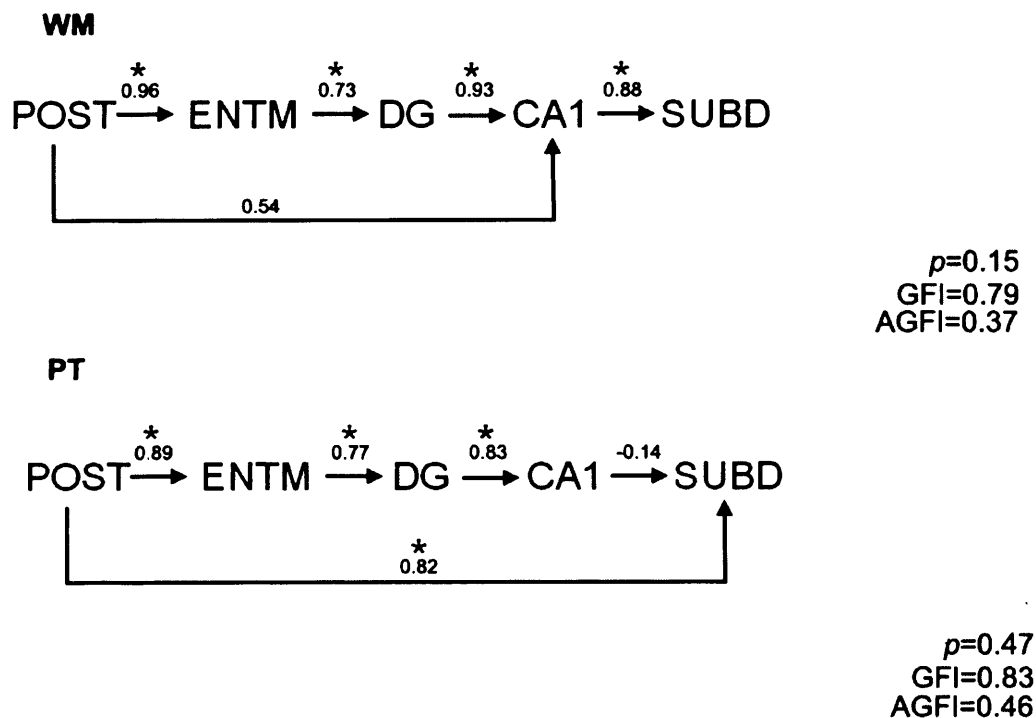
Figure 93
Postrhinal to dorsal subiculum model for c-Fos including significant path coefficients and indices of fit (p=, GFI and AGFI). p= represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (POST-postrhinal cortex, ENTM-medial entorhinal cortex, DG-dentate gyrus, SUBD-dorsal subiculum).

4.2.2.6 c-Fos Perirhinal model

For the perirhinal to subiculum model both groups have a pathway through the hippocampus but the Procedural Task group's (PT) terminates in the CA1 region and does not progress to the subiculum like the Working Memory group (WM) (Fig. 94).

WM

$$\text{PERI} \xrightarrow[0.90]{*} \text{ENTL} \xrightarrow[0.60]{*} \text{DG} \xrightarrow[0.71]{*} \text{CA1} \xrightarrow[0.86]{*} \text{SUBD}$$

ENTL $\xrightarrow{-0.35}$ CA1

p=0.058
GFI=0.76
AGFI=0.29

PT

$$\text{PERI} \xrightarrow{0.51} \text{ENTL} \xrightarrow{-0.20} \text{DG} \xrightarrow[0.83]{*} \text{CA1} \xrightarrow{0.36} \text{SUBD}$$

ENTL $\xrightarrow[0.92]{*}$ DG

p=0.41
GFI=0.83
AGFI=0.49
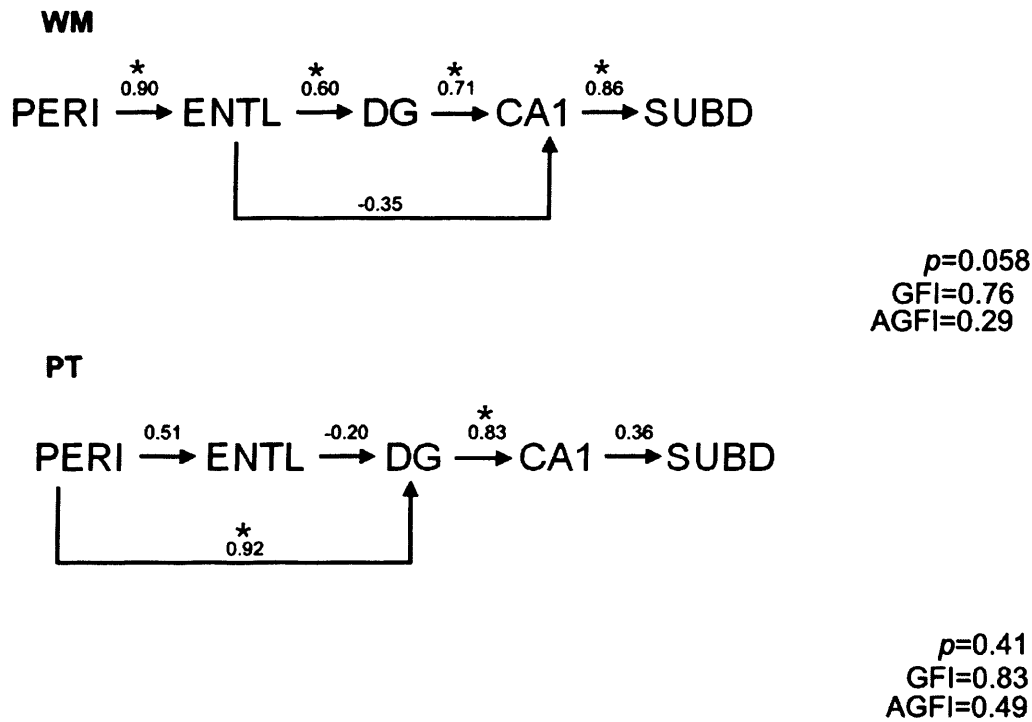
Figure 94
Perirhinal to dorsal subiculum model for c-Fos including significant path coefficients and indices of fit (p=, GFI and AGFI). p= represents the significance of chi-squared testing with non-significant values indicating good fit. Values close to one represent good fit for GFI (goodness of fit indices) and AGFI (adjusted goodness of fit indices). (PERI-perirhinal cortex, ENTL-lateral entorhinal cortex, DG-dentate gyrus, SUBD-dorsal subiculum).

4.3 Discussion

Structural equation modelling analysis of two distinct tasks in the water maze resulted in two different patterns of connections within the sampled brain areas. Models terminating in the retrosplenial cortex had less significant pathways in the Working Memory group as compared to the Procedural Task group. For both groups, models commencing in the postrhinal cortex had a significant pathway through the hippocampus proper to the subiculum whereas in the perirhinal models this was not the case. Removing the retrosplenial cortex from the model had some interesting results. For the postrhinal model, as seen previously, the Working Memory group had fewer significant pathways compared to the Procedural Task group and again both groups had a significant pathway that travelled through the hippocampus. For the perirhinal model a number of changes were seen without the presence of the retrosplenial cortex. The Working Memory group now had the most significant pathways as compared with the Procedural Task group. The Procedural Task group again did not have a pathway through the hippocampus whereas the Working Memory group now did.

4.3.1 Zif268 Models

In the case of 3 out of 4 models tested, data from the Working Memory group have fewer significant pathways than the Procedural Task group, this could indicate that they have strong connections within the network whereas in the Procedural Task group they are a greater number of weak connections. The perirhinal model which terminates in the subiculum is interesting because it gives the opposite result seen in the other three Zif268 models, where the Working Memory group has fewer significant pathways through to the subiculum or retrosplenial cortex as compared to the Procedural Task group. However it does replicate findings seen in Jenkins et al. (2003) where their working memory model had connections between the parahippocampal cortices and the hippocampus proper whereas their 'control' group did not. The only significant pathway seen in the Procedural Task group was from perirhinal cortex to lateral entrorhinal cortex and then directly to the subiculum. In all Working Memory models connections between the parahippocampal region and the hippocampus were seen. This was not the case in half the models for the Procedural Task group.

The fewer significant pathways seen in the Working Memory group as compared to the Procedural Task group suggests that there is a streamlining of the network in the former group. The animals in the Working Memory group are well learned at the task and this may have led to the lack of 'noise' encountered between the connections.

In the case of models represented in this experiment a unidirectional relationship between the variables is assumed. This assumption is made based on what is known about the anatomy in the brain and how the stronger synaptic connections follow this pattern. However a number of the regions presented, for example the retrosplenial cortex, have bi directional connections. This supposition based on anatomy can be tested by performing the SEM analysis backwards and showing that the model does now no longer fit the data as it did when tested forwards. For example, the perirhinal-lateral entorhinal model to the dorsal subiculum for Zif268 has fit indices of p=0.48, GFI=0.90 and AGFI=0.64 forwards but p=0.00, GFI=0.66 and AGFI=-0.18 backwards. This indicates that the model fits the data well when examined forward but testing with connections travelling from the dorsal subiculum to the perirhinal cortex creates a poor fit.

4.3.2 c-Fos models

The c-Fos models tested here attempted to be a direct comparison with the results seen in Jenkins et al. (2003). In the case of the Working Memory group both the perirhinal and postrhinal models had direct linear connections to the dorsal subiculum through the hippocampus. For the Procedural Task group in the case of the model tested that commenced in the postrhinal cortex a connection to the subiculum was seen but this evaded the hippocampus proper. For the perirhinal model a significant pathway was unable to reach the subiculum and instead terminated in the · CA1 region. This pattern does replicate what was seen by Jenkins et al. (2003). In their group "place", the equivalent of group Working Memory in the present study, a connection between the parahippocampal regions and the hippocampus was seen although this did not progress to the subiculum. In Jenkins et al. (2003) the "control" group, i.e. group "landmark", had no significant connections between the parahippocampal regions and the hippocampus because of disconnection between the entorhinal cortex and the hippocampus. A connection was seen between the parahippocampal regions and the subiculum but this bypassed the hippocampus. This

pattern was also seen in the current experiment in the Working Memory group, where there were direct connections between the parahippocampal cortices and the hippocampus, and unlike Jenkins et al. (2003), between the hippocampus and the subicular region. No such connections were seen in the Procedural Task group which either completely bypassed the hippocampus or failed to reached the forward projecting subiculum.

4.3.3 The importance of the hippocampus in our models

In the case of all models using both Zif268 and c-Fos as neuronal markers there is the obvious central importance of the hippocampus. For the postrhinal models using Zif268 counts the Working Memory group has a significant pathway through the hippocampus whereas the significant pathways seen in the Procedural Task group bypasses this structure. The perirhinal models are more complex with the one model that terminates in the retrosplenial cortex having no significant pathways through the hippocampus in either group. The Working Memory group in this case did have a pathway through the hippocampus to the dorsal subiculum which was not seen in the Procedural Task group. With the model that terminates in the dorsal subiculum the Working Memory group now have a significant pathway through the hippocampus whilst the Preocedural Task group does not. For all the c-Fos models the Working Memory group had direct pathways through the hippocampal subfields that end in the subiculum whereas the Procedural Task group do not.

4.3.4 Why is the hippocampus so key in the working memory task?

There is a lot of evidence that implicates the hippocampus in working memory tasks (see 2.1.4 and 3.1.2). However, there has been little research into the dissociation in function between the subfields and their specific role in working memory tasks. As described earlier (2.1.4), Lee and Kesner (2003) looked at delayed non-matching-to-place at short and intermediate delays. They found that lesions of DG and CA3 impaired performance at the short delay and all subfields disrupted performance at the intermediate delay. At the short delay the recurrent networks (neural networks that keep a representation active whilst it is required) in CA3 and dentate gyrus are disrupted by the lesions but as CA1 has no recurrent network no deficit is seen. Whilst information is passing through these networks efficient spatial representations are being formed, related to computational processes in the dentate

162

gyrus and auto association of visual landmark, vestibular and kinsthetic stimuli in CA3. As CA1 was required for longer delays the authors (Lee and Kesner, 2003) suggest the information has to circulate through a bigger network loop with the recurrent networks at the epicentre. Therefore the CA1 region may be involved in converting short term representations from the dentate gyrus-CA3 network into longer term representations in extrahippocampal regions.

There is also a body of evidence that dissociates CA1 and CA3 function in relation to pattern separation and pattern completion. Pattern completion is the "ability of a network to respond to a degraded input pattern with the entire previously stored output pattern" (Guzowski et al., 2004). Pattern separation is the ability of a network to make two similar inputs more dissimilar to prevent recall errors. Lee et al. (2004) recorded single unit activity from CA1 and CA3 simultaneously whilst animals ran around a track surround by distal cues with cues present on the track also. Cues were rotated at a probe test with track cues being rotated anti-clockwise and distal cues clockwise. When the mismatch between the cues was small, cells fired in the same configuration as seen before the cues were rotated in both CA1 and CA3. With larger mismatches some cells in CA1 followed the proximal cues and some the distal the majority remapping from the previous representation. In CA3, however, most cells followed the proximal cues with only a few remapping. In other words CA3 showed pattern completion with CA1 showing mismatched output pattern to coincide with the mismatched input pattern.

Leutgeb et al. (2004), however, showed that cells in CA3 were more likely to perform pattern separation. Animals were exposed to three environments in three similar but distinguishable rooms. The environments were either identical (large box), slightly different (large box vs. small box) and very different (large square vs. small circle). Cells recorded in CA1 had significant overlap in all three environments. In CA3 cells had distinct firing patterns in each room even when the environments were identical showing that CA3 neurons performed pattern separation.

Even though the above two studies (Leutgeb et al., 2004; Lee et al., 2004) have conflicting findings Guzowski et al. (2004) state that the differences between the way animals were tested led to this discrepancy. They went onto to declare that the studies taken together show that when sensory inputs were changed to a small extent (as in the rotation of cues in a familiar environment in the Lee et al. study) CA3 would

perform pattern completion or when the sensory inputs changed greatly (as in the different environments used in the Leutgeb et al. study) pattern separation.

A dissociation between the different sub-regions within the dorsal hippocampus is beginning to become evident, There is an emerging body of evidence that suggests how the function of these regions differ although further research is required to clarify this issue.

Taking the results of the Zif268 and c-Fos models together it becomes evident that in every model produced for the Working Memory group there is a connection between the parahippocampal regions and the hippocampus that progresses on to the dorsal subiculum. This is not the case in most of the models of the Procedural Task group. This suggests that although decreases in activation are seen in the Working Memory group, in the case of Zif268, the hippocampus is not becoming disengaged rather it is becoming recruited more efficiently. As shown by a number of studies (Morris et al., 1982; Cassel et al., 1998; Bannerman et al., 2002; Pouzet et al., 2002) the integrity of the hippocampus is required to perform a spatial working memory task in the water maze. This suggests that an intact hippocampus would not be required to perform the procedural task.

Chapter Five

Behavioural analysis of a *zif268*-EGFP transgenic rodent model

5.1 Introduction

The value of using immediate early gene expression as a tool for studying learning and memory is widely documented in the literature and demonstrated by this thesis. However, there are limitations to the technique; tissue must be fixed and expensive antibodies/probes used. A technique that allows us to visualise the protein without the need for immunohistochemical amplification would reduce the time of processing and the expense. It would also make many double labelling procedures easier. A transgenic animal has been created by Man (2004) that has the immediate early gene *zif268* driving the bioluminescent green fluorescent protein (GFP) and this would allow such a direct visualisation. In this chapter I will describe my studies with a novel secondary model that is based on the original *zif268/egr-1*-GFP transgenic rat.

5.1.1 What is GFP?

Green Fluorescent Protein is a bioluminescent protein that is found in the jellyfish *Aequorea victoria* and was first cloned by Prasher et al. (1992) and introduced into another host organism by Chalfie et al. (1994). As it is bioluminescent GFP naturally glows under the correct excitation source (~490nm). Its use in biochemistry and neuroscience has increased exponentially in the past years because of its universal and non-toxic properties. It can be easily integrated into a cell or vector and can be tagged onto any gene to act as a reporter. Also it can be quantified with out any further processing i.e. immunohistochemistry, *in situ* hybridization etc.

5.1.2 The uses of GFP in biological research

GFP has been used in a number of different ways in biological research due to its versatility. It has been utilised in organ transplantation research. Hakamata et al. (2001) created a GFP-actin fusion protein which meant that GFP was expressed in nearly all cells and organs of the rat's body. These organs were then used for transplantation and the migration of GFP cells in the blood was studied to assess acceptance of the transplanted organ. Ikawa et al. (1998) also produced mice that

expressed GFP in nearly every cell. They believed that because GFP was expressed in sperm and in fertilized eggs up until the blastocyst stage it could be used to assess integration of any transgene into an embryo. This would remove the need for PCR analysis of a transgenic line and if only embryos expressing GFP were implanted all mice born would express the desired transgene.

Another application is in the area of cell sorting for taxonomy and differential gene expression analysis. The use of different fluorescent markers fused to promoters only expressed in certain cell types would increase ease of sorting instead of using other methods such as morphology of cell type. For example, Sugino et al. (2006) used GFP and YFP (yellow fluorescent protein) to look at different neuronal populations seen in the cortex of the brain. These markers were under the control of a number of differing genes that were only expressed in certain neurons. This allowed cells to be sorted from different parts of the cortex based on gene expression, morphology, laminar distribution and electrophysiological recordings. These cells then underwent microarray analysis to determine other genes that were differentially expressed in the neuronal populations.

Van Praag et al. (2002) used GFP to tag a retroviral vector that only labelled dividing cells to study neurogenesis in live hippocampal slices. The GFP tagged vectors were injected into the dentate gyrus and their migration was studied. GFP+ cells were present in the dentate gyrus in brain slices taken 48 hours and 4 weeks after injection. The cells' phenotype was also studied using immunolabelling. At 48 hours cells were immature neurons, neural precursors or glia; none were mature neurons. At 4 weeks the GFP+ cells had developed into mature neurons. Because of the nature of GFP being expressed throughout the cytoplasm, dendrites and axons were able to be visualised in these mature neurons. Electrophysiological recordings were also carried out in these GFP+ neurons and were shown to have normal neuronal firing properties. GFP allows new cells to be traced over a time period and permits them to still be visualised after they have become mature and integrated into the surrounding tissue. As these cells are still visible certain properties can be studied e.g. phenotyping of the cells, in a number of ways.

One advantage of GFP is that it allows GFP-tagged transplanted cells to be studied. Often fluorochromes that are used for such experimentation are cytotoxic. Schmitt et al. (2005) used GFP in an experiment that tried to recover function in a GluR-Alpha knockout mouse. These mice were unable to show tetanus induced LTP

in the CA1 and CA3 regions of the hippocampus and were impaired on a spatial working memory task. GFP was used to tag a "Glur-Alpha expression system" that was transferred into the deficient mice. These cells were integrated into the CA1 region and it was now possible to induce LTP in these cells. Animals were tested on a standard working memory task in the radial arm maze a task at which Glur-A knockout mice are normally poor. The GFP-Glur-A mice showed some recovery of function as compared to the knock out mice but their performance was still poorer than wild types.

5.1.3 GFP as a reporter gene for IEG's

As described above GFP has a number of applications. However it is very commonly used as a reporter of gene transcription or protein expression. GFP has been fused with many different proteins for application in many different fields of research. One class of genes where very little work has been carried out utilising GFP as a reporter gene is immediate early genes. The first to be produced was an *egr-1* or *zif268*-EGFP transgene by Slade et al. (2002) which will be discussed in much greater detail below. Another was created by Barth et al. (2004) who produced a transgenic mouse line with a c-FosGFP C-terminus fusion protein. Basal expression of the transgene was seen in the hippocampus, cerebellum, olfactory bulb and neocortex. An increase in the protein of the transgene was seen in the magnocellular and parvocellular neurons in the paraventricular nucleus after dehydration. Induction of GFP protein was also seen in a number of brain areas after Clozapine injection. This was all seen as direct expression in fixed tissue.

5.1.4 The *egr-1*-EGFP or *zif268*-EGFP transgene

The *zif268*-EGFP transgenic model was first created and characterised by Slade et al. (2002). A derivative of this transgenic model (Man, 2004) was characterised by research described in this chapter. The original model was created by inserting pd4EGFP downstream of the *egr-1* or *zif268* promoter (Fig. 95). Four lines were produced with line 57C being selected for further analysis. Using *in situ* hybridization constitutive mRNA expression was seen in the retrosplenial, parietal cortex and in the CA1 subfield of the hippocampus. Similar expression of endogenous *zif268* was also seen in these areas. Up-regulation of transgene mRNA expression was seen after administration of the convulsant Metrazole in the

hippocampus, retrosplenial, parietal and perirhinal cortex and amygdala as compared to vehicle controls. No induction of transgene protein expression was seen using this paradigm. Also the ability of physiological stimuli to up-regulate the transgene was studied using a circadian, light pulse paradigm. After this stimulation up-regulation of transgene mRNA was seen in the suprachiasmatic nucleus and within the hypothalamic periventricular nucleus.

Rat egr -I
promoter   egr -I intron   d4EGFP
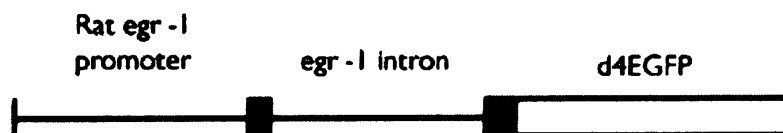
Figure 95
Picture taken from Slade et al. (2002) to show design of the original *egr-1* or *zif268*-EGFP transgene.

This 57C line was also used to study transgene expression in the pituitary gland in the female rat after oestrogenic stimulation (Man and Carter, 2003). Animals were either ovariectomised and injected with vehicle, ovariectomised and injected with oestradiol or tissue samples were taken on different days of the oestrus cycle. An increase in GFP mRNA expression was seen in the anterior pituitary in the ovariectomised rats which was reversed with injections of oestradiol. An increase in GFP expression was also seen in intact rats during pro-oestrus and reduced on other days of the oestrus cycle.

Due to the 57C line not expressing EGFP at the protein level further lines were created using the pd2EGFP which has a shorter half life than the original pd4EGFP used. A number of different lines were produced called the Z lines (Man, 2004). These all expressed GFP at the protein level in a number of brain areas including the cortex and the CA1 subfield of the hippocampus. The line Z27-B was selected for further behavioural analysis due to its strong GFP expression particularly in the hippocampus, which is the area of most interest.

Evidence has shown that this *zif268*-EGFP transgene has constitutive protein expression of GFP and that this co-localises with Zif268 protein (Man, 2004). GFP mRNA has also been shown to be induced by stimulation (light stimulation and metrazole; Man, 2004). It is now of interest to see if GFP is able to be induced at the

protein level by other stimuli and particularly by a learning and memory paradigm. Zif268 has been shown to be induced in a number of such paradigms including spatial working memory (see 3.1.4). Results of previous experiments in this thesis suggest that due to the problems encountered using the radial arm maze with the Sprague Dawley strain of rats (the strain in which the transgenic has been produced) the water maze is a better choice of paradigm. The water maze task researched previously (chap 3) is well characterised in relation to Zif268 activation and the pattern of results has been replicated. Transgene regulation will be studied initially in the CA1 region of the hippocampus as GFP expression has been shown to exhibit particularly high basal levels in the *zif268*-EGFP transgene. It is also one region which showed a large difference between the experimental and control group in previous experiments (3.2.2.2). Zif268 expression will also be studied in these animals as will the colocalization of Zif268 and GFP immunohistochemistry.

5.2 Experiment Five

5.2.2 Method

Subjects

Animals were fourteen non-pigmented Sprague Dawley transgenic rats of the strain Zif268-d2EGFP at the age of 3 months at the time of testing. Transgenic rats were generated as described below. They were housed on a light/dark cycle of 14/10 hours. Food and water was available *ad libitum* throughout the course of the experiment. Animals were maintained in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Transgenic line production

The new transgenic model was produced by P.Man (School of Biosciences, Cardiff University; Man, 2004).The strain egr-1or Zif268-d2EGFP was produced in a similar manner to the line egr-1-d4EGFP (Slade et al, 2002) apart from the Zif268 intron which was replaced with a chimaeric intron derived from the pSTEC-1 vector (Stec et al., 2001), and the d4 variant of EGFP was replaced with the shorter half-life d2EGFP (Clontech, Palo Alto, Cal, USA). In brief, a rat *egr-1/NGFIA/zif268* genomic clone (pJDM290, gift of Jeffrey Milbrandt, Washington University, St.Louis, MI, USA) containing 1.58 kb of 5' flanking sequence was incorporated into the p-STEC cloning vector using standard procedures (Sambrook et al., 1989). A 0.92kb *SalI-NotI* fragment of pd2EGFP (Clontech) was then inserted between the intron sequence and the polyA addition site in pSTEC-1. The full length transgene fragment was isolated with a combination of *AatII* and *BstXI* restriction enzymes, and purified for oocyte microinjection as described (Murphy and Carter, 1993).

Six transgenic founders were generated and these were used to establish seven independent hemizygous lines that exhibited single chromosomal integration sites and stable inheritance of the transgene. Initial genotyping and subsequent integration site analysis were conducted by Southern blot analysis as described (Slade et al., 2002) using a 736bp *BamHI-HindIII* fragment of pd2EGFP as probe. Subsequent genotyping was done with genomic DNA PCR amplifications (Murphy and Carter, 1993) as described below.

Genotyping

<u>DNA isolation</u>

Ear or tail biopsies were taken from each animal between 10 and 14 days of age and DNA was isolated. Briefly, tissue was incubated overnight at 55°C in Proteinase K/STE until it had degraded. Phenol was then added to the sample and mixed by vigorous shaking for three minutes and then centrifuged. The upper aqueous phase was then decanted to a fresh tube. Phenol/chloroform/isoamyl alcohol (P/C/IAA; 25:24:1) was then added to the decanted liquid and centrifuged. The upper phase was removed again and 3M sodium acetate and 100% ethanol added and mixed until the DNA precipitate was seen. This was then centrifuged for 10 minutes. The supernatant was decanted and the pellet washed with 70% ethanol. Again the supernatant was removed and the pellet left to dissolve in TE buffer overnight. This genomic DNA was then used for genotyping by polymerase chain reaction (PCR).

<u>PCR analysis.</u>

PCR analysis was carried out for all samples for the actin gene and EGFP transgene. A master mix for the PCR reaction was produced, this consisted of: purified sterile water; 1x reaction buffer (Promega); dNTP mix (consisting of 200μM of each dATP, dCTP, dGTP and dTTP) (Promega); 1.5mM magnesium chloride (Promega); Primer mix (100μM EGFP forward primer [5'- cggcatcaaggtgaacttcaagatccg- 3'], 100μM reverse EGFP primer [5'- cttgtacagctcgtccatgccg-3'], 100μM actin forward primer [tcatgccatcctgcgtctggacct], 100μM actin reverse primer [ccggactcatcgtactccttg]) and 0.04 units/μl Taq DNA polymerase (Promega).

For each sample, 24.5μl of master mix was added to 0.5μl of genomic DNA. A positive, negative and blank control were also added to the samples. The microcentrifuge tubes were then placed in the PCR machine. Amplifications were conducted using the following thermal cycle: 95°C for 2 minutes; 95°C for 30seconds then 62°C for 2minutes for 30 cycles and then 72°C for 7minutes.

<u>Agarose gel electrophoresis</u>

Gels were prepared by dissolving 1% agarose in 1x TAE buffer by heating in a microwave oven for two minutes. The agarose solution was left to cool slightly before 100ng/ml of ethidium bromide was added. This was then poured into the gel templates with a comb inserted to produce the wells. This was then allowed to cool

until solid. The gel tray was then placed into the electrophoresis tank and submerged in 1x TAE buffer. Orange-G loading buffer was added to 0.5 volume of PCR product and then each sample was loaded into the wells in the gel. The gel was run at 90-120 volts until the DNA was sufficiently resolved. The bands on the gel were visualised and images taken using GeneGenius™ Imaging system (Syngene) (Fig. 96).
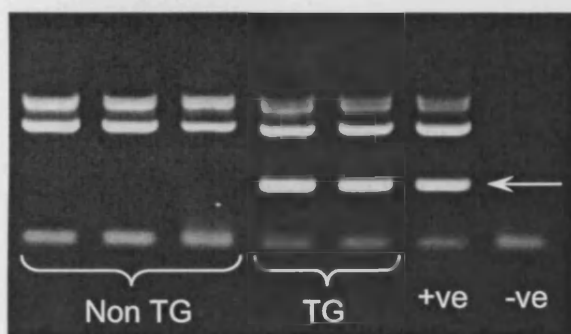
Figure 96
An example of agarose gel electrophoresis for the EGFP transgene (band indicated by white arrow) including a positive (previous sample known to be transgenic) and negative (sterile water) control.

Water maze apparatus

This apparatus was the same as described previously (3.2.1).

Procedure

Animals were split into 2 groups with seven animals in each group, group Working Memory (WM) and group 5cm Procedural Task (5cmPT). All animals performed 8 consecutive trials lasting for no longer than 90 seconds each, with an inter-trial interval of 30 seconds. Upon reaching the platform animals remained on it for 10 seconds. All animals received 20 sessions. In all cases swim paths were recorded. Animals were placed into a quiet and dark room for twenty minutes before the experiment began and for an hour and half afterwards. This was to reduce any extraneous stimulation the animals may have received. When animals were moved from room to room they were placed in a covered carry box measuring 28x45x21cm to maintain the dark conditions.

Pretraining

Animals were pretrained for 2 days before the experiment began in the same manner as experiment two, Chapter three.

<u>Working Memory group</u>

For the Working Memory group the platform position remained constant from trial to trial but moved session to session. The platform could be placed in a possible 12 different positions in the maze; both near the periphery of the pool and nearer the centre. These positions were balanced throughout the sessions. Animals were released from the edge of the pool and the start points differed on each trial. Latencies to find the platform were recorded as was the swim paths taken. On day 20, the final test day the Working Memory group performed the task as previously with the platform in a position that had never been experienced before.

<u>5cm Procedural Task group</u>

For the 5cmPT group the platform position was always around the perimeter of the water maze 5cm from the edge to encourage thigamotaxic behaviour. The platform could be in a possible 8 different positions. As the platform position altered trial to trial it was in all of the positions on any given day but the order of presentation differed from day to day in a pseudorandom manner. The animals were released from the centre of the maze. On the final test day the platform position remained in the same place on each trial and was in the equivalent position as for the Working Memory group.

Post hoc animals in the Working Memory group and the Procedural Task groups were matched on total time spent in the pool and processed for immunohistochemistry together.

Immunohistochemistry

Ninety minutes after the beginning of the final session animals were killed by cranial stunning and dislocation. The brains were removed and placed in 4% PFA in 0.1M PBS for 24hours and then in 20% sucrose solution overnight. Sections were cut coronally at 10μm on a cryostat and one in four sections were mounted directly onto gelatine covered slides. Slides were stored at -70°C and removed to perform immunohistochemistry as needed. Immunohistochemistry was performed for GFP and Zif268. Slides were washed with 0.01M PBS and then permeabilized in Methanol for 2 minutes at -20°C. After four washes in PBS, slides were incubated in blocking solution (10% goat serum in PBST) for 20 minutes. Slides were then incubated in the primary antibody, either anti EGFP (1:200 in PBST, A-11122, Molecular probes) or

anti Zif268 (1:400 in PBST, C19, Santa Cruz) for 1 hour at room temperature. Slides were again washed four times in PBS then incubated in the conjugated secondary antibody, Alexa Fluor 488 for GFP (1:500 in PBST, A-11008, Molecular probes) Cy3 for Zif268 (1:500 in PBST, C2306, Sigma) for 30 minutes at room temperature. After washing with PBS, excess liquid was removed from the slides and mounting medium (vector shield with DAPI, vector laboratories) and a coverslip was added. Double labelling of sections was also performed for GFP and Zif268 using the same method as above but with the primary (anti GFP, A-11120, Molecular probes and anti Zif268, C19, Santa Cruz) and secondary antibodies (Alexa Fluor 488, A-21202, Molecular probes and Cy3, C2306, Sigma respectively) for both markers being added at the same time. Control experiments (Man, 2004) have shown that GFP immunoreactivity is not observed in the brains of non-transgenic animals.

Direct GFP detection

Tissue was cut at 10μm on a cryostat and mounted onto gelatine coated slides. They were then stained with DAPI (Vectorshield with DAPI, Vector laboratories) and cover-slipped.

Image Analysis

Sections were scanned onto a PC (IBM) computer using a Leica DMRB microscope and an Olympus DP70 camera. Counts of the stained nuclei were carried out using the program analySIS (Soft Imaging Systems). Counts were taken from 4 consecutive sections at x20 magnification.

5.2.3 Results

5.2.3.1 Behavioural results

On the final day of testing, half of the animals performed a standard version of the working memory task in the water maze, whilst the others performed a procedural task (Fig. 97). No effect of group was seen (p=0.196) (Fig. 98) with the expected group by trial interaction being found (F (7, 84) = 10.8, p <0.001). An overall effect of trial was seen (F (7, 84) = 10.1, p <0.001) with latencies in the Working Memory group reducing over the trials. Simple effect analysis revealed that differences were significant between some trials in the Working Memory group (t1 vs. t3: p=0.001; t1 vs. t4: p=0.001; t1 vs. t5: p=0.002; t1 vs t6: p=0.002; t1 vs. t7: p=0.002; t1 vs. t8: p=0.001) with no significant differences seen between any trials in the Procedural Task group.
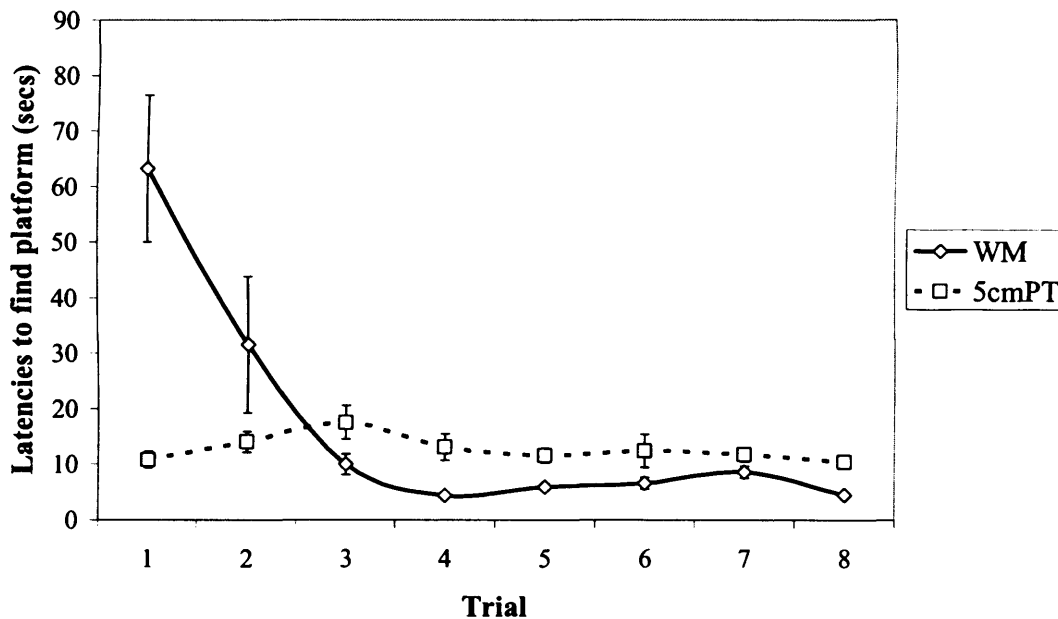
Figure 97
Latencies to find platform in the Working Memory (WM) and Procedural Task (5cmPT) groups over eight trials on the final day of testing. The Working Memory group show a decrease in latencies to find the platform over trials whilst the Procedural Task group do not.

Total time spent in maze (Secs) — 180, 160, 140, 120, 100, 80, 60, 40, 20, 0

WM   5cmPT
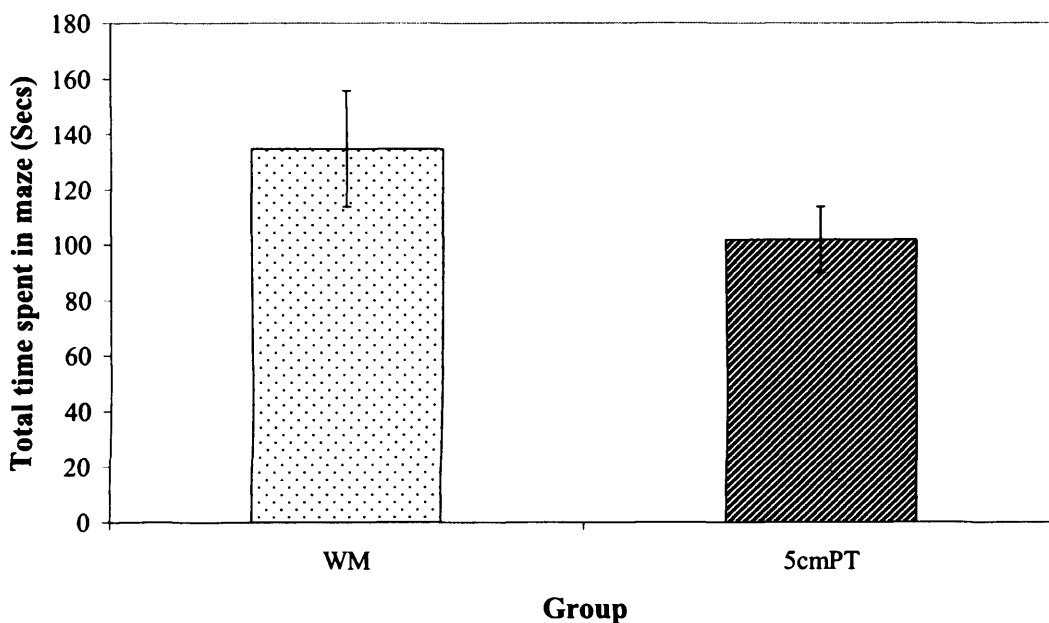
Group

Figure 98

Total time spent in the water maze by the Working Memory (WM) and Procedural Task (5cm PT) groups. No difference is seen between the two groups in the total time spent in the maze on the final test day.

5.2.3.2 Immunohistochemical results

GFP

For the 5cm Procedural Task group in 6 out of 7 animals a number of GFP+ve neurons were present within the CA1 region of the hippocampus (Fig. 99 and 101). All sections were double labelled with DAPI which stains DNA and allows visualisation of the cell population present in the section. These images were merged to show the localization of the GFP cells. No GFP was present in CA1 in the Working Memory group (Fig. 99 and 102). There was however GFP seen in other areas in both groups including the amygdala and some cortical regions (Fig. 103 and 104).

Zif268

Zif268 expression was also seen in the CA1 region in 6 out of 7 animals in the control group and in 4 animals in the Working Memory group (Fig. 105 and 106). For Zif268 expression a one way ANOVA revealed a significant effect of group (F (1, 12) =90.331, $p<0.001$) with the Working Memory group having significantly less Zif268 in CA1 than the Procedural Task group (Fig. 100). Analysis showed that GFP does co localise with Zif268 within the CA1 region (Fig. 107).
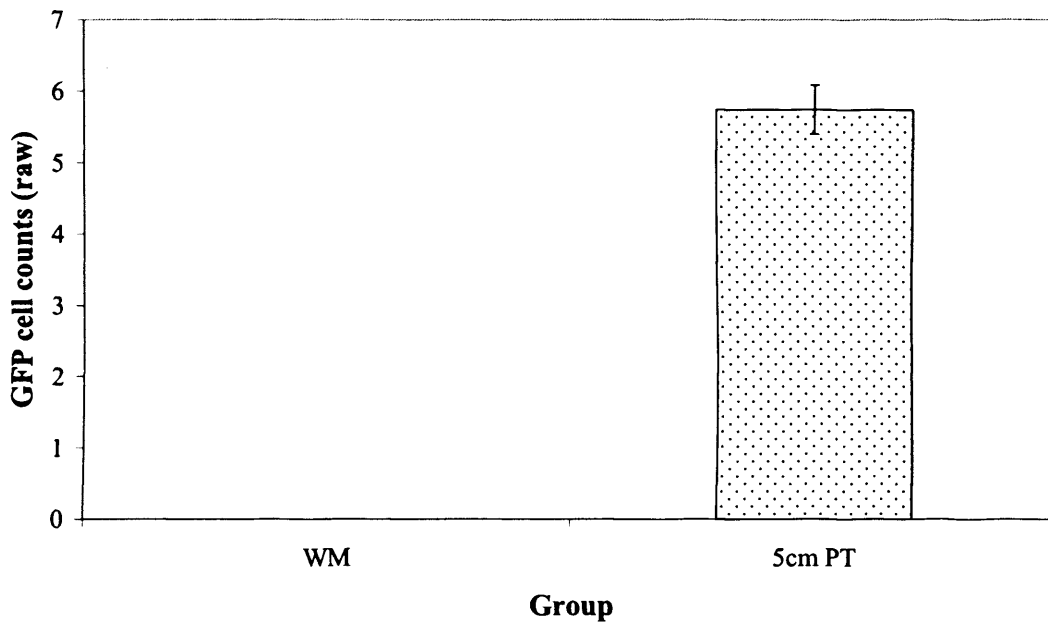
Figure 99
Mean number of GFP immunoreactive cells in the CA1 region in the Working Memory (WM) and Procedural Task (5cmPT) group.
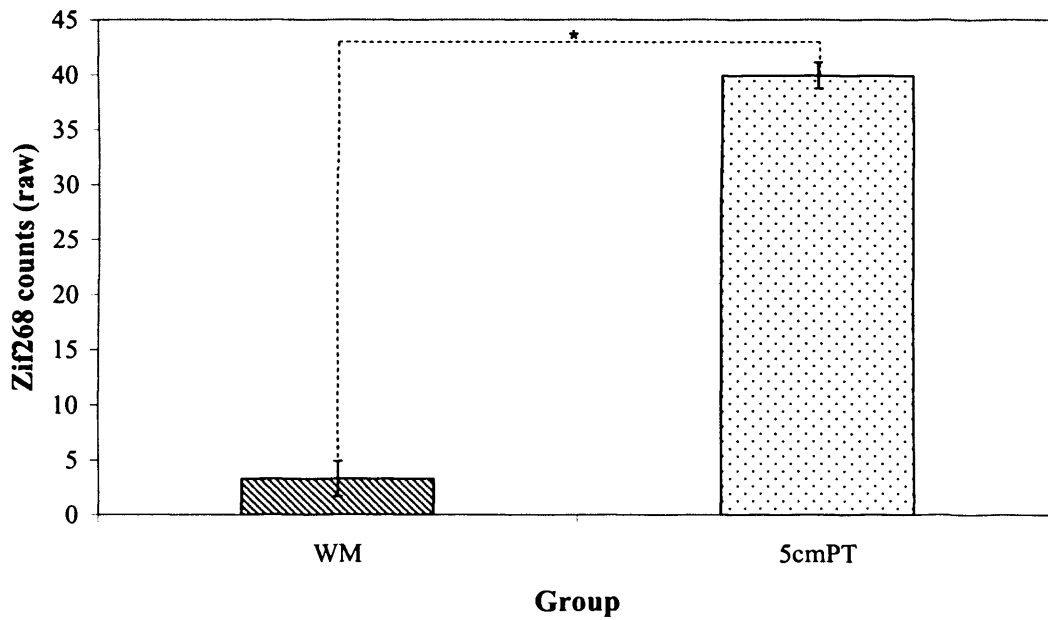
Figure 100
Mean number of Zif268 immunoreactive cells in the CA1 region in the Working Memory (WM) and Procedural Task (5cmPT) groups.

5.2.3.3 Direct GFP fluorescence analysis.

GFP fluorescence was also directly visualized. Although very few cells were evident under direct visualization, a few neurons were observed in CA1 and the dentate gyrus of animals in the Procedural Task group (Fig. 108 and 109). No cells were evident in the Working Memory group (images not shown).
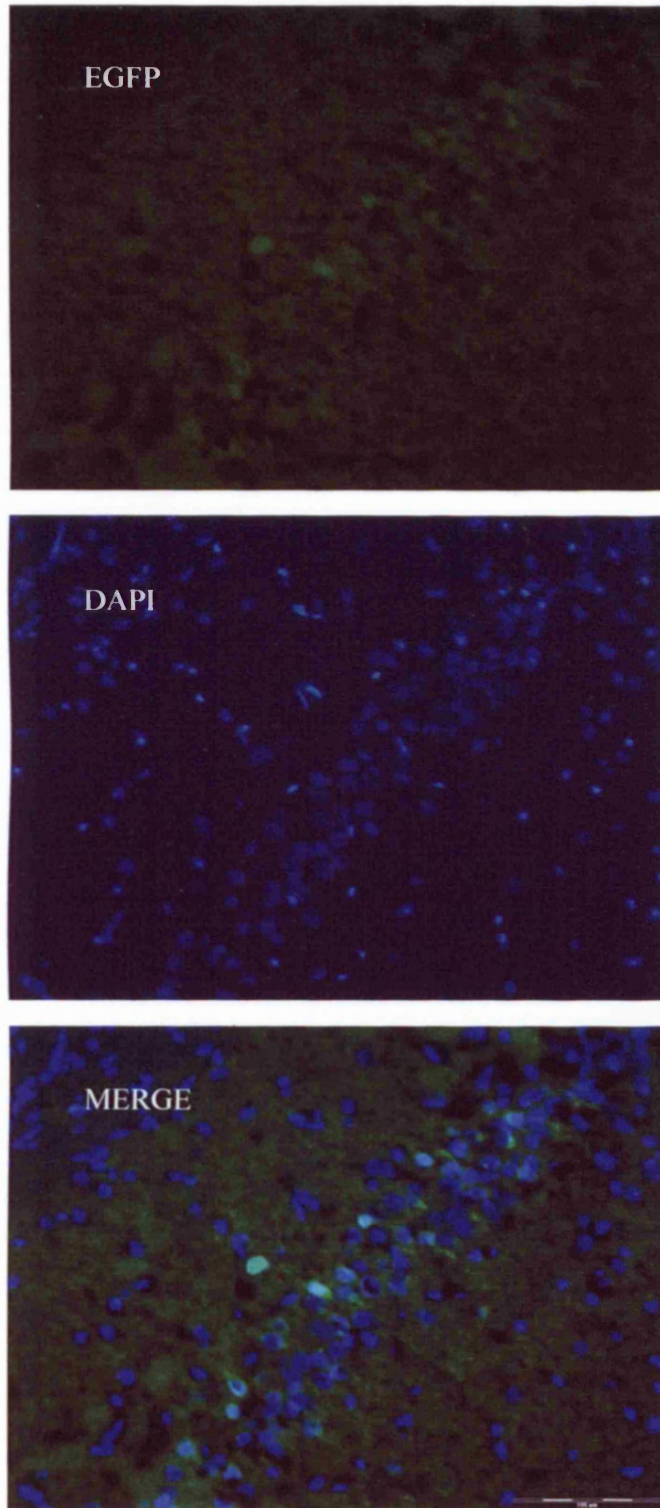
Figure 101
Images of GFP immunoreactive (top) and DAPI stained (middle) cells in the CA1 region at x20
magnification in a Procedural Task animal. The bottom picture is a merge of both images.
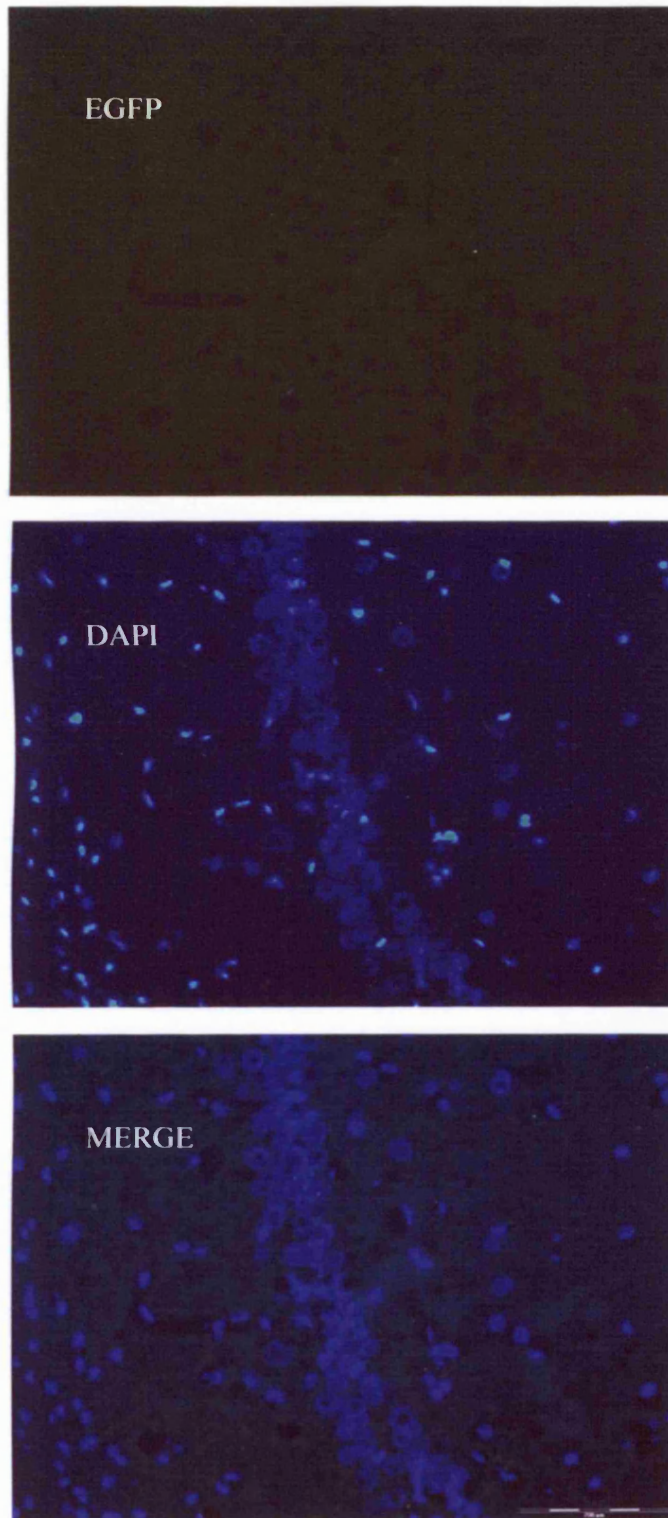
Figure 102
Images showing absence of GFP immunoreactivity (top) and maintenance of DAPI-stained cells (middle) in the CA1 region at x20 magnification in a Working Memory animal. The bottom picture is a merge of both images.
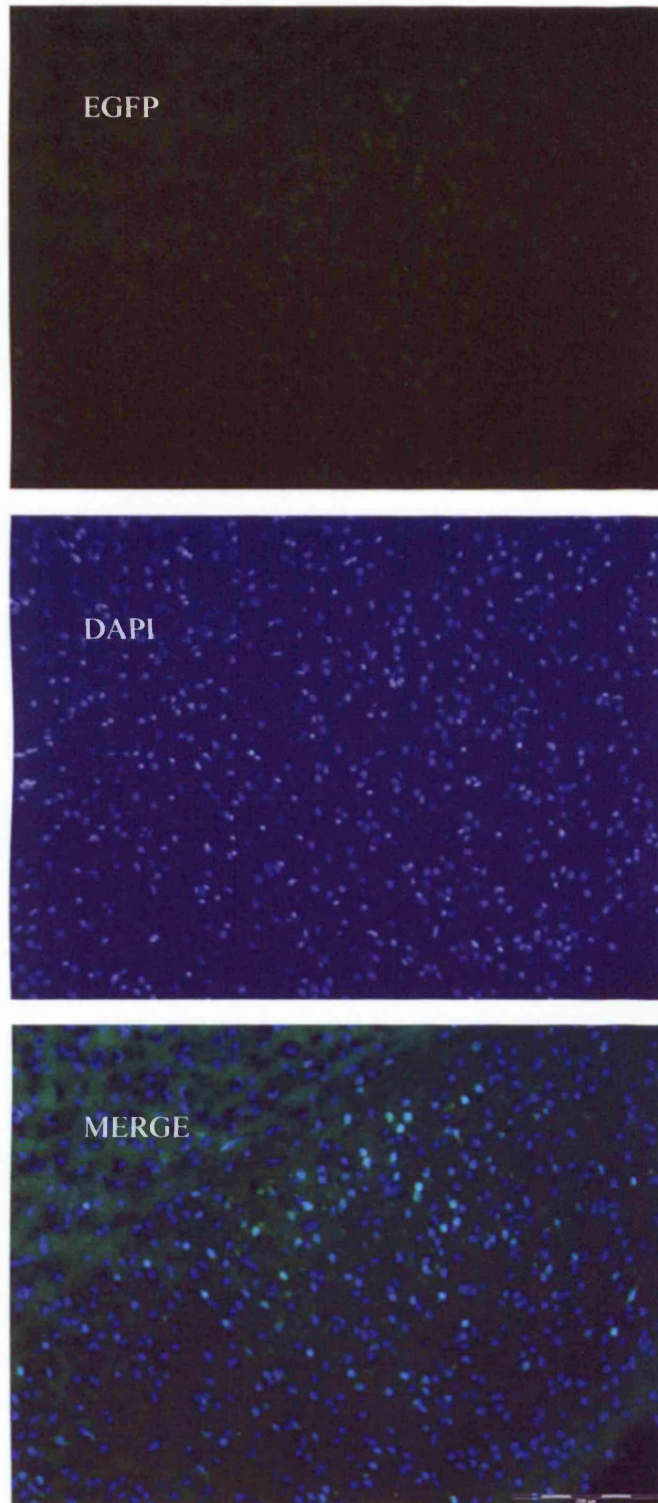
Figure 103
Images of GFP immunoreactive (top) and DAPI stained (middle) cells in the basolateral amygdala at x10 magnification in a Procedural Task animal. The bottom picture is a merge of both images.
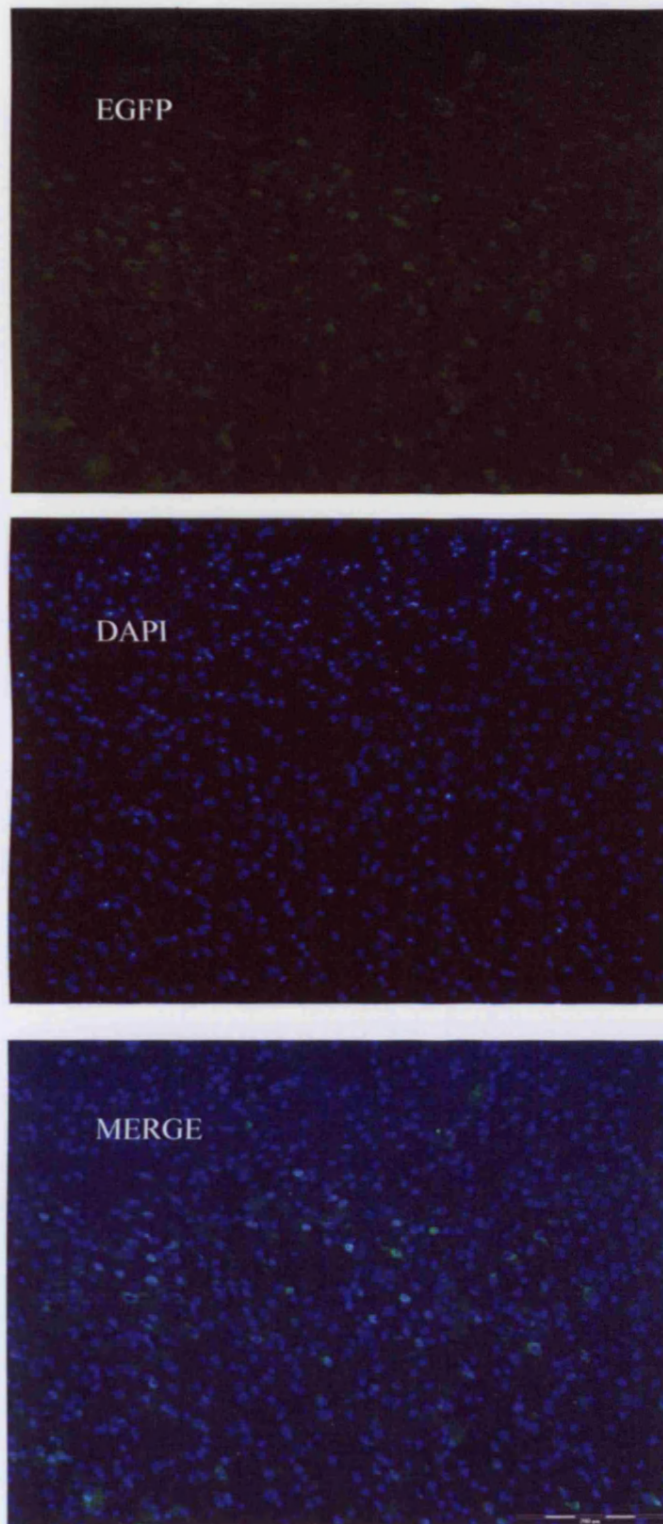
Figure 104
Images of GFP immunoreactive (top) and DAPI stained (middle) cells in the basolateral amygdala at x10 magnification in a Working Memory animal. The bottom picture is a merge of both images.
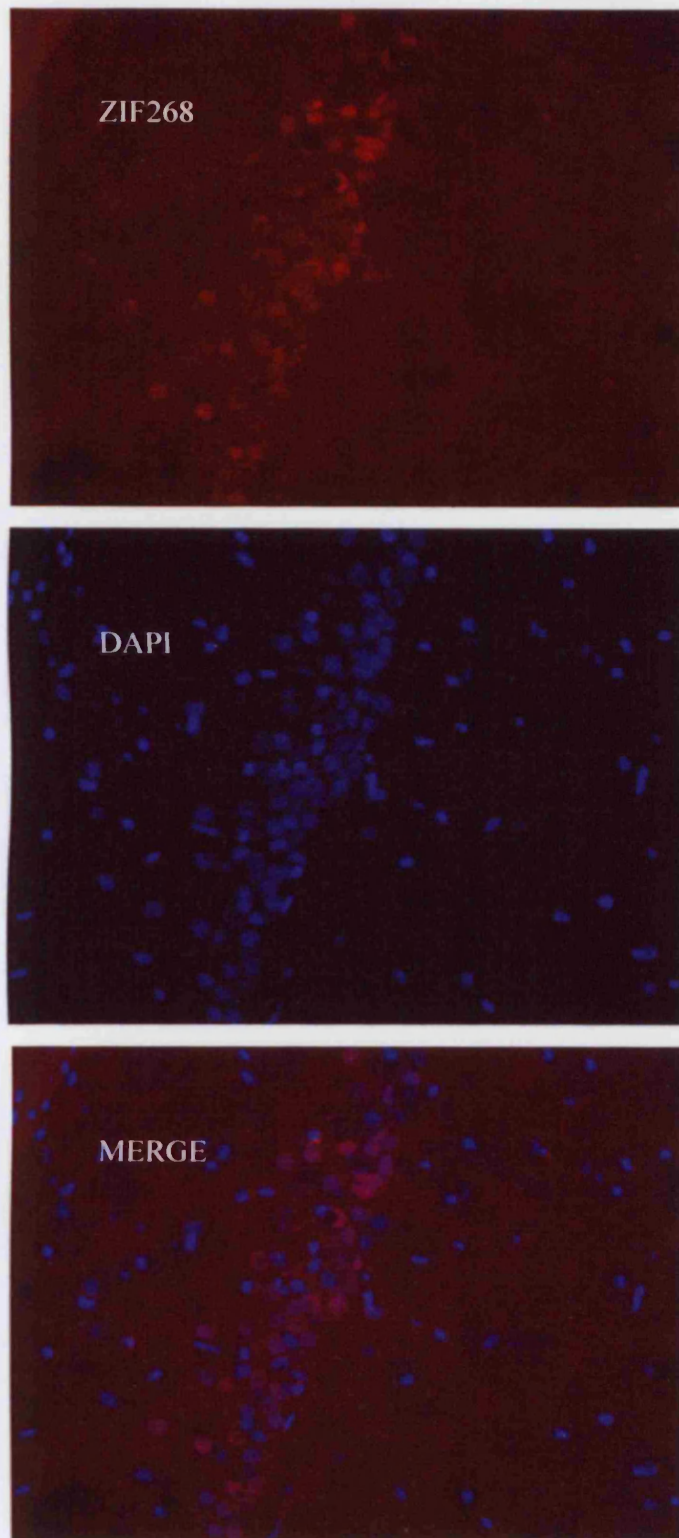
Figure 105
Images of Zif268 immunoreactive (top) and DAPI stained (middle) cells in the CA1 region at x20 magnification in a Procedural Task animal. The bottom picture is a merge of both images.
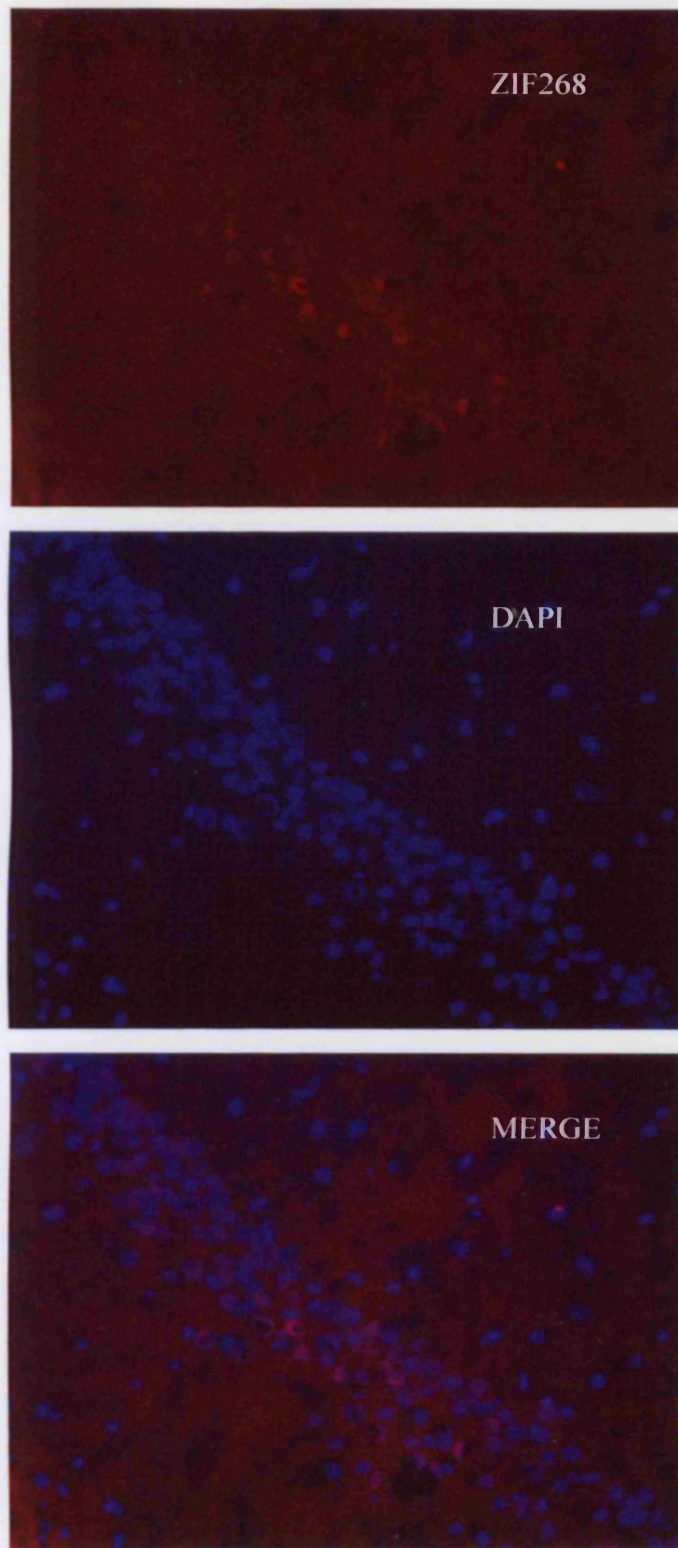
Figure 106
Images of Zif268 immunoreactive (top) and DAPI stained (middle) cells in the CA1 region at x20
magnification in a Working Memory animal. The bottom picture is a merge of both images.

Figure 107
Images of colocalized GFP (top) and Zif268 (middle) immunoreactive cells in the CA1 region at x20 magnification in a Procedural Task animal. The bottom picture is a merge of both images white arrows show colocalised GFP and Zif268 cells.
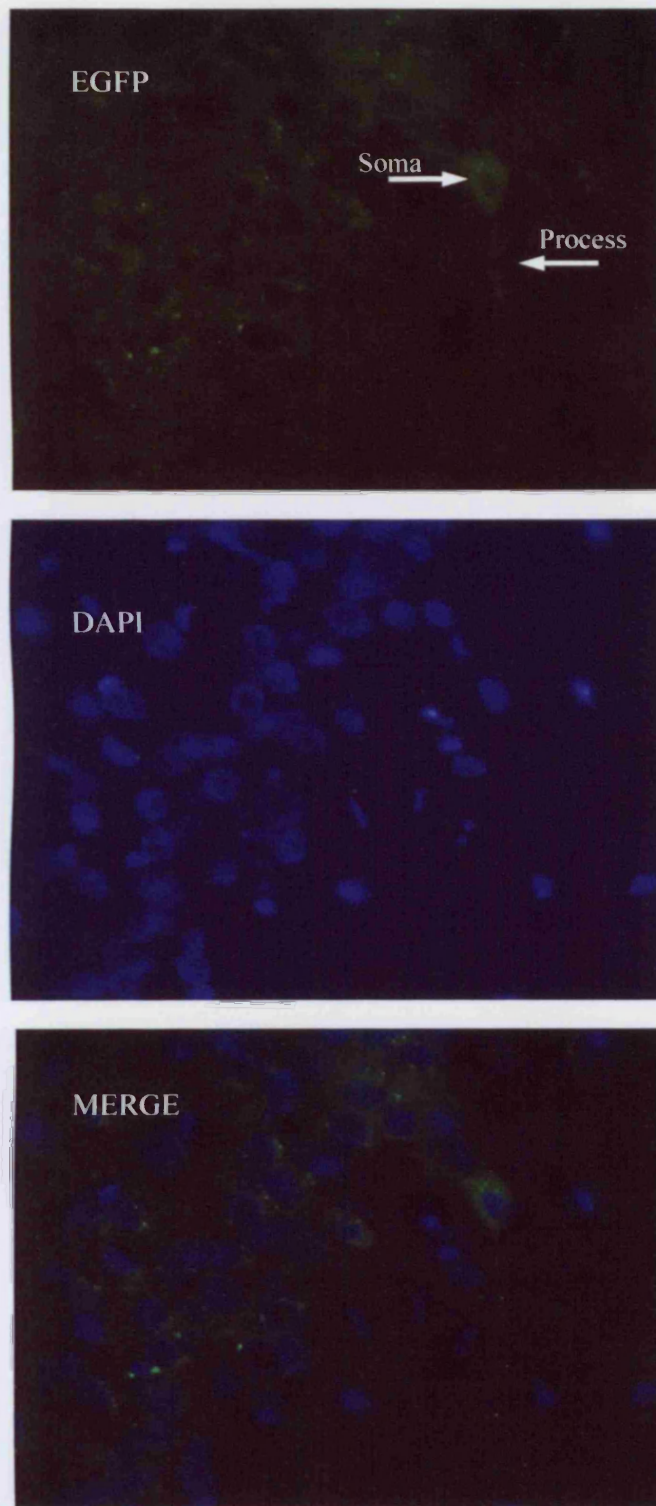
Figure 108
A neuron expressing GFP in the CA1 region of an animal in the Procedural Task group (top) and DAPI stained cells (middle) and a merge of the two images (bottom). The white arrows points to the GFP neuron.
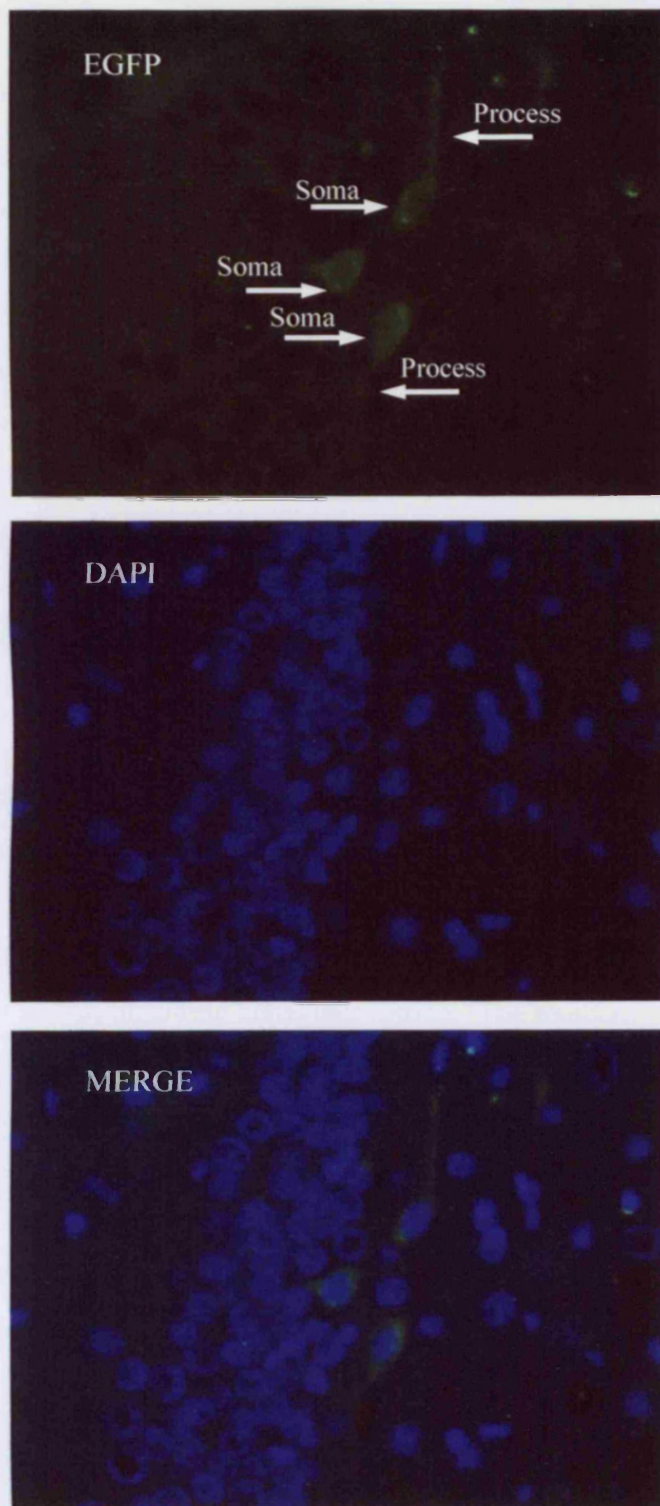
Figure 109
A cluster of neurons expressing GFP in the dentate gyrus of an animal in the Procedural Task group (top) and DAPI stained cells (middle) and a merge of the two images (bottom). The white arrows point to the GFP neurons.

5.4 Discussion

A novel transgenic rodent model was used to investigate its utility in the study of a working memory paradigm in the water maze. A well characterized task was used (see Chap 3) to assess whether the model was suitable to study Zif268 neuronal activation after behavioural testing. Activation of GFP neurons was seen in the Procedural Task group after training in the CA1 region of the hippocampus. No such activation was seen in the Working Memory group. The Working Memory group did, however, have expression in a number of other brain regions including the amygdala.

These results replicate findings of previous experiments in this thesis (chap 3) where a reduction of Zif268 immunoreactivity was seen in a working memory paradigm in the water maze in the CA1 region. The implications of this have been discussed elsewhere (3.6).

5.4.1 Viability of the transgenic line for behavioural testing

This transgenic line has previously been used for circadian rhythm research with results showing that the transgene can be markedly up-regulated by light stimulation (Man, 2004). The next question was whether the line could be used for more subtle manipulations, including behaviour testing. The transgenic line would afford a certain amount of improvement on current behavioural-IEG experiments as the GFP can be visualised without the need for any further processing. This would cut down on the time and cost of such experiments, and facilitate additional forms of analysis such as *ex vivo* sorting of neurons for microarray analysis (Sugino et al., 2006) and electrophysiological recording (Barth et al., 2004). The current experiment does indeed show that differences are seen between an experimental group and a 'control'.

This GFP transgenic line has another advantage over native immunohistochemical approaches. Whereas both Zif268 and c-Fos are expressed only in the nucleus of the cell GFP is expressed more widely including in neuronal projections (see Figure 109). This allows differing cell types to be visualised easily with out the need for different staining techniques.

Previously it has been demonstrated that GFP positive cells colocalised with Zif268 immunoresponsive cells (Man et al., 2004). However, the images presented in this thesis show poor co-localization and further work is need with better quality

tissue to provide a more thorough evaluation of EGFP and Zif268 co-localization. Also as demonstrated by the cell counts obtained here more cells expressed Zif268 than did GFP. The next step in analysis would be to determine if it is only cells that posses a strong Zif268 signal that are expressing GFP, and whether this accounts for the discrepancy between the expression levels. It is also possible that GFP is more unstable than Zif268 and therefore a majority of GFP-expressing cells are not detected at the time of sampling. This seems unlikely as Waters et al. (1990) showed that the Zif268 protein has a half-life of less than 90 minutes whereas D2EGFP is anticipated to be two hours. Nevertheless, further studies should be performed to assess the relative half-lives of GFP and Zif268 in this cellular context.

As immunohistochemical techniques were initially used to visualise the GFP in this experiment the next step was to see if changes in expression associated with spatial learning would permit direct visualization of GFP fluorescence. As can be seen in figures 108 and 109 it is possible to detect GFP neurons without any further immunohistochemical processing. Although these cells in this experiment are small in number, characterization of new lines of this transgenic model is being carried out and it may be possible to optimize expression further. The fixation protocol may also require further optimization. If this occurs this transgenic line could be used for learning and memory IEG studies, which would negate the need for expensive and time consuming tissue processing. The only caveat is that although this transgenic model would remove the need for tissue processing the animals still need to be genotyped which itself is a time consuming and expensive process. However recent work has shown that it is possible to visualise GFP in live animals in a non invasive and inexpensive manner. Tyas et al. (2003) used a readily available blue LED flashlight with filters for the correct emission and visualisation light wavelength to study a GFP tau fusion transgenic. Transgenic and non transgenic pups were easily able to be distinguished using this technique. Tau is a protein that is expressed at a high level and is in most tissues. Zif268 is also widely expressed so it could be possible to use such a technique to genotype the *zif268*-EGFP transgenic line. Although initial genotypic confirmation would be required to validate the accuracy of the method this could be stopped once its accuracy had been established. The cost of setting up a breeding colony is low and maintenance is no more difficult than that of general laboratory rat husbandry. Taken together this all tentatively suggests a new

and interesting addition to our tools for studying immediate early gene expression in learning and memory paradigms.

Chapter Six

Transcriptome analysis of a dysfunctional brain network

6.1 Introduction

As described in the introduction (1.8) changes in IEG activation occur not only in a functioning but also in a dysfunctioning brain network. The research carried out in this chapter was based on results found from a study performed by Jenkins et al. (2004). They investigated the induction of immediate early gene activation after lesions of the anterior thalamic nuclei. This lesion produced a severe reduction in c-Fos expression in the superficial layers of the retrosplenial cortex, with a 90% reduction in the number of c-Fos positive cells as compared to the sham hemisphere. However, Nissl stains revealed no cell loss in the same area. This pattern of expression was seen after survival times of 6 to 13 weeks after both NMDA and electrolytic lesions. After survival times of 19 months reduction of c-Fos expression was beginning to be seen in the deeper layers of the granular cortex and also in the dysgranular cortex. This decrease in IEG expression in the retrosplenial cortex is of medical relevance because parallels can be drawn with an effect seen in early Alzheimer's disease as discussed below.

6.1.1 What is Alzheimer's disease?

Alzheimer's disease is a progressive neurodegenerative disorder of older adults. It is characterized by symptoms such as subtle decline in cognitive capacity that worsens as the disease progresses, personality changes, deficits in language abilities and eventually dysfunction of the motor system (Braak et al., 1997). Clinically it is characterized by the presence of neurofibrillary tangles and amyloid plaques throughout the brain. These show a distinct pattern of presentation with cognitive symptoms only becoming evident once much of the brain has been invaded by tangles and plaques (Braak et al., 1997). A definitive diagnosis of Alzheimer's disease can only be made at the post mortem stage where the presence of these abnormalities can be seen. This is obviously problematic as early diagnosis could potentially allow therapies to be administered that could slow down the progress of the disease.

6.1.2 Where is damage seen in early Alzheimer's disease?

One way early diagnosis can be attempted to be made *ante mortem* is to use neuroimaging techniques and note if a decrease in function can be seen in any brain region.

PET scans were employed by Minoshima et al. (1997) to study metabolic activity in 66 patients with probable Alzheimer's disease as well as 23 patients who had memory impairment without general cognitive decline, 8 of whom went on to develop Alzheimer's disease at a follow up test. These were compared against 22 age matched normal controls. Marked metabolic reduction in posterior cingulate cortex and cinguloparietal transitional area was seen in Alzheimer's disease and memory impaired patients. In the memory impaired patients the area with the greatest loss was the posterior cingulate cortex. This region compares to the retrosplenial cortex in the rat. This loss of function indicates that the retrosplenial cortex may be a key brain area that becomes hypofunctional in very early Alzheimer's disease and also in mild cognitive impairment, a common precursor to Alzheimer's disease. Further support came from Nestor et al. (2003) who discovered that the most consistently hypometabolic region in patients suffering from Mild Cognitive Impairment was the retrosplenial cortex (BA29/30).

Huang et al. (2002) compared cerebral blood flow in patients suffering from mild cognitive impairments (MCI) which progressed to Alzheimer's disease and those that did not. The conversion rate to Alzheimer's disease was 13.7%. The earliest deficit seen in patients that had progressive MCI was hypoperfusion in the posterior cingulate cortex. The posterior cingulate cortex, however, shows no pathological changes in preclinical Alzheimer's disease. It was stated that in preclinical Alzheimer's disease the isolation of the posterior cingulate from temporal lobe structures may be an important mechanism for cognitive impairments. These disconnections may lead to the decrease in blood flow seen in the posterior cingulate cortex. Another area that shows very early changes in Alzheimer's disease is the anterodorsal nucleus of the thalamus (Braak and Braak, 1991). The authors' stated that according to their disconnection theory lesions of the anterodorsal nucleus in early Alzheimer's disease could lead to the hypoperfusion seen in the cingulate cortex.

Also of great interest are studies that have been carried out in people that are at risk for developing Alzheimer's disease. There are a number of gene mutations that are said to be a risk factor for familial or early onset Alzheimer's disease; these

mutations involve presenilin 1 and 2 and amyloid precursor protein and for sporadic or late onset Alzheimer's disease the epsilon 4 allele of the apolipoprotein E gene. Cingulate regions of patients with familial Alzheimer's disease were found to be significantly smaller than controls by MRI scan (Jones et al., 2006). Also, Reiman et al. (1996) showed that subjects that were homozygous for the (epsilon) e4 allele of apolipoprotein gene and who had yet to develop Alzheimer's disease had a reduction in glucose metabolism in the posterior cingulate, parietal, temporal and prefrontal regions with maximum reduction being seen in posterior cingulate cortex. Many of the patients went on to develop Alzheimer's disease. This result was replicated by Fox et al. (2001) who studied subjects who were at risk for Alzheimer's disease but at the time of scanning displayed no symptoms. All subjects went on to develop the disease between 5 and 8 years after the first scan. These subjects showed atrophy before they showed symptoms in the medial temporal lobe and the posterior cingulate.

All the above studies indicate the importance of the retrosplenial cortex in pre-clinical Alzheimer's disease, as changes can be seen in this region in MCI before it progresses to Alzheimer's disease and even before symptoms are present in those at risk for Alzheimer's disease. It shows that changes in the retrosplenial cortex may be a good early indicator of Alzheimer's disease and that the connection between the retrosplenial cortex and anterior thalamic nuclei is of potentially great importance.

6.1.3 The anterior thalamic nuclei, the retrosplenial cortex and the connections between the two

The anterior thalamus consists of three main nuclei; the anterodorsal (AD), anteroventral (AV) and anteromedial (AM). These nuclei are bordered caudally by the lateral dorsal nucleus (LD), which is closely related to the anterior nuclei, and it is important to note as lesions of the anterior thalamic nuclei can result in damage in this area. The AV nucleus can be subdivided depending on the cell body size into the ventromedial or magnocellular part and a dorsolateral or parvicellular part (Van Groen et al., 1993). The AM and AV nuclei contains multipolar neurons whilst the AD nucleus contains fusiform neurons (Bentivoglio et al., 1993).

The retrosplenial cortex in the rat runs half of the length of the brain and consists of two parts, granular and dysgranular. In the human the granular part is named area 29 a-c and the dysgranular portion 29d. The granular part is further subdivided into granular a (Rga) and granular b (Rgb), areas 29a and b and 29c in the

human. The retrosplenial cortex is arranged in distinct layers in both the granular and dysgranular parts.

The anteromedial nucleus has sparser projections to the retrosplenial cortex as compared to the other anterior thalamic nuclei. AM projects to primarily to anterior cingulate region IR with other connections to granular a of the retrosplenial cortex and area 18b. In Rga it terminates primarily in layers I and IV. Rostromedial AM projects to caudal retrosplenial and caudal AM to rostral Rga (Van Groen et al., 1993). The AV nucleus projects to the retrosplenial cortex, postsubiculum, presubiculum and subiculum. Connections to Rgb terminate in layers I and IV and to Rga cortex terminate in layer I. Again connections are topographically organized (Van Groen et al., 1993). The AD nucleus projects to the retrosplenial cortex, postsubiculum, presubiculum and parasubiculum. Projections in Rga and Rgb terminate in layers I and III/IV. The LD nucleus has much broader connections connecting with IR, precentral agranular, retrosplenial dysgranular, retrosplenial granular a and b, area 18b, postsubiculum, presubiculum, parasubiculum and entorhinal cortex. In the dysgranular retrosplenial LD projections terminate in layers I, III and IV and in Rgb and in Rga they terminate in layer I (Van Groen et al., 1993).

Within the retrosplenial cortex layer II is the only region which contains fusiform pyramidal cells (Van Groen et al., 1993). These cells have apical dendrites that extend into layer I. The AV nucleus projects mainly to regions that contain the dendritic bundles (layers 1a and IV) and these connections are highly specific. The AD nucleus projects mainly to layers I and III/IV whereas LD projections terminate in layer Ia (Van Groen et al., 1993).

6.1.4 Changes in IEGs in Alzheimer's disease

Marcus et al. (1998) looked at c-Fos and c-Jun activation in the hippocampus of post mortem tissue from Alzheimer's disease brains. A significant increase in c-Jun levels were seen in all hippocampal subfields in the Alzheimer's patients as compared to age-matched controls. An increase was also seen in c-Fos apart from in CA1 where no difference from controls was seen. Anderson et al. (1994) also found an increase in c-Fos and c-Jun immunoreactivity in Alzheimer's post mortem tissue in both the hippocampus and the entorhinal cortex. However, c-Jun activation showed a much greater increase in Alzheimer's disease brain than c-Fos. MacGibbon et al. (1997) studied c-Fos, Fra1, Fra2, c-Jun and Krox 20 and Krox 24 (Zif268) activation

194

in post mortem Alzheimer's disease tissue. Only c-Jun and Krox 24 (Zif268) showed an increase in the hippocampus of Alzheimer's disease brains.

As described above a number of studies show changes in gene expression in Alzheimer's disease. However these studies only focus on how a few genes change at anyone time. A technique that quickly allows us to assay the expression of many genes in a given sample at one time would be advantageous. Microarray technology affords a more global view of gene expression, assessing many thousands of genes simultaneously. Because of the striking changes seen in the Jenkins et al. (2004) study it would be of interest to see if this is an IEG specific change or whether more global changes in gene expression would be seen. Microarray analysis of retrosplenial tissue after lesion of the anterior thalamic nuclei would allow us to answer this question.

6.1.5 What are microarrays?

Micro or gene arrays are "solid substrates onto which hundreds or thousands of DNA molecules are affixed. ...and are capable of monitoring alterations in gene expression..." (Nisenbaum, 2002). They are a useful research tool that allows a vast amount of data to be collected at once. A tissue sample can be assessed to see which genes are up-regulated, down-regulated or remain the same after experimental manipulation. Microarrays are available in two main types: cDNA or spotted arrays and oligonucleotide arrays. Spotted arrays are made by fixing DNA fragments to a platform, usually glass or nylon. The genes of interest are identified by these DNA probes. Commonly used oligonucleotide arrays have sets of oligonucleotide 'features' that consist of multiple 'perfect match' oligos and a 'mismatch' as a control for each gene.

Each of these arrays has its own specific advantages and disadvantages. Custom cDNA arrays are extremely versatile allowing the genes used to be modified to include any that are required including those that have been newly discovered (Barlow and Lockhart, 2001). Oligonucleotide arrays can be manufactured without physical intermediates and can include a large number of probes (Barlow and Lockhart, 2001). There is also an ability to carry out unplanned *post hoc* analyses and these arrays are also good at distinguishing genes from the same family (Luo and Geschwind 2001). Oligonucleotide arrays have a very particular advantage in that because they are mass produced they have very low variability because of quality

control (Barlow and Lockhart, 2001). cDNA arrays have the disadvantage that they depend on physical intermediates and that there is a large variability due to the spotting techniques used (Nisenbaum 2002). Oligonucleotide arrays on the other hand are not as flexible if a particular set of genes is required on the array and they are also more expensive than cDNA arrays (Luo and Geschwind, 2001). In this experiment oligonucleotide arrays will be utilised because of their advantageous factors and because we have no *a priori* indications of the genes we would expect to find changes in and hence want all those available to us.

6.1.6 False positives and microarray analysis

Another issue related to high throughput methods such as microarray technology is the incidence of false positives in analysis, i.e. flagging a gene as having significantally differential expression when it does not. When an analysis is performed on gene lists a comparison could be being made between 15, 000 or so genes all of which are compared individually and as the number of comparisons increases the incidence of false positives also increases. There are a number of methods available when performing such analyses to help reduce the incidence of false positives.

The Bonferroni correction is very conservative and is calculated by multiply the number of genes in the gene list by the p-value of each gene. If this new p-value is still below the cut off point the gene will be seen to be significant. However because this method is so stringent it can lead to an increased occurrence of false negatives i.e. calling a gene that does show differential expression not significant.

The Bonferroni step-down (Holm) correction is similar to the Bonferroni correction. P-values for each gene are ranked smallest to largest and then the first p-value is multiplied by the number of genes in the list. This pattern continues multiply by one less gene every time down the list until no more significant genes are found. This correction is less stringent than Bonferroni but still can lead to an increase in frequency of false negatives.

The Westfall and Young Permutation unlike the previous two methods treats the gene list as a whole and does not focus on each gene individually. Initially the p-values are ranked and then a pseudo-data set is created by splitting the data into imitation treatment and control groups. The p-values for this data set are then calculated and compared to the original p-values. This process is repeated a number

196

of times and the adjusted p-value is the proportion of the data sets where the smallest pseudo p-value is less than the original p-value.

The Benjamini and Hochberg false discovery rate is calculated by ranking the p-values for each gene from smallest to largest. The largest p-value remains unadjusted, the second p-value is taken and is multiplied by the total number of genes divided by it's rank. This process continues for all genes.

Although a correction like Bonferroni is very stringent it also leads to an increased incidence of false negatives something that also wants to be reduced in such an analysis. This is also true of the Holm and Westfall and Young corrections which are all based on the family wise error rate method of control. The Benjamini and Hochberg method is based on the false discovery rate error control method and although is less stringent it also reduces the incidence of false negatives and provides a nice balance between the two.

6.1.7 Previous microarray analyses of dysfunctional brain

In recent years microarray technology has become widely used in biomedical research. This review will focus on their usage in studying dysfunction and disease within the brain. Ying et al. (2004) used the technique to look at gene expression in the hippocampus after connections with the entorhinal cortex were severed. Expression was studied via microarray ten days after lesion. 152 genes were identified that had 1.5 to 2.8 fold changes in the lesioned brain. Eight candidate genes were confirmed by northern blotting and *in situ* hybridization. Four genes associated with the immune response were up-regulated by deafferentation (MHC I, MHC II, $\beta 2$-M, 1FN-γ). MHC genes have also been shown to be involved in neural plasticity (Huh et al., 2000). Also up-regulated was osteonectin which is involved in axon growth. Three other genes involved in the actin cytoskeleton interaction (thymosin-$\beta 4$, profilin, gelsolin) were up-regulated and the authors state that they could be involved in the remodelling of the dendrites and axonal sprouting of hippocampal neurons following deneveration.

Microarray analysis has also been used to study transcript changes in Alzheimer's disease. Colangelo et al. (2002) conducted a transcriptome analysis of post mortem tissue from the CA1 region of Alzheimer's disease suffers. A general decrease in gene activity was seen as compared to normal brains. Decreases were seen in synaptic and cytoskeletal proteins, transcription factors and signal transduction

197

elements related to synaptic plasticity. Increases were seen in genes associated with sustained inflammation. Emilsson et al. (2006) found that genes associated with calcium signalling were down regulated in post mortem tissue from the frontal cortex and Brodman areas 8 and 9 of Alzheimer's disease brains.

The microarray approach has also been used to study Alzheimer's disease murine models. Reddy et al. (2004) compared Tg2576 mice (an amyloid precursor protein transgenic that mimics the pathology of Alzheimer's disease) with wild type age matched controls. Samples were taken from the cortex at 3 time points; when animals were 2 months old, 5 months old and 18 months old. These time points were chosen because 2 mths is long before any plaque pathology, 5mths is just before pathology is seen and 18 mths is long after pathology is apparent. Genes that were up regulated in the Tg2576 brains were associated with mitochondrial energy metabolism, the cytoskeleton, ubiqutination and apoptotic cell death. A number of genes seen in Alzheimer's disease were down-regulated in this mouse model including arc, proteosome 26s subunit, S100 calcium binding protein, synapsin 1 and cadherin 1. Also down- regulated were a number of genes associated with multiple cellular pathways. These results were somewhat surprising because genes related to energy metabolism are up-regulated in this experiment and are often down regulated in Alzheimer's disease. The authors suggest this may be because these genes are compensating for oxidative damage done to the mitochondria by APP and the problems associated with post mortem tissue collection.

Vast gene changes are seen after experimental brain injuries including ischemia and cerebral occlusion. Rall et al. (2003) carried out microarray analysis on tissue from the cortex and hippocampus of animals that had received a cortical injury 24 hours previously. In the cortex genes associated with the cell cycle and neuropeptides were up-regulated, and genes associated with reactive oxygen species and metabolism were down-regulated. The greatest changes were seen in genes related to signal transduction, metabolism and transcription/translation in the frontal cortex after injury. Lu et al. (2004) analysed transcripts 30min and 2hours after middle cerebral artery occlusion. Tissue was taken from the injured hemisphere 30 minutes, 4hours, 8 hours, 24 hours, 3 days and 7 days after injury. Genes that were up regulated over the time course were: immediate early genes, transcription factors and heat shock proteins at 30 minutes and apoptosis, inflammation, growth factors, cytoskeletal structure and metabolism after 24 hours. At 7 days an increase in

neurotrophic factors was seen. Down-regulated genes included ion channel genes and neurotransmitter receptor genes between 8 and 24 hours after injury and synaptic protein genes between 3 and 7 days. Of the up-regulated genes IEGs are shown to mediate downstream apoptotic death and heat shock proteins are protective during apoptosis. The increase in inflammatory genes contributes to further neuronal damage. The increase in growth factors seen at 7 days helps to enhance neuronal survival and repair any damage that the brain has undergone. Reduction of ion channel genes may lessen any further damage by reducing the imbalance of cellular ions that is commonly seen with brain injury (Lu et al., 2004).

The vulnerability of the retrosplenial cortex to injury and its hypofunction in early Alzheimer's disease suggests a very important role. The findings of Jenkins et al. (2004) show an area-specific decrease in c-Fos activation once the anterior thalamic nuclei are disconnected. It would be of interest to see if this disconnection effect is restricted to immediate early genes or whether is has more global effects on retrosplenial gene activity with other genes showing similar changes. Microarray technology will allow us to answer this question.

The microarray experiment conducted in this thesis was a collaborative effort with the sampling procedure and initial statistical analysis of genes being carried out jointly with another research student. Predictive patterning of genes was carried out and will be presented elsewhere. Any further interpretation and assessment of gene ontology was performed as individual work.

6.2 Experiment six

6.2.1 Materials and Methods

Subjects

Subjects were 12 male pigmented rats (Dark Agouti) weighing between 210g and 250g at time of surgery. Animals were housed in pairs under standard light conditions (14h light/10h dark) and tested during the light phase. Animals had free access to water throughout. Animals were assigned to two groups depending on when surgery occurred so a recovery period of 4 weeks could be observed for all animals. Both groups consisted of six animals. Within these groups animals were split again into one of 3 time delay groups, 30 minutes, 2 hours or 8 hours. All groups were counterbalanced for lesion side and strength of NMDA infusion used to produce the lesion.

Surgery

Animals were deeply anaesthetised by intraperitoneal injection of sodium pentobarbital. Each animal was placed into a stereotaxic apparatus with the nose bar set at +5.0. The scalp was cut and retracted to expose the skull. All rats under went counterbalanced unilateral NMDA lesions of the anterior thalamic nuclei. Injections of between 0.18 and 0.20μl of 0.12M NMDA were made using a 1μl Hamilton syringe in two sites, the anteromedial (AM) and anteroventral (AV) thalamic nuclei. Differences in the amounts of NMDA infused occurred to ensure that lesions of an appropriate size were created fearing that initial 0.18μl infusions may produce sparing. The stereotaxic co-ordinates relative to ear-bar zero were, for AM (AP) +5.2; lateral (L) +/-1.0, for AV, (AP) +5.2, (L) +/-1.6 with the height for AM being +4.8 and +5.3 for AV. At the completion of surgeries, the skin was sutured and an antibiotic powder (Aureomycin, Fort Dodge) was topically applied. Animals also received subcutaneous injections of 5ml glucose saline (sodium chloride 0.9% w/v and glucose 5% w/v) and paracetamol (0.005%) in their drinking water.

Apparatus

A square room measuring 290cm by 290cm was used as a secondary holding room; this was used to provide a familiar room for the animals to be housed in prior to the

commencement of the experiment as described below. The walls were covered with visual cues and contained a foraging table apparatus. Animals were placed in a rack in their home cages. The room was set to the same light/dark cycle that the animals experienced in their holding rooms.

Novelty exposure

Animals were left 4 weeks post-surgery before testing began. A week before sampling animals were split from their cage mate and housed in separate cages. Animals were habituated to the secondary holding room for three hours each day 5 days prior to the experiment beginning. Twenty four hours before sampling began animals were placed into the now familiar secondary holding room in their home cages with free food and water. After this 24 hour period each animal was placed into a novel room within a novel holding cage for twenty minutes, a procedure that is known to up-regulate immediate early gene expression. Upon finishing this task rats were returned to the familiar holding room and kept there for the appropriate time interval (30 minutes, two hours or eight hours). The time interval commenced from the onset of the animal being placed in the novel activity chamber (Fig. 110). Once the time interval had elapsed animals were removed and killed using a schedule one method.
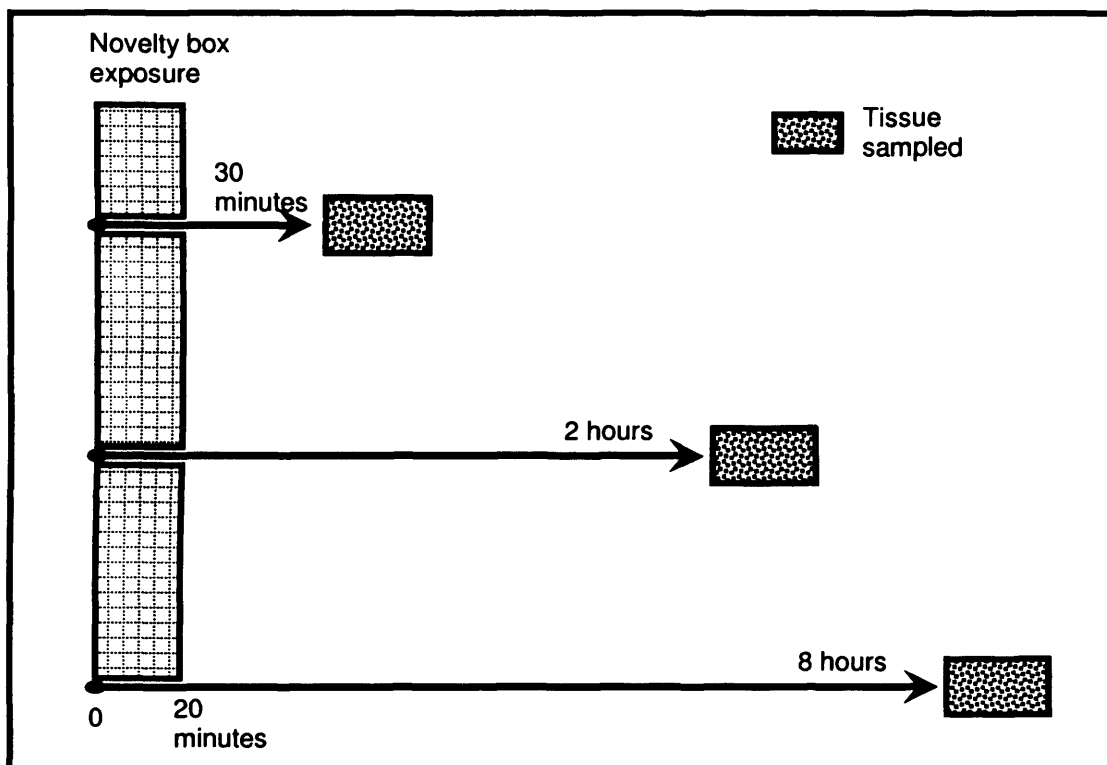
Figure 110
Diagram of experimental design

Microdissection of tissue

Tissue was sampled between the hours of 5pm and 8pm on three separate days, with a different time interval group being sampled on each day (Fig. 111). Before commencing the experiment the bench surfaces were thoroughly cleaned with 70% alcohol and all instruments sterilised using an ultrasonic bath. Access to the room was restricted to prevent contamination of samples and for the same reason the experimenters wore hair nets, overshoes and gloves at all times. Rats were killed by cranial stunning and cervical dislocation. After rapid removal of the brain it was rinsed in ice cold DEPC treated PBS and placed in a brain matrix. Two slices of retrosplenial cortex tissue 2 mm thick were then taken by placing razor blades in 10th, 11th and 12th slots from the front of the matrix. The rostral part of the brain was retained and placed in paraformaldehyde (4%) to assess the accuracy of the lesions. The slices taken were then frozen using solid CO_2. A punch was taken from both hemispheres of the retrosplenial cortex (i.e. the side on which the lesion had been performed and the side which had received no lesion) using a 19 gauge blunted needle. Punches were placed in separate eppendorf tubes and frozen on dry ice. Tissue was pooled for RNA extraction after the validity of the lesions was assessed.

202

After each animal was processed the instruments and brain matrix were cleaned using 70% alcohol and the DEPC treated PBS changed. New razor blades were used for each brain and needles were cleaned with alcohol between each punch. A replication of the tissue sampling was carried out two weeks later following the same protocol. For each replication the punches from each hemisphere from the two rats sampled at each time point were pooled, so the final sample consisted of four punches for each hemisphere.
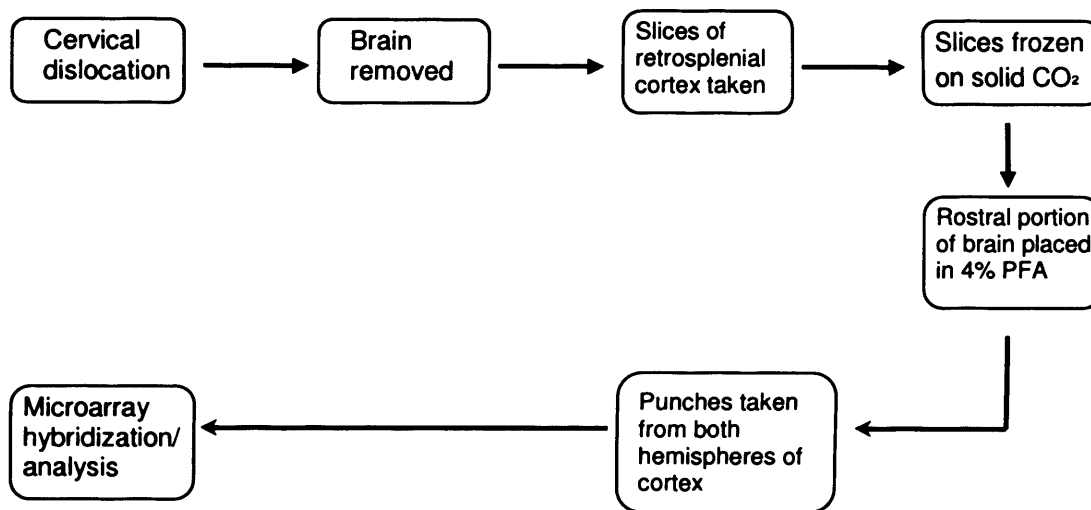
Figure 111
Diagram to show design of tissue sampling

RNA extraction and microarray hybribization.

RNA was extracted as described previously (Chomczynski and Sacchi, 1987). This was performed by Dr.D.Carter (School of Biosciences, Cardiff University). Briefly, the sample was homogenised in the denaturing solution (guanidinium isothiocyanate/mercaptoethanol) and then sodium acetate, phenol, and chloroform-iso-amyl alcohol mixture were added and the solution shaken vigorously. After a 10 minute incubation on ice, samples were centrifuged for 10 minutes at 14000rpm at 4°C. At this point the RNA was present in the aqueous phase. This phase was removed and mixed with ethanol and placed at -20°C for an hour for the RNA to precipitate. This solution was centrifuged again for 10 minutes and the resulting pellet was dissolved in the denaturing solution and then precipitated with ethanol at -20°C for 1 hour. After further centrifuging the pellet was washed in 95% ethanol, dried and dissolved in DEPC-treated water. Yields of total cellular RNA extracted from the

pooled punches in each sample ranged from 1.40 – 2.52µg. A 3 µl aliquot (approx. 0.6 µg) of each sample was supplied to the Wales Gene Park Affymetrix GeneChip Expression Profiling Service (Department of Pathology, UWCM). Before further processing, each RNA sample was quality controlled using Agilent RNA6000 chips. Following this check, 100ng of each sample was used to amplify biotinylated cRNA targets using Affymetrix GeneChip protocols and reagents. The biotinylated cRNA (target) was probed with rat genome 230A GeneChips. Hybridization and washing was performed using a GeneChip fluidics station 400 (Affymetrix). After scanning the microarrays, initial data processing was conducted with Microarray Suite 5.0 (Affymetrix). The average signal intensity of each array was scaled to 100. This procedure normalizes the raw data and corrects for technical variation between the arrays (e.g. Differences in hybridization conditions).

Microarray data analysis

Microarray data were analysed using the software Genespring (Version 6, Silicon Genetics). Samples were normalized using a per chip normalization, normalizing to the 50th percentile and a per gene normalization which was normalizing to the median. Candidate gene lists were initially derived through filtering steps. First, all genes flagged as absent in 12 out of 12 samples were removed. Genes were then filtered on control signal with those retained that fell within the 25.73 and 4,244.89 normalized intensity range in six out of six conditions. This was performed because data measurements that fall below a certain control signal as defined by the cross gene error model are seen to be less precise than those above that point. For each time point comparing treatments genes that fell below the 1.8 fold change were removed and these lists were combined. A two way ANOVA (time x lesion) was performed on the resulting list with genes of $p=0.05$ and below being seen as significant. Genes were functionally annotated using GO ontologies from Genecards (www.gencards.org) and the rat genome database (http://rgd.mcw.edu/) and literature searches on Pubmed (www.ncbi.nlm.nih.gov/entrez/).

Histology

Brains were left in 4% PFA for at least 48 hours and then placed in 25% sucrose overnight. Coronal sections were cut at 40µm on a cryostat. One in three sections

were mounted onto gelatine-coated slides and stained using cresyl violet. Sections were also collected for immunohistochemistry.

Immunohistochemistry

Sections were blocked using 0.3% hydrogen peroxidase (quenches endogenous peroxidase activity) in PBST (Phosphate Buffered Saline with 2% Triton-X) for 10 minutes. After four 10 minute washes in PBST sections were incubated in the primary antibody, anti c-Fos (1:500 Ab5, Calbiochem) for 48 hours at 4°C. Sections were again washed four times in PBST then incubated in the secondary antibody with goat serum (1:2000 in PBST, vector laboratories) for two hours at room temperature. After washing with PBST, sections were incubated in avidin-biotinylated horseradish peroxidase complex diluted in PBST (Elite kit, Vector laboratories) for one hour at room temperature. Sections were washed again in PBST and then in 0.05M tris buffer. The sections were then stained using diaminobenzidine (DAB kit, Vector laboratories). The reaction was stopped by washing in cold PBS. Sections were then mounted on gelatine-covered slides, dehydrated using a series of increasing alcohol concentrations and then cover slipped using DPX (Fisher scientific) as a mountant.

Image Analysis

Images were viewed on a Leica DMRB microscope and photographed using an Olympus DP70 camera, and transferred to a computer. Counts of the stained nuclei were carried out using the program analySIS D (Soft Imaging Systems). The threshold was set at the same level for all sections and counts were made in a frame area of 0.84 x 0.63 mm using a 10x magnification. Where possible counts were taken from 4 consecutive sections from both hemispheres of the retrosplenial cortex.

QPCR validation of array candidate genes

For each sample, 0.5ug of total RNA was processed in a reverse transcription reaction either in the presence (RT+) or absence (RT-) of reverse transcriptase (Superscript II protocol, Invitrogen). Then 15μl of each cDNA was diluted with 135ul of 10*g/ml tRNA, and 5μl was retained. Real time quantitative (Q) PCR analysis of transcript levels was performed by Dr David Sugden (School of Biomedical Sciences, King's

College, London) according to established protocols (e.g. Sugden, 2003). The sense and antisense primers for each gene can be seen in appendix B.

6.2.2 RESULTS

6.2.2.1 Lesion analysis
Lesion analysis of the 12 animals used for microarray analysis revealed very conservative lesions (Figs. 112 and 113). The smallest lesion reveals some anteroventral nucleus sparing throughout, though mainly in the rostral part of the nucleus with less sparing further caudally. Complete loss of the anterodorsal nucleus was seen with little damage in the anteromedial nucleus or the lateral dorsal nucleus. For the smallest lesion a small amount of fornix damage was seen mainly due to track marks from the needle insertion. The largest lesion removed all of the anteroventral and anterodorsal nuclei. There was some damage in the lateral dorsal nucleus more rostrally. A quantity of cells were lost in the anteromedial nucleus but it was not completely removed. There was also a small amount of fornix damage.
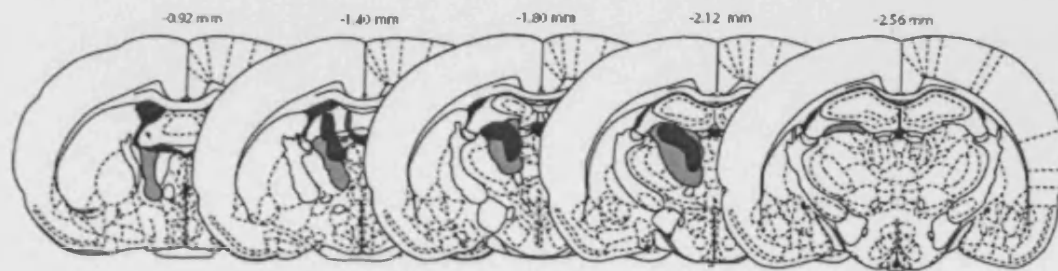
Figure 112
Extent of smallest (shown in black) and largest (shown in grey) anterior thalamic nuclei lesion with distances from Bregma shown.
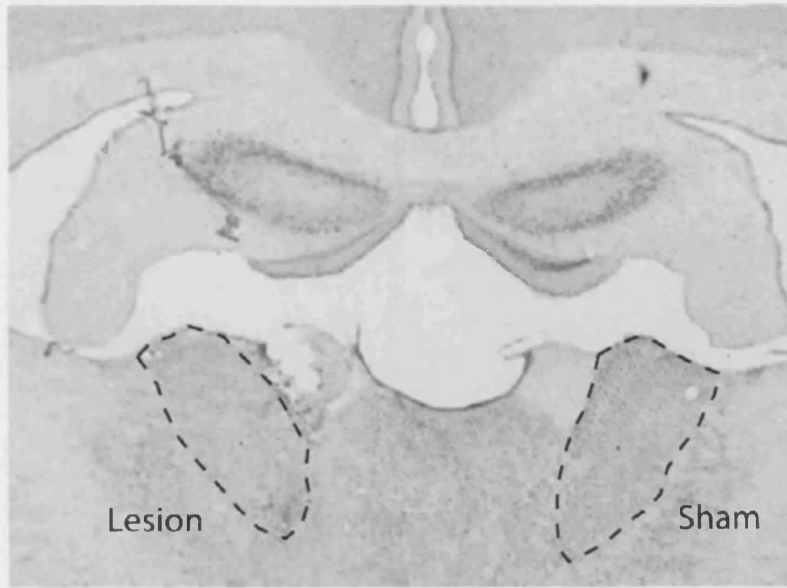
Figure 113
Example of a unilateral anterior thalamic nuclei lesion visualized with cresyl.

6.2.2.2 c-Fos cell counts in the retrosplenial cortex
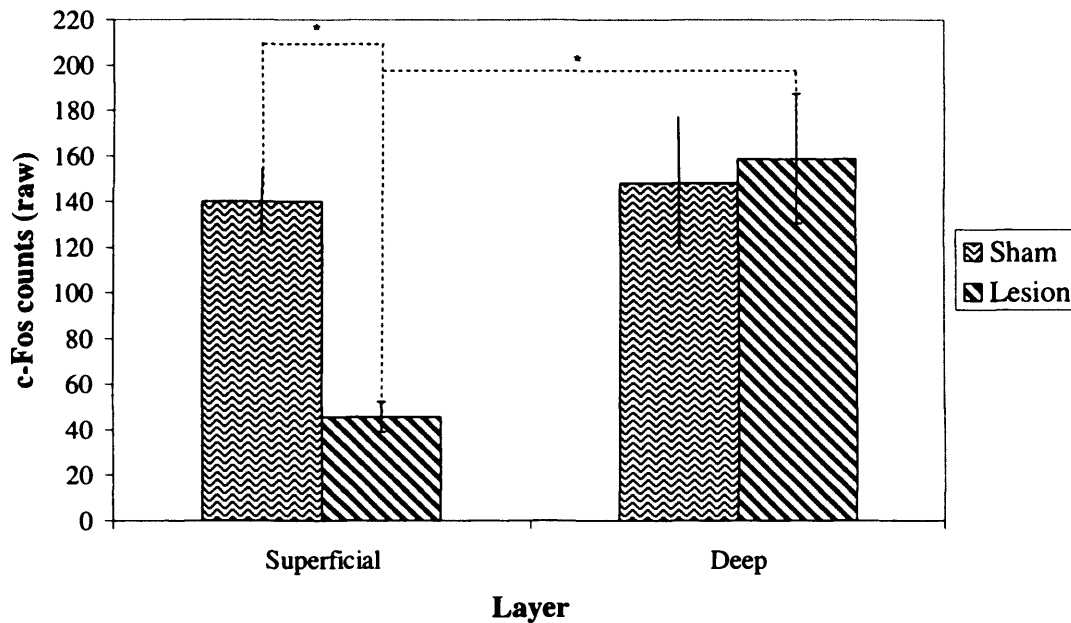
Figure 114

Graph showing c-Fos counts in superficial and deep layers of the retrosplenial cortex in both the sham and lesion hemispheres. Significant differences were seen between c-Fos counts in the sham and lesion hemispheres in the superficial but not deep layers. A significant difference was also seen between the superficial and deep layers in the lesion hemisphere but not on the sham hemisphere.

Analysis of c-Fos positive cell counts in the superficial and deep retrosplenial layers revealed an overall effect of layer (F (1, 22) = 16.2, p=0.001) and a layer by lesion interaction (F (1, 22) = 12.04, p=0.002) (Fig. 114). No overall effect of lesion was seen (F (1, 22) = 2.4, p=0.132). Simple effects analysis revealed significant differences between the lesion and sham hemisphere in the superficial layers (p<0.001) with no difference between the two in the deep layers (p=0.799). Differences were also seen between the layers in the lesion group (p<0.001) with no differences in the sham group (p=0.701) (Fig. 114).

Sham   Lesion

c-Fos x3

c-Fos x10
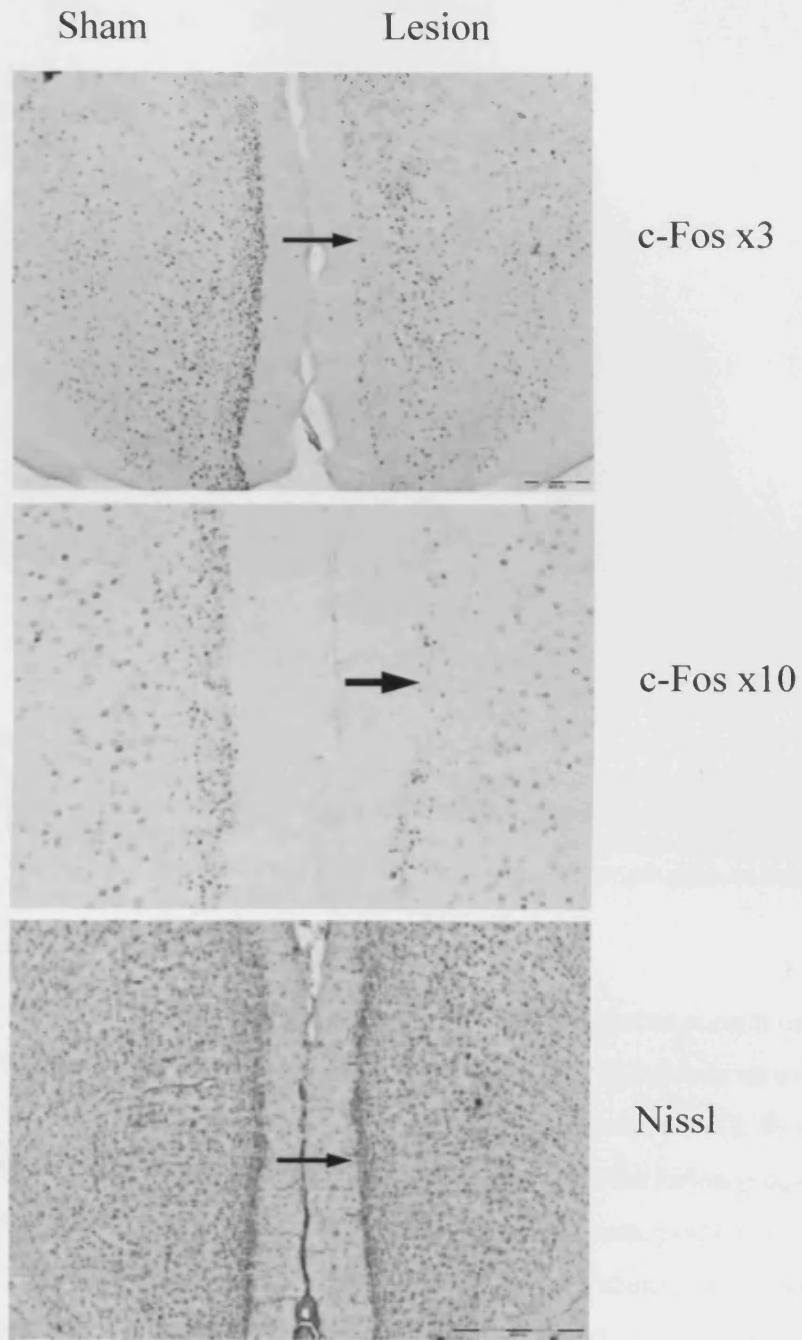
Nissl

Figure 115
Images of c-Fos immunoreactive at x3 magnification of both the lesion and sham hemisphere
with the arrow pointing to the loss of cells in the superficial layers (top) and c-Fos at x10
magnification (middle). The bottom image shows Nissl staining at x5 magnification in both
the lesion and sham hemispheres. The arrow points to the lack of cell loss on the lesion side.
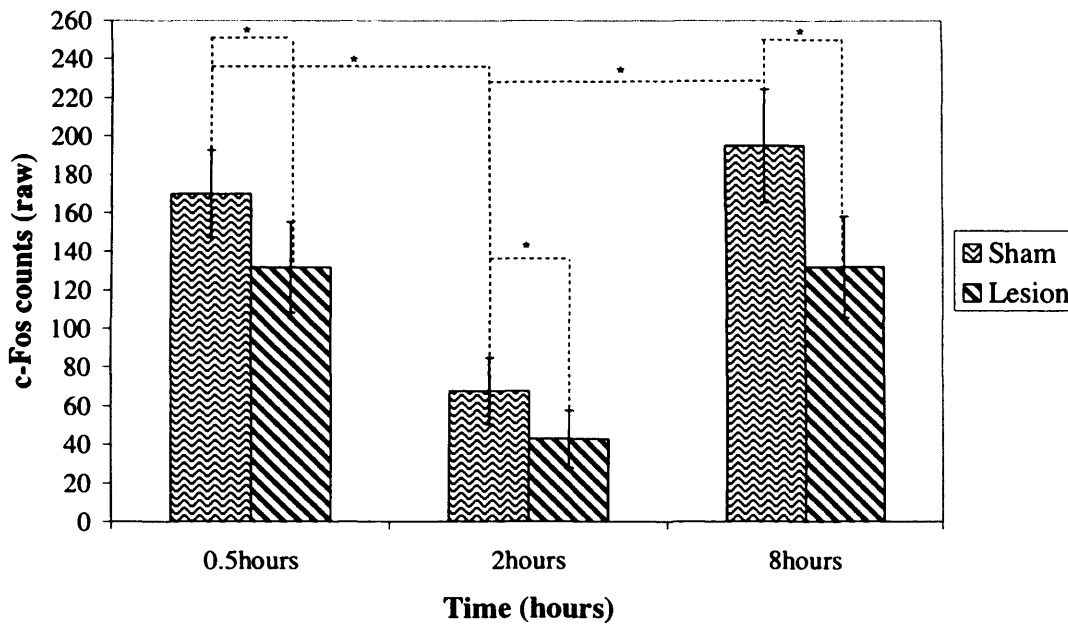
Figure 116
Graph showing c-Fos+ve cell counts across all layers of the retrosplenial cortex in both the sham and lesion hemispheres over time. Significant differences were seen between c-Fos counts in the sham and lesion hemispheres at all time points. A significant difference was also seen between the 0.5 hrs and 2hrs time points and the 2hrs and 8hrs time points in the sham hemisphere.

Comparisons between cell counts in the sham and lesion hemispheres at each time points revealed an overall effect of lesion (F (1, 9) =67.7, $p<0.001$) as was an overall effect of time (F (2, 9) =7.0, $p=0.015$) and a time by lesion interaction (F (2, 9) =4.78, $p=0.039$). Simple effects analysis showed that at all time points the lesion group was significantly lower than the sham group (0.5hours, $p=0.002$; 2hours, $p=0.02$; 8hours, $p<0.001$). For the sham group the counts for 2hours were significantly lower than those for 0.5hours and 8hours ($p=0.40$ and $p=0.012$ respectively). No difference was seen between the time points in the lesion group (Fig. 116).
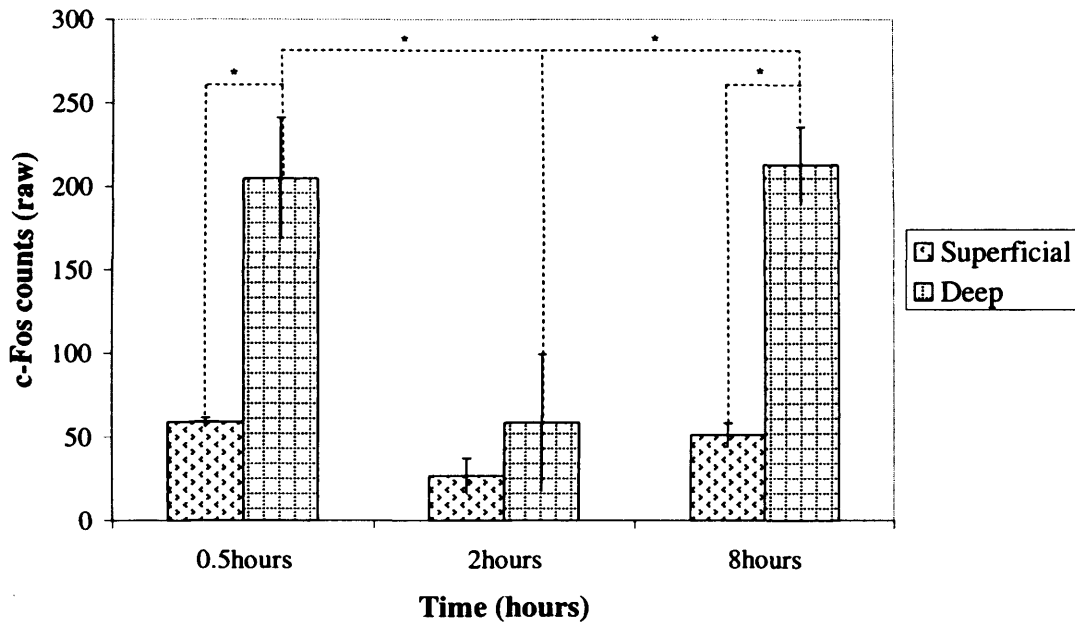
Figure 117

Graph showing c-Fos+ve cell counts in superficial and deep layers of the retrosplenial cortex in the lesion hemisphere over time. Significant differences were seen between c-Fos counts in the superficial and deep layers at the 0.5 hours and 8 hours time points. A significant difference was also seen between the 0.5 hrs and 2hrs time points and the 2hrs and 8hrs time points in the deep layers.

A three way ANOVA analysis (time x lesion x layer) reveals a significant overall effect of layer (F (1, 18) = 24.789, $p<0.001$), lesion (F (1, 18) =5.076, $p=0.037$) and time (F (2, 18) = 13.479, $p<0.001$). A layer by lesion (F (1, 18) = 18.471, $p<0.001$) and a layer by time (F (2, 18) = 7.455, $p=0.004$) interaction were seen. No three way interaction was seen (F (2, 18) = 0.426, $p=0.660$). For the lesion hemisphere (Fig. 117) simple effects analysis revealed that at 0.5hours and 8hours the counts in the superficial layers were significantly lower than the deep layers ($p<0.001$ and $p<0.001$ respectively). In the superficial layers no change was seen over time whereas counts were significantly different between 30minutes and 2hours ($p=0.030$) and 2hours and 8hours ($p=0.021$) in the deep layers (Fig. 117).
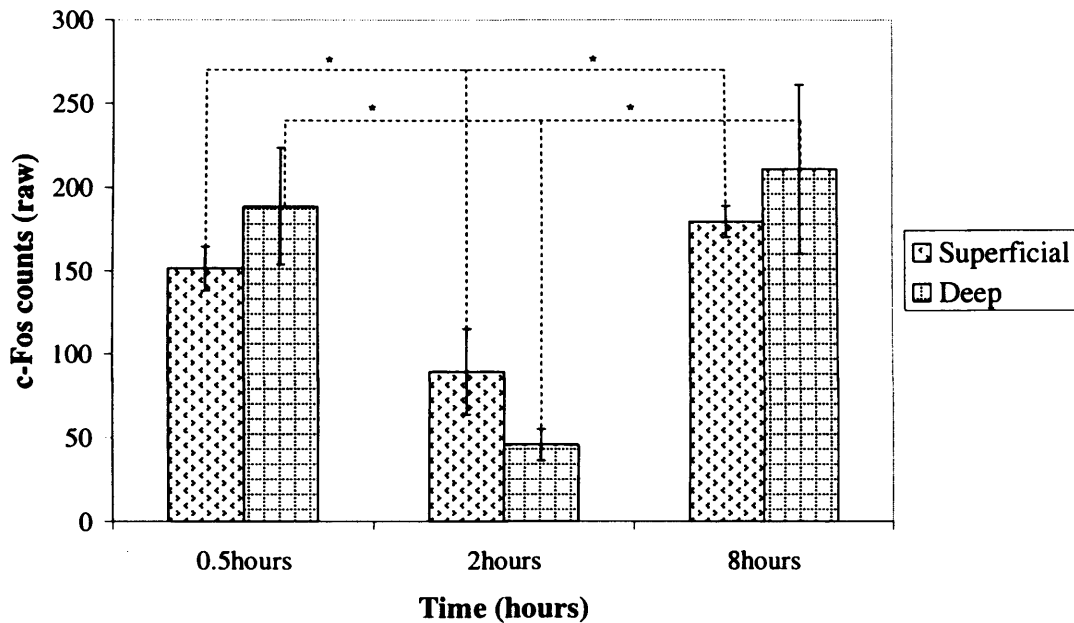
Figure 118

Graph showing c-Fos+ve cell counts in superficial and deep layers of the retrosplenial cortex in the sham hemisphere over time. Significant differences were seen between c-Fos counts in the deep layers between the 0.5 hours and 2 hours time points and the 2 hours and 8 hours time points. A significant difference was also seen in the superficial layers between the 0.5 hours and 2 hours time points and the 2 hours and 8 hours time points.

Simple effect analysis for the sham hemisphere showed no difference between the layers at any time point. For the superficial layers significant changes in c-Fos expression were seen between 0.5hours and 2 hours (p=0.020) and 2hours and 8hours (p=0.001). In the deep layers changes were seen between 0.5hours and 2hours (p=0.030) and 2hours and 8hours (p=0.021) (Fig. 118).

6.2.2.3 Microarray analysis

After filtering, 319 genes were differentially expressed at the >1.8 fold level. This list was collapsed over time and a two way ANOVA (time x lesion) was then performed which generated a list of 32 genes that changed between the lesion and sham hemisphere. QT (Quality Threshold) clustering, which clusters together genes with similar expression patterns, was performed on this list of 32 genes which generated 3 clusters (Figs. 119 and 123). QT clustering groups genes together based on a similarity measure and a minimum similarity stipulated by the user. A minimum cluster size is also predetermined and clusters that fall below this measure are discarded. Clusters that are Figures 120-122 show the average pattern of genes over the time points for each cluster. A one-way ANOVA was also performed using lesion as the parameter which produced a list of genes that differ at one of the time points (Fig. 124). Ten genes were ESTs (Expressed Sequence Tags), which are unidentified genes and Blast searches (www.ncbi.nlm.nih.gov/BLAST) were performed to find homologous sequences which would aid gene identification. Of the 32 genes that showed significant differential expression, analysis revealed that more genes were up-regulated on the lesion side than the sham, 25 compared to 7. Of all the genes differentially expressed 26% were shown to be associated with neurogenesis and cell adhesion with the next highest classification being associated with inflammation and the immune response (17%) (Fig. 125). Other genes differentially expressed were transcription factors (9%) or genes associated with ischemia/apoptosis (9%) or receptor activity (9%) (Fig. 125). In relation to cellular localization of the genes a large proportion were integral to the cell membrane (37%) (Fig. 126).

Figure 119
Table to show ANOVA-generated list of 32 genes that differentially change over condition

Cluster	Common Name	Synonym	LvS	Function	Accession number
1	Voltage-gated sodium channel subunit beta1-A (SCN1B)		L>S	ion transport	AF182949
	Nell1		L>S	cell adhesion, neurogenesis	NM_031069
	Kruppel-like factor 5 (intestinal) (Klf5)	Iklf5, Bteb2	L>S	transcription factor (cell proliferation)	NM_053394
	Netrin-G1a (Ntng1a) (EST)	Lmnt1	L>S	axon guidance, neurogenesis	BM391312
	Cerebellin 1 precursor protein (cbln1) (EST)	Precerebellin-1 gene	L>S	synaptic transmission, neurogenesis	AI227829
	Chemokine (C-X-C motif) ligand 14 (Cxcl14) (EST)	KS1, Kec, BMAC, BRAK, NJAC, MIP-29, bolekine, Scyb14	L>S	signal transduction, inflammatory response	BG380414
	Matrix metalloproteinase 9 (MMp9)	Gelatinase B, 92-kDa type IV collagenase	L>S	zinc ion binding, ischemia (breaks down extracellular matrix)	NM_031055
2	Chromogranin B (Chgb)	Parathyroid secretory protein; secretogranin I	L>S	hormone activity, disease	NM_012526

LvS = Lesion vs. Sham

215

Cluster	Common Name	Synonym	LvS	Function	Accession number
2	Olfactomedin 3 (olfm3)	Optimedin form B	L>S	eye photoreceptor development	AF442822
	Limbic system associated membrane protein (Lsamp)	LAMP	L>S	neurogenesis	U31554
	Fibronectin type III domain containing 5 (Fndc5); LOC260327 (EST)	Peroxisomal protein (pepgene, Pep), Pxp, FRCP2	L>S	cell adhesion, metabolism, wound healing, acute phase response ?	AI172165
	5-HT2C receptor (5-HT2cR) (EST)	Htr1c	L>S	synaptic transmission	BF285539
	BAC clone RP23-312H15 from 16 (EST)	LOC360483 mRNA	L>S	?	AI412090
	Retinoblastoma binding protein 5 (RBBP-5) (EST)	RBQ 3	L>S	apoptosis, disease	AA946518
3	Adrenergic receptor, beta 3 (Adrb3)		S>L	receptor activity	NM_013108
	Cysteinyl leukotriene receptor 1 (Cyslt1, Cysltr1)		S>L	inflammatory response	NM_053641
	Zyxin	HED-2	S>L	regulation of inflammatory response	AA943537

LvS = Lesion vs. Sham
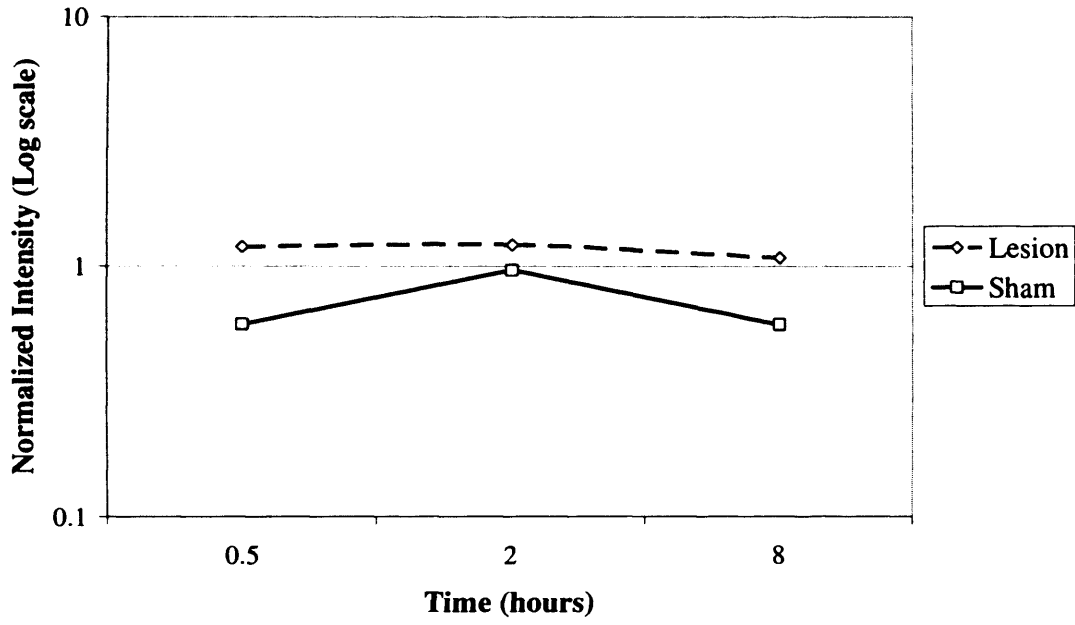
Figure 120
Average gene expression for cluster one
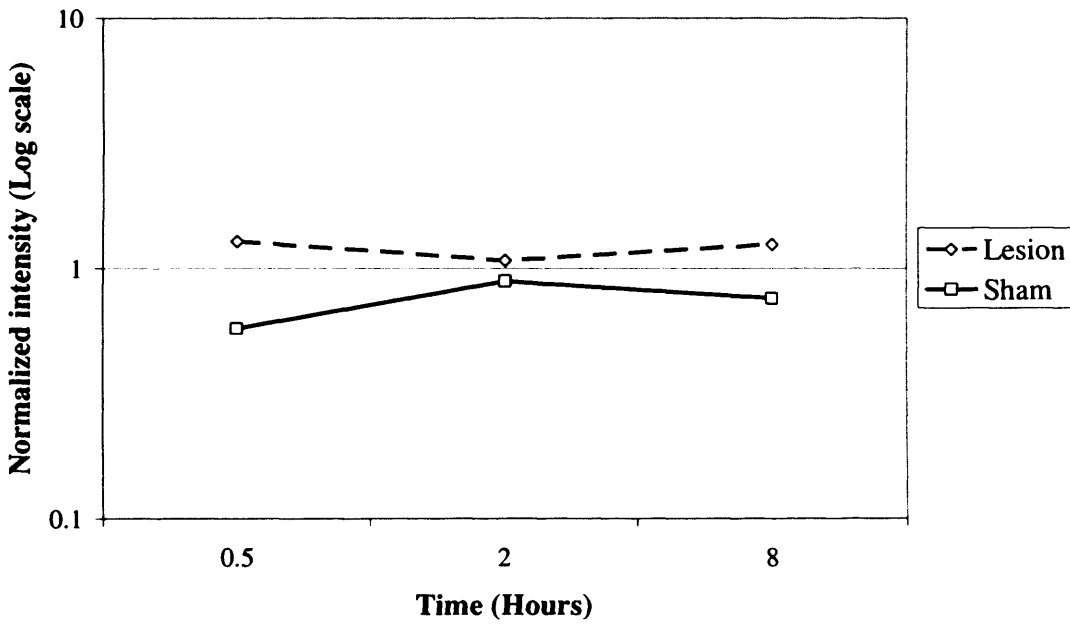
Figure 121
Average gene expression for cluster two
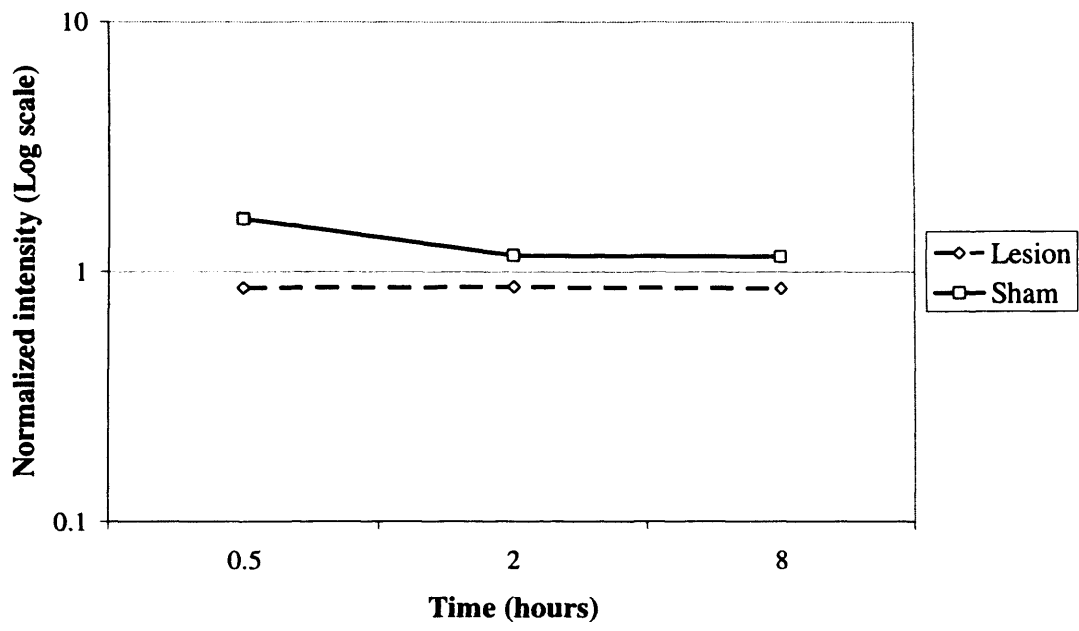
Figure 122
Average gene expression for cluster three

Figure 123
Table to show un clustered genes from 32 gene list

Cluster	Common Name	Synonym	LvS	Function	Accession number
UC	Calponin, acidic isoform (calponin 3)	Cnn3	L>S	actin/calmodulin binding, neuroprotection?	AW916491
	c-fos		L>S	DNA binding; cell growth and/or maintenance; inflammatory response	BF415939
	Hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1)		L>S	metabolism	NM_017080
	Non-processed neurexin I-alpha (Nrxn1)	Nrxn1b	L>S	protein binding; cell adhesion	NM_021767
	ERM-binding phosphoprotein	Slc9a3r1	L>S	protein binding	NM_021594
	Zinc finger protein 91 (PZF)	ZFP91	L>S	transcription, anti-apoptosis?	BE112093
	Lysophosphatidic acid acyltransferase, theta (EST)	MGC11324	L>S	metabolism	AI599365
	Fos-related antigen, exon 4 (Fra-2) (EST)		L>S	regulation of transcription, cell death	AI031032
	Speckle-type POZ protein (Spop) (EST)		L>S	mRNA processing; protein binding	BF283504
	Ubiquitin specific protease 3 (Usp3)		L>S	ubiquitin-specific protease activity; peptidase activity; hydrolase activity	AI411205

LvS = Lesion vs. Sham

219

Cluster	Common Name	Synonym	LvS	Function	Accession number
UC	CD74	DHLAG, HLADG, la-GAMMA, INVG34	S>L	immune response, protein binding	NM_031069
	Desmuslin (Dmn)		S>L	protein binding	BG373779
	Protein S (ProS1)		S>L	calcium ion binding, cell adhesion	U06230
	Cytoskeleton-associated protein 4 (Ckap4)	P63, CLIMP63	L>S	apoptosis	BI278813
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	HLA-DRA	S>L	immune response	Y00480

LvS = Lesion vs. Sham

Figure 124
Table to show genes that differ across condition at particular time points

Time	Common Name	Synonym	LvS	Function	Accession number
30 minutes	Limbic system associated membrane protein (Lsamp)	LAMP	L>S	neurogenesis	U31554
	Kruppel-like factor 5, intestinal (Klf5)	Iklf5, Bteb2	L>S	transcription factor (cell proliferation)	NM_053394
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	HLA-DRA	S>L	immune response	Y00480
	CD74	DHLAG, HLADG,la-GAMMA,INVG34	S>L	immune response, protein folding	NM_013069
	Fibronectin type III domain containing 5 (Fndc5); LOC260327 (EST)	Peroxisomal protein (pepgene, Pep), Pxp, FRCP2	L>S	cell adhesion, metabolism, wound healing, acute phase response ?	AI172165
	Retinoblastoma binding protein 5 (EST)		L>S	apoptosis, disease	AA946518
	Adrenergic receptor, beta 3 (Adrb3)		S>L	receptor activity	NM_013108
	Cytoskeleton-associated protein 4 (Ckap4)	P63, CLIMP63	L>S	apoptosis	BI278813

LvS = Lesion vs. Sham

Time	Common Name	Synonym	LvS	Function	Accession number
30 minutes	Voltage-gated sodium channel subunit beta1-A (SCN1B)		L>S	ion transport	AF182949
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)		L>S	protein binding	NM_053954
	Cerebellin 1 precursor protein (cbln1) (EST)	Precerebellin-1 gene	L>S	synaptic transmission, neurogenesis	AI227829
	Chromogranin B (Chgb)	Parathyroid secretory protein; secretogranin I	L>S	hormone activity, disease	NM_012526
	Zyxin (Zyx)	HED-2	S>L	regulation of inflammatory response	AA943537
	5-HT2C receptor (5-HT2cR) (EST)	Htr1c	L>S	synaptic transmission	BF285539
2 hours	Putative homeodomain transcription factor 2 (Phtf2) (EST)		S>L	?	AI410924
	Spectrin beta 1 (spnb1) (EST)	SPTBN1, SPTB2	L>S	actin binding	AI172054
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)		L>S	protein binding	NM_053954

LvS = Lesion vs. Sham

Time	Common Name	Synonym	LvS	Function	Accession number
2 hours	RT1 class histocompatability antigen, B-1 beta chain precursor (RT1.B beta 1)	HLA-DQB1	S>L	immune response	AI715202
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	HLA-DRA	S>L	immune response	Y00480
	Lysophosphatidic acid acyltransferase, theta (EST)	MGC11324	L>S	metabolism	AI599365
	CD74	DHLAG, HLADG,la-GAMMA, INVG34	S>L	immune response, protein folding	NM_013069
8 hours	Olfactomedin 3 (olfm3)	Optimedin form B	L>S	eye photoreceptor development	AF442822
	Putative homeodomain transcription factor 2 (Phtf2) (EST)		S>L	?	AI410924
	Chemokine (C-X-C motif) ligand 14 (Cxcl14) (EST)	KS1, Kec, BMAC, BRAK, NJAC, MIP-29, Bolekine, Scyb14	S>L	signal transduction, inflammatory response	BG380414
	Retinoblastoma binding protein 5 (RBBP-5) (EST)	RBQ 3	L>S	apoptosis, disease	AA946518
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)		L>S	protein binding	NM_053954

LvS = Lesion vs. Sham

Time	Common Name	Synonym	LvS	Function	Accession number
8 hours	RT1 class histocompatability antigen, B-1 beta chain precursor (RT1.B beta 1) (EST)	HLA-DQB1	S>L	immune response	AI715202
	Cerebellin 1 precursor protein (cbln1) (EST)	Precerebellin-1 gene	L>S	synaptic transmission, neurogenesis	AI227829
	Desmuslin (Dmn)		S>L	protein binding	BG373779
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	HLA-DRA	S>L	immune response	Y00480
	CD74	DHLAG, HLADG,la-GAMMA, INVG34	S>L	immune response, protein folding	NM_013069

LvS = Lesion vs. Sham

224
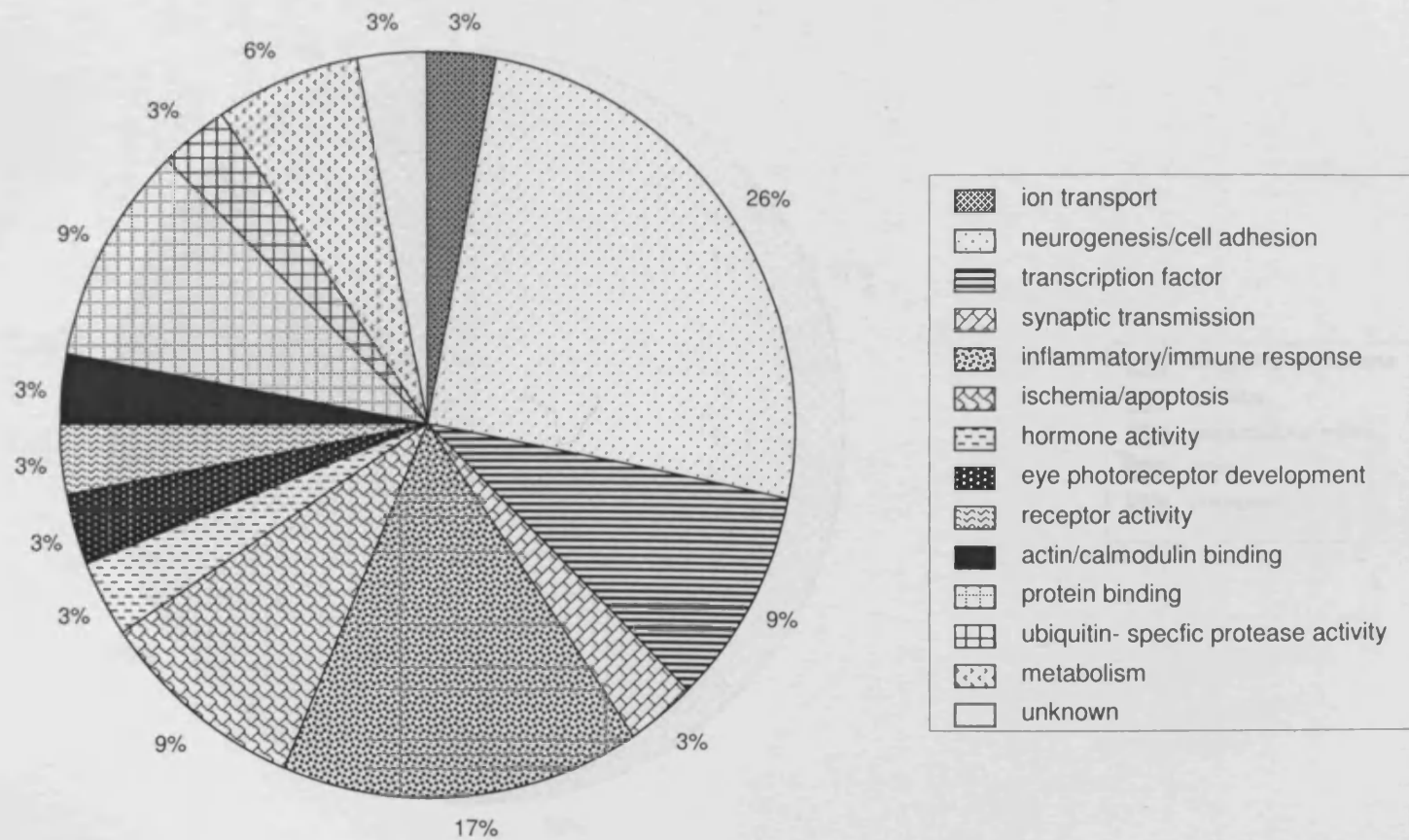
Figure 125
Functional annotation of genes in the 32 list (Fig. 97)
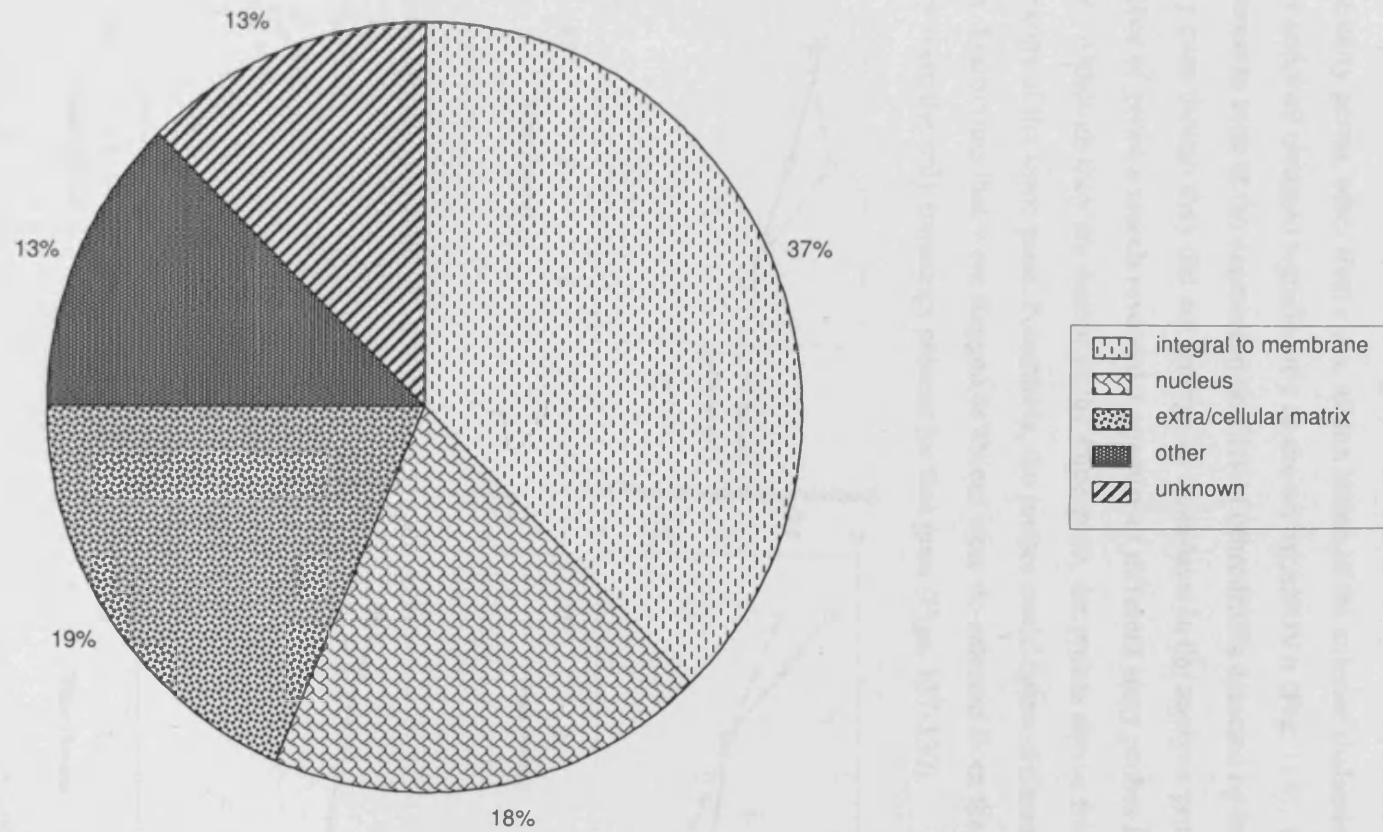
Figure 126
Cellular localization of the genes in the 32 list (Fig. 97) as shown by gene ontology labelling (genecards.org)

Expression of Immediate Early Genes

One of the aims of the present experiment was to study the expression of immediate early genes, other than c-fos, after a lesion of the anterior thalamic nuclei. Only c-fos and fra2 changed significantly as shown by ANOVA (Fig. 119). However, it is of interest to look at the expression profiles of other IEG's detected by the microarray even though they did not emerge as candidates in the analysis protocol. For a number of genes a search revealed a quantity of different array probes for the same gene. Although they are named as the same gene, the probes derive from different exons of the same gene. Potentially, the probes could detect different transcripts. Transcripts that were flagged as absent were de-selected from the analysis unless they were the only transcript present for that gene (Figs. 127-130).
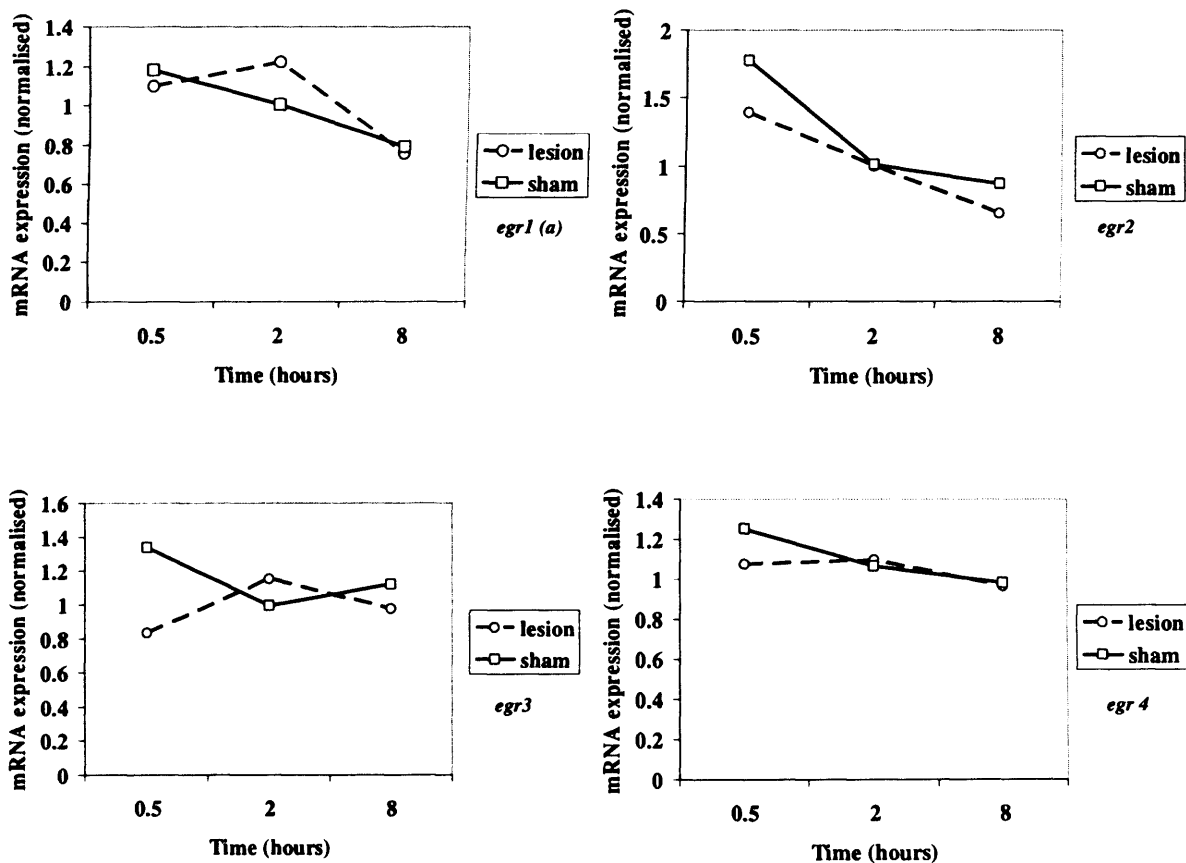
Figure 127
Patterns of mRNA expression of immediate early genes across the time points. An a = gene transcript flagged as absent by the Genespring program.

Most IEG transcripts showed a decrease over time. For *fra1*, expression on the lesion side decreases over time but on the sham side expression increases. For *homer 3* both hemispheres showed an increase in expression over time. For *egr3* and *c-jun* the sham side shows a pattern whereby it has dual peaks of expression at 0.5 and at 8 hours with the lesion side doing the opposite and peaking once at 2 hours.
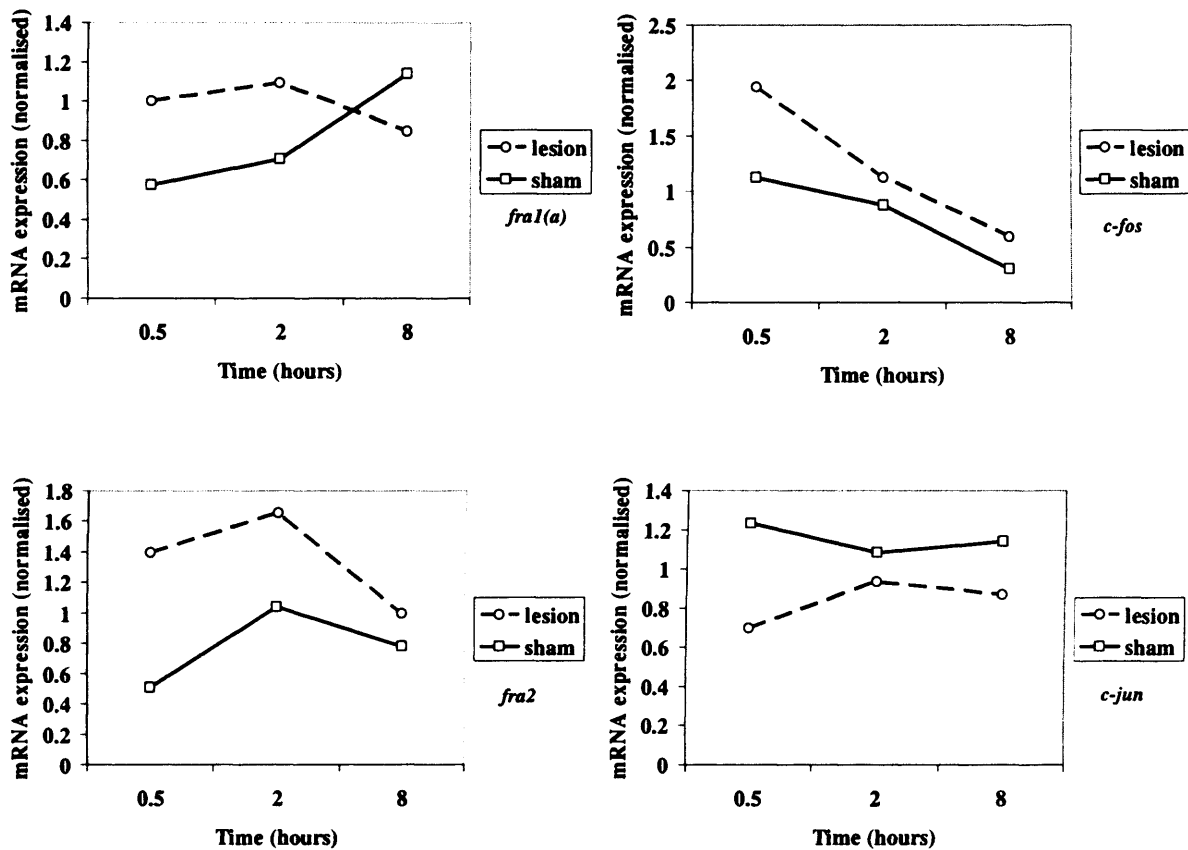
Figure 128
Patterns of mRNA expression of immediate early genes across the time points. An a = gene transcript flagged as absent by the Genespring program.

Patterns of expression appear to split into two groups with a few exceptions. The first pattern is that both sides have the same configuration of activation but at different expression levels e.g. *c-fos*. The second pattern is that one side shows a linear decrease or increase in activation whilst the other does not.

228
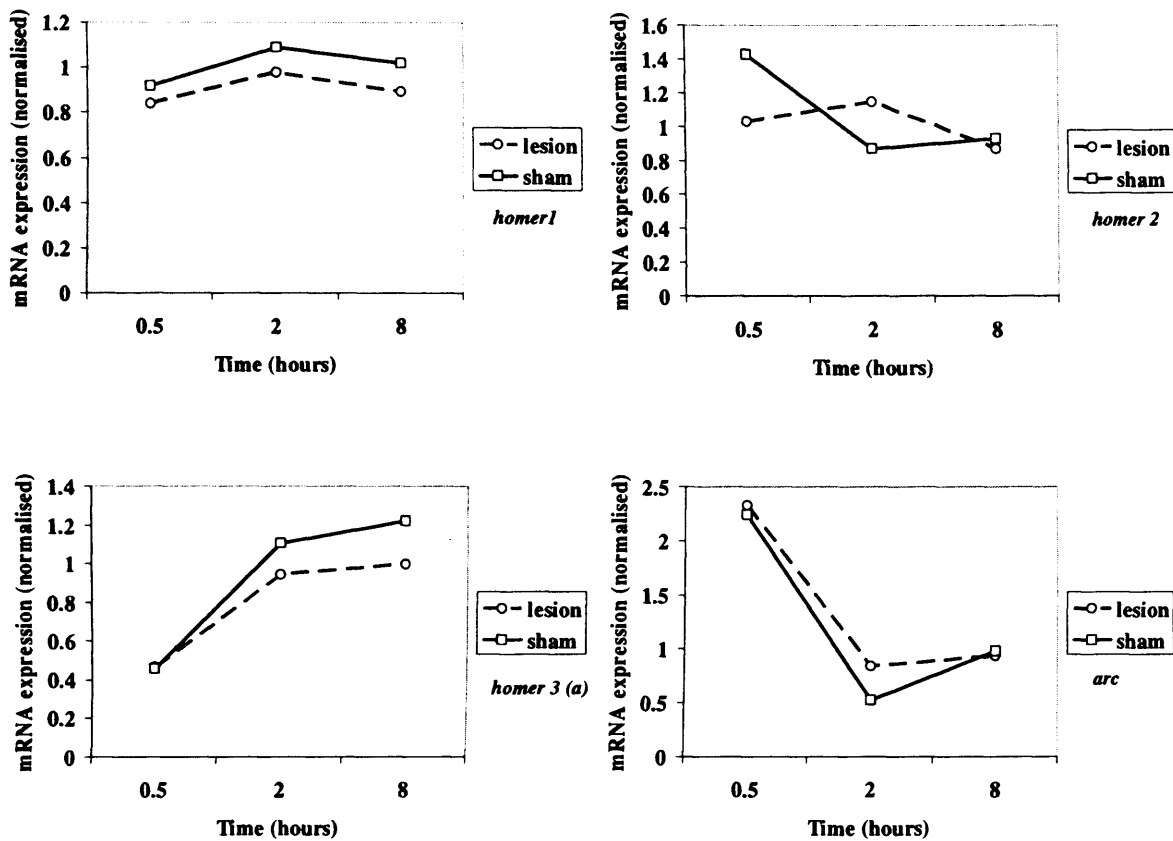
Figure 129
Patterns of mRNA expression of immediate early genes across the time points. An a = gene transcript flagged as absent by the Genespring program.

The number of transcripts showing higher activation on the lesion side is comparable to the sham side. It is evident that certain genes are globally more highly activated on one side than the other. On the sham side of the brain the *egr* family of genes are more highly activated apart from *egr1* (*zif268*) which is higher on the lesion side. The fos family, including *c-fos* itself, *fra1* and *fra2* are highest on the lesion side. Also increased on the lesion side are *arc* and *bdnf*. On the sham side *c-jun* is increased as is the Homer family of genes (*homer 1, 2* and *3*). This is interesting specifically as the genes that have the most commonly been shown to correlate with learning and memory (*egr1(zif268), c-fos, arc, bdnf*) are highest in the lesion hemisphere whilst the genes on the sham side shown to be associated with other functions. It is however possible that the genes on the sham side are those that are not so well characterised in relation to learning and memory.
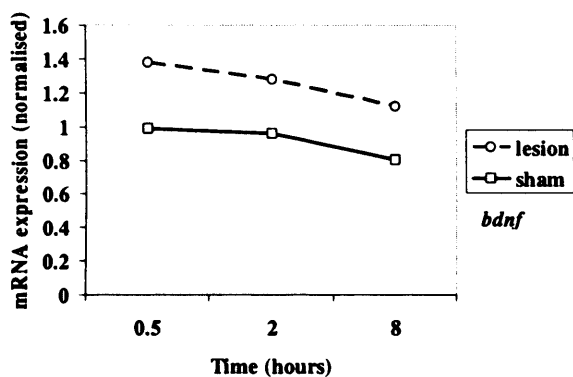
Figure 130
Patterns of mRNA expression of immediate early genes across the time points. An a = gene transcript flagged as absent by the genespring program.

In summary, my results show that lesions of the anterior thalamic nuclei cause disruption and changes in the expression of other immediate early genes in the retrosplenial cortex. This indicates that this is not a phenomena specific to *c-fos* and that it is a more widespread disruption of the immediate early gene transcription. It is of interest to note that whereas the protein level of c-Fos shows a dramatic decrease in the retrosplenial cortex after lesion the mRNA levels increase (at least at the 30 minute post-novelty time point). Possible reasons for this will be discussed below.

6.2.2.4 QPCR validation of candidate genes

QPCR (quantitative polymerase chain reaction) analysis revealed comparable patterns of expression to those seen in the microarray (Figs.131-140). A number of candidate genes were chosen from all clusters obtained from the QT clustering algorithm. Of the candidate genes selected all genes with two exceptions revealed the same pattern of expression across time and treatment. Zyxin at the first time point (0.5 hours) had the opposite pattern of expression; in the microarray sample the sham was higher than the lesion but the lesion was higher in the QPCR sample. For Usp3 at 2 hours the sham hemisphere showed a small increase compared to the lesion where as the QPCR sample showed a slight increase in the lesion group at that time point. However, these differences between the microarray and QPCR mRNA levels are of findings that were small in magnitude at the outset and may reflect an initial unreliable result.
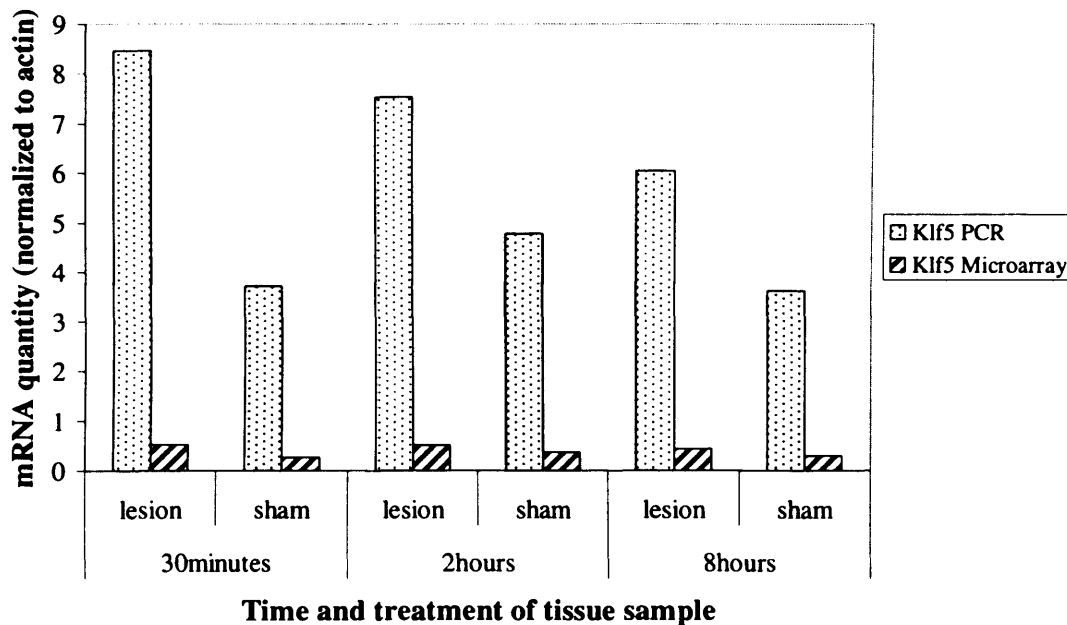
Figure 131
Quantities of mRNA from QPCR and microarray analysis for Klf5
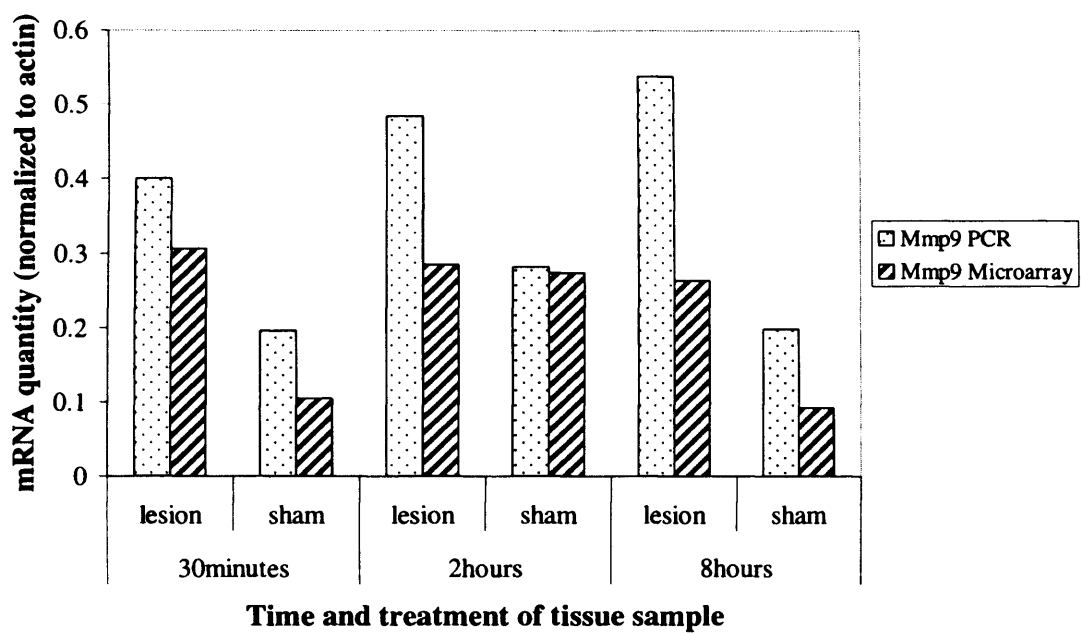
Figure 132
Quantities of mRNA from QPCR and microarray analysis for Mmp9
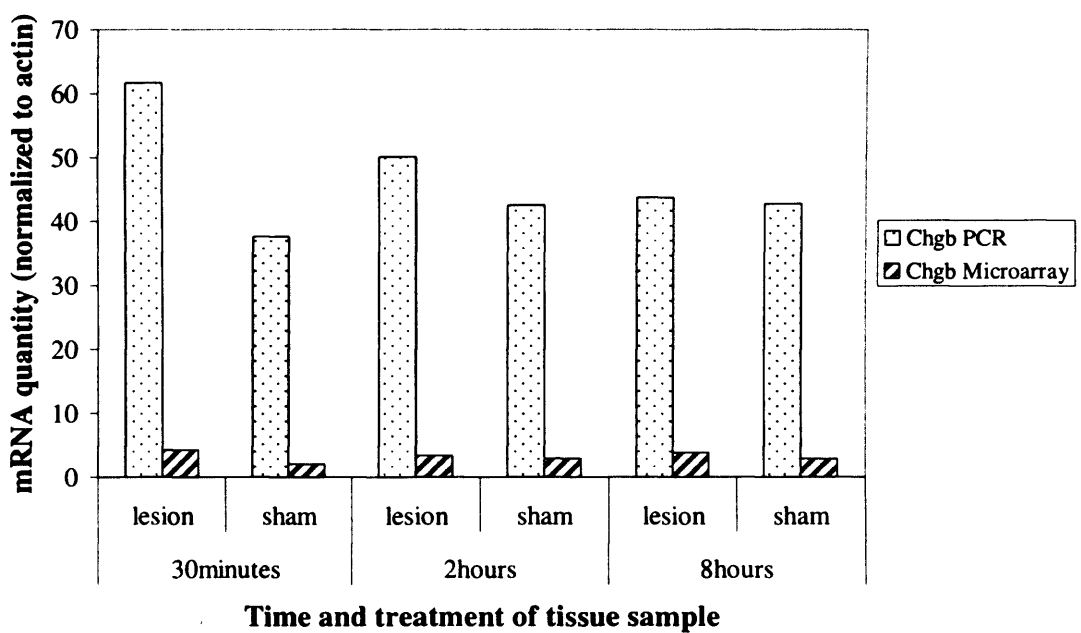
Figure 133
Quantities of mRNA from QPCR and microarray analysis for Chgb
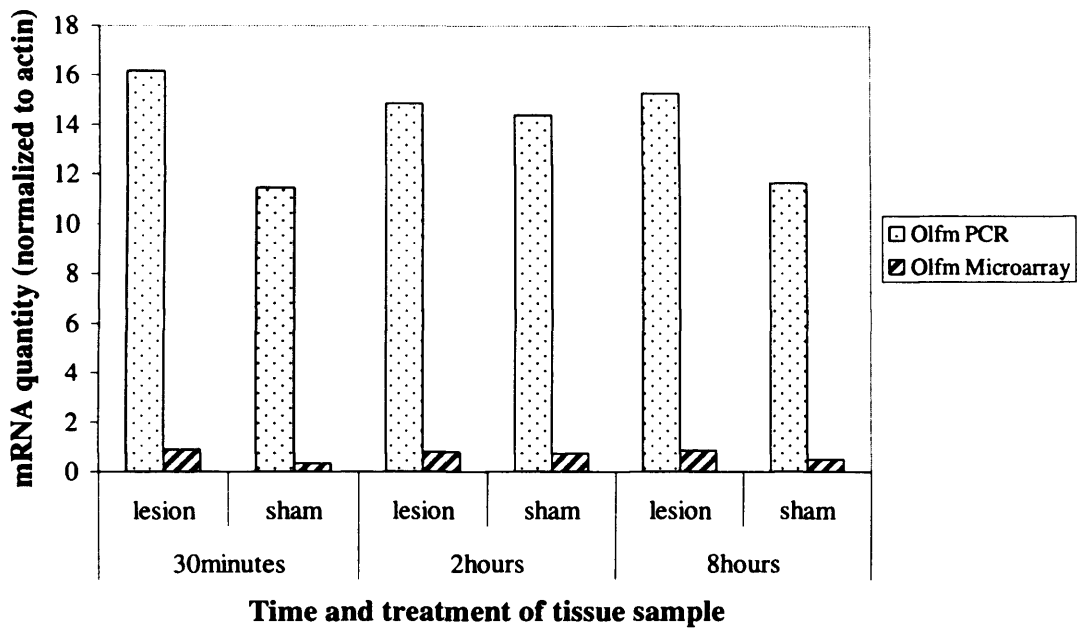
Figure 134
Quantities of mRNA from QPCR and microarray analysis for Olfm
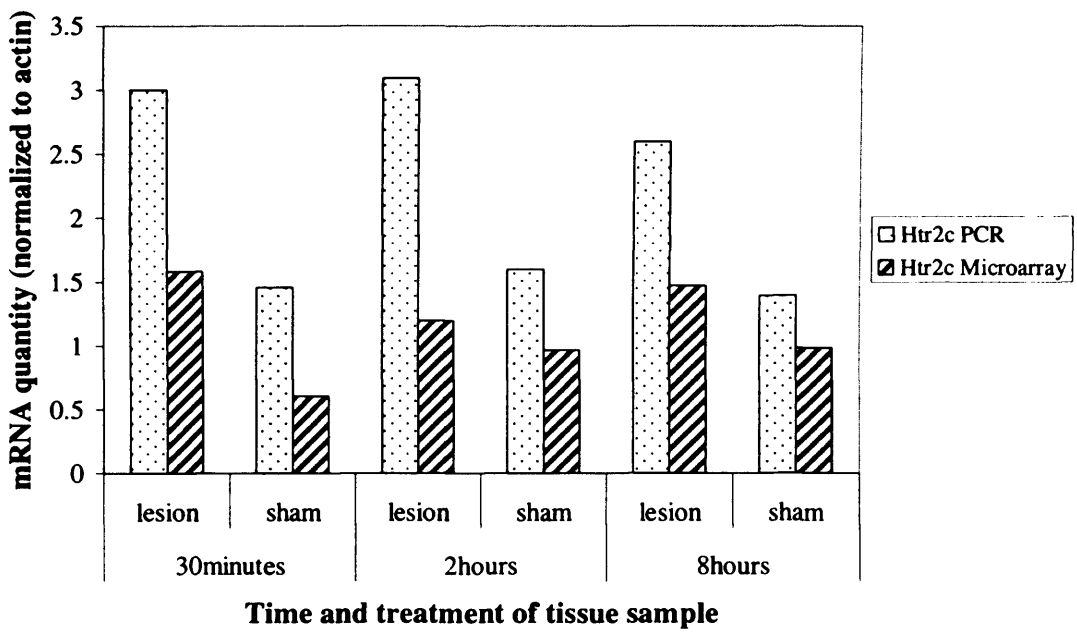
Figure 135
Quantities of mRNA from QPCR and microarray analysis for Htr2c

233
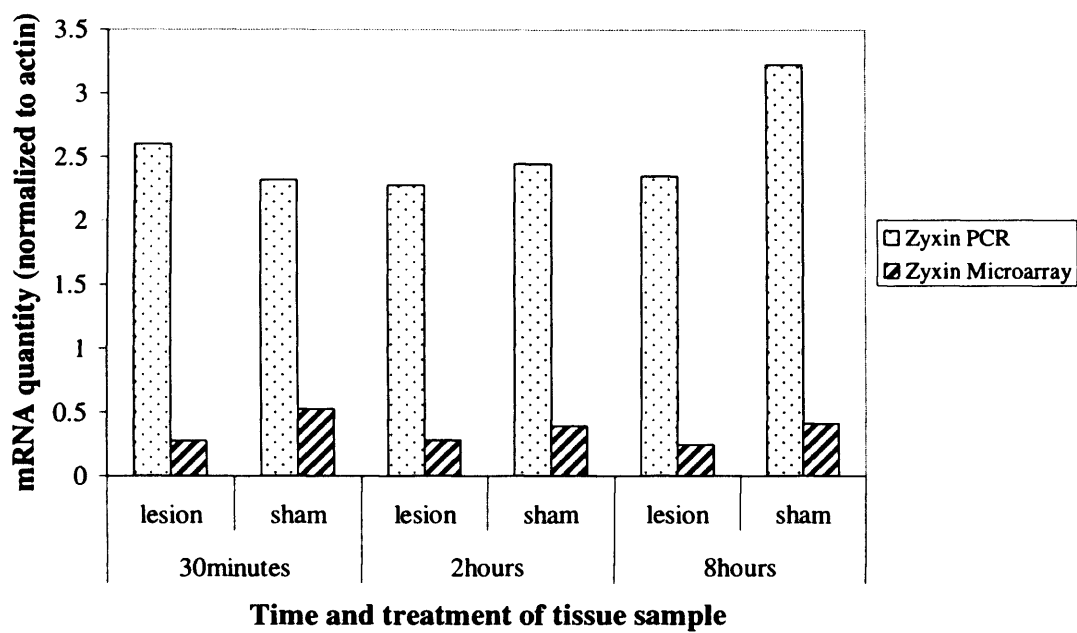
Figure 136
Quantities of mRNA from QPCR and microarray analysis for Zyxin
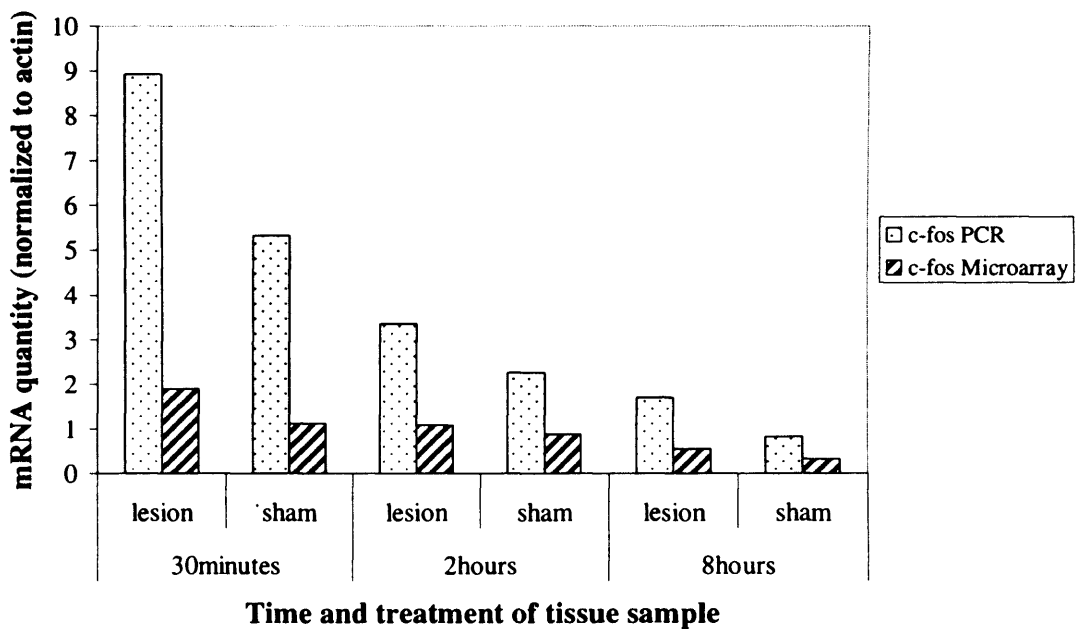
Figure 137
Quantities of mRNA from QPCR and microarray analysis for c-fos
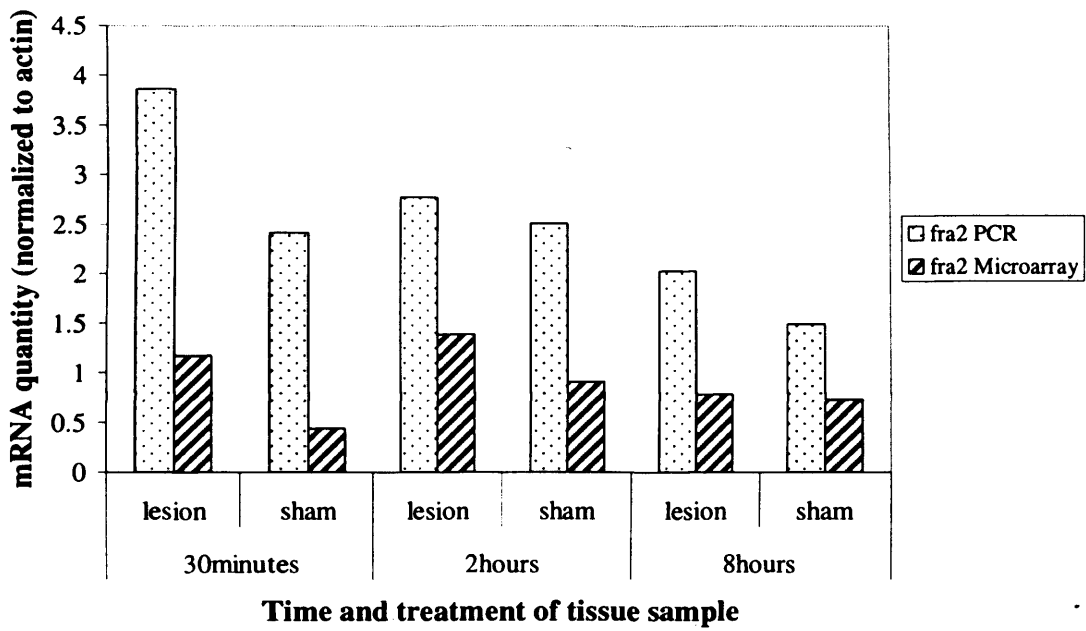
Figure 138
Quantities of mRNA from QPCR and microarray analysis for Fra2
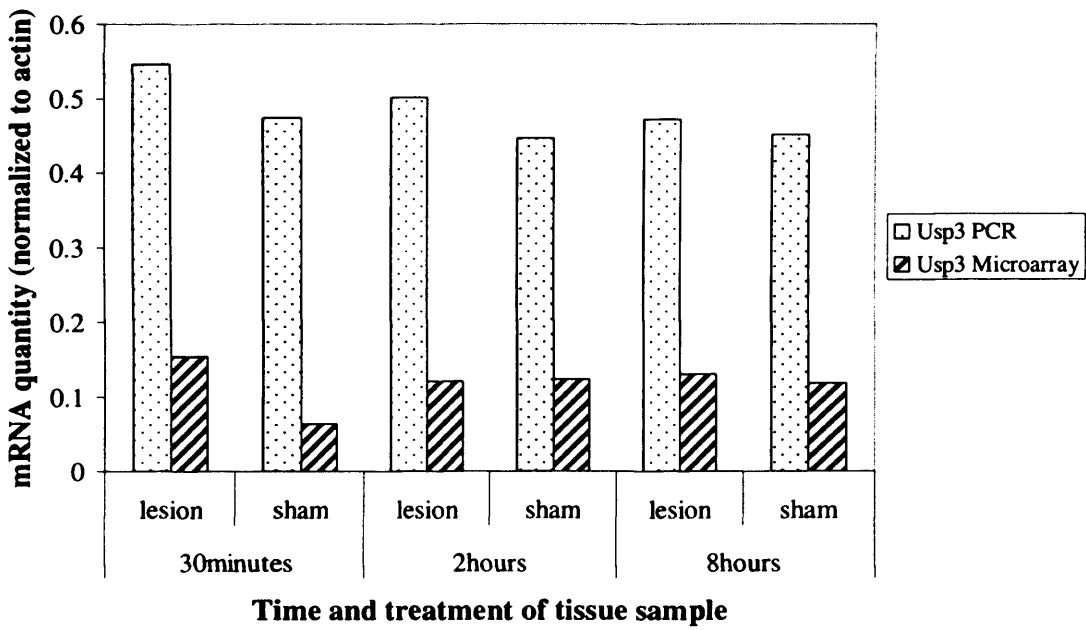
Figure 140
Quantities of mRNA from QPCR and microarray analysis for Usp3

6.3 DISCUSSION

6.3.1 Gene changes seen in the damaged retrosplenial cortex

Results from microarray analysis of the retrosplenial cortex after anterior thalamic nuclei lesion reveal that genes other than *c-fos* show changes. Therefore, the decrease in c-Fos protein expression observed previously (Jenkins, 2004) is accompanied by multiple changes in cellular gene expression.

Perhaps surprisingly, the lesion side of the retrosplenial cortex generally exhibits higher transcript levels of the candidate genes identified here. Of these a large number of genes are involved in cell adhesion and neurogenesis and other aspects of potential regeneration. These include Nell1, Lsamp, and Nrxn1. Nell1 is a thrombospondin 1 like protein which are a group of proteins that are responsible for cell and cellular environment interactions and regulate cell adhesion and are expressed when tissue is formed (Adams, 1997). Lsamp is a member of the immunoglobulin super family group of cell adhesion molecules. It is responsible for mediating neuronal growth and axon guidance and for remodelling of existing circuits particularly in the limbic system (Pimenta et al., 1995). Nrxn1 forms intracellular junctions by binding to neuroligins and could be involved in forming synaptic junctions in synaptogenesis (Rowen et al., 2002).

There were also changes in a number of genes involved in general growth and restructuring of tissue. For example the sodium channel transcript (SCN1B) which plays a role in propagation of the action potential and also interacts with members of the immunoglobulin super family, which are a group of cell adhesion molecules, and together they promote neurite extension (Davis et al., 2004). Klf5 is a transcription factor that stimulates cell proliferation and growth (Sun et al., 2001). Netrin-g1a promotes neurite outgrowth and increases cortical branching which is important for creating connectivity in the central nervous system (Tang and Kalil, 2005). Cerebellin 1 has a neuromodulatory function (stimulating noradrenaline release) and may be involved in neurogenesis. Mmp9 is involved in remodelling of the extracellular matrix and is found to increase after trauma and in neurodegenerative disorders (Magnoni et al., 2004; Adair et al., 2004; Lorenzl et al., 2003). More specifically it is involved in breaking down the blood brain barrier after an insult and propagating further damage (Gidday et al, 2005). Calponin induces changes in cell morphology if

over expressed by remodelling the actin filament (Ferhat et al., 2003). c-Fos is a transcription factor that has a critical role in signal transduction and in cell proliferation. It is also been shown to increase after ischemia and seizure and in neurodegenerative disorders (see 1.8 and 6.1.4). Hsd11beta1 reduces levels of glucocorticoids which is known to be anti-inflammatory, which with higher activation in the lesion hemisphere suggests a role in reducing inflammation. Slc9a3r1 connects the plasma membrane to ERM proteins and regulates surface expression of these proteins. ERM proteins are responsible for cell adhesion and motility due to their ability to link actin filaments with the plasma membrane (Louvet-Vallee, 2000). ZFP91 is a zinc finger transcription factor which is involved in cell proliferation but also has anti-apoptotic properties. Fra2 is another transcription factor which is commonly induced in and has prolonged expression after ischemia (Butler and Pennypacker, 2004; Pennypacker, Eidizaden et al., 2000). This extended expression could allow induction of genes related to repair and regeneration (Pennypacker, Kassed et al., 2000). This could be true of many transcription factors which have increased expression here (Klf5, c-fos, ZFP91). A number of genes that show an increase in the lesion hemisphere have also been shown to increase in Alzheimer's disease. These include the immediate early gene c-fos and others such as mmp9, Chromogranin and Hsd11b1 (Marcus et al., 1998; Lorenzl et al., 2003; Marksteiner et al., 2000; de Quervain et al., 2004)

The transcript levels of some genes were found to be decreased on the lesion side of the brain. For example, a decrease in Cysteinyl leukotriene receptor 1 is seen. This is of interest because antagonists of cyslt1 have a neuroprotective effect after ischemia (Yu et al., 2005), so a reduction of cyslt1 on the lesion side would indicate the initiation of damage-limiting mechanisms in the brain. In the lesioned hemisphere a number of genes associated with inflammation are down-regulated including CD74, RT1D and Zyxin. Zyxin is also associated with focal adhesion and the regulation of cell growth and differentiation.

6.3.2 How could the damage in the retrosplenial cortex occur?

Studies have shown that the anesthetic Ketamine when injected systemically produces neurodegeneration and vacuolization within the retrosplenial cortex (Olney et al., 1989). This and other NMDA antagonists such as PCP and MK-801 have begun to be seen as a possible model of Alzheimer's disease and schizophrenia.

These drugs cause NMDA receptor hypofunction that has been shown to be increased in the aging brain and is much more exaggerated in Alzheimer's disease patients (see Olney et al., 1998). Glutamate acting through NMDA receptors maintains inhibitory control over a number of excitatory circuits that terminate in the retrosplenial cortex. Administration of an NMDA antagonist abolishes this control and causes disinhibition on these circuits leading to hyperactivation of the neurons in the retrosplenial cortex and damage. (Olney et al., 1998)

Farber et al. (2003) used muscimol a GABAergic agonist to test this hypothesis. Muscimol was injected into different areas in the brain under inhalation anesthesia. Upon anesthesia being discontinued animals were injected subcutaneously with the NMDA antagonist MK-801, which induces maximal neurotoxicity in the retrosplenial cortex. Injections of muscimol into areas of the brain which have direct projections with the retrosplenial cortex protected against the neurotoxicity. These include the diagonal band of broca, lateral dorsal thalamic nuclei, anterior cingulate cortex, subiculum, ventral orbital cortex and the contralateral retrosplenial cortex. The strongest protection was seen upon injection into the anterior dorsal and ventral thalamic nucleus, with similar reactions seen in the lateral dorsal thalamus (Fig. 141). The mechanism would appear to involve the GABA agonist removing the imbalance created by the NMDA antagonist. The GABA neurons regain their inhibitory control and damage in the retrosplenial cortex is prevented.

This NMDA hypofunction reflects damage seen in Alzheimer's disease where it would be caused by another mechanism (Olney et al., 1998). Olney et al.(1998) proposed a two stage theory as to how this occurs. In stage one, low concentrations of amyloid are present that interact with NMDA receptors and cause the disinhibition seen with NMDA antagonists. As these neurons die, amyloid deposits are laid down but only in the areas where these inhibitory connections are present. (e.g. in the ATN where the first deposits of amyloid are seen). At this point few symptoms would be seen. In stage two, the disinhibition of these neurons leads to destruction of neurons in other areas (i.e. retrosplenial cortex) and spreads this 'disinhibition excitotoxicity syndrome' throughout neurons in the cortex and limbic structures. It is at this point that cognitive function would begin to be disturbed. Also at this point as neurons die neurofibrillary tangles would be produced. This could occur because of increased activation of secondary messenger systems that are related to protein phosphorylation

and may activate the hypophosphorylation of the tau protein that is required to produce the tangles.

Much of this work with NMDA receptor antagonists centres on the effects of systemic injections. However work carried out by Tomitaka et al. (2000) focused on the blockade of NMDA receptors in the anterior thalamic nuclei and its effects on the retrosplenial cortex. Animals were bilaterally injected with the NMDA antagonist MK-801 in the anterior thalamus or the retrosplenial cortex and allowed to survive for 24 hours after surgery. Injections into the anterior thalamus induced expression of HSP70, a measure of cell injury, in the retrosplenial cortex and most abundantly in the posterior retrosplenial cortex. This was primarily in layer III of the granular cortex. Injections of MK-801 into the retrosplenial cortex did not induce HSP70 in the contralateral hemisphere.
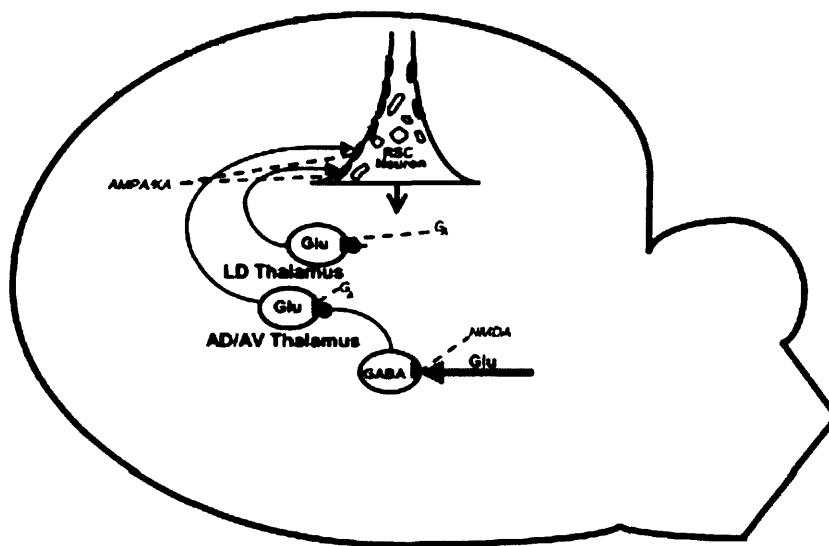
Figure 141
Figure adapted from Farber et al. (2003) to show the NMDA hypofunction hypothesis. Administration of NMDA antagonists prevents the inhibitory control these neurons (e.g. in the AD/AV thalamus) ordinarily have over excitatory circuits in the retrosplenial cortex. This leads to hyperactivation of the neurons within the retrosplenial cortex and damage to this region.

6.3.3 Differential expression of c-Fos protein and *c-fos* mRNA

An interesting finding of the current experiment is the discrepancy seen in the effect of the lesion on c-Fos protein and mRNA. Immunohistochemical analysis revealed dramatic reduction in c-Fos protein in the superficial layers of the retrosplenial cortex after anterior thalamic nuclei lesion. However *c-fos* mRNA expression was increased after lesion as shown by microarray and QPCR analysis. The levels of mRNA still decrease over the time course of the experiment but levels remain much higher on the lesion, compared to the sham side. This could mean that because of the *c-fos*' negative feedback loop, i.e. the ability of the mRNA to prevent protein induction (Sassone-Corsi et al., 1988) the mRNA is being produced at an increased level because the protein is either not produced at all or is produced in a non recognized form. Immunohistochemical analysis does show however that the superficial layers of the cortex are in fact unreactive to stimulation as expression levels do not change over time (see 6.2.2.2, Fig. 94). Therefore because of the nature of the sampling for the microarray where both superficial and deep layers of the retrosplenial cortex were taken this mRNA change could be occurring in the deep layers of the cortex. However as no difference was seen in c-Fos protein induction between the lesion and sham side in the deep layers there is still an obvious issue related to the discrepancy.

This decrease in protein in the superficial layers of the retrosplenial cortex has also been observed with another immediate early gene, Zif268 (Jenkins et al., 2004). Evidence from the microarray analysis does suggest that the levels of *zif268* mRNA are slightly increased in the lesioned hemisphere (Fig. 105). However as this transcript was flagged as absent and was not shown to significantly change by statistical analysis this result is unreliable and further work is needed to confirm this finding.

One reason the discrepancy between mRNA and protein expression could have occurred is due to faulty transcripts in the dysfunctional brain. Liu et al. (2001) discovered that oxidative stress in the brain following ischemia can lead to faulty transcripts of genes being produced. Reactive oxygen species (ROS) are created by oxygen metabolism in the normally functioning brain but are removed by DNA repair. However after damage to the brain such as ischemia and in Alzheimer's disease the ROS are not successfully removed. They can instigate changes to DNA and RNA by modifying base pairs, producing sites without bases or causing strand breaks and

could possibly cause a change in the coding sequence of transcripts. The gene would still be functional at the level of mRNA expression but the transcript would not be translated into the protein. This would mean that because c-fos negatively autoregulates, excessive mRNA would be produced because the level of protein is too low to generate the signal to stop mRNA production.

Another similar idea suggests that the damage is caused directly to the protein, not the mRNA. After ischemia, protein aggregation was seen to occur. (Liu et al., 2005), mainly arising in cells that are destined to die but are undergoing delayed cell death. This leads to irreversible inhibition of protein synthesis in this cell population, and happens co-translationally so mRNA production is normal. This protein aggregation occurs because of problems with protein quality control after depletion of ATP associated with ischemia. This could suggest that protein is being produced after ATN lesion but because it is aggregated the antibody used for immunohistochemical analysis does not recognize it. Also, this damage to the protein could change the expression of downstream targets and may cause modifications to the cell.

As described above (6.3.2) administration of NMDA antagonists most likely causes damage to the retrosplenial cortex by abolishing inhibitory control. Sharp and colleagues (Hisanaga et al.,1992; Sharp et al., 1993) studied the difference between c-fos mRNA and protein induction levels with differing stimuli after NMDA receptor blockade. They found that blockade of NMDA receptors prevented induction of c-Fos like protein but did not prevent induction of mRNA with the blockade not reducing overall protein synthesis. This could mean that NMDA receptors are involved in the translation from mRNA to protein possibly regulated by calcium entry through the membrane ion channels. Hisanaga et al. (1992) suggested that the c-Fos protein could be synthesised but degrade very quickly (in their experimental paradigm) but also suggested as above that NMDA receptors may be needed for translation.

If NMDA receptors are needed for c-Fos induction it does suggest that damage to them would produce a deficit in protein production. NMDA lesions arise because the neurons within the region are caused to fire until they die. The strong connections between the ATN and the retrosplenial cortex mean that a vast influx of NMDA into ATN must have some effect on the retrosplenial cortex possibly damaging but not destroying the NMDA receptors. This could therefore lead to a decrease in c-Fos protein due to the requirement for functioning NMDA receptors for translation (Sharp

et al., 1993). However, further work would need to be carried out to examine this idea.

Recent work by Garden et al. (2006) set out to discover if unilateral lesions of the anterior thalamic nuclei affected plasticity in the retrosplenial cortex. Recordings were taken in layer II fusiform pyramidal neurons after stimulation. Long term depression (LTD) could be induced in sham animals but was unable to be induced in layer II neurons in ATN lesioned animals. LTD was however able to be induced in layer IV in the same slice and in layer II on the contralateral side to the lesion. This provides further evidence for the unresponsiveness of superficial layers in the retrosplenial cortex after anterior thalamic nuclei lesions.

6.3.4 Relations of our findings to previous microarray studies

Our results show a down regulation of genes associated with inflammation after lesion. This pattern is the opposite of what has been seen previously. Ying et al. (2004) found an up-regulation of genes associated with inflammation after deafferentation between the hippocampus and the entorhinal cortex. However the time course of their experiment is vastly different from the present experiment with Ying et al. (2004) sampling 10 days after lesion compared with our four weeks post-lesion sampling. A number of gene functions that were up-regulated in the Ying et al. (2004) study have been seen in the current experiment with genes involved with axon growth and the actin cytoskeletal interaction, suggesting remodeling of dendrites and axons after deafferentation.

Our results are somewhat in contrast to what has been seen using post mortem brain tissue from Alzheimer's disease sufferers. In the case of this tissue a general decrease in transcript activity is seen (Colangelo et al., 2002) unlike the current experiment where a general increase in gene expression is seen. However again genes associated with inflammation were up-regulated as compared to normal controls. Gene functions that were down-regulated were transcription factors and synaptic and cytoskeletal proteins, gene functions which are up-regulated in the current study.

Genes up-regulated after ischemia (Lu et al., 2004) at early time points (30 minutes post injury) are immediate early genes and transcription factors which are up-regulated in the current experiment. However at later time points (24hours post injury) up-regulated genes are associated with inflammation and metabolism which are down-regulated in the retrosplenial cortex after lesions of the anterior thalamus.

The genes that are most commonly up-regulated after injury and in Alzheimer's disease including mouse models of Alzheimer's disease are related to energy metabolism (Reddy et al., 2004; Rall et al., 2003; Lu et al., 2004). Genes related to this function show no changes in expression in the current experiment.

Lesions of the anterior thalamic nuclei produce a vast change in gene expression in the retrosplenial cortex. This extends work from Jenkins et al. (2004) to show that not just the IEG c-fos shows differential expression after lesion. This research also produced the interesting discrepancy between c-fos mRNA and protein which needs further study to be fully understood.

Chapter seven
General discussion

7.1 Introduction

The aim of this thesis was to study immediate early gene (IEG) activation in a functioning and dysfunctioning brain network. The studies were motivated by a desire to obtain fresh insights into IEG expression in the context of learning and memory paradigms in rodent models, and if possible to apply these insights to our understanding of human pathologies. The work described here mainly focuses on IEG expression after spatial working memory as regards the functioning brain, and the aftermath of anterior thalamic nuclei lesion as regards the dysfunctioning network. Also studied were the interconnections between different brain regions after performing a working memory task using structural equation modelling.

7.2 Summary of findings

7.2.2 Chapter two

Investigation of c-Fos induction using a working memory paradigm in the radial arm maze was studied. No significant differences were seen between any of the groups in any of the brain regions examined. However a very low number of subjects were used in this experiment which could have led to this lack of significant result. However in a number of areas there was a trend towards a decrease in c-Fos levels in the Working Memory group as compared to the two control groups. This result is opposite to that found by Vann et al. (2000) who used a similar task. These potential differences could have occurred because of the different strains of rats used in each experiment.

7.2.3 Chapter three

Due to the problems encountered with testing animals in the radial arm maze a water maze task was used in the next group of experiments. These experiments tested a number of novel 'control' groups in comparison to a Working Memory group in the water maze. Both studies revealed decreases in Zif268 activation in the Working Memory group in the hippocampus proper and parahippocampal regions. Experiment 3a also looked at c-Fos activation in the same task and found increases in the Working Memory group as compared to the Procedural Task group in prefrontal regions. The

decrease in Zif268 levels was thought to occur because the animals were very proficient at the task, leading to a streamlining of the required brain network. Thus, there was a reduction of Zif268 levels that could be attributed to a loss of 'noise' and now any activation seen could be attributed to the occurrence of the learning experience.

7.2.4 Chapter four

Structural Equation Modelling analysis of the data obtained from the experiments from Chapter three allowed us to look at the distinct networks of brain regions involved in the Working Memory group and the Procedural Task group. Models tested using c-Fos as a marker showed that in the Working Memory group a significant pathway was seen through the hippocampus to the dorsal subicular region. In the Procedural Task group connections were seen to the dorsal subiculum that by-passed the hippocampus or no significant connections were formed that passed to the dorsal subiculum.

Models tested using Zif268 as a marker generally found the same results as c-Fos, with the Working Memory models having connections between the parahippocampal regions to the dorsal subiculum via the hippocampus. In contrast, the connections between the parahippocampal regions and the dorsal subiculum in the Prodedural Task group generally did not pass through the hippocampus. This suggested that the hippocampus was engaged by the working memory task but not by the procedural task. Also by and large fewer significant pathways were seen in the Working Memory group as compared to the Procedural Task group which is indicative of a streamlining of the brain network.

7.2.5 Chapter five

This chapter focused on a novel transgenic rodent model in which the *zif268* promoter drives expression of the fluorescent protein, GFP. Previous analysis of the transgene showed basal expression at the protein level in a number of brain regions (Man, 2004). Induction had also been shown to increase after light stimulation showing that the transgene was able to be up-regulated by a stimulus (Slade et al., 2002). Animals were tested on the spatial working memory task studied in chapter three. It was revealed that a number of GFP immunoreactive neurons were seen in the CA1 region of the hippocampus in the Procedural Task group. No such activation

was seen in the Working Memory group but induction was seen in other regions of the brain. Direct GFP fluorescence was also seen in CA1 in the Procedural Task group whereas none was seen in the Working Memory group. This demonstrates that results seen from experiments 2 and 3a were replicated using the novel transgenic rodent model and that this strain could be used to study Zif268 expression without the need for immunohistochemical procedures.

7.2.6 Chapter six

Microarray technology was used to explore gene activation in the retrosplenial cortex after unilateral lesion of the anterior thalamic nuclei. A dramatic decrease in c-Fos immunoreactive cells was seen in the superficial layers of the retrosplenial cortex in the lesion hemisphere, confirming previous results (Jenkins et al., 2004). Microarray analysis revealed that there were also changes in expression of other genes on the lesion side of the brain. A number of these transcripts were concerned with cell adhesion, neurogenesis and repair. A discrepancy was seen between the induction of c-fos mRNA and the protein. Higher c-fos mRNA was seen in the lesion hemisphere in contrast to the decrease in protein expression. The reasons for this discrepancy are not fully understood.

7.3 Relation to previous findings

The decreases seen in Zif268 expression after performance of a spatial memory task are at first sight the opposite of what has previously been found using similar paradigms (Guzowski et al., 2001). However, the control groups used in previous experiments have been typically inadequate so results have been difficult to interpret. Improving the stringency of the control group led to the discovery of a decrease in Zif268 immunoreactivity in the experimental group. There is evidence of decreases in IEG expression in recent and remote memory research. A decrease in immediate early gene activation was correlated with remote memories as compared to recent ones in the hippocampal subfields (Maviel et al., 2004). Decreases have also been seen with extended training (Gusowski et al., 2001; Kelly & Deadwyler, 2003; Passino et al.,2002) and reflect a consolidation process and a streamlining of the brain network. This seems to be the most likely explanation as results from the structural equation modelling experiment suggest that animals in the Working Memory group

generally had fewer significant connections within the hippocampal and parahippocampal network as compared to the Procedural Task group but with the hippocampus still being engaged. However, connections between the parahippocampal regions and the subiculum by-passed the hippocampus in the Procedural Task group. Findings from the structural equation modelling experiment are similar to those found in the Jenkins et al. (2003) study who observed a connection between the parahippocampus and the hippocampus in the "place" task; their equivalent of the working memory task. In their "Landmark" task connections were seen between the parahippocampus and the subiculum that by-passed the hippocampus.

The decrease in hippocampal Zif268 in the Working Memory task was also replicated using the *zif268*-EGFP transgenic animal. A number of cells expressing GFP were seen in the CA1 region in the Procedural Task group using both immunohistochemical detection and direct visualisation whereas none were seen in the Working Memory group. GFP immunoreactive cells were present in the Working Memory group in other brain regions. The results found in the two experiments in the water maze allowed us to create a task that had replicable results that demonstrate that the novel rodent transgenic is viable for behavioural testing.

The decrease seen in c-Fos protein in the superficial layers of the retrosplenial cortex after anterior thalamic nuclei lesions replicates previous findings by Jenkins et al. (2003). This decrease in protein was however, associated with an *increase* in c-*fos* mRNA levels in the lesion hemisphere. At the same time, a greater number of transcripts were up-regulated in the lesion hemisphere including genes associated with cell adhesion/neurogenesis and repair. This is somewhat similar to a microarray experiment that looked at hippocampal gene expression after connections with the entorhinal cortex were severed (Ying et al., 2004). Increases in the expression levels of genes associated with inflammation axon growth, and sprouting were seen. However the up-regulation of a large number of transcripts in the lesion hemisphere in the current experiment is opposite to the trend observed in microarray experimentation with post mortem tissue from Alzheimer's patients (Colangelo et al., 2002; Emilsson et al., 2005).

7.4 Are decreases in IEG expression really decreases?

In chapter 2, 3 and 5 of this thesis decreases in Zif268 immunoreactivity were observed in a number of brain regions after a working memory task compared to appropriate control groups. However it could be questioned whether this really reflect decreases in gene activation. In comparisons with basal control counts it can be seen that in a number of regions Zif268 activation in the Working Memory group is higher. However there are also a number of regions that show no difference and in the case of CA3 and the perirhinal cortex a decrease below basal level is seen. This occurrence of gene activity being below basal level is unusual but has been seen previously (Maviel et al., 2004). However in this case because such a large number of comparisons have been made it is possible that these results could be anomalous. Decreases in gene activity suggest repression of the gene which in this group of experiments is not believed to be the case. As stated elsewhere (3.6.6) this reduction in gene activity is seen as a streamlining of the appropriate brain networks and a reduction of noise. Therefore, it could be interpreted that there is in fact less of an increase in Zif268 activation in the Working Memory groups as compared to controls or a relative decrease as compared to controls.

7.5 What does Zif268 do?

Zif268 has been shown to be up-regulated in learning paradigms (Rosen et al. 1998; Tokuyama et al., 2002) and transgenic knockouts of the gene lead to deficits in spatial learning tasks (Jones et al., 2001; Bozon et al., 2002). However a more specific role in learning and memory has yet to be established. Jones et al. (2001) tested *zif268-/-* and *zif268+/+* mice on a spontaneous alternation task in the T maze and found no deficits with both a 30 second and ten minute delay between runs. A massed training protocol in the water maze showed that *zif268-/-* mice took longer to find the platform compared to the *zif268+/+* mice. However, with extended training the *zif268-/-* mice were able to perform the spatial task. These results led the authors to state that *zif268* was not required for short-term memory tasks such as the T-maze but may be needed for retention of spatial information. This was further shown by Bozon et al. (2002) using *zif268* knock-out mice on an object recognition task. After exploring an array of three novel objects one of them was displaced. On the third day of testing mice were given three new objects to explore. Twenty four hours later they

were tested with one of the familiar objects being replaced with a novel one. For the spatial aspect of the experiment, the *zif268+/+* mice spent more time exploring the moved object, with *zif268-/-* mice spending the same amount of time exploring all objects equally, showing that *zif268* may in fact be implicated in long term memory.

The notion that Zif268 is required for retention but not the learning aspects of the task does not accord with the results found here. However, it would be difficult to demonstrate the retention aspect of a working memory task as it is over a very short time frame these animals have to retain the necessary information. The results of the current experiments seem to suggest that *zif268* is induced by novelty and levels decline as training progresses. This has been demonstrated by Mello et al. (1995) using the avian equivalent of Zif268, Zenk. Initially exposure to a song induced Zenk activation in the caudo-medial neostriatum which peaked after 30 minutes of exposure and declined back to baseline after 180 minutes. An introduction of a novel song re-induced the Zenk activation, showing that Zenk activation occurred with exposure to novelty. More importantly it could be suggested that Zif268 is activated when animals first pay attention to novel visual landmarks and as these landmarks become less important less attention is paid to them and levels of Zif268 activation declines. As proposed by the Pearce-Hall model (1980) animals pay more attention to stimuli that do not have a predictable outcome as seen in the Procedural Task group in the water maze experiments (chap 3) and in the 'control' groups in the radial arm maze experiment (chap 2). This greater attentiveness leads to greater amounts of Zif268 activation.

7.6 How does the decrease in Zif268 affect the brain?

As has been previously discussed (1.6) Zif268 has been linked with synaptic plasticity. It is assumed that Zif268 up-regulates downstream targets and it is these that cause the plastic changes in the brain. However, there is evidence to show that induction of Zif268 causes down regulation of a vast number of genes. A microarray analysis of PC12 cells transfected with Zif268 showed that a considerable number of genes were down-regulated by the over expression on the Zif268 construct (James et al., 2005). These include genes associated with synaptic plasticity and genes that regulate the proteasome (James et al., 2005; 2006). There is also evidence to show that Zif268 acts with co-repressors such as NAB 1 and 2 to suppress a subset of genes

(Svaren et al., 1996; Thiel et al., 2000). Therefore, a change that lowers levels of Zif268 could remove an inhibitory influence of the protein. This removal could consequently have an influence on synaptic plasticity and the proteasome. The function of the proteasome in learning and memory processes is somewhat contentious. Evidence demonstrates that inhibition of the proteasome will increase synaptic strength and lead to an increase in postsynaptic potentials (Zhao et al., 2003). However it has also been suggested that these proteasome inhibitors may suppress plasticity and diminish learning and memory (Moss et al., 2003; Lopez-Salon et al., 2002). For the current group of experiments this could mean one of two things. If inhibitors increase plasticity the removal of the inhibitory influence of Zif268 on the proteasome would reduce plasticity and therefore information from the task would not be stored. This is in line with the idea of working memory as active forgetting, the information does not produce plastic changes because it will no longer be required or be helpful to the animal beyond that days training. However, if inhibitors suppress plasticity the decrease in Zif268 removing the inhibitory influence would increase plasticity and facilitate learning and memory. Both of these explanations are possibly plausible and further research would be required to clarify these findings. James et al. (2006) state that, this discrepancy in the function of the possible role of the proteasome in synaptic plasticity may be because of the balance between proteins some of which cause repression and others that cause activation.

7.7 Further directions

Further experiments could be carried out to try and show that decreases in immediate early gene activation occur with extended training. A simple way to prove this would be to access immediate early gene activation on different days of training in animals performing a standard working memory task and compare the relative amount of Zif268 activation. If the decreases seen could be attributed to extended training a peak of immediate early gene activation may be seen at an earlier point in training with levels beginning to decline as training progresses. It would also be of interest to study a synaptic marker such as synaptophysin to study synaptogenesis in both the Working Memory and Procedural Task group. As has been suggested by previous studies, an increase in synaptogenesis is seen with extended training which also coincides with a decrease in immediate early gene activation (Kleim et al., 1996).

It would be expected that an increase in synaptogenesis would be seen in the Working Memory group as compared to the Procedural Task group. The other possibility is that the decrease in Zif268 activation could be seen due to active forgetting. Animals that are well trained on the task may have learnt to forget that day's platform position because they know that a new position will occur on the next session. This explanation could be examined by performing a probe test a period of time after a sessions training to see if animals still remember that day's platform position or if it is quickly forgotten.

Results from the structural equation modelling experiment show that connections are seen through the hippocampus in the Working Memory group but not in the Procedural Task group. This suggests that the integrity of the hippocampus is required for the working memory task and this has been shown on a number of occasions (Morris et al., 1982; Cassel et al., 1998; Bannerman et al., 2002; Pouzet et al., 2002). However animals should be able to perform the procedural task with a hippocampal lesion and it would be interesting to see if this is true. There has been some suggestion that the medial prefrontal cortex (de Bruin et al., 1997) or the caudate nucleus (Packard and McGaugh, 1996) are involved in procedural or response learning, the caudate nucleus not being studied in the current experiments. Studying immediate early gene activation in these areas would be interesting and it could be hypothesised that lesions of these areas could lead to an inability to learn the procedural task in the current group of experiments.

Optimization of the *zif268*-EGFP transgenic line and the fixation protocol used could improve the expression of GFP and particularly the direct visualisation of the protein. If this was possible the line could used for further learning studies using direct visualisation. It would also be of use to see if other methods of genotyping other than PCR could be used, for example whether the torch described by Tyas et al. (2003) could be used for detection of GFP in live animals. Also, although the results of the Zif268 decreases seen in a working memory paradigm in the water maze could be replicated using the *zif268*-EGFP transgene, only activation in the CA1 region was studied; other brain regions should be sampled to see if this is a global replication.

The *zif268*-EGFP transgenic could also be used to improve tissue sampling for microarray analysis. Tissue sampling in the microarray experiment was done using a punch which meant that both superficial and deep layers of the granular cortex were sampled. Also some of the sample may have included tissue from the dysgranular

cortex. One better sampling technique would be to use laser capture micro dissection which would allow both the superficial and deep layers of the retrosplenial cortex to be sampled separately. This would permit one to look at the layers individually and to try and discover why the superficial layers are particularly vulnerable whereas the deep layers are not. The *zif268*-EGFP transgenic model could also be used and allow even greater specificity. The work carried out by Sugino et al. (2006) shows that GFP can be used to sort small populations of different cell types. It would allow us to use these transgenics to select out neurons in the retrosplenial superficial layers to allow better characterisation to further understand why they may be so vulnerable to damage. Along with DAPI staining of the tissue it would also allow one to select the cells in the retrosplenial cortex that still express Zif268 and those that don't and access how they may be different from each other.

The increase seen in the expression of genes associated with neurogenesis and cell adhesion in the lesion hemisphere of the retrosplenial cortex after lesions of the anterior thalamic nuclei suggests that it would be of interest to study the localisation of other neurogenic markers, and perhaps conduct BrdU studies. It would be expected that an increase in neurogenesis would be seen in the superficial layers on the lesion and side and not the deep. With regard to the differential effects of lesions on c-Fos protein and mRNA levels, the work by Hisanaga et al. (1992) suggests that blockade of NMDA receptors prevents the induction of c-Fos protein with no effect on *c-fos* mRNA levels. However there has been no further work into such a phenomenon. It would be interesting to see if this result could be replicated. Also, as this study was conducted in-vitro, the effect of specific NMDA receptor blockade in vivo in the retrosplenial cortex could be informative. It would also be possible to look at the amounts of NMDA receptor protein and mRNA within each hemisphere to see whether the discrepancy between the *c-fos* mRNA and protein is due to loss of NMDA receptors. This analysis would allow the spatial pattern of NMDA receptors to be visualized with a working hypothesis that a decrease in NMDA receptors would be seen in the superficial layers of the retrosplenial cortex and not the deep. In fact recent work by Garden et al. (2006) demonstrated an inability to produce LTD in the superficial layers of the retrosplenial cortex in the lesion hemisphere. These workers are carrying out further research to see if there are changes related to ion channels and receptors. The other possibility is that a discrepancy between the *c-fos* mRNA and protein could have occurred because of faulty transcripts of the mRNA as suggested

by Liu et al. (2001). It would be possible to discover if this was the case by sequencing the *c-fos* gene from the lesion hemisphere and seeing if it was abnormal in any way.

This thesis demonstrates the differential regulation of immediate early genes in a functional and dysfunctional brain network. Decreases in Zif268 activation in the hippocampus and parahippocampal regions were seen following a working memory task in the water maze as compared to procedural controls. This decrease in Zif268 activation in the hippocampus was replicated using a novel transgenic rodent model (*zif268*-EGFP). Structural equation modelling revealed that although decreases were seen in Zif268 activation in the working memory task the hippocampus was still engaged by the task unlike the procedural control group. In contrast, c-Fos was up-regulated in the working memory task in prefrontal regions. In a dysfunctioning brain network the effect of anterior thalamic nuclei lesions on the retrosplenial cortex were studied using microarray technology. This approach revealed changes in the expression of many genes indicating that the molecular mechanisms underlying the dysfunction are complex and will require much more investigation.

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256

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Appendix A

Figure 1
Table to show raw c-Fos cell counts and standard error for Working Memory, Passive and 1-arm groups

Region	Group		
	WM	**P**	**1-arm**
IL	150.3 ± 27.2	137.8 ± 11.2	147.7 ± 16.1
PL	135.6 ± 30.5	115.6 ± 12.5	146.8 ± 10.3
Aca	94.5 ± 36.5	99.3 ± 10.9	120.0 ± 17.2
Subd	71.5 ± 8.2	74.6 ± 11.7	74.8 ± 11.7
CA1	53.5 ± 9.9	62.8 ± 16.1	60.3 ± 9.2
CA3	35.6 ± 4.5	54.9 ± 12.0	46.7 ± 7.9
DG	35.0 ± 10.4	32.2 ± 3.3	36.6 ± 10.0
Peri	53.2 ± 8.8	51.1 ± 2.9	62.9 ± 9.0
Por	55.8 ± 16.1	93.1 ± 16.0	89.7 ± 16.2
Entl	72.3 ± 26.5	54.7 ± 8.2	74.8 ± 14.4
Entm	68.4 ± 18.4	41.5 ± 7.3	61.8 ± 22.0
Retro	164.8 ± 38.6	148.4 ± 25.8	200.5 ± 38.1
Ssp	109.0 ± 25.5	110.9 ± 19.8	132.7 ± 27.1
Mop	117.3 ± 42.0	86.7 ± 10.4	93.1 ± 12.8

Figure 2
Table to show raw Zif268 cell counts and standard error for Working Memory and Procedural Task groups

Region	Group	
	WM	**PT**
IL	838.6 ± 56.5	924.6 ± 65.5
PL	950.2 ± 65.9	1024.2 ± 69.6
Aca	1172.9 ± 114.0	1198.3 ± 88.9
Subd	278.5 ± 54.3	321.1 ± 69.9
CA1	234.7 ± 12.6	308.0 ± 30.8
CA3	163.0 ± 10.2	283.1 ± 19.9
DG	108.0 ± 6.9	149.2 ± 13.3
Peri	486.2 ± 45.7	487.4 ± 49.5
Por	176.9 ± 26.3	259.9 ± 39.7
Entl	113.8 ± 14.5	186.2 ± 24.6
Entm	81.0 ± 9.0	137.3 ± 33.3
rRSP	1435.9 ± 115.9	1360.5 ± 93.5
Ssp	1049.8 ± 63.4	610.3 ± 47.8
Mop	1078.7 ± 81.0	1056.1 ± 26.3
Bfd	2641.3 ± 127.1	2952.7 ± 173.6

Figure 3
Table to show raw Zif268 cell counts and standard error for Working Memory, 5cm Procedural Task and 13cm Procedural Task groups

Region	Group		
	WM	**5cmPT**	**13cmPT**
IL	248.7 ± 37.4	287.2 ± 35.0	291.0 ± 23.9
PL	312.6 ± 37.1	308.0 ± 39.7	340.7 ± 31.9
Aca	492.1 ± 38.1	511.8 ± 37.0	553.4 ± 39.9
Subd	183.0 ± 12.6	227.2 ± 16.8	276.6 ± 18.2
CA1	527.1 ± 25.6	654.3 ± 22.5	645.3 ± 27.6
CA3	154.4 ± 5.3	183.6 ± 10.0	176.3 ± 11.7
DG	59.9 ± 3.4	66.7 ± 3.3	35.4 ± 1.2
Peri	203.4 ± 20.0	224.5 ± 19.3	233.6 ± 12.5
Por	216.7 ± 15.2	246.4 ± 20.5	208.3 ± 9.9
Entl	217.9 ± 13.9	225.5 ± 22.0	240.6 ± 16.6
Entm	84.2 ± 10.9	118.9 ± 20.3	114.0 ± 8.1
Retro	427.7 ± 19.1	495.7 ± 32.2	482.7 ± 35.6
Ssp	602.5 ± 66.9	610.3 ± 47.8	622.8 ± 50.6
Mop	576.7 ± 52.6	547.4 ± 38.3	542.9 ± 36.6
Bfd	621.4 ± 27.1	549.7 ± 57.6	644.8 ± 55.6

Figure 4
Table to show raw c-Fos cell counts and standard error for Working Memory, 5cm Procedural Task and 13cm Procedural Task groups

Region	Group		
	WM	**5cmPT**	**13cmPT**
IL	339.6 ± 17.9	278.2 ± 20.5	276.5 ± 11.9
PL	329.2 ± 19.2	262.0 ± 9.7	261.3 ± 15.3
Aca	383.2 ± 35.5	265.0 ± 20.6	231.7 ± 19.2
Subd	174.0 ± 15.0	163.8 ± 5.1	188.6 ± 15.0
CA1	153.3 ± 12.6	164.2 ± 13.9	164.9 ± 10.7
CA3	102.1 ± 11.0	109.7 ± 9.2	99.0 ± 11.6
DG	36.6 ± 2.1	41.8 ± 2.7	41.3 ± 4.2
Peri	132.8 ± 8.0	119.6 ± 6.9	116.5 ± 9.4
Por	155.3 ± 11.8	141.6 ± 9.9	146.0 ± 6.1
Entl	141.3 ± 9.8	128.3 ± 11.1	105.4 ± 9.5
Entm	129.8 ± 13.7	108.8 ± 12.6	98.2 ± 9.4
Retro	476.6 ± 15.5	440.9 ± 19.6	465.1 ± 19.1
Ssp	309.4 ± 26.2	287.6 ± 16.5	285.3 ± 18.7
Mop	405.6 ± 29.0	294.2 ± 20.0	339.0 ± 27.7
Bfd	359.6 ± 25.2	328.1 ± 29.3	317.7 ± 19.7

Figure 5
Table to show raw Zif268 cell counts and standard error for Working Memory, Cage Control and Free Swimming groups

Region	Group		
	WM	CC	FS
IL	292.5 ± 23.4	198.0 ± 18.4	324.3 ± 23.9
PL	332.4 ± 27.4	260.6 ± 35.2	449.3 ± 27.0
Aca	559.0 ± 34.2	425.9 ± 27.3	635.6 ± 38.7
Subd	235.4 ± 18.8	246.9 ± 26.7	356.3 ± 12.2
CA1	503.7 ± 25.8	568.2 ± 31.0	700.3 ± 30.0
CA3	147.2 ± 7.5	190.9 ± 13.9	181.4 ± 14.8
DG	62.4 ± 3.8	73.8 ± 7.2	72.5 ± 6.9
Peri	257.2 ± 15.1	289.1 ± 8.5	279.4 ± 11.5
Por	228.0 ± 15.2	120.2 ± 19.5	243.7 ± 11.1
Entl	228.5 ± 20.5	233.9 ± 17.9	320.7 ± 13.5
Entm	92.7 ± 9.0	88.6 ± 11.3	125.5 ± 11.2
Retro	468.3 ± 19.7	449.6 ± 23.2	679.3 ± 36.2
Ssp	690.0 ± 67.4	455.7 ± 39.5	676.9 ± 42.9
Mop	642.0 ± 56.7	348.2 ± 25.5	629.0 ± 53.0
Bfd	663.8 ± 27.4	456.9 ± 33.8	667.8 ± 40.5

Figure 6
Table to show raw c-Fos cell counts and standard error for Working Memory, Cage Control and Free Swimming groups

Region	Group		
	WM	CC	FS
IL	373.1 ± 20.6	73.6 ± 13.9	244.5 ± 14.6
PL	383.2 ± 30.6	82.4 ± 15.9	266.6 ± 24.1
Aca	456.3 ± 23.2	116.1 ± 27.6	414.6 ± 35.8
Subd	175.5 ± 10.3	173.1 ± 19.2	238.5 ± 23.4
CA1	163.7 ± 8.8	87.3 ± 10.0	147.1 ± 17.9
CA3	108.5 ± 12.0	102.8 ± 7.3	112.2 ± 12.5
DG	40.3 ± 1.7	28.8 ± 4.1	47.9 ± 5.3
Peri	123.4 ± 9.8	66.2 ± 5.8	157.0 ± 9.9
Por	150.2 ± 4.1	76.1 ± 9.6	173.6 ± 17.0
Entl	139.4 ± 11.1	70.1 ± 8.5	169.7 ± 14.5
Entm	118.9 ± 8.5	24.4 ± 7.3	96.3 ± 10.3
Retro	490.4 ± 6.9	297.0 ± 45.8	517.4 ± 23.3
Ssp	338.9 ± 17.9	161.2 ± 22.5	371.2 ± 36.3
Mop	463.8 ± 20.1	76.3 ± 13.0	360.5 ± 60.7
Bfd	386.6 ± 25.2	104.2 ± 16.2	487.6 ± 45.5

Appendix B

Figure 1
Table to show *p* values of clustered genes from 32 gene list

Cluster	Common Name	P value	Genbank number
1	Voltage-gated sodium channel subunit beta1-A (SCN1B)	0.026086	AF182949
	Nell1	0.026086	NM_031069
	Kruppel-like factor 5 (intestinal) (Klf5)	0.04713	NM_053394
	Netrin-G1a (Ntng1a) (EST)	0.04713	BM391312
	Cerebellin 1 precursor protein (cbln1) (EST)	0.037745	AI227829
	Chemokine (C-X-C motif) ligand 14 (Cacl14) (EST)	0.04713	BG380414
	Matrix metalloproteinase 9 (MMp9)	0.037745	NM_031055
2	Chromogranin B (Chgb)	0.02614	NM_012526
	Olfactomedin 3 (olfm3)	0.04713	AF442822
	Limbic system associated membrane protein (Lsamp)	0.037745	U31554
	Fibronectin type III domain containing 5 (Fndc5); LOC260327 (EST)	0.04713	AI172165
	5-HT2C receptor (5-HT2cR) (EST)	0.026086	BF285539
	BAC clone RP23-312H15 from 16 (EST)	0.04713	AI412090
	Retinoblastoma binding protein 5 (RBBP-5) (EST)	0.04713	AA946518
3	Adrenergic receptor, beta 3 (Adrb3)	0.02614	NM_013108
	Cysteinyl leukotriene receptor 1 (Cyslt1, Cysltr1)	0.04713	NM_053641
	Zyxin	0.04713	AA943537

271

Figure 2
Table to show *p* values of unclustered genes from 32 gene list

Cluster	Common Name	P value	Genbank number
UC	Calponin, acidic isoform (calponin 3)	0.048634	AW916491
	c-fos	0.047658	BF415939
	Hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1)	0.04713	NM_017080
	Non-processed neurexin I-alpha (Nrxn1)	0.04713	NM_021767
	ERM-binding phosphoprotein	0.04713	NM_021594
	Zinc finger protein 91 (PZF)	0.037745	BE112093
	Lysophosphatidic acid acyltransferase, theta (EST)	0.034477	AI599365
	Fos-related antigen, exon 4 (Fra-2) (EST)	0.02614	AI031032
	Speckle-type POZ protein (Spop) (EST)	0.026086	BF283504
	Ubiquitin specific protease 3 (Usp3)	0.026086	AI411205
	CD74	0.026086	NM_031069
	Desmuslin (Dmn)	0.026086	BG373779
	Protein S (ProS1)	0.026086	U06230
	Cytoskeleton-associated protein 4 (Ckap4)	0.026086	BI278813
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	0.026086	Y00480

272

Figure 3

Table to show genes that differ across condition at particular time points

Time	Common Name	P value	Genbank number
30 minutes	Limbic system associated membrane protein (Lsamp)	0.039807	U31554
	Kruppel-like factor 5, intestinal (Klf5)	0.039807	NM_053394
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	0.015595	Y00480
	CD74	0.004786	NM_013069
	Fibronectin type III domain containing 5 (Fndc5); LOC260327 (EST)	0.043852	AI172165
	Retinoblastoma binding protein 5 (EST)	0.043852	AA946518
	Adrenergic receptor, beta 3 (Adrb3)	0.043852	NM_013108
	Cytoskeleton-associated protein 4 (Ckap4)	0.043852	BI278813
	Voltage-gated sodium channel subunit beta1-A (SCN1B)	0.043852	AF182949
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)	0.043852	NM_053954
	Cerebellin 1 precursor protein (cbln1) (EST)	0.043852	AI227829
	Chromogranin B (Chgb)	0.039807	NM_012526
	Zyxin (Zyx)	0.039807	AA943537
	5-HT2C receptor (5-HT2cR) (EST)	0.039807	BF285539
2 hours	Putative homeodomain transcription factor 2 (Phtf2) (EST)	0.038342	AI410924
	Spectrin beta 1 (spnb1) (EST)	0.038342	AI172054
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)	0.025595	NM_053954
	RT1 class histocompatability antigen, B-1 beta chain precursor (RT1.B beta 1)	0.02528	AI715202
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	0.002699	Y00480
	Lysophosphatidic acid acyltransferase, theta (EST)	0.002116	AI599365
	CD74	0.001243	NM_013069

273

Time	Common Name	P value	Genbank number
8 hours	Olfactomedin 3 (olfm3)	0.04978	AF442822
	Putative homeodomain transcription factor 2 (Phtf2) (EST)	0.047493	AI410924
	Chemokine (C-X-C motif) ligand 14 (Cxcl14) (EST)	0.044204	BG380414
	Retinoblastoma binding protein 5 (RBBP-5) (EST)	0.035425	AA946518
	Potassium voltage-gated channel, subfamily S, 1 (kcns1)	0.033969	NM_053954
	RT1 class histocompatability antigen, B-1 beta chain precursor (RT1.B beta 1) (EST)	0.032209	AI715202
	Cerebellin 1 precursor protein (cbln1) (EST)	0.032209	AI227829
	Desmuslin (Dmn)	0.032209	BG373779
	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	0.006448	Y00480
	CD74	0.001979	NM_013069

274

QPCR primers

NM_053394.1
Rattus norvegicus Kruppel – like factor 5 (intestinal) (Klf5), mRNA

Left   5' –cagagcctggaagtcctgat- 3'
Right   5' –agcagcataggacggaggt- 3'

NM_031055.1
Rattus norvegicus matrix metalloproteinase 9 (gelatinase B, 92-kDa type IV collagenase) (Mmp9), mRNA

Left   5' –tccgcagtccaagaagattt- 3'
Right   5' –agcctagccccaacttatcc- 3'

NM_012526.1
Rattus norvegicus chromogranin B (Chgb), mRNA

Left   5' –aggcagaagatgaaaaggacag- 3'
Right   5' -cgccaagttctccagttcc- 3'

AF442822.1
Rattus norvegicus optimedin form B mRNA, complete cds; alternatively spliced

Left   5' –cgctaatgaccaagcattttc- 3'
Right   5' –gcgtcggttttgtactgctc- 3'

NM_012765.2
Rattus norvegicus 5-hydroxytryptamine (serotonin) receptor 2C (Htr2c), mRNA [substituting for BF285539 (rat EST) 5-hydroxytryptamine (serotonin) receptor 2C (Htr2c)]

Left   5' –atcatgtggtgcccgttt- 3'
Right   5' –tacaggccttcccacaaaga- 3'

AA943537
Zyxin [using the affymetrix target sequence]

Left   5' -caaccctctgggaacgtg- 3'
Right   5' –aacctagcagcacagagcaag- 3'

X06769.1
Rat c-fos mRNA
[substituting for BF415939 (rat EST) c-fos mRNA]

Left   5' –cagcctttcctactaccattcc- 3'
Right   5' –acagatctgcgcaaaagtcc- 3'

AI031032
Rattus norvegicus Fra-2

Left   5' –agaggaggagaagcgtcgaatc- 3'
Right   5' –ctccttctgcagctcagcgatt- 3'

XM_343415.1
Rattus norvegicus similar to Ubiquitin specific protease 3 (LOC363084, mRNA [substituting for AI411205 (rat EST) similar to Ubiquitin specific protease 3]

Left   5' –cgacagttgctgtatgacc- 3'
Right   5' –tgtgtaatgcccagaaccaa- 3'