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Neuroenhancement in normal ageing: preserving cognitive
status through pharmacological interventions.

Boon Wan Wang

May 2, 2012

Declaration

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Thesis 9605

Summary

The preclinical work presented here seeks to establish an enhancement of memory functions in healthy ageing rats via the manipulation of behavioural tasks. This series of studies compared behavioural deficits between thirteen and three months olds male Wistar rats. The motive of using middle-aged animals was to ascertain if cognitive functions were intact prior to severe ageing and, more importantly, if procognitive therapy was able to prevent consequential damage to memory due to advancing age. The task employed was a Water Plus Maze task which was consisted of two different types of trials (fixed and multiple-start) spanning three separate sessions. The extended paradigm allows further insight into different time-points especially the acute (beginning of trials) and chronic (end of trials) drug effects. We were able to demonstrate that manipulating the central cholinergic system and maintaining glucoregulatory functions can alleviate age-related cognitive deficits.

Donepezil, an acetylcholinesterase, was able to alleviate age-related behavioural inflexibility demonstrated in the more challenging multiple-start trials. Rosiglitazone, on the other hand, failed to induce similar effects. However, acquisition was markedly elevated in the initial fixed-start trial suggesting an acute effect of rosiglitazone is sufficient to promote an improvement in cognitive function. Notably, learning and memory were augmented even in younger rats, with no distinct neuromodulatory dysfunction, giving further support for the procognitive abilities of these agents.

The neuroenhancement success in ageing animals with the singular drug treatments of rosiglitazone and donepezil led to a separate study utilising the Spontaneous Object Recognition task. We investigated if a combination of both treatments could augment recognition memory. Although the adjunct therapy ameliorated memory deficits in middle-aged rats, it appears that the joint administration of both drugs produced a dampened effect and was unable to produce the prominent memory enhancement as we theorised. To fully understand the mechanisms behind these drug effects, several molecular components were investigated at the end of each task.

In summary, results from our current work indicated an amelioration of cognitive deficit in middle-aged rats which led to an improved spatial working memory via the enhancement of neurotrophins and an upregulation of the nicotinic alpha 7 ($\alpha 7$) receptor within the hippocampus. Additionally, the heightened cognitive abilities in middle-aged rats under rosiglitazone treatment seemed to be managed through an upregulation of Glucose Transporter-3 receptors in the hippocampus. We propose that enhancing such systems before the onset of ageing might be a potential procognitive method for alleviating the decline of mental functions associated with advancing age.

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To Cheryl, thanks for switching on the lights in the tunnel. Your poem belongs here.

its true
there's no point
i agree
its all blue
be a doctor
be the best for you
don't forget you said
it was the best for you
it hurts
you know how time flies
only yesterday he was born
this summer haze
finish it
remind yourself
never mind how long it will take
do it
and please you

List of abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's Disease
ANOVA	Analysis of variance
ATP	Adenosine-5-triphosphate
BADGE	Bisphenol-A-diglycidyl ether
BchE	Butyrylcholinesterase
BDNF	Brain-derived neurotrophic factor
CA	<i>Cornu Ammonis</i>
ChAT	Choline acetyltransferase
ChEI	Cholinesterase inhibitors
CJD	Creutzfeld-Jakob disease
CNS	Central nervous system
COMBI	Combination
CR	Caloric restriction
CREB	cAMP-response element binding protein
CT	Cycle number
DMTP	Delayed non-matching to position
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DON	Donepezil
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron Transport Chain
FTD	Frontotemporal dementia
GLUT	Glucose Transporter
HD	Huntington's
IDE	Insulin-degrading enzyme

IGF	Insulin-like growth factors
IL	Interleukin
LDL	Low-density lipoprotein
LTD	Long-term depression
LTP	Long-term potentiation
MA	Middle-aged
mAChR	Muscarinic receptor
MCA	Middle cerebral artery
MCI	Mild cognitive impairment
MEM	Memantine
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple-start
MTL	Medial temporal lobe
MWM	Morris Water Maze
NMDA	N-methyl-D-aspartate
O ²	Oxygen
ODR	Object discrimination ratio
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFC	Prefrontal cortex
PI/IL	Prelimbic/Infralimbic
PPAR	Peroxisome proliferator-activator receptor
RAM	Radial Arm Maze
RAWM	Radial Arm Water Maze
RNA	Ribonucleic acid
ROSI	Rosiglitazone
S	Session
SNMTS	Spatial non-matching to sample
SOR	Spontaneous Object Recognition
STS	Short-term store
T2DM	Type 2 Diabetes Mellitus
TZD	Thiazolidinedione
VGCC	voltage-gated Ca(2+) channel
WPM	Water Plus Maze
YA	Young adult

$\alpha 7$	Nicotinic alpha 7 receptor
β NGF	Nerve growth factor

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Chapter 1

The repercussion of ageing on mnemonic functions

“Age is an issue of mind over matter. If you don’t mind, it doesn’t matter.” Mark Twain

1.1 Ageing and its social implications

Ageing is an unavoidable process and encompasses the entire physiological entity. The most recent evidence on population ageing indicated that the proportion of people in Ireland aged 65 years or older is projected to increase from 11% of the overall population in 2006 to 20% by 2036 (Ageing in Ireland, 2007). The impact of ageing spans from an individual’s capacity to becoming a social responsibility. Based on the current characteristics of the Irish public pension system, it might be necessary to carry out reforms to ensure its viability, and therefore, the welfare of the Irish population in the long term (Agudo & García, 2010). Cognitive ageing, itself, can be a liability and recent developments have focused on procognitive measures to aid intellectual repair as a tool to combat the unwavering and persistent ageing population (Flight, 2011; Daffner, 2010). Discrimination against the elderly has led to initiatives in 2005 to counter ageism and elder abuse in Ireland such as the annual “Say no to ageism” week¹. Such prejudice viewed against ageing and the aged themselves is perpetuated by the portrayal of older people as frail, ill, suffering mental deterioration, poor and dependent (Bytheway & Johnson, 1990). It is of no surprise that the core research areas in gerontology studies are aimed at improving ageing or securing “anti-ageing” prospects.

One of the fundamental aims of gerontology studies is to wind back time and build a cognitive reserve by increasing the possibility that age-related cognitive impairments could be restored by drug treatments. “Successful ageing” the book itself (Rowe *et al.*, 1998) has been cited in 1408 times in different articles since 1998, giving grounds to the unyielding need to maintain cognitive functions during ageing. Investigating the ageing brain can be a troublesome area of research as it is often difficult to separate the effects of normal ageing

¹Equality Authority/Health Service Executive, “Say No To Ageism” (<http://www.equality.ie/index.asp?docID=798>)

from those of pathological processes that compromise cognition. For example, something as general as the circulatory system can have an important impact in the central nervous system (CNS). In a longitudinal study neuropsychological assessments demonstrated a link between vascular health and normal brain function which may be agitated by a vascular disease (Knopman *et al.*, 2001). Moreover, diabetes and hypertension were shown to be associated with erosion of cognitive function, as early as late middle age validating the delicate relationship between the circulatory system and brain functions.

1.2 Defining memory systems

Memory is, "the record of experience represented in the brain [whereby] the strength of memory is modulated by emotional arousal and declines in ageing," (Eichenbaum, 2008). Historically, the study of the memory system was split into two main categories: short-term and long-term memory. Short-term memory was originally described by Atkinson and Shiffrin in 1970s as short-term store (STS). This happens when environmental information is entered into STS where it remains temporarily. Any information that remains may be copied into long-term store (LTS). They also go on to suggest that a single unitary STS can also function as working memory which is necessary for learning and the performance of other cognitive tasks. This idea has now been abandoned as there are irregularities in this concept, it is now known that patients with STS impairments had few cognitive problems and have capacity for long-term learning (Baddeley, 1992). The term working memory has since evolved and is commonly used to emphasise the differences between a three-component model and earlier unitary models of short-term memory (Baddeley, 2003). Baddeley proposed a tripartite system encompassing a control system of limited attentional capacity, termed the central executive, assisted by two subsidiary storage systems: the phonological loop and the visuospatial sketchpad (Baddeley *et al.*, 1974). The phonological loop is mainly involved in short-term verbal memory, and it deals with acoustics and verbal information. The visuospatial sketchpad, as the term described, is capable of temporarily maintaining and manipulating visuospatial information which play a role in spatial orientation and the solution of visuospatial problems (Baddeley, 1998). The central executive, on the other hand, plays a crucial role in the working memory framework in coordinating the two other systems. In recent years, Baddeley has suggested a fourth component, the episodic buffer, to represent a storage system using a multimodal code. It holds integrated episodes or scenes and acts as a buffer in providing a limited capacity to interface between systems using different codes (Baddeley, 2003).

Historical neuropsychological studies propel our current understanding of memory components and its inherent properties. Our understanding of declarative memory first begun with studies on patient H.M, who had a bilateral surgical resection of the medial temporal lobes as a treatment from intractable epilepsy by William Scoville. Although he recovered from his epilepsy, he suffered from anterograde amnesia and could no longer form long-term memories. At that time when the H.M case was first described, the physiology of the medial temporal lobe was still not clearly understood. In the last fifty years, we have come to understand that the surgery cost him the ability to both to acquire new information and to recall information learned previously (Scoville & Milner, 1957; Corkin, 2002). He seemed to retain memory that was acquired prior to the surgery (Schmolck *et al.*, 2002), and demonstrated intact primary- or immediate-memory capacity leading scientists to conclude that it was his memory, and only that, that was impaired (Eichenbaum, 1997). Preclinical work can be considered the crux of memory experiments. A decade ago, Goldman-Rakic proposed that the sustained delay-period activity in prefrontal cortex (PFC) found in monkeys during a delayed-response task (Fuster, 1973) and the neuropsychological model short-term memory storage is fundamentally the same mental phenomenon (Goldman-Rakic, 1990) which has proven to be an influential idea (Postle, 2006). In humans, the dorsal and ventral PFC play different roles in component processes of working memory which were revealed in various

imaging techniques paired with memory tasks (Cabeza *et al.*, 1997; Logothetis *et al.*, 2001; Graf & Schacter, 1985; Grady, 2000). Contrastingly, a system for permanently storing, managing, and retrieving information for later use is widely accepted to be long-term memory. This can be further divided into declarative and non-declarative / procedural memory (Squire *et al.*, 2004). Declarative memory refers to the capacity for conscious recollection about facts and events while non-declarative memory is dispositional and is expressed through performance (Squire *et al.*, 2004). It is a well established fact that medial temporal lobe (MTL) structures, including the hippocampus, are critical for declarative memory (Tulving & Markowitsch, 1998). Facts (semantic) and events (episodic) are the further division for declarative memory, although they do overlap. Episodic memory, on the other hand, is concerned with remembering past experiences while semantic memory is related with storage and utilization of knowledge about words and concepts, their properties, and interrelations (Tulving & Thomson, 1973).

1.3 Impact of ageing on cognitive ability

Impairments brought about by ageing can be broadly categorised into three distinct patterns of age-related cognitive decline (life-long declines, declines that occur late in life and relative stability across life) (Hedden & Gabrieli, 2004). In the first category, cognitive disabilities are evident in encoding new memories of episodes or facts, working memory (the simultaneous short-term maintenance and manipulation of information involving executive processes) and processing speed (the speed with which information can be processed) (Craik, 1994). These are the basic mechanisms of cognitive function. One possible cause of memory failures is the insufficient engagement of brain regions (e.g., medial temporal lobe (MTL) regions) important for encoding new information (the process of physical sensory input into one's memory) (Stevens *et al.*, 2008). A model proposed by Iain and colleagues suggested that the reorganisation in aged memory-impaired individuals is apparent and that small concurrent changes during ageing strengthen the auto-associative network of the CA3 subregion at the cost of processing new information coming from the entorhinal cortex. The result of such a reorganisation is that information that is already stored would become the dominant pattern of the hippocampus to the detriment of the ability to encode new information (Wilson *et al.*, 2006).

By contrast, late-life decline lies in short-term memory (a component process of working memory), autobiographical memory, semantic knowledge and emotional processing remain relatively stable until later in life (about the 7th decade). Well-practised tasks or tasks that involve knowledge show little or no decline in performance until very late in life and have relative stability across lifespan. Lastly, the idea that "all is lost" when it comes to ageing is also fraudulent since autobiographical memory, emotional processing and automatic memory processes seem to be unchanged throughout life (Hedden & Gabrieli, 2004; Mather & Carstensen, 2005). One might argue that an advantage of ageing comes with a gain of experience and strategies with time where a greater use of selection, compensation, and optimisation strategies in everyday functioning give rise to more efficient or effective strategies when performing tasks (Lang *et al.*, 2002). Although still debatable, there is also convincing evidence that cognitive training and other interventions (by continued engagement in mentally stimulating activities) can have an immediate beneficial effect on the level of performance in the trained tasks in adults of all ages and may also prevent age-related cognitive decline (Van Praag *et al.*, 2000). This may not be wholly truthful as there is currently little scientific evidence that differential engagement in mentally stimulating activities alters the rate of mental ageing (Salthouse, 2006).

1.4 Mechanistic hypothesis in relation to ageing

The idea of cell senescence is cultivated by the accumulative damages in cellular changes. Oxidative damage is a popular hypothesis in brain ageing which postulated that degenerative changes are arising from free radical reactions. Briefly, oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. How this affects ageing is believed to be the progressive accumulation of ubiquitous free radicals resulting from exposure to ionising radiation, from both non-enzymatic reactions, and enzymatic reactions, particularly those of the two major energy-gaining processes employed by living things-photosynthesis and the reduction of oxygen (O_2) to water (Harman, 1981). An impaired electron transport chain (ETC) leads to the core of modulatory mitochondrial deoxyribonucleic acid (DNA) mutation (Calabrese *et al.*, 2001). With an already impaired ETC, a few related changes are taking place in the cells: decreased ATP production, increased formation of toxic free radicals, and altered calcium homeostasis. Further mitochondrial damage is instigated with these products of ETC dysfunction resulting in cell degeneration and death through oxidation of mitochondrial DNA, proteins, lipids, and opening of the mitochondrial permeability transition pore (Calabrese *et al.*, 2001).

Since mitochondrial respiratory chain is the major cellular producer of reactive oxygen species (Ambrosio *et al.*, 1993), it is inevitable that mitochondrial dysfunction is another critical aspect of cellular changes which accompanies ageing. Modulatory influence of mitochondrial function is important to the ageing process and mutations in them are capable of slowing metabolism resulting in increased or decreased production of reactive oxygen species (Sedensky & Morgan, 2006). Modulatory differences such as a reduction of mitochondrial function, is expected to impair health and shorten lifespan. On the other hand, augmentation of mitochondrial function has been shown to extend lifespan (Bishop *et al.*, 2010). In relation to brain ageing, such dynamics in mitochondrial function might also contribute to age-dependent functional deficits in neurons by causing them to be vulnerable to age-related pathology (Chan, 2006). In a recent review on the neural mechanisms of ageing, reduced mitochondrial function, in terms of mitochondrial gene expression observed during brain ageing, was suggested to be part of an active compensatory mechanism. This occurrence may be useful in resisting transient stress but with persistent stress, a further reduction in mitochondrial function may lead to a cycle of decline (Bishop *et al.*, 2010)

1.4.1 Protein oxidation accompanying the ageing process

Protein oxidation is another mechanistic hypothesis for several neurodegenerative diseases where protein aggregation occurs. Neurodegenerative diseases due to protein oxidations are Alzheimer's disease (AD), Huntington's disease (HD), prion disorders such as Creutzfeld-Jakob disease (CJD), and disorders with aggregated α -synuclein, such as Parkinson's disease (PD) and frontotemporal dementia (FTD). This area of experiments was built upon the basis that ageing and the incidence of oxidative stress increase due to diminished antioxidant defence capabilities, goes hand in hand. The reason why the brain is so susceptible to such oxidative insults is because it requires a large amount of oxygen, is rich in polyunsaturated fatty acids, accumulates redox metal

ions, is relatively low in antioxidants, and is composed largely of non-mitotic cells (Butterfield & Kanski, 2001). An interesting model has been proposed by Ehud Cohen and Andrew Dillin (Cohen & Dillin, 2008) based on the idea that a threshold amount of toxic protein species is required to initiate disease, especially protein-aggregation-mediated neurodegenerative diseases. With this model, it is proposed that toxic protein aggregation and the cellular detoxification capacity determines the age at which the amount of toxic aggregates will cross the threshold level that is required for disease onset. A higher aggregation load and lower protective activities will lead to early disease onset, such as in familial neurodegenerative diseases, whereas lower aggregation load and higher protective capabilities will postpone the age of disease onset. This model proposed an age-related decline in natural counter-proteotoxic activities is the leading cause of similar age-of-onset of different neurodegenerative diseases.

1.4.2 Cell death occurrence in brain ageing

Another widely accepted hypothesis for cellular changes in ageing is apoptosis (the process of programmed cell death) which is central to many neurological disorders as the average lifespan gets longer. It is thought that ageing enhanced apoptosis and susceptibility to it in several types of intact cell. However, it is still debatable whether ageing suppresses or enhances apoptosis in vivo (Higami & Shimokawa, 2000). Apoptosis is up-regulated during ageing in various cells of the CNS and this loss of neurons has been reported in several regions of the senescent brain in human (Anderson *et al.*, 1983). Apart from normal ageing, several neurodegenerative diseases are affected by apoptosis: neuronal death in frontal and hippocampal cortices is responsible for the symptoms of Alzheimer's disease (AD) (Li *et al.*, 1997); death of midbrain neurons that use the neurotransmitter dopamine underlies Parkinson's disease; Huntington's disease involves the death of neurons in the striatum, which control body movements; and death of lower motor neurons manifests as amyotrophic lateral sclerosis (Goodall & Morrison, 2006). The triggers for the neuronal apoptosis are as follows: the lack of neurotrophic factor support, over-activation of glutamate which usually involve calcium influx, increased oxidative stress and finally, metabolic stress (conditions in which levels of glucose, oxygen and other molecules required for ATP [energy] production are decreased) (Mattson, 2000). The relationship between neuronal loss and volumetric changes is a prominent area of clinical ageing research. The morphology of the ageing brain is important in relation to the behavioural component of human ageing. Early post-mortem investigation into the human brain shows robust neuronal loss due to ageing (Brody, 1955; Ball, 1977). On the contrary, current studies demonstrated that age related changes in the morphology of neurons were selective and it seemed that there was no universal pattern across the entire brain. In fact, in most brain areas neuronal loss does not have a significant role in age-related cognitive decline (Burke & Barnes, 2006; West *et al.*, 1994). Such evidences are parallel in rodent model where no loss of principle neurons in the dentate gyrus, CA1 and CA3 of the hippocampus or cortical areas of the medial temporal lobe was demonstrated which normally characterised ageing (Rapp & Gallagher, 1996). Dendritic branching in normal ageing can be extensive in certain area of the hippocampus (Buell & Coleman, 1981) for example in CA1 and subiculum and dendritic extent of apical and

basal trees was found to be stable in normal ageing (Flood & Hanks, 1991).

These changes in neuronal population and brain structure might have an effect on brain volume. In a 5 year clinical study measuring brain volume, hippocampus shrinkage rate observed in older adults was comparable with the rates reported for healthy subjects in the seventh to ninth decades of their lives (Raz *et al.*, 2004). Correspondingly, another imaging study has shown a substantial decline in the rates and regional distribution of gray and white matter tissue loss throughout the brain involving multiple selected brain regions (age 59 to 85 years at baseline), reflecting demyelinating changes in older individuals (Resnick *et al.*, 2003). There was also evidence of an increase in ventricular volume, reflecting central brain atrophy which was significantly greater in older compared with younger individuals. Shrinkage was also distinct in several regions of the brain: depreciation in the cerebellum, hippocampus, the entorhinal cortices, the inferior temporal cortex and the prefrontal white matter increased with age (Raz *et al.*, 2005). In relation to behavioural outcome, it was also observed that hippocampus volumes were significantly smaller for elderly individuals with declining cognitive function compared to stable memory performance over time (Persson *et al.*, 2006). It is of the general consensus that there is indication of a structural and functional pattern of change in ageing that may reflect detrimental processes, the emergence of compensation, or both.

The theories for memory impairment as a result to ageing are numerous and the few that we covered does not justify the global changes that proceed and precede with ageing. In the past years, epidemiological studies established that exercise, intellectual activity, social relationships and a healthy diet all lead to a lower risk of dementia (Deweerd, 2011). Although healthy ageing seems to be our only ally in combating the persistent process of ageing, evaluation of putative anti-ageing therapies might give additional encouragement and hope to the ageing individuals. Enhancement of memory through physiological manipulation, irrespective of any given condition is still rare (Gräff & Tsai, 2011). Notably, in a rodent model, administering insulin-like growth factor II (IGF-II, also known as IGF2) significantly enhances memory retention suggesting a generalised cognition-enhancing qualities (Chen *et al.*, 2011). Fundamental to our work is the preservation of glucoregulatory system. Rosiglitazone, an anti-diabetic drug, is the choice of pharmaceutical manipulation in our ageing rodent utilising middle-aged rats free from any known pathological disorders. Presently, there are several drugs on the market that improve memory, concentration, planning and reduce impulsive behaviour and risky decision-making, and many more are being developed (Sahakian & Morein-Zamir, 2007). Thus, cognitive-enhancing drugs are a useful therapy option for cognitive disorders, including AD. At the current research level, no treatments for AD that can stop or reverse the decline in brain function are available. Cholinesterase inhibitors are being used to ameliorate the impaired neural transmission in the cholinergic system (Heydorn, 1997). Such drugs aim to increase the levels of acetylcholine, a neurotransmitter important for maintaining attention and in forming new memories, and may have additional neuroprotective effects. Another area of our research is directed at manipulating the cholinergic system within an ageing rodent model with, donepezil, an anticholinesterase inhibitor. Such measures might give an indication of how we can rebuild a cognitive reserve and increase the possibility that age-related cognitive impairments could be restored by different drug treatments.

Chapter 2

Materials and Methods

2.1 Materials

Animals

Wistar Rats (3 and 14 month old males)	B&K, UK
Laboratory rat diet	Red Mills, Ireland

ELISA: Plastics and Kits

Rat BDNF ELISA kit	Promega, UK
Rat NGF ELISA kit	R&D Systems, Ireland
Substrate Solution	R&D Systems, Ireland
IL-1 β	R&D Systems, Ireland
Insulin Plasma Kit	DRG, Ireland
Plasma Adiponectin	Phoenix Pharmaceuticals, Inc., US
Plasma Corticosterone	ids, Ireland

General Laboratory Chemicals

Acrylamide	Sigma, Ireland
Ammonium persulphate	Sigma, Ireland
Bio-Rad dye reagent concentrate	Bio-Rad, UK
Bis-acrylamide	Sigma, Ireland
Bovine serum albumin	Sigma, Ireland
Bromophenol blue	Sigma, Ireland
Calcium chloride	Lennox, Ireland
Diethyl Pyrocarbonate (DEPC)	Sigma, Ireland
Dimethyl sulphoxide	Sigma, Ireland

Di-Sodium hydrogen orthophosphate (Na_2HPO_4)	Sigma, Ireland
DL-Dithiothreitol (DTT)	Sigma, Ireland
Ethanol	Lennox, Ireland
Glucose	Lennox, Ireland
Glycerol	Sigma, Ireland
Glycine	Sigma, Ireland
Hydrochloric acid	Lennox, Ireland
Hydrogen peroxide	Sigma, Ireland
Hyperfilm	Amersham, US
Hyperfilm	Pierce, US
Leupeptin	Sigma, Ireland
Magnesium sulphate	Sigma, Ireland
Magnesium Chloride (MgCl_2)	Sigma, Ireland
Mercaptoethanol	Sigma, Ireland
Methanol (MeOH)	Lennox, Ireland
Nitrocellulose membrane	Amersham US
Potassium chloride (KCL)	Sigma, Ireland
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma, Ireland
Potassium hydroxide	Sigma, Ireland
Potassium phosphate	Sigma, Ireland
2-Propanol	Sigma, Ireland
Sodium carbonate (Na_2CO_3)	Sigma, Ireland
Sodium bicarbonate (NaHCO_3)	Sigma, Ireland
Sodium chloride (NaCl)	Sigma, Ireland
Sodium dodecylsulphate (SDS)	Sigma, Ireland
Sodium hydrogen carbonate	Lennox, Ireland
Sodium hydroxide (NaOH)	Lennox, Ireland
Sodium phosphate (monobasic)	Sigma, Ireland
Sodium phosphate (dibasic)	Sigma, Ireland
Sucrose	Sigma, Ireland
Standard grade No. 3 filter paper	Whatman, Ireland
Tetramethyl-diamine (TEMED)	Sigma, Ireland
Tetramethylbenzidine (TMB)	R&D Systems
Tris-base	Sigma, Ireland
Tris-HCl	Sigma, Ireland
Tween-20	Lennox, Ireland

General Laboratory Products

Microtest 96-well flat bottomed plates	Sarstedt, Ireland
Pipette Tips	Sarstedt, Ireland
Microtubes	Sarstedt, Ireland
Plastic transfer pipettes	Sarstedt, Ireland
Laboratory roll	Parafilm, US

Molecular Reagents

Absolute ethanol	Sigma, Ireland
Agarose	Sigma, Ireland
Biosphere filter pipette tips	Sarstedt, Ireland
Ethidium Bromide	Sigma, Ireland
High Capacity cDNA archive kit	Applied Biosystems
Loading Dye (6X)	Promega, UK
Molecular Grade Water	Sigma, Ireland
Optical adhesive covers	Applied Biosystems, Ireland
PCR tubes	Sarstedt, Ireland
RNAlater TM	Ambion, Ireland
RNase away	Invitrogen, Ireland
RNase-free microtubes	Ambion, Ireland
RNase Zap wipes	Ambion, Ireland
Total RNA isolation kit	Macherney-Nagel, Germany
Taqman gene expression assays (see table 2.2)	Applied Biosystems, Ireland
Taqman universal PCR master mix	Applied Biosystems, Ireland
10 X TBE buffer	Invitrogen, Ireland
96-well optical reaction plates	Applied Biosystems, Ireland

Western Blotting Reagents and Antibodies

Glucose Transporter GLUT3	Abcam, Ireland
Anti-mouse IgG HRP	Sigma, Ireland
Anti-rabbit IgG HRP	Sigma, Ireland
Nicotinic Acetylcholine Receptor alpha 7	Abcam, Ireland
Hyperfilm	Pierce, US
Nitrocellulose membrane	Amersham, US
Prestained dual band molecular weight standard	Biorad, UK

ReBlot Plus strong antibody stripping solution

Chemicon

SuperSignal

Pierce, US

Drug dosing

Pure Canadian Maple Syrup

Newforge, Ireland

Rosiglitazone

GlaxoSmithKline, UK

Aricept

Pfizer, UK

Behavioural Equipment and Software

EthoVision 3.1

Noldus Information Technology, Netherlands

Plusmaze

Made to measure, Trinity College, Ireland

2.2 Animals

2.2.1 Water Plus Maze task

Young adult (YA; 3 months) and middle-aged (MA; 12-14 months) Male Wistar rats (B&K, UK) were used. At the start of the experiments, rats weighed between $400 \text{ g} \pm 4$ (YA) and $598 \text{ g} \pm 10$ (MA). The rats were housed in groups of two or three in a controlled environment (temperature: $20\text{-}22^\circ\text{C}$, 12/12 h light/dark cycle) with free access to food and water. In total, six subgroups were used (Donepezil; MA and YA, $n = 8$), (rosiglitazone; MA and YA, $n = 10$) and (control; MA and YA, $n = 10$). All experiments were carried out under a license from the Department of Health and Children (Ireland) and with ethical approval from Trinity College Dublin Animal Users Ethical Committee.

2.2.2 Object Recognition task

Male Wistar rats were used and obtained from Bioresources on campus in Trinity College. Middle aged (MA) rats were aged between 13-14 months (average weight between $635 \pm 20\text{g}$) and young adult (YA) rats were between 3-4 months old (average weight between $331 \pm 0\text{g}$). The rats were housed in groups of two in a controlled environment (temperature: $20\text{--}22^\circ\text{C}$ Celsius, 12/12 h light/dark cycle) with free access to food and water. Rats were then randomly assigned to DON-treated, ROSI-treated, DON- and ROSI-treated (ROSI+DON) and CTL. There were 64 rats in total, 32 MA and 32 YA split further into four groups of eight for each age group giving a total of eight subgroups (MACTL, MADON, MAROSI, MACOMBI, YACTL, YADON, YAROSI, YACOMBI). Rats were handled 3 days prior to experimentation to familiarise them with the surroundings and handling in general. All experiments were carried out under a license from the Department of Health and Children (Ireland) and with ethical approval from Trinity College Dublin Animal Users Ethical Committee.

2.3 Methods for Water Plus Maze task

2.3.1 Drug dosing and regime

All rats were handled for one week pre-drug treatment and fed 0.2mL of maple syrup daily (Pure Canadian Maple Syrup, Newforge) to familiarise them with feeding by syringe. Donepezil Hydrochloride is an acetylcholinesterase inhibitor and is marketed by Pfizer. This treatment was administered orally mixed in a solution of maple syrup. Treated rats were given an oral suspension of maple syrup and Donepezil (0.3mg/kg/day; *p.o.*) while control group of rat were given maple syrup only (0.2mL/rat/day). Rosiglitazone is a thiazolidinedione (5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione) and is marketed by GlaxoSmithKline as Avandia®. Rosiglitazone was also administered orally mixed in a solution of maple syrup. Treated rats were given an oral dose of maple syrup and rosiglitazone suspension (6 mg/kg/day; *p.o.*) while controls were given maple syrup only. The dose of 6 mg/kg/day was chosen based on an effective dose range of 3-10 mg/kg/day in rodents reported in the literature for peripheral insulin sensitisation (Kramer *et al.*, 2001; Roy *et al.*, 2007) and improvement in cognitive performance (Gemma *et al.*, 2004; Pedersen *et al.*, 2006; Pathan *et al.*, 2008). Rosiglitazone in rats typically has a T_{max} of 2 hours in blood following oral dosing. Based on unpublished PK/PD studies conducted by GSK colleagues, there has been no difference between measured PK profiles when comparing aged, middle-aged and young adult rats. Chronic treatment of rosiglitazone and donepezil was maintained daily for 40 days and drug was administered 14 hours before the start of each behavioural testing.

2.3.2 Water Plus Maze

The water plus maze (see Figure 2.1) was a black, fibreglass plus-shaped water-filled pool with four arms with a hidden platform at the end of one arm. (Dimensions: 110 cm long; 50 cm deep; arm width 10 cm; arm length 50 cm). The water was maintained at room temperature (21°C). A platform (10x10x40cm) was placed at the end of one arm, hidden approximately 5 cm below the water surface. The decision zone was the intersection between the arms of the plus maze; it was 15 cm in diameter measured from the centre of the arm projecting outwards to each of the 4 sides of the maze. The hidden platform remained in a fixed position for the duration of the task. The maze was located in a quiet room and was surrounded by black curtains on which large visual cues were attached, while a personal computer was situated on a bench behind the curtains.

Each trial was a maximum of 60 seconds long. The trial was stopped when the animal either found the platform or entered one of the incorrect arms (an arm in which the platform was not situated). The animal was subsequently removed and placed in a holding area for an inter-trial interval which was 30 seconds long. The experimenter remained out of sight behind the curtains during each trial. There were 3 test sessions (commencing on days 1, 18 and 37 of dosing) in total; each session consisted of 3 days of fixed-start trials followed by a multiple-start probe test (see Figure 2.2 for an overview of the experimental design).

Briefly, fixed-start comprised of rats being released from a predetermined arm (e.g. South). The animal

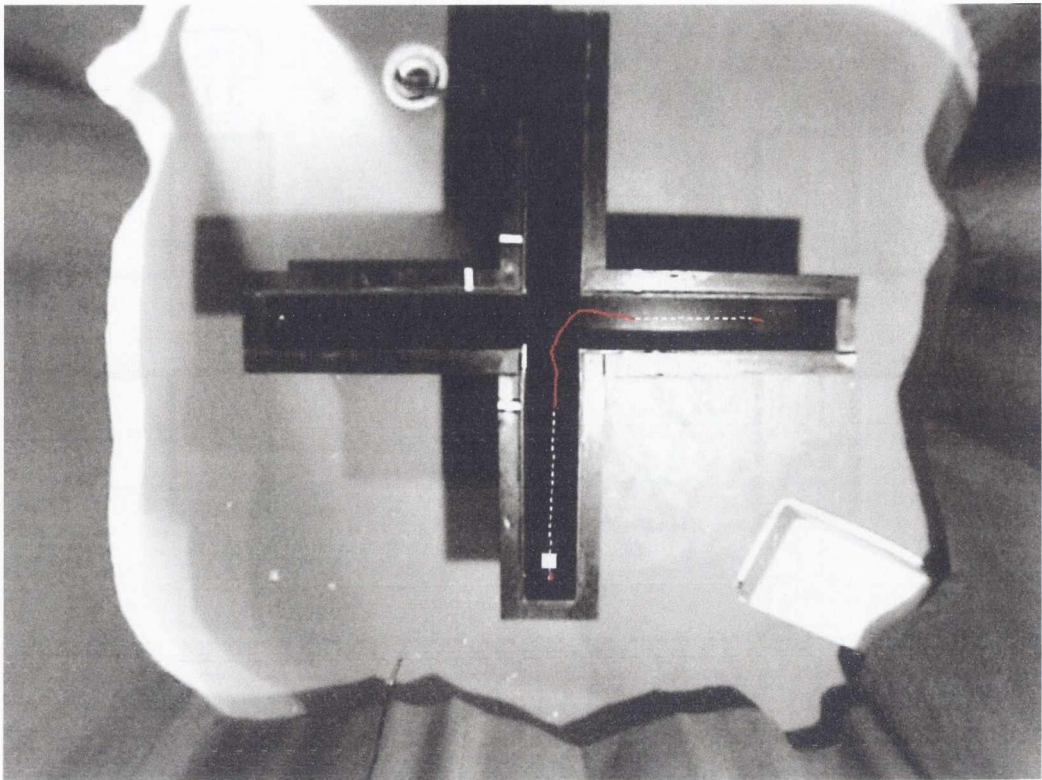


Figure 2.1: **Diagram of water plus maze.** A photograph of the Water Plus Maze (WPM). Spatial cues were provided although not visible in this picture. The track indicates the recorded swim behaviour of the rat. In this case, the rat made a right turn response to locate escape platform during fixed-start trials.

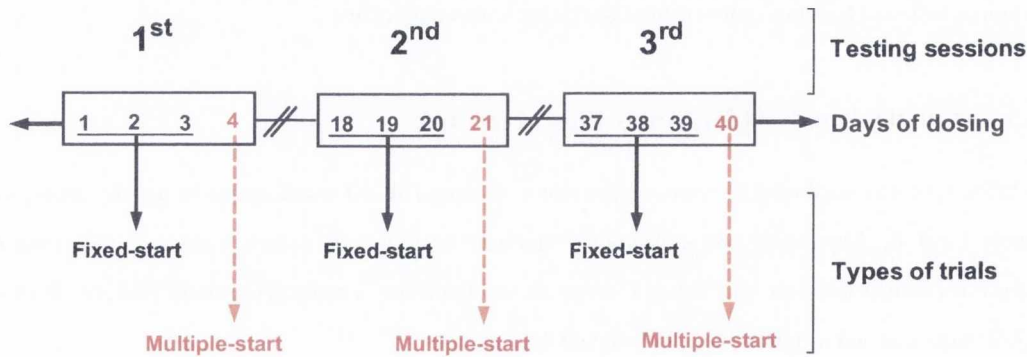
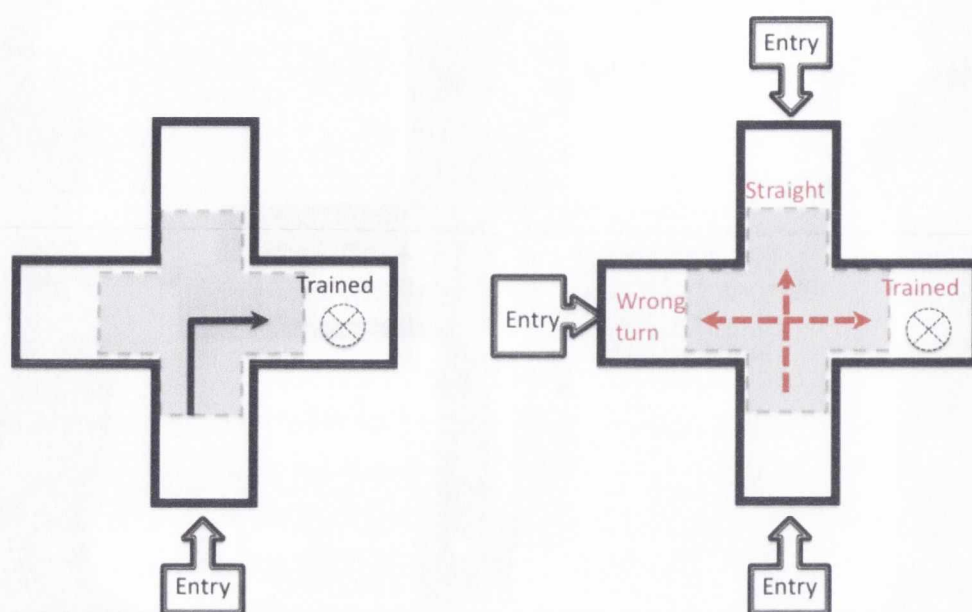


Figure 2.2: **Water plus maze timeline for dosing and behavioural testing.** Timeline representation for the dosing regime pertaining to the chronic administration of Donepezil. There were 3 sessions in total and within each session were 4 testing days (within boxed area). The first 3 days were fixed-start trials (10 trials/day) while the last day was the more difficult multiple-start trials. The parallel lines (//) between the boxes represented days of rest period between behavioural testing although dosing was continuous throughout all 40 days.



Fixed start trial

Multiple-start trial

E.g. South entry, trained right turn response

E.g. South entry, 3 types of response available

Figure 2.3: **Arrangement of fixed- and multiple-start trial.** (A) Plus-shaped maze with 4 separate arms and designated decision zone (grey area). (B) Procedure for entries and escape paths in Multiple-start (MS) trials. Red arrows indicate the swim behaviour and responses relevant to MS trials. The black arrow demonstrated the trained response for fixed-start trials. Protocol design was randomly allocated for rats and entry for all arms is balanced within, and for all trials. (not drawn to scale)

was required to find the allocated escape platform, which was also predetermined and remained consistent for both types of trials (eg., East). This start arm was held constant for the rat throughout the first 3 days of testing, which consisted of 10 trials/day. On the fourth day, multiple-start trial begins when the animal was released randomly from the remaining 3 arms that did not contain the hidden platform for a total of 30 trials. This harder task (as demonstrated from the data collected) was completed in one day. Trials were recorded and analysed by a computer-based tracking system using EthoVision Software (Noldus).

2.3.3 Statistical analysis for Water Plus Maze task

The WPM task was employed in assessing the effect of donepezil and rosiglitazone in healthy ageing (see chapters 3 and 4). Importantly, the same control rats were used in both studies in the attempt to meet the standards for animal use under the Trinity College 3R requirements. A comparison study between all groups (control, donepezil and rosiglitazone) can be found in chapter 6.

Normality tests were conducted prior to nonparametric repeated measures ANOVA. Data from young adult control (YACTL), young adult treated [donepezil (DON)/ rosiglitazone(ROSI)], middle-aged control (MACTL) and middle-aged treated [donepezil (DON)/ rosiglitazone(ROSI)] were first analysed between-sessions followed by a within-session analysis (how each group performed within S1, 2 and 3 respectively). Post-hoc tests

were conducted using Bonferroni multiple comparisons test and significance levels were set at 0.05. Individual training days were pooled into the relevant fixed-start trials for each session. Within each session data from fixed-start and multiple start trials were analysed separately. Non-parametric ANOVAs with repeated measures were conducted using performance scores with sessions as between-subjects variable and treatment groups ($n = 10$ rats for each group) as within-subjects variable. The same analysis was used for multiple start tests conducted at the end of each session after fixed-start trials [3 individual sessions (within subjects) X 4 age-treatment groups (between subjects)]. Dunns multiple comparisons test was used as a post-hoc test. Correct responses and swim speeds for fixed-start trials were pooled from the 3 days of training for each session which consists of 30 trials per rat (10 trials for each day of fixed-start training). The multiple start test was conducted in a single day and consisted of 30 trials per rat (see Figure 2.3). ANOVA was also used for the analysis of errors during multiple start trials with type of errors as between-subjects variable and treatment groups as within-subjects variable.

2.4 Methods for Spontaneous Object Recognition task

2.4.1 Drug dosing and regime

ROSI is a thiazolidinedione (5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione) and is marketed by GlaxoSmithKline as Avandia. DON is an acetylcholinesterase marketed by Pfizer as Aricept. Maple syrup was used as a control (Pure Canadian Maple Syrup, Newforge). All drugs were administered orally mixed in a solution of maple syrup via a 1ml syringe. Each ROSI rat received 0.2ml of a 6mg/kg/day ROSI and maple syrup suspension. Each DON rat received 0.2ml of a 0.3mg/kg/day of DON and maple syrup suspension. Rats receiving a combination of the two drugs got 0.2ml of a mixed 6mg/kg/day ROSI and 0.3mg/kg/day DON with maple syrup suspension. Rats were dosed for 5 consecutive days and were not dosed on the last day of testing i.e. on day of sacrifice.

2.4.2 Methods for behavioural paradigm

A circular arena, 45cm high x 50cm wide, made from black cardboard was used. Sawdust was used as a base. A recording camera was mounted on the ceiling providing an aerial view of the arena. Recording was done and was linked to a computer behind the curtain. CyberLink Powerdirector software was used to record the rats. The arena was located in a quiet room surrounded by black curtains with two 60 watt lamps during experimentation. Each rat was habituated with the arena for a period of 10 minutes in the 2 days preceding testing. On the first testing day two identical objects were placed in the arena (sample object A). Each rat was placed in the centre of the arena, with its nose pointing away from the objects, to avoid bias. Sample exposure was for 10 minutes. 1 hour later each rat was placed in the arena with sample object A and an unseen i.e. novel object B, for 3 minutes. 24 hours later the same was done but with novel object C. Objects used were made from Lego and cleaned with 70% alcohol spray between each animal.

2.4.3 Analysis of recordings

A timer was assigned to each object. The appropriate timer was started when the animal's nose was directed at the object and stopped when animal ceased contact. Data was not recorded for any duration when animal was on top or leaning against the objects. This was continued for the duration of the recording. The scoring methods used here were closely adapted from Bevins et al (Bevins & Besheer, 2006). Times were converted to milliseconds and discrimination ratio, total exploration time and difference scores were found using Microsoft Excel.

2.4.4 Statistical analysis for OR task

Three main observations were analysed in the OR task. An actual exploration span is firstly analysed which presented the measure of interaction the rats have, in total, with both objects during each phase within the arena. This method of comparison enabled an accountability for what rats were doing in each because some rats



Figure 2.4: **Picture of the Spontaneous Object Recognition task.** Testing arena for the SOR task. A total of 3 different objects were used depending on phase of the task. A black curtain was used to surround the arena.

might not explore and remain stagnant within the arena. The percentage of time spent for the phases between familiarisation and delays trials were different (e.g. 10 minutes for familiarisation while only 3 minutes for delay).

Secondly, an object discrimination ratio was determined as a measurement of novel object interaction time as a ratio to the actual exploration span (see section 5.3.1). Chance level is set at 0.5 which means rats spend half the exploration time on the novel object. Recognition memory and ability to discriminate between novel and sample object are considered intact when ODR is above chance. Lastly, a preference score (difference in time spent between novel and sample) was analysed. A negative score represents more time spent on the sample object instead. A score of zero indicates that animal spent an equal amount of time on both the novel and sample objects. For all the parameters analysed, a one-way ANOVA for within group measures was used. Tukey's Multiple Comparison post hoc test revealed further interactions for each age group at different phases

2.5 Methods for hippocampal tissue analysis

2.5.1 Tissue and blood preparation

Rats were sacrificed by decapitation six hours after the last dose of drug administration at the end of the behavioural experiments. Trunk blood and brain tissue was collected and stored for subsequent analysis. Plasma preparation from whole blood was conducted as follows: Anti-coagulant tubes were prepared by coating falcon tubes with NaOH-EDTA. Trunk blood dispensed into the prepared tubes was immediately mixed and kept on ice. Tubes were centrifuged at 3000 rpm at 4°C for 10 minutes and plasma was removed and frozen at -80°C.

Hippocampus tissue extraction was done on ice. Samples were homogenized in the appropriate buffers and stored for further analysis. Individual tissue samples for ELISA were homogenized in 500 μ L Krebs buffer [composition: 7.95 g NaCl, 0.19 g KCL, 0.16 g KH_2PO_4 , 0.27 g MgSO_4 , 1.34 g NaHCO_3 , 1.8 g Glucose; pH 7.3 add CaCl_2 (1:5000 dilution before use)] while samples for gel electrophoresis were stored in 500 μ L Lysis buffer (composition: 0.158 g Tris-HCL, 0.292 g NaCl, 0.446 g $\text{Na}_2\text{PO}_7 \cdot 10\text{H}_2\text{O}$, 0.21 g NaF, 1% IGEPAL, pH to 7.4 add Na_3VO_4 1:100 dilution, PMSF 1:100 dilution, protease inhibitor cocktail 1:100 dilution). All homogenized samples were stored at -20°C for later analysis.

2.5.2 Protein quantification using the Bradford assay

A dye reagent was first prepared by making a 1:5 dilution of Bio-rad in double distilled water followed by filtration. A 1000 μ g/ml Bovine serum albumin (BSA) stock was prepared and standard comprising a range of 0-1000 μ g/ml was made up. 10 μ L of each dilution of standard and sample was added in duplicate in to a sarstedt 96-well plate. 200 μ L of dye was added and plates were incubated for 5 minutes at room temperature before absorbance was read at 595 nm using a plate reader.

2.5.3 Preparation of samples for gel electrophoresis

Samples of homogenate were equalized for protein concentration (Bradford, 1976) and aliquots of sample (100 μ l) were added to sample buffer (100 μ l; composition: Tris-HCL pH 6.8, 0.5M; SDS, 10

2.5.4 Gel electrophoresis

Acrylamide gels, 7.5% or 10% (see Appendix) were cast between 2 glass plates and inserted into the electrophoresis unit (BioRad Mini-PROTEAN 3, BioRad Laboratories, Hertfordshire, England). Electrode running buffer (composition: Tris base, 25 mM; glycine, 200 mM; SDS 17 mM) was added to the inner and outer reservoirs. Prepared samples (10 μ L) or pre-stained molecular weight markers (5 μ L; BioRad) were loaded into the wells and run at 32 mA for approximately 40 minutes then transferred to Polyvinylidene Fluoride (PVDF) membrane.

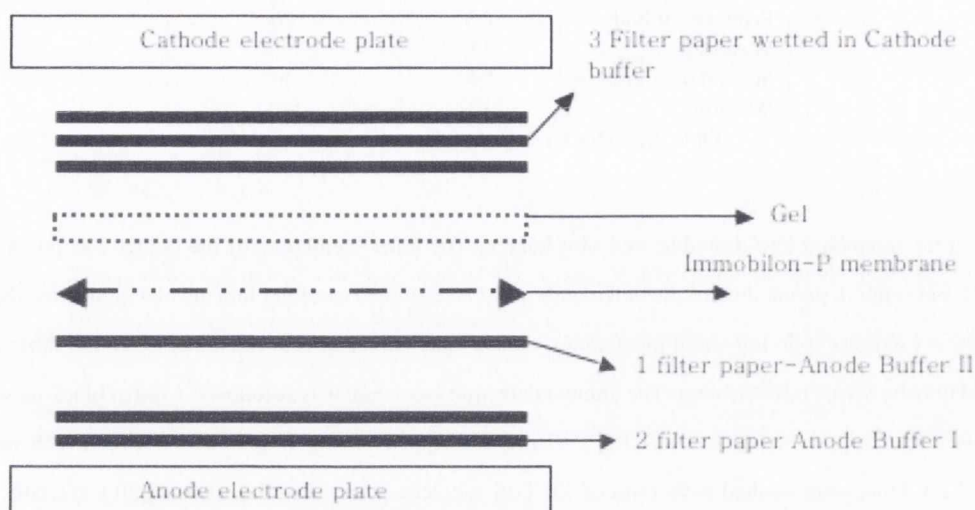


Figure 2.5: **Transfer stack for western blotting using SNAP i.d.® System.** PVDF Immobilon-P membrane was used in place of the more traditional nitrocellulose membrane. A total of six filter paper (dampen in its respective buffers) were used in addition to the gel, itself. A roller provided by the system was used to remove air bubbles between stacks.

2.6 Methods for western immunoblotting

2.6.1 Western blotting

Three separate buffers were prepared (see Appendix); Cathode, Anode I and Anode II. Gel was immersed in 100 μ L of cathode buffer and left to equilibrate for 15 minutes. The membrane was incubated in 100% methanol for 15 seconds and quickly transferred to autoclaved water for 2 minutes and lastly incubated in Anode II buffer to equilibrate for 5 minutes or more. The transfer stack was subsequently assembled (see Figure 2.5). The lid, containing the cathode, was placed firmly on top and the transfer was carried out at 225 mA for 80 min.

Following the transfer, the gel was discarded along with the filter papers. PVDF membrane was left to soak in 100% methanol for 10 seconds in order to drive water out. The membrane was placed on top of filter paper and allowed to dry fully (around 15 minutes) until it appeared opaque. Drying the membrane enhances protein binding to the surface and reduces background noise. The membrane was rehydrated with 100% methanol and rinsed with autoclaved water prior to usage.

2.6.2 Protein detection

Millipore's SNAP i.d.® Protein Detection System was used to quantify proteins of interest. Using a vacuum-driven system, all immunodetection steps after the protein transfer to a membrane (i.e., blocking, washing, and primary and secondary antibody incubations) were done utilizing the SNAP i.d.® Protein Detection System.

Steps	Volume (ml)	Duration (minutes)
Blocking	15	10
Washing	15	0.5
Primary antibody	1.5	20
Washing	15	0.5
Secondary antibody	1.5	20
Washing	15	0.5

Table 2.2: SNAP i.d.[®] Protein Detection Protocol

Blots were assembled into a double well blot holder. The inner membrane of the holder was pre-wet with autoclaved water in preparation of the blot membrane. Once aligned carefully into the blot holder together with the spacer (to prevent drying out of membrane), excess water was removed with a blot roller and subsequently placed into the SNAP i.d.[®] System. The immunodetection procedure was as follows: 15ml of blocking solution was used for each well (0.05% BSA-TBS-TWEEN; see Appendix) for 10 minutes. Between each step (see table 2.2), blots were washed with 15ml of 1X TBS supplemented with 0.1% Tween[®]20 surfactant. Three sequential washes were done with the vacuum left continuously on to ensure optimal performance. The primary antibody solution was evenly applied to each blot and incubated for 20 minutes with the vacuum pump off. Immediately after washing off the primary antibody solution, the secondary antibody was applied to blot. The vacuum was activated once again and the blots washed three times with TBST prior to visualization of the immunoreactive proteins. After the final wash, the blots were incubated for 5 minutes at room temperature with SuperSignal West Dura Extended Duration Substrate. A 1:1 ratio of the two substrates were mixed and applied evenly to each blot. The blots were exposed to the photographic film in the dark and the film was developed using a Fuji X-ray processor. Following Western immunoblotting for proteins of interest, all blots were stripped with an antibody stripping solution (1:10 dilution in dH₂O; ReBlot plus Strong Antibody Stripping Solution; Millipore) and reprobed for β -actin expression, an endogenous control gene used to confirm equal loading of protein.

2.6.3 Hippocampal nicotinic alpha 7 ($\alpha 7$) receptor protein detection

The expression of the hippocampal $\alpha 7$ receptor was assessed on 7.5% SDS Page gels. The nitrocellulose membrane was blocked for non-specific binding by incubation with TBS-T containing 5% BSA for 15 minutes in the blot holder at room temperature. The blot was washed with 15ml of 1X TBS supplemented with 0.1% Tween[®]20 surfactant via the vacuum pump. TBST was used to wash the membrane, three times in 5 ml each time TBS-T and then incubated with the primary antibody (see Table 2.3; 1:300 dilution in TBS-T containing 2% BSA) for 20 minutes at room temperature. The blot was washed again with 15ml of 1X TBS supplemented with 0.1% Tween[®]20 surfactant. Blot was further incubated with the secondary antibody [Anti-Rabbit IgG (whole molecule) peroxidase antibody raised in goat ((see Table 2.3; 1:10,000 dilution in TBS-T containing 2% BSA) for 20 minutes at room temperature. The blot was washed again with 15ml of 1X TBS supplemented with 0.1% Tween[®]20 surfactant, before SuperSignal was added for 5min. The membrane was exposed to the photographic film in the dark and the film was development using a Fuji X-ray processor.

1°Antibody	Dilution	2°Antibody	Dilution
Glucose Transporter GLUT3 antibody (abcam: ab41525)	1:700	Anti-Rabbit IgG (whole molecule) Peroxidase antibody produced in goat (Sigma: A0545)	1:10,000
Nicotinic Acetylcholine Receptor alpha 7 antibody (abcam: ab23832)	1:300	Anti-Rabbit IgG (whole molecule) Peroxidase antibody produced in goat (Sigma: A0545)	1:10,000
Monoclonal Anti-Actin antibody produced in mouse (Sigma: A3853)	1:2000	Anti-Rabbit IgG (whole molecule) Peroxidase antibody produced in goat (Sigma: A0545)	1:10,000

Table 2.3: List of Antibodies used for western Immunoblotting for proteins of interest

2.6.4 Hippocampal glucose transporter protein-3 (GLUT-3) receptor protein detection

Protein detection method for hippocampal GLUT-3 receptor was detailed in the SNAP i.d.[®] protocol (listed in Table 2.2). Between each steps the vacuum pumped was activated to wash the membrane and remove the blocking agent, primary and secondary antibodies (listed in Table 2.3).

2.6.5 Hippocampal interleukin-1 beta (IL-1β) receptor protein detection

Protein detection method for hippocampal IL-1β receptor was detailed in the SNAP i.d.[®] protocol (listed in Table 2.2). Between each steps the vacuum pumped was activated to wash the membrane and remove the blocking agent, primary and secondary antibodies (listed in Table 2.3).

2.6.6 Densitometric Analysis

All protein bands were quantitated by densitometric analysis using the ZERO-DScan Image Analysis System (Scanalytics, Fairfax, USA) or the Gel Doc It Imaging System (UVP, Medical Supply Company,Ireland) in conjunction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

2.7 Methods for enzyme-linked immunosorbent (ELISA) assay

2.7.1 Preparation of Samples for ELISA

Homogenized samples were thawed and spun down at 500g for 30 seconds. This was to separate the pellet from the supernatant. The supernatant was used for all ELISA assays.

2.7.2 Analysis of brain-derived neurotrophin factor (BDNF) concentration by ELISA

The levels of neurotrophin BDNF were investigated in supernatant prepared from previously equalized supernatant prepared from homogenized brain samples of the hippocampal brain region using ELISA. A 1:500 dilution of anti-BDNF monoclonal coating antibodies was prepared by adding 10 μ l of capture antibody to 9.99 ml of carbonate coating buffer (pH 9.7). 100 μ l of this reagent was added to each of the 96-well plates (Nunc-Immuno plate with maxiSorp surface) and left to bind overnight at room temperature. The plates were washed in TBS-Tween (0.05%, pH 7.2-7.4) and reagent diluent was subsequently added to all the wells and left to incubate for an hour at room temperature. The plates were washed as mentioned above before the addition of duplicates for each standard (100 μ l; 0-500 pg/ml; BDNF Standard; Promega, UK), and individual samples. Plates were left to incubate for 2 hours followed by 5 washes of TBS-Tween. The detection antibody provided in the kit is prepared from Anti-Human BDNF polyclonal antibodies. This reagent (100 μ l; 100 ng/ μ l in Block and Sample Buffer) was added to the plates and left to incubate for 2 hours and then rinsed out with TBS-Tween. 1:200 dilution of Anti-IgY HRP Conjugate was prepared by adding 50 μ l of Anti-IgY HRP Conjugate to 9.95 ml of Block and Sample 1X Buffer. 10 μ l of this solution was added to each well and incubated for an hour. 100 μ l of TMB One solution was added into each well and incubated for 10 minutes. Reaction was stopped (100 μ l; 1N hydrochloric acid) and the plates were read at 450nm using a 96 well plate reader (Labsystems, Multiskan RC). BDNF concentrations were estimated following extrapolation from the standard curve and the results were expressed as pg BDNF/ml.

2.7.3 Analysis of beta-nerve growth factor (β -NGF) concentration by ELISA

NGF expression was analyzed in supernatant prepared from previously equalized homogenized samples of the hippocampus using ELISA. 96-well plates (Nunc-Immuno plate with maxiSorp surface) were coated with capture antibody (100 μ l; 0.4 μ g/ml in PBS; R&D Systems) and incubated at room temperature overnight. Plates were washed in PBS-Tween (0.05%, pH 7.2-7.4), a blocking buffer, reagent diluent was then added to the wells (300 μ l; 1% BSA in PBS) and the plates were incubated for 1 hr at room temperature without shaking. Plates were washed in PBS-Tween and duplicate (100 μ l) sample and standards (100 μ l; 0-1000 pg/ml; NGF Standard; R&D Systems), were added to plates and incubated for 2 hrs at room temperature. Following washing, detection antibody was added to the plate (100 μ l; 100 ng/ml in reagent diluent) was added to each well and incubated at room temperature for 2 hrs. The plates were washed and Streptavidin-HRP (10 μ l; 1:40 dilution in reagent diluent; R&D Systems) was added to each well and incubated for 20 minutes at

room temperature away from direct light. Plates were again washed with PBS-Tween (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2 - 7.4, 0.05% Tween®20) and Substrate Solution (100 µl) was added and incubated at room temperature (away from direct sunlight) for 20 minutes or until the colour developed. The reaction was stopped using 50 µl of 2N sulphuric acid. Plates were read at 450 nm using a 96 well plate reader (Labsystems, Multiskan RC) NGF concentrations were estimated following extrapolation from the standard curve and results were expressed as pg NGF/ml.

2.7.4 Analysis of plasma insulin concentration by ELISA

All blood specimens used were generated under non-fasting conditions. Endogenous plasma insulin levels were determined with a rat/mouse insulin ELISA kit (EMD Millipore (NYSE: MIL), Billerica, Massachusetts). Assay procedure was carried out as in the following steps. 10X wash buffer concentrate provided was diluted 10 fold with 450ml deionised water. Each well was washed 3 times with 300µl of diluted wash buffer per wash. All washes were carried out using the labtech LT-3000 microplate washer. Residual wash buffer was removed by inverting the plate and tapping it onto absorbent tissue. All wells were done in duplicate. 10µl assay buffer was added to the blank and sample wells. 10µl matrix solution was added to the blank, standard and quality control wells. 10µl rat insulin standards were added to the standard wells in descending order (10ng/mL, 5ng/mL, 2ng/mL, 1ng/mL, 0.5ng/mL and 0.2ng/mL). 10µl of quality control 1 was added to its wells on plate 1 and 10l of quality control 2 to plate 2. To sample wells 10µl of the plasma sample to be tested was added. 80µl detection antibody was added to all wells. The plate was covered with a plate sealer and incubated at room temperature for 2 hours on a plate shaker (Stuart Scientific, UK) that rotated at 400rpm. The plate sealers were removed and solutions decanted. Plates were tapped as before to remove residual solution. Wells were washed 3 times with 300 µl diluted wash buffer per well per wash. 100µl enzyme solution was added to each well. Plate was sealed and incubated at room temperature for 30 minutes on the plate shaker. The sealer was removed and solutions decanted. Wells were washed 6 times with 300µl diluted wash buffer per well per wash. Plates were tapped and residual solution removed as before. 100µl substrate solution was added to each well and the plate was sealed and shaken by hand to gently mix solution in wells. When appropriate blue colour was seen in most wells 100µl stop solution was added. The plate was shaken to ensure complete mixing of stop solution which turned blue colour to yellow (acidification). Any bubbles were burst with a needle. The absorbance was read at 450nm and 595nm in a plate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments Inc. U.S.A).

2.7.5 Analysis of plasma adiponectin concentration by ELISA

Plasma samples were diluted 1:500 with 1X assay buffer provided in the Millipore rat adiponectin ELISA kit (EMD Millipore (NYSE: MIL), Billerica, Massachusetts, USA). The dilution was made by adding 10µl plasma sample to 990µl 1X assay buffer, then 100µl of this diluted sample was added to 400µl 1X assay buffer to give a 1:500 dilution. Rat adiponectin standards were prepared by adding 0.5 ml distilled water into the glass vial

of standard provided to reconstitute a 200ng/mL concentration of adiponectin standard. The vial was inverted, allowed sit for 5 minutes then vortexed (GV Lab, Gilson) gently. Rat adiponectin quality controls 1 and 2 were also reconstituted with 0.5 ml distilled water. They were inverted, allowed sit for 5 minutes then mixed well. All reagents were pre warmed to room temperature prior to assay setup. 10X wash buffer was diluted 10 fold by mixing its contents with 900 ml distilled water. Plates were assembled and labelled accordingly then washed 3 times with 300 μ l diluted wash buffer. A plate washer (labtech LT-3000 microplate washer) was used for all wash steps. 80 μ l assay running buffer was added to all wells. An additional 20 μ l assay running buffer was added to blank wells. 20l rat adiponectin standard was added in descending order to the standard wells. 20 μ l QC1 was added to plate 1 and 20 μ l QC2 was added to plate 2. 20 μ l of each sample of rat plasma was added to the remaining wells. The plate was covered with the plate sealer provided and incubated at room temperature for 2 hours on a plate shaker (Stuart Scientific, UK). The plate sealer was removed, solutions decanted and tapped on absorbent tissue to remove residual solution. Each well was washed 3 times as before. 100 μ l detection antibody was added to all wells. The plate was covered with a plate sealer and incubated at room temperature for 1 hour on a plate shaker (Stuart Scientific, UK). The plate sealer was removed, solutions decanted and plates tapped as before. Each well was washed 3 times as before. 100 μ l enzyme solution was added to each well, plate was covered and incubated at room temperature for 30 minutes on a plate shaker. The plate sealer was removed, the solutions decanted and tapped on tissue to remove any residual solution. Each well was washed 3 times with diluted wash buffer as above. 100 μ l substrate solution was added to each well. The plate was covered and shaken by hand. A blue colour formed in the standard wells with intensity proportional to increasing concentrations. When a blue colour was seen in most wells the reaction as stopped with 100 μ l stop solution. Air bubbles were burst with a needle and plates were read at 450nm and 590nm (control) in a plate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments Inc.).

2.7.6 Analysis of plasma corticosterone concentration by ELISA

Plasma samples were obtained as above and diluted 1:22 by adding 10 μ l plasma sample to 210 μ l sample diluent provided by the kit. An IDS Corticosterone ELISA kit was used (Immunodiagnostic Systems Limited, UK). All samples were vortexed (GV Lab, Gilson) to mix thoroughly. To the antibody coated plate 100 μ l each of the standards, quality control and samples were added to the appropriate wells in duplicate. 100 μ l of enzyme conjugate solution was added to all wells using a multichannel pipette. The plate was covered with a plate sealer and incubated for 18 hours at 5°C. All wells were washed 3 times with 300l wash solution as above with an automated plate washer (labtech LT-3000 microplate washer). The plate was tapped on absorbent tissue to remove excess wash buffer. 200 μ l of TMB substrate was added to all wells and the plate was incubated at room temperature for 30 minutes. 100 μ l of stop solution was added to all wells. The absorbance was measured at 450nm and 650nm (control) using a plate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments Inc.).

2.7.7 Analysis of hippocampal acetylcholine (ACh) ELISA

An initial Bradford protein assay (see section 2.5.2 on page 32) was carried out to assess the level of protein in each sample. The samples were equalised by diluting the tissue sample in the appropriate volume of krebs buffer. A Wuhan EIAab Science kit was used (Wuhan EIAab Science Co., Ltd, China). All reagents were brought to room temperature before use. Wash buffer was made up by diluting 30ml of wash buffer concentrate in 750ml distilled water. The standard was reconstituted with 1ml of sample diluent giving a stock standard of 200nmol/L. The remaining standards were made using a serial dilution method and the sample diluent acts as a zero standard. 100l of either standard, blank or sample was added to the appropriate wells. The plate was covered with a plate sealer and incubated for 2 hours at 37°C. The liquid was removed from the wells but the wells were not washed. 100l of detection reagent A was added to each well. The plate was covered with a plate sealer and incubated at 37°C for 1 hour. Each well was washed 3 times with 400l wash buffer in an automated plate washer (labtech LT-3000 microplate washer). Excess liquid was removed by tapping the plate on absorbent tissue. 100l detection reagent B was added to every well, the plate was covered with a plate sealer and incubated at 37°C for 1 hour. The plate was washed 5 times and tapped dry. 90l of substrate solution was added to every well. The plate was sealed with a plate sealer and incubated at 37°C for 15-30 minutes. The plate was protected from light by placing paper over it. 50l stop solution was added to every well and the plate tapped to ensure uniform mixing. The plate was read at 450nm in a plate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments Inc.).

2.8 Methods for mRNA quantitation

2.8.1 RNA extraction procedure

A total RNA isolation kit (Macherey-Nagel, Germany) was used to extract RNA. Briefly, samples were removed from freezer, placed in 350 μ l of RA1 buffer and 3.5l of β mercaptoethanol and homogenised using a homogeniser (Polytron, Kinematica Inc). Sample homogenate was added to NucleoSpin Filter® units and filtered by centrifugation at 13000rpm for 1 minute. 350 μ l of 70% ethanol was added to each sample lysate and mixed by pipetting up and down approximately 5 times. Each sample mix was placed in NucleoSpin®RNA II columns and centrifuged at 13,000 rpm for 30 seconds to bind the RNA to the silica membrane. Following the centrifugation the column was placed in a new collecting tube and 350 μ l of membrane desalting buffer (supplied) was added. The column was then centrifuged at 13,000 rpm for 1 minute. DNA was digested using rDNase and DNase Reaction Buffer (supplied). rDNase was diluted 1:10 in DNase Reaction Buffer and 95 μ l of this solution was pipetted directly into the centre of the silica column. Samples were incubated with DNase mix for 15 minutes at room temperature. 200 μ l of buffer RA2 was added to the column and centrifuged at 13000 rpm for 30 seconds, following which, the column was placed in a new collecting tube. 600 μ l of RA3 buffer (50ml of ethanol was added to 25ml of RA3 buffer concentrate) was added to the column and centrifuged at 13000 rpm for 30 seconds. The flow-through was discarded and the collecting tube re-used for the second RA3 wash. 250 μ l of RA3 buffer was added to the column and centrifuged at 13000 rpm for 2 minutes. Column was placed in fresh RNase free microtube and RNA eluted by addition of 60 μ l of H₂O and centrifugation at 13000rpm for 1 minute. Eluted RNA was then frozen and stored at -85C for qualification, quantification and reverse transcription.

2.8.2 RNA Quantification

The Nanodrop spectrophotometer was first initialised by adding 1 μ l of deionized water and this was done again to blank the readings. This should give a measurement of zero or very close to it. Between each addition of sample, the pedestal was cleaned with 70% alcohol on a paper towel. 1 μ l of each sample was added and read. The measurement was automatically stored on the computer attached to the Nanodrop.

2.8.3 Reverse Transcription of RNA

A high capacity cDNA archive kit (Applied Biosystems, Ireland) was used to reverse transcribe samples to extracted using the total RNA isolation kit (Macherey-Nagel, Germany). Briefly, 20-50l of 0.5-2.5g of RNA was mixed in a PCR mini-tube with an equal volume of 2X master mix that was made up as follows: 1.5 dilution of 10X Reverse Transcription Buffer, 1:12.5 dilution of 25X dNTPs, 1:5 dilution of Random Primers, 1:10 dilution of MultiScribe Reverse Transcriptase and 1:2.381 dilution H₂O. Samples were then placed in thermal cycler and incubated at 25°C for 10 minutes followed by 2 hr incubation at 37°C. Resultant cDNA was frozen at -20°C until ready for real time polymerase chain reaction (PCR) analysis.

Gene Name	Assay Number	NCBI Gene Reference
IL-1 β	RN00580432_m1	NM_198741.1
IL-1 β	RN00580432_m1	NM_031512.1
IL-4 II	RN01456866_m1	NM_29270.1
PPAR γ	Rn00440945_m1	NM_001145367.1

Table 2.4: List of Gene assays used

2.8.4 Real Time PCR

Gene expression¹ of targets (see table 2.4 for list) was assessed using off the shelf Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes. β actin gene expression was used to normalise gene expression between samples, and was quantified using a β -actin endogenous control gene expression assay containing specific primers, and a VIC-labeled MGB probe for rat β actin.

Plate set-up for multi-target (multiplex) QPCR: Briefly. cDNA was diluted 1:4 and 10 μ l of diluted cDNA was pipetted onto a PCR plate, to which 1.25 μ l of target primer/probe and 1.25 μ l β actin primer/probe and 12.5 μ l of Taqman master mix was added (25 μ l reaction volume). Samples were run in duplicate, and electronic (EDP3 20-200 μ l, 2-20 μ l, and 10-100 μ l) were used to ensure pipetting accuracy.

Samples were placed in a real time PCR thermocycler (Applied Biosystems 7300, UK) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by 1 minute at 60°C . Step two was repeated 40 times, and fluorescence read during the annealing and extension phase (60°C for the duration of the program.

During step two of the PCR reaction, the double stranded cDNA is denatured at 95°C for 15 seconds. As the temperature begins to fall to 60°C (annealing and extension) the target probe is first to anneal to the single-stranded cDNA as it has a higher melting temperature than the target primers (Applied Biosystems, Ireland). This probe contains FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this dye prevents the dye from emitting a fluorescence signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems, Ireland). At 60°C the primers anneal and the strand is extended by 5' nuclease activity of Taq polymerase. This displaces the FAM/VIC-labeled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescence signal. Due to the specificity of the probe and primers for the cDNA sequence, one fluorescence signal is generated for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

2.8.5 Real-time PCR analysis

The $\Delta\Delta$ CT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method was used to assess relative gene expression by comparing gene expression of experimental samples to a normal/control sample, rather than quantifying the exact copy number of the target gene. In this manner the fold-difference (increase-decrease) can be assessed between experimental and control samples. The fold-difference was assessed using the cycle number (CT) difference

¹Gene reference as listed on the national centre for biotechnology information (NCBI) Entrez-Nucleotide website: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide>

Treatment groups	Target CT	β -actin CT	Δ CT	$\Delta\Delta$ CT	2- $\Delta\Delta$ CT Fold Difference
Control	20	16	20-16=4	4-4=0	$2^{-0} = 1$
Sample 1	21	16	21-16=5	5-4=1	$2^{-1} = 0.5$
Sample 2	22	16	22-16=6	6-4=2	$2^{-2} = 0.25$
Sample 3	19	16	19-16=3	3-4=-1	$2^{-(-1)} = 2$
Sample 4	18	16	18-16=2	2-4=-2	$2^{-(-2)} = 4$
Sample 5	17	16	17-16=1	1-4=-3	$2^{-(-3)} = 8$

Table 2.5: Relative quantification with $\Delta\Delta$ CT method.

between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is 100% efficient. Thus samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (22), similarly a 5-fold cycle difference is a 32-fold difference (25).

To measure this fold-difference relative to control, the CT of the endogenous control (β -actin) was subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the (Δ CT). The CT difference (Δ CT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the Δ CT value. The $\Delta\Delta$ CT (cycle difference corrected for β -actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the $\Delta\Delta$ CT (difference in control and sample CT corrected for actin) gives the fold-difference in gene expression between the control and experimental samples. The control sample always has a $\Delta\Delta$ CT value of 0, thus 2^{-0} gives a 2- $\Delta\Delta$ CT of 1, against which all other samples are referenced, as outlined in Table 2.5

2.8.6 Statistical analysis for molecular endpoint experiments.

Data obtained from tissue sample analyses were investigated using either Students t-test for independent means or a two-way analysis of variance (ANOVA) followed by a post-hoc NewmanKeuls test to determine which conditions were significantly different from each other. Data are expressed as mean $\pm$ standard errors of the mean (S.E.M) and deemed statistically significant when $p < 0.05$.

Chapter 3

Modulating the cholinergic system in normal ageing

3.1 Rationale

As the human body ages, general alterations within brain regions threaten cognitive performance with changes in neuromodulatory systems (Li *et al.*, 2001). Well known and frequently discussed changes include increased oxidative stress, disturbances in energy metabolism (Prolla & Mattson, 2001), occurrence of inflammation-like processes (Sarkar & Fisher, 2006) and reduced glucose metabolism (Arnaiz *et al.*, 2001). Such molecular changes can be of a varying and extensive nature, therefore, elucidating the precise nature of these pathological alterations should significantly enhance our understanding of age-dependent cognitive decline. The role of the cholinergic system in regulating memory and learning has been a central theme in understanding age-dependent cognitive decline for the past thirty years. Studies conducted strongly implicated a vulnerability in the hippocampal cholinergic circuit leading to memory decline in old age and dementia (Perry *et al.*, 1977). Further examination of postmortem brain tissue revealed a continuity in the AD-type pathology (Perry, 1980). The general conclusion, from extensive and diverse researches, has indicated that deficiencies in the cholinergic function is the main culprit in causing general memory decline during ageing (Bartus *et al.*, 1982).

Preventive therapy in early ageing is the main aim of this study. With this in mind, healthy middle-aged animals were used in the current behavioural model to investigate changes in cognitive function. Donepezil is the gold standard drug therapy to improve memory in AD (Geldmacher, 2004) and it is speculated that such a treatment in healthy middle-aged rats might alleviate memory decline and prevent cognitive deficits. Donepezil is part of a large family of N-benzylpiperidine- based acetylcholinesterase (AChE) inhibitors developed, synthesised and evaluated by the Eisai Company in Japan (Kryger *et al.*, 1999). As a cholinesterase inhibitor, this drug has a high affinity for AChE and a high degree of selectivity for AChE versus butyrylcholinesterase (BChE). Treatment with donepezil can delay the breakdown of acetylcholine (ACh) released into synaptic clefts, thus enhancing cholinergic neurotransmission (Ogura *et al.*, 2000).

The scope of this chapter extends from the exploration of the cholinergic hypothesis and its implication on geriatric memory which was initiated almost thirty years ago (Bartus *et al.*, 1982) to the development of symptomatic and palliative therapy using donepezil in the treatments of AD and other demented conditions, such as PD and vascular dementia (Geldmacher, 2004). The behavioural experiments chosen aimed to give an insight in memory retention and consolidation using a version of a water maze task, known as the Water Plus Maze (WPM). The biological effects from the chronic treatment of donepezil was further elucidated via pharmaceutical endpoints that were examined. It was hypothesised that donepezil treatment can attenuate age-related deficits in memory and learning demonstrated in healthy ageing animals.

3.2 Introduction

The origins of the cholinergic hypothesis started back in the 1970s with the postulation of the involvement of central cholinergic function in learning and memory. Early work demonstrated that cholinergic synapses can be modified by learning itself and such alterations might be due to increased sensitivity to ACh in postsynaptic membranes (Deutsch, 1971). Ten years after this discovery, Bartus and colleagues published an extensive review detailing the formulation of the cholinergic hypotheses of geriatric memory dysfunction (Bartus *et al.*, 1982). A large pool of evidence was mentioned in this review whereby choline acetyltransferase activity (ChAT) (an enzyme responsible for the formation of the neurotransmitter ACh) decreased significantly in aged animals and humans. It was also around this period of time that neuropathologists started to look with increasing interest to the neurochemical alterations present in samples. These were obtained from post-mortem brain of patients with diagnosis of various pathologies of the nervous system. This resulted in the emergence of cholinergic hypothesis which began with the examination of brain samples from AD patients (Contestabile, 2010).

In the United States, the age-specific prevalence of AD doubles every five years from less than 5% in sixty year olds to a prevalence of over 20% in eighty year olds (Katzman & Fox, 1999). As one of the most common neurodegenerative disorders associated with ageing, most experimental reviews follows the progression of AD and its associated changes in the cholinergic system. Normal ageing, on the other hand, also demonstrates such changes but the extent of the dysfunction in the cholinergic system may not be that obvious. Postmortem studies in normal human brain have suggested modification in number, size and function of cholinergic neurons with age (Bartus *et al.*, 1982). Within these cholinergic neurons, choline is crucial for the synthesis of ACh and membrane phosphatidylcholine synthesis (Wurtman, 1992). Decreased choline uptake in the brains of older adults has been suggested to be a reason for neuronal vulnerability as the cholinergic neurons may become more susceptible to degeneration (Cohen *et al.*, 1995). Age-dependent changes in cholinergic receptors within the brain are not consistent and are not as obvious as changes in AD. In fact, both decreases and no change in the muscarinic (mAChR) number has been reported in the cerebral cortex, hippocampus and caudate nucleus (Nordberg *et al.*, 1992). Prominent evidence for cholinergic dysfunction in normal ageing (between non-demented young, non-demented old (> 65 years) was shown in a study comparing abnormalities in magnocellular basal forebrain cholinergic neurons and their axons. Moreover, pathological changes in cholinergic axon abnormalities (i.e., thickened fibres and ballooned terminals) found in pathologically mild and severe AD cases were also evident even in middle-aged non-demented subjects (Geula *et al.*, 2008). DeKosky and colleagues found elevated hippocampal choline acetyltransferase (ChAT) activity levels in individuals with mild cognitive impairment (MCI) as compared to normal older adults. ChAT levels were preserved in subjects with mild AD suggesting that a compensatory mechanism might be at work in preventing damage in other brain sites. This phenomenon might extinguish when MCI progresses, resulting in decreased hippocampal and/or frontal cortical ChAT contributing to dementia (DeKosky *et al.*, 2002). This is in agreement with a previous study where there was failure in demonstrating a large or reliable change in ChAT activity in normal ageing

(Bartus *et al.*, 1982).

A consistent pathology found in post-mortem material from AD patients was of a specific cholinergic deficit. Such deficits involved cholinergic projections from a basal forebrain neuronal population, the nucleus basalis magnocellularis of Meynert, to the cortex and hippocampus (Davies & Maloney, 1976). Additionally, a decrease in ChAT activity from the cortex and hippocampus was also found in brains of AD patients (Bartus *et al.*, 1982). Many of the existing views on cholinergic dysfunction seemed to be of an AD-specific nature including the correlation of clinical dementia ratings with reductions in cortical cholinergic markers such as ChAT, muscarinic and nicotinic ACh receptor binding as well as levels of ACh (Schliebs & Arendt, 2006). The two hallmarks of AD, β -amyloid ($A\beta$) plaques (in cortical and hippocampal areas) and neurofibrillary tangles (within the cytoplasm of cortical pyramidal neurons) are also linked to cholinergic functions. α - and β -secretory ($A\beta$) precursor protein (APP) processing can be differentially regulated through muscarinic receptor (mAChR) subtypes (Liskowsky & Schliebs, 2006) and the ability of acetylcholinesterase (AChE) to affect aggregation of $A\beta$ peptides suggested a particular role of the ACh-degrading enzyme in the pathogenesis of AD (Zhang, 2004). The question pertaining to our aim in prophylactic therapy is whether these cholinergic dysfunctions occur in normal ageing and then proceed to manifest as an AD pathology or only in AD-specific pathologies.

The abundance of ACh within the brain could potentially impact many different systems. Manipulating the cholinergic system allowed researchers to gain further insight into its relevance in memory and ageing. One commonly used experimental tool is scopolamine, which is an anticholinergic drug. Clinically, Drachman and colleagues found that young subjects treated with scopolamine exhibited memory deficits similar to those found in naturally aged participants tested on the same clinical battery (Drachman & Leavitt, 1974). Furthermore, in a preclinical experiment utilising the Morris Water Maze (MWM), Radial Arm Maze (RAM), delayed non-matching to position (DMTP) tasks, and fixed ratio five bar pressing, scopolamine was shown to disrupt all working memory measures but not reference memory measures (Hodges *et al.*, 2009). In addition, scopolamine also perturbed attention and psychomotor function before affecting working memory. The low doses used in that study implied that even a small disruption of the central cholinergic system could lead to some profound effects on attention and/or psychomotor control before any measurable mnemonic disruption. In terms of neuronal vulnerability, neuronal loss was located in the nucleus basalis of Meynert in an AD patient and was elevated as compared to age-matched controls (Whitehouse *et al.*, 1981). Such changes in this region may not be specific to AD patients as MR analysis reveals shrinkage of the substantia innominata, reflecting degeneration in the nucleus basalis of Meynert even in normal ageing (Hanyu *et al.*, 2002).

In a preclinical model, AChE-positive neurons in the forebrain cholinergic nuclei were found to be reduced in both size and population in older rats which was correlated with impaired performance in the MWM task (Fischer *et al.*, 1989). Unlike human studies utilising postmortem tissues, animal studies can be better adapted to assessing cholinergic system failure in ageing as the use of lesions is possible in enhancing our understanding of brain networks. Structurally, the brain undergoes modifications in the basal forebrain cholinergic system during ageing (Giacobini, 1990). A depreciation in cholinergic neuronal population and their atrophic and/or

hypertrophic natures are characteristics of aged animals that perform poorly in learning and memory tasks assessing the limbic/cortical systems (Gallagher & Colombo, 1995).

A cell transplantation study was done with NGF receptor-bearing neurons, in the ageing basal forebrain of rats. This resulted in a reversal in neuronal atrophy and improvements in behavioural impairments suggesting that memory enhancing effects of NGF, mediated via a direct action on the NGF receptor-bearing neurons (Martinez-Serrano *et al.*, 1995). Not only did NGF change the neuronal structure in the basal forebrain, this alteration also brought about positive changes in the cognitive status of an aged animal model which has been demonstrated in a plethora of similar experiments (Bäckman *et al.*, 1996; Fischer *et al.*, 1989; Klein *et al.*, 2000; Fischer *et al.*, 1994). It is noteworthy to mention that cholinergic neurons outside the basal forebrain showed signs of deterioration in aged brains as well (Fischer *et al.*, 1989; Stroessner-Johnson *et al.*, 1992). Besides, even within the same region, different strains of rats reveal different manifestations of ageing, for example, the aged Wistar-Kyoto rats have a reduction in choline uptake and ACh release and pyramidal cell loss as compared to aged Brown-Norways rats (Gilad *et al.*, 1987).

There have been several contrasting evidences in relation to the presence of ChAT activity in aged brain. It has been demonstrated that cognitive impairments were accompanied by decreased activity in the vertical diagonal band nucleus, the dentate gyrus, and striatum (Luine & Hearn, 1990). However, some reports on ageing rodent cortical ChAT activity were inconclusive since research so far indicated decrease, increase and no change (Sarter & Bruno, 1998; Sherman & Friedman, 1990). Such diverse observations could be a result of compensatory mechanisms that existed within each ageing individual. Deterioration in basal forebrain cholinergic neurons occurs along with many other changes in limbic/cortical systems (Gallagher & Colombo, 1995). Although the cholinergic system is central to ageing studies, other monoaminergic systems could also be at play when it comes to ageing. Diminished robustness in dopamine, serotonin and noradrenaline pathways were also evident in animals suffering from age-related memory decline (Luine *et al.*, 1990). It has been recently suggested that the remodelling of mesocortical dopaminergic fibres during ageing occurs in relation to the prevailing cognitive status of the subject. In cognitively unimpaired animals, there is an increase in dopaminergic boutons while in cognitively impaired animals, this is the opposite (Allard *et al.*, 2010a). Interestingly, the densities of the presynaptically located monoamine transporters (dopamine, serotonin and noradrenaline) evolves throughout the life span of the rat, from weaning to late adulthood, and within different regions of the brain, a plateau in the density is reached at different stages (Moll *et al.*, 2000). In early maturing brain regions, such as the midbrain and brain stem, densities of these monoamine transporters reach a plateau at weaning, while for later maturing regions such as the frontal cortex and striatum, the densities may continue to evolve until late adulthood. This may explain the regional differences in neuronal structures during ageing and its corresponding effects on behavioural impairments.

Lesion studies, such as the induction of selective lesions of the basal forebrain cholinergic neurons, is another popular experimental tool in understanding the process of ageing and its dysfunctional cholinergic system. These studies might differ in the type of toxin used, the selection of injection sites and the microinjection parameters (e.g., toxin concentration and volume) thus producing different results. Due to the varying

subpopulations within the basal forebrain cholinergic neurons, it is important to note that the administration of 192 IgG-saporin (an immunotoxin targeted at the low affinity NGF receptor (Waite *et al.*, 1994)) into the nucleus basalis of Meynert or substantia innominata region removed only the cortical cholinergic projections, while leaving intact the majority of the cholinergic innervation of the amygdala (Gallagher & Colombo, 1995). Hence, to fully understand the implications of lesion studies for cholinergic functions, a more complex background must be considered in terms of natural ageing. These include regional differences in subpopulations of cholinergic neurons, numerous other changes in limbic/cortical circuitry on top of existing alterations in the basal forebrain and other brain cholinergic systems, such as the striatal and brain stem cholinergic neurons.

More recently, advancing experimental techniques are able to reveal further insights from these lesion studies. For example, gene expression changes through cDNA microarray analysis were observed in the rat brain after 192 IgG-saporin immunoablation of cholinergic basal forebrain (Paban *et al.*, 2010). These gene changes were associated with neurotrophins, inflammatory response, neuronal apoptosis, regulation of angiogenesis, and synaptic plasticity. Therefore, an ageing process was found to be associated with the regulation of glial proliferation and apoptosis (Paban *et al.*, 2010). Upregulation of a neurotrophin, Brain-Derived Neurotrophic Factor (BDNF) was demonstrated after cholinergic damage (in the medial septum, dorsal hippocampus, and the frontal and entorhinal cortices), validated by several studies (De March *et al.*, 2008; Imai *et al.*, 2006). Such an upregulation might be an attempt to rescue striatal neurons from degeneration. Further studies such as these expand our knowledge on gene expression-profiling during ageing which might aid future pharmaceutical developments in procognitive therapies.

Since the loss of cholinergic neurons and its associated decrease in the level of cholinergic neurotransmission have been associated with the cognitive impairment observed in AD patients (Evans *et al.*, 1989), it seems reasonable from a pharmaceutical point of view to enhance the performance of the cholinergic system. Sugimoto presented a detailed review in the therapeutic options in this area which consisted of six main classes of drugs: (1) Cholinesterase inhibitors (ChEI) that block AChE resulting in increased cholinergic activity. (2) Choline precursors for supplementing availability for cholinergic transmission. (3) ACh releasers to aid release of ACh from presynaptic terminals. (4) Muscarinic receptors agonists (M1 and M3) which can mimic ACh on postsynaptic terminals. (5) M2 and M3 receptor antagonists known as auto-receptors facilitating the release of ACh through negative feedback. (6) Nicotinic agonists or substances that have nicotine-like effects in enhancing ACh release (Sugimoto, 2001a).

The rationale for the use of acetylcholinesterase inhibitors (AChEIs) in the treatment of AD was to correct cholinergic hypo-function by preventing ACh hydrolysis within CNS, thus prolonging its bioavailability (Scali *et al.*, 2002). Donepezil, also known as donepezil hydrochloride (E2020), historically is, the second drug approved by the US FDA for the treatment of mild to moderate AD. Its chemical structure, as an AChE inhibitor, consists of N-benzylpiperidine and an indanone moiety, which shows longer and more selective action than other ChEIs (Sugimoto, 2001a). In humans, donepezil is one of the main therapeutic options for AD because of its utility in slowing down deterioration of cognition and global function in patients (Román & Rogers, 2004). Besides AD, donepezil demonstrates its cognitive enhancing effects with other diseased states such as vascular

dementia; perhaps showing potential therapeutic benefit for other neurological conditions. Mild cognitive impairment (MCI) has been thought to be a preceding condition that leads to AD (Morris & Cummings, 2005) and donepezil offers a small but significant improvement on the primary measures of cognition (Doody *et al.*, 2009).

Other AChEIs used in the pharmaceutical field in treating cognitive dysfunction include Galantamine, Huperzine A, Neostigmine, Physostigmine and Rivastigmine (Kosasa *et al.*, 1999b). Neuroenhancement using AChEIs provided contradictory insights into the benefits of this type of drug. In a recent comparative review, based on the use of AChEIs, neuroenhancement in healthy individuals might not be as sustainable as previously suggested (Repantis *et al.*, 2010). Neuroenhancement, or rather, 'brain doping' is not a novel concept (Larrieviere *et al.*, 2009) and it involves the use of drug therapy to optimise a normally functioning cognitive system. In the few studies that have been recently conducted on healthy individuals, one was able to show an enhancement of retention memory in older non-demented adults (specifically pilots) in the training on complex aviation tasks (Yesavage *et al.*, 2002). On the other hand, in a subset of 30 young healthy individuals, only episodic memory was found to be enhanced (Grön *et al.*, 2005). Conflicting results have been demonstrated where donepezil was unable to attain the expected cognitive enhancing capabilities in healthy volunteers (range 25-49 years) (Beglinger *et al.*, 2004).

In animals, donepezil failed to show an attenuation of memory impairments in aged rats during a RAM task even though the density of nicotine binding sites in both the hippocampus and PFC of the treated rats significantly elevated (Barnes *et al.*, 2000). In this particular study, the drug-induced increase in nicotinic receptor number was found to be greater in the cortex than in the hippocampus which might explain the lack of behavioural improvements observed. Additionally, the RAM task employed might not be sensitive enough to elucidate the neocortical cholinergic function. In a similar experiment using young rats, scopolamine impaired both working and reference memory in the partially baited RAM which was ameliorated by the treatment of donepezil (Wang *et al.*, 1998).

Memantine (MEM) is the first in a novel class of Alzheimer's disease medications acting on the glutamatergic system by blocking NMDA glutamate receptors (Reisberg *et al.*, 2003). In humans, a combination therapy of MEM (which increases ACh availability) and donepezil improved cognitive functions in AD patients (Grossberg *et al.*, 2008). A similar study was done in animals which revealed a surge in ACh release; however, in this preclinical model, increased ACh levels did not directly correlate with improved cognitive performance (Ihalainen *et al.*, 2011). Hernandez and colleagues recently exploited the chronic administration of donepezil in another spatial task using the MWM. They were able to demonstrate that donepezil, at 0.75mg/kg, attenuated learning deficits in aged rats, providing positive effects on the acquisition (and potentially, sustained effects on the retention) of a spatial learning task (Hernandez *et al.*, 2006). Such conflicting studies suggest that the cholinergic system has yet to be elucidated in terms of the neuromodulatory changes in consequence of donepezil treatment.

The work in this chapter provides insights into the therapeutic effects of donepezil in a rodent model of ageing. It was hypothesised that manipulating the cholinergic system in healthy subjects with an acetyl-

cholinesterase might enhance acquisition of task and memory retention. A chronic drug paradigm of 40 days was chosen while investigations into memory and learning were carried out at different time-points. The behavioural task employed assessed spatial learning and memory in a plus-shaped water maze. The work was concluded with analyses into endpoint biological changes.

3.3 Results for behavioural experiments

3.3.1 Validity of water plus maze protocol.

The water plus maze (WPM) design used in the current study was adapted from the more widely used Morris water maze (MWM). Although there are several advantages to the MWM (D'Hooze *et al.*, 2001), there are also certain aspects of the WPM that deviate from the more traditional MWM. The plus-shaped maze has already been used in laboratories to test working memory (Wahlsten *et al.*, 2005; Vloeberghs *et al.*, 2006). Fundamentally, the main aims were to investigate age-related memory deficits (between middle aged and young adult rats) and to elucidate the effects of drug treatment in the present task. Our hypothesis was that non-treated middle aged rats (13 months, MA) would not be able to acquire the task as quickly, nor at the same level, as the young adult rats (3 months old, YA), and that drug intervention may recover performance deficits observed in the task for MA rats.

Task layout and protocol was sensitive to ageing effects

In order to ascertain if both the task and protocol layout were sensitive to age effects, an initial analysis was crucial in the comparison between MA and YA rats' performance level. As described in the methods, a total of 3 sessions were carried out and each session comprised of fixed-start trials (of 3 continuous days) and a multiple-start trial at the end of each session (see Figure 2.3). A two-way ANOVA analysis with sessions and trials as between-subjects variable and age as within-subjects variable demonstrated a statistically significant relationship between age factor [$F_{(1,3588)} = 182.50, p < 0.0001$], sessions and trials factor [$F_{(5,3588)} = 156.33, p < 0.001$] and a high interaction between these two factors [$F_{(5,3588)} = 22.65, p < 0.0001$]. As shown in figure 3.1, YACTL rats performed significantly above chance level (33.33%) while MACTL rats took a longer period of time to acquire the task from the first fixed-start trials session (S1) ($t = 11.06, p < 0.001$). The multiple-start trials that follow directly after three days of fixed-start trials presented a different trend where by performance remained at chance level for both the YA and MA group of rats with no significant difference between groups ($t = 1.581, p > 0.05, ns$). This might reflect the difficulty of the task.

The following session (S2), again demonstrated the same high disparity in performance between the YA and MA groups ($t = 9.852, p < 0.001$) during the fixed-start trials. Once again, subsequent multiple-start trials failed to reveal differences in performance between age groups ($t = 0, p > 0.05, ns$), with both groups performing at the chance level. This trend changed by the last session (S3) whereby YACTL rats could perform the multiple-start task but MACTL could not ($t = 8.271, p < 0.001$). S3 may have brought about a significant difference during multiple-start trials but this was not evident for fixed-start trials whereby both the MA and YA group of rats have attained a very high level of performance (93% and 84% for YA and MA respectively) and there is no significant difference among them ($t = 2.33, p > 0.05$). In summary, between age groups, the general pattern is an increase in an ability to perform fixed trials over the sessions (for both groups of rats). Additionally, with more sessions, the ability to solve the multiple-start task was observed for YA rats but not for MA rats which only performed at chance level.

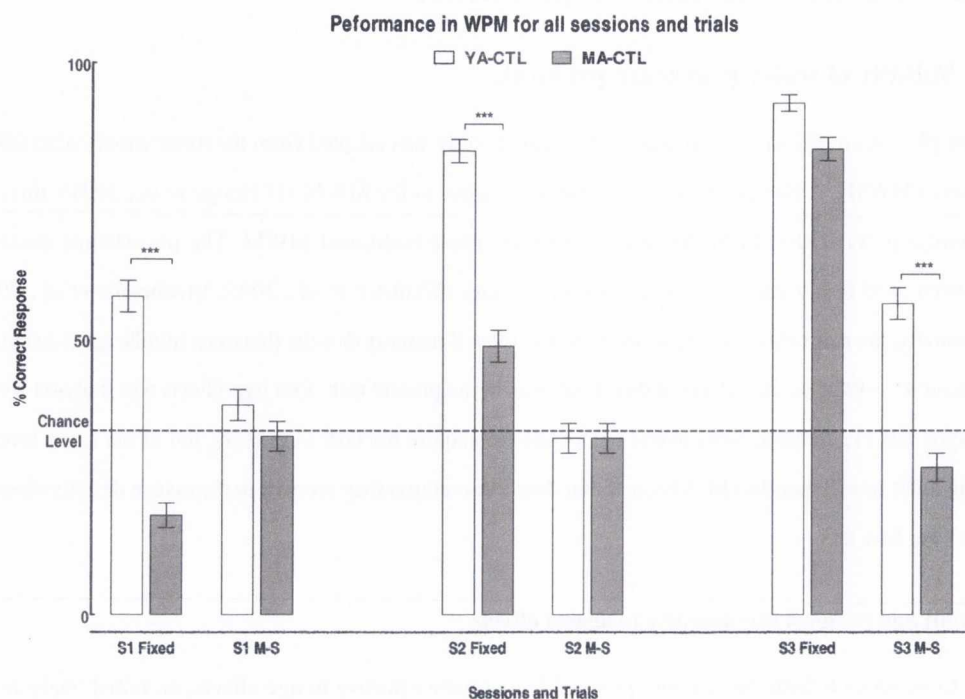


Figure 3.1: **Water Plus Maze design and protocol are sensitive to age effects in non-treated rats.** Data presented here are in the percentage of correct responses attained during task. The dotted line represents chance level of 33.33% due to the choice the rat is given when entering maze (1 out of 3 arms) to find the platform during multiple-start trials. Since the chance of re-entering the start arm is slight, this is not considered as one of the choice. Multiple-start trials are labelled as M-S. Within fixed-start trials, control rats are all able to improve their performance and attain the task gradually throughout the sessions. Notably, the MA rats acquire task at a lower rate than YA rats. Data is represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Latency within designated zone differed between age groups

The time spent by each animal within the designated decision zone was analysed (see Figure 3.2). Statistical analysis revealed significance for age factor [$F_{(1,3580)} = 33.14, p < 0.0001$], sessions and trial factor [$F_{(5,3580)} = 21.84, p < 0.0001$] and an interaction [$F_{(5,3580)} = 16.05, p < 0.0001$]. In contrast with performance level, the MACTL group of rats attained a higher latency compared to YACTL in both S1 ($t = 9.571, p < 0.001$) and S2 ($t = 1.522, p < 0.001$) during fixed-start trials (see Figure 3.2). There is very little variation in the time spent in the decision zone for the YACTL rats (a range of 2.5 to 3 seconds) for all sessions and type of trials. By S3, both MA and YA spent the same amount of time within the decision zone for both trial types. Overall, there was no change in latency within the zone for YA group of rats while the MA cohort of rats spent most time in S1 which declined in S2 and 3. In summary, YA rats managed to acquired the fixed-start trials at a higher level although both age groups of rats did reach a ceiling effect by the last session. Compared to younger rats, MA rats performed poorly at the multiple-start trials and were unable to perform at a level higher than chance. MA rats tended to spend more time in the decision zone although this behaviour decrease over the sessions. It is interesting to note that multiple-start trials did not bring about much changes in the time spent in decision zone between age groups.

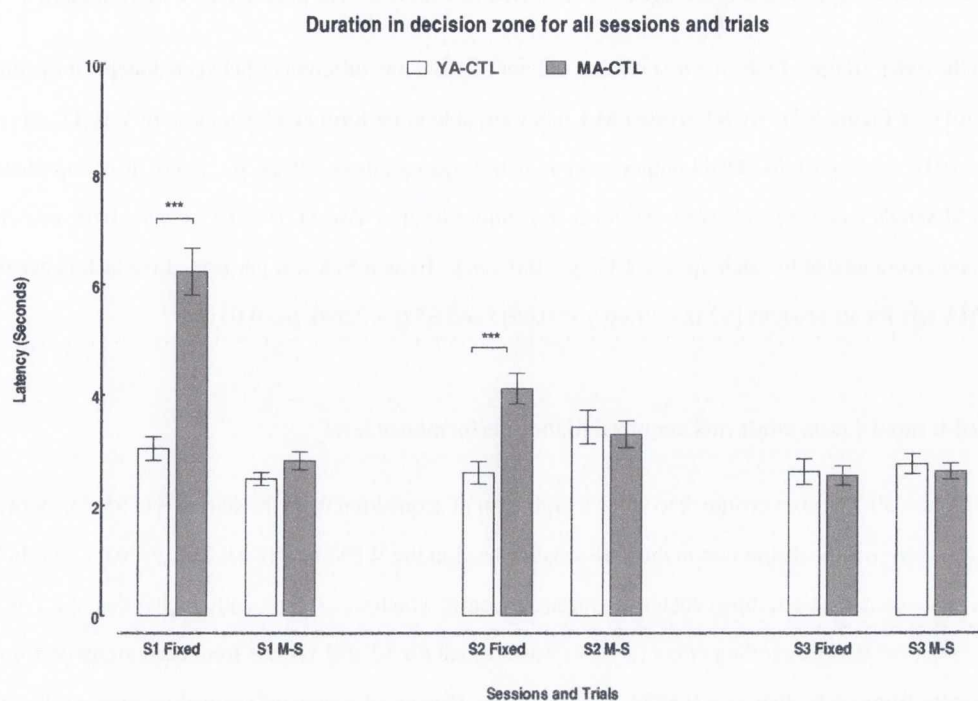


Figure 3.2: **Middle-age rats spent longer in the designated decision zone.** During the fixed-start trials, older rats required more time than YA rats in the initial sessions (S1 and S2) and this declined as the sessions progress. Latency is recorded in seconds and all data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

3.3.2 Learning progression during fixed-start trials

There are several ways to investigate the effect of donepezil on the level of performance in the WPM task. Levels of acquisition of the task was first determined by the percentage of correct responses. Two-way ANOVA analysis reported a high statistical significant effects for treatment and age factor [$F_{(3,3228)} = 119.5$, $p < 0.0001$], sessions factor [$F_{(2,3228)} = 546.4$, $p < 0.0001$] and interaction for both these factors [$F_{(6,3228)} = 28.91$, $p < 0.0001$] for fixed-start trials.

Donepezil rescued age-related impairments in task performance

By comparing the trend between untreated YA and MA rats it was possible to see the general pattern of behaviour and changes that might occur with ageing (see Figure 3.3). The pattern of acquisition throughout the sessions seemed similar for donepezil and control rats, with younger rats accomplishing the task at a higher rate. In the initial fixed-start trial session (A), young adult rats treated with donepezil were better at the task, producing a higher percentage of correct response as compared to its paired MADON group ($t = 10.79$, $p < 0.001$). Such a distinction in performance levels between MA and YA rats persisted in S2 ($t = 2.55$, $p < 0.05$). Similar to control rats, the difference in level of performance was negligible for S3 between YADON and MADON groups.

Treatment with donepezil in middle-aged rats resulted in superior task performance after session 1

Between the older groups of rats, the first session did not disclose any differences between donepezil treatment and control (see Figure 3.3). By S2, treated MA rats were able to perform at a level close to YACTL rats ($t = 0.819$, $p > 0.05$, *ns*). In S3, MADON outperformed control ageing rats ($t = 2.89$, $p < 0.05$). It is important to note that MADON rats were still able to perform at a similar level to YACTL ($t = 0.33.13$, $p > 0.05$, *ns*) while MACTL rats were unable to catch up ($t = 2.17$, $p > 0.05$, *ns*). Treated MA rats performed the task better than control MA rats for all sessions [S2 ($t = 11.66$, $p < 0.001$) and S3 ($t = 2.890$, $p < 0.05$)].

Donepezil-treated young adult rats acquired higher performance level

Unlike MA rats, all YA rats were quick to attain a high level of acquisition from the start of S1 (55 %). Between the YA rats, there was no distinction in the performance level in the WPM task ($t = 0.715$, $p > 0.05$, *ns*). In S2, YADON rats scored the highest percentage of correct response, which was significantly higher than the YACTL rats ($t = 3.503$, $p < 0.01$). A ceiling effect ($> 90\%$) was reached for S3 and YA rats from each group performed no differently from each other ($t = 0.5859$, $p > 0.05$, *ns*). Donepezil treatment seemed to augment learning even in younger rats with no prior learning deficits. The acquisition during fixed-start trials was superior for donepezil-treated rats.

3.3.3 Latency in decision zone during fixed-start trials

Latency in the decision zone was another quality of the task that was able to help us further understand the learning capabilities of the rats. Two-way ANOVA analysis reported a high statistically significant effect for the treatment and the age factor [$F_{(3,3223)} = 32.39.5$, $p < 0.0001$], sessions factor [$F_{(2,3223)} = 71.94$, $p < 0.0001$] and interaction for both these factors [$F_{(6,3223)} = 7.812$, $p < 0.0001$] for fixed-start trials

Older rats persisted in decision zone for a longer duration

Latency within the decision zone revealed an opposite pattern to the performance levels. While task performance levels were increasing and improved over sessions, latency decreased over time. Although older rats persisted longer within the decision zone, the age-related difference for time spent in decision zone was not significant between donepezil and control rats for all sessions ($p > 0.05$, *ns*).

Young adult rats demonstrated lower latency in decision zone as compared to older rats

The level of acquisition was higher for YA rat and this was accompanied by a lower latency in decision zone when comparing between age-groups. Treatment with donepezil did not reveal an obvious effect in the tendency to remain in decision zone. All YA rats behaved similarly in this aspect ($p > 0.05$, *ns*).

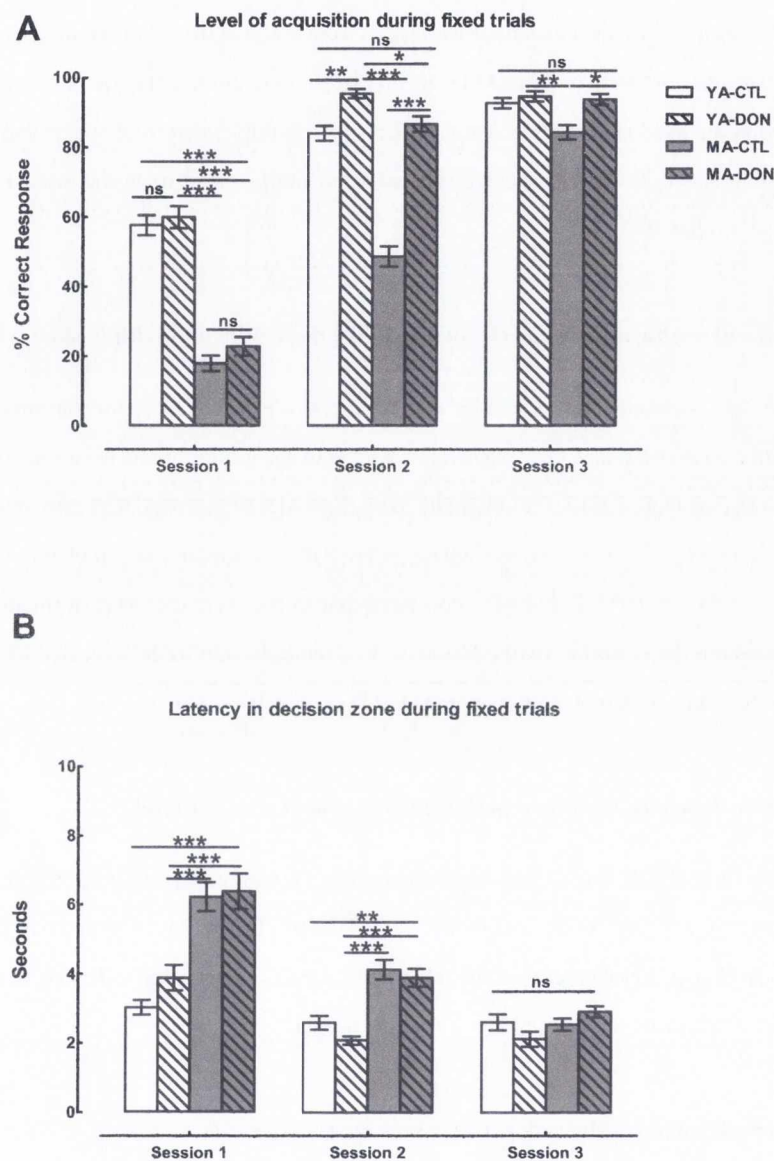


Figure 3.3: Effects of donepezil treatments on learning and dormancy within decision zone during fixed-start trials in the Water Plus Maze task. Performance level is established by percentage of correct response (in finding hidden platform) for each session. Fixed-start trials for each session comprised of an average of data collected over 3 days of fixed-start training. Latency was recorded for the duration that animal enters and remain within designated decision zone. (A) Acquisition of task was higher in treated rats after initial session. (B) Latency was longer for the MA rats, especially MADON. All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.3.4 Learning progression during multiple-start trials

On the other hand, the high level of task performance in the fixed-start trials was not present in the multiple-start trials (see Figure 3.4A). In the treated groups of rats, there was a lack of difference between performance levels for the multiple-start trials. In this much harder trial, rats were released from 3 different arms (excluding the arm with hidden platform) and were required to locate an escape route through the use of spatial cues. The ceiling effect observed in the fixed-start trials was not evident in the multiple-start trials suggesting that this is a much harder task for the rats to learn. The average values for the multiple-start trials was around 50% while the fixed-start trials was around 80%.

Treatment with donepezil augmented task performance in the more difficult multiple-start trials

Notably, in untreated rats, performance was similar and below chance level (33%) for the initial sessions. donepezil treatment affected performance level positively where both age groups of rats were able to rise above chance from S2 onwards (see Figure 3.4A). Additionally, both the YADON and MADON rats were acquiring the task at a similar rate ($t = 0.3773$, $p > 0.05$, *ns*), although YADON was superior at completing the MS trial by the final session ($t = 2.84$ $p < 0.05$). Essentially, donepezil-treated rats were able to perform above chance level consistently throughout the sessions. By the last session of multiple-start trials, untreated MA rats were the only group of rats that were performing below chance level.

Middle-aged rats under donepezil treatment performed the task at a higher level

In S1, task performance in MADON was slightly higher than MACTL with no significant differences between them ($p > 0.05$, *ns*)(see Figure 3.4A). As the session progressed, MACTL rats did not improve in learning the MS trial. MA rats under donepezil treatment, on the other hand, surpassed untreated older rats in the level of task performance in S2 ($t = 5.11$ $p < 0.001$) and 3 ($t = 4.89$ $p < 0.001$).

Treatment with donepezil enhances the task performance for younger rats

Acquisition of the MS trial was similar in the first session between YADON and YACTL rats ($p > 0.05$, *ns*). In the subsequent session, the large discrepancy in performance level was mainly due to the huge improvement in task acquisition in YADON and the lack of improvement in YACTL rats ($t = 4.71$ $p < 0.001$)(see Figure 3.4A).

3.3.5 Latency in decision zone during the multiple-start trial

Variation in data collected for multiple-start trials was statistically significant whereby treatment and age factor [$F_{(3,3219)} = 53.36$, $p < 0.0001$], sessions [$F_{(2,3219)} = 23.17$, $p < 0.0001$] and interaction between both factors [$F_{(6,3219)} = 11.43$, $p < 0.0001$] are all highly significant. Post hoc Bonferroni tests further revealed the differences between treatment and age groups for both types of trials.

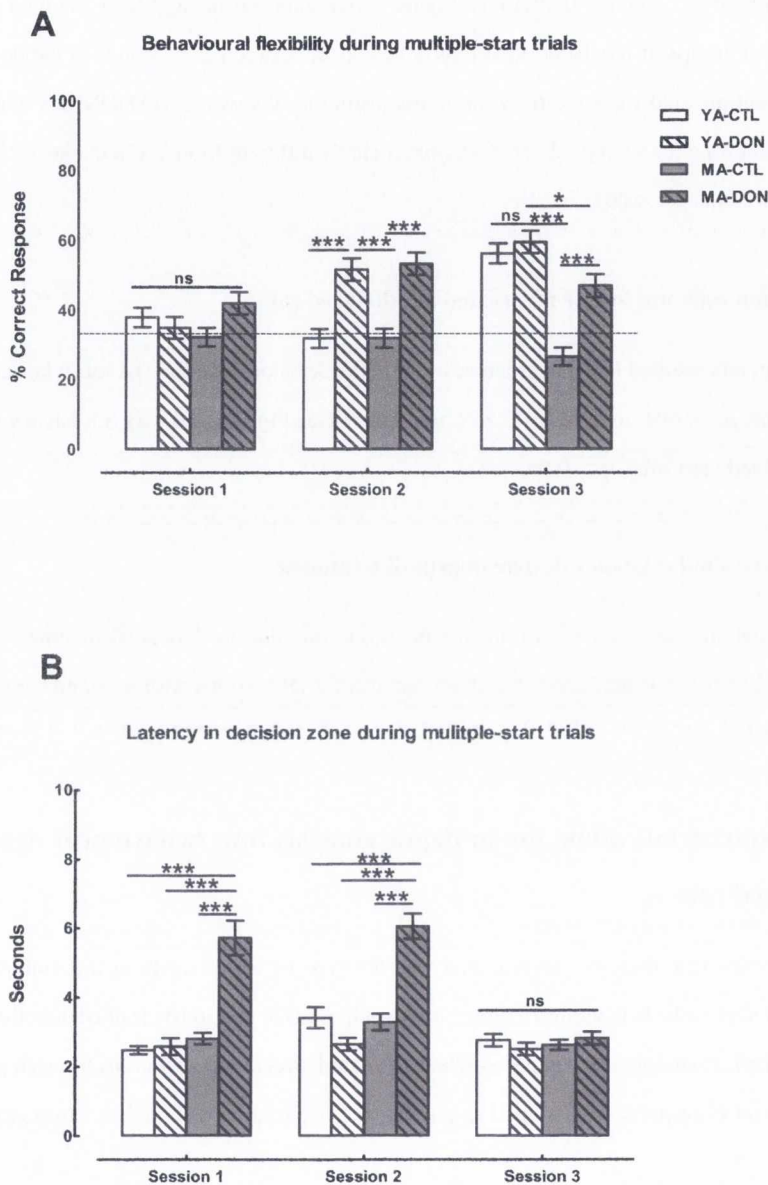


Figure 3.4: **Effects of donepezil treatments on learning and dormancy within the decision zone during multiple-start trials in the WPM task for all sessions.** Each session is composed of 30 trials completed within a single day. The performance level is established by the percentage of correct responses in finding the hidden escape platform. The chance level was set at 33% and is represented as the dotted line across the graph. Latency was recorded in seconds for each session. (A) MADON rats were more superior at solving the MS trials. (B) MADON rats spent more time in decision area. All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Age-related differences in dormancy within the decision zone was present within donepezil-treated rats

In S1 and S2, MADON rats demonstrated a higher latency in the maze as compared to YADON rats [S1 ($t = 5.896$, $p < 0.001$) and S2 ($t = 4.203$, $p < 0.001$)](see Figure 3.4B). This was negligible by the final session ($t = 1.833$, $p > 0.05$) and all groups of rats behaved similarly and spent between 2-3 seconds in the decision zone ($p > 0.05$, *ns*). The multiple-start trials produced more interesting results where MADON rats spent the most time within the decision zone for S1 and S2. This was significantly different from YADON rats [S1 ($t = 8.473$, $p < 0.001$) and S2 ($t = 9.208$, $p < 0.001$)].

Latency in the decision zone was longer for treated middle-aged rats

Donepezil treatment in rats resulted in a longer latency within the decision zone for MA rats when compared to controls [S1 ($t = 8.268$, $p < 0.001$) and S2 ($t = 7.877$, $p < 0.001$)](see Figure 3.4B). By S3, latency was similar for all groups in the fixed-start trials ($p > 0.05$, *ns*).

Younger rats produced similar latency despite donepezil treatment

No difference in behaviour was observed within the decision zone due to donepezil treatment in younger rats(see Figure ??B). Latency was between 2.5 and 3.5 seconds for all sessions with no significant differences between groups ($p > 0.05$, *ns*).

3.3.6 Multiple-start trials allow for in-depth analysis into behavioural flexibility in middle-aged rats

Another parameter of the task that was investigated was the type of errors made in the multiple-start trials. Trained response refers to the behavioural action corresponding to the previously trained direction for fixed-start trials (e.g. left turn). A straight error happens when a forward directional swim fails to reach platform. A directional turn different from previously trained responses (e.g. right turn) is scored as a turn error (refer to figure 2.3).

One way to ascertain the strategies are used during the multiple-start trials was to focus on the behavioural responses generated for both YA and MA rats. A two way ANOVA analysis demonstrated statistical significance for the type of errors factor [$F_{(2,54)} = 8.319$, $p < 0.001$] and for the factor interaction [$F_{(2,54)} = 7.21$, $p < 0.01$] but none for the age factor [$F_{(1,54)} = 0$, $p = 1$, *ns*] in S1. Posthoc analysis further revealed the variation in the errors produced (see Figure 3.5) for each session. In the first session (see Figure 3.5 A), YA rats appeared to demonstrated more trained response during multiple-start trials as compared to the older rats ($t = 2.70$, $p < 0.05$). On the other hand, MA rats preferred the straight response ($t = 2.67$, $p < 0.05$). Both ages performed the same percentage of turning errors ($t = 0.23$, $p > 0.05$, *ns*). Also in the first session (S1), MA rats demonstrated a preference for straight swim errors and this was shown to be significantly different from trained response ($t = 3.174$, $p < 0.01$) and turn response ($t = 4.03$, $p < 0.001$).

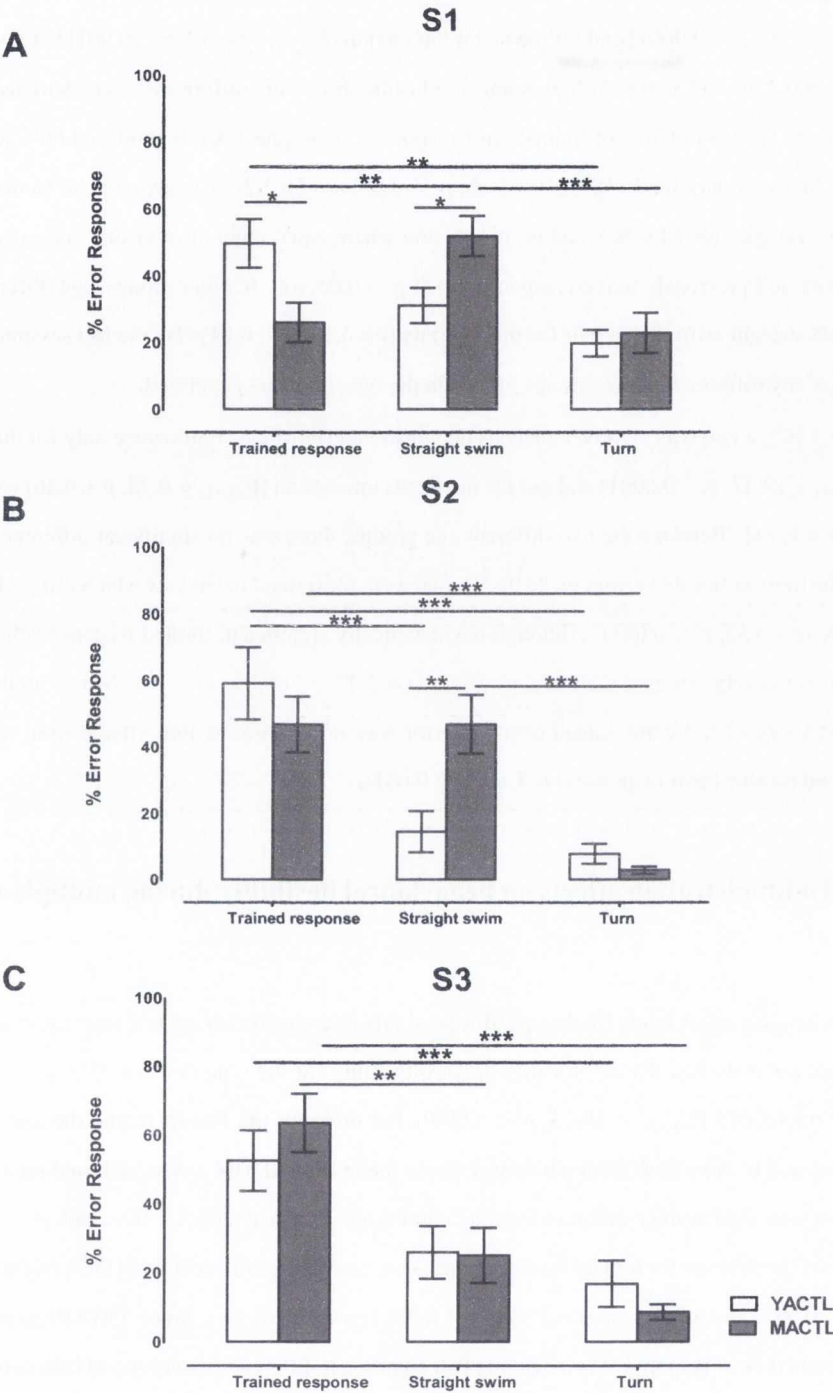


Figure 3.5: Age-related effects demonstrated by the type of errors generated during multiple-start trials. Further analysis on multiple-start trials where the type of errors generated are analysed. MA rats generated more straight swim errors as compared to YA rats which made more trained error responses. All sessions are represented (A; S1, B; S2 and C; S3). Data represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Within S2 (see Figure 3.5 B), two way ANOVA analysis demonstrated statistical significance for the type of errors factor [$F_{(2,54)} = 23.91, p < 0.0001$] and for the factor interaction [$F_{(2,54)} = 6.212, p < 0.001$] but none for the age factor [$F_{(1,54)} = 0.3, p = 0.58, ns$]. Although not significant, observable differences were demonstrated between the different age groups in terms of trained error responses made (the YA rats produced 66% trained error response while MA rats only made 48%) ($t = 1.66, p > 0.05, ns$) for S2. It is also crucial to note the different behavioural strategies used by MA rats in this session where equal amount of errors were made for straight swim behaviour and previously trained response ($t = 0, p > 0.05, ns$). In other groups, age differences pointed to a prominent straight swim behaviour for the MA rats ($t = 3.12, p < 0.01$). By the last session (S3), there WERE no longer any differences between age groups in the type of errors generated.

In S3 (see Figure 3.5C), a two way ANOVA analysis demonstrated statistical significance only for the type of errors factor [$F_{(2,54)} = 19.37, p < 0.0001$] and not for the factor interaction [$F_{(2,54)} = 0.78, p = 0.46$] and age factor [$F_{(1,54)} < 0, p = 1, ns$]. Between the two different age groups, there was no significant differences for all types of errors. The most noticeable errors made by YA rats were for trained behaviour which differed from turning error response ($t = 3.58, p < 0.001$). Although not statistically significant, trained response behaviour in these rats was also noticeably stronger than straight swim ($t = 2.47, p > 0.05, ns$). This behavioural trend was also evident in MA rats whereby the trained response error was most recurrent and differed from straight ($t = 3.575, p < 0.01$) and turning error response ($t = 5.16, p < 0.001$).

3.3.7 Donepezil administration effects on behavioural flexibility during multiple-start trials

The indepth analyses into the errors made by donepezil-treated rats as compared to control rats was done and a two-way ANOVA revealed statistically significant relationships only for the type of errors [$F_{(2,96)} = 35.18, p < 0.0001$] and the interaction [$F_{(6,96)} = 10.23, p < 0.0001$] but none for the treatment and the age factor [$F_{(3,96)} = 0, p = 1, ns$] in S1. An exceptional amount of errors made by YADON rats were based on trained response (80%) which was significantly different from its control age-matched subjects ($t = 3.60, p < 0.01$). Moreover, YADON rats' preference for trained responses was also statistically different from the straight swim errors ($t = 6.435, p < 0.001$) and turn errors ($t = 7.611, p < 0.001$) (see Figure 3.6). Since YADON generated the most amount of trained error response it is inevitable that significant differences were found between them and both older groups of rats (MADON: $t = 3.452, p < 0.01$, MACTL: $t = 6.149, p < 0.001$). Between the 13 month old rats, MADON rats demonstrated an elevated trained response error compared to MACTL rats ($t = 2.51, p < 0.05$). Although not statistically significant, MADON rats performed less straight swim errors as compared to MACTL rats ($t = 2.10, p > 0.05, ns$). Another interesting observation was that YADON rats attained a majority of errors for trained response, they have the least amount of straight and turn errors ($p > 0.05, ns$). This might reflect the high performance in the fixed-start trials and the tendency for YADON rats to retain its trained response.

In the proceeding session (S2) statistical analysis with two-way ANOVA once again revealed significance

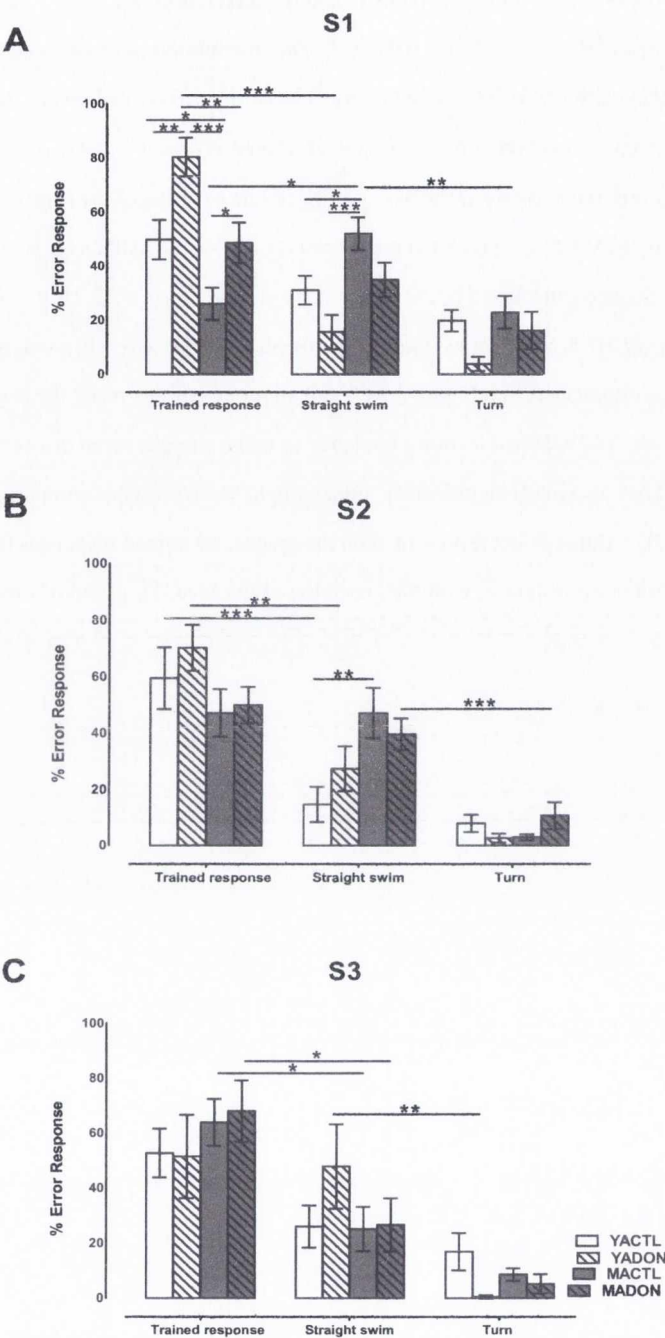


Figure 3.6: Donepezil administration alleviated behavioural inflexibility in middle-aged rats during multiple-start trials. Straight swim errors were much lower in donepezil treated middle-aged rats than control. Trained response refers to the behavioural action corresponding to previously trained direction for fixed-start trials (e.g. left turn). A straight error happens when a forward directional swim failed to reach platform. A directional turn different from previously trained response (e.g. right turn) is scored as a turn error (refer to figure 2.3). Both donepezil and control groups are represented together for each session (A; S1, B; S2 and C; S3). Data represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

only for the type of errors [$F_{(2,96)} = 54.9, p < 0.0001$] and the interaction [$F_{(6,96)} = 3.52, p < 0.0001$] but not for treatment and age factor [$F_{(3,96)} = 0.2, p = 0.91, ns$]. The distribution of errors made by MADON rats in the S2 was similar to that of the MACTL rats whereby 50% of the errors made were trained behaviour (see Figure 3.6 B). Both YA rats generated similar amount of trained errors ($t = 0.42, p > 0.05, ns$). Although not significant, the only difference between the MA groups of rats in terms of the type of errors made was for turning response whereby MADON accumulated more errors ($t = 0.76, p > 0.05, ns$). By the final session (S3), the only statistical significance generated by ANOVA was for errors [$F_{(2,96)} = 32.21, p < 0.0001$] and none for both the interaction [$F_{(6,96)} = 1.3, p = 0.26, ns$] and the treatment factor [$F_{(3,96)} = 0, p = 1, ns$]. Most variation in the data at this stage was diminished (see Figure 3.6 C) and all groups of rats made the same amount of trained error responses. However, YADON had a strong tendency to make straight swim errors as compared to other groups ($p > 0.05, ns$). They also made significantly more straight swim errors as compared to turning response errors ($t = 3.48, p < 0.01$). Although not represented on the graphs, all trained responses (for all sessions) were significantly different from turn response, with the exception of the MACTL group of rats in S1.

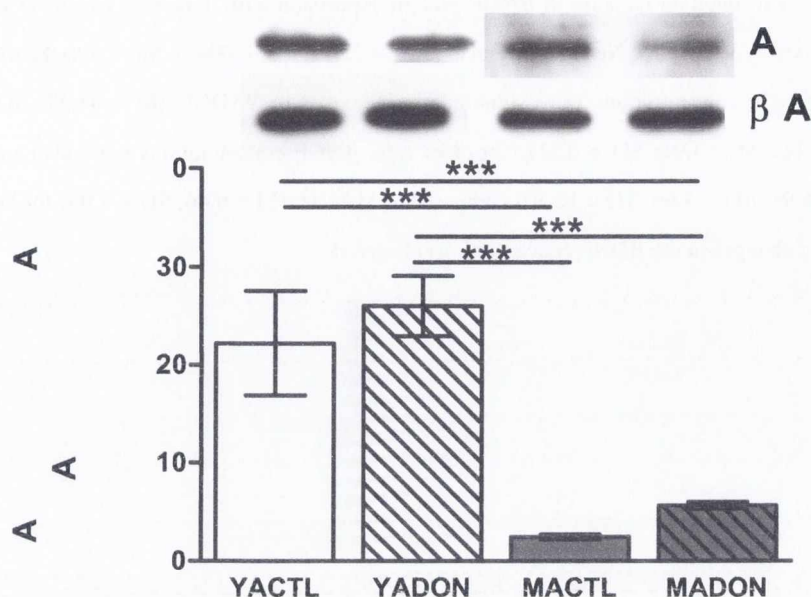


Figure 3.7: Rats under chronic donepezil administration demonstrated elevated protein expressions of α -7 nicotinic receptor within the hippocampus. Donepezil administration increased α 7 receptor in the hippocampus for both ages. Data represented by means $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.4 Molecular alterations within the hippocampus

3.4.1 Donepezil treatment upregulated hippocampal alpha7 (α 7) nicotinic receptor levels

An assessment of the nicotinic receptor expression was conducted using western blot (see Figure 3.7). Although treatment has no effect within each age group ($p > 0.05$), there is a significant effect of treatment on the levels of α 7 receptors [ANOVA, post hoc Newmann Keuls, $F_{(3,25)} = 30.87$, $p < 0.0001$]. Post hoc comparisons using Newmann Keuls test indicated that the mean level of α 7 receptors for YACTL rats ($M = 22.23$, $SD = 10.61$) was significantly higher than in MACTL rats ($M = 2.47$, $SD = 0.67$). Similarly in donepezil-treated rats, MA rats had a lower α 7 receptor levels in the hippocampus ($M = 5.69$, $SD = 0.99$) as compared to younger rats ($M = 26.02$, $SD = 7.48$).

3.4.2 Treatment with donepezil increased neurotrophin expression levels

Chronic donepezil treatment increased the BDNF protein expression (see Figure 3.8A) in all treated rats regardless of age (ANOVA, post hoc Newmann Keuls, $F_{(3,31)} = 5.69$, $p < 0.0001$). BDNF concentrations were significantly increased in YADON ($M = 350.6$, $SD = 87.17$) compared to YACTL ($M = 110.5$, $SD = 100.1$). Similarly BDNF concentrations were significantly increased in MADON ($M = 322.5$, $SD = 98.90$) compared to MACTL ($M = 46.25$, $SD = 35.99$). There was no significant age-related changes in BDNF concentrations

observed.

Figure 3.8B demonstrated an increase in β NGF protein expression with donepezil treatment in both the YG and MA rats (ANOVA, post hoc Newmann Keuls, $F_{(3,31)} = 22.11, p < 0.0001$). Similar to the trend shown in BDNF levels, β NGF concentrations were significantly increased in YADON ($M = 41.57, SD = 11.96$) compared to YACTL ($M = 5.91, SD = 6.31$). In older rats, β NGF concentrations were also significantly increased in MADON ($M = 25.66, SD = 12.50$) compared to MACTL ($M = 9.46, SD = 7.03$) for both. There were no age related changes in the BDNF concentrations observed.



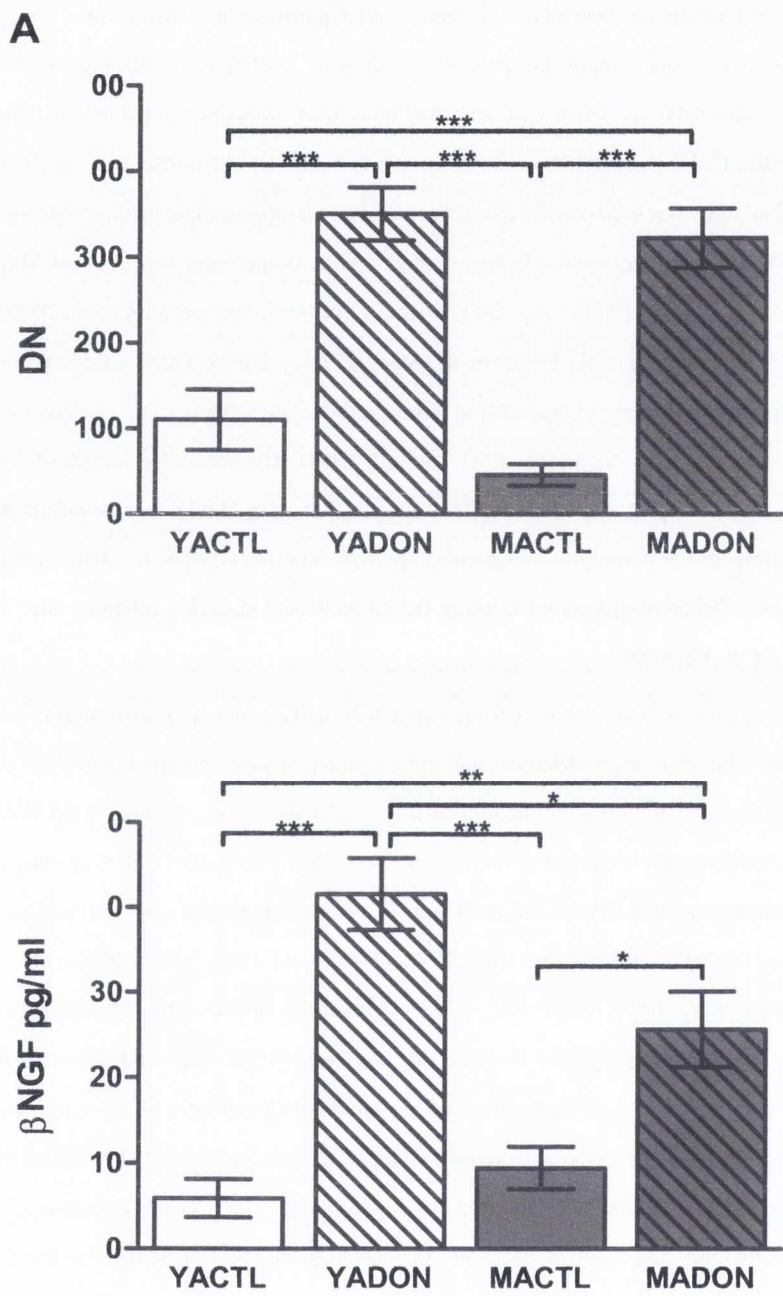


Figure 3.8: Donepezil administration elevated protein expressions of neurotrophin levels within the hippocampus. BDNF (A) and β Nerve growth factor (β NGF) protein levels (B) in the hippocampus were significantly elevated compared to control groups. Data represented by means $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.5 Discussion

In both humans and animals, several lines of evidence suggest that learning and memory gradually decline with advancing age. This is supported by evidence from age-related impairment in spatial learning and memory tasks (Altun *et al.*, 2007). The spatial component of memory in rodents has been investigated with the application of the Morris water maze (MWM) which was described more than 25 years ago (Morris, 1984). Since then it has become a central tool for the investigation of ageing in behavioural neuroscience. A disruption in the spatial learning and memory has been demonstrated in animals with hippocampal damage (Morris *et al.*, 1982; Olton & Samuelson, 1976). Correspondingly, age-related deficits in the more conventional MWM has been demonstrated on several accounts (Wyss *et al.*, 2000; Frick *et al.*, 1995; Schoenbaum *et al.*, 2002; Rapp *et al.*, 1987; Lindner, 1997; Shukitt-Hale *et al.*, 1998; van der Staay & Staay, 1997). The inability of older animals to solve the task lies in the poor learning of the task and errors pertaining to reference and working memory failure (Van Der Staay & de Jonge, 1993; Rapp *et al.*, 1987; Lindner, 1997). The generalised deficit of the aged rats in solving mazes revealed the importance of the hippocampus for recalling highly specific information and also indicated a possible role for the frontal lobes in learning and remembering nonspecific skill-related information (Wincur *et al.*, 1990). Hence compromised memory functions related to ageing might be due to both frontal lobe and hippocampal dysfunction.

Structural differences in our maze demanded the animals to make a choice of entry during the location of the escape platform. This required a different strategy as compared to the traditional WMW. Although our current experimental design was built upon the foundations laid by the more commonly used MWM, there are several discernible discrepancies between the two mazes. The water plus maze (WPM) or plus-shaped maze, a variation from the more standard MWM, has been used in recent years for the assessment of spatial memory impairment in animal models of behavioural studies (Wahlsten *et al.*, 2005; Vloeberghs *et al.*, 2006; Locchi *et al.*, 2007; Diamond *et al.*, 1999; Chen *et al.*, 2004). Alterations in the configuration of the WPM from the MWM were to serve several purposes. Because of differences in the shape and structure, the strategies employed by experimental subjects differ because of the more restricted entry and escape zone within the WPM. A combination of both egocentric and allocentric strategies were suggested to be utilised when subjects are required to solve the MWM task (McGauran *et al.*, 2005; Gerlai *et al.*, 2002; Kealy *et al.*, 2008). In our WPM configuration, memory investigation might also be more cognitively demanding in terms of task solving strategies. Here, rats were required to learn the single association between the escape platform and a particular distal environment to solve the WPM task. Whereas in the MWM, animals had to learn to form multiple spatial relations (Vloeberghs *et al.*, 2006). Structurally, an additional restriction was present in the WPM whereby there was a possibility between a “right” or “wrong” entry into the arms during the search for the hidden platform. In contrast to the MWM, rats would not be able to employ an alternative strategy of swimming in large circles until they bump into the platform (Wyss *et al.*, 2000). Once again due to the architectural differences between the two mazes, such a strategy proposed may not adequately cover the learning and memory mechanisms behind the WPM protocol. This leads us to designate the centre of the WPM as the decision zone and giving allowance

for rats to accurately assess all spatial cues provided before making an entry into any arms. In a similar study using the plus-shaped maze, this central area has shown to aid spatial learning whereby mice were demonstrated to perform less straight swim errors (Wahlsten *et al.*, 2005).

This chapter is primarily dedicated to understanding the effects of chronic donepezil on behavioural and molecular outcomes. The specific strategies and behavioural conditions in relation to the application of the WPM task will be provided in chapter 6, page 119. In brief, younger animals were able to attain the task right from the start and this trend increased gradually over the 3 sessions. For middle-aged animals, improvement in acquiring the task was present but was achieved at a slower rate. The multiple-start protocol was very difficult for untreated middle-aged rats to learn (below chance level) while young animals managed to score above chance but only at the final session. Treatment with donepezil caused some deviation from this pattern of behaviour and attenuated learning deficits in middle aged rats.

Research leading to the development of donepezil began in 1983 (Sugimoto, 2001a). Ever since there has been an immense interest in the therapeutic nature of this acetylcholinesterase drug. Fundamentally, donepezil is used mainly to improve cognitive functions relating to dementia (Howland, 2011), mild cognitive impairment (MCI) (Doody *et al.*, 2010) and AD (Burns *et al.*, 1999). The prescription use of donepezil therapy is not only limited to neurodegenerative diseases and has been used with patients with MS (Krupp *et al.*, 2011), individuals with Down Syndrome (Kishnani *et al.*, 2010) to children with autism (Handen *et al.*, 2011). Improvements seen in these studies are mainly due to increase the concentration of ACh as it is a relatively specific inhibitor of AChE (Sugimoto, 2001a).

Clinically, donepezil treatment did improve cognitive functions in young adults (Zaninotto *et al.*, 2009; Ginani *et al.*, 2011). Physiologically, inhibition of cholinesterase activity in the brain was more potent in aged animals than young when donepezil treatment was given (Kosasa *et al.*, 1999b). By studying the effects of donepezil on young animals we were able to investigate the specific profile of the donepezil mechanism on cognitive functions in the presence of unaltered cholinergic neurotransmission systems. In our study, donepezil increased acquisition as compared to control young animals. Donepezil-induced improvement in memory in young animals is not novel and was supported by a recent study where chronic treatment ameliorated memory functions and explorative strategies, sped up the acquisition of localizing knowledge, augmented responsiveness to the context, and reduced anxiety levels (Cutuli *et al.*, 2008). It is important to note that the enhancement of acquisition we observed was not immediate (i.e., during the first session). One possibility for such a delay in the improvement might be due to alterations in neurogenesis which is a process that requires some time (Nilsen *et al.*, 1999). The presence of newly formed neurons have been demonstrated to be involved in memory processes and age-related cognitive alterations (Drapeau *et al.*, 2003). Interestingly, a chronic treatment (four weeks) of donepezil was able to alter the survival of hippocampal neurogenesis, particularly cells in the dentate gyrus of the hippocampus (Kotani *et al.*, 2006). This phenomenon of enhanced newborn cells survival may be partly associated with cognitive improvement since several studies have suggested that neurogenesis in the adult hippocampus plays a crucial rule in cognitive function (Kempermann *et al.*, 2004; Kaneko *et al.*, 2006). It is reasonable to speculate that the resulting neuroprotective ability of donepezil needs at least a twelve hour

pretreatment condition and that such responses produced might be via gene expression as they tend to involve a delay of this order (Akasofu *et al.*, 2003).

In our older animals, donepezil treatment was hypothesized to improve memory and task performance. This was evident right from the beginning of the experiments. Evidence from recent studies also supports our hypothesis that improving ACh concentration in the ageing brain can reverse the age-induced memory impairments (Tronche *et al.*, 2010). It had been suggested that such improvements in memory functions might be due to the preventive measures of donepezil in the alterations of the neuronal dendrite morphology caused by aging. Besides increasing ACh concentration in the synaptic cleft, donepezil may also ameliorate progressive mental decline by inhibiting subsequent neuronal cell death. Hence the therapeutic nature of donepezil is of two different natures and has been suggested to be a cognitive enhancer even in normal animals (Spowart-Manning & van der Staay, 2004) which is supported by our study. The basis of such neuroprotection might be due to decreased glutamate toxicity through down-regulation of N-methyl-D-aspartate (NMDA) receptors, following stimulation of alpha7 nAChRs (Shen *et al.*, 2010).

The multiple-start trials gave us an idea on how learning was achieved and maintained in the WPM task. Both middle age and young adult rats were able to learn the task even though their performance was not at the same levels as those attained during fixed-start trials. From the present study, it would appear that MACTL animals were unable to perform at the level of YA rats, unlike MADON. Behavioural flexibility has always been suggested to be an accompaniment to ageing. Latencies in duration zone together with analysis in the type of errors made might give an insight into behavioural inflexibility. Rats in the WPM upon reaching the decision zone have a 'choice' to make in order to escape and find the hidden platform. This delay within the designated zone might give us two different views on the pattern of behaviours in the WPM task. The longer delay in the zone observed in middle aged animals was not due to a general motor impairment due to ageing as an analysis into swim speeds did not show any differences between groups (see chapter 6, page 119).

We hypothesized that the longer the latency in the decision zone, the more accurate the choice for the escape route. In older control animals, latency was longer compared to the YACTL groups but performance levels were not much higher. This led us to investigate the possibility of behavioural inflexibility. More time in the decision zone did not enhance middle-aged rats ability to acquire the task. On the other hand, MADON rats displayed exactly this behaviour whereby a long latency in the decision zone resulted in a high performance level in the multiple start trials. One might also infer from these experiments that behavioural inflexibility occurs when rats consistently choose the straight response error when given a choice to escape from the WPM which was a behaviour exhibited by MACTL rats. A recent study on reversal learning supported our claims that inhibition of the central acetylcholinesterase by donepezil can alter and treat behavioural inflexibility (Chen *et al.*, 2009). In this instance the central cholinergic system was suggested to be important in the suppression of previously reinforced responses and has less influence on the acquisition of new rules. In young animals (both treated and untreated), latency was constant throughout the sessions and this might suggest that once acquisition of the task was stable, time required in the decision zone was reduced. This was consistent with learning progression in the MWM (Morris, 1984) where latency is a sensitive index for the efficiency of rats performance in the task.

The molecular targets that were investigated in this study provided evidence of upregulation of neurotrophins following chronic donepezil treatment. It seems possible that the upregulation of $\alpha 7$ receptors in the brain may represent only one facet of chronic donepezil treatment. The interest in $\alpha 7$ receptors has been shown in several AD studies citing it as a therapeutic target (Kem, 2000). The presence of $\alpha 7$ receptors in regions of the brain is important for cognition especially in the cerebral cortex and the hippocampus. Other studies suggest that the pathophysiological consequences of A β 42 binding to $\alpha 7$ nAChR on neuronal surfaces stem from excessive intraneuronal A β 42 accumulation, the $\alpha 7$ nAChR could be an important therapeutic target for treatment of AD (D'Andrea & Nagele, 2006). In rats the $\alpha 7$ receptor was found to be important in influencing processes as diverse as cell migration, dendritic elaboration and apoptosis during hippocampal maturation (Adams *et al.*, 2002). Hence, the augmented performance levels seen in treated animals were accompanied by an upregulated $\alpha 7$ receptor expression in the hippocampus.

Adult neurogenesis has been implicated in affecting cognitive performance and more importantly, hippocampal neurogenesis was found to be drastically reduced in aged rats (Kuhn *et al.*, 1996). Hence the alleviated cognitive deficit in ageing might reveal another aspect of donepezil treatment. The bridge between the manipulation of the cholinergic system and increased hippocampal neurogenesis have been demonstrated recently (Kotani *et al.*, 2006). Activation of the cholinergic system by chronic donepezil treatment amplified adult neurogenesis in the hippocampus by an increased expression level of phosphorylated cAMP response element binding protein (CREB), which is related to cell survival in the dentate gyrus (Kotani *et al.*, 2006). In rats neurotrophins such as BDNF support the survival of neurons in the adult rat forebrain and may act as a permissive factor for neuronal recruitment in adulthood (Kirschenbaum & Goldman, 1995). In terms of cognitive functions, BDNF together with exercise can enhance learning ability and activate hippocampal neurogenesis (Cotman & Berchtold, 2002). These evidences support the hypothesis that improved cognitive abilities through donepezil treatment may, in part, be due to increased neurotrophins. Additionally, treatment with donepezil was shown to be accompanied with an increase of BDNF serum concentration in AD patients reaching the level of healthy controls (Leyhe *et al.*, 2008). An investigation into neurotrophin levels in the hippocampus demonstrated that donepezil, indeed, is capable of augmenting neurotrophin synthesis and might be the mechanism behind better cognitive functions. Both young and middle-aged rats in our study exhibited an increased level of BDNF after a chronic administration of donepezil.

NGF, another neurotrophin, is an agent of the neurotrophic growth factor and is intimately related to the maintenance of function of the cholinergic basal forebrain system (Higgins *et al.*, 1989). Forebrain cholinergic neurons are the only cells in the adult brain that express high amounts of the low-affinity p75 receptors for NGF. Clinically, NGF might be offered as a neuroprotective treatment in AD as NGF increases hippocampal ACh and prevents cholinergic cell loss and atrophy after fornix lesions (Olson, 1993). In our case, it might be possible that the administration of donepezil mediates NGF levels which in turns offer neuroprotection against age-related cognitive deficits. NGF affords neuroprotection against cytotoxicity as cortical neuronal cultures incubated with NGF for 1-24 hours were protected from delayed cytotoxicity induced by brief exposure to glutamate (Kume *et al.*, 2000). The cholinergic system can also manipulate levels of this neurotrophin as ACh

is known to potentiate the effect of NGF (Knipper *et al.*, 1994). Additionally, an agonist of nAChR, nicotine, not only enhanced spatial memory it also increased high-affinity tropomyosin-receptor kinase A NGF receptors (TrkA) and their phosphorylation (Hernandez & Terry, 2005). Hence, donepezil as an AChE-inhibitor, might offer neuroprotective effects in our ageing rats. Complimentary to BDNF analysis, investigations in to NGF levels also revealed an elevated expression induced by donepezil treatment, regardless of age.

3.5.1 Conclusion

The novel water plus maze task we used in this study gave an insight into age-related differences in memory, cognitive functions and strategies used. In healthy and untreated rats, young rats were superior in completing the task and required less time in the decision zone. In control rats, the swim strategies employed to locate the platform were different between middle-aged and young rats. Middle-aged rats during the harder multiple-start trials lacked behavioural flexibility and in a majority of the trials employed a straight swim response and failed to reach the platform. Learning deficits were obvious as this pattern of behaviour was held constant for two sessions. Lastly, donepezil attenuated learning deficits in healthy middle-aged rats even though the effect was not observed until 18 days later (S2). Such a delay in effect might suggest that donepezil-induced neurogenesis has a part to play in the enhancement of memory and cognitive functions. In terms of behavioural flexibility, donepezil alleviated learning deficits and the straight swim response diminished in treated middle-aged rats. The molecular targets we examined in the study also suggest that an upregulation of neurotrophins, specifically BDNF and NGF, was present after chronic treatment with donepezil. Such a result ties in with the behavioural component of the task. In summary, chronic donepezil treatment alleviated learning deficits seen in middle-aged rats possibly through the enhancement of neurotrophins and the upregulation of $\alpha 7$ receptor in the hippocampus.

Chapter 4

Maintaining glucoregulatory functions in normal ageing

4.1 Rationale

An extensive epidemiological study involving 2.7 million participants recently revealed a rising trend in glycaemia and diabetes globally which is driven by ageing and increased age-specific prevalence (Danaei *et al.*, 2011). Disturbances in glucose metabolism and glucose tolerance generally declines with advancing age (Facchini *et al.*, 2001), most notably as a consequence of peripheral insulin resistance caused by a post receptor deficit in target tissue insulin action (Fink *et al.*, 1983). Insulin resistance may precede Type 2 diabetes mellitus (T2DM) onset, (Reaven, 1995), hence, a clear therapeutic approach is to reduce such resistance in humans. Individuals with insulin resistance already demonstrate similar reductions in cognitive performance as those described in T2DM (Bruehl *et al.*, 2010), giving support that cognitive functions may be impaired before glucoregulatory impairment reaches abnormal levels consistent with a type 2 diabetes diagnosis (Messier *et al.*, 2010).

As an anti-diabetic agent, rosiglitazone binds and activates peroxisome proliferator-activator receptor gamma (PPAR), and alters expression of genes involved in glucose uptake and disposal, as well as lipid metabolism. In other words, rosiglitazone heightens responses to insulin in target tissues without stimulating insulin secretion (Lebovitz *et al.*, 2001; Wagstaff & Goa, 2002). In the present study, alterations in memory and learning under rosiglitazone treatment are being investigated in healthy middle-aged rats (thirteen months old). The WPM behaviour paradigm required the rats to acquire associations between the escape platform and a constellation of distal cues. It was hypothesised that treatment with rosiglitazone can enhance learning and memory abilities in ageing rats by regulating glucose metabolism. To complete the study, analysis of several molecular markers will help elucidate the link between chronic rosiglitazone treatment and an augmented hippocampal function in ageing animals.

4.2 Introduction

Age-related changes in physiological conditions can be accompanied by alterations in glucose regulation (Facchini *et al.*, 2001). The maintenance of glucose within the brain is an essential factor for basic cognitive functions since the glucose carried by blood accounts for 99% of the brain energy requirements. Clinically, glucose uptake and utilisation in the brain (Maher *et al.*, 1994) are impaired in aged (Gumbiner *et al.*, 1992) and diabetic subjects (Awad *et al.*, 2004). In fact, with the increasing prevalence of obesity and an ageing population, T2DM is rapidly becoming the most common chronic disease in the US (Bogaert & Schrier, 2011). More importantly, diabetes is now known to be a risk factor for cognitive decline (Cukierman *et al.*, 2005) and concomitant physiological changes with insulin resistance can improve memory functions (Yanagawa *et al.*, 2011).

Glucose regulation compared between elderly subjects demonstrated that individuals with good glucoregulation outperformed others with poor glucoregulation in memory tasks evaluating working memory, verbal declarative memory and executive functions (Messier *et al.*, 1997; Messier *et al.*, 2003; Craft *et al.*, 1994; Craft *et al.*, 1992). Comparable results showing poor performance have been demonstrated in older subjects with high blood glucose in several other tasks such as paragraph recall, word list learning (Kaplan *et al.*, 2000) and verbal memory (Convit *et al.*, 2003). There is a general idea that glucose regulation has a crucial impact on memory processing, especially in ageing humans. A central theme in ageing is the erosion of memory capacity because of loss or change in brain structures and/or neuronal components. An alternative theory states that the essential mechanisms for learning and memory remain largely intact with age, but the neuroendocrine regulators that engage the mechanisms of memory storage are deficient (Korol & Gold, 1998). This would fit the idea of glucose dysfunction nicely as the hardware necessary to create a new memory is present, but the specific mechanisms instructing or signalling the brain when to activate the processes necessary for memory formation are absent or impaired (dysfunctional glucoregulation). It is probable that the cognitive deficits observed in aged persons may reflect a problem of diminished regulatory mechanisms in addition to a loss of storage mechanisms.

Ameliorations of cognitive decline found in older individuals with T2DM and MCI has demonstrated with the administration of rosiglitazone (Abbatecola *et al.*, 2010; Watson *et al.*, 2005). Improvement in circulating insulin levels was key to the enhanced cognitive function and rosiglitazone treatment can be beneficial in protecting against cognitive decline. Thus, the decline of mnemonic abilities in older individuals might be compensated by adequate glucose conservation. As a member of the thiazolidinedione (TZD) class of drugs, rosiglitazone operates as an insulin sensitiser. Recent observations suggested that rosiglitazone also modulates the CNS. Because current experiments support observations that defects in cerebral glucose metabolism may underlie the pathology of AD, insulin sensitisers such as rosiglitazone might stabilise or improve the clinical course of the disease. This was shown in a population of mild to moderate AD patients, exhibiting cognitive and functional improvement in response to rosiglitazone (Risner *et al.*, 2006). Alterations in peripheral blood glucose resulting from rosiglitazone administration may increase brain glucose availability, leading to relative

improvements in performance on cognitive tasks, as demonstrated by improved working memory performance in a cohort of adults with T2DM under rosiglitazone treatment (Ryan *et al.*, 2006).

A more recent small pilot study utilising pioglitazone in AD and amnesic MCI patients, who were treated for T2DM, demonstrated improved cognitive performance and peripheral insulin sensitivity relative to a baseline assessment (Hanyu *et al.*, 2009). Although the mechanism of alleviating cognitive functions in these clinical studies is still unclear, it has been proposed that the role of an insulin sensitiser may aid cerebral glucose metabolism. It has been established that attaining steady-state glucose level could help improve memory functions in both humans and animals. Patients with T2DM suffer from chronically elevated glucose and/or insulin levels and this might affect cognitive functions in two forms. The first being failed glucoregulation (as discussed above) and the second, elevated insulin levels.

The presence of insulin receptors and insulin-second messengers systems in neurons in the central nervous system is widespread (Havrankova *et al.*, 1978) and animal model research indicates that insulin deficiency might be responsible for impairments in synaptic plasticity and cognitive processes. There are some evidence that insulin itself can mediate memory-enhancing effects in both animal and human models (Benedict *et al.*, 2004; Biessels *et al.*, 1998; Biessels *et al.*, 1996; Park *et al.*, 2000; Reger *et al.*, 2006; Zhao *et al.*, 1999). Clinical studies suggested that insulin insensitivity might affect cognitive processing (Park, 2001). There are no current studies that investigate the effects of rosiglitazone on the normal ageing human population. One can only deliberate about the changes in normal ageing in terms of glucoregulation and hypothesise that rosiglitazone might improve cognitive functions in elderly in the absence of any neurodegenerative diseases or metabolic failures. Since central glucose uptake and utilisation are impaired in aged and diabetic subjects it could be implicated that a decreased uptake of glucose in the brain can be attenuated by increased circulating glucose levels in these subjects (Manning *et al.*, 1990). From these clinical studies, it seems plausible that the modulatory role of rosiglitazone on insulin levels does affect glucoregulation leading to improved cognitive functions.

Preclinical studies where memory tasks are improved by glucose administration are in abundance (Flint & Riccio, 1997; Flint & Riccio, 1999; Messier & White, 1984; Packard & White, 1990; Ragozzino *et al.*, 1996). More than a decade ago, Winocur and colleagues assessed spatial learning and memory in aged rats on three separate tests of hippocampal functions which included the radial arm maze (RAM) task, the spatial non-matching to sample (SNMTS) task and a cue-preference test that compares associative strength between different cues (local or spatial) and food reward (Winocur & Gagnon, 1998). Animals were required to utilise spatial information in different ways with these tasks which are sensitive to ageing and glucose influence. Improvement of tasks was observed only for the RAM and SNMTS tasks (both requiring memory for highly specific experiences) suggesting that glucose effects do not generalise to all aspects of hippocampal function that are affected in old age. Moreover, glucoregulation via administration of glucose in aged rats with depleted brain extracellular fluid glucose can also enhance memory function (McNay & Gold, 2001; Winocur & Gagnon, 1998; Winocur, 1995).

In rodent models, rosiglitazone administration appears to improve neurocognitive function in ageing and obesity research (Gemma *et al.*, 2004; Kramer *et al.*, 2001). In AD mice models, rosiglitazone attenuated

learning and memory deficits by affecting brain Insulin-Degrading Enzyme (IDE) levels, and brain A β 42 levels (Pedersen & Flynn, 2004; Pedersen *et al.*, 2006). Cellularly, dendritic spines density was increased in a dose-dependent manner when incubated for 24 hours with rosiglitazone which was prevented by PPAR specific antagonist, suggesting that this modulation involves PPAR pathway (Brodbeck *et al.*, 2008). Additionally, certain molecular changes are observed with rosiglitazone administration in rats, such as up-regulation of glucose transporter protein (GLUT-1 and 4) (Kramer *et al.*, 2001; Young *et al.*, 1995). Restoration of vascular function by rosiglitazone has been linked to adiponectin levels, a protein hormone that modulates a number of metabolic processes, where it is required to mediate the PPAR γ effect (Wong *et al.*, 2011). Moreover, adiponectin is found at a higher level plasma and CSF from MCI and AD suggesting a critical role in the onset of AD (Une *et al.*, 2011).

Besides poor glucoregulation, many age-associated neurodegenerative diseases are manifested by an underlying chronic inflammatory state mainly due to local infiltration of inflammatory cells, such as macrophages, and higher circulatory levels of pro-inflammatory cytokines, complement components and adhesion molecules (Sarkar & Fisher, 2006). Levels of adiponectin, one of the most important adipocytokines released from adipose tissue, have been associated with mental decline such as MCI and AD (Une *et al.*, 2011). In particular, plasma adiponectin level was significantly higher in MCI and AD compared to cognitive normal controls. Adiponectin was also recently demonstrated to have anti-inflammatory, antiatherogenic properties in vitro and beneficial metabolic effects in animal. Interestingly, rosiglitazone increases plasma adiponectin levels in type 2 diabetic subjects which might in turn provide putative antiatherogenic effects.

Rosiglitazone, as a PPAR agonist, could have other influence on inflammation as well, as PPARs agonists can suppress the inflammatory response in peripheral macrophages (Bouhlef *et al.*, 2007) and in several models of human autoimmune diseases (Lovett-Racke *et al.*, 2004). Interleukin levels are also strongly associated with the ageing brain. Importantly, rosiglitazone treatment in aged rats can attenuate aged-related increase in hippocampal IL-1 β and can restore long-term potentiation (Loane *et al.*, 2009). Such evidences support the idea that PPAR agonists might be beneficial for CNS disorders possessing an inflammatory component, including AD (Kummer & Heneka, 2008; Mohanty *et al.*, 2004). PPAR activators exert these anti-inflammatory actions in different immunological and vascular wall cell types such as monocyte/macrophages, endothelial, epithelial and smooth muscle cells in which PPARs are expressed (Chinetti *et al.*, 2000). In an extensive study involving rosiglitazone and the antagonist of PPAR, Bisphenol-A-diglycidyl ether (BADGE), it was proposed that the activation of PPAR reduces the development of acute inflammation, and contributed to the anti-inflammatory effects of rosiglitazone (Cuzzocrea *et al.*, 2004). On the other hand, there have been numerous reports stating that the anti-inflammatory effects of TZDs are PPAR independent, suggesting that other off-target effects or mechanisms may be relevant (Feinstein *et al.*, 2005).

Despite evidence that rosiglitazone improve memory in a population of diabetics, no research has been made in the assessing the therapeutic advantages of rosiglitazone on *memory* in DM nor STZ rats. The therapeutic nature of rosiglitazone in insulin resistance state includes the prevention of post-ischemic injury in isolated hearts (Khandoudi *et al.*, 2002), normalisation of glycaemia in Zucker rats (Maier *et al.*, 2000) and

protective nature against the cerebral infarction and neurological dysfunctions induced by embolic middle cerebral artery (MCA) occlusion in the rat (Allahtavakoli *et al.*, 2006). In summary, rosiglitazone may be beneficial in aiding insulin resistance but that may not be its primary purpose. Besides its well known vascular properties affecting glucose metabolism and adipocyte differentiation (Spiegelman, 1998; Ricote *et al.*, 1998; Jiang *et al.*, 1998), as a part of the TZD class of drugs, it targets the nuclear receptor PPAR γ also exhibit potent anti-inflammatory and anti-oxidant actions and inhibit both neural excitotoxicity and apoptosis (Griffin *et al.*, 2006; Wolf *et al.*, 2004). In the face of all these advantageous effects of rosiglitazone, it is still not known how rosiglitazone may affect memory. Glucose regulation has been demonstrated in a rodent model involving stress where rosiglitazone increased neuronal glucose metabolism (via improved glucose uptake and expression of the GLUT-3 neuronal glucose transporter), restored brain Adenosine-5'-triphosphate (ATP) levels and prevented impairments in glutamate uptake mechanisms (García-Bueno *et al.*, 2006). Rosiglitazone directly altering neuronal structures by increasing dendritic spine in cultured primary cortical rat neurons provided further implications in its ability to improve cognition (Brodbeck *et al.*, 2008). Lastly, the cognitive enhancing properties of rosiglitazone might be due to a peripheral effect (Gemma *et al.*, 2004) or perhaps rosiglitazone may affect the level and/or activity of factors that readily cross the blood-brain-barrier (Wagstaff & Goa, 2002).

4.3 Results for behavioural experiments

4.3.1 Learning progression during fixed-start trials

During fixed-start trials, rats were required to acquire the single association between the escape platform and a constellation of distal cues. In each session three consecutive days of training were appointed and each day consisted of ten trials for each animal. To successfully complete the fixed-start trial, it was essential for the subject to master the trained response. For example, rats were released only from south arm to find fixed-start and constant escape platform in west arm (see Figure 2.3).

Rosiglitazone rescued age-related differences in task performance

In this study, age-related differences in performance levels were found for treated rats which persisted throughout all sessions (S1: $t = 6.17$, $p < 0.001$, S2: $t = 5.75$, $p < 0.001$, S3: $t = 2.72$, $p < 0.05$) (see Figure 4.1A). As previously mentioned, in control rats, the level of performance in young rats is more advanced compared to older rats. This trend was parallel to rosiglitazone treated groups of rats where YA rats acquired the task at a much higher level compared to the older rats.

Rosiglitazone-treated middle-aged rats acquired task at a higher level than controls

Between the groups of older rats, treatment with rosiglitazone was able to rescue age-related deficits observed in learning the task (see Figure 4.1A). MAROSI was able to attain a higher level of correct response in both session 1 ($t = 6.27$, $p < 0.001$) and 2 ($t = 5.33$, $p < 0.001$). In the final session, rosiglitazone treatment could not alleviate learning deficit to the level of YA rats ($t = 0.103$, $p > 0.05$). It was interesting to note if rosiglitazone treatment could rescue learning deficits and improve task performance to the level of young rats. Although the differences in score between MAROSI and YACTL diminished gradually throughout the trials (S1: $t = 6.169$, $p < 0.001$, S2: $t = 5.748$, $p < 0.001$, S3: $t = 2.719$, $p < 0.05$), rosiglitazone treated middle-aged rats still could not perform the task to a similar standard as YACTL rats.

Treatment with rosiglitazone enhanced task performance for younger rats as well

Rosiglitazone treatment also elevated task performance in young adult rats (see Figure 4.1A). For younger rats, acute and chronic treatment of rosiglitazone improved task performance in session 1 ($t = 4.91$, $p < 0.001$) and 2 ($t = 3.346$, $p < 0.01$). Although there is a lack of statistical significance in performance levels in the last session ($t = 2.089$, $p > 0.05$, *ns*), crucially it is noted that YAROSI group of rats managed a mean score of 99%. In order to get a sense of the extent of learning among all groups of rats, learning in the young treated group (the hypothesised best performers) and the untreated middle-aged group (the hypothesised worst performers) was analysed. Overall, the acquisition of the task was lead by YAROSI rats which was maintained for all sessions and trials (not shown on graph). Such a distinction in the level of correct response was evident between YAROSI and MACTL group (S1: $t = 17.35$, $p < 0.001$, S2: $t = 14.43$, $p < 0.001$, S3: $t = 4.704$, $p <$

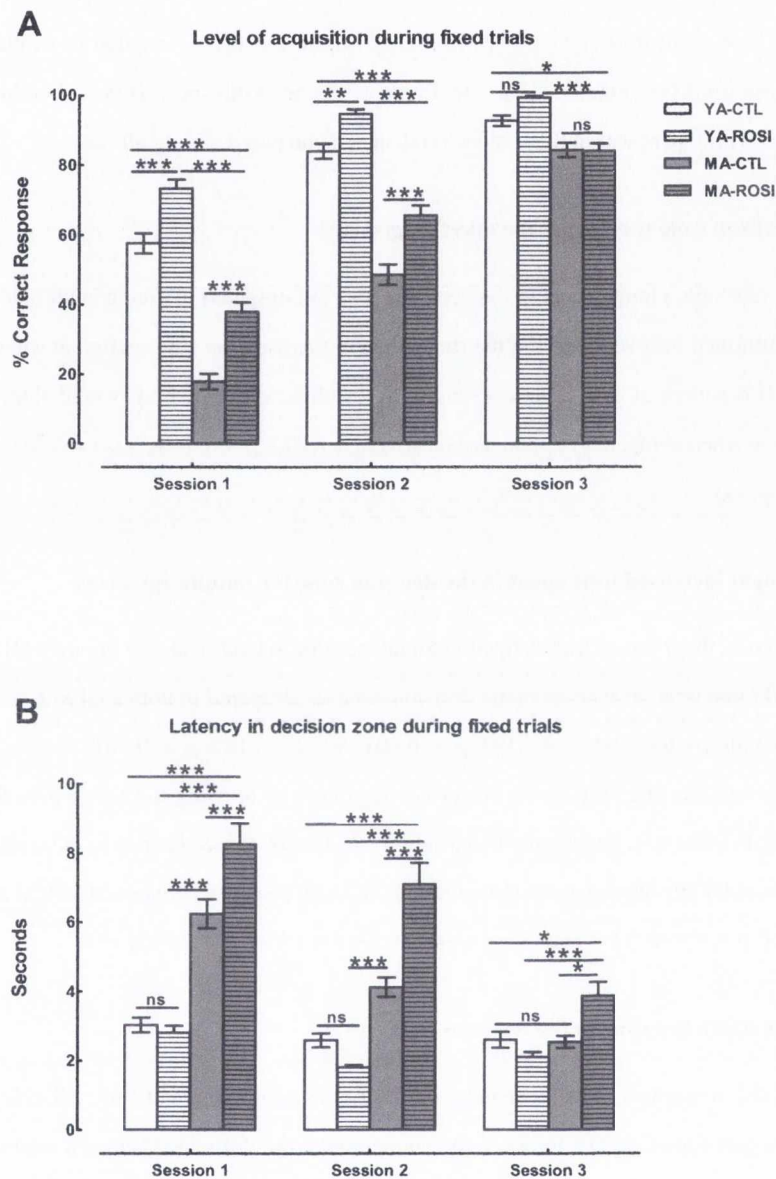


Figure 4.1: **Effects of rosiglitazone treatment on learning and dormancy within decision zone during fixed-start trials in the Water Plus Maze task.** Each session is composed of an average of data collected over 3 days of fixed-start training. Performance level is established by percentage of correct response in finding hidden escape platform. (A) Acquisition of the task was significantly higher in treated rats for both ages. (B) Middle-aged rats generally spent more time in designated decision zone which was recorded in seconds. All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

0.001).

4.3.2 Latency in decision zone during fixed-start trials

An analysis into the time spent in designated decision zone within the WPM revealed to us the pattern of learning during the task itself (see Figure 4.1B). The designated zone in this maze is described in detailed in the Donepezil chapter. Time spent within this zone was taken and compared among all rats.

Period of time in decision zone was longer for middle-aged rats

In control rats, older rats took a longer time in the decision zone as compared to young adult rats (see Figure 4.1B). This delay diminished over sessions and the time taken in final session was negligible when compared to young adult rats. This pattern of behaviour was similar in rosiglitazone treated rats where older rats persist longer in decision zone when compared to younger ones (S1: $t = 6.272$, $p < 0.001$, S2: $t = 5.331$, $p < 0.001$, S3: $t = 0.104$ $p > 0.05$, *ns*).

Rosiglitazone treatment increased time spent in the decision zone for middle aged rats

Between middle-aged rats, there was a clear distinction for latency due to treatment (see Figure 4.1B). MAROSI group of rats required more time on average in the decision zone as compared to untreated MA rats. This was maintained throughout all sessions (S1: $t = 4.182$, $p < 0.001$, S2: $t = 6.187$, $p < 0.001$, S3: $t = 2.758$, $p < 0.05$). It is speculated that rats that produced a longer latency might be utilising the area within the decision zone to solve the task. In older rats, treatment with rosiglitazone did not reduce latency to the levels similar to young control rats. As older rats require much more time in the zone, disparity between MAROSI and YACTL was high (S1: $t = 5.21$, $p < 0.001$, S2: $t = 4.487$, $p < 0.010$, S3: $t = 1.254$, $p < 0.015$).

Younger rats require much less time in the decision zone

During fixed-start trials, young rats did not hesitate within the decision zone and was still able to attain a high level of response (see Figure 4.1B). Between the younger rats, the difference between time spent in the decision zone is not statistically significant for all sessions ($p > 0.05$, *ns*). However, in the second session, YAROSI noticeably spent lesser time in the zone as compared to its control group. By comparing YAROSI and MACTL rats, the pattern of behaviour between the best and worst performers for the WPM task was present (see above). With YAROSI (best task performers), time spent in the zone was significantly lower than MACTL (worst performers) for the first two sessions (S1: $t = 7.14$, $p < 0.001$, S2: $t = 4.80$, $p < 0.001$) but not in the last (S3: $t = 0.9$, $p > 0.05$, *ns*).

4.3.3 Learning progression and dormancy within decision zone during MS trials

Unlike fixed-start trials, rats in the MS trials require more than a single association between the escape platform and a constellation of distal cues. The designated escape platform is constant for both fixed-start and MS trials

and it located in one of the arms. Rats were released from either one of the remaining three arms during each trial and were required to find the previously encountered (from fixed-start trials) escape platform. For example, rats could be released from either North, South, or East arm, to find fixed-start and constant escape platform in West arm.

Rosiglitazone could not rescue age-related differences observed in task performance

During MS trials, treatment with rosiglitazone have an effect on task improvement (see Figure 4.2A). Between 2 age groups of control rats, performance was similar and very close to chance level (33 %) for the first two sessions. In this case, an improvement beyond chance was only found in the last session for young rats. Treatment with rosiglitazone improved the rats performance to above chance levels for young rats. This caused a distinct difference in performance level within rosiglitazone treated groups. In the present study, YAROSI rats were able to perform task at a higher level when compared to MAROSI group. Such a disparity not only persisted but increased throughout the sessions (S1: $t = 2.477$, $p < 0.05$, S2: $t = 3.926$, $p < 0.001$, S3: $t = 3.842$, $p < 0.001$).

Middle-aged rats acquired task at similar levels despite treatment

In the more difficult MS trials, rosiglitazone was unable to rescue age-related learning deficits in middle-aged rats as they performed at the same level as MACTL rats throughout all sessions($p > 0.05$, *ns*). Percentage of correct response recorded for these rats were only around chance levels (see Figure 4.2A). In the first two sessions, MAROSI rats performed the task at a rate that was similar to YACTL rats (S1: $t = 1.024$, $p > 0.05$, *ns*, S2: $t = 0.17$, $p > 0.05$, *ns*). Since MAROSI rats could not perform above chance level, YACTL rats acquired the task at a level significantly higher than MAROSI rats in the final session(S3: $t = 6.403$, $p < 0.001$).

Treatment with rosiglitazone enhanced task performance for younger rats

Between young rats, treatment enhanced multiple-start task performance (S1: $p > 0.05$, *ns*, S2: $t = 4.098$, $p < 0.001$). YAROSI rats were able to score above chance level however this level of task acquisition remained throughout all the sessions and did not improve (see Figure 4.2A). The result of this was a difference in treatment between rosiglitazone treated young rats and control in the final session as control rats were able to progress and improve (S3: $t = 2.561$, $p < 0.05$). The distinction in performance levels demonstrated in the fixed-start trials for YAROSI and MACTL rats was also present in these trials (S1: $t = 2.90$, $p < 0.05$, S2: $t = 4.1$, $p < 0.001$, S3: $t = 5.035$, $p < 0.001$). While YAROSI rats were able to perform well above chance level, MACTL struggled to keep up.

4.3.4 Latency in decision zone during multiple-start trials

MS trials required rats to use different strategies to find the escape platform more so than in fixed-start trials. In this case, time spent in decision zone is even more crucial in deciphering the pattern of learning and behavioural

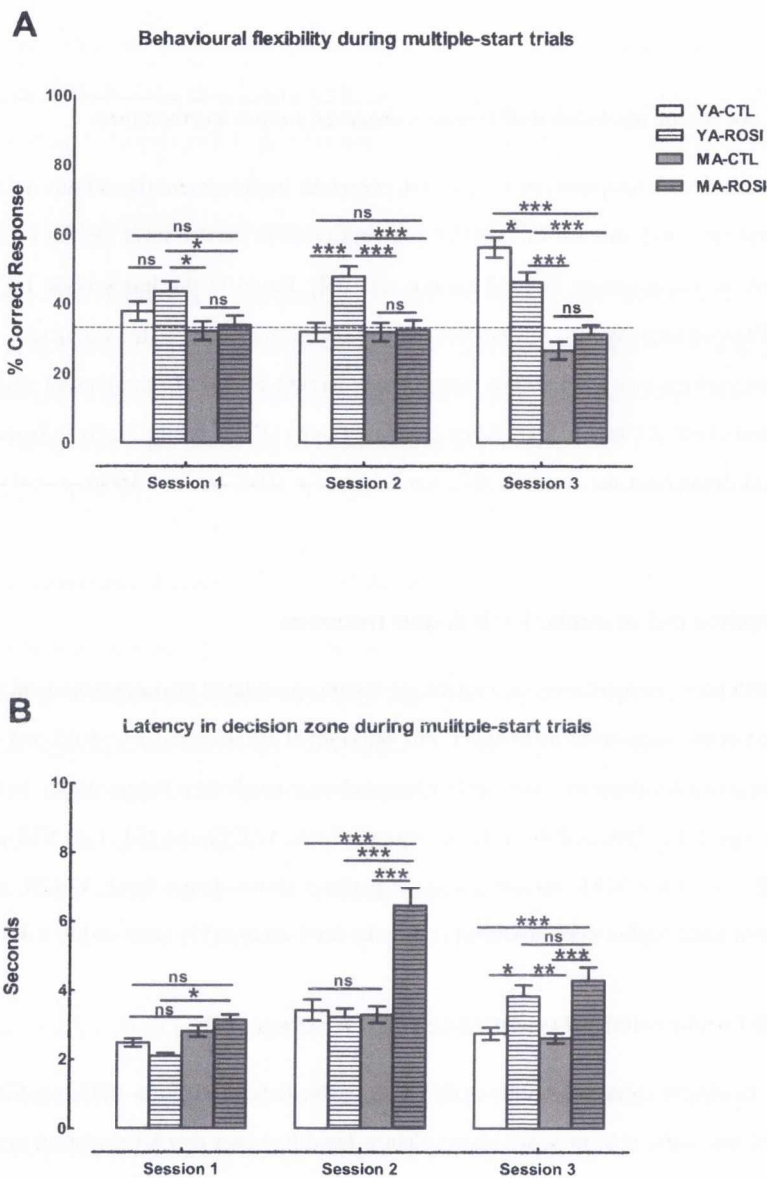


Figure 4.2: Effects of rosiglitazone treatment on learning and dormancy within decision zone during MS trials in the Water Plus Maze task. Each session is composed of 30 trials completed within a single day. Performance level is established by percentage of correct response in finding hidden escape platform. (A) Treatment with rosiglitazone did not improve task performance for all sessions. (B) In the last 2 sessions, MAROSI spent significantly more time in decision zone compared to all other rats. Chance level is determined at 33.33% while all data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

inflexibility that might occur in ageing rats.

Latency in decision zone was particularly longer for treated middle-aged rats

In untreated rats, time spent in decision zone was the same for both age group throughout the sessions (see Figure 4.2B). Rosiglitazone treatment caused an increase in latency for older rats and this resulted in a difference in latency between middle-aged and young adult groups (S1: $t = 2.768$, $p < 0.05$, S2: $t = 8.542$, $p < 0.001$, S3: $t = 1.205$, *ns*). Middle-aged rats required slightly more time in session one although this was not statistically significant ($p > 0.05$, *ns*). In the subsequent sessions, MAROSI spent an augmented amount of time in designated zone which was very distinct from untreated older rats (S2: $t = 8.353$, $p < 0.001$, S3: $t = 4.41$, $p < 0.001$). Comparison between MAROSI and YACTL rats might reflect treatment effect where rosiglitazone in middle-aged rats encourage behaviour similar to control younger rats. This was not revealed in the MS trials where latency for MAROSI grew progressively while MACTL rats spent a relatively the same amount of time in all sessions. Discrepancy for these groups, in terms of latency, was high (S1: $t = 1.75$, $p > 0.05$, *ns*, S2: $t = 8.02$, $p < 0.001$, S3: $t = 4.05$, $p < 0.001$).

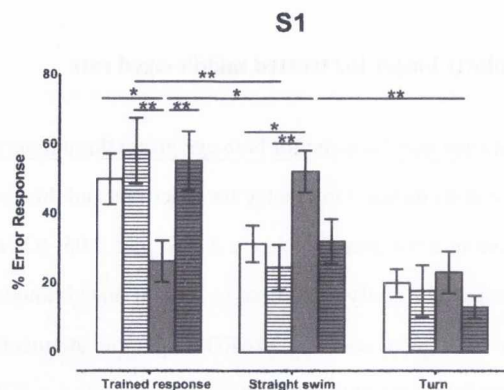
Younger rats required less time in decision zone

A short amount of time was spent in decision zone for young adult rats (see Figure 4.2B). Treated young rats spent more time in decision zone but only in the last session (S3: $t = 2.85$, $p < 0.05$). Previous sessions did not draw out any differences in behaviour among all young adult rats and untreated old rats ($p > 0.05$, *ns*). However, in session 3, YAROSI rats spent a significant amount of time in designated zone. Latency recorded in this instance was higher than those from untreated older rats ($t = 3.21$, $p < 0.01$).

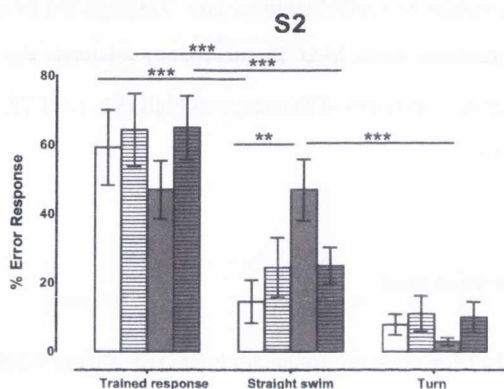
4.3.5 Analysis into type of errors made during multiple-start trials revealed behavioural inflexibility in ageing rats

The WPM task has so far allowed us to provide an in-depth analysis into behaviour during each trials in terms of correct response made and time spent in decision zone. Additionally, the type of errors generated during MS trials might give an insight into behavioural inflexibility in ageing rats. Due to the nature of the task, during MS trials, rats were given an option to find an escape route from one of the 3 available arms. Swim behaviour for each rat can be categorised under a straight swim, trained turn response or a wrong turn response. For example, a trained response involved a rat escaping from South arm to the West (left turn) during MS trials. Importantly, prior to the MS trials, the subject had already been give 30 trials (in 3 separate days) to acquire the task of making a left turn. It was hypothesised that MA rats are unable to provide any flexibility in making different swim response other than the trained version (e.g., left turn).

A



B



C

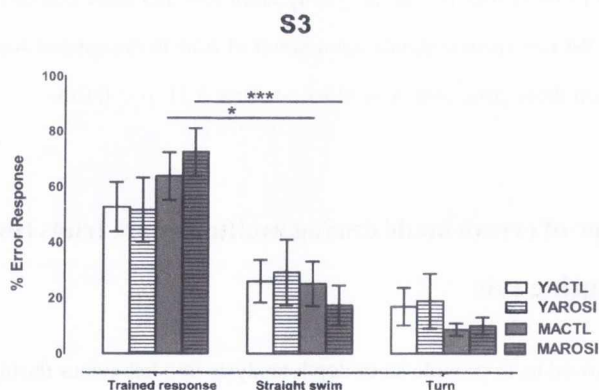


Figure 4.3: Rosiglitazone administration attenuated behavioural inflexibility demonstrated by MA rats during multiple-start trials. MAROSI rats behaved similarly to YA rats as compared to untreated older rats. Trained response refers to behavioural action corresponding to previously trained direction during fixed-start trials (e.g. left turn). A straight error happens when a forward directional swim failed to reach platform. A directional turn different from previously trained response (e.g. right turn) was scored as a turn error (refer to figure 2.3). Both treated and control groups are represented together for each session (A; session 1, B; session 2 and C; session 3). Data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Rosiglitazone administration attenuated behavioural inflexibility demonstrated by middle-aged rats during multiple-start trials

The proportion of variability found in the type of errors made by rosiglitazone treated rats were significantly different from control rats. In the initial session (S1), two way ANOVA revealed significance for type of errors [$F_{(2,108)} = 20.76, p < 0.0001$], interaction between errors and treatment groups [$F_{(6,108)} = 4.21, p < 0.001$] but not for treatment and age factor [$F_{(3,108)} = 0, p = 1, ns$]. Modification of behavioural response with rosiglitazone administration was evident in MAROSI rats. MAROSI made more trained error response as compared to MACTL rats ($t = 3.02, p < 0.01$) (see 4.3A). Notably, this difference in trained error response was not present between YA rats ($t = 0.94, p > 0.05, ns$). There was no differences in terms of preference for straight swim errors ($t = 0.72, p > 0.05, ns$) nor turning error response ($t = 0.22, p > 0.05, ns$) observed for all younger rats.

Within the second session (see 4.3B) ANOVA revealed significance for type of errors [$F_{(2,108)} = 50.08, p < 0.0001$], interaction between errors and treatment groups [$F_{(6,108)} = 2.39, p < 0.05$] but not for treatment and age factor [$F_{(3,108)} = 0.15, p = 0.9, ns$]. Except for MACTL rats, it is evident that trained behaviour was still the most commonly generated errors when comparing all groups of rats. Preference for a straight swim before failing to reach platform was persistent in the second session for control older rats. MACTL rats' inclination to perform a straight swim also differed when compared to YAROSI rats ($t = 3.1, p < 0.01$). There were no differences in the measure of turn error response between all groups of rats. Additionally, trained error responses made by all groups were differ significantly to turn responses ($p < 0.001$) (not shown on graphs).

In the final session, all rats behaved very similarly. ANOVA revealed significance only for the type of errors [$F_{(2,108)} = 33.8, p < 0.0001$] and none for interaction [$F_{(6,108)} = 1.02, p = 0.42$] and treatment and age factor [$F_{(3,108)} = 0, p = 1, ns$]. Although not significant, MACTL rats performed the least amount of turning response ($p > 0.05, ns$) as compared to all other groups. Treatment with rosiglitazone in MA rats resulted in significantly more trained errors when compared to straight swim ($t = 3.77, p < 0.01$) and turning behaviour ($t = 5.17, p < 0.001$). Trained error responses made by all groups were significantly different to turn responses ($p < 0.001$) (not shown on graphs). The majority of errors made at the final stage (S3) was for trained errors. There seemed to be an increase in the number of turn error responses for the YA rats as compared to the MA rats in the final session (between 18 to 20 % of responses were for turning behaviour while MA rats had 10%).

4.4 Locating biochemical changes in selected targets after task completion

4.4.1 Chronic administration of rosiglitazone and its effect on plasma insulin, corticosterone and adiponectin levels

Following the completion of the WPM task, all rats were sacrificed and several different type of analyses were done on samples collected (plasma, hippocampal supernatant and mRNA (see methods). These samples were used for biochemical analysis of selected targets.

To ascertain if rosiglitazone treatment affected stress levels which might in turn affect task performance, plasma levels of corticosterone was analysed. A one way ANOVA demonstrated no statistically significant changes [$F_{(3,36)}=1.59$, $p = 0.2$, *ns*] in the levels of plasma corticosterone among all groups at the end of the task. However, middle-aged rats had slightly elevated levels of plasma corticosterone, though of no statistical significance (see Figure 4.4A).

Changes in basal insulin levels in the plasma was analysed to study the effect of ageing and chronic rosiglitazone treatment (see Figure 4.4B). In untreated rats, plasma insulin levels increase with age (ANOVA, post hoc Newman Keuls, $F_{(3,38)} = 7.98$, $p < 0.001$). Since both YA groups have low insulin level, MACTL rats had significantly higher insulin level as compared to treated YA rats ($p < 0.01$). Importantly, treatment with rosiglitazone appears to reverse age-related increase in plasma insulin. Statistical analysis of this trend revealed that MAROSI has significantly reduced levels of plasma insulin compared to MACTL (ANOVA, post hoc Newman Keuls, $F_{(3,38)} = 7.98$, $p < 0.05$).

Chronic rosiglitazone treatment affected plasma adiponectin levels (see Figure 4.4C). Levels of adiponectin were much lower in untreated rats and both YACTL and MACTL had very similar amount of plasma adiponectin ($p > 0.05$, *ns*). On the other hand, rosiglitazone treatment significantly increased plasma adiponectin levels in both YG and MA groups when compared to their age-matched controls (ANOVA, post hoc Newmann Keuls, $F_{(3,39)} = 70.32$, $p < 0.0001$). Between the treated rats, plasma adiponectin levels were much higher in young adult rats ($p < 0.001$).

4.4.2 Chronic rosiglitazone did not alter mRNA expression levels in several molecular targets

Gene expression changes for mRNA targets of interest were analysed in the hippocampus tissue as illustrated in figure 4.5. In the first instance, pro-inflammatory targets such as IL-1 β (ANOVA, $F_{(3,39)} = 0.28$, $p = 0.84$, *ns*) and MHC II (ANOVA, $F_{(3,39)} = 0.6$, $p = 0.62$, *ns*) did not show any variations in relation to age nor drug treatment (see Figure 4.5A and B). This was also true for anti-inflammatory target, IL-4 (ANOVA, $F_{(3,38)} = 0.31$, $p = 0.82$, *ns*) (see Figure 4.5C). mRNA levels of PPAR- γ remained unchanged in relation to experimental protocol associated with rosiglitazone treatment and age ($p > 0.05$, *ns*) (see Figure 4.5D).

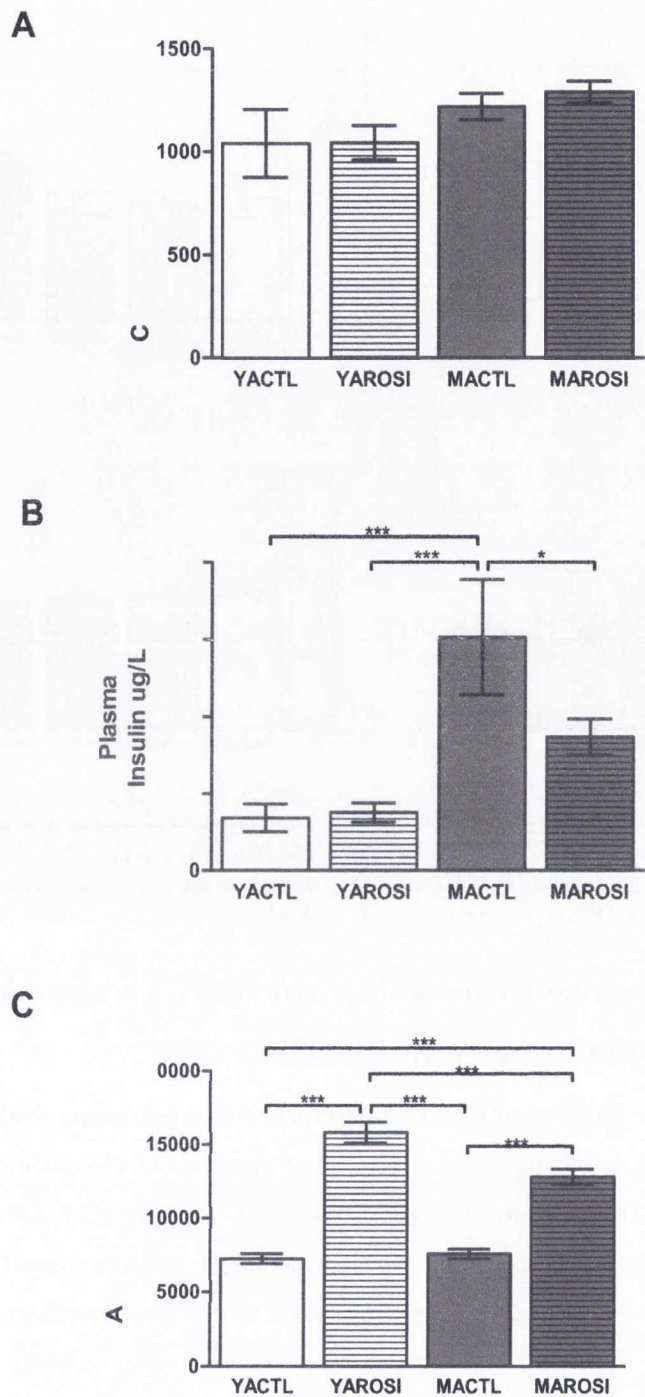


Figure 4.4: Rosiglitazone restored elevated insulin levels in older rats. Treated rats showed elevated adiponectin levels but no changes in plasma targets for corticosterone. Changes in plasma targets (Corticosterone (A), Insulin (B) and Adiponectin (C)) in relation to chronic rosiglitazone administration and age effects (37 days, n=10 per group). Data represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

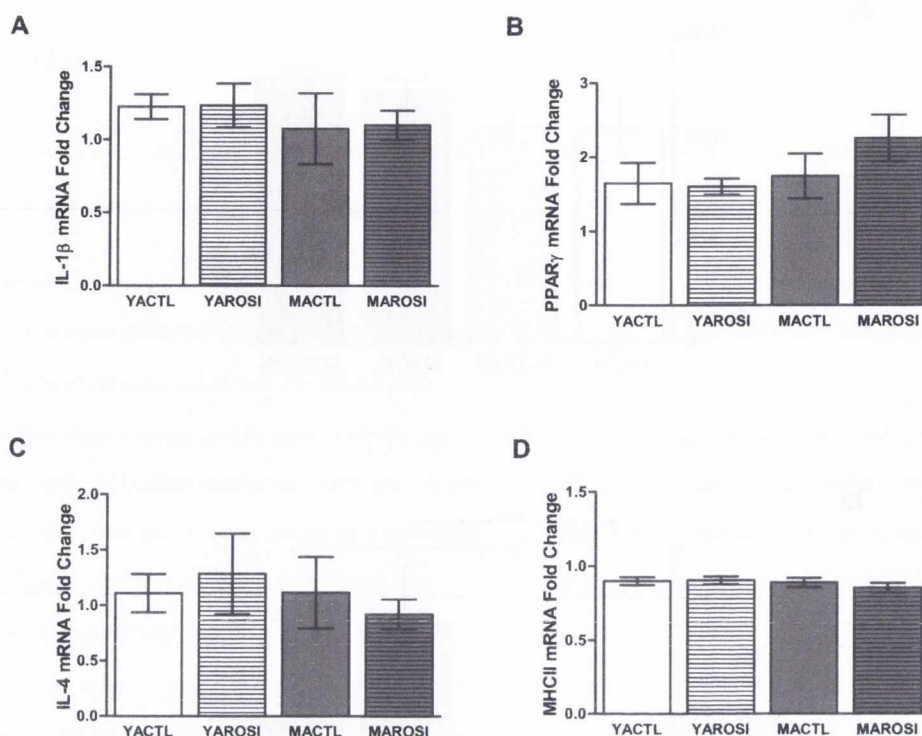


Figure 4.5: **Chronic rosiglitazone did not alter mRNA expression levels in several molecular targets.** Pro-inflammatory levels (A and B) (interleukin-1 β (IL-1 β) and major histocompatibility complex (MHC) II) and anti-inflammatory levels (C) (interleukin-4 (IL-4)) were investigated in the rats' hippocampus together with PPAR- γ (D), (n=10 per group). Data represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4.3 Age-related changes and treatment effect were observed in hippocampal protein expression of glucose transporter-3 (GLUT3)

The sample immunoblot illustrates a decrease in GLUT3 expression with age and an increase with rosiglitazone treatment compared to age match controls. As shown in 4.6, rosiglitazone treatment has an effect on hippocampal protein expression of GLUT 3 as compared to untreated rats (ANOVA, $F_{(3,27)} = 3.2$, $p < 0.05$). Post hoc study demonstrated this difference was only significant in the MA rats ($p < 0.05$) and none between the YA rats. Notably, control rats also differed in GLUT 3 levels within the hippocampus depending on age. MACTL possessed a lower expression of GLUT 3 as compared to younger control rats ($p < 0.05$). This significant difference between age groups was not present in the rosiglitazone treated groups of rats ($p > 0.05$, ns).

4.4.4 Age-related changes in hippocampal interleukin-1 β (IL-1 β) levels

In terms of protein expression of IL-1 β observed in the hippocampus (see 4.6B), there were no prominent changes between treated and untreated rats within the same age group (ANOVA, $F_{(3,36)} = 4.31$, $p < 0.05$). However, there was an increase in IL-1 β protein expression with age which was not attenuated by rosiglitazone treatment. YACTL rats, on the other hand, maintained the lowest expression for IL-1 β and this was subsequently different from MA rats' levels ($p < 0.05$).

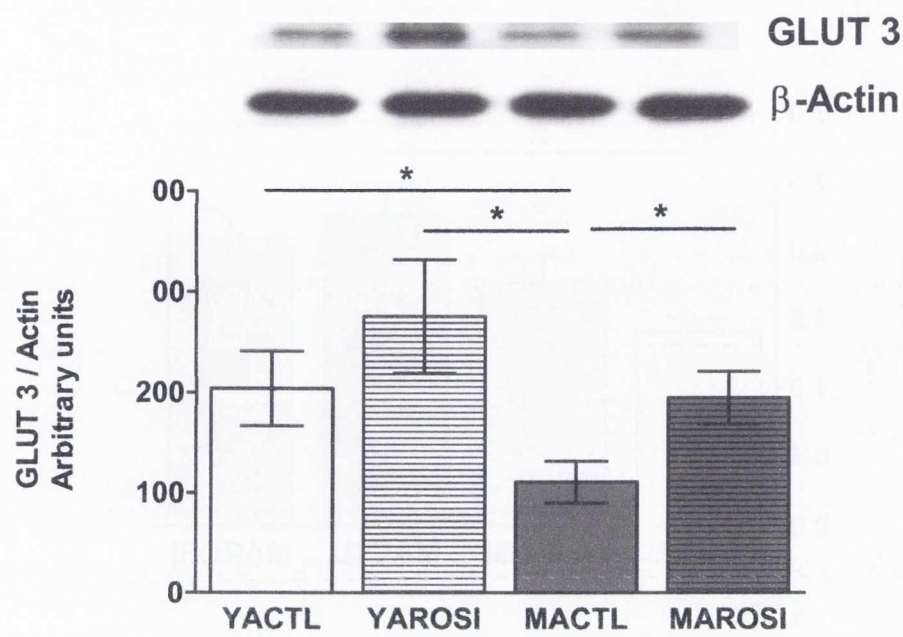


Figure 4.6: Rosiglitazone increased hippocampal in protein expression of glucose transporter (GLUT3) for both ages. Hippocampal GLUT3 protein levels were elevated under rosiglitazone treatment. Data are represented by means ± S.E.M. * $p < 0.05$

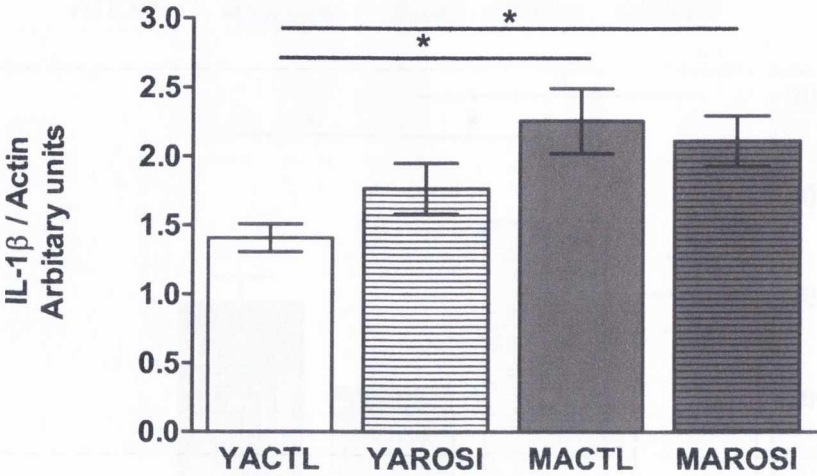


Figure 4.7: **Hippocampal interleukin-1 β (IL-1 β) levels were increased in middle-aged rats.** Rosiglitazone treatment has no significant effect on hippocampal IL-1 β levels. Data are represented by means $\pm$ S.E.M. * p < 0.05

4.5 Discussion

In the current study, an augmentation in mnemonic functions was demonstrated in middle-aged rats which might bear a moderate insulin-resistance background. Task performances during fixed-start trials were significantly improved in both middle-aged and young adult rats under rosiglitazone administration suggesting that cognitive enhancement observed was not only limited to ageing rats. Surprisingly, attenuation of learning deficits in middle-aged rats were not alleviated during MS trials by rosiglitazone treatment. This reflected a direct effect of the task difficulty in MS trials for these rats. In a way, the MS trials functioned similarly to the reversal protocol found in the common Morris Water Maze experiments. This design required the rearrangement of hidden platform and the consequential behavioural condition following such a shift was then investigated since the allocentric spatial relationship of the distal room cues remains stagnant. The current work also focuses on the accuracy of such spatial representations, however, the rats were released from different start arms while locating a previously designated escape platform. The logic behind the use of such a challenging trial was partly due to the need to investigate the learning progression in fixed-start trials. Previous exposure to fixed-start trials allowed rats to associate escape platform using both egocentric and allocentric cues. The more sophisticated MS task demanded more load on memory context leading to a lower accurate response in all rats. Importantly, cognitive requirements of the MS trials were also raised as rats were required to switch from praxis strategy (learning a sequence of movements, the trained response) to taxis strategy (using proximal cues).

Importantly, the diminished ability in ageing rats to complete MS trials was not present during fixed-start trials (although progression is much slower than young adult rats). Learning was stunted since responses for all treated and untreated middle-aged rats were only around chance level for MS trials. Seemingly, when several cues had to be remembered, the older rats had difficulty switching from the retention of a previously learnt cues to the learning of a new one. This phenomenon has also been demonstrated in an odour discrimination task in aged rats (Roman *et al.*, 1996). It could be argued that the difficulty MA rats found in the MS trials is a direct evidence of behavioural inflexibility. Such behavioural inflexibility during MS trials might be a result of impairments in the prefrontal regions affecting executive functions. It has been shown that aged animals have a high preservation at choice points and needs more shaping period as behaviour can be described as rigid, or preservative, or susceptible to interference in older animals (Sarter, 1987).

In a task similar to the MS trials, rats were trained extensively to one task (turn left response) and during a behavioural switch, rats were required to have maintained a “place” strategy rather than a “response” strategy (Rich & Shapiro, 2007). The prelimbic (PL) and infralimbic (IL) regions of the rat PFC were shown to contributed to memory for task switches and perhaps even by modulating activity between memory systems. Our study indicated that MA rats might have frontal lobe and hippocampal dysfunction even in normal ageing. The generalised deficit aged rats possess in solving mazes revealed the importance of the hippocampus for recalling highly specific information and also indicate a possible role for the frontal lobes in learning and retention (Winqur *et al.*, 1990). Although all middle-aged rats performed similarly in the MS trials, the type of errors

analysed revealed that behaviour leading to completing the task was different between the treated and untreated groups. Rosiglitazone administration might alleviate behavioural inflexibility as MAROSI rats generated the same type of errors as YA rats while MACTL rats preferred the straight swim response. It is possible that with increased MS trials, MAROSI rats might improve and acquire the task above chance while untreated MA rat might not be able to.

Other animal models utilising rosiglitazone administration demonstrated similar observations to the study where treatment can modify several physiological components in animals. Zucker fatty rats are helpful in understanding a widely studied model of obesity and insulin resistance (Kurtz *et al.*, 1989). They have also been used to study a wide variety of disorders such as insulin resistance, hypertension and obesity (Kasiske *et al.*, 1992). In these animal models, scientists were able to piece together the *in vivo* mechanisms of insulin sensitisation following rosiglitazone administration. Evidence from such studies have shown that rosiglitazone can normalise glucose tolerance in insulin-resistant animal models, mostly through an upregulation of glucose transporter 4 (GLUT4) protein in adipose tissues (Young *et al.*, 1995; Hofmann *et al.*, 1991). A seven day treatment of rosiglitazone in Zucker rats substantially improves peripheral insulin sensitivity *in vivo* associated with normalising GLUT-4 protein in fat and increasing GLUT-1 protein levels in both fat and skeletal muscle (Kramer *et al.*, 2001). Although activation of PPAR γ receptors seemed to exhibit its primary effects in adipose tissue increasing GLUT1 and 4 proteins, rosiglitazone might have a direct effect in skeletal muscle of the obese animals by increasing GLUT-1 (Kramer *et al.*, 2001). The study was able to demonstrate that rosiglitazone treatment reversed basal plasma insulin abnormalities and increased hippocampal GLUT-3 expression in middle-aged rats. Taken together, these results suggest that rosiglitazone modulates hippocampal circuitry effectively to promote an improvement in cognitive function, possibly via a glucose transporter-3 mechanism (Wang *et al.*, 2011a).

These studies give support to the changes in molecular markers analysed in the present study. Results demonstrated that chronic rosiglitazone treatment increased basal plasma adiponectin, revealing that the peripheral PPAR γ receptor was activated for both young and middle-aged groups. Chronic treatment with rosiglitazone increased plasma adiponectin levels which has been reflected in clinical studies as well. It has also been observed that a high concentrations of adiponectin were associated with a substantially reduced relative risk of T2DM (Spranger *et al.*, 2003). Moreover, T2DM patients on rosiglitazone treatment benefits from an increased in plasma adiponectin levels, possibly mediated through the mechanism involving increased transcription of adiponectin gene through the activation of the transcription factor PPAR γ (Yang *et al.*, 2002).

As a member of the TZD class of drugs, rosiglitazone operates as an insulin sensitiser. In the current study, elevated insulin levels were rescued in treated older rats. This was in accordance with the therapeutic effect rosiglitazone, which acts primarily by increasing insulin sensitivity. A large multicenter clinical trial demonstrated that rosiglitazone is an effective and well tolerated monotherapy for T2DM patients and improved illness through observed reductions (or lack of increase) in plasma immunoreactive insulin, proinsulin, split proinsulin, and C peptide, all of which reflected a decrease in insulin resistance (Lebovitz *et al.*, 2001). Beside insulin resistance, another clinical manifestation for T2DM patients (and other metabolic syndromes) is the low

levels of plasma adiponectin levels (Hotta *et al.*, 2000). Furthermore, reduced levels of neuronal GLUT-3 in the hippocampus of middle-aged rats compared to young adult rats was demonstrated. This was significantly reversed by chronic rosiglitazone treatment in middle-aged rats. The increase in expression of GLUT-3 within the hippocampus coupled with a higher performance level in the WPM tasks suggest that rosiglitazone treatment has an important therapeutic role in ageing and enhancing memory. Therefore, these data indicate that, in a preclinical model at least, both acute and chronic treatment with rosiglitazone can reverse age-related deficits in neurocognitive function. The results also suggest that rosiglitazone may exert its pro-cognitive effects on a more insulin-resistant background, in that improvements in peripheral insulin sensitivity and glucose uptake into neurons (via expression of GLUT-3) may offer a plausible explanation to the mode of action of rosiglitazone in reversing age-related cognitive deficits. Such evidences give support that rosiglitazone administration can normalise effects on glucose regulation and that may be the reason for improved cognitive functions in aged rats.

In aged rats, rosiglitazone-assisted PPAR γ activation could prevent age-related deficits in hippocampal function by restoring contextual fear conditioning (Gemma *et al.*, 2004). However, rosiglitazone's effect on memory improvement might not be receptor-dependent as there was no evidence of modulation in pro-inflammatory cytokines (IL-1 β hippocampal levels) in oxidative functions (expression of protein carbonyl) nor reversal of age-dependent decreases in NMDA receptors (Gemma *et al.*, 2004). Although cognitive benefits demonstrated here are central, peripheral mechanisms might be involved since rosiglitazone does not cross the blood-brain barrier (Gemma *et al.*, 2004). Another line of thought was that rosiglitazone might affect the level and/or activity of factors that readily cross the bloodbrain barrier (Wagstaff & Goa, 2002). Rosiglitazone administration (six weeks) was also able to reverse age-dependent onset of insulin resistance in a transgenic AD mouse model by increasing peripheral insulin sensitivity and restoring insulin response back to wild type levels (Pedersen & Flynn, 2004). In the same transgenic model, the activation of PPAR γ was able to attenuate spatial and reference memory deficits (Pedersen *et al.*, 2006). Better regulation of glucocorticoid levels and improved glucocorticoid responses in cortical and hippocampal regions is speculated to be the underlying mechanism for this particular study. Reduced A42 levels were also demonstrated in this study which is thought to be due increased levels of IDE in the hippocampus. IDE plays a key role in degrading A β and since deficient insulin signalling (decreased phosphatidylinositol 3-kinase (PI3- kinase) subunit P85) was correlated with reduced IDE, it has been suggested that strategies to enhance insulin signalling could be used as an intervention for AD (Zhao *et al.*, 2004a) and perhaps in improving cognitive functions in animals and humans as discussed above. Rosiglitazone and pioglitazone are both under the same prescription drug of the class TZD and are both equally effective in achieving glycemic control in patients with T2DM and dyslipidemia although they appear to have differential effects on lipids. In a rodent model, Pioglitazone not only overcomes cerebrovascular dysfunction and altered neurometabolic coupling in aged APP mice, but also counteracts cerebral oxidative stress, glial activation, and, partly, cholinergic denervation (Nicolakakis *et al.*, 2008).

The speculation that higher brain glucose levels in T2DM patients are responsible for cognitive defects have been demonstrated in experimental diabetes mellitus (DM) rats induced by intraperitoneal injections of

streptozotocin (STZ) (Dou *et al.*, 2005; Popović *et al.*, 2001; Blokland & Jolles, 1993; Shoham *et al.*, 2003). Interestingly, in DM rats impaired long-term potentiation (LTP) has also been observed (Biessels *et al.*, 1996). Such associations suggest that the development of learning deficits might be directly related to the level of glycemic control. As an anti-diabetic drug, rosiglitazone affects insulin resistance and the next step is to explore the relationship between insulin and memory. STZ disrupts glucose utilisation through an insulin-dependent mechanism in the cerebral cortex and hippocampus by reducing activity of several enzymes involved in the glycolytic metabolism of glucose (see (Plaschke & Hoyer, 1993) for details). Another part of the puzzle in understanding how impaired glucose metabolism affects memory is the involvement of insulin receptor (IR). Since IR is abundantly distributed in the brain, insulin levels themselves may affect cognitive functions which has been shown in a rat model of spatial memory (Zhao *et al.*, 1999). Most importantly, there is a very high density of IR in the choroid plexus which may be required for transport of glucose and peripheral insulin across the blood-brain barrier. An upregulation of IR mRNA was found after a series of water maze training, suggesting that IR may play a role in the regulation of synaptic activities (such as neurotransmission and/or synaptic plasticity) during memory formation (Zhao *et al.*, 1999). Only minor memory impairments were observed in a particular study assessing MWM performance in DM rats and this may suggest that the role of insulin/IR in the brain is most likely a modulatory one and activates specific signal transduction cascades affecting memory formation (Dou *et al.*, 2005). Another approach to these results is the theory of compensatory mechanisms for the brain to achieve its functions important for survival, such as activation of other signalling pathways (for details see (Dou *et al.*, 2005).

An interaction between diabetes and ageing has been shown in a rat model whereby learning impairments were significant not only in young diabetic rats, but even further pronounced in aged ones (Kamal *et al.*, 2000). Disturbed hippocampal synaptic plasticity in aged diabetic rats was one of the important finding from this study demonstrated by a failure to induce LTP and a higher depression of the synapses (LTD). Effects of both advancing age and diabetes were prominent in spatial learning deficits. Although the nature for such an adverse memory function is not clear, alterations in oxidative stress, microvascular changes in the brain and disrupted neuronal Ca^{2+} homeostasis has been suggested (Kamal *et al.*, 2000). Within the research group, a restoration of synaptic plasticity in the dentate gyrus of rosiglitazone treated middle-aged rats was demonstrated after a single dose intake, parallel to the enhanced acquisition of learning found in the WPM task. Additionally, the spatial receptive fields of hippocampal CA1 place cells were significantly improved by chronic rosiglitazone administration (Wang *et al.*, 2011a).

Another contribution of rosiglitazone administration might lie in its anti-inflammatory properties. There is a crucial link in which inflammation, a key mechanism of atherogenesis, appears to have particular relevance to diabetic vascular complications, as well as in the development of diabetes itself. It is thought that the activation of these PPARs may also suppress inflammation and atherosclerosis (Libby & Plutzky, 2007). Hence more recently there has been more interest in the anti-inflammatory properties of rosiglitazone in both human and animal experimental models (Stocker *et al.*, 2007; Cuzzocrea *et al.*, 2004; Sanchez-Hidalgo *et al.*, 2005; Mohanty *et al.*, 2004; Liu *et al.*, 2005; Esposito *et al.*, 2006). Cellular anti-inflammatory effect was found

to occur directly before insulin sensitisation or the glucose-lowering effect of rosiglitazone, through a rapid and consistent suppression of intranuclear content of the pro-inflammatory transcription factor NF κ B (Mohanty *et al.*, 2004). Ageing has also been associated with increased production of pro-inflammatory cytokines (Griffin *et al.*, 2006), which in turn impairs LTP (Maher *et al.*, 2005). With rosiglitazone treatment impaired LTP was restored in aged rats hippocampi due to an increase in hippocampal interleukin (IL-4), which in turn down-regulates IL-1 β expression *in vivo* and *in vitro* (Loane *et al.*, 2009). PPAR activators exert these anti-inflammatory actions in different immunological and vascular wall cell types such as monocyte/macrophages, endothelial, epithelial and smooth muscle cells in which PPARs are expressed (Chinetti *et al.*, 2000). In an extensive study involving rosiglitazone and the antagonist of PPAR γ , Bisphenol-A-diglycidyl ether (BADGE), it was established that an activation of PPAR γ reduced the development of acute inflammation and contributed to the anti-inflammatory effects of rosiglitazone (Cuzzocrea *et al.*, 2004). It is plausible that anti-inflammatory properties of rosiglitazone could rescue cognitive deficits in aged individuals. However, there have been numerous reports suggesting that the anti-inflammatory effects of TZDs are PPAR γ independent, suggesting that other off-target effects or mechanisms may be relevant such as a direct effect on the mitochondrial (Feinstein *et al.*, 2005).

4.6 Conclusion

Despite evidences that rosiglitazone improve memory in a population of diabetics, there is currently no animal model in assessing the therapeutic advantages of rosiglitazone on *memory* in DM nor STZ rats. The therapeutic nature of rosiglitazone in insulin resistance state have been demonstrated where elevated level of basal insulin was lowered in MA rats. In summary, rosiglitazone is primarily beneficial in aiding insulin resistance but that might not be its sole purpose. As a part of the TZD class of drugs, it targets the nuclear receptor PPAR γ and have been reported recently to exhibit potent anti-inflammatory and anti-oxidant actions and inhibit both neural excitotoxicity and apoptosis (Griffin *et al.*, 2006; Wolf *et al.*, 2004). Although the results did not show an altered expression level of PPAR γ and changes in cytokine levels, metabolic change in plasma adiponectin levels was present. There is also a possibility that rosiglitazone does not modulate entirely through PPAR γ receptors and might have a more peripheral effect (Wang *et al.*, 2011a) (Refer to Appendix). In the face of all these advantageous effects of rosiglitazone, it is still not know how rosiglitazone may affect memory. It is suggested that the procognitive abilities in rosiglitazone treatment might be linked to the upregulation in GLUT-3 within the hippocampus. Together with the findings in this study, rosiglitazone, might offer a potential treatment option to reverse or halt age-related decline in cognitive function, relevant to a number of neurological diseases.

Chapter 5

Preventive adjunct therapy in normal ageing

5.1 Rationale

Previous studies presented evidences that task performance in the WPM was significantly improved in both donepezil-treated and rosiglitazone-treated rats than in untreated ones. Such a phenomenon was mainly attributed to ageing rats acquiring better strategy outcomes and improved behavioural flexibility under both treatments (see section 4.3.5 and 3.3.6). Nevertheless, it is important to note that therapeutic advantages from donepezil treatment was only demonstrated in the later sessions (day 14 onward) suggesting an initial structural change in plasticity beforehand. The natural progression of the current study is based on the procognitive abilities of both donepezil and rosiglitazone, as discussed in previous chapters. Hence, combination therapy that improves cognition under the influence of both rosiglitazone and donepezil represents an attractive potential treatment for procognitive benefits in an ageing model. It was hypothesised that an adjunct therapy of donepezil and rosiglitazone might alleviate memory deficits found in ageing rats from the onset of treatment and seek to understand the relationship between these two known cognitive enhancing drugs.

Clinical studies have investigated the effects of rosiglitazone in addition to an acetylcholinesterase inhibitor (AChEI) in apolipoprotein (APO) E4-positive subjects with Alzheimer's disease. In this case, adjunct therapy utilising both donepezil and rosiglitazone failed to detect a significant benefit in cognition (Caspi *et al.*, 2003). The present investigations into the combination of the drugs therapies in an ageing rodent model, are seminal into the relationship between a complex networks of both the cholinergic and metabolic system.

With an employment of a spontaneous object recognition (SOR) task, the current experiments deviates from previous studies in terms of the type of memory investigated and the length of treatment. The effect of an acute 5 day treatment utilising donepezil (0.6mg/kg/day; p.o.) in addition to rosiglitazone (6 mg/kg/day; p.o.) is presented in a task exploring recognition memory performed on young (YA; 3-4 months) and middle aged (MA; 13-14 months) rats. The SOR task is a reliable and efficient behavioural paradigm that can succinctly

demonstrate alterations in discrimination strength between healthy ageing rats and younger rats. Each age group was divided into 4 treatment groups with 8 rats in each group [untreated, donepezil, rosiglitazone and combination (rosiglitazone + donepezil)]. Treatment was carried out for 5 days with testing commencing on day 3 and rats sacrificed on day 6, between one to two hours after the last test. Dosing was not received on the last day of testing. Under donepezil-only treatment, rat received 0.3mg/kg/day; (*p.o.*) while rosiglitazone-only treatment consisted of 6mg/kg/day (*p.o.*). A drug suspension (0.2ml/day; *p.o.*) was given for the adjunct therapy. Under the combination group each animal received rosiglitazone (6mg/kg) and donepezil (0.3mg/kg) treatment suspended in maple syrup.

The SOR task revealed MA rats did not learn the task but rosiglitazone-only treatment increased recognition of novel object. YA rats were able to discriminate between objects and learn the task without effort. However, with the combination treatment, these rats failed to identify the novel object to a significant level. Additionally, alterations at the molecular level were investigated, particularly relating to systems of interests. Plasma corticosterone levels were diminished in MA rats while hippocampal ACh levels were increased in YA rats, as a direct effect of donepezil-only treatment. In summary, the combination drug therapy did not rescue memory and learning in the SOR task. The drugs do not complement each other and fail to have a synergistic effect, in fact they seem to inhibit expected normal behaviour from both respective drugs.

5.2 Introduction

The approach took in this study arrived from the clear evidences acquired from both the donepezil and rosiglitazone experiments. The procognitive capacity for these drugs instigated the idea on combining both therapies, manipulating both the glucoregulatory and cholinergic systems. Rosiglitazone, itself, has been utilised in several combination therapies primarily aim to regain metabolic control in subjects with impaired glucoregulation. Currently, there are numerous studies investigating the effects of rosiglitazone in combination with metformin, another anti-diabetic drug associated with increased insulin sensitivity (Kahn *et al.*, 2011; Li *et al.*, 2011; Torres *et al.*, 2011; Smiley & Umpierrez, 2007). The central finding in these adjunctive experiments with rosiglitazone was the effects of the drugs on lipid and glycemic control, usually in T2DM (Ahmed *et al.*, 2011; Lee *et al.*, 2011b; Ceriani *et al.*, 2010). As antihyperglycaemic drugs, both Metformin and thiazolidinediones (the likes of rosiglitazone, troglitazone and pioglitazone), lower blood glucose concentrations in type 2 diabetes without causing overt hypoglycaemia. These drugs act without stimulating insulin secretion but require the presence of insulin to generate their therapeutic effects (Derosa *et al.*, 2009). Additionally, as a PPAR γ agonist, combination therapies with rosiglitazone have been employed to demonstrate anti-inflammatory properties (Wakui *et al.*, 2010; Thal *et al.*, 2011; Wang *et al.*, 2009a). Importantly, neuroprotective effects found in some of these studies are not solely related to PPAR γ -dependent mechanism (Thal *et al.*, 2011; Gemma *et al.*, 2004).

Most of the beneficial characteristics of the rosiglitazone treatment discussed in the previous chapter (see chapter 4) arrived from a chronic application of the drug. However, acute exposure (20 minutes) in cell culture to the rosiglitazone administration significantly improved many of the adverse effects of gluco- and lipotoxic conditions on insulin secretory responsiveness. This gave an insight into the direct action rosiglitazone has on beta-cell viability and function during gluco- or lipotoxicity (Irwin *et al.*, 2011). Furthermore, the cardioprotective nature of rosiglitazone has been demonstrated with an acute therapy. Evidence was given for reduced ischemic injury in a non-diabetic mouse heart which was attenuated by a PPAR γ inhibitor, giving support for PPAR γ activation (Morrison *et al.*, 2011). Another important impact on anti-inflammatory response was demonstrated when an acute pretreatment with rosiglitazone suppressed pro-inflammatory cytokines in a model of nephrotoxicity (Kim *et al.*, 2010). These studies show the superiority rosiglitazone has in anti-inflammatory actions in cellular studies. Hence, acute rosiglitazone administration was an efficacious treatment for glucose dysregulation. It is important to determine if short term therapy with rosiglitazone can affect both memory and glucoregulation in an *in vivo* rodent ageing model.

In terms of exploiting the cholinergic system to gain a further insight into ageing and its accompanying alterations, donepezil was chosen as it is a popular candidate drug in treating cognitive deficits. As an AChEI, donepezil is currently the gold standard therapy in AD. Treatment with donepezil inhibits AChE, an enzyme responsible for the destruction of one neurotransmitter ACh, leading to increased concentrations of ACh in the brain which can rescue cognitive deficits (Kryger *et al.*, 1999). Clinically, donepezil is a forefront runner as a procognitive drug and has been tested vigorously on healthy adults (Ginani *et al.*, 2011; Nathan *et al.*, 2001; Zaninotto *et al.*, 2009). There are several properties of this drug that makes it a profoundly significant drug

treatment option in neurodegenerative diseases (Wang *et al.*, 2009b; Ravina *et al.*, 2005; Krupp *et al.*, 2011). In a recent mouse model of AD, donepezil has been demonstrated to possess neuroprotection by amelioration of neuroinflammation, tau pathology, synaptic loss, and neuronal loss, as well as decreased tau insolubility and phosphorylation (Yoshiyama *et al.*, 2010). Clinically, a link between enhanced neuronal transmission by donepezil and cytokines has been established. In AD patients who are treated with donepezil, there was an increased level of expression of the anti-inflammatory cytokine, IL-4 and a decrease in IL-1 β levels and expression (Gambi *et al.*, 2004). Memantine, as an NMDA-receptor antagonist (Motawaj *et al.*, 2011), is a common addition to any of the AChEIs (donepezil, galantamine or rivastigmine) for the treatment of AD (Patel & Grossberg, 2011). Clinically, this combination of memantine and donepezil significantly improves measures of behaviour and cognition (Cappell *et al.*, 2010). In animals, this adjunctive therapy produced a significantly greater increase in ACh levels in neocortex and hippocampus than giving either drug alone (Ihalainen *et al.*, 2011).

Microdialysis was used to study the effects of acute administration of drugs (donepezil and memantine) on extra-neuronal concentration of different transmitters and their metabolites (Shearman *et al.*, 2006). It was established recently that acute doses of donepezil and memantine alter not one, but a number of neurotransmitters irrespective of whether they are primarily cholinergic or glutamatergic compounds (Shearman *et al.*, 2006). This coincides with clinical studies where positive cognitive effects of acute donepezil can be observed in various cognitive domains including mood (Zaninotto *et al.*, 2009). Another study demonstrated in young healthy males, acute donepezil can improve sustained attention, reaction times, dual-task performance and the executive component of digit span (Ginani *et al.*, 2011). Preventing a failure in both glucoregulation and cholinergic systems proves to be the central link in the study of an adjunct therapy of donepezil and rosiglitazone in a healthy ageing rodent model. Perhaps by improving the glucoregulatory system ACh transmission is enhanced. Animal research has revealed that glucose may improve memory through a facilitation of ACh synthesis and release in the brain (Kopf *et al.*, 2001; Ragozzino *et al.*, 1996; Ragozzino *et al.*, 1998). Importantly, both animal models and studies with T2DM elderly patients have shown that altered glucose regulation impairs learning and memory processes. It appears that poor glucoregulation may exert a deleterious effect by potentiating the neuronal death produced by other pathological processes taking place such as amyloid deposition (Messier & Gagnon, 1996).

In this combination study, the SOR task was employed to investigate a 5 day treatment utilising donepezil (0.3mg/kg/day; p.o.) in addition to rosiglitazone (6 mg/kg/day; p.o.). In studies such as this, a behavioural index is used in this task to test for object recognition based on the natural preference for novel objects displayed by rats. The study of memory and learning using the recognition task began in 1988 based on the differential exploration of familiar and new objects (Ennaceur *et al.*, 1988). The recognition memory task can be briefly broken down to three phases; a comparison between presented items (or events) and previously stored information must be allocated. The fundamental protocol consists of a sample phase (presentation of stimulus), a delay phase (subject to retain information) and a choice phase (comparison against previously stored information). After which, depending on the design of the experiment, responses follow two basic rules, matching or

non- matching (Steckler *et al.*, 1998). The advantage of the SOR task, especially with aged animals, is the lack of reinforcement such as food or electric shock since it is based on spontaneous exploratory behaviour (Lukaszewska & Radulska, 1994). Similarly there is no confoundment in results due to stimulus (or response)-reward associations. This is agreement with the previous study utilising the WPM where the task aimed to be free from motivational rewards.

Unlike tasks that rely solely on spatial cues, such as the MWM, the hippocampus is not thought to be a central component of the SOR task. Several lines of studies have demonstrated that the perirhinal cortex is mainly responsible for the "remembering" in SOR task. However, it has been also been suggested that the hippocampus has a role in recognition memory when such memory involves information that has a spatial component (Brown & Aggleton, 2001). Age-related deficits in SOR task have been previously demonstrated (Bartolini *et al.*, 1996; Pietá Dias *et al.*, 2007; de Lima *et al.*, 2005; Shukitt-Hale *et al.*, 2001). Fundamentally, even though aged rats spent significantly less time exploring objects, age does not affect discrimination ability involved in object recognition. It does, however, impair some component of the mechanism mediating responses to the displacement of a familiar object since they are unable to recall the location of objects over a 30 min delay period even though both the objects and environment are familiar (Liu *et al.*, 2004).

Currently there are several ageing experiments using the SOR task. Most recruit the use of pharmacological intervention to aid cognitive functions associated with ageing. L-deprenyl is a selective irreversible inhibitor of monoamino oxidase B (MAO-B) that displays antioxidant properties shown to be effective in treating symptoms of Parkinson's disease (Marin *et al.*, 1995; Thomas, 2000). In aged rats, have shown reversal of age-related deficit in novel object recognition memory measured 24 hours after training (de Lima *et al.*, 2005). Some recent drug interventions that proved efficient in attenuating aged-related deficits in object recognition tasks are molsidomine (Pitsikas *et al.*, 2005), tumor necrosis factor- synthesis inhibitor,(Baratz *et al.*, 2011), serotonin 5-HT₆R blockade (Da Silva Costa-Aze *et al.*, 2011) and nerve NGF-related proteins (Terry Jr *et al.*, 2011). Notably, some studies have shown that memory involved in object recognition is not affected by ageing (Lukaszewska & Radulska, 1994; Willig *et al.*, 1987). The rational behind these different outcomes, even though the same behavioural paradigms had been employed, could be based on differences in the difficulty imposed on the task, for example, discrimination between objects could be based on several dimensions (for detailed review see (Cavoy & Delacour, 1993)).

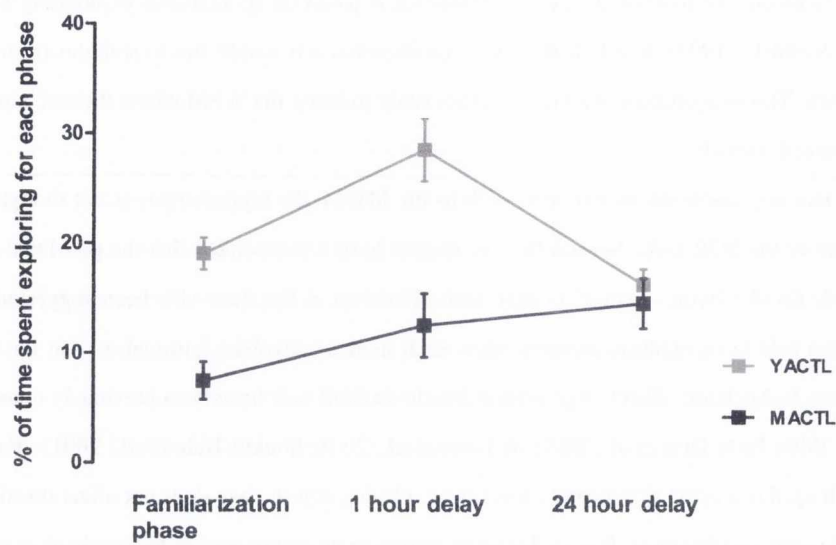


Figure 5.1: **Untreated middle-aged rats spent less time exploring objects during all phases.** The actual exploration span plotted here is the measure of time rats spent interacting with both objects within each phase. The percentage represents a ratio of time spent exploring all objects / entire test phase duration. Grey symbol represents YA rats and black symbol represents MA. Younger rats exploration increased at 1 hour delay but not at 24 hour delay. Data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

5.3 Results for behavioural experiments

5.3.1 Percentage of time rats spent on object exploration

The data for the actual exploration span that is presented here is the measure of interaction the rats have, in total, with both objects during each phase within the arena. This method of comparison enabled an accountability for what rats were doing in each because some rats might not explore and remain stagnant within the arena. The percentage of time spent for the phases between familiarisation and delays trials were different (e.g. 10 minutes for familiarisation while only 3 minutes for delay).

In figure 5.1, an actual exploration span is being investigated as rats might be immobile within the arena. In this case, a measure of seconds alone cannot account for the bigger picture of what the rats are doing within each testing phase. One-way ANOVA for within group measures was used. Tukey’s Multiple Comparison post hoc test revealed further interactions for each age group at different phases. Time spent for MA rats in all three phases are similar [$F_{(2,23)} = 2.398$, $p > 0.05$]. Although not significant, exploration times for MA rats during familiarisation phase (7.5 seconds) increased at the 1 hour delay (12.5seconds) but did not continue to increase at the longer delay (14.5 seconds).

In the younger rats, there was more variance in exploration span [$F_{(2,23)} = 10.05$, $p < 0.0001$]. Post hoc analysis revealed that exploration time during 1 hour delay is much longer than in familiarisation phase ($q =$

4.649, $p < 0.01$) and in 24 hour delay ($q = 6.044$, $p < 0.001$). Two-way ANOVA revealed significance in the age factor [$F_{(1,42)} = 29.66$, $p < 0.0001$], type of phase factor [$F_{(2,42)} = 5.775$, $p < 0.01$] and an interaction as well [$F_{(2,42)} = 5.56$, $p < 0.01$]. Further differences between the age groups for each phases were revealed by Bonferroni posttests. During familiarisation ($t = 3.7$, $p < 0.01$). and one hour delay ($t = 5.15$, $p < 0.001$) trials, young rats spent significantly more time exploring all objects as compared to older rats.

Exploration within the arena was more sustained for untreated YA rats when compared to MA, as shown in figure 5.2. Comparing all treatments within younger rats, two-way ANOVA revealed significance for phase [$F_{(2,84)} = 7.02$, $p < 0.01$] and interaction [$F_{(6,84)} = 2.76$, $p < 0.05$] but not for treatment factor [$F_{(3,84)} = 1.18$, $p = 0.32$, *ns*]. In the familiarisation phase, animal encounters two identical objects for a total of 20 minutes. In this instance, rosiglitazone-treated rats spent a longer time exploring when compared to YADON rats ($t = 2.496$, $p < 0.05$). In the one hour delay, there was no statistical differences between the groups although YAROSI spent the least time exploring. Once again in the final phase, YAROSI spent the most amount of time exploring ($t = 2.87$, $p < 0.05$). Variability in exploration within each YA group of rats was assessed between phases using a one-way ANOVA. Besides the control group, donepezil-treated rats spent significantly more time during familiarisation phase when compared to one hour delay ($q = 3.90$, $p < 0.05$).

Differences in exploration times in the older rats were present at one hour delay but not at 24 hours (see Figure 5.2B). Although not statistically significant, MADON and MAROSI spent more time exploring at the shorter delay. Variability among data for all treatment group is low for MA rats, even among the different phases.

5.3.2 Implications of age measured by object discrimination ratio (ODR)

The object discrimination ratio is a measurement of novel object interaction time as a ratio to the actual exploration span (see section 5.3.1). Chance level is set at 0.5 which means rats spend half the exploration time on the novel object. Recognition memory and ability to discriminate between novel and sample object are considered intact when ODR is above chance.

Two-way ANOVA revealed statistical differences for delays in untreated rats [$F_{(1,28)} = 4.32$, $p < 0.05$] and interaction [$F_{(1,28)} = 9.27$, $p < 0.01$], but not for age [$F_{(2,28)} = 2.71$, $p = 0.11$, *ns*]. At the one hour delay, both age groups of rats were able to discriminate between novel and sample objects and have a preference for the unfamiliar object ($t = 0.989$, $p > 0.05$, *ns*). The older rats were less able to differentiate between novel and sample after a 24 hour delay ($t = 3.32$, $p < 0.01$). In fact, the ability to discriminate dropped significantly (below chance) in the latter delay for MA rats ($t(14) = 3.14$, $p = 0.0072$). YA rats were able to perform above chance level of 0.5 for both delays.

Two-way ANOVA revealed that treatment in YA rats (see Figure 5.4A) significantly affects ODR [$F_{(3,56)} = 7.37$, $p < 0.001$] while the type of phase [$F_{(1,56)} = 3.39$, $p = 0.07$, *ns*] and interaction between them does not [$F_{(3,56)} = 1.63$, $p = 0.19$, *ns*]. Surprisingly, combination therapy did not augment discrimination to a level higher than untreated rats for both delays (1 hour delay : $t = 1.92$, $p > 0.05$, *ns*, 24 hour delay: $t = 1.14$, $p > 0.05$,

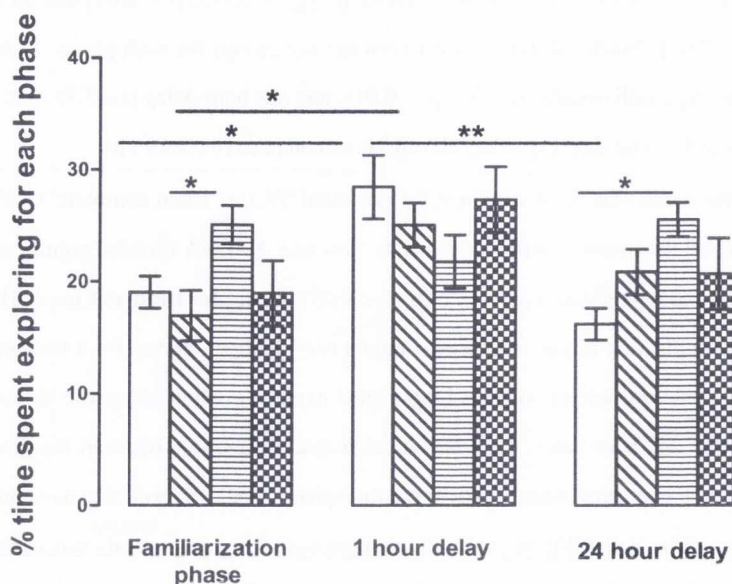
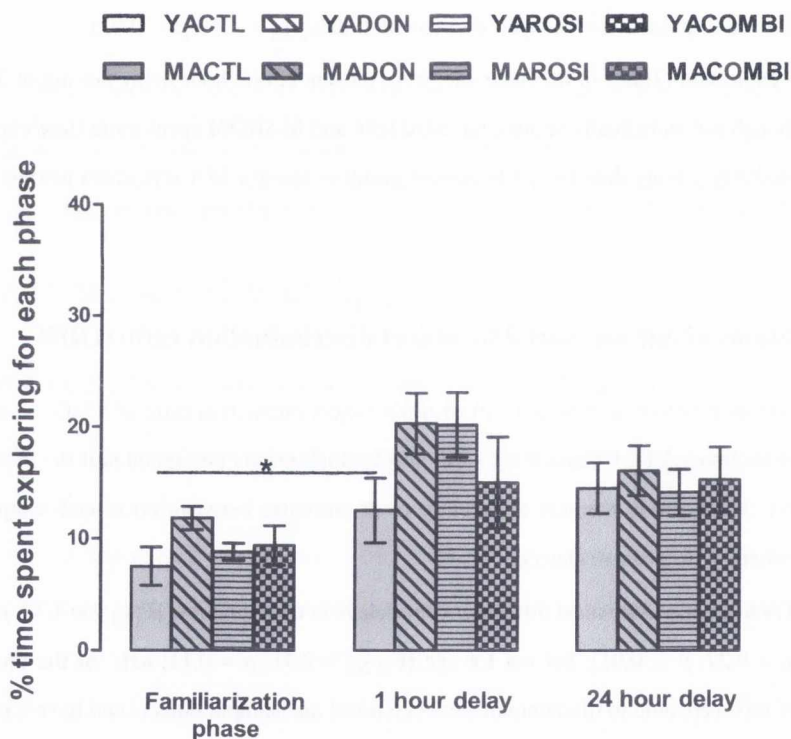
A**B**

Figure 5.2: **Monotherapy increases exploration times in older rats but not combination treatment.** The percentage of actual exploration span gives a direct comparison to how rats behave for each phase. Combination treatment in YA rats did not produce drastic changes in exploration times. At 1 hour delay, MACOMBI rats explore at a rate comparative to untreated older rats. Data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

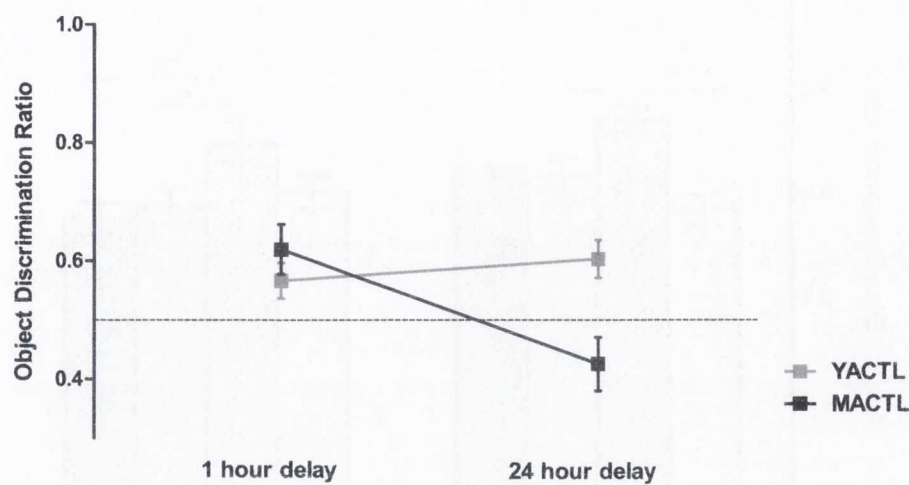


Figure 5.3: **Ability to discriminate between objects decreased for untreated MA rats at longer delay.** The ODR is a ratio of the duration of time spent on the novel object within the actual exploration span. Recognition of object is reflected when ODR is above 0.5 (represented by the dotted line). Between untreated rats, age does not play a role at shorter delay. Data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

ns). Furthermore, rosiglitazone-treated rats failed to perform better than control rats on both occasions (1 hour delay : $t = 1.48, p > 0.05$, *ns*, 24 hour delay: $t = 0.61$, $p > 0.05$, *ns*). Only donepezil treated rats were able to produce a significant change in ODR scores. YADON, with the highest ODR of 0.74, attained a much higher ratio than control ($t = 3.86$, $p < 0.001$) and rosiglitazone-treated YA rats ($t = 2.38$, $p < 0.05$) with the shorter delay. With the longer delay, ODR was still above chance level. In this instance, donepezil treatment augments ODR when compared to YAROSI ($t = 2.68$, $p < 0.05$) and YACOMBI ($t = 3.201$, $p < 0.01$).

In older rats, treatment effect [$F_{(3,56)} = 1.12$, $p = 0.3494$, *ns*] and interaction between delay and treatment on ODR was not significant [$F_{(3,56)} = 1.44$, $p = 0.2408$, *ns*]. Type of delay, however, was significant [$F_{(1,56)} = 16.96$ $p < 0.0001$]. MA rat were less able to separate between novel object and sample at the 24 hour delay. This was particularly so for both control ($t(14) = 3.14$, $p = 0.0072$) and combination-treated rats ($t(14) = 2.34$, $p = 0.0347$). In fact, both groups of rats perform below chance level. Similarly to exploration span, monotherapy of donepezil and rosiglitazone in MA rats were able to increase ODR when compared to control rats.

5.3.3 Preference score gives insight into middle-aged behaviour towards sample object

A reduction in ODR is mainly a result of a lower exploration of novel object. An additive effect to a lowered ODR is an increased exploration of the sample object. Hence, it is crucial to observe the preference score

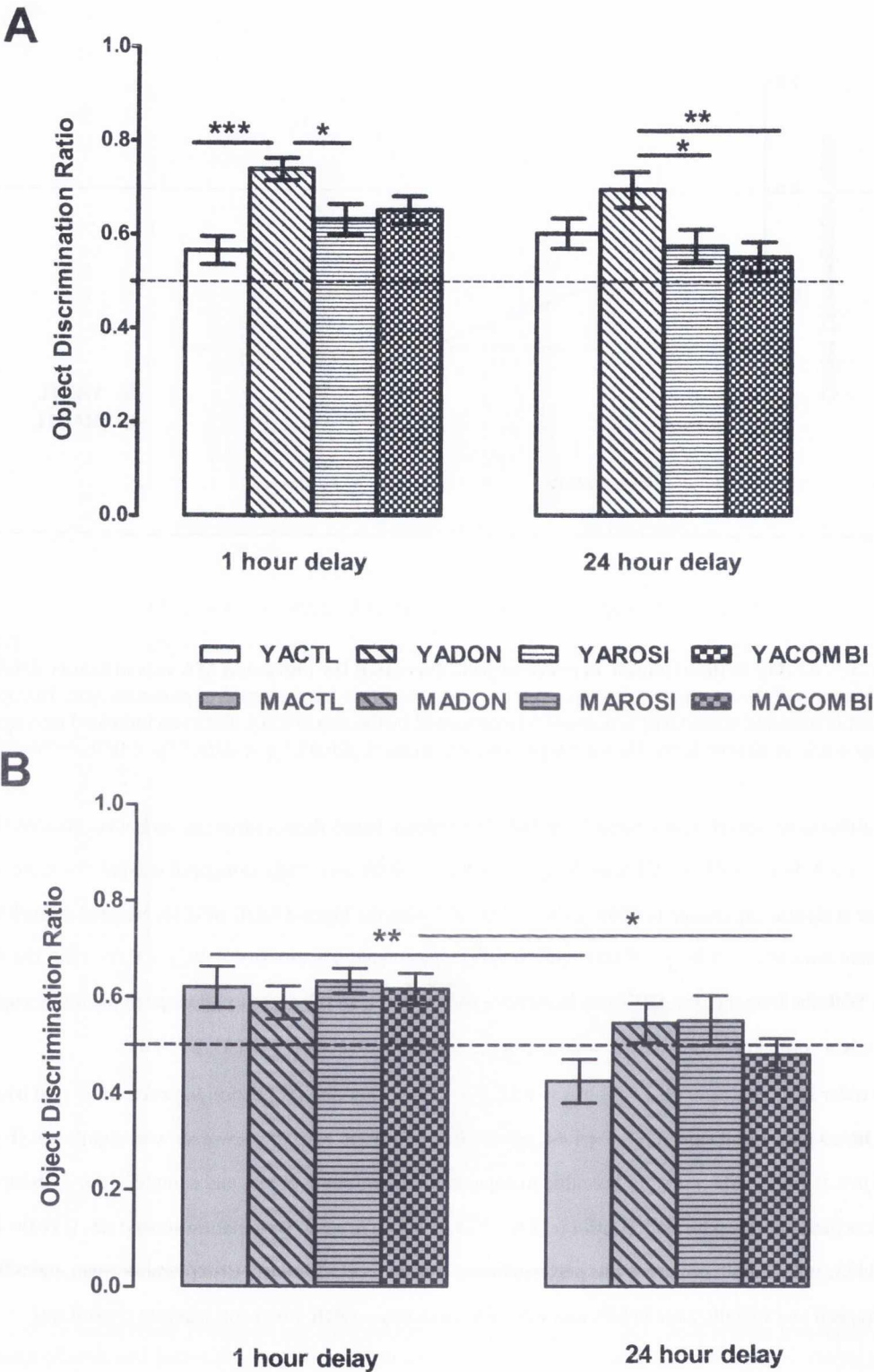


Figure 5.4: At longer delay, monotherapy of rosiglitazone and donepezil in middle-aged rats improve object discrimination ratio above chance. Recognition of an object is reflected when ODR is above 0.5 (represented by the dotted line). Within young treated rats (A), YADON was most able to separate between novel and sample objects for both delays. MA rats were able to discriminate above chance at shorter delay. With the 24 hour delay, monotherapy of rosiglitazone and donepezil both improve ODR to above chance. Data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

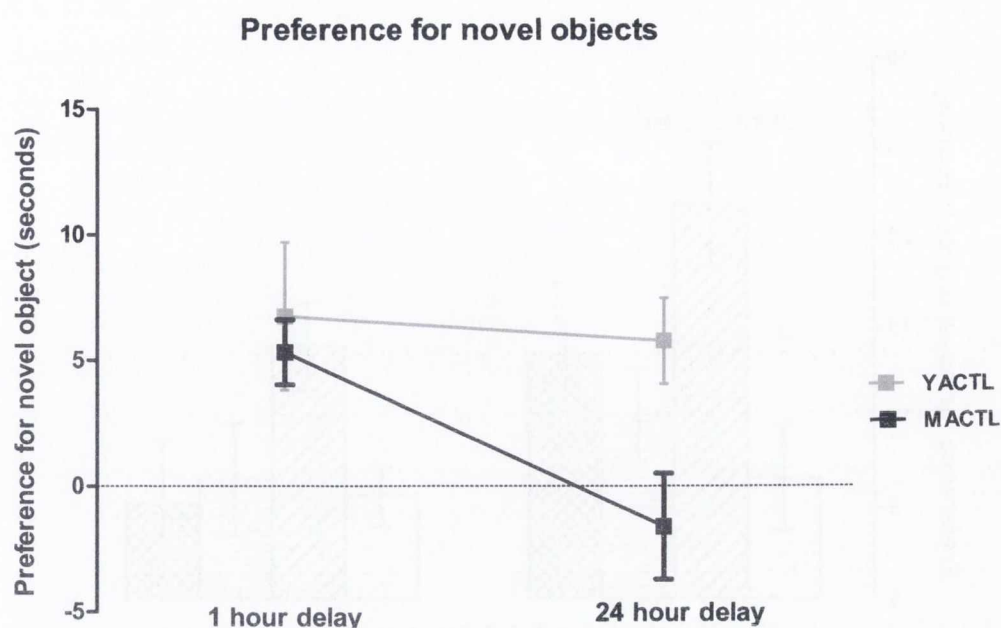


Figure 5.5: **Untreated middle-aged rats were more inclined to explore the sample object at longer delay.** YA rats behaved similarly for both delays and were able to seek the novel object. A 24 hour delay resulted in a preference switch from novel to sample object in MA rats. The grey symbol represents the score for younger rats while the black squares exhibit MA scores. Individual scores are here with mean $\pm$ S.E.M.

(difference in time spent between novel and sample). A negative score represents more time spent on the sample object instead. A score of zero indicates that animal spent an equal amount of time on both the novel and sample objects.

Age played a major factor in the differences presented by the data [$F_{(1,28)} = 4.39, p < 0.05$]. Two-way ANOVA revealed that delay [$F_{(1,28)} = 3.53, p = 0.0707, ns$] and interaction did not [$F_{(1,28)} = 2, p = 0.1683, ns$]. At the longer delay, the majority of MA rats failed to notice the novel object and subsequently acquired a negative preference score which was significantly different to YA rats ($t = 2.48, p < 0.05$).

Inclination to interact with novel object was demonstrated for all YA rats at both delays. Two-way ANOVA revealed statistical differences in treatment and age [$F_{(3,56)} = 6.87, p < 0.05$] but not interaction between them [$F_{(3,56)} = 0.7928, p = 0.5030$]. In the shorter delay, YADON's score was significantly different from control ($t = 3.847, p < 0.001$) and rosiglitazone ($t = 2.958, p < 0.01$). Although not statistically significant, preference for novel object was higher than YACOMBI also ($t = 2.118, p > 0.05, ns$). Exposure to a second novel object after 24 hours also resulted in a high preference for novel object, especially so in YADON rats. Due to the small sample size and high variability among the data statistical significance might be lost. The trend that persists was that donepezil-treated YA rats had a higher novelty seeking preference.

Two-way ANOVA demonstrated differences in types of delay [$F_{(3,56)} = 12.82, p < 0.001$] but not for treatment [$F_{(3,56)} = 1.497, p = 0.2254, ns$] and the interaction between them [$F_{(3,56)} = 0.06441, p = 0.9785, ns$]. Due to large variance between the data, some important differences between treatment groups are lost. However, the main effect of combination treatment in MA can be clearly demonstrated at the 24 hour delay.

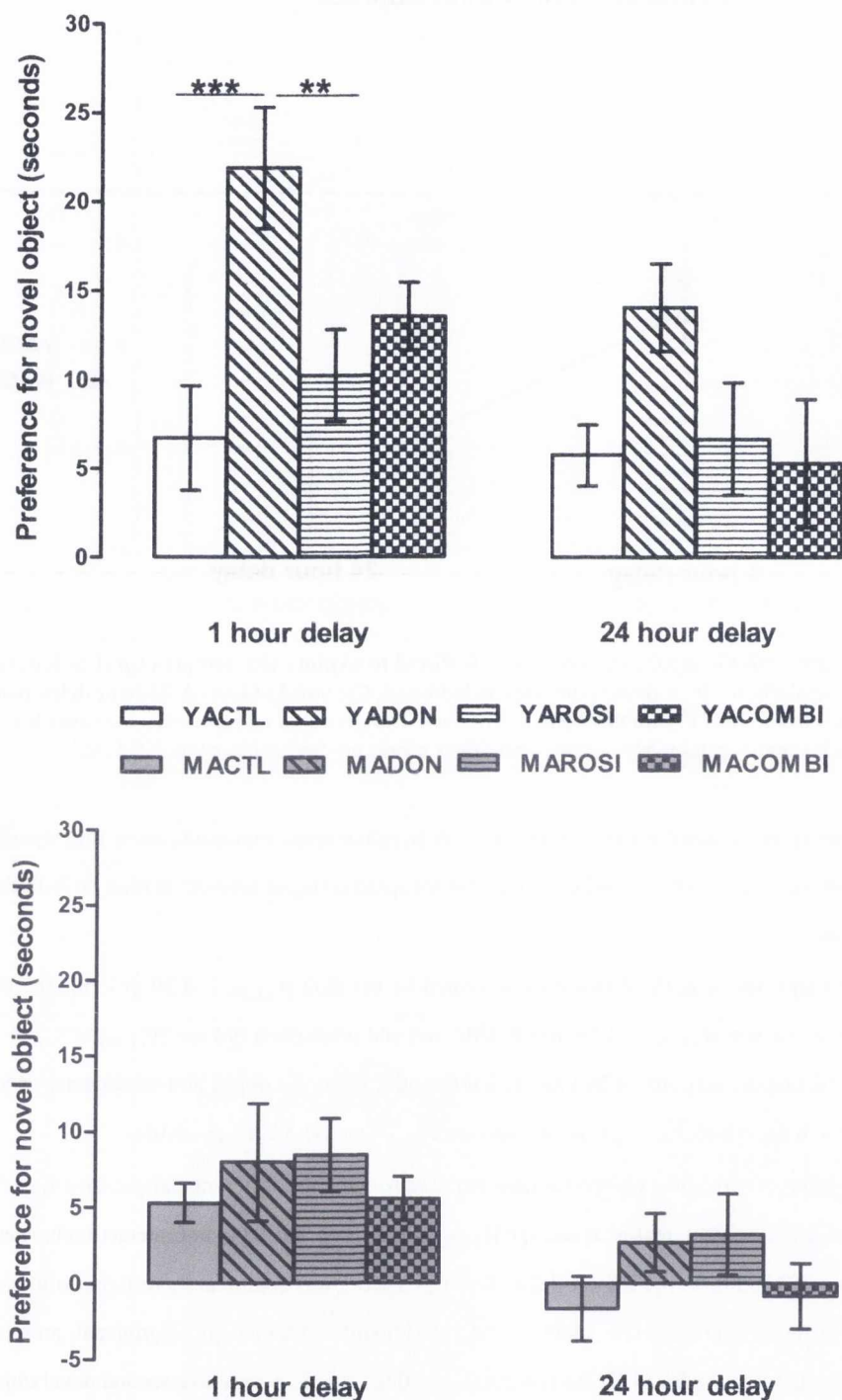


Figure 5.6: **Combination therapy failed to preserve novelty seeking behaviour in middle-aged even at long delay.** All treated YA rats had a higher preference for the novel object. Donepezil-treated rats produced the highest preference score among all YA rats in both delays. Untreated rats and MACOMBI performed similarly at the longer delay and preferred the sample object. Data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

MACOMBI behaved similarly to MACTL rats and had no preference for novel object at 1 hour delay and after 24 hours delay explored sample object more. Treatment with donepezil and rosiglitazone individually ameliorated this effect and produced a positive score.

5.4 Effect of drug treatments in molecular components after 5 days therapy

5.4.1 Neurotrophin levels in hippocampus after short-term treatments

Up-regulation of brain-derived neurotrophic factor (BDNF) in serum has been demonstrated in AD patients undergoing donepezil treatment (Leyhe *et al.*, 2008). Treatments have an effect on hippocampal BDNF levels [One-way ANOVA: $F_{(7,63)} = 7.38$, $p < 0.0001$] (see Figure 5.7 A). Although not significant, it is interesting to note that both donepezil and combination therapies increased BDNF levels compared to untreated and rosiglitazone-treated older group of rats. Donepezil treatment ($M = 972.6$, $SD = 286.8$) and combination therapy ($M = 921.0$, $SD = 57.40$) in MA rats increased BDNF levels as compared to YADON ($M = 594.4$, $SD = 54.50$), YACTL ($M = 520.7$, $SD = 46.28$), YAROSI ($M = 521.6$, $SD = 55.81$) and YACOMBI ($M = 606.1$, $SD = 44.09$) rats.

A short term treatment (15 days) with donepezil has resulted in significant behavioural changes but no alterations in NGF levels in brains of aged rats (Hernandez *et al.*, 2006). The 5 day treatment plan has shown significant changes in NGF levels within the hippocampus (see Figure 5.7 B) where a singular treatment with donepezil demonstrated an elevated hippocampal NGF levels in YA rats ($M = 242.5$, $SD = 8.50$). Since hippocampal NGF levels were the highest in YADON group of rats, this differed significantly from YAROSI ($M = 177.0$, $SD = 10.15$), YACOMBI ($M = 179.9$, $SD = 9.53$) and MACTL ($M = 177.7$, $SD = 17.82$) rats.

5.4.2 Changes in plasma insulin, corticosterone and adiponectin levels after treatments

It has been recently demonstrated that adiponectin is required to mediate rosiglitazone PPAR γ effect on vascular endothelium of diabetic mice (Wong *et al.*, 2011). An adiponectin ELISA was performed on trunk blood to test its levels in YA and MA rats (see Figure 5.8B). A one-way ANOVA gave means to be significantly different [$F_{(7,63)} = 15.94$, $p < 0.0001$]. Monotherapy with donepezil did not change adiponectin levels in YA or MA rats but posthoc tests revealed that rosiglitazone significantly increases plasma adiponectin levels ($p < 0.001$) compared to control groups and donepezil treated groups in both ages. Rosiglitazone-treatment alone produced the most pronounced effects in plasma adiponectin in both ages (mean concentrations of 27651ng/mL in MA rats and 31904ng/mL in YA rats, $n=8$). Notably, this definite effect in adiponectin is lost when rosiglitazone is given together with donepezil (mean difference = 7928ng/mL).

Treatment with donepezil has been demonstrated to reverse the chronic stress-induced decrease in neurogenesis (Kume *et al.*, 2000). In the current study, donepezil relieved elevated plasma corticosterone levels in MA rats only (see Figure 5.8B). A one-way ANOVA gave significance of means [$F_{(7,63)} = 3.142$, $p < 0.01$]. Post hoc test revealed that MACTL rats had significantly higher corticosterone levels compared to YACTL. In MA rats, donepezil significantly reduced corticosterone levels compared to controls ($q = 4.65$, $p < 0.05$). Although not statistically significant, it is important to note that rosiglitazone and the combination therapies

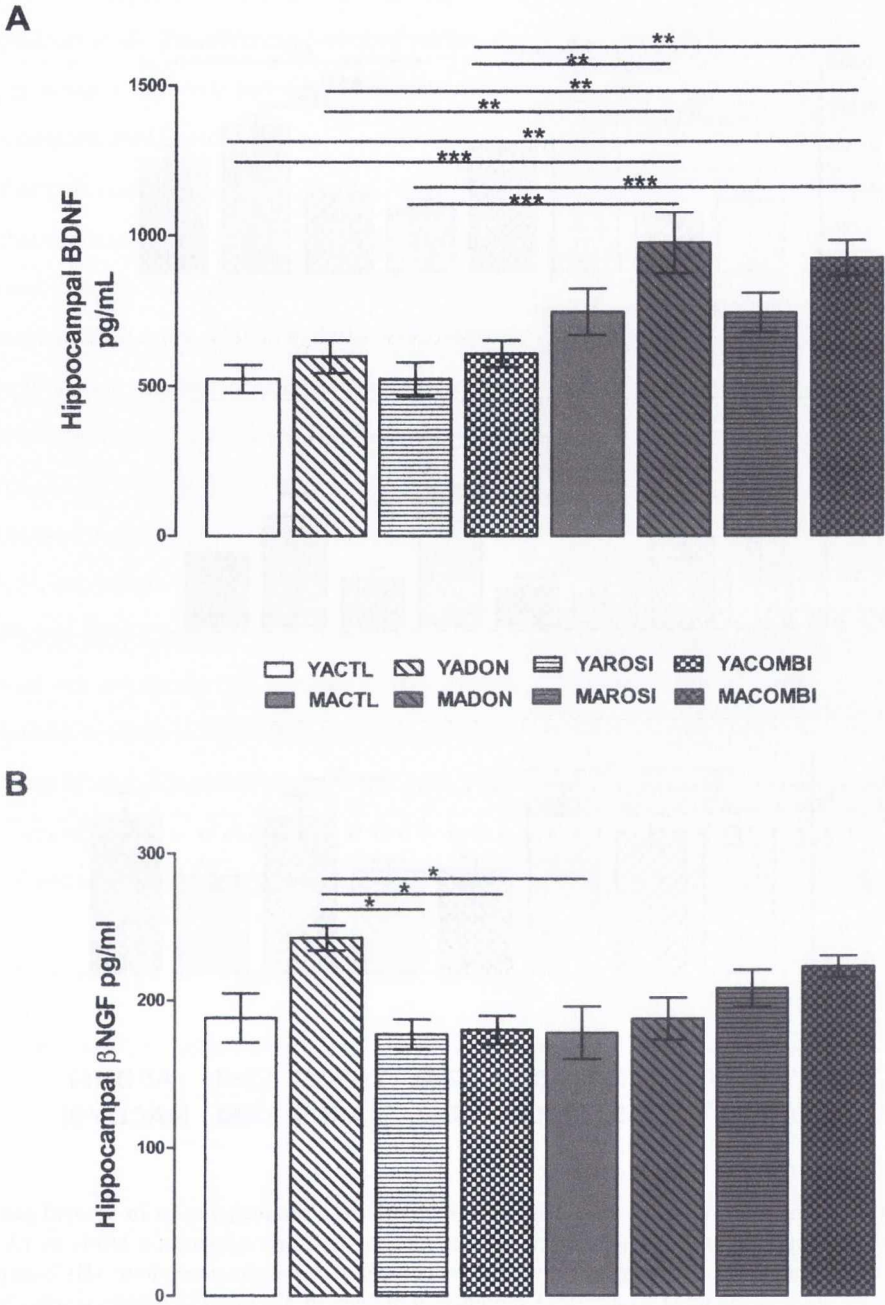


Figure 5.7: **Treatments employed had no effect on hippocampal neurotrophins levels.** There are no differences in BDNF within each age group (see Figure 5.7 A). Alterations in NGF levels were present only in YA rats (see Figure 5.7B). Data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

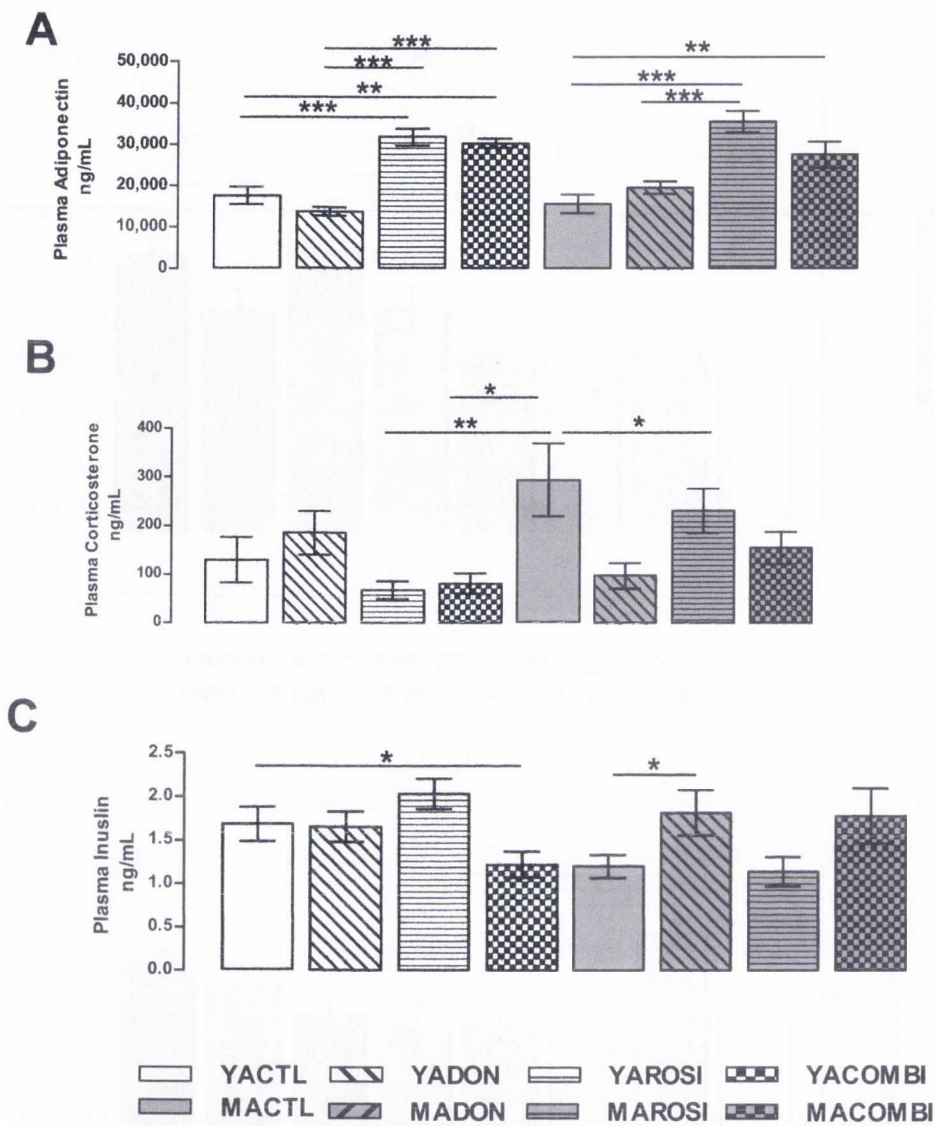


Figure 5.8: **Combination therapy fail to give distinct advantage over monotherapies in several plasma targets.** (A) Both rosiglitazone and combination treatments increased plasma adiponectin levels in YA and MA rats. Although the combination effects were muted when compared to rosiglitazone alone. (B) Donepezil significantly reduces stress levels in MA rats. (C) Combination therapy in YA lowered plasma insulin levels but raised it in MA rats. Data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

reduced corticosterone levels in MA rats. Among YA rats donepezil increased corticosterone levels but not to a significant level. Rosiglitazone decreased plasma corticosterone levels in both YA and MA rats. From the graphs, it seems that combination treatment produced an effect that was an average effect of both rosiglitazone and donepezil used singularly.

Rosiglitazone as an anti-diabetic drug mainly works by affecting basal insulin levels. It is interesting to see that combination therapy in YA lowered plasma insulin levels but raised it in MA rats (see Figure 5.8C). A one-way ANOVA demonstrated that treatments had an effect on plasma insulin levels [$F_{(3,28)} = 6.97, p < 0.05$] although posthoc analysis failed to reveal where these differences are. It seemed to us that MACTL rats have a lower level of plasma insulin compared to YACTL which was attenuated when donepezil treatments (including combination therapy) were given. Plasma insulin in donepezil-treated MA rats were present at a much higher level than MACTL ($t(14) = 2.126, p < 0.05$, one-tailed t-test). Levels of plasma insulin was similar in MACTL and MAROSI rats which was surprising considering rosiglitazone is an anti-diabetic drug ($t(14) = 0.25, p > 0.05$, *ns*, one-tailed t-test).

Plasma insulin was elevated in rats where donepezil was given in conjunction with rosiglitazone. This increase was not significantly different to MACTL levels ($t(14) = 1.713, p > 0.05$, *ns*, one-tailed t-test) and comparable to levels in MADON rats ($t(14) = 0.073, p > 0.05$, *ns*, one-tailed t-test). In YA rats, combination-treatment failed to bring plasma levels up to control levels ($t(14) = 1.9, p < 0.05$, one-tailed t-test). Monotherapy of donepezil and rosiglitazone individually did not demonstrate any large elevation of insulin levels from YACTL rats ($p > 0.05$, *ns*, one-tailed t-test).

5.4.3 Levels of ACh present in hippocampus as an effect of treatments

Acute donepezil treatment can increase ACh in several brain regions (Shearman *et al.*, 2006). In the present study, hippocampal ACh levels can be manipulated by donepezil treatments.

The different treatments employed in this study has a direct effect on hippocampal ACh levels in rats [$F_{(7,63)} = 2.997, p < 0.01$]. Post hoc analysis revealed donepezil treatment significantly increased ACh levels ($t(14) = 4.61, p < 0.001$) in YA rats compared to control. This trend, however, was not revealed in MA rats. The levels of ACh in MA rats does not significantly change between treatment groups but there is a small reduction in ACh when rosiglitazone is given in MA rats. Donepezil-induced levels of ACh was lessen when donepezil is given in conjunction with rosiglitazone in younger rats. On the other hand, the combination treatment in MA seemed to increase ACh levels when compared to rosiglitazone-only treatment.

5.4.4 mRNA expression of AChE, $\alpha 7$, PPAR γ , GLUT3, IL-1 β and IL-4 analysed in hippocampal tissues.

An investigation into the changes in hippocampal mRNA expression was conducted for several molecular targets in relation to the 5 day treatments. However, there are no significant alterations observed in this study. AChE mRNA expression ($[F_{(7,55)} = 1.1246, p > 0.05, ns]$) is increased in MARON whereas in YADON, it was

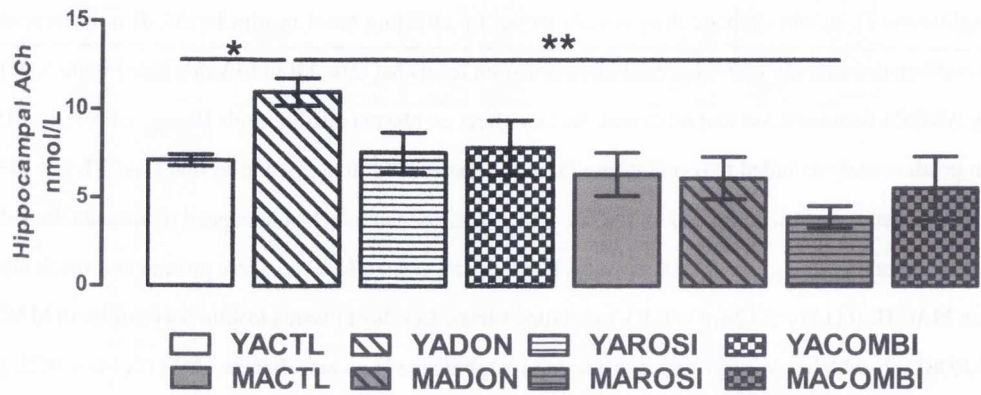


Figure 5.9: **Donepezil increases acetylcholine (ACh) levels in YA rats.** Elevated ACh levels found in monotherapy was attenuated by combination treatment. Data are represented as mean $\pm$ S.E.M. ** $p < 0.01$

found to be decreased. $\alpha 7$ mRNA expression was not influenced by treatments ($[F_{(7,55)} = 0.87, p > 0.05, ns]$). In untreated rats, age-related changes in $\alpha 7$ was not demonstrated. GLUT3 mRNA expression was similar across all groups and treatments ($[F_{(7,55)} = 0.7174, p > 0.05, ns]$) although a slight increase was found in YADON rats. PPAR γ expression in hippocampus was slightly elevated in MA rats but not to a statistical level ($[F_{(7,55)} = 1.246, p > 0.05, ns]$). In the the hippocampus, cytokines (IL-1 β and IL-4) levels were similar across groups and treatments ($p > 0.05, ns$).

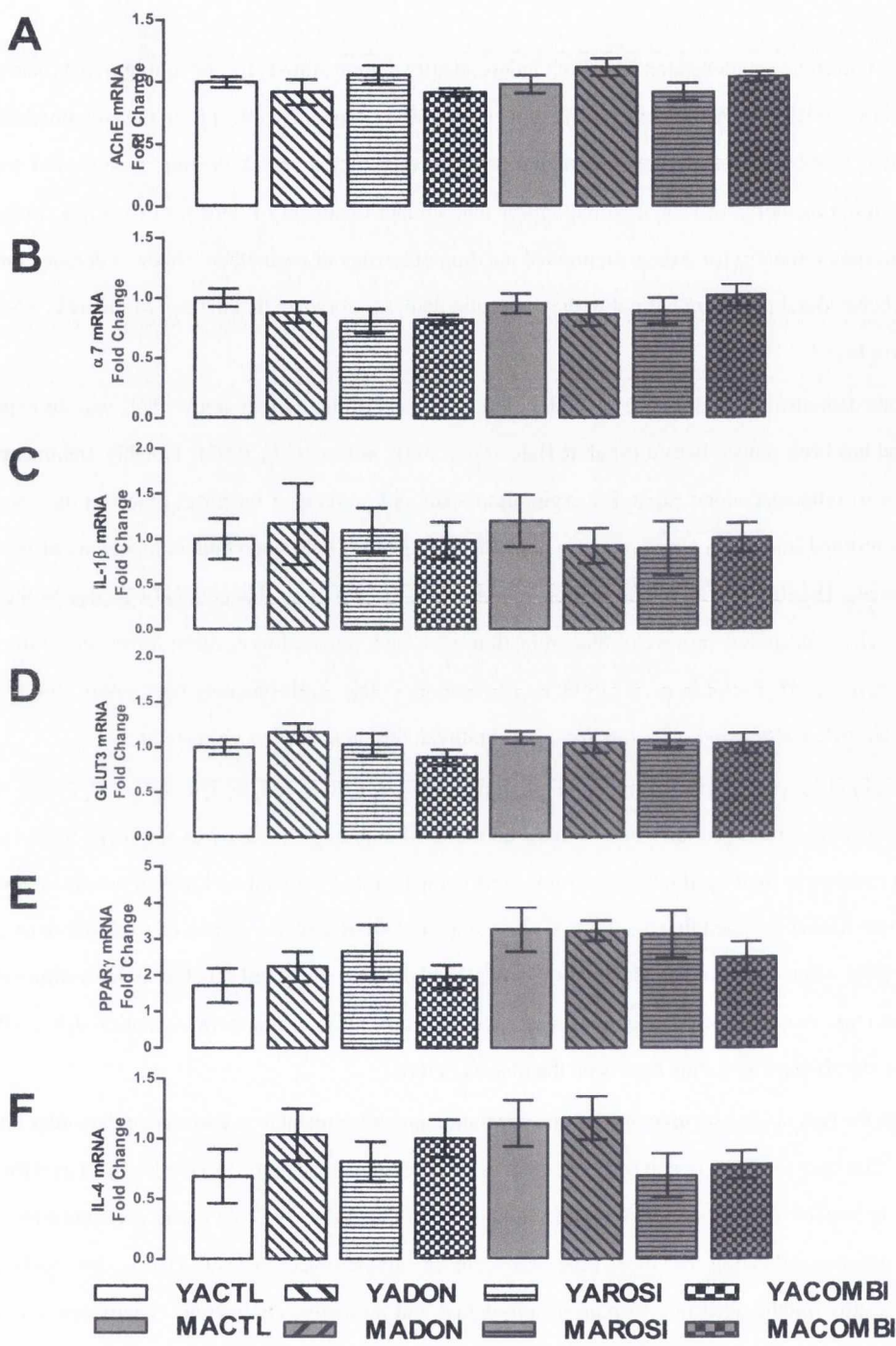


Figure 5.10: **Treatment with rosiglitazone and donepezil does not significantly alter the levels of mRNA in targets analysed.** AChE and $\alpha 7$ mRNA expression did not rely donepezil-treatment effects (A and B). GLUT3 and PPAR γ mRNA expressions (C and D) did not alter after 5 day rosiglitazone treatment. Cytokines levels in hippocampus (IL-1 β and IL-4) did not alter between groups and treatments (E and F). Data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

5.5 Discussion

Although rosiglitazone and donepezil have both independently demonstrated efficacy in improving memory in the behavioural models, no preclinical study has thus far shown a cooperative effect from the co-administration of both drugs. Combination therapy manipulating both glucoregulation and cholinergic systems did not improve recognition memory. In fact, it would appear that adjunct treatment of donepezil (0.3mg/kg) together with rosiglitazone (6mg/kg) for 5 days suppressed the drug efficiency of each other. This was demonstrated in the current behavioural paradigm but not in the molecular analyses completed at the end of the task, where no changes were found.

Older rats demonstrated a decrease in exploration of objects in the current study. This was an expected outcome and has been proven before (Shukitt-Hale *et al.*, 2001; Soffie *et al.*, 1992). Notably, treatment with donepezil or rosiglitazone alone expanded exploration span and preference for novel object at the one hour delay. This resulted in no major differences within MA groups for ODR since both components of the ratio were increased. The dropped in discrimination ratio in MA rats to below chance level was due to the long delay. Age-related decreases in novelty discrimination have been reported to occur at longer delay intervals (Pietá Dias *et al.*, 2007; Bartolini *et al.*, 1996; de Lima *et al.*, 2005). Unfortunately the combination therapy did not rescue such a deficit and older rats were performing at the same level as untreated rats.

Burke and colleagues (2010) manipulated the commonly used SOR task to determine the source of the recognition impairment in aged rats. In this study, an identical-objects procedure was employed to test object recognition memory in their aged rats, allows for direct comparison of exploration times of novel and familiar objects. It was further revealed that aged rats with an impaired ODR also show reduced exploration of a pair of novel objects when tested in the identical-objects procedure as well. Aged rats fail at separating sample objects from representations of other objects they encountered because of a pattern separation deficit (Burke *et al.*, 2010), they behave as if they have seen the objects before.

Although the task did not set up to assess this phenomenon, it is crucial to acknowledge that older rats did not "forget" their previous experience with an object as such but that they approach sample object as if they are novel. This is evident for untreated MA and the combination-treated rats (see 5.6) where preference for novel object was negative indicating that more time was spent on sample object instead. Hence, the combination therapy in healthy middle-aged rats with an insulin-deficit and normative cholinergic system (see 5.8), does not rescue memory deficits demonstrated in SOR task. In YA rats with no glucoregulatory and cholinergic dysfunction, procognitive treatment with rosiglitazone and donepezil, alone, was able to afford an increase in SOR task ability. Once again, the combined treatment failed to elicit such a response as expected (see Figure 5.6 and 5.4). To understand why such a trend exists in a treatment that was aimed at boosting cognitive functions, investigations into several molecular targets that might give an insight into the mode of drug actions in a combined donepezil and rosiglitazone treatments.

Neurotrophins are proteins that are crucial for inducing the survival, development, and function of neurons (Hempstead, 2006). The link between neurotrophins and memory functions has strengthened over the last

decade, especially within the ageing model (Bizon *et al.*, 2004; Siwak-Tapp *et al.*, 2007; Bondolfi *et al.*, 2004; Frielingsdorf *et al.*, 2007; Mattson & Magnus, 2006). In fact, memory deficits associated with aging can be reversed by several members of the neurotrophin family. It has been demonstrated that the reversal effect may be mediated through the activation of multiple neurotrophin receptors associated with cholinergic and possibly non-cholinergic systems in the brain (Fischer *et al.*, 1994). The previous study has demonstrated a chronic donepezil-induced effect on both neurotrophins, BDNF and β NGF levels in the hippocampus. This has instigated the approach in searching for changes in neurotrophin levels within the hippocampus at the end of the SOR tasks. In the current study, BDNF levels within the hippocampus were similar for all ages although a slight increase was revealed in MADON rats. The lack of difference between groups might be due to the short 5 day treatments which might not have been sufficient to elicit a noticeable increase in BDNF levels in the hippocampus. Although we did not explore neurotrophin changes in the previous study utilising rosiglitazone, it has recently been demonstrated that rosiglitazone treatment decreases immunoreactivities of markers for neurogenesis in the mouse and was responsible for the decreased BDNF levels (Lee *et al.*, 2010). Additionally it has been recently suggested that changes in transcriptional expression of BDNF and GDNF may be indirectly affected by the PPAR pathway (Lee *et al.*, 2010). Other studies utilising chronic donepezil treatment have demonstrated a modulation of adult hippocampal neurogenesis (Kotani *et al.*, 2008; Kotani *et al.*, 2006).

On the other hand, it appears that a 5 day treatment with donepezil was sufficient to increase β NGF hippocampal levels in younger rats. Adjunct therapy with rosiglitazone seems to be responsible for suspending increased hippocampal levels of β NGF as combination therapy failed to project similar higher levels of β NGF. In fact, levels of β NGF were comparable between rosiglitazone-only treatment and combination therapy. A similar observation has been demonstrated in canine white adipocytes where rosiglitazone reduced NGF secretion (Ryan *et al.*, 2008), while pro-inflammatory cytokine increased NGF levels suggesting that the neurotrophin is strongly linked to the inflammatory response. On the other hand, donepezil caused a dose-dependent increase in neurite outgrowth induced by NGF (Ginestet *et al.*, 2007). As an acetylcholinesterase, donepezil augments the lifetime of ACh and has been suggested to have neuroplastic activity (Oda *et al.*, 2007). In a rat model of encephalomyelitis, inflammation of the brain and spinal cord, chronic treatment with donepezil restores cognitive performance and ngf mRNA expression (D'Intino *et al.*, 2005). It appears that rosiglitazone, alone or in the combination therapy, is unable to manipulate β NGF levels in the hippocampus. We propose that a 5 day treatment was insufficient for rosiglitazone to affect neurotrophin levels.

Heightening the cholinergic system with an acetylcholinesterase such as donepezil increased hippocampal ACh levels in young adult rats. This result was further supported by the combination therapy. This is not unfounded as donepezil has been demonstrated to increase extracellular ACh levels in the rat cerebral cortex and hippocampal region in a dose-dependent level primarily through AChE inhibition (Kosasa *et al.*, 1999a; Kosasa *et al.*, 1999a). Surprisingly, not only was donepezil unable to elevate ACh levels in middle-aged rats, rosiglitazone-only treatment seemed to decrease it. In patients with diabetes, rosiglitazone appears to improve ACh dose response in terms of vascular functions (Natali *et al.*, 2004; Pistrosch *et al.*, 2004). Within the

CNS, rosiglitazone caused a reduction of microvascular damage and inflammation (Wang *et al.*, 2009a; Sain *et al.*, 2011). PPAR γ activation can affect the cholinergic system directly by its downstream manipulation on AChE. This was demonstrated in a STZ-induced mouse dementia model where treatment with pioglitazone significantly prevented STZ-induced rise in brain AChE activity (Kaur *et al.*, 2009). In the current experiment it could be deduced that rosiglitazone, in a well maintained cholinergic system, can reduce ACh levels in the hippocampus due to its effect on AChE. In terms of mRNA expression, different treatments did not result in any differences between groups. A 5 day treatment plan might not be sufficient to illicit a downstream effect and result in any changes in mRNA expressions that was investigated in the current study.

Although no changes was observed in the hippocampal AChE mRNA expression levels in the current study, it has been demonstrated that its activity in the CNS can be augmented with chronic donepezil treatment (28 days, 2mg/kg s.c) (Zivin & Pregelj, 2008). In the previous study, a chronic 40 day treatment of 0.3mg/kg was able to increase $\alpha 7$ protein levels within the hippocampus of healthy middle-aged rats. However, with a shorter treatment plan, hippocampal $\alpha 7$ mRNA expression levels demonstrated were similar with no differences between groups. The lack of apparent alterations in mRNA levels has also been demonstrated in a study where 4 days treatment with donepezil (10 M) in rat cortical neurons induced the up-regulation of nicotinic receptors without alteration in the mRNA level (Kume *et al.*, 2005). It is possible that donepezil modulates the up-regulation of nicotinic receptors at post-transcriptional levels.

Ageing and inflammation occurs together in the hippocampus where cytokines, such as IL-1 β , increase with age (Lynch & Lynch, 2002). As a pro-inflammatory cytokine IL-1 β has been demonstrated to affect synaptic plasticity by inhibiting hippocampal LTP (Lynch & Lynch, 2002; Hwang *et al.*, 2008). Inflammation, itself, is not the main cause of LTP changes where an imbalance between pro-inflammatory and anti-inflammatory cytokines in the aged brain can contribute to age-related deficits in synaptic function. Increased hippocampal concentration of IL-1 β and IL-1 β -induced signalling, together with decreased IL-4 concentration and IL-4 induced signalling in the aged rat profoundly affects synaptic function (Maher *et al.*, 2005). There is a close relationship between both cytokines where in aged rats an increase in hippocampal IL-1 β is accompanied by a decrease in IL-4, anti-inflammatory cytokine (Loane *et al.*, 2009). Interestingly, under stressful conditions, an increase in IL-1 β can subsequently increase AChE activity resulting in a magnified attack by both inflammation and cholinergic dysfunction (Tagliari *et al.*, 2011). The lack of any discernible changes in both cytokine mRNA expression levels was predominately due to the age of the rats used in the current study. Animals used in the ageing studies mentioned above were around 22-24 months old. It is possible that noticeable changes in inflammation within the CNS are not yet present in middle-aged rats at the age of 13 months.

In terms of the effect of rosiglitazone, alone or with donepezil, we investigated several markers for glucoregulation. To elucidate if a 5 day treatment with rosiglitazone was able to achieve any alterations in managing glucoregulatory systems, we investigated plasma insulin and adiponectin levels. By comparing results between the combination therapy and monotherapies of rosiglitazone and donepezil, we were able to decipher how large an effect does adjunct therapy can administer, if they have an effect at all. In the case of plasma adiponectin, rosiglitazone alone was able to significantly raise levels in both ages. Donepezil, alone, has no

effect on adiponectin as expected and levels were similar to control rats. Rosiglitazone and donepezil, given in conjunction, also raised levels of adiponectin but not at a high level as compared to rosiglitazone-alone treatment. This demonstrates that a combination therapy of this nature seemed to impede both drug actions to a certain level. Rosiglitazone treatment singularly did not affect basal insulin levels severely as they were comparable to untreated rats. Although insulin was slightly raised under the effect of an insulin-sensitising agent. Notably, the profound effects of the combination therapy on rats were unprecedented. Basal insulin levels were lowered in young adult while raised in older rats. Essentially, in a non-fasted young adult rat, plasma insulin level is around 1ng/mL (Stein *et al.*, 1996). By combining treatments, levels of plasma insulin were recovered to a similar level of around 1ng/mL regardless of age, demonstrating no change. We are not certain of the exact mechanism behind such an occurrence but this is the first proof we have that combination therapy has a positive effect at a physiological level

Plasma corticosterone levels were examined to ascertain an effect of stress from the behavioural task employed in the current study. Age-related changes in plasma corticosterone has already been demonstrated where a higher basal level of corticosterone was present in older animals due to age-associated decline of immune responses (Kizaki *et al.*, 2000). Elevated plasma corticosterone levels in middle-aged animals were rescued by all 3 treatments although donepezil-only therapy was the most effective. There is a tight relationship between the cholinergic system and corticosterone production where cholinomimetic drugs increased corticosterone production and secretion (Cattaneo *et al.*, 1993). Additionally, by activating the hypothalamo-pituitary-adrenal axis, plasma corticosterone levels were elevated after donepezil treatment (Umegaki *et al.*, 2009). Although the effect of glucocorticoids on memory has been controversial (Roozendaal, 2002), we demonstrated that elevated plasma corticosterone levels in middle-aged rats resulted in below chance performance in the SOR task. In younger animals, it appears that rosiglitazone lowered plasma corticosterone levels in both rosiglitazone-only and the combination treatment. This result is consistent with a recent study where rosiglitazone was demonstrated to have an anti-depressive effect in a murine model through the decline of plasma corticosterone (Eissa Ahmed *et al.*, 2009).

Further investigations revolving around rosiglitazone and its pharmacological properties include GLUT-3 and PPAR γ mRNA expression levels within the hippocampus. Although the current study did not reveal any apparent differences between groups for both targets, previous study (Wang *et al.*, 2011a) determined that rosiglitazone actions were mediated through GLUT-3 regulation where up-regulation of GLUT-3 protein levels were demonstrated in rosiglitazone-treated animals. Additionally, rosiglitazone was also capable of increasing in GLUT-1 and GLUT-4 protein levels in adipose tissue (Kramer *et al.*, 2001). Similar to prior studies on rosiglitazone administration, PPAR γ mRNA expression was not affected after a 5 day treatment with rosiglitazone nor combination therapy. Evidence that PPAR γ activation is clear as adiponectin and PPAR nuclear receptor signaling pathways have already been demonstrated (Chinetti *et al.*, 2000) and changes in adiponectin levels in this study reflects PPAR γ activation. Recent studies are still in support of rosiglitazone and its ability to alleviate inflammation through PPAR γ activation (Wang *et al.*, 2011b; Sayan-Ozacmak *et al.*, 2011)

5.6 Conclusion

Neurochemical alterations occurring in the brain during ageing is unavoidable which led us to correcting glucoregulation and enhancing cholinergic transmission. It might be crucial that the main aim in procognitive therapy in ageing is to restore the complex interplay between acetylcholine and several other neurotransmitters systems, such as dopamine, serotonin, noradrenaline, or glutamate, that are likely involved in the progressive deterioration of several cognitive functions such as attention, memory, and learning (Bogaert & Schrier, 2011). In the current study, an investigation into different molecular targets gave an insight into the pharmacological intervention and benefits of a combination therapy manipulating both cholinergic and glucoregulatory systems. Combination therapy was unable to attenuate age-related learning and memory deficits explored in a SOR task. It appears that both drugs impede each other's procognitive benefits as explored in previous studies. Molecular analyses revealed a similar trend in neurotrophin and plasma insulin levels. It is important to mention at this point that all targets were primarily exploring changes within the hippocampus. Recent studies have supported the notion that solving the SOR task involves an overlapping networks of brain structures for components of both spatial and recognition memory (Vann & Albasser, 2011). We are aware that it would not be scientifically fair to concentrate solely on alterations within the hippocampus. The primary reason behind this motive was for the continuation in comparison among the chapters (see chapter 6). It is likely that further experiments based on tissue samples from the perirhinal cortex might be more relevant to the task involved. Nevertheless, results from the current study still holds its strength as 1) object recognition memory is not a distinct entity in itself and 2) spatial components of the task was not deliberately eliminated and the involvement of the hippocampus cannot be ruled out.

Chapter 6

Comparative analyses between tasks and treatments

6.1 Rationale

In this chapter, a comparison was done between the two behavioural paradigms employed in previous studies. In order to elucidate the effects of pharmacological manipulations on both the glucoregulatory and cholinergic systems, an understanding of the memory components underlying the two tasks undertaken in the ageing studies, the WPM and the SOR. The WPM task was primarily made up of two separate and distinct trials fixed-start and MS this was aimed at identifying task solving abilities relating to age-related memory deficits. As mentioned previously, the current experimental design was built upon the foundations laid by the more commonly used MWM. The age-related deficits observed in the MWM task were conclusive. The impairments in spatial learning in aged rats were mainly due to the slower acquisition of the spatial cues (Gallagher & Nicolle, 1993; Frick *et al.*, 1995; Lindner, 1997; Brandeis *et al.*, 1989). Notably, these deficits in the spatial task are not attributable to general motivation or to sensorimotor deficits. The type of memory responsible for acquiring the MWM task has been famously linked to spatial memory since the task was first described more than 20 years ago (Morris, 1984). Although variations in methodical details are present, the MWM task has been and is being used by research groups in many different applications, giving us an insight into spatial learning and memory (D'Hooze *et al.*, 2001). It has been suggested that the MWM is well suited to the study of selective visuospatial factors in place learning and working memory, but less suitable for repeated measures or for assessment of long-term memory deficits (Hodges, 1996). The task may have disadvantages where immersion in water might induce undetermined stress effects (Morris, 1984; Heyser *et al.*, 1995). However, it has been argued that stress would be minimal when water was maintained at a reasonable temperature (Sandi *et al.*, 1997).

The investigation into adjunctive therapy with both donepezil and rosiglitazone was accomplished using the SOR task (see chapter 5 on page 95). The origins of the recognition task was based on the differential exploration of familiar and new objects (Ennaceur *et al.*, 1988) where recognition is considered a judgement

of the prior occurrence. The advantage of employing the task, especially with aged animals, is the lack of reinforcement such as food or electric shock since it is based on spontaneous exploratory behaviour (Lukaszewska & Radulska, 1994). It is the current view that the hippocampus is involved in object recognition memory and that object recognition processes of parahippocampal structures (e.g., perirhinal cortex) are sufficient to support object recognition memory over short retention intervals (Hammond *et al.*, 2004). An age-related decline in recognition memory has demonstrated functional specialisation of structures within the medial temporal lobe memory system, as well as the independence of age-related deficits in different cognitive domains, particularly impairments in perirhinal cortex function (Burke *et al.*, 2010). Our analysis of recognition memory under pharmacological manipulations revealed that a combination therapy with donepezil and rosiglitazone failed to boost memory functions in middle-aged animals, although separate and singular treatments of donepezil and rosiglitazone does. Such a finding was not expected and analyses of molecular changes revealed that both drugs inhibited each others ability to provide procognitive aid during ageing.

In an attempt to bridge cognitive alterations observed in an ageing rodent model and neuromodulatory changes, work was committed to investigate the alterations present in several neurobiochemical targets with ageing rats. The ageing process is relentless and prone to diseases. Thus, the intricate relationship between physiological aging and pathological aging must also be considered. Well demonstrated changes in biological systems due to ageing have been established in several human and animal models. In normal ageing, there are reduced concentrations of some neurotransmitters (Gottfries, 1990). The dopaminergic system, for example, besides having a central role in efficient motor functioning (Freed & Yamamoto, 1985), has also been, critically implicated in many higher-order cognitive abilities (Bäckman *et al.*, 2006). In humans, neuroimaging studies have documented an age-related decline in striatal dopamine transporters as a marker of dopaminergic neurodegeneration (Innis *et al.*, 2002). Increased dopaminergic varicosities was demonstrated in the rat cortex which correlates to higher cognitive abilities in ageing animals (Allard *et al.*, 2010a). The cholinergic system is another well studied area in the gerontology field where the cholinergic hypothesis of geriatric memory dysfunction proposed that functional disturbances in cholinergic activity occur in the brains of healthy older adults and demented patients and that these disturbances play a role in memory loss and related cognitive problems (Bartus *et al.*, 1982). It has been recently proposed that the cholinergic changes that occur with ageing is caused by the change of functional circuitry where compensation will recruit the cholinergic system and associated cortical areas to maintain good performance on a task in ageing adults leading to an increase in frontal activation for older adults (Dumas & Newhouse, 2011). Besides dynamic changes in the neurotransmitter system, metabolic functions can undergo drastic alterations as well resulting in cognitive impairments. One such example is the strong relationship between type 2 diabetes mellitus and cognitive impairments (Huber, 2008; Strachan *et al.*, 2008; Abbatecola *et al.*, 2010). This comparative chapter is directed at rationalising changes demonstrated in several molecular targets pertaining to both the cholinergic and glucoregulatory systems. Both chronic (40 day; rosiglitazone and donepezil) and short-term (5 day; rosiglitazone, donepezil and combination) dosing demonstrated downstream effects in the targets analysed. Close comparisons among the targets examined might give insight into the difference between treatment and drugs mediation for the neuromodulatory

changes accompanying healthy ageing.

6.2 Introduction

It is difficult for an animal model to incorporate the complexity of human ageing and the cognitive alterations that accompanies it. Ageing, an unavoidable ongoing process, encompasses a varying degree of changes. Studies into ageing and memory are generally focused on neurodegeneration and pathological changes (Mattson & Magnus, 2006; Semsei, 2000; Lu *et al.*, 2004; Hedden & Gabrieli, 2004). It was aimed to understanding ageing by studying alterations in a fundamentally normal rodent model before severe age-related impairment sets in. The purpose of using middle-aged animals is to ascertain if cognitive functions were intact prior to severe ageing and, more importantly, if procognitive therapies were able to prevent consequential damage to memory due to advancing age.

Experimental behavioural work on ageing includes several constraints that must be made clear before commencing the study. In an ageing model, the accuracy of age for subjects used is fundamental. There is a significant amount of variation in how different laboratories define the categories of aged, middle-age and adults for their animal populations. In an extensive review, McCutcheon and Marinelli went through a systematic review of published articles on LTP and behaviour studies using Morris water maze. They highlighted a problem when neuroscientists might have overlooked age as a critical experimental variable (McCutcheon & Marinelli, 2009). The two problems that arose under such circumstance were; firstly, animals used to model adulthood might not have reached the suitable age, and secondly, the difficulty in defining older ages of adulthood, middle age, aged and senescent. The stages of rodent maturation is crucial for the basis of the research because of the changes that occur in rats preceding severe and acute ageing.

Understandably, the most common method a laboratory uses to gauge an age category is employing a weight range assumption together with the birth date of the animals used. Despite this common practice, a comparison of weights and ages from websites of two major vendors showed that there is considerable variability between vendors and even between different colonies from the one single vendor (McCutcheon & Marinelli, 2009). Hence, it is imperative to highlight that the weight of the animal can only be an approximate marker of age. Furthermore, standardized procedures and criteria are required to provide consistent intra-laboratory values and reduce inter-laboratory differences in sexual maturation observations. Specifically, the day birth occurred was designated day one postpartum (PD 1, day 1 of lactation). Sexual maturation becomes apparent at the onset of puberty and is usually assessed by the presence of balanopreputial separation (in male rodents; PD 45 to 48) or vaginal opening (in female rodents; PD 32 to 34) at the postnatal age (Lewis *et al.*, 2002).

In the comprehensive review mentioned above, 60% of the studies defined young animals incorrectly as 'adults' and astonishingly 5% did not even specify the age of the experimental subjects used. Accuracy of age is crucial because developmental changes can be sudden and the different neuromodulatory systems matures at a varying degree for rodents. Neural circuits mature during transition to adulthood. For example, dopamine D2 receptor activation were observed in slices from adult (postnatal day [PD] > 50) but not preadolescent (PD < 36) animals. Notably, neuromodulatory changes do occur at a late time period (after PD 50) and developmental processes might not have matured as commonly believed (Tseng & O'Donnell, 2007) which might impact

the experimental outcomes. Thus, it is the researchers' responsibility to ascertain the age and maturity of the animals used before the onset of any experiments, as this is fundamental in exploring age-related changes in any animal models.

More than two decades ago, Martin Sarter (Sarter, 1987) investigated differences in cognitive abilities between young and aged animals by analysing several behavioural paradigms. When exploring age-related behavioural research there are several non-cognitive issues that may discount the power of the study. Motivation, for example, might differ between a young, inquisitive animal from a senescent one. Prominent physicality differences can lead to varying skills and effort required for an aged animal (which may be 40-80% heavier) than its younger counterpart. There are several factors that come into play when considering the completion of a behavioural task. Aged animals have high inter-individual variability, require more time to complete a task, high preservation at choice points and needs more shaping period. Speed-dependent responses are one of the fundamental problems in older rats as behaviour can be rigid, or perservative, or susceptible to interference. It has been demonstrated that an aged animal show reduced resistance to extinction and have less behavioural rigidity compared to young animals (Sarter & Markowitsch, 1983). Additionally, rodents that go through normal ageing do not show neurodegenerative diseases such as AD, unless they are genetically modified to show such pathology (Oddo *et al.*, 2003). Normal ageing models give scientists the opportunity to compare the trajectory of changes in the brain that lead to impaired or preserved memory in terms of ageing.

An important connection, i.e., a negative correlation, has been made between ageing and MWM performance. The impairments seem to manifest themselves early in place training in the MWM task by a profound lack of acquisition of spatial information (Rapp *et al.*, 1987). However, with a prolonged exposure to the maze, these aged animals were able to eventually learn the task. Young animals, unlike aged rats, were able to navigate using spatial cues. Another hypothesis for the poor performance might be due to an age-related deficit in the retention of information important for task performance across days (Rapp *et al.*, 1987). Although aged animals are known to be spatially impaired, it is important to account for individual variability which is another parameter in behaviour studies that mirrors ageing humans (Rapp & Amaral, 1992). This occurrence was shown when a subset of the aged animals performed as well as young rats while the remaining rats performed poorly, outside the range of young rats (Robitsek *et al.*, 2008). It has been suggested that there are different ageing progressions even within the same strain of animals. In an extensive experiment encompassing 36 trials over 71 weeks, a high variability in the learning curves was observed in 24 month old rats. This demonstrated that ageing in the Wistar strain of rats was found not to be a homogeneous process but can be differentiated on the basis of individual processes. This could be due to differences in viability between shipments and the possible occurrence of genetic drift (van der Staay & Staay, 1997). Critically, performance during initial acquisition seems to be more sensitive to individual differences than later trials where the performance of less severely impaired aged rats has approached the levels of performance within the range of the younger rats (Lindner, 1997).

Several measures of behavioural outcomes can determine the severity of the ageing process for the MWM task. A proximity measure can assess the search error on training trials and also determines the spatial accuracy

during the search for platform during probe trials. This measure of accuracy in spatial memory demonstrated that aged rats acquire spatial bias more slowly than young rats. Additionally, a learning index score was composed by Gallagher providing a graded measure of spatial learning suitable for correlation analyses with other behavioural data (Gallagher *et al.*, 1993). In this study in particular, age-impaired group of animal performed poorly in place training but they were good at the cued version of the task, suggesting an impairment only in the spatial component of the task. It is important to point out that unimpaired-aged groups of animals perform similarly to young animals in terms of path length and escape latencies (Gallagher *et al.*, 1993).

The application of mazes in a task is standard in exploring changes in spatial memory. Beyond the traditional use of the MWM, there are several other versions of mazes that probe for spatial learning and memory performance in ageing rats. Maze tasks can differ in a few qualities, i.e. types of apparatus (MWM, plus shaped maze, T-maze radial maze, complex maze), presence of distal or local visuospatial cues, associative sensory cues, complexity of the environment, and motivation (aversive escape, encountering novel object, food or water reward) (Hodges, 1996). In a recent experiment assessing remote spatial memory, a novel maze was used in which a "village" scenario was used for the assessment of spatial memory when subjects are trained to find specific reward locations (Winocur *et al.*, 2008). Testing remote spatial memory needs to be confined to two primary requisites; firstly, adequate experience should be given to grant formation of memory representations to facilitate recall after long intervals. Next, subjects should be denied contact with the environment after primary experience until memory testing stage. Two groups of old rats are used for this elegant design; one cohort of rats were raised from three months in the environment context while the other is naive to the same environment. Old rats reared in the first group exhibited preserved remote memory for learned spatial locations in that environment and were substantially better than old rats tested for the first time. The study revealed that old rats were capable of forming initial episodic memories and once established, the information gained is integrated with pre-existing knowledge to form less detailed semantic or schematic memories (Winocur *et al.*, 2008).

Additionally, both short-term and long-term memory can be assessed within a maze structure. Efficiency of patrolling the maze in terms of number of reentries into arms can be assessed for short-term memory, while long-term memory includes reintroducing the animal back in to the maze after a period of time intervals (Sarter, 1987). Twenty five years ago, Ingram demonstrated that aged mice are impaired in a Stone maze (a multiple T-maze with restricted availability of extreme cues) task due to higher perservation errors at choice points in the maze (Ingram, 1985). One of the striking features of Stone maze performance is the tendency to demonstrate an alternation strategy in solving the maze (Ingram, 1988). The RAM task was first devised as an eight-arm maze in a paradigm in 1976 (Olton & Samuelson, 1976). Animals have choice alternatives presented sequentially and the animal is faced with only one subgroup (usually a pair) of the entire set of alternatives at any given time in order to solve the task. The common response described in this paper suggested that rats were predisposed not to repeat their choices in this situation, presumably reflecting the tendency toward spontaneous alternation. Acquisition of the RAM, particularly those with a subset of rewarded arms to dissociate reference and working-memory components, can be a lengthy process (Hodges, 1996). Although the RAM is a spatial

task, the memory system tapped relates to working memory which involves the retention of trial specific or trial unique information for short periods (generally up to 30 seconds) (Hodges, 1996). A modified version of the RAM was recently present in a study whereby a combination of the MWM and RAM is used to measure spatial learning as a function of age in young and old rats by assessing reference and working memory errors simultaneously (Shukitt-Hale *et al.*, 2004). The RAWM, has a few advantages over its two original design. The use of proximal strategy is eliminated since the animal is forced to swim in either the central open area or one of the arms, there is no chance co-incidence of bumping into the platform unlike in the MWM and motivational reinforcement is not required unlike the RAM. Young rats in this study were able to learn the maze more quickly and thus solving the task with a shorter latency. Aged rats showed deficits in the rate of acquisition of the task by committing more errors while finding and remembering the platform location which could be due to the non-spatial strategies utilized by them. Additionally, older rats made more reference memory errors (entries into an arm that did not contain the platform) and more working memory errors (reentries into an arm)(Shukitt-Hale *et al.*, 2004).

According to the Oxford English dictionary, recognition is an identification of a thing or person from previous encounters or knowledge. The memory expected from recognition requires a capacity for both identification and judgement of the prior occurrence of what has been identified (Mandler, 1980). In humans, the impairment in object recognition is related to age is clear where older subjects were not only slower but also less accurate at matching objects at varying viewpoints induced by increasing angular disparity between objects (Pilz *et al.*, 2011). Human imaging studies have revealed that there are qualitatively different populations of neuronal activity involved because these occur in different locations and also have different properties, reflecting the likelihood of using recollection or familiarity to distinguish target items (Brown & Aggleton, 2001). There are several conflicting evidences from animal studies on which systems are responsible for recognition memory. Memory impairment arising from lesions in the perirhinal cortex has resulted in significant evidences that perirhinal cortex is critically involved in object- or stimulus-recognition memory (Albasser *et al.*, 2011). Furthermore, it has been demonstrated that the transient blockade of AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid) receptor-mediated synaptic transmission within the perirhinal cortex disrupted encoding for short- and long-term memory, as well as retrieval and consolidation (Winters & Bussey, 2005). Additionally, rats with bilateral perirhinal lesions were able to perform a delayed non-matching-to-position task for short intervals suggesting that perirhinal cortex is involved in maintaining representations of trial-specific information over time (Wiig & Burwell, 1998). Severe impairment in object-in-place memory was demonstrated in monkeys that underwent disconnection surgery (unilateral perirhinal cortex ablation in one hemisphere, unilateral fornixfimbria transection in the contra-lateral hemisphere, and partial forebrain commissurotomy)(Gaffan & Parker, 1996). This supports the view that in episodic memory, the perirhinal cortex provides input of visual object information to the subiculum, hippocampus, and fornix. On the other hand, differential neuronal activation determined by immunohistochemistry revealed that the hippocampus is importantly involved in processing information essential to recognition memory concerning the relative familiarity of arrangements of items, as needed for episodic memory of scenes, whereas the perirhinal cortex processes such

information for individual items (Wan *et al.*, 1999). More specifically, hippocampal damage impairs memory for contextual or spatial aspects of an experience, whereas memory for objects that were part of the same experience are left relatively intact (Mumby *et al.*, 2002). It would appear that a system centering on the perirhinal cortex is concerned with discriminating the familiarity and recency of occurrence of individual stimulus items, whereas a system centring on the hippocampus is concerned with judging the prior occurrence of constellations of stimuli (Brown & Aggleton, 2001).

Individual differences in cognitive decline, corresponding to the human ageing process are pivotal in understanding the course of ageing. Strong associations between test scores in young and middle-aged adults, contrasting with increasing disassociations between test scores in older groups, provide evidence that most individuals' biological ageing affects all parts of the brain, and thus, all mental functions to a similar extent (Rabbitt & Lowe, 2000). Frontostriatal changes such as decreases in dopamine, noradrenaline and serotonin, and declines in the volume and function of the prefrontal cortex are a consequence of normal neurocognitive processes that are related to ageing (Hedden & Gabrieli, 2004). It has been suggested that a higher-order systems biology of the brain can be significantly altered by normal ageing in the absence of disease where separate brain regions that interact to subserve higher-order cognitive functions show less-coordinated activation with ageing. Neuroimaging revealed a compensatory response where neural activity becomes less integrated and and less localised and aged individuals who exhibit delocalized activity show better cognitive performance (Bishop *et al.*, 2010).

It is equally critical to connect behavioural findings to physiological alterations underlying basic molecular mechanisms of ageing. For example, studies examining the septohippocampal cholinergic system in behaviorally-characterized rodents have shown that there is a decline in many markers for these cholinergic neurons that coincides with severity of spatial learning impairment (Gallagher & Nicolle, 1993). Understanding age-related changes in both behavioural and neurobiological measures can help distinguish between those changes that are functionally detrimental and those that may represent compensatory adaptations to maintain cognitive function. Our primary interests in the neurobiological mechanism of ageing have led us to investigate both the presence of cholinergic dysfunction and conservation of glucoregulatory system in normal middle-aged rats under drug manipulation.

Pharmaceutical intervention by means of manipulating cholinergic innervation is a common route in treating cognitive impairments. The physiological reason is the existence of high-density cholinergic innervations in both the cortex and hippocampus which originate from several regions in the basal forebrain (Lysakowski *et al.*, 1989; Rye *et al.*, 1984). Donepezil is a specifically designed piperidine derivative with reversible acetylcholinesterase inhibitor activity (McGleenon *et al.*, 1999). Consequently, neuromodulation via donepezil administration might have an impact on ACh, itself, AChE and Nicotinic Acetylcholine receptors (AChRs). ACh modulates. In patients with AD, chronic donepezil treatment (10mg/day for 2 years) led to an increase in the protein level of the AChE-R (a variant of AChE) which corresponded to a high AChE inhibition in CSF and subsequently protected against cognitive deterioration (Darreh-Shori *et al.*, 2006). One repercussion underlying a long-term inhibition of AChE is the activation of compensatory mechanisms to reduce the effect of

inhibition. Importantly, donepezil exposure in rats at 3mg/kg/day for 14 days did not inhibit brain AChE continuously during the day, suggesting that this dose will not lead to tolerance development (Haug *et al.*, 2005). The development of tolerance for chronic ChEI, such as donepezil, progresses with a downregulation of muscarinic receptors (Scali *et al.*, 2002). Importantly, levels of extracellular ACh can persist after 21 days of ChEI treatment, and there appears to be no inhibition in ACh synthesis since further ChEI administration can still increase ACh levels (Scali *et al.*, 2002).

In terms of neuromodulation within an ageing brain, a study revealed that impaired spatial learning ability in a subset of aged rats was accompanied by decreased ChAT in the basal forebrain, striatum, and frontal cortex (Gallagher *et al.*, 1990), suggesting that intact cholinergic activity is crucial for memory. Among the rodent studies, a loss of markers selective for cholinergic neurons is consistently reported in aged rat brains compared to young, including Fisher 344, Sprague-Dawley, and Long-Evans strains (Fischer *et al.*, 1989; Bieganski *et al.*, 1986; Fischer *et al.*, 1992; Gallagher *et al.*, 1990). The general consensus accompanying these studies was that the cholinergic phenotype in this system is particularly vulnerable to the effects of aging (Gill & Gallagher, 1998). A close relationship was also established between decreased muscarinic (M2) receptor binding and individual cognitive ability in aged rats, suggesting that this cognitive decline may be a consequence of degeneration within the septohippocampal cholinergic system (Gill & Gallagher, 1998; Gallagher & Nicolle, 1993). However, the conclusion here may not be as definite as it is made out to be.

Microdialysis in the rodent model revealed that the inhibitory effects of donepezil on cholinesterase activity are greater in aged than in young rats (Kosasa *et al.*, 1999a). Several studies are in agreement with recent findings that the neuroprotective effects of donepezil were mediated by nicotinic receptors activation (Takada-Takatori *et al.*, 2008c; Fujiki *et al.*, 2005; Takada *et al.*, 2003). Protecting the neural system from degeneration involves the up-regulation of $\alpha 7$ -nicotinic receptor, specifically, and two intracellular signaling pathways, phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathway (Takada-Takatori *et al.*, 2008c; Arias *et al.*, 2005; Akaike *et al.*, 2006). It has also since been suggested that donepezil's neuroprotective mechanism against A β 42-induced neurotoxicity was mediated via the activation of phosphoinositide 3 kinase/Akt (PI3-K/Akt) pathway and inhibition of GSK-3 (Noh *et al.*, 2009).

Donepezil has numerous downstream cellular and molecular effects that influences AD progression (Takada-Takatori *et al.*, 2008b). However, the principal disease-modifying mechanism of donepezil has not yet been identified. Neuroprotection was offered by AChE-inhibitors via the upregulation of BDNF expression in both preclinical and clinical studies (Leyhe *et al.*, 2008). BDNF belongs to the family of nerve growth factors and plays an important role in neuronal survival, differentiation, and synaptic plasticity in the CNS (Hennigan *et al.*, 2007). Inhibiting AChE in patients with AD ameliorates pre-existing down-regulation of BDNF in the serum and brain of AD patients to levels similar to healthy controls (Leyhe *et al.*, 2008). Similarly, the mechanism behind BDNF neuroprotection comes from mediation of simultaneous contribution of the PI3-K and MEK/ERK pathways (Almeida *et al.*, 2005; Fumagalli *et al.*, 2005).

Evidence for neuronal differentiation has been described in several cell culture studies where donepezil potentiated NGF-induced neurite outgrowth (Oda *et al.*, 2007; Ishima *et al.*, 2008). However, in vivo cor-

relations between analyses of molecular markers and behavioural studies in rodents revealed that donepezil was unable to affect NGF and NGF receptor levels, even though sustained positive effects were present under the behavioural paradigm (Hernandez *et al.*, 2006). NGF binding to its high-affinity receptor TrkA causes TrkA dimerization, followed by autophosphorylation of multiple tyrosine residues, which in turn initiates signal transduction events that generally promote the survival of cholinergic neurons. Conversely, signaling via the low-affinity p75 NTR typically (but not exclusively) activates pathways leading to cell death (Barker & Shooter, 1994; Deppmann *et al.*, 2008). Additionally, potentiation of NGF-induced neurite outgrowth has also been linked to sigma-1 receptors and interaction with IP₃ receptors

In a recent review on cholinergic neuromodulation of cognition, AChE inhibitors do not merely increased cholinergic neurotransmission but they also uncouple presynaptic from postsynaptic information transmission and produced complex changes in local and efferent circuitry (Hasselmo & Sarter, 2011). This phenomenon occurs due to high sustained levels of extracellular ACh, leading to excessive stimulation of presynaptic M2 receptors which in turn inhibits ACh and attenuates presynaptic signalling (Sarter *et al.*, 2009). Extracellular ACh binds to cholinergic muscarinic receptors which may couple with cytosolic phospholipase A2 (cPLA2) activation and arachidonic acid (AA) release from synaptic membrane phospholipid (Clark *et al.*, 1995). Quantitative autoradiography which revealed donepezil mode of action where an increased brain AA signal which is mediated by ACh acting at muscarinic receptors (Basselin *et al.*, 2009).

Lastly, in addition to neuroprotective mechanisms mentioned above, donepezil has a direct relationship with intracellular calcium concentration $[Ca^{2+}]_i$. Chronic treatment in cortical neuron culture enhances nicotine-induced increase in $[Ca^{2+}]_i$ (Takada-Takatori *et al.*, 2008b). In terms of neural protection against NMDA toxicity, pretreatment with donepezil significantly decreased the NMDA-induced increase of $[Ca^{2+}]_i$ (Akasofu *et al.*, 2003). In a depolarization-induced neuronal cell injury model, donepezil assert neuroprotective activity via inhibition of the rapid influx of sodium and calcium ions, and decreased glutamate and glycine release (Akasofu *et al.*, 2008).

Calcium dysregulation, presented as disturbances in calcium homeostasis in cells, plays a central role in AD pathogenesis (Canzoniero & Snider, 2005). Calcium is essential for basic signalling processes. Thus altered calcium signaling could influence the increase of susceptibility of cells to apoptosis, synaptic plasticity, mitochondrial dysfunction, membrane excitability, oxidative stress, APP processing, and Tau phosphorylation (Yu *et al.*, 2009). Coincidentally, TZDs were able to modulate $[Ca^{2+}]$ -dependent pathways in the brain. Rosiglitazone, specifically, reduced voltage-gated Ca^{2+} channel (VGCC) currents, potentially conferring neuroprotection in cultured hippocampal neurons (Pancani *et al.*, 2009). Cognitive benefits attained from rosiglitazone administration is genuine for both the studies and independent work in the neuroscience field. The mechanism for such an outcome has been linked to anti-inflammatory properties of the drug treatment. Activation of PPAR γ by rosiglitazone induces neuroprotection in an in vivo mouse model of Parkinson disease which arrested the degenerative process in particular parts of the brain by reversing TNF α expression to control levels (Carta *et al.*, 2011).

Regulation of inflammatory processes have been well demonstrated with rosiglitazone therapy (Orasanu

et al., 2008; Chinetti *et al.*, 2000; Sanchez-Hidalgo *et al.*, 2005; Mohanty *et al.*, 2004; Loane *et al.*, 2009). More recently, rosiglitazone has been demonstrated to halt neuronal damage by interfering with glial activations and increases anti-inflammatory cytokines in response to ischemic damage (Lee *et al.*, 2011a; Fatehi-Hassanabad & Tasker, 2011) which can be reversed by PPAR γ antagonist (Zhang *et al.*, 2010). The mechanisms behind such ameliorative effects come from activating PPAR expression. Since PPARs are ligand-activated transcription factors belonging to the nuclear receptor family, they have a wide variety of functions. They are key regulators in various pathophysiological processes related to energy metabolism including lipid, carbohydrate metabolism, cellular proliferation, differentiation, apoptosis and inflammation (Chinetti *et al.*, 2000; Sung *et al.*, 2004). Rosiglitazone is also known for modulating vascular changes and improving insulin resistance in diabetic patients, and has now become one of the most popular anti-diabetic drugs in the developed countries (Yamagishi, 2010). In a rodent model of chronic cerebral hypoperfusion, the PPAR γ agonist was able to rescue cerebral hypoperfusion-induced brain damage through suppressing glial activation and preserving synaptic plasticity (Sayan-Ozacmak *et al.*, 2011). The pathway responsible for acute cardioprotective effect was mainly mediated through the inhibition of Jun NH2-terminal kinase phosphorylation (Khandoudi *et al.*, 2002). Additionally, rosiglitazone has been demonstrated to activate Wnt signalling in vivo (Toledo & Inestrosa, 2009) as well as in cultured hippocampal neurons (Inestrosa *et al.*, 2005). The Wnt signaling pathway plays a significant role in cellular metabolism where abnormalities in this pathway may impart increased risk for type 2 diabetes mellitus (Maiese *et al.*, 2008).

The pathways through which the PPARs control inflammatory responses is mediated through negative interference between PPARs and other nuclear factors such as NF- κ B, AP-1 and C/EBP. However, PPAR γ agonists may regulate inflammation either through activation of PPAR γ or through a PPAR γ -independent mechanism (Genolet *et al.*, 2004). For example, in a genetically modified mouse model repressing PPAR γ gene in macrophages, a major transcriptional role of PPAR γ is negative regulation of specific subsets of genes that are activated by T helper 1 cytokines (Welch *et al.*, 2003). PPAR-independent effects have been demonstrated where an equivalent antagonists is unable to inhibit direct effect of rosiglitazone in blood vessels (Ryan *et al.*, 2004). In human lung cancer cells, rosiglitazone inhibits cell proliferation by affecting downstream secondary pathways both directly and indirectly via upregulation of the PTEN/AMPK and down-regulation of the Akt/mTOR/p70S6K signal cascades (Han & Roman, 2006).

6.3 Contrasting procognitive effects between donepezil and rosiglitazone treatments

6.3.1 Acquisition trend throughout all sessions in the Water Plus Maze paradigm

The WPM or plus-shaped maze, a variation from the more standard MWM, has been used in recent years for the assessment of spatial memory impairment in animal models of behavioural studies (Wahlsten *et al.*, 2005; Vloeberghs *et al.*, 2006; Locchi *et al.*, 2007; Diamond *et al.*, 1999; Chen *et al.*, 2004) (see 3.5 on page 66). The work done with the WPM is seminal in its design using a chronic oral administration of donepezil and rosiglitazone¹. Age of subjects at the start of each behavioural task experiments, for all studies mentioned hereafter, are in the range of 12-14 months, for middle-aged rats, and 3-4 months for young adult rats.

It was previously demonstrated that the WPM task can be sensitive to age-related memory and learning impairments (see section 3.3 on page 51). These results here are organised differently from previous chapters in order to achieve a further insight into the progression of learning in the animals for each session. Instead of representing fixed-start performance as an average for each sessions, all trials are exhibited allowing new attributes of the task to be analysed. This might help elucidate the specific advantage for both procognitive drugs.

6.3.2 Statistical analysis for behavioural and molecular results

Non-parametric ANOVAs with repeated measures were conducted using performance scores with sessions as between-subjects variable and treatment groups ($n = 10$ animals for each group) as within-subjects variable. The same analysis was used for multiple start tests conducted at the end of each session after fixed-start trials [3 individual sessions (within subjects) 4 age-treatment groups (between subjects)]. Dunns multiple comparisons test was used as a post-hoc test.

Correlations were drawn between the two different models. In the first instance, the relationship between latency and task ability was investigated. This would enable a direct comparison between the two parameters and assess how large an effect latency had on the performance level. Secondly, analyses from all molecular experiments were correlated with behavioural outcomes (WPM performance, latency in decision zone, ODR at 1 and 24 hour delays). Such a comparison gave us further insight into individual groups of rats and the relationship between behavioural outcomes and molecular components. It is important to specify that the analyses utilised in the current study was Pearson R correlation, which indicated the magnitude and direction of the association between two variables. Although the Bonferroni correction was not used, an additional statistical method can be used to counteract the problem of multiple comparisons. This method alters the alpha value which is commonly set at 0.05. A modification is done by dividing 0.05 by the number of tests that were carried out. Although this leads to a stricter measurement, it tends to lead to from Type I errors.

¹Descriptions for the WPM procedure and task layout have already been discussed previously (see section 2.3.2 on page 26). A short introduction will be detailed here in preparation for the examinations of behavioural results

6.3.3 Latency declined in untreated rats as correct responses increased during fixed-start trials in session 1

The 3 consecutive days of fixed-start trials revealed an increasing trend for the percentage of responses, which is accompanied by a decreasing trend for latency within the decision zone. This might suggest that, in control rats, rats spent less time in the decision zone as they gradually learn to locate the escape platform. In general, YA rats performed at a much higher level than MA in terms of task acquisition. Latency was prolonged for older rats when compared to young adult rats. Two-way ANOVA revealed statistical differences in correct response for both age [$F_{(1,72)} = 63.44, p < 0.001$], type of trials [$F_{(3,72)} = 2.97, p < 0.05$] and the interaction between them [$F_{(3,72)} = 6.243, p < 0.0001$]. YA rats accomplished the task with more correct responses than MA rats from day 1 ($t = 3.71, p > 0.01, ns, df = 29\%$). This trend persisted for all fixed-start trials (day 2 : $t = 6.65, p < 0.001, df = 52\%$, day 3 : $t = 4.86, p < 0.001, df = 38\%$)

MA remained in the decision zone for longer than YA rats, although this behaviour diminished over time. Two-way ANOVA revealed statistical differences in duration for both age [$F_{(1,71)} = 39.49, p < 0.0001$], type of trials [$F_{(3,71)} = 21.74, p < 0.0001$] and the interaction between them [$F_{(3,71)} = 6.598, p < 0.001$]. Differences between ages were only present in day 1 ($t = 6.018, p < 0.001$) and 2 ($t = 3.94, p < 0.01$). It appears that as MA rats improved in acquiring the task, duration in the decision zone dropped (from 9.4 seconds in the first day to 2.5 seconds on MS trial). Latency declined for both ages as correct responses increased throughout session 1. Notably, when task performance was comparable for MS trial, latency was also similar between ages.

6.3.4 Progression in acquisition was present for both ages

A rest period of 14 days between testing sessions did not affect young rats' ability to accomplish the task at a high level. This might suggest that the memory for acquiring the task was intact even after 14 days. Strikingly, MA rats were able to acquire a higher percentage of correct response even after the rest period when comparing between day 3 (23%) and day 18 (44%) of fixed-start trial. Two-way ANOVA revealed statistical differences in correct response for age [$F_{(1,72)} = 41.22, p < 0.001$], type of trials [$F_{(3,72)} = 22.72, p < 0.001$] and the interaction between them [$F_{(3,72)} = 5.64, p < 0.0001$]. Throughout the second session, acquisition of task was on a much higher level for YA rats [(day 18 : $t = 3.271, p < 0.01, df = 27\%$), (day 19 : $t = 5.69, p < 0.001, df = 47\%$), (day 20 : $t = 3.88, p < 0.001, df = 32\%$)].

Latency did not change between the two MS trials (S1 and 2), suggesting that rats were not using the same strategy as in fixed-start trials. Hence, latency is an indicator for performance level only for the fixed-start trials. This is further supported by data represented on day 20 where an increase in latency for MA rats coincided with an increased in the correct response. This is represented by the grey circle on both graphs. This gives support to the view that a different strategy is at place for the much harder MS trial.

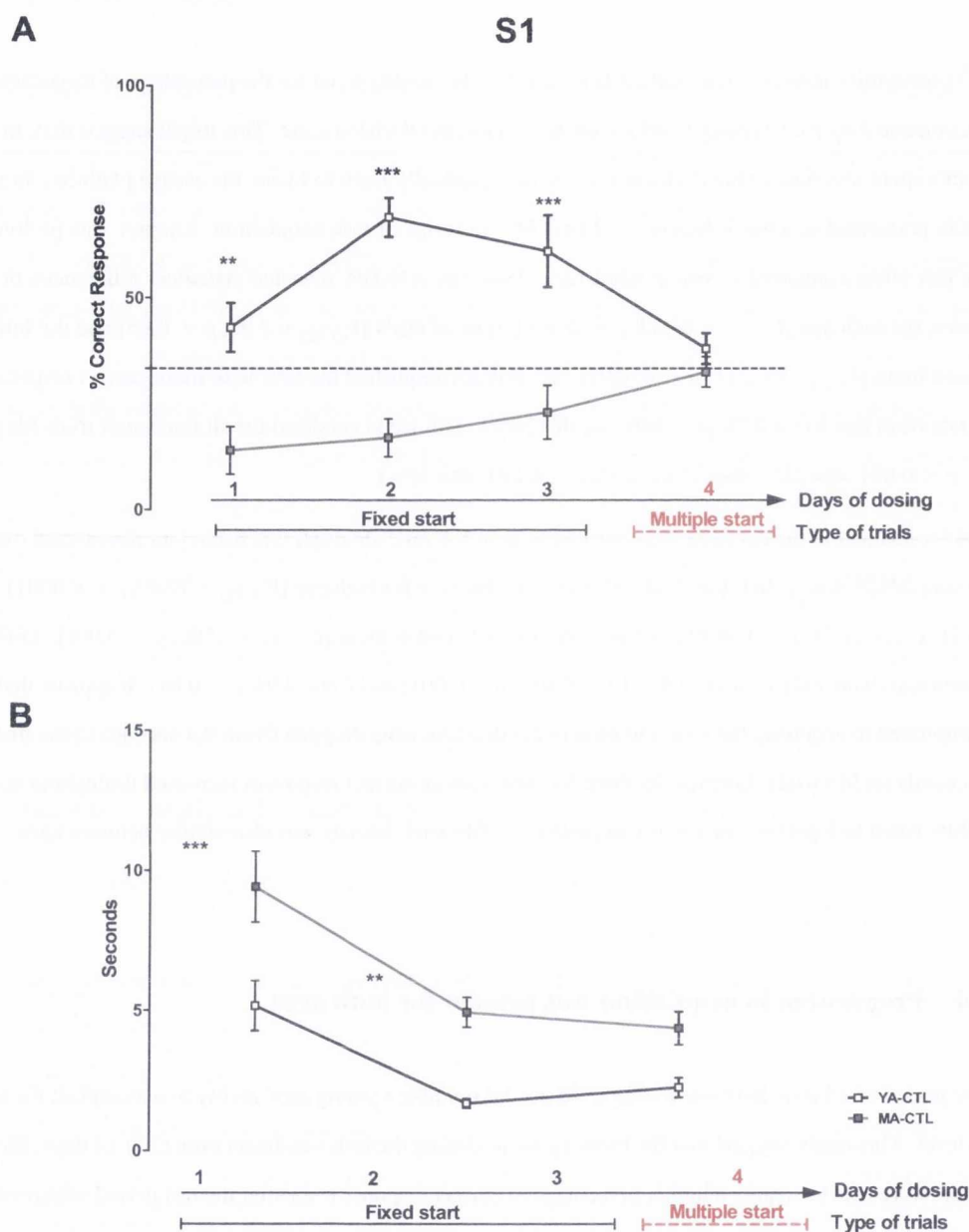


Figure 6.1: **Trend of acquisition in untreated rats for session 1.** The basic progression for each sessions was as follows: 3 days of fixed-start trial (10 trials/day) and multiple-start trial after (30 trials in 1 day). (A) The results here represent the increasing performance level in terms of correct response for middle-aged (MA) and young adult (YA) rats. The dotted line on the graphs denotes chance level during a MS trials. (B) A decline in latency (seconds) was demonstrated throughout the session. All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

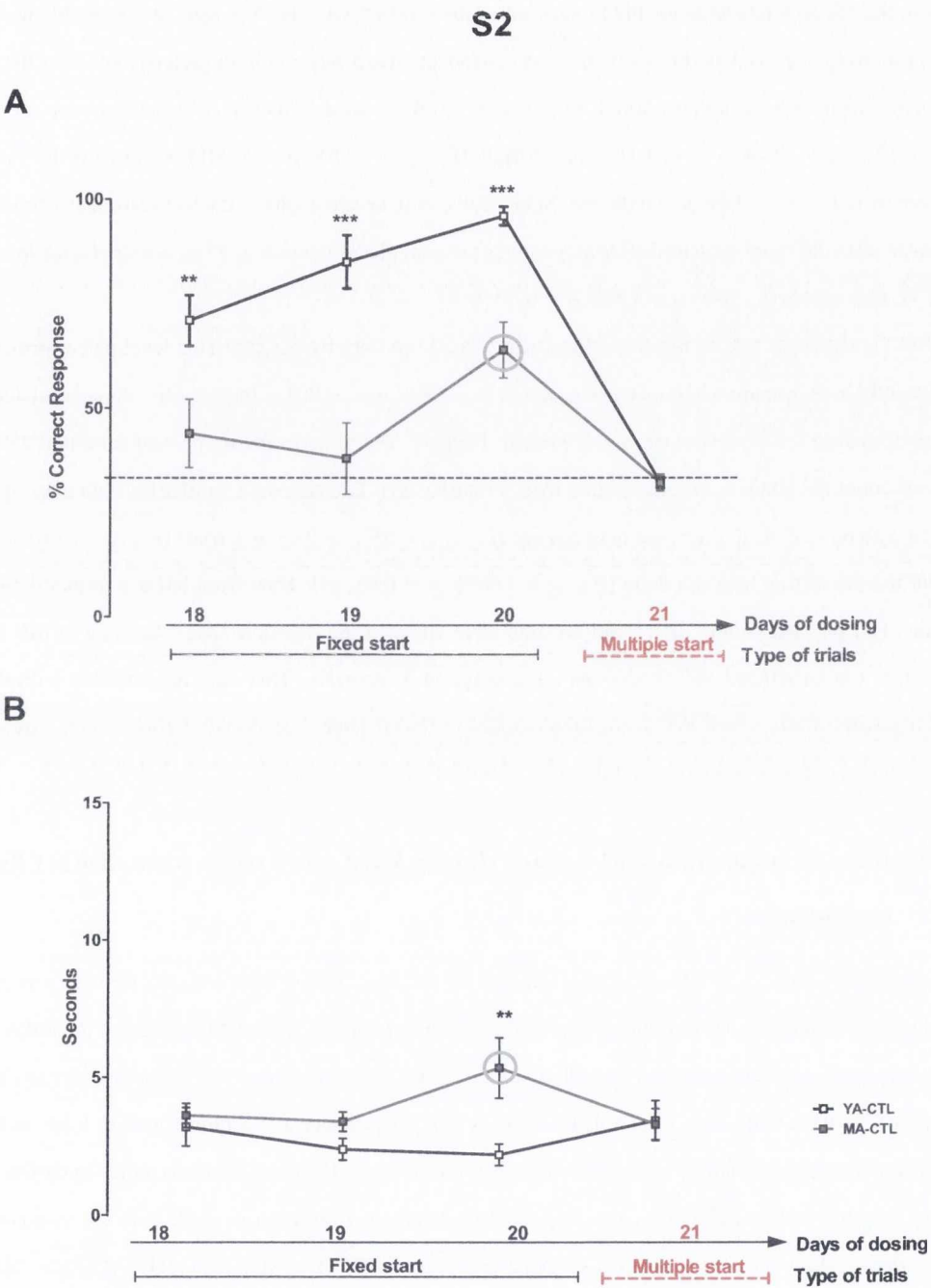


Figure 6.2: **Improvement in task performance even after 14 day rest period** Between sessions, rats were maintained on a dosing regime. (A) Performance increase throughout the session once again. YA maintained a higher correct response compared to MA rats. (B) There is no change in latency for YA rats. A peak in latency for MA rats (grey circle) corresponded to a peak in performance level. All data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

6.3.5 Young adult rats reached ceiling effect for fixed-start trials by day 20

Even in the S3, MA rats were unable to perform to the level of YA rats. Younger rats were able to maintain high proficiency for fixed-start trials by the last session and there was no dip in performance after the 16 days rest period with no behavioural testing. Two-way ANOVA revealed statistical differences in correct response for age [$F_{(1,72)} = 12.78, p < 0.001$], type of trials [$F_{(3,72)} = 37.55, p < 0.001$] but not for the interaction between them [$F_{(3,72)} = 2.09, p > 0.05, ns$]. Statistically, both ages completed the last session at a similar level. However, with the more difficult MS trial, younger rats completed the task at 57%, above chance level, while MACTL was unable to (27%) ($t = 3.923, p < 0.001$).

YACTL rats were able to reach a ceiling effect in S2 but only by day 20. This level of performance was significantly higher than its age-matched controls ($t = 3.877, p < 0.001$). Importantly, the age-related gap in task performance for fixed-start trials was evident. Notably, YA rat performance peaked at around 95%, while MA rats could not attain a response higher than 89% (day 39). Latency were similar for both ages by the last session with no statistical difference between age [$F_{(3,72)} = 0.05, p > 0.05, ns$], trials [$F_{(3,72)} = 0.467, p > 0.05, ns$] nor the interaction between them [$F_{(3,72)} = 0.6674, p > 0.05, ns$]. Data from latency further demonstrate that rats had reached ceiling effect and no time were spent in the decision zone. Latency in MS trials, in particular, was unchanged for all sessions (an average of 3 seconds). This does not coincide with the trend from fixed-start trials where low acquisition equates to longer latency in decision zone. Such a trend might indicate that a different strategy to a different trial was at work.

6.3.6 Rate of acquisition and latency during fixed-start trials were distinct between treatments

Comparative analyses in the current chapter uncover the extent of procognitive capacity for both donepezil and rosiglitazone treatments. Distinction in terms of acquisition for fixed-start and behavioural flexibility for MS trials were previously demonstrated for both donepezil (refer to chapter 3) and rosiglitazone (refer to chapter 4) treatments. In brief, both drugs were able to augment task performance and acquisition of task was heightened due to cholinergic manipulation and glucoregulatory conservation. Hence, it is important in the current chapter to gain a further understanding into how this outcome emerged. Comparisons made here are based on differences in acquisition between treatments for both ages (e.g. YAROSI vs MADON and YADON vs MAROSI) and difference between ages for separate treatments (e.g. YAROSI vs YADON and MAROSI vs MADON). All other comparisons not mentioned here would have been described in the relevant chapters (see chapter 3 and 4).

6.3.7 Session 1 revealed acute drug response for rosiglitazone-treated rats

Although not statistically significant, rats given an acute treatment (on day 1) with rosiglitazone excelled in the task, in both ages, compared to treatment with donepezil. MAROSI were performing at the same level as YACTL rats ($t = 1.67, p > 0.05, ns, \text{diff} = 17\%$). Older rats on their first donepezil administration were unable

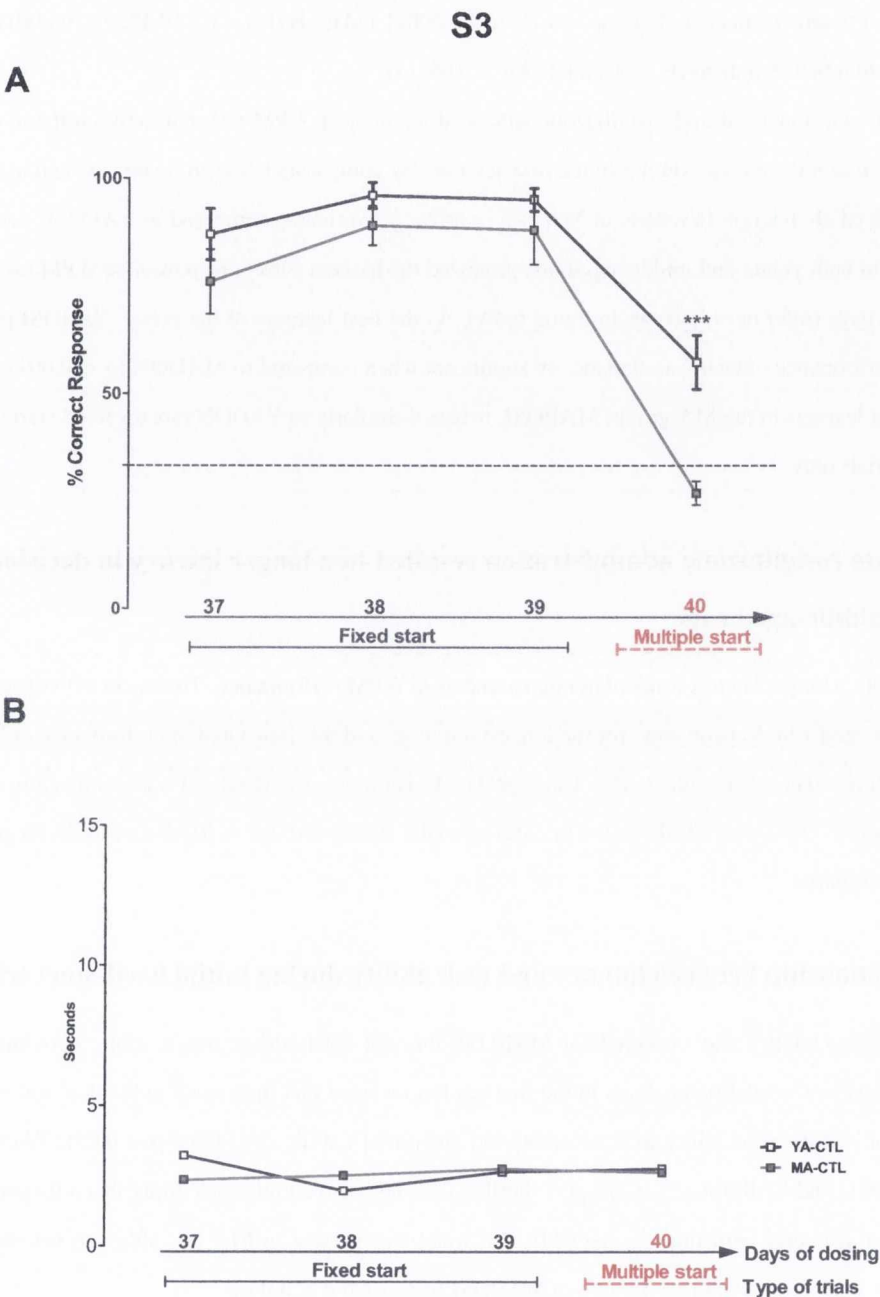


Figure 6.3: **Ceiling effect reached in young adult rats in session 3** Between sessions, rats were maintained on a dosing regime. (A) Performance increased throughout the session once again. YA maintained a ceiling effect of a higher correct response compared to MA rats. MA rats were unable to acquire MS trial at higher than chance level. (B) There is no change in latency for all rats. All data are represented as mean $\pm$ S.E.M. *** $p < 0.001$

to acquire the task at the same level as YACTL rats ($t = 3.056$, $p < 0.05$, difference = 33%). Rosiglitazone treatment produced the best performance within each age group in the first session. Two-way ANOVA revealed significance for treatment and age [$F_{(5,150)} = 26.76$, $p < 0.0001$] and trials [$F_{(2,150)} = 19.42$, $p < 0.0001$] but not for the interaction between them [$F_{(10,150)} = 1.183$, $p > 0.05$, *ns*].

Treatment with donepezil and rosiglitazone enhanced learning of WPM task but between them, the progression of task acquisition was similar in the first session. By comparing between treatments and age, it was clear if enhanced cholinergic functions in MA rats improve performance compared to YAROSI. Seemingly, rosiglitazone in both young and middle-aged rats produced the highest correct response for WPM task during the first three days (refer to red arrows in figure 6.4A). As the best learners of the group, YAROSI produced the highest performance, which was statistically significant when compared to MADON ($p < 0.001$) for all 3 days. The best learners in the MA group, MAROSI, behaved similarly to YADON rats for fixed-start trials for the first two trials only.

6.3.8 Acute rosiglitazone administration resulted in a longer latency in decision zone in middle-aged rats

In untreated rats, a longer latency resulted in improvements in WPM performance. Treatment with rosiglitazone caused MA to persist in decision zone for the longest duration, and this translated in performance in WPM as expected (refer to red arrow in figure 6.4B). Time spent in decision zone for MAROSI was significantly different from YADON ($t = 3.839$, $p < 0.001$) on the first day of trials. By the last day of fixed-start trials, all groups of rats behaved similarly.

6.3.9 Relationship between latency and task ability during initial fixed-start trial

Although a higher latency was observed for MAROSI, this did not translate into a strong correlation with performance levels. Correlation analyses in the first session revealed that time spent in the decision zone was an indicator of performance ability in three month old rats only [YACTL ($r = -0.54$, $p < 0.05$); YADON ($r = -0.67$, $p < 0.001$) and YAROSI ($r = -0.62$, $p < 0.001$)]. The negative correlations imply that a longer latency was needed for a poorer performance. Notably, this trend was present in MA rats although not significant [MACTL ($r = -0.29$); MADON ($r = -0.36$) and MAROSI ($r = -0.13$) $p > 0.05$].

6.3.10 Donepezil-mediated effects on learning task were demonstrated in session 2

As demonstrated in previous chapter (see 3 on page43), donepezil treatment was unable to elicit a positive influence on WPM performance in session 1 for both ages. By the following session, significant statistical differences in performance were evident in MADON rats for all three days compared to untreated rats (day 18: $t = 4.025$, $p < 0.001$, difference = 37%, day 19: ($t = 5.214$, $p < 0.001$, difference = 48%, day 20: $t = 3.586$, $p < 0.01$, difference = 33%). Outstandingly, donepezil-treated rats were able to reach ceiling effect, represented by performance levels $> 90\%$, much earlier than all other groups of rats. Notably, MADON rats reached ceiling

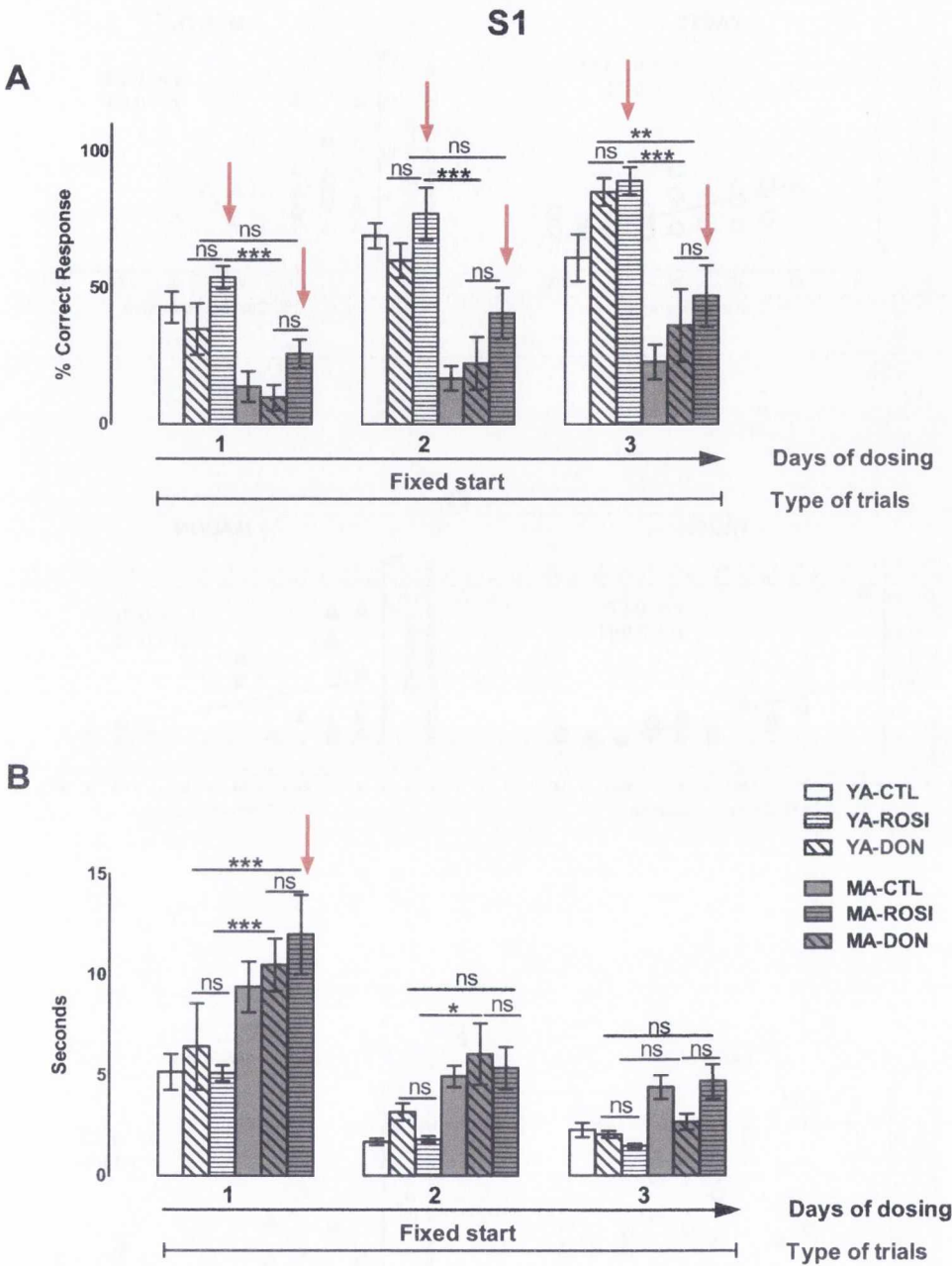


Figure 6.4: **Initial session revealed acute rosiglitazone effect on task performance for both ages.** A total of 6 groups are depicted in these graphs. Middle-aged rats are represented by grey bars while YA rats are in white. Blank, stripe and horizontal bars represent control, donepezil and rosiglitazone treatment groups respectively. Red arrows indicate acute effect of rosiglitazone evident from day 1 and increased latency by acute donepezil treatment. All data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

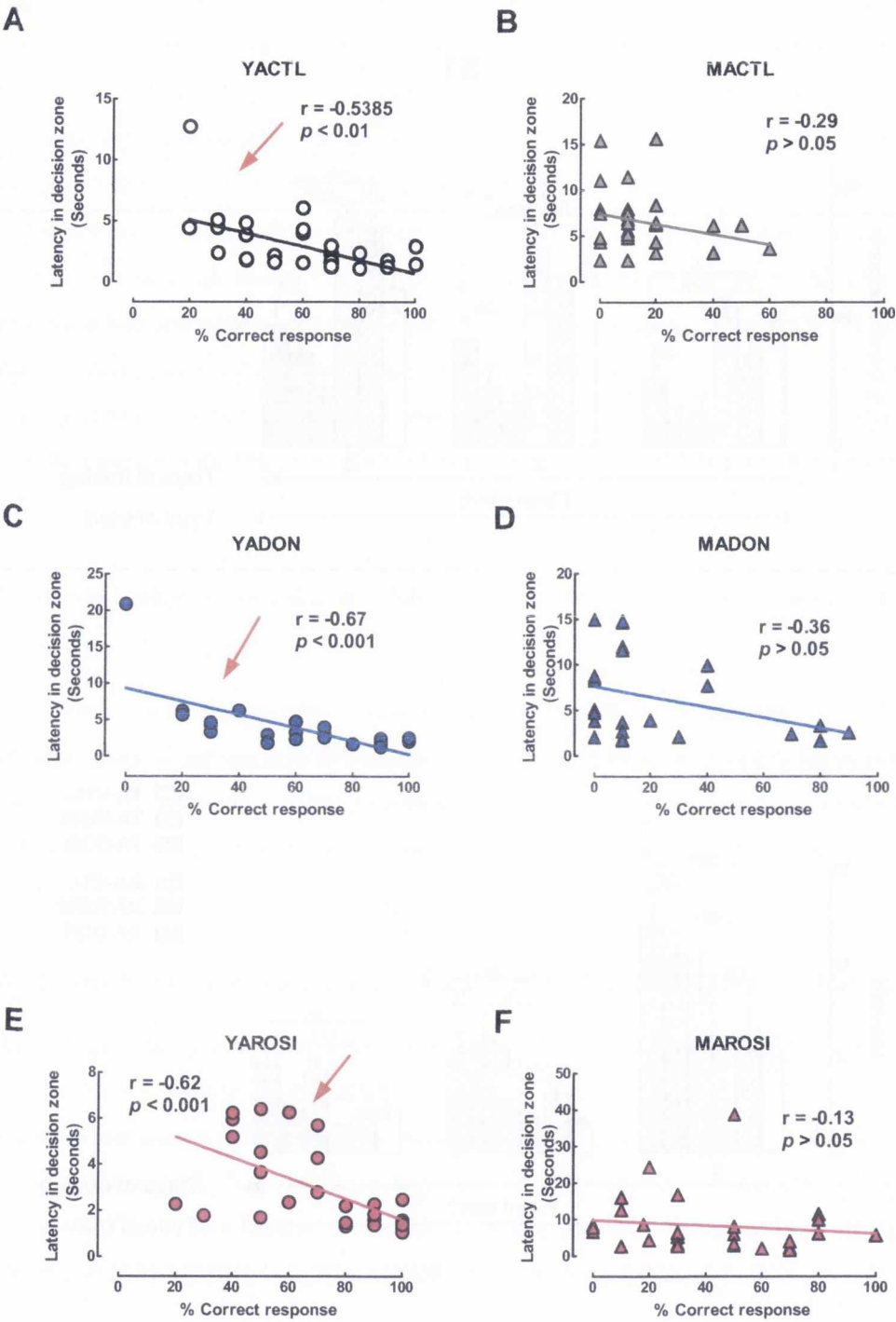


Figure 6.5: Associations between latency in decision zone and performance level. A strong relationship exists for all YA rats between latency and performance ability. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for all 30 fixed-start trials are represented here while each data point represents an individual trial. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

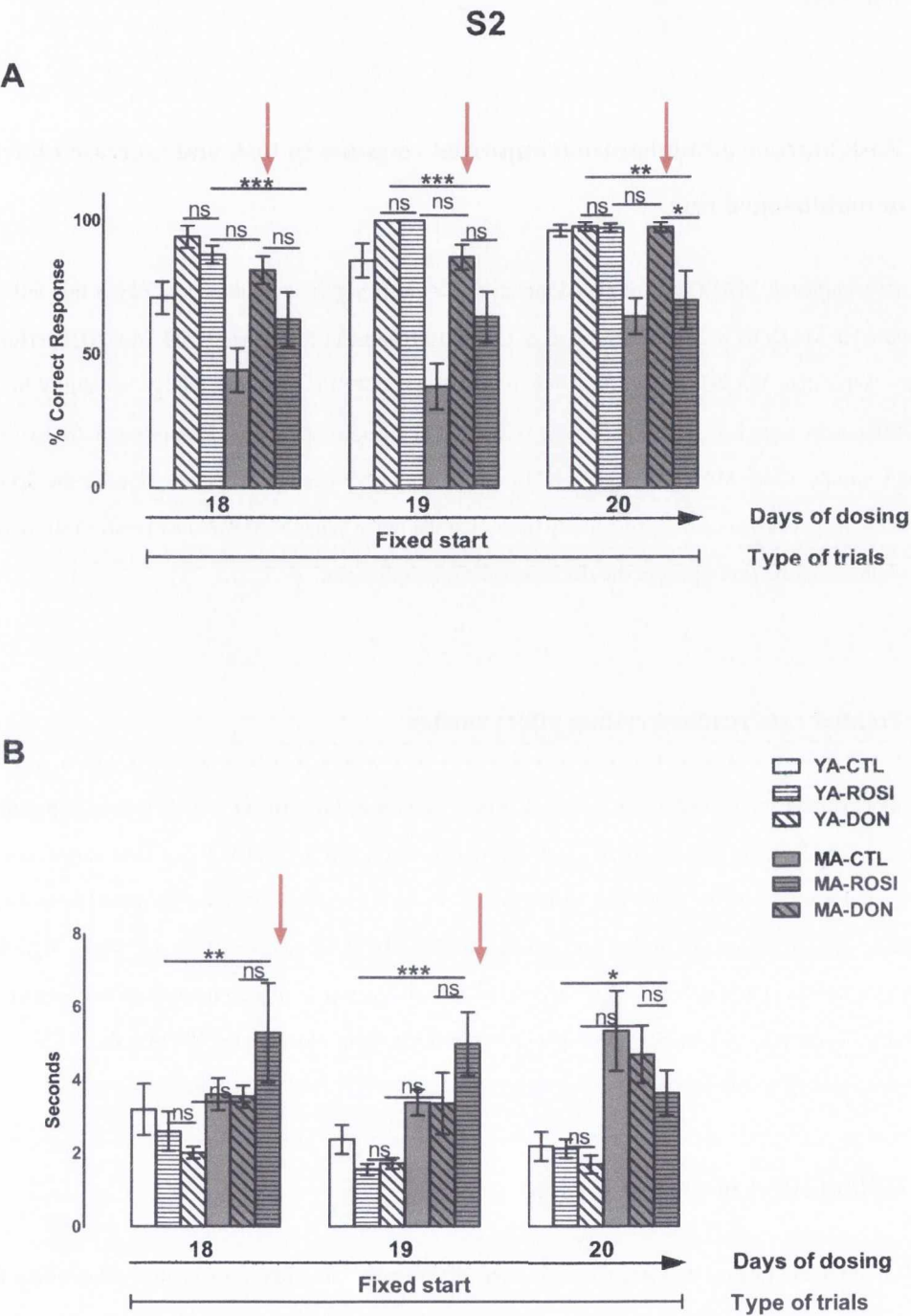


Figure 6.6: **Effects of donepezil appear in session 2.** Within MA groups of rats, donepezil treatment increased percentage of correct response. Latency in decision zone was most pronounced in MAROSI rats. All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Red arrows indicate statistically significant outcomes

effect at the same rate as YACTL rats at day 20. This ceiling effect was maintained throughout the sessions for donepezil treated rats.

6.3.11 Rosiglitazone administration improved response in task and increased latency in middle-aged rats

Although not significant, MAROSI completed the task at a much higher level than control but this fell short when compared to MADON ($p > 0.05$, *ns*) on days 18 and 19. On the last fixed-start trial, MADON performed significantly better than MAROSI rats ($t = 2.938$, $p < 0.05$, $df = 27\%$). Due to the large variability in data, statistical differences were lost. However, latency recorded in the second session demonstrated drastic differences in MA groups where MAROSI consistently produced a higher latency. This behaviour in the decision zone improved the performance level on a daily basis. On the other hand, MADON rats behaved similarly to all young adult rats in the time spent in the decision zone ($p > 0.05$, *ns*).

6.3.12 Treated rats reached ceiling effect earlier

The scatterplot exhibits a trend that exists in YA rats where data for ceiling effect ($> 90\%$) was more prominent for treated rats, rosiglitazone and donepezil. Task abilities for untreated YA and MA rats were varied, covered a wide range and were unable to reveal any strong trends. In the second session, the consistent higher latency in the decision zone correlated to a higher performance level in MAROSI rats ($r = -0.53$, $p < 0.01$). Significant correlation was present in YADON ($r = -0.45$, $p < 0.05$) as well, demonstrating an interaction between latency and task ability, where a longer latency accompanies lower performance level in the WPM task.

6.3.13 Ceiling effect in the last session

On day 37 in the final session, it is interesting to note all YA rats were able to maintain ceiling effect from previous session except control rats (YADON = 97.5%, YAROSI = 98%, YACTL = 87%). Similarly in MA rats, untreated rats were performing at a lower level similarly to MAROSI (MADON = 91.11%, MAROSI = 77%, MACTL = 76%). YAROSI with donepezil treatment were significantly better at fixed-start than MAROSI rats ($t = 2.928$, $p < 0.05$). In fact, MAROSI performed at the same level as MACTL ($p > 0.05$, *ns*). Although not statistically significant, among all MA rats, MADON rats were able to attain a higher level of response throughout session 3. Two way ANOVA failed to produce any differences between groups for latency recorded in the final session ($p > 0.05$, *ns*). As the ability to complete the task was near 100%, the time needed in the zone was as low as 2.6 seconds.

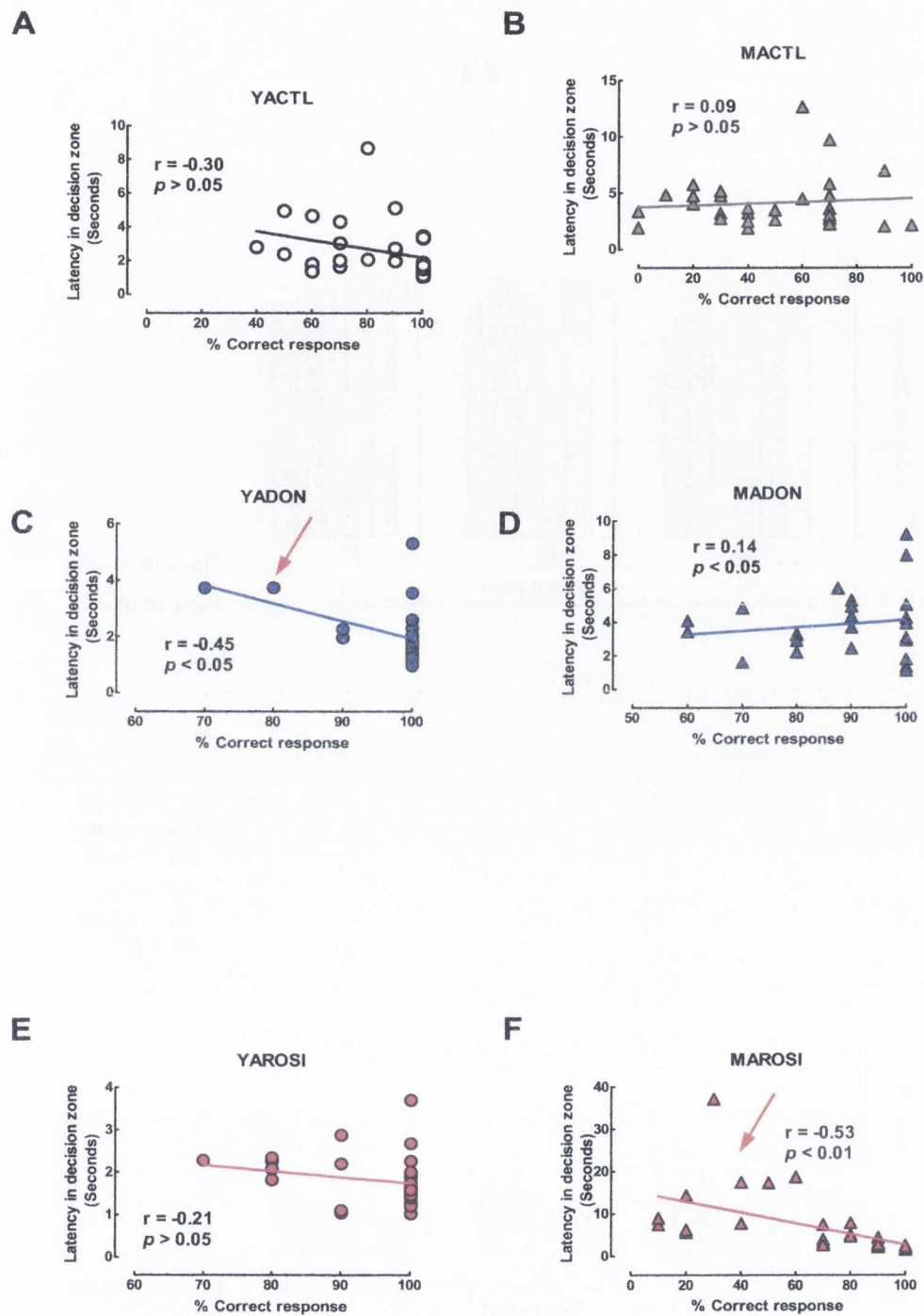
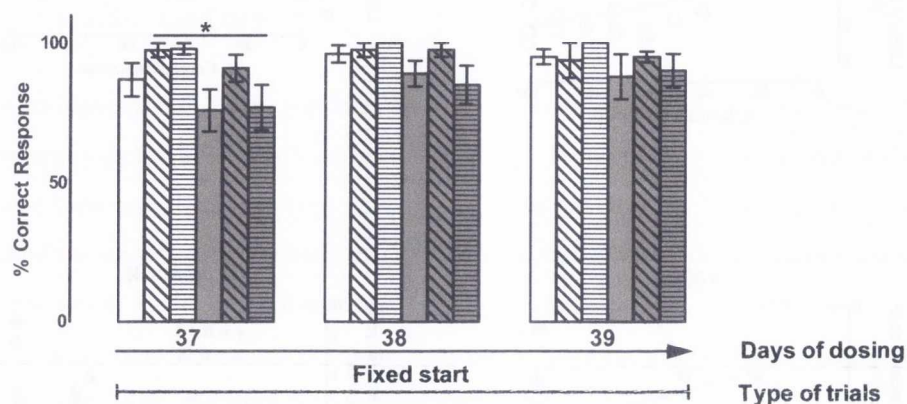


Figure 6.7: **Treated young adult rats arrive at ceiling effect ahead of older rats** Negative correlations were revealed for YADON and MAROSI rats. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for all 30 fixed-start trials are represented here while each data point represents an individual trial. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

S3

A



B

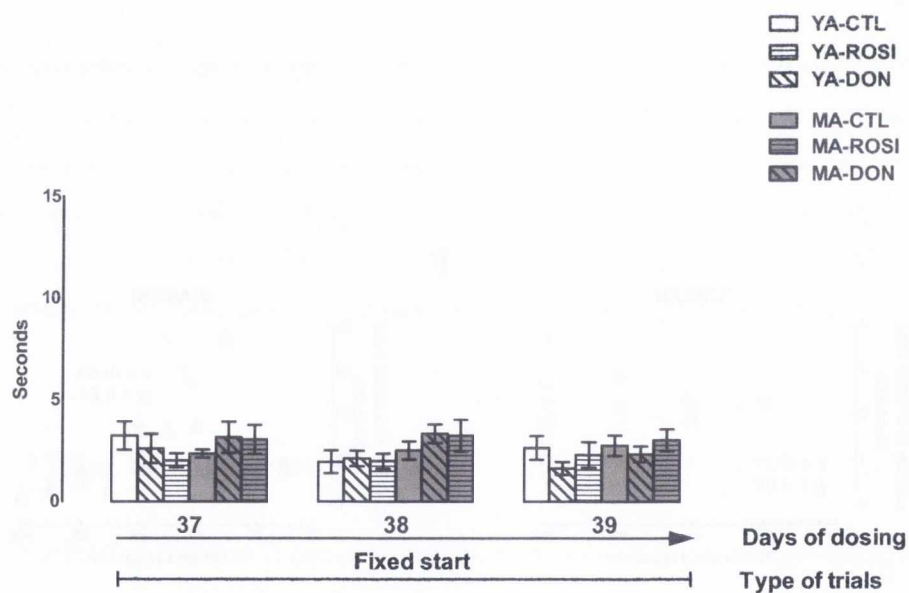


Figure 6.8: **Final session revealed a ceiling effect for fixed-start trials.** All age and treatment groups were able to acquire task at a high level by the final session. Latency was comparable in all groups as well. All data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

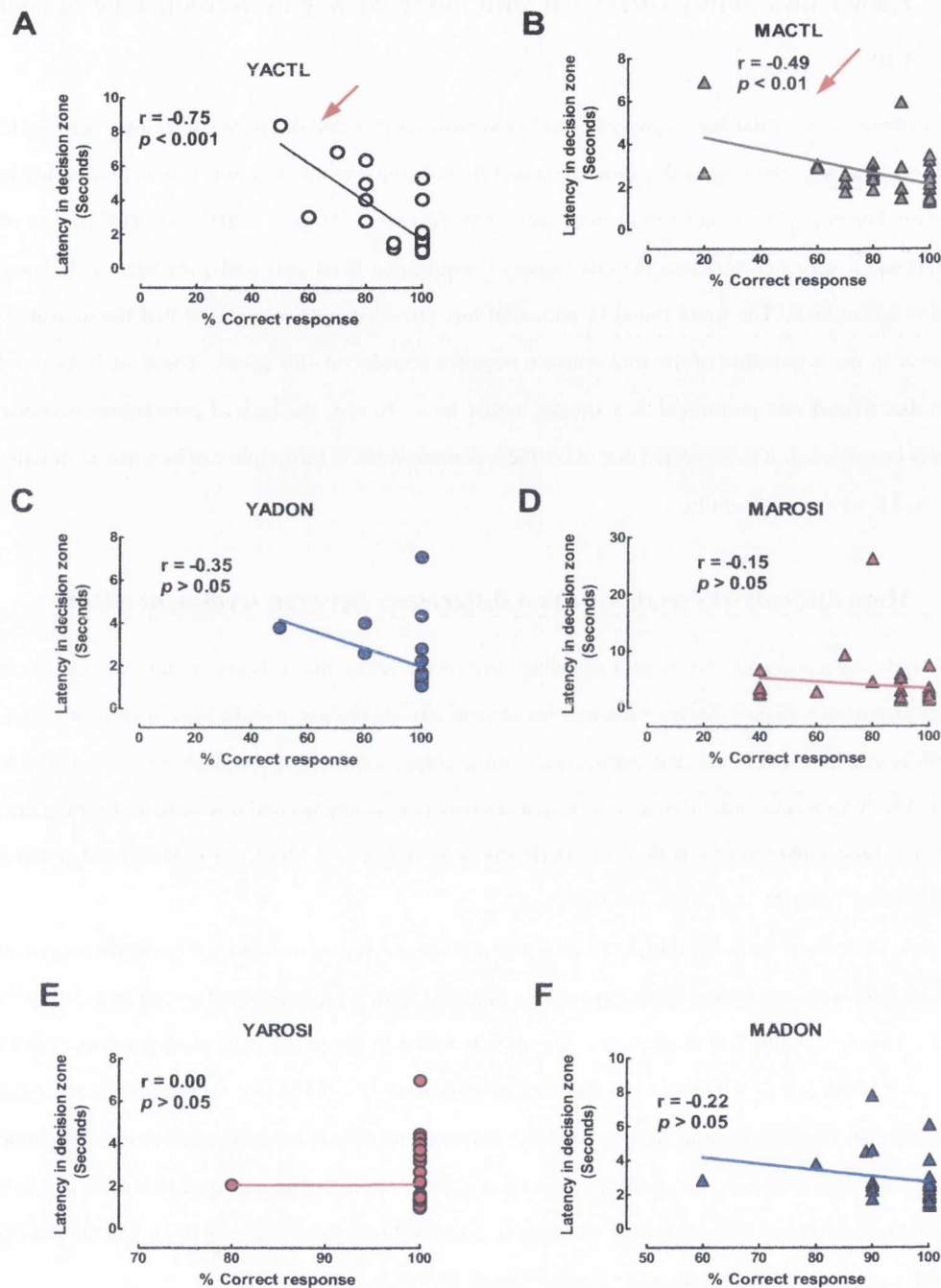


Figure 6.9: **Correlations between latency and performance were revealed only in untreated rats.** Latency recorded was comparable in all groups in the final session. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for all 30 fixed-start trials are represented here, while each data point represents an individual trial. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

6.3.14 Higher task ability correlated with lower latency in decision zone in control rats

Although latency was similar across groups, correlation analyses revealed different interactions between them. Interestingly, a strong relationship that failed to reveal itself in the second session is now demonstrated in the last session. For example, control rats of both ages [YACTL ($r = -0.75$, $p < 0.001$) and MACTL ($r = -0.49$, $p < 0.01$)] had a strong correlation. As rats began to acquire the fixed-start trials, the latency declines and correlation diminished. The trend found in untreated rats provided further evidence that the untreated rats were slower in the acquisition of the trial where a negative correlation still exists. These analyses provided evidence that treated rats performed at a similar higher level. Hence, the lack of correlations between the parameters investigated. It is suggested that the outliers demonstrated in the graphs can be a true representation of individual behavioural diversity.

6.3.15 More difficult MS trials revealed differences between treatment effects

The MS trials were proven to be more demanding, demonstrated by the inability in most groups of rats to locate platform above chance levels. Although not statistically significant, rosiglitazone treatment in YA and donepezil-treated MA rats in the first session were, on average, acquiring MS task above 33% chance level. One-way ANOVA revealed no differences in responses between treatments and rats were performing the first MS trial at a comparable level for both older (MAROSI vs MADON : $t = 1.932$, $p > 0.05$, *ns*) and young adult rats (YAROSI vs YADON : $t = 2.073$, $p > 0.05$, *ns*).

The lack of differences in the initial MS trial was not unexpected as rats had not learnt the requirements of the task. Rats were previously trained to locate a platform from a predetermined escape arm during fixed-start trials. During a multiple start trial, rats were then required to locate the same platform using a different strategy. Both donepezil ($q = 6.646$, $p < 0.001$) and rosiglitazone ($q = 5.734$, $p < 0.001$) treatments enhanced task performance in YA rats compared to controls, although there was no differences between treatments. Between treated MA rats, only donepezil treatment was able to improve above chance ($q = 6.98$, $p < 0.001$), while rosiglitazone-treated rats performed similarly to untreated ones (see Figure 6.10A). Notably, untreated rats of both ages were still unable to attain a performance above chance.

Latency recorded revealed that MAROSI spent a significant amount of time in the decision zone (see Figure 6.10A). However, this behaviour did not translate into an increase in performance. There was a significant effect on latency on the type of treatment administered [$F_{(5,1670)} = 29.94$, $p < 0.0001$]. Type of error analysis revealed that most rats preferred to reproduce a trained turn response in the first MS trial. Two way ANOVA demonstrated significant differences for interaction between treatment and errors [$F_{(10,522)} = 5.847$, $p < 0.0001$] and type of errors made [$F_{(2,522)} = 49.88$, $p < 0.0001$] but none for treatment alone [$F_{(5,522)} = 0.0026$, $p > 0.05$, *ns*]. The only group of rats that differed from this trend was untreated older rats which preferred the straight swim response (see Figure 6.10C). Between treatments, statistical differences were revealed for YA rats ($t = 2.42$, $p > 0.05$) but not MA rats ($t = 0.6947$, $p < 0.05$, *ns*).

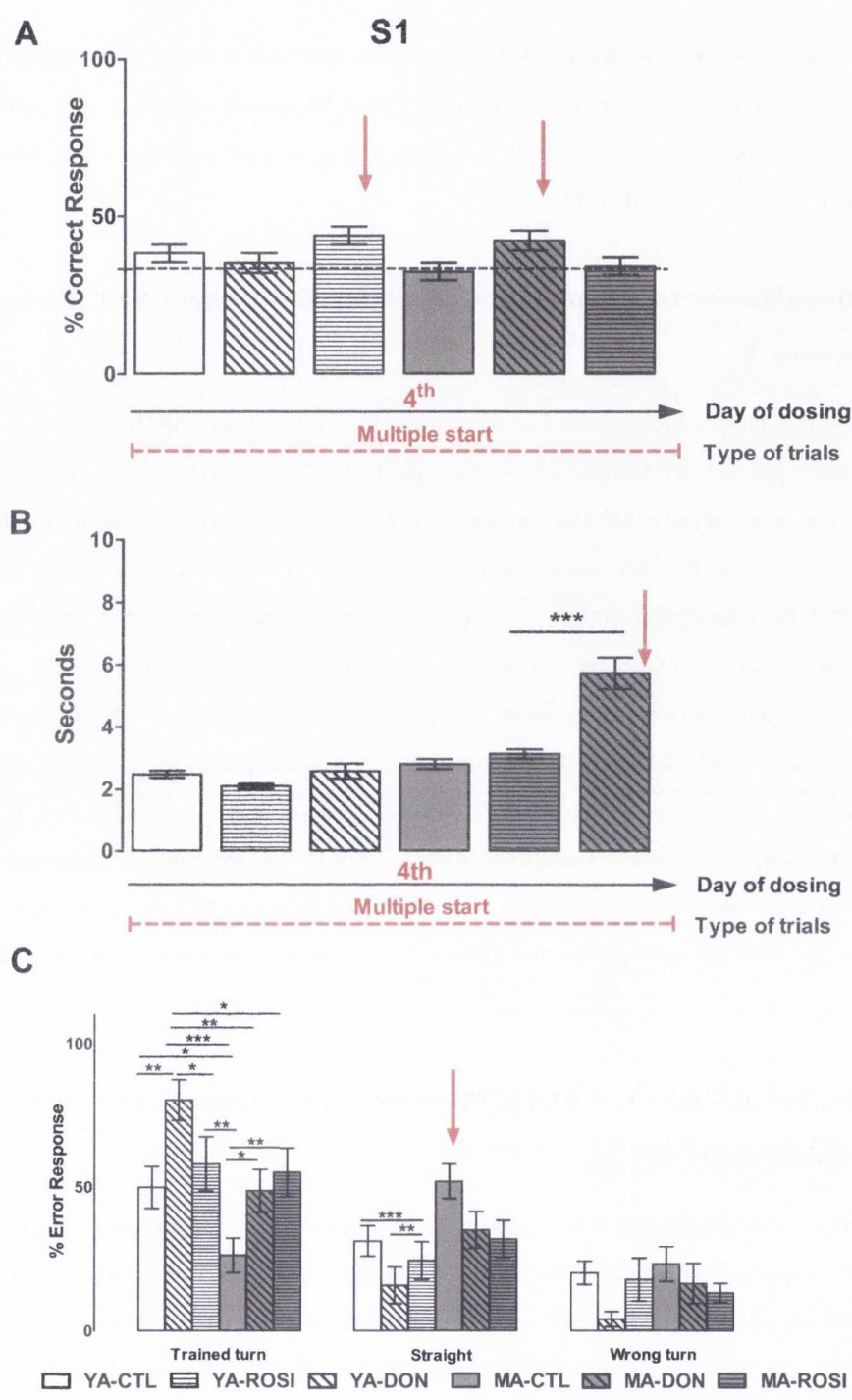


Figure 6.10: **Initial multiple-start trial revealed difficulty for rats to score above chance level.** (A) YAROSI and MADON were able to score above chance level. (B) Latency in MAROSI was distinctly longer than other groups. (C) MA rats with no treatment demonstrated behavioural inflexibility. Red arrows indicate noteworthy differences. All data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

6.3.16 Latency during multiple-start trial not an indicator of task ability

There was a lack of interaction between the two parameters measured in the MS trials for the WPM task. However, the data spread depicted in the graphs revealed that YA rats were behaving more similarly (lesser spread of data) compared to MA rats (large data spread). This trend might suggest that individual variability was present and was more evident in older rats.

6.3.17 Rosiglitazone-treated middle-aged rats unable to acquire multiple-start trial in session 2

The treatment type had a significant effect on latency as revealed by one way ANOVA [$F_{(5,1672)} = 23.04, p < 0.0001$]. Latency increased for both MA treatment groups of rats. However, an increase in latency for MAROSI did not imply an improvement in MS trial performance. Two way ANOVA demonstrated that treatment did not result in significant differences between groups [$F_{(5,522)} = 0.35, p > 0.05, ns$]. Type of errors made [$F_{(2,522)} = 81.3, p < 0.0001$] and the interaction [$F_{(10,522)} = 2.173, p < 0.05$] produced significant differences in data. The high percentage of trained error responses might be responsible for the low performance level.

There was a significant effect of treatment on acquisition in MS trial [$F_{(5,1674)} = 12.66, p < 0.0001$]. Acquisition in the second MS trial revealed differences between treatment groups for MA rats. Under donepezil treatment, increased latency equates to a marked increase in correct response compared to MACTL ($q = 7.206, p < 0.001$). In younger rats, there was no change in latency even though performance improved in treated rats. Hence, latency as an indicator for improvement in MS trial was only true for MA rats. There are no differences in latency between YA rats and it appears that a longer duration in decision zone was not required to solve the task.

6.3.18 Higher multiple-start trial performance was accompanied by lower latency in middle-aged rats

Data from MA rats fell close to the linear regression line suggesting a strong correlation between the parameters investigated, as opposed to YA rats where data was spread apart. This observation deviated from the previous session indicating that different behavioural strategies might be occurring in these MA rats. Rats that acquire the MS trial at a higher level usually spend less time within the designated zone. Although not significant, in treated MA rats, data suggest that In YA rats, there was no particular trend and pattern, different level of performance occurred at different latencies.

6.3.19 Untreated rats unable to acquire strategies to solve multiple-start trial

The final MS trial revealed several differences between age and treatments. Although the percentages of response were not as great as those acquired during fixed-start trials, all young adult rats were able to perform above chance. Age and treatment [$F_{(5,1674)} = 21.16, p < 0.0001$] significantly affected the correct response

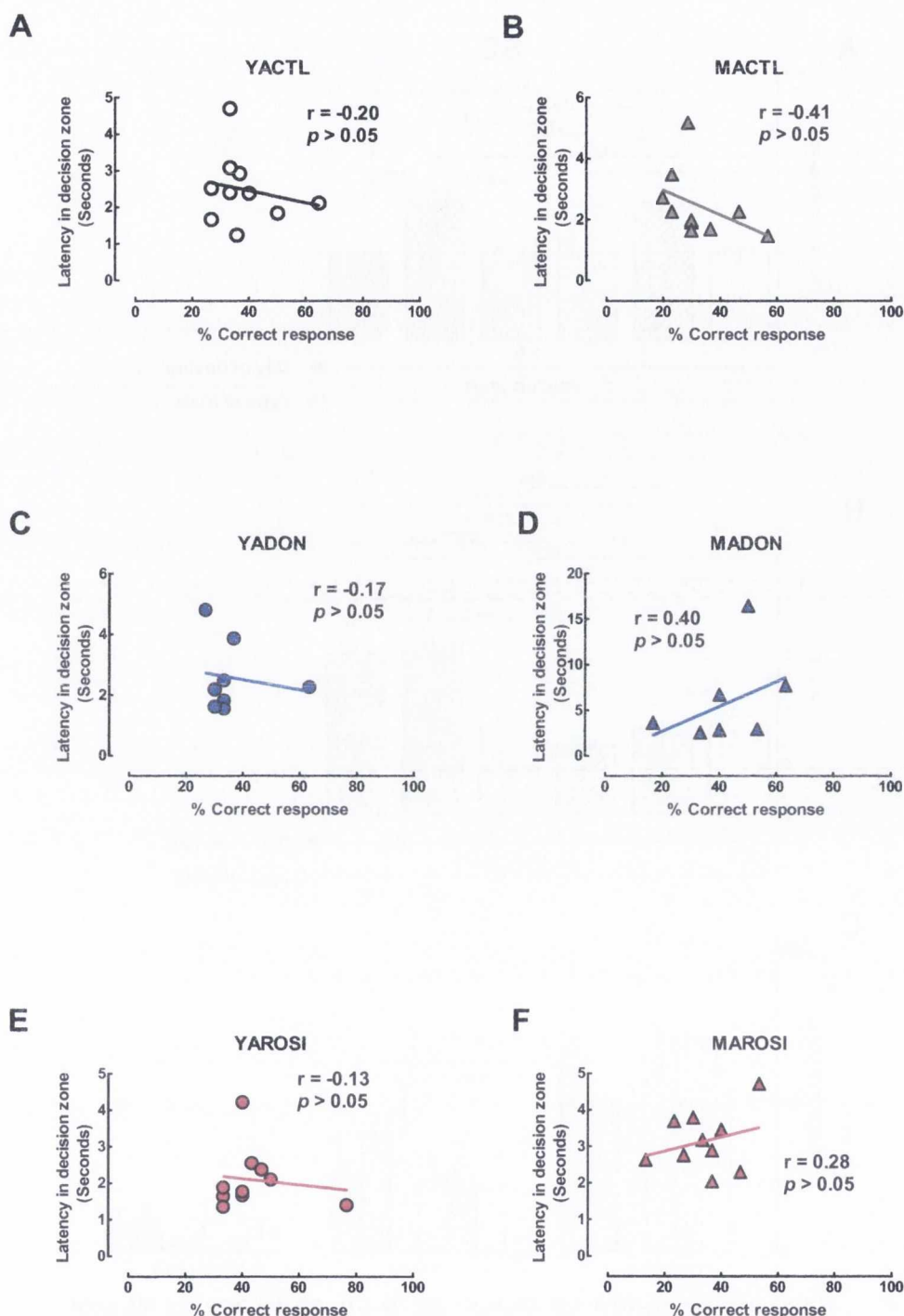


Figure 6.11: Latency in the harder multiple-start trial not an indicator of performance. Latency recorded was comparable in all groups in the initial session. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for MS trials are represented here while each data point represents an individual rat. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

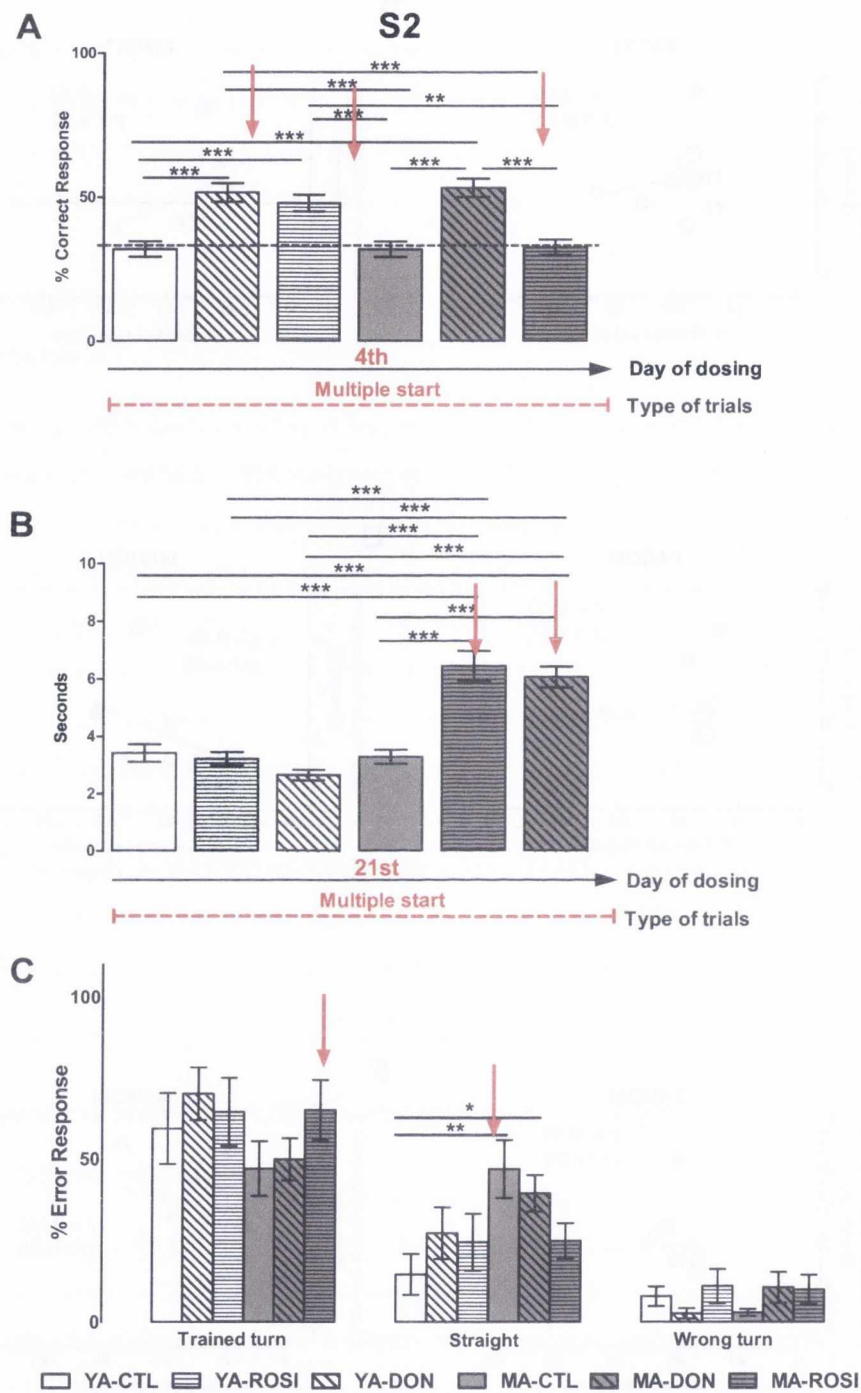


Figure 6.12: Rosiglitazone-treated middle-aged and untreated rats unable to perform above chance in second multiple-start trial trial. All treated rats were able to perform above chance except for MAROSI (A). A longer duration in the decision zone was recorded for treated MA rats (B). Behavioural inflexibility persisted in MACTL rats resulting a low acquisition in MS trial (C). All data are represented as mean $\pm$ S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Red arrows indicate statistically significant outcomes.

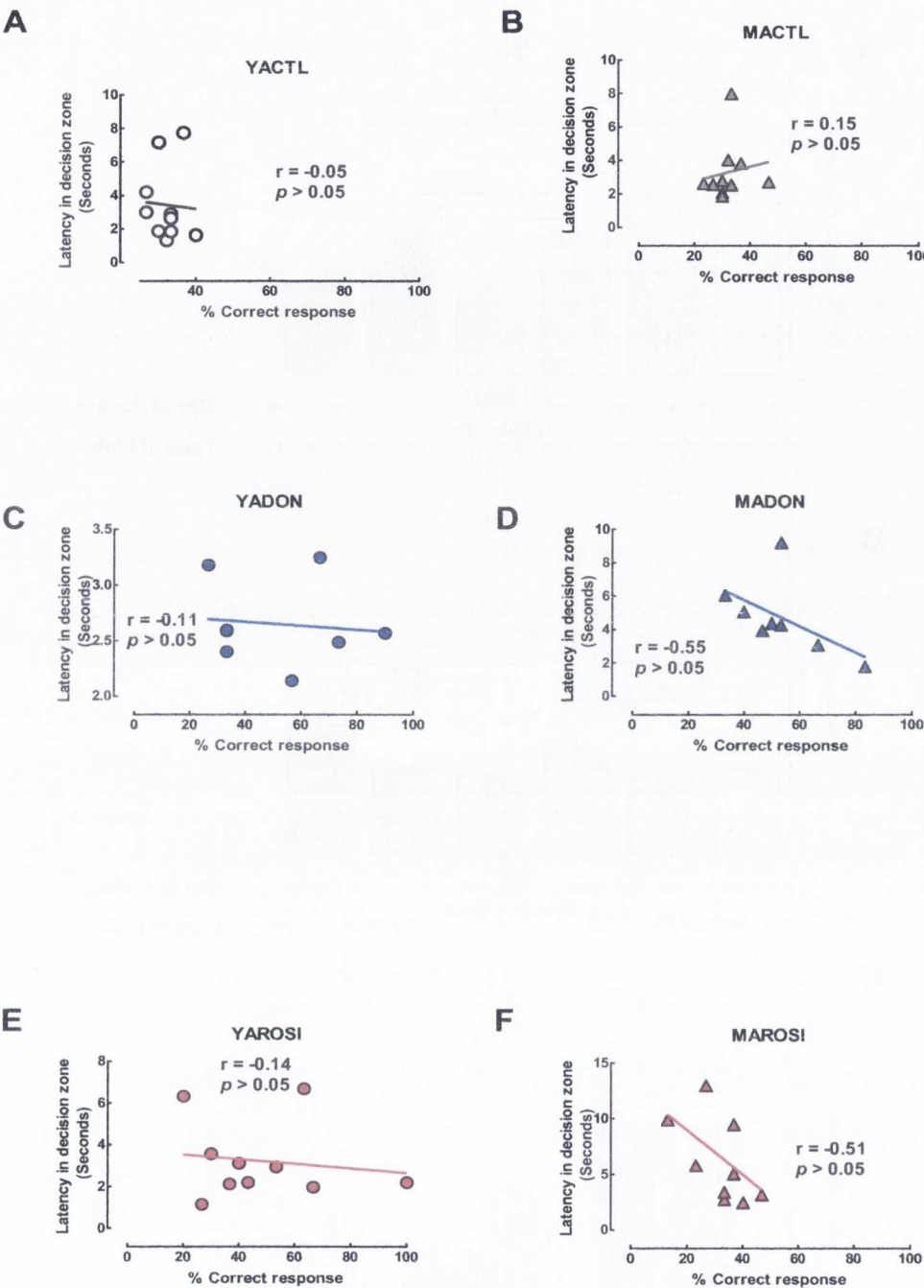


Figure 6.13: **Data for middle-aged rats fell close to trend line in second session.** Latency recorded was higher in treated MA rats in all groups in the second session. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for MS trials are represented here while each data point represents an individual rat. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

level. The MADON group of rats was still superior at MS trial as compared to untreated MA ($t = 4.89$, $p < 0.001$) and MAROSI ($t = 3.77$, $p < 0.01$) rats. Remarkably, rosiglitazone treatment in MA did not escalate MS performance and response level was comparable to MACTL ($t = 1.184$, $p > 0.05$, *ns*). Cholinergic manipulation with donepezil administration allowed MA rats to perform the harder MS trials similar to YACTL ($t = 2.215$, $p > 0.05$, *ns*). MA rats without any treatment is unable to acquire MS task throughout all sessions.

MAROSI continued to persist in the decision area during the MS trial but this did not increase the percentage of correct response. Indeed, MAROSI not only failed to acquire MS trial, these rats also acquired the highest percentage of trained error response. It appears that YADON rats were most able to complete the MS trial. They have a low latency and a variable amount of error (trained, straight and wrong). The ability to provide any behavioural flexibility seemed to be absent in MACTL rats as it is only in the last session where straight swim behaviour was diminished.

6.3.20 Positive correlations between latency and task ability in middle-aged rats.

Older rats acquired the last MS trial at a lower level as compared to YA rats. However, statistically significant correlations between latency in the decision zone and the level of task performance were only evident in MADON ($r = 0.92$, $p < 0.05$) and MACTL rats ($r = 0.64$, $p < 0.05$). In these cases, a higher task ability was accompanied by a longer duration within the decision zone. This gives an indication as to how MADON rats were able to perform on a par with YA rats. Correlation analysis provided graphic evidence that MAROSI rats even though persisting in the decision area did not acquire the MS task.

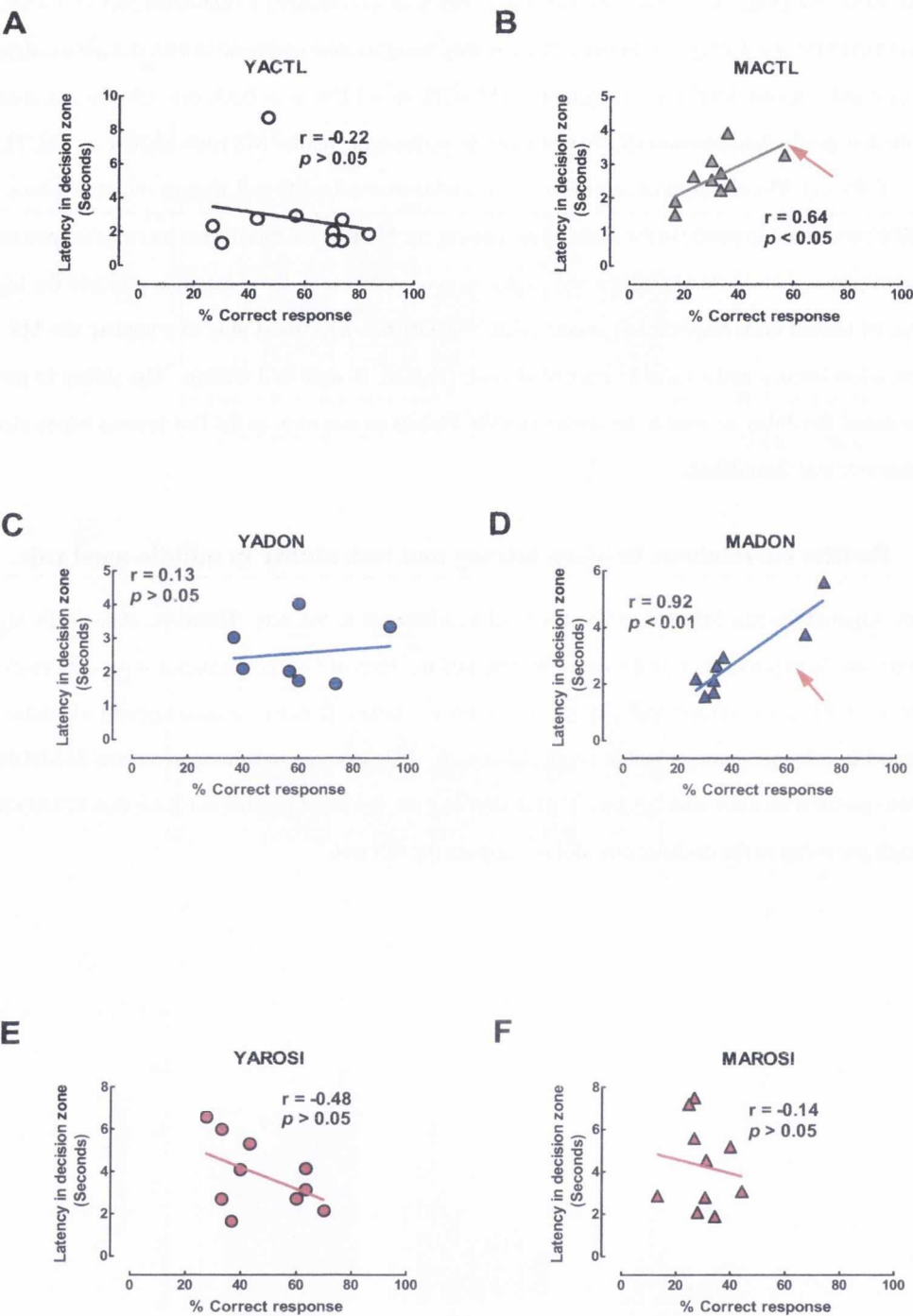


Figure 6.15: **Higher task performance was indicative of a longer latency in older rats.** Longer latency persisted in MAROSI rats although this did not correlate with an increased task ability. All groups of YA (A, C and E) and MA rats (B, D and F) are being represented. (N = 10 for control groups, N = 8 for donepezil-treated groups) Data for MS trials are represented here while each data point represents an individual rat. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

6.4 Clarifying the relationship between molecular changes and behavioural outcomes.

The current chapter also attempts to evaluate physiological alterations witnessed in the studies in regard to the several relevant molecular targets that were analysed. Relevant changes in one target from the chronic study will be discussed in relation to alterations found in the short-term therapy. Correlations between augmented behavioural performances (for both SOR and WPM task) and molecular analyses have also been carried out. Such analyses are crucial in translating behavioural outcomes to physiological alterations, giving indications of the span of drug treatment benefits associated with learning and memory. Under the SOR paradigm, the parameter taken into account for the analysis was for the ODR as it gave the best indication to the task solving ability. For the WPM task, both latency and task scores were taken into consideration. Only data from the MS task (S3) were taken into the analyses as these behavioural outcomes were the closest behavioural conditioning prior to the time of tissue collections (14 hours prior).

To avoid repetition, this chapter concentrates mainly on the biological changes demonstrated in the three main studies, chronic donepezil, rosiglitazone and short-term combination therapy. Results discussed in this section will exclude all others that fail to produce any significant findings which would otherwise be discussed in their relevant chapters. Plasma adiponectin, for example, failed to reveal any changes when compared to donepezil-treated effects. This is not unexpected as current literature have not reported any links between them. Statistical analyses in search of correlations between behavioural and molecular findings were conducted by quantifying the relationship between the two variables. Pearson correlation coefficient (r) determines the relationship between the two parameters selected. A positive value indicates positive correlation while a negative r value means an inverse relationship. A value equating to close to, or equal to zero, predicts that the variables do not affect each other at all. A r value close to one, predicts a perfect correlation. A linear regression was carried out to locate the best line where data are modeled using linear functions, and unknown model parameters are estimated from the data.

6.4.1 Relationship between task performance and neurotrophins levels

Hippocampal brain-derived neurotrophic factor (BDNF) levels

Chronic donepezil administration elevated BDNF levels within the hippocampus of young and middle-aged rats. A strong statistical correlation between these factors were not located for these treated rats (see Figure 6.16). It is important to note that the data spread was close to the line of regression, suggesting that a higher task ability was accompanied by the low BDNF levels within the hippocampus. The two behavioural parameters applied in the correlation analyses were the level of correct response produced and latency within the designated decision zone. A correct response, but not latency, demonstrated positive interactions. YACTL rats produced a strong correlation effect between the levels of BDNF and level of response ($r = 0.66$, $p < 0.05$) where the enhanced ability to solve WPM task is accompanied by high hippocampal BDNF protein levels.

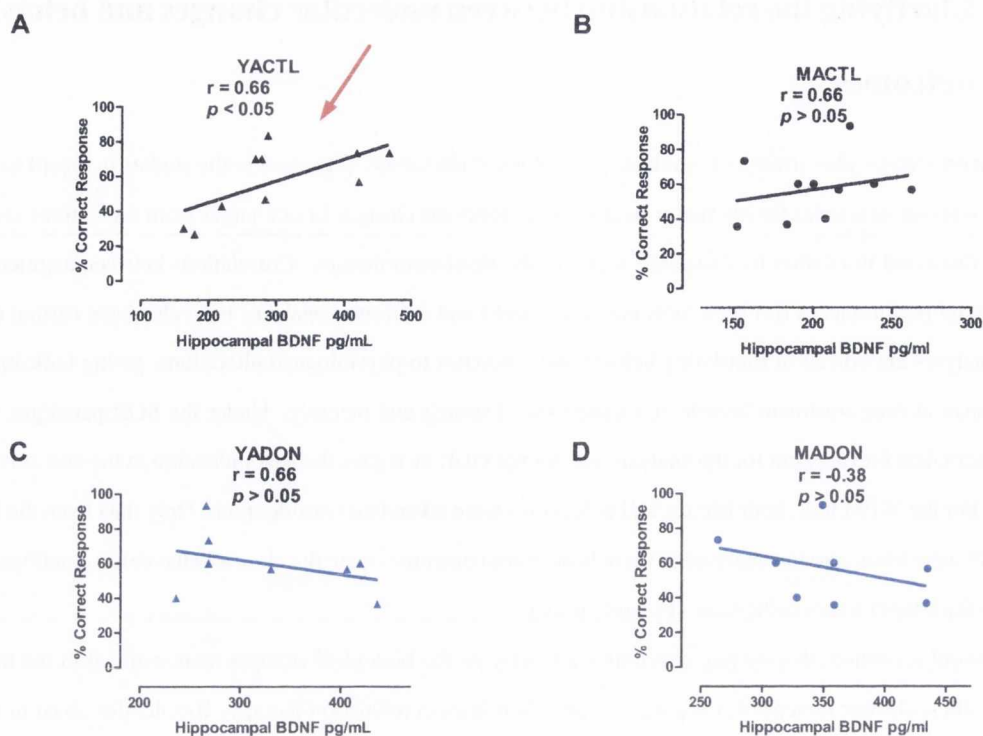


Figure 6.16: Behavioural outcome for Water Plus Maze task correlated with brain-derived neurotrophic factor levels. Hippocampal BDNF protein levels were correlated to level of performance in WPM task. There was a lack of strong correlations in donepezil-treated groups. Data from YACTL rats were strongly correlated where an increase in performance matches the high BDNF levels. YA (A and C) and MA rats (B and D) are being represented ($N = 10$ for control groups, $N = 8$ for donepezil-treated groups). Line across graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

In order to distinguish a clear correlation between task performance and end-point hippocampal BDNF levels, all data from different age and treatment groups were combined for analysis. Even though data from YACTL rats were strongly correlated where an increase in performance matches the high BDNF levels (see Figure 6.16), the lack of correlation failed to suggest an overall connection between these two parameters ($r = 0.13$, $p > 0.05$).

Hippocampal β Nerve growth factor (β NGF)

It has previously been demonstrated that the levels of NGF protein were decreased by 40% in the hippocampus of aged rats (Lärkfors *et al.*, 1987). In the current work, hippocampal β NGF protein levels were elevated under chronic (40 days) donepezil treatment for both young adult and middle-aged rats as compared to control rats. The short-term treatment regime induced an elevated β NGF level only in hippocampus of YADON rats. This trend was absent in middle-aged rats and β NGF levels were similar across treatment groups.

Behaviourally, rats under chronic donepezil treatment acquired an enhanced acquisition and improved behavioural flexibility. Correlation between the level of correct response for the MS trials and β NGF levels was apparent in MACTL rats only ($r = 0.86$, $p < 0.05$). A relationship between these two parameters was not

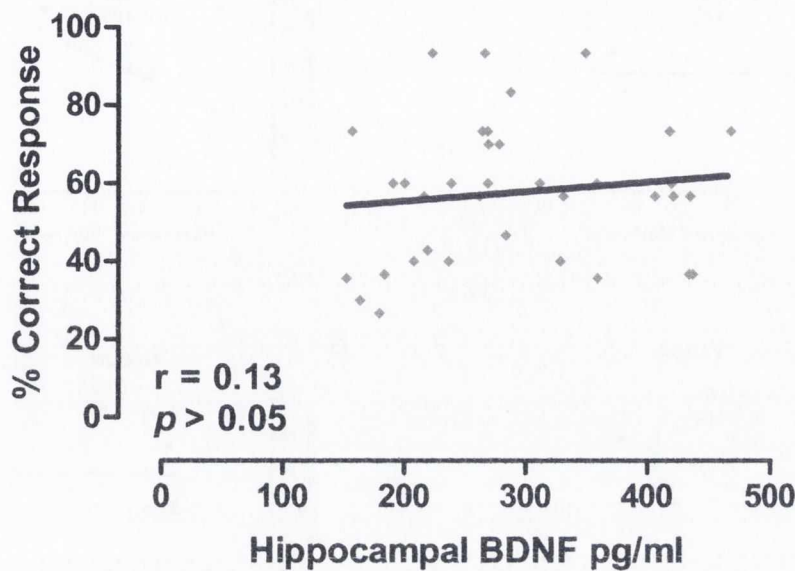


Figure 6.17: No correlation between performance score and brain-derived neurotrophic factor levels. The level of performance in the WPM task has no strong relationship to the hippocampal BDNF protein levels when all groups of rats are pooled together. Line across graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

present in MADON rats ($r = 0.03, p < 0.05$)). Behavioural outcomes in SOR task failed to reveal any interaction with levels of β NGF protein levels.

6.4.2 Relationship between latency in decision zone and hippocampal interleukin-1 β levels

Further analyses revealed an effect of IL1 β expression levels on latency in the decision zone (see Figure 6.20). Augmented IL1 β expression levels indicated a longer latency in the zone for MAROSI rats ($R^2 = 0.46, p < 0.05$). Correspondingly, lower expression levels of the cytokine IL1 β were an indicator of a shorter latency in the designated zone. In untreated MA rats, IL1 β expression levels were unable to give an indication of behavioural outcome ($R^2 = 0.01, p > 0.05$). In YA rats, the correlation analyses failed to reveal any important findings ($p > 0.05$).

In order to distinguish a clear correlation between task performance and endpoint IL-1 β expression levels, all data from different age and treatment groups were combined for analysis. Even though data from MAROSI rats were strongly correlated where augmented IL1 β expression levels indicated a longer latency in the zone (see Figure 6.20), the lack of correlation failed to suggest an overall connection between these two parameters ($r = 0.15, p > 0.05$).

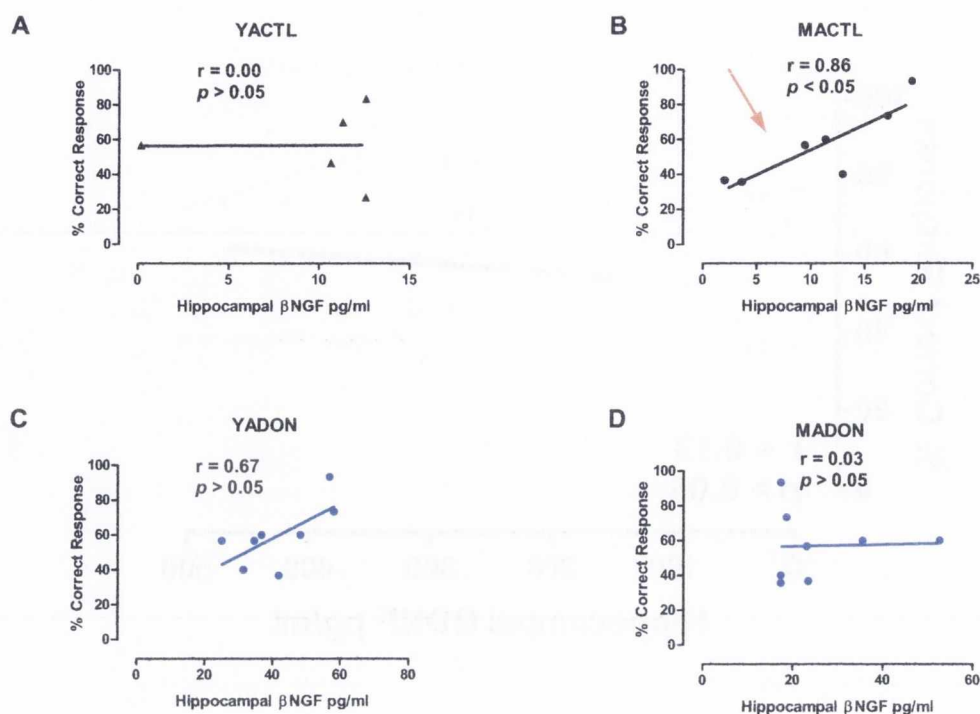


Figure 6.18: **Water Plus Maze task performance correlates to hippocampal β nerve growth factor levels in control middle-aged rat.** Endpoint molecular changes in β NGF protein levels were analysed and correlated with percentage of correct response acquired in WPM task. YA (A and C) and MA rats (B and D) are all represented. Each data point represents an individual rat. Outliers YACTL 1,3,4 and MACTL 18 were removed. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

6.4.3 Administration of different drugs did not affect swim speeds in Water Plus Maze task

ANOVA was performed to determine if drug treatments were having an effect on swim speeds.² In the fixed-start trials, two way ANOVA demonstrated a lack of significant differences for interaction between treatment and swim speeds [$F_{(8,124)} = 1.72$, $p > 0.05$, *ns*]. On the other hand, statistically significant results were present for treatments alone [$F_{(4,124)} = 24.76$, $p < 0.0001$] and none for treatment alone [$F_{(2,124)} = 17.43$, $p < 0.0001$]. Although there are several differences present among the groups, an effect of drug treatment on speed was not demonstrated within the same age group (see Figure 6.22 A).

As for MS trials, two way ANOVA demonstrated statistically significant differences for interaction between treatment and swim speeds [$F_{(8,124)} = 2.11$, $p < 0.05$], for treatments alone [$F_{(4,124)} = 21.33$, $p < 0.0001$] and sessions none for treatment alone [$F_{(2,124)} = 18.15$, $p < 0.0001$]. Once again, MADON rats were able to perform similarly to YA rats and were much quicker in the task than all other MA rats (see Figure 6.22. For example, MADON rats were much quicker in the first two sessions when compared to both MAROSI [S1: ($t = 3.38$, $p < 0.001$); S2: ($t = 3.60$, $p < 0.001$)] and MACTL [S1: ($t = 4.28$, $p < 0.001$); S2: ($t = 5.14$, $p < 0.001$)]

²Unable to locate the original data for swim speeds recorded for YADON rats.

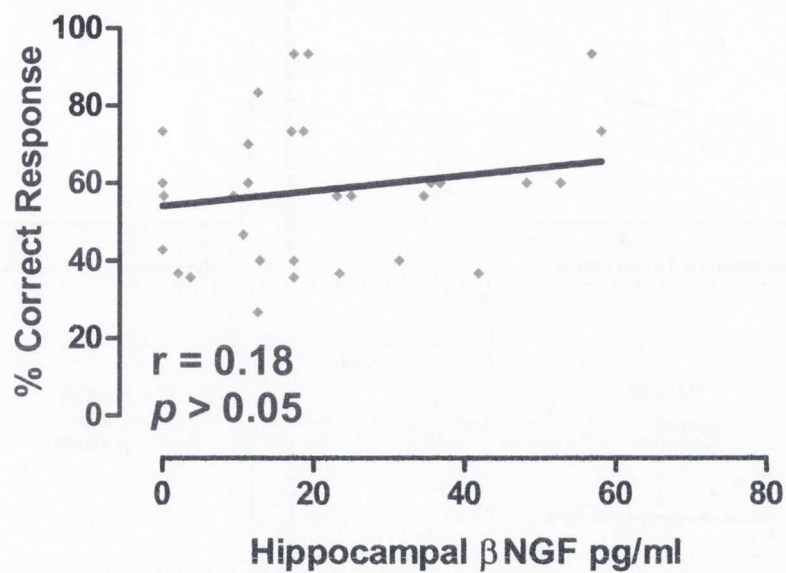


Figure 6.19: **Lack of relationship between Water Plus Maze task performance and hippocampal β nerve growth factor levels.** Endpoint molecular changes in βNGF protein levels were analysed and correlated with percentage of correct response acquired in WPM task. The level of performance in the WPM task has no strong relationship to the hippocampal βNGF protein levels when all groups of rats are pooled together. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

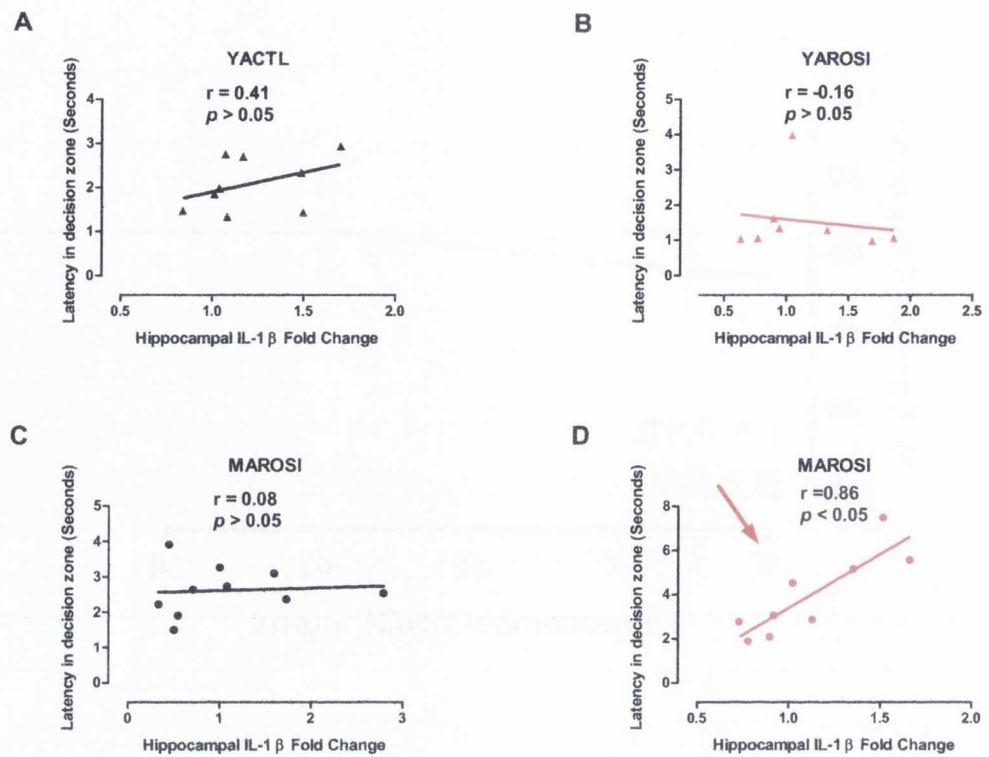


Figure 6.20: **Hippocampal Interleukin-1 β expression levels as an indicator of latency.** Latency in designated zone for the WPM task was correlated with hippocampal cytokine IL-1 β expression levels. All groups of rats were represented here and latency was revealed to be longer for rats with a higher IL-1 β expression levels, particularly for MAROSI rats. Each data point represents an individual rat. Outliers for IL-1 β levels were removed; YACTL 7, YAROSI 20 and MAROSI 37. The line on each graph indicates linear regression (N = 10 per group) Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

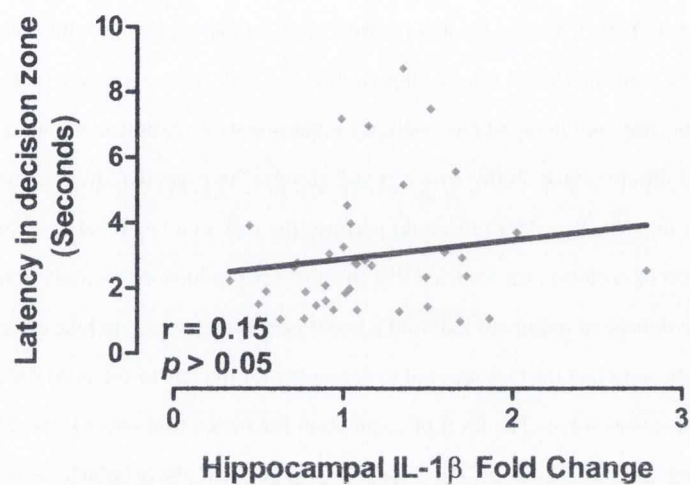


Figure 6.21: No correlation between overall hippocampal interleukin-1 β expression levels and time spent in decision zone. The latency in designated zone for the WPM task has no strong relationship to the hippocampal cytokine IL-1 β expression levels when all groups of rats are pooled together. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

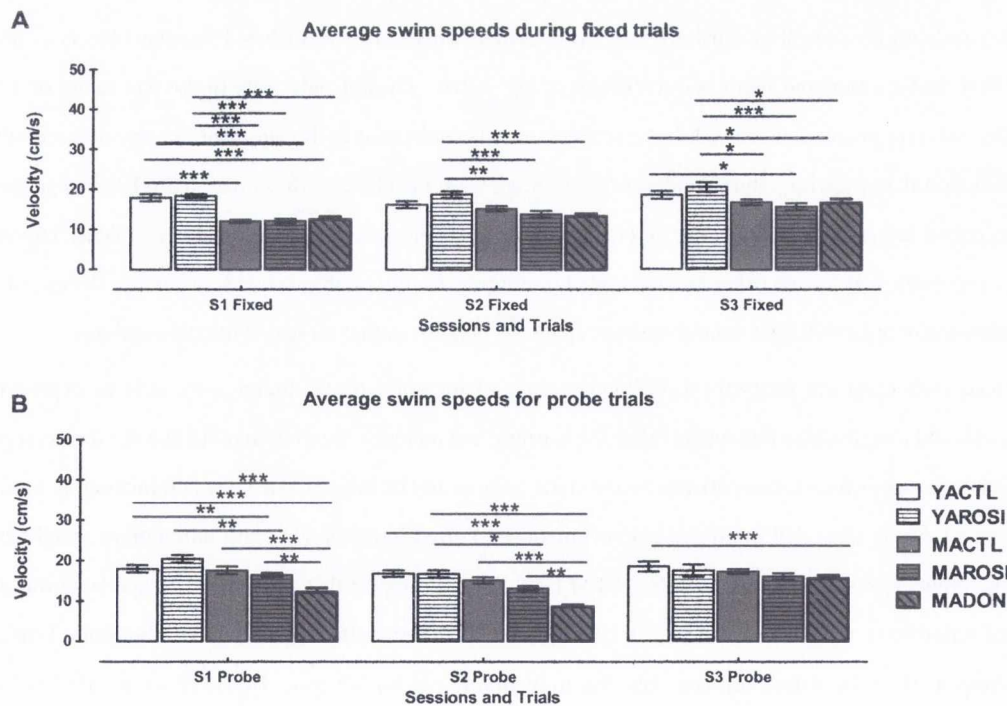


Figure 6.22: Treatment did not affect swim velocity between treatments within the same age groups. Although differences in speed were present between MA and YA rats, treatments did not affect the physiological conditions in these rats. All data are represented as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

6.5 Contrasting outcomes between two different tasks

Detailed analyses of learning and memory between donepezil and rosiglitazone administrations were carried out and several important findings were revealed from the application of the WPM task. Firstly, both drugs improved acquisition in both ages. This demonstrates that pharmacological manipulations with donepezil and rosiglitazone in young adult rats, with predicted functioning cholinergic and glucoregulatory systems, could still augment performance. In healthy middle-aged rats, memory impairments were noticeable with the WPM task employed and this showed different task ability between age groups. As expected, drug administrations enhanced acquisition and more importantly, older rats could perform the task to a level that is comparable to YA rats. Notably, the progression of performance revealed that rosiglitazone influenced memory enhancement acutely (session 1, day 1) while donepezil treatment required a latent period (session 2) to take effect. Ceiling effect (>90%) was achieved indicating that the task was not too complex for the rats to solve. MS trials, on the other hand, were demanding for rats to solve. The level of acquisition for such a trial was 41.2% (average for all MS trials for all sessions and all rats). However, most were able to perform above chance, except control and rosiglitazone-treated middle-aged group of rats. One could argue that even with a high threshold of 90% for the fixed start trial, the MS trial was still too difficult to accomplish completely.

In general, the SOR task employed in the study showed age-related decline in the ability to discriminate between objects, especially at the 24 hour delay. Exploration among the three phases (familiarisation, 1 hour delay and 24 hour delay) was also much lower compared to younger rats. Preference for novel object was in the negative range which implied that rats were exploring the sample object more than the novel one. Such a behaviour could be a result of a pattern separation deficit, where older rats treated familiar objects as novel, as if they had encountered them before (Burke *et al.*, 2010). Overall older rats in the age range of 13-14 months old were unable to accomplish the recognition task and failed to discriminate the object successfully. Combination therapy with administration of both drugs were unable to address this age-related impairment. However, monotherapy with donepezil and rosiglitazone alone increased exploration at 1 hour delay, enhanced object recognition at longer delay by augmented preference for novel objects. MA rats under donepezil and rosiglitazone treatment did not exhibit pattern separation deficits, unlike untreated middle-aged rats.

Since both tasks are focused on different aspects of memory, these studies were able to expose how donepezil and rosiglitazone treatments influence learning and memory. Bearing in mind that the dosing regime for both tasks are different, comparisons between the tasks would be limited to a 5 day administration timeline. It is speculative to state that improved glucoregulation occurred immediately upon initiation of rosiglitazone dosing. Nonetheless, the results from the WPM task did give support that learning to escape from the plus-shaped water maze was enhanced in rats for both ages under an acute effect (1 day) of rosiglitazone. A 6mg/kg rosiglitazone dose for 5 days did not alter discrimination levels for YA rats. However, in middle-aged rats, ability to differentiate between novel and familiar object was significantly augmented.

Since both donepezil and rosiglitazone were able to enhance the WPM task performance individually, it was hypothesised that a combination therapy could boost cognitive functions as well. In fact, such a combination

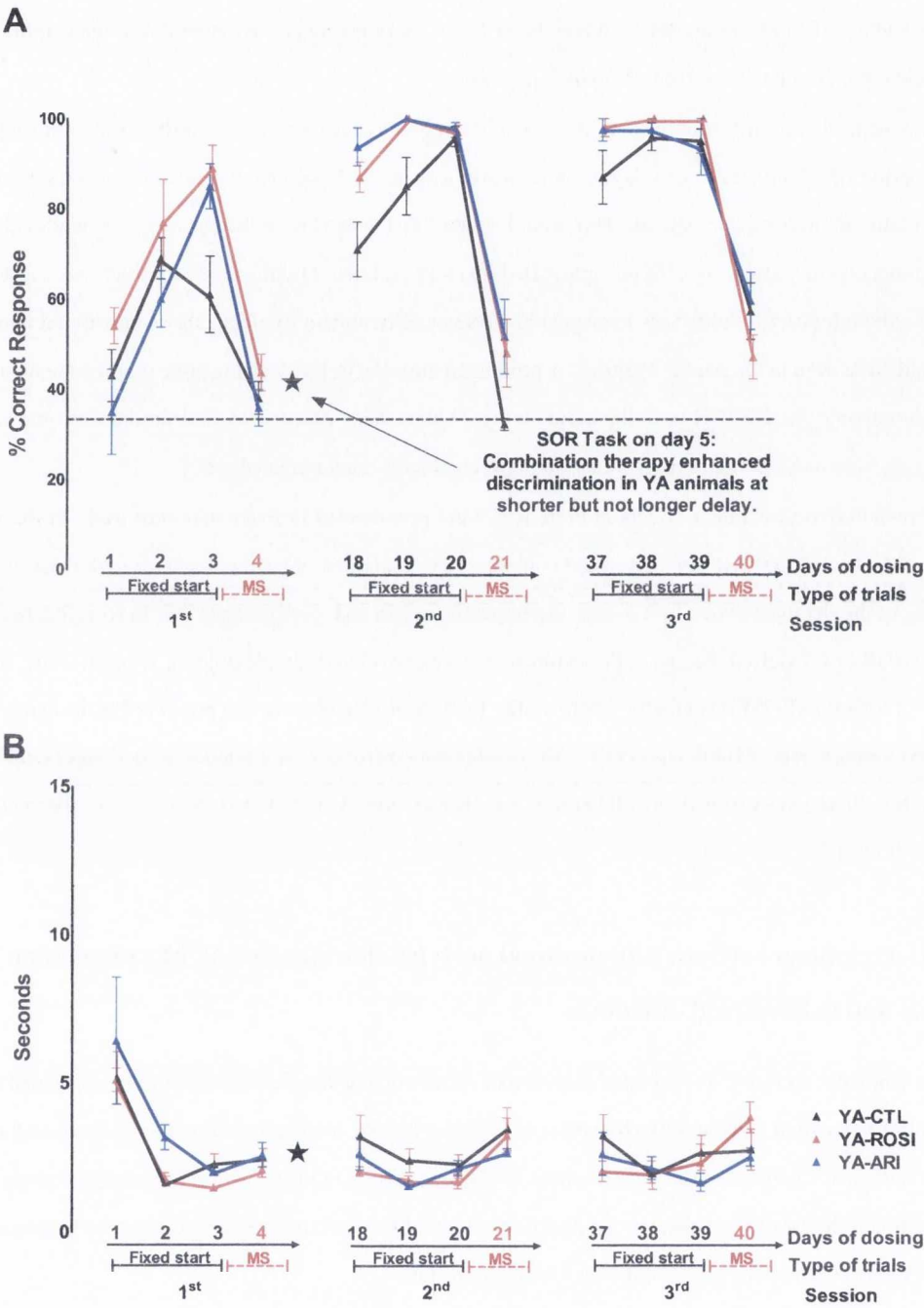


Figure 6.23: **Comparative analyses of behavioural tasks for young adult rats.** Acquisition for all trials and sessions for all groups are represented here. Rosiglitazone treatment resulted in an increased acquisition of WPM task for both ages. Combination therapy failed to improve discrimination in YA rats at longer delay. ★ symbol indicate when SOR task began on the same drug dosing timeline. Detailed analysis see Figure 6.25. All data are represented as mean ± S.E.M.

therapy only succeeded in enhancing SOR task performance in MA rats at longer delay while none of the three treatments had an effect at shorter delay. When the task was more taxing at the longer delay, the combination therapy failed to enhance the discrimination in younger rats.

While donepezil administration in YA rats was able to elicit a correct response in the fixed-start trials for the short period of administration (4 days), an improvement in the SOR task was also demonstrated by an increased ability to discriminate objects. This would suggest that a short term dosing was sufficient to elevate cognitive functions in young rats with no age-related memory deficits. Middle-aged rats were less capable in both tasks although task difficulty was manageable. Donepezil treatment in older rats demonstrated a higher rate of acquisition even in the harder MS trial. A prolonged duration in the decision zone might be responsible for such an outcome. In the SOR task, the more taxing 24 hour delay phase delivered similar outcome where MADON rats were much better at discrimination between objects than untreated rats.

Acute rosiglitazone treatment effects in both ages were pronounced in fixed-start start trial. In the more demanding MS trial, MAROSI rats performed comparably to untreated older rats for both performance level and latency in the decision zone. In YA rats, the improvement in task performance due to rosiglitazone was slight. The SOR task required the rats to discriminate between novel and sample objects. Rosiglitazone, in this case, did not affect YAROSI rats greatly where ability to discriminate objects was preserved at the same level as untreated younger rats. Middle-aged rats with rosiglitazone treatment fared better at the longer delay and preference for objects was distinctively higher than all other groups. As mentioned above, MA at shorter delay performed similarly.

6.5.1 Correlations between hippocampal acetylcholinesterase (AChE) expression levels and behavioural outcomes

Within the senescent rat brain, a substantial decrease in AChE content was demonstrated to be correlated to an impairment in acquisition and memory (Biegon *et al.*, 1986). Notably, in the previous work (see section 5.10 on page 113), statistical significant effect of treatment on AChE expression levels within hippocampal tissue was not demonstrated. In the present chapter, it was possible to establish several correlations between hippocampal AChE mRNA levels and behavioural outcomes for the SOR task.

An inverse correlation was demonstrated for middle-aged rats where a high discrimination ratio corresponded to low AChE expression levels in untreated ($r = -0.89$, $p < 0.01$) and rosiglitazone-treated ($r = -0.91$, $p < 0.01$) rats hippocampus (see Figure 6.26A and C). Interestingly, in younger rats treated with donepezil, a similar trend was demonstrated but of a positive nature ($r = 0.74$, $p < 0.05$). An augmented ability to discriminate between objects correlates to higher AChE expression but only for the longer delay. Taking into consideration for the small data sample, all other groups fail to reveal a trend between AChE levels and all other behavioural indexes.

The inverse correlation demonstrated for middle-aged rats where a high discrimination ratio corresponded to low AChE expression levels was not reflected when all data are combined for analysis ($r = -0.17$, $p > 0.05$).

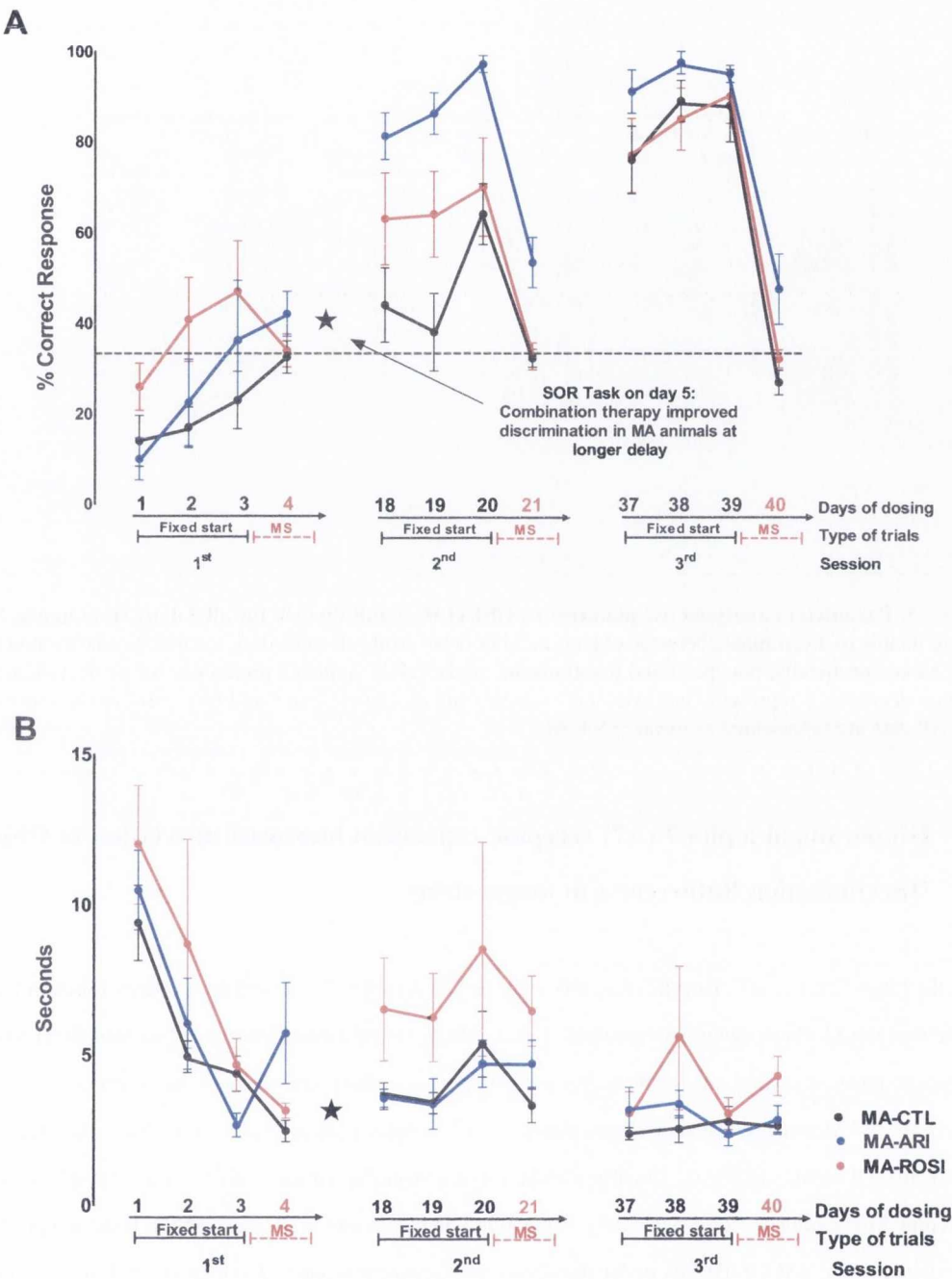


Figure 6.24: **Comparative analyses of behavioural tasks for middle-aged rats.** Acquisition for all trials and sessions for all groups are represented here. In older rats, both treatments improved recognition memory. Combination therapy improved discrimination in MA rats at longer delay. ★ symbol indicates when SOR task began on the same drug dosing timeline. Detailed analysis see Figure 6.25. All data are represented as mean ± S.E.M.

NOR		Ability to discriminate novel object (O.D.R > 0.5)		Exploration span (% time spent exploring)		Preference for novel objects (seconds)	
		1 hr delay	24 hour delay	1 hr delay	24 hour delay	1 hr delay	24 hour delay
Young adults (3 months)	CTL	Yes (0.6 ± 0.03)	Yes (0.6 ± 0.03)	High (28.4 ± 2.9)	Less compared to 1hr delay (16.2 ± 1.4)	(6.75 ± 3)	↔ compared to 1 hr delay (5.7 ± 1.7)
	DON	↑ (0.7 ± 0.02)	↑ (0.7 ± 0.04)	↓ (25.0 ± 2.0)	↑ (20.8 ± 2.0)	↑ (21.9 ± 3.4)	↑ (14.0 ± 2.5)
	ROSI	↔ (0.6 ± 0.03)	↔ (0.6 ± 0.04)	↓ (21.8 ± 2.4)	↑ (25.5 ± 1.5)	↑ (10.3 ± 2.6)	↑ (6.7 ± 3.2)
	COMBI	↑ (0.7 ± 0.03)	↓ (0.5 ± 0.03)	↔ (27.3 ± 2.9)	↑ (20.7 ± 3.2)	↑ (13.6 ± 1.9)	↔ (5.3 ± 3.6)
Middle-aged (13 months)	CTL	Yes (0.6 ± 0.04)	Fail ± 0.05)	(0.4 Low ± 2.9)	(12.5 ↔ compared to shorter delay (14.5 ± 2.3)	(5.3 ± 1.3)	much less compared to shorter delay (- 1.6 ± 2.1)
	DON	↔ (0.6 ± 0.03)	↑ (0.5 ± 0.04)	↑ (20.3 ± 2.7)	↔ (16.1 ± 2.2)	↑ (8 ± 3.9)	↑ (2.7 ± 1.9)
	ROSI	↔ (0.6 ± 0.03)	↑ (0.6 ± 0.05)	↑ (20.2 ± 2.9)	↔ (14.2 ± 2.1)	↑ (8.5 ± 2.4)	↑ (3.3 ± 2.7)
	COMBI	↔ (0.6 ± 0.03)	↑ (0.5 ± 0.03)	↔ (15.0 ± 4.1)	↔ (15.3 ± 3.0)	↔ (5.6 ± 1.4)	↔ (-0.8 ± 2.1)

Figure 6.25: **Parameters analysed in Spontaneous Object Recognition task for all 3 drug treatments.** MA rats were unable to discriminate between objects at 24 hr delay while all three drug treatments ameliorated this deficit. More specifically, donepezil and rosiglitazone increased the animal’s preference for a novel object. ↓ represents decrease, ↑ represents increase and ↔ represents no change compared to control in the same age group. All data are represented as mean ± S.E.M.

6.5.2 Hippocampal alpha 7 (α7) receptor expression increased in relation to Object Discrimination Ratio scores at longer delay

Within the hippocampus, α7 nicotinic receptors were demonstrated to be critical for memory function while nicotinic receptor blockade can impair memory (Levin, 2002). Investigations into the α7 receptor level within the hippocampus was carried out for both chronic (40 days) and short-term (5 days) treatment regime. The data from the WPM study revealed an augmentation of α7 receptor protein levels within the hippocampus of donepezil-treated middle-aged rats. Notably, short-term treatment did not raise any significant findings under the different drug administrations. Separately, behavioural outcomes and α7 expression levels did not produce a large disparity for YACOMBI rats under the short-term treatment regime. Correlation analyses, however, revealed a positive correlation between α7 receptor expression levels and ODR scores at the longer delay. This trend was found in all YA rats, particularly so for YACOMBI ($r = 0.87$ $p < 0.001$). Although not statistically significant, it is important to note that the correlations trend demonstrated in MA rats were of a negative nature as compared to younger rats (see Figure 6.28 E to F). Further correlations for the short delay and WPM behavioural outcomes were not evident in the statistical analyses.

There is a lack of a significant relationship when all data from all age groups and treatment groups is combined for analysis ($r = 0.097$, $p > 0.05$). Levels of α7 receptor expression had no indication for ODR scores.

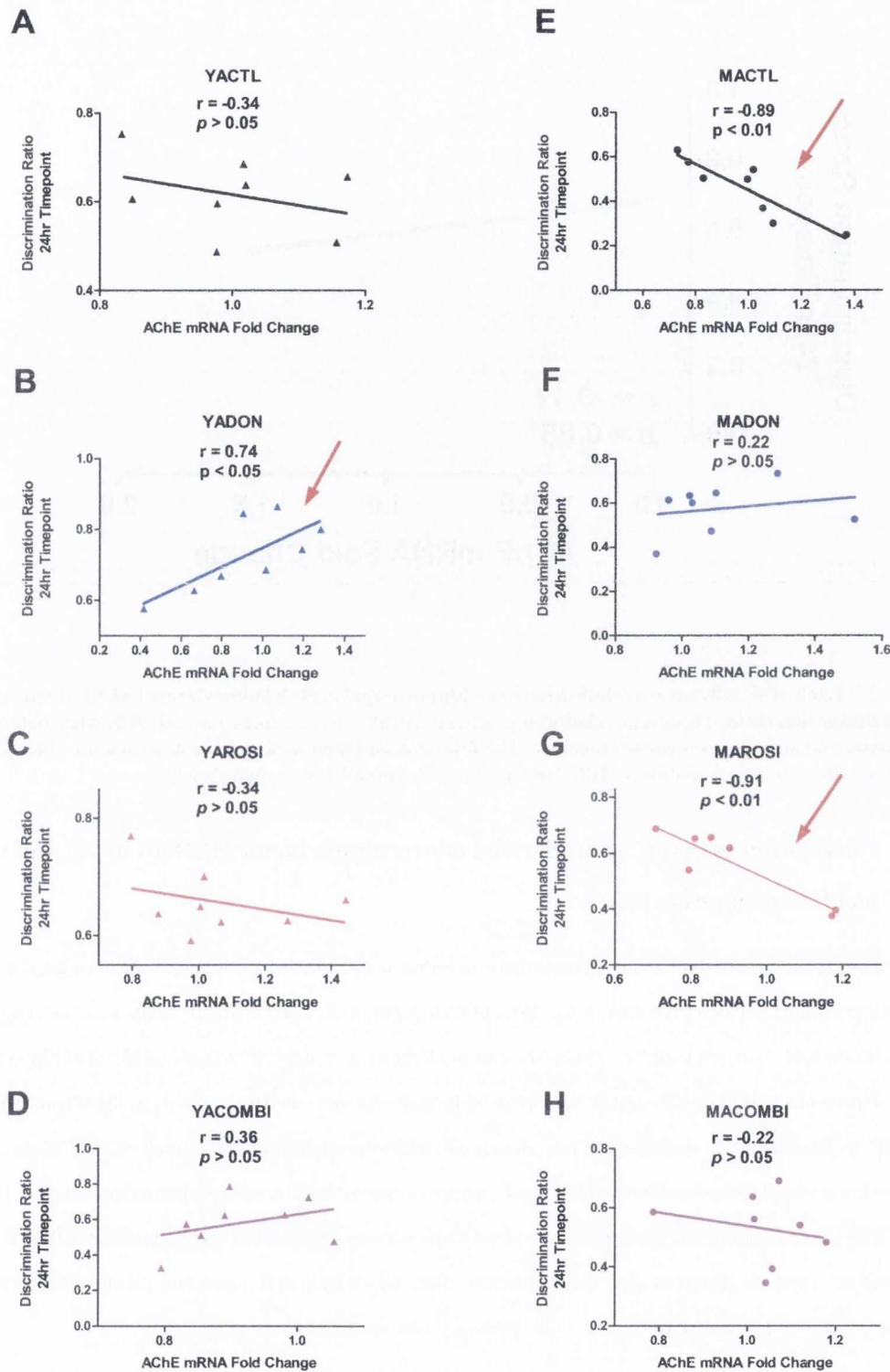


Figure 6.26: **Inverse correlation between hippocampal acetylcholinesterase expression and discrimination ratio in middle-aged rats.** In younger rats, the positive correlation between AChE levels and discrimination ability was revealed. Correlation analyses for YA (A to D) and MA (E to H) rats are represented here. Each data point represents an individual rat. ($n = 8$ per group) The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

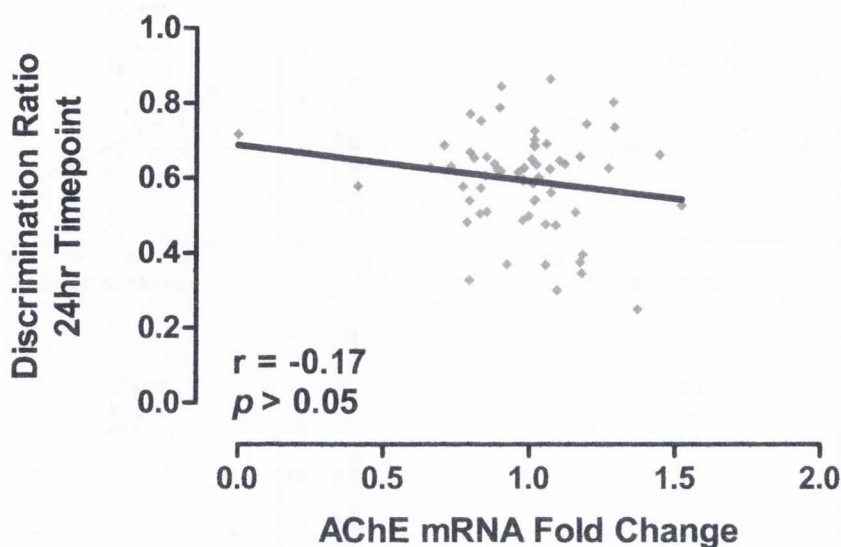


Figure 6.27: **Lack of significant correlation between hippocampal acetylcholinesterase (AChE) expression and discrimination ratio.** There is no relationship between AChE mRNA expression and ODR when data from all treatment and age groups is pooled together. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

6.5.3 Correlation between brain-derived neurotrophic factor (BDNF) levels and Object Discrimination Ratio

Increased expression of BDNF in the hippocampus, together with exercise, have been well correlated to enhanced hippocampal plasticity (Griffin *et al.*, 2009; O'Callaghan *et al.*, 2007). BDNF levels were investigated in both chronic and short-term studies. Under the chronic treatment, donepezil induced an elevated hippocampal BDNF protein levels in middle-aged rats. The short-term treatments failed to produce any significant effects on BDNF levels although in middle-aged rats, donepezil induced a slight increase as well. Correlations analyses between molecular data and behavioural performances demonstrated a strong relationship between ODR at both delays (see Figure 6.30), percentage of correct response (see Figure 6.16) and hippocampal BDNF levels. However, when all groups of data was combined, there was a lack of a significant relationship between hippocampal BDNF levels and the ability to discriminate between objects (see Figure 6.31).

6.5.4 Relationship between object discrimination ratio and plasma insulin levels

The presence of insulin receptors and insulin-second messengers systems in neurons in the central nervous system is widespread (Havrankova *et al.*, 1978) and animal model research indicates that insulin deficiency might be responsible for impairments in synaptic plasticity and cognitive processes. In the 5 day treatment study, there was no change in plasma insulin levels when comparing between age and treatment groups (see

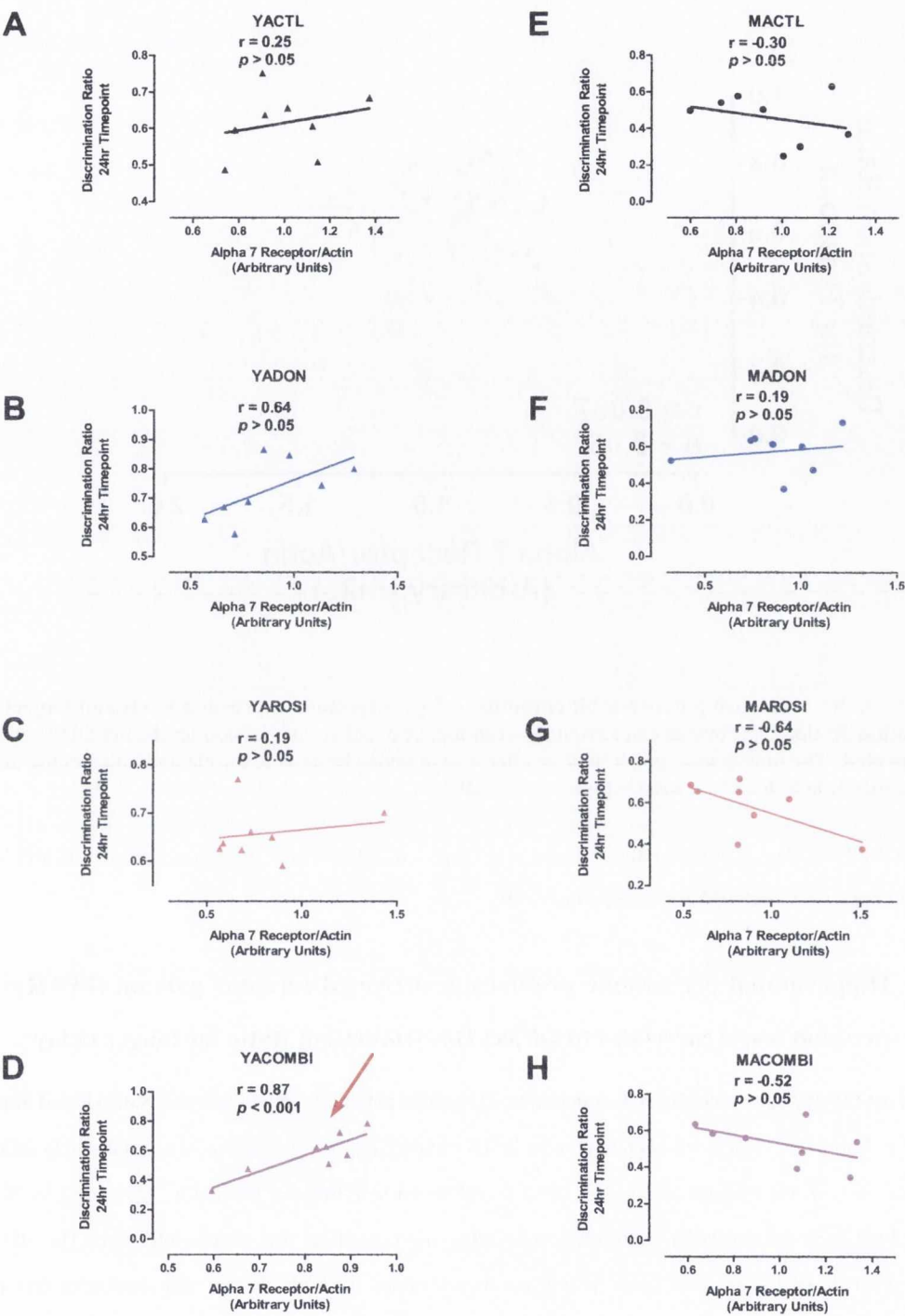


Figure 6.28: **Positive correlation between hippocampal $\alpha 7$ ($\alpha 7$) receptor expression levels and Object Discrimination Ratio.** At the longer delay, ability to discriminate in younger rats corresponded positively to $\alpha 7$ receptor expression levels. Outlier MAROSI 24 and YAROSI 20 removed from data set. Each data point represents an individual rat. (n = 8 per group) The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Red arrows indicate statistically significant outcomes.

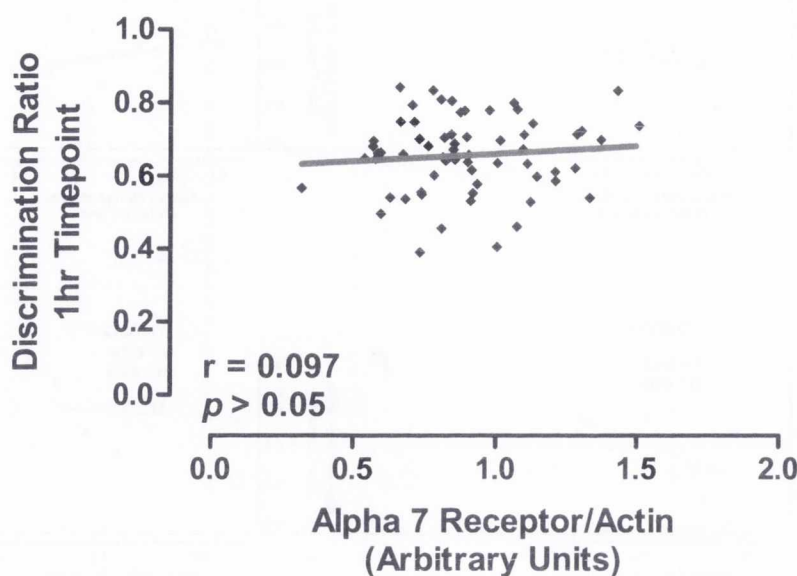


Figure 6.29: **No relationship between hippocampal $\alpha 7$ ($\alpha 7$) receptor expression levels and Object Discrimination Ratio.** No obvious connection between hippocampal $\alpha 7$ expression levels and ODR when all data is pooled. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

Figure 5.8). However, a positive relationship ($r = 0.77$, $p < 0.05$) was present between plasma insulin levels and ODR scores for YAROSI rats (see Figure 6.32).

6.5.5 Hippocampal peroxisome proliferator-activated receptor gamma ($PPAR\gamma$) expression levels correlates to Object Discrimination Ratio for longer delay

Agonists of $PPAR\gamma$ have been shown to ameliorate AD-related pathology in animal models of AD and improve cognition (Landreth, 2007). Investigations into $PPAR\gamma$ expression levels at behavioural end-points failed to reveal any significant findings under both chronic and 5 day rosiglitazone treatment. Comparisons of data collected between the two tasks is not recommended as the task effect was unaccounted for. The effect of treatment on $PPAR\gamma$ expression levels within the hippocampus for both chronic and short-term therapy is described in their relevant chapters.

Correlation analyses were carried out to decipher the link between the behavioural outcomes observed and the physiological changes within the hippocampus. Although rosiglitazone is a $PPAR\gamma$ agonists, there was a failure to locate any relationship between these two parameters. However, analyses for YACOMBI ($r = 0.73$, $p < 0.05$) and MADON ($r = -0.9$, $p < 0.01$) revealed a strong interaction between ODR at 24 hour delay and hippocampal $PPAR\gamma$ expression levels. Increases in hippocampal $PPAR\gamma$ mRNA levels corresponded to an increase in ODR. This trend was inverse in MADON rats since the r value was negative. Notably, this inverse pattern was also present for all other YA groups and MACTL rats. However, there is a lack of relationship

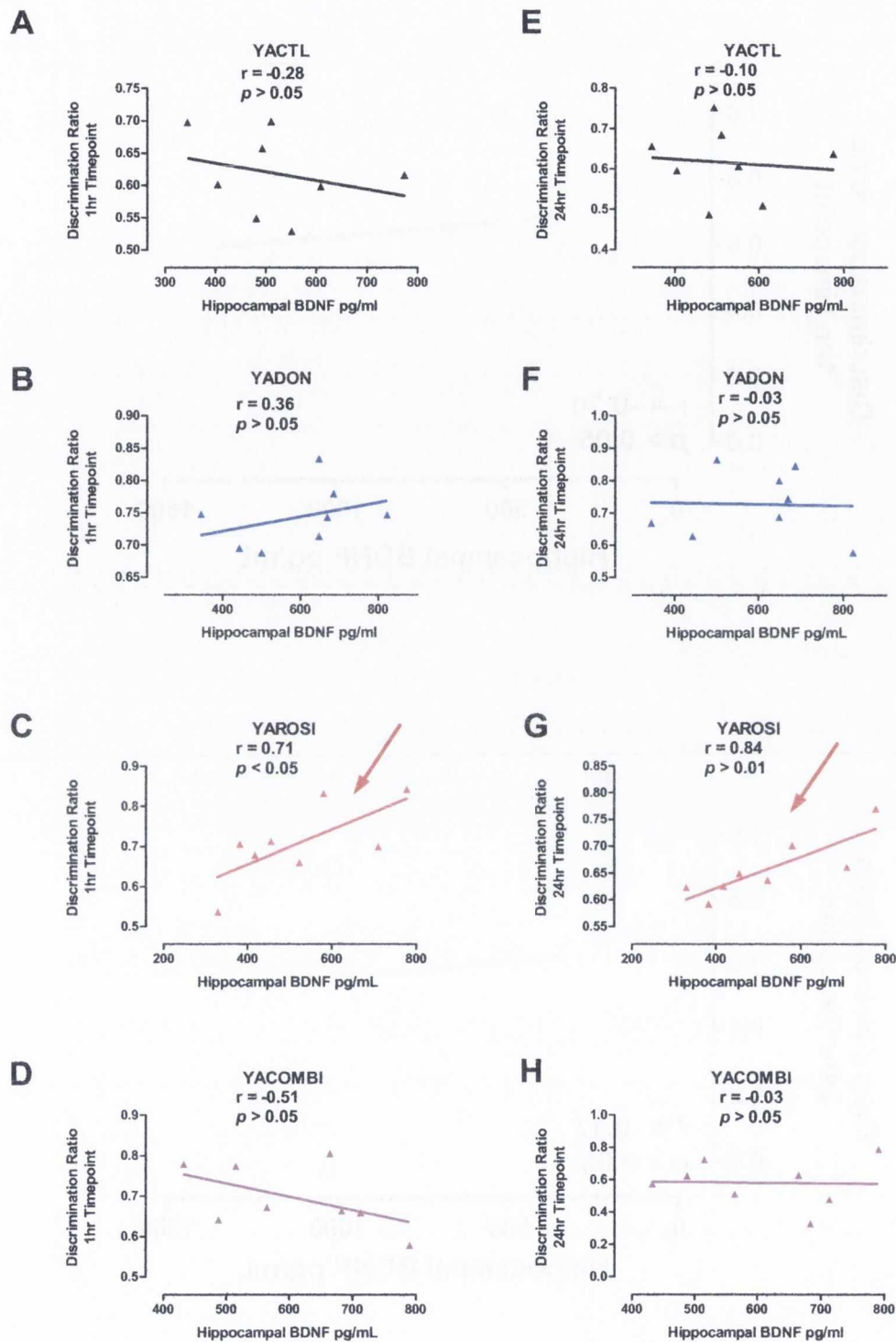
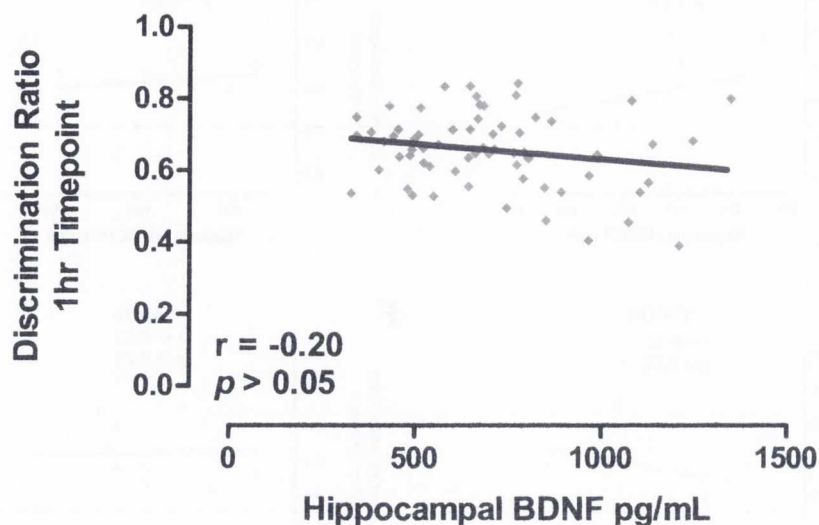


Figure 6.30: **Strong positive relationship between hippocampal brain-derived neurotrophic factor levels and ODR** Correlation between ODR and BDNF levels were analyses for both 1 hour (A to D) and 24 hour (E to H) delays. Hippocampal BDNF levels in YAROSI rats, on both occasion, demonstrated strong correlation to ODR score. The better the discrimination, the higher the protein expression levels. Each data point represents an individual rat. (n = 8 per group) The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. The significance of regression is represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Red arrows indicate statistically significant outcomes.

A



B

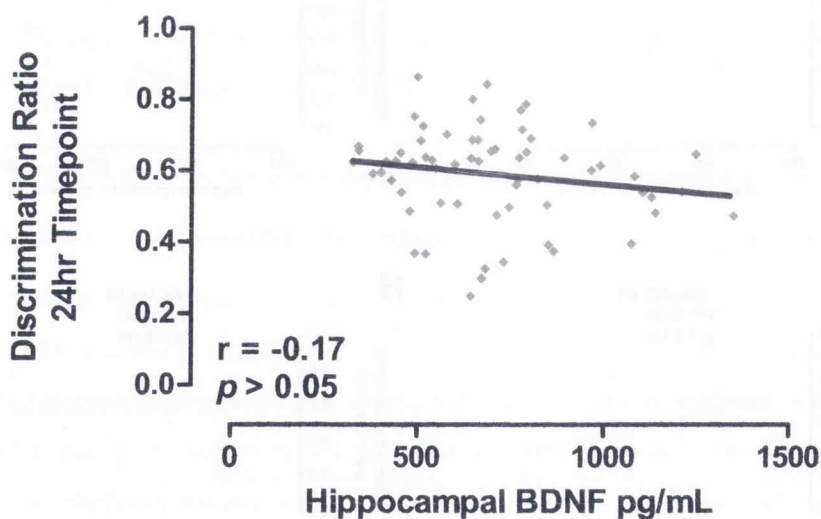


Figure 6.31: No relationship between hippocampal brain-derived neurotrophic factor (BDNF) levels and Object Discrimination Ratio. There is no clear relationship between hippocampal BDNF levels and ODR. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

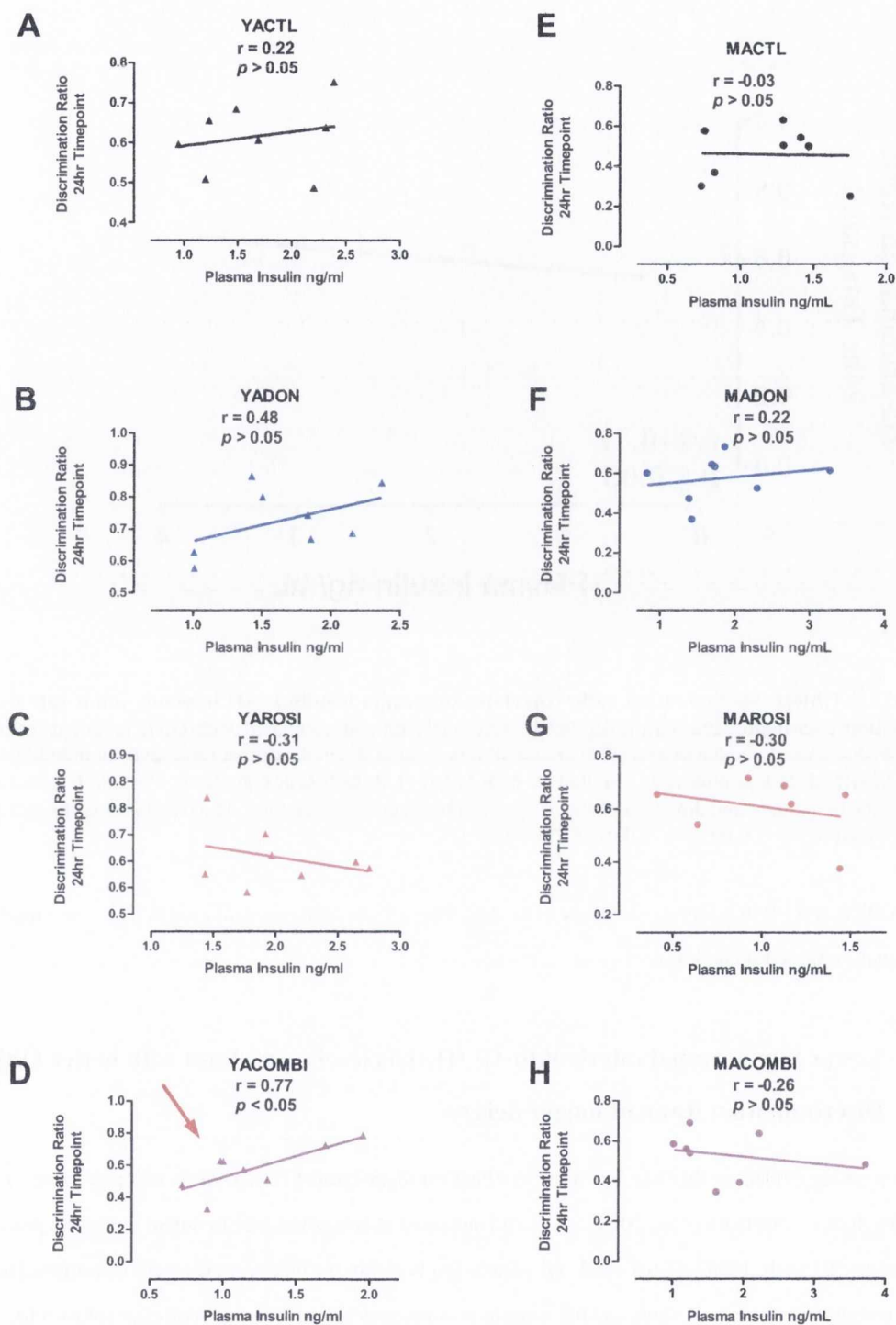


Figure 6.32: **Object discrimination ratio correlates to plasma insulin levels in young adult rats under combination treatment.** Plasma insulin levels were analysed and correlated with ODR recorded from the SOR task. YA (A to D) and MA rats (E to H) are all represented. Each data point represents an individual rat. Outlier MAROSI 21 was removed. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. The significance of regression is represented by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Red arrows indicate statistically significant outcomes.

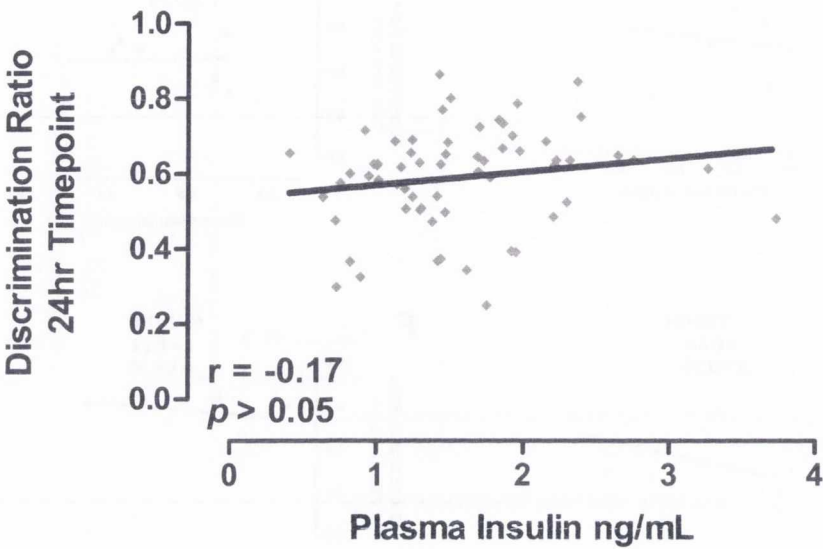


Figure 6.33: **Object discrimination ratio correlates to plasma insulin levels in young adult rats under combination treatment.** Plasma insulin levels were analysed and correlated with ODR recorded from the SOR task. YA (A to D) and MA rats (E to H) are all represented. Each data point represents an individual rat. Outlier MAROSI 21 was removed. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. The significance of regression is represented by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

between ODR and PPAR γ expression levels when data from all age and treatment groups were combined and further analysed (see Figure 6.35).

6.5.6 Lower hippocampal interleukin-1 β (IL-1 β) levels correlates with better Object Discrimination Ratio at longer delays

There are strong evidences that age has a direct effect on hippocampal IL-1 β levels (Murray *et al.*, 1997; Rachal Pugh *et al.*, 2001; Jin *et al.*, 2008) where and increased in concentration can impair long-term potentiation (Murray & Lynch, 1998). Cytokine IL-1 β protein levels within the hippocampus were investigated in the chronic rosiglitazone treatment study and the short-term combination study. Therapeutic effect of rosiglitazone on age-related IL-1 β levels were not demonstrated in the chronic study. Similarly, hippocampal IL-1 β expression levels were unaltered in these rats. Under the short-term study, mRNA IL-1 β levels were not significantly different between treatment groups.

Analyses in the current chapter indicated that lower cytokine expression levels within the hippocampus accompanies a higher discrimination ability in YACTL ($r = -0.85$, $p < 0.01$) and MADON ($r = -0.87$, $p < 0.01$) rats under short-term treatments. This trend demonstrated that an increased ability to discriminate between objects was accompanied by a lower hippocampal IL-1 β level for in YA and MA groups. However, there

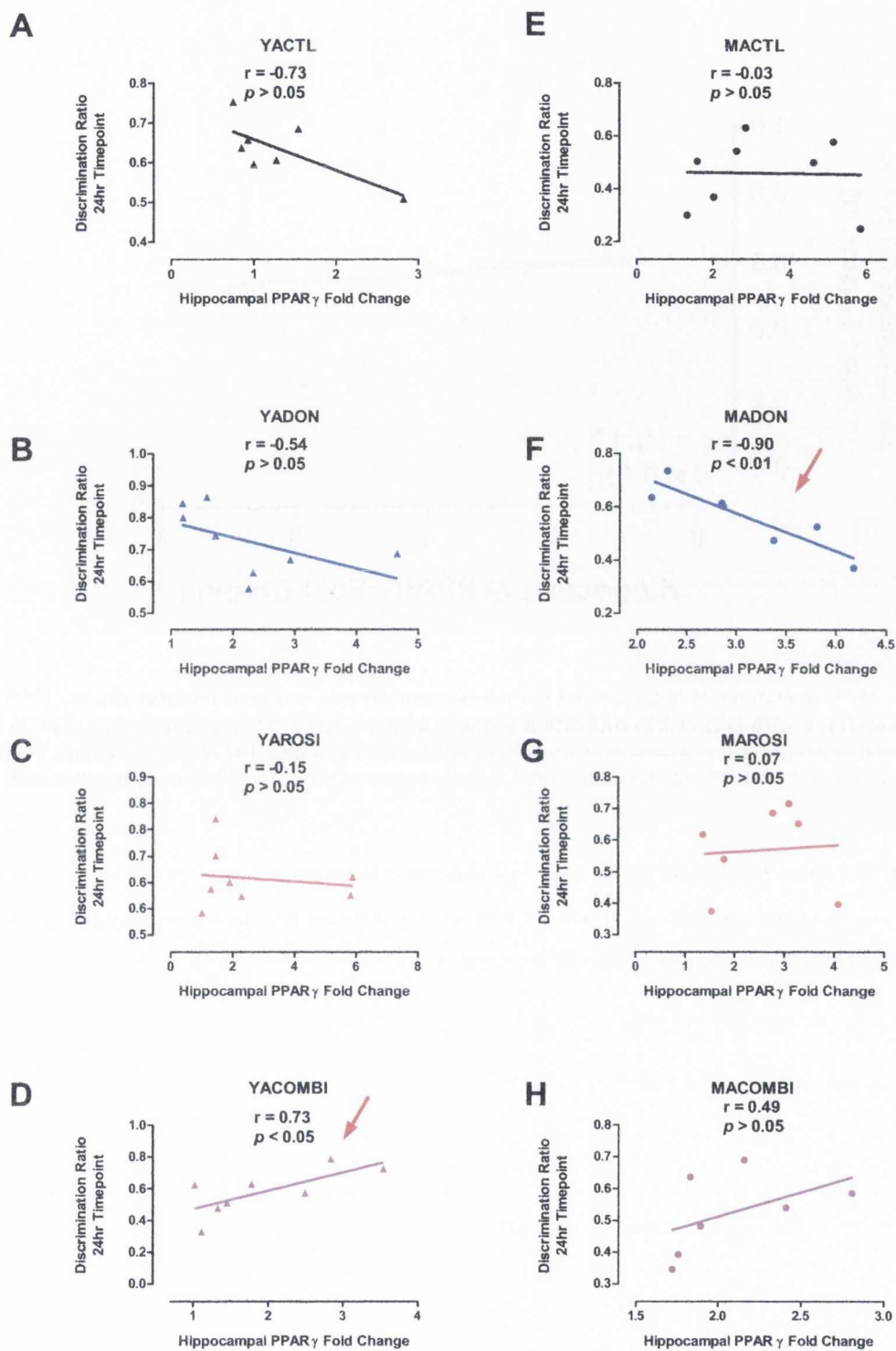


Figure 6.34: Correlations of hippocampal peroxisome proliferator-activated receptor gamma (PPAR γ) expression levels with Object Discrimination Ratio at 24 hour delay. PPAR γ mRNA expression in hippocampal tissues collected was correlated with discrimination ability of rats. YA (A to D) and MA rats (E to H) are all represented. Each data point represents an individual rat. Outliers YACTL 3 and MADON 15, MAROSI 19 and MACOMBI 30 are removed. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. The significance of regression is represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Red arrows indicate statistically significant outcomes.

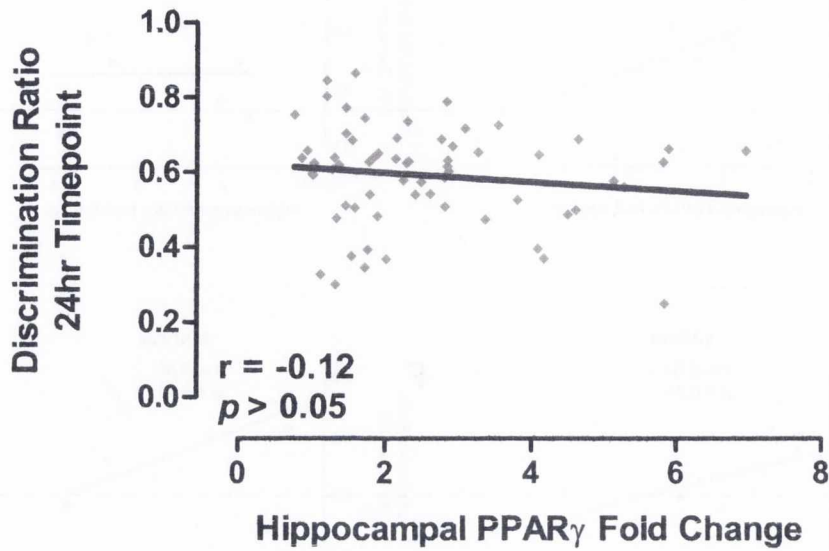


Figure 6.35: **Correlations of hippocampal peroxisome proliferator-activated receptor gamma (PPAR γ) expression levels with Object Discrimination Ratio at 24 hour delay.** PPAR γ mRNA expression in hippocampal tissues collected was correlated with discrimination ability of rats. The line on each graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

is a lack of relationship between ODR and IL-1 β expression levels when data from all age and treatment groups were combined and further analysed (see Figure 6.37). All other treatment groups failed to produce a correlation trend between the two parameters investigated³.

³Alterations in targets that did not correlate to behavioural outcomes are detailed in the appendix.

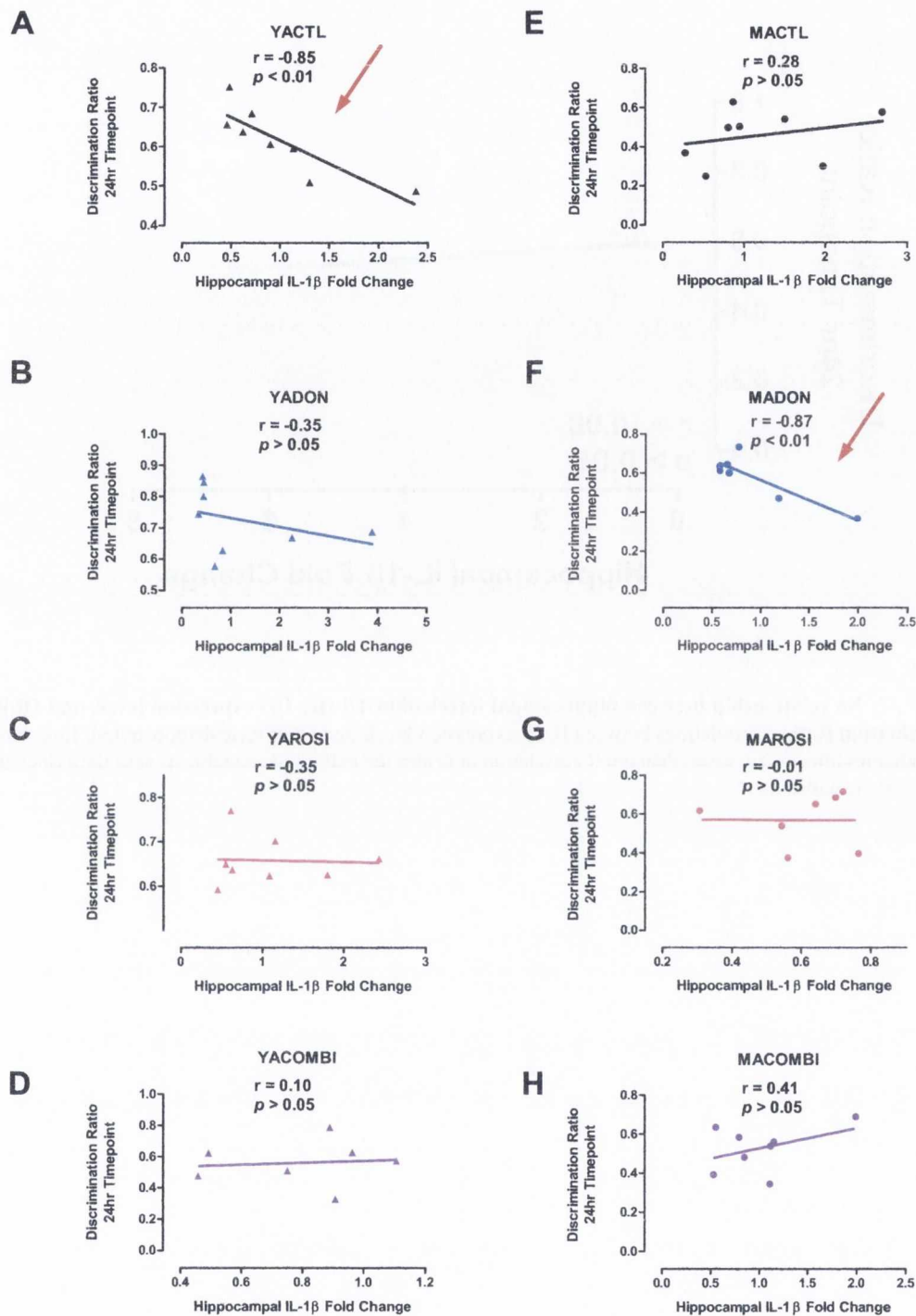


Figure 6.36: **Hippocampal interleukin-1 β expression levels correlated with Object Discrimination Ratio in untreated young adult rats.** Correlations between IL1 β expression levels and ODR were demonstrated. A positive relationship between the parameters investigated was produced for YACTL and MADON rats. YA (A to D) and MA rats (E to H) are all represented. Each data point represents an individual rat. Outliers for IL1 β levels were removed; YACOMBI 27, MADON 12 and MAROSI 19(N = 8 per group). Line across graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables. Significance of regression represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Red arrows indicate statistically significant outcomes.

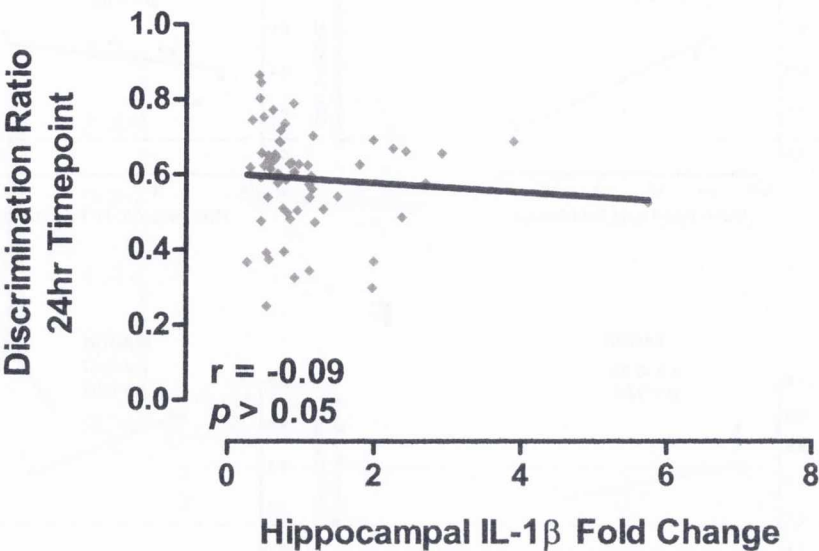


Figure 6.37: **No relationship between hippocampal interleukin-1 β (IL-1 β) expression levels and Object Discrimination Ratio.** Correlations between IL1 β expression levels and ODR were demonstrated. Line across graph indicates linear regression. Pearson R correlation indicates the magnitude and direction of the association between two variables.

Targets encompassing the	Target:	Source:	Study involved:	Figure in text	Significant correlations
		Protein levels	Chronic DON		None
Targets encompassing the cholinergic system	α7 receptor	mRNA expression	Short-term Combination	5.26	@ 24 hour delay : YACOMBI [(r = 0.87), p < 0.001]
	AchE	Protein levels	Short-term Combination	5.25	@ 24 hour delay : YADON [(r = 0.74), p < 0.05], MACTL [(R = -0.89), p < 0.05], MAROSI [(R = -0.91), p < 0.01]
	Ach	Protein levels	Short-term Combination		None
Targets encompassing the glucoregulator system	PPARγ	mRNA expression	Chronic ROSI		None
	GLUT 3	mRNA expression	Short-term Combination	5.29	@ 24 hour delay : YACOMBI [(r = 0.73), p < 0.01], MADON [(r = -0.90), p < 0.01]
		Protein levels	Chronic ROSI		None
	Insulin	mRNA expression	Short-term Combination		None
		Plasma concentration	Chronic ROSI		None
	Adiponectin	Plasma concentration	Short-term Combination	5.28	@ 24 hour delay : YACOMBI [(r = 0.77), p < 0.05]
Targets involving age-related physiological changes	IL-1B	mRNA expression	Chronic ROSI	5.19	Latency: MAROSI [(r = 0.86), p < 0.01]
		mRNA expression	Short-term Combination	5.3	@ 24 hour delay : YACTL [(r = -0.85), p < 0.05], MADON [(r = -0.87), p < 0.01]
		mRNA expression	Short-term Combination		None
	MHC II	mRNA expression	Chronic ROSI		None
	IL-4	mRNA expression	Chronic ROSI		None
		mRNA expression	Short-term Combination		None
	Corticosterone	Plasma concentration	Chronic ROSI		None
		Plasma concentration	Short-term Combination		None
	BDNF	Protein levels	Chronic DON	5.18	% Correct response : YACTL [(r = 0.66), p < 0.05]
		Protein levels	Short-term Combination	5.27	@ 1 hour delay : YAROSI [(r = 0.71), p < 0.05], 24 hour delay : YAROSI [(r = 0.84), p < 0.01]
Targets encompassing neurogenesis	βNGF	Protein levels	Chronic DON	5.2	% Correct response: MACTL [(r = 0.86), p < 0.05]
		Protein levels	Short-term Combination		None

Figure 6.38: **Summary of correlations between behavioural outcomes and expression of molecular targets.** Molecular targets examined at the end of all behavioural experiments are all listed and categorized under three different systems: cholinergic, glucoregulatory and other age-related physiological changes. All mRNA and protein levels are collected from hippocampal tissues unless otherwise stated. Significant correlation analyses are detailed corresponding with figures indexed within the chapter.

6.6 Discussion

6.6.1 Augmenting cognitive status with donepezil and rosiglitazone treatments

This chapter provided analyses between treatments and its effect in both the WPM and SOR task. As yet, the intricate memory components that could underlie the performance in WPM task have not been discussed. A large part of the task requires spatial awareness where the animal was required to form a single association between the start arm and the escape platform. There are several strategies that are in place to help the animal find its way out of the maze. Praxis strategy (the learning of a sequence of movements to the platform), taxis strategy (using proximal cues to escape) and spatial strategy (using spatial configuration of distal cues to learn about platform location) are the three main strategies that may be employed by the subject to reach the hidden platform during a trial (Brandeis *et al.*, 1989). Upon completion of 3 days of fixed-start trials, the animal would have encountered the maze thirty times and given an opportunity to form the association between start and escape arm. The standard probe trial in the original water maze employed the reversal training where the platform could either be moved to a new location within the maze, or a transfer test where the platform was removed completely (Morris, 1984). Hence in a reversal training, the animal has to learn the new position of the platform using the spatial cues given. The probe, MS trial, used a completely different procedure where the entry point into the maze was switched randomly for 30 trials. In this case, a strong association between the spatial cues and the subject's most recent position is required to solve the task, proving the task to be difficult. The animal would need to retain information about where it currently is, where it was in the previous trial and if that previous location and strategy resulted in a successful escape. Such taxing parameters demanded the animal to possess intact spatial working memory and reference memory in the initial MS trial.

Analyses of latency in the decision zone and the type of errors produced gave several insights into the strategies an animal used to escape from WPM. Age-related deficit in the MS trial was accompanied by a high straight swim error and a low latency in the decision zone. Giving support that the animal persevered in swimming directly across to the opposite facing arm without hesitation within the decision zone to retrieve spatial information from distal cues (Tolman, 1948). This is an unexpected behaviour because even in the prior fixed-start trials, rats were trained to turn, either left or right, to find the escape platform. There can be two reasons for such persistent straight swim behaviour; 1) rats swam ahead due to a few escapes encountered through straight swims, 2) rats gave up and realise that an immediate reaction to leave arm results in escape. Since all groups and trials are balanced and randomly assigned, the proposition that rats preferred a straight swim response was not observed. This might indicate that the older rats had "given up" on the trials and performed a straight swim to escape from the arm quickly. Notably, in the final session where rats reached a high criterion (>90%), untreated middle-aged rats failed to solve the MS trial. Similar behaviour have been demonstrated in rats with lesions of the PI-IL cortices and the dorsal anterior cingulate cortex (Dias & Aggleton, 2000), suggesting that the middle-aged rats might exhibit a lack of inhibitory control over behaviour, corresponding to a prefrontal dysfunction.

Generally, an impairment of this nature might present itself as a behavioural inflexibility which has been

demonstrated in marmoset where the animal produced perservative responding to the previously rewarded stimulus without any significant effects on either retention of a discrimination learned pre-operatively or acquisition of a novel discrimination postoperatively (Clarke *et al.*, 2007). In humans, normal aging exhibits measurable deterioration of prefrontal brain function where decreased ability to regulate behavior on decision making was demonstrated (Daigneault *et al.*, 1992). The failure to acquire the harder MS trials could be an indicator of a defective working memory capability as well. In an effort to replicate reversal deficit described in the MWM, learning and memory was examined in a plus-shaped water maze (Van Dam *et al.*, 2000). Reversal deficit in finding the changing platform was explained by a decreased response exibility where subjects have difculties in changing a learned spatial navigation strategy as opposed to a more generalised working memory impairment. Performance of MS trials eventually increased above chance level for most rats, except MACTL and MAROSI, suggesting that behavioural inflexibility is not the only explanation. Rats that were able to rise above chance in the last session imply that rats were switching strategies in the latter sessions. This view was further supported by the trend in the type of errors made where YA rats made more trained errors in the beginning and in the end, made a variable amount of different errors. This could mean that the swim behaviours for these rats have switched from one type of turn (e.g. trained right turn) to several other swim behaviours (e.g. straight, untrained left turn).

Age-related impairments in spatial tasks have been previously demonstrated (Fischer *et al.*, 1992; Gage *et al.*, 1984; Morris, 1984; Barnes *et al.*, 1980; Gallagher *et al.*, 1993; Rapp *et al.*, 1987). Frick and colleagues launched an extensive study using a large number of rats (a total of 99) to investigate the impairments associated with ageing rats within the water maze task (Frick *et al.*, 1995). Spatial reference memory is targeted by a place discrimination protocol where the rat is required to locate a submerged platform using spatial cues in the environment. In this scenario, ageing is severely affected by the 24 months old rats while middle-aged rats at eleven months produced a mild memory deficit in platform crossings. Assessment of spatial working memory involves the rearrangement of the platform from the previous session, such that learning can be assessed by both within and between sessions. Senescent rats at 24 months old were demonstrated to have spatial working memory deficits (Van Der Staay & de Jonge, 1993; Means & Kennard, 1991).

Another crucial characteristic of behavioural studies is the discrepancy between age and strain which might influence the outcome of tasks. Different strains of rats at the same chronological age might not be behaviorally or neurobiologically equivalent as demonstrated in a comparison study of strains where 28 months old Long-Evans rats had no impairments in solving the MWM task unlike Sprague-Dawley and Fischer344 rats which were unable to adequately complete the tasks (Lindner & Schallert, 1988). A large behavioural study involving three separate paradigms and four different strains of rats have demonstrated that there are adequate task and strain-dependent differences in performance in tasks of learning and memory (Andrews *et al.*, 1995). Besides strain variability, differences between rats of the same strain and the same age has been reported. Individual variability associated with learning in aged rats have been first demonstrated in a complex maze task (Goodrick, 1968) where groups of impaired and unimpaired learners were identified among aged rats. Although there were several outliers in the data, such a trend in the studies due to the small number of rats in each group (n=10)

was not located. Critically in a larger study, reliable individual differences should be correlated to biochemical analyses in an attempt to acknowledge genuine individual differences in an ageing population.

One aspect of the task that needs to be discussed was the concern of over-training. Fixed-start training occurred for a total of nine days in three separate sessions resulting in a total of 120 trials for each rat. With the MS trial, the animal was expected to engage in a combination of allocentric (e.g. head towards arm in a fixed position of the room) and egocentric strategies (e.g. turn right when in North arm). Arguably, in the fixed-start trial, egocentric strategy based on self-movement information alone might prevail (e.g. turn right when entering maze) (Morris, 1981). In an attempt to investigate animals use of egocentric strategy alone to escape from the MWM, Kealy and colleagues have employed several protocols that were similar to ours (Kealy *et al.*, 2008). Rats trained for 12 days from the same starting point were released from different starting points during probe trials. With the lack of distal cues (in the dark), animals did not employ the use of egocentric strategy as expected. Instead, animals either simply swam around the side of the pool or stayed at their respective starting position. The authors above were unable to demonstrate a correlation between over-training and an employment of dominant egocentric strategies due to the maze they chose to work with. The study was able to confront this predicament as the design of the maze caused on the animal to make a choice. Although investigating procedural learning was not the fundamental aim, it appears that with ninety trials over a period of forty days, animals might engage in allocentric strategies when learning locations, then switch to egocentric strategies after over-training (Chang & Gold, 2003). It has been suggested that the hippocampus and caudate nucleus selectively mediate expression of place and response learning, respectively. Interestingly, neuromodulation by intracerebral glutamate infusion can influence the brain towards the use of a specific memory system to control learned behavior and thereby influence the timing of the switch from the use of cognitive memory to habit learning to guide behavior in a maze shaped liked ours (Packard, 1999). Elucidating the long-term memory capability in middle-aged rats was another objective of the task. The evidence that long-term memory is intact for middle-aged animals was demonstrated between sessions where a latent period of around fourteen days enforced. Elevated levels of correct responses on the first trial days of subsequent session (days 18 and 37) reflect intact long-term memory for trained response. Age-related deficits in memory and learning have been previously described in middle-aged rats. Specifically, middle-aged rats exhibited a temporal-graded retrograde amnesia associated with consolidation (Ward *et al.*, 1999). Impairment in spatial memory for middle-aged rats arises from a longer escape latency and failure to locate the hidden platform (Klein *et al.*, 2000). Such changes in memory components described in middle-aged rats seemed to evolve from impairments in hippocampal LTP (Rex *et al.*, 2005). Changes in synaptic plasticity have been identified in dendritic spines within the prefrontal cortex of middle-aged rats where a loss of stress-induced spine plasticity was demonstrated (Bloss *et al.*, 2011).

Our investigations into procognitive drug therapy highlighted it's effect on the innate preference for novelty. The memory for recognition and subsequent discrimination between novel and familiar objects underlie the basis of the SOR task. The design of this task began in 1988 and was entirely based on the spontaneous exploratory behaviour of rats towards objects (Ennaceur *et al.*, 1988). In this seminal study, several measures of the task can be investigated and were used to represent 'pure' working memory. As there are no positive or

negative reinforces such as food or electric shocks, the test is a useful tool for pharmacological and neurological studies on the memory of rats. Manipulations of retention period between phases seemed to have a direct effect on recognition memory. It is apparent that exposure to distractive stimuli during the retention delay can impair object recognition (Ennaceur & Meliani, 1992). Although it has also been suggested that delay-independent effects might result from altered acquisition/encoding rather than from altered retention (Steckler *et al.*, 1998). Exposure time to objects and inter-trial delay are also demonstrated to have a close relationship with the exploration of a novel object more than the familiar object, as the inter-trial delay decreased and/or the duration of familiarisation phase increased (Dodart *et al.*, 1997). Importantly, the selection of different delays allows variation of mnemonic load, i.e. the study of retention of information over time (Steckler *et al.*, 1998).

There are several evidences that object recognition, itself, is intact in aged rats (22 months old) as compared to adult rats (8 months old) (Lukaszewska & Radulska, 1994; Willig *et al.*, 1987). It has been suggested that in aged rats spatial but not object recognition was impaired in Wistar rats (Cavoy & Delacour, 1993). However, impairment in object recognition was clear in the same strain of rats at eighteen months (Bartolini *et al.*, 1996). In this case, acquisition more than memory processes appear to be affected since rats were unable to discriminate at one minute delay even though there were no impairments found in the familiarisation phase. In the current study, slightly younger rats, at thirteen months old, were able to discriminate at one hour delay but not the longer delay at 24 hours. Hence, acquisition of the task for middle-aged rats was not impaired and recognition memory was intact at 1 hour delay. Importantly, all 3 treatments employed in the task were able to ameliorate such a deficit. These findings demonstrate that an augmentation in cholinergic and glucoregulatory functions can enhance recognition memory in middle-aged rats who were impaired at longer delays.

In recognition tasks manipulating placement of objects, aged rats (22-24 months old) were severely impaired, suggesting that senescent rats have decrements in the ability to build spatial representations of the environment and to use this information to detect such changes, even though object recognition is not impaired with age (Shukitt-Hale *et al.*, 2001). Exploratory activity in aged animals (26 months old) seemed to be affected by age as performance in open field activity and were deficient in the object displacement task (Schulz *et al.*, 2007). Reduction in exploratory behaviour has been reflected in another study where age does not affect memory involved in object recognition (able to distinguish novel from familiar) but impairs some component of the mechanism mediating responses to the displacement of a familiar object since they are unable to recall the location of objects over a 30 min delay period even though both the objects and environment are familiar (Liu *et al.*, 2004). Recent studies with SOR revealed that recognition memory deficits in a mouse model was present at 18 months of age and this particular memory impairment can be ameliorated by blockade of a serotonin receptor, 5-HT₆R (Da Silva Costa-Aze *et al.*, 2011). Additionally, in a accelerated senescence mouse model, there were obvious functional deficits in hippocampal and prefrontal cortical circuits directly related to the acquisition and storage of new motor and cognitive abilities where animals were interacting with familiar objects rather than novel (López-Ramos *et al.*, 2011). The behaviour where old animals treat novel objects as familiar is reminiscent of behavioral impairments in young rats with perirhinal cortical lesions suggesting that alterations in the perirhinal cortex may be responsible for reducing aged animals' ability to distinguish new

stimuli from ones that have been encountered previously (Burke *et al.*, 2010). Data from the WPM suggest that functional deficits in the prefrontal cortex might be present in middle-aged animals as they lack behavioural flexibility to solve the MS trials. Evidences from the SOR task also indicate that older animals were relating to familiar objects as novel, demonstrated by a negative preference score. Importantly, only rosiglitazone and donepezil monotherapy were able to rescue such an impairment while a combination therapy resulted in a failure to discriminate between novel and familiar at longer delay.

The type of assessments made from the SOR are varied, i.e., memory for place (recognition that an object is in a location where previously there had been no object), memory for object in place (recognition that a specific object has changed position with another object) and memory for context (recognition that a familiar object is in a context different to that in which it was previously encountered) (Dix & Aggleton, 1999). Since the fundamental protocol in recognition task consists of a sample phase (presentation of stimulus), a delay phase (subject to retain information) and a choice phase (comparison against previously stored information). Several alterations can be made in the methodical aspect such as post-training pharmacological manipulations, which seemed to have an effect on recognition memory where adrenaline can facilitates long-term memory for standard object recognition (Jurado-Berbel *et al.*, 2010). Studies employing drug treatments to seek an understanding into memory in SOR tasks will be further discussed in section 5 on page 114. Recognition in different type of mazes, instead of open field, has also demonstrated age-related impairments. Recognition in the Y-maze, on the basis of place or object exploration, was impaired in older animals at 18 months old (Dellu *et al.*, 1992). In a comparison between the employment of a standard SOR and a radial maze, spatial recognition was demonstrated to be more vulnerable than the object recognition because various visual and physical features of the object are probably coded on more dimensions than a spatial place (Ennaceur & Meliani, 1992). This might be echoed in the experiments although impairments at longer delay were evident in middle-aged animals, this was not as severe as the learning deficits demonstrated in the spatially dependent- WPM task.

Another adaption to assessing recognition memory is the use of matching-to-sample or non-matching-to-sample criteria as mentioned above. This usually takes place in an operant chamber with the presentation of two levers for rats to choose. Although recognition memory is associated with task, it is also critical to note that these levers also serve as a spatial stimulus (Steckler *et al.*, 1998). Aged rats showed no impairments when they are required to choose between matching or non-matching choice response. However, with delay as an added interference, 24 months old rats were unable to acquire the task (Dunnett *et al.*, 1988). This short-term memory impairment was not due to the effects of proactive interference (responses made on the preceding trials can interfere pro-actively with current-trial performance) (Dunnett *et al.*, 1990). Prefrontal and hippocampus dysfunction has been suggested to be the culprit for such an age-related memory decline (Winocur, 1992). Similarly there are several pharmaceutical drugs that can intervene with learning in a delayed matching to position performance in a rat model leading us to understand the neurochemical principle behind this task. An infusion of scopolamine, a cholinergic antagonist, into the prefrontal cortex has shown to impair DMTP task performance (Herremans *et al.*, 1996) which can be reversed by donepezil (Poorheidari *et al.*, 1998). This gives support to the data from both the WPM and SOR tasks where donepezil-treated animals were superior in terms

of learning and memory. Additionally, the cholinergic system is critical in the short-term memory processes supported by the medial PFC (Broersen *et al.*, 1995) which was translated in the both fixed-start and MS trials.

The combination of both tasks allow us to gain an insight into different memory systems that suffered from age-related impairments in middle-aged rats. More sophisticated task using contextual association can be utilized to clarify brain regions that support memory for associations between specific cues and their respective context. Recently, a task has emerged using both contextual and olfactory cues in deciphering changes in the aged rodent. The basis for such associative learning presents itself when the rat becomes familiarized with the object in the sample phase and will show preference for a novel object or environment (Mumby *et al.*, 2002). Functional impairments in the medial temporal lobes and particularly in the hippocampus have been attributed to the failure to establish associations between a stimulus and a context (Luu *et al.*, 2007). Rats with hippocampal lesion studies are able to display a novelty preference for objects (two identical sample objects, one is replaced by novel object) but fail to discriminate between the objects on Place trials (normal rats more time exploring the object in the novel location than the one in the familiar location) or Context trials (normal rats spent more time exploring the object in a context different from that during familiarization) (Mumby *et al.*, 2002). Conclusions from the study represents that the formation or the retrieval of associations involving the objects and the particular contexts in which they were encountered is disrupted due to the lesions. The SOR task employed demonstrated slight impairments in middle-aged rats and might not be adequate enough in giving more indepth details about other functional impairments that might exist in them.

In light of the several inadequacies described in middle-aged rats pertaining to task accomplishment in both the WPM and SOR experiments, the study was able to gain important knowledge of how donepezil and rosiglitazone administration can improve cognitive capabilities in ageing rats. Data from the WPM experiments provided support that treatment with rosiglitazone and donepezil both improved long-term memory demonstrated by the elevated response level found on the initial trial for session 2 and 3. Behavioural flexibility was sufficiently increased for MADON rats to complete MS trials on the same level as younger rats. Rats, regardless of age, on either drugs were able to accomplish the task at a higher level. Between treatments it appears that acute rosiglitazone has a prompt effect on acquisition. Findings imply that acute rosiglitazone does have an immediate effect on glucoregulation. Secondly, an increased in insulin sensitivity can directly affect memory and learning functions.

A direct injection of glucose into the dorsal hippocampus enhanced hippocampal functions and accelerated the rate of learning and the early adoption of a response strategy in a T-maze task (Canal *et al.*, 2005). Canal and colleagues suggested that accelerated learning could be accompanied by an earlier transition from place to response solutions (Restle, 1957), and an early selection of a response strategy leads to accelerated learning of the T-maze. This is very similar to what was observed from the three sessions. While acquisition in fixed-start trials is accelerated, the equivalent probe trials resulted in a much lower response. This highlights the difference between treatments for donepezil and rosiglitazone. Although both were able to accelerate learning and acquire a high ceiling effect in the final session, only MADON rats were able to score higher than chance for the probe trial. This indicate that manipulation of the cholinergic system produced a superior effect in

learning and memory, compared to a maintained glucoregulatory system.

6.6.2 Investigating the physiological alterations evolving from prescribed treatments and augmented behavioural outcomes.

This chapter also aims to marry behavioural outcomes established in the experimental studies and the molecular endpoints examined in previous corresponding work. In order to gain a greater understanding of the mechanistic basis of the ageing process, the bridge between behavioural changes in the rodent model should be linked to the molecular basis of brain ageing. Despite the small sample size ($n = 8$ or 10), there were some clear evidence of a straight forward relationship between behavioural performance and molecular results. The fallacy of *um hoc ergo propter hoc* (Latin for "with this, therefore because of this"), must be clarified before attending to the correlation results. Evidence of correlations between the parameters involved does not imply the causation of another. More appropriately, correlation analyses help to identify how strongly and in what direction two variables co-vary in an environment. Therefore, augmentation of a particular molecular biological component which correlates with a particular behavioural parameter demonstrates a strong relationship between both variables within a particular drug-induced setting. In order to keep the discussion succinct, this section is dedicated to understanding the interaction between biological variation and behavioural outcomes encountered in the current work. A thorough review of molecular and behavioural results demonstrated from specific experiments utilising chronic donepezil (see chapter 3 on page 51), rosiglitazone (see chapter 4 on page 84) and combination therapy (see chapter 5 on 100) are excluded from the current discussion. Additionally, correlation analyses that failed to locate any significant outcomes are reviewed in their relevant result sections (see Appendix).

Rosiglitazone treatment rescued memory deficits in middle-aged rats which complemented the augmented physiological changes in the targets investigated. Briefly, the results demonstrated that chronic rosiglitazone treatment increased basal plasma adiponectin and rescued elevated plasma insulin levels without PPAR γ activation. Additionally, treatment with rosiglitazone failed to alleviate an elevated pro-inflammatory cytokine, IL-1 β , in older rats suggesting that anti-inflammatory properties of the drug might not be solely responsible for the improved acquisition and behavioural flexibility involved in acquiring the task. Importantly, work in the laboratory has demonstrated that improvements in glucose uptake and utilization within the hippocampal neuronal circuitry may contribute to the improvements in cognitive function observed in rosiglitazone-treated middle-aged rats since an increased GLUT-3 expression within the hippocampus was observed coupled with changes in hippocampal electrophysiology and synaptic plasticity (Wang *et al.*, 2011a).

Within the targets investigated encompassing the glucoregulatory system, there was no strong relationships and interactions between the improved behavioural results and changes in the molecular targets as hypothesised. Surprisingly, MADON rats demonstrated a strong negative correlation between PPAR γ mRNA expression and ODR acquired at the longer delay. As yet, there is no evidence that donepezil has any effect on PPAR γ expression. However, recent work in vascular dementia has demonstrated that donepezil can reverse diabetes induced impairment of learning and memory behaviour (Sharma & Singh, 2011; Rajeshkumar Ukabhi Ko-

ladiya, Amteshwar Singh Jaggi, Singh Nirmal, n.d.). Whether such an effect on vascular tone might affect PPAR γ expression is still unknown. Although rosiglitazone treatment itself failed to produce any correlations between PPAR γ expressions and behavioural data, YACOMBI demonstrated correlations within these parameters where an increased hippocampal PPAR γ expression was accompanied by improved object recognition. It is difficult to provide an explanation for such an outcome although it is not possible to rule out the effect of combination therapy between PPAR γ expression and SOR ability. The failure to locate expected correlations between altered physiological status and cognitive capacity could be avoided in the future if tissues were taken at separate sessions correlating to behavioural testing. This, however, requires a much bigger population of rats and these experiments might not meet the standards for animal use under the Trinity College 3R requirements.

In terms of insulin sensitivity brought on by rosiglitazone treatment, direct intracerebroventricular administration of insulin has been demonstrated to improve memory formation in rats in a passive avoidance task, (Park *et al.*, 2000). Considering the high concentrations of insulin receptors within the brain, such an effect is not unexpected. In humans, these findings are replicated where intranasal insulin improved hippocampus dependent declarative memory function (Benedict *et al.*, 2004). However, it is important to note that memory improvement by insulin/insulin receptor within the CNS is not due to a direct effect on glucose metabolism (Zhao *et al.*, 2004b). Improvements in memory and consolidation have been linked to insulin receptor signalling modulates synaptic plasticity acting on glutamatergic and gamma-Aminobutyric acid (GABAergic receptors) (Paulsen & Moser, 1998). Additionally, the insulin/insulin receptor-mediated signal transduction has been put forward as a molecular basis underlying learning and memory (Zhao *et al.*, 2004b). The cognitive enhancing properties of rosiglitazone might be due to a peripheral effect (Gemma *et al.*, 2004) or perhaps rosiglitazone may affect the level and/or activity of factors that readily cross the blood-brain-barrier (Wagstaff & Goa, 2002). In the current analyses, there was no evidence of a direct interaction between altered plasma insulin levels and WPM task performance. However, YACOMBI rats with higher plasma insulin levels performed better at the SOR task. Elucidating the mechanism behind such a trend might require a larger data sample. Additionally, a closer investigation into the insulin receptor signalling systems might help provide a clearer picture of the therapeutic effects of the combination therapy.

Alterations within the cholinergic neural systems can be correlated to the process of learning in which the magnitude of ACh release in different neuroanatomical regions can predict task solving behaviour in rats (McIntyre *et al.*, 2003). More specifically, *in vivo* microdialysis revealed the magnitude of ACh release in different neuroanatomical regions, even when measured before initial exposure to the T-maze. In rats that used a place strategy, instead of a response strategy, to solve the task produced a higher ratio of ACh release in the hippocampus compared to dorsal striatum (McIntyre *et al.*, 2003). Differential activation of the neural systems seem to promote varying strategy for maze solving. For example, increases in ACh release in the hippocampus precede increases in ACh release in the striatum during training on a task in which learning proceeds sequentially from place to response strategies (Chang & Gold, 2003). Chang and colleagues also demonstrated that the process of learning, itself, coincides with ACh release in the hippocampus which increased and maintained throughout training indicating that this brain region was engaged at onset of learning and remained engaged

even with extended training.

These studies give support to the view that manipulation of the cholinergic system by AChE inhibition directly enhances cognitive functions by affecting learning and task solving abilities. Findings from the current studies demonstrated superior task solving abilities by donepezil-treated rats of both ages. The blockade of AChE by donepezil treatment works by invigorating cholinergic activity to enhance cognitive function (Sugimoto, 2001a). A surplus of ACh in the hippocampus led to a higher acquisition in the fixed-start trials, and increased behavioural flexibility in the multiple-start trial. As mentioned above, a higher ACh release indicated that rats were able to locate the goal in the maze by employing the place strategy (e.g. go to a particular part of the room to approach the goal). This would be particularly helpful in solving the harder MS trials. Indeed, rats that rose above chance levels for the MS trials were under donepezil treatment. However, this effect was more pronounced in the MA rats, suggesting that procognitive effects of donepezil was more therapeutic in an already impaired cholinergic system. Additionally, some age-related differences in memory might reflect changes in preferred solution to a problem on the basis of activation of different neural systems, rather than on the basis of differences in learning or memory capacity (Chang & Gold, 2003).

We searched for alterations pertaining to the the cholinergic system which might correlate to augmented cognitive function as demonstrated in the previous study. In neuronal culture, chronic donepezil treatment (10 μ M, 4 days) was effective in upregulation $\alpha 7$ nicotinic receptor (Takada-Takatori *et al.*, 2008a). The current *In vivo* experiment with a 5 day application of donepezil failed to produce similar results. Correlations between $\alpha 7$ nicotinic receptor, ACh, AChE levels and behavioural outcomes were not successful in elucidating effects in donepezil-treated rats. The only positive interactions between these targets was present in YADON rats where interaction between the rats ability to discriminate objects at 24 hour delay and AChE mRNA levels was strong and of a positive nature. Even though hippocampal AChE mRNA levels were similar for all groups under the short-term treatment study, within the YADON group of rats, a higher AChE expression levels indicated an improved ODR. It is difficult to explain this finding as it is counterproductive to donepezil treatment. In fact, a recent study demonstrated that donepezil was able to rescue spatial memory impairment by preventing a rise in brain AChE activity that accompanies streptozotocin induced diabetes in rodents (Sharma & Singh, 2011).

Although correlations for donepezil-treated rats were not substantial, other drug treatments did reveal some interaction with molecular targets that comprised the cholinergic system. Negative correlations in MA rats suggest that a low AChE expression levels was accountable for the improved ODR observed. This is in accordance to the cholinergic pathway where neurotransmitter ACh can directly affect synaptic plasticity subsequently affecting learning and memory (Rasmusson, 2000). A thorough review of age-related changes in AChE activity within the rat brain demonstrated a decrease in 18-22 month old rats (Das, 2001). Although this was not observed in the current work, the decline in AChE expression in the hippocampus provided a better ODR for untreated and rosiglitazone-treated rats, but not donepezil-treated one. This finding is unexpected and it is hard to determine what are the reasons behind these trends. Drugs influencing cognitive mechanisms induce changes in a number of neurotransmitters and the current work presented is supportive of the idea that other neurotransmitters influenced by a 5 day donepezil treatment might lead to the improved ODR which has no

correlation to targets in the cholinergic system. Although ACh and AChE levels were unchanged in the study, acute donepezil treatment has demonstrated to affect other neurotransmitters within the CNS, such as an increase in extracellular DA in the cortex and in the dorsal hippocampus (Shearman *et al.*, 2006). This might be a reasonable explanation for the current observations where an improved ODR in donepezil-treated rats did not have a strong relationship with AChE and ACh levels.

Importantly, combination therapy did not improve rats' ability to complete the object recognition task drastically. Molecular alterations that correlated with behavioural outcomes seems to suggest that $\alpha 7$ up-regulation in YACOMBI rats was crucial. Fundamentally, activating $\alpha 7$ receptor can have profound, acute and long-lasting beneficial consequences for cognition through neural circuitry of working memory and synaptic plasticity (Castner *et al.*, 2011). A profound, sustained effect on novel object recognition have been demonstrated to correspond with an increased receptor number through the activation of $\alpha 7$ receptor via a partial agonist AZD0328 (Werkheiser *et al.*, 2011). The beneficial effects of activating the nicotinic $\alpha 7$ receptors seemed to affect synaptic plasticity directly. In fact, activating $\alpha 7$ -nAChRs recovered the presynaptic deficits in *beta*-Site amyloid precursor protein-cleaving enzyme 1 (BACE1) knock-outs in murine models (Wang *et al.*, 2010). Relevancy of such information is particularly important to the treatment of AD where the neuroprotective function of nAChR activation is blocked by the antagonizing effect of A β peptides (Hernandez *et al.*, 2010). Importantly, it appeared that nAChRs are neuroprotective both by modulating the neurotrophic system crucial for the maintenance of cholinergic neuron integrity as well as stimulating signal transduction pathways that support neuron survival (Parri *et al.*, 2011).

Additionally, $\alpha 7$ -nAChRs can be up-regulated by neurotrophins through glutamatergic transmission (Massey *et al.*, 2006). In the study, neurotrophins, both BDNF and β NGF, protein concentrations were elevated under a chronic influence of donepezil within the rat hippocampus. Importantly, this occurrence was irrespective of age suggesting that a direct manipulation of the cholinergic system by an AChE inhibitor can single-handedly augment neurotrophin levels. Failure to demonstrate a positive correlation between BDNF and β NGF levels with behavioural outcomes meant that the two factors are unrelated. Donepezil-treated rats with an unregulated neurotrophin levels were not responsible for the increased task performance. The failure to find a correlation between neurogenesis and memory enhancement is not new. Although the mechanism behind the findings is unclear, it has been proposed that neurogenesis can occur without learning enhancement and is otherwise an alternative function of neurogenesis in the control of motor behaviour (Rhodes *et al.*, 2003).

6.7 Conclusion

We have described noticeable age-related changes in spatial and recognition memory with the tasks employed in the studies. Behavioural flexibility was impaired in middle-aged rats and this might, in part, be directly linked to the type of errors made. Perservative errors produced by older rats were diminished by the an effect of drug treatments. The solution rats used trying to solve the MS trial was mostly a response strategy which might be attributed to the elevated level of acquisition and total number of fixed-start trials employed. However, donepezil treatment in middle-aged rats ameliorated the performance deficit in the WPM task and these rats function comparably to younger rats in terms of trial acquisition. Rosiglitazone, on the other hand, did not produce similar effects. Rosiglitazone administration led to a prompt increase in acquisition but this did not translate to enhanced behavioural flexibility. The SOR task revealed an augmented discrimination ratio for treated rats at the longer 24 hour delay. Since all rats were able to discriminate at 1 hour delay, memory impairment was not at a critical level for middle-aged rats. Extrapolating the delays might give further insight into how extensive memory impairments are in middle-aged rats. Importantly, combination therapy failed to reach the expected level of procognitive potential and treated rats behaved comparably to untreated rats (see chapter 5 on page 114). There are several aspects of both tasks that could be improved to aid the understanding of the ageing process in rats. In the WPM task, analytical measures in the progression of directions utilised during MS trials could give an indication if working memory was intact. The type of response that varying from trial to trial could give us an "online" acuity into how rats behave when in different arms and after acquiring a high level of trained response.

Physiological alterations pertaining to drug administration of rosiglitazone and donepezil suggested that enhancing the cholinergic system and stabilising glucoregulatory metabolism can preserve cognitive status in older rats and enhance memory and learning in younger ones. Correlative data exploited in this chapter could not fully account for the interaction between the molecular targets investigated and their effects on augmented task abilities. To further elucidate the relationship between behavioural and molecular parameters a larger population sample might be necessary. Amelioration of age-related spatial working memory deficit was described under chronic rosiglitazone treatment. Investigations in the molecular endpoint targets led us to propose that the principal mechanism behind the results was due to an up-regulated hippocampal GLUT 3 expression. Glucose delivery and utilization in the mammalian brain is mediated primarily by GLUT3 in all neuronal populations (Simpson *et al.*, 2008). Acquiring the tasks employed required a high cognitive demand and the presence of an up-regulated GLUT3 level within the hippocampus might facilitate this high neuronal energy demand. Donepezil, on the other hand, appears to facilitate memory enhancement through enhancement of neurotrophins and an up-regulation of $\alpha 7$ receptor within the hippocampus. Lastly, combination therapy failed to reach the expectations in augmenting both recognition memory and specific molecular targets in MA rats.

Chapter 7

Main discussion

In an attempt to establish an improvement in age-related memory deficits in healthy ageing individuals, procognitive therapies were provided through the route of enhancing the cholinergic system and stabilising glucoregulatory status in a preclinical rodent model. Three procognitive therapies were utilised in the experiments. The first of the hypotheses inferred that stabilising the glucoregulatory status in ageing rats might alleviate learning and memory difficulties associated with ageing. Rosiglitazone is a TZD that reduces insulin resistance and works to preserve insulin secretion (Lebovitz *et al.*, 2001). Additionally, investigations into the cholinergic system were established based on the cholinergic theory which states that a dysfunction of ACh containing neurons in the brain contributes substantially to the cognitive decline observed in those with advanced age and AD (Sugimoto, 2001b). Donepezil is the gold standard treatment in treating AD and we demonstrated an attenuation of memory impairment in middle-aged rats attributed to an enhanced cholinergic transmission. Lastly, we hypothesise that the combination of both drugs might amplify memory enhancement since both systems, glucoregulatory and cholinergic, were jointly manipulated.

By utilising the different tasks and drug treatment regime, we were able to gain further insight into the effects of both short-term combination therapy and chronic treatments. The water plus maze (WPM) task was designed to explore the limitations of spatial working memory in ageing rats, while a discrimination paradigm between novel and previously encountered object tapped into recognition memory known as SOR task. In terms of treatments used in these studies, several aspects of the therapies can be elucidated. Donepezil did not spark an acute improvement in WPM task ability within the first few trials as demonstrated by rosiglitazone treated rats. Such a latency might suggest that the downstream effects of donepezil treatment are required before it exhibits its therapeutic benefits in terms of task performance. Notably, donepezil treatment in middle-aged rats ameliorated performance deficits in the WPM task and these rats function similarly to younger rats in terms of trial acquisition. Although rosiglitazone administration enhanced task ability this was at a much lower level compared to treatment with donepezil. Remarkably, rosiglitazone administration led to a prompt (one day treatment) increase in acquisition even if this enhancement did not translate to a higher calibre in behavioural flexibility. Equally, treatments with donepezil and rosiglitazone resulted in an augmentation of

memory and learning in younger rats. This provided further support that such therapies can alter memory systems even in healthy younger rats with intact central organisation, such as the cholinergic system, and the peripheral glucoregulatory system. Evidence gathered from the work revealed that memory and learning can be augmented to a noticeable extent in healthy ageing rats. Consequently there were several prominent physiological alterations that accompanied the behavioural outcomes witnessed. Administration with donepezil revealed substantial evidences that invigorating cholinergic activity can lead to alterations in both physiological and cognitive terms. Results from the current work indicated an amelioration of cognitive deficit in middle-aged rats which lead to an improved spatial working memory via the enhancement of neurotrophins and an up-regulation of nicotinic $\alpha 7$ receptor within the hippocampus. Additionally, the heightened cognitive abilities in middle-aged rats under rosiglitazone treatment seemed to be managed through an up-regulation of GLUT3 receptors in the hippocampus.

The hypothesis of neuroenhancement through drugs in healthy ageing was substantiated in the current study. The work accomplished so far suggest that drug treatments utilised in the study ameliorated age-related cognitive deficits observed in middle-aged rats as well as improving spatial working memory in young adult rats. These results would suggest that both drugs could be used as “procognitive” therapeutic options as cognitive enhancement was achieved even in the absence of an age-dependent background dysfunction of glucose and cholinergic systems. A reasonable follow up study was to include a combination study of utilising both donepezil and rosiglitazone in a cognitive deficient animal model. Unfortunately the combination therapy consisting of both rosiglitazone and donepezil did not improve recognition memory and failed to reach hypothesised level of procognitive potential. When both drugs are given concomitantly, they appear to dampen each other’s effect in both molecular targets and behavioural outcomes analysed. The neuroendocrine function of glucose seems to play a part in augmenting or impairing neurotransmitter functions in relation to the cholinergic system. As an essential metabolic compound, glucose also provides substrates (acetyl-CoA) for the synthesis of ACh and other neurotransmitters and this in turn contributed to memory enhancement (Ragozzino *et al.*, 1996). There is much support for such a study as systemic glucose injections can enhance spontaneous alternation performance and consequentially potentiate ACh output (Ragozzino *et al.*, 1996). A crucial observation was that glucose administered under resting condition does not modify hippocampal ACh output. Hence, it is tangible that glucose may modulate ACh output only under conditions where cholinergic neurons are activated. Other studies using antagonists of the cholinergic system have demonstrated the relationship between glucose and ACh bio-availability. Scopolamine, a nonspecific muscarinic receptor antagonist, causes memory deficits in several animal modes which can be attenuated by a peripheral glucose injection (Messier *et al.*, 1990). Additionally, there is strong evidence that glucose availability and ACh levels are highly related when a task of a highly demanding cognitive activation is required (Messier, 2004; Ragozzino *et al.*, 1996; Korol & Gold, 1998).

7.0.1 Age-related behavioural impairments revealed

The tasks employed in the study were not novel by design and have been applied in understanding memory in several different studies. Discriminating between newly encountered and previously encountered objects was first demonstrated in the early 1990s (Ennaceur & Meliani, 1992). Similarly, the WPM or plus-shaped maze, a variation from the more standard MWM, has been used in recent years for the assessment of spatial memory impairment in animal models of behavioural studies (Wahlsten *et al.*, 2005; Vloeberghs *et al.*, 2006; Locchi *et al.*, 2007; Diamond *et al.*, 1999; Chen *et al.*, 2004). Elucidating between which memory systems are deteriorating during advancement of age is an important area of research. The tasks we used in the studies gave important insights into behavioural flexibility and behavioural strategies employed by ageing rats. Rodent models exploring the prefrontal model of normal ageing are mainly focused on the orbitofrontal region because age-related deficits involving this region are unlikely to be confounded by contributions from the effects of ageing on the medial temporal lobe structures (Schoenbaum *et al.*, 2002). The appropriateness of response was curbed due to impulsiveness or disinhibited behaviour in monkeys with damage to prefrontal cortex (Meunier *et al.*, 1997) suggesting that preservative errors found in aged clinical studies may be due to prefrontal cortex impairment (Rapp & Heindel, 1994; Daigneault *et al.*, 1992). However, a rat model has shown that orbitofrontal cortex is not necessary for inhibiting responses unless responses were altered to reflect changing relationships between cues and outcomes (Schoenbaum *et al.*, 2002). Inactivation of the PI-IL areas in rats was shown to impair strategy switching but not reversal learning (Ragozzino *et al.*, 1999). A more recent study confirms this and suggest that the PI-IL region contributes to the maintenance of task switches, perhaps by modulating activity between memory systems (Rich & Shapiro, 2007). A recent study has demonstrated that rats with PL-IL lesions were disrupted in spatial navigation when trained from four different start positions (Fitz *et al.*, 2008). This behaviour is similar to the work we presented, suggesting that intact PI-IL cortices might be necessary for adequately planning spatially guided responses when a high level of flexibility is required, which is lacking in middle-aged rats.

One of the more prominent findings in the current work is the consequence of behavioural inflexibility in untreated middle-aged rats and the strategies used by them to solve the WPM task. The harder multiple-start trials in the WPM task were aimed at unravelling strategy switching in middle-aged rats. Evidences from the work suggested that cholinergic manipulation with donepezil was able to ameliorate age-related impairments in this aspect. In another study, choice impulsivity, a decision making deficit, was exemplified by the tendency of middle-aged rats to choose an immediate, but small reward (Dalley *et al.*, 2004). We found a similar behaviour consisting of a straight swim response in the presence of three different escape routes. When selective lesions on septal cholinergic neurons were done, acquisition of T-maze task was slower and rats employed a persistent turn and utilized a response strategy. Hence a selective loss of cholinergic neurons can impair hippocampal function by affecting the ability to switch from one strategy to another (Fitz *et al.*, 2008). This gives support to the work with donepezil where an attempt to boost cholinergic system resulted in an augmented acquisition and mnemonic abilities. On the other hand, improving insulin sensitivity in middle-aged rats did not appear

to affect behavioural flexibility in the current work. Although we were unable to demonstrate that improving glucoregulatory system has a direct effect on behavioural strategies, a direct injection with glucose into the hippocampus have been demonstrated to boost acquisition by a rapid adoption of a response solution for the T-maze (Canal *et al.*, 2005).

7.0.2 Neuromodulatory alterations in the systems investigated

The relationship between GLUTs and memory is a close one since GLUT expression is plastic and consistently adapts to changes in brain neuronal activation and glucose utilization. Plastic changes in GLUT-1 expression have been associated with learning and memory processing in the hippocampus and sensorimotor cortex but not GLUT-3 (Choeiri *et al.*, 2005). Molecular findings under rosiglitazone treatment suggest that improvements in peripheral insulin sensitivity and glucose uptake into neurons (via expression of GLUT-3) might offer a plausible explanation to the mode of action of rosiglitazone in reversing age-related cognitive deficits. Importantly, glucoregulatory functions are fundamental to most mammalian cells, and the transport of glucose across cell membranes is facilitated by a family of integral membrane transporter proteins, the GLUTs (Simpson *et al.*, 2008). GLUT-3, in particular, is highly and specifically expressed in neurons, making it an essential for mediating and utilising glucose within the brain. There is a marked reduction in cerebral glucose utilisation associated with AD (De Santi *et al.*, 2001) and a prominent loss of GLUT3 protein expression in parietal, occipital, and temporal cortex, together with caudate nucleus and hippocampus (Simpson *et al.*, 1994). Under stressful conditions, rosiglitazone has a therapeutic effect on restoring brain glucose uptake via a restoration of protein expression of GLUT-3 (García-Bueno *et al.*, 2006).

Molecular alterations within the hippocampus that emerged from chronic donepezil treatment involved $\alpha 7$ receptor up-regulation and increased neurotrophins (BDNF and β NGF) expressions. It would appear that the involvement of $\alpha 7$ -nicotinic receptor, the PI3-K pathway and the mitogen-activated protein kinase pathway are all dominant in mediating the effect of chronic donepezil treatment (Tyagi *et al.*, 2010). Up-regulated nicotinic receptors have been demonstrated to enhance the neuronal response to nicotine and enhance the sensitivity to the neuroprotective effect of donepezil (Takada-Takatori *et al.*, 2008a). In a way, donepezil affords neuroprotection through a mechanism that is unrelated to AChE inhibition and is dependent on $\alpha 7$ nicotinic receptors and the PI3K-Akt pathway (Arias *et al.*, 2005; Tyagi *et al.*, 2010). Notably, donepezil is effective in modulating hippocampal neurogenesis in normal rats via CREB signalling (Kotani *et al.*, 2008). The activation of CREB signalling might be relevant to the up-regulated BDNF levels demonstrated in the hippocampus of donepezil-treated rats. Additionally, the neuroprotective effect of donepezil might be mediated by an upregulation of NGF since chronic treatment was able to restore cognitive performance, choline acetyltransferase activity, and NGF mRNA expression (D'Intino *et al.*, 2005).

Although the study is mainly focused on two central systems pertaining to glucose regulation and cholinergic transmission, other systems within the ageing brain should not be discounted. Recent researches suggest that cholinergic dysfunction does not provide a complete account of age-related cognitive deficits (Abe *et al.*,

2003) and that other systems may be involved. For example, serotonergic impairments in the brain are also involved in causing cognitive deficits in aged rats and a combination therapy of inhibiting AChE and serotonin transporter (SERT) improved spatial memory in aged rats. This suggests that both cholinergic and serotonergic systems are involved in maintaining brain arousal and improving memory (Abe *et al.*, 2003). Glutamatergic neurotransmission has a pivot role in an ageing brain where reduced glutamate uptake capacity, as well as a loss in the number of high affinity glutamate transporters in glutamatergic terminals have been described in aged rats (Segovia *et al.*, 2001). Importantly, the observed cognitive impairment in aged rats has been correlated with an imbalance inhibitory changes in GABAergic appositions on cortical pyramidal neurons (Allard *et al.*, 2010b). Although this is beyond the scope of research, other areas of perturbations within the brain homeostasis includes calcium dysregulations (Toescu *et al.*, 2004) and dopaminergic regulation (Morcom *et al.*, 2009),

7.0.3 Gene assay and tailored therapies

Hence, it is evident that the changes within the brain in terms of neuromodulation is expansive and diverse. Microarray studies of gene expression involve a high-capacity system developed to monitor the expression of many genes in parallel (Schena *et al.*, 1995). Such a technique involves a high-speed robotic printing of complementary DNAs on glass which are then used for quantitative expression measurements of the corresponding genes. Additionally, differential expression measurements are usually distinguished by means of simultaneous, two-colour fluorescence hybridisation (Schena *et al.*, 1995). The ability to sequence and map thousands of genes provides data that reflects the order and logic of the genetic program, rather than the physical order of genes on chromosomes (Brown & Botstein, 1999). In mice, ageing resulted in a differential gene expression pattern, indicative of a marked stress response and lower expression of metabolic and biosynthetic genes (Weindruch *et al.*, 2001). Moreover, the gene-expression profile of ageing mice is indicative of an inflammatory response, oxidative stress and reduced neurotrophic support in brain regions such as the neocortex and cerebellum (Lee *et al.*, 2000). Under the more general umbrella of ageing and gene changes, the involvement of insulin-like hormones suggests that the plasticity in life spans evident in animal phylogeny may be due to variation in the timing of release of hormones that control vitality and mortality as well as variation in the response to those hormones (Finch & Ruvkun, 2001). In future, drug treatment may be better tailored to individuals through a better understanding of how genes influence the body's response to drugs. In a way, the knowledge of the molecular basis of disease can lead to novel target identification, toxicogenomic markers to screen compounds and improved selection of clinical trial patients (Ginsburg, 2001). To date, gene profiling in node-positive breast cancer patients has made critical advances where assays are available to detect the HER2 protein receptor or copies of the HER2 gene sequence to determine their eligibility for herceptin treatment or adriamycin treatment (Ginsburg, 2001). The application of physiologically-based pharmacokinetic (PBPK) modeling is another aspect of tailored therapy where pharmacokinetic (PK) parameters from human *in vitro* data can help predict the processes of drug clearance, distribution, and absorption (Rowland *et al.*, 2011).

7.0.4 Individual differences in ageing

Apart from unique and personalised therapies, innate cognitive abilities also differ in aged individuals. Memory difficulties in middle or old age are not necessarily a harbinger of future dementia but can be part of the normal pattern of cognitive ageing (Craik, 1994). Clinical work established by cross-sectional and longitudinal studies have given neuroscientists a chance at elucidating age-related memory impairments or alterations that accompany it. Notably, the declines in mental abilities can vary for aged individuals and can also be of different memory type (TuckerDrob & Salthouse, 2011). In most clinical studies, that not all elderly subjects perform memory tasks at a lower level as compared to younger participants. The variability in test performance between the young and aged individuals increases with age (Ylikoski *et al.*, 1999). A recent study has suggested that the increased variability in memory performance with age is an indication of pathological processes, while similar performance levels between younger and older samples indicates normal ageing (Small, 2001). The reason for such a variability of memory performance between ages may include, increases in strategic options through life-long learning, plasticity in response to varied life experience, or greater variation in physical or mental activity levels (for example, before and after retirement) (Hedden & Gabrieli, 2004; Starr *et al.*, 1997). Structurally, cortical recruitment in aged individuals is prevalent and additional recruitment of these cortical areas is of a compensatory in nature for older adults (Cabeza *et al.*, 2002).

7.0.5 The age of neuroenhancement

The enhancement of normal neurocognitive function by pharmacological means seemed like an apparent route in rescuing memory decline in older adults. Laborious research in memory led to a deeper understanding of synaptic changes that encodes for memory. Such information gathered can be applied to manufacturing memory-enhancing drugs. For example, manipulating AMPA-type glutamate receptors can accelerate the encoding of memory and have positive effects on models of cognitive dysfunction in both preclinical and human studies (Lynch, 2004). Ampakines, as cognitive enhancer, are primarily positive modulators that facilitate excitatory transmission and network level activity. They work by enhancing and prolonging synaptic currents which can be generated by the release of glutamate from axon terminals through the desensitisation and deactivation of AMPA receptors. Notably, several studies have confirmed that ampakines and other positive modulators elevate production of BDNF as well (Lauterborn *et al.*, 2000; Lauterborn *et al.*, 2003). Demonstrations of elevated neurotrophin levels might directly have facilitated LTP and subsequently encourage profound effects on the substrates of cognition. Most recent work in rat cortical neurons revealed that AMPA potentialisation can potentially activate genes and functional processes involved in neuroprotective and cognitive effects such as (Mourlevat *et al.*, 2011).

Under the span of improving mental capacity in healthy beings, pharmaceutical advances are aimed at augmenting the consolidation process itself. Most efforts at promoting consolidation deal with the late, protein synthesis-dependent phase which involves the cAMP-response element binding protein (CREB) family of transcription factors (Lynch, 2002). CREB's role in memory is tightly linked to its participation in long-term forms

of synaptic plasticity (Soderling & Derkach, 2000) where its expression in postsynaptic neurons of the Schaffer collateral pathway can directly facilitate the establishment of long-lasting LTP in hippocampal slices (Barco *et al.*, 2002). Since enhancing CREB functions can lead to stable encoding of long-term memory (Suzuki *et al.*, 2011; Yiu *et al.*, 2011), biotechnology companies (Memory Pharmaceuticals and Helicon) are focused on at developing drugs that facilitate CREB contributions to long-term memory (Lynch, 2002). Neuroenhancement with pharmaceutical drugs are available for purposes other than restoring memory capabilities. In terms of executive functions, drugs that target the dopamine and noradrenaline neurotransmitter systems are effective at improving deficient executive function (Farah *et al.*, 2004). Although methylphenidate, also known as Ritalin, is prescribed for the management of attention-deficit hyperactivity disorder, treatment in healthy young volunteers have been demonstrated to augment working memory performance (Mehta *et al.*, 2000). Modafinil is generally prescribed for sleep disorders, such as narcolepsy. Young healthy man, specifically pilots, on modafinil acquired higher test scores of performance and physiological arousal than they did while on placebo, and also had improved self-ratings of vigour, energy, alertness, talkativeness, and confidence (Caldwell *et al.*, 2000).

7.0.6 Improving memory through limiting calories

On the other hand, there are other theories about protecting and preserving cognitive function from the ageing process that are free from pharmaceutical interventions. Lifelong caloric restriction (CR) has been reported to reduce some of the effects of ageing in both humans and animals (Mattson *et al.*, 2001). Aged animals and the declined synaptic function might consequently lead to deficits in learning. CR has been demonstrated to effective in preventing age-related deficit in LTP by preventing the loss of expression of NR1 (an essential NMDA receptor subunit). In aged animals significant less potentiation was demonstrated as compared to those on calorie restriction (Eckles-Smith *et al.*, 2000). The positive relationship between dietary restriction and alteration in neuronal plasticity is due to several molecular changes in the ageing brain, i.e., expression of genes that encode proteins involved in innate immune responses, oxidative stress and energy metabolism (Lee *et al.*, 2000). In humans, the risk of developing a neurodegenerative disorder such as AD may be lower in subjects with low calorie intake (Mayeux *et al.*, 1999). Similarly in a mouse model, restrictions on diet can ameliorate age-related deficits in learning and memory (Ingram *et al.*, 1987). Dysfunction in neurons that comes with ageing can be narrowed down to three major alterations involved in the neurodegenerative process: increased oxidative stress, perturbed cellular ion homeostasis and impaired energy metabolism (Mattson, 2000). With increased oxidative stress, subsequent damage to proteins, lipids and nucleic acids follows. Dysregulations of cellular ion homeostasis can be further agitated by aberrant increases in intracellular calcium levels. Lastly, impaired energy metabolism can result from, and contribute to, mitochondrial dysfunction (Mattson, 2000) and all of these changes will most likely lead to apoptosis. With CR, neurons acquired some resistance to the change that accompanies ageing. Two major classes of genes affected by CR include those encoding cellular stress proteins and neurotrophic factors (Mattson *et al.*, 2001). In rat brains, protective measures

adopted due to dietary restriction includes: increased levels of the stress proteins HSP-70 and GRP-78 (a neuroprotective mechanism), preservation of glucose uptake following oxidative and metabolic insults, and loss of mitochondrial function was prevented (Guo *et al.*, 2000). Apart from preserving neurons from oxidative stress, neurotrophic factors (Duan *et al.*, 2001) such as BDNF levels are found to be increased just by reducing food intake. Ageing animal models of CR have shown positive associations through memory and learning (Ingram *et al.*, 1987). Two spatial memory tasks were recruited, the eight-arm radial maze and the Morris water maze, to understand the correlations between CR and memory systems (Stewart *et al.*, 1989). Aged rats on restricted diets performed both better and quicker as compared to aged rats on *ab libitum* in these spatial tasks. Resistance to excitotoxin-induced degeneration in hippocampal neurons is shown in a separate study, supporting the hypothesis that resistance of the brain to insults that involve metabolic compromise and excitotoxicity has increased (Bruce-Keller *et al.*, 1999).

7.0.7 The need for regulatory science

The controversy about rosiglitazone originated from a meta-analysis of controlled clinical trials which concluded that rosiglitazone was associated with a significant risk of myocardial infarction and with an increase in the risk of death from cardiovascular causes (Nisse & Wolsk, 2007). Although the authors were unable to ascertain the mechanism behind such a finding, it was suggested that the potential contributing factor may be the adverse effect of the drug on serum lipids. Indeed, rosiglitazone has been demonstrated to increase low-density lipoprotein (LDL) cholesterol in 18.6% of patients under 26 weeks treatment (8mg/day). The alteration in LDL is associated with an increase in adverse cardiovascular outcomes (Nisse & Wolsk, 2007). Rosiglitazone is still kept on the shelves in the United States of America, although efforts have been made to inform patients and providers about potential cardiovascular risk (Rosen, 2010). In Europe, however, The European Medicines Agency has recommended suspending sales of rosiglitazone (Blind *et al.*, 2011). The controversy surrounding an innovative drug, such as rosiglitazone, reflects the extreme difficulty of making the regulatory decision that will best balance the benefits and risks of a drug. In recent times, more emphasis have been put on regulatory science which is a specialised research area aimed to improve assessment of experimental therapies, preventives, and diagnostics (Halffman, 2003). For example, a partnership has been forged between the Department of Health and Human Services, the National Institutes of Health, and the Food and Drug Administration where a program was installed to aid researchers' understanding of regulatory parameters for drugs and devices developed from their work (Pratt, 2010). By definition, regulatory science encompasses rules that are neutral and open to public scrutiny must be created through cooperative research among industry, academic and regulatory scientists (Tominaga *et al.*, 2011). Lessons from the FDA advisory committee meeting in 2007 are crucial not only to diabetic drugs but to all future drug developments. Three basic guidelines were mentioned in a review written by the chairman of the advisory meeting (Rosen, 2007). A clear understanding of the pathogenesis of disorders is firstly required. Followed by, treatment options that should be clarified through an evidence-based system; lastly, a uniform approach should be used to determine the societal benefits and risks associated with a

given intervention.

7.0.8 The future of successful ageing

The ageing process is unrelenting and has consequences on both physical and mental capabilities. The attainment of successful ageing appears to be the light at the end of the tunnel for most of us. As the ageing population is on the increase, more people experience the period of old age to be a time of growth and productivity. The term "successful ageing" is multidimensional, encompassing the avoidance of disease and disability, the maintenance of high physical and cognitive function, and sustained engagement in social and productive activities (Rowe *et al.*, 1998). Recent profiling of 5,688 persons aged 50-74 years in New Jersey revealed factors that contributed to successful ageing. The researchers found that people are more likely to age successfully if they are educated, have never been incarcerated, are married, consume only moderate amounts of alcohol and either work for pay or do volunteer work (Pruchno *et al.*, 2010). Additionally, recent clinical trials suggest that caloric restriction, physical activity, cognitive intervention, stress reduction, and social programs may enhance the cognitive and emotional health of older people (Depp *et al.*, 2010). Emerging interests in promoting states of cognitive and emotional health in older adults are relevant since intact mental faculties are crucial and have serious repercussions in physical abilities as well (Depp *et al.*, 2010). Pursuing successful ageing might enhance the quality of living in future days by preserving the cognitive capabilities.

7.1 Future directions

Attempting to preserve memory in ageing is a fundamentally attractive idea to most pharmaceutical companies. There are already some over-the-counter therapies which are marketed as having the ability to improve memory, attention, and related cognitive function. For example, the package insert for one brand of Ginkgo biloba, Ginkoba, claims that it will enhance mental focus and improve memory and concentration. Unfortunately, clinical studies failed to demonstrate similar procognitive effects in a six weeks trial where no measurable benefit in memory or related cognitive function was found (Solomon *et al.*, 2002). Evidences provided in this preclinical model of ageing provided clear procognitive evidences for rosiglitazone and donepezil. We suggest that neuroenhancement can be adapted as part of the preventive therapy in ageing. The procognitive effects demonstrated in this preclinical model give support that cognitive impairments do appear even before severe ageing takes place. On top of that, modulating distinct neuromodulatory systems can ameliorate such impairments.

7.1.1 Recommended improvements to the work presented so far

7.1.2 Further analyses into different brain regions

There are several ways to obtain a clearer picture of the mechanisms behind the procognitive therapies we have observed. Undeniably, the biological targets we analysed do not encompass the entirety of the neuromodulatory

systems relating to the cholinergic and glucoregulatory systems we are investigating. At the end of the studies there are some molecular targets, in different brain regions, that we could investigate in the future providing further insights into the mechanisms underlying these procognitive therapies. Besides the hippocampus, several other brain regions are implicated in the learning and memory demonstrated in the spatial MWM task. Lesions caused by injection of quinolinic acid into the striatum lead to specific deficits in motor performance and spatial learning reflecting categories of cognitive function that are disturbed in Huntington's disease (Block *et al.*, 1993; Furtado & Mazurek, 1996). Similar with regions within the hippocampus, variation in subregions of the striatum contributes differently to MWM performance. Dorsal and lateral striatal lesion does not lead to sensorimotor or spatial deficits and can perform the MWM task unlike medial striatum lesions which leads to increased thigmotaxis in rats (Thullier *et al.*, 1996; Devan & White, 1999). An intact cholinergic system within the basal forebrain is required to perform the MWM tasks. Lesion such as the bilateral excitotoxic lesions of the nucleus basalis magnocellularis brings about deficit in the water maze task regardless of training parameters (Mandel *et al.*, 1989). Another disruption in the cholinergic system pertaining to lesions in the entorhinal cortices which has shown to reduce acetylcholinesterase positive fibres in the CA3 and stratum moleculare of the hippocampal formation, resulting in spatial discrimination deficits (Spowart-Manning & van der Staay, 2005). Cortical areas also play a role in planning and executive functions as lesions to such areas impaired MWM performance as well (D'Hooze *et al.*, 2001). Importantly, cerebellar connections involve a large system that includes frontal, posterior parietal, inferior temporal cortices, hippocampus and basal ganglia. All of these form an allocentric spatial system and an egocentric control system that interlock to process the information involved in representing spatial memory (Petrosini *et al.*, 1998). The behavioural inflexibility exhibited in the middle-aged group of rats suggested a prefrontal dysfunction where rats preferred a straight swim behaviour without associating with available spatial cues. It would be interesting to locate the mechanisms involving the therapeutic effects of donepezil in the prefrontal region. More specifically, the PI-IL cortices and the dorsal anterior cingulate cortex, since lesions in these parts resulted the inability to switch strategies (Dias & Aggleton, 2000). A through investigations into these frontal regions might give further clues into how donepezil alters behavioural flexibility.

7.1.3 Expanding the targets analysed in evaluating physiological alterations under drug treatments

The molecular targets analysed in the study were chosen as they reflected important physiological components in the understanding of ageing within the behavioural models. For example, an indepth analysis into the secondary systems and receptors involved in neurogenesis would provide more knowledge in the drug actions we observed. Additionally, locating changes in insulin/IR within the work presented might provide clues underlying the mechanisms of procognitive effects of rosiglitazone. It would be interesting to discover why an adjunct therapy involving both systems failed to further boost acquisition and memory abilities. Microarray techniques assay numerous targets using high-throughput screening methods. Probing into the gene changes accompany-

ing such alterations might be helpful in elucidating the muted effect observed under the combination therapy. Biomedical research would certainly be an interesting path to take with the current study as it could give a more distinct and absolute answer for the changes occurring during ageing and the therapeutic implications of the drugs used. However, it is also important to note that this technique also brings with it several major bioinformatics and resource problems such as generating extremely large and unwieldy data sets which are subject to high false positive and false negative rates (Blalock *et al.*, 2005). Although we have speculated that rosiglitazone's procognitive effects were mainly regulated via an improvement in peripheral insulin sensitivity and the increased GLUT-3 expression in the brain, there is an query as to the PPAR γ action. An extension of the current study employing the use of a antagonist of PPAR γ , BADGE, might reveal some potentially important explanations PPAR's action in memory enhancement.

7.1.4 Achieving more with the WPM task

The study using the adjunct therapy might achieve more information under the WPM task protocol. It is likely that similar neuroenhancement could be distinguished in a chronic study, corresponding to the work presented under donepezil and rosiglitazone treatments. In a way, deploying the WPM task in investigating effects of combination therapy might be more accurate in terms of comparison between the groups we already have. It is interesting to ascertain if the improved behavioural flexibility persists when both treatments are given in conjunction and to what extent. Currently we have presented the type of errors made for the trials given. More complex analyses into the pattern of swim behaviour in the multiple-start trials could provide a clearer picture of how the animal makes a decision when the starting position is altered. For example, recording sequential swim behaviour in relation to its start position might indicate when the animal switches from "response" strategy to "place" strategy. In short, the research presented in the separate studies are efficient in elucidating age-related learning impairments. In conclusion, the fascination with science is that it is incomplete. We have suggested some relevant additions to the study that could further the understanding in the ageing behavioural model.

Chapter 8

Main conclusion

There are several conclusions that can be drawn from this series of studies. Firstly, the design and protocol of the WPM task was adequate for the assessment of cognitive deficits in healthy ageing rats and the memory dysregulations we observed were distinct from young adult rats. The novel paradigm we enlisted in this study was able to track noticeable changes in memory improvements with the time-points designated in the chronic study of rosiglitazone and donepezil treatments. Results from the WPM task revealed that middle-aged rats were slower in acquiring both the fixed-start and multiple-start trials. Spatial representation in these rats might not be intact as they failed to complete the harder multiple-start task beyond chance levels. Secondly, the observed decline in cognitive functions within these rats can be ameliorated by manipulating the cholinergic and glucoregulatory systems. Evidently, assisting the cholinergic transmission through the augmentation of ACh by donepezil can diminish behavioural inflexibility observed in the older rats. Although treatment with rosiglitazone failed to produce similar behavioural alterations, maintaining glucoregulatory functions seemed to alleviate memory impairments in middle-aged rats. Notably, an acute dose of rosiglitazone enhanced task acquisition in young rats which presented itself as an advantageous lead in solving the WPM task throughout all the entire study. Alongside these observations, it appears that the therapeutic options we investigated can be advantageous even in younger rats with no discernible cognitive deficits. The next step was the employment of a combination therapy utilising both donepezil and rosiglitazone in search of changes in the recognition memory exhibited by the SOR task. Unfortunately, the combination therapy did not prove to be successful despite the prominent memory enhancements observed from singular chronic therapies of rosiglitazone and donepezil. In fact, the administration of both drugs seems to impede the therapeutic effects observed for each drug. Lastly, we further extended our work into investigating the biological alterations that accompanied these behavioural paradigm in the hope of identifying the mechanism behind the drug actions. We suggest that the procognitive properties observed from donepezil might be partly due to an upregulation of the nicotinic $\alpha 7$ receptor and neurotrophins within the hippocampus. As for rosiglitazone, the upregulation of GLUT 3 hippocampal receptors might be an important mechanism for the procognitive effects of the PPAR γ agonist. We propose that enhancing such systems before the onset of ageing might be a potential procognitive method

of addressing the decline of mental functions accompanied by advancing age.

Appendices

Recipes for buffers and reagentsCarbonate Coating Buffer pH 9.7

Sodium hydrogen carbonate	0.025M
Sodium carbonate	0.025M
Distilled Water	0.025M

Electrode Running Buffer

Tris Base	25 mM
Glycine	200mM
SDS	17mM

Krebs Solution

NaCl	136mM
KCl	2.5mM
KH ₂ PO ₄	1.18mM
MgSO ₄	1.18mM
NaHCO ₃	16mM
Glucose	10mM
Containing CaCl ₂	2mM

Phosphate-buffered Saline (PBS) pH 7.4

Na ₂ HPO ₄	80mM
NaH ₂ PO ₄	20mM
NaCl	100mM

PBS-Tween (PBS-T)

Tris-HCL	0.5M, pH 6.8
Glycerol	10% v/v
SDS	0.05% w/v
B-mercaptoethanol	5% w/v
Bromophenol	0.05% w/v

Separating Gel

Bisacrylamide	33% w/v
Tris-HCL	1.5M, pH 8.8
SDS	1% w/v
Ammonium persulphate	0.5% w/v
Distilled Water	
TEMED	0.1% w/v

Stacking Gel

Bisacrylamide	6.5% w/v
Tris-HCL	0.5M, pH 6.8
SDS	1% w/v
Ammonium persulphate	0.5% w/v
Distilled Water	
TEMED	0.1% w/v

Transfer Buffer pH 8.3

Tris Base	25mM
Glycine	192mM
Methanol	20% w/v
SDS	0.05% w/v
Distilled Water	

TBS-Tween Wash Buffer

Tris-HCL (pH 7.6)	20mM
NaCl	150mM
Tween-20	0.05% w/v
Distilled Water	

Alterations in targets that did not correlate to behavioural outcomes.

Rosiglitazone-induced increased in hippocampal Glucose Transporter Protein 3 (GLUT-3) levels.

There is an age-dependent decrease in GLUT 3 expression in discrete areas of rat hippocampus (28 months old) (Fattoretti *et al.*, 2001). In our chronic rosiglitazone study, age-related decline in hippocampal GLUT 3 protein levels were ameliorated in middle-aged animals under treatment. Notably, this phenomenon was also present in YA animals. Behaviourally, the ability to solve the WPM task was augmented in these animals. Correlations between behavioural parameters in the final session (latency and level of correct responses) and GLUT 3 levels failed to produce a distinct effect. Under the short-term treatments, GLUT 3 mRNA levels were investigated and remained unchanged among all groups. Additionally, rosiglitazone treatment failed to produce a significant effect on ODR for both delays. In the absence of any variation in both data sets, correlation analyses did not produce any pronounced effects (r value close to 0, $p > 0.05$).

Elevated Acetylcholine (ACh) concentration in hippocampal tissues

The decline of ACh release contributes to a reduction in cholinergic function in the senescent brain (Gibson & Peterson, 1981). Donepezil have been demonstrated to increase ACh concentration in the synaptic cleft of the hippocampus, mostly through AChE inhibition (Kosasa *et al.*, 1999a). In this short-term study, 5 days of dosing with donepezil and COMBI treatments elevated hippocampal ACh in young adult rats compared to untreated animals. In the older animals, short-term treatments did not produce any significant alterations between groups. Molecular endpoint analyses did not correlate with any behavioural parameters (r value close to 0, $p > 0.05$).

Hippocampal major histocompatibility complex (MHC) II expression levels consistent between groups.

In normal aging, an age-related increase in hippocampal MHC II mRNA levels was described in 24 month old rats (Frank *et al.*, 2006). To understand the micro-environment in the ageing CNS, we investigated MHC II expression levels in middle-aged animals. Under the chronic rosiglitazone paradigm, no change was presented among the groups of animals. Correspondingly, analyses between these results and behavioural outcomes did not deliver any correlations (r value close to 0, $p > 0.05$).

Similar interleukin-4 (IL-4) mRNA expression in hippocampus despite age differences in rats.

Rosiglitazone reversed the age-related decrease in hippocampal IL-4 concentration, giving strength to the theory of rosiglitazone's anti-inflammatory properties (Loane *et al.*, 2009). Deviations in experimental methods might explain why these findings were not demonstrated here. The dosing regime and method were distinct, Loane and colleagues added rosiglitazone to rat chow and these animals were given a prolonged treatment of 8 weeks. On the other hand, rosiglitazone was administered orally and for a shorter period of time. Additionally, middle-aged animals might not present an inflammatory phenotype within the CNS, unlike old rats (22 month old). IL-4 mRNA expression was similar in tissue prepared from all of the treatment groups for both tasks. Correlation failed to reveal any interaction between IL-4 and behavioural outcomes for both tasks (r value close to 0, $p > 0.05$).

Plasma corticosterone levels has no effect on behavioural outcomes.

Imposed physiological stress and long-term exposure to elevated plasma corticosterone can lead to spatial learning deficits in middle-aged rats (Bodnoff *et al.*, 1995). Chronic studies like ours, especially in a water maze, can affect the physiological state of the animals. Molecular endpoints, such as, plasma corticosterone, revealed similar plasma corticosterone concentration for all groups. In the short-term study, corticosterone levels differ within groups where donepezil treatment was able to relieve elevated levels in MA animals. None of the behavioural outcomes used in the correlation analyses described any relationship to plasma corticosterone levels (r value close to 0, $p > 0.05$).

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