METHYLENE BLUE, SPATIAL MEMORY AND ANTERIOR THALAMIC LESIONS RELEVANT TO AMNESIC DISORDERS

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ABSTRACT

While recent studies have focused on methylene blue's interaction with amyloid plaques and neurofibrillary tangles in Alzheimer's disease, no studies have investigated the efficacy of methylene blue in animal-lesion models of regional pathology relevant to AD. The goal of this dissertation was to examine the effects of methylene blue on spatial memory in aged Wistar rats with lesions to the anterior thalamic nuclei (ATN), part of an extended episodic memory system that shows early pathology in AD and diencephalic amnesia. First, 12 ATN rats were compared to 14 aged shams (18 to 22 months old) to assess spatial memory acquisition in a standard water maze task (10 days). Rats in each group then received intraperitoneal injections of methylene blue (1 mg/kg) or placebo 1 hour after each daily trial for 10 days in which acquisition of a new platform position was examined, followed by a probe trial 5 days later. Anterior thalamic lesions impaired initial acquisition of the reference memory task. In the subsequent acquisition and probe trial, methylene blue treatment vs. placebo improved spatial learning in ATN rats, but there was no effect in sham rats. These results provide the first evidence that methylene blue may prevent the learning impairments observed in rats with lesions to the anterior thalamus and supports methylene blue as a potential therapeutic intervention for older humans with memory disorders associated with injury to the ATN and the extended episodic memory system.

1. INTRODUCTION

1.1 General Introduction

Recent research has demonstrated that methylene blue can enhance memory in both normal and impaired animals. Furthermore, there is evidence from human clinical trials that methylene blue may prevent the symptoms of Alzheimer's disease, and these findings have been supported by animal literature that has revealed that methylene blue has a range of positive effects on Alzheimer's pathology. However, there have been no studies that have examined the effects of methylene blue on animals that model the pathology of older humans who have episodic memory deficits. The purpose of the present study was to establish whether a short chronic administration of methylene blue could enhance spatial memory in older rats with brain lesions in a region that is believed to play a critical role in the severe memory deficits evident in people who suffer from Alzheimer's disease and other memory disorders.

1.2 Methylene Blue

Methylene Blue (MB) is a phenothiazine that has been used in medicine for more than 100 years in the treatment of various diseases such as malaria, schizophrenia, and methemoglobinemia (Atamna et al., 1996; Bodansky & Gutmann, 1947; Deutsch et al., 1997). It is also a redox dye that has cationic and lipophilic properties that attract it to the mitochondrial membrane, and based on these qualities it is used as a selective supravital stain for neurons (Visarius et al., 1997). Its redox couple feature allows it to be an electron mediator between enzymes and substrates. Low dose methylene blue enters mitochondria,

where it diverts a portion of the electron flow to molecular oxygen (Hassen & Fridovich, 1979). Additionally, MB acts as a potent antioxidant that competitively inhibits the reduction of molecular oxygen to superoxide by acting as an alternative electron acceptor (Riedel et al., 2003). The molecular formula for MB is $C_{16}H_{18}N_3SCl$ (Fig. 1).

The half-life of MB is 5-6.5 hours and research has demonstrated that it is able to cross the blood brain barrier when administered intraperitoneally to rats (Peter et al., 2000). While high doses of MB can have dangerous effects in humans (Martinez, Jr., et al., 1978), it has been demonstrated that therapeutic doses of MB have little to no side effects that could affect the behaviour or health of subjects. Animal studies have indicated that MB may have both anxiolytic and antidepressant effects. For example, Eroglu & Caglayan (1997) have reported that doses of 7.5 - 30mg/kg MB increased entries to the open arms of the elevated plus maze in mice and doses of 15 and 30mg/kg significantly decreased immobility time in the forced swim test. However, Bruchey & Gonzalez-Lima (2008) suggest that it is unlikely that these behavioural effects induced by MB influence performance on memory tasks.

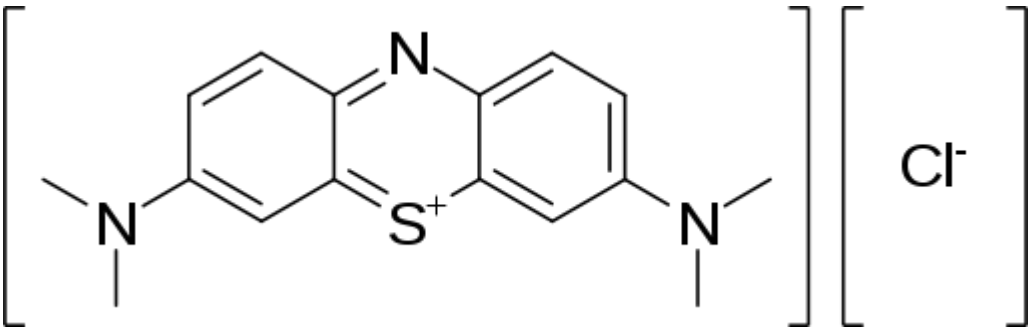


Figure 1: The structural formula of methylene blue.

1.3 Methylene Blue and Memory Enhancement

Recent research on MB's neurobiological and behavioural actions suggests that MB has powerful metabolic enhancing effects that facilitate memory. The memory enhancing effects of MB were first reported by Martinez, Jr. et al. (1978), who discovered that low dose post-training administration of MB improved memory retention in an inhibitory avoidance task in rats. They showed that 1 mg/kg MB given immediately post-training improved memory retention tested one day later, whereas animals given a 50 mg/kg dose of MB showed impaired learning. These results suggested that low-dose MB administration could facilitate the memory consolidation occurring in the brain following a learning session and that higher doses had a negative impact on memory.

These findings led Callaway and colleagues (2002) to propose that the effects of MB on memory retention demonstrated in the study by Martinez, Jr. et al. (1978) may have been due to MB increasing mitochondrial respiration during a critical period of memory consolidation. MB increases mitochondrial oxygen consumption by shuttling electrons to oxygen in the electron transport chain (Visarius, et al. 1997). Callaway et al. (2002) found that post-training administration of 1 mg/kg of MB reversed spatial memory deficits in a hole-board maze produced by impaired mitochondrial respiration in rats given sodium azide, an inhibitor of cytochrome c oxidase (CO). CO is the terminal enzyme in the mitochondrial electron transport chain and is responsible for the utilisation of oxygen for metabolic energy production in the brain (Wong-Riley, 1989). Impaired CO activity has been associated with memory deficits, and is linked to various neurodegenerative diseases, such as Alzheimer's disease (Gonzalez-Lima, 1998).

There is also evidence from animal research that during acquisition of spatial memory tasks there is an increase in CO activity in brain regions associated with spatial learning. For example, Mendez-Lopez and colleagues (2010) recently found that CO activity was highest in the dentate gyrus, medial prefrontal cotrex, and the mammilary region following spatial memory training in the water maze. Thus, Callaway and colleagues conducted a follow up study (2004) where they demonstrated that low dose MB enhanced brain CO levels, as tested *in vitro* in brain homogenates and *in vivo* 24 hours after administration to rats. Rats administered 1 mg/kg MB in this study also showed significantly improved memory retention in a baited hole-board task compared to those treated with saline. Together, these studies suggest that an increased oxidative metabolic capacity in the brain, as measured through increased CO activity, is the potential mechanism whereby MB improves memory retention.

Further studies have investigated the effects of MB on more complex learning tasks. Gonzalez-Lima & Bruchey (2004) found that memory retention of extinction of Pavlovian fear conditioning was enhanced with post-extinction administration of MB. Moreover, this enhancement was related to an increase in CO activity in the prefrontal cortical regions, brain regions that are associated with mechanisms of extinction memory. This study was extended by Wrubel et al. (2007a) who found that MB administration improved retention of the extinction memory as demonstrated by significant decreases in fear renewal in an animal model for susceptibility to learned helplessness. Therefore, Wrubel and colleagues' results suggest that low-dose MB administered in conjunction with exposure behavioural therapy in humans may be a useful therapeutic agent to facilitate retention of extinction of conditioned fear or other traumatic memories (Gonzalez-Lima & Bruchey, 2004). Other research has examined the effects of MB on discrimination learning tasks that require repeated days of

training. While previous studies using the hole-board maze focussed purely on MBs effect on spatial memory, Wrubel et al. (2007b) used a task where the goal was not simply to find where the baited holes were but also to discriminate "when" non-reward trials were given. This study therefore tested temporal memory as well as spatial memory. They found that, following three days of discrimination training, rats treated daily with post-training MB (1 mg/kg) reliably discriminated rewarded and non-rewarded trials, and that no such discrimination effects were observed in controls.

MB has a half-life of 5-6.5 hours (Peter et al., 2000). Therefore, Bruchey & Gonzalez-Lima (2008) have suggested that it is unlikely that an increase in memory retention observed more than 24 hours after the last injection of MB will reflect a continued direct action of the drug. Rather, they propose that such changes to memory are probably due to secondary brain metabolic effects occurring at a critical time in memory consolidation. MB increases CO enzymatic activity in a use-dependent manner, with brain regions requiring highest metabolic demand during memory consolidation in a particular task showing the largest increases in CO activity (Gonzalez-Lima & Bruchey, 2004). Although MB administration leads to enzyme induction after a few hours, enhanced metabolic capacity in the brain is not evident untill the following day (Bruchey & Gonzalez-Lima, 2008). Thus, MB does not show memory enhancing effects instantly, and MB treated subjects tend to demonstrate day to day improvement in performance. This indicates that MB should show beneficial effects in acquisition when the learning task requires daily testing and drug administration is given on a daily basis. However, very few studies of this nature have been undertaken thus far. Based on previous studies (summarised in Table 1.) demonstrating the memory enhancing effects of MB, it appears to be a useful compound not only in models of metabolic impairment, but also in normal subjects. It is therefore a very promising drug for memory improvement and, if

Table 1. Summary of animal studies that examine the memory enhancing effects of methylene blue

Year	Study	Impairment/ Species/ Treatment Protocol	Behavioural Tasks	Outcomes
1978	Martinez Jr., Jensen, Vasquez, McGuiness, & McGaugh	Non-impaired mice/ Single dose following training (Either 0.01, 0.1 or 1 mg/kg)	Inhibitory avoidance response task	-Memory improvement following 1 mg/kg dose
2002	Callaway, Riha, Wrubel, McCollum, & Gonzalez-Lima	Metabolically-impaired rats using sodium azide/ Single dose following training (1 mg/kg)	Spatial reference memory task using baited holeboard maze	-Memory Improvement, equal to non-impaired rats
2004	Callaway, Riha, Bruchey, Munishi, & Gonzalez-Lima	Non-impaired rats/ 5 days repeat administration (1 mg/kg)	Spatial reference memory task using baited holeboard maze	-Memory Improvement -CO activity increased
2004	Gonzalez-Lima & Bruchey	Non-impaired rats/ 5 days repeat administration (4 mg/kg)	Fear acquisition/extinction memory task using tone-shock pairing	-Improved retention of the extinction memory -CO activity increased
2005	Riha, Bruchey, Echevarria, & Gonzalez-Lima	Non-impaired/ rats/ Single dose following training (4 mg/kg)	-Open field habituation -Object Recognition	-Improved behavioural habituation -Improved recognition memory -CO activity increased
2007a	Wrubel, Barret, Shumake, Johnson, & Gonzalez-Lima	Congenitally helpless rats (learned helplessness model)/ 5 days repeat administration (4 mg/kg)	Fear acquisition/extinction memory task using tone-shock pairing	-Improved retention of the extinction memory
2007b	Wrubel, Riha, Maldonado, McCollum, & Gonzalez-Lima	Non-impaired rats/ 3 days repeat administration (1 mg/kg)	Spatial discrimination memory task using baited holeboard maze	-Memory Improvement -CO activity increased

administered correctly and in low doses, could provide a potent resource for those looking for memory enhancing compounds with little to no side effects for the treatment of neurodegenerative disorders.

1.4 Methylene Blue and Alzheimer's Disease

Recently, the potential of MB to slow down cognitive decline in Alzheimer's disease (AD) has attracted attention. AD is the most common cause of dementia in the elderly affecting approximately 13% of adults over 65 in the United States (The Alzheimer's Association, 2010). Clinically, it is characterised by progressively severe memory loss, neuropsychiatric disturbances, and eventual death (Barten & Albright, 2009). Histological hallmarks are extracellular deposits of β-amyloid protein in neuritic plaques, intracellular neurofibrillary tangles caused by the abnormal aggregation of tau protein, and neuronal cell loss, particularly affecting the cholinergic system. Recent studies have demonstrated that MB has action on many of the cellular and neurotransmitter pathologies observed in AD, and these findings highlight its potential as a therapeutic intervention not only for AD but for a range of human memory disorders.

Strong evidence exists that the accumulation of the β-amyloid protein plays a major role in AD pathogenesis. β-amyloid protein aggregation, which leads to extracellular fibrillar deposits known as amyloid plaques, is a complex process involving either an increase in the protein's production or a decrease in its degradation (Hardy & Selkoe, 2002). Although the mechanisms underlying the oligomerization and fibril formation of amyloid plaques is not clearly understood, it is pertinent that MB has been shown to inhibit β-amyloid oligomerization in vitro (Necula et al., 2007). In addition, MB administered to preformed β-

amyloid have been expanded on in animal studies using transgenic mice. Medina et al. (2010) examined MB using the transgenic mouse model 3xTg-AD, which develops age-dependent accumulation of β-amyloid and tau. They observed that chronic dietary MB treatment reduced β-amyloid protein levels and improved the learning and memory impairments observed in this mouse model. This study concluded that the mechanisms underlying the effects of MB on β-amyloid pathology was mediated by an increase in proteasome function. The proteasome system is involved with the cleavage, and subsequent clearing, of β-amyloid, and decreased proteasome activity has been reported in human AD brains (Oddo, 2008). However, exactly how MB effects proteasome function is yet unknown and the authors have recommended that further research needs to be undertaken in order for the mechanisms by which MB increases proteasome activity to be better understood (Medina et al., 2010).

Neurofibrillary tangles (NFTs) consist of paired helical filaments and straight filaments, which are made of the microtubule-associated protein tau in a hyperphosphorylated state (Iqbal & Novak, 2006). The accumulation of hyperphosphorylated tau protein correlates with neuronal loss and the severity of cognitive impairment in AD. In an early study by Wischik et al. (1996), a number of phenothiazines were investigated for their potential to inhibit aggregates of truncated tau protein. MB was among those phenothiazines that were found to be successful in this task through reversing the proteolytic stability of paired helical tau filaments. Similar results were observed in a more recent study, where it was found that MB inhibits heparin-induced assembly of tau protein into filaments in vitro (Taniguchi et al., 2005). It is acknowleged that a four-repeat peptide in the microtubule-binding domain of tau protein plays an essential role in filament formation (Friedhoff et al., 2000), and therefore, the importance of this repeat domain in the inhibitory effects of MB on filament formation was also recently investigated in an in vitro study (Hattori et al., 2008). In this study, it was

demonstrated that the inhibitory responses with respect to heparin-induced filament formation to the second and third repeat peptides of the mictotubule-binding domain were not altered by MB. This suggests the importance of the first and fourth repeat peptides in the inhibitory activity of MB for tau filament formation.

Given its tau-dissolving properties, the efficacy of MB as a therapeutic for AD has been tested using transgenic mouse models of human tau pathology. Harrington et al. (2008) examined the effects of MB on tau pathology using a transgenic mouse model that overexpressed the repeat domain of tau. Post-mortem examination showed that these mice presented with abundant tau pathology in the hippocampus and entorhinal cortex, while those transgenic mice treated with 2 weeks of oral MB showed a significant reduction in counts in tau positive neurons in these regions. In addition to these findings, a separately published study using the same sample showed that the transgenic mice treated with MB showed a reduction in spatial problem solving deficits in a water maze task (Melis et al., 2008). Nevertheless, these results have been challenged in more recent transgenic animal studies. A study by O'Leary et al. (2010) showed that while MB is capable of reducing tau formation and improving cognition at high doses (1 mM infused directly into the hippocampi), it did not affect pathology in mice with pre-existing tangle formation and neurodegeneration. Similarly, MB failed to inhibit abnormal tau phosphorylation and neuronal cell loss, and did not ameliorate a swimming defect in a zebrafish model of tauopathy (van Bebber, 2010). Therefore, these contrary studies suggest that MB may not reduce exisiting tau pathology, but may provide neuroprotection by reducing soluble tau levels.

Methylene blue has also been investigated in a phase 2 human clinical trial with a formulation called Rember™ (Wischik et al., 2008). An exploratory, placebo-controlled trial

conducted in 332 individuals diagnosed with mild to moderate AD found that after six months those receiving the compound experienced a significant improvement in cognitive function as compared to those receiving placebo. Over the course of a year, MB slowed the progression of AD by 81%, with SPECT- and PET-scan evidence showing increased glucose metabolism in brain regions affected by tau pathology (Murray et al., 2008). However, while these results appear promising, criticism has been raised concerning the clinical trial's validity. The results have, up until now, been presented at a conference but have not yet been published in a peer-reviewed journal. In addition, the gelatine capsules containing the highest MB dose were reportedly defective, and the data of this dosage group was folded into the placebo group (Sullivan, 2008). Furthermore, issues with study blinding have been put forward as MB turns the subjects urine blue (Sullivan, 2008). There has been little information released on the progress of these human trials following 2008 and, consequently, further clinical studies are required to investigate the potential of MB as a therapeutic intervention for human AD.

While the majority of research investigating MB as a therapeutic for AD has focussed on its effects on tau and amyliod pathology, there is also evidence that the drug interacts with the cholinergic system, which is the target of current AD medications. The cholinergic system plays an important role in the regulation of learning and memory and studies have linked cholinergic dysfunction to aspects of the neuropathology and cognitive dysfunction in AD (Nordberg, 2006). The primary medications currently prescribed to AD patients are various acetylcholinesterase inhibitors (AChEIs), although treatment effects are often mild. It is generally recognised that these treatments offer only temporary symptomatic benefit and there is little evidence that they modify the course of the disease (van Dam et al., 2008). The cost-effectiveness of these AChEI medications have also been questioned (Kaduszkiewicz et

al., 2005). There is evidence that MB acts as a cholinesterase inhibitor (Pfaffendorf et al., 1997), and thus currently prescribed AChEIs have been compared with MB using a pharmacological animal model of AD. In a study by Deiana and colleagues (2009), it was demonstrated that MB was superior to the commonly-used AChEI, rivastigmine, in reversing a spatial-learning impairment induced by anti-cholinergic scopolamine treatment. Furthermore, when combined with rivastigmine the effect of MB was potentiated, suggesting that some of the side effects associated with higher rivastigmine doses could be avoided by adding low doses of MB to lower doses of rivastigmine (Deiana et al., 2009).

Another medical benefit for using MB in the treatment of AD has been proposed through its neuroprotective effects via enhanced mitochondrial function and increased heme synthesis. As explained previously, MB improves neuronal metabolic energy production through increasing CO activity. Impaired CO activity is a recognised feature of AD (Gonzalez-Lima, 1998), with a decrease in CO activity leading to cytotoxicity, increased oxidant production, and decreased energy charge of the mitochondria (Barros et al., 2004). Mitochondrial dysfunction and oxidative stress are thought to be key abnormalities that lead to cellular senescence and aging (Atamna et al., 2008). MB may therefore be useful in delaying mitochondrial dysfunction with aging and the decrease in CO in AD. Furthermore, MB increases heme synthesis (Atamna et al., 2008). An increase in heme synthesis is necessary to support the assembly of CO, and also supports other metabolic functions involved in decreased oxidant production (Carr & Winge, 2003).

Another proposed mechanism of action of MB that could contribute to its positive effects on neurodegenerative disorders involves its antioxidant properties. MB inhibits superoxide by accepting electrons from tissue oxidases (Kelner et al., 1988; Salaris et al.,

1991). It is also an inhibitor of nitric oxide synthase (Mayer et al., 1993) which can form free radicals after reacting with superoxide. A recent study has examined the effects of MB as an antioxidant to protect against rotenone-induced neurodegeneration in an animal model of optic neuropathy (Zhang et al., 2006). Rotenone is a neurotoxin that induces severe neurodegeneration, and previous studies have shown that it is associated with motor function impairment in the rat when selectively infused into the striatum (Sindhu et al., 2006). Neurodegeneration in the retinal ganglion cell layer 24 hours after rotenone injection was completely prevented by the injection of methylene blue plus rotenone. The author proposed that MB administration enhanced oxygen consumption and acted as an antioxidant in this study, preventing oxidative damage and cell death. The neuroprotective effects of MB have also been examined using rats with neurotoxic lesions to the striatum using rotenone (Rojas et al., 2009). Rotenone induced large anatomical lesions resembling "metabolic strokes", and the size of these lesions was greatly reduced in MB-treated rats. In addition, MB prevented the decrease in CO activity and the perilesional oxidative stress associated with rotenone infusion in the striatum. These findings support the notion that MB could be a valuable intervention against neural damage associated with neurodegenerative disorders.

Prior to the study by Rojas et al. (2009), there was no research that examined the effects of MB using animal lesion models that attempt to represent the regional pathology of AD and other amnestic disorders. However, a recent study by Riha et al. (2010) investigated the memory-enhancing properties of MB in rats that received an infusion of sodium azide, a CO inhibitor, directly into the retrosplenial cortex. Retrosplenial cortex hypometabolism is a common feature in neurodegenerative disorders and results in oxidative damage, neurodegeneration and memory deficits (Mosconi et al., 2006; Vann & Aggleton, 2002). Riha et al. (2010) found that while sodium azide infusions into the retrosplenial cortex induced

impairments in visuospatial memory in a holeboard food-search task, an intraperitoneal dose of 4 mg/ kg MB immediately following surgery prevented such impairments. Administration of MB to rats with the metabolic lesions also reduced the retrosplenial cortex lesion volume and partially restored CO activity in brain regions disrupted by the lesion, such as the thalamus and hippocampus. This study is relevant to mild cognitive impairment and AD as it is the first to demonstrate that systemic MB administration can prevent the hypometabolic damage and impaired behavioural effects induced by mitochondrial insult. These findings support further investigation of MB using animal lesion models of neurodegenerative disorders.

As summarised in Table 2., the positive effects of MB on memory have been demonstrated in animals expressing both β-amyloid pathology (Medina et al., 2010) and tau pathology (Harrington et al., 2008; Melis et al., 2008), and also in rats with cholinergic dysfunction (Deiana et al., 2009). Other recent studies have shown that MB is affective in ameliorating memory impairment in animals with "metabolic" lesions to brain regions associated with memory. Considering that the most recent study by Riha et al. (2010) examined animals with lesions to the retrosplenial cortex, a region that plays an integral part in normal episodic memory (Aggleton, 2008). it is therefore important that research extends upon their findings by examining MBs effect on animals with lesions to other regions of the brain involved in learning and memory, and that are implicated in human memory disorders. The current study aimed to examine the effect of MB on spatial memory in rats with neurotoxic lesions to the anterior thalamus. Rats with lesions to the anterior thalamus make an ideal model for amnestic disorders as this region has been heavily implicated in memory and learning (Aggleton, 2008; Kopelman, 2002). The following is an introduction to the anterior thalamus and a review of animal experimental findings pertaining to anterior

thalamic involvement in memory functioning in order to demonstrate the rationale for using this lesion model to examine the memory enhancing effects of MB.

1.5 The Anterior Thalamus and Diencephalic Amnesia

The thalamus is a part of a group of brain structures that constitue a major part of the diencephalon. The thalamus consists of a large number of nuclei, including the Anterior Thalamic Nuclei (ATN) (Fig 2.). The importance of the ATN is that they form part of a circuit that includes the hippocampus, mammilary bodies and cingulate/retrosplenial cortices. It has been proposed that this circuit is primarily involved in episodic memory (Aggleton & Brown, 1999). The ATN receive bilateral input from the hippocampus either directly via the subicular complex through the fornix (Aggleton, 1986) or indirectly through the mammilary bodies and the mammilothalamic tract (Vann et al., 2007). The ATN also have reciprocal connections with the retrosplenial cortex. This cortical region provides a route for indirect projections from the diencephalon to the temporal lobe (Amaral & Price, 1984). The ATN project directly back upon the hippocampal formation (De Vito, 1980). Aggleton and Brown (1999) suggest that within this "extended hippocampal system" the ATN play a major role in diencephalic amnesia and that they function in a reciprocal fashion with the hippocampus.

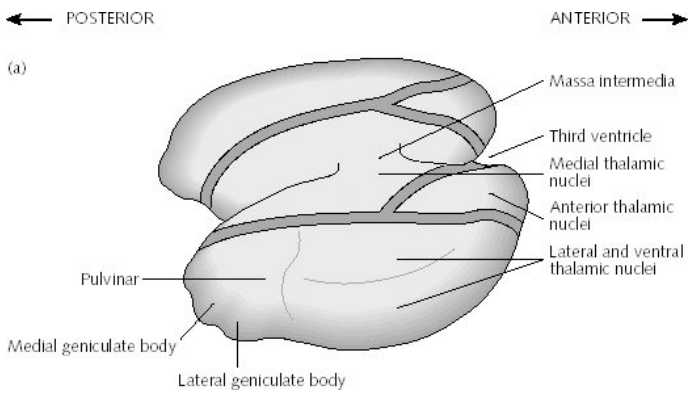


Figure 2. Representation of the thalamus and its nuclei.

Table 2. Summary of animal studies examining the effects of methylene blue in animal models of Alzheimer's disease/ neurodegeneration

Year	Study	Sample- Impairment/ Treatment Protocol	Behavioural Tasks	Outcomes
2006	Zhang, Rojas, & Gonzalez-Lima	Rotenone-induced optic neuropathy in rats/ local microinjection of MB (70 µg/kg)	none	Optic neurodegeneration prevented in a dose-dependent manner.
2008	Harrington, Rickard, Horsley, Harrington, Hindley, Riedel, Theuring, Seng, & Wischik/ Melis, Deiana, Zabke, Stamer, Harrington, Riedel, Theuring, Seng & Wischik	Transgenic mice expressing tau-pathology/ 14 days repeat oral administration (15 or 45 mg/kg)	Spatial reference memory in the water maze	-Reduction in tau pathology -Memory improvement
2009	Deiana, Harrington, Wischik, & Riedel	Anti-cholinergic scopolamine-induced amnesia in mice/ Single dose of 0.15-4 mg/kg MB in mono or in combination with rivastigmine	Spatial reference memory in the water maze	-Memory improvement in a dose dependent manner when MB administered alone. -Enhanced memory improvement when combined with rivastigmine
2009	Rojas, Simola, Kermath, Kane, Schaller, & Gonzalez-Lima	Rotenone-induced striatal neuropathy in rats/ Local microinjection of MB (70 µg/kg)	-Open field task -Dot removal test -Vibrissae-evoked forelimb placing	-Reduction in lesion volume -Increased CO activity and functional connectivity of motor regions. -Prevention of sensorimotor dysfunction elicited by rotenone
2010	Medina, Caccamo, & Oddo	Transgenic mice expressing tau and Aβ-pathology/ 16 weeks of repeat oral administration (25 mg/kg)	Spatial reference memory in the water maze	-Memory improvement -Reduction in Aβ-pathology -No reduction in tau-pathology -No effect on CO activity

2010	O'Leary, Li, Marinec, Blair, Congdon, Johnson, Jinwal, Koren, Jones, Kraft, Peters, Abisambra, Duff, Weeber, Gestwicki, & Dickey	Transgenic mice expressing tau pathology/ Single administration of with 1mM or 0.1mM MB infused into the hippocampi.	Spatial reference memory in the water maze	- Memory improvement only after high dose. -No reduction in existing tau-pathology. -Reduction in further tau formation after high dose.
2010	van Bebber, Paquet, Hruscha, Schmid, & Haass.	Transgenic zebrafish expressing tau pathology/ Embryos were cultured in a solution containing either 10µM or 100µM MB for up to 6 days.	Escape response test	-No reduction in tau pathology -No effect on dysfunctional swimming behaviour
2010	Riha, Rojas, & Gonzalez-Lima	Sodium-azide induced retrosplenial cortex hypometabolism in rats/ Single dose (4 mg/kg) following surgery	Spatial reference memory in the baited holeboard maze.	-Memory improvement demonstrated in probe trial -Reduction in lesion volume -Increased CO activity in regions connected to the retrosplenial cortex

Until recently there has been a firmly held belief that the thalamus functions primarily as a relay station which transmits information from the limbic regions to the cortex (Jones, 1985), and that memory deficits observed after thalamic lesions may arise from a disconnection to the information flow between the brain regions involved in memory (Macchi & Jones, 1997). However, more recent research suggests the anterior thalamus contributes to episodic memory operation and that its function is not just limited to passively processing hippocampal outputs (Aggleton, 2008). It is now almost universally agreed that the hippocampal formation is a crucial region implicated in anterograde amnesia (Squire et al., 2004), a condition defined by the presence of a severe deficit for the recall of recent events that contrasts with intact short-term memory, semantic memory IQ, and skill learning (Parkin, 1991). While early reports suggested that hippocampal damage and diencephalic damage might produce qualitatively different kinds of memory dysfunction (Huppert & Piercy, 1978), the current view is that damage to either region results in an amnesic syndrome that is characterised by a common impairment in anterograde and retrograde declarative memory but intact non-declarative memory (Gold & Squire, 2006).

Diencephalic amnesia is most readily observed in cases of Korsakoff's syndrome, a disorder arising from alcohol abuse and thiamine deficiency (Kopelman, 1995), as well as in other cases of thalamic damage produced by tumours, cysts and thalamic infarcts. Cases of Korsakoff's syndrome in particular provide strong evidence of the contribution of the ATN to memory functioning. For example, a study by Harding et al. (2000) analysing the post-mortem brain tissue of Korsakoff's syndrome concluded that neuronal loss in the ATN was the most critical site for the presentation of amnesia, and not degeneration of the mammillary bodies or other thalamic regions. Alzheimer's dementia affects most regions in the limbic system, including the ATN. It has been suggested that these changes in the ATN play a

critical part in the emergence of the severe memory problems associated with AD (Braak & Braak, 1991a; Johnson et al., 1998). Post-mortem examination reveals that both anterior thalamic and hippocampal changes occur relatively early in Alzhiemer's pathology. Braak & Braak (1991a) suggest that these neuropathological thalamic changes may be responsible for hampering the transmission of information through limbic circuits and that this may lead to the cognitive deficits observed in the disease. Evidence from these neuropathological conditions highlights the role of the ATN in memory impairment. However, damage to the ATN in humans is often accompanied by damage to other thalamic nuclei, as well as white matter tracts that transverse the area, making direct relationships between structure and function hard to ascertain (Aggleton, 2008). Therefore, animal research using precise lesion placing is essential in clarifying the functional role of the ATN and has been used to create an animal model that replicates the cognitive impairments present in human memory disorders.

1.6 Experimental Anterior Thalamic Lesions

Permanent lesions to the ATN as well as lesions to the individual sub-nuclei in rats produce impairments in spatial memory as observed in behavioural tasks such as the water maze (Sutherland & Rodriguez, 1989; Van Groen et al., 2002; Wolff et al., 2008), radial maze (Mitchell & Dalrymple-Alford, 2006; Sziklas & Petrides, 2007), and T maze (Ward-Robinson et al., 2002) (Table 3.). These deficits remain despite the rats being extensively pre-trained before surgery, at least as observed in T-maze and standard water maze tasks (Warburton et al., 1999). There is little indication that ATN lesions impair memory of other classic non-spatial tasks, such as object recognition (Aggleton et al., 1995), sensory preconditioning (Ward-Robinson et al., 2002), configural learning (Moran & Dalrymple-Alford, 2003) or egocentric (motoric) learning (Wolff et al., 2008).

The ATN consists of three subnuclei: anteroventral (AV); anteromedial (AM) and anterodorsal (AD). Questions have been raised as to whether damage to all or just some of the subcomponents is sufficient to produce the observed deficit in spatial memory. A study by Aggleton et al. (1996) has demonstrated that long-term persistent deficits on spatial memory tasks, such as a T-maze alternation task, are more likely to be obtained when damage to the whole complex is sustained, whereas AM only or AV plus AD lesions result only in delayed task acquisition. Van Groen et al. (2002) have also demonstrated that while selective damage to the anterior thalamus' individual components produces impairments on working and reference memory tasks in the water maze, the animals were able to acquire the tasks over time. Total ATN damage, on the other hand, produced severe impairment with no improvements evident across trials. Such evidence strongly suggests that lesions involving all three anterior thalamic nuclei are required to produce severe longer-lasting deficits on tests of spatial memory processing. However, relatively minor non-specific damage to the ATN (i.e., when passing fibres are also affected) can produce severe deficits and may potentially contribute to the severity the observed diencephalic amnesia when the insult is sustained elsewhere (Byatt & Dalrymple-Alford, 1996).

From such studies as those summarised in Table 3, it has been acknowledged that the ATN is involved in spatial-working memory processing as well as the acquisition of spatial reference memory. This evidence highlights the functional similarity between the ATN and the hippocampus (Aggleton, 2008). Other evidence that supports the notion that the ATN and hippocampus are part of a functional system comes from studies that employ a cross-lesion method. In cross-lesion studies a unilateral lesion is placed in both structures of interest but in different cerebral hemispheres. The rationale behind such experiments is that unilateral

Table 3. Summary of animal studies examining the effects of anterior thalamic lesions on memory.

Year	**Study**	**Lesion Method**	**Behavioural Tasks**	**Outcomes**
1989	Sutherland & Rodriguez	ATN/ Electrolytic/ Mice	1. Working memory in the Water maze 2. Reference memory in the Water maze	1. Lesion= impaired 2. Not impaired for same platform position, but impaired for position change.
1989	Beracochea, Jaffard & Jarrard	ATN/ Ibotenic/ Rats	1. 12-arm Radial maze 2. Spatial reversal task	1. Lesion = non-impaired 2. Lesion = non-impaired
1991	Peinado-Manzano & Pozo-Garcia	ATN/ Electrolytic/ Rats (Mediodorsal thalamus also damaged)	Operant delayed alternation task	Lesion = impaired
1991	Beracochea & Jaffard	ATN/ Ibotenic & Alcohol/ Mice	Spontaneous alternation task in T maze	Lesion = impaired
1991	Aggleton, Keith & Sahgal	ATN + Fornix/ NMDA/ Rats	Operant delayed matching to postion	Lesion = impaired
1994	Beracochea & Jaffard	ATN/ Ibotenic/ Mice	1. Delayed alternation task in T maze 2. Sequential alternation task in T maze	1. Lesion = impaired after long delay, but not after brief delay. 2. Lesion = non impaired
1996	Byatt & Dalrymple-Alford	Anterventral and Antermedial nuclei of ATN/ Radiofrequency/ Rats	1. Working memory in 12-arm Radial maze 2. Reference memory in 12-arm Radial maze	1. Lesion = impaired 2. Lesion = impaired

1996	Aggleton, Hunt, Nagle & Neave	ATN, Antermedial nucleus only, or Anterventral & Anterodorsal nuclei only/ NMDA/ Rats	1. Forced alternation task in T maze 2. Egocentric discrimination task 3. Reference memory in 8-arm Radial Maze	1. All lesions impaired, but full ATN lesions showing more impairment. 2. All lesions non-impaired. 3. Full ATN and Anteroventral/Anterodorsal lesions impaired, but Antermedial-only lesion non-impaired.
1999	Warburton & Aggleton	ATN or ATN+Fornix/ NMDA & Radiofrequency/ Rats	1. Reference memory in Water maze + Probe Trial. 2. Forced alternation task in T maze 3. Spontaneous object recognition task	1. Both lesions impaired. 2. Both lesions impaired, but ATN+Fornix worse. 3. Both lesions non-impaired.
1999	Sziklas & Petrides	ATN/ Electrolytic/ Rats	1. Reference memory in 8-arm radial maze 2. Spatial visual assocation task 3. Visual egocentric task in T maze	1. Lesion = impaired 2. Lesion = impaired 3. Lesion = non-impaired
2000	Warburton, Baird, Morgan, Muir & Aggleton	ATN+Fornix Ipsilateral, ATN+Fornix Contralateral, or ATN+Fornix+Hippocampus Contralateral/ Cytotoxic/ Rats	1. Spontaneous object recognition 2. Object location in place task 3. Forced alternation task in T maze 4. Reference memory in Water maze 5. Reference memory in 8-arm Radial maze 6. Working memory in T maze	1. All lesions non-impaired 2. All lesions impaired 3. All lesions impaired 4. All lesions impaired 5. All lesions impaired 6. All lesions impaired

2002	Ward-Robinson, Wilton, Muir, Honey, Vann & Aggleton	ATN/ NMDA/ Rats	1. Non-spatial sensory pre-conditioning to fear 2. Conditioned taste aversion 3. Spatial forced Alternation task in T maze	1. Lesion = non-impaired 2. Lesion = non-impaired 3. Lesion = impaired
2002	Van Groen, Kadish & Wyss	Anterodorsal+Anteroventral nuclei, or Anterdorsal+Anteroventral+ Anteromedial nucei/ Ibotenic/ Rats	1. Reference memory in Water maze	1. All lesions = impaired
2003	Moran & Dalrymple-Alford	ATN or Perirhinal cortex/ NMDA/ Rats	1. Reference memory in 12-arm Radial maze 2. Configural learning task 3. Spontaneous object recognition task	1. ATN lesion only = impaired 2. Perirhinal cortex lesion only= impaired 3. Both lesions = non-impaired
2004	Sziklas & Petrides	ATN/ Electrolytic/ Rats	1. Conditional associative learning task in the T maze	1. Lesion = non-impaired
2004	Henry, Petrides, St-Laurent & Sziklas	ATN unilateral + Hippocampus unilateral/ Electrolytic/ Rats	1. Spatial conditional associative task 2. Delayed forced alternation task in T maze	1. Combined lesion = impaired 2. Combined lesion = impaired
2005	Mitchell & Dalrymple-Alford	ATN, Lateral Thalamus, or Medial Thalamus/ NMDA/ Rats	1. Working memory in 8-arm Radial maze 2. Reference Memory in 8-arm Radial maze 3. Conditional associative learning task	1. ATN & Lateral Thalamus = impaired; Medial Thalamus = non-impaired 2. ATN & Lateral Thalamus = impaired; Medial Thalamus = non-impaired 3. ATN & Lateral Thalamus = non- impaired; Medial Thlamus = impaired

2006	Wolff, Gibb & Dalrymple-Alford	ATN/ NMDA/ Rats	1. Memory for temporal order of list of odours	1. Lesion = impaired
2006	Mitchell & Dalrymple-Alford	ATN or Lateral Thalamus/ NMDA/ Rats	1. Working memory in the Plus maze 2. Working memory in the 8-arm Radial maze	1. Lateral thalamus only = impaired 2. ATN only = impaired
2006	Gibb, Wolff, & Dalrymple-Alford	ATN, Lateral Thalamus, or Medial Thalamus/ NMDA/ Rats	1. Odour-place paired-association task 2. Spatial and odour discrimination	1. ATN and Lateral Thalamus = impaired; Medial Thalamus = non-impaired 2. All lesions = non-impaired
2007	Sziklas & Petrides	ATN/ Electrolytic/ Rats	1. Visual-spatial conditional associative learning task 2. Working memory in 8-arm Radial maze	1. Lesion = non-impaired 2. Lesion = impaired
2007	Loukavenko, Ottley, Moran, Wolff & Dalrymple-Alford	ATN/ NMDA/ Rats	1. Working memory in T maze	1. ATN lesion = impaired, but non-impaired if housed in enriched environment at both 5 and 40 days following surgery
2008	Wolf, Gibb, Cassel & Dalrymple-Alford	ATN or Intralaminar nulei of thalamus/ NMDA/ Rats	1. Reference memory in the Water maze 2. Left/Right discrimination in the Y maze	1. ATN lesion only = impaired 2. Both lesions = non-impaired
2009	Lopez, Wolff, Lecourtier, Cosquer, Bontempi, Dalrymple-Alford & Cassel	ATN or Intralaminar nuclei+Lateral Thalamus/ NMDA/ Rats	1. Reference memory in Water maze and delayed retesting	1. ATN lesion= impaired across conditions; Intralaminar nuclei+Lateral Thalamic lesion = impaired only at retesting

2009	Wolff, Loukavenko, Will, & Dalrymple-Alford	ATN/ NMDA/ Rats	1. Reference memory in Water maze 2. Working memory in Water maze	1. ATN lesion = impaired, but non-impaired if housed in enriched environment. 2. ATN lesion = impaired, but non-impaired if housed in enriched environment.

lesions in both structures in opposite hemispheres will only have a disruptive effect if the two regions form a part of a functional system, whereas unilateral lesions made in the same hemisphere will have relatively little effect on behaviour. Studies performed by Warburton and colleagues (2000; 2001) found that unilateral lesions to the ATN and opposite hemisphere unilateral lesions to the hippocampus or fornix produced impairments in spatial memory on T-maze, water maze and radial arm maze tasks. However, same hemisphere lesions only produced mild impairments in spatial memory. Warburton and colleagues suggested that this evidence provides direct support for the notion that the ATN and hippocampus operate in conjunction as part of an integrated neural network during learning.

Declarative memory, as tested through associative learning tasks, is also affected by ATN lesions in rats. For example, ATN lesions result in severe deficits on spatial-visual tasks which require learning an association between scenes and objects embedded in them (Sziklas & Petrides, 1999), or between different odours and locations (Gibb et al., 2006). It has been demonstrated that the hippocampus also plays a role in the formation of associations between arbitrary visual stimuli and places or scenes (Sziklas et al., 1998). However, It should be noted that ATN deficits on associative learning are task dependent, and in some cases deficits are observed following hippocampal but not ATN lesions (e.g., Sziklas & Petrides, 2007). Further studies have shown that the functional connection between the ATN and hippocampal formation may rely on a direct route through the fornix, and indirectly between both the ATN and hippocampus through the retrosplenial cortex. When lesions are restricted to either the fornix (Sziklas & Petrides, 2002) or the retrosplenial cortex (St-Laurent et al., 2009) no impairments on associative learning tasks are observed, but associative learning is severely impaired following combined damage to both the fornix and retrosplenial cortex (Dumont et al., 2010). Dumont and colleagues (2010) propose that this evidence supports the notion that

the ATN and the hippocampus function interdependently in this type of spatial learning, and the interaction between these structures relies on both the fornix and the retrosplenial cortex.

These animal lesion studies, have led to models of the ATN involvement in amnesia have been proposed. Aggleton and Brown (1999) have suggested that the medial temporal lobe and diencephalic structures work in parallel through highlighting the similarity of the amnesic syndrome produced by damage each structure. They have proposed a model in which they argue that an "extended hippocampal system" comprising the hippocampus, fornix, mammillary bodies, and ATN, is essential for the encoding and subsequent recall of episodic information (Fig. 3). As a consequence, damage to the component structures of this system can result in anterograde amnesia (Aggleton & Brown, 1999).

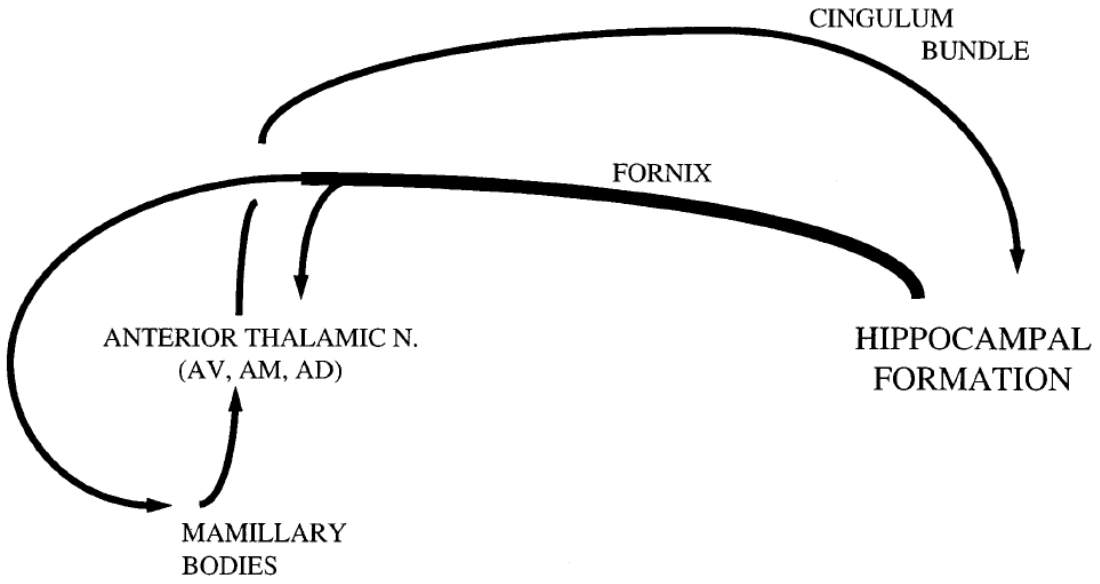


Figure 3. Schematic diagram showing the primary group of interconnections underlying spatial memory in the rat (Aggleton & Brown, 1999).

Damage to the extended hippocampal system causes deficits in spatial memory and in memory for relational information that characterises memory for autobiographical episodes,

but spares recognition based only on familiarity (Aggleton, Vann, Oswald & Good, 2000; Mayes et al., 2003; Yonelinas et al., 2002). Aggleton and Brown (1999) have further proposed that a reciprocal interaction exists between the hippocampus and the ATN. Support for this model comes largely from the disconnection studies explained above, which have demonstrated that the ATN and hippocampus are interdependent for some aspects of spatial learning. Other support comes from studies that have demonstrated that ATN lesions can induce abnormalities in hippocampal activity as measured by the immediate-early gene activity (Jenkins et al., 2002a; Jenkins et al., 2002b) and reductions in hippocampal acetylcholine levels (Savage et al., 2003). There is also evidence that navigation mechanisms that rely on head direction cells are dependent on the projections from the mammillary bodies to the ATN and on to the hippocampus (Basset et al., 2005).

Aggleton (2008) has revised the "extended hippocampal system" model by proposing that the observed similarity between temporal lobe and diencephalic amnesia may be emphasising the fact that both the hippocampus and ATN are connected to a third region, the retrosplenial cortex, which possibly functionally links both structures. He asserts that dysfunction in the retrosplenial cortex is seen to contribute to both temporal and diencephalic amnesia. The retrosplenial cortex has extensive connections with the ATN (Van Groen & Wyss, 1995). The AV nuclei especially have prominent connections with the retrosplenial cortex, while the AM has more connections with the dorsal anterior cingulate than the retrosplenial cortex (Van Groen et al., 1999). Human studies have shown that damage to the retrospenial cortex can result in anterograde amnesia (Maguire, 2001; Rudge & Warrington, 1991). In the animal literature, retrosplenial cortex lesions produce impairments in spatial memory (Aggleton & Vann, 2004; Lukoyanov et al., 2005; Vann & Aggleton, 2003), although contrary results have also been reported. For example, retrospenial cortex lesions

have been noted to produce only mild spatial memory impairments on a T-maze alternation task (Pothuizen et al., 2008) and on water maze tasks (Vann & Aggleton, 2004; Van Groen et al., 2004). However, it has been suggested that retrosplenial lesions may have a more selective effect on spatial working memory, disrupting only some of the strategies the animal can use to solve the maze (Pothuizen et al., 2008).

Aggleton (2008) argues that the retroplenial cortex is seen to represent an example of "covert pathology" in anterograde amnesia. Covert pathology is used to refer to a brain region that appears normal by the standard histological means and yet is functionally lesioned. In order to establish whether the retrosplenial cortex does represent covert pathology in diencephalic amnesia it is necessary to use a marker for neural function in this region, such as immediate-early genes (c-*fos*). Trans-synaptic activation can stimulate slow long-term changes in the post-synaptic neuron that involve the induction of gene expression. One of the categories of genes that are responsible for trans-synaptic activation are the immediate-early genes. The early activation of c-*fos*, combined with the fact that they are involved in the transcription of other down-stream genes, places c-*fos* as a potential candidate marker for covert pathology (Aggleton, 2008). Examination of c-*fos* activity after ATN lesions has revealed significant losses in early gene activation in the retrosplenial cortex (Jenkins et al., 2004). It was also observed that the ATN lesions did not alter the appearance of the retrosplenial cortex as determined by standard histological techniques. Interestingly, further studies have also shown that the extent of c-*fos* activity loss increases depending on the post-surgical interval, suggesting that retrosplenial cortex dysfunction is enhanced with longer survival after ATN lesion surgery (Poirer & Aggleton, 2009). There is also evidence that ATN lesions result in a loss of synaptic plasticity in the retrosplenial cortex, as

demonstrated through a decrease in long-term depression induction and GABA transmission in the region (Garden et al., 2009).

Lesions to the hippocampus also produce similar changes in c-*fos* activation in the retrosplenial cortex (Albasser et al., 2007). This evidence reinforces Aggleton's (2008) suggestion that both temporal lobe and diencephalic amnesia are characterised by hidden pathology in the retrosplenial cortex. This hypothesis has interesting implications for human neurodegenerative disorders. For example, hypoactivity in the retrosplenial cortex of Alzheimer's disease patients is often one of the first metabolic changes noted on a PET scan (Minoshima et al., 1994), and similar changes are often noted in patients with Korsakoff's disease (Nestor et al., 2003). Thus, retrosplenial cortex dysfunction is a common feature in both temporal lobe amnesia and diencephalic amnesia and, considering no neuronal loss is found in this region, provides a basis for examining potential metabolic-enhancing medications such as MB.

In summary, animals with lesions to the ATN offer an exceptional model with which to examine therapeutic interventions for human memory disorders. Animal research has provided extensive evidence that the ATN constitutes a part of the extended hippocampal system involved in spatial memory processing, and therefore any beneficial effects demonstrated from medication/interventions will suggest application as treatment for humans with memory disorders where this neural network is damaged. Recent suggestions that impaired ATN functioning can negatively affect other brain regions offers interesting prospects when researching new medications, where functional gains may be obtained through reactivation of regions presenting covert pathology. These findings provide rationale for investigating the efficacy of a pharmacological intervention, such as MB, using ATN

lesioned rats as a model for diencephalic amnesia, AD, and other human memory disorders. The first step, however, is to determine the influence of MB in animals with lesions to the extended hippocampal system.

1.7 The Present Study

Current therapeutic intervention studies using the ATN lesion model have focussed mainly on environmental enrichment (Loukavenko et al., 2007; Wolff et al., 2008). This research has demonstrated that functional recovery to memory can be observed in rats with ATN lesions when they are housed in a stimulating environment following surgery. The present study is the first to examine a pharmacological intervention for potential recovery of function after ATN lesions. To date, the only study that has attempted to explore the memory-enhancing effects of MB using a lesion model that replicates the regional pathology of AD, diencephalic amnesia or other related memory disorders is that published recently by Riha and colleagues (2010). The lesions used in that study were "metabolic" in nature, in that the neuronal tissue itself was not damaged and that the memory impairment observed resulted from a temporary disruption to CO activity in the retrosplenial cortex. The ATN lesion model used in this study produced results in permanent damage to the thalamo-limbic system, which can be expected to produce dysfunction in other interconnected neural regions, such as the retrosplenial cortex (Garden et al., 2009). Therefore, examining MB using this model provides a valid representation of the drug's potential for treating amnestic disorders in which these neural circuits are disrupted.

The present study focussed on a preliminary investigaion of the effects of daily administration of MB to ATN-lesioned rats on spatial reference memory in the Morris water

maze. Previous research has demonstrated that MB has a positive effect in both normal and impaired rats on tasks that test reference memory, such as the baited hole-board maze and the water maze (e.g., Callaway et al., 2002, 2004; Deiana et al., 2009; Medina et al., 2010). Spatial memory in animals is considered a suitable model of key aspects of episodic memory in humans (Dere et al., 2006). The current study therefore employed a standard behavioural procedure in the water maze that tests acquisition of allocentric spatial reference memory, and this was followed by a probe trial which assesses long-term memory retention. The current study is the first to examine the effects of repeat administration of MB using animals with permanent lesions that model the regional pathology of human memory disorders. Based on prior lesion research, we hypothesised that rats with anterior thalamic lesions would display impaired spatial reference memory in the water maze compared to controls, and that these impairments would be ameliorated in those lesioned rats treated daily with MB. We also predicted that MB would enhance long-term memory retention in ATN-lesioned rats, as demonstrated in the probe trial. Older rats (19 to 22 months old) were employed, so the study mimics the additional influence of ATN dysfunction in the aging brain. Due to literature documenting the general memory-enhancing effects of MB, we also predicted that control rats treated with MB would perform better in the water maze than those treated with saline. Results from this study may aid the development of MB as new medication for the treatment of impaired memory in humans. Due to time contraints, *c-fos* or CO changes were not investigated. Evidence of behavioural improvements after MB administration in ATN rats would in any case be a necessary first step.

2. MATERIALS AND METHODS

2.1 Subjects

Subjects were thirty nine male Wistar rats were used, bred in house and weighing between 550 and 865g on the day of surgery. They were between 19 and 22 months old at time of surgery and, apart from one week for post surgery recovery, were housed 3-4 per cage under standard laboratory conditions with a reversed 12 hour light/dark cycle and free access to food and water. All testing was undertaken during the dark period. Rats were handled for 2 weeks prior to surgey and thereafter. The study conformed to the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University of Canterbury Animal Ethics Committee. In full thirteen rats were excluded from the study, due to insufficient lesions and surgery comlpications, leaving the final analysis with twenty six rats. For the first part of the experiment rats were divided into 2 groups with final sample sizes of: 12 with ATN lesions and 14 Shams. For the second part of the experiment each group was subdivided into 4 groups: 6 with ATN lesions were treated with MB (MB-ATN), 6 with ATN lesions were treated with saline (SA-ATN), 7 Shams were treated with MB (MB-SHAM), and 7 Shams were treated with saline (SA-SHAM).

2.2 Drug Administration

Methylene blue (USP-grade; ScienceLab Inc, Houstan, TX, USA) was dissolved in saline and administered intraperitoneally (IP) at a dose of 1 mg/kg. This dosage has been established from previous research to be most effective in enhancing memory retention in normal rats when administered repeatedly (Bruchey & Gonzalez-Lima, 2008). USP-grade

MB was used to avoid any impurities, with arsenic measuring a maximum limit of 8ppm, and copper and zinc measuring under 0.02%. Rats were administered MB 2 hours after testing to avoid immediate symptomatic effects at the time of testing. Blue food colouring was added to the saline, and the MB and saline were labelled either solution A or B in order to blind the experimenter.

2.3 Apparatus

The water maze used was a 1.8 metre diameter, 60 cm high white fibreglass pool. The pool was situated in a room (5.4 x 4.0 metres) that contained allocentric cues that were both 2-dimensional (posters attached to the wall) and 3-dimensional (various objects attached to the walls including plastic containers and cones, gardening tools, buckets). Other distal cues included a computer, a drawn curtain, sink unit, tables and a tall lamp. The water in the maze was made opaque by the addition of 1 litre of non-toxic white paint. The pool was virtually divided into four equal quadrants with four starting points indentified as north (N), east (E), south (S), and west (W). An escape platform (20 cm diameter) was placed 1 cm beneath the water surface and kept in a constant position in the pool during each of the acquisition trials. The temperature of the water was kept between 20ºC and 23ºC during the testing period, with the temperature checked regularly. The swim paths of the rats were tracked using a video camera suspended directly above the pool, and all sessions were recorded on videotape. Data such as latency, path length and velocity were collected and analysed using the animal tracking software Ethovision® XT (Noldus Information Technology, The Netherlands). Due to the fact that white Wistar rats were used in this experiment, their heads were stained black so that they could be accurately tracked against a white background when using the tracking software.

2.4 Surgical Procedure

Prior to commencing the final surgeries, 13 pilot surgeries were undertaken. These were used to adjust lesion coordinates and NMDA volume in order to produce optimal anterior thalamic lesions. Criteria for an optimal lesion were: 1. Minimal Fimbria Fornix and Intralaminar Nuclei damage; 2. Lesion is bilateral and does not extend too far anterior or posterior; 3. At least 50% of the bilateral AT was damaged. Rats were anaesthetised with ketamine (90 mg/kg IP) followed by domitor (1 mg/kg IP). This was supplemented with subcutaneous mepivicane (3 mg/kg) during surgery. The anaesthetics were counteracted using antisedan (1 mg/kg IP) at the end of surgery. Rats were placed in a stereotaxic frame using atraumatic ear bars (Kopf, Tujunga, CA) and the incisor bar was set at -7.5 mm below the interaural line to minimise damage to the fornix. Bilateral lesion were directed at the anteroventral nucleus (AV) and the anteromedial nucleus (AM). To improve target accuracy, one of five anterior-posterior (AP) coordinates was used based on an individual rat's bregma to lamda (B-L) distance (in millimetres). For the AV lesion, the AP coordinates from bregma were: -2.55 for B-L = 7.0 or less; -2.60 for B-L = 7.1 to 7.4; -2.65 for B-L = 7.5 to 7.8; -2.70 for B-L = 7.9 to 8.2; and -2.75 for B-L = 8.2 and over. Two depths were used for the AV lesion, with coordinates placed ±1.52 mm lateral from the midline, and -5.68 mm ventral (from dura) for the upper site followed by -5.82 mm ventral for the lower site. The AM lesions followed an identical scheme except that only one depth was used and lesions were placed ±1.20 mm lateral from the midline, -5.95 mm ventral from the dura and the AP was 0.1 mm more anterior than for the AV site. Either 0.15 µl (for each AV lesion depth) or 0.18 µl (AM lesion) of 0.12 M N-methyl-d-aspartic acid (NMDA; Sigma, Castle Hills, NSW, Australia) in phosphate buffered saline (pH 7.2) was infused over at a rate of 0.03 µl/minute

at each site, using an automated Stoelting microinfusion pump and a 1 µl Hamilton syringe (Reno, NV, USA). The needle was left in situ for a further 3 min for diffusion at each site and retracted slowly. Sham controls received the same surgical procedure, but the needle was lowered to 1.50 mm above the lesion coordinates and no NMDA was infused. All rats were given at least two weeks to recover following surgery, during which period they were monitored daily.

2.5 Behavioural Training

Table 4. Experimental Design.

Procedure	Duration	MB or Saline	Duration Since Previous Test
Habituation	5 Days		0
Visible Platform	1 Day		6 Weeks
1st Reference Memory Task	10 Days		1 Day
2nd Reference Memory Task	10 Days	1 mg/kg following training each day	5 Days
Probe Trial	1 Day		7 Days

2.5.1 Habituation Phase

Subjects were habituated to the water maze prior to surgery. This included initial familiarisation and training to escape on to a visible platform. A visible cue was attached to the platform, which was submerged 1 cm below the water surface and curtains were drawn around the maze to help the rats focus on the visible escape. The platform was located 15 cm perpendicular to a start point and the rat was released at one of four of these points located around the maze periphery. Animals were tested with four trials per day on each of the

consecutive five days prior to surgery to ensure all rats were swimming comfortably. Each habituation trial was terminated once the animal located the visible platform, at which point the rat was allowed to remain on the platform for 30 seconds. During the first few days some rats had to be led to the escape platform by the experimenter's hand. By the fifth day all rats were swimming comfortably and were able to reach the escape platform.

2.5.2 Acquisition Phase

2.5.2.1 Visible Platform

Once all rats had recovered from surgery they were given the same procedure as conducted preoperatively to ensure that all rats were swimming correctly and could escape from the water on to the platform. The platform was placed in a different location to that used preoperatively and all rats were tested on a single day. Two rats were excluded from the study at this point due to swimming difficulties, possibly as a result of inner ear damage during surgery.

2.5.2.2 Part 1: Post-Surgical Spatial Reference Memory Task

This procedure was used to establish the memory impairment observed in rats with ATN lesions compared to the sham rats. The finidings were then used to equate groups to receive either MB or saline treatment. During 10 consecutive days, the platform was placed in the centre of the north-west quadrant and the curtain surround was drawn back to reveal room cues. Each day, the rats were given four 60 second trials in which they were released from the four starting points (N, E, S, W) in a random order for each rat. The trial was

terminated when the rat reached the hidden platform and had remained there for over 1 second, at which point it was left on the platform for a further 30 seconds before being removed. If the rat did not find the platform within 60 seconds, it was led to it and placed on the platform if necessary. All rats were given approximately 4 to 5 minutes between trials. The latency was recorded for each trial with a stop watch, and these data used to (a) balance spatial memory performace of the control rats that would later receive MB vs saline condions (part 2); and (b) to do the same for the ATN rats. Allocation to the two conditions (MB or saline treatment) was made by first rank ordering latency (for example, within the controls) and randomly allocating one of each successive rank-ordered pairs.

2.5.2.3 Part 2: Post-Surgical Spatial Reference Memory Task and Methylene Blue Treatment

Following a five day break, the rats were tested again over 10 consecutive days using the same procedure as used during the first reference memory task, except the platform was now placed in the south-east quadrant. For part 2, on the day prior to commencing training, then 2 hours after each day of training, the rats were given an i.p. injection of either MB or saline according their treatment group. Treatment was administered in this fashion as prior research has demonstrated that MB has a peak effect on memory using this procedure (Bruchey & Gonzalez-Lima, 2008).

2.5.2.4 Probe Trial

Following a 7 day break all rats were tested on one single day to measure the rat's long-term spatial memory for the previous location of the platform (no additional drug was

given). During this probe trial the platform were removed from the water maze and the rat was allowed to swim for 60 seconds in the pool. The amount of time that the rat spent in the previously correct quadrant of the maze and the previous platform location and the frequency that the rats reached these areas indicated the strength and precision of their spatial memory.

2.6 Histology

On completion of behavioural testing, all rats were euthanized using pentobarbitone and transcardially perfused with cold saline followed by 4% formalin. The brains were post-fixed for two days, cytoprotected in 30% sucrose, and every coronal 50-µm section throughout the thalamic region was collected using a vibratome for cresyl violet staining of cell bodies. Lesion extent was estimated on electronic copies of the Paxinos and Watson (1998) atlas. Automated pixel counts of the estimated damage relative to the relevant intact brain region were used to generate percent lesion volumes by factoring the pixel areas and the distances provided in the atlas. Acceptable lesions were defined as having more than 50% bilateral damage to the ATN, but not more than 40% damage to the corresponding adjacent thalamic regions. Previous work suggests that ATN lesions with 50% or more damage are consistently assocociated with severe memory deficits (Mitchell & Dalrymple-Alford, 2006).

2.7 Statistical Analysis

Statistica 10 software was used for statistical analysis. All data were analysed with analysis of variance (*ANOVA*), and *p*-values with an alpha value of .05 regarded as significant. *Post-hoc Newman-Keuls* tests were used to establish pair-wise comparisons.

Priori planned comparisons were used to examine the differences between treatment groups following group balancing, and to examine whether the treatment groups performed significantly different from chance levels in the probe trial.

3. RESULTS

3.1 Lesion Evaluation

The largest and smallest acceptable lesions in the two treatment groups are shown in Figures 4A & B. Only rats with ATN lesions meeting the Histology criteria (6 MB and 6 Saline rats) were included in the behavioural analyses (see Table 5). One lesion case was rejected because only 12% ATN damage was sustained. In addition, one Sham was excluded due to a large hypothalamic tumour.

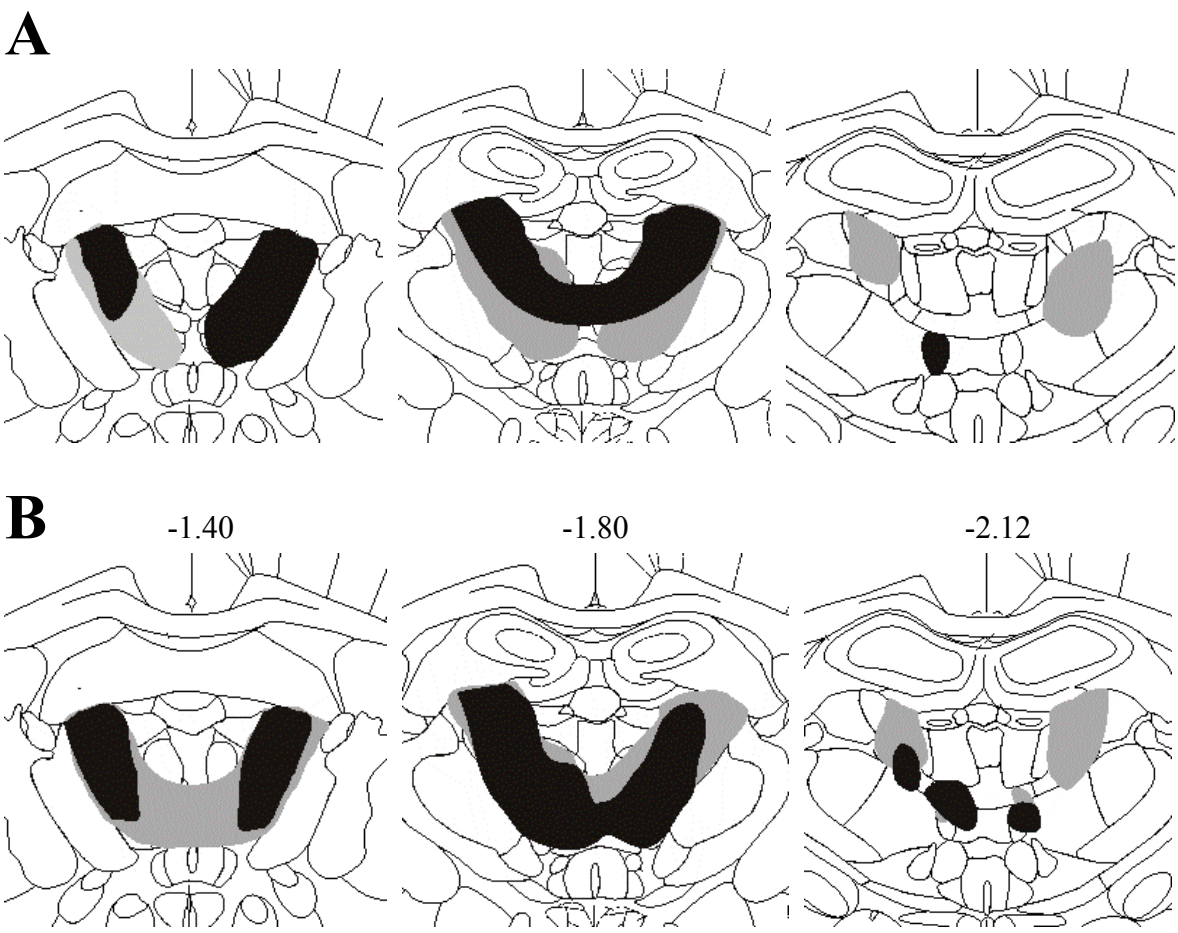


Figure 4. Lesion Size. Schematic representation of the largest (grey) and smallest (black) lesion in the **A**: MB-treated ATN group and **B:** Saline-treated ATN group. Numbers indicate distance from bregma in millimetres (from Paxinos & Watson (1998)).

Table 5. Percentage of bilateral damage (volume) to selected areas for each of the rats in the study.

	ATN and components												
RATS	AD	AM	AV	ATN	MT	LT	IAM	LD	PT	PVA	PV/PVP	Re	Rh
MB-ATN													
AG2AG	70	43	90	54	0	2	29	1	11	1	0	0	0
CG11KR	87	70	90	77	0	1	33	5	6	0	0	0	7
EG15BB	77	57	94	72	0	0	26	2	42	1	0	1	4
FB17FB	62	39	94	58	0	0	25	0	1	0	0	0	2
FG18RG	99	63	92	73	10	14	26	3	17	0	0	0	3
HR24NG	73	40	98	66	0	4	10	7	1	0	0	0	1
MB-ATN Median	**75**	**50**	**93**	**69**	**0**	**1.5**	**26**	**2.5**	**8.5**	**0**	**0**	**0**	**2.5**
SA-ATN													
AB1AB	86	62	87	50	3	9	30	10	8	0	17	0	5
DB10JR	84	40	92	57	2	10	52	4	9	0	0	0	0
EB14RR	74	72	92	58	5	13	9	5	7	0	0	0	0
GG19GG	93	55	94	76	0	0	32	4	19	0	0	0	2
HB20KB	91	63	98	80	0	6	41	5	20	1	0	0	0
LR28OG	95	70	93	79	2	7	59	4	20	0	0	0	6
SA-ATN Median	**88.5**	**62.5**	**93**	**67**	**2**	**8**	**36.5**	**4.5**	**14**	**0**	**0**	**0**	**1**

Abbreviations: AD= anterodorsal nucleus; AM= anteromedial nucleus; ATN = anterior thalamic aggregate comprising the anterodorsal, anteromedial and anteroventral thalamic nuclei; ATN median= median percent damage for all included rats; AV= anteroventral nucleus; EE-ATN = rats with anterior thalamic lesions housed in enriched cages; IAM= interanterodorsal nucleus; LT= lateral medial thalamic aggregate comprising the intralaminar nuclei (centrolateral, paracentral and rostral central medial nuclei) and lateral mediodorsal thalamic nuclei (lateral and paralamellar nuclei); LT median= median percent damage for all included rats; MB-ATN = rats with anterior thalamic lesions treated with methylene blue; MT= posteromedial thalamic aggregate comprising the central and medial mediodorsal nuclei and the intermediodorsal nucleus; MT median= median percent damage for all included rats; PT= paratenial nucleus; PVA= anterior paraventricular nucleus; PV/PVP= paraventricular nucleus/posterior paraventricular nucleus; Re= reunions nucleus; Rh= rhomboid nucleus; SA-ATN = rats with anterior thalamic lesions treated with saline/control.

3.2 Part 1: Post-Surgical Spatial Reference Memory Task

As expected, the ATN lesion group displayed poor spatial memory performance, with little improvement in the amount of time taken to reach the platform or the path length to the platform over the 10 days of training. The Sham group, on the other hand, showed improvements in spatial memory over the 10 days of training. Time taken to reach the escape platform was analysed using a 2-way repeated measures *ANOVA* (Lesion by Day), with the average of the 4 trials from each training session used as the repeated measure. Assumptions of normality and homogeneity were satisfactory, and the alpha was set at .05. A significant main Lesion effect was evident ($F(9,24) = 6.86$; $p < 0.015$), with poorer performance in the ATN group throughout training (Fig. 5A). There was no significant interaction between Lesion x Day ($F(9,24) < 1.0$). *ANOVA* for the path length to reach the platform also revealed a significant Lesion main effect ($F(9,24) = 5.70$; $p < 0.025$) (Fig. 5B), with no significant interaction between Lesion x Day ($F(9,24) < 1.0$). Analysis of the swim speed of the two groups revealed that there were no significant differences in velocity ($F(9,24) = .541$; $p > .05$) (Fig. 5C), indicating that the differences in latency and path length were not due to differences in swim speed.

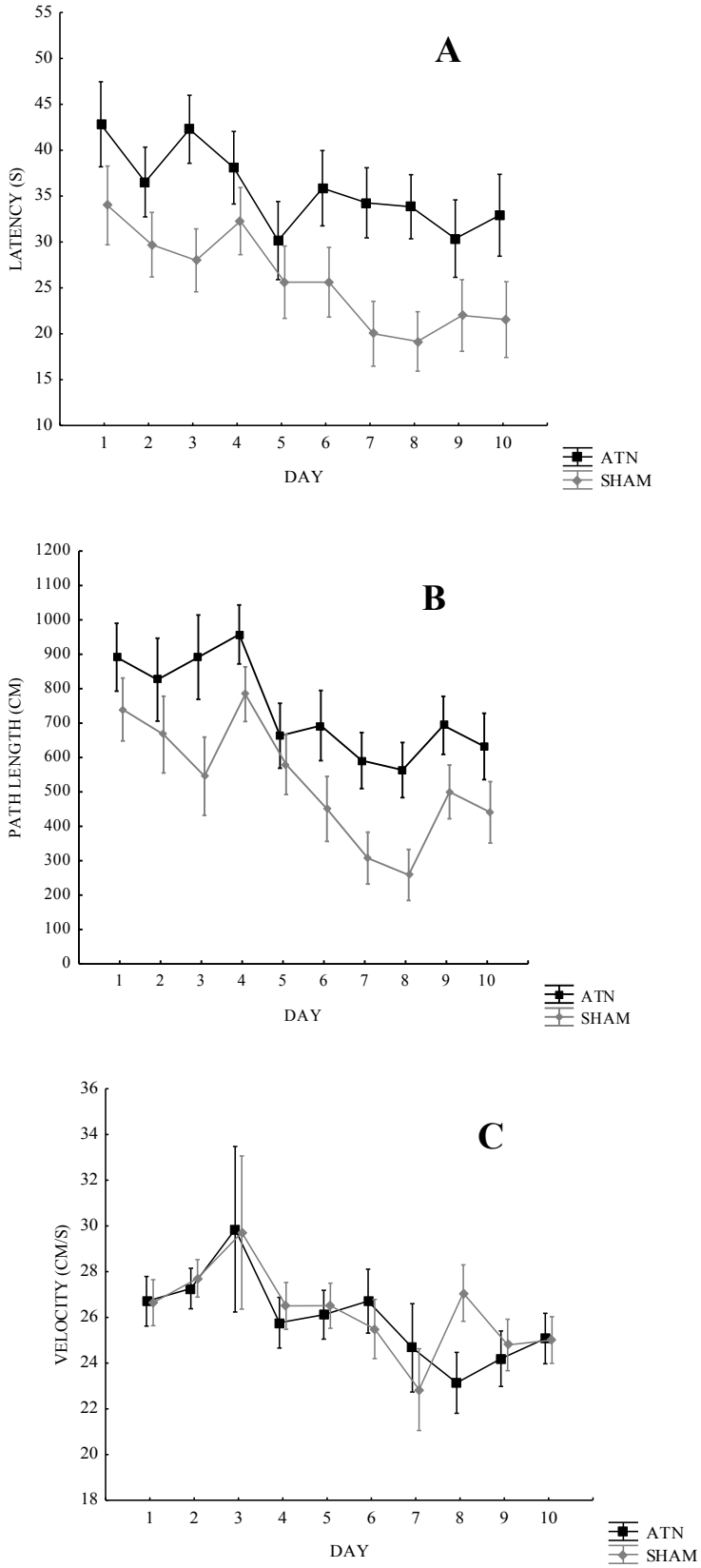


Figure 5. Anterior Thalamic Lesions and Spatial Reference Memory Performance in the Morris Water Maze. (**A**) Latency: Mean (± SEM) time (seconds) to reach the escape platform. (**B**) Path Length: Mean (± SEM) distance (centimetres) to reach the escape platform. (**C**) Velocity: Mean (± SEM) swim speed (centimetres per second) during platform searching. ATN = neurotoxic lesion of the anterior thalamic nuclei.

3.3 Treatment Allocation/ Group Balancing

Rats were allocated to treatment groups according to their latency performance in Part 1. They were first rank-ordered into pairs and then rats from each pair were randomly allocated to each treatment group. A *t*-test for path length differences between treatment groups in Part 1 revealed that there were no significant differences between the two ATN treatment groups ($t(10) = -1.07$, $p > .05$) and the two Sham groups ($t(12) = -1.24$, $p > .05$). The similarity in path length scores in Part 1. indicate that the four treatment groups were balanced before commencing MB or saline administration, as path length is generally less likely than latency to be influenced by swim speed.

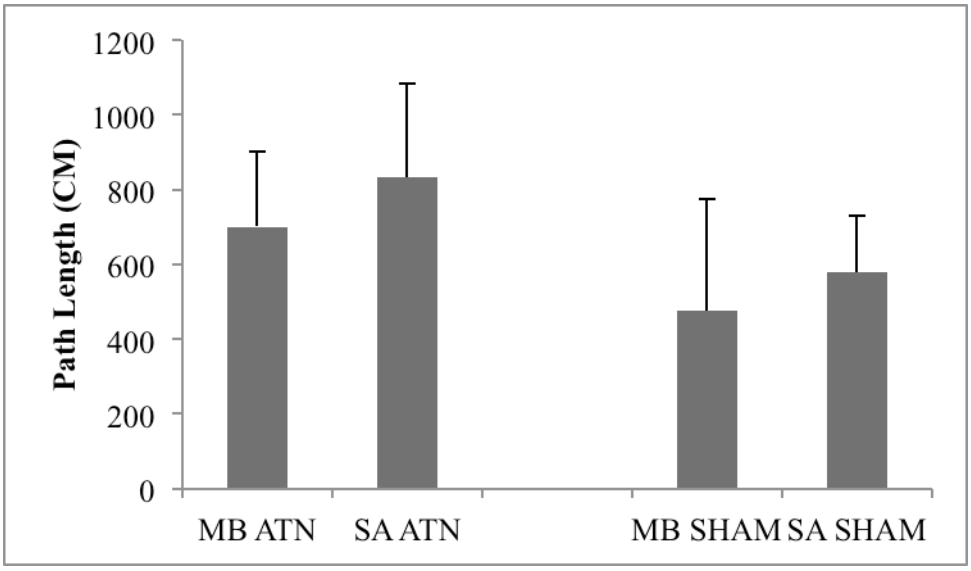


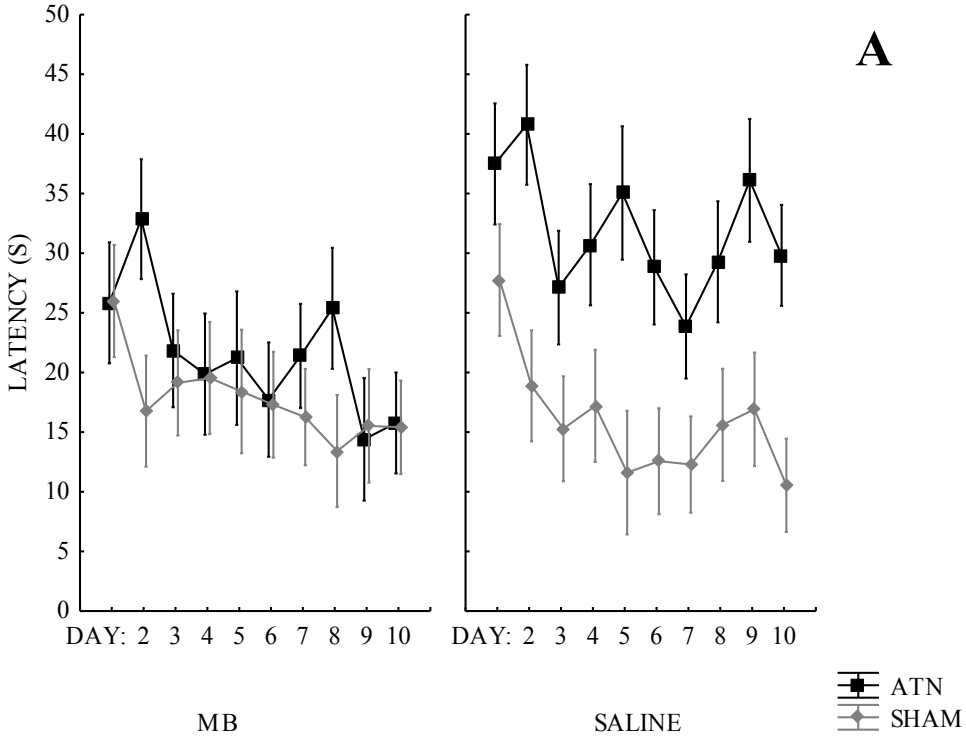
Figure 6. Spatial Memory Performance Differences Between Treatment Groups Prior to Drug Administration. Path Length: Mean (± SEM) distance (centimetres) to reach the escape platform across the ten days of training. MB ATN = Rats with neurotoxic lesions to the anterior thalamic nuclei treated with methylene blue; SA ATN = Rats with neurotoxic lesions to the anterior thalamic nuclei treated with saline; MB SHAM = Rats that underwent sham/control surgeries treated with methylene blue; MB SHAM = Rats that underwent sham/control surgeries treated with saline.

3.4 Part 2: Post-Surgical Spatial Reference Memory Task and Methylene Blue Treatment

Rats with ATN lesions treated with MB displayed improvements in spatial memoy performance across the 10 days of testing, performing at a similar level to the two Sham groups. The ATN-lesioned rats treated with saline, on the other hand, showed little improvement in spatial memory and performed similary to the ATN group in Part 1 (Fig. 7A). Sham rats treated with MB did not perform differently from those Sham rats treated with saline, although both Sham groups generally performed better than in Part 1. A 3-way repeated measures *ANOVA* (Lesion by Treatment by Day) for the time taken to reach the escape platform revealed a significant Lesion main effect ($F_{(9,\ 24)} = 6.66$; $p < 0.017$), reflecting a ATN lesion deficit. In addition, there was a significant Lesion by Day interaction for latency ($F_{(9,\ 24)} = 2.50$; $p < 0.05$), because the Sham group overall showed more improvement in escape time across sessions, while the ATN lesion group overall showed less improvement across sessions. The *ANOVA* revealed that there was no significant main effect for treatment ($F_{(9,\ 24)} = 1.17$; $p > 0.05$) and no significant interactions between Lesion x Treatment ($F_{(9,\ 24)} = 2.50$; $p > 0.05$) and Lesion x Treatment x Day ($F_{(9,\ 24)} = 1.28$; $p > 0.05$). However, *Post-hoc Newman-Keuls* tests verified that the saline-treated ATN group performed significantly worse than the other three groups, whereas the MB-treated ATN group did not perform significantly different from the Sham groups.

Regarding path length (Fig. 7B), a 3-way repeated measures *ANOVA* (Lesion by Treatment by Day) revealed that there was a significant difference between the lesion groups ($F_{(9,\ 24)} = 8.40$; $p < 0.008$), and a significant Lesion by Day interaction ($F_{(9,\ 24)} = 1.93$; $p < 0.049$), mostly because the Sham group swam shorter distances to reach the escape platform

across sessions, while the ATN grouped showed less improvement. Furthermore, there was a significant 3-way interaction between Lesion x Treatment x Day ($F(9, 24) = 1.93$; $p < 0.049$). While the MB-treated ATN group and the two Sham groups all improved in performance across the sessions, the saline-treated ATN group showed little consistent improvement in path length across sessions. There was no significant interaction between Lesion x Treatment ($F(9, 24) < 1.0$). Again, a *Post-hoc Newman-Keuls* test verified that the MB-treated ATN group did not significantly differ from the Sham groups in path length, while the saline-treated ATN group significantly differed from the three other groups (Fig. 7B). Analysis of the swim speed of the four groups in Part 2 revealed no significant differences in velocity ($F(9, 24) < 1.0$) (Fig. 7C), indicating that the differences in latency and path length were not due to differences in swim speed.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MB
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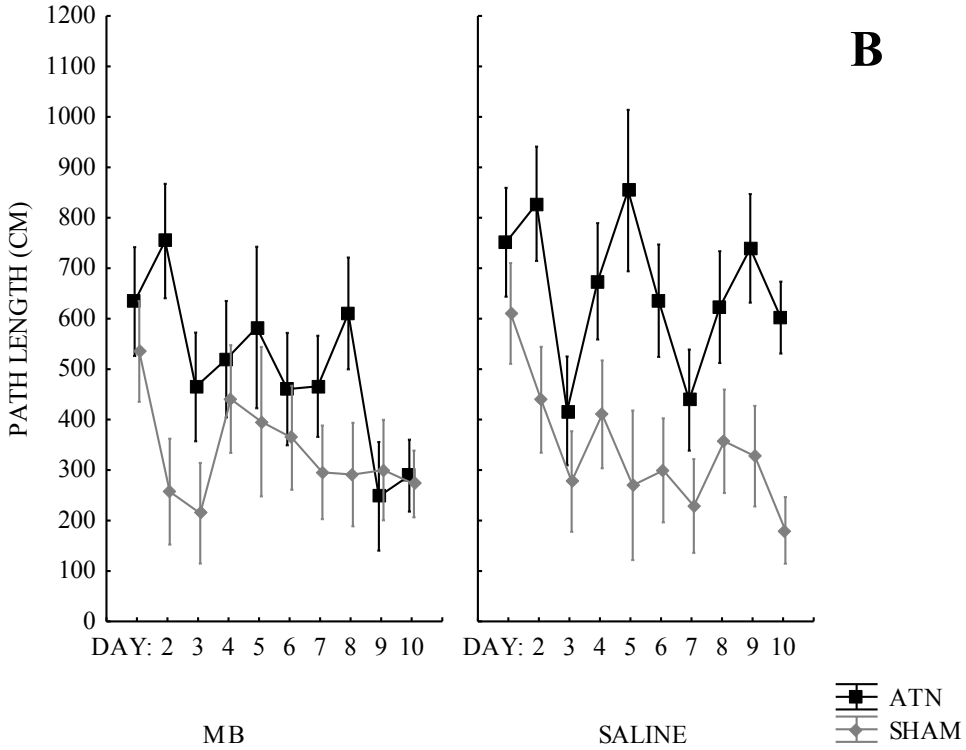
B
PATH LENGTH (CM)
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MB
SALINE
ATN
SHAM

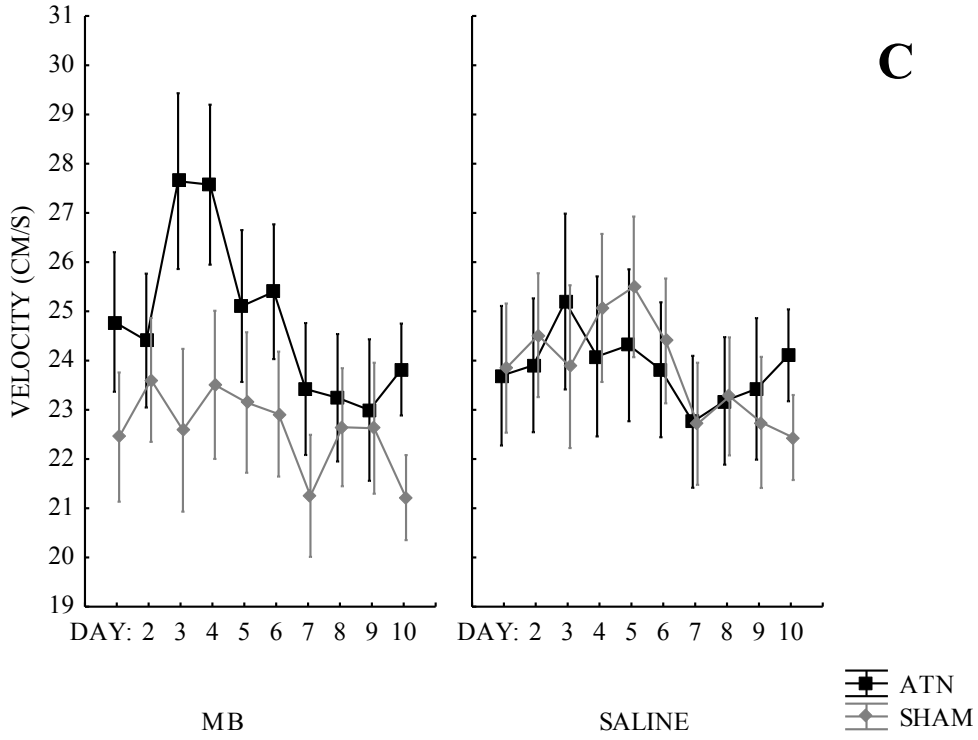

Figure 7. The Effect of Methylene Blue on Spatial Reference Memory Performance in Rats with Anterior Thalamic Lesions in the Morris Water Maze. (**A**) Latency: Mean (± SEM) time (seconds) to reach the escape platform across the ten days of training. (**B**) Path Length: Mean (± SEM) distance (centimetres) to reach the escape platform across the ten days of training. (**C**) Velocity: Mean (± SEM) swim speed (centimetres per second) during platform searching. ATN = neurotoxic lesion of the anterior thalamic nuclei; MB = Treatment with methylene blue.

3.5 Probe Trial

The graphs for correct quadrant duration, correct zone duration, and zone crossings suggest that ATN-lesioned rats treated with saline performed worse than the other three groups, whereas the performance of ATN-lesioned rats treated with MB was similar to that of the Sham groups (Fig. 8A, B, & C). In addition, the ATN-lesioned rats treated with saline also performed at a below chance level (mean second was not significantly different to 15 seconds in correct quadrant; $t(5) = -0.35$, $p > 0.05$), while the other three groups all performed above chance level (means were significantly different from 15 seconds in the correct quadrant; MB-ATN ($t(5) = 2.92$, $p < 0.05$); MB SHAM ($t(6) = 2.70$, $p < 05$); SA SHAM ($t(6) = 2.62$, $p < 0.05$)) (Fig. 8A). However, a 2-way *ANOVA* (Treatment by Lesion) revealed that there were no significant main effects or interactions for either the amount of time the rats spent in the correct quadrant, the amount of time the rats spent in the previous platform zone, or the frequency that the rats cross into the previous platform zone. The main effects for treatment were bordering on significance for both the amount of time spent in the correct platform zone ($F(9, 24) = 3.24$; $p < 0.086$), and the frequency of correct platform zone crossovers ($F(9, 24) = 3.55$; $p < 0.073$), while the main treatment effect for amount of time spent in the correct quadrant did not approach significance ($F(9, 24) < 1.0$) Analysis of the swim speed of the four groups in this task revealed that there were no significant differences in velocity ($F(9, 24) = .01$; $p > 0.05$), indicating that the differences in latency were not due to differences in swim speed.

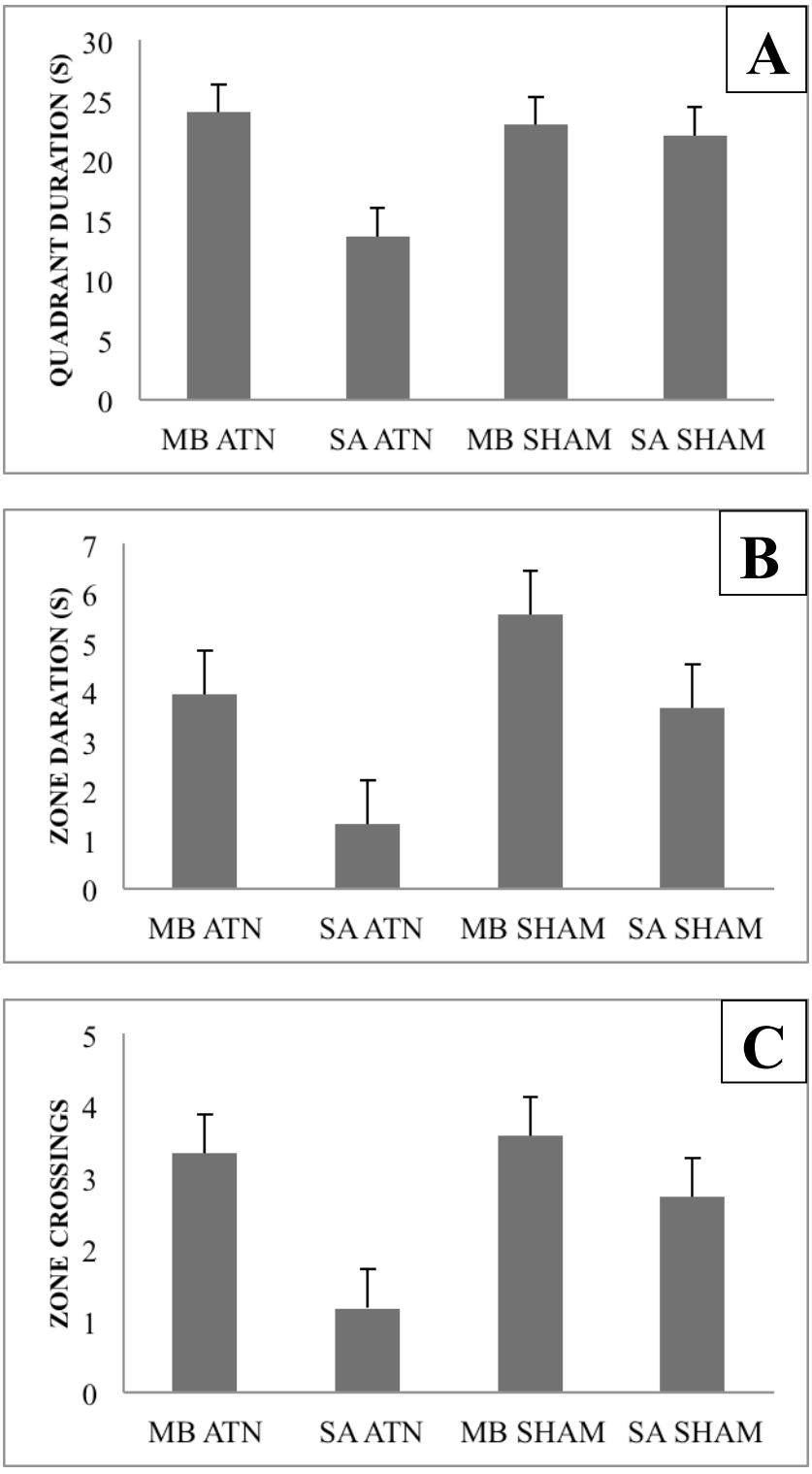

Figure 8. The Effect of Methylene Blue on Probe Trial Performance in Rats with Anterior Thalamic Lesions in the Morris Water Maze. (**A**) Mean duration (seconds) spent in the previously correct quadrant during the probe trial. (**B**) Mean duration (seconds) spent in the zone where the previous platform was located. (**C**) Mean frequency that the rats crossed over the zone where the previous platform was located. MB ATN = Rats with neurotoxic lesions to the anterior thalamic nuclei treated with methylene blue; SA ATN = Rats with neurotoxic lesions to the anterior thalamic nuclei treated with saline; MB SHAM = Rats that underwent sham/control surgeries treated with methylene blue; MB SHAM = Rats that underwent sham/control surgeries treated with saline.

5. DISCUSSION

4.1 General Summary

The present study aimed to establish whether repeat post-trial administration of MB could enhance spatial reference memory in rats with lesions to the anterior thalamus, a region implicated in human memory disorders. We established that rats with ATN lesions were significantly impaired in the water maze when compared with shams, and that those ATN-lesioned rats treated with MB showed significantly improved learning compared to those treated with saline. While there were no statistically significant differences in the probe trial, which examined the long-term memory of the rats, there was a clear trend for the saline-treated lesioned rats to perform worse than the MB-treated lesioned rats and both of the sham groups. A discussion of the findings, strengths and limitations of the study, implications of the findings for research and clinical practice, and possible future directions for research are described below.

4.2 Anterior Thalamic Lesions and Spatial Memory

As was expected, ATN lesioned rats in the first experiment performed worse than the sham group on a measure of spatial reference memory in the water maze. This was demonstrated through both a longer duration and a longer swim distance taken to reach the escape platform for the ATN lesioned rats compared with the sham rats. These findings are consistent with previous research and emphasise the robustness of memory deficits observed after ATN lesions. The permanent memory deficits that follow bilateral ATN lesions such as those used in the present study, together with similar impairments observed after disconnection lesions of the ATN and the hippocampal system, point to the potential

importance of the thalamic region as a core part of a system supporting episodic memory (Aggleton & Brown, 2006; Byatt & Dalrymple-Alford, 1996; Warburton et al., 2000, 2001). The findings support the notion that the ATN serves as a gateway by which information from subcortical structures and the hippocampus can be sent to the cortex (Aggleton, 2008). Learning to navigate a place in the water maze is also sensitive to damage to both the hippocampal system (Morris et al., 1982) and the retrosplenial cortex (Lukoyanov et al., 2005), and the impairments observed in this study after ATN lesions therefore further highlight the anatomical relationships between the three regions and their involvement in the episodic memory system.

There is evidence from other studies that while damage to the ATN results in impairment in allocentric learning, the ability to navigate based on spatial cues, it does not impair performance on egocentric tasks, where the animal learns to use body direction for navigation (Sziklas & Petrides, 1999, 2004). Therefore, the impairments demonstrated by the ATN rats on the reference memory task may be largely due to an inability of the animals to employ an allocentric type strategy in order to solve the maze, and instead a tendency to rely on an egocentric strategy to navigate to the platform. This may explain why a minor improvement in performance can be observed for the ATN group across the 10 days of training, although not at the superior level of the sham group, which would have likely used an allocentric strategy to navigate to the escape platform. Another observation made from the latency results is that while the sham rats learnt the position of the escape platform over the 10 days of training, they did not reach the reduced latency scores usually found in other lesion studies using the reference memory task in the water maze (e.g., Warburton & Aggleton, 1999; Wolff et al., 2008). A possible explanation for this may come from the fact that the rats used in this study were older than that typically used in ATN lesion studies, and

therefore their general learning abilities may be somewhat naturally impaired with the effects of aging. It should also be noted that the sham group performed better in terms of both path length and latency on the first day of testing, and this may be due to either an improved ability to learn the platform position across the first four trials, or that the ATN rats found it more difficult to switch from previous platform positions used in the habituation phase of testing.

Both clinical and animal research has previously pointed to the fact that structures within the thalamus contribute to memory. Evidence from human cases strongly implicate disruption of the ATN in the amnesic syndrome associated with both diencephalic amnesia (Van der Werf et al., 2000; 2003) and AD (Braak & Braak, 1991b). Animal studies have also confirmed that the ATN are implicated in episodic-like memory processes (Aggleton & Brown, 1999; Mair et al., 1999; Wolf et al., 2006). The anterior thalamic nuclei have direct connections with both the retrosplenial cortex and hippocampal formation (Aggleton, 2008). Therefore, the finding that ATN lesioned rats performed worse than the control group on a measure of spatial memory should be interpreted as supporting the notion that such deficits are due to a disconnection of the ATN from the extended hippocampal system, as supported by previous disconnection studies (e.g., Warburton et al., 2000; 2001). Overall, the findings of this study therefore further validate ATN-lesioned rats as a model of human memory disorders and reinforces the view that the ATN is a critical component of an integrated hippocampal-diencephalic system.

4.3 The Effect of Methylene Blue on Rats with Anterior Thalamic Lesions

As was consistent with our primary hypothesis, 10 days of continuous 1 mg/kg MB treatment resulted in rats with lesions to the anterior thalamus performing better on a measure of spatial reference memory than those treated with saline. The MB-treated lesioned rats performed equal to the sham groups in their ability to navigate to the escape platform in the water maze, while the saline-treated lesioned rats performed worse than the other three groups in terms of both path-length and latency. These findings suggest that the application of daily low dose MB injections to ATN-lesioned rats can reverse the spatial memory impairments induced by the lesion, resulting in learning abilities similar to that of normal, aged rats without any observable side effects caused by the drug. This study is therefore the first to provide evidence that memory impairments associated with damage to the ATN may be ameliorated through a pharmaceutical intervention. Furthermore, this study provides evidence that MB may provide beneficial effects for human memory disorders where there are disruptions to the thalamo-hippocampal circuit, such as in diencephalic amnesia and AD. This indicates that thalamic pathology in such diseases may be a potential target through which MB can improve human cognition, as was demonstrated in the Wischik et al. (2008) clinical study investigating the application of MB to AD patients.

Given the age and poor acquisition in sham rats, there was no difference in memory performance between the MB and saline treated rats in the sham group, suggesting that MB had no effect on reference memory in normal rats in the water maze. Previous research, such as that conducted by Callaway et al. (2004) and Wrubel et al. (2007b) have demonstrated that repeat administration of low dose MB can improve spatial memory in normal rats, and therefore we would expect to see a difference in performance between the sham groups. A

potential explanation for why no differences were observed may be that both of the sham groups performed at an optimal level in the water maze, and no further improvements in learning were possible through the application of a memory-enhancing agent such as MB. Considering the older age of the rats used in the present study, it is also plausible that normal, older rats may not be as responsive to the general memory-enhancing effects of the drug, and that such effects are only noticeable in younger rats such as those used in previous MB research (e.g., Callaway et al., 2002; Riha et al., 2005; Wrubel et al., 2007a).

Interestingly, the ATN lesioned rats administered MB did not significantly differ from those administered saline on the probe trial. While this may suggest that MB does not affect long-term memory in ATN lesioned rats, the fact that the graphs display a clear trend for the saline-treated ATN rats to perform worse than both the MB-treated ATN rats and the two sham groups indicates that MB may have had some effect on the rats' memory for the previous platform location. In addition, the scores of the MB-treated ATN rats was near identical to that of the two sham groups, suggesting that the administration of MB resulted in normal long term memory for the previous platform position despite anterior thalamic damage. Furthermore, the amount of time the saline-treated ATN rats spent in the correct quadrant was at a level equivalent to chance, while all three other groups performed at levels above chance. There are a number of possible reasons why the differences between the four groups on the probe trial did not reach statistical significance. Firstly, there is the possibility that MB has only a minimal effect on recall over a longer period, and that its effects may be limited to memory consolidation during learning. Additionally, it is possible that that the differences were only minimal because the rats were no longer under the influence of the drug during the probe trial. The peak of MBs influence on neuronal metabolic activity is 24 hours following administration (Bruchey & Gonzalez-Lima, 2008), and it is unlikely that any

residual effect on metabolism would be present 7 days following the last administration when the probe trial was given. Therefore, as no other studies examining the effect of MB on spatial memory have employed a probe trial, the findings of the present study may indicate that MB only exerts effect on memory during the period where it is metabolically active. Nevertheless, the visible differences between the scores of saline administered ATN rats and the other three groups imply that further research investigating long-term spatial memory needs to be undertaken.

Considering that the ATN lesion model only diplays damage to the anterior thalamus while leaving the remaining neural substrate unimpaired, there are a number of possible mechanisms through which MB may enhance spatial memory in ATN lesioned rats. Firstly, as mentioned previously it has been demonstrated that MB increases neural metabolic activity, through increasing CO activity in the brain, and that these effects correspond with memory improvements (Callaway et al., 2004; Gonzalez-Lima & Bruchey, 2004; Riha et al., 2005; Wrubel et al., 2007a). Researchers have found that some mitochondrial genes, including transcripts for CO, are up-regulated and over-expressed during memory consolidation (Pinter et al., 2005), and increased CO activity results in more oxygen consumption and ATP production in the brain (Zhang et al., 2006). With there being no damage to either the hippocampus or the retrosplenial cortex in the ATN-lesioned rats, it is possible that MB enhances spatial memory performance through increasing CO activity in both of these regions. Spatial training in the water maze requires an increase in CO activity in both the hippocampus and retrosplenial cortex (Conejo et al., 2010), and there is also evidence that ATN lesions results in metabolic impairment in both of the hippocampus (Jenkins et al., 2002) and the retrosplenial cortex (Amin et al., 2010; Garden et al., 2009; Poirier et al., 2008; van Groen et al., 1993). Therefore, MB may restore the metabolic

functioning of these two regions following ATN lesions, resulting in an improved functionality of the hippocampal network and, thus, normal spatial memory processing. Evidence in support of this notion is provided by the recent study by Riha and colleagues (2010), where MB administration resulted in metabolic restoration to the extended hippocampal after extensive damage to the retrosplenial cortex.

Another possible way that MB may have improved spatial memory in the ATN-lesioned rats is through its function as a cholinesterase inhibitor (Pfaffendorf et al., 1997). It has been demonstrated that MB can improve reference memory in the water maze in rats cholinergically-impaired with scopolamine, and that this memory improvement is superior to the commonly used AChEI, rivastigmine (Deiana et al., 2009). Recent research has also shown that water maze-tested rats administered with scopolamine show a decrease in CO activity in the anterior thalamus, suggesting that the memory deficits induced by scopolamine may be due to an impairment in cholinergic function in the ATN and its neural connections (Mendez-Lopez et al., 2010). Therefore, the ATN lesions used in the present study may have resulted in disruptions to the cholinergic system, and these disruptions may have been ameliorated following MB administration, resulting in memory improvement.

The considerations discussed above validate the findings of the present study and gives possible explanations as to why MB restores spatial memory in ATN-lesioned rats. Other possible mechanisms whereby MB may restore spatial memory are through its action as a potent antioxidant, where neuroprotective effects may have been exerted preventing further oxidative stress and cell death following the lesions. Also the drugs properties as a nitric oxide synthase and guanylate cyclise inhibitor (Deutsch et al., 1996) and influence on the glutamatergic system (Vutskits et al., 2008) may also contribute to the drugs positive

effects on memory. Although in need of replication with a larger sample size, the current findings suggest that repeat low-dose MB administration may prevent the disruptions to the thalamo-hippocampal memory system induced by ATN lesions and supports the ongoing research investigating MB as a potential candidate for the treatment of human neurodegenerative disorders.

4.4 Strengths

Along with demonstrating that MB has a positive effect on memory in rats with lesions to the anterior thalamus, this is also the first study to explore a number of other research avenues. To begin with, to our knowledge this is the first time an ATN-induced memory deficit has been demonstrated in older rats. The standard rat age at time of surgery used in research using the ATN lesion model is between 3-6 months old (e.g., Poirier & Aggleton, 2009; van Groen et al., 2002; Warburton et al., 1997; 2001), with the oldest rats used in any study being 10 months old (Wolff et al., 2008). Similarly, this study presents the first time that the memory-enhancing effects of MB has been examined using older rats. The rats used in the present study, on the other hand, were all aged between 19-22 months old at the time of surgery. Considering that the neurodegenerative disorders, such as diencephalic amnesia and AD, most commonly occur in older patients, it can be argued that the rats used in the present study offer a more valid model of human memory disorders present in the geriatric population. Additionally, this study demonstrates that lesion induced differences in memory are present in older rats despite potential existing natural memory impairments that may be present due to the rat's age.

Previous research, especially that focussing on MB as a therapeutic agent for neurodegenerative disorders, has used either a single dose (e.g., Callaway et al., 2002; Riha et al., 2010) or a maximum of 5 days continuous treatment (Callaway et al., 2004; Wrubel et al., 2007b). However, the present study used a treatment regime of 10 days continuous administration of 1 mg/kg MB, with no apparent side effects being noted by the researcher. This is highly relevant when the findings are considered in the context of human treatment, where patients would potentially be treated with MB over a prolonged period. Current human AD trials have found beneficial effects following 6 months of continuous oral treatment (Wischik et al., 2008). Therefore, future animal and human research should aim to use longer administration periods when investigating AD as a potential treatment for memory disorders.

The present study demonstrates that MB is effective in improving spatial memory using a reference memory procedure of this sort in the water maze. Studies examining the memory-enhancing effects of MB in normal rats have typically used the baited holeboard maze, where odour cues can confound results (e.g., Callaway et al.,2002; Wrubel et al., 2007b). An exception is the study examining MB using rats administered scopolamine by Deiana and colleagues (2009), where a variation of the reference memory procedure was used in the water maze. However, this study did not examine memory improvement over time, and instead examined short-term memory through employing a probe trial after 6 consecutive training trials. The present study can therefore be considered the first study to examine methylene blue using a reference memory training protocol over a extended period.

4.5 Limitations

The present study also has a number of limitations worth acknowledging that should be addressed in future research. Firstly, the sample size used was smaller than intended which may affect the validity of the findings. Unfortunately, due to the nature of the lesion surgery and the advanced age of the rats, during histology two rat were found to have large brain tumours that impinged on the thalamic region and others had swimming difficulties. Therefore, these rats were not included in the study. It is possible that if the sample sizes were increased we may have seen even further discrepancies between the treatment groups. Therefore, the current study could be viewed as a pilot study for a similarly designed study with a larger sample size.

In addition, while the advanced age of the rats can be considered a strength of the study in terms of increasing the lesioned rat's validity as a model of AD, it is also possible that performance in the water maze may have been impaired due to the older age of the rats. This is supported by literature that has demonstrated that aged rats can vary in water maze performance, where some show clear spatial reference memory deficits while others are not impaired and show cognitive capacities similar to those of younger rats (Gallagher & Nicolle, 1993). It has been demonstrated that this variability may be due to impaired hippocampal neurogenesis in some older rats (Drapeau et al., 2003). While the MB vs. Saline groups appeared to be balanced in terms of performance scores prior to drug administration, there were some minor differences, with the rats receiving MB generally performing slightly better in the first testing segment. Therefore, the older rats used in the present study may have varied in pre-existing hippocampal impairment prior to surgery, and thus, some may have shown more spatial memory impairment than others during testing.

Another limitation to the current study is that only one dose level of MB was investigated. A number of recent studies have examined the effects of a range of MB doses (0.5-4 mg/kg) on spatial memory in rats, with general findings that higher doses of up to 4 mg/kg result in greater improvements in spatial memory (Deiana et al., 2009), while doses exceeding 4 mg/kg have a negative impact on spatial memory and can produce anxiogenic effects (Bruchey & Gonzalez-Lima, 2008). This demonstrates the hormetic dose-response of MB, where effects are diminshed with higher doses until they reach baseline (Bruchey & Gonzalez-Lima, 2008), and such dose responses are of great importance when investigating optimal treatment dosage. The present study would have benefited from including a range of MB doses to the treatment regime as it would be interesting to discover the differing effects that smaller and larger doses of MB may have on spatial memory in ATN-lesioned rats, and such findings would contribute greatly to the development of MB as a human pharmacological intervention.

Although the present study demonstrated beneficial effects on memory of MB in ATN-lesioned rats following MB administration, there is no definite way to infer from the results the potential mechanism through which MB exerted its effects. Previous research has spefically examined CO levels in conjunction with memory-enhancing effects of MB, and there is evidence to suggest that increased CO levels is the mechanism through which MB exerts its beneficial effects (Callaway et al., 2004; Gonazalez Lima & Bruchey, 2004; Riha et al., 2005; Wrubel et al., 2007b). As stated above, there are various ways through which MB could have increased CO levels in the ATN lesioned rats which may have contributed to the observed memory improvement, and therefore, it is important that future research looks at CO levels in ATN-lesioned rats and what changes occur after MB administration.

4.6 Implications

To our knowledge, no attempt has been made prior to the current study, apart from research by our lab using the drug cerebrolysin (in prep), to use a pharmacological intervention to ameliorate the behavioural deficits observed after lesions to the anterior thalamus, which is strongly implicated in contributing to the severity of the amnestic disorder in both AD and diencephalic amnesia. The current study therefore demonstrates that MB has beneficial effects for memory following ATN lesions, and these findings have a number of implications relating to research for human memory disorders. As stated previously, there are currently human clinical trials that are investigating the efficacy of MB as a treatment for human AD patients, and findings so far have been impressive (Wischik et al., 2008). These trials have focussed on the action of MB on neurofibrillary tangles, with the authors suggesting that MB's tau-dissolving properties are largely responsible for the improved memory performance in AD patients administered the drug. However, research following the publication of the AD trials has demonstrated inconsistent effects of MB on NFTs, and also shown that MB has potential action on a number of other pathologies associated with AD. The current study therefore adds to this research by suggesting that MB may ameliorate memory disturbances associated with damage to the anterior thalamus, and that this action may also contribute to the beneficial effects observed in the human clinical trials.

Pharmaceutical grade (USP) MB is an FDA approved drug with powerful antioxidant activity that is currently most commonly prescribed for metheglobinemia (Bradberry, 2003). While chemical grade MB is an industrial dye that may contain contaminants with potential toxicity (Auerbach et al., 2010), purified pharmaceutical grade MB used at low doses has no

toxic effect in humans (Bradberry, 2003). The ability of MB to permeate the blood-brain barrier and its unique pharmokinetic properties make it attractive as a potential therapeutic agent. MB presents with a pharmacological profile that differs from the commonly prescribed cholinesterase inhibitors and may provide a novel approach for the treatment of cognitive impairments in neurodegenerative diseases. Due to its metabolic-enhancing and neuroprotective effects, MB could have disease-modifying effects for such diseases without the side effects and high costs associated with currently prescribed pharmaceutical treatments.

4.7 Future directions

There are a number of ways in which the current study can be built upon in future research. To begin with, the effects of MB should also be examined in young rats with ATN lesions. While using older rats in the present study may have resulted in a rodent model that is more representative of human neurodegenerative diseases, their use may also have had a negative impact on performance in the water maze. In addition, the majority of research on the behavioural effects of thalamic lesions has used young rats and it would be interesting to see if even greater improvement in memory are observed in rats that are not already cognitively impaired to varying extents due to old age. Furthermore, while the current study assessed MB using a reference memory procedure, future research should trial MB in ATN-lesioned rats using a variety of memory procedures, such as working memory and fear conditioning. A working memory procedure would be particularly useful in that it would allow the experimentors to assess various doses of MB using the same subjects through administering a different dose for a different platform position following a sufficient wash-

out period. A variety of different doses should be examined in future research as this is valuable information that can be applied to clinical research.

The only other study to date that has demonstrated recovery of function in ATN-lesioned rats following a therapeutic intervention is that by Loukavenko et al. (2007), which showed that rats housed in a stimulating environment or given cerebrolysin (in prep) following surgery showed improved memory performance compared with those lesioned rats housed in a standard environment. Therefore, ATN-lesioned rats exposed to a combination of both MB and an enriched environment may show further recovery of function in terms of memory impairment. Pharmaceutical combination therapies could also be investigated, such as combining currently used memory-enhancing medications with MB. The recent study by Deiana et al. (2009) has provided evidence that the combination of the AChEI rivastigmine with MB resulted in further improvements in spatial memory in scopolamine-impaired rats when compared to those treated with either drug alone. Therefore, such improved performance from combined therapy may also be observed in rats with ATN lesions, with findings being particularly useful in the development of a new pharmaceutical interventions.

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