Unimpaired Spatial Working Memory Following Mammillothalamic Tract Damage in Rats: Implications for the Neuroanatomy of Memory

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Table of Contents

List of Figures

List of Tables

Abbreviations

AP	anterior-posterior coordinate
ATN	anterior thalamic nuclei
ANOVA	analysis of variance
B	bregma
CCB	cingulum bundle
DG	dentate gyrus
DNMP	delayed non-matching to place
DNMS	delayed non-matching to sample
Dtg	dorsal tegmental nucleus
DV	dorsoventral
E (#)	number of days after fertilisation
EC	entorhinal cortex
FF	fimbria fornix
Fx	fornix
Hipp	hippocampus
IEG's	immediate early genes
ITI	inter-trial interval
L	lambda
LMn	lateral mammillary nucleus
MB	mammillary bodies
MBR	mammillary body region
MD	mediodorsal nucleus of the thalamus
MMn	medial mammillary bodies
MS	medial septum
MTg	mammillotegmental tract
MTT	mammillothalamic tract
NAcc	nucleus accumbens
NMDA	n-methyl-d-aspartic acid (neurotoxin)
P(#)	number of days post natal
PTD	pryathiamine depletion
RAM	radial arm maze
RE	nucleus reuniens
RH	retrohippocampal
SD	standard deviation
SE	standard error
VTNg	ventral tegmental nucleus

In humans, damage to the mammillothalamic tract (MTT) as a result of localised strokes, tumours or alcohol abuse has consistently been implicated in the severe anterograde amnesia evident in these patients. This small neural pathway, which connects the mammillary bodies (MB) to the anterior thalamic nuclei (ATN), is thought to provide one important link in a larger extended hippocampal circuit involved in encoding and retrieval of episodic memory. Brain damage in clinical cases is, however, typically diffuse and contributions from additional sites of pathology cannot be ruled out. There are also inconsistencies within a limited animal literature on MTT lesions. The current study made MTT lesions in female rats and used multiple 'episodic - like' memory tasks relevant to the proposed importance of this pathway. The project also intended to test whether enrichment reduces any impairments after MTT lesions. None of the lesions resulted in complete bilateral disconnection of the MTT, but many had moderate to large bilateral (n = 6) (81% to 50%), or unilateral MTT damage (n = 4). Rats with bilateral lesions were compared to controls (n = 14, including 4 other lesion rats in which no lesion occurred). The severe working memory deficit in the water maze expected for rats with MTT lesion was not found and only a slight deficit in reference memory in the water maze was observed (so enrichment was not implemented). Although none of the bilateral MTT lesions were complete, they are also often incomplete in clinical cases and previous research has shown that lesions to the ATN in excess of 50% are sufficient to induce severe behavioural deficits in rats. Therefore, if the MTT is critical to memory then substantial but not total bilateral disconnection should be sufficient to induce profound deficits in rats, at least on spatial working memory. Taken together these findings suggest a less crucial role for the MTT in memory than previously suggested. Future research needs to resolve the inconsistencies observed in the animal literature by repeating the present study, using larger MTT lesions and both male and female rats.

1. Introduction

1.1 General

Anterograde amnesia is characterised by a severely impaired ability to acquire new episodic information, that is after the onset of brain injury plus some retrograde amnesia. This debilitating disorder creates substantial burden for both patients and their families. In more severe cases patients may even forget their own marriages, births of their children, or the death of family members while anterograde memory can be virtually non-existent (Rosenbaum, Murphy & Rich, 2012). Understandably there is confusion and frustration for the patient as their memory no longer corresponds to reality. This disorder results from damage to the medial temporal lobe, diencephalon or basal forebrain and prognosis is poor (Markowitsch & Staniloiu, 2012). There is currently no standardised therapeutic approach for human amnesic disorders. However, the ATN lesion animal model of diencephalic amnesia (Loukavenko, Ottley, Moran, Wolff & Dalrymple-Alford, 2007; Wolff, Loukavenko, Will & Dalrymple-Alford, 2008) suggests that exposure to enriched environments may help to attenuate memory deficits, while other work using the PTD model of Korsakoff's syndrome, which is associated with diecephalic injury, suggests that cholinomimetic drugs may be helpful (Savage, Hall & Rescende, 2012)

As alluded above, the pathology associated with anterograde amnesia most commonly is found in two distinct brain regions, the medial temporal lobe and the diencephalon (Aggleton & Brown, 1999). Traditionally, medial temporal and diencephalic amnesias were often observed as two separate conditions. In fact, the core features of both medial temporal and diencephalic amnesia are strikingly similar. For instance, both share a particularly severe and persistent loss of new episodic learning, while other cognitive abilities, such as priming,

procedural learning and short term memory appear largely intact (Aggleton & Brown, 1999; Aggleton, 2008). The consistent symptomatic overlap observed between medial temporal and diencephalic amnesias was noted some time ago and led to the suggestion that the neural basis for episodic memory is represented by an extended memory circuit (Delay & Brion, 1969). In this extended memory system, areas of the hippocampal formation project via the fornix to the mammillary bodies and the ATN. The mammillary bodies in turn project information to the anterior thalamic nuclei (ATN) via the mammillothalamic tract (MTT) and the ATN project to the medial limbic cortex (Van der Werf, Jolles, Witter & Uylings, 2003a). The notion that these limbic structures function together in an integrated circuit had previously been raised in a different context in a paper by Papez (1939), but his focus was a limbic system circuit providing the basis of emotion, rather than memory.

More recently Aggleton and colleagues (Aggleton & Brown, 1999; Aggleton, 2008) developed the formal hypothesis that an extended hippocampal system is critical for the efficient encoding and normal recall of new episodic information. From this perspective, damage to any component structure can result in anterograde amnesia. That is, damage to different parts of this system may produce similar memory impairments, although not necessarily identical ones, as some key structures will have a larger contribution to normal memory function (Aggleton & Brown, 1999). Later Aggleton (2008) made the important addition of a diaschisis effect throughout this extended memory system when any of its components received injury. Diaschisis supposes that, in addition to the temporary effects of trauma, the mere disconnection of an area could induce impairment in the function of distal regions, especially those with strong functional connections, and that some of these impairments can be long lasting. The last claim followed extensive research showing hypo-activation of immediate early genes (which provide an estimate of neural activation) in the

retrosplenial cortex, after lesions of the hippocampal formation, the ATN and the MTT (Jenkins, Vann, Amin & Aggleton, 2004; Poirier & Aggleton, 2009; Vann & Albasser, 2009).

1.2 Critical Structures within the Medial Temporal Lobe and Diencephalon

Figure 1.1 shows the key structures in both the diencephalon and the medial temporal lobe as well as the fibre tracts connecting the two structures. The diencephalon itself includes the thalamus, the hypothalamus and mammillary bodies. The fornix provides the primary link that connects the hippocampus to the ATN via two routes, the first directly through the fornix (a reciprocal route) and the second indirectly via the MB and MTT (Aggleton, 2008). Importantly, Vann & Aggleton (2003) suggest that it is critical to understand the role of the MTT because it is the only structure with connections confined within the Delay and Brion circuit. The MTT projects up from the lateral and medial MB to innervate and terminate in the ATN, but there is no reciprocal connection (Vann, 2010). Aggleton, Vann & Saunders (2005) have suggested that the ATN are the only efferent target of the MB and that the MTT must therefore reflect core aspects of MB function. Clearly, it is vital to understand the effect of both damage to the MTT itself and the nuclei that give rise to it.

There is almost universal agreement that the critical region for temporal lobe amnesia is the hippocampal formation and its adjacent cortex (Aggleton & Brown 1999; Aggleton 2008). Diencephalic amnesia, in the context of Wernicke Korsakoff syndrome, was investigated much earlier than temporal lobe amnesia yet its neural basis remains less certain (Aggleton 2008). Neuropsychological studies have failed to provide definitive evidence concerning the basis of diencephalic amnesia, primarily because such evidence would have to come from patients with well characterized amnesia that is pathologically restricted to just one brain structure and confirmed by post mortem (Aggleton & Brown, 1999; Van der Werf et al, 2003a). Unfortunately brain injuries resulting from neurodegeneration or traumatic insults are often diffuse and non-specific making it difficult to attribute dysfunction to

individual structures. The various sub-regions implicated in diencephalic amnesia include the MB, the MTT and selective thalamic nuclei. Aggleton (2008) suggested that the MB and the ATN are of particular importance. This suggestion derives primarily from the conjunction of neuroanatomical and neuropsychological findings. These two diencephalic regions have an exceptionally close anatomical relationship. Despite the association of multiple subcortical regions with memory loss, the clinical evidence most consistently implicates the MTT/MB as among the most common diencephalic sites, as summarized below.
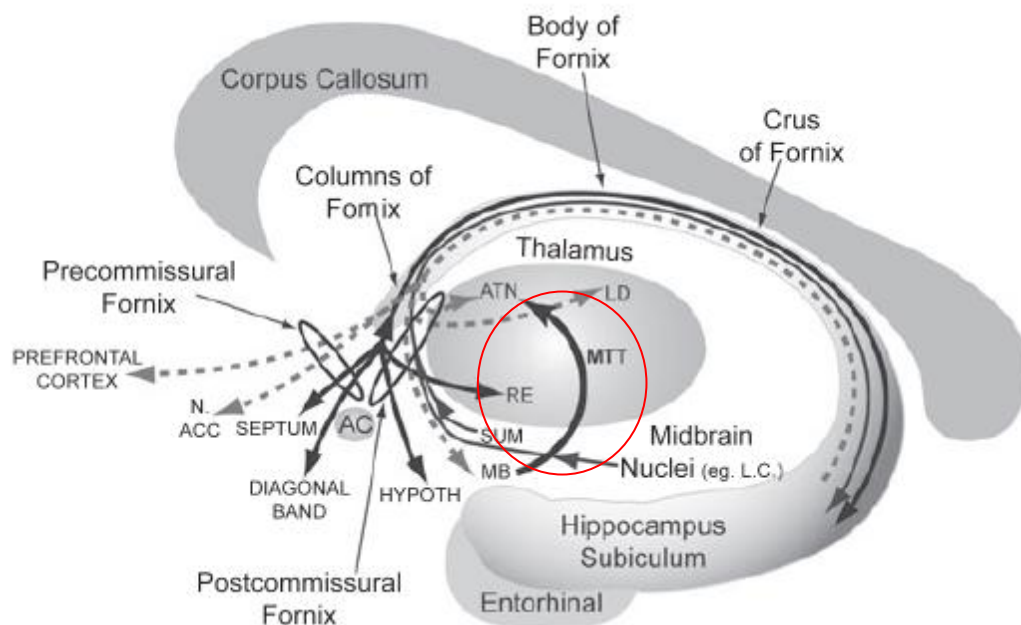
Figure 1.1. Diagrammatic representation of the human limbic system showing the mammillary bodies, the mammillothalamic tract, and the anterior thalamic nuclei and the major fibre tracts running between them. Adapted from Aggleton, Omara, Vann, Wright, Tsanov & Erichsen (2010) pg, 2293. Specifically, it shows that the fornix connects the subiculum of the hippocampal formation to the ATN and MB and the MTT (circled in red) connects the MB to the ATN.

1.3 Thalamic Infarcts

Cases of thalamic infarction are of significant interest as damage to the anterior portion of the thalamus not only disturbs the afferent nuclei of the MTT but often the trajectory of the MTT itself. Examination of the resulting dysfunction may help to elucidate the functional contribution of specific diencephalic structures. Although the studies that follow provide a compelling case for the involvement of the MTT in human amnesic syndromes, it must be stressed that in no case does disruption of the MTT occur in isolation. The close anatomical relationship between the MTT and its afferent structures the anterior thalamic nuclei of the thalamus means that concurrent damage to both structures is usually present.

An excellent review by Van der Werf, Witter, Uylings & Jolles (2000) suggested that lesions restricted to the thalamic region regardless of aetiology can cause cognitive disturbances. The pattern of symptomatology present is not consistent among all cases of thalamic lesions with the resulting behavioural disturbances showing some regional specificity (Van der Werf et al 2000). Van der Werf et al (2000) grouped cases according to the location of their lesions either in the anterior third of the thalamus, the middle third of the thalamus, or cases in which pathology extended into both the anterior and middle portions. They found that in all but two cases, regardless of group assignment, "the occurrence of an amnesic syndrome (was) associated with the lesioning of the MTT" (Van der Werf et al, 2000, pg 622). This quote is important because it suggests that merely damaging the MTT is sufficient to induce the amnesic syndrome and implies that complete bilateral transection is not necessary. The relevance of the partial nature of this injury will become apparent later in the thesis.

A later study by Van der Werf, Scheltens, Lindeboom, Witter, Uylings & Jolles (2003b) provided information on thalamic structures associated with executive function, but also attention, and confirmed the previously established structure function relationships for

memory. Van der Werf et al (2003b) tested 22 cases of thalamic infarction on a fixed battery of tests so individual cases could be compared. MRI scans were taken from each patient and lesion sites were plotted both in standard stereotactic space and in an atlas of the thalamus. They found that memory performance within their group of patients varied from intact to severely impaired. A clear relationship between a specific kind of memory disorder, i.e. the amnesic syndrome, and structural damage to the MTT was found. Van der Werf et al (2003b) also suggested that, as the MTT contains fibres bound for the ATN, it is to be expected that infarctions affecting the ATN produce the same deficit as damage to the MTT, but AT infarctions are rarely encountered. No simple relationships were found between other thalamic structures and executive functioning or attention.

A subsequent review by Carrera & Bogousslavsky (2006) examined the effect of anatomically distinct strokes in the thalamus on behaviour and reinforced Van der Werf et al's (2003b, 2000) findings. That is, they concluded that anatomic studies suggest that the amnesic syndrome results primarily from interruption of the MTT and its projections to the ATN and hence their influence on the cingulate gyrus, hippocampus, orbitofrontal and prefrontal cortex.

A more recent review by Carlesimo, Lombardi & Caltagirone (2011) extended this earlier work by including a larger sample of patients and giving particular emphasis to Aggleton & Brown's (1999) recollection/familiarity distinction in the domain of declarative memory. Carlesimo et al (2011) examined 41 papers published between 1983 and 2009. These studies provided data on a total of 82 patients with lacunar infarcts in the mesial and anterior regions of the thalamus, both frequently associated with amnesic syndromes. Nearly all the patients with a neuroradiologically documented lesion to the MTT presented with a clinically relevant memory disorder confirmed by a formal neuropsychological evaluation. In agreement with Van der Werf et al (2000), Carlesimo et al (2011) also suggested that

complete bilateral disconnection of the MTT was not necessary to induce the amnesic syndrome. They state, "The presence of an amnesic syndrome (with both an anterograde and retrograde memory deficit) is strongly predicted by the involvement of the MTT" (Carlesimo et al, 2011, pg 787). Calesimo et al's (2011) study also lends support for Aggleton & Brown's (1999) conclusion regarding the extended hippocampal system as they found that the qualitative pattern of memory impairments observed after a focal lesion to the medial and anterior regions of the thalamus is very similar to that observed after medial temporal lobe damage. That is the chronic amnesic syndrome includes a prevalent deficit for long term anterograde memory, a less consistent deficit in long term retrograde memory and largely spared short term and implicit memory, but with relative sparing of familiarity based recognition memory. Carlesimo et al (2011) found that the data deriving from a few single case reports supported the hypothesis of a differential memory role of thalamic regions connecting to different areas of the medial temporal lobe. They suggested that the MTT/ATN axis is mainly implicated in recollective memory processes and that, by contrast, the ventro-amdalofugal pathway/MD axis underlies familiarity processes.

Two recent studies, Nishio, Hashimoto, Ishii & Mori (2011) and Edelystyn, Mayes, Denby & Ellis (2012), have provided partial support for the relationship between MTT damage and amnesia observed by Van Der Werf et al (2003, 2000) and Calesimo et al (2011). Support from these two studies was only partial because other circuits could not be ruled out or may have had an additional contribution to the deficit caused by the MTT damage alone.

Despite the considerable evidence implicating MTT damage in clinically defined cases of the amnesic syndrome, not all reports of MTT damage have resulted in a memory deficit. Duprez, Serieh & Raftopoulos (2005) stereotactically implanted stimulation electrodes within the MB of three patients, passing through the MTT, in order to treat chronic refractory epilepsy. They state that none of the three patients experienced any memory deficit

either immediately after the surgical implantation, or during the global or elective stimulations. Additional comprehensive cognitive testing was performed all of which failed to reveal any early or delayed mental decline after implantation. Unfortunately, Duprez et al (2005) did not specify how many cognitive tests were performed or at which time points following implantation these were administered. Duprez et al (2005) suggest that perhaps the lack of memory impairment can be attributed to the intact direct route from the hippocampus via the fornix to the ATN and this alternative pathway may explain why MB or MTT lesions, even if they are bilateral, are not as disruptive as ATN lesions. This work also suggests that when previous studies imply MTT involvement, the location of the injury might be such that fornix-ATN connections are also affected.

1.4 Traumatic Brain Injury and Anterograde Amnesia.

There are many case studies examining memory function after traumatic brain injury to the temporal lobes and other cortical sites. By contrast, reports of anterograde amnesia resulting from traumatic brain insults to the diencephalon are rare. Traumatic brain injury provides an important perspective as an exact time course for the insult can be established. Two cases are often cited in the literature (Squire, Amaral, Morgan, Kirtchovsky, & Press, 1989; Dusior, Kapur, Byrnes, Mckinstry & Hoare, 1990). While these cases are interesting to consider both are confounded because damage to multiple diencephalic sites occurred as a result of the penetrating injuries.

Squire et al (1989) described patient N.A. who sustained a penetrating brain injury when a miniature fencing foil was thrust up his right nostril. A series of MRI studies conducted more than 20 years after the insult revealed three major areas of brain damage. A large lesion was observed in the left thalamus that interrupted the intralaminar and mediodorsal nuclei, but which also likely transected both the MTT and the post commissural fornix. The posterior hypothalamus was also markedly disrupted and the mammillary bodies

appeared to be missing bilaterally. There was also damage to the anterior temporal lobe which extended to the amgydaloid complex. Patient N.A. presented with a verbal memory impairment that was considered to be unusually pure, differentiating him from patients with Korsakoffs syndrome. Additionally, unlike patients with Korsakoff syndrome, he showed good insight into his memory problem, accurately predicting his subsequent performance on a recognition memory test. Although Squire et al (1989) attributed the memory dysfunction to the internal medullary laminae and mammillothalamic tract damage many structures relevant to memory were disrupted by the trajectory of the penetrating object making attribution of the amnesic syndrome to a single structure difficult.

The second key instance of diencephalic damage resulting in severe memory impairment concerned patient BJ (Dusior, Kapur, Byrnes, Mckinstry & Hoare, 1990). Patient B.J received a penetrating brain injury caused by a snooker cue which entered his left nostril into the basal regions of the brain. In the initial period after his injury his memory disorder had the clinical features of a dense amnesic syndrome with both retrograde and anterograde amnesia. However, formal memory testing 21 months after the insult showed marked verbal memory impairment, but relatively intact non-verbal memory and the retrograde amnesia had regressed to mainly affect the period 6 months before the injury. MRI showed bilateral damage to the MB. Importantly there was no damage to the body of the thalamus implicating MB injury as the primary cause of this memory loss.

Aggleton and collegues (Aggleton, O'Mara, Vann, Wright, Tsanov & Erichsen, 2010; Aggleton, 2008) have long suggested that an important consideration in understanding the functional relationship between temporal lobe amnesia and diencephalic amnesia depends on determining the role of the fornix, the major interlinking tract (see figure 1.1). A recent line of research by Tsivilis, Vann, Denby, Roberts, Mayes, Montaldi & Aggleton (2008) related fornix volume with memory impairment following the removal of colloid cysts. A colloid

cyst is a benign tumour that develops in the third ventricle adjacent to the fornix. These cysts are normally surgically removed, but this often causes substantial damage to the fornix accompanied by memory loss. Fornix status was assessed directly by fornix volume and indirectly by MB volume, as atrophy is observed in this structure following fornix damage. Overall fornix volume was not consistently correlated with memory performance. Unexpectedly Tsvilis et al (2008) found that MB volume significantly predicted the recall of episodic information and correlated significantly with 13 of 14 recall memory tests and 7 of 8 memory indices. Furthermore individuals with colloid cysts and the smallest MB volume remaining performed significantly worse on tests of recall than those with the largest MB volumes. Another much older line of evidence for the involvement of the mammillary bodies and their efferent pathway in anterograde amnesia has come from the neurodegenerative disorder, the Wernicke Korsakoff syndrome.

1.5 Wernicke's Encephalopathy and Korsakoff's Syndrome

Wernicke's encephalopathy is an acute neuropsychiatric reaction to thiamine deficiency that is characterised by nystagmus (involuntary eye movement) and opthalmoplegia (paralysis of extraocular muscles), mental status changes and unsteadiness of stance and gait (Sechi & Serra, 2007, Kopelman, Thomson, Guerrini & Marshall, 2009). It is diagnosed more commonly in alcoholics at post mortem than it is in life (Kopelman et al, 2009). Around 80% of those who survive Wernicke's encephalopathy develop the Korsakoff syndrome which is defined as a disproportionate deficit in memory relative to other features, due usually to thiamine deficiency. Thiamine (or vitamin B1) is an essential co-enzyme for intermediate carbohydrate metabolism, lipid metabolism and production of amino acids and glucose-derived neurotransmitters such as GABA (Sechi & Serra, 2007). Wernicke's encephalopathy is most prevalent in patients who abuse alcohol, but also occurs in various illnesses where the absorption of nutrients has been compromised such as AIDs, anorexia nervosa (purging) and

peptic ulcers (Sechi & Serra, 2007). Brain lesions are often restricted to selective and vulnerable areas with a high thiamine turnover and can occur in as little as 2-3 weeks which correlates with the time taken to deplete the body's stores of thiamine as these are only sufficient for up to 18 days (Sechi & Serra, 2007). Sechi & Serra (2007) suggest that up to 50% of patients have extensive midline pathology located primarily in the periaquaductal grey matter, the mammillary bodies and medial thalamus. Furthermore, autopsies show that approximately 82% of patients with mental status changes are associated with the involvement of the thalamus and the mammillary bodies (Sechi & Serra, 2007).

The Korsakoff syndrome is characterised by a chronic and striking loss of everyday memory (Sechi & Serra, 2007). Patients with Korsakoff's present with severe anterograde amnesia and are unable to remember events even within the last half hour, but retain implicit learning so are still able to learn new motor skills or develop conditioned reactions to stimuli. A recent study by Jung, Chanrand & Sullivan (2012) suggested that mammillary body shrinkage is observed in upwards of 60-80% of neuorpathological studies and this damage has been proposed as a specific macroscopic lesion of Chronic Wernicke Korsakoff syndrome. However Jung et al (2012) also report that MRI findings show that mammillary body shrinkage has been observed in cases without amnesia and the correlations between mammillary body volume and memory impairment are inconsistent.

Although mammillary body atrophy is most often associated with amnesia in the Korsakoff's syndrome, other diecenphalic structures have also been suggested to be critical. Victor, Adams & Collins (1989) argued that the mediodorsal nucleus of the thalamus was the only other structure together with the MBs to be affected in 100% of patients suffering from Korsakoff's or Wernicke Korsakoff's syndrome, therefore pointing to a possible role for the mediodorsal nucleus in the memory disturbances of these patients. More recently Harding Halliday, Caine & Krill (2000) reported that neurodegeneration in the anterior thalamus was

the only consistent lesion found in alcoholics with Korsakoff's, which differentiated them from other alcoholics with only Wernicke's encephalopathy. These findings suggest that structural or neurochemical abnormalities within a wider circuit involving the mammillary bodies, mammillothalamic tract, and the anterior thalamus may account for the anterograde amnesia observed in Wernicke Korsakoff syndrome (Sechi & Serra, 2007). Recently however the MTT has been directly implicated in memory impairment associated with Wernicke's encephalopathy.

Kim, Ku, Namkoong et al (2009) compared 7 chronic alcoholics recovering from Wernicke's encephalopathy, with 14 alcoholics without Wernicke's encephalopathy and 14 healthy controls. The participants underwent functional connectivity fMRI scans, as well as verbal and non-verbal memory tests. A resting state functional connectivity strength between the ATN and the MB was generated over the duration of a five minute viewing task. Kim et al (2009) found the memory function in patients recovering from Wernicke's encephalopathy paralleled the level of MTT connectivity between the MB and ATN.

A follow up study by Kim, Ku, Jung et al (2010) reinforced this finding by showing that improvement in delayed verbal and non-verbal recall memory after high dose thiamine replacement therapy paralleled MTT function connectivity between the MB and ATN. Kim et al (2010) followed an individual with Wernicke's encephalopathy over 20 months both before and after high dose thiamine replacement therapy. A direct transfer function analysis showed significant information flow between the MB and thalamus (in the direction of the thalamus) except in the acute illness state.

Collectively, the mounting clinical evidence concerning anterograde amnesia following damage to the diencephalon, whether from stroke, trauma or neurodegeneration, all suggest a critical role for the MTT and MB in normal episodic memory function. Although the MTT and MB are the most consistently involved regions in the human amnesic syndrome

it is impossible to rule out the involvement of additional structures and/or neural circuits. This is especially true in Korsakoff's syndrome as thiamine deficiency causes diffuse change throughout the brain. This highlights the need for animal studies that can create localised lesions in single structures within the brain and measure the behavioural changes that result in order to elucidate specific functional importance of these structures. The next section summarizes the findings of animal models of diencepahlic amnesia, including thiamine deficiency and lesions to the ATN, MB and MTT. Particular consideration is given to the MB and MTT because of their consistent implication in a clinical setting.

1.6 Animal Models of Diencephalic Damage

1.61 Thiamine Deficiency

A recent review by Savage (2012) shows that animal models of thiamine deficiency have increased understanding into the mechanisms associated with thiamine deficient neurodegeneration. These animal models are the result of treatment with the thiamine antagonist pyrathiamine (PTD). Similar to patients with Wernicke Korsakoff syndrome, the pathology induced in the PTD rat model is relatively diffuse and in addition to diencephalic damage there is also damage to major white matter tracts including the corpus callosum and the internal capsule (Vann, 2010). Savage (2012) suggests that induced neuropathy to three diencephalic regions, the AT, internal medullary laminae and the MB, primarily affect the performance of tasks that have heavy demands on spatial episodic working memory, but generally spare short term and implicit memory. PTD in rats also disrupts hippocampal and cortical acetylcholine and noradrenalin levels (Vann, 2010; Savage, 2012).

1.62 The Anterior Nuclei of the Thalamus

In contrast to the human literature, studies in rats and monkeys have shown that damage to the anterior nuclei more consistently leads to more severe memory deficits than lesions of the MTT or MB. These deficits can be seen in paradigms of delayed non-matching to place,

delayed non-matching to sample, associative memory, allocentric learning and radial maze learning (Aggleton & Mishkin, 1983; Aggleton, Hunt, Nagle & Neave, 1996; Byatt & Dalrymple-Alford, 1996; Sziklas & Petrides, 1999; Mitchell & Dalrymple-Alford, 2005; Loukavenko et al, 2007).

1.63 A Therapeutic Approach to Diencephalic Damage

Recent research has suggested that enriched environments may provide a possible therapeutic approach to diencephalic damage (Loukavenko et al, 2007; Wolff et al, 2008). Loukavenko et al (2007) found ATN lesions in rats housed in standard cages produces long-lasting memory deficits, but these were ameliorated by postoperative exposure to enriched environments. Loukavenko et al (2007) found regardless of latency, either at day 5 (expt 1) or day 40 (expt 2) exposing rats to an enriched environment after bilateral ATN lesions dramatically improved their spatial working memory compared to ATN standard housed rats. Correct performance in the tasks used required the rats to visit the alternate arm in a cross maze from that previously visited on the sample run of the trial. This cross-maze task controls for the use of egocentric cues. Additionally, the spatial memory gains observed in experiment 2 were maintained for 4 months post-surgery despite no further enrichment.

Subsequently, Wolff et al (2008) compared allocentric spatial memory (the use of relational spatial representations) recovery in rats with ATN lesions housed in enriched environments compared to lesion rats housed in standard environments. Rats were tested also on reference memory task in the water maze which used a fixed start point and fixed platform, followed by probes from novel start points. Wolff et al (2008) found that standard housed rats with ATN lesions showed a substantial deficit when probe trials utilised a novel starting position, whereas the ATN enriched rats' performance only mildly decreased when the novel probe trials were introduced, demonstrating that the latter rats were able to make flexible use of their spatial representation of the test room.

1.64 The Mammillary Bodies

Despite being consistently implicated in amnesia resulting from Korsakoff's syndrome, the functional contribution of the mammillary bodies to memory is still not clear (Vann & Aggleton, 2004). Table 1.1 summaries a number of studies that tested either mice or rats in spatial memory tasks following MB lesions. The crucial observation from table 1.1 is that lesion studies that have involved ablation of the MB to varying degrees do not always produce consistent results. Lesion size seems to be an important consideration, with the greatest deficits being observed when the lesions extend beyond the MB to include adjacent structures and fibre tracts (Sziklas & Petrides, 1993; see table 1.1).

The effects of MB damage are not as severe as a hippocampectomy, and typically less severe than ATN damage. The resulting deficit of MB lesions also seems to diminish with training although possibly through the use of alternative routes/ strategies (Vann & Aggleton, 2004). Traditionally, theories of MB function emphasised their connections with the hippocampal formation as the MB receive substantial input via the fornix from the hippocampus. Thus they were thought to provide a relay, passing information from the hippocampus to the ATN (Vann, Erichsen, O'Mara & Aggleton, 2010).

A recent study by Vann et al (2010) suggests that the medial MB may have unique input into this memory system. Vann et al (2010) cut the descending component of post-commissural fornix, which disconnects the dorsal subiculum from the MB, thus leaving intact the direct connection between the dorsal subiculum and the ATN. If the MB were an important hippocampal relay, then severing the post-commissural fornix should induce a marked behavioural deficit. In contrast, they found that cutting the post-commissural fornix only had a mild or sometimes no apparent effect on performance of spatial memory tasks, much less than impairments found previously after direct MB or MTT lesions (Vann &

Aggleton, 2003). Hence, the MB and MTT appear to have an independent contribution to the extended hippocampal memory system.

Additionally, Aggleton et al (2010) reported that connections from the subiculum to the MB and ATN arise from two different populations of subicular neurons. Furthermore, the segregated property of the subiculum extends dorsally into the retrosplenial cortex and ventrally into the entorhinal cortex as well as the post-pre and para-subiculum. Wright, Erichsen, Vann, O'Mara & Aggleton (2010) suggest that hippocampal projections via the fornix to the MB and ATN are potentially capable of providing independent information despite the strong likelihood of a convergence in the ATN.

The behavioural deficit following MB damage is suggested to be a result of the loss of head direction information, and/or the disruption of theta rhythms (Vann & Aggleton 2004). Head direction cells aid navigation by selectively firing when a rat is facing a certain direction on a horizontal plain (Vann & Aggleton 2004). These cells are concentrated in the lateral mammillary bodies and form a system with the dorsal tegmental nucleus (Dtg) and the anterior dorsal nucleus of the ATN. Theta activity refers to the regular bursts of firing of cells, which in conjunction give rise to theta rhythms. The interest in theta rhythms arose from their possible links with memory. For example Vann & Aggleton (2004) state that long-term potentiation in the hippocampus can be elicited by stimulation at theta frequency. Therefore theta activity may act as a significance signal. Additionally recordings made in the medial MB reveal neurons that fire rhythmically in phase with hippocampal theta, thus the medial MB are considered to relay hippocampal theta rhythms to the ATN and beyond (Vann & Aggleton 2004).

As selective disconnection of the fornix innervation to the MB did not result in "MB or MTT like" lesion deficits (Vann et al, 2010), another line of research has examined the behavioural outcomes of lesions to the ventral tegmental nucleus of Gudden (Vann, 2009).

This nucleus projects to the medial mammillary bodies via the mammillotegmental tract a route which is reciprocal. Rats with VTNg lesions were tested on a battery of standard memory tasks including the t-maze, water maze and radial arm maze (RAM). Vann (2009) found that VTNg lesions appeared to produce deficits on the same array of spatial memory tasks and to a similar degree as medial mammillary nucleus lesions. This study suggests that the VTNg and not the fornix afferents to the MB maybe critical to normal memory function. Unfortunately there is only one reported case of a man with amnesia that was attributed to pathology in the VTNg region (Goldberg, Antin, Bilder, Gerstman, Hughes & Mattis, 1981).

In contrast to the large amount of experimental research that has been conducted into the functional contribution to memory of the MB and the ATN, there has been little research directed at the MTT specifically. Given the mounting clinical evidence consistently implicating the MTT in cases of amnesia it would be a logical step to examine in detail the behavioural effects of MTT transection in an animal model. Table 1.2 shows the scarcity of the animal research examining the effects of "selective" MTT lesions conducted to date. Unfortunately vast differences in lesion methodology and histological outcome make the three earlier studies hard to interpret. Lesions in these studies ranged from small (Kreickhaus & Randall, 1968) to large lesions surrounded by large areas of necrotic tissue (Field et al, 1978 and Thomas & Gash, 1985). The later studies (Vann, Honey & Aggleton, 2003; Vann & Aggleton, 2003) seem to have taken more care to minimise damage to the tissue surrounding the MTT. However these studies only confirmed this observation with a nissl stain. Considering the MTTs consistent implication in human amnesia it is surprising that no primate studies creating MTT lesions have been conducted to date.

Table 1.1 Summary of studies on spatial memory tasks in rats following MB lesions

Year	Authors	Lesion site/s	Lesion method	Behavioural tasks	Deficits
2005	Vann	LMN	Ibotenic acid	1. T-Maze WM 2. Water maze WM	LMN not impaired on #1, impaired on #2
2001	Gaffan, Bannerman, Warburton, & Aggleton	FX MB ATN RH	Severed NMDA NMDA NMDA	1. Visual scene discrimination	RH not impaired on #1 Fx, ATN and MB enhanced performance on #1
1999	Santin, Rubio, Begega, & Arias	MB	Electrolytic	Water-maze 1. Reference memory	MB not impaired on #1
1997	Neave, Nagle, & Aggleton	MB FX CCB	NMDA, Radio frequency	1.T-maze, 2.Cross maze 3. RAM	MB impaired on #1, #2 & #3 but not in #2 & #3 in egocentric discrimination. FX/CCB impaired on #1 and #3, but not #2
1995	Aggleton, Neave, Nagle, & Hunt	ATN MB FX	NMDA NMDA Radio frequency	T-maze 1. Forced Alternation task 2.Object recognition	ATN impaired on #1 MB impaired on #1 FX impaired on #1
1993	Sziklas & Petrides	MB MB-R Hipp A	Electrolytic Electrolytic Electrolytic Electrolytic	1.RAM (working memory) 2. RAM extended ITI 3. non-spatial DNMS 4. Conditioned taste aversion	MB no impairment on #1, #2, #3 or #4 MB-R impaired on #2, but not #1,#3 or #4 Hipp impaired on #1 & #2 (not tested on #3 & #4) A no deficit on #3 but impaired on #4
1990*	Beracochea, & Jaffard	MB	Ibotenic acid	T-Maze 1.Spontaneous Acquisition 2. Sequential delayed alternation	MB impaired on #1 but not #2, until ITI's extended from 50s to 3mins

1989	Sutherland & Rodriguez	FF ATN NAcc MS MB	Electrolytic	Water-maze, Reference memory 1. Retention 2. Acquisition	FFC impaired on #1 and #2 NAcc impaired on #2 ATN impaired on #2 MB modest impairment on #2 MS modest impairment on #2
1987*	Beracochea & Jaffard	MMn	Radio Frequency, or Kainic acid	T-Maze 1.Spontaneous Alternation (S.A) 2.S.A reduced ITI 30s to 5sec 3.Adding external cue	MM impaired on #1 not on #2 or #3
1984	Jarrad, Okaichi, Steward & Goldschmidt	FF EC DG MB	Electrolytic Electrolytic Colchicine Electrolytic	RAM 1. Place task 2. Cue task	FF impaired on #1 and #2 EC impaired on #1 and #2 DG not impaired on #1 or #2 MB not impaired on #1 or #2

Abbreviations: ATN = Anterior Thalamic Nuclei, FX= fornix, FF= fimbria fornix, EC= entorhinal cortex, DG=dentate gyrus, NAcc =nucleus accumbens, MB= mammillary bodies, MMn= medial mammillary nucleus, LMn=lateral mammillary nuclei, MS= medial septum, CCB = cingulum bundle, RH= retrohippocampal region, Hipp = hippocampus, MB-R= mammillary body region (includes supramammillary nucleus and fibre tracts), A=amygdala, RAM = radial arm maze, DNMS = delayed non-matching to sample and NMDA= N-Methyl-D-Aspartic acid (neurotoxin)
* indicates studies that used mice.

1.7 The Mammillothalamic tract

1.71 Anatomical Trajectory and Connectivity of the MTT

Efferent fibres of the MTT project from both the medial and lateral mammillary nuclei to all three elements of the anterior nuclei of the thalamus (Powell & Cowan, 1954; Cruce, 1975; and Seki & Katuyazyo, 1984). More specifically, the medial mammillary bodies project unilaterally via the MTT to both the anterior medial and anterior ventral nuclei, while the lateral mammillary bodies project bilaterally to the anterior dorsal nuclei of the thalamus (Cruce, 1975). Furthermore, it is believed that the neurons contained within the various nuclei of the mammillary bodies have very few, if any, interconnecting neurons suggesting that their primary function is to pass information on to the ATN (Vann & Aggleton, 2004).

1.72 The Developmental Course of the MTT in the Rat Brain

Recently Alpeeva & Makarenko (2009) described the schedule of mammillothalamic tract development in the rat by using carbocyanine dye tracing. The fibres of the MTT are initially formed as collaterals of the mammillotegmental tract, and axons start bifurcating from the mammillotegmental tract 17 days after fertilization (E17). Subsequently the MB axons of the MTT grow simultaneously and reach the ventral region of the anterior thalamus where they first start to form terminal arborisations from E 20-21. Ipsilateral projections form the medial mammillary nucleus to the anteromedial and anteroventral thalamic nuclei develop from E20 to post-natal day 6 (P6). Finally the bilateral projections from the lateral mammillary bodies to the anterodorsal nuclei develop later, between P3-P6, after the formation of the thalamic decussation of the mammillary body axons. Alpeeva & Makarenko (2009) suggest the possible importance of timing in the development of the MB fibres within the MTT, because it had previously been shown that the neurons of the thalamus develop between E15 and E17 and separate thalamic nuclei can be defined by E21. As later development in the thalamus

coincides with MTT development Alpeeva & Makarenko (2009) suggest that the unique spatial and temporal pattern of the perinatal development of the ascending mammillary body connections to the ATN may reflect the importance of these connections within the limbic circuitry.

1.73 Behavioural Studies

The three early studies shown in table 1.2 (Krieckhaus & Randal, 1968; Field et al, 1978 and Thomas & Gash, 1985) are confounded by considerable variation in lesion size. For example Kriekhaus & Randall (1968) produced relatively localised MTT lesions that did not substantially encroach on surrounding areas. In contrast Field et al (1978) and Thomas and Gash (1985) both had very large lesions extending significantly beyond the outer boarder of the MTT. All three studies conducted their behavioural testing in the T-maze apparatus, but only Thomas & Gash (1985) used a working memory task.

The behavioural procedures adopted by third Kreickhaus & Randall (1968), are hard to interpret due to lack of detail and poor rationale. The simplest interpretation is they were trying to measure whether MTT lesions interfered with behavioural flexibility between days and between trials. In their first experiment Kreickhaus & Randall (1968) trained rats to go to a single place, either the left of the right arm of the t-maze for water. Training for each day concluded when rats had successfully entered the correct arm for ten consecutive trials. For each day of training thereafter the correct arm was reversed from the previous day. Kreickhaus & Randall (1968) found rats with bilateral MTT lesions showed no decrement in daily alternation compared to controls. The first task seems to suggest that rats with MTT lesions were able to alter their behavioural strategy between days at a level comparable to controls. For their second experiment both arms were initially rewarded and the rat was allowed free choice. Following this trial the rat had to alternate to the opposite arm in order to receive water. If the rat failed to alternate and went to the same arm twice it was allowed to

go to the correct arm (an informing trial) and on the next trial the rat was required to visit this arm again before returning to alternating. Rats with MTT lesions were found to be significantly inferior at returning to the rewarded arm on a trial after an informing trial (i.e. failure to persevere). The results of this second task are somewhat difficult to interpret as not returning to the same arm on a trial after an informing trial suggests the adoption of the "correct" alternation strategy necessary for the main task, whereas the control rats seem to show a perseverance of behaviour returning to the previously rewarded arm. Kriechaus & Randall (1968) concluded that the findings of their two experiments offer no support for the notion that the MTT mediate short-term memory.

Field et al (1978) compared the behavioural outcomes of MB, mammillotegmental tract (MTg) and MTT lesion rats on a massed alternation task in the t-maze. In this task the rats were initially given a choice trial where they could select either arm for a water reward following which they had to alternate across the subsequent 20 trials. The completion criterion was set at 18 of 20 responses correct over two days. Testing was terminated if the rat had not reach criterion at the end of day eight. All lesion groups made significantly more errors compared to control rats in this task and there were no differences between the lesion groups. Field et al (1978) also measured ambulation in the open field, as hyperactivity is commonly reported after lesions to the hippocampus and fornix. Both the MB and MTg lesion rats showed significantly increased levels of activity compared to controls. By contrast the MTT group seemed relatively lethargic, initially showing less activity than the control group; however, this difference was not significant.

A later study by Thomas & Gash (1985) used a standard delayed non-matching to place procedure in the t-maze. In this procedure each trial consists of two parts, a forced run and a choice run. In the forced run one of the arms is blocked off forcing the rat to enter the open arm for a food reward. The rat was then immediately returned to the starting area for the

choice run. In the choice run the block was removed and both arms were open. A correct choice required the rat to alternate from the forced run and enter the previously closed arm. Thomas and Gash (1985) found that with sufficient training the initial severe deficit they observed in MTT lesion rats substantially improved. In fact five of the nine rats that sustained complete bilateral damage to the MTT eventually (after 72 trials) reached a high level of performance 80% + correct. In the remaining four, two reached 80% + performance but not consistently and the other two showed near chance performance for the entire testing period. No effect of partial MTT lesions was observed. Partial lesions included any damage that failed to destroy the MTT bilaterally, ranging from complete sparing to unilateral MTT destruction.

More recent studies made a concerted effort to clarify the behavioural impact of MTT lesions with the addition of well validated paradigms and more localised and consistent lesions (Vann & Aggleton, 2003; Vann, Honey & Aggleton, 2003). Vann & Aggleton (2003) compared both MB and MTT lesion rats to sham operated controls in the t-maze, radial arm maze and water maze. As indicated in Table 1.2 MB and MTT lesions impaired acquisition of all three spatial tasks but a closer examination of the results suggests a more complex story. The performance deficits found in t-maze alternation for both lesion groups were only transient (as in Thomas & Gash, 1985) and over the final four days of testing (12 days in total) there were no significant differences between the lesion and control groups. Furthermore, when proactive interference was increased for this task by using massed trials accuracy decreased for all groups, but neither lesion group was differentially affected by this manipulation and remained at control levels. The MTT group showed a more persistent deficit in the radial arm maze (RAM) whereas the MB rats were only impaired for the first few sessions. With training, both lesion groups improved on the RAM but subsequent maze rotation showed that neither lesion group performed the task in the same way as the control

group, which suggests, that the transient impairment on this task may have hidden a more permanent abnormality in spatial learning. The most robust behavioural deficit for MB and MTT groups was observed during the delayed matching to place working memory procedure in the water maze. Both lesion groups had significantly longer escape latencies than the control group, but there were no significant differences between the MB and MTT groups over the 12 days of standard testing. Increasing the interference in the water maze by extending the inter trial interval between trial 1 and 2 to 30 minutes only revealed a significant effect of group when examined with path length. There was also a significant effect between MTT and control when water maze testing was conducted in a novel room. This study suggests that damage to the MTT/MB impairs the learning of new spatial tasks but does not necessarily increase sensitivity to proactive interference or delay. According to Vann & Aggleton (2003) this pattern of results points to an encoding deficit for spatial memory tasks that includes learning new locations in familiar settings.

Vann, Honey & Aggleton (2003) extended the previous study in the same rats (Vann & Aggleton, 2003) by showing that MTT lesions disrupted the acquisition of a contextual conditioned discrimination when visual cues served as context, but not when thermal cues were used. Importantly, as in their previous study, acquisition was retarded but not precluded altogether. The findings from this study indicate that MTT lesions may have a disproportionate influence on the encoding of visuospatial information rather than resulting in a general deficit in encoding or using contextual information per se. As the same rats as in Vann & Aggleton (2003) were used the rats already had extensive training on spatial tasks in the t-maze, RAM and water maze.

During the course of the present study in this thesis a recent paper by Winter, Wagner, Mc Millin & Wallace (2011) came to light suggesting that the MTT may not be as crucial in spatial learning as the last two studies suggested. Winter et al (2011) examined the effect of

MTT lesions on spatial orientation in the food hoarding paradigm and in the water maze. In the food hoarding paradigm rats carried items of food to a refuge under conditions where access to various environmental cues was controlled. In this procedure the MTT lesion rats were only found to be impaired when having to rely on self-movement cues to return to the refuge, but not when having to rely on the use of environmental cues. Following the food hoarding procedure rats were trained on a standard reference memory and a delayed matching to place task in the water maze. In the reference memory task, MTT lesion rats had significantly longer escape latencies over the 5 days of testing compared to control and unilateral lesion rats. Further examination suggests this effect was weak as the bilateral lesion group showed similar level of performance to controls and the unilateral group on days 1, 2 and 5. It is entirely possible that given an extended training period the group difference would not persist. Unlike Vann & Aggleton (2003), Winter et al (2011) found no effect of group in the previously sensitive delayed non-matching to place task across the four days of testing. There was an indication that rats with bilateral MTT lesions out performed both the control and unilateral lesion group on trial 1 of this task. This may suggest an increased swim speed or more effective swim strategies.

1.74 Suggestion of Circuit Wide Dysfunction Following MTT Damage

As alluded to previously, the concept of diaschisis suggests that damage to one part of the brain can affect a wider neural circuit. Vann & Albasser (2009) recently examined how MTT damage may influence the wider memory system. They measured the impact of MTT lesions on the expression of c-fos, an immediate early gene (IEG), in three key regions: the hippocampus, the prefrontal cortex and the retrosplenial cortex. Vann & Albasser (2009) found that MTT damage produced pervasive c-fos hypoactivity in the hippocampus, retrosplenial cortex and prelimbic cortex sites, which have all been critically linked to the encoding and recall of episodic memory. Furthermore this study suggests that c-Fos

hypoactivity can occur in the hippocampus or retrosplenial cortex without direct deafferentaion, as the MTT is only indirectly connected to these two regions. Vann & Albasser (2009) concluded that as MTT lesions that produced severe persistent deficits on tasks such as delayed matching to place in the water maze, in the Vann & Aggleton (2003) study, a task which is sensitive to hippocampal and retrosplenial cortex lesions, it is possible that MTT lesion effects on c-fos underlie functional disturbances in this network of structures.

Table 1.2 Summary of studies on spatial memory tasks in rats following MTT lesions

Year	Authors	Lesion site/s	Lesion method	Behavioural tasks	Deficits
2011	Winter, Wagner et al	MTT	Electrolytic	1. Food Hoarding Paradigm Water maze 2. reference memory 3. DNMP	MTT impaired on #1, but only when using self-movement cues, Impaired on #2, no impairment on #3
2003	Vann & Aggleton	MTT, MB's	Radio frequency, NMDA	T-maze 1. DNMP Radial Arm Maze 2. DNMP Water maze 3. DNMP	MTT transient deficit on #1, deficit in #2 & #3 MB transient deficit on #1, no deficit on #2, deficit on #3
2003	Vann, Aggleton & Honey	MTT	Radio frequency	Contextual discrimination 1. Spatial cues 2. Thermal cues	MTT impaired acquisition on #1, but not on #2
1985	Thomas & Gash	MTT	Electrolytic	T-maze 1. DNMP	MTT transient deficit on #1
1978	Field, Rosenstock, King and Greene	MTT MTg MB	Electrolytic Electrolytic Electrolytic	1. T-maze, massed trials 2. Ambulation in the open field	MTT impaired on #1, equivalent to control on #2. MTg impaired on #1, hyperactivity on #2 MB impaired on #1, hyperactivity on #2
1968	Krieckhaus & Randall	MTT	Electrolytic	T-maze 1. Alternation between days 2. Alternation between trials	MTT not impaired on #1, but impaired on #2

Abbreviations: MTT = mammillothalamic tract, MTg = mammillotegmental tract, MB = mammillary bodies, DNMP = delayed non-matching to place, and NMDA = N-Methyl-D-Aspartic acid (neurotoxin). No mouse studies in this table.

1.8 Aims of the Present Study

There is considerable clinical evidence consistently implicating the mammilothalamic tract in cases of anterograde amnesia. Despite these clinical findings there are few studies examining the behavioural effects of MTT lesions in animal models. Some of the scant early studies are hard to interpret because of the tasks used and large variations in non-target damage. More recently, Vann & Aggleton (2003) found rats given restricted MTT lesions showed a substantial acquisition deficit in working memory versions of the t-maze, radial arm maze and especially water maze. A subsequent study also found a deficit in spatial contextual discrimination following MTT lesions (Vann, Honey & Aggleton, 2003). A recent study however (Winter et al, 2011) did not support Vann & Aggleton's (2003) findings. As the behavioural deficits following MTT lesions may be similar to those observed after ATN lesions, together with evidence that these structures are strongly connected, it is also reasonable to assume that enriched environments would ameliorate the behavioural deficits associated with MTT lesions in a similar manner to that observed after ATN lesions. If environmental enrichment is hoped to provide a therapeutic approach for anterograde amnesia in humans, then it is important to validate a similar effect in the MTT, the structure most often implicated in human diencephalic amnesia.

The primary aim of the present study was to create localised lesions to the MTT in line with those of Vann & Aggleton (2003) and Winter et al (2011). Furthermore as this study intended to expose half of the lesion and half of control rats to enriched environments it was important to establish a behavioural deficit before examining recovery effects associated with enrichment. For this reason the delayed non-matching to place procedure in the water maze was the primary task investigated as Vann & Aggleton (2003) found this task to have the most robust behavioural deficit for both MTT and MB lesion groups. This task was run in two parts: phase one which followed Vann & Aggleton's (2003) procedure in an attempt to

replicate their results. In phase two, instead of being tested individually with massed trials, rats were tested in squads of three (plus one group of four), all rats in a squad completing one trial before moving on to the next.

Standard reference memory testing in the water maze was used to clarify the effect of MTT damage on this task. Rats with lesions to the ATN showed a persistent and severe impairment on this task (Warburton, Morgan, Baird, Muir & Aggleton, 1999; Warburton & Aggleton, 1999; Lopez, Wolff, Le Courtier, Cosquer, Bontempi, Dalrymple-Alford & Cassel, 2009; Wolff et al, 2008), but rats with lesions to the mammillary bodies show only a transient deficit on this task (Sutherland & Rodriguez, 1989). A weak effect was observed for MTT lesions on this task (Winter et al, 2011), but testing was not conducted long enough to draw absolute conclusions.

It is also possible that the MTT plays a role in memory long term memory consolidation. Thus, following reference memory training the rats were pseudo randomly assigned to a 5 day or 25 day retention interval after which they completed a probe trial, testing memory consolidation of the last experienced platform location. Lopez et al (2009) found that rats with rostral intralaminar thalamic lesions showed no deficit in acquisition of the reference memory task or on a probe trial 5 days post-training, but were significantly impaired on a probe trial 25 days after reference memory training. It is possible that the ATN also contribute to consolidation of memory, but rats with ATN lesions performed so poorly on the reference memory task that detecting any differences in later probe trials was not feasible.

Previous studies giving MTT lesions have found a transient deficit on the delayed-non matching to place procedure in the t-maze, but only in the simpler version of this task (Vann & Aggleton, 2003; Thomas & Gash, 1985). Because both components of a trial, the sample and choice run, were started from the same end then the rat can use egocentric (body turn)

information to correctly alternate from the sample to choice run. The present study substantially increased task demand by using a t-maze embedded in a cross-maze. This meant that the sample and choice runs of each trial could be started from any of two opposite ends. To maximise rewards the rat must use allocentric and directional (environmental) information to alternate when the choice run starts from an opposite start area to that used for the sample run. Rats with ATN lesions show a substantial deficit in this task and control rats take longer to learn this task than they do the simple version (Loukavenko et al, 2007).

Diencephalic amnesia in the clinical setting is characterised by a severely impaired ability to acquire new episodic information (Aggleton, 2008). It is therefore important to test rats with tasks with some analogy to episodic memory. Episodic memory essentially comprises three subcomponents, "what", "when" and "where" (Tulving & Markowitsch, 1998). A recent study using an object recognition task compared mice with hippocampal and prefrontal cortex lesions to controls using a combination of two different sets of four identical objects (Devito & Eichenbaum, 2010). Including this procedure also allows a test of Aggleton & Browns (1999) extended hippocampal memory system as MTT lesions would be expected to show a similar albeit reduced pattern of results. Mice with hippocampal lesions were found to be significantly impaired on all three components compared to controls, whereas mice with PFC lesions were only impaired on the "where" component. Object recognition tasks rely on the rodent's innate preference for novelty, and as such these tasks do not require any extensive pre-training and are relatively quick to administer.

Exposure to four objects concurrently may diminish rats' performance on components of the episodic memory task, so further testing broke this task down in to its constituent parts, only requiring the rats to discriminate between two objects. The object location test replicated the "where" component of episodic memory. For this task the rats had to discriminate a moved object from a stationary one. Previous studies have found lesion to the fornix,

cingulate gyrus and (Ennaceur, Neave & Aggleton, 1997) hippocampus (Mumby, Gaskin, Glenn, Schramek & Lehmann, 2002) significantly impaired performance on this task. The temporal order task replicated the "when" aspect of the episodic memory task exposing rats to two different previously experienced objects; they then had to discriminate the "older" object from the more "recent" one. In contrast to the hippocampal findings of De Vito and Eichenbaum (2010) a previous study reported that rats with ATN lesion were not impaired at discriminating between an earlier and more recently presented object (Mitchell & Dalrymple-Alford, 2005)

As well as including tasks that would be expected to show a deficit in the lesion group it is also helpful to include tasks that should not show a difference between groups to ensure the lesion rats are relatively specific. For this reason the rats were also tested on a novel object task. This task required the rat to distinguish between a "novel" and previously experienced or "familiar" object. Hippocampal lesions generally fail to disrupt judgments of familiarity in animals (review by Mumby, 2001). Additionally rats with ATN and MB lesions discriminate at a level comparable to controls (Aggelton et al 1995; Mitchell & Dalrymple-Alford, 2005).

1.9 Expected Findings

It was expected that rats with MTT lesions and sham surgery rats would show a similar level of preference for the novel object in the object recognition test given that previous research shows no effect of hippocampal or ATN lesions. In the "what", "where" and "when" episodic memory test it was expected that the rats with MTT lesions would perform significantly worse than controls on all aspects of this task, in line with the deficit found following hippocampal lesions on this task. Similarly, as the object location and temporal order tasks essentially replicate the "where" and "when" aspects of the episodic memory test it was expected the MTT lesion would show a significantly lower level of discrimination than

controls in the object location task given that both hippocampal and ATN lesions significantly impaired discrimination on this task. However, ATN lesions did not disrupt temporal order memory when rats had to discriminate between a "recent" and "less recent" object, so it was expected that the rats with MTT lesions might discriminate at a rate comparable to controls.

Given the robust and consistent spatial working memory deficit observed by Vann & Aggleton (2003) it was expected that the MTT lesion group would take significantly longer to locate the submerged platform in the Morris water maze, the primary measure for this thesis. This deficit should be apparent in both phase one and phase two. Groups may perform better in phase two because they had time to consolidate learning in between trials. It was expected that the MTT lesion group would perform significantly worse than controls in the standard reference memory task in the Morris water maze, in line with previous research (Winter et al, 2011), but this deficit was not expected to be as pronounced as that observed in rats with ATN lesions in other studies. Furthermore there is as yet no information whether the MTT plays a role in consolidation and remote memory retrieval, so the comparison of the probe trial at 5 days or 25 days post training is highly novel.

As discussed above, various studies have indicated a transient deficit in t-maze alternation with lesions to the MB or the MTT. These studies, however, used a relatively simple version of the t-maze requiring the animal to only start from one place for each trial. The present study increased task difficulty by starting the rats from either of two opposing ends of a t-maze embedded within a cross maze, and used trials in which the test run began from the opposite start area to that used for the sample run. It was predicted MTT lesions would produce a particularly severe deficit in these "opposite start" trials because they emphasis the uses of allocentric and directional visual cues for successful performance.

Following testing it was intended that matched pairs of rats would be randomly allocated to either standard or enriched housing for 30 days, before being retested on the complete battery of tests.

2. Method

2.1 Subjects and Housing Conditions

Fifty two female PVGc hooded rats were used (3 were subsequently lost due to complications associated with surgery) all were approximately 12 months old and weighed between 185g and 245g at the time of surgery. The rats were randomised to either lesion or sham surgery: 28 rats received MTT lesions and 24 received sham surgery. Prior to surgery all rats were housed in standard housing conditions of three or four rats per opaque plastic cage (50 cm long by 30 cm wide by 23 cm high), with reversed lighting conditions (lights off from 8am to 8pm) during which behavioural testing was conducted. Following surgery all rats were housed individually for a recovery period of approximately 4 weeks. Food and water were available ad libitum during surgery, recovery and the initial behavioural tasks. The final task required the rats to be deprived to 85% of their free feeding body weight with water was still available ad libitum. Unfortunately the deficit expected for the MTT lesion group in the delayed matching to place task in the water maze was not found so the use of enrichment housing was abandoned.

2.2 Surgical Procedure

Aseptic conditions were used. An intra-peritoneal (IP) injection of ketamine and domitor (for doses see table 2.1) were administered (half the dose of ketamine first, followed by the remaining half of ketamine with domitor added), followed by Hartman's saline (sodium lactate) IP. Methopt Forte eye drops were given plus a moist gauze placed above and clear of the eyes was used. The rats were given local analgesia (mepivacaine) to the scalp during the course of the surgery and skull was exposed before Bregma, Lambda and the mid sagittal suture were identified to locate the MTT coordinates (see table 2). Lesions were made using a Radionics TCZ radio frequency electrode, with a 0.3 mm tip length and a 0.25 mm diameter

(Radionics, Burlington, VT). The electrode was lowered vertically to the mammillothalamic tract coordinate and the tip was raised to 58°c and maintained at that temperature for 60 seconds using a RFG4-A Lesion Maker (Radionics). Sham animals received the same procedure except the electrode was lowered 1mm above the site and the temperature was not raised. The rat's body was kept warm during surgery. Additional ketamine (only) was given if necessary. The rat's condition was monitored carefully throughout surgery and immediately after surgery. Emla analgesic cream was applied to the scalp area following suturing and the rat was given additional Hartman's saline, followed by antisedan (table 2.1) to promote recovery. Post-operatively, especially during the first week, both the researcher and laboratory technicians monitored recovery to check that the rats were drinking, eating and remained bright, alert and responsive. All procedures complied with the University of Canterbury animal ethics guidelines and were subject to AEC approval.

Table 2.1. Doses for the various drugs used during surgery

Drug	Dose (solution)	Dose (mg/kg)	Dose (surgery)
Carprofen	5mg/ml	5mg/kg	
Ketamine	50mg/ml	75mg/kg	
Domitor	0.35mg/ml	0.245mg/kg	
Hartmans solution			1ml (half at start half at end)
Mepivacaine	2mg/ml		0.2ml
Antisedan	2.5mg/ml	1.75mg/kg	

Table 2.2. MTT Surgery Coordinates

Distance B to L	AP	Laterality	DV (from dura)
< 0.65	-0.26	+/- 0.088	-0.7
0.65-0.67	-0.265		-0.6 (shams)
>0.67	-0.27		

B= bregma, L= lambda, AP= anterior posterior (relative to bregma), DV= dorsoventral

2.3 Object Recognition Tasks

2.31 Apparatus

All testing in the activity box (including habituation, object location and the temporal order tasks) was recorded using a webcam mounted on a beam 1 meter above, in a windowless room (4m by 4.7m). The rectangular boxes (30×30×60cms) were made of custom wood and painted with black gloss enamel. Different flat geometric stimuli made from laminated coloured paper were stuck on each of the 4 internal walls (Figure 2.1) to make each side of the box distinct. The boxes were situated on a table 70 cm above the floor and were placed approximately 50cm apart. For the entire duration of testing the rats were tested in pairs (singly per box) with diffuse lighting being provided by overhead florescent lights. The experimenter was not present in the room during testing.
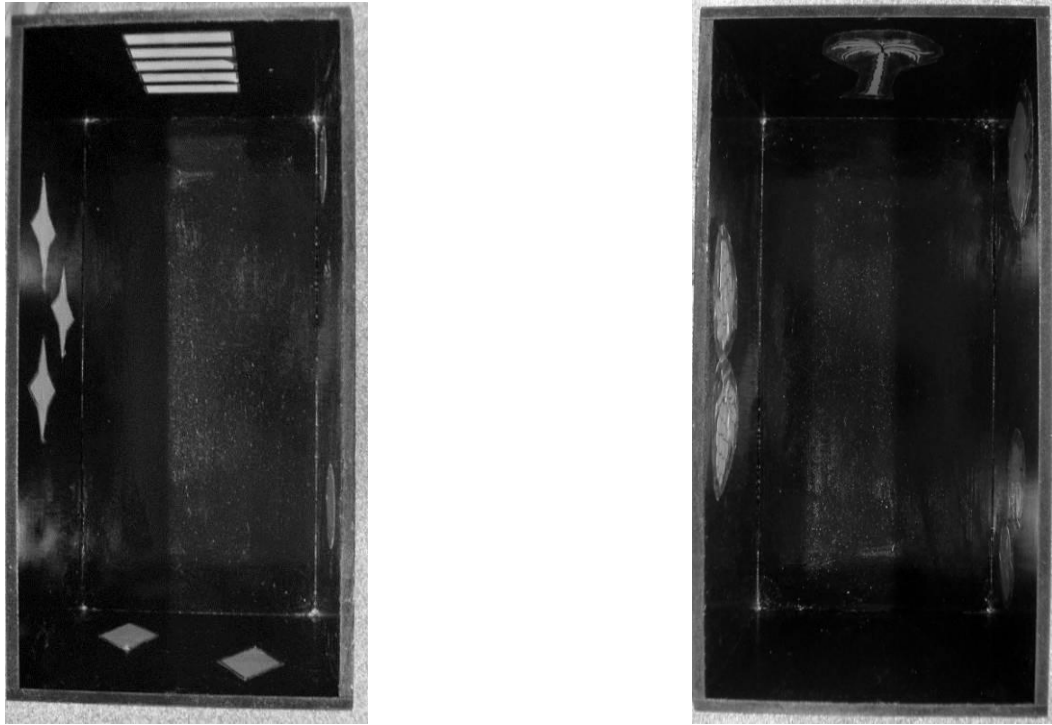
Figure 2.1. Photograph of the rectangular boxes used for all object recognition testing showing the internal cues adhered to the walls. The photograph on the left depicts the configuration used for the novel object and episodic memory task and the photograph on the right depicts the configuration used for the object location and temporal order tasks.

2.32 Novel Object Task

The rats were split into two groups (n = 24 & 25) and tested a week apart. Each group consisted of a number of lesion and control animals. The order in which the rats were tested was randomised for each of the two groups. For the first two days the rats were placed in the centre of the empty box to habituate for a five minute period. On day 3 the rats received a novel object recognition task (figure 2.2). For this they were released in the centre of the open field and presented with two identical objects occupying adjacent corners along one of the larger walls. They were allowed five minutes to explore these objects before being returned to their home cages. Each rat was then given a 50 minute retention interval before being returned to the box where one of the previously encountered objects had been replaced with a novel object and an old object was replaced with a replica and allowed to explore for five minutes. Between trials each of the boxes and objects were thoroughly cleaned with

powerquat blue 2% and dried to remove any residual odour cues. Multiple copies of each

object were used to ensure quick resetting for the next rat.

Sample   Test

50
minute
delay
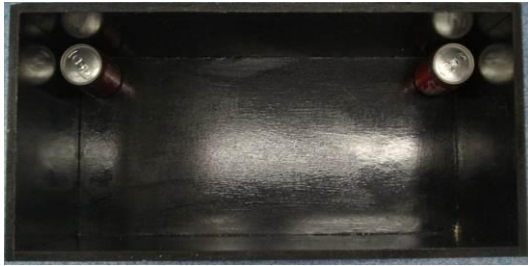
Figure 2.2. Representation the novel object tasks in the sample phase the rat is presented with two identical objects and then removed for a 50 minute delay. In the 'test" a novel objects is present with one of the previous objects.

Simple discrimination indices and discrimination ratios were calculated to assess

object preference. The discrimination index was expressed as the time exploring the "novel"

object minus the time spent exploring the "familiar" object. A positive score indicates

discrimination for the "novel" object. The discrimination ratio was expressed as the time

spent exploring the "novel" object minus the time spent exploring the "familiar" object

divided by the sum of those two times. A score greater than zero indicated a preference for

the "novel" object over the "familiar" one. The rat had to be within 2cm of an object and be

actively engaged in exploration i.e. head oriented towards the object without touching it.

Sitting beside an object was not considered to be exploration. This criterion was followed for

all of the subsequent object exploration tests

2.33 Episodic Memory Task

This procedure was based on DeVito & Eichenbaum's (2010) task and consisted of two

sample phases and a "test" phase (figure 2.4). In sample phase one, four identical objects

were arranged in a triangular configuration in the rectangular box with three equidistant along

the northern (long) wall (two in corners one in the middle) and one in at the centre of the

southern wall. The rats were placed in the centre of the field and allowed to explore for five

minutes. After a 50 minute delay the rats were exposed to sample phase two in which four

new, identical objects were arranged in a square formation in the open field (one in each

corner) and again allowed to explore for five minutes. After another 50 minute delay the rats

were exposed to the test phase in which two objects from each of the sample phases were

presented in a square formation in the open field. The two objects from sample phase one

now occupied the northwest and southeast corners of the open field and the more recent

objects (sample phase two) occupied the northeast and southwest corners relative to their

previous positions. Hence, this test phase configuration consisted of one "old object" (sample

phase one) in a stationary position as well as a sample of one "old object" in a displaced

position, while the two "recent objects" (sample phase two) remained stationary (see figure

2.4). Between trials each of the open fields and objects were thoroughly cleaned with

powerquat blue 2% and dried to remove any residual odour cues. Multiple copies of each

object were used to ensure quick resetting for the next rat.
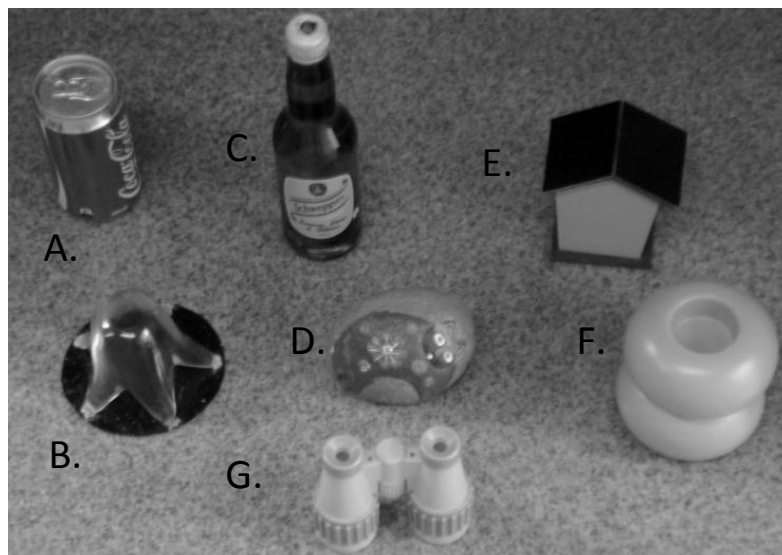
Figure 2.3. The objects used for the object recognition tasks novel object (A & B), episodic
memory task (C&D), object location (E) and temporal order (G&F).

Sample 1   Sample 2   Test

50 minute delay   50 minute delay
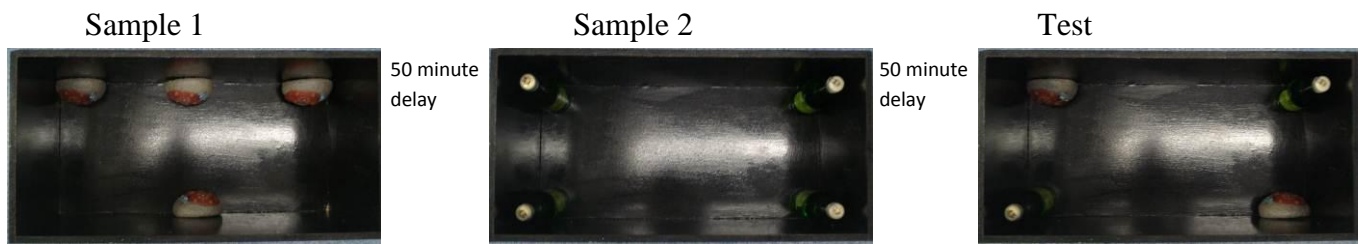
Figure 2.4. Representation of the episodic memory task. In sample one the rats were presented with four identical copies of object D (from figure 2.3) in a triangular formation. They were then removed for a 50 minute delay. In sample two they were exposed to four identical copies of object C (from figure 2.3) in a rectangular formation, before being removed for another 50 minute delay. Finally, in the test phase they were exposed to a combination of objects D and C, some in familiar and some in novel locations.

2.34 Measures of Object Preference in the Episodic Memory Task

Discrimination ratios (DeVito & Eichenbaum 2010) were calculated based on exploration of particular combinations of objects in the test session and were used to provide different memory measures. The discrimination ratio for "what" memory was calculated as the difference between the average exploration times for both sample one objects minus that of the sample two objects, divided by the sum of those times. "What" memory was indicated by a greater than zero "what" discrimination ratio, which reflects greater exploration time for sample one objects compared to sample two objects. The discrimination ratio for "where" memory was calculated as the difference between the exploration time for the displaced sample one object and that for the stationary sample one object, divided by the total time exploring both objects. "where" memory is indicated by a greater than zero "where" discrimination ratio, which reflects greater exploration time for the displaced sample one object compared to the "stationary" sample one object. The discrimination ratio for "when" memory was calculated as the difference between the exploration time for the "stationary" sample one ("old") and that for the average exploration times for sample two ("recent"),

divided by the sum of those times. "When" memory is indicated by a greater than zero "when" discrimination ratio, which reflects greater exploration time for the "stationary" sample one compared to sample two objects.

2.35 Object Location Apparatus

This procedure used the same rectangular box as the novel object and episodic memory task, but different intra maze cues were used (figure 2.1). The rats were tested in the same two groups and same randomised order as before, approximately a week after the novel object and episodic memory tasks. To control for any order effect the rats were counterbalanced within their groups, so half completed the object recognition first and the other half completed the temporal order task first. Prior to testing the rats received only one five minute habituation sessions (per rat) due to their previous experience in the apparatus.

2.36 Object Location Task

This occurred on either the 2nd or 3rd day after habituation (depending on allocation). The rats were placed in the centre of the testing box which contained two identical objects in adjacent corners and allowed to explore for five minutes. The rat was then removed from the testing box and placed back in its home cage for a 50 minute retention interval. In the test phase of this procedure both objects were removed and replaced with replicas, one in the top right hand corner as before and the other was placed in a novel location i.e. the bottom left corner (see figure 2.5). As before the rat was placed in the centre of the box and allowed five minutes to explore.

Sample   Test

50
minute
delay

Figure 2.5. Representation of the object location task. In the sample phase the rat was presented with two identical objects in adjacent corners of the box. Following a 50 minute delay the rats was exposed to the same two objects, but one had been moved to a novel location so the two objects were now diagonal to one another. The red lines were added to highlight the location of the objects.

2.37 Temporal Order Apparatus

This procedure utilised the same rectangular box and habituation procedure as the object

location task.

2.38 Temporal Order Task

The rats experienced this task on the 2nd or 3rd day after habituation depending on their

allocation. This task consisted of two sample phases where rats were presented with two

different sets of two identical objects and one test phase with a replica object from each of the

sample phases. In the first phase, the rat was placed into the testing box which contained two

identical objects occupying adjacent corners and allowed five minutes to explore. The rat was

then removed and placed back in its home cage for 50 minutes. On their return, the test box

now contained a different pair of identical objects in the same position as the previous pair.

Again the rat was allowed five minutes to explore then removed for another 50 minute

period. In the test phase, one replica object from each of the previous two phases was placed

in the box and the rat was again allowed five minutes to explore before being removed and

returned to its home cage.

Sample 1		Sample 2		Test
	50 minute delay		50 minute delay	
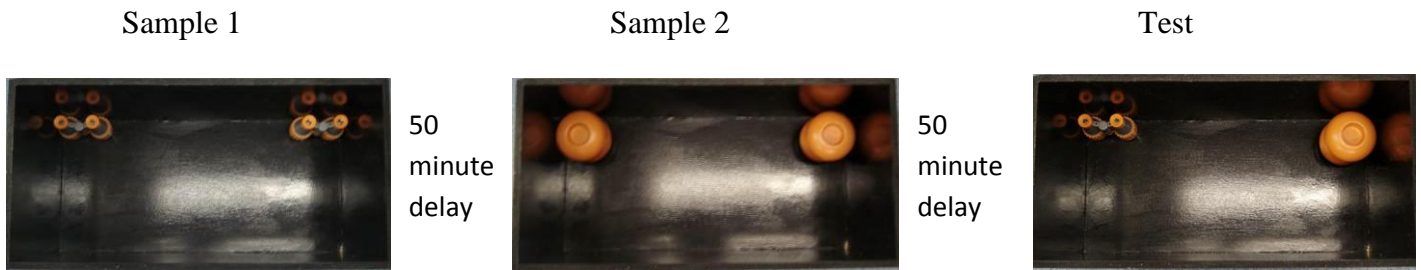
Figure 2.6. Representation of the temporal order task. In sample one rats were presented with a pair of identical objects were then removed for a 50 minute delay. In sample two they were exposed to a different pair of identical objects and subsequently removed for another 50 minute delay. In the test the rats were presented with a replica of one of each of the objects from the two sample phases in the same spatial configuration.

For both the temporal order and object location task discrimination indices and simple discriminations ratios were calculated for each rat. The discrimination index was expressed as the time exploring the target object ("moved" or "old") minus the time exploring the non-target object ("stationary" or "most recent"). A positive value indicates discrimination for the target object. The discrimination ratio was calculated for each rat by subtracting the exploration time of the non-target object ("stationary" or "most recent") from that of the target object ("displaced" or "old") and dividing this by the total of both times. A value greater than zero indicates the rat has a preference for the target object over the non-target object.

2.4 The Morris Water-maze

2.41 Apparatus

The water maze was constructed out of white rigid plastic and had an internal diameter of 180cm with a height of 45cm with an outer lip protruding 5cm. It was located off centre on the on the floor of the same windowless room used in the object recognition tests. The water maze was filled to a height of 30cm with water that was $21 \pm 2°c$ made opaque by the addition of acrylic non-toxic paint (Super Tempera, Fine Art Supplies, New Zealand). The water maze was divided into 4 virtual quadrants (1, 2, 3, 4) using four compass points on the

rim of the pool. Each quadrant was further divided evenly in half by four release points (R1, R2, R3, R4) (see figure 2.8), thus dividing the pool into 8 equal sectors. A 10cm circular white perspex escape platform was placed in the pool at various positions and distances from the pool edge and sat 2cm below the surface of the water. During the testing phase the room's salient visual cues were visible, such as geometric shapes and high contrast visual stimuli placed on the walls, e.g. small road cones, sink unit, a computer, tables and posters. The testing room also contained a beige curtain hanging from the ceiling on a circular track around the pool that could be opened or closed. A camera fixed to the ceiling above the centre of the pool was used to track swim pathways (Ethovision XT 5.0.212, Noldus Information Technology, The Netherlands). Measures recorded were path-length, escape latency and swim speed. Three CPUs were placed around the room and left running; one of which was the data recording computer. Lighting was provided primarily by a large upward facing lamp (300 watts) on a stand approximately 180cms tall and positioned 40cms from R1 (see figures 2.7 & 2.8). Two additional lamps (60w) located in the corner of the room opposite R1 (see figures 2.7 & 2.8) that were used to keep the rats warm during testing provided an additional light source.

2.42 Pre-surgery Training

All 52 rats received three days of training prior to surgery, consisting of four swims per day. The curtain surrounding the pool was drawn closed, and both the start position and platform position were changed for each swim. For the first two days, each swim was terminated when the rat either located the platform or 120 seconds had elapsed. If the rat did not locate the platform within the 120 seconds it was guided there by the experimenter's hand. Rats remained on the platform for 15 seconds. On the third day of pre-training a swim was terminated when the rat had located the platform or 60 seconds had elapsed in which case the

rat was guided to it. Following their four trials the rats were placed back into opaque cages and kept warm with towels and the heat of a 60w lamp.

Figure 2.7. Photograph depicting the spatial layout of the testing room for the Morris water maze. The lamp in the left hand corner of the picture was the primary light source for all procedures. The beige curtain in the background could be drawn closed around the pool to obscure all extra-maze cues

The rats all received a one day "reminder" session after they had recovered from surgery. This "reminder" session followed the same procedure as the first two days of pre-surgery training described above.

2.43 Spatial Working Memory: Delayed Matching to Place: Phase 1

(2 second ITI)

For the spatial working memory task the curtain was drawn from around the pool. The rats were split into two groups (n = 25 & 24) and the testing was staggered so that the first day of testing for the second group commenced on the fifth day of testing for the first group. The testing order of the rats was randomised and the same order was used over all the days of testing. The rats were given four trials a day for 10 sessions (40 trials in total). The task used

10 of a 16 possible platform positions, which varied in their distance from the pool perimeter and 8 possible release points (see figure 2.8). The location of the platform remained constant across the four trials of a given day but varied between days. The same start position was used for the first two trials of each session but was changed for the remaining two trials. Start points could only be in the 4 sectors directly opposite to the platform location e.g. if the platform was located in Q3 (see figure 2.8) then the four possible start points for that day would be Q2, R2, Q1, and R1. This insured the rats were never released in a sector to the left or right of the platform. Keeping the release point the same for trial 1 and trial 2 allowed a direct comparison of the latencies and distances. As before the trials were terminated after the rat located the platform or 120 seconds had elapsed at which point they were guided to the platform by the experimenter's hand. The animals were then left on the platform for 15 seconds to allow for spatial orientation. The next trial began almost immediately afterwards giving an ITI of ~2 seconds.

2.44 Spatial Working Memory Phase 2 (4-6 minute ITI)

After the first 10 days of testing in the water maze all of the rats were given a three day break before returning to the water maze for an additional six days of delayed matching to place testing. Phase 2 of water maze testing utilised the same procedure as phase one. Additional lighting was however added to one relatively dark corner of the room to help improve the rats' recognition of room cues. The inter trial intervals were extended to 4-6 minutes, by testing the rats in squads of three (plus one squad of four). Instead of massed trials, all three rats completed a given trial before moving on to the next one. Spaced trials were implemented to allow the rats a chance to dry off, warm up and for consolidation of the spatial information acquired. Again, the trials were terminated when the rat located the submerged platform or 120 seconds had elapsed, in which case they were guided to the

platform by the experimenter's hand. In this procedure the rats remained on the platform for

30 seconds before being removed from the pool.
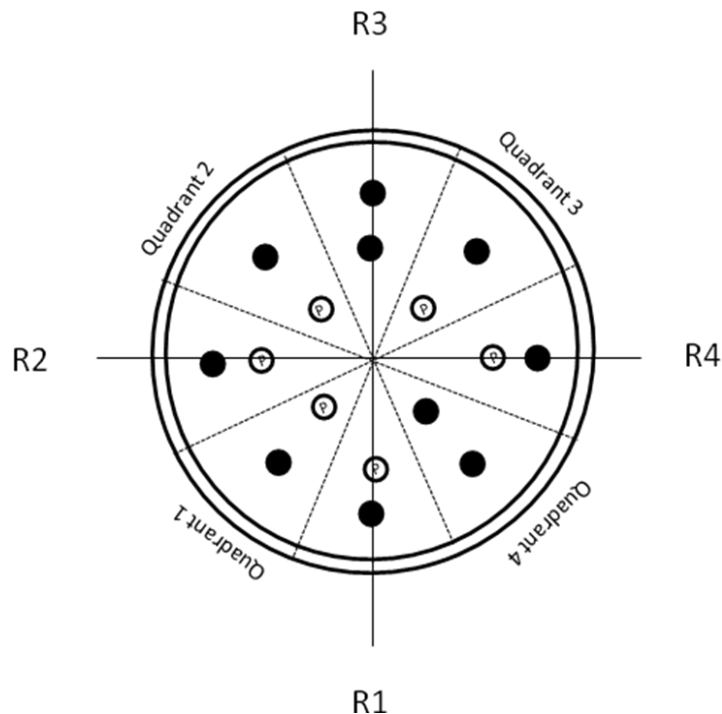
Figure 2.8. Representation of the Morris water maze showing the 10 (from a possible 16) platform positions used (solid black circles) for both phase 1 and phase 2 of testing. R1, R2, R3, R4 as well as Quadrant 1, 2, 3 & 4 represent the 8 release points around the edge of the pool used for all trials. The pool was also divided into 8 arbitrarily placed sectors (broken lines) in each of which two possible platform locations were placed. P = platform positions not used.

2.45 Reference Memory

Following the working memory procedure the rats were given a five day break before being

trained on a standard reference memory task in the water maze. For this task the rats were

split into four squads based on equal performance during the last three days of working

memory testing. Each group was assigned to one of four platform locations (NE, NW, SE,

SW), as all rats swimming to a single location may provide a strong odour cue aiding

navigation. Each group was only trained to go to one platform position which remained

constant over the 8 days of training. Each rat received four trials per day from four different

starting points N, S, E, W. The order of these starting points was varied each day so each rat

did not receive the same order of start points over the 8 days of testing. Release points were

also varied within each group per day to reduce systematic error. On the first day of testing

trials were terminated when either the rat located the hidden platform or 120 seconds had

elapsed, in which case they were guided there by the experimenter as per their previous

training. From days 2-8 trials were reduced to 60seconds which is the standard protocol for

reference memory. Once on the platform they remained there for a further 30 seconds before

being removed. The rats were trained in squads of three giving an ITI of ~2-4 min.
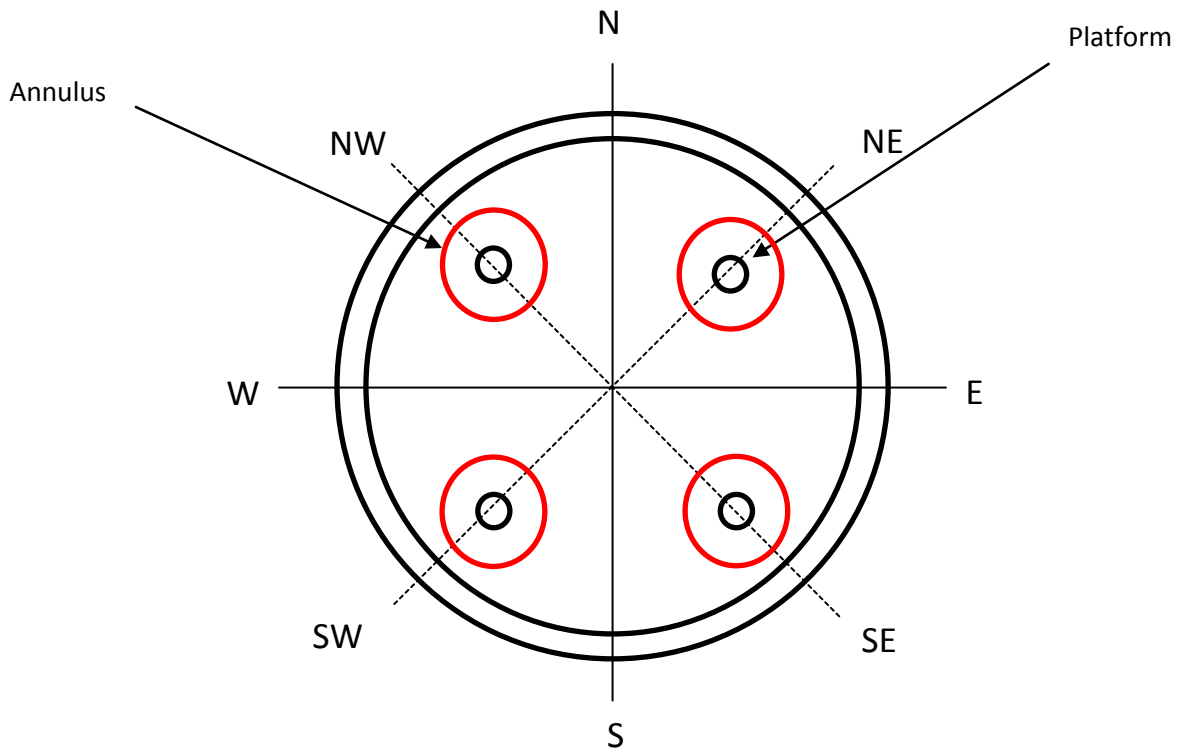
Figure 2.9. Water maze configuration for the reference memory and probe trials. Small
circles indicate the possible platform locations used, larger red circles indicate the annulus
used for the probe trials. N, S, E, W represent the 4 release points used for the duration of the
reference memory procedure. NW, NE, SE, SW the 4 platform locations used. The cardinal
compass points were also used to split the pool into 4 equally sized quadrants each containing
a platform so the time spent by each rat in different areas of the pool could be quantified.

2.46 Remote Memory Probes

The rats were ranked by their performance for the 7 days of reference memory training and randomly assigned to only one of two probe conditions, a short retention interval (5 days after training) and a long retention interval (25 days after training). No testing was conducted during these retention intervals and all rats remained in their standard housing conditions. The probe retention test on days 5 and 25 consisted of a 60 second trial with the platform removed from the water maze. Rats were released directly opposite to the platform location they were trained to either, (NE, NW, SE, SW). The primary retention measure used was the accuracy ratio (Lopez et al 2009) time spent in the target quadrant multiplied by 3 and divided by the time spent in all other quadrants. An additional index of memory precision was the number of crossings in the target area, corresponding to the platform diameter enlarged by 20cms (see figure 2.9).

2.5 Cross Maze Spatial Working Memory

2.51 Apparatus

Spatial working memory was tested in a cross-maze with two stems (one at each end of the arms) so the rats were released from either north or south towards a "T" intersection (the opposite stem was blocked). The cross-maze sat on a stand raised 75cm above the floor. The wooden runways were 10.5 cm wide and painted gray, with 2.5 cm high galvanised steel walls. The two stems were 1 m long with a guillotine door located 28 cm from either end to create a North and a South starting area. The two goal arms were 40 cm long at the end of which was a raised wooden food well (2.5 cm diameter, 1cm deep) with some inaccessible food to control odour cues. Wooden blocks (10.5cm wide by 30cm high by 10cm deep) were used to restrict access to any stem or arm. The maze was located in a diagonal orientation in a windowless room (3 by 3.5m) which contained a number of distal cues including high contrast stimuli on the walls such as posters, small road cones, a curtain, tables and a

television and VCR recorder. Diffuse lighting was provided by overhead fluorescent lights.

The maze was rotated every 3-4 days to reduce the influence of odour cues.

Figure 2.10. Photograph of the elevated cross-maze. The picture shows the two possible starting areas with removable guillotine doors leading down the stem to two reward arms each with a small food tray at the end. Both the stems and arms could be blocked off with use of a large wooden block shown in the picture.

2.52 Food Deprivation and Reward Habituation

Prior to testing rats were deprived to 85% of their free feeding weight. This ensured the rats

were sufficiently motivated to perform the task. To habituate the rats to the food reward 20

small chocolate drops (1g) were placed into the rats home cages each day for the week prior

to pre-training.

2.53 Pre-training

All rats then received a minimum of 6 days (up to 8 days) of pre-training in the T-maze as

follows. On days 1 and 2 the rats were put on the apparatus in cage groups (3-4 rats) and

allowed to roam freely for 10 minutes, with chocolate drops scattered in the middle of each

stem close to the reward arms and scattered down each arm ending with piles in each food well. All doors and arms were open. On day 3 the rats were placed individually in the T-maze for 3 minutes in total, first in one start arm and then after 90 seconds they were placed in the other start arm. A trail of chocolate drops was left in each of the goal arms with a small pile in each of the food wells. On day 4 the rats were placed individually in the maze for up to 10 minutes, each rat received a maximum of 12 chocolate drops. The rats were placed individually in a start area and the door was lifted. Once the chocolate pieces were eaten the rat was placed back in the opposite start area and so on until all 12 chocolate pieces had been eaten or 10 minutes had elapsed. Day 5 followed the same general procedure as day 4 but the session time was reduced to 6 minutes and the rat only started from one start area. Day 6 was the same as day 5 but this time the rats started in the opposite start area. Day 7 and 8 repeated days 5 and 6 for rats that were slow to run.

2.54 Delayed non-Matching to Place Testing

Testing in the cross-maze was run for a maximum of 16 days with six trials per rat per day (96 trials in total). Each trial consisted of two parts, a 'sample' and a 'test' run. Correct performance on the test run required the rat to choose the alternate arm form that previously visited during the sample run of the trial (reinforced spatial alternation). To ensure the rats were not simply using an egocentric strategy from "sample" to "test" runs a pseudorandom half of the trials used the opposite start area across "sample" and "test" runs (e.g. S for the "sample" run and N for the "test" run of a given trial). At the start of each trial two chocolate drops (1g) were placed in each of the food wells in the reward arms. A wooden block was then placed in the neck of one of the two reward arms blocking it off. In each sample run the rat was forced to enter the open arm and confined there for 5-10 sec while it ate the chocolate pellets, it was then returned to the appropriate start area for a delay of 5-10 sec while the arm barriers at the choice point were removed or repositioned as required. When the door was

raised for the test run the rat was thus allowed a free choice between the two maze arms (Hind foot down that arm; no retracing). If the rat entered the previously blocked arm it was rewarded with two (1g) chocolate drops and confined to the arm for 5-10 seconds while it ate the reward and was then returned to the holding cage. If the rat chose the same arm it had entered on the sample run it received no reward and was confined to the arm for 10 seconds before being returned to the holding cage. The correct arm for the sample run was determined on a pseudorandom basis so that each rat experienced both the left and right being correct equally. The rats were tested in groups of three or four with each rat completing a trial before moving on to the next trial giving an inter trial interval of 3-4 minutes.

2.55 Completion Criteria

A rat was deemed to have reached criterion if over 3 consecutive days (from day 11 onwards) it achieved 14 out of 18 trials correct with each day having a score of 4/6 or above. Any rat that did not meet these criteria was tested for the full 16 days.

2.6 Histology

2.61 Perfusion

The rats were euthanized with an overdose of sodium pentobarbital (1ml of 300mg/ml, i.p.) and transcardically perfused with ~150mls of chilled saline solution (4˚c) followed by ~100mls of 4% paraformaldehyde solution. The brains were post-fixed in 4% paraformeldehyde for a minimum of seven days. Coronal sections (50μm) were taken through the mammillothalamic tract from ~-2.80 AP to -4.20 AP using a vibratome (Campden Instruments). The extent of the MTT lesion was assessed using a black gold II myelin stain and a light creysl violet counter stain.

2.62 Black Gold II Myelin Staining

Coronal slices were mounted from distilled water onto gelatine coated slides and then air dried over night at ambient temperature. The following day the slides were rehydrated in distilled water for 3 minutes. 150mg of black gold II powder (Histochem, Arkansas) was added to 50mls of 0.9% saline solution and stirred until dissolved. At this point the black gold II solution was transferred into a water bath set at 65°c and allowed to heat until the solution reached a stable temperature between 60-65°c. The rehydrated slides were placed in to the beaker containing the black gold stain five at a time and allowed to incubate for approximately 6 minutes while monitoring the degree of labelling of the slides under a microscope (for full black gold II staining protocol see Appendix A). The slides were considered to be impregnated as soon as the finest myelin fibres in the first layer of the cerebral cortex had been labelled. At this point the slides were immediately removed from the stain and submerged in distilled water for 2 minutes to rinse off excess stain. The slides were then fixed in a 1% solution of sodium thiosulfate (1g to 100mls of 0.9% saline) and incubated for 3 minutes at room temperature. After being removed from the thiosulfate solution the slides were transferred into three 5 minute washes of cold tap water, to remove any residual thiosulfate and to weaken any background staining. Finally the slides were delipidised, first with 10 dips in 70% ethanol and then they were left in 70% ethanol for 2 minutes before being counterstained with cresyl violet acetate.

2.63 Cresyl Violet Counter-staining

After being removed from the 70% ethanol the slides were rinsed in distilled water for 1 minute before being submerged in 250mls of 0.5% Cresyl violet acetate solution and incubated for 5 minutes at room temperature. The sections were then rinsed in 2×2 minute dips of distilled water to remove excess stain. The slides were then dehydrated and differentiated using a 70% ethanol solution for 2 minutes, then a 95% ethanol solution for 2

minutes. Followed by 95% acid alcohol solution for 40 seconds (400mls of 95% ethanol with 1ml of glacial acetic acid added). The dehydrated sections (2×100% ethanol for 2 minutes) were then cleared in xylene for 5 minutes before being cover slipped with DPX.

2.64 Lesion Verification

Photomicrographs were taken for the MTT of each rat at 4 × magnification with a Nikon camera (DS Fi1) mounted to a Nikon microscope (Eclipse E800). For sham rats photomicrographs of the MTT were taken at approximately -3.3 and -3.6 from bregma. The relative anterior and posterior coordinates for each section were obtained from the rat brain atlas (Paxino & Watson, 1998). For the lesion rats, photomicrographs were taken from the first evidence of lesion related damage until damage was no longer apparent (approximately -3.3 through to -3.8 from bregma). Photomicrographs were also taken for the MB of sham and lesion surgery rats from – 4.30 to -5.20 from bregma. The MTT areas were quantified by loading the photos in to UTHSCSA Image Tool (University of Texas) and calibrated with a 1000 micrometer slide (Nikon). The outer perimeter of each tract (left and right) was traced for all rats.

3. Results

3.1 Histology

On removal of the brains for histology many unexpectedly had tumours protruding from the ventral surface of the brain. These pituitary gland adenomas occasionally occur in female rats over 12 months old (Gilbert, Gillman, Loustalot and Lutz, 1958; Crain, 1957). The tumours were usually a few millimetres in diameter but one was $12\times7\times6$ mm, weighed half a gram and had dramatically distorted the adjacent brain tissue. Surprisingly, 14 out of 49 rats were found to have a pituitary gland tumour. After sections were stained, any rat with visual distortion in the region of the mammillary bodies was removed from further analysis to rule out any deficits that might be related to the tumours. The final sample for analysis consisted of 14 sham surgery rats and 21 lesion surgery rats.

3.11 Lesion Verification and Quantification

Myelin staining showed that complete bilateral destruction of the MTT did not occur in any of the rats with lesions. This was unexpected because trial surgeries produced large amounts of tract damage (see figure 3.3). Nonetheless many rats in the lesion group had bilateral or unilateral MTT damage (figure 3.3). Total left and right MTT damage was compared to the mean tract areas of the control rats. The total area of the left and right MTT tract at approximately -3.3 (see appendix A) and -3.6 from bregma (see figure 3.1) was calculated in micrometers squared for all rats, including sham controls. Some damage was found posterior to -3.6 from bregma but because the tract starts to descend more rapidly toward the MB posterior to this point these cases produced lesions that were too dorsal. Additionally, at this posteriorty the tract becomes poorly defined making quantification of tract size too difficult (figure 3.2). No lesions occurred anterior of -3.3 from bregma.
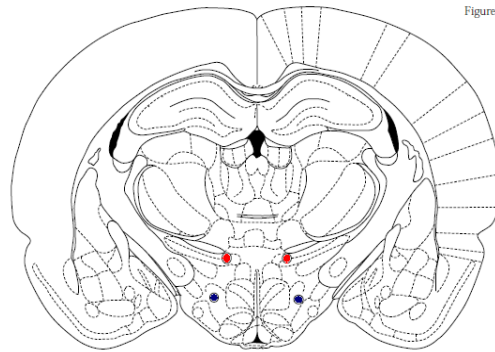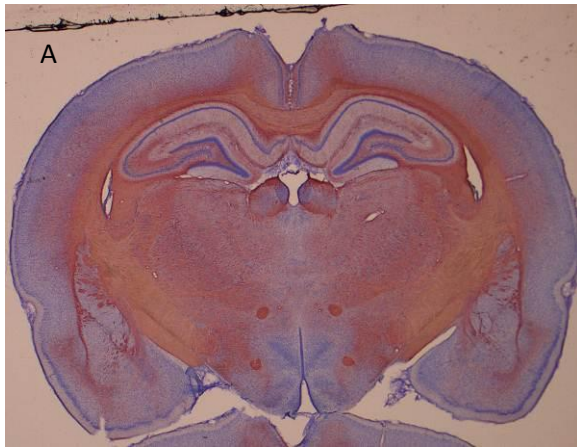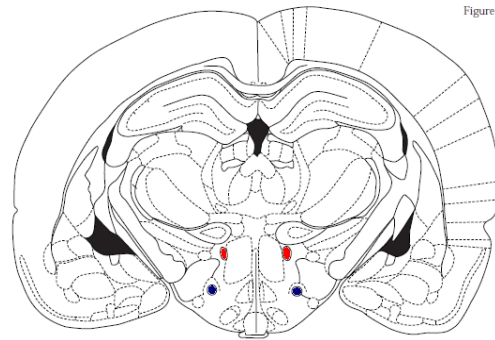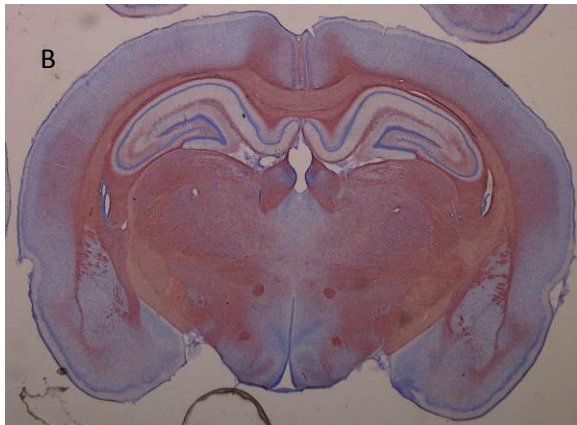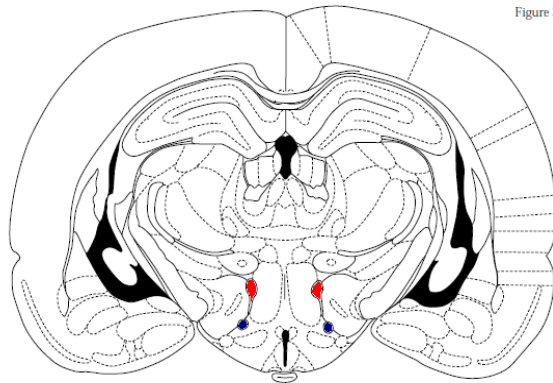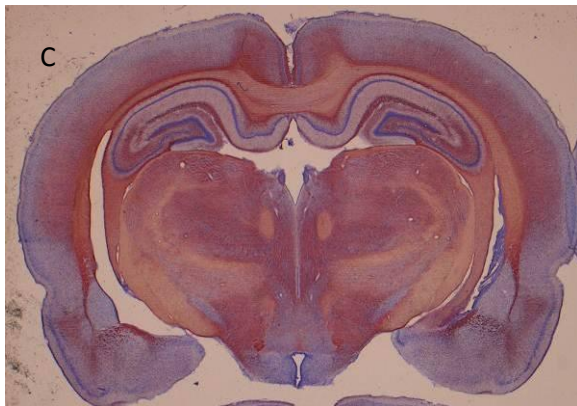
Figure 3.1. Photomicrographs (left) of control rat brains at approximately -3.3 (A) -3.6 (B) and -3.8 (C) from bregma. The sections were stained with Black Gold II myelin stain (myelin stain is red) and light counter stain with cresyl violet (purple blue colour). Photomicrographs are presented with the corresponding plate (33, 34, 35) from the rat brain atlas (Paxinos & Watson, 1998). The MTT is shown in red and the post commissural fornix in blue in the schematics. The MTT region corresponds to the atlas plates, but note that the angle of the cut means that dorsally the hippocampus appears more posterior than shown in the plate.
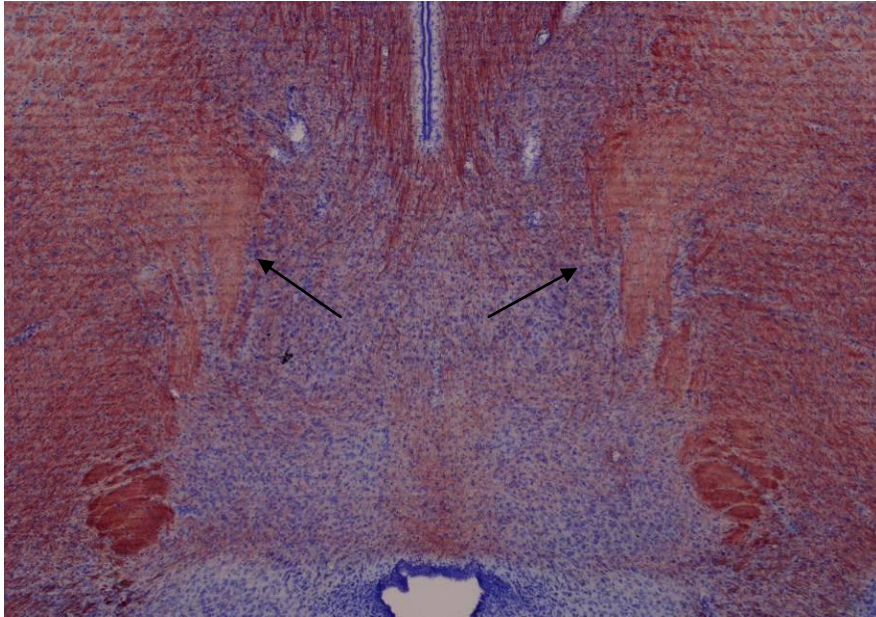
Figure 3.2. Photomicrograph at 4×magnification of a coronal section from a control rat at approximately -3.8 from bregma the MTT (arrows) and post commissural fornix (dark red spots below) are shown. At this AP it becomes difficult to distinguish the MTT from surrounding tissue.

Because of variability in lesion size, the MTT rats were classified on the basis of the amount of tract damage present at -3.6 from Bregma (for group means see table 3.1), which was the primary target for the MTT lesions. Anterograde atrophy of the MTT was also found at -3.3 from bregma and generally mirrored the pattern of damage observed at -3.6 (see Appendix B). Rats (n=6) with a total of 50% or more damage across the left and right MTT, with at least 30% damage on each side were labelled as a 'moderate' damage group (moderate in table 3.1). Rats with at least 35% damage on one side only (n= 4) formed the 'unilateral' MTT damage group (unilateral in table 3.1). Of the remaining twelve rats seven had only minor damage to the tract with 9-23% overall reduction (minimal in table 3.1). These last two groups of rats were removed from further analysis because they did not fit within the lesion or control groups. The MTT in the remaining four rats (listed with the controls in table 3.1) did not differ from controls in that estimates of their MTT areas were within the 2.5- 97.5 percentile range of those found in the sham controls so these rats were

assumed to have no lesion and were included in the control group for analyses. There was also some evidence of MB atrophy in the moderate lesion group following MTT damage as shown in figure 3.4, but this observation was not quantified due to time constraints.

Table 3.1 Percentage decrease in both left and right tract volumes for all the rats in the lesion surgery group at approximately -3.6 from bregma (the closest quantifiable site to the lesions). These values were calculated from the control tract means for the left and right tracts separately.

Group	Rat ID	Left volume	% reduction	right volume	% reduction	overall % reduction	Mean Volume for Group	
Moderate	N6	14310.43	0.85	21468.63	0.77	0.81	left	39602.63
(included in	B3	42540.74	0.54	35534.59	0.62	0.58	right	39347.23
Analyses)	N13	53993.56	0.42	29507.81	0.68	0.55		
	N2	41776.03	0.55	49278.57	0.47	0.51		
	R8	48437.83	0.48	44181.97	0.52	0.50		
	N12	36557.19	0.61	56111.80	0.40	0.50		
Unilateral	R13	79409.49	0.15	35257.33	0.62	0.38	left	73610.48
(excluded from	N1	82241.76	0.12	47380.95	0.49	0.30	right	55348.95
analyses)	P12	53163.26	0.43	78643.28	0.15	0.29		
	B12	79627.39	0.14	60114.25	0.35	0.25		
Minimal	P13	72841.59	0.22	70076.4	0.25	0.23	left	79896.22
(excluded from	B1	74841.59	0.20	69210.32	0.26	0.23	right	73340.33
analyses)	B2	67226.24	0.28	77933.72	0.16	0.22		
	B13	77072.11	0.17	70750.18	0.24	0.21		
	B8	78114.09	0.16	74797.35	0.20	0.18		
	N5	93596.19	-0.01	74794.37	0.20	0.09		
	P6	95581.76	-0.03	75819.95	0.18	0.08		
Control-like	P9	90744.54	0.03	83875.53	0.10	0.06	left	99586.08
(included with	N11	96628.21	-0.04	89550.51	0.04	0.00	right	88353.51
control group)	N3	98904.47	-0.06	93791.47	-0.01	-0.04		
	B7	112067.08	-0.20	86196.51	0.07	-0.07		

Mean of left volume in controls = 93071.69, mean of right volume in controls = 92960.53, Percentile boundaries (left and right combined) of control tracts 2.5% = 76823.17 and 97.5% = 120112.2
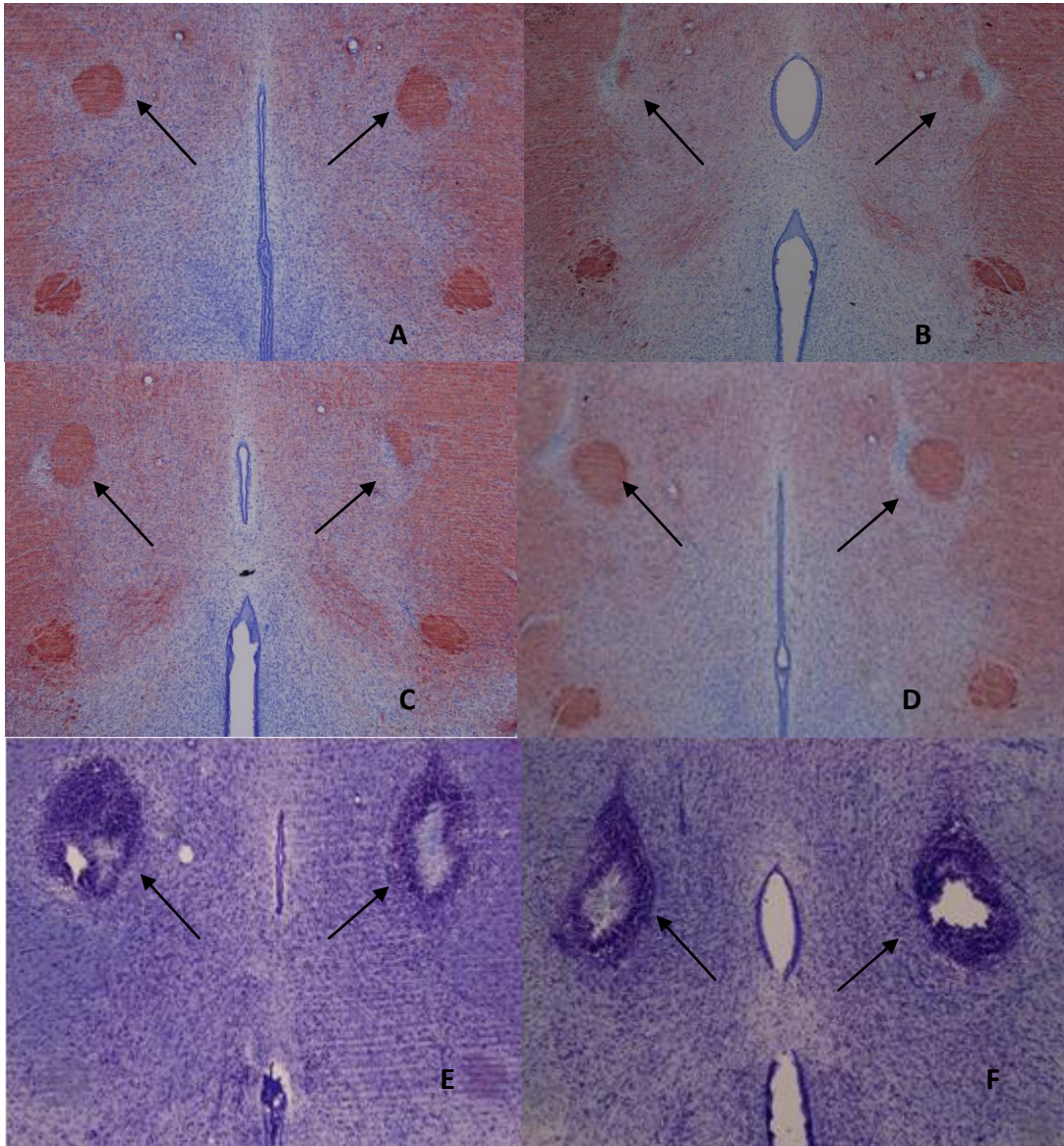
Figure 3.3. Photomicrographs at 4× magnification of coronal sections stained with Black Gold II and/or Cresyl Violet, at approximately -3.6 AP showing the MTT (arrows) in a control rat (A), the rat with the largest extent of MTT damage from the moderate damage group (B), a rat from the unilateral damage group (C), and lesion surgery rat with MTT estimates within the 2.5-97.5% percentile range of those found in controls (D). The two coronal sections at the bottom (E & F) show the extent of the lesions created during trial surgeries (Cresyl only).
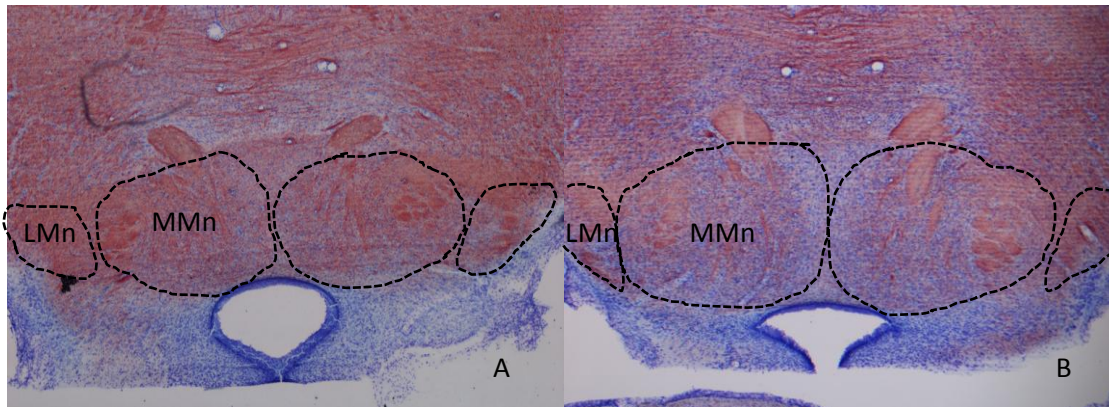
Figure 3.4. Photomicrographs at 4.0 × magnification showing the mammillary bodies the moderate lesion rat with the largest extent of MTT damage (A), and a control rat (B), at approximately -4.52 from bregma. The broken lines give an approximate outline of the boundaries of the lateral mammillary nuclei (LMn), and the medial mammillary nuclei (MMn). There is an apparent increase in ventricle size of the MTT lesion rat. Additionally the medial MB's are visibly smaller in this rat.

3.2 Behavioural Results

Only behavioural data from the control (+ no damage) and the moderate lesion groups (referred to as the MTT group from this point on) were analysed. Although the group with unilateral MTT damage was not included in the primary analyses, the mean and standard deviation for this group are presented and discussed separately at the very end of the results section. Given the small size of the final lesion group individual data were plotted along with group means wherever possible to more accurately compare the performance of individual lesion rats to the distribution of control scores. Additionally, the results of the rat with the greatest extent of MTT damage are highlighted (circled in black) where individual data have been presented. The results of this rat have been considered separately at the end of the results section because any behavioural deficits resulting from MTT damage should be the most pronounced in this rat. An alpha value of 0.05% was used for all significance testing.

3.3 Episodic Memory Task

Group mean total exploration time for all objects across the ten consecutive 30 second intervals of testing was analysed using the between subject factors group and interval as the repeated measure (figure 3.5) . For all three components of this task, "what", "where" and "when" mean discrimination ratios for the control and MTT group were analysed in the same manner as the total exploration data (figure 3.6). Finally cumulative mean discrimination ratios for the "what", "where" and "when" components across the entire five minutes of testing were analysed (figure 3.6) and these were used as the primary performance measure for this task per De Vito & Eichenbaum, (2010). For the latter, t-tests for independent means were performed on each component to assess group effects and a series of one sample t-tests were performed (for each group) on each component to test whether any group was discriminating between the target and non-target objects significantly above chance (i.e. relative to zero).

3.31 Episodic Memory Task: Total Object Exploration

Figure 3.5 shows the mean total object exploration for the control and MTT group across the ten consecutive 30 second intervals of testing. The two peaks in activity observed in the MTT group at 120 and 180 seconds were not representative of the group in general (in both intervals extreme values from the same two rats (N2 & N12), pulled the average up. There was a significant difference between the groups in total exploration, $F(1,22) = 6.06$, $p<0.03$, indicating the MTT group spent a greater amount of time exploring the objects than the control group. There was also a significant effect of interval, $F(9,198) =7.3$, $p<0.001$ reflecting the reduction in exploration over time. The interval by group interaction was not significant, $p = 0.39$.
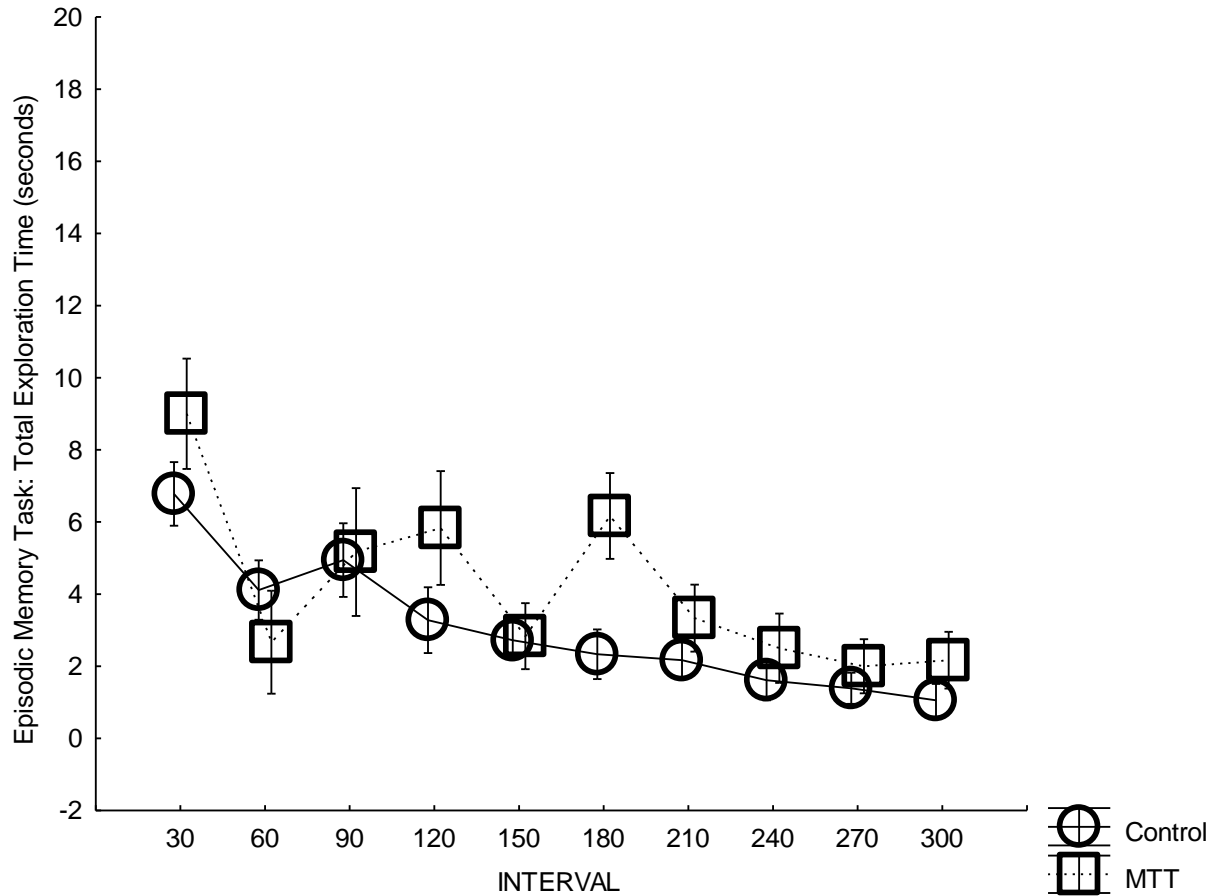
Figure 3.5. Episodic memory task: mean exploration time +/- SE for the control and MTT groups across the ten consecutive 30 second intervals of testing. There was a significantly higher level of exploration shown by the moderate lesion group. There was also a significant reduction in total exploration by both groups over time.

3.32 "What", "Where" and "When" Memory

"What" memory reflected a greater exploration time for all less recently experienced objects by comparison to the more recently experienced objects irrespective of location, whereas "where" memory reflected a greater exploration time for objects in novel places and "when" memory reflected a greater exploration time for objects that were experienced earlier during testing, but in the same location. The top row panels in figure 3.6 show the mean '"what", "where" and "when" discrimination ratios for the control and MTT groups across the ten consecutive 30 second intervals of testing. There were no significant main effects of group on any of the components($F<1$), nor were there any significant interval by group interactions ($F<1$). However the "where" and "when" memory components both had a significant effect of interval $F(9,198) = 2.05$, $p<0.05$ and $F(9,198) = 3.54$, $p<0.001$ respectively, reflecting the alternating pattern of preferences exhibited by both groups across the intervals.

The box plots on the bottom panels of figure 3.6 display the cumulative mean "what", "where" and "when" memory discrimination collapsed across the whole of the five minute testing period. Both groups exhibited weak discrimination on all components of this task. Neither group discriminated significantly above the level of chance on the "where" or "when" memory components ($p > 0.05$) and only the controls discriminated significantly above the level of chance in the "what" memory component, $t(17) = 2.98$, $p<0.01$, (moderate group had a $t<1$). The mean group discrimination ratio's did not differ significantly for any of the memory components ($p>0.1$).
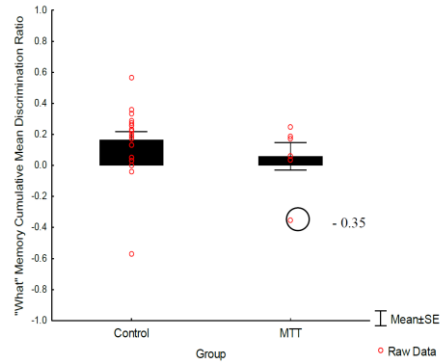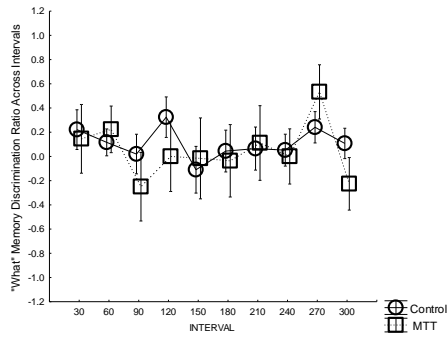
"What" Memory   "Where" Memory   "When" Memory
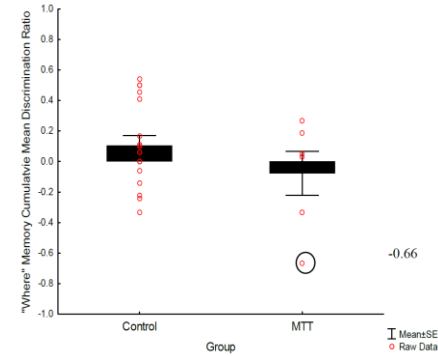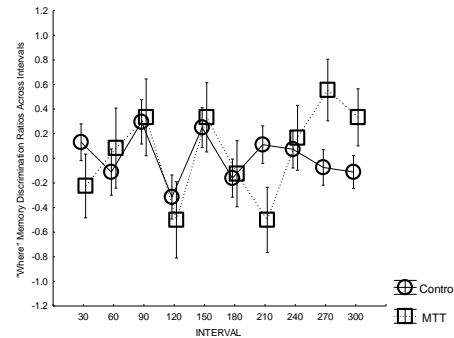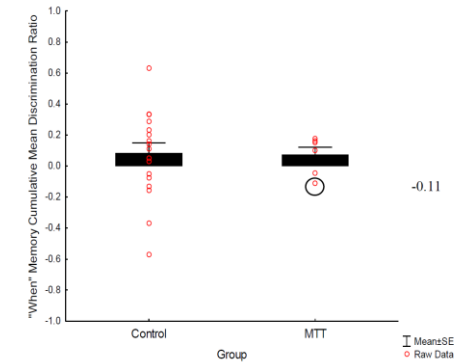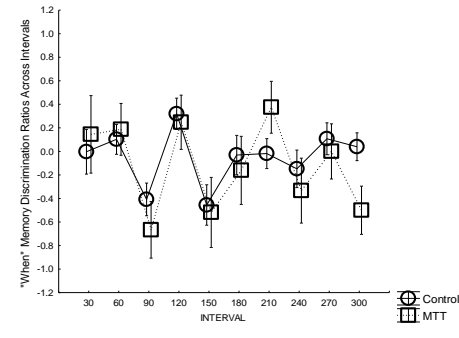
Figure 3.6. The top panels show the mean discrimination ratio +/- SE for the control and MTT groups on the "what", "where" and "when' components of the episodic memory task across the ten consecutive 30 second intervals of testing. There were no significant differences between the groups on any of these components, but there was a significant effect of interval for "where" and "when" memory reflecting the alternation in object preference found across the intervals. The box plots on the bottom display the cumulative mean discrimination ratio's +/- SE for the "what", "when" and "where" memory collapsed across the total five minute testing period. There was no significant effect of group for any of the components, and all groups exhibited weak discrimination for the target objects across the components. The control group only discriminated above the level of chance on the "what" component. For both groups "where" and "when" discrimination were not significantly greater than chance.

3.4 Novel Object Task

 The data for this task were analysed in four ways. First, the mean total exploration time for the objects was plotted for each group in ten consecutive 30 second increments across the 5 minute testing period (figure 3.7) and analysed with a repeated measures ANOVA using the factors interval and group. The same procedure was followed for the discrimination index means (figure 3.8) and the discrimination ratio means (figure 3.9, left). The discrimination index is a widely used measure in object recognition tasks (see Akkerman, Prickaerts, Steinbusch & Blokland, 2012) which gives a gross indication of time spent exploring the target object over and above the non-target object. In contrast, the discrimination ratio indicates the proportion of time spent exploring the target over the non-target object. Finally, cumulative mean discrimination ratios (figure 3.9, right) were analysed (for each group) following two minutes and five minutes of testing, and these were the primary performance measures for this task. An analysis following two minutes of testing was included because Dix & Aggleton (1999) suggested that discrimination for objects significantly declines after this time point over the five minutes of the test period. For the cumulative mean discrimination ratios group effects were analysed with t-tests for independent means and a series of one sample t-tests compared the total mean discrimination ratio (for each group) at two and five minutes to chance discrimination (i.e. zero).

3.41 Novel Object Task: Exploration and Discrimination

There were no main effects of group for total object exploration times (figure 3.7) or discrimination index values (figure 3.8) ($p>0.2$), but both groups exhibited a significant reduction in exploration time and discrimination indices across the intervals of testing $F(9,189) = 10, p<0.001, \ F(9,189) = 4.09, p<0.001,$ respectively. The Interval by Group interaction was not significant for either measure, ($F<1$). In terms of the discrimination ratios

across intervals, there were no significant effects of group, interval, or interval by group interaction (figure 3.9a) ($p > 0.1$).

For the cumulative mean discrimination ratios (figure 3.9b) both groups exhibited strong preference for the novel object over the two minutes control, $t(16)=9.36$, $p<0.001$; MTT, $t(5)=5.3$, $p<0.01$) and five minutes of testing control, $t(17) = 13.78$, $p<0.00$; MTT $t(5) = 8.44$, $p<0.001$. No significant effect of group was found across either two minutes or five minutes ($t<1$).
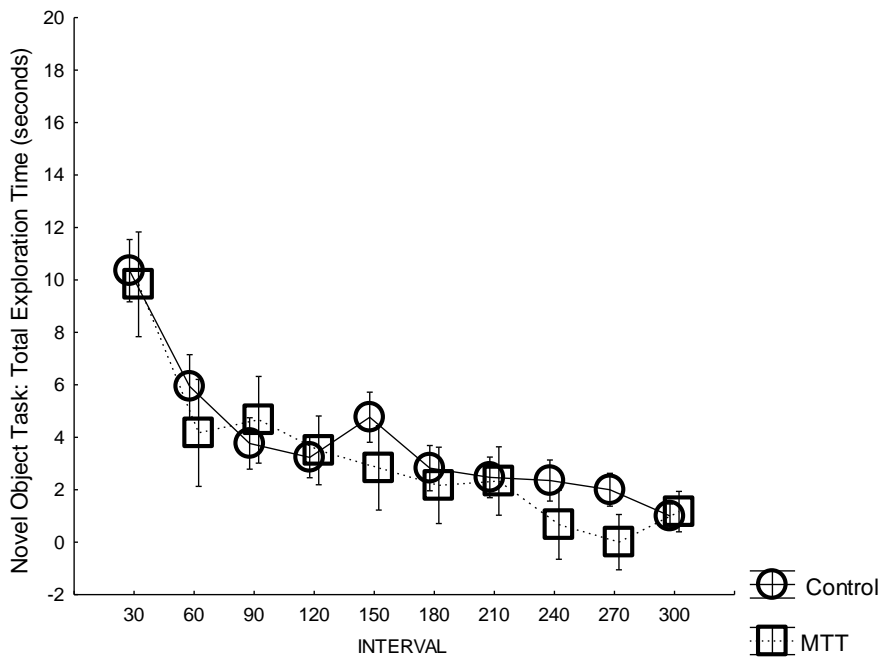
Figure 3.7. Novel object task: total object exploration mean +/- SE for the control (minus one control rat, recording error) and MTT groups across the ten consecutive 30 second intervals of testing. There was no significant difference between the groups in object exploration over the testing period, but both groups showed a significant reduction in object exploration across intervals.
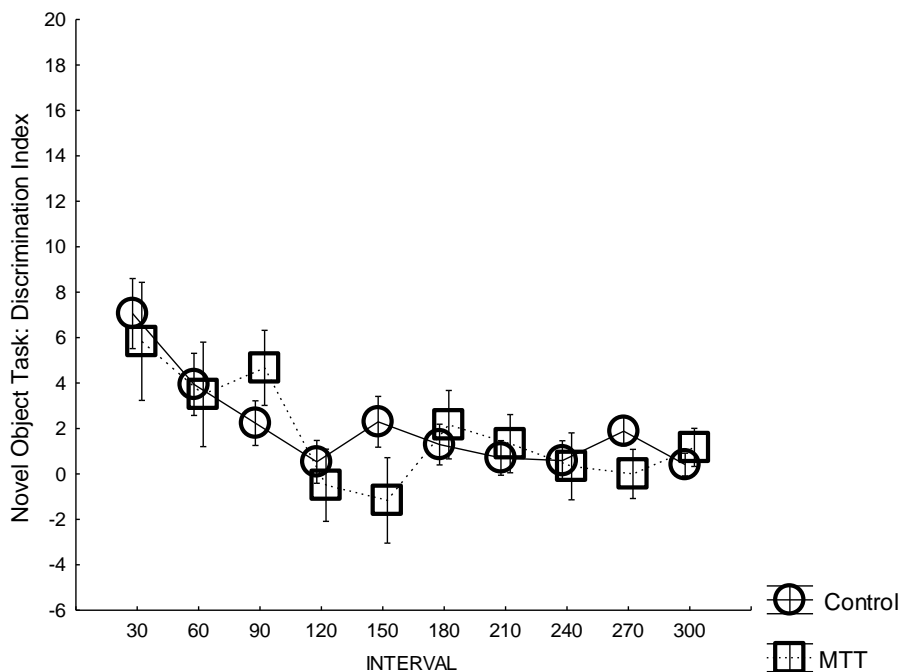
Figure 3.8. Novel object task: mean discrimination index +/- SE for the control and MTT groups across the ten consecutive 30 second intervals. There was no significant difference between the group discrimination indices. Both groups exhibited a significant reduction in preference for the novel object over time.
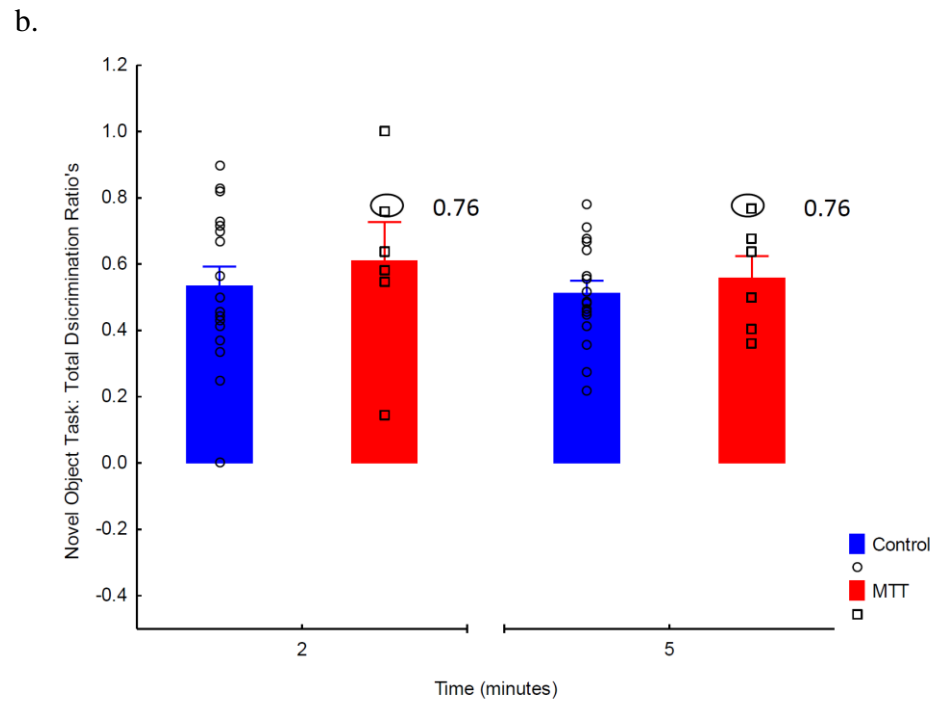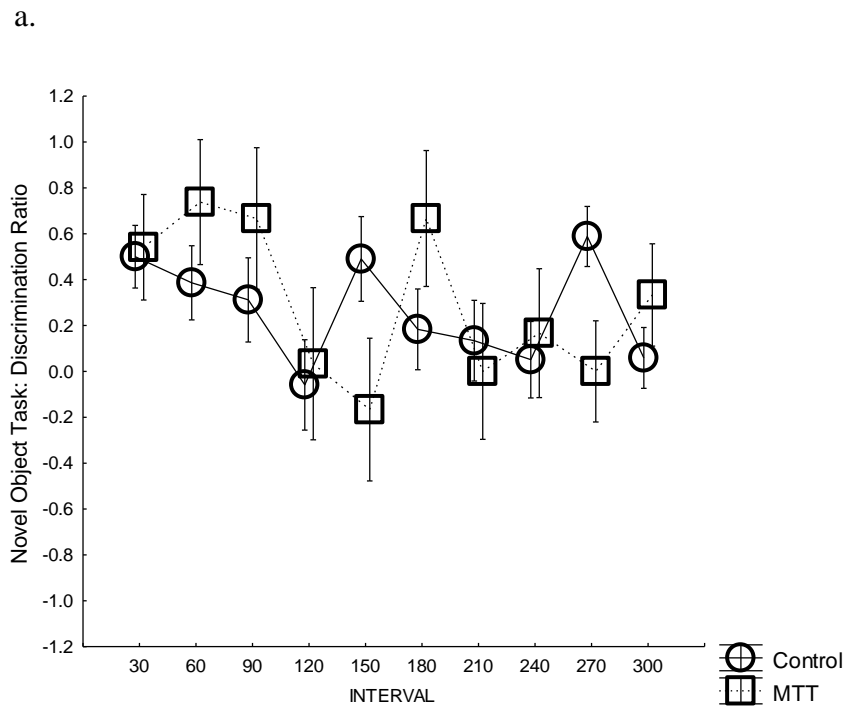
Figure 3.9a. Novel object task: mean discrimination ratio +/- SE for the control and MTT groups over the ten 30 second intervals of testing. There were no significant effects of group, interval, or group by interval interaction. Figure 3.9b. Novel object task: cumulative mean discrimination ratio+ SE (columns) for the control and MTT groups collapsed across two minutes (left) and five minutes (right) of testing. The overlaying dot plot shows the raw scores for individual rats. The black circles show the performance of the rat with the largest extent of MTT damage and its raw score has been presented to the right of the column. Both group show discrimination ratios significantly above the level of chance whether the data was analysed after two or five minutes of testing. There was no effect of group at either time point..

3.5 Object Location Task

The same procedure of data collection and analysis was used for this test as detailed in the novel object test. However, Dix & Aggleton (1999) suggest that, in object recognition tests involving a change of location, rats only show significant discrimination in the first minute of testing, so cumulative discrimination ratios were analysed following one and five minutes of testing.

3.51 Object Location Task: Exploration and Discrimination

No significant effects of group were found for total object exploration (figure 3.10) or discrimination indices (figure 3.11), but there was a significant effect of interval on both measures, $F(9,198) =7.73$, $p<0.001$ and $F(9,198) = 2.76$, $p<0.01$, respectively, which reflects a reduction in exploration and preference over time. The peak in activity observed at 240 seconds by the MTT group relates to one rat (B3) and is not representative of the rest of the group. Interestingly this task had the lowest initial exploration for both groups (first 30 seconds) for all object recognition tasks, which likely reflects the consecutive exposure to identical objects in a novel configuration. There was no significant effect of group for discrimination ratios (figure 3.12a) across intervals, $F<1$, but a significant effect of interval $F(9,198)=2.05$, $p<0.05$, reflecting the variability in preference found in both groups across the testing period. The interval by group interaction was not significant ($F<1$).

The analysis of the cumulative mean discrimination ratios (figure 3.12b) revealed that the control group discriminated significantly above the level of chance following one minute $t(18)=3.5$, $p<0.01$ and five minutes; $t(18)=3.2$, $p<0.05$ of testing, but the MTT group did not at either time ($p >0.1$). However, given there was very little difference between the means of the groups the fact that the moderate lesion group did not discriminate significantly above chance is likely due to a lack of power from a small sample size and variability in

performance with poor discrimination by one rat. The cumulative mean discrimination ratios

did not differ significantly from one another at either point in time ($t<1$).
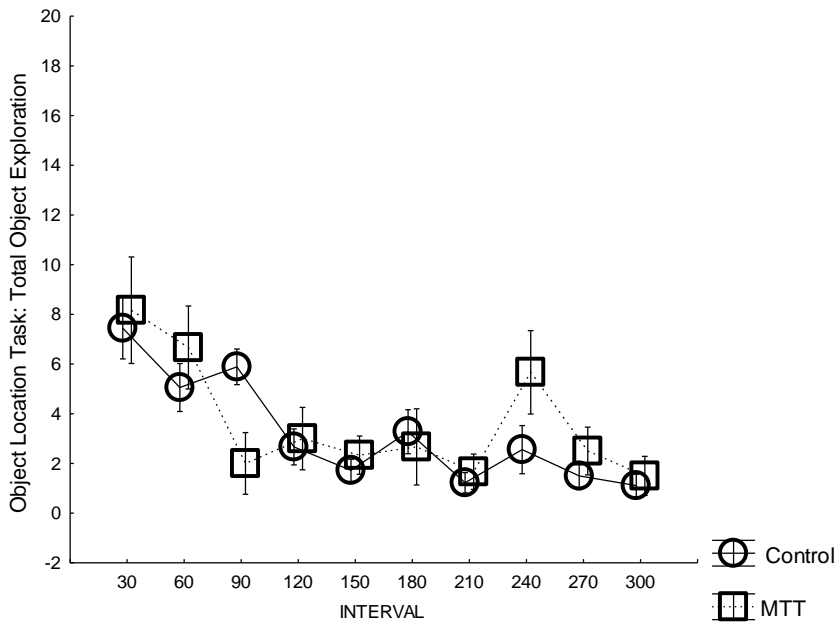
Figure 3.10. Object location task: mean exploration time +/- SE for the control and MTT groups across the ten consecutive 30 second intervals of testing. There was no significant difference between the groups in total exploration time, but both showed a significant reduction in object exploration across the intervals of testing.
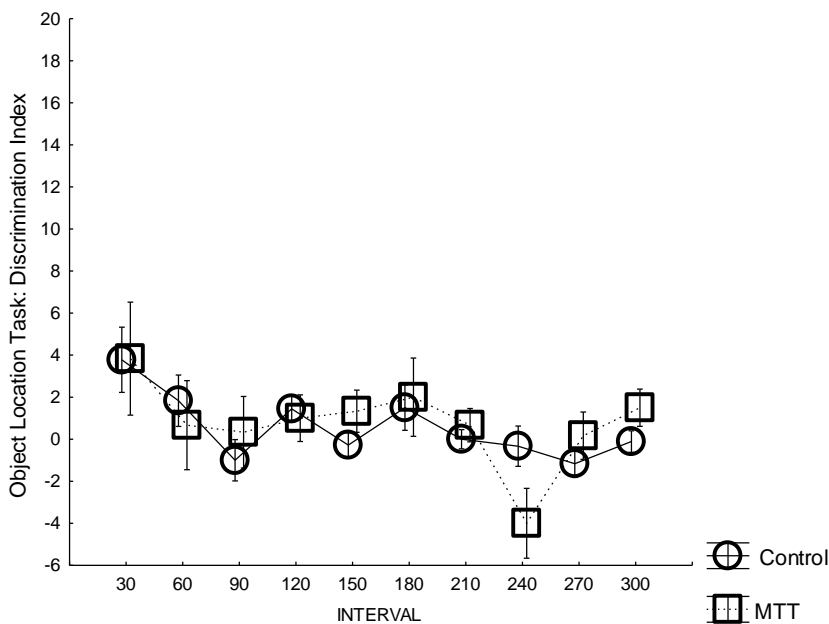
Figure 3.11. Object location task: mean discrimination index +/- SE for the control and MTT groups across each of the ten consecutive 30 second intervals. Both groups generally exhibited low preference for the displaced object across all intervals of testing. The groups did not differ significantly on this measure, but a significant effect of interval suggested a reduction in preference for the displaced object across intervals.

Figure 3.12a. Object location task: mean discrimination ratio +/- SE for the control and MTT groups across the ten 30 second intervals of testing. There was no significant effect of group, but there was a significant effect of interval reflecting the varied discrimination ratios across the intervals. Figure 3.12b. Object location task: cumulative mean discrimination ratio +SE (columns) for the control and MTT groups collapsed across one (1), and five minutes (5), of testing. The overlaying dot plots show raw scores for each rat. The black circles indicate the performance of the rat with the largest extent of MTT damage and the raw score for this rat has been presented to the right of the columns. There was no significant effect of group and only the control group discriminated significantly greater than chance at both time points.

3.6 Temporal Order Task

The same method of data analysis was used for this procedure as for the novel object task.

3.61 Temporal Order Task: Exploration and Discrimination

No significant effect of group was found for total object exploration (figure 3.13) on this task ($p>0.2$) but the groups differed significantly on the discrimination index for this task (figure 3.14, $F(1,19) = 5.08$, $p<0.05$), indicating that the MTT group had a mildly stronger preference for the target object than the control group on this measure. A significant effect of interval was found for both measures reflecting a reduction in total exploration over time, $F(9,191) = 7.32$, $p<0.001$, and a reduction in the magnitude of preference for the target object over time, $F(9,171) = 2.53$ $p<0.01$. Neither measure had a significant interval by group interaction ($F<1$). The peak observed at 180 seconds (figures 3.13, 3.14) by the MTT group was caused by two rats (B3 and R8) that showed substantially more exploration than the remaining four rats in the moderate lesion group. For the discrimination ratio across intervals (figure 3.15a), however, there were no effects of group, interval or group by interval ($p >0.1$). Interestingly, the strong preference for the incorrect object by the MTT group at 180 seconds (figure 3.15a) was a reflection of group performance as only one rat (N6) had a discrimination ratio more positive than -0.7 for this interval.

The analysis of cumulative mean discrimination ratios (figure 3.15b) revealed that the MTT group discriminated significantly above the level of chance following both two minutes, $t(5) = 3.2$, $p<0.05$, and five minutes, $t(5) = 3.2$, $p<0.05$, of testing. In contrast, the control group discriminated above the level of chance for the first two minutes of testing $t(15)= 2.19$, $p<0.05$, but not after five minutes of testing, $p>0.1$. There were no significant effects of group at either point in time ($p >0.1$).
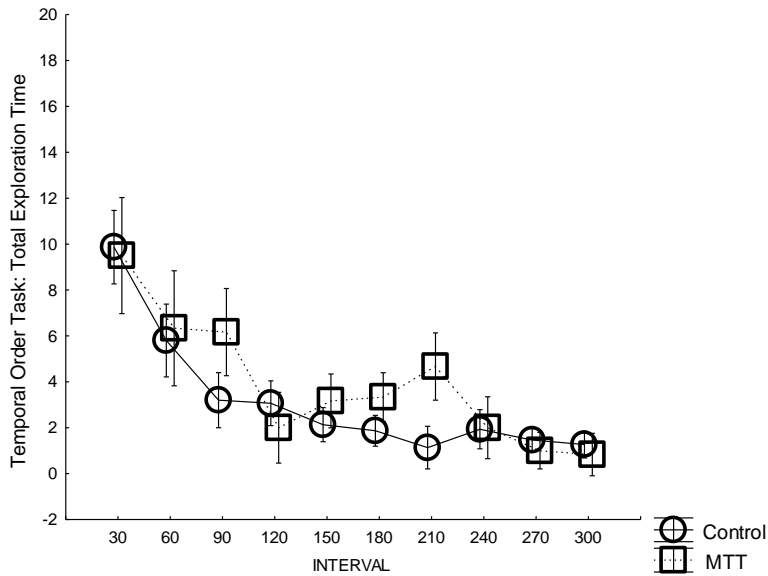
Figure 3.13. Temporal order task: mean total exploration times +/- SE for the control and MTT groups (minus two control rats, escaped testing box) across the ten consecutive 30 second intervals of testing. There was no significant difference between the groups, but a significant effect of interval indicated that group exploration reduced across the intervals of testing.
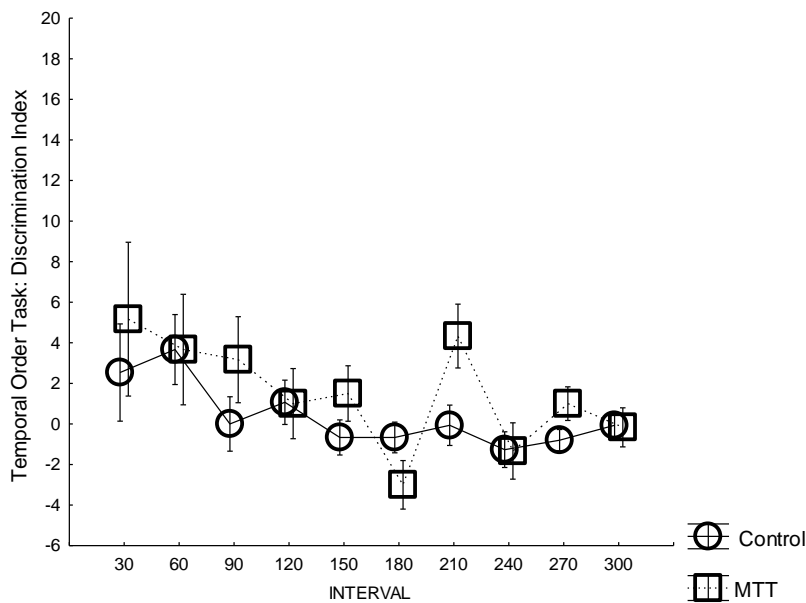
Figure 3.14. Temporal order task: mean discrimination index +/- SE for the control and MTT group across the ten consecutive 30 second intervals of testing. The MTT group showed a mildly stronger preference for the target object than the control group for the duration of testing. Additionally there was a significant effect of interval indicating that preference for the target object reduced across the intervals for both groups.
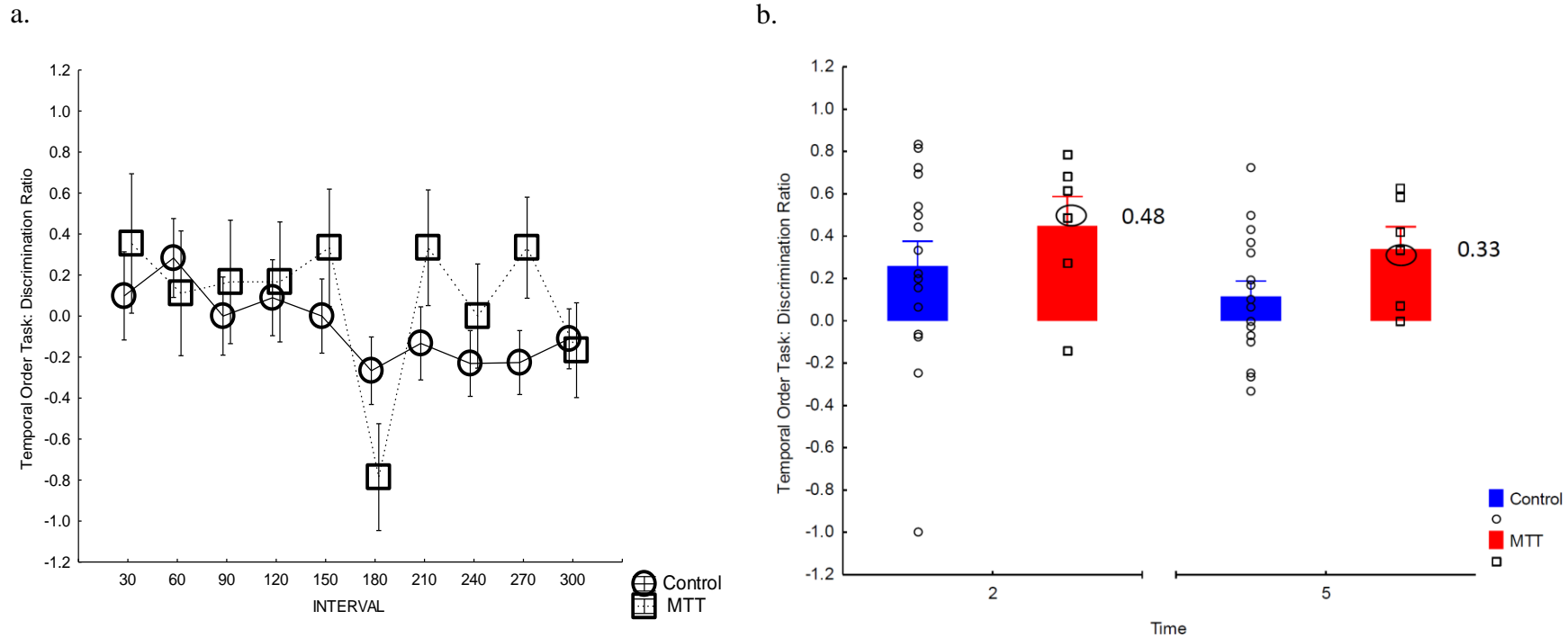
Figure 3.15a. Temporal order task: mean discrimination ratio +/- SE for the control and MTT groups across each of the ten consecutive 30 second intervals of testing. There were no significant effects of group or interval or group by interval interaction. Figure 3.15b. Temporal order task: mean cumulative discrimination ratios + SE (columns) for the control and MTT groups collapsed across two (2), and five minutes (5), of testing. The overlaying dot plots show raw scores for each rat. The score of the rat with the largest extent of MTT damage has been circled in black and its raw score has been presented to the right of the columns. There were no significant differences between the groups, but only the MTT group discriminated significantly above the level of chance following five minutes of testing.

3.7 Water-maze Testing

For all tasks in the water maze, escape latency, path length and swim speed were obtained from the tracking software. No substantial differences were observed in mean swim speed in phase 1, phase 2 (control =27.08cm/s; MTT = 25.93cm/s) or (control = 25.7 cm/s; MTT = 26.9 cm/s) of working memory testing, or during reference memory training (control = 30.07cm/s; MTT = 30.29 cm/s). Both escape latency and path length are shown but only path length analyses are described as the conclusions were generally the same across these measures.

3.71 Working Memory Phase 1 (Short ITI)

The first 9 days of testing in the working memory task in the water maze involved massed trials with a short ITI (~2 seconds) after the rat had rested on the platform. The 9 days of acquisition were blocked in groups of three and a repeated measures ANOVA was performed using the factors group, block and trial. As shown in figure 3.16, there was a significant effect of block $F(2,210) = 7.5$, $p<0.001$ reflecting a significant reduction in path lengths with training. The significant effect of trial $F(3,630) = 35.69$, $p<0.001$ shows improved performance suggesting that both groups learned within each session. There was no significant effect of group, and no significant interactions between any factors ($p >0.05$) with the exception of trial by block $F(6,630) = 2.19$, $p = 0.04$. This interaction was primarily due to improved performance on the first day's trial across blocks reflecting improved search strategies for the new daily location.

Path length
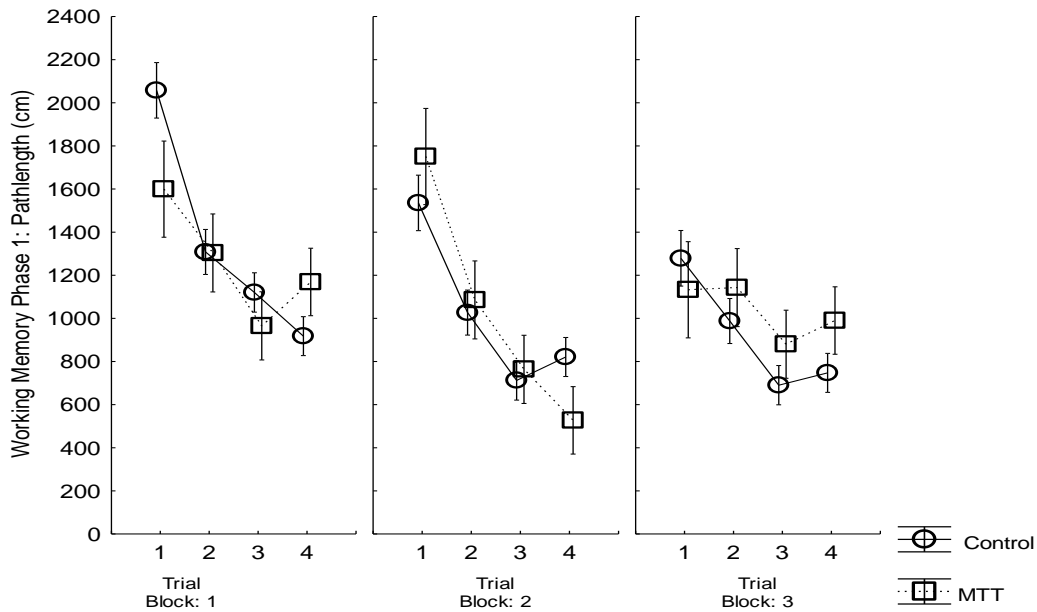
Escape Latency
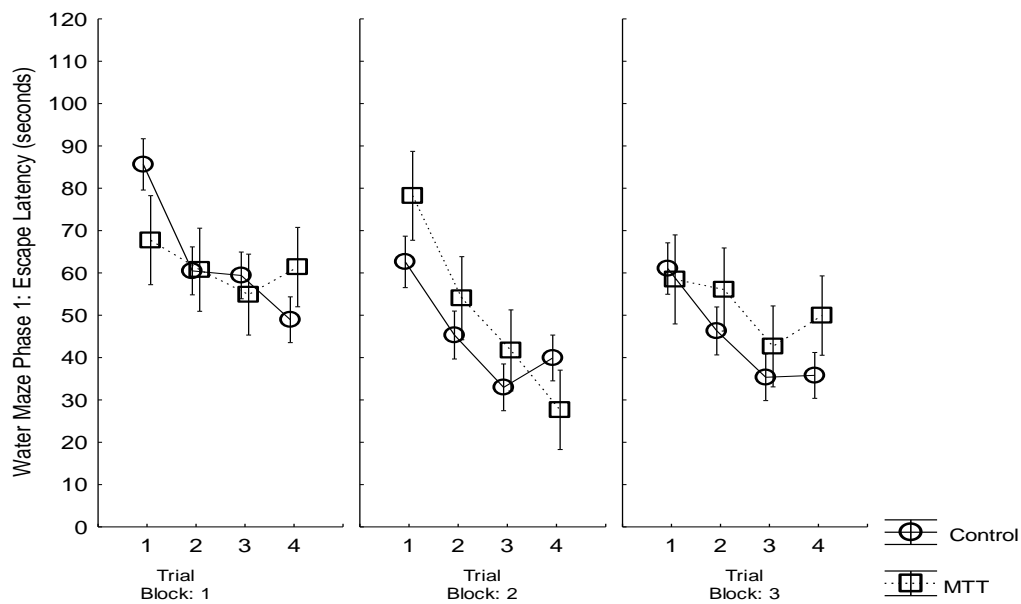
Figure 3.16. Mean path lengths (top) and mean escape latencies (bottom) +/- SE, for the control and MTT groups across trials 1-4, for each of the three blocks of testing on phase 1 of working memory in the water maze. There was no significant difference between the groups, both performing at a similar level over the three blocks of training. However, both groups showed a significant reduction in path lengths across trials 1-4, which indicated they were learning within a session. A significant effect of block indicated that both groups improved significantly with training.

3.72 Working Memory Phase 2 (Long ITI)

Testing for phase 2 of the working memory task in the water maze, which occurred 3 days after phase 1, involved running the rats in squads of 3 to extend the inter-trial interval to 4-6 minutes. The 6 days of testing in were blocked into two groups of 3 days. Figure 3.17 shows the mean path lengths (top) and escape latencies (bottom) for trials 1-4 over the two blocks of testing. A repeated measures ANOVA revealed there was no significant effect of block, $F<1$, or group, $p = 0.24$, but there was a significant effect of trial $F(3,420) = 83.5$, $p<0.001$, which reflected the reduction in path length to the hidden platform after trial 1 especially, showing that both groups showed excellent spatial working memory within a session. No interaction terms were significant (all $F<1$).

To assess whether the rats performed significantly better with longer inter-trial intervals, the final block of training in phase one was compared to the first block of training in phase two. There was a significant effect training phase, $F(1,140) = 4.31$, $p<0.05$, reflecting a reduction in path lengths by both groups when the inter–trial interval was extended. The significant trial by training phase interaction, $F(3,420) = 9.7$, $p<0.001$, reflected superior performance trials 2 (especially), 3 and 4 when the ITI was extended. The reduction in path length from trial 1 to trial 2 is the strongest measure of working memory in this protocol.

Path length
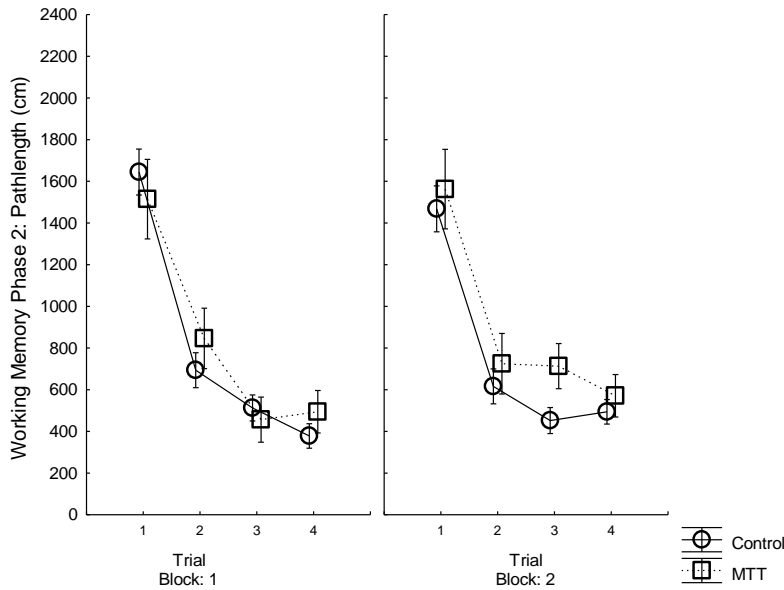
Escape Latency
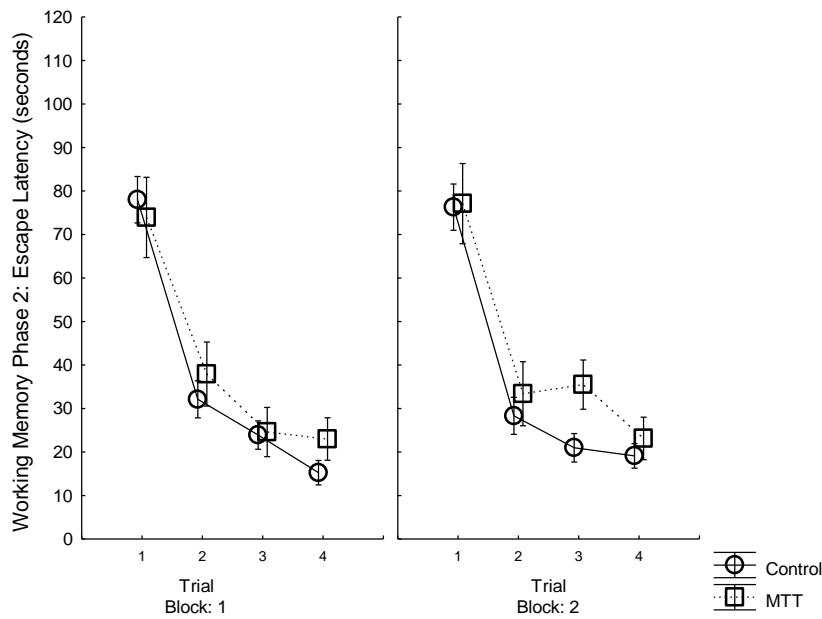
Figure 3.17. Mean path lengths (top) and mean escape latencies (bottom) +/- SE, for the control and MTT groups across trials 1-4, during the two blocks of testing on phase 2 of working memory in the water maze. There was no significant difference between the group path lengths for either block, but there was a significant effect of trial which suggested that both groups learnt within a session.

3.73 Reference Memory

All rats were then given 7 days of training on the standard reference memory task in the water maze. The rats were run in squads of 3 giving a 2-4 minute inter-trial interval. Figure 3.18 shows the path length (top) and escape latencies (bottom) for each group over the 7 days of training in the reference memory task. A repeated measures ANOVA (group by day) revealed a significant effect of group, $F(1,21) = 4.5$, $p<0.05$, indicating a modest effect in which the control group had shorter path lengths to the hidden platform across training. There was also a significant effect of day, $F(6,126) = 3.6$, $p <0.01$, indicating a reduction in path lengths by both groups to the platform with training. The day by group interaction was not significant ($F <1$).

Path length
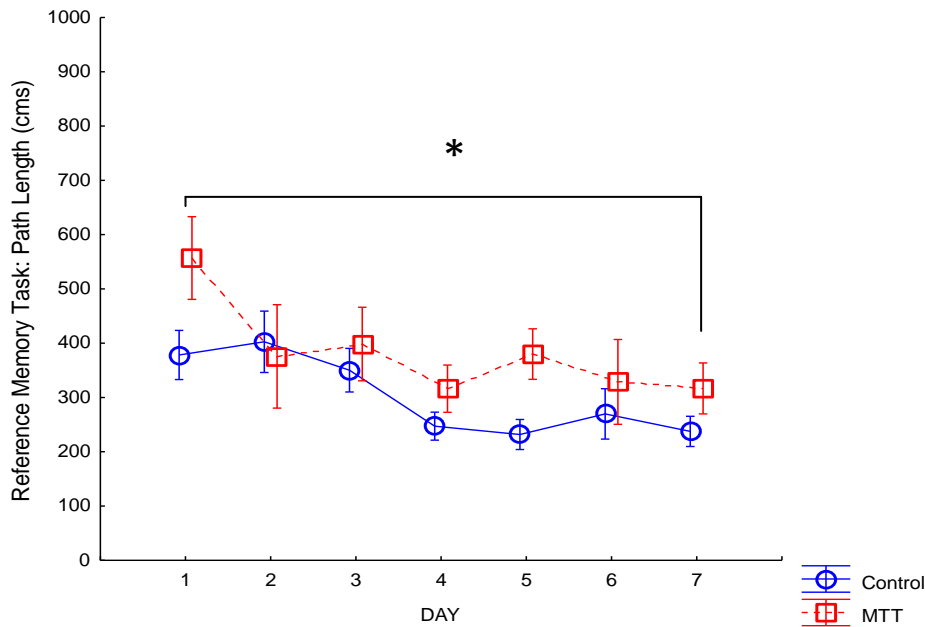
Escape Latency
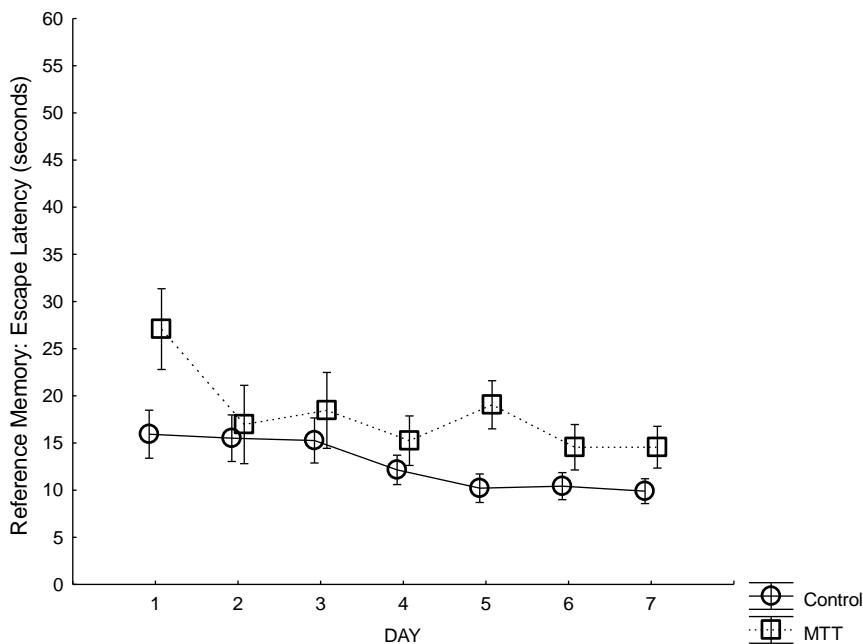
Figure 3.18. Mean path lengths (top) and mean escape latencies (bottom) +/- SE, to reach the hidden platform for the control and MTT groups across the 7 days of training in the reference memory task. The control group had significantly shorter path lengths to the hidden platform across the 7 days of training. Both groups significantly reduced the distance swum to reach the hidden platform with training. * Indicates a significant effect of group at $p<0.05$.

3.74 Five and Twenty Five Day Reference Memory Probes

Pairs of rats from the sham and MTT groups were matched for performance for the last 3 days of reference memory training and randomised to either a 5 or 25 day probe, testing their memory of the location used in the reference memory test. Two measures were used: the accuracy ratio, which gives an indication of the proportion of time spent in the correct quadrant of the pool; and crossings of the annulus region, which is the diameter of the platform enlarged by 20 centimetres to provide an estimate of search accuracy. Mean group performance was plotted for both measures and due to the small sample size the raw data are also shown to compare the performance of individual rats. Examples of swim paths from rats with median performance (on both retention intervals) and the rat with the largest lesion (25 day retention interval) are also presented (figures 3.21 & 3.22). The two delays were analysed with a 2×2 factorial ANOVA using the factors retention interval and group. A regression analysis was also performed to see if mean path length for the final 3 days of testing in the reference memory task was a significant predictor of accuracy ratios or crossings of the annulus (separately), first in the 5 day groups and then in the 25 day groups.

3.75 Accuracy Ratio

As shown in figure 3.19 there was significant effect of retention interval, $F(1,20) = 4.77$, $p<0.05$, providing a manipulation check in which rats were more accurate if they completed the 5 day retention interval than if they completed the 25 day retention interval. The groups did not differ significantly in their accuracy for the last known location of the platform ($F<1$) and there was no significant interaction between retention interval and group ($p = 0.3$). Performance for the last 3 days on the reference memory task did not significantly predict the accuracy ratio for rats completing the 5 day retention interval (b -0.19, $p>0.5$), but it did significantly predict the accuracy ratio of rats completing the 25 day retention interval (b -.71, $p<0.01$). This suggests that the strength of the memory formed for the location of the hidden

platform by the end of training was more important when retrieval was weaker after a long delay.
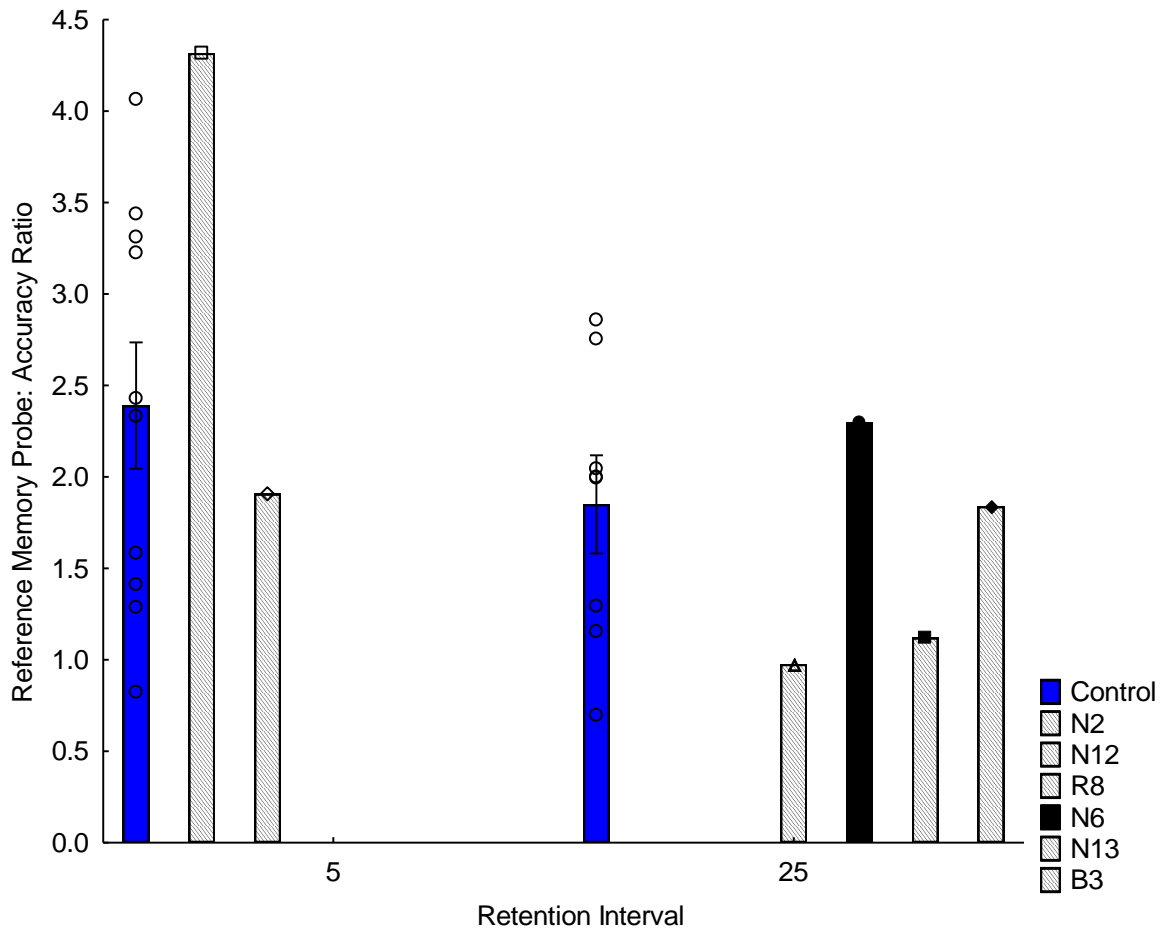
Figure 3.19. Mean accuracy ratios + SE (columns) for the control group and the individual rats in the MTT group following a 5 day (left), or 25 day retention interval (right). Dot plots of the raw data for the control group is also shown. The black column (N6) indicates the rat with the largest extent of MTT damage. The groups that completed the 5 day probe were significantly more accurate than the groups that completed the 25 day probe. There was no significant difference between the control and MTT groups in terms of accuracy for the platform location for either retention interval.

3.76 Crossings of the Annulus

There were no significant differences in annulus crossings between the retention intervals ($F=$ 1.26) and no group ($F<1$) or group by retention interval ($p = 0.29$) in figure 3.20. Mean path lengths for the final three days of reference memory training did not significantly predict the performance of rats completing the 5 day retention interval, ($b =-0.18$, $p>0.5$), but approached significance for rats that completed the 25 day retention interval, ($b = -0.55$, $p = 0.06$).
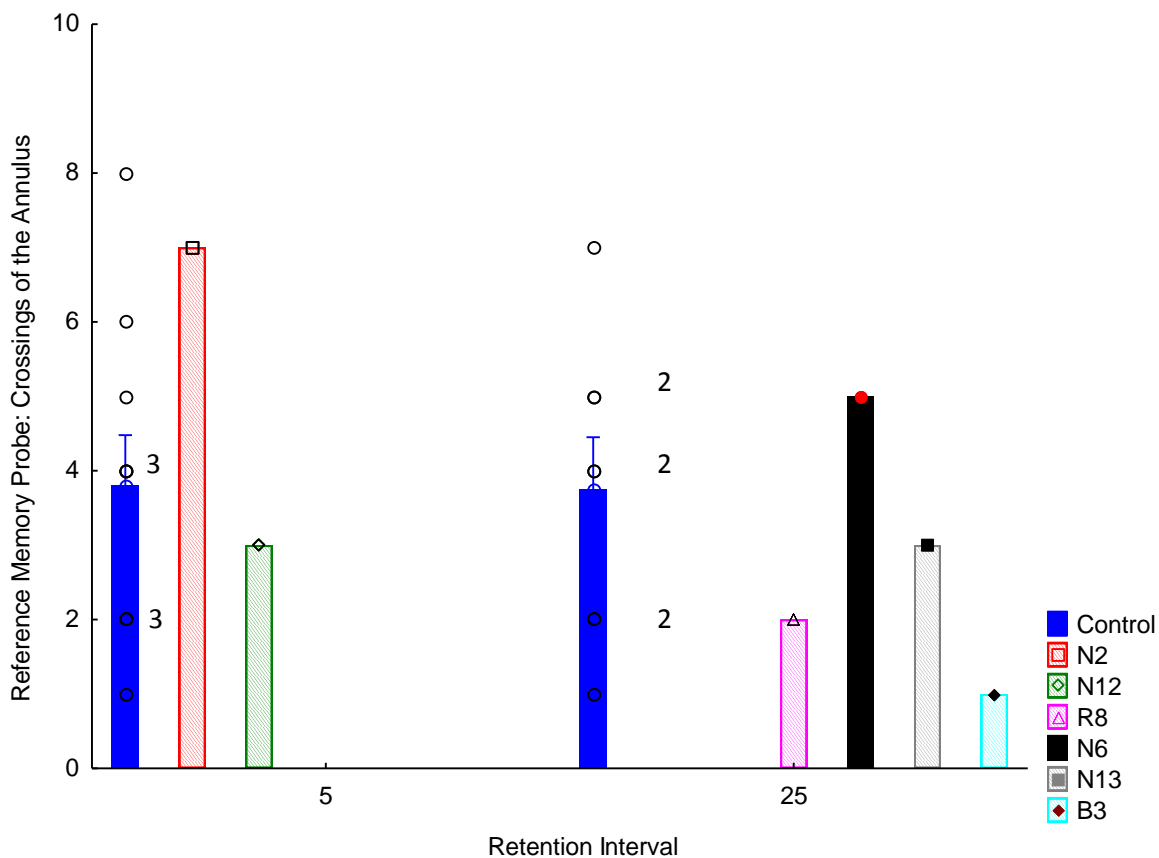
Figure 3.20. Mean crossings of the annulus (platform diameter area enlarged by 20 centimetres) for the control group and the individual MTT rats following a 5 day (left), or a 25 day retention interval (right). Dot plots of the raw data for the control group is also shown. Where a marker represents more than one rat the frequency is presented to the right of the column. The black column (N6) indicates the performance of the rat with the largest extent of MTT damage. There were no significant effects of retention interval or group in annulus crossings.

Figure 3.21 Examples of the swim path of the median control (A), and median MTT rat (B), on the 5 day reference memory probe. The control rat's swim path is more concentrated around the platform location than the rat in the MTT group.
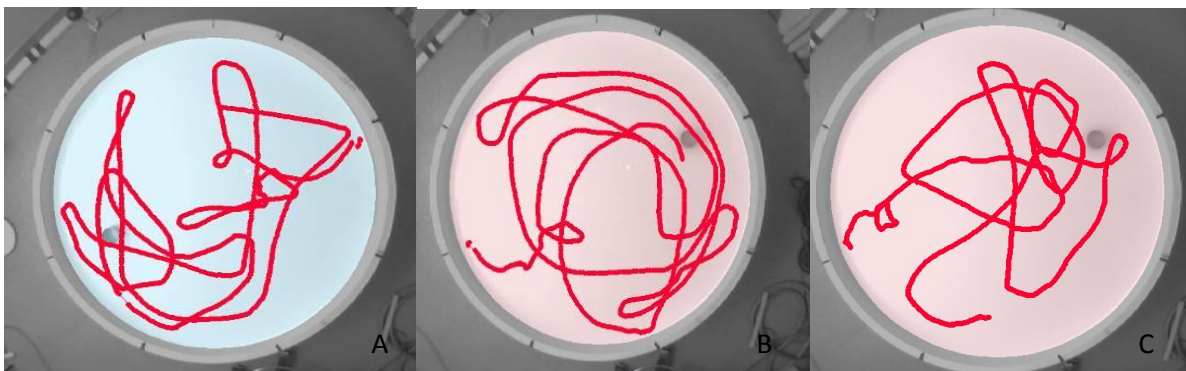
Figure 3.22 Examples of the swim path of the median control rat (A), the median MTT rat (B), and the rat with the largest extent of MTT damage (C), on the 25 day retention probe. The control rat showed the most accurate swim pattern with the majority of the swim path within the platform quadrant. In contrast, the rat in the MTT group showed a less precise swim strategy. As with the control rat, the rat with the largest extent of MTT damage spent the majority of time in the correct quadrant, but seems to have covered less total distance during the probe trial.

3.8 Spatial Working Memory in the Cross-maze

Two rats did not complete this task (1 control and 1 moderate) because they refused to leave the start area and were disrupting the performance of the other rats and were dropped after 12 trials. The 15 days of testing were blocked into 5 three day groups and analysed using a repeated measures ANOVA with the factors block and group. Data for this task were analysed in three ways. First group performance was compared for all trials over the 5 blocks of testing. Next group performance was analysed separately for those trials that used the same start end for both the sample and choice runs (standard t - maze task) and those that used different start ends. Finally, the mean performance of the few rats that reached criterion across the five blocks of testing have been plotted in figure 3.25 to observe the progression of responding exhibited by these rats .

3.81 Group Performance All Trials

As indicated in figure 3.23 all groups performed poorly on this task, as it was expected that the control group would reach at least 85% correct over two consecutive days before the end of testing. The control rats made more correct arm choices on average than the moderate lesion group for the duration of testing, but this difference did not approach significance, ($p=$ 0.12). In addition, the mean for the MTT group was the same as that for the control group on the last block. There was a non- significant effect of block ($p>0.05$) reflecting the relatively stable level of performance observed in both groups across blocks. The block by group interaction was not significant $F<1$.
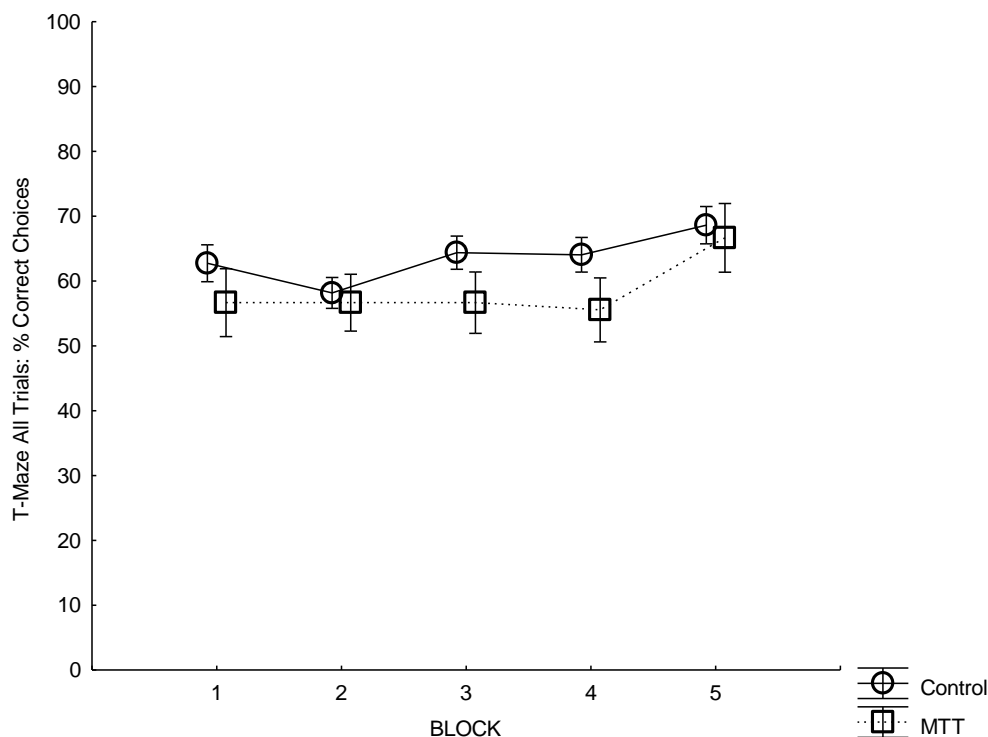
Figure 3.23. Group mean percentage of correct choices for total trials (same and different) +/- SE, across the 5 blocks of testing on the spatial working memory task in the cross maze. The control group on average made more correct arm choices than the MTT group, but this difference was not significant. Neither group made significantly more correct choices with training. Unfortunately, most controls did not reach the expected level of performance for this task (85% over two consecutive days).

3.82 Group Performance: Same and Different Start Trials

As shown in figure 3.24 both groups performed better on trials where both the sample and choice run were started from the same end of the cross-maze (top) and worse when the runs were started from different end (bottom). The MTT group appeared to have been more affected by different start trials than the control group, but there were no significant effects of block ($p > 0.2$) or group ($p > 0.1$) or block by group interactions ($p > 0.3$) for either trial type.

Same Start Trials
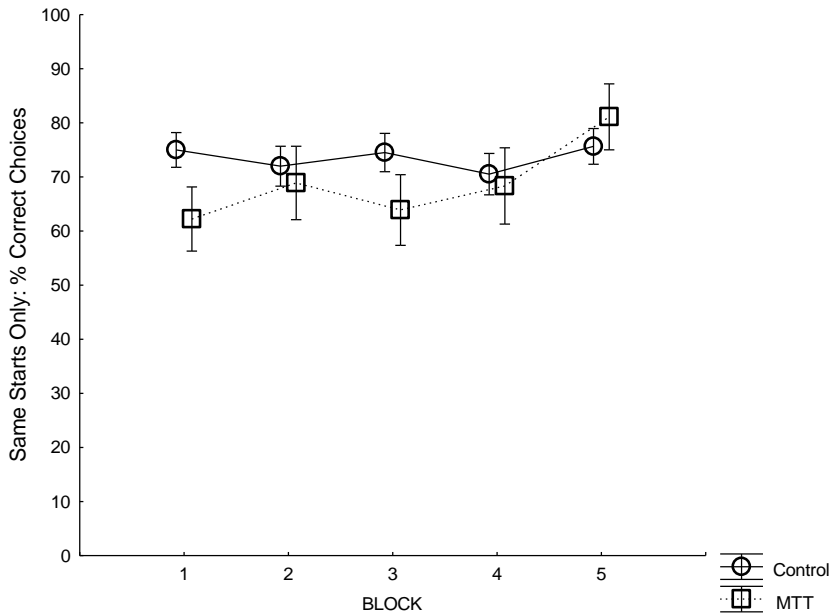
Different Start Trials
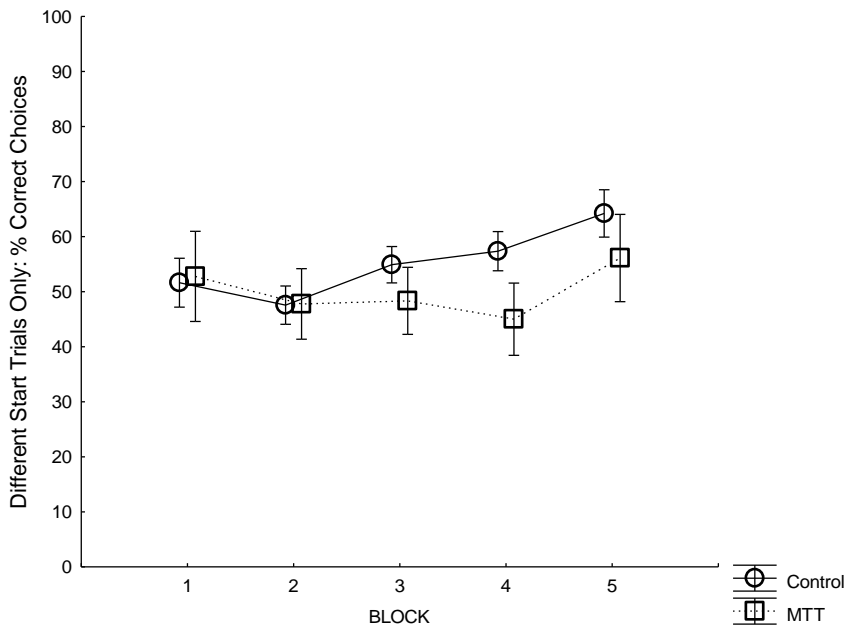
Figure 3.24. Mean percentage of correct choices for same (top) different start trials (bottom) +/- SE, for the control and MTT groups across the 5 blocks of testing for spatial working memory testing in the cross maze. Both groups performed better on same start trials and the MTT group appear to be more affected by different start trials than the control group, but there were no significant effects of block or group for either trial type.

3.83 Individual Completion Criteria

Unfortunately only six rats (five controls and one MTT rat "R8" 2nd smallest lesion in this group) reached criterion, three on day 12, one on day 13 and two on day 15. Figure 3.25 shows the mean percentage of correct choices of the six rats that reached the completion criteria over the 5 blocks of testing. Generally these rats showed acquisition that peaked in the final block of testing. A fishers exact chi square test showed the proportion of rats that reached criterion was not significantly different between the groups ($p = 0.58$). Interestingly, the MTT rat that reached completion criteria had the highest percentage of correct choices in the last block of training (6 out of 6 trials correct for three consecutive days).
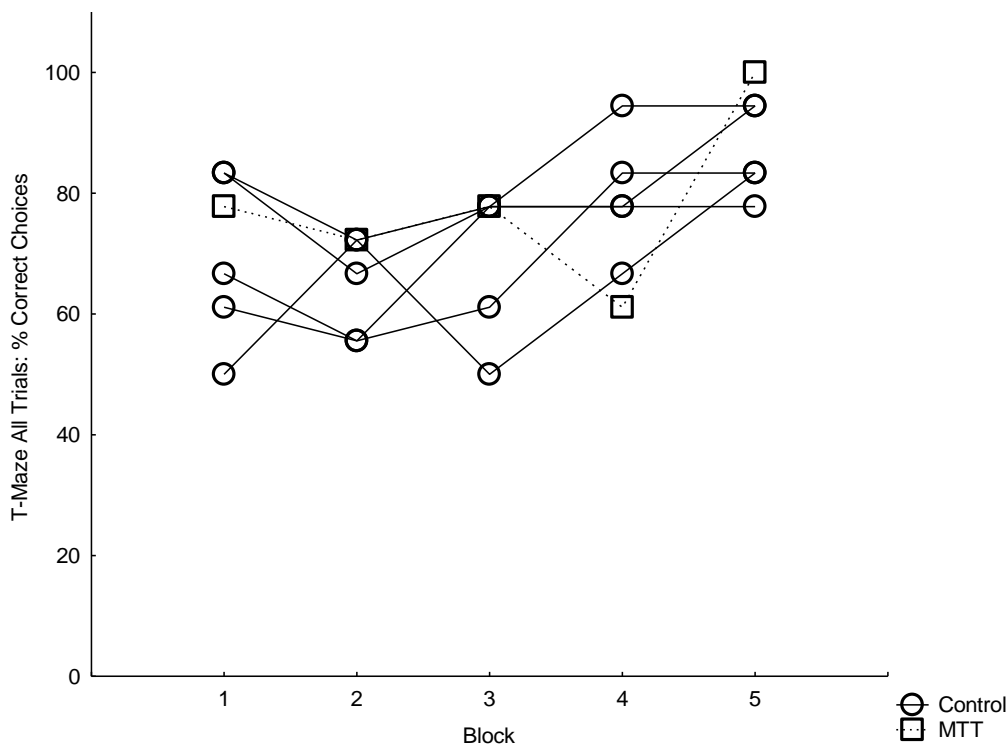
Figure 3.25. Individual percentage of correct choices for the six rats that reached completion criteria across the 5 blocks for the spatial memory task in the cross maze. Generally, these rats showed gradual improvement that peaked in the final block of testing. There was no significant difference in the proportion of control and MTT rats that reached criterion

3.9 Results of the Rat with the Largest Extent of MTT Damage (Rat # N6)

In general N6 performed unexpectedly well across the behavioural tasks and often had comparable if not better performance than many control rats, and with few exceptions consistently performed in the middle of the MTT rats. One exception was the episodic memory task, for the "what", "when" and "where" memory components when N6 was the poorest performer in the MTT group (see figure 3.6 bottom row). When compared to the control rats N6 was the second to worst performer on the "what" memory component, the worst performer on the "where" memory component, but fell within the spread of control discrimination values on the "when" memory component. However when N6 was tested on the object location (figure 3.12b) and temporal order tasks (figure 3.15b), which replicated "simplified" aspects of the "where" and "when" components of the episodic memory task, no impairment was observed. At both testing intervals (one or five minutes) on the object location task N6 discriminated appropriately and had the strongest preference for the target object of all rats after five minutes of testing. Similarly in the temporal order task N6, showed a strong preference for the target object at both testing intervals (two & five minutes) and outperformed the majority of the control rats. For the novel object task (figure 3.9b), although this is a task where no impairment with MTT would be expected, N6 had a strong preference for the target object comparable to the better performing control rats at both testing intervals (two & five minutes).

For phase 1 of working memory testing in the water maze N6 was slightly impaired compared to the control group mean and had longer path lengths for block 1 (trials 1-4: 2814cm, 1634cm, 1206cm & 1573cm, compare with figure 3.16). Improvement was observed in block 2 and N6 had path lengths comparable to the control group mean (trials 1-4: 1227cm, 989cm, 921cm & 409cm). In block 3 N6 had comparable path lengths to control mean and performed well on (trials 1 and 3: 858cm & 586cm), but struggled on trials 2 and 4

(1110cm & 1172cm). For phase 2 of working memory testing (compare with, figure 3.17) N6 was initially impaired in block 1, especially trial 2 (trials 1-4: 1159cm, 1086cm, 585cm & 814cm), for which it swam nearly twice the distance of the control mean to find the platform. This is important because the reduction in path length from trial 1 to 2 is considered the strongest measure of working memory. However, by block 2 N6 had similar path lengths to the control group (trials 1-4: 1351cm, 326cm, 459cm & 745cm).

Across the seven days of reference memory training (figure 3.18) N6 had similar path lengths to the platform as the control group mean (days 1-7: 319cm, 429cm, 322cm, 299cm, 344cm, 399cm, & 247cm) despite this the MTT group as a whole showed a weak deficit. N6 completed the completed the 25 day reference memory probe test and had a comparable performance to the superior control rats for the accuracy ratio (figure 3.19) and crossings of the annulus (figure 3.20).

Finally, N6 acquired the delayed non matching to place task in the cross maze (blocks 1-5: 33%, 55%, 44%, 61% & 66%) at a slower rate than the control group (compare with figure 3.23). Furthermore, N6 was disproportionately affected by different start trials (blocks 1-5: 33%, 44%, 33%, 52% & 52%) compared to same start trials (blocks 1-5: 33%, 72%, 52%, 80% & 88%). However, it made more correct choices than the control mean across the final two blocks of training for the same start trials (compare with figure 3.24).

3.10 Results of the Unilateral MTT Damage Group

The unilateral MTT damage group (uniMTT) had a variable pattern of results across tasks. In some tasks they outperformed the moderate lesion and control group and in others they were more impaired than the MTT group. For the episodic memory task the uniMTT group had stronger mean discrimination on the "what" and "where" memory components (mean = 0.38, SD = 0.06) and (mean = 0.19, SD = 0.7) than the control and MTT groups (compare with figure 3.6 bottom row). However they exhibited virtually no discrimination (mean = 0.07, SD

= 0.85) for the "when" memory component. The uniMTT group were not impaired in the novel object recognition task had a comparable mean level of discrimination to the control group (figure 3.9b) after two (mean = 0.52, SD = 0.2) and five minutes of testing (mean = 0.52, SD = 0.28). However, in the object location task the uniMTT group had weaker mean discrimination than the control group and MTT group (compare with figure 3.12b) after one minute (mean = 0.18, SD = 0.11) and five minutes of testing (mean = 0.03, SD = 0.22). For the temporal order task the unilateral group showed greater mean discrimination than the control group, but not the MTT group (compare with figure 3.15b) after both two (mean = 0.31, SD =0.15) and five minutes (mean = 0.25, SD = 0.20).

For the first phase of working memory testing in the water maze the uniMTT group tended to have longer path lengths across trials 1-4 on block 1 (trials 1-4: 2615cm, 1721cm, 1233cm & 1047cms) than the control and MTT groups (compare with figure 3.16), but comparable path lengths to both groups in block 2 (trials 1-4: 1369cm, 770cm, 684cm & 951cm). In the final block of testing the uniMTT group had shorter path lengths than the MTT group, but not the controls (trials 1-4: 1845cm, 915cm, 801cm & 858cm). For phase 2 of working memory of the water maze (Compare with figure 3.17) the uniMTT groups mean path lengths to the platform fell between the control and MTT groups in block 1 (trial 1-4: 1671cm, 566cm, 420cm, & 596cm), but were longer than both groups in block 2 especially on the second trial (trial 1-4: 1798cm, 936cm, 502cm & 490cm).

The uniMTT group generally had longer path lengths than the control and MTT groups (compare with figure 3.18) across the seven days of reference memory training (days 1-7: 608cm, 445cm, 389cm, 403cm, 385cm, 335cm & 226cm). Coincidentally, two rats from the uniMTT group performed the 5 day reference memory probe and two performed the 25 day probe. The two rats that completed the 5 day probe had a comparable level of accuracy to the control group (mean = 2.20, SD = 0.89). In addition, they made more crossings of the

annulus (mean = 4, SD = 1.4) than the control group, but not the MTT group (compare with figure 3.19 and figure 3.20). However, the two rats that completed the 25 day probe test were less accurate (mean = 1.3, SD = 0.44) and made fewer crossings (mean = 0.44, SD = 0.7) than the control and MTT groups.

Finally, the uniMTT group generally made fewer correct choices than the control or MTT groups on the spatial working memory task in the cross maze (compare with figure 3.23) (blocks 1-5: 55%, 48%, 47%, 48% & 55%). Like the control and MTT groups (compare with figure 3.24) the uniMTT group made more correct choices on same start only trials (blocks 1-5: 74%, 61%, 55%, 58% & 63%) but performed poorly on different start trials (blocks 1-5: 37%, 36%, 42%, 42% & 52%).

4. Discussion

4.1 Summary of Results

This study provides evidence that the MTT may not be as critical to the normal functioning of the extended hippocampal memory circuit as previously suggested (Vann & Aggleton, 2003; Vann, Honey & Aggleton, 2003; Vann, 2010). A deficit in spatial working memory in the water maze represented the clearest deficit in a previous MTT lesion study (Vann & Aggleton, 2003). In the present study, no deficit was evident for this task despite moderately large damage to the MTT, consistent with the negative result in another recent study (Winter et al, 2011). As with that recent study, damage to the MTT produced a mild deficit in the standard reference memory task in the water maze (Winter et al, 2011), which was not tested by Vann & Aggleton (2003). Also, in contrast to previous studies, no deficit was found for the spatial working memory task in the cross maze (Vann & Aggleton, 2003; Thomas & Randall, 1985). Despite the mild reference memory deficit, the additional recent (5 day) and remote (25 day) reference memory probes provided no evidence that the MTT has a significant role in memory consolidation. No study has previously examined long term retrieval of spatial memory after MTT lesions. In line with previous reports following lesions to the MB and ATN in rats (Aggleton et al, 1995; Mitchell & Dalrymple-Alford, 2005) rats with MTT damage showed no impairment in their ability to discriminate between a "novel" and a "familiar" object or the temporal order of objects. Another contrast to the predicted set of findings, however, subsequent object recognition tasks testing "episodic like" memory and memory for the location of objects also found no significant differences between rats with MTT damage and controls. The overall lack of effect of MTT lesions was reinforced by the fact that the rat with the largest extent of MTT damage, 81%, performed unexpectedly well during the spatial working memory task in the water maze and across reference memory

training. In Addition, this rat had an accurate memory for the last known platform position in the 25 day retention interval probe test. These findings when combined with those of Winter et al (2011) suggest that more research is needed before any definitive conclusion regarding the role of the MTT in memory can be reached.

4.2 Behavioural Tasks

Each of the behavioural tasks in now examined in more detail.

4.21 Water Maze: Working Memory Task

As stated, it was unexpected that there were no significant differences between the groups in either phase 1 or phase 2 of working memory testing in the water maze. Of particular relevance, the significant effect of trial across all blocks of testing indicated that both groups could learn a novel platform position within a session and use this information to reduce the distance they had to swim to find the platform on subsequent trials. More generally, this evidence suggests that both groups successfully used environmental and spatial cues to navigate to the platform. Previous work by Vann & Aggleton (2003) found rats with either MTT or MB lesions had severe deficits in the acquisition of this task, so rats with MTT lesions in the present study were expected to have a severe and pervasive acquisition deficit here too. Whilst completing behavioural testing for the current study, a new study by Winter et al (2011) was published. Winter et al (2011) also found that rats with complete bilateral MTT lesions were unimpaired on this task and performed at the level of their control rats.

It is possible that the discrepancy between the present study and that of Vann & Aggleton (2003) was the result of subtotal MTT lesions but this was not true of the MTT lesions in the study by Winter et al (2011). The idea that the MTT requires complete disconnection before a behavioural deficit emerges, is consistent with the suggestion in the context of MB damage made by Sziklas & Petrides (1993) who found that marked behavioural deficits in rats were only observed following MB lesions if damage was total and

98

extended to a wider region, including adjacent nuclei and fibre tracts. However, if the MTT is as critical to memory, as Vann and Aggleton (2003) suggest, substantial damage would be expected to cause a robust deficit. Less than total damage to other diencephalic structures has been shown to produce robust and severe behavioural deficits. For example, Byatt & Dalrymple-Alford (1996) deliberately created small radio frequency lesions in either the anteromedial or anteroventral components of the ATN. This substantial, but incomplete ATN damage produced a robust behavioural deficit in working and reference memory versions of the RAM. This is an important consideration given the MTT/MB are more consistently implicated in human amnesic syndromes than the ATN. Furthermore, it is not known whether the remaining myelinated MTT tissue in the MTT group in the current study was healthy, so it is possible that the MTT tract damage is greater than reported.

The point at which the MTT is interrupted may also be a factor to consider, as it is possible that the deficit observed by Vann & Aggleton (2003) resulted from damage to midline thalamic structures in addition to the MTT. The surgical procedure used Vann & Aggleton (2003) produced lesions at approximately -2.8 from bregma (see Vann & Albasser, 2009). At -2.8 from bregma the MTT is directly adjacent to the nucleus reuniens (RE) of the midline thalamus. Given the close proximity of these structures, lesion to the MTT at this level would likely cause damage to the RE. A recent study has shown that the RE is the principal source of thalamic input to the hippocampus (Vertes, Hoover, Szigeti-Buck & Leranth, 2007). In addition, they showed that the RE may represent a critical link between the medial prefrontal cortex and the hippocampus, structures which are consistently implicated in memory.

Although Winter et al (2011) only ran their spatial working memory task for four days there were no noticeable differences in escape latencies between their lesion and control groups. By contrast, the deficit reported in the comparable task by Vann & Aggleton (2003)

was immediately apparent with controls locating the platform with substantially shorter path lengths than either the MB or MTT rats after only three days of training. Vann & Aggleton (2003) suggested that rats with MTT lesions have an acquisition deficit on this task and if this is true then it would be expected that any group differences should emerge early in training. Winter et al (2011) proposed that a strain difference may explain the contrast between their study and Vann & Aggleton (2003) as they used Long Evans compared to Dark Agouti rats, respectively. Long Evans rats generally exhibit superior performance to other strains on spatial memory tasks suggesting this factor may have exaggerated any differences between their groups. However, this premise seems unlikely given that there is no discernible difference between the mean escape latencies for Winter et al's (2011) lesion or control group. Interestingly, both the present study and Winter et al (2011) used female rats, in contrast to the male rats used by Vann & Aggleton (2003). It is possible that the discrepancies between the findings of the present study and Winter et al (2011) and the findings of Vann & Aggleton (2003) may also relate to sex differences.

The control group in the present study had a slower rate of acquisition of spatial working memory than is typically expected for this task. After nine days of training in phase 1 in the water maze, the control rats still had an average path length of ~950 cms (on trial two) to locate the platform in the 180cm diameter pool. In contrast, after the same amount of training the control rats used by Vann & Aggleton (2003) were swimming approximately 300 cms to reach the platform in a 200cm diameter pool. However, the present study used a more demanding spatial working memory procedure, which required the rats to locate ten different platform locations across training, compared to Vann & Aggletons (2003) four platform locations. In addition, the quicker task acquisition shown by Vann & Aggletons (2003) rats' may have reflected more extensive training, as their rats received testing in the t-maze and radial arm maze before being trained on the spatial working memory task in the water maze.

That is, the rats of Vann & Aggleton (2003) had perhaps already learnt to pay attention to environmental cues which in turn helped them acquired the water maze working memory task in a shorter period.

As expected all groups performed significantly better when the inter-trial intervals were extended from ~2 seconds to 4-6 minutes and the rats were allowed an additional 15 seconds on the platform to orientate themselves to the environment. The quick improvement was likely a result of both these factors. The rats' performances on massed trials in phase 1 may also have been retarded by the use of a lower water temperature in the present study (19-21°c) compared to (23-25°c) used by Vann & Aggleton (2003), in combination with the use of smaller female rats. A lower water temperature was used in this study is commonly used to motivate the rats to escape from the water.

4.22 Reference Memory Task

As expected the moderate lesion group took significantly longer path lengths to find the platform, but this deficit was unexpectedly very mild in contrast to markedly severe and often persistent deficit evident with ATN lesions (Warburton et al, 1999; Warburton & Aggleton, 1999; Wolff et al, 2008; Lopez et al, 2009). It is possible given the trend in the data that the observed deficit may have resolved with more than seven days of training. A previous study also found rats with complete bilateral MTT lesions took significantly longer to locate the hidden platform in a spatial reference memory test (Winter et al, 2011). However, Winter et al's (2011) analyses similarly indicated that this deficit was mild and their MTT lesion group was trending back towards control performance by the final day (of five days) of training. Lesions of the MB have produced mixed results for this task. Sutherland & Rodriguez (1989) found a transient deficit in rats with MB lesions, but Santin et al (1999) found rats with MB lesions were not impaired on this task.

4.23 Reference Memory Probe Trials

There were no significant group differences observed for the accuracy ratio or crossings of the annulus after either a 5 day or 25 day retention interval. Individually, the rats in the moderate lesion group performed within the range of controls on both measures, which suggests that the MTT does not play a significant role in the retrieval of remote memory. It was not previously known whether rats with MTT lesions would be impaired in recent (5day) or remote (25 day) memory consolidation, but this task was included because previous work (Lopez et al, 2009) found that rats with a rostral intralaminar thalamic lesion, which showed no deficit during reference memory training or after a 5 day retention interval, were significantly impaired following a 25 day retention interval. It is also thought that the ATN may play a role in long-term retrieval, but rats with ATN lesions are so severely impaired during reference memory acquisition that any decrement in performance following a retention interval would be difficult to detect.

There was however, a significant effect of retention interval on accuracy for the last known location of the platform suggesting that the general manipulation was successful in that the rats performing the task after five days were more accurate than rats performing the probe after 25 days. Interestingly, mean path lengths over the final three days of the reference memory task did not predict accuracy for platform location after a 5 day retention interval, but significantly predicted accuracy after a 25 day retention interval. This novel finding suggests that the strength of the memory formed for the platform location by the end of training was more important if rats had to retrieve spatial information after a longer delay. This association has never been examined in the literature previously to the experimenter's knowledge.

4.24 Spatial Working Memory in the Cross-maze

The MTT group, on average, made fewer correct choices than control rats over the 15 days of delayed non-matching to place training in the cross maze. However this deficit did not approach significance, perhaps because of the poor performance of the control group. Furthermore, no deficit was observed when trials where the sample and choice runs were started from the same or different ends of the apparatus were analysed separately. Both groups had a greater percentage of correct choices in same start only trials, likely reflecting the possible benefit of egocentric information for arm selection by simply alternating body turn with the trial. Rats with MTT lesions were expected to have a severe behavioural deficit on the delayed non-matching to place task in the cross-maze when all trials were included in the analysis, and especially when different start trials when the sample and test runs began from opposite start areas. Previous research has shown that rats with ATN lesions are severely impaired on this task (Loukavenko et al, 2007). Given the transient deficit observed by Thomas & Randall (1985) and Vann & Aggleton (2003) on a simplified t-maze task, it was expected that a similar transient impairment would be found when only same start trials were analysed.

Unfortunately, only 5 of the 17 rats in the control group managed to reach the completion criteria for this task and many of the controls were still performing at the level of chance (fifty percent) on the last block of trials. As a result group performance for all trials in the control group peaked just below 70 % correct even in the last block. Previous research using a similar procedure (Loukavenko et al, 2007) found that control rats reached a stable level of performance above 80% after 10 sessions. However, Loukavenko et al's (2007) rats were much younger, approximately 6 months old at the time of surgery, in contrast to 12 month old rats used in the present study. Due to time constraints training in this task had to be

cut short and a greater proportion of the control rats may have reached criterion with additional training.

4.25 Episodic Memory task

The MTT group did not discriminate significantly above chance on any of the components of the episodic memory task, while the control group also performed poorly and only discriminated above chance on "what" memory, but there were no significant effects of group. The control group but not the MTT group was expected to discriminate significantly above the level of chance across all the "what", "where" and "when" memory components in this task. Previous research by De Vito & Eichenbaum (2010) found mice with hippocampal lesions were significantly impaired in all three memory components.

The control rats in the present study were not able to replicate the robust discrimination shown by mice in the De Vito & Eichenbaum (2010) study discriminating marginally lower in comparison on the "what" memory component but approximately half as much on the "where" and "when" memory components. The most obvious difference between the present study and De Vito & Eichenbaum's (2010) is the species of rodent used (rat vs. mouse). Perhaps the parameters of testing used for this task were not directly transferable across species and the procedure needed to be adapted for use with rats. Additionally, the objects used may be relevant as the rats may have had a particular preference for one set of objects over the other and as all the analyses for this task relied on the same data expressed in different ways which would interfere with the results of all components.

The inability to find a group difference in any of the components of this task could be related in this instance to the size of the MTT lesions. The rat with the largest lesions was the poorest performer in the MTT group on all three measures. It is also important to consider that MTT lesions would not be expected to disrupt performance to the same degree as

hippocampal lesions. Also, the small group size of the MTT group made the group means sensitive to extreme values in this task especially.

4.26 Temporal Order Task

In the temporal order task, both sham and MTT groups discriminated significantly greater than chance after two minutes of testing, but only the MTT group discriminated significantly above the level of chance after five minutes of testing. There were, however, no significant differences in group performance at either time point. Rats with MTT lesions would be expected to discriminate between a "less recent" and "recent" object at a rate significantly greater than chance. A previous study found that rats with ATN lesions did not differ significantly in their ability to discriminate between a "recent" and "less recent" object compared to controls (Mitchell & Dalrymple-Alford, 2005). In contrast, lesions of the perirhinal and medial prefrontal cortex significantly impaired temporal order discrimination in rats but, these two regions are suggested to support different memory processes than the MTT (Barker, Bird, Alexander & Warburton, 2007).

This task replicated a "simplified" version of the "when" memory component of the episodic memory task with only two objects. Comparatively, both groups performed better on this task than the "when" component of the episodic memory task which likely relates to decreased task demands i.e. discrimination between two as opposed to four objects.

4. 27 Object Location Task

Only the controls were able to significantly discriminate between the two objects after one minute and five minutes of testing in the object location task, but there were no significant differences between the groups at either time point. Given the similarity of the group means the fact that the MTT group did not discriminate significantly above chance is most likely due to a small sample size, variability in performance and lack of statistical power, rather than a behavioural deficit. It was expected that rats with MTT lesions would not be able to

discriminate between the "stationary" and "displaced" objects in this task. Previous research found that rats with hippocampal lesions were not able to discriminate between a displaced and stationary object and performed significantly worse than the control group (Mumby et al, 2002). As mentioned previously, MTT lesions would not be expected to disrupt performance to the same degree as hippocampal lesions, which may account for the non-significant group difference found in the present study. However these results did not reflect a particularly poor performance of the control rats in general as they performed at a similar level following one minute of testing as displayed by control rats in previous studies (Dix & Aggleton, 1999; Barker et al, 2007).

Both groups exhibited a mildly greater mean preference for the target object after one minute of testing than five minutes of testing, but the results did not support the findings of Dix & Aggleton (1999) who suggested that, for object recognition tasks that use a change of location, intact rats only discriminate significantly in the first minute of testing. This may relate to differences in testing environments and the use of female as opposed to male rats. Interestingly, this task showed a different pattern of results to the "where" memory component of the episodic memory task, as the controls discriminated significantly between the objects in this task but not in the episodic memory task. As for the temporal order task, this likely relates to a reduction in task demands.

4.28 Novel Object Task

While there was no effect of group for this task, both sham and MTT groups showed a robust and significant preference for the "novel" over the "familiar" object, after two minutes (as per Dix & Aggleton, 1999) and five minutes of testing. Both groups exhibited a mild reduction in mean discrimination for the novel object following five minutes of testing, but the discrimination ratios of both groups remained significantly above chance. Previously, Dix & Aggleton (1999) reported that intact rats fail to discriminate significantly above the level of

chance after two minutes of testing. This difference could relate to factors such as strain differences, the objects used, differences in the apparatus and testing environment and sex differences as female rats (used here) are generally more active than male rats (used by Dix & Aggleton, 1999). Lesions of the MTT would not be expected to disrupt discrimination in a simple object recognition task, which has been shown to be dependent on the perirhinal cortex (Mumby, 2001). Previous studies have found that lesions of the hippocampus, ATN and the MB do not disrupt these familiarity-based discriminations (Mumby, 2001; Aggleton et al, 1995; Mitchell & Dalrymple-Alford, 2005).

4.3 Results of N6 (*Largest extent of MTT damage*)

With the exception of the episodic memory task and low performance for the different start trials in the spatial working memory task in the cross maze, N6 was generally unimpaired. N6 showed slightly poor performance in phase 1 and phase 2 of working memory in the water maze and a slower acquisition in the cross maze compared to mean of the control group, but this was also true of many individual rats in the control group that performed either comparatively or worse than N6 on these tasks. Given the extent of the damage bilaterally, it would be expected that N6 would be impaired in the working memory version of the water-maze especially. There are at least two possible explanations for the performance of this rat. Either the MTT is not as important to memory as previously thought and rather damage to multiple diencephalic sites including the MTT results in the human amnesic syndrome or complete bilateral transection is required for a deficit to appear. The latter seems unlikely in humans given the diffuse but often incomplete pattern of brain damage to different structures including the MTT following stroke, trauma, or disease related neurodegeneration in human amnesic syndromes. Additionally, it is important to note that Vann & Aggleton (2003) and Winter et al (2011) verified their lesions with a nissl stain only, so it is entirely possible that not all of the MTT was destroyed in their studies either. Despite not using a myelin specific

stain, both studies explicitly stated that rats included in their MTT lesion groups had complete bilateral lesions.

4.4 Results of Unilateral MTT Damage Group

While it is interesting to consider this group because unilateral MTT damage is common in human cases, the small size of this group and variability of performance across behavioural tasks make definitive conclusions difficult. It is not clear whether the poor performance of these rats on spatial working memory in the cross maze or reference memory training was related to MTT damage or another extraneous common elements within rats tested in this study. Previous research would suggest that the results of this group are not a attributable to unilateral MTT damage. Winter et al (2011) reported no behavioural differences between rats with unilateral MTT lesions and control rats during working memory or reference memory training in the water maze (Winter et al, 2011).

4.5 Limitations

When brains were extracted it was found that several rats had tumours protruding from the ventral surface of the skull up into the brain. In total, 14 out of 49 rats used in this study had a tumour of some description. These ranged in size from a few millimetres in diameter through to one that was $12 \times 7 \times 6$ mm and weighed half a gram. Interestingly more control surgery rats than lesion surgery rats were found to have tumours and the tumour bearing rats were asymptomatic until just prior to all rats being sacrificed. A search of the literature revealed that the tumours were most likely anterior pituitary adenomas which are relatively common in older female rats (Gilbert, et al 1958; Crain, 1957). Studies have shown that diet, strain and age have a significant influence over tumour development. A low carbohydrate, high protein diet has been shown to significantly reduce the occurrence of all tumour types including pituitary tumours without reducing total life expectancy (Gilbert et al, 1958). Furthermore it has been suggested by others (Ross, Bras & Ragbeer, 1969) that long term food restriction is

sufficient to delay the time of occurrence and reduces the overall incidence of spontaneous tumours in mice and rats. These tumours are thought to be caused by growth hormones including estrogen and prolactin (Lloyd, 1983), as long term estrogen exposure leads to hyperplasia of the prolactin cells in the pituitary gland and a 100% incidence of pituitary gland tumours (Lloyd, 1983). It seems likely that the age of these rats in combination with free feeding up until the last few months of their lives may have contributed to the high incidence of tumours in this particular cohort.

For reasons beyond the control of the experimenters at the time of surgery the rats were already 12 months old (Christchurch quakes). This was not expected to be a problem as rats typically live two years and testing was expected to conclude after approximately 3-4 months. Generally the controls in this experiment performed poorer than expected and the most likely explanation is their age. It is also possible that some pathological changes in the rats associated with pituitary gland adenomas could underlie the poor performance even in cases that had not yet developed a tumour. In any case it would be advisable to use much younger rats in future.

Although full bilateral destruction of the mammillothalamic tract was not achieved in any of the lesion rats, many had a substantial localised damage of MTT. Trial lesion surgeries conducted prior to the start of the experiment suggested that 58°Celcius for a total of 60 seconds was sufficient to extensively damage and disconnect the MTT. Lesion verification in these cases consisted of a nissl stain (cresyl violet acetate) of the damaged region and the rats were culled a few days after receiving the lesion as per normal practice, but it is possible that inflammation of the damaged region may have made the lesion appear larger than it actually was. Additionally, without the inclusion of a myelin stain which visualises the myelinated tissue in the brain, it is hard to tell whether any small remnant of the tract remained although this seemed unlikely. Another explanation for the incomplete lesions in this study may have

been a malfunction of the radio frequency generator which occurred sometime in between the last trial surgery and the first experimental surgery. The latter is probable given the vast differences in lesion extent between the trial and experimental surgeries as the exact same methodology was used for each.

Many lesions, however, were extremely accurate given the small and difficult target, but a few lesions missed because they were located too posterior in the brain. Lesions that posterior are problematic because caudal to -3.6 from bregma the MTT starts to disperse as it descends rapidly into the MB so any lesions that hit beyond this AP level were too high and resulted in little or no damage to the tract. Given the small margin for error there were also a few occurrences where the midline coordinate was not correct so the lesions hit the side of the tract. A very methodical approach was taken to locate the midline of the brain using the mid sagittal suture and the midpoints of both bregma and Lambda, but this did not prove successful in all cases. One accurate approach to establish midline would be to remove a bone flap and use the mid sagittal sinus as a midline, but variation can still occur and this greatly increases the chances of extensive bleeding, so is not the preferred method. Additionally using female rats made determining the anterior-posterior coordinate more difficult as most previous studies creating MTT lesions, except one (Winter et al, 2011), used male rats. Winter et al (2011) had some success with an absolute AP coordinate of -1.9 from bregma, but the method used in the present study is preferable as it accounts for head size variations within a given cohort of rats.

The present study created a novel method for verification/quantification of MTT damage as all previously published studies creating MTT lesions simply categorized their lesions into either full bilateral lesion, full unilateral lesion or a miss. To quantify the tract in the present study an AP coordinate was chosen that was as close as possible to the intended lesion site which allowed accurate quantification. Furthermore it is not clear whether the

remaining myelin that was quantified was pathogenic, so it is possible that the damage observed in the MTT group was greater than reported. It is possible to visualise neural degeneration in brain tissue through specialised stains such as amino cupric silver stain and flouro jade C stain, but the potential value of doing this in the present study only became apparent after the histological procedures had been carried out (Wozniak, Hartman, Boyle, Vogt, Brooks, Tenkova et al, 2004; Schmued, Stowers, Scallet & Xu, 2005).

4.6 Contributions of the Current Study

Given the relatively small sample size of the MTT lesion group in the present study, conclusions must be tentative. However, there are sufficient observations that suggest the MTT may not be as important to memory as previously suggested, especially when the findings of the present study are combined with those of Winter et al (2011), at least when female rats are used.

One important issue is that, if the MTT is critical to memory, then extensive but subtotal damage to the MTT should be sufficient to induce a severe memory deficit. There is now a wide range of evidence strongly implicating the MTT in human amnesic syndromes. Importantly, neither of the large reviews examining behavioural and cognitive deficits following thalamic infarction (Van der Werf et al, 2000; Carlesimo et al, 2011) suggested that complete bilateral destruction of the MTT was required to induce the amnesic syndrome. In fact many of the studies included in these reviews reported anterograde memory deficits following unilateral MTT damage. Furthermore, in none of the cases examined in these clinical reviews was damage restricted solely to the MTT, but rather damage extended into various nuclei of the thalamus. It is possible then, despite the fact the MTT is consistently implicated in the human literature (Van der Werf et al, 2000, 2003b; Calesimo et al, 2011; Kim et al, 2009, 2010), the diffuse pathological changes in adjacent brain regions that accompany the MTT damage may account for the memory impairment, not just the MTT

injury. Indeed, one seldom reported study suggested that localised damage to the MTT produced no cognitive or behavioural changes in their three patients (Duprez et al, 2005). Unfortunately, it is hard to substantiate how much if any physical damage was caused to the MTT in that study as electrodes were inserted into the tracts, which may have simply moved or displaced, rather than damaged them.

Given the anatomical trajectory of the MTT it is inevitable that in cases of amnesia involving MTT damage, nuclei within the adjacent thalamus are also damaged. This is especially true for the ATN. Paradoxically, studies in rodents have consistently shown severe memory deficits in working memory tasks following lesions to the ATN (for a review see Van der Werf et al, 2003a) and these deficits appear to be more pervasive and severe than those observed following MB or MTT lesions (see review by Vann, 2010). Furthermore, a study by Harding et al (2000) suggested that neurodegeneration in the anterior thalamus was the only consistent lesion found in alcoholics with Korsakoff's syndrome that differentiated them from other alcoholics with Wernicke's encephalopathy. In their review Van der Werf et al (2000) concluded that the MTT was the critical region for human amnesic syndromes, but they also suggested that because MTT contains fibres bound for the ATN it is to be expected that infarctions affecting the ATN would produce the same deficits as damage to the MTT. This suggests an important role for both of these structures in diencephalic amnesia but actually places a greater precedence and emphasis on the MTT. It cannot be disputed that the MTT is associated with in the amnesic syndrome, but given the diffuse nature of brain injury and additional damage present in patients, it is not sufficient to suggest that damage to the MTT alone results in amnesia. Rather it may be that damage to the MTT in combination with damage to surrounding thalamic nuclei more accurately describes the resulting memory impairment.

Although none of the lesions in the present study resulted in complete bilateral transection of the MTT it would still be expected that damage to over 50% of the tract should cause pervasive deficits, especially in spatial working memory tasks. In previous studies any lesions that failed to destroy the tract bilaterally were removed from the lesion groups and usually combined with controls (Krieckhaus & Randall, Field et al 1978, Thomas & Gash 1985; Vann & Aggleton, 2003). In contrast, studies giving ATN lesions to rats have shown that less than total damage is sufficient to induce severe behavioural deficits (Byatt & Dalrymple-Alford, 1996; Loukavenko et al, 2007; Aggleton et al, 1996; Warburton et al, 1999). Furthermore, Loukavenko et al (2007) found that, with lesions that were >50%, there was no correlation between the size of an their ATN lesions in their female rats and the resulting behavioural deficit for the last three days of spatial working memory in the cross maze. If the MTT is as critical to memory as suggested by Vann & Aggleton (2003) and the clinical literature, then substantial but subtotal damage would be expected to result in a profound and chronic behavioural deficit in rats.

There is now a substantial body of research concerning behavioural impairments following lesions to the MB in rats and mice. Despite early inconsistent findings, it is widely accepted that MB lesions create task-dependent spatial working memory deficits. Given the results of the present study, and those of Winter et al (2011), it is important to consider why lesions to the MTT would not produce equivalent deficits to MB lesions. One interesting feature is that the MB are not solely connected to the ATN, but also have a reciprocal connection with the tegmentum nucleus of Gudden (Aggleton et al, 2010). Disconnection of the MTT removes the MB input into the ATN, but does not disrupt communication between the MB and the tegmentum nucleus. A recent study by Vann (2009) showed that lesions to the ventral tegmentum nucleus (VTNg) produced deficits on a similar array of tasks and to a similar degree as do medial MB lesions. These considerations lead to

113

the unusual and important suggestion that the loss of VTNg input or disruption of VTg function caused by deafferentation following MB lesions may better account for the behavioural deficit than the loss of MB information per se. Of course, disconnecting the MTT would also remove the indirect VTNg input to the ATN, but the VTNg also projects to numerous peripheral sites including, the prefrontal cortex, cingulate cortex, and entorhinal cortex (Simon, Le Moal & Calas, 1979). Perhaps these other pathways are able to compensate for the loss of MB information through the MTT.

4.7 Future Directions

Surprisingly, there are still very few studies that have examined the behavioural impact of MTT lesions. This is somewhat puzzling given the mounting implication of this fibre pathway in human amnesic disorders. Vann & Aggleton (2003) found that male rats given MTT lesions had an acquisition deficit on working memory versions of the water maze, t-maze and RAM. However, a more recent study by Winter et al (2011) found no deficit in female rats with MTT lesions on the working memory task in the water maze, but their rats showed impaired reference memory acquisition and in a food hoarding task where they had to rely on self-movement cues to navigate. It is clear that bilateral MTT damage or disconnection results in behavioural deficits, but given the inconsistent nature of these findings absolute conclusions cannot be reached without further investigation.

For future work to elucidate the functional role of the MTT in memory the present study needs to be replicated with a younger population of male and female rats and variation in lesion size. If a sex difference was confirmed, then we would still need to explain why ATN lesions substantially impair spatial memory in both sexes. The most important behavioural paradigm would be the working memory version of the water-maze as this discrepancy in findings must be resolved. Furthermore, subtle alterations in the water-maze task such as number of platform positions used could be employed to determine the

conditions necessary and sufficient to induce a behavioural deficit. If the deficit reported by Vann & Aggleton (2003) is established then further studies attempting to ameliorate this deficit through environmental enrichment or other neuroprotective strategies would be warranted.

Another important line of enquiry would be to compare directly the behavioural outcome of MTT, MB and ATN lesions on standard spatial working memory tasks within a single study. This is of interest because in human cases of diencephalic amnesia the MTT and the MB are more consistently implicated than the ATN. However, in the animal literature lesions to the ATN have been found to induce a more severe and persistent memory deficit than either MTT or MB lesions on several types of spatial and non-spatial learning tasks.

It is also important to untangle the functional contributions of the hippocampal inputs into the ATN, i.e. via the MTT and the fornix. A recent study by Vann et al (2010) found that the hippocampal inputs in to the MB are not as functionally important as previously thought. Vann et al (2010) disconnected the post-commissural fornix and removed the hippocampal input to the MB and found the subsequent behavioural deficits to be much less severe than either MB or MTT damage. Their finding is important because it suggests that the MB are not merely a relay of hippocampal information but provide a unique input into the extended hippocampal memory system which it is suggested is through the MTT. This being the case it would be of interest to compare in a single study the effect of MTT lesions and lesions that transect the hippocampal inputs into the ATN (pre-commissural fornix) thus severing the hippocampal inputs to the ATN either directly through the fornix or indirectly through the MTT. This could be tested with four groups of rats, bilateral MTT lesions, bilateral pre-commissural fornix lesions, a mixed lesion group with a disconnection lesion to the MTT on one side and a unilateral lesion to the pre commissural fornix on the other, and a control

group. It would be best to test these groups on the same procedures used by Vann & Aggleton (2003) i.e. working memory versions of the t-maze, RAM and water maze.

Finally, given that human cases of diencephalic amnesia involving the MTT report diffuse diencephalic damage, usually involving various thalamic nuclei, it seems unlikely that MTT damage alone is responsible for the human amnesic syndrome. Rather, damage to the MTT either bilaterally or unilaterally in combination with damage to other memory specific regions may be responsible for the severe memory impairments observed. This speculation could be tested in mixed lesion animal models. For example, it would be beneficial to find out whether contralateral MTT/ATN lesions and ipislateral MTT/ATN lesions produce a greater behavioural deficit than bilateral MTT lesions alone. The same procedure could also be adopted for MTT lesions combined with mediodorsal or intralaminar thalamic lesions. This could increase understanding of how damage to multiple diencephalic structures may interact to compound memory deficits. Furthermore the impact of these mixed lesion models on the functioning of other regions important for memory, such as the hippocampus and the retrosplenial cortex, could also be measured by looking at c-fos or acetylcholine expression (Vann & Albasser, 2009; Jenkins et al, 2004; Poirier & Aggleton, 2009; Savage et al, 2012).

4.8 Conclusions

The results of this study suggest the contribution of the MTT to the extended hippocampal memory system may not be as pronounced as previously suggested. It is clear from the reviewed literature that the MTT is consistently implicated in the human amnesic syndrome, yet little attention has been paid to this structure in the animal literature. One key study found severing the MTT bilaterally results in a transient deficit in the working memory versions of the t-maze and RAM, but a robust acquisition deficit in the working memory version of the water maze (Vann & Aggleton, 2003). However a recent study (Winter et al, 2011) was unable to replicate the robust deficit found previously in working memory, but found rats

with MTT lesions showed a mild impairment in reference memory learning and when they had to use self-movement cues for navigation. Although it seems the MTT has at least some involvement in spatial memory formation, the inconsistencies in results from this scant animal literature certainly suggests that the MTT may not be as important as previously thought. Damage to the MTT in the present study did not result in the robust behavioural deficit in the working memory version of the water maze previously reported, but again found very mild deficits in reference memory in the water maze. Future research needs to resolve the inconsistent results found in the animal literature and further delineate the functional role of the MTT in the extended memory circuit. Clearly, this has important implications for our understanding of the neuroanatomical circuits associated with episodic memory. As the MTT is consistently implicated in human amnesic syndromes, understanding its role in memory formation may suggest possible therapeutic approaches to improve the prognosis of patients with diencephalic amnesia.

5. References

Akkerman, S., Prickaerts, J., Steinbusch, H. W. M., & Blokland, A. (2012). Object recognition testing: Statistical considerations. *Behavioural Brain Research, 232*, 317-322.

Aggleton, J. P., O'Mara, S. M., Vann, S. D., Wright, N. F., Tsanov, M., & Erichsen, J. T. (2010). Hippocampal-anterior thalamic pathways for memory: uncovering a network of direct and indirect actions. *European Journal of Neuroscience, 31*, 2292-2307.

Aggleton, J.P. (2008).Understanding anterograde amnesia: Disconnections and hidden lesions. *The Quarterly Journal of Experimental Psychology,* 1-31.

Aggleton, J. P., Vann, S. D., & Saunders, R. C. (2005). Projections from the hippocampal region to the mammillary bodies in macaque monkeys. *European Journal of Neuroscience*, *22*, 2519–2530.

Aggleton, J. P., and Brown, M. W. (1999). Episodic memory amnesia and the hippocampal-anterior thalamic axis. *Behavioral and Brain Sciences, 22*, 425-489.

Aggleton, J. P., Hunt, P. R., Nagle, S., & Neave, N. (1996). The effects of selective lesions within the anterior thalamic nuclei on spatial memory in the rat. *Behavioural Brain Research*, *81*, 189–198.

Aggleton, J.P., Neave, N.J., Nagle, S. & Hunt, P.R. (1995) A comparison of the effects of anterior thalamic, mamillary body and fornix lesions on reinforced spatial alternation. *Behavioural Brain Research*, *68*, 91–101.

Aggleton, J. P., & Mishkin, M. (1983). Memory impairments following restricted medial thalamic lesions in monkeys. *Experimental Brain Research, 52,* 199-209.

Alpeeva, E. V., & Makarenko, I. G. (2009). Perinatal development of the

mammillothalamic tract and innervation of the anterior thalamic nuclei. *Brain

Research, 1248*, 1-13.

Barker, G., Bird, F., Alexander, V., & Warburton, E. (2007).Recognition memory for

objects, place, and temporal order: a disconnection analysis of the role of the medial

prefrontal cortex and perirhinal cortex. *J Neurosci, 27* (11), 2948– 57.

Beracochea, D. J., & Jaffard, R. (1990). Effects of ibotenic lesions of mammillary bodies

on spontaneous and rewarded spatial alternation in mice. *Journal of Cognitive

Neuroscience*, *2* (2), 133–140.

Beracochea, D. J., & Jaffard, R. (1987). Impairment of spontaneous alternation behavior

in sequential test procedures following mammillary body lesions in mice:

Evidence for time-dependent interference-related memory deficits. *Behavioral

Neuroscience*, *101*(2), 187–197.

Byatt, G., & Dalrymple-Alford, J. C. (1996). Both anteromedial and anteroventral thalamic

lesions impair radial-maze learning in rats. *Behavioral Neuroscience, 110*, 1335–

1348.

Carlesimo, G. A., Lombardi, M. G., & Caltagirone, C. (2011). Vascular thalamic amnesia :

A reapprasial. *Neuropsychologica, 49*, 777-789.

Carrera, W., & Bogousslavsky, J. (2006). The Thalamus and Behavior: Effects of

Anatomically Distinct Strokes. *Neurology,* 1817-1823.

Crain, R. C. (1958). Spontaneous Tumors in the Rochester Strain of the Wistar Rat. *Amer J.

Pathology, 34,* 311-335.

Cruce, J. A. F. (1975). An Audiographic Study of the Projections of the Mammillothalamic

Tract in the Rat. *Brain Research*, *85*, 211-219.

Delay, J., & Brion, S. (1969). *Le syndrome de Korsakoff*. Paris: Mason

DeVito, L. M., & Eichenbaum, H. (2010). Distinct contributions of the hippocampus and medial prefrontal cortex to the "what-where-when" components of episodic like memory in mice. *Behavioural Brain Research, 215*, 318-325.

Dix, S. L., & Aggleton, J. P. (1999). Extending the spontaneous preference test of recognition: evidence of object-location and object context recognition. *Behavioural Brain research, 99*, 191-200.

Duprez, T. P., Serieh, B. A., & Raftopoulos, C. (2005). Absence of Memory Dysfunction after Bilateral Mammillary Body and Mammillothalamic Tract Electrode Implantation: Preliminary Experience in Three Patients. *AJNR Letters, 26*, 195-197.

Dusoir, H., Kapur, N., Brynes, D. P., McKinstry, S., & Hoare, R. D. (1990). The role of diencephalic pathology in human memory disorder. *Brain, 113*,1695–1706.

Edelstyn, N. M. J., Mayes, A. R., Denby, C., & Ellis, S. J. (2012). Impairment in material-specific long-term memory following unilateral mediodorsal thalamic damage and presumed partial disconnection of the mammillo-thalamic tract. *Journal of Neuropsychology, 6*, 119-140.

Ennaceur, A., Neave, N.J. & Aggleton, J.P. (1997). Spontaneous object recognition and object location memory in rats: the effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. *Exp Brain Res., 113*, 509–519.

Field, T. D., Rosenstock, J., King, C., & Greene, E. (1978). Behavioral role of the mammillary efferent system. *Brain Res Bull, 3*,451–456.

Gaffan, E. A., Bannerman, D. M., Warburton, E. C., & Aggleton, J. P.(2001). Rats processing of visual scenes: effects of lesions to fornix, anterior thalamus, mammillary nuclei or the retrohippocampal region. *Behaviorual Brain Research, 121*, 103-117.

Gilbert, C., Gillman, J., Loustalot, P., & Lutz, W. (1958). The Modifying Influence of Diet
and the Physical Environment on Spontaneous Tumour Frequency in Rats. *Brit J
Cancer, 12*, 565-593.

Goldberg, E., Antin, S. P., Bilder, R. M., Jr., Gerstman, L. J., Hughes, J. E., & Mattis, S.
(1981). Retrograde amnesia: Possible role of mesencephalic reticular activation
in long-term memory. *Science, 213*(4514), 1392–1394.

Harding, A., Halliday, G., Caine, D., & Kril, J. (2000). Degeneration of anterior thalamic
nuclei differentiates alcoholics with amnesia. *Brain, 123*,141–154.

Jarrard, L. E., Okaichi, H., Steward, O., & Goldschmidt, R. B. (1984). On the role of
hippocampal connections in the performance of place and cue tasks: Comparisons
with damage to hippocampus. *Behav Neurosci, 98*(6), 946–954.

Jenkins, T. A., Vann, S. D., Amin, E.,& Aggleton, J. P. (2004). Anterior thalamic lesions
stop immediate early gene activation in selective laminae of the retrosplenial cortex:
Evidence of covert pathology in rats? *European Journal of Neuroscience*, 19, 3291–
3304.

Jung, Y-C., Chanraud, S., & Sullivan, E. V. (2012). Neuroimaging of Wernicke's
Encephalopathy and Korkakoff's Syndrome. *Neuropsychol Rev, 22*, 170-180.

Kim, E., Ku, J., Jung, Y-C., Lee, H., Kim, S. I., Kim, J-J., Namkoong, K., & Song, D-H.
(2010). Restoration of mammillothalamic functional connectivity through thiamine
replacement therapy in Wernicke's encephalopathy. *Neuroscience Letters, 479*, 257-
261.

Kim, E., Ku, J., Namkoong, K., Lee, W., Lee, K. S., Park, J-Y., Lee, S. Y., Kim, J-J., Kim,
S. I., & Jung, Y-C. (2009). Mammillothalamic functional connectivity and memory
function in Wernicke's encephalopathy. *Brain, 132,* 369-376.

Kopelman, M. D., Thomson, A. D., Guerrini, I., & Marshall, J. E. (2009). The Korsakoff Syndrome: Clinical Aspects, Psychology and Treatment. *Alcohol & Alcoholism, 44* (2), 148-154.

Krieckhaus, E.E., & Randall, D. (1968). Lesions of the mammillothalamic tract in rat produce no decrements in recent memory. *J Neurol 91*, 369–378.

Lloyd, R. V. (1983). Estrogen-Induced Hyperplasia and Neoplasia in the Rat Anterior Pituitary Gland: An Immunohistochemical Study. *Am J Pathology, 113*, 198-206.

Lopez, J., Wolff, M., Lecourtier, L., Cosquer, B., Bontempi, B., Dalyrymple-Alford, J., & Cassel, J. C. (2009). The Intralaminar Thalamic Nuclei Contribute to Remote Spatial Memory. *The Journal of Neuroscience, 29* (10), 3302-3306.

Loukavenko, E. A., Ottely, M. C., Moran, J. P., Wolff, M., and Dalrymple-Alford, J. C. (2007). Towards therapy to relieve memory impairment after anterior thalamic lesions: improved spatial working memory after immediate and delay postoperative enrichment. *European Journal of Neuroscience*, *26*, 3267-3276.

Markowitsch, H.J., & Staniloiu.A. (2012). Amnesic disorders. *Lancet Review, 11*, 1-12.

Mitchell, A. S., & Dalrymple-Alford, J. C. (2005). Dissociable memory effects after medial thalamus lesions in the rat. *European Journal of Neuroscience, 22*, 973–985.

Mumby, D., Gaskin, S., Glenn, M., Schramek, T.,& Lehmann, H. (2002). Hippocampal damage and exploratory preferences in rats: memory for objects, places, and contexts. *Learn Mem, 9*,49–57.

Mumby, D. G. (2001). Perspectives on object-recognition memory following hippocampal damage: Lessons from studies in rats. *Behavioural Brain Research, 127*, 159–181.

Neave, N., Nagle, S., & Aggleton, J. P. (1997). Evidence for the involvement of the mammillary bodies and cingulum bundle in allocentric spatial processing by rats. *Eur J Neurosci, 9* (5), 941–955.

Nishio, Y., Hashimoto, M., Ishii, K., & Mori, E. (2011). Neuroanatomy of a

Neurobehavioural disturbance in the left anterior thalamic infarction. *J Neurol*

Neurosurg Psychiatry, 1-6.

Papez, J.W. (1937). A proposed mechanism of emotion. *Arch. Neurol. Psychiatry, 7,* 103–

112.

Paxinos, G., & Watson, C. (1998). *The rat brain in stereotaxic coordinates* (4th ed.). San

Diego: Academic Press.

Poirier, G. L., and Aggleton, J. P. (2009). Post-surgical interval and lesion location within

the limbic thalamus determine extent of retrosplenial cortex immediate-early gene

hypoactivity. *Neuroscience*, *160*, 452-469.

Powell, T. P. S., & Cowan, W. M. (1954). The Origin of the Mammillothalamic Tract in the

Rat. *Journal of Anatomy, 88*, 489-497.

Rosenbaum, R. S., Murphy, K. J., & Rich, J. B. (2012). The amnesias. *Cogn Sci, 3*, 47-63.

Ross, M.H., Bras, G., & Ragbeer, M. S.(1969). Influence of Protein and Caloric Intake upon

Spontaneous Tumor Incidence of the Anterior Pituitary Gland of the Rat. *J Nutrition,*

100, 177-189.

Santin, L. J., Rubio, S., Begega, A., & Arias, J. L. (1999). Effects of mammillary

body lesions on spatial reference and working memory tasks. *Behav Brain Res,*

102 (1–2), 137–150.

Savage, L. M., Hall, J. M., & Rescende, L. S. (2012). Translational Models of Korsakoff

Syndrome Reveal the Critical Neuroanatomical Substrates of Memory Dysfunction

and Recovery. *Neuropsychol Rev, 22*, 195-209.

Schmued, L. C., Stowers, C. C., Scallet, A. C., & Xu, L. (2005). Fluoro-Jade C results in

ultra high resolution and contrast labelling of degenerating neurons. *Brain Research,*

1035, 24-31.

Sechi, G. P., & Serra, A. (2007). Wernicke's Encephalopathy: New Clincial Settings and Recent Advances in Diagnosis and Management. *Neurology, 6,* 442-455.

Seki, M., & Zyo, K. (1984). Anterior Thalamic Afferents from the Mammillary Body and the Limbic Cortex in the Rat. *The Journal of Comparative Neurology, 229*, 242-256.

Simon, L., Le Moal, M., & Calas, A. (1979). Efferents and Afferents of the ventral tegmental A10 region studied after local injection of [3H] leucine and horseradish peroxidise. *Brain Research, 178*, 17-40.

Squire, L. R., Amaral, D. G., Zola-Morgan, S., Kritchevsky, M., & Press, G. (1989). Description of brain injury in the amnesic patient N.A. based on magnetic resonance imaging. *Experimental Neurology, 105*, 23–35.

Sutherland, R. J., & Rodriguez, A. J. (1989). The role of the fornix/fimbria and some related subcortical structures in place learning and memory. *Behav Brain Res, 32*(3), 265–277.

Sziklas, V., & Petrides, M. (1999). The effects of lesions to the anterior thalamic nuclei on object- place associations in rats. *European Journal of Neuroscience*, *11,* 559-566.

Sziklas, V., & Petrides, M. (1993). Memory impairment following lesions to the mammillary region of the rat. *Eur. J. Neurosci, 5*, 525–540.

Thomas, G. J., & Gash, D. M. (1985). Mammillothalamic tracts and representational memory. *Behav Neurosci. 99*, 621–630.

Tsivilis, D., Vann, S.D., Denby, C., Roberts, N., Mayes, A.R., Montaldi, D. & Aggleton, J.P. (2008). The importance of the fornix and mammillary bodies for human memory: a disproportionate role for recall versus recognition. *Nat.Neurosci.,11*, 834–842.

Tulving, E., & Markowitsch, H.(1998). Episodic and declarative memory: role of the hippocampus. *Hippocampus,8*, 198–204.

Vann, S. D., Erichsen, J. T., O'Mara, S. M., & Aggleton, J. P. (2010).Selective

disconnection of the hippocampal formation projections to the mammillary bodies

produces only mild deficits on spatial memory tasks: Implications for fornix function.

Hippocampus, *00*, 1-13.

Vann, S.D. (2010). Re-evaluating the role of the mammillary bodies in memory.

Neuropsychologia, *48*, 2316-2327.

Vann, S.D., & Albasser, M. M. (2009). Hippocampal, Retrosplenial,and Prefronatal

Hypoactivity in a model of Diencephalic Amnesia: Evidence towards an

Interdependent Subcortical-cortical Memory Network. *Hippocampus*, *19*,1090–1102.

Vann, S. D. (2009). Gudden's ventral tegmental nucleus is vital for memory: Reevaluating

diencephalic inputs for amnesia. *Brain, 132*, 2372–2384.

Vann, S.D. (2005). Transient spatial deficit associated with bilateral lesions of the lateral

mammillary nuclei. *Eur. J. Neurosci., 21*, 820–824.

Vann, S. D., and Aggleton, J. P. (2004). The mammillary bodies: two memory systems in

one? *Nature Reviews/Neuroscience*, *5*, 35-44.

Vann, S. D., Honey R. C., & Aggleton, J. P. (2003). Lesions of the mammillothalamic tract

impair the aqusition of spatial but not nonspatial context conditional discriminations.

European Jounal of Neuroscience, *18*, 2413-2416.

Vann, S. D., & Aggleton, J. P. (2003). Evidence of a spatial encoding deficit in rats with

lesions to the mammillary bodies or the mammillothalamic tract. *The Journal of

Neuroscience*, *23* (8), 3506-3514.

Van der Werf, Y., Jolles, J., Witter, M. P., & Uylings, H. B. M. (2003a). Contributions of

thalamic nuclei to declarative memory functioning. *Cortex, 39*, 1047–1062.

Van der Werf, Y., Scheltens, P., Lindeboom, J., Witter, M. P., Uylings, H. B. M., & Jolles, J. (2003b). Deficits of memory, executive functioning and attention following infarction in the thalamus: A study of 22 cases with localised lesions. *Neuropsychologia, 41*, 1330–1344.

Van der Werf, Y., Witter, M. P., Uylings, H. B. M., & Jolles, J. (2000). Neuropsychology of infarctions in the thalamus: A review. *Neuropsychologia, 38*, 613–627.

Vertes, R. P., Hoover, W. P., Szigeti-Buck, K., & Leranth, C. (2007). Nucleus reunions of the midline thalamus: Link between the medial prefrontal cortex and the hippocampus. *Brain Research Bulletin, 71*, 601-609.

Victor, M., Adams, R. D., & Collins, G. H. (1989). *The Wernicke–Korsakoff syndrome and related neurologic disorders due to alcoholism and malnutrition (2nd ed.).* Philadelphia: Davies.

Warburton, E. C., & Aggleton, J. P. (1999). Differential deficits in the Morris water maze following cytotoxic lesions of the anterior thalamus and fornix transection. *Behavioural Brain Research, 98*, 27-38.

Warburton, E. C., Morgan, A. Baird, A.L., Muir, J. L., & Aggleton, J. P. (1999). Does Pretraining Spare the Spatial Deficit Associated With Anterior Thalamic Damage in Rats? *Behavioural Neuroscience, 113, (5),* 956-967.

Winter, S. S., Wagner, S. J., Mc Millin, J. L., & Wallace, D. G.(2011). Mammillothalamic lesions disrupt dead reckoning in the rat. *European Journal of Neuroscience, 33*, 371-381.

Wolff, M., Loukavenko, A. E., Will, B. E., and Dalrymple-Alford, J. C. (2008). The extended Hippocampal-diencephalic Memory system: Enriched housing promotes recovery of the flexible use of spatial representations after anterior thalamic lesions. *Hippocampus*, 1-12.

Wozniak, D. F., Hartman, R. E., Boyle, M. P., Vogt, S. K., Brooks, A. R., Tenkova, T., et al. (2004). Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functionally recovery in adults. *Neurobiology of Disease, 17*, 403-414.

Wright, N., Erichsen, J.T., Vann, S.D., O'Mara, S. & Aggleton, J.P. (2010). Parallel but separate inputs from limbic cortices to the mammillary bodies and anterior thalamic nuclei in the rat. *J. Comp. Neurol., 518*, 2334–2354.

6. Appendices

6.1 Appendix A: Black Gold II Staining Protocol

Equipment

"Subbed" slides (gelatine coated)

2×50ml beakers (for the stain and fixing solutions)

Rectangular staining trays (for the counter stain and reagents)

Water bath

Slide holders

Cover slips

Stains and Reagents

Black Gold II – Myelin stain (can be purchased in powder or liquid concentrate) We got it from biosensis initially in a kit and then later in a powdered from histo-chem a company based in Jefferson Arkansas. The black gold II can be re-used multiple times even after a fine black precipitate starts to form. 150mg should process approximately 50-60 slides. Stop using stain when myelin impregnation takes in excess of 20 minutes.

Sodium Thiosulfate- fixative (can be purchased in powder or liquid concentrate). Again initially it came in the biosensis kit but later from Sigma Aldrich as a powder (Reagent grade).

Cresyl Violet Acetate - counterstain (tech's always have some made up)

70% ethanol solution

95% ethanol solution

100% ethanol solution

95% Acid Alcohol Solution (95% alcohol with 1ml of glacial acetic acid added)

Xylene (MUST BE USED IN FUME HOOD)

DPX mounting media (also used in fume hood)

Preparation of the Specimens for staining

IMPORTANT: this type of stain does not work on fresh or paraffin embedded tissues. The rat must be perfused with either 10% formalin or 4% paraformaldehyde (preferable).

Following perfusion the brains should be left to post fix for at least 24 hours.

NOTE: If brains are left to post fix for more than 12 months the black gold may fail to capture the finest myelin fibres.

50µ coronal sections can be cut on either the vibrotome or sliding microtome. If using the sliding microtome brains should be transferred to long term solution after 24 hours in post fixative then allowed to sink before cutting. The slices should then be mounted onto the subbed slides from distilled water and allowed to dry in ambient temperature overnight.

<u>Myelin staining (after the slides have been allowed to dry)</u>

Switch on water bath and set both temperature and max temperature at 70˚c. Give the water bath at least 45mins to warm up. Setting the temp at 70˚c seems to result in a stable water temperature of 65˚c. NOTE always check the temperature with a thermometer first.

For concentrated black gold II solution (keep dark and at 4˚c):

 Add 1 part Black Gold II to 9 parts distilled water -. in a clean 50ml beaker add 5 mls of Black Gold II to 45 mls of distilled water (use a pipette/cyclinder as markings on the beaker are only a rough guide). This makes a 0.3% Black Gold II solution. If using powder 150mg of black gold II get dissolved in to 0.9% saline solution (it dissolves very easily).

In a second clean beaker add 5 mls of sodium thiosulfate to 45mls of distilled water (again 1:9). This makes a 1% Sodium Thiosulfate solution. Or powder 0.5 g to 50mls of distilled water. Put on stirrer with flea.

First rehydrate the slides in distilled water for approximately 3 minutes(if slides are dried overnight, longer periods may be necessary for extended drying times)

Place the beakers containing the Black gold into the water bath and allow it to reach 60˚ +. The stain in the beaker will not be as hot as the water in the water bath! Having the water bath at 65˚c results in a stain temperature between 60 and 62˚ (which is fine). It will take at around 10-15 minutes for the stain to reach this temperature from room temperature. NOTE the black gold will not stain unless it is at the right temperature so check the temperature before staining. The hotter the stain becomes the quicker it will impregnate the myelin so make sure to keep the stain at a constant temperature otherwise it becomes very hard to achieve the desired level of staining.

Place slides in slide holder and submerge them in the black gold solution: staining should take approximate 8-9 minutes but check under microscope every 2-3minutes until desirable level of satin is obtained. You will most likely not see any colour changes in the tissue for at least 4-5 minutes but once something starts to happen it progresses fast so keep a close eye on it. Slides are said to be ready when the finest myelin fibre in the cortex are stained. The tissue should be red in colour – pink needs longer- black/ purple too much. NOTE: the appearance of a conspicuous lavender coloured background stain indicates the tissue is becoming over stained and should be removed from the stain at once.

Rinse the slides by submerging them in a rectangular staining dish of distilled water for 2 minutes.

Remove the slides from the distilled water and submerge them in the sodium thiosulfate and incubate for 3 minutes.

Rinse the slides in 3×5minute changes of tap water.

Then into distilled water for 1 minute to remove any impurities

<u>Cresyl Violet Counterstain</u>

After being removed from the distilled water the slides (10 at a time in glass slide holder) are dipped 10× in 70% ethanol

They are then submerged in 70 % ethanol solution for two minutes

Before being rinsed for 1 minute in distilled water

Then into 250mls of 0.5% Cresyl violet acetate solution (in a rectangular staining dish) and incubated for 5-7 minutes (depends on the strength of the cresyl) at room temperature.

The slides are then rinsed in distilled water for 2 minutes each.

The slides are then dehydrated and differentiated

First, in a 70% ethanol solution for 2 minutes

Then, a 95% ethanol solution for 2 minutes

Then a 95% acid alcohol solution for 40 seconds (400mls of 95% ethanol with 1ml of glacial acetic acid added).

They are then submerged in 2× 100% ethanol for 2mins.

The dehydrated sections are then cleared in xylene for 5 minutes before being cover slipped with DPX.

Let them dry for 24 hours before use.

6.2 Appendix B

Table 6.1 Left and Right MTT tract Volume for all rats in the lesion surgery group at -3.3 from bregma.

Rat ID	Left Volume	% Reduction	Right Volume	% Reduction	Overall % Reduction
N13	59989.04	0.31	33699.58	0.61	0.46
N6	49517.08	0.43	47808.77	0.45	0.44
B3	51128.49	0.41	52699.66	0.39	0.40
R8	56943.60	0.34	57044.96	0.34	0.34
N12	46766.79	0.46	72527.06	0.16	0.31
N2	54895.42	0.37	65468.74	0.24	0.30
B12	59999.47	0.31	64350.74	0.26	0.28
R13	72436.13	0.16	53424.12	0.38	0.27
P12	66234.94	0.23	60203.69	0.30	0.27
P13	58195.76	0.33	73248.55	0.15	0.24
B13	71652.04	0.17	62813.86	0.27	0.22
B8	71021.48	0.18	65616.31	0.24	0.21
N5	77565.53	0.10	65771.34	0.24	0.17
N1	78155.83	0.10	65435.94	0.24	0.17
B2	74315.87	0.14	69828.95	0.19	0.17
P6	67664.50	0.22	77107.89	0.11	0.16
N3	75696.23	0.12	73329.04	0.15	0.14
P9	79072.59	0.09	73598.85	0.15	0.12
B1	91337.83	-0.06	67752.45	0.22	0.08
N11	84683.48	0.02	75484.55	0.13	0.07
B7	81615.68	0.06	82921.50	0.04	0.05

Control left volume mean = 86458.01 and Control right volume mean = 86484.42