The Role of Endocannabinoids in Alzheimer's Disease

Nazia Maroof, BSc MRes

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Abstract

The endocannabinoid system (ECS) comprises the endocannabinoids (ECs), including anandamide (AEA) and 2-arachidonoyl glycerol (2AG), which interact with the G protein-coupled type-1 and type-2 cannabinoid receptors (CB1 and CB2 respectively). The ECS is thought to have a role in a number of central processes including neuroinflammation, neurogenesis, neuroprotection, learning and memory. Due to its influence on a diverse number of processes, it has been suggested that modifying the ECS may be therapeutically beneficial in Alzheimer's disease (AD). AD is an age-related neurodegenerative disorder characterised by the presence of extracellular amyloid β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) resulting in impairments in learning in memory.

The aim of this thesis was to determine the status of the brain ECS in the APPswe/PS1ΔE9 mouse model of AD and wildtype littermates at 4, 6 and 8 months of age and the performance of these animals in a behavioural test battery. The results of this study indicated that APPswe/PS1ΔE9 animals were hyperactive compared to their wildtype counterparts at all ages and that they also displayed deficits in behavioural flexibility. EC levels increased with age in both wildtype and APPswe/PS1ΔE9 mice. Cannabinoid receptor coupling was increased in the frontal cortex and striatum of APPswe/PS1ΔE9 mice relative to wildtype. This study concluded that the status of the brain ECS is altered in AD.

Modifications to the performance of the ECS were made in the form of chronic administration of a CB1 receptor antagonist (SR141716A/rimonabant)

and a CB2 receptor agonist (JWH133). Chronic administration of SR141716A was able to reverse some learning impairments in APPswe/PS1ΔE9 animals. In contrast, chronic administration of JWH133 resulted in impaired memory extinction in both wildtype and APPswe/PS1ΔE9 mice. The results support the potential benefit of modulating the endocannabinoid system in the treatment of memory impairment in AD.

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Contents

Abbreviations

Δ9-THC	Δ9-tetrahydrocannabinol
2AG	2-arachidonoylglycerol
AA	Arachidonic Acid
ABHD	αβ hydrolase
ACh	Acetyl Choline
AD	Alzheimer's Disease
ADDLs	Aβ Derived Diffusable Ligands
AEA	Anandamide
AmCH	Amyloid Cascade Hypothesis
ANOVA	Analysis of Variance
APOE4	Apolipoprotein 4
APP	Amyloid Precursor Protein
Aβ	Amyloid β
BDNF	Brain Derived Neurotrophic Factor
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBR	Cannabinoid Receptors
CFC	Contextual Fear Conditioning
CFC	Contextual Fear
ChAT	Choline Acetyl Transferase
CNS	Central Nervous System
COX2	Cyclooxygenase 2

CR1	Complement Receptor 1
DAGL	Diacylglycerol Lipase
DAGs	Diacylglycerols
DSI	Depolarisation Induced Suppression of Inhibition
ECLs	Endocannabinoid-like Ligands
ECs	Endocannabinoids
ECS	Endocannabinoid System
FAAH	Fatty Acid Amide Hydrolase
FLAT	FAAH-Like Anandamide Transporter
GABA	γ Amino Butyric Acid
GPCR	G Protein Coupled Receptor
GSK-3β	Glycogen Synthase Kinase 3β
IHC	Immunohistochemistry
IL	Interleukin
IPSCs	Inhibitory Post Synaptic Current
KI	Knock-Ins
KO	Knock-Outs
LC	Liquid Chromatography
LMA	Locomotor Activity
LOX	Lipoxygenase
LTD	Long Term Depression
LTP	Long Term Potentiation
MAGL	Monoacylglycerol Lipase
MAGs	Monoacylglycerols

MAPK	Mitogen Activated Protein Kinase
MCI	Mild Cognitive Impairment
MS	Mass Spectrometry
NAAA	N-acylethanolamine Hydrolysing Acid Amidase
nAChRs	Nicotinic Acetyl Choline Receptors
NAEs	N-acylethanolamines
NAPE	N-arachdonoylphosphatidylamines
NAPE-PLD	N-acylphospatidylethanolamine phospholipase D
NFTs	Neurofibrillary Tangles
NMDA	N-Methyl-D-Aspartate
OEA	N-oleoylethanolamide
PCA	Principal Component Analysis
PEA	N-palmitoylethanolamide
PKA	Protein Kinase A
PLC	Phospholipase C
PPARs	Peroxisome Proliferator Activated Receptors
PS	Presenelin
ROS	Reactive Oxygen Species
TNFα	Tumour Necrosis Factor α
TRPV1	Transient Receptor Potential Vanilloid Type 1

Chapter 1

General Introduction

1. 1 THE ENDOCANNABINOID SYSTEM

1.1.1 What are Endocannabinoids?

The psychoactive mind altering effects and medical attributes of *cannabis sativa* have been known for centuries, with some attributing its use as far back as the second millennium (Campbell, 1949). However, it was only in 1964 that Yechiel Gaoni and Raphael Mechoulam (Gaoni et al. 1964) were able to isolate the compound responsible for the mind altering effects of *cannabis sativa*: Δ9-tetrahydrocannabinol (Δ9-THC). This was followed 24 years later by the discovery of a G protein coupled cannabinoid receptor in the brain, the existence of which suggested the presence of endogenous cannabinoids (endocannabinoids) which interact with it (Devane et al. 1988; Matsuda et al. 1990). This was confirmed in 1992 with the discovery of anandamide (Devane et al. 1992) and the endocannabinoid system was born.

Endocannabinoids (EC) are neuromodulators and immunomodulators (Marchalanta et al. 2008) derived from arachidonic acid (AA), phosphatidylethanolamine and diacylglycerol (Maccarrone et al. 2007). The principal ECs known to date are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) with 2-AG being the most abundant form. AEA was first isolated from porcine brain in 1992 and 2AG in 1995 from canine intestines (Devane et al. 1992; Mechoulam et al. 1995). Both AEA and 2AG are members of the larger classes of lipid signalling molecules N-acylethanolamines (NAEs) and monoacylglycerols (MAGs). In addition to the ECs, these classes comprise a number of endocannabinoid-like ligands

(ECL) which include N-oleoylethanolamide (OEA), N-palmitoylethanolamide (PEA) and N-arachidonoylglycine (AraGly) (figure 1.1) amongst others (Devane et al. 1994; Alexander et al. 2007). ECLs are thought to facilitate the activity of AEA and 2-AG, as well as possessing important biological functions of their own (Ben-Shabat et al. 1998; De Petrocellis et al. 2001; Di Marzo et al. 2007).

1.1.2 Mechanism of EC synthesis

ECs are synthesised post-synaptically on demand in response to a range of stimuli including elevations in intracellular calcium (Ca^{2+}). Although AEA and 2AG are both derived from arachidonic acid-containing phospholipids (Okamoto et al. 2004; Bisogno et al. 2003; Wang et al. 2009) their formation utilises different synthetic routes.

The main synthetic pathway for AEA involves an N-acyltransferase converting phospholipids to N-arachidonoylphoshatidylamines (NAPE) which are subsequently acted on by N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) to form AEA (Okamoto et al. 2004; Ahn et al. 2008). In addition to this, 3 other synthetic pathways are proposed to exist (Wang et al. 2009; Luchicchi et al. 2012) involving phospholipase C (PLC), lyso phospholipase D and αβ hydrolase 4 (ABHD4). These pathways are depicted in figure 1.1 (taken from Luchicchi et al. 2012).

Similarly, 2AG synthesis also involves multiple synthetic routes. The primary route (Bisogno et al. 2003) involves the formation of diacylglycerols (DAGs) by PLC which are then acted on by diacylglycerol lipase α (DAGLα) or β

(DAGLβ) to form 2AG (figure 1.1). A lesser known pathway utilizing phospholipase A1 (PLA1) and a lysophosphatidyl inositol-specific PLC has also been proposed (Ueda et al. 1993).

1.1.3 Endocannabinoid Metabolism

EC actions are terminated by cellular uptake, although the precise mechanism by which this occurs is disputed, and rapid catabolism. The existence of an EC transporter has been hypothesised but solid evidence remains elusive to date (Moore et al. 2005). An alternative hypothesis is centred on the enzyme responsible for the degradation of AEA, fatty acid amide hydrolase (FAAH). FAAH is thought to maintain an inwardly directed concentration gradient enabling facilitated diffusion of AEA through the plasma membrane (Maccarrone et al. 2007). This concept has received support from the discovery in 2011 of a catalytically silent form of FAAH termed FAAH-like anandamide transporter (FLAT). FLAT has been shown to facilitate AEA transport into cells and is blocked by known AEA transport inhibitors such as AM404 (Fu et al. 2011).

AEA metabolism, like its synthesis, can occur through multiple catabolic pathways (figure 1.1). The main route of degradation involves hydrolysis by FAAH but alternative mechanisms include hydrolysis by N-acylethanolamine hydrolysing acid amidase (NAAA) (Ueda et al. 2010) and oxidation by lipoxygenase (LOX), cyclooxygenase 2 (COX2) and cytochrome P450 (Ueda et al. 1995; Yu et al. 1997; Bornheim et al. 1993).

2AG is hydrolysed (figure 1.1) primarily through the action of monoacylglycerol lipase (MAGL) although the enzymes αβ hydrolase 6 (ABHD6) and αβ hydrolase 12 (ABHD12) also contribute to some extent (Marrs et al. 2010; Savineinen et al. 2012).

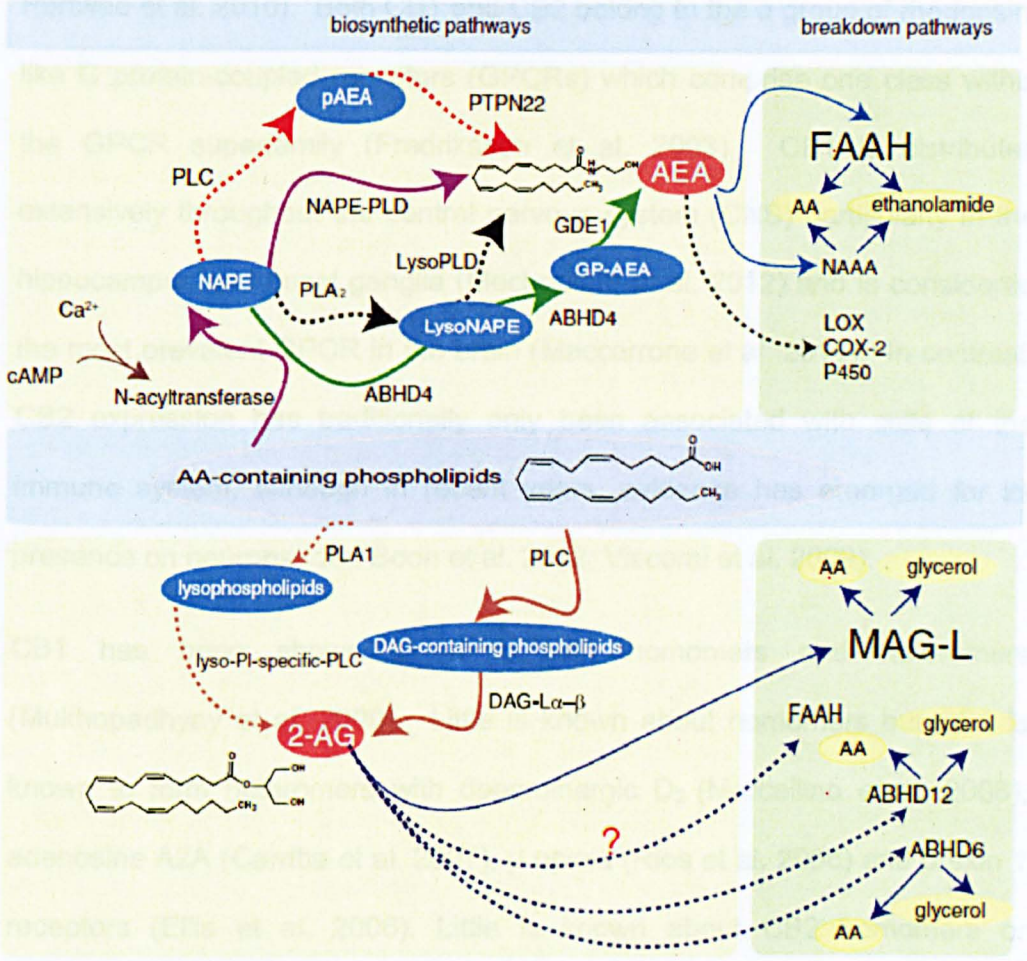

Figure 1.1: Routes of synthesis and breakdown for AEA and 2AG (taken from Luchicchi et al. 2012). NAPE = N-arachidonoylphosphatidylethanolamine; PLC = phospholipase c; pAEA = phosphoAEA; PTPN22 = protein tyrosine phosphatase N22; NAPE-PLD = NAPE-hydrolysing phospholipase D; PLA1, PLA2 = phospholipase A1,A2; GP-AEA = glycerophospho AEA; GDE1 = glycerophosphodiesterase1; DAG-Lα-β = diacylglycerol lipase α-β; FAAH = fatty acid amide hydrolase; AA =arachidonic acid; NAAA = N-acylethanolamine hydrolysing acid amidase; LOX = lipoxygenase; COX-2 = cyclooxygenase 2; P450 = cytochrome P450; MAG-L = monoacylglycerol lipase; ABHD4, 6, 12 = αβ hydrolase 4, 6, 12.

AEA and 2-AG bind to 7 transmembrane G protein-coupled type-1 (Matsuda et al. 1990) and type-2 (Munro et al. 1993) cannabinoid receptors (CB1 and CB2 respectively). CB1 and CB2 exhibit high sequence homology of 44% and are thought to originate from a common ancestor (Katona et al. 2012; Pertwee et al. 2010). Both CB1 and CB2 belong to the α group of rhodopsin-like G protein-coupled receptors (GPCRs) which comprise one class within the GPCR superfamily (Fredriksson et al. 2003). CB1 is distributed extensively throughout the central nervous system (CNS) particularly in the hippocampus and basal ganglia (Mechoulam et al. 2012) and is considered the most prevalent GPCR in the brain (Maccarrone et al. 2011). In contrast, CB2 expression has traditionally only been associated with cells of the immune system, although in recent years, evidence has emerged for its presence on neurons (den Boon et al. 2012; Viscomi et al. 2009).

CB1 has been shown to form both homomers and heteromers (Mukhopadhyay et al. 2000). Little is known about homomers but CB1 is known to form heteromers with dopaminergic D_2 (Marcellino et al. 2008), adenosine A2A (Carriba et al. 2007), μ opioid (Rios et al. 2006) and orexin 1 receptors (Ellis et al. 2006). Little is known about CB2 homomers or heteromers although a recent study demonstrated the formation of functional CB1/CB2 heteromers in transfected neuronal cells and in brain tissue (Callen et al. 2012). Of particular interest was their observation of bidirectional cross-antagonism in the heteromers with CB1 antagonists able to block the effects of CB2 agonists and vice versa.

CB1 receptors are located both pre- and post-synaptically, whereas CB2 receptors are thought to display a preferentially post-synaptic distribution (Callen et al. 2012; Brusco et al. 2008). CB1 but not CB2 receptor distribution is also affected by membrane cholesterol content, with lower cholesterol levels resulting in increased CB1 signalling (Maccarone et al. 2011; Bari et al. 2005).

CB1 and CB2 both signal through the Gi/o class of heterotrimeric G proteins (Stella et al. 1997; Bosier et al. 2010). AEA acts as a partial agonist at CB1 and CB2, whereas 2AG is able to interact as a full agonist at both receptors (Stella et al. 1997). Both AEA and 2AG are considered to possess slightly greater affinity for CB1 than CB2 receptors (Pertwee et al. 2010).

Activation of CB1 or CB2 through binding of ECs or synthetic agonists such as HU210, JWH133, WIN-55-212, results in dissociation of the Gα subunit from the Gβγ and the consequent initiation of a number of intracellular signalling cascades. Both CB1 and CB2 activation result in inhibition of adenlyl cyclase and the associated inactivation of protein kinase A through the Gα subunit, and activation of elements of the mitogen-activated protein kinase (MAPK) signalling mediated by Gβγ (Howlett, 2005). In addition, CB1 stimulation also mediates activation of inwardly rectifying and type A potassium channels (K_{ir} and K_A respectively) inhibition of L, N, P/Q type voltage gated calcium channels (VGCCs) and can cause a rise in intracellular calcium by means of its actions on phopholipase C (PLC). These processes are clearly depicted in figure 1.2 taken from Bosier et al.

2010. In addition to the $G_{i/o}$ class of G proteins, CB1 is also known to signal through G_s (Glass et al. 1997) and G_q (Lauckner et al. 2005) subunits to promote a rise in intracellular Ca^{2+}.

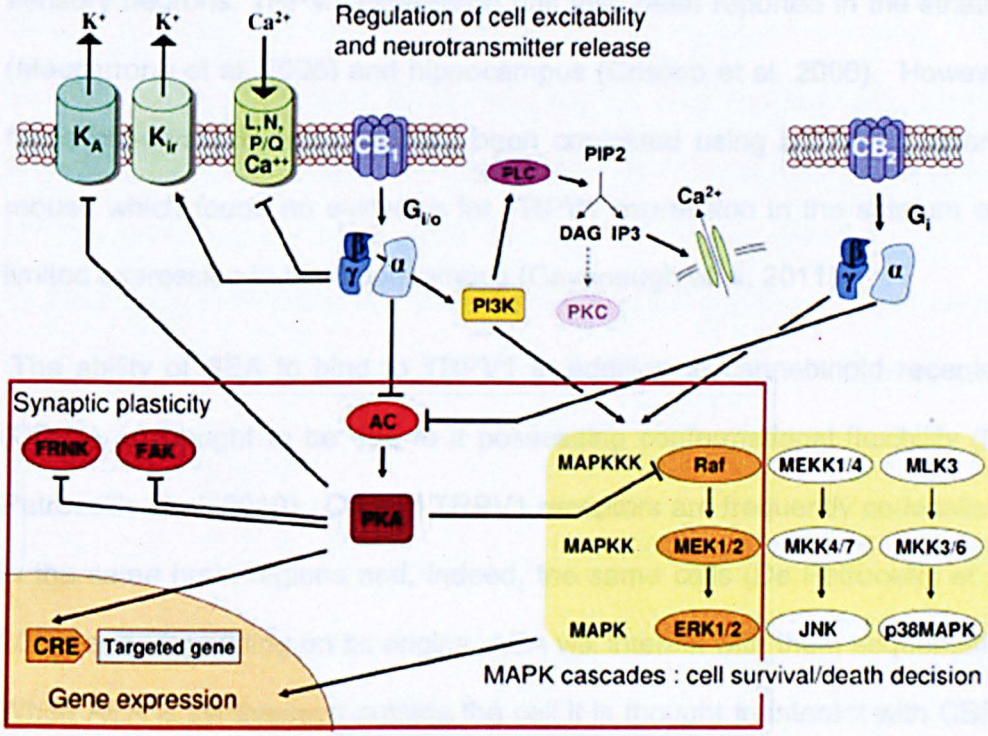

Figure 1.2: Signalling pathways activated by CB1/CB2 activation (figure taken from Bosier et al. 2010). Binding of endocannabinoids to CB/CB2 results in the dissocation of Gα from the Gβγ subunit. Gα goes on to inactivate adenylate cyclase (AC) whilst Gβγ activates the MAPK signalling cascades. In addition, Gβγ units from CB1 activation are able to inhibit A-type potassium channels (K_A) and L, N, P, Q type calcium channels (L,N, P, Q Ca^{2+}) and activate inwardly rectifying potassium (K_{ir}) channels.

1.1.5 Transient Receptor Potential Vanilloid 1 Receptors

AEA, but not 2AG, in addition to PEA (De Petrocellis et al. 2001) and OEA (Ahern, 2003) are able to bind to and activate transient receptor potential vanilloid type 1 (TRPV1) channels (De Petrocellis et al. 2010; Smart et al. 2000). TRPV1 is a poorly selective cation channel, although changes in permeability upon activation result in it exhibiting a 10 fold preference for

Ca^{2+} ions over monovalent cations (Ho et al. 2012), primarily expressed in nociceptive sensory neurons and is activated by high temperatures and noxious stimuli (Battista et al. 2012; De Petrocellis et al. 2010). In addition to sensory neurons, TRPV1 expression has also been reported in the striatum (Maccarrone et al. 2008) and hippocampus (Cristino et al. 2006). However, these observations have recently been contested using a TRPV1 reporter mouse which found no evidence for TRPV1 expression in the striatum and limited expression in the hippocampus (Cavanaugh et al. 2011).

The ability of AEA to bind to TRPV1 in addition to cannabinoid receptors (CBRs), is thought to be due to it possessing conformational flexibility (De Petrocellis et al. 2010). CB and TRPV1 receptors are frequently co-localised in the same brain regions and, indeed, the same cells (De Petrocellis et al. 2010) and, depending on its origins, AEA will interact with them sequentially. When AEA is synthesised outside the cell it is thought to interact with CBRs first followed by TRPV1; however, when it is synthesised within the cell it interacts with TRPV1 prior to CBRs. When CB1 is located pre-synaptically and TRPV1 post-synaptically, AEA is able to downregulate 2AG production and its subsequent retrograde signalling at presynaptic CB1, by activating TRPV1 (Maccarrone et al. 2008; De Petrocellis et al. 2010).

Other consequences of TRPV1 activation by ECs and ECLs include a rise in intracellular Ca^{2+}, increased expression of caspases and pro-apoptotic kinases, mitochondrial uncoupling and release of cytochrome c (Battista et al. 2012).

1.1.6 Peroxisome Proliferator Activated Receptors

Peroxisome proliferator activated receptors (PPARs) are also activated by ECs and ECLs (Kozak et al. 2002; Fu et al. 2003). PPARs are members of a nuclear receptor superfamily comprising a deoxyribonucleic acid (DNA) binding domain, which form dimers with retinoid X receptors (RXRs) and are subsequently able to interact with peroxisome proliferator response elements (PPREs) in a range of target genes to regulate their expression (Aspostoli et al. 2012). Three PPAR isoforms are known to date: α, β/δ and γ with varying expression patterns (Wahli et al. 2012). PPARα is predominantly expressed in tissues catabolising a high volume of fatty acids such as liver, heart and kidney (Auboeuf et al. 1997; Vidal-Puig et al. 1997). PPARβ/δ displays the broadest expression pattern with levels in certain tissues determined by cell proliferation and differentiation (Michalik et al. 2006). PPARβ/δ is found in the skin, gut, adipose tissue, brain, skeletal and heart muscle amongst others (Auboeuf et al. 1997). PPARγ has two isoforms: γ1 found in the brain, vascular, immune and inflammatory cells, and γ2 expressed predominantly in adipose tissue (Vidal-Puig et al. 1997; Michalik et al. 2006).

In addition to the ECs and ECLs, PPARs are able to bind fatty acids, eicosanoids, and phospholipids as well as a number of synthetic compounds such as non-steroidal anti-inflammatory drugs (Kliewer et al. 1997; Lehmann et al. 1997; Pertwee et al. 2010). Both ECs and ECLs are able to activate PPARα whilst only ECs can activate PPARγ (Bouaboulg et al. 2005; O'Sullivan et al. 2010).

1.1.7 Functions of endocannabinoids

1.1.7.1 Retrograde Signalling

One of the most well established functions of ECs is their role as retrograde messengers (Wilson et al. 2001; Ohno-Shosaku et al. 2001; 2012). ECs released from the post-synaptic neurons traverse the synaptic cleft and bind to pre-synaptic CB1 receptors reducing Ca^{2+} and potassium (K^{+}) conductance and ultimately decreasing release of neurotransmitter (Ohno-Shosaku et al. 2012). Historically, both AEA and 2AG have been associated with retrograde signalling, however, recent evidence suggests this role may primarily be mediated by 2AG (Tanimura et al. 2010; Pan et al. 2011).

As a consequence of their role as retrograde messengers, ECs are intimately associated with the phenomena of long-term potentiation (LTP) and depression (LTD), 2 alternative forms of synaptic plasticity underlying learning and memory (Iremonger et al. 2011). LTP is defined as a persistent strengthening of a synapse whilst LTD in contrast refers to the persistent weakening of a synapse. Both LTP and LTD are dependent on glutamate-mediated activation of the N-methyl-D-aspartate (NMDA) receptor.

ECs activate presynaptic CB1 receptors located on γ amino butyric acid (GABA) interneurons (Carlson et al. 2002), thus suppressing inhibitory post synaptic currents (IPSCs), a process termed depolarisation-induced suppression of inhibition (DSI). IPSCs maintain the magnesium (Mg^{2+}) plug preventing the activation of NMDA receptors. By suppressing IPSCs, ECs permit a given excitatory input to cause a greater depolarisation,

subsequently activating the NMDA receptors resulting in a rise in intracellular Ca^{2+} levels pre-requisite for the generation of LTP (Pallas, 2009). The opposite process, LTD involves the same sequence of events except the process involves depolarisation-induced suppression of excitation (DSE) in which case the CB1 receptors are located on glutamatergic neurons. Support for the involvement of the ECS in LTP and LTD is provided by observations in CB1-null mice which show enhanced LTP and no LTD (Varvel et al. 2009).

The role of ECs in learning and memory has also been demonstrated by a number of behavioural experiments assessing recent memory. Intra-hippocampal administration of the CB1 antagonist SR141716A (rimonabant) facilitates learning when given during a watermaze task (Robinson et al. 2008). Intrahippocampal administration of SR141716A has also been shown to negate the memory-disrupting effects of the main psychoactive constituent of marijuana Δ^9-THC and the cannabinoid receptor agonist CP-55,940 (Wise et al. 2009). Chronic intrahippocampal infusion of SR141716A or the agonist WIN 55.212-2 has been shown to improve or impair performance respectively in a delayed non-matching to sample task (Hampson et al. 2011). In particular, ECs have been shown to be critical for the extinction of aversive memories (Marsciano et al. 2002; Niyuhire et al. 2007;Ruehle et al. 2012) and are, therefore, thought to underlie aspects of behavioural set shifting (Pamplona et al. 2008).

Studies using knockout animals have indicated $MAGL^{-/-}$ mice show enhanced LTP accompanied by improved performance in the novel object recognition and water maze paradigms relative to wildtype mice (Pan et al. 2011). Similarly, $FAAH^{-/-}$ animals display enhanced acquisition of an aversively motivated Barnes maze task (Wise et al. 2009). In conclusion, experimental evidence shows that CB activation by ECs impairs or facilitates learning and memory depending on the route and timing of administration.

1.1.7.2 Anti-Inflammatory Mediators

Another function of ECs and ECLs is as anti-inflammatory mediators by means of activation of CB2 and PPARs. Some studies have shown that CB2 expression is up-regulated in activated microglia during neuroinflammation (Ramirez et al. 2012; Stella, 2009). CB2 agonists have been shown to attenuate blood brain barrier (BBB) dysfunction by attenuating BBB permeability; partly through an increase in tight junction proteins (Ramirez et al. 2012). AEA has been shown to prevent synthesis of biologically active pro-inflammatory cytokines interleukin (IL) 12 (IL-12) and IL-23 (Correa et al. 2009). A number of studies have demonstrated that ECs and synthetic cannabinoids are able to prevent the release of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and IL-1β (Ortega-Gutierrez et al. 2005), whilst simultaneously promoting the release of anti-inflammatory cytokines such as IL-4 (Hickman et al. 2008) and the IL-1 receptor antagonist (Molina-Holgado et al. 2003). Furthermore, CB2 activation by 2AG has been

shown to increase microglial proliferation in response to an inflammatory insult (Carrier et al. 2004).

In addition to CB2 stimulation, non-selective synthetic CBR agonists such as WIN55,212-2 and HU210 have been shown to possess anti-inflammatory actions through prevention of microglial activation following neurotoxic insults (Chung et al. 2012).

In addition to CB2, the anti-inflammatory effects of ECs and ECLs can also be mediated through PPARα (O'Sullivan et al. 2010). Both OEA and PEA and have been shown to reduce inflammation through a PPARα-dependant mechanism (D'Agostino et al. 2007; Sun et al. 2007). 2AG has been shown to inhibit IL-2 expression through PPARγ activation (Rockwell et al. 2006). Additionally, 2AG is able to suppress the expression of cyclooxygenase-2 (COX2), an inflammatory marker enzyme, by means of CB1-dependant restoration of PPARγ expression (Du et al. 2011).

1.1.7.3 Anti-Oxidants

Phenolic cannabinoids (Δ^9-THC, cannabinol, cannabidiol, CP 55,940, HU 210 and AM 404) have also been shown to display anti-oxidant properties in a CB1-independent manner (Marsicano et al. 2002). More recent studies have demonstrated the involvement of CB1-mediated inhibition of PKA in counteracting oxidative stress following stimulation by WIN,55 212-2 and AEA (Kim et al. 2005). Further support for a role of cannabinoids in oxidative stress is provided by a recent study indicating that HU210 and WIN55,212-2 are able to inhibit NADPH oxidase (Chung et al. 2012) and consequently,

the associated generation of reactive oxygen species (ROS). In addition to CB1, CB2 selective stimulation has also been shown to reduce oxidative stress in neurons by inducing the expression of Bcl2 (an anti-apoptotic protein) and Hsp 70 (which protects against stress) whilst concomitantly promoting neuroprotection by preventing glial activation (Oddi et al. 2012).

1.1.7.4 Neuroprotection

The neuroprotective effects of cannabinoids, ECs and ECLs have been well documented (Viscomi et al. 2009; Garg et al. 2010; Kreutz et al. 2009; Scuderi et al. 2012; Galan-Rodriguez et al. 2009). Neuroprotection by synthetic and endogenous cannabinoids and their associated ligands occurs in two main forms: protection from excitotoxiclty and prevention of microglial activation in the absence of inflammation. AEA has been shown to confer CB1-dependant neuroprotection in a rat model of excitotoxicity (Hansen et al. 2001; Veldhuis et al. 2003). CB2 activation by the synthetic CB2 selective agonist JWH-015 is able to prevent remote cell death through prevention of cyctochrome c release from mitochondria (an early indication of apoptosis) and involves a PI3K/Akt signalling mechanism (Viscomi et al. 2009). Additionally, the neurons of CB1-null ($CB1^{-/-}$) mutants are more susceptible to neurodegeneration induced by excitotxic cell death than those from wildtypes (Marsicano et al. 2003).

In addition to AEA, OEA (Sun et al. 2007; Galan-Rodriguez et al. 2009) and PEA (Hansen, 2010; Scuderi et al. 2012) have also been shown to provide neuroprotection through a PPARα-dependant mechanism following neuronal

insult. OEA and PEA prevented glial activation (Scuderi et al. 2012; Sun et al. 2007) in addition to reducing neuronal apoptosis (Galan-Rodriguez et al. 2009). 2AG, apparently through its actions on the abnormal cannabidiol-sensitive receptor found on microglia, is able to protect neurons against excitotoxic cell death (Kreutz et al. 2009).

As a consequence of the manifold roles described above, ECs beginning to be considered as viable treatment options for neurodegenerative diseases including Alzheimer's disease (AD) (Marchalant et al. 2012). As this project will investigate the role endocannabinoids may play AD pathology, the next section provides a brief description of the disease process.

1.2 ALZHEIMER'S DISEASE

1.2.1 Alzheimer's Disease General Background

AD is a progressive age-related neurodegenerative disorder affecting approximately 33.9 million people worldwide with the incidence estimated to triple in the next 40 years (Barnes et al. 2011). In the UK in 2012, 800,000 individuals, including 17,000 under the age of 65, are affected by a form of dementia of which AD is the most common, (Alzheimer's Society 2012).

AD represents the most common cause of dementia worldwide (Alzheimer's Society 2012) characterised by impairments in learning and memory. Memory can be classified into 2 broad categories: procedural and declarative. Procedural memory refers to the memory of 'how to do things' and requires no conscious attention during its activation leading to the suggestion that it

lies below the level of consciousness. In contrast, declarative memory can be subdivided into semantic and episodic memory. Semantic memory refers to memory for facts and general knowledge about the world and forms of it can exist in animals other than humans whereas episodic memory can broadly be defined as the memory of past experiences (Tulving, 2002).

In the pre-symptomatic phase of AD, disturbances in semantic memory are detectable at least a decade prior to clinical presentation (Amieve et al. 2008). Disturbances in episodic memory are detected closer to AD diagnosis and are usually classified as mild cognitive impairments (MCI) which are defined by the inability of an individual to retain information over a period of delay (Albert, 2011). An example would be recalling a conversation or appointment or learning something new such as a shopping list. MCI individuals also display impairments in behavioural flexibility, language functions and visuo-spatial abilities leading to disorientation (Albert, 2011). Emotional and sleep disturbances accompanied by changes in personality are also observed. The decline in performance continues until patients are no longer able to function independently.

1.2.2 Genetics of Alzheimer's disease

Until 2009, the only known genetic associations for AD were for the genes encoding amyloid precursor protein (APP), preselinin (PS) 1 (PS1), PS2 and the ε4 allelle of apolipoprotein 4 (APOE4) (Tanzi et al. 2012; Bertram et al. 2010). Of these, only APOE4 was associated with the most common, late onset form of AD whilst the others were linked to the rare early presenting

(<60 years) familial form which only represents 5% of AD cases (Tanzi et al. 2012). To date, more than 40 point mutations have been discovered for APP resulting in the over-production of amyloid β (Aβ), approximately 80 in PS1 and 10 in PS2 (Ashe et al. 2010). For an up to date list of familial AD-related mutations the reader is referred to: http://www.alzforum.org/res/com/mut/asp.

Twin studies have indicated that as much as 60% of the susceptibility to late onset AD (LOAD) may be accounted for by genetics (Lambert et al. 2009). The LOAD genes first identified by Harold et al. (2009) are APOE, clusterin (CLU) also known as APOJ, PICALM, complement component (3b/4b) receptor 1 (CR1), bridging integrator 1 (BIN1) and disabled homolog 1 (DAB1). APOE, CLU and CR1 were also replicated in an independent study by Lambert et al. (2009).

Since publication of the above, another 5 genes conferring genetic susceptibility for LOAD have been identified. These are ATP-binding cassette, sub-family A, member 7 (ABCA7), membrane spanning 4A gene cluster (MS4A), CD2 associated protein (CD2AP), CD33 and ephrin type-A receptor 1 (EPHA1) (Hollingworth et al. 2011; Naj et al. 2011). For up to date information on LOAD associated genes the reader is referred to: http://www.alzgene.org/TopResults.asp.

These genes can be broadly subdivided into 3 main classes according to their functions. CLU, CR1, ABCA7, CD33 and EPHA1 can all be linked to the immune system, PICALM, BIN1, CD33, CD2AP are associated with processes at the cell membrane and APOE, CLU and ABCA7 are thought to

play roles in lipid processing (Hollingworth et al. 2011). APOE and CLU are thought to be the most abundantly expressed lipoproteins in the brain and have both been detected in Aβ plaques and are able to bind to Aβ and, hypothetically, clear it (Lambert et al. 2009). APOE alone, of the 10 genes listed, is thought to account for up to 50% of the genetic susceptibility for LOAD, and as its main role in the brain is as a cholesterol-carrier protein, its influence in AD is thought to be related to impaired cholesterol transport (Lambert et al. 2009; Gamba et al. 2012).

1.2.3 Risk Factors for Alzheimer's Disease

Age is the biggest risk factor for AD followed by possession of the genes, APOE4 in particular, indicated above. However, there are a number of other potentially modifiable risk factors which increase susceptibility (Barnes et al. 2011). These are stress, diabetes, hypertension, obesity, depression, physical inactivity, smoking and cognitive activity/low educational attainment (Pardon et al. 2008; Barnes et al. 2011).

1.2.4 Neuropathology of Alzheimer's Disease

AD is characterised by the presence of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) caused by hyperphosphorylation of the microtubule-associated protein tau (Liebscher et al. 2012).

Aβ is derived by proteolytic cleavage of APP. APP cleavage can follow 2 main pathways of which one is pathogenic and the other is not (figure 1.3).

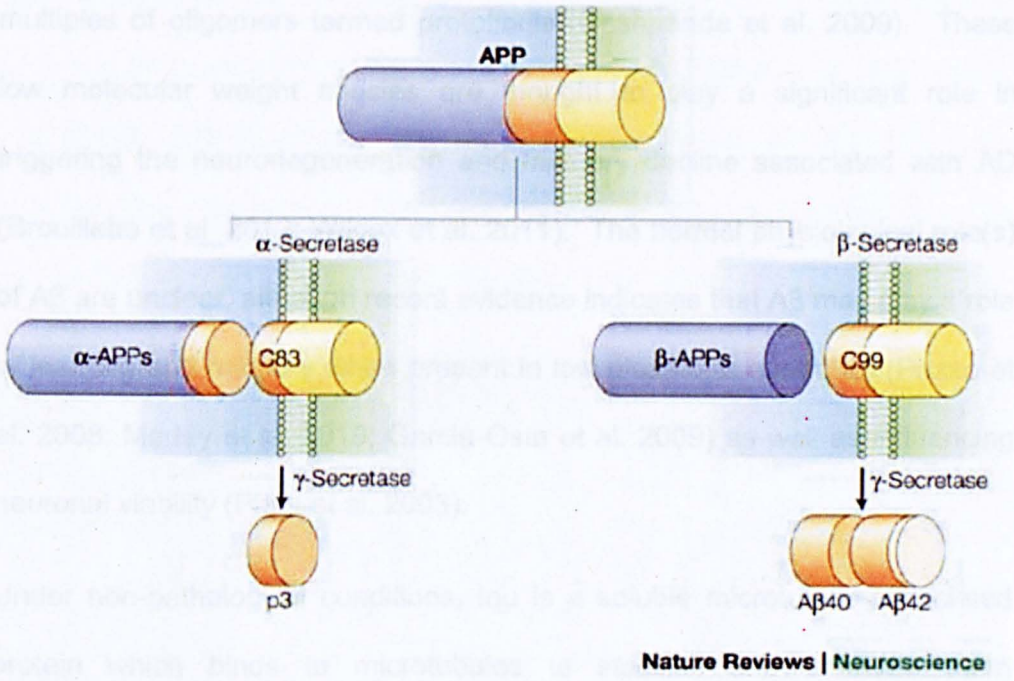

Figure 1.3: Amyloid precursor protein (APP) processing pathways (taken from Citron, 2004). APP is processed either along the α-secretase or β-secretase pathway. In the non-pathogenic α-secretase pathway, α-secretase cleaves in the middle of the β-amyloid region (orange) to release soluble APP, α-APPs (blue), and the C83 peptide which is metabolised to p3 by γ-secretase. In the β-secretase pathway, β-secretase cleaves APP to generate β-APPs (blue) and C99 peptide. C99 is then cleaved by γ-secretase at several positions to generate $A\beta_{40}$ and the aggregation prone $A\beta_{42}$.

The non-pathogenic pathway involves cleavage of APP by α secretase to generate soluble APP and C83 peptide. In the pathogenic pathway, however, APP undergoes sequential cleavage by β and γ secretase to produce $A\beta_{40}$ and $A\beta_{42}$ (Liebscher et al. 2012; Citron et al. 2004). Of the two forms, $A\beta_{42}$ is the more aggregation-prone species (Liebscher et al. 2012; Jankowsky et al. 2004). Once generated, Aβ monomers aggregate in a number of ways ultimately leading to the formation of Aβ plaques. The intermediate steps result in the formation of a number of dimeric, trimeric species as well as Aβ-derived diffusible ligands (ADDLs), oligomers (15-20 monomeric units) and

multiples of oligomers termed protofibrils (Deshpande et al. 2009). These low molecular weight species are thought to play a significant role in triggering the neurodegeneration and memory decline associated with AD (Brouillette et al. 2012; Wilcox et al. 2011). The normal physiological role(s) of Aβ are unclear, although recent evidence indicates that Aβ may play a role in learning and memory when present in low picomolar quantities (Puzzo et al. 2008; Morley et al. 2010; Garcia-Osta et al. 2009) as well as influencing neuronal viability (Plant et al. 2003).

Under non-pathological conditions, tau is a soluble microtubule-associated protein which binds to microtubules to stabilise and orientate them (Shahpasand et al. 2012; Iqbal et al. 2009). Tau is referred to as a phosphoprotein which means that its biological activity is regulated by its degree of phosphorylation (Shahpasand et al. 2012; Iqbal et al. 2009). Under normal circumstances tau is moderately phosphorylated, but becomes hyperphosphorylated in AD, resulting in the loss of its ability to bind to microtubules. As a consequence, the microtubule network becomes destabilised, resulting in impaired axonal transport. Hyperphosphorylated tau subsequently becomes insoluble and forms paired helical filaments and eventually NFTs.

1.2.5 Amyloid Cascade Hypothesis

Two main theories exist to explain AD pathology. The most prominent and widely accepted is the amyloid cascade hypothesis (AmCH) first heralded in

1992, which states that the major cause of neuronal dysfunction and death in AD is due to the production and aggregation of Aβ (Wirths et al. 2004; Reitz et al. 2012). The hypothesis has undergone modification since its inception to include the impact of soluble Aβ oligomers and figure 1.4 illustrates the most up to date version of the hypothesis for both familial and late onset AD.

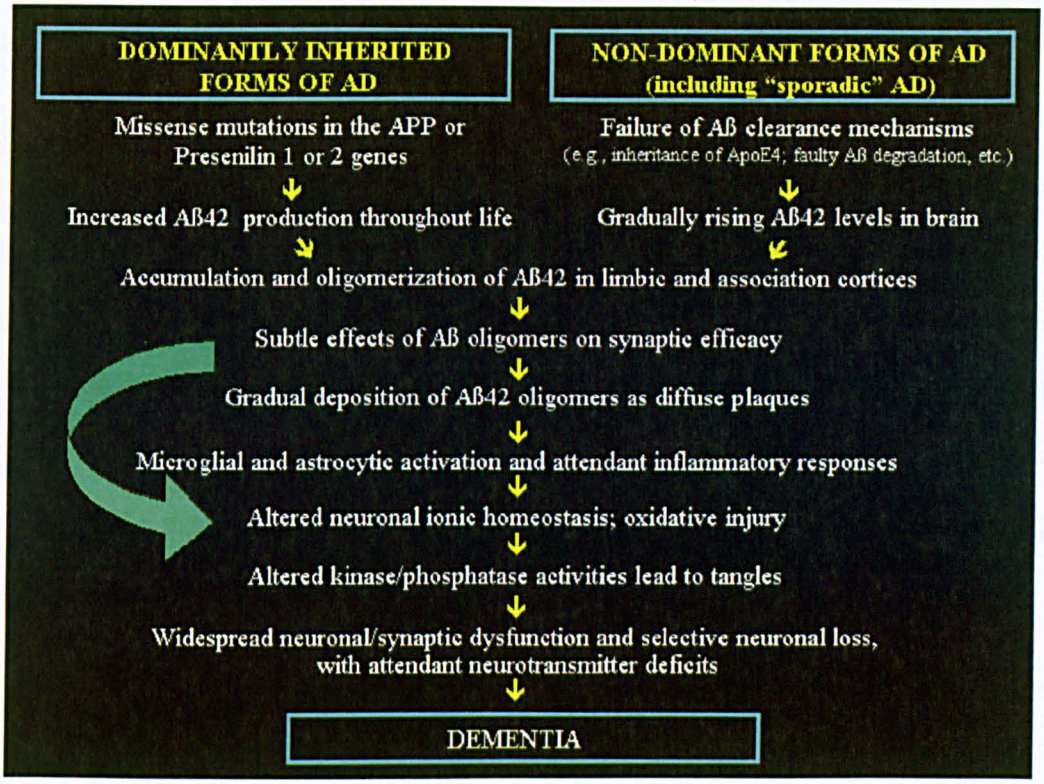

Figure 1.4: Modified Version of the Amyloid Cascade Hypothesis taken from http://www.alzforum.org/res/adh/cur/knowntheamyloidcascade.asp.

A number of factors strongly support the AmCH including the observations that administration of Aβ *in vitro* and *in vivo* leads to neurodegeneration, and mutations in the genes involved in the rarer genetic form of AD (see below), APP, PS1, PS2 over-expression results in overproduction and accumulation of Aβ (Reitz et al. 2012). Additionally, mice expressing a mutation in the tau

gene alone show fewer NFTs than mice co-expressing the P301L tau and APP_{635} Swedish mutations. This observation suggests a role for Aβ in NFT formation, possibly by increasing the levels of enzymes such as glycogen synthase kinase 3β (GSK-3β) responsible for the hyper-phosphorylation of tau seen in AD (Zhang et al. 2011). Indeed, GSK-3β inhibitors have been shown to reduce neuronal death and cognitive decline in a mouse model recapitulating some aspects of AD (Engel et al. 2006).

However, in recent years the AmCH has become increasingly contentious with the continuing failure of multiple clinical trials targeting Aβ (Castellani et al. 2011) in addition to the continued absence of a correlation between Aβ plaque load and cognitive decline (Giannakopoulos et al. 2003). Further objections to the AmCH come in the form of the suggestion that Aβ plaques and NFTs are a consequence of neurodegeneration rather than cause (Reitz, 2012; Armstrong, 2011).

The second hypothesis is centred on tau hyper-phosphorylation and suggests that NFTs are the root cause of the disease (Verdile et al. 2004). This second theory has fewer proponents but does receive support from the correlation between NFT density and AD severity (Bierer et al. 1995; Giannakopoulos et al. 2003). Additionally, NFTs have been found in the brains of patients expressing symptoms of dementia in the absence of Aβ (de Paula et al. 2009). However, despite these caveats, the AmCH continues to predominate as the primary explanation for AD.

1.2.5 How does Aβ Contribute to Alzheimer's Disease?

Exactly how Aβ causes the deficits observed in AD remains controversial. This is not helped by the finding that deficits in learning and memory occur prior to the appearance of Aβ plaques and NFTs. In relation to this, a relatively recent study suggested that neurons which degenerate in AD re-enter the cell cycle prior to visible Aβ deposition (Varvel et al. 2008). Neurons in AD patients displaying MCI also show cell cycle re-entry (Varvel et al. 2008). Hence, neuronal cell cycle re-entry can be regarded as a pre-plaque indicator of neuronal distress (Varvel et al. 2008) and may be partially responsible for the occurrence of memory impairments prior to visible Aβ deposition.

The physiological effects of Aβ include excessive inflammation, oxidative stress, excitotoxicity and reduced forebrain cholinergic transmission.

1.2.5.1 Inflammation in AD

A key feature of the AD brain is neuroinflammation. Neuroinflammation is caused by activated microglia releasing a host of compounds which go on to activate astrocytes and cause effects such as excitotoxicty and the generation of reactive oxygen species (ROS) amongst others.

Microglia

Microglia are the phagocytic immune cells of the brain. Studies have shown that microglia are activated early in the process of AD (Crehan et al. 2012) during which they take on anti-inflammatory roles (alternative activation; Jimenez et al. 2008) including the clearance of Aβ by macropinocytosis (Mandrekar et al. 2009). Additionally, infiltrating T cells in the CNS promote the release of the anti-inflammatory cytokine IL-4 from astrocytes. IL-4 causes microglia to adopt an alternative activation phenotype in AD which promotes the release of anti-inflammatory agents and neurotrophic factors (Jimenez et al. 2008).

However, with disease progression and the continued rise in Aβ load, it is thought that the capacity of microglia to clear Aβ is overwhelmed and they switch to a classical activation phenotype (Jimenez et al. 2008). Classical activation supports the release of a number of pro-inflammatory cytokines and chemokines including TNFα and IL-1β (Couturier et al. 2012). Additionally, microglia also release complement factors, chemokines, ROS, neurotoxic secretory products and nitric oxide (Couturier et al. 2012; Jimenez et al. 2008). Microglia mediate their actions through a number of signalling pathways including that of NFκB which is stimulated by Aβ (Rubio-Perez et al. 2012). Prevention of NFκB activation has been shown to reduce the release of pro-inflammatory cytokines in addition to limiting apoptosis (Coutirier et al. 2012; Delgado et al. 2008). Furthermore, as stated above, genetic variations in the CR1 gene encoding complement receptor 1 have been associated with

AD and CR1 levels are raised in AD (Crehan et al. 2012). CR1 is involved in regulating complement activation including that of complement C3, deficiency of which is associated with enhanced Aβ deposition, apoptosis and a non-phagocytic phenotype in microglia (Crehan et al. 2012). Through their release of chemokines microglia encourage the homing of other glial cells to their location, thereby exacerbating the inflammatory process (Westin et al. 2012). C-C motif ligand 2 (CCL2) is one such chemokine, the levels of which are raised in the cerebrospinal fluid of AD patients and are associated with an accelerated rate of cognitive decline (Westin et al. 2012). Additionally, CCL2 signalling has previously been associated with increased Aβ deposition in an AD animal model (Yamamoto et al. 2005).

In contrast to the above, Hickman et al. (2008) showed that alternatively activated microglia are still present in the immediate region of Aβ plaques, whilst the classically activated microglia are found almost everywhere else in the CNS, including regions devoid of Aβ. Their results suggest that soluble forms of Aβ (ADDLs) may be responsible for the initiation of the classical response involving TNF-α release as it coincides with a large increase in ADDLs.

In summary, microglia appear to play a dual role in AD. In the early stages of AD they provide a supportive function by clearing Aβ and releasing neurotrophic and anti-inflammatory agents. However, with disease progression, the microglial phenotype switches such that it is now detrimental and exacerbates pathology.

Astrocytes

Astrocytes are thought to play a number of roles in AD including clearance and degradation of Aβ, providing trophic support to neurons in addition to serving as barriers between Aβ and neurons (Rubio-Perez et al. 2012). However, despite their beneficial role, in stressful conditions, they can prolong neuroinflammation by expressing inducible nitric oxide synthase (iNOS) which generates nitric oxide (NO) excessive levels of which are neurotoxic. Additionally, exposure of astrocytes to Aβ results in increased levels of TNFα, IL-1β and IL-6 (Wyss-Coray et al. 2012).

One of the most important functions of astrocytes is the regulation of glutamate levels in the CNS. Glutamate released into the extracellular space is transported into astrocytes where it is degraded by glutamine synthetase into glutamine. Glutamine is subsequently shipped to neuronal presynaptic terminals where it is used to synthesise glutamate for neurotransmission. However, Aβ disrupts this process through an adenosine A2A receptor-dependant mechanism (Matos et al. 2012).

A number of other functions have also been ascribed to astrocytes in recent years including roles in LTP mediated by the release of D-serine in a calcium-dependant manner (Hennerberger et al. 2010). Astrocytes are also capable of providing neuroprotection through the release of neurotrophins (Barres 2008).

1.2.5.2 Excitotoxic Insult:

Neuroinflammation, in addition to direct effects of Aβ, causes the release of glutamate from neurons and glia. Glutamate activates NMDA receptors on neurons resulting in influx of Ca^{2+}, thereby increasing intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Additionally, Aβ is capable of forming pores in the lipid bilayer which are permeable to Ca^{2+} thereby further enhancing $[Ca^{2+}]_i$. Excessively raised $[Ca^{2+}]_i$ leads to cellular depolarisation, mitochondrial dysfunction, production of ROS and activation of DNAases, proteases and lipases amongst others.

Memantine, a non-competitive NMDA receptor antagonist is used, albeit with limited efficacy, in the treatment of moderate to severe AD, thereby providing further corroboration for excitotoxic cell death being an important factor in AD pathology. Memantine acts by inhibiting pathological but not physiological actions of the NMDA receptor by reducing the neuronal signal to noise ratio during excessive glutamate release (Marchalant 2008). Additionally, it promotes the release of the brain-derived neurotrophic factor (BDNF). BDNF is thought to be pre-requisite for LTP and memory consolidation (Bekinschtein et al. 2008; Lu et al. 2008) and has been shown to have a number of beneficial effects in animal models of AD (Nagahara et al. 2009). Mice injected with lentiviral vectors constitutively expressing BDNF showed significant improvements in behavioural paradigms designed to test spatial and contextual memory (Nagahara et al. 2009). Additionally, BDNF was

shown to attenuate dysregulation of hippocampal and entorhinal gene expression and to reverse synaptic degeneration (Nagahara et al. 2009).

Unfortunately, memantine provides only minor clinical improvements when given to moderate to severe AD cases, and benefits are virtually undetectable when given to patients with mild to moderate AD (McShane et al. 2006).

1.2.5.3 Oxidative Stress in Alzheimer's Disease

Oxidative stress is thought to occur early in the course of AD and manifests in the form of raised levels of oxidation of proteins, lipids, DNA and the formation of toxic compounds such as peroxides (Feng et al. 2012). Lipid peroxidation results in the generation of a number of toxic species including the highly reactive 4-hydroxy 2,3-nonenal (4HNE), levels of which are raised in AD (Feng et al. 2012; Lovell et al. 2012). 4HNE has a number of detrimental effects including impairing the uptake of glutamate by astrocytes (Lovell et al. 2012) in addition to depleting membrane phospholipids, inhibition of neuronal glutamate and glucose transporters, sodium/potassium ATPases and dysregulation of Ca^{2+} homeostasis amongst others (Feng et al. 2012; Massaad, 2011). These processes are likely to cause the generation of further ROS and neuronal apoptosis leading to a self-perpetuating process (Feng et al. 2012). In addition, it has recently been demonstrated that neuronal apoptosis following oxidative stress is associated with an increase in lysosomal Aβ accumulation mediated by macroautophagic mechanisms (Zheng et al. 2011; 2009).

Further detrimental effects occur as a consequence of ROS resulting in excessive influx of glutamate leading to excitotoxicty, damage to DNA in the region of Aβ and NFTs (Feng et al. 2012), and generation of the hydroxyl (OH•) radical following Aβ mediated reduction of copper (Cu^{2+}) ions from Cu^{2+} to Cu^{+} (Massaad, 2011).

1.2.5.4 Cholinergic Transmission in Alzheimer's Disease

Acetylcholine (ACh) is one of the primary neurotransmitters in the brain and has roles in a number of processes including memory and executive functions (Bernier et al. 2009). ACh activates two classes of receptors: muscarinic and nicotinic. In AD, the expression of the presynaptic nicotinic ion channel receptors (nAChRs) is reduced (Schliebs et al. 2011). Deficits in levels of the enzyme required to manufacture ACh, choline acetyl transferase (ChAT), are observed in the course of AD and in animal models of AD, due to degeneration of ChAT-positive neurons in the basal forebrain (Schliebs et al. 2011). The death of these neurons results in degeneration of the cortical neurons to which they project. It has been suggested that Aβ is able to bind directly to nAChRs, in particular the α7 subtype, with high affinity, prompting the suggestion that, in certain cases, deficits in cholinergic transmission may be due to inactivation of nAChRs as a consequence of Aβ binding, rather than cell death (Parri et al. 2011; Schliebs et al. 2011). In addition, α7nAChRs have been shown to co-localise with $A\beta_{42}$ in Aβ plaques in post-mortem human AD brains and the addition of $A\beta_{42}$ results in the death of human neuroblastoma cells over-expressing α7nAChRs whilst the addition of

an α7nAChRs agonist protects them (Dziewczapolski et al. 2009). Furthermore, deletion of α7nAChRs in the PDAPP mouse model of AD resulted in the rescue of cognitive deficits, the preservation of synaptic markers and reduced gliosis (Dziewczapolski et al. 2009). It has also been proposed that α7nAChRs are present on microglia where their activation is associated with reduced release of TNFα. In AD, microglial α7nAChRs are thought to be chronically inactivated by Aβ binding, thereby contributing to the inflammatory process (Parri et al. 2011).

Agonists of nAChRs have been shown to improve cognitive performance whilst antagonists worsen it (Schliebs et al. 2011). In addition, the muscarinic acetylcholine receptor antagonist scopolamine has long been associated with amnesic effects. As a result, the cholinesterase inhibitors such as galantamine and donepezil are used as current therapies for AD with the aim of prolonging the amount of time for which ACh is active. However, whilst their use modestly alleviates the symptoms of AD it does not halt or reverse the disease process. Additionally, both of these compounds have extensive side effect profiles limiting their use to some extent (Sozio et al. 2012). Due to the effects discussed above, the α7nAChRs has recently become a prime target for pharmacological intervention and the search is underway for suitable agonists (Pohanka, 2011).

In summary, the pathological consequences of AD arise as a result of perturbations in multiple CNS pathways. Currently available therapies are only able to act on one or two of these systems and, therefore, can only

attenuate symptoms whilst providing no cure. Moreover, current therapies (NMDAR antagonists/AChEI) treat the symptoms of AD but offer no disease modifying benefits. Thus, there is a need for therapies which modify disease progression and also offer a means of halting or reversing neurodegeneration. The most successful clinical trials to date have centred on Aβ immunotherapy, although trials had to be halted due to 6% of participants developing meningoencephalitis (Lemere et al. 2010; Menendez-Gonzalez et al. 2011). However, despite this setback, Aβ pathology was reduced to some extent and modest improvements in cognition were observed. Given that impairments in cognition are observed prior to the visible plaque pathology, by tackling the problem only when Aβ plaques are observed, immunotherapy is unlikely to provide the permanent solution needed to reverse AD. Hence, there is an urgent requirement to find a therapeutic approach which is able to act on the multiple systems involved in AD and potentially reverse pathological changes. The development of animal models of AD has enabled significant advances in exploring the therapeutic potential of various compounds and some of these models are described in the following section.

1.2.5.5 Animal Models of Alzheimer's Disease

Studying AD in humans is limited by the fact that tissue is only available post-mortem and this will have undergone a number of unpredictable changes as a consequence. Furthermore, variations in the manner in which the tissue was processed, the age of the subject, the stage of AD they had

reached, the variations in treatment regimens and the presence of co-morbidities further complicate research. As a result of these limitations, animal models have become increasingly popular for studying diseases such as AD. Advantages of animal models include the ability to control and manipulate their environment, a high throughput rate and the ability to apply experimental intervention strategies.

There are a number of animal models of AD that have been developed. These models are mainly based on replicating the mutations in the APP and PS genes associated with familial AD. As stated earlier, APP is cleaved by β and γ secretases to form pathogenic Aβ. PS1 and 2 are both essential components of the core γ secretase complex in addition to 3 other molecules. It has been suggested that the active site of the γ secretase complex may be formed by 2 aspartate residues located on the presenelins (Tanzi et al. 2005). Additionally, presenelins play a number of other important physiological roles including the maintenance of neuronal function and survival, modulation of calcium entry and protein trafficking and turnover. The presence of APP and PS genes results in the formation and deposition of Aβ but these animals fail to develop NFT pathology (Ashe et al. 2010; Kokjohn et al. 2009). This precipitated the use of the frontotemporal dementia (FTD) associated mutation P301L in tau protein being used alongside either APP or APP/PS to generate animals which express NFT pathology in (Kokjohn et al. 2009). For comprehensive reviews, the reader is referred to Duyckaerts et al. 2008; Hall, 2012 and Ashe et al. 2010.

APP transgenics

The genetic manipulations employed include abolishing the expression of a protein coded for by a gene (knock-outs, KOs), introducing mutations into a gene so that the protein it expresses is altered (knock-ins, KIs) and introducing foreign genes into the mouse genome (transgenics) (Mineur et al. 2005). The transgenic approach for the study of AD was the first that was successful (Games et al. 1995). It involved the insertion of a mutant form of the human APP (hAPP) gene into the mouse genome resulting in APP over-expression and subsequently enhanced levels of Aβ with some of the associated cognitive defects.

To date, there are 8 APP transgenics (Duyckaerts et al. 2008) which have been developed and characterised, all of which show defects across a range of behavioural tests conducted in a variety of laboratories.

Presenelin transgenics

Another strategy has been to focus on PS1 and 2 and a number of transgenic and KO models have been generated. In general PS1 KO are not viable unless a conditional KO is used, which means that the gene is inactivated post-natally (Mineur et al. 2005). Wildtype and mutant PS1 KI, on the other hand, are viable but show no obvious behavioural abnormalities or Aβ plaques (Dineley et al. 2002).

APP Presenelin double transgenics

Expression of mutant PS1 in the presence of the hAPP transgene results in an accelerated Aβ deposition rate (Borchelt et al. 1997). These APP/PS1 double transgenic mice show Aβ as early as 2-3 months of age whilst none is seen in the APP single transgenics at this stage (Borchelt et al. 1997). Furthermore, by 9 months of age, the plaque load in the cortex and hippocampus of APPswe/PS1 resembles those seen in 18 month old APPswe alone (Borchelt et al. 1997). These observations led Borchelt et al. to suggest that APP and PS1 mutations show a synergistic interaction resulting in enhanced Aβ deposition, as well as promoting production of the more aggregation-prone $A\beta_{42}$ isoform. Berezovska et al. (2005) suggested that the enhanced production of the $A\beta_{42}$ isoform may be due to PS1 mutations resulting in an alteration in the distance between the N and C termini of PS1. This results in a change in the alignment between PS1 and APP and its presentation to the γ secretase active site leading to $A\beta_{42}$ being produced instead of $A\beta_{40}$.

APP PS1 double transgenic mice show impaired cognition

APP/PS1 mutants display a number of cognitive abnormalities which support their validity as models for AD. These include deficits in spatial reference memory as measured by performance in the radial arm (Gordon et al. 2001; Volianskis et al. 2008) and Morris water mazes (Jankowsky et al. 2005). Additionally, deficits are also observed in attention and recognition memory in a novel object paradigm (Howlett et al. 2004; Scullion et al. 2009) and

impaired contextual fear memory (Dineley et al. 2002; Pardon et al. 2009; Rattray et al. 2009).

APP Tau double transgenic mice

Double mutants for APP and tau have also been generated (Lewis et al. 2001). These mice show Aβ plaques similar in number and distribution to single APP mutants and NFTs morphologically resembling those observed in tau mutants (Lewis et al. 2001). However, large numbers of both Aβ plaques and NFTs are only seen at advanced ages in these animals (Lewis et al. 2001). Additionally, they also show non-AD-related motor deficits and generally tend to breed poorly and have a short life-expectancy (Laferla et al. 2005). Hence, they are not deemed to be very suitable for age-related AD research.

APP PS1 Tau triple transgenic mice:

In addition to APP/PS1 double mutant mice, triple transgenic mice (3xTg) harbouring mutations in APP, PS1 and tau protein genes have also been generated. These mice develop Aβ plaques at approximately 6 months of age and NFTs by 10-12 months (Laferla and Oddo, 2005). Impaired cognition is first seen in these mice at 4 months and manifests as retention deficits observed in the contextual fear memory paradigm (Billings et al. 2005).

APPswedish/Presenelin 1 Deleted Exon 9

For the purposes of the studies described in this thesis, a double transgenic mouse model comprising the human APP_{695} Swedish transgene encompassing the FAD-related mutations K595N/M596L (APPswe), and the deleted exon PS1 (PS1ΔE9) variant transgene (APPswe/PS1ΔE9) were used (Borchelt et al. 1997; Jankowsky, 2001). This model was chosen for a number of reasons, including the fact that it is amongst the best characterised. Additionally, the APPswe/PS1ΔE9 model shows an accelerated rate of Aβ deposition compared to other APPswe/PS1 models such as the APPswe/A246E PS1 (Jankowsky et al. 2004). Aβ is present before 3 months of age (Volianskis et al. 2008) and Aβ plaques are present in APPswe/PS1ΔE9 mice by 4 months of age and progressively increase with age, particularly between 6 and 8 months (Garcia-Alloza et al. 2006). The APPswe/PS1ΔE9 model demonstrates a higher deposition rate for the more aggregation prone $Aβ_{42}$ such that the ratio of Aβ40:$Aβ_{42}$ is 0.75:1 (Jankowsky et al. 2004), which may partially explain the aggressive course of the disease in this model, as $Aβ_{42}$ is considered to be more toxic than $Aβ_{40}$ (Berezovska et al. 2005). Importantly, it is the only AD model to date in which there is some neurodegeneration (Richner et al. 2009; Liu et al.2008), the absence of which has provoked debate about the validity of other AD models. At 4 months of age APPswe/PS1ΔE9 mice exhibit forebrain monoaminergic axon density similar to wildtype animals but by 12 and 18 months, these axons have progressively degenerated with a concomitant increase in Aβ plaque pathology (Liu et al. 2008). Tyrosine hydroxylase

positive (TH^+) axons appear to be more prone to degeneration than 5-hydroxytryptamine (5-HT)-containing neurons (Liu et al. 2008). All monoaminergic neurons which project axons to the cortex and hippocampus appear to be susceptible to neurodegeneration, with neuronal atrophy visible at 12 months followed by neuronal death by 18 months (Liu et al. 2008). In contrast to monaminergic axons and neurons, no loss is seen in cholinergic neurons (Perez et al. 2007). However, in common with human AD, these mice show defects in cholinergic transmission which may be due to impairments in vesicular transport and muscarinic receptor-effector coupling (Perez et al. 2007; Machova et al. 2008). Neuronal loss is also observed in the striatum of 12 month old APPswe/PS1ΔE9 animals, whilst 6 month APPswe/PS1ΔE9 exhibit levels similar to wildtype (Richner et al. 2009). The latter authors suggested a causal relationship between neuronal loss and the progressive increase in Aβ levels.

AD is associated with altered neurogenesis. In common with human AD patients, APPswe/PS1ΔE9 animals also show evidence of impaired neurogenesis, with 7 month old APPswe/PS1ΔE9 animals showing a dramatic reduction in the survival rates of newly generated neurons compared to wildtypes (Verret et al. 2007).

The APPsweΔE9PS1 model also displays evidence of progressive cognitive decline in a number of behavioural tests assessing episodic and reference memory. Savonenko et al. (2005) demonstrated impaired episodic memory but intact reference memory in 18 month old APPswe/PS1ΔE9 animals using

the radial water maze. However, 6 month old APPswe/PS1ΔE9 animals displayed no deficits in either. Jankowsky et al. (2005) found evidence of impairments in spatial memory in 8 month old female APPswe/PS1ΔE9 in the water maze paradigm. Reiserer et al. (2007) detected impairments in spatial memory in 7 month old APPswe/PS1ΔE9 in the Barnes maze and Zhang et al. (2011) detected impairments in long-term contextual memory using the step-down passive avoidance test. Additionally, APPswe/PS1ΔE9 animals display multiple inflammatory responses associated with an early pre-plaque age corresponding to the formation of soluble Aβ species (Zhang et al. 2012).

A further benefit of the APPswe/PS1ΔE9 model is that unlike the TASTPM (APPswe/PS1M146V) mouse model of AD, it was not developed by the pharmaceutical industry, and hence is not subject to any intellectual property issues.

A double transgenic model was chosen for study in preference to a 3xTg model for a number of reasons. Aβ pathology in the 3xTg precedes that of tau hyperphosphorylation which does not usually appear in the hippocampus until 12 months of age, thereby mimicking the situation in aging humans (Oddo et al. 2003; Pietropaolo et al. 2008). In human AD, cognitive decline correlates best with NFT formation (Verdile et al. 2004) but deficits in LTP and cognition in 3xTg mice were shown to proceed from intra-neuronal Aβ and not tau (Oddo et al. 2003; Laferla et al. 2005). Additionally, at 6 months 3xTg did not show deficits in the conditioned fear (CF) paradigm designed to

test for associative learning which is impaired early in the course of human AD (Pietropaulo et al. 2008). Furthermore, P301L is a susceptibility gene for fronto-temporal dementia and not genetically linked to AD (Laferla et al. 2005; Garcia-Alloza et al. 2006) making the 3xTg an artificial model which does not mimic the genetic basis of AD.

In summary, animal models enable recapitulation of some of the features of AD in a laboratory setting. It is important to emphasise that there are no animal models which display the full range or neuropsychiatric and neuropathological features of human AD (Hall et al. 2012). The ideal model would be one which displayed behavioural and cognitive deficits accompanied by Aβ plaques, NFTs, gliosis, loss of synapses, axonopathy and substantial neurodegeneration (Hall et al. 2012). Until such a model is generated, however, research is limited to the ones currently available, most of which do display impairments in cognition and Aβ pathology. By manipulating the processes underlying these features, for example by activating or inhibiting certain hippocampal memory circuits through the application of various drugs, it may be possible to gain a better understanding of the disease process. Once a better understanding is gained, it will be possible to search effectively for compounds which effectively halt or reverse the pathology. One approach which might be beneficial in AD is manipulation of the endocannabinoid signalling system in the brain.

1.3 ENDOCANNABINOIDS AND ALZHEIMER'S DISEASE

1.3.1 Endocannabinoid Actions

ECs have been shown to have anti-inflammatory and antioxidant actions as well as protecting against excitotoxicity and enhancing neurogenesis. The main mechanisms involved in AD have been defined earlier. As ECs are able to impact upon all of these systems to various extents, they may potentially have beneficial effects in AD (Marchalant et al. 2012; Benito et al. 2007).

1.3.2 Evidence supporting a role for the endocannabinoid system in AD

Support for the involvement of the ECS in AD pathology is provided by a number of human studies which show upregulation and nitrosylation of CB1 and CB2 receptors on microglia in the post-mortem AD brain (Ramirez et al. 2005). CB1 expression was reduced whilst CB2 expression was enhanced in post-mortem AD brains (Ramirez et al. 2005; Solas et al. 2012). Solas et al. also found that cortical CB2 expression was correlated to $A\beta_{42}$ levels and senile plaque score. In contrast to Ramirez et al. (2005) and Solas et al. (2012), Lee et al. (2010) found no differences in CB1 levels in post-mortem AD brain, but they did observe a correlation between frontal cortical CB1 levels and cognitive test scores assessed in the year prior to death. Additionally, Lee et al. (2010) demonstrated that CB1 receptors in these patients were functionally intact and may have played a role in the preservation of cognitive function.

Additionally, levels of AEA and its lipid precursor 1-stearoyl, 2-docosahexaenoyl-*sn*-glycerophosphoethanolamine-*N*-arachidonoyl (NArPE) were reduced in the mid-frontal and temporal cortices of AD patients post-mortem (Jung et al. 2012). Furthermore, AEA levels in these patients were found to be inversely correlated to $A\beta_{42}$ but not $A\beta_{40}$, and positively correlated with measures of speed of information processing and language abilities (Jung et al. 2012). In contrast, Jung et al. observed no differences in 2AG levels.

Raised levels of FAAH are also found in brain areas containing plaque pathology (Benito et al. 2003). In normal brains, FAAH immunoreactivity is primarily detected in neuronal elements whereas, in AD brains, hypertrophied astrocytes show the most intense staining (Benito et al. 2003). Astrocytic FAAH is hypothesised to degrade AEA, thereby releasing AA and resultant pro-inflammatory eicosanoids into the surrounding area leading to an exacerbation of inflammation (Maccarrone et al. 2007). These changes are more pronounced in the vicinity of the plaques and are observed less frequently, or not at all, at more distant sites. Furthermore, FAAH gene expression and protein levels are raised in the peripheral blood mononuclear cells of AD sufferers (D'Addario et al. 2012). FAAH inhibition has, therefore, been suggested as a logical therapeutic strategy for reducing AD-related neuroinflammation (Benito et al 2003). However, when testing this hypothesis by incubating astrocytes derived from wildtype and FAAH knockout ($FAAH^{-/-}$) animals with Aβ, Benito et al (2012) surprisingly observed

an increase in the expression of pro-inflammatory cytokines by $FAAH^{-/-}$ astrocytes. This effect, however, was not reproduced using pharmacological blockade of FAAH (Benito et al. 2012), thereby supporting the underlying proposal of potential beneficial effects of FAAH inhibition. In support of this proposal, Murphy et al. (2012) observed that pharmacological blockade of FAAH resulted in attenuation of the age-related increase in inflammatory markers and partial restoration of a deficit in LTP.

In contrast to FAAH deletion, genetic deletion of the main enzyme responsible for 2AG degradation, MAGL, has also been proposed to have beneficial effects in AD (Piro et al. 2012). Piro et al (2012) based this on their observation that MAGL controls the release of arachidonic acid which is used to synthesise eicosanoids such as prostaglandins. Genetic deletion of MAGL in an APP/PS1 double transgenic mouse model of AD resulted in reduced eicosanoid levels, attenuated glial activation and associated neuroinflammation and reduced amyloid plaque burden (Piro et al. 2012). Conversely, Mulder et al. (2011) observed impaired recruitment of MAGL to biological membranes of post-mortem AD patients leading them to suggest that 2AG signalling is terminated more slowly in these patients. This observation lead them to suggest that this impairment in 2AG signalling may cause prolonged DSI, resulting in synapse silencing as was observed by them in experiments involving the superfusion of mouse hippocampi with Aβ (Mulder et al. 2011). This suggests that the use of MAGL inhibitors should be approached with caution when assessing their potential for therapy in AD.

Primary cell culture experiments using cortical neurons incubated with Aβ in the presence of AEA, 2AG, or the FAAH inhibitor URB597, displayed reduced apoptosis and lysosomal destabilisation (Noonan et al. 2010). The application of exogenous 2AG or MAGL inhibitors to primary hippocampal neurons incubated with Aβ also resulted in reduced apoptosis and neurodegeneration in a CB1-dependant manner (Chen et al. 2011).

Aβ-induced hippocampal degeneration and cognitive deficits are accompanied by increased synthesis of 2AG, leading to the proposal that neuronal damage up-regulates EC synthesis (van der Stelt et al. 2006). This has prompted the suggestion that the production of ECs and the resultant CBR activation may be an attempt by the CNS to protect against damage (Campbell et al. 2007). Further support for this theory is provided by the observation that intraperitoneal administration of the EC transport inhibitor VDM-11 to rodents previously injected with Aβ peptide in the frontal cortex, alleviated memory impairments and attenuated the resultant neurotoxicity (van der Stelt et al. 2006). However, the exact timing of VDM-11 administration was critical. For VDM-11 to have any beneficial effects it had to be administered early in the disease process. Given later in the course of AD it actually impaired memory retention, possibly as a result of reducing hippocampal ACh levels (Campbell et al. 2007).

CB1-null mutants show enhanced release of ACh, prompting the suggestion by Davies et al (2002) that a degree of tonic inhibition of ACh release mediated by the ECS must exist in the normal CNS. In contrast to this, a

different study showed that the intravenous administration of a small amount of CBR agonist such as WIN-55,212-2, can also increase the release of ACh *in vivo*, which could be diminished by the CB1 antagonist SR141716A (Acquas et al. 2000). In this instance, upregulation of the ECS may be beneficial in AD, particularly in light of the fact that one of the main treatments for AD involves the use of acetylcholinesterase inhibitors.

CB2 levels are raised in microglia in AD brains by neuroinflammation (Benito et al. 2007). The synthetic cannabinoid receptor agonists HU210, WIN55,212-2 and JWH 133 were able to return activated microglia to their resting state morphology *in vitro* whereby they were no longer secreting pro-inflammatory cytokines (Ramirez et al. 2005). Additional effects attributed to CB2 include down-regulation of the CD40 ligand which may be involved in Aβ deposition and inhibition of NO production (Maccarrone et al. 2007). Oral administration of the CB2 selective agonist JWH133 for 4 months rescued recognition memory impairments in 11 month old APP mice in addition to normalizing cerebral glucose metabolism as measured by FDG-PET and counteracting microglial activation (Martin-Moreno et al. 2012). Similarly, in 6 month old APP/PS1 animals, 5 weeks of intraperitoneal JWH133 administration resulted in reduced levels of microglial reactivity and release of pro-inflammatory cytokines as well as alleviation of memory impairments (Aso et al. 2012).

Inhibition of NO generation by inhibiting iNOS reduces lipid peroxidation and is thought to be part of the mechanism by which cannabinoids function as

anti-oxidants. Furthermore, Law et al. (2001) suggested that NO may be involved in the development of plaques and NFTs, and that its inhibition would influence multiple pathological parameters.

Another feature of AD that can be modified by cannabinoids is excitotoxicity. A number of studies have shown that activation of CB1 receptors reduces excitotoxic cell death of hippocampal neurons by a number of mechanisms (Skaper et al., 1996; Shen et al. 1998). Under normal circumstances, one of the primary roles of the ECS is the regulation of neuronal ion channels (Alexander et al. 2007). Hence, cannabinoids acting through CB1 located in the vicinity of the NMDA channel are able to attenuate its activation. Thus, they are able to inhibit the release of Ca^{2+} from intracellular calcium stores, thereby preventing a further toxic rise in $[Ca^{2+}]_{i}$. Additionally, elevations in Ca^{2+} levels would be predicted to increase generation of ECs which feedback negatively to reduce transmitter release. In support of these concepts, CB1 agonists have previously been shown to reduce glutamate release by attenuating presynaptic Ca^{2+} entry (Shen et al. 1998; Twitchell et al. 1997). The synthetic cannabinoid analogue HU-211 is also able to prevent excitotoxicity probably by direct inhibition of NMDA receptors. Furthermore, cannabinoids are able act on microglial CB2 receptors to inhibit their activation and, hence, prevent excess glutamate release, thereby reducing excitotoxic cell death in AD (Ramirez et al. 2005).

Other mechanisms by which cannabinoids are potentially able to attenuate AD pathology include the inhibition of GSK-3β and subsequent

hyperphosphorylation of tau (Esposito et al. 2006), the release of neurotrophic factors such as BDNF (Khaspekov et al. 2004) and stimulation of anti-apoptotic pathways (Tyler et al. 2002; Maccarrone et al. 2007).

Finally, cannabinoids are also involved in adult neurogenesis in the subventricular zone and dentate gyrus of the hippocampus. Neurogenesis is defective in animal models of AD, although post-mortem examination of human AD brains suggests increased neurogenesis (Jin et al. 2004). A study by Beatriz Gonclaves et al. (2008) suggested that it is the activity of the CB2 receptor in partnership with that of DAGL which is of primary importance in neurogenesis. This was based on the observation that the DAGL inhibitor RHC-80267 and CB2-selective antagonist JTE-907 reduced neural progenitor cell proliferation while the CB2-selective agonist JWH-133 enhanced it. Hence, it may be possible to manipulate the ECS to enhance adult neurogenesis in a bid to replace the neurons lost in AD, and potentially attenuate the rate of cognitive decline.

1.3.3 Cannabinoids and AD

In addition to ECs, a number of external cannabinoids such as the constituents of *cannabis sativa* have also been shown to have beneficial effects in AD. These include the phytocannabinoid cannabidiol (Esposito et al. 2007; 2011), the active component of *cannabis sativa* Δ9 tetrahydrocannabinol (Δ9 THC) (Eubanks et al. 2006) and a constituent of Δ9 THC, dronabinol (Volicer et al. 1997).

In vitro cannabidiol has been shown to protect PC12, N13 and rat primary microglial cells from Aβ induced neurotoxicity through a combination of anti-apoptotic, anti-oxidative and reduced intracellular calcium influx (Iuvone et al. 2004; Martin-Moreno et al. 2011). These effects were shown to be mediated by the inhibition of the phosphorylated form of p38 MAPK and the transcription factor NFκB (Esposito et al. 2006). In vivo studies examining the effects of cannabidiol treatment prior to intrahippocampal injections of Aβ into 3-5 month old mice supported in vitro studies by demonstrating attenuated measures of inflammation and oxidative stress (Esposito et al. 2007). The same group subsequently showed the neuroprotective effects of cannabidiol to be mediated by PPARγ activation (Esposito et al. 2011) although other groups have suggested a role for the cannabinoid and adenosine A_{2A} receptors (Martin-Moreno et al. 2011).

In addition to cannabidiol, studies have also demonstrated potential beneficial effects of Δ9THC in AD. As stated previously, one of the main mechanisms for treating AD currently is through the use of AChE inhibitors. Δ9THC has been shown to competitively inhibit AChE through its peripheral anionic site whereby it serves a dual function in preventing ACh metabolism and also diminishing Aβ aggregation (Eubanks et al. 2006). Furthermore, Δ9THC is a much more effective inhibitor of AChE induced Aβ aggregation than either Tacrine or Donepezil as it is able to prevent aggregation to a greater extent at a lower concentration (Eubanks et al. 2006).

In addition to its role in AChE inhibition, a primary constituent of Δ9THC, dronabinol has been shown to be effective in stimulating appetite and alleviating disturbed behaviour in patients with a clinical diagnosis of probable AD who were refusing food (Volicer et al. 1997).

1.3.4 Summary

In summary, ECs may act as a double-edged sword in the pathophysiology of AD. Activation of CB1 has been shown to exacerbate memory impairments unless it occurs within a narrow spatial and temporal window (Van der Stelt, 2006). In contrast, the neurogenesis and anti-inflammatory actions associated with CB2 activation may be of benefit. Hence, blockade of CB1 receptors concomitantly with enhanced CB2 activation could provide a feasible treatment option for attenuation and possibly reversal of AD pathology. Additonally, as cannabis smoking is linked to memory impairments (Solowij et al. 2008), it is possible that CB1 antagonism may provide some short-term benefits even in the absence of the ECS having a role in AD.

1.4 Experimental Hypothesis

It is hypothesised that activation of the ECS produces multiple, possibly opposing, effects in AD. It can potentially attenuate neuroinflammation and AD progression through CB2 activation but will disrupt memory and cognition through CB1 stimulation.

1.5 Aim and Objectives

The main aim of this project is to determine the role of ECs in AD. This will be achieved by characterising the state of the brain ECS in the APPswe/PS1ΔE9 mouse model of AD by correlating changes in EC levels, catabolic enzyme activity and receptor/effector coupling with age-related deposition of Aβ, cognitive impairments and inflammatory markers. Initial studies will course the age-related changes in the ECS and behavioural performance of wildtype and APPswe/PS1ΔE9 mice at 4, 6 and 8 months. Following this, CB1 antagonist and CB2 agonist administration preceding/following behavioural tests assessing short-term memory will be used to determine the feasibility of targeting the ECS in AD.

Chapter 2

Behavioural Analysis

2.1 Introduction

One of the earliest presenting features of human AD are impairments in episodic memory (Backman et al. 2001). The subject of whether animals possess episodic memory is contentious although it is generally accepted that they exhibit 'episodic-like' memory (Morris, 2001). This can be assessed in rodents using the novel object/location tests (Ennaceur et al.1988; 1992), spontaneous alternation in the T-maze (Gerlai, 1998) and contextual fear conditioning (Maren, 2001). These tasks have also been used extensively in laboratories all over the world for many years and as such are well established validated protocols for testing learning and memory in rodents. Additionally, all these tasks are hippocampal-dependant to some extent (Gerlai, 1998; Tronson et al. 2009), as a result of which they are particularly useful in studying Alzheimer's related memory decline which is partially attributed to hippocampal neurodegeneration. One of the advantages of these tests is that, in contrast to others such as the Morris water maze, there is no requirement for rule learning. Hence, the behaviour being measured is based on the natural behaviour of the rodent to complex environmental stimuli.

Novel object and novel location recognition tests are based on the natural tendency of rodents to explore novel objects or environments more than familiar ones (Ennaceur et al. 1988; 1992). Novel object or location recognition paradigms comprise 2 phases: 1) the habituation phase and 2) the testing phase. For novel object recognition, the habituation phase

involves the placement of an animal into an arena containing 2 identical objects which it is permitted to explore freely for a given amount of time, with the intention of it becoming 'habituated' to the objects. The animal is subsequently removed from the arena for *x* amount of time (termed the inter-trial-interval) following which it is placed back in the arena for the testing phase. In the testing phase, one of the previous objects has been replaced with a novel object. The amount of time the animals spends exploring the familiar and novel object is recorded. The novel location test is based upon a similar principle, with the exception that the location of one of the objects from the habituation phase is changed in the testing phase and no novel object is introduced. Both these tasks are referred to as one trial tests due to there being no requirement for rule learning as a result of which the tasks can be administered in one testing session. Ennaceur et al. 1992 cite the lack of rule learning as being a distinct advantage of these tests based on the observation that memories of a unique event are more vulnerable to damage than more robust memories of repetitive events. Furthermore, they argue that the lack of reinforcement learning means that the motivational and arousal states of the animal more closely replicate the conditions under which human memories are measured (Ennaceur et al. 1992).

The T-maze spontaneous alternation task is also based upon the principle of animals preferring to explore a novel environment over a familiar one (Lalonde, 2002). Animals are expected to become familiar with the goal arm they enter first so that they select the opposite goal arm to explore next time as it will be novel to them, and hence alternate between them. Performance

in the task appears to depend on the presence of extra-maze cues to a certain extent (Gerlai, 1998). Mice naturally tend to show alternation at levels significantly above chance (Lalonde, 2002) although differences in performance have been reported between inbred and out bredstrains (Gerlai, 1998).

Contextual fear conditioning (CFC) is a form of Pavolvian associative learning whereby an animal forms an association between a conditioned stimulus (CS) and an unconditioned stimulus (US) (Kim et al. 2006). In the case of CFC the CS is the context, and the US a mild footshock (Rudy et al. 2004). The animal learns to fear the context even in the absence of the US which manifests as freezing behaviour when the animal is in the context. CFC measures 2 main components: learning and memory. The animal is initially required to 'learn' the association between CS and US and later remember the connection when it is placed back into the context. Furthermore, the test is able to measure new learning in the extinction phase whereby the CS-US pairings become disassociated to the extent that a new memory is formed where CS and US are not linked (Kim et al. 2004). This process is referred to as 'cognitive flexibility' by some and is frequently impaired in AD patients (Albert, 1996).

As CFC uses immobility as an indicator of contextual fear (CF), baseline activity levels of animals are recorded prior to administration of the CFC paradigm to ensure there are no underlying differences in activity to confound the CFC outcome measures. Baseline activity levels are

measured either immediately preceding the administration of the first footshock, or in a separate open field task whereby the animal is placed in an arena or permitted to explore for a given length of time.

This study utilised all of the above methods for characterising the behaviour of wildtype and transgenic APPswe/PS1ΔE9 mice at a range of ages. The study is presented in two parts, an initial characterisation followed by a more in-depth behavioural examination.

2.2 Aims and study design; part 1

The first part of this chapter details an initial study which aimed to ascertain the behaviour of the APPswe/PS1ΔE9 mice at 4 and 5.5 months of age in the contextual fear conditioning (CFC) paradigm and the locomotor activity (LMA) task. This study was conducted due to this being the first instance that the APPswe/PS1ΔE9 mouse model of AD had been used at the University of Nottingham, and to confirm that it showed similar deficits to those displayed by the TASTPM model used previously. The TASTPM mouse model displayed deficits in cognitive flexibility and episodic memory which are also affected early in the course of human AD (Pardon et al. 2009; Rattray et al. 2009). Both these parameters can be tested using the CFC paradigm. Additionally, this study aimed to confirm 4 months as being a suitable baseline from which to initiate future studies.

2.3 Materials and Methods

2.3.1 Animals

Double transgenic mice backcrossed on a C57 background, over-expressing the human APP_{695} Swedish gene encompassing the familial AD-related mutations K595N/M596L (APPswe), and the deleted exon PS1 (PS1ΔE9) variant gene (Borchelt et al. 1997; Jankowsky et al. 2001) were generated in-house following purchase of the initial breeding stock from Jackson Laboratories (Bar Harbor, ME). The APPswe/PS1ΔE9 double transgenic animals were subsequently mated with wildtype C57 mice to produce heterozygote double mutants (APPswe/PS1ΔE9) or wildtype littermates. Due to possible sexual dimorphisms in pathology, only male mice were used for the study. All animals were housed in the same room on a 12h light:dark cycle with room temperature, humidity and air exchange all being automatically regulated. Animals were initially group-housed in cages supplied with nesting material and a play tube, with ad-libitum access to food and water until 3 months of age. At 3 months, animals were isolated due to age-related increases in aggression commonly observed in male mice. Animals were randomly allocated to 2 independent groups which were tested at either 4 (n=13-14) or 5.5 (n=10-11) months.

All animal procedures were carried out in accordance with the UK Animal Scientific Procedures Act under project license 40/3238.

2.3.2 Experimental design

Animals underwent a LMA task on day 1 followed by the CFC paradigm 3 days later comprising 3 phases which were conducted over 3 days: acquisition (day 4), retention (day 5) and extinction (day 6). Following the extinction trial, animals were placed back in their home cage for 48 hours to allow physiological parameters to return to baseline. Animals were subsequently killed (Schedule 1) and the frontal cortices and hippocampi were removed, snap frozen in liquid nitrogen, and stored at -80°C to determine endocannabinoid levels and cannabinoid receptor-effector coupling (figure 2.1).

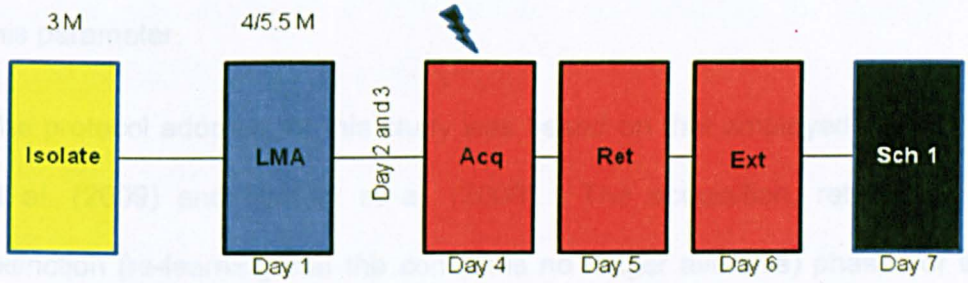

Figure 2.1: Timeline of behavioural experiments; LMA = Locomotor Activity. Acq = CF acquisition. Ret = CF retention. Ext = CF extinction.

2.3.3 Locomotor activity

LMA was conducted to ensure that any potential differences observed between the groups in the CFC paradigm were not due to differences in activity levels, as immobility levels are used to indicate CF. Animals were placed in Perspex activity boxes (30cm x 35 cm x 25 cm) for 30 minutes during which their behaviour was recorded and subsequently analysed using

Ethovision software (Noldus, Wagenigen, Netherlands). The total distance moved in 30 minutes was recorded and used for statistical analysis.

2.3.4 Contextual Fear Conditioning

CFC is a form of Pavlovian conditioning which analyses the pairing of a conditioned stimulus (context) with a noxious electric foot shock (unconditioned stimulus). It has previously been established that the hippocampus is crucial for the acquisition and retrieval of CFC which can be regarded as a test for episodic memory (Matus-Amat et al. 2004). As impairments in episodic memory are amongst the earliest traits observed in human AD patients, CFC was considered an appropriate tool for assessing this parameter.

The protocol adopted for this study was based on that employed by Pardon et al. (2009) and Rattray et al. (2009). The acquisition, retention and extinction (re-learning that the context is no longer aversive) phases of the task were all conducted in the same chamber (25cm x 25cm x 38cm) consisting of 3 grey stainless steel walls and a transparent Perspex wall, with stainless steel rods placed 1 cm apart forming the floor. The steel rods on the floor of the chamber were connected to a shock generator (Campden Instruments, Loughborough, UK) and formed the means by which the shock was administered to the animals. A camera placed above the chamber was used to monitor and record the behaviour of the animals. The recordings were subsequently used to determine the amount of time the animal spent immobile in each trial. Immobility levels were manually scored twice per trial.

If the difference between measurements was greater than 5 seconds, the trial was re-scored a third time and the two closest values used for data analysis. The average values obtained were used for statistical analysis.

Acquisition (Trial 1): Animals were placed in the chamber (conditioning context) for 1 minute prior to receiving their first foot shock (1 sec, 0.4mA). Following this, a further 9 foot shocks were administered at 1 minute intervals for 9 minutes, giving a total of 10 foot shocks. The animals were then removed from the chamber and placed back in their home cage. Increasing immobility (e.g. lack of displacement, body movement, head turn and grooming (Pardon et al. 2009; Rattray et al. 2009) in response to repeated administration of foot shocks was used as an indicator of acquisition (learning) of fear conditioning.

Retention (Trial 2): 24 hours after the acquisition trial, animals were placed back in the chamber for 3 minutes to test their recent CF memory. No foot shocks were applied and their behaviour was recorded as before. The amount of time the animal spent immobile was used as an indication of fear memory i.e. how well the animal remembered the aversive experience received 24 hours earlier.

Extinction (Trial 3): A further 24 hours later (48 hours after acquisition) the animal was placed back in the chamber for 3 minutes and its behaviour recorded. As before the total amount of time spent immobile was determined. It was expected that the animal would show less immobility in this trial due to relearning that the context was no longer aversive in trial 2. An extinction

index value was calculated by subtracting the time spent immobile in the retention trial from that in the extinction trial. The more negative the extinction index value, the better the extinction.

It has previously been shown that the increasing immobility observed in all 3 trials is due to the aversive experience. Animals that did not receive foot shocks were active at least 80% of the time in all 3 trials compared to animals that did receive foot shocks and displayed increasing immobility with time in trial 1 (Rattray et al. 2009).

2.3.6 Statistical Analysis:

LMA: Total distance travelled was analysed using a two-way ANOVA with age and genotype as between group factors.

CFC: Immobility during the acquisition trial was assessed by means of a two-way repeated measures ANOVA with age and genotype as the between-subjects factors and time (1 shock per minute, 10 modalities) as the within-subjects factor. Intra-group differences in retention and extinction were also analysed using a two-way repeated measures ANOVA with age and genotype as between-subjects factors and trial (2 modalities) as the within-subjects factor. A one-sample t-test was used to determine whether the extinction index is significantly different from 0 which would indicate no extinction has taken place.

Post-hoc testing:

Planned comparisons were conducted post hoc when appropriate using the least significant difference (LSD) test. The main focus of this study was to identify differences between wildtype and APPswe/PS1ΔE9 animals at each age group. Hence, planned comparisons identified to be of interest prior to the start of the experiment were those between wildtype and APPswe/PS1ΔE9 at 4 months, and 5.5 months.

Rationale for statistical tests:

One sample t-tests were conducted to establish whether an animal has performed the requirements of the task, in the case of CFC this would be indicated by an extinction index significantly different from 0. Repeated measures and two way ANOVAs were conducted in order to determine differences within and between groups as a factor of age and genotype irrespective of whether an animal has performed the requirements of the task.

2.5 Results

2.5.1 Locomotor Activity

The results of the LMA test indicated that APPswe/PS1ΔE9 mice were significantly hyperactive compared to their wildtype littermates [$F_{(1,45)} = 25.96$; $p<0.001$]. APPswe/PS1ΔE9 animals consistently travelled a greater distance during the 30 minute test than wildtype animals at both ages (figure 2.2a). Post-hoc analysis applied to the main effect of genotype indicated significant differences between 4 month old wildtype and APPswe/PS1ΔE9 and 5.5

month old wildtype and APPswe/PS1ΔE9 mice ($p<0.001$ in both cases). No significant effect of age or genotype x age interaction was observed.

2.5.2 Contextual Fear Conditioning

Acquisition of contextual fear conditioning

Shock-induced immobility was measured during the acquisition of contextual fear as an indicator of how well the animal learned the task. All groups showed a significant increase in immobility with time [$F_{(9,396)} = 121.45$; $p< 0.001$] indicating that they were all able to associate the context with the aversive stimulus (figures 2.2b-c). No significant effects of age or genotype were observed.

Retention and extinction of contextual fear conditioning

APPswe/PS1ΔE9 showed significantly higher immobility levels compared to wildtype animals during the retention [$F_{(1, 45)} = 4.19$; $p=0.046$] and extinction [$F_{(1, 45)} = 8.22$; $p=0.006$] trials (figures 2.2d-e). No significant effects of age or an age x genotype interaction were observed in the retention or extinction trial. Post-hoc tests revealed a significantly higher levels of immobility in APPswe/PS1ΔE9 compared to wildtype animals at 5.5 months of age [pairwise comparison: $p=0.004$] and a non significant increase in immobility during the retention trial at 5.5 months [pairwise comparison: $p=0.069$].

Statistical analysis of immobility levels during retention compared to extinction revealed significant main effects of trial [$F_{(1, 45)} = 8.51$; $p=0.006$] and genotype [$F_{(1, 45)} = 7.26$; $p=0.010$]. Post hoc analysis applied to the main

effects of genotype and trial indicated that 4 month old wildtype animals showed significantly lower levels of immobility in the extinction trial compared to the retention [pairwise comparison: p=0.039]. Similarly, 4 month old APPswe/PS1ΔE9 showed significantly lower immobility levels during the extinction trial compared to the retention [pairwise comparison: p=0.030]. In contrast no significant differences were observed in immobility levels during retention compared to extinction for 5.5 month old wildtype or APPswe/PS1ΔE9 animals.

To determine whether an animal had successfully extinguished contextual fear, an extinction index value was calculated by subtracting immobility during the retention trial from that in the extinction trial. For successful extinction to have taken place the value of the extinction index needs to be negative and significantly different from 0 (figure 2.2f). Wildtype animals at 4 months (one sample t-test: p=0.016) and 5.5 months of age (one sample t-test: p=0.009) demonstrated successful extinction in contrast to APPswe/PS1ΔE9 which did not. In the case of 4 month WT and APPswe/PS1ΔE9 animals, the mean extinction index values were very similar. However, the standard deviation for the APPswe/PS1ΔE9 group was almost double that of the wildtype group, hence why the wildtype value is significantly different from 0 whilst that for the APPswe/PS1ΔE9 is not.

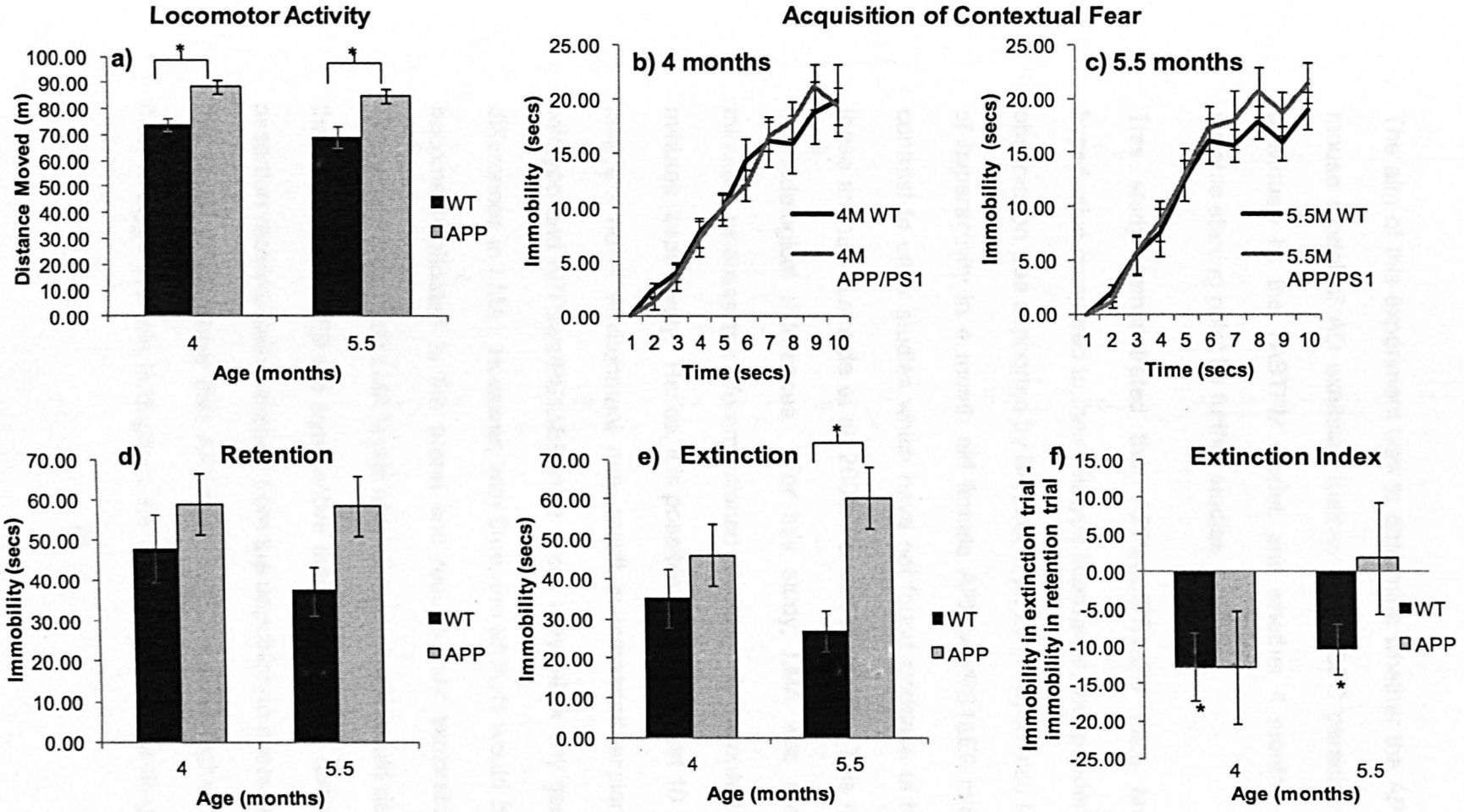

Figure 2.2 (data presented as mean ± SEM): (a) Locomotor activity. Total distance travelled in 30 minutes by wildtype (WT) and APPswe/PS1ΔE9 (APP) mice aged 4 (n=13-14) and 5.5 (n=11) months. Pairwise comparisons: *p=0.002. **Contextual fear conditioning b-f; (b-c)** All animals were able to learn the task at 4 (n=13-14) and 5.5 (n=10-11) months demonstrated by immobility increasing significantly with time (p<0.001); **(d)** APP animals display significantly higher levels of immobility compared to wildtype animals independent of age during the retention trial (p=0.046); **(e)** APP animals show significantly greater levels of immobility compared to wildtype animals at 5.5 months (pairwise comparison *p=0.004); **(f)** only wildtype animals showed successful extinction of contextual fear. One-sample t-test *p=0.016 (4 months) and *p=0.009(5.5 months) compared to 0 (no extinction).

2.6 Discussion

The aim of this experiment was to determine whether the APPswe/PS1ΔE9 mouse model of AD exhibited deficits in the CFC paradigm as displayed previously by the TASTPM model, and whether 4 months of age was a suitable starting point for further studies.

This study demonstrated that APPswe/PS1ΔE9 mice are significantly hyperactive compared to their wildtype littermates, independent of age. This observation was supported by Bonardi et al. 2011 which also found evidence of hyperactivity in 4 month old female APPswe/PS1ΔE9 mice. This is in contrast to other studies which have not found evidence of hyperactivity in these animals (Lalonde et al. 2004; Park et al. 2010). This may be due to methodological differences. For this study, LMA was recorded for 30 minutes, whereas the aforementioned studies only recorded for 5 and 10 minutes respectively. Hence, it is possible that for the first 10 minutes of the task the novel environment may result in increased exploration by both wildtype and APPswe/PS1ΔE9 mice which may mask any genotype related differences in LMA. However, with time, the animals would be expected to become habituated to the arena and reduce their exploratory behaviour. This would mean that LMA levels of wildtype animals would decrease but as the APPswe/PS1ΔE9 are hyperactive their levels would remain high. This assertion receives some support from the unpublished observations made in this study which show that APPswe/PS1ΔE9 exhibit higher activity levels than wildtype animals throughout the whole 30 minute testing period, even

when broken down and examined in 5 minute time bins. Furthermore, both wildtype and APPswe/PS1ΔE9 animals show highest activity levels in the first 5 minutes of the task followed by relatively steady levels for the remainder of the 30 minutes testing session. This would indicate that animals become habituated to the arena after 5 minutes. Hence, when animals are only tested for 5 minutes the activity levels will be reflective of the fact that the animal is in a novel environment and thus more likely to exhibit exploratory behaviour rather than being an indication of steady state activity levels. However, by testing for 30 minutes this caveat is overcome and a genuine estimate of steady state activity levels is obtained.

The increased activity levels may be due to APPswe/PS1ΔE9 mice possessing a greater exploratory drive, or reflect evidence for behavioural disinhibition as suggested by Lalonde et al. (2005), although their data were from 12-month-old APPswe/PS1ΔE9 mice of both genders. Alternatively, it may be related to an alternative mechanism hitherto undiscovered such as increased excitability of striatal neurons although this hypothesis would need to be tested through the use of electrophysiology or microdialysis.

APPswe/PS1ΔE9 animals were able to learn CF at the same rate as their wildtype counterparts at both 4 and 5.5 months thereby demonstrating that learning is not impaired in these animals. Hence, any differences observed during the retention trial must be due to impaired recall and not learning defects. Both wildtype and APPswe/PS1ΔE9 displayed high levels of

immobility in the retention trial, thus confirming recall of the aversive experience from the previous day and therefore no memory impairments.

However, contrary to expectations APPswe/PS1ΔE9 exhibited no impairments in recent contextual fear memory at 4 or 5.5 months. This was demonstrated by APPswe/PS1ΔE9 mice showing greater levels of immobility compared to their wildtype littermates, and not lower, as would be expected if there were impairments in retention of contextual fear. This outcome receives support from 2 recent studies conducted using the same mouse model, which also found no differences in the freezing behaviour of both male and female APPswe/PS1ΔE9 over 8 months and 4 month old females 24 hours after acquisition of CF (Park et al. 2010; Bonardi et al. 2011). In contrast, another study conducted using a different APP/PS1 model (TASTPM) did show impairments in contextual fear memory at 5.5 months (Pardon et al. 2009; Rattray et al. 2009). However, studies conducted in the APPswe/PS1ΔE9 using different tests of hippocampal memory such as the Morris water maze or radial arm water maze also do not show short-term episodic memory deficits in animals at 6 (Savonenko et al. 2005) and 7 months (Reiserer et al. 2007; Volianskis et al. 2010). Additionally, suggestions that hyperactivity may mask impairments in CF memory can be refuted by the observation that despite their hyperactive nature APPswe/PS1ΔE9 display higher levels of immobility compared to non-hyperactive wildtype mice. In light of these factors, it is possible that recent memory is not impaired in the APPswe/PS1ΔE9 model until a later age. Alternatively, it is possible that memory deficits are rescued in this model of

AD due to their hyperactive nature. It has been shown that exercise reduces the levels of Aβ in a mouse model of AD (Adlard et al. 2005) as well as inducing synaptogenesis in hippocampal CA1 and dentate gyrus (Dietrich et al. 2008). Thus, it is theoretically possible that the increased activity of APPswe/PS1ΔE9 result in beneficial effects which counteract the synaptic dysfunction.

As mentioned earlier, contrary to expectations, APPswe/PS1ΔE9 mice displayed higher levels of immobility in both the retention and extinction trials relative to wildtype mice at both 4 and 5.5 months. Furthermore, 5.5 month APPswe/PS1ΔE9 animals actually display higher immobility levels in the extinction trial than the retention.

Under normal circumstances, animals would be expected to re-learn during the retention trial that the context is no longer aversive and, therefore, show increased mobility during the extinction trial. This was seen for the wildtype but not APPswe/PS1ΔE9 animals, indicating the presence of a deficit in extinction. This suggests that APPswe/PS1ΔE9 mice, in common with the TASTPM model, are unable to inhibit previously learned associations that are no longer relevant (Pardon et al. 2009). This is supported by Bonardi et al. 2011 which also found impairments in CF extinction in 4 month old female APPswe/PS1ΔE9 mice. Additional support is provided by the demonstration by Savonenko et al. (2005) that during the radial arm watermaze procedure 18 month old APPswe/PS1ΔE9 mice showed a strong tendency to visit the previous day's platform location. Savonenko et al. (2005) suggested this

may be due to APPswe/PS1ΔE9 being unable to quickly learn information related to the new episode of training and also postulated this defect may be accompanied by old memory traces being continuously activated. These observations correlate well with changes seen early in human AD where defects in cognitive flexibility are also apparent early in the course of the disease. Tests for cognitive flexibility are amongst the most reliable indicators for distinguishing between controls and MCI individuals and MCI and mild dementia patients (Gualtieri et al. 2005; Albert, 1996).

2.7 Aims and study design: part 2

The second part of this chapter describes a longitudinal study examining the behaviour of APPswe/PS1ΔE9 animals at 4, 6 or 8 months of age (n=18-20 determined by power analysis of data from part 1 using an α error probability of 0.05, effect size of 0.25 and power of 0.96). Animals were subjected to a battery of behavioural tests over a 12 day testing period (figure 2.3a). Animals underwent LMA testing on day 1, followed by a novel location/novel object recognition test (Nov) on day 2 and spontaneous alternation in the T-maze on day 4. After a recovery period of 3 days, animals were subjected to the acquisition (day 8), retention (day 9) and extinction (day 10) trials of the CF task. Following the extinction trial, animals were placed back in their home cage for 48 hours to allow physiological parameters to return to baseline. Animals were subsequently killed (Schedule 1) and the frontal cortex, striatum and hippocampi were removed, snap frozen in liquid nitrogen, and stored at -80°C for further analysis (see Chapter 3).

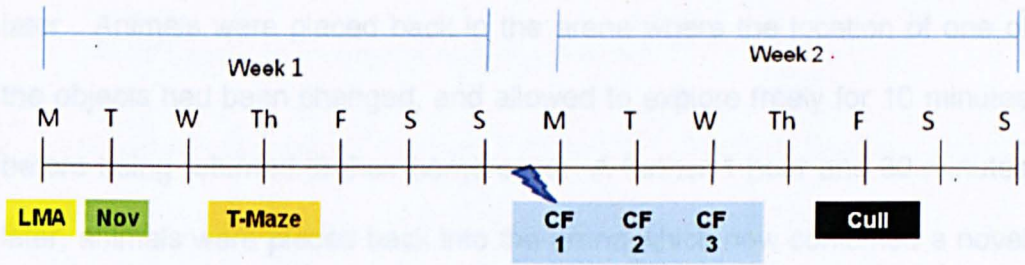

Figure 2.3a: Timeline for 12 day behavioural testing protocol.

2.8 Materials and Methods

2.8.1 Locomotor Activity

The LMA task was performed as detailed in 2.3.3

2.8.2 Novel Location and Novel Object Recognition

This test had two components, novel location, which measures spatial memory, and novel object recognition which measures recognition memory. The test is based on the tendency of animals to spend more time exploring novel situations more than familiar ones. The tests were carried out in the same arena as described previously for LMA. Two sets of objects were used; these were circular or triangular wooden blocks. It has previously been shown that animals display no preference for one shape over another (Scullion et al. 2009). Animals were subjected to 3 trials each; acquisition, novel location and novel object recognition. During the acquisition trials, animals were placed in an arena containing 2 identical objects and permitted to explore freely for 10 minutes following which they were placed back in their home cage. The novel location trial took place 1 hour and 20 minutes

later. Animals were placed back in the arena where the location of one of the objects had been changed, and allowed to explore freely for 10 minutes before being returned to their home cage. A further 1 hour and 30 minutes later, animals were placed back into the arena which now contained a novel object in addition to the familiar one, and were allowed to explore for 10 minutes (figure 2.3b). The position of the objects was counterbalanced throughout the procedure and all objects and the arena were cleaned using 20% ethanol between each trial to eliminate odour cues. All of the trials were recorded by a camera positioned directly above the arena and later used to determine the amount of time spent exploring each object. Exploration was defined as the amount of time spent by an animal with its nose pointing towards the object at a distance of $\leq$ 0.5 cm. The distance moved, total exploration time and exploration time for each object were recorded for each trial. To determine an animals' preference for the novel object, the amount of time spent exploring the novel object was divided by the total exploration time and multiplied by 100% to obtain a preference index value.

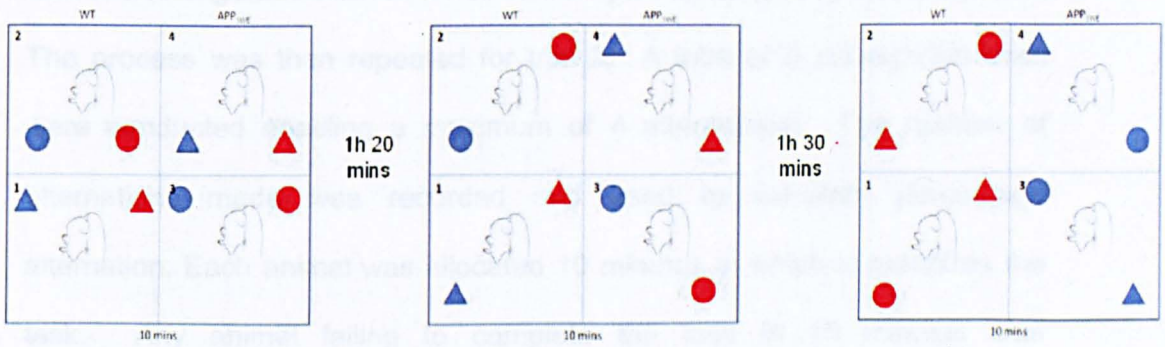

Figure 2.3b: Novel Location and Novel Object Recognition Protocol.

2.8.3 Spontaneous Alternation in the T-maze

Spontaneous alternation in the T-maze is a test of working memory performance measuring the exploratory behaviour of mice. The procedure used was adapted from Spowert-Manning et al. (2004). The T-maze comprised three grey Perspex arms each measuring 41.5 cm in length and 6 cm in width surrounded by 15 cm high transparent Perspex walls. The start box 6 cm x 7.5 cm was located in the lower central arm of the maze. The entrance to the start box and the arms of the maze could be closed using vertical sliding doors.

Animals were placed into the start box and permitted to explore freely for 1 minute after which they were guided back to the start box and the door lowered. For trial 1, the door was lifted and the animal was given a free choice to enter either the left or right arm of the maze. Once an animal had entered one arm the entrance to the other arm was closed off using the vertical doors. The animal was allowed to explore down the length of the arm and then guided back to the start box by sectioning off the area behind it. The process was then repeated for trial 2. A total of 5 consecutive trials were conducted enabling a maximum of 4 alternations. The number of alternations made was recorded and used to calculate percentage alternation. Each animal was allocated 10 minutes in which to complete the task. Any animal failing to complete the task in 10 minutes was subsequently excluded. Animals were not handled during the task to minimise stress.

2.8.4 Contextual Fear Conditioning

The CFC paradigm was identical to that detailed in 2.3.4

2.8.5 Statistical Analysis

LMA: Total distance travelled was analysed using a two-way ANOVA with age and genotype as between group factors.

CFC: Immobility during the acquisition trial was assessed by means of a two-way repeated measures ANOVA with age and genotype as the between-subjects factors and time (1 shock per minute, 10 modalities) as the within-subjects factor. Intra-group differences in retention and extinction were also analysed using a two-way repeated measures ANOVA with age and genotype as between-subjects factors and trial (2 modalities) as the within-subjects factor. A one-sample t-test was used to determine whether the extinction index is significantly different from 0 which would indicate no extinction has taken place.

T-maze: Percentage alternation was analysed using a two-way ANOVA with age and genotype as between-subject factors. A one sample t-test was used to determine whether percentage alternation, within each group, was significantly different from 50% which represents chance levels.

Novel location and novel object recognition: A two-way repeated measures ANOVA was used to determine differences in total exploration times between the 3 trials with age and genotype as between-subjects factors and trial (3 modalities) as within-subjects. A two-way ANOVA was used to examine

differences in preference indices for the novel location task with age and genotype as between-subjects factors. The same test was also conducted to analyse preference indices for the novel object task. Novel location and novel object discrimination were assessed individually by means of a two-way repeated measures ANOVA for exploration times with age and genotype as between-subjects factors and object familiarity (2 modalities) as within-subjects factors. One sample t-tests were conducted to determine if preference indices were above the level of chance (50%).

Post-hoc testing:

Planned comparisons were conducted post hoc when appropriate using the least significant difference (LSD) test. As previously, the performance of wildtype and APPswe/PS1ΔE9 animals at 4, 6 and 8 months was compared.

2.8 Results

2.8.1 Locomotor Activity:

APPswe/PS1ΔE9 animals were significantly hyperactive compared to their wildtype counterparts [$F_{(1,106)} = 93.34$; $p<0.001$] at 4, 6 and 8 months (figure 2.4a). Activity declined significantly with age [$F_{(2,106)} = 4.45$; $p=0.014$], but at all ages, APPswe/PS1ΔE9 were hyperactive compared to their age-matched wildtype littermates ($p<0.001$ at 4 and 6 months and $p=0.001$ at 8 months).

As APPswe/PS1ΔE9 animals were found to be hyperactive in the first part of this study, it was determined that the possibility of these animals exhibiting anxiety-like traits which may influence behavioural testing should be

excluded. This was assessed by comparing the ratio of activity in the centre of the arena to that in the periphery. No significant differences in this ratio were observed between wildtype and APPswe/PS1ΔE9 mice as a function of age or genotype (figure 2.4b).

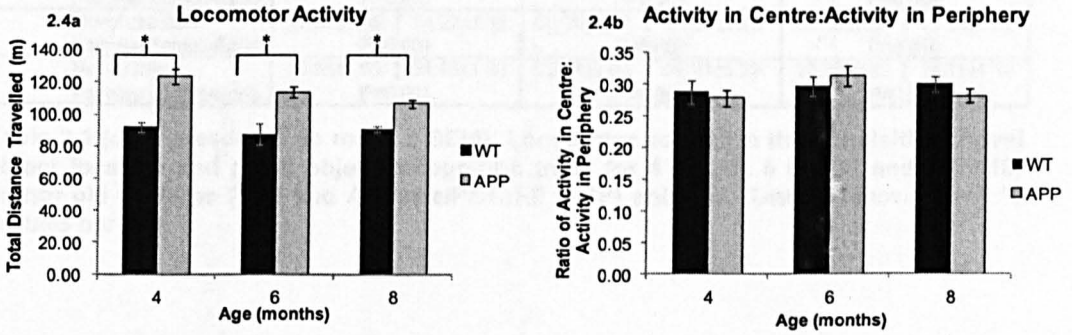

Figure 2.4 (data presented as mean ± SEM): (a) Locomotor Activity). Total distance travelled in 30 minute testing session of 4 (n=20), 6 (n=20) and 8 (n=18) wildtype (WT) and APPswe/PS1ΔE9 (APP) mice. APP mice display significant hyperactivity compared to their WT counterparts which decreased with age. ($p<0.001$ and $p=0.014$ respectively). Pairwise comparisons:*$p \leq 0.001$. **(b)** Ratio of activity in the centre of the arena:time:activity in the periphery. No significant differences were observed as a function of age or genotype.

2.8.2 LMA and Exploration Times During Acquisition, Novel Location and Novel Object Recognition Trials

Locomotor activity during the acquisition, novel location and novel object recognition trials was also analysed. In line with the LMA data above, APPswe/PS1ΔE9 animals were significantly hyperactive [$F_{(1,106)} = 54.50$; $p<0.001$] compared to their wildtype counterparts (Table 2.1) but activity declined significantly with age [$F_{(1,106)} = 11.76$; $p<0.001$]. A significant effect of trial [$F_{(2,212)} = 323$; $p<0.001$] and a trial x genotype interaction [$F_{(2,212)} = 5.55$; $p=0.005$] were also observed indicating activity decreased with increasing trial number. Post hoc pairwise comparisons indicated that APPswe/PS1ΔE9 exhibited higher activity than wildtype animals during the

acquisition trial at 4, 6 and 8 months ($p<0.001$), the novel location trial at 4, 6 ($p<0.001$) and 8 months ($p=0.016$) and the novel object recognition trial at 4 ($p=0.001$), 6 ($p=0.008$) and 8 ($p=0.036$) months (Table 2.1).

Distance Moved (m)	4MWT	4MAPP	6MWT	6MAPP	8MWT	8MAPP
Acquisition	35.13±1.13	42.43±1.42	31.46±1.12	38.86±1.47	31.23±0.86	37.99±1.32
Pairwise comparisons	P<0.001		P<0.001		P<0.001	
Novel Location	25.58±1.05	34.22±1.35	23.74±1.18	31.08±1.73	23.48±0.85	27.95±1.32
Pairwise comparisons	P<0.001		P<0.001		P=0.016	
Novel Object	25.59±1.03	31.10±1.30	22.54±1.05	27.01±1.22	20.22±0.72	24.11±1.10
Pairwise comparisons	P=0.001		P=0.008		P=0.036	

Table 2.1 (data presented as mean ± SEM): Locomotor activity in the acquisition, novel object location and novel object recognition trials for 4 (n=20), 6 (n=20) and 8 (n=18) month old wildtype (WT) and APPswe/PS1ΔE9 (APP) animals. Distance moved over 10 minutes per trial.

There was a significant overall reduction in total exploration time as a function of age [$F_{(2,110)} = 11.03$; $p<0.001$], genotype [$F_{(1,110)} = 14.50$; $p<0.001$] and trial [$F_{(2,110)} = 169.80$; $p<0.001$]. APPswe/PS1ΔE9 displayed greater total exploration times at all ages and for all 3 trials compared to their wildtype counterparts (Figure 2.5a) as indicated by significant age x trial [$F_{(4,220)} = 2.83$; $p=0.025$] and genotype x trial [$F_{(2,220)} = 3.65$; $p=0.028$] interactions. Post hoc analysis demonstrated that APPswe/PS1ΔE9 aged 4 ($p<0.001$), 6 ($p=0.036$) and 8 ($p=0.032$) months displayed significantly higher total exploratory activity in the acquisition trial compared to their age matched wildtype counterparts.

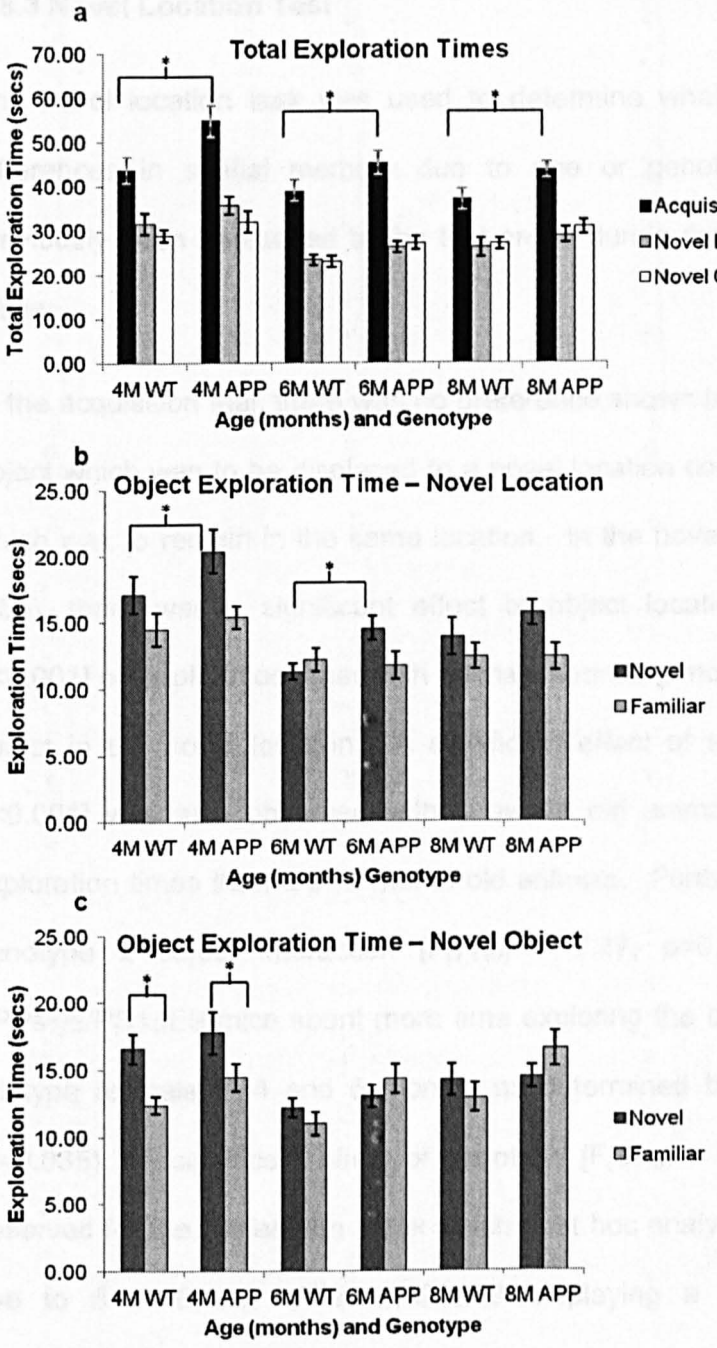

Figure 2.5 a-c (data presented as mean ± SEM): Total and Object Exploration Times during the acquisition , novel location and novel object recognition trials for 4 (n=20), 6 (n=20) and 8 (n=18) month old wildtype (WT) and APPswe/PS1ΔE9 (APP) animals.

(a) Total exploration time over each 10 minute trial. Exploration time decreased significantly with age and trial ($p<0.001$). APP displayed significantly higher exploration times compared to WT animals at all ages ($P=0.025$) and in all trials ($p=0.028$). Pairwise comparisons: $p<0.001$ (4 months) $p=0.036$ (6 months), $p=0.038$ (8 months).

(b) Animals spent significantly more time exploring the object in the novel location ($p<0.001$) than the familiar with 4 month old animals displaying significantly higher exploration times than 6 and 8 month old ($p<0.001$) and APP more than wildtype ($p=0.007$). Pairwise comparisons: $p=0.035$ (4 and 6 months).

(c) Animals spent significantly more time exploring the novel object than the familiar ($p=0.022$). Exploration times decreased significantly with age ($p=0.005$). APP displayed greater exploratory activity than wildtype mice ($p=0.008$). Pairwise comparisons: 4 month old WT ($p<0.001$) and APP ($p=0.006$) displayed significantly higher exploration times for the novel object that the familiar.

2.8.3 Novel Location Test

The novel location task was used to determine whether there were any differences in spatial memory due to age or genotype. Animals had previously been habituated to the test arena during the LMA task 24 hours earlier.

In the acquisition trial, there was no preference shown by the animals for the object which was to be displaced to a novel location compared to the object which was to remain in the same location. In the novel location trial (figure 2.5b), there was a significant effect of object location [$F_{(1,110)}$ = 24.31; $p<0.001$] on exploration times with animals spending more time exploring the object in the novel location. A significant effect of age [$F_{(2,110)}$ = 12.83; $p<0.001$] was also observed with 4 month old animals displaying higher exploration times than 6 or 8 month old animals. Furthermore, a significant genotype x object interaction [$F_{(1,110)}$ = 7.47; $p=0.007$] indicated that APPswe/PS1ΔE9 mice spent more time exploring the displaced object than wildtype animals at 4 and 6 months as determined by post hoc analysis ($p=0.035$). A significant effect of genotype [$F_{(1,110)}$ = 5.65; $p=0.019$] was observed for the preference index which post hoc analysis determined to be due to 6-month-old APPswe/PS1ΔE9 displaying a significantly greater preference than wildtype animals for the displaced object ($p=0.014$). One sample t-testing demonstrated preference indices to be significantly above chance for all groups except 6 and 8 month old wildtype animals (figure 2.5d).

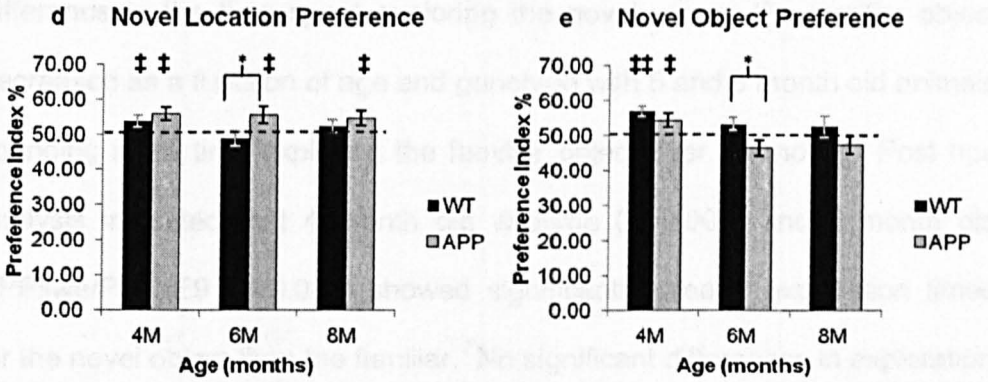

Figure 2.5 d-e (data presented as mean ± SEM): Preference Index for the novel location and novel object recognition trials for 4 (n=20), 6 (n=20) and 8 (n=18) month old wildtype (WT) and APPswe/PS1ΔE9 (APP) animals. (d) Preference index is calculated as a percentage using the following formula: novel location exploration time/total exploration time x100%. APP display significantly greater preference indices than wildtype animals (p=0.019). *Pairwise comparisons: 6 months p=0.014. A one sample t-test compared preference index for each test to those obtained by chance (50%) ‡p<0.05 **(e)** Preference index is calculated as a percentage using the following formula: novel object exploration time/total exploration time x 100%. Preference index decreased significantly with age (p=0.009) and genotype (p=0.005). *Pairwise comparisons: 6 months p=0.022. A one sample t-test compared preference index for each test to those obtained by chance (50%) ‡p<0.05; ‡‡p=0.000.

2.8.4 Novel Object Recognition

The novel object recognition task was used to determine whether there were any differences in recognition memory due to age or genotype. A significant main effect of object was observed [$F_{(1,110)}$ = 5.38; p=0.022] indicating that animals spent more time exploring the novel object than the familiar one (Figure 2.5c). Exploration times decreased significantly with age [$F_{(2,110)}$ = 5.66; p=0.005] but APPswe/PS1ΔE9 displayed significantly greater exploratory activity than wildtype mice [$F_{(1,110)}$ = 7.38; p=0.008].

Significant age x object [$F_{(2,110)}$ = 7.25; p=0.0051] and genotype x object [$F_{(2,110)}$ = 4.91; p=0.029] interactions were also observed indicating that the

difference in the time spent exploring the novel versus the familiar object decreased as a function of age and genotype with 6 and 8 month old animals spending more time exploring the familiar object over the novel. Post hoc analysis indicated that 4 month old wildtype ($p<0.001$) and 4 month old APPswe/PS1ΔE9 ($p=0.006$) showed significantly greater exploration times for the novel object than the familiar. No significant differences in exploration times were observed for 6 or 8 month old animals. Significant main effects were observed for age [$F_{(2,110)} = 4.91$; $p=0.009$] and genotype [$F_{(1,110)} = 8.09$; $p=0.005$] on the preference index (figure 2.5e). Post hoc analysis indicated this was due to a significant difference in preference indices between 6 month old wildtype and APPswe/PS1ΔE9 animals ($p=0.022$). Only 4 month old wildtype and APPswe/PS1ΔE9 animals displayed a preference index significantly above chance ($p=0.000$ and 0.048 respectively) whilst all other animals displayed values close to 50%.

2.8.5 T-Maze

Animals were assessed for spatial memory using spontaneous alternation in the T-maze (figure 2.6). There was a significant genotype x age interaction [$F_{(2,99)} = 3.19$; $p<0.05$] reflecting the observed decrease in alternation rates with age in wildtype animals and its increase in APPswe/PS1ΔE9 mice. No significant differences were observed in alternation rates between wildtype and APPswe/PS1ΔE9 animals at 4 ($p=0.68$), 6 ($p=0.74$) or 8 ($p=0.48$) months. No group showed alternation rates significantly different from

chance (50%) although the 8 month old wildtype group approached significance (p=0.059).

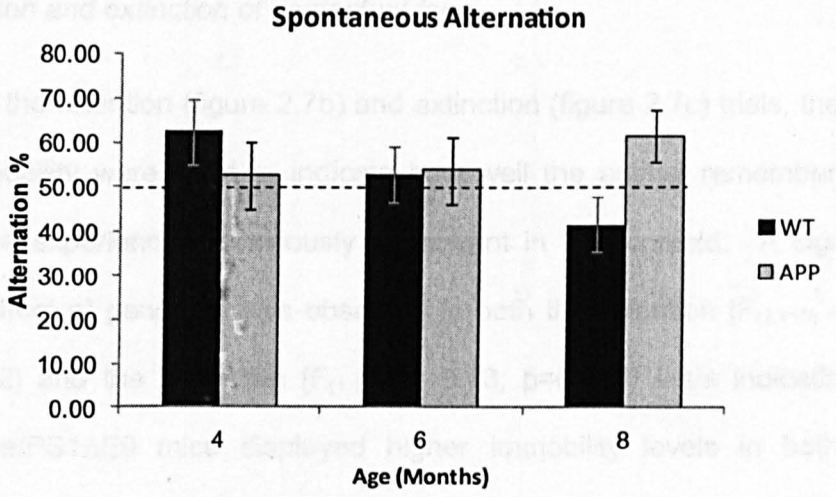

Figure 2.6 (data presented as mean ± SEM): Spontaneous alternation in the T-Maze. Percentage alternation displayed by wildtype (WT) and APPswe/PS1ΔE9 (APP) animals of 4 (n=20), 6 (n=20) and 8 (n=18) months. There are no significant differences in percentage alternation due to genotype or age. A significant genotype x age interaction ($p<0.05$) was observed. Alternation rates are not significantly different from chance (50%).

2.8.6 Contextual Fear Conditioning (CFC)

Acquisition of contextual fear

Shock-induced immobility was measured during the acquisition task (figure 2.7a) as an indicator of how well the animal has learned the task. All groups showed increasing immobility with time indicating that they were able to associate the context with the aversive stimulus [$F_{(8,793)} = 296.61$; $p<0.001$]. A significant main effect of genotype was also observed [$F_{(1,108)} = 4.20$; $p\leq0.05$] which post hoc analysis attributed to 4 month old APPswe/PS1ΔE9 mice displaying significantly lower immobility levels than wildtype mice at the

5^{th}, 7^{th}, 8^{th} and 9^{th} minutes ($p \leq 0.05$). There were no other significant main effects indicating that age did not influence acquisition of contextual fear.

Retention and extinction of contextual fear

During the retention (figure 2.7b) and extinction (figure 2.7c) trials, the levels of immobility were used to indicate how well the animal remembered the aversive experience it previously underwent in that context. A significant main effect of genotype was observed in both the retention [$F_{(1,110)} = 6.41$; $p=0.012$] and the extinction [$F_{(1,110)} = 8.73$; $p=0.004$] trials indicating that APPswe/PS1ΔE9 mice displayed higher immobility levels in both trials compared to wildtype animals. A significant main effect of trial was also observed demonstrating that both APPswe/PS1ΔE9 and wildtype mice displayed lower immobility levels in the extinction trial than the retention [$F_{(1,110)} = 6.20$; $p=0.014$]. Post-hoc analysis indicated a significant difference between wildtype and APPswe/PS1ΔE9 mice at 8 months of age in both the retention ($p=0.014$) and the extinction ($p=0.004$) trials.

To determine whether an animal had successfully extinguished contextual fear, an extinction index value was calculated (as described in part 1) by subtracting immobility during the retention trial from that in the extinction trial. For successful extinction to have taken place, the value of the extinction index needs to be negative and significantly different from 0 (Figure 2.7d). There were no significant differences between wildtype and APPswe/PS1ΔE9 mice as a function of age, genotype or age x genotype interactions for extinction index (figure 2.7d). One sample t-test analysis

demonstrated that only wildtype animals at 6 months of age were successfully able to extinguish contextual fear memory (p=0.016).

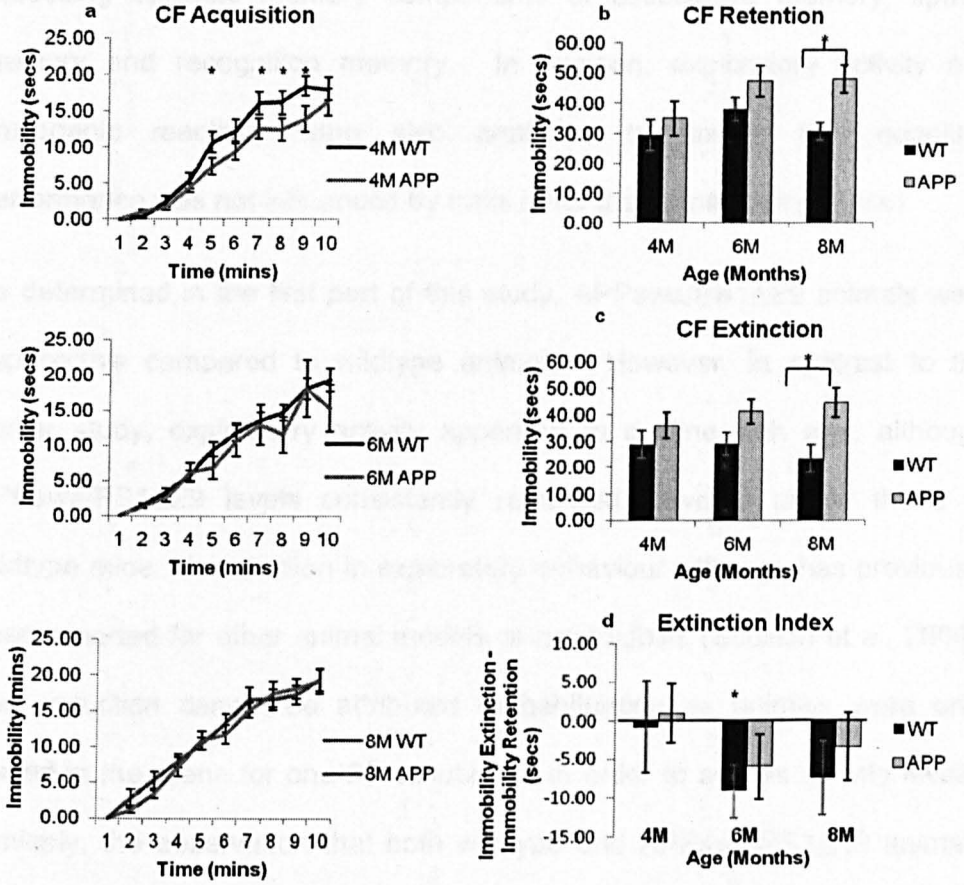

Figure 2.7 (data presented as mean ± SEM): Contextual Fear Memory. Immobility levels displayed by wildtype and APPswe/PS1ΔE9 animals of 4 (n=20), 6 (n=20) and 8 (n=18) months in the contextual fear memory task. (a) Both wildtype and APPswe/PS1ΔE9 animals similarly acquired contextual fear. (b-c) APPswe/PS1ΔE9 displayed significantly higher immobility levels than wildtype mice in the CF retention and extinction trials. Post hoc *p≤ 0.01. Both genotypes displayed significantly lower immobility levels in the extinction trial than the retention. (d) Only 6 months wildtype animals displayed successful extinction. One sample t-test *p=0.016 compared to 0 (no extinction).

2.9 Discussion

Animals underwent a battery of behavioural tests at 4, 6 and 8 months of age to determine their performance in a range of different memory tests assessing episodic memory components of associative memory, spatial memory and recognition memory. In addition, exploratory activity and anxiogenic reactivity were also analysed to ensure that cognitive performance was not influenced by traits other than those being tested.

As determined in the first part of this study, APPswe/PS1ΔE9 animals were hyperactive compared to wildtype animals. However, in contrast to the earlier study, exploratory activity appeared to decline with age, although APPswe/PS1ΔE9 levels consistently remained elevated above those of wildtype mice. A reduction in exploratory behaviour with age has previously been reported for other animal models of amyloidosis (Scullion et al. 2009). The reduction cannot be attributed to habituation as animals were only placed in the arena for one 30 minute trial in order to assess activity levels. Similarly, the observation that both wildtype and APPswe/PS1ΔE9 animals displayed reduced activity levels across trials in the novel location and novel object paradigm argues against the suggestion that APPswe/PS1ΔE9 mice may be failing to habituate. Alternative explanations suggested in section 2.6 may also apply.

As these animals were found to be hyperactive in the earlier study (2.5.1), their anxiogenic reactivity was measured to ensure that they did not exhibit anxiety-like traits which could influence their performance in other

behavioural tests. A comparison of the ratio of activity in the centre of the arena to that in the periphery demonstrated no differences due to genotype or age. This is in line with previous reports indicating that APPswe/PS1ΔE9 mice do not exhibit anxiety like behaviour in the plus maze at 7 or 12 months of age (Lalonde et al. 2004; 2005). Hence, any changes observed in the behavioural test parameters are unlikely to be due to the animals being anxious.

Spatial memory performance was assessed using the novel location and spontaneous alternation tasks. No genotype-related differences were observed in novel location exploration times. Spatial memory performance in the novel location task diminishes with age as demonstrated by 6 and 8 month old wildtype animals exhibiting exploratory activity at chance levels, whilst it remained greater than chance for APPswe/PS1ΔE9 mice at all ages. In the case of the spontaneous alternation task, a significant interaction between age and genotype reflected an increase in alternation with age in APPswe/PS1ΔE9 and decrease in wildtype animals. This may be due to spatial alternation requiring an optimal level of anxiety (Lalonde, 2002) based on which, the evidence for behavioural disinhibition in APPswe/PS1ΔE9 animals may explain why these animals perform well in this task. However, the results of this study do not support this concept as no differences in anxiety were observed between wildtype and APPswe/PS1ΔE9 animal. These results are consistent with previous studies employing this model at 7 and 12 months (Lalonde et al. 2004, 2005; Reiserer et al. 2007) which also did not find any differences in spatial alternation and, indeed, found 12 month

old APPswe/PS1ΔE9 to display alternation rates significantly above chance in contrast to age-matched wildtype animals (Lalonde et al. 2005).

These findings would suggest that spatial memory performance remains intact in these animals between 4 and 8 months of age. Previous studies using alternative methods such as the Morris water maze or radial arm maze, for testing spatial memory awareness suggest that this may be the case as animals in those studies do not typically show impairments until later in life (Savonenko et al. 2005; Volianskis et al. 2008).

Recognition memory using the novel object task indicated that all animals were able to distinguish between the novel and familiar objects. However, exploration times decreased significantly with age and genotype such that 6 and 8 month-old APPswe/PS1ΔE9 animals spent more time exploring the familiar object than the novel although the difference was not significant. This may indicate animals are beginning to find it more difficult to distinguish between the novel and familiar object. Preference index of 6 and 8 month-old animals did not vary much within genotypes. Only 4 month old animals showed a preference index greater than chance, although in the case of APPswe/PS1ΔE9 this was only just significant, whilst all other groups did not. This could possibly be due to animals becoming confused between the objects after exposure to them for a total of 20 minutes during the acquisition and novel location trial. Although the novel object was a different shape to the familiar object, they were still exposed to 2 objects per trial and it is possible by the third trial that they would simply have lost interest in exploring

them, but as exploration times remain relatively high in the third trial this proposal is not supported by the data. A more straightforward novel object test uses one object in the acquisition trial and subsequently adds in another object in the test phase. Hence, it is a lot more apparent that there is a new object in the arena. As the animals seem to do comparatively better in the novel location test, it suggests that their spatial memory remains intact; hence, when they enter the arena in a novel object test which excluded a novel location trial previously, the fact that there is a second object in there now may register more strongly, than in an arena where there are still 2 objects (except that the shape of one has changed). An alternative explanation may be related to the inter-trial interval of 1 hour 30 minutes being too short, as a consequence of which the animal becomes confused or loses interest as it has not 'dis-habituated' from the previous test trial (novel location). This hypothesis could be tested by repeating the protocol using different groups of animals with increasing inter-trial-intervals e.g. 1.5/3/6 hours. The fact that only the 4 month old wildtype and APPswe/PS1ΔE9 animals displayed a preference index greater than chance would suggest that any confusion which arises is age related and not genotype specific although the protocol itself may have an influence. It is also possible that these factors apply differently to each group. For instance, the failure of 6 and 8 month old wildtype animals to display a preference index greater than chance may be due to a combination of habituation, and confusion or reduced motivation to explore. This suggestion receives support from the reduction in total exploration times observed in these animals with age in the

acquisition trial. In contrast, 6 and 8 month old APPswe/PS1ΔE9 mice may not exhibit a preference index greater than chance due to them being unable to discriminate between the novel and familiar object. This interpretation is supported by the observation that, at these ages, APPswe/PS1ΔE9 mice spent more time exploring the familiar object than the novel, a trait which was not observed in wildtype animals.

Furthermore, both the wildtype and APPswe/PS1ΔE9 animals exhibited a reduction in total exploration time and LMA with age and increasing trial number, with the decline being more apparent in wildtype animals than APPswe/PS1ΔE9. This indicates that animals become habituated to the arena and, thereby, display reduced motivation to explore, the more they are exposed to it. As the novel object trial was the last of three trials, at this point, an animals' motivation to explore would have been at its lowest level and, thus, may potentially have resulted in the observed decline in discrimination of the novel object from the familiar. In future experiments, it would be advisable to use an alternative test for spatial memory and use only one object in the acquisition trial and two in the test phase. Alternatively, switching the order of the trials so that the object recognition trial is second and the location trial last may be of benefit. Additionally, increasing the inter-trial interval to 4 hours may prove beneficial in discerning behavioural differences between the different groups.

The contextual fear memory paradigm, in line with earlier studies (2.5.2), demonstrated that both wildtype and APPswe/PS1ΔE9 mice were able to

learn an association between the footshock and the chamber in which they received it at all ages. APPswe/PS1ΔE9 animals of 4 months of age appeared to learn slower than their wildtype counterparts at the 5, 7, 8 and 9 minutes time-points. However, as they appear to demonstrate a similar rate of learning to wildtype animals at all other times, and at 6 and 8 months of age, this effect is most likely due to variation in individual data points comprising the group mean rather than a slower task acquisition rate. Hence, APPswe/PS1ΔE9 animals did not exhibit learning impairments at 4, 6 or 8 months of age. However, although both wildtype and APPswe/PS1ΔE9 animals were able to recall CF in the retention trial, APPswe/PS1ΔE9 mice appeared to display better recall as demonstrated by higher immobility levels than wildtype animals. The difference in recall between the two genotypes was particularly pronounced at 8 months of age. A similar pattern was observed for the extinction trial in which animals would be expected to display lower immobility levels than in the retention trials as a practical illustration of them re-learning that the context is no longer aversive. Whilst animals did display lower immobility levels in most cases (4 month old APPswe/PS1ΔE9 animals actually display slightly higher immobility levels), the reduction was not significant for any of them except 6-month-old wildtype animals. This is reflected in the extinction index data, whereby only 6 month old wildtype animals were able to successfully extinguish CF. Hence, in this study, contrary to expectations, 4 and 8 month-old wildtype and APPswe/PS1ΔE9 mice at all ages were unable to extinguish CF. This is in contrast to the observations described in section 2.5 in which wildtype

animals aged 4 and 5.5 months were able to successfully extinguish CF. This difference may be accounted for by the presence of large individual variations in immobility levels in the groups examined in this study as illustrated by the presence of relatively large variance in the data. In the case of 4 and 5.5 month-old animals (section 2.5) and of 6 month old wildtype animals in this study, the variance was comparatively smaller, thereby suggesting lower levels of individual variation and, hence, successful extinction. The individual variation is particularly apparent in the 4 month old group. In the case of failure to extinguish in the 8 month old wildtype group, in addition to it being due to the variation in individual scores, it is possible that, at this age, the animals are naturally becoming cognitively impaired due to advancing age. In the case of APPswe/PS1ΔE9 animals, based on the results described in the first half of this chapter and demonstrated by other studies (e.g. Bonardi et al. 2011), it was expected that they would demonstrate a failure to extinguish CF memory and thus show impairments in cognitive flexibility. However, despite increasing age and, therefore, advancing pathology, these animals still did not display evidence for recent memory impairments even at 8 months of age.

In summary, the strongest outcomes from the battery of behavioural tests conducted in the course of this study indicate that APPswe/PS1ΔE9 mice are hyperactive and demonstrate impairments in cognitive flexibility. Both of these traits are apparent at 4 months and can still be detected at 8 months of age. Additionally, both of these tasks involve aspects of striatal circuitry (Clarke et al. 2008; Brooks et al. 1995). This is of particular interest as an

age-related decrease in the number of neurons in the striatum of 12, but not 6 month old APPswe/PS1ΔE9 mice in response to increasing Aβ load has previously been reported (Richner et al. 2009). It is, therefore, possible that the behavioural deficits detected during the course of this study represent a means by which damage to striatal neurons preceding degeneration could be assessed.

Spatial memory appears to remain intact in APPswe/PS1ΔE9 animals up to 8 months of age whilst recognition memory indices suggest the possibility of impairment in APPswe/PS1ΔE9 mice. This is interesting as lesion studies have previously demonstrated that a smaller lesion volume of the hippocampus is required to induce impairments in spatial memory (~50%) whilst a greater lesion (75-100%) is necessary to cause defects in recognition memory (Broadbent et al. 2004). However, the results of this study suggest that spatial memory remains intact whilst recognition memory may be impaired in APPswe/PS1ΔE9 animals. Alternatively, it is possible that the recognition memory impairments observed may not be related to an inability to distinguish between novel and familiar objects but may be due to habituation to the test arena as suggested for wildtype animals.

2.10 Conclusion

The results presented in both parts of this chapter suggest that the APPswe/PS1ΔE9 mouse model of AD is an appropriate one for investigating behavioural impairments arising from the presence of the genetic insertion of the APPswe and PS1ΔE9 transgenes into the mouse genome. In particular,

the presence of impairments in cognitive flexibility present in these animals as early as 4 months of age allows its use for investigating the efficacy of any potential therapeutic interventions at an early stage of the disease process, prior to the manifestation of extensive neuronal damage and global cognitive decline. Furthermore, an impairment in cognitive flexibility is one of the earliest effects distinguishable in human AD, using this parameter to study drug effects may enable better translation from a preclinical to clinical stage. However, despite these benefits, the conclusions drawn above should be tempered by the wildtype animals not exhibiting the behavioural phenotype expected.

Chapter 3

Endocannabinoid System and Lipidomics

3.1 Introduction

Alterations in lipid metabolism are thought to play a role in Alzheimer's disease (AD). Two groups of lipids which have received particular attention are the plasmalogens (Rothhaar et al. 2012) and cholesterol (Hartmann et al. 2007). Plasmalogens are glycerophospholipids which are the primary constituents of neuronal cell membranes as well as having roles in membrane fusion, ion transport and cholesterol efflux (Rothhaar et al. 2012). In relation to AD, plasmalogen levels are found to be decreased in the post-mortem brains of human AD patients, particularly in areas displaying extensive neurodegeneration (Ginsberg et al. 1995). Further analysis by Han et al. (2001) demonstrated this deficiency to be more prevalent in white matter than grey matter. Rothhaar et al. (2012) recently demonstrated that reduced plasmalogen levels result in increased activity of γ-secretase, thereby promoting enhanced production of amyloid beta (Aβ).

In addition to glycerophospholipids, cholesterol is also an essential component of neuronal membranes. Cholesterol metabolism is essential for efficient neurotransmission and repair (Bales, 2010). In the CNS, cholesterol is primarily supplied by glia after development as its synthesis is an energetically demanding process which is thought to confer too great a burden on neurons (Pfriegar, 2003). Instead, neurons rely on lipoproteins to provide them with cholesterol, in particular apolipoprotein E (APOE) (Gamba et al. 2012). The APOE type 4 allelle (ε4) was one of the earliest and most persistent risk factors discovered for sporadic AD (Strittmatter et al. 1993)

thus implicating perturbations in cholesterol transport. In addition, it has been suggested that high levels of cholesterol promote processing of APP along the amyloidgenic pathway and may associate with APOE to provide a seeding platform for Aβ aggregation (Gamba et al. 2012).

Other lipid entities associated with AD include alkylacylglycerophosphocholine second messengers which are thought to increase tau phosphorylation and initiate signalling cascades leading to neuronal cell death (Ryan et al. 2009) and endocannabinoid production (Pardon et al. 2009).

The role of endocannabinoids in AD has been extensively covered in Chapter 1. As stated earlier, a substantial body of research implicates beneficial roles of endocannabinoids in AD resulting from their multimodal functionality as anti-inflammatory (Murphy et al. 2012; Piro et al. 2012), anti-oxidant (Oddi et al. 2012), neuroprotective (Sun et al. 2007; Chen et al. 2011) and pro-neurogenic agents (Soltys et al. 2010; Goncalves et al. 2008). Amongst the evidence suggesting that endocannabinoid levels have an influence in AD, is the observation by Pardon et al. (2009) of differences in regional endocannabinoid levels at 5.5 months between wildtype animals and the TASTPM mouse model of AD (Pardon et al. 2009).

Aberrant lipid metabolism has also been implicated in a number of diseases other than AD (Han et al. 2001; 2002) resulting in an urgent need for developing sensitive MS techniques enabling detection, characterisation and quantification of components of the cellular lipidome (Oresic et al.2008).

These requirements have been addressed in recent years by the emerging field of lipidomics. Lipidomics is a branch of metabolomics, which is the study of global metabolite changes in cells, tissues and biofluids (Oresic et al. 2011). Hence, lipidomics can be defined as an attempt to analyse and characterise lipids and the molecules they interact with by unravelling individual pathways and combining them to generate a picture of how the system operates as a whole (Wenk et al. 2003). A major advantage of lipidomics is that it enables the application of an unbiased approach as no prior assumptions are programmed into the method. Hence, it provides a global output of all the elements of the lipidome as it has not been instructed to monitor only certain m/z values as with many other analytical approaches. This can result in the revelation of novel mediators previously not associated with the conditions being evaluated.

Ultra performance liquid chromatography (UPLC) coupled to MS is a recent technique developed for application in research into lipidomics (Wenk et al. 2005). UPLC involves the use of particles less than 2.5μm in size to line a column enabling greater efficiency, resolution and speed of separation (Scwartz, 2005). However, because of the diverse array of lipid structures and their complex interactions, analysing and interpreting the information gained from MS studies is currently still a work in progress, hindered by the lack of availability of reliable internal standards for several lipid classes, although rapid advances are being made (Oresic et al. 2008; Shevchenko et al. 2010).

The limitations associated with accurately measuring lipid levels are particularly exacerbated when applied to the small tissue quantities associated with regional mouse brain areas such as the frontal cortex, hippocampus and striatum. This is particularly problematic in the case of endocannabinoids and related compounds as their levels are relatively low in the brain compared to other lipid entities (Buczynski et al. 2010). It has previously been reported by Muccioli et al. (2008) that a minimum tissue weight of 30 mg is necessary for accurate detection of N-acylethanolamines using gas-chromatography coupled with mass spectrometry (GC-MS). However, regions such as the striatum can weigh as little as 6 mg when using samples from one hemisphere only (Maroof et al. unpublished observations). In addition to this, the absence of functional groups conferring electrical activity, fluorescence or permitting UV absorption increases the difficulty of isolating endocannabinoids from other lipid mediators (Buczynski et al. 2010; Zoerner et al. 2011). As a consequence, most quantitative analysis of endocannabinoids is conducted using mass spectrometry coupled to liquid chromatography (LC-MS). LC-MS enables separation of the various endocannabinoids and related molecules on the LC column followed by their fragmentation and detection of the fragmented component using MS (Le Blanc et a. 2003; Buczynski et al. 2010). The use of LC-MS enhances the signal to noise ratio of the sample, thereby reducing interference from other constituents of the biological matrix (Buczynski et al. 2010).

Due to manipulations of the endocannabinoid system being increasingly endorsed as potential treatment strategies for neurodegenerative disorders, and much of this being based on pre-clinical work undertaken in rodent models, it is essential to determine regional levels of endocannabinoids in the absence of pharmacological manipulations. At present, much of the work assumes the changes observed are due to alterations in endocannabinoid tone but little is known regarding the innate state of the system prior to external manipulations, or as a function of age. This is particularly important as ageing is known to be affected by endocannabinoids based on the observation that CB receptor knockout mice display features of accelerated ageing (Bilkei-Gorzo et al. 2012).

Many other techniques are used in conjunction with the above methods to obtain a comprehensive understanding of processes driving the cellular and systemic aberrations associated with pathology. Amongst these are assays enabling measurement of the functional consequences of receptor activation by the analytes measured using MS, such as the [^{35}S] GTPγS binding assay (Harrison et al. 2003). The binding of a ligand to a G protein-coupled receptor (GPCR) results in the activation and subsequent dissociation of a $G_{\alpha}(GDP)G_{\beta\gamma}$ heterotrimer complex. Upon activation GDP dissociates from G_{α} and is replaced by GTP which results in the G_{α} subunit separating from the $G_{\beta\gamma}$ and activating downstream signalling cascades (Figure 1.2; Harrison et al. 2003). The reformation of the $G_{\alpha}GDP$ complex by the action of GTPase and its subsequent binding to $G_{\beta\gamma}$ terminates the process. The [^{35}S] GTPγS binding assay replaces normal GTP with a non hydrolysable ^{35}S

labelled GTPγS. As it cannot be hydrolysed $G_{\alpha}[^{35}S]$ GTPγS complexes accumulate in the sample and can be measured by quantifying the amount of radioactivity present.

In addition to assessing G protein activation, the $[^{35}S]$ GTPγS binding assay can also be used to determine the efficacy and potency of ligands at a particular GPCR. As a result, the assay is a particularly useful tool for analysing the pharmacodynamics of new drug candidates which have usually been identified using MS-based approaches. Coupling these techniques to those used in metabolomics approaches enables a picture to be built of a given disease process as well as providing means for assessing potential treatments.

3.2 Aims

The aims of this chapter were to determine the levels of AEA, OEA, PEA and 2AG in the mouse hippocampus (HC), frontal cortex (FCTX) and striatum (STR) using LC-MS/MS in order to determine whether alterations are observed in the ECS with advancing age. A lipidomic analysis was also conducted to determine whether there were any group differences in the lipid metabolome as a consequence of either age or genotype. Additionally, the efficiency of cannabinoid receptor-effector coupling in these regions was investigated using the $[^{35}S]$ GTPγS binding assay.

3.3 Materials and Methods

3.3.1 Chemicals

Acetonitrile (far UV grade), methanol, ethanol, ethyl acetate, hexane, ammonium acetate and formic acid were obtained from Fisher Scientific (Loughborough, UK). Sodium chloride was purchased from MP Biomedicals Inc (Ohio, USA). AEA, OEA, PEA, 2AG, AEA-d8 and 2AG-d8 were obtained from Cambridge Bioscience (Cambridge, UK). High pressure liquid chromatography (HPLC) grade water was used for all experiments (Elga, High Wycombe, UK).

3.3.2 Standard preparation

Stock solutions were prepared in ethanol to give a final concentration of 14mM AEA, 2.8mM AEA-d8, 15mM OEA, and 17mM PEA. 13mM 2AG and 259 µM 2AG-d8 were prepared in acetonitrile. All stock solutions were stored at -80°C. Stock solutions were diluted in a solution of ethyl acetate:hexane (9:1 v/v), to prepare standard concentrations ranging from 500nM to 100pM for extraction and analysis.

3.3.3 Tissue acquisition

As stated in Chapter 2.3.2, animals underwent Schedule 1 killing following which the brain was removed, the frontal cortex, hippocampus and striatum were dissected out, snap frozen in liquid nitrogen and stored at -80°C until use.

3.3.4 Sample extraction

The analytical method used is based on that of Richardson et al. (2007) with further optimisation and developments introduced to enable quantification of endocannabinoids using the smaller tissue weights obtained from mouse brain regions. Since the sample size used was typically in the region of 10-20 mg, the main changes involved a significant scaling down in volumes of solvents and solutions used compared with previous methodology intended for rat brain tissue. In addition, water as a sample diluent was replaced with 0.9% NaCl to better mimic the physiological tissue environment, and homogenisation of tissue samples was performed directly in Eppendorf tubes without silanisation using a pestle pellet cordless motor. Furthermore, both standards and internal standards were prepared in ethyl acetate:hexane (9:1 v/v). These changes to the sample preparation procedure significantly improved the sensitivity and reliability of the method to measure ECs and other lipids in the small samples sizes as can be observed in table 3.3. Tissue samples were weighed and homogenised in 400 µl 0.9% sodium chloride (NaCl) using a handheld pellet pestle cordless motor with disposable pestles (Sigma Aldrich). Once homogenised, 750 µl of ethyl acetate:hexane (9:1 v/v) and internal standards (8.4 pmol AEA-d8 and 1 nmol 2AG-d8) were added followed by vortexing and placement on the shaker for 30 minutes. Following this, samples were centrifuged at 13,000 RPM for 10 minutes and the ethyl acetate:hexane layer removed and placed in fresh tubes. The remaining pellet was resuspended in 9:1 ethyl acetate:hexane and the process repeated. The supernatants were pooled and subsequently

evaporated to dryness using a centrifugal evaporator (Jouan RC10.32). The same procedure was used for the extraction of EC standards and human plasma QC (quality control) samples. Human plasma was used for QC due to the difficulty associated with obtaining large quantities of standardised brain tissue for this purpose. However, as a substitute, human plasma contains all of the endocannabinoids and related molecules being investigated in addition to a range of other mammalian lipids. Plasma QC samples were included for each batch of study samples in accordance with bioanalytical QC guidelines and used to ensure analytical results were valid for each sample set. Following evaporation, samples were concentrated by re-constituting in 200 µl acetonitrile and evaporated to dryness again, after which they were reconstituted in a final volume of 50 µl acetonitrile and 10 µl injected for analysis..

3.3.5 HPLC-MS/MS conditions for EC identification and quantification

All samples were analysed using Applied Biosystem MDS SCIEX 4000 Q-Trap hybrid triple-quadrupole–linear ion trap LC/MS/MS mass spectrometer model 1004229-F (Applied Biosystem, Foster City, CA, USA) in conjunction with a Shimadzu series 10AD VP LC system (Shimadzu,Columbia, MD, USA) comprising a cooled autosampling unit. A Thermo Scientific HyPurity Advance C8 column (100mm length, 2.1mm ID, 3µm particle size) fitted with a guard column was used for LC separation. The column was maintained at a temperature of 40°C, a flow rate of 0.3ml/minutes and maximum pressure set to 350 bar. Mobile phases comprising aqueous (water, 1g/L ammonium

acetate, 0.1% formic acid, 10% acetonitrile) and organic (acetonitrile, 1g/L ammonium acetate, 0.1% formic acid, 10% water) solutions were passed through the column using a stepwise gradient (Table 3.1). Samples were maintained at 4°C in the auto sampler prior to analysis and an injection volume of 10µl was used.

Analytes and internal standards were quantified using tandem electrospray mass spectrometry in positive ion mode (ES^+) using multiple reaction monitoring (MRM) to enable simultaneous detection of multiple analytes of interest. A source temperature of 550°C was used. Protonated $[M+H]^+$ and product ion mass to charge (*m/z*) values were used to identify endocannabinoids and deuterated forms of the internal standards. The declustering potential (DP), collision cell entrance (CE) and exit (CXP) potentials are listed in Table 3.2.

3.3.5.1 Analyte Quantification

Samples were quantified using Analyst 1.4.1 (Applied Biosystems, Foster City, CA, USA). Compounds were identified by their LC retention times combined with *m/z* values. Peaks for each individual compound were integrated and used to determine area under curve (AUC). Data obtained were subsequently imported into Microsoft Excel. A ratio of the AUC for the extracted standard to the AUC of the internal standard (y axis) was plotted against concentration (x axis) for each standard. Linear regression analysis was subsequently applied and used to generate an equation for each standard. The ratio of sample AUC to internal standard AUC was put into

Time (mins)	[Pump A] %	[Pump B] %
0.01	45	55
2.00	35	65
6.50	35	65
8.00	30	70
8.01	0	100
9.00	0	100
9.01	45	55
15.00	Stop	

Table 3.1: Gradient conditions for LC/MS/MS.

Analyte	Retention Time (mins)	Q1 Mass	Q3 Mass	EP (V)	DP (V)	CE (V)	CXP (V)
AEA	5.78	348.28	62.06	10	40	30	8.56
OEA	7.55	326.30	62.06	10	40	32	8.19
PEA	6.60	300.28	62.06	10	40	30	9.22
2AG	6.97	379.28	287.22	10	88	22	4.90
AEA-d8	5.70	356.33	63.03	10	80	45	15.00
2AG-d8	6.89	387.33	95.11	10	70	61	7.08

Table 3.2 Retention times, m/z values for precursor (Q1), product (Q3) ions and MS/MS conditions for AEA, OEA, PEA, 2AG, AEA-d8 and 2AG-d8. EP = Entrance potential, DP = Declustering potential, CE = Collision cell entrance potential, CXP = Collision cell exit potential.

	Lowest Standard Detected				% CV AEA-d8			% CV 2AG-d8		
Modification	AEA	OEA	PEA	2AG	QC	Ext Stds	Samples	QC	Ext Stds	Samples
Same procedure as Richardson et al. 2007 but with all components scaled down and using Q-Trap instead of Quattro Ultima.	5 nM	2 nM	5 nM	100 nM	3.46	16.74	71.2	8.4	22.8	61.48
As above but standards and internal standards made up in ethyl acetate:hexane (9:1 v/v). Additionally, water has been replaced with 0.9% NaCl.	500 pM	1 nM	2 nM	10 nM	2.14	8.3	7.84	1.64	3.06	11.05
AEA-d8 dilution altered from 1:100 to 1:5000	100 pM	100 pM	1 nM	5 nM	3.58	5.11	8.77	0.6	4.08	9.81

Figure 3.3: Effects of modifications made to protocol of Richardson et al. 2007. Modifications resulted in standards of lower concentration being detectable and improvements in coefficients of variation (% CV) for the internal standard content of plasma QCs, extracted standards and tissue samples.

the equation and used to determine the concentration of each analyte of interest present in the sample. AEA-d8 was used as the internal standard for AEA, OEA and PEA and 2AG-d8 for 2AG analysis.

3.3.5.2 Statistical Analysis

The concentration of each endocannabinoids was analysed using two way ANOVA with age and genotype as between subject factors. Planned comparisons were conducted post hoc when necessary using the least significant difference (LSD) test for multiple comparisons.

3.3.6 Lipidomics Analysis

The frontal cortical, hippocampal and striatal samples prepared for the above study were subsequently used to conduct a lipidomics analysis using UPLC linked to high resolution mass spectrometry (HRMS). This analytical work was conducted by Srinivasarao Ravipati using the protocol outlined below.

3.3.6.1 Chemicals

Acetonitrile (far UV grade), chloroform, methanol, ethanol, isopropanol and ammonium acetate were all purchased from Fisher Scientific (Loughborough, UK). AEA, OEA, PEA, 2AG, AEA-d8, 2AG-d8, 5,6-dihydroxyeicosatrienoic acid (5,6-DHET), 8,9-dihydroxyeicosatrienoic acid (8,9-DHET), 11,12-dihydroxyeicosatrienoic acid (11,12-DHET), 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), 9-hydroxyeicosatetraenoic acid (9-HETE), 11-hydroxyeicosatetraenoic acid (11-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE), 16-

hydroxyeicosatetraenoic acid (16-HETE), 19- hydroxyeicosatetraenoic acid (19-HETE), 20-hydroxyeicosatetraenoic acid (20-HETE), arachidonic acid (AA). 5,6-epoxyeicosatrienamide (5,6-EET-EA), 2-(14,15-epoxyeicosatrienoyl)-glycerol (14,15-EET-G), Prostaglandin D_2 ethanolamide (PGD_2-EA), prostaglandin E_1 ethanolamide (PGE_1-EA), prostaglandin E_2 ethanolamide (PGE_2-EA) and prostaglandin $F_{2\alpha}$ ethanolamide ($PGF_{2\alpha}$-EA) were obtained from Cambridge Bioscience (Cambridge, UK). 5-hydroxyeicosatetraenoic acid (5-HETE), 8-hydroxyeicosatetraenoic acid (8-HETE), 5,6-epoxyeicosatrienoic acid (5,6-EET), 8,9-epoxyeicosatrienoic acid (8,9-EET), 11,12-epoxyeicosatrienoic acid (11,12-EET), 14,15-epoxyeicosatrienoic acid (14,15-EET), 12-hydroperoxyeicosatetraenoic acid (12-HPETE) were all purchased from Biomol International (Exeter, UK). High pressure liquid chromatography (HPLC) grade water was used for all experiments (Elga, High Wycombe, UK).

3.3.6.2 Standard and QC Preparation

Standard preparations comprising the ECs, eicosanoids and prostaglandins listed in 3.3.9 were prepared and diluted to a final concentration of 1μM in ethanol for use in quantitative determination

Plasma lipids from QC samples were extracted using 500 μL of ice-cold (-20°C) chloroform/methanol (1:2 v/v) to 50 μL of blood plasma, followed by vortexing and the addition of 500 μL of water. Samples were placed on a shaker for 10 minutes followed by centrifugation at 13000 rpm for 10 minutes at 4°C. Lower lipophilic and upper hydrophilic phases were separated by a

central protein layer in this procedure. For the lipid metabolomics measurements, 100µL of lower lipophilic phase was removed and mixed with an equal amount of 100% isopropanol prior to injection.

3.3.6.3 UPLC-MS Conditions

Lipidomic profiling was conducted using a Accela High speed UPLC-MS system comprising an Orbitrap Exactive Mass Analyser, Accela Exactive Pump, Accela Exactive Column Oven and Accela Exactive autosampler with Xcalibur v2.1 operating system (Thermo Fisher Scientific, USA). An ACE 2 C18 column (150×2.1mm, 2µm)

UPLC separation was performed using an ACE Excel C18 reverse phase column (150mm length, 2.1mm ID, 2µm particle size). The column was maintained at a temperature of 30°C and flow rate of 300µl per minute with maximum pressure set to 350 Bar. Mobile phases comprising aqueous (60:40 ACN/10mM ammonium acetate in 100% H_2O), and organic (90:10 IPA/10mM ammonium acetate in 100% ACN) solutions were passed through the column using a stepwise gradient to elute lipid species of variable polarity. An injection volume of 10µl was used for all samples.

Analytes were identified using tandem electrospray in both positive (ES^+) or negative (ES^-) ion mode. Capillary voltage was set to 25 V in ES^+ mode and 27 V in ES^- ion mode. Tube lens and skimmer voltages in ES^+ were 115V/22V and 140V/28V in ES^-. Flow rates of sheath gas, auxiliary gas and sweep gas for both ion modes were set to 30, 15 and 5 (arbitrary units) respectively and capillary and heater temperature were maintained at 300°C.

Data was collected in full scan ion mode using m/z values 100-1200. The maximum scan inject time was 100ms per micro scan.

3.3.6.4 Data Analysis

Data acquired by Xcalibur were imported and processed using Sieve (Thermo Scientific, USA). Sieve aligns and extracts the relative ion chromatograms (RICs) for every aligned ion. It gives specific differences between groups for all the extracted RICs. The data given by Sieve is exported to SIMCA-P (MKS Umetrics AB, Umea, Sweden) to produce principle component analysis of the sample sets. PCA provided a ratio for each *m/z* value which was used to determine differences between wildtype and APPswe/PS1ΔE9 mice.

3.3.6.5 Principle Component Analysis

Principal component analysis (PCA) is a multivariate statistical technique used to reduce large volumes of data comprising many variables into a smaller number of artificial variables (principal components) which enable the identification of similarities and differences in the data set. Each variable is assigned a weighting score which determines how much of the variance present in the data set is due to that variable. Hence, the variable (A) with the highest weighting score accounts for the greatest amount of variance in the data set. The variable (B) with the second highest weighting score accounts for second greatest amount of variance in the data set that is not accounted for by the first variable and so on. Importantly, A and B are not correlated.

3.3.6.6 Compound Identification

The *m/z* values corresponding to the top fifty PCA weighting scores for each group were selected for compound identification. Compounds were identified by entering the m/z value into either the human metabolome database (HMDB: http://www.hmdb.ca) or Lipid Maps (http://www.lipidmaps.org)

3.3.7 [^{35}S] GTPγS Binding Assay

Cannbinoid receptor-effector coupling efficiency was determined using the [^{35}S] GTPγS binding assay following optimisations.

3.3.7.1 Reagents

Tris, hydrated magnesium chloride ($MgCl2.6H_2O$), bovine serum albumin (BSA) Guanosine diphosphate (GDP), theophyliine and Guanosine 5'-[γ-thio]triphosphate tetralithium (GTPγ) salt were purchased from Sigma Aldrich (UK), Sodium chloride (NaCl) from Fisher Scientific, HU-210 from Tocris Bioscience (Bristol, UK) and [^{35}S]Guanosine 5'-[γ-thio]triphosphate from Perkin Elmer (MA, USA).

3.3.7.2 Brain Membrane Preparations

Membrane fractions were prepared using tissue from the frontal cortex, striatum and hippocampus. Tissue was weighed and homogenised using a pestle pellet cordless motor in a 20x volume of 50mM Tris, pH 7.4 at 4°C. Following homogenisation, samples were centrifuged at 20,000g for 10

minutes and the supernatant was removed. Samples were re-suspended in a further 20x volume of 50mM Tris and the process repeated 4 times in total. Following the final centrifugation step, samples were re-suspended to give a final concentration of 1mg/ml and stored at -20°C until needed.

3.3.7.3 [^{35}S] GTPγS binding assay

Membrane preparations were re-suspended in binding buffer (50mM Tris, 100mM NaCl, 10mM MgCl2.6H_2O, 0.2mg/ml BSA in distilled water). 50µg of tissue was added to each tube as optimisation studies indicated that a lower tissue concentration provides the greatest increase in activity above basal (figure 3.1a) along with 40µM GDP (optimised value, figure 3.1b) and 2mM theophylline. Samples were vortexed and incubated at 30°C for 30 minutes. Following incubation samples were separated into one of three conditions: basal, non-specific binding and HU210 (i.e. cannabinoid receptor activated). Each sample condition was conducted in triplicate for each animal. Drug buffer was added to basal samples, 1µM unlabelled GTPγ to non-specific binding samples and 1µM HU-210 to HU-210 samples. All samples were subsequently incubated with 0.02nM [^{35}S] GTPγS for 90 minutes at 30°C to attain steady state. Following incubation, the reaction was terminated by rapid filtration of membrane samples through a Brandel Tissue Harvester (Gaithersburg, MD, USA) onto Whatman GF/B glass-fiber filters (Clifton, NJ) and the filters were washed four times using distilled water at 4°C. Filter discs were subsequently transferred to scintillation vials and covered with Ultima Gold XR scintillant (Perkin Elmer, MA, USA) and the radioactive

decay measured using a Packard Tri-Carb 2100 TR scintillation counter (Isotech, Chesterfield, UK) with a count time of 3 minutes.

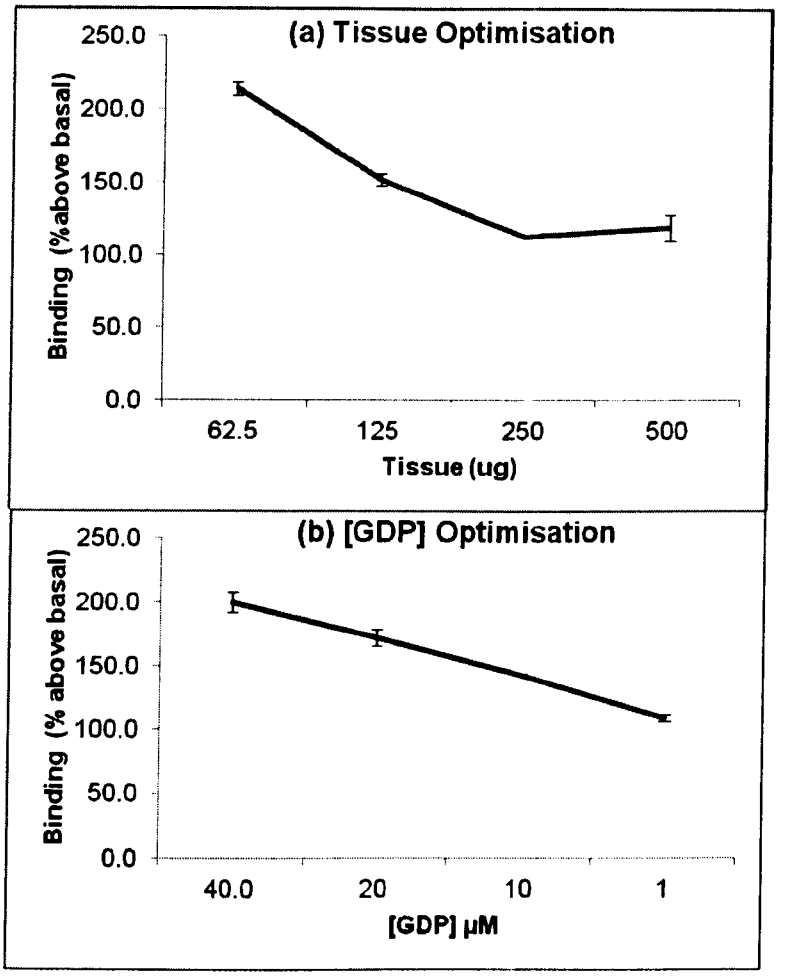

Figure 3.1: HU210 induced [^{35}S] GTPγS binding with increasing tissue content (a) and GDP concentration (b) (mean ± SEM). Percentage binding above basal elicited by the cannabinoid receptor agonist HU210 to cannabinoid receptors in the cortex of 5.5 month old wildtype mice. (a) Lower tissue quantities yielded greater binding. (b) Higher concentrations of GDP elicited greater binding.

3.3.7.4 Data Analysis

[^{35}S] GTPγS specific binding was determined by subtracting the non-specific binding from the basal and HU210 conditions. The specific binding in the HU210 condition was subsequently divided by that of the basal samples to enable percentage activity over basal to be calculated.

3.3.7.5 Statistical Analysis

The percentage activity over basal was analysed using two way ANOVA with age and genotype as between subject factors. Planned comparisons were conducted post hoc when necessary using the least significant difference (LSD) test for multiple comparisons.

3.4 Results

3.4.1 Endocannabinoid Concentrations

Improvements to Assay Method

Reducing the volume of solvents did not significantly increase sensitivity as indicated by the lower standard ranges not being detected (Table 3.3). However, by preparing the internal standards and standards in ethyl acetate: hexane (9:1 v/v), in addition to replacing water with 0.9% NaCl, dramatically increased the peak areas for both the extracted standards and internal standards (data not shown). Furthermore, these alterations enabled lower standard concentration ranges to be detected whilst simultaneously lowering the coefficient of variation (CV) for internal standards in plasma QCs,

extracted standards and tissue samples thus indicating reduced variation in their levels between different samples. Finally, the peak area determined by LC-MS/MS for AEA-d8 when diluted 1:100 in ethyl acetate:hexane (9:1 v/v) was in the region of 10^6 a very high value which could potentially lead to interference from minor contaminants in the AEA-d8 solution. To normalise this peak area to that routinely obtained in other protocols the dilution factor for AEA-d8 was changed to 1:5000 which resulted in more reasonable peak areas in the expected region of 10^5. This step resulted in a further increase in sensitivity, possibly as a reduction in unwanted background intereference, as demonstrated by the limit of detection being lowered further so that more standards in the lower concentration ranges could be detected (Table 3.3).

Endocannabinoid analysis

Concentrations of the two principal endocannabinoids AEA and 2AG, and the endocannabinoid-related acylethanolamines OEA and PEA were determined in the hippocampus, frontal cortex and striatum (figure 3.3). There was a significant age-related increase in the concentrations of AEA [$F_{(2,54)} = 15.41$; $p<0.001$], OEA [$F_{(2,54)} = 9.37$; $p<0.001$] and PEA [$F_{(2,54)} = 22.22$; $p<0.001$] in the hippocampus of both wildtype and APPswe/PS1ΔE9 animals. APPswe/PS1ΔE9 exhibited significantly higher hippocampal AEA concentrations than wildtype animals [$F_{(1,54)} = 4.70$; $p=0.03$].

A similar significant age-related increase was also observed in the frontal cortex for AEA [$F_{(2,53)} = 17.54$; $p<0.001$], OEA [$F_{(2,53)} = 13.62$; $p<0.001$] and PEA [$F_{(2,53)} = 10.91$; $p<0.001$]. No significant differences were observed for

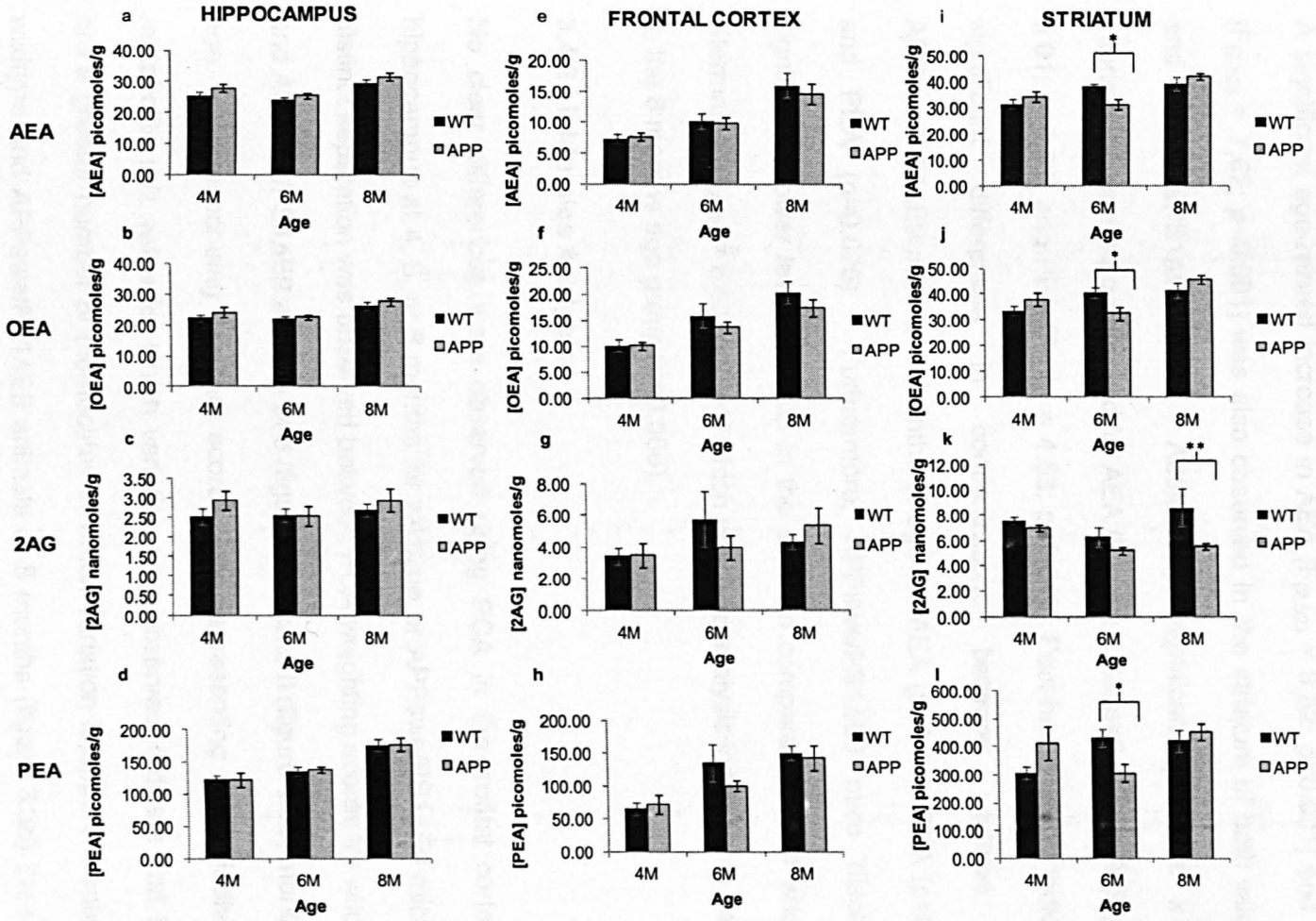

Figure 3.2: Endocannabinoid Levels in Hippocampus, Frontal Cortex, and Striatum (mean ± SEM). Concentrations of AEA, OEA, 2AG and PEA in the hippocampus, frontal cortex and striatum of wildtype and APPswe/PS1ΔE9 mice at 4, 6 and 8 (n=10) months. (a-h) AEA, OEA and PEA increase significantly with age in the hippocampus and frontal cortex of wildtype and APPswe/PS1ΔE9 ($p<0.001$); no significant differences were observed for 2AG; (i-k) AEA and OEA increase significantly with age in the striatum ($p \leq 0.001$) of wildtype and APPswe/PS1ΔE9 mice; post hoc *$p \leq 0.01$. (k) 2AG concentration is significantly lower in APPswe/PS1ΔE9 ($p=0.014$); post hoc **$p=0.006$. (l) Post hoc *$p=0.03$.

the concentration of 2AG in the hippocampus or frontal cortex as a function of age or genotype.

A significant age-related increase in AEA [$F_{(2,52)}$ = 9.82; p<0.001] and OEA [$F_{(2,52)}$ = 7.62; p=0.001] was also observed In the striatum of both wildtype and APPswe/PS1ΔE9 mice. Additionally, significant genotype x age interactions were also observed for AEA [$F_{(2,52)}$ = 4.63; p=0.01], OEA [$F_{(2,52)}$ = 5.01; p=0.01] and PEA [$F_{(2,53)}$ = 4.63; p=0.01]. Post-hoc analysis indicated significant differences in concentrations between wildtype and APPswe/PS1ΔE9 mice at 6 months of age for AEA (p=0.01), OEA (p=0.01) and PEA (p=0.025). Furthermore, APPswe/PS1ΔE9 mice displayed significantly lower levels of 2AG in the striatum compared to their wildtype littermates [$F_{(2,52)}$ = 6.53; p=0.01] which post hoc analysis indicated to be due to the 8 months age group (p=0.006).

3.4.2 Lipidomics Analysis

No clear differences were observed using PCA in the frontal cortex or hippocampus at 4, 6, or 8 months for wildtype or APPswe/PS1ΔE9 mice. A distinct separation was observed between PCA weighting scores for wildtype and APPswe/PS1ΔE9 animals at 6 (figure 3.3c) and 8 (figure 3.3b) months of age. Corresponding loading score plots representing each individual metabolite m/z value for which variation was observed, indicate that there are a greater number of metabolites in which variation is apparent between wildtype and APPswe/PS1ΔE9 animals at 8 months (figure 3.3d) than at 6 (figure 3.3.c). The 50 metabolite m/z values showing the greatest amount of

variability for each group were selected for identification due to time constraints. These were further refined following identification to exclude those m/z values which displayed complex adducts which were unlikely to have formed. As compounds were analysed using an untargeted MS rmethod rather than a targeted MS/MS approach it was not possible to identify a given m/z value as corresponding to a specific metabolite. Thus, for the m/z values remaining after exclusions, a number of different possible metabolites were identified as shown in the appendix. However, in certain cases HMDB only identified one metabolite as corresponding to a given m/z value, such as for m/z 708.664 (figure 3.4) which corresponds to cholesteryl ester only (appendix). Table 3.4 lists some of the lipid metabolites showing the greatest variation in each of the four groups. Scatter plots for signal intensity were subsequently generated for the m/z values listed in table 3.4 and t-tests conducted (figure 3.4) to further corroborate the results obtained by PCA. A metabolite which is listed as high for one group e.g. 6 month wildtype will by default be considered to be lower in the corresponding APPswe/PS1ΔE9 animals. Variations in diglycerides levels were observed in all groups ($p<0.01$ 6 and 8 month old APP; $p<0.001$ 6 month wildtype; figure 3.4a; m and n respectively) except 8 month old wildtype animals. Monoglyceride levels were only significantly high in 8 month old wildtype animals, and, thus, low in 8 month APPswe/PS1ΔE9 ($p<0.01$; figure 3.4r-s). Variations in N-acylethanolamines and related compounds were observed at 6 ($p<0.001$; figure 3.4j; $p<0.05$ 3.4k) and 8 months ($p<0.01$; figure 3.4t) with levels being significantly higher in wildtype animals than in APPswe/PS1ΔE9

mice. Levels of fatty acids in general were higher in 6 month wildtype animals than APPswe/PS1ΔE9 ($p<0.001$; figures 3.4h-i). Compounds derived from cholesterol such as bile acids were raised in both wildtype ($p<0.001$) and APPswe/PS1ΔE9 ($p<0.05$) mice at 6 months of age but the individual identities of the bile acids differ between groups (see appendix for m/z 394.3293 wildtype and 473.3185 APPswe/PS1ΔE9). Cholesteryl ester, the major transport and storage form of cholesterol was significantly higher in 6 month old APPswe/PS1ΔE9 relative to their age matched wildtype counterparts ($p<0.05$; figure 3.4d). Components of the cholesterol ($p<0.01$; figure 3.4q) and steroid biosynthethic pathways ($p<0.01$; figure 3.4q) were also raised in 8 month wildtype animals relative to APPswe/PS1ΔE9. Additonally, 6 month old APPswe/PS1ΔE9 exhbited significantly raised levels of corticosteroids ($p<0.05$; figure 3.4 e), antioxidant activity ($p<0.05$; figure 3.4 b) and potential apoptotic inducers ($p<0.01$; figure 3.4g). 8 month old APPswe/PS1ΔE9 displayed significantly higher levels of glutathione conjugates ($p<0.001$; figure 3.4p) and leukotriene B4 ($p<0.05$; figure 3.4o). Levels of glycerol-3-phosphate were also raised in 6 month APPswe/PS1ΔE9 animals ($p<0.05$; figure 3.4f).

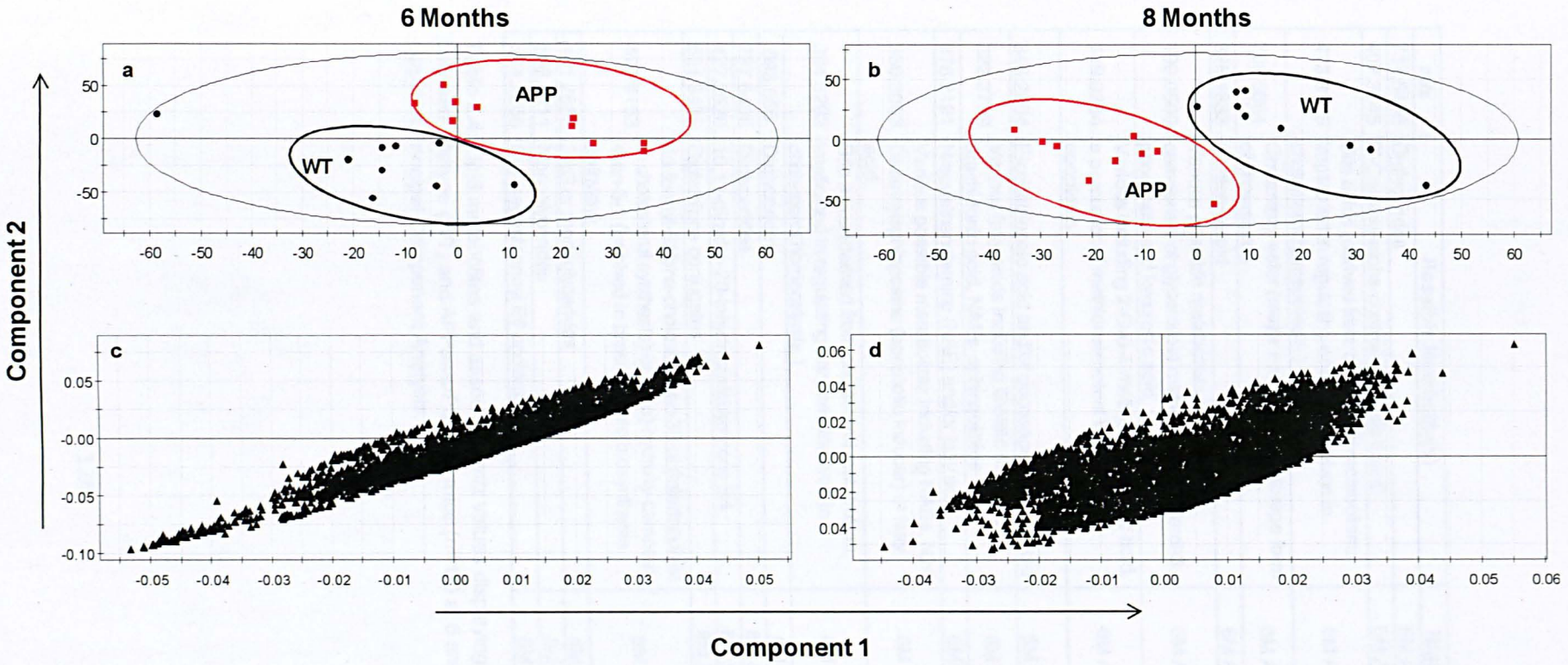

Figure 3.3: Principle component analysis weighting score plots for striatum samples for 6 (a) and 8 (b) month old wildtype (WT) and APPswe/PS1ΔE9 mice display distinct separations in terms of variation in metabolites detected in the samples from each genotype. Corresponding loading plots at 6 (c) and 8 (d) months representing each individual metabolite m/z value suggest variation in a greater number of metabolites at 8 (d) months than at 6 (c).

m/z	Possible Metabolite(s)	High in	Figure 3.4
737.5466	Diglycerides	6M APP	a
407.3045	9'-Carboxy-alpha-chromanol (antioxidant)	6M APP	b
473.3185	Bile acids (derived from cholesterol metabolism; implicated in regulating enzymes involved in cholesterol homeostasis)	6M APP	c
708.6664	Cholesteryl ester (major transport and storage form of cholesterol)	6M APP	d
325.1833	Corticosteroid	6M APP	e
190.0500	Various possible metabolites including a component of glycerolipid metabolism, glycerol-3 phosphate and kynurenic acid	6M APP	f
149.0234	Various including 2-Oxo-4-methylthiobutanoic acid, a precursor of methional=potent inducer of apoptosis.	6M APP	g
348.2864	Eicosatrienoic acid and/or docosapentaenoic acid	6M WT	h
322.2709	Various fatty acids including linoleic acid and arachidonic acid, NAEs, sphingolipids	6M WT	i
376.3191	N-acylethanolamine (NAE) and/or fatty acid	6M WT	j
350.3053	Various possible metabolites including NAEs, N,N-Dimethylsphingosine (apoptotic inducer) or fatty acid.	6M WT	k
394.3293	Bile acids (derived from cholesterol metabolism; implicated in regulating enzymes involved in cholesterol homeostasis)	6M WT	l
698.665	Diglycerides	6M WT	m
737.5466	Diglycerides	8M APP	n
427.2526	10,11-dihydro-20-trihydroxy-leukotriene B4	8M APP	o
661.3425	Glutathione conjugates	8M APP	p
475.4153	4α-formyl-5alpha-cholesta-8-en-3β-ol (intermediate in cholesterol synthesis) or (22α)-hydroxy-campest-4-en-3-1 (involved in brasinsteroid biosynthesis pathway)	8M WT	q
401.2655	2AG or monoglycerides	8M WT	r
506.4211	Monoglycerides	8M WT	s
384.2256	Eicosapentaenoyl Ethanolamide	8M WT	t

Table 3.4: Lipid metabolites and associated m/z values displaying significant variation between wildtype (WT) and APPswe/PS1ΔE9 mice (n=10) at 6 and 8 months based on the output of Principal Component Analysis.

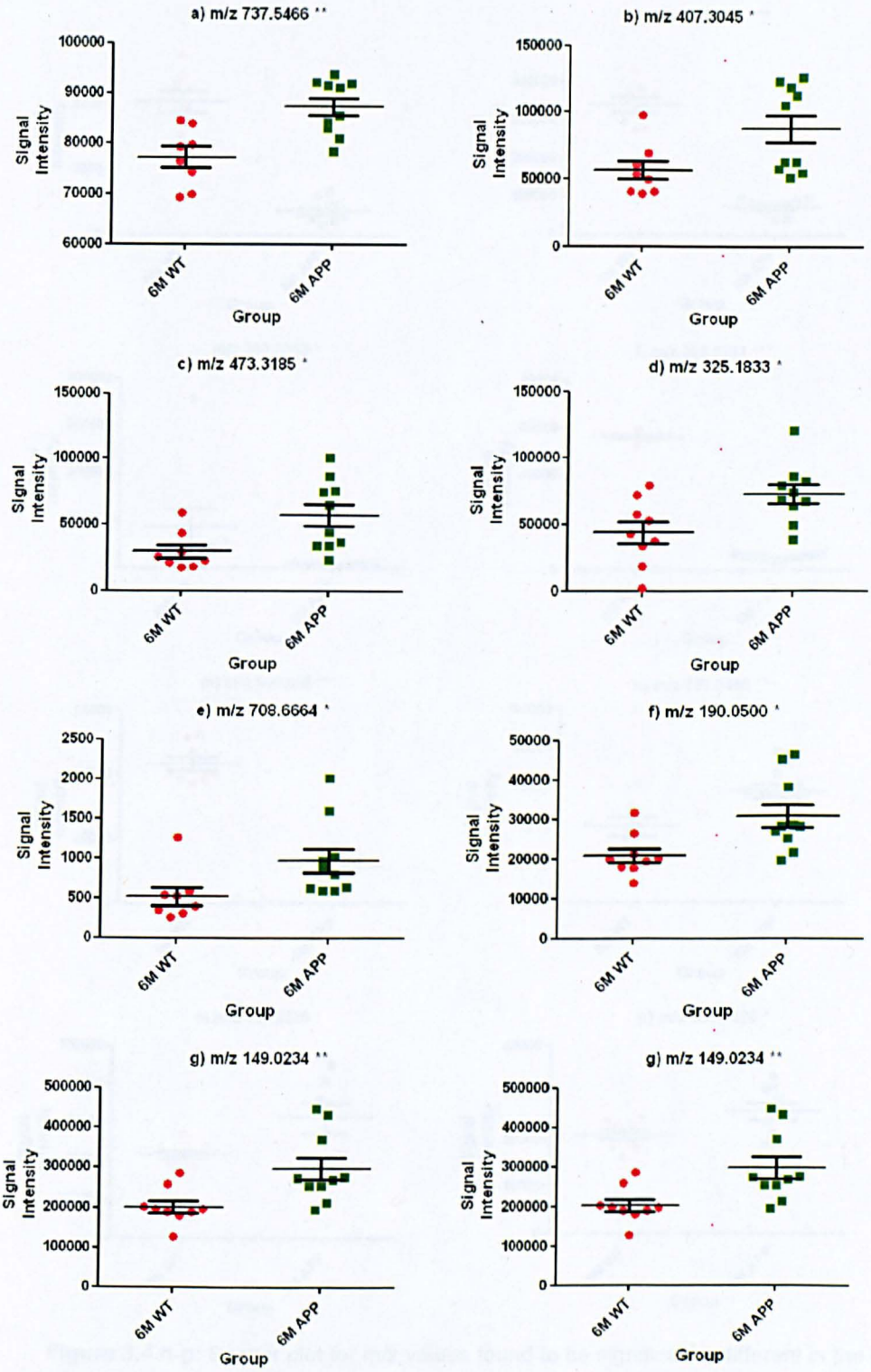

Figure 3.4 a-g: Scatter plot for m/z values found to be significantly different in the striatum of wildtype (WT) and APPswe/PS1ΔE9 mice at 6 or 8 months n=9-10 per group). Unpaired t-test: *p<0.05 **p<0.01 ***p<0.001

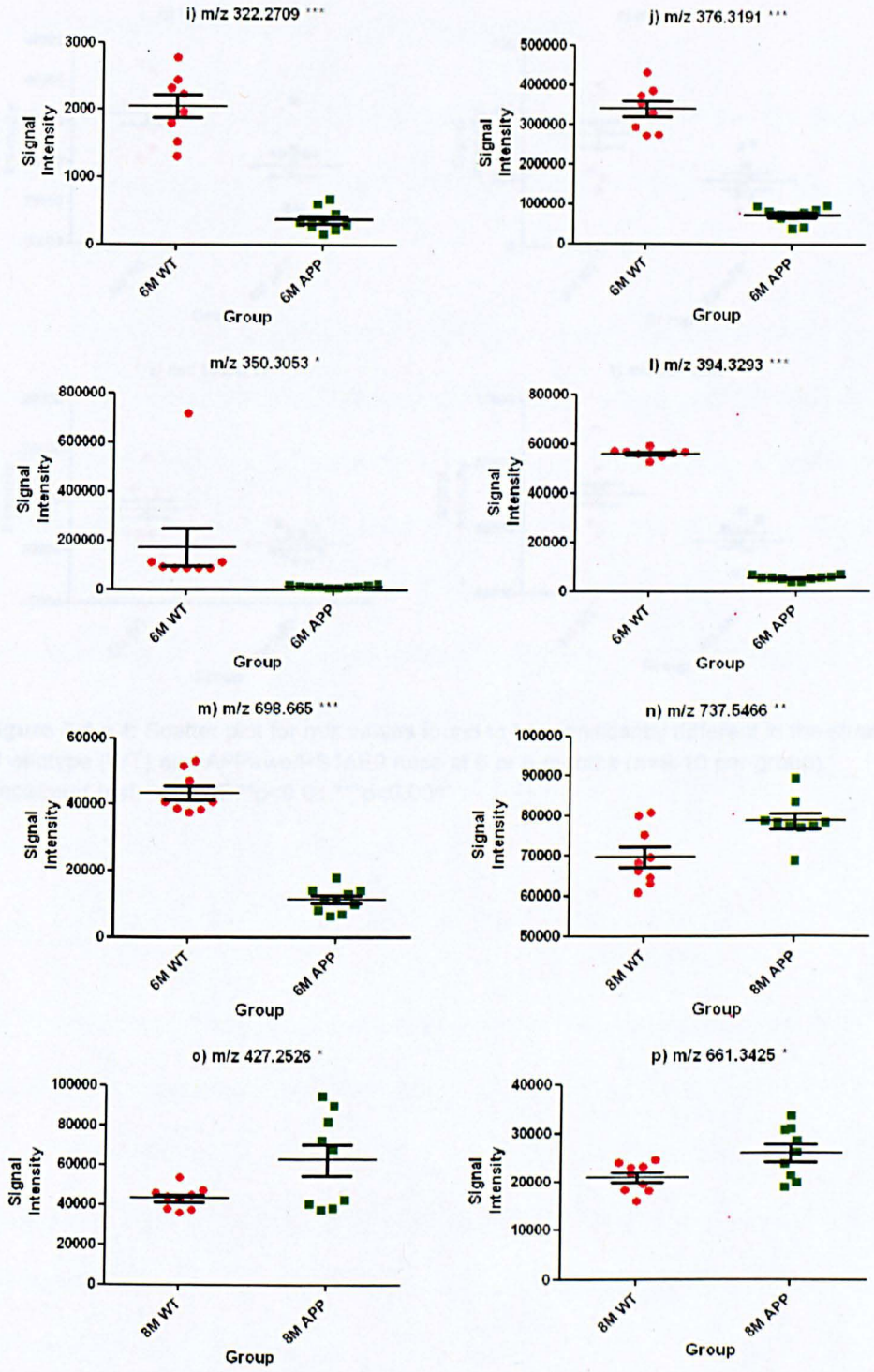

Figure 3.4 h-p: Scatter plot for m/z values found to be significantly different in the striatum of wildtype (WT) and APPswe/PS1ΔE9 mice at 6 or 8 months (n=9-10 per group). Unpaired t-test: *p<0.05 **p<0.01 ***p<0.001

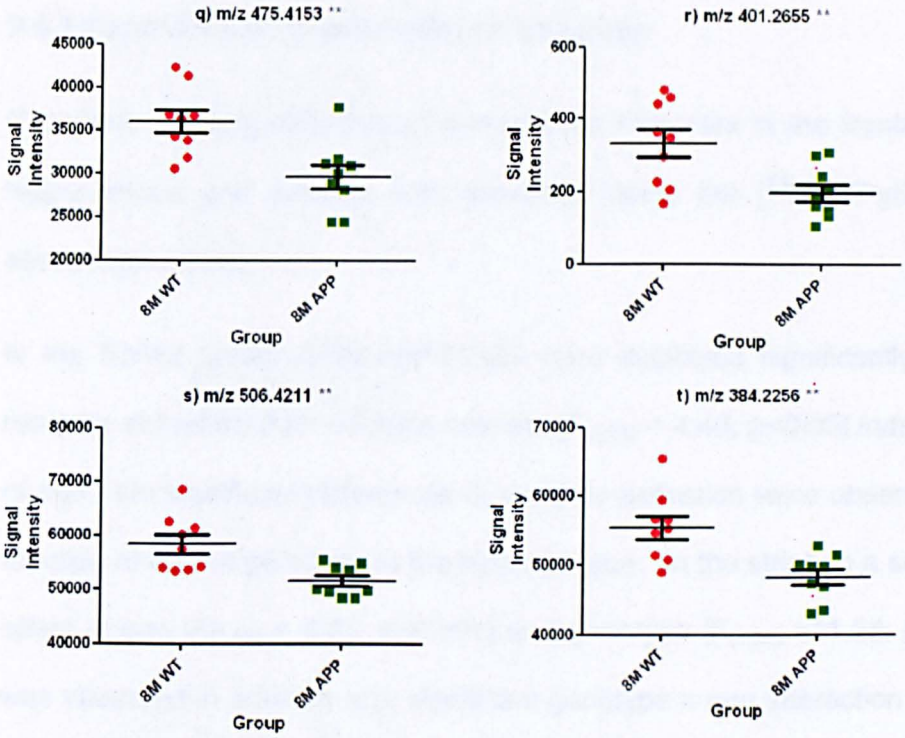

Figure 3.4 q-t: Scatter plot for m/z values found to be significantly different in the striatum of wildtype (WT) and APPswe/PS1ΔE9 mice at 6 or 8 months (n=9-10 per group). Unpaired t-test: *p<0.05 **p<0.01 ***p<0.001

3.4.3 Cannabinoid receptor-effector coupling:

G protein coupling efficiency of cannabinoid receptors in the frontal cortex, hippocampus and striatum was assessed using the [^{35}S]GTPγS binding assay (figure 3.4).

In the frontal cortex APPswe/PS1ΔE9 mice displayed significantly greater receptor activation than wildtype animals [$F_{(1,51)}$ = 4.40; $p<0.05$] independent of age. No significant differences in receptor activation were observed as a function of age or genotype in the hippocampus. In the striatum a significant effect of age [$F_{(2,52)}$ = 8.81; $p<0.001$] and genotype [$F_{(1,52)}$ =11.26; p=0.002] was observed in addition to a significant genotype x age interaction [$F_{(2,52)}$ = 6.94; p=0.002]. Post hoc analysis indicated this to be due to APPswe/PS1ΔE9 mice displaying significantly greater receptor activation compared to their wildtype littermates at 8 months of age.

3.4.4 Correlation analysis

No correlations were observed between the concentration of 2AG and cannabinoid receptor effector coupling efficiency in the striatum of wildtype and APPswe/PS1ΔE9 animals at 4 (r^2=0.017 p=0.733 wildtype; r^2=0.013 p=0.309 APPswe/PS1ΔE9), 6 (r^2=0.023 p=0.700 wildtype; r^2=0.027 p=0.650 APPswe/PS1ΔE9) and 8 (r^2=0.020 p=0.700 wildtype; r^2=0.058 p=0.531 APPswe/PS1ΔE9) months.

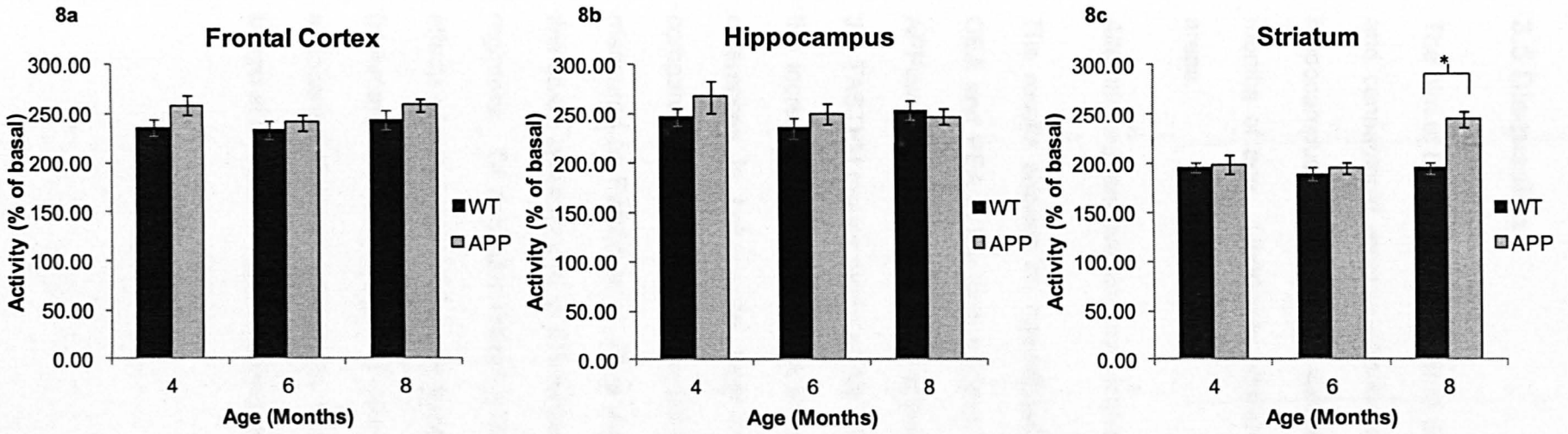

Figure 3.4: HU210 induced [^{35}S] GTPγS binding (mean ± SEM). Percentage of activity above basal elicited by the binding of the cannabinoid receptor agonist HU210 to receptors in (a) frontal cortex (b) hippocampus and (c) striatum of 4, 6 and 8 months (n=9-10) wildtype and APPswe/PS1ΔE9 mice . (a) APPswe/PS1ΔE9 show significantly enhanced receptor-effector coupling activity ($p<0.05$) compared to wildtype animals. (b) No significant differences were observed in the hippocampus between wildtype and APPswe/PS1ΔE9. (c) APPswe/PS1ΔE9 display significantly enhanced receptor-effector coupling activity compared to wildtype ($p\leq0.001$) with age ($p<0.001$). Pairwise comparisons*$p=0.002$.

3.5 Discussion

The aim of this experiment was to determine the levels of endocannabinoids and cannabinoid receptor-effector coupling efficiency in the frontal cortex, hippocampus and striatum of wildtype and APPswe/PS1ΔE9 at 4, 6 and 8 months of age. Lipodomic profiles were also obtained for all three brain areas.

Alterations in endocannabinoid levels

The results showed an age-related increase in the concentration of AEA, OEA and PEA in the hippocampus and frontal cortex of both wildtype and APPswe/PS1ΔE9 animals in a similar pattern (Table 3.3) to that observed in the TASTPM mouse model of AD (Pardon et al. 2009). However, the size of the increase is much larger in Pardon et al. 2009, most likely due to the differences in behavioural regimens undergone by 5.5 month animals compared to 4 months. Furthermore, the levels of endocannabinoids measured by Pardon et al. 2009 were much higher then those observed in this study, possibly due to differences in mouse strain used and behavioural regimens. Of particular import is that Pardon et al. 2009 were testing the effects of stress which is known to promote the release of endocannabinoids (Hohmann et al. 2005). However, support for the validity of the endocannabinoid concentrations measured in this study is provided by Di Marzo et al. 2000 whose measurements were broadly in line with ours.

Frontal Cortex	4M WT (1)	4M TASTPM (1)	4M WT	4M APP	5.5M WT (1)	5.5M TASTPM (1)	6M WT	6M APP	8M WT	8M APP	2-4M WT (2)
AEA (pmol/g)	63.00	75.00	7.31	7.63	875.00	1416.00	10.16	9.90	15.98	14.59	U
OEA (pmol/g)	680.00	554.00	10.15	10.06	7070.00	19649.00	15.82	13.69	20.37	17.39	U
PEA (nmol/g)	15.37	11.92	0.07	0.07	19.65	23.98	0.14	0.10	0.15	0.14	U
2AG (nmol/g)	69.93	72.00	3.40	3.47	418.29	537.82	5.77	3.98	4.34	5.38	U
Hippocampus											
AEA (pmol/g)	146.00	153.00	25.37	27.75	144.00	146.00	24.20	25.46	29.58	31.56	26.00
OEA (pmol/g)	991.00	955.00	22.40	24.28	756.00	738.00	22.25	22.61	26.42	27.72	U
PEA (nmol/g)	17.97	17.77	0.12	0.12	13.29	12.79	0.14	0.14	0.18	0.18	U
2AG (nmol/g)	63.93	56.12	2.53	2.96	225.01	221.65	2.57	2.56	2.70	2.96	5.00
Striatum											
AEA (pmol/g)	U	U	31.44	34.16	U	U	38.21	31.19	39.18	42.10	28.00
2AG (nmol/g)	U	U	7.58	6.97	U	U	6.27	5.20	8.60	5.53	6.00

Table 3.3: Comparison of regional endocannabinoid levels in wildtype (WT), APPswe/PS1ΔE9 (APP) and TASTPM mice measured using LC-MS/MS.
(1) Figures from Pardon et al. 2009. (2) Figures from Di Marzo et al. 2000. U = unavailable.

In contrast to 5.5 month old TASTPM animals, AEA, OEA and 2AG levels were not significantly higher in the frontal cortex of APPswe/PS1ΔE9 compared to wildtype at any age, although hippocampal AEA levels appeared to be higher in APPswe/PS1ΔE9 animals than in wildtype. No differences were observed in 2AG levels in the frontal cortex or hippocampus as a function of age or genotype in APPswe/PS1ΔE9, in contrast to TASTPM animals in which age and genotype related increases were observed in the frontal cortex, along with an age-related increase in the hippocampus (Pardon et al. 2009). In addition to the strong influence of behavioural regimes stated previously, these discrepancies could also be due to strain differences (C57bl/6J versus C57) or the PS1 mutations expressed by the TASTPM (A246E) and APPswe/PS1ΔE9 (PS1ΔE9). Support for this observation is provided by the existence of phenotypic differences between the two genotypes with APPswe/PS1ΔE9 consistently displaying hyperactivity in contrast to TASTPM animals at similar ages (Scullion et al. 2011). Alternatively, they may be due to differences in extraction techniques as this study used an extraction method optimised for the small tissue samples associated with mice whereas that of Pardon et al. (2009) was undertaken using the procedure of Richardson et al. 2007 which was optimised for rat. Additionally, the sensitivity of the HPLC-MS/MS systems used differed between the two studies as the Q-Trap used in this study is considered to be a more sensitive instrument than the Quattro Ultima.

AEA and OEA levels also increased with age in the striatum of both wildtype and APPswe/PS1ΔE9 animals whilst PEA levels increased as a

consequence of interplay between genotype and age. However, at 6 months of age, levels of AEA, OEA and PEA were significantly higher in wildtype animals than APPswe/PS1ΔE9. In contrast to the hippocampus and frontal cortex, 2AG levels were found to be lower in APPswe/PS1ΔE9 animals compared to wildtype animals, this difference was most apparent at 8 months of age.

The involvement of N-acylethanolamines in neuroprotection has previously been established in multiple experiments including the demonstration of the ability of AEA to confer neuroprotection in a rat model of excitotoxicity (Veldhuis et al. 2003), OEA has been shown to reduce infarct volume following middle cerebral artery occlusion in mice (Sun et al. 2007) and PEA was able to protect against oxidative stress in a neuronal cell line (Duncan et al. 2009). Based on this, the age-related increases in AEA, OEA and PEA may represent a response by the CNS to protect against oxidative stress and neuroinflammation, both of which are known to increase in normal (Park et al. 2009; Lee et al. 2000) and pathological ageing (Du et al. 2008; Cameron et al. 2010). In support of this proposal, Hamilton et al. (2012) demonstrated an increased expression of 8-oxoguanine (a marker of oxidative stress), in the hippocampus of both wildtype and APPswe/PS1ΔE9 animals with age independent of genotype. Hamilton et al. detected the presence of 8-oxoguanine as early as 3 months in both wildtype and APPswe/PS1ΔE9 animals. Similarly, Zhang et al. 2012 have reported the presence of $GFAP^+$ astrocytes in both wildtype and APPswe/PS1ΔE9 animals at 3 months of age. Both oxidative stress and neuroinflammation increase with age in these

animals (Hamilton et al.2012; Zhang et al. 2012), an observation which is paralleled by the rise in the levels of N-acylethanolamines in the present study. Additionally, the presence of markers of oxidative stress such as 8-hydroxyguanine in 6 month and glutathione conjugates and leukotriene oxidation products in 8 month old APPswe/PS1ΔE9, in addition to high levels of prostaglandins in both groups, provides further support for the presence of oxidative stress and neuroinflammation in this model of AD. Furthermore, these results provide support for the proposed involvement of the ECS in certain aspects of ageing, as suggested by the observation that CB1 null mutants display accelerated cognitive decline and skin histology associated with ageing compared to wildtype age-matched controls (Bilkei-Gorzo et al. 2012).

Although both wildtype and APPswe/PS1ΔE9 animals display indicators of oxidative stress and neuroinflammation, the rate at which they proceed is higher in APPswe/PS1ΔE9 animals (Zhang et al. 2012). Based on this, the significantly higher levels of AEA observed in the hippocampus of APPswe/PS1ΔE9 mice may represent an attempt by the CNS to combat the higher levels of neuronal stress they may be experiencing. This is in contrast to a study that examined post-mortem tissue from human AD patients which found lower levels of AEA in the temporal cortex (Jung et al. 2011). This may be due to several reasons. Firstly, it could be due to species differences, as mouse and humans are known to possess different amounts of white matter (40% humans, 10%mouse). It has recently been suggested that microglia in white matter are more responsive to inflammatory stimuli

than those in grey matter (Hart et al. 2012). Hence, as humans possess more white matter than mice, they are likely to experience a more aggressive pathology. This difference in white matter content may be partially responsible for one of the significant caveats of using animal models of AD which is their failure to recapitulate the extensive neurodegeneration seen in human AD patients. Alternatively, the discrepancy between the 2 studies may be due to differences in the brain regions studied (hippocampus versus the whole of the temporal cortex), the extraction protocol (ethyl acetate:hexane (9:1 v/v) versus chloroform:water (2:1 v/v) , the mobile phases used for elution (acetonitrile versus methanol) and the instruments used for detection (MS/MS versus MS).

No interactions between genotype and age were observed in relation to AEA, OEA and PEA levels in the frontal cortex and hippocampus but an interaction was observed in the striatum. This observation was due to APPswe/PS1ΔE9 animals exhibiting significantly lower levels of AEA, OEA and PEA at 6 months of age compared to wildtype animals and higher levels at 4 and 8 months. In contrast, wildtype animals appear to have higher levels of AEA, OEA and PEA at 6 months than at 4 months, with levels remaining consistent between 6 and 8 months. At 6 months, APPswe/PS1ΔE9 animals have been shown to have prominent Aβ pathology and deposits were frequently found in close proximity to tyrosine hydroxylase immuno-reactive fibres (Perez et al. 2005). It is possible that the CNS immune response is initially able to overcome the initial Aβ insult which may result in N-acylethanolamine levels decreasing by 6 months. However, as Aβ

deposition increases exponentially with age (Perez et al. 2005) the bulk CNS immune response changes from an anti-inflammatory to a pro-inflammatory phenotype As a result, there is a requirement for the anti-inflammatory actions of N-acylethanolamine causing levels to rise again by 8 months. Alternatively, N-acylethanolamines are able to signal through intracellular peroxisome proliferator-activated receptors (PPARs) which are able to sense alterations in lipid dynamics. The lipidomic analysis indicates the presence of high levels of fatty acids and cholesterol in APPswe/PS1ΔE9 animals at 6 months. These changes may be sensed by PPARs and result in altered gene transcription which may ultimately impact upon reduced N-acylethanolamine production. Finally, as stated earlier, the number of Aβ plaques increases exponentially between 4 and 6 months which may result in a subsequent reduction in the levels of soluble Aβ oligomers and ADDLs. If, as some have suggested, these soluble oligomers and ADDLs are the primary cause of neuroinflammation then their recruitment into Aβ plaques will reduce the unbound concentration of these ligands and thereby may result in reduced inflammation and therefore a subsequent decrease in the release of anti-inflammatory mediators.

In line with previous work conducted by Pardon et al. (2009) no differences were found in hippocampal 2AG levels between wildtype and APPswe/PS1ΔE9 animals at any age. There were also no differences in frontal cortical 2AG levels observed between wildtype and APPswe/PS1ΔE9 animals. Studies examining the CSF of human AD patients also found no differences in 2AG levels between control and AD patients (Koppel et al.

2009). This is in contrast to other studies which have observed an elevation in 2AG levels in rats injected with $A\beta_{1-42}$ fragments in the frontal cortex (van der Stelt et al. 2006). However, protein profiling studies conducted by Mulder et al. (2011) found no difference in DAGL and MAGL levels in APPswe/PS1ΔE9 mice. Alternatively, the differences between this study and that of van der Stelt et al. (2006) may be methodological, as the acute exposure to Aβ used by van der Stelt et al. (2006) is likely to have a different pathological profile compared to the gradual build up of Aβ in the APPswe/PS1ΔE9 model used in this study. It has also been suggested that pathological stimulation of neurons results in a substantial rise in 2AG levels with only modest effects on AEA (Walter et al. 2003). Thus, it is possible that neuronal distress at the ages studied has not reached a level which triggers a rise in 2AG synthesis. Based on this speculation it would be interesting to determine whether AEA and 2AG have differing actions in response to different distress signals. For example does inflammation initially trigger the release of AEA and related acyl ethanolamines which are able to interact with TRPV1 and PPAR receptors in addition to CB1 and thus recruit their anti-inflammatory effects too, whilst neuronal distress signals generated from more severe cellular stress such as excitotoxicity results in 2AG accumulation?

In contrast to the frontal cortex and hippocampus, APPswe/PS1ΔE9 animals showed lower 2AG levels in the striatum at 8 months compared to their wildtype counterparts. This would result in reduced neuroprotection as well as reduced inhibitory control of GABA neurons resulting in excessive

glutamate release and may contribute to the neurodegeneration observed in the striatum of these animals at 12 months (Richner et al. 2009).

Lipid metabolism

PCA of the lipodomic profiles for the frontal cortex and hippocampus showed no distinct separation of lipids between wildtype and APPswe/PS1ΔE9 animals at 4, 6 or 8 months. However, a clear separation was observed for a number of lipid classes in the striatum of 6 and 8 month old animals. In particular, PCA which is an unsupervised clustering method requiring no prior knowledge, provided support for the targeted endocannabinoid analysis by indicating that AEA, OEA and PEA levels are higher in the striatum of wildtype animals at 6 months than APPswe/PS1ΔE9. Similarly, in the 8 month age group, PCA indicated that 2AG was higher in wildtype animals than APPswe/PS1ΔE9.

The lipidomics analysis indicates that there are global disturbances in lipid metabolism present in the striatum of APPswe/PS1ΔE9 animals at 6 and 8 months. Due to time constraints these differences could not be fully investigated. The presence of high levels of cholesterol and fatty acids in 6 month old APPswe/PS1ΔE9 and 8 month old wildtype animals suggests both aging and Alzheimer's disease impact upon these factors. As these comprise components of cell membranes and lipid rafts their altered levels suggest disturbance in membrane fluidity and protein activation (Niemala et al. 2009) as a function of age (Yechiel et al. 1985) and AD-related pathology (Qiu et al. 2011). Indeed, Qiu et al. (2011) suggested that the presence of

cholesterol results in a more ordered lipid domain which protects against Aβ-induced membrane disruption and formation of a β-sheet structure which they hypothesise acts as a seeding platform for toxic Aβ oligomers. This is in contrast to other studies which suggest that high cholesterol levels are a risk factor for developing AD (Papolla et al. 2003).

Wildtype animals appear to show high levels of N-acylethanolamines products which are therefore, by default, lower in APPswe/PS1ΔE9, suggesting impairments in synthesis or degradation of these lipid mediators in AD.

High levels of glycerophospholipids and cholesterol present in 6 month APPswe/PS1ΔE9 animals may cause alterations in membrane fluidity and lipid rafts. Modification of the cholesterol content of lipid rafts has previously been shown to control CB1 receptor function in the striatum (De Chiara et al. 2010) although whether this has any influence on N-acylethanolamines synthesis is currently unknown.

All groups showed high levels of glycerides although the composition and chain length varies between them. This observation provides further support for disturbed lipid metabolism being a feature of age and AD pathology as well as emphasising the need for accurate analytical techniques for measuring lipids.

Influence of post-mortem delay interval

The post-mortem delay interval has been shown to have a substantial impact on levels of endocannabinoids and other fatty acids in a number of different animals (Schmidt et al. 1995). Studies examining the influence of post-mortem delay on levels of AEA in rat brain samples have found levels begin to rise steadily after a 3 hour interval but found very little effects prior to this time point (Kempe et al. 1996). Further studies modelling post-mortem delay intervals ranging from 0 to 80 hours at room temperature and 4°C using human brain extracts to measure fatty acid composition, demonstrated no change up to 72 hours (Fraser et al. 2008). Specifically, studies using human tissue and measuring AEA and 2AG content found a steady increase in AEA at 1-6 hours post-mortem and a converse decrease in 2AG content (Palkovits et al. 2008). Similar studies in C57bl/6J mice have found AEA to increase significantly 5 hours after decapitation but not before, whilst 2AG levels were found to decrease for the first few hours although not significantly (Patel et al. 2005). However, Palkovits et al. 2008 observed that once frozen at -80°C, sample endocannabinoid content was stable up to 13 years following collection.

The post-mortem interval between decapitation and freezing in liquid nitrogen for this study was approximately 5 minutes. Additionally, all regional brain dissections were undertaken by the same researcher to reduce variability due to individual differences in collection strategy and duration. Thus, whilst some changes may have been inevitable based on the literature

cited above, as all the samples were collected in very similar time intervals by the same researcher, any post-mortem changes will apply equally to all of them. Based on this, any differences observed in regional endocannabinoid levels are due to experimental effects rather than an influence of post-mortem delay although this caveat cannot be wholly discounted.

Cannabinoid receptor coupling

No differences in CBR coupling were observed between wildtype and APPswe/PS1ΔE9 animals in the hippocampus. As AEA levels were raised in APPswe/PS1ΔE9 in this brain area compared to wildtype animals, the lack of alteration in CBR activation would provide support for the idea that AEA may be acting through non-CBR pathways such as TRPV1 (Veldhuis et al. 2003). The lack of change in hippocampal CBRs is supported by a recent study in human AD patients where no changes in CB1 expression were detected (Mulder et al. 2011).

In contrast to the hippocampus, APPswe/PS1ΔE9 had higher receptor-effector coupling activity compared to wildtype animals in the frontal cortex and striatum. The increased CBR activity in the striatum was most apparent at 8 months of age corresponding with the observed reduction in 2AG levels in APPswe/PS1ΔE9 compared to their wildtype counterparts at 8 months. The enhanced receptor activation may represent an attempt to compensate for reduced 2AG levels although a correlation analysis does not support this as no correlations was observed between concentrations of 2AG and receptor-effector coupling at any age in the striatum. These observations

contrast with those of Ramirez et al. (2005) and Westlake et al. (1994) in human AD patients where they observed reduced coupling in the cortex, hippocampus and striatum. However, Westlake et al. (1994) suggested the reduction in CBR activation was due to advancing age and other non-AD related pathology. These differences are possibly due to this study examining CBR coupling in a mouse model of AD which recapitulates some, but not all, aspects of AD pathology whereas the human AD sufferers will express the full pathological cohort. Additionally, the CBR agonists used in each study varied. For this study HU210 was used, whilst Ramirez et al. 2005 used WIN55, 212-2 and Westlake et al. [³H]CP-55,940. WIN55, 212-2 has also been shown to interact with PPARα (Downer et al. 2012) implying that non-CB receptors may potentially have influenced the results of Ramirez et al. (2005).

3.6 Conclusion

The results presented in this chapter provide support for the involvement of endocannabinoid system in the ageing process. Some tentative support for the involvement of endocannabinoids in Alzheimer's disease is also provided, although a more thorough examination of the system involving biochemical studies examining the sub-cellular distribution and activity of major synthetic and degradation enzymes is required. Lipidomics analysis reveals that disturbed lipid metabolism is a feature of both ageing and pathology; hence it is crucial to ensure differentiation between the two when investigating diseases such as AD. Additionally, it highlights the usefulness of lipidomics

as a tool for unbiased research with no prior expectations, thereby potentially revealing the influence of factors previously not associated with a given condition.

Chapter 4

Drug Intervention Study

4.1 Introduction

It has long been established that CB1 receptor blockade alleviates memory impairments (Wolff et al. 2003; Thiemann et al. 2007; Lichtman, 2000; Wise et al. 2009). As a result, it was thought that it might be beneficial in Alzheimer's disease (AD) which is defined by impairments in learning and memory. In support of this hypothesis, Mazzola et al. 2003 demonstrated that the selective CB1 receptor antagonist SR141716A (rimonabant) prevents memory impairments in a step-through passive avoidance test in mice which had received intracerbroventricular injections of Aβ peptide fragments.

In contrast to CB1 blockade, CB2 receptor stimulation is also thought to be potentially beneficial in AD. Ramirez et al. (2005) demonstrated that the CB2 selective agonist JWH133 prevented toxicity mediated by microglia following treatment with Aβ peptide. Furthermore, Aso et al. (2012) recently reported improvements in memory following 5 weeks of JWH133 treatment (0.2 mg/kg) in 6 month old APP/PS1ΔE9 mice. They attributed their observations to reductions in the pro-inflammatory cytokines IL-6 and interferon γ as well as to reductions in microglial reactivity.

As can be observed from the preceding information pharmacological manipulation of an animals' CNS is frequently used to test hypotheses about the influence of a given exogenous or endogenous compound on behaviour. Two procedures commonly used for administering drugs to mice are by means of injection into the peritoneal cavity (i.p) or subcutaneously (s.c)

(Leenaars et al. 1998). However, both these procedures are unsuitable for long-term dosing regimens, particularly with regard to i.p injections which can cause peritonitis and carry the risk of intestinal puncture (Diehl et al. 2001). Injection stress is also a potential confounder. Additionally, neither of these methods enable plasma levels of the compound to be maintained at a steady concentration.

Osmotic minipumps are a viable alternative to the above procedures. Osmotic minipumps can be implanted subcutaneously (Paria et al. 1998) or intraperitoneally (Murikinati et al. 2010). Among the benefits of using osmotic minipumps is a reduced requirement for animal handling and, hence, reduced levels of stress related to handling and injection discomfort (Balcombe et al. 2004). Additionally, they enable substances to be continuously infused, thereby ensuring stable plasma levels of the compound are maintained (Alzet.com). Furthermore, osmotic minipumps reduce experimental variability. Due to these benefits, osmotic minipumps are increasingly popular as research tools in pharmacological intervention studies.

A number of techniques can be used to assess the effects of drug intervention upon brain physiology including behavioural paradigms and immunohistochemistry (IHC). IHC is a well established (first reported in 1941 by Coons et al.) commonly used technique based upon the amalgamation of immunology, histology and chemistry (Ramos-Vara, 2005). The principles of IHC are centred on the binding of a specific antibody to its cognate brain

antigen. The antibody-antigen complex can subsequently be visualised in a number of ways, most frequently involving the use of a fluorescent tag or enzyme-chromogen complex. Behavioural testing regimens are crucial in determining the functional consequences of central manipulation. A number of memory tests have been used in the studies mentioned above including object recognition and passive avoidance learning. The present study utilised similar testing protocols to those described in Chapter 2 and the reader is referred to section 2.1 for a reminder.

This study administered vehicle, SR141716A or JWH133 via subcutaneous implantation of osmotic minipumps to female wildtype and APPswe/PS1ΔE9 mice for 28 days preceded and followed by behavioural testing. Representative brain slices from APPswe/PS1ΔE9 mice from the vehicle and SR141716A treated groups also underwent IHC for Aβ to confirm its presence in the APPswe/PS1ΔE9 mice. In addition, a separate, smaller study examining the effects of acute administration of JWH133 on the behaviour of female wildtype and APPswe/PS1ΔE9 mice, also at 7 months, was undertaken.

4.2 Aims

An underlying hypothesis in this thesis is that endocannabinoids have potentially opposing actions in Alzheimer's disease, with CB1 receptors mediating acute detrimental effects on memory, whilst CB2 receptors mediate positive effects on disease progression via control of neuroinflammatory processes.

The aims of this chapter were, therefore, to observe the effects of chronic administration of the CB1 receptor antagonist SR141716A or the CB2 receptor agonist JWH133 via subcutaneously implanted osmotic minipumps, on cognitive deficits in 7 month old female wildtype and APPswe/PS1ΔE9 mice. Brain slices from some of the APPswe/PS1ΔE9 animals were used to confirm the presence of Aβ. The remainder of the brain slices were intended for IHC experiments examining various components of the endocannabinoid system. Due to time constraints the IHC is still an on-going process as a consequence of which the results of this part of the study will not be presented in this chapter.

4.3 Materials and Methods

4.3.1 Study Design

Animals underwent behavioural testing at 4 and 6 months in a two day test battery comprising the locomotor activity (LMA) task, spontaneous alternation in the Y maze and novel object recognition paradigm. Two days after the behavioural testing at 6 months of age, animals underwent surgery for subcutaneous (s.c) osmotic minipump implantation. Prior to surgery the osmotic minipumps were filled with either vehicle, SR141716A or JWH133, and remained in place for 28 days following which they were surgically removed. Two days after pump removal surgery, animals underwent behavioural testing in the locomotor (to enable recovery from surgery and drug washout) activity task, spontaneous alternation in the Y maze, novel object recognition and contextual fear conditioning (CFC). Following CFC

animals were placed back in their home cages for 2 days followed by terminal anaesthesia whilst undergoing cardiac perfusion. The brains were removed and placed in 4% paraformaldehyde followed by placement in 30% sucrose (Figure 4.1).

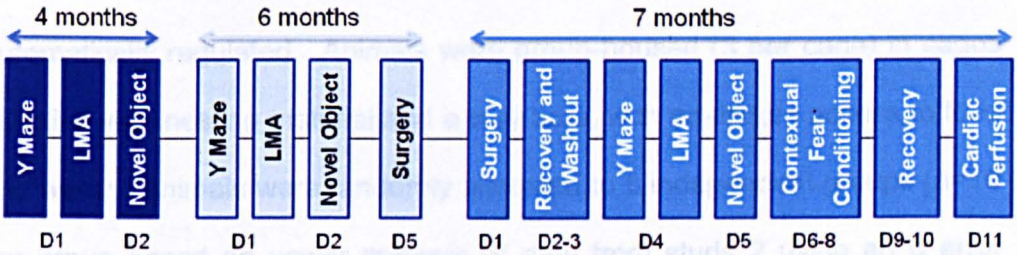

Figure 4.1: Timeline for study. LMA = locomotor activity;

In addition, a separate smaller study was conducted observing the effects of acute administration of JWH133 on the behaviour of wildtype (n=5) and APPswe/PS1ΔE9 (n=8) female mice between 6 and 7 months of age. Animals were injected with either vehicle or JWH133 intraperitoneally (i.p) followed by behavioural testing of spontaneous alternation in the Y maze 30 minutes later. 25 minutes after completing the spontaneous alternation in the Y maze, animals undertook the LMA task. Three days after the test session, the process was repeated with animals which had been administered JWH133 in the first testing session receiving vehicle in the second testing session and vice versa.

4.3.2 Animals

The animals used in this study were of the same background and genotypes (wildtype and APPswe/PS1ΔE9) as those detailed in chapter 2.3.1. However, whilst the previous studies used male animals, this study used female animals. All animals were housed in the same room on a 12h light:dark cycle with room temperature, humidity and air exchange all being automatically regulated. Animals were group-housed (3 per cage) in cages supplied with nesting material and a play tube, with ad-libitum access to food and water. Animals were randomly allocated to 6 independent groups (n=10 per group based on power analysis of data from study 2 using an α error probability value of 0.05, effect size of 0.25 and power of 0.99) which were tested at 4, 6 and 7 months in a battery of behavioural tasks. The existence of sexual dimorphisms in AD means that females tend to show a more rapid decline than males (Finch et al. 1999; Clinton et al. 2007) as a result of which this study used female animals only.

4.3.3 Drugs

SR141716A (kindly donated by the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (North Carolina, USA)), was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) at a concentration sufficient for final delivery of a dose of 3 mg/kg which has been found to be sufficient for CB1 blockade and alleviating memory impairments (Lichtman, 2000; Robinson et al. 2008). For use in osmotic minipumps (Alzet® model 2004, mean pumping rate 0.25 µl/hour, reservoir volume of

200 µl) JWH133 (Tocris Bioscience, Bristol, UK) was prepared in 20% DMSO and 5mg/ml bovine serum albumin (BSA) (Sigma Aldrich, UK). For use in acute intraperitoneal (i.p) injections, JWH133 (Tocris Bioscience, Bristol, UK) was prepared in a solution of ethanol (Sigma Aldrich, UK):tween 80 (Sigma Aldrich):saline (1:1:18 v/v). In both cases JWH133 was made up to a concentration calculated to deliver a dose of 1.2 mg/kg (Goncalves et al. 2008). Vehicle was either 100% DMSO or 20% DMSO added to 5mg/ml BSA in the chronic study using osmotic minipumps and ethanol:tween 80:saline (1:1:18 v/v) in the acute study. A stock solution of 50 mg/ml Rimadyl (Pfizer, Kent, UK) was diluted 1:100 in saline.

For the acute study, drugs were freshly prepared on the day of use. For the chronic studies, drugs were prepared on the day on which the minipumps were to be implanted.

4.3.4 Surgery

Animals were anaesthetised using isofluorane (3.5% for induction; 1.5- 2% for maintenance) in 100% oxygen. Once an animal was fully anaesthetised rimadyl (analgesic) was administered (s.c), a small area in the upper back where the surgical incision was to be made was shaved, wiped with chlorohexidine (Ecolab Ltd, Leeds, UK) and a topical analgesic (lidocaine 25 mg/g; Astra Zeneca, Luton, UK) applied. The animal was subsequently transferred to the sterile surgical area located over a heated mat. A small incision was made in the skin on the upper back, a sterile osmotic minipump (Alzet® 2004, supplied by Charles River, UK) containing vehicle,

SR141716A or JWH133 was inserted into the area underneath the incision. The outlet for the osmotic minipump was placed so it was facing away from the incision site. Absorbable sutures (Ethicon, UK) were used to close the incision and the animal was placed into a pre-heated new cage and monitored until it had recovered from anaesthesia. Animals were weighed daily and monitored post-surgically for a minimum of 5 days.

4.3.5 Spontaneous Alternation in the Y Maze

Spontaneous alternation in the Y maze is very similar to that in the T maze detailed earlier (section 2.8.2) and operates on similar principles in that it is based on the innate tendency of mice to explore a novel maze arm over a familiar arm which they have just vacated, and thus to alternate between arms (Hughes, 2004). As with the T maze, it is used as an indicator of spatial working memory performance.

The Y maze was constructed from transparent Perspex and comprised 3 identical arms measuring 7.5cm in width, 46 cm in length and 25 cm in height. Animals were placed in the centre of the Y maze and permitted to explore for 5 minutes following which they were placed back in their home cage. The number of alternations and arm entries the animals made during the 5-minute period were recorded and subsequently used for statistical analysis. The same protocol was used for both the chronic and acute studies.

4.3.5 Locomotor Activity

The LMA task for the chronic study was identical to that detailed in section 2.3.3. In the case of the JWH133 acute study, the procedure was identical with the exception of the time in the arena being 15 minutes rather than 30 minutes.

4.3.6 Novel Object Recognition

The principles of the novel object recognition (NOR) paradigm are explained in section 2.8.2. The protocol used was as described in Scullion et al. (2009) with modifications in the form of automated scoring replacing manual scoring and object exploration defined as a distance of ≤2cm rather than ≤0.5cm. The NOR paradigm was conducted in the same arena in which the animals had undergone LMA testing the previous day, thus habituating them to the arena. Animals were placed in an arena containing one object (wooden circular or triangular block) and permitted to explore freely for 10 minutes after which they were returned to their home cage. After 4 hours, the animals were placed back in the arena which now contained a novel object in addition to the object that was present in the first trial. As previously, animals were permitted to explore freely for 10 minutes before being placed back in their home cage (figure 4.2). All of the trials were recorded by a camera positioned directly above the arena and later used to determine the amount of time spent exploring each object using a validated protocol for Ethovision XT automatic tracking software (Noldus, Wagenigen, Netherlands). Exploration was defined as the amount of time spent by an animal with its nose pointing towards the object at a distance of $\leq$ 2 cm. The distance

moved, total exploration time and exploration time for each object were recorded for each trial. To determine an animals' preference for the novel object, the amount of time spent exploring the novel object was divided by the total exploration time and multiplied by 100% to obtain a preference index value.

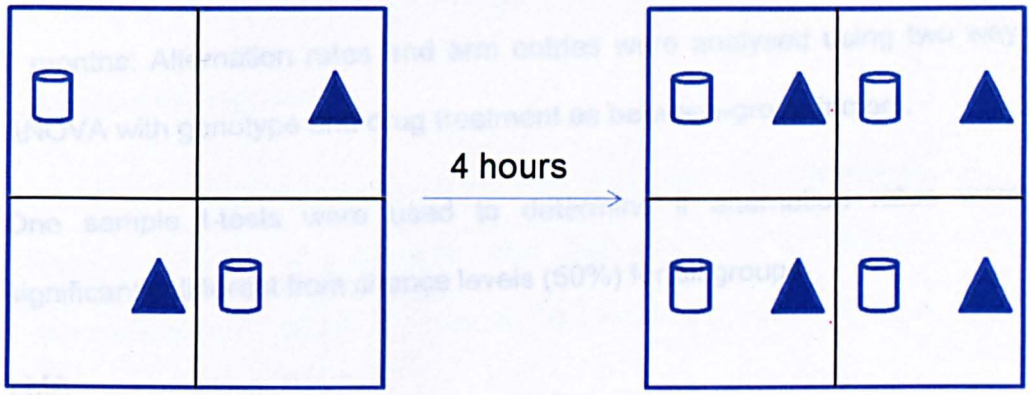

Figure 4.2: Novel Object Recognition Paradigm. Animals are placed in the arena (A) in trial 1 and permitted to explore freely. For 10 minutes after which they are removed. After 4 hours animals are placed back into the same arena which they occupied earlier (B) which now has a novel object in it. Animals are again permitted to explore freely for 10 minutes prior to removal.

4.3.7 Contextual Fear Conditioning

The contextual fear conditioning paradigm was identical to the one described in section 2.3.4.

4.3.8 Statistical Analysis

Due to the dual nature of this study, statistical analysis was conducted in 2 parts. Animals from the 4 and 6 month groups were compared to assess the effects of age on behavioural outcomes, whilst animals from the 7 month

group undergoing drug treatments were assessed separately to analyse the effects of pharmacological manipulation of the ECS.

Y maze

4-6 months: Alternation rates and arm entries were analysed using repeated measures ANOVA with age and genotype as between-group factors.

7 months: Alternation rates and arm entries were analysed using two way ANOVA with genotype and drug treatment as between-group factors.

One sample t-tests were used to determine if alternation rates were significantly different from chance levels (50%) for all groups.

LMA

4-6 months: The total distance travelled and the ratio of time spent in the centre of the arena to the periphery were analysed using repeated measures ANOVA with age and genotype as between-group factors.

7 months: Total distance travelled and the ratio of time spent in the centre of the arena to the periphery were analysed using two way ANOVA with genotype and drug treatment as between-group factors.

Novel Object Recognition

4-6 months: Repeated measures ANOVA with age and genotype as between-group factors were used to analyse the total distance moved, object exploration times, total exploration times and preference index.

7 months: Repeated measures ANOVA was used to analyse total distance moved, object exploration times and total exploration times. Two way ANOVA was used to analyse preference index measures. Genotype and drug treatment were used as between-group factors.

One sample t-tests were used to determine if preference index was significantly different from chance levels (50%) for all groups.

Contextual Fear Conditioning

7 month: Acquisition of contextual fear was analysed using two-way repeated measures ANOVA with genotype and drug treatment as between-group factors and time (1 shock per minute, 10 modalities) as the within-subjects factor.

Retention, extinction and extinction index were analysed using two way ANOVA with genotype and drug treatment as between-group factors. A two way repeated measures ANOVA comparing immobility in the retention trial to that in the extinction was also conducted, with genotype and drug treatment as between group factors and trial (2 modalities) as the within-subjects factor.

A one-sample t-test was used to determine whether the extinction index was significantly different from 0 which would indicate that no extinction had taken place.

Post-hoc testing:

Planned comparisons were conducted post-hoc when appropriate using the least significant difference (LSD) test. The main focus of this study was to

identify differences between wildtype and APPswe/PS1ΔE9 animals between 4 and 6 months, and the effect of drug treatment at 7 months. Hence, planned comparisons identified to be of interest prior to the start of the experiment were those between wildtype and APPswe/PS1ΔE9 at 4 and 6 months, and between vehicle, SR141716-treated and JWH-treated wildtype and APPswe/PS1ΔE9 animals at 7 months.

4.3.9 Cardiac Perfusion

Animals were anesthetised using isofluorane (3.5% for induction; 1.5-2% for maintenance) in 100% oxygen. Once fully anaesthetised, an incision was made in the chest area to expose the ribcage. The ribcage was cut away to expose the heart. A needle attached to a hydraulic pump was placed in the left ventricle, a small incision was made in the right atrium, and the pump turned on. The systemic circulation was initially perfused with 0.1 M phosphate-buffered saline (PBS) to clear the system of all blood. This was followed by perfusion with 4% paraformaldehyde (Sigma Aldrich, UK) until the liver became pale in colour and the limbs of the animal became rigid. Following perfusion, the brain was removed and placed in 4% paraformaldehyde for 7 days at 4°C after which it was placed in 30% sucrose solution (30% sucrose in 0.1 M PBS (Oxoid Ltd, Hampshire, UK) supplemented with sodium azide) and stored at 4°C until sectioned.

4.3.10 Amyloid β immunohistochemistry

All Aβ immunohistochemistry-related sectioning, staining and imaging was undertaken by the laboratory of Professor James Lowe, Department of Histopathology, Queen's Medical Centre, University of Nottingham.

Following storage in 30% sucrose, APPswe/PS1ΔE9 mouse brains for the vehicle and SR141716A treated groups (n=3) were placed back in 4% paraformaldehyde for 48 hours prior to paraffin wax infusion using a LEICA TP 1050 tissue processor, (Leica Microsystems, Milton Keynes, UK). Following processing, brains were embedded in paraffin wax using a LEICA EG 1160 paraffin embedding station and 5 µm sections cut using a LEICA RM 2135 rotary microtome. Slices were obtained 10 µm apart from the frontal cortex, striatum and hippocampus for each animal.

Sections were stained using the Leica Bond Polymer Refine Detection Kit as follows. Sections were dewaxed in xylene (2 x 5 minutes) followed by rehydrating in industrial methylated spirit (IMS) and rinsed with tap water. Sections were subsequently placed in concentrated formic acid for 2 minutes followed by washing in tap water for 10 minutes and rinsed in Bond wash buffer prior to loading onto the Dako Techmate 500 Plus immunostainer (Ely, Cambridgeshire, UK). Antigen retrieval was conducted using ER2 (Leica Microsystems, Milton Keynes, UK) followed by washing in Dako REAL wash buffer 1. Sections were stained automatically using a standard protocol designed by the Department of Histopathology. The protocol is outlined below; each step listed was followed by washing in Dako REAL wash buffer

1. Sections were peroxide blocked for 5 minutes, followed by washing, incubated in Aβ primary antibody (Leica Vision Biosystems) diluted 1:25 for 15 minutes, washed, incubated with a dual link secondary antibody for 8 minutes (Dako REAL link biotinylated secondary antibody, anti mouse and anti rabbit), washed, exposed to horseradish peroxide (HRP) (DAKO REAL streptavidin peroxidase) for a further 8 minutes and washed. The HRP was subsequently stained with the chromogen diaminobenzadine exposure (DAB) for 10 minutes, washed, exposed to a DAB enhancer for 5 minutes, washed and counterstained with haematoxylin for 5 minutes prior to alcohol dehydration and xylene cleaning followed by mounting in DPX glue. Sections were subsequently scanned using the Nanozoomer Digital Pathology slide scanner at x20 magnification to confirm the presence of Aβ.

IHC was only conducted on sections from APPswe/PS1ΔE9 animals' brains as wildtype mice do not naturally possess the APP gene and hence to not produce Aβ.

4.4 Results

4.4.1 Y Maze

Spatial memory was assessed using spontaneous alternation in the Y maze. There were no statistically significant differences between wildtype and APPswe/PS1ΔE9 animals as a function of age or genotype alone between 4 and 6 months of age. However, a significant age x genotype interaction was observed [$F_{(1,60)}$=4.51; p=0.038] indicating that alternation performance decreased with age in wildtype animals whilst increasing with age in

APPswe/PS1ΔE9 mice (figure 4.3a). Post-hoc analysis indicated a significant difference between wildtype and APPswe/PS1ΔE9 animals at 4 months ($p=0.042$). In contrast, no significant differences in alternation rate were observed as a function of drug treatment or genotype in the 7 month old animals (figure 4.3a). All groups alternated significantly above chance levels (50%).

APPswe/PS1ΔE9 animals showed a significantly greater number of arm entries [$F_{(1,60)}=11.66$; $p=0.001$] compared to wildtype animals (figure 4.3b) as a function of age [$F_{(1,60)}=7.07$; $p=0.01$]. Post-hoc analysis revealed a significantly reduced number of arm entries in wildtype compared to APPswe/PS1ΔE9 at both 4 ($p=0.001$) and 6 months ($p=0.025$). No effects of drug treatment were observed on the number of arm entries made, but, in line with the observations made in 4 and 6 month old animals, APPswe/PS1ΔE9 also made a significantly greater number of arm entries [$F_{(1,56)}=5.91$; $p=0.018$] than wildtype animals at 7 months (figure 4.3b). Post-hoc analysis indicated this to be due primarily to differences between wildtype and APPswe/PS1ΔE9 animals in the vehicle-treated condition ($p=0.01$) since both treatments significantly reduced the number of arms visited in the 5 min period.

4.4.2 Locomotor Activity

APPswe/PS1ΔE9 animals were significantly hyperactive compared to their wildtype counterparts at 4, 6 [$F_{(1,59)}=62.65$; $p<0.001$] and 7 months [$F_{(1,55)}=31.99$; $p<0.001$]. Overall activity levels decreased significantly with

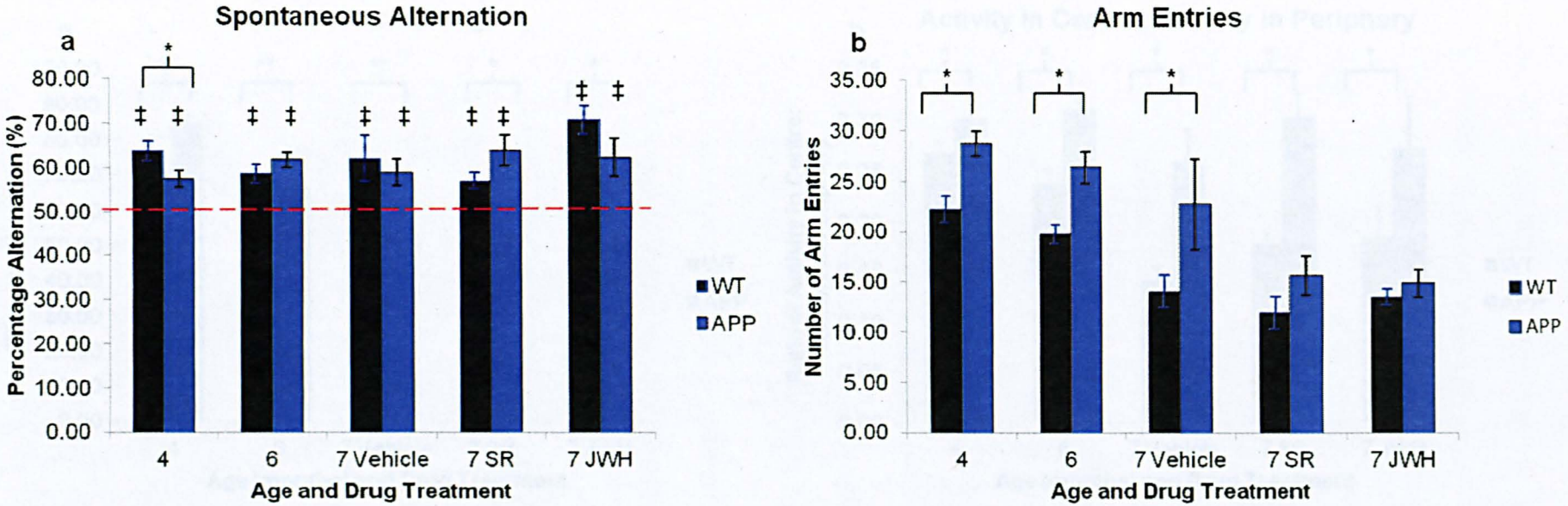

Figure 4.3 (data presented as mean ± sem): Spontaneous alternation and number of arm entries in the Y Maze. (a) Percentage alternation in the Y maze at 4 (n=35 WT;39 APP), 6 (n=33 WT; 38APP) and 7 (n=10) months of age for wildtype (WT) and APPswe/PS1ΔE9 (APP) animals. Pairwise comparisons *p=0.042. All animals alternated significantly above chance ‡ p<0.05. **(b)** Number of arm entries at 4 (n=35 WT;39 APP), 6 (n=33 WT; 38APP) and 7 (n=10) months of age for wildtype (WT) and APPswe/PS1ΔE9 (APP) animals. Pairwise comparisons *p= 0.001 (4 months); 0.025 (6 months) and 0.01 (7months vehicle).

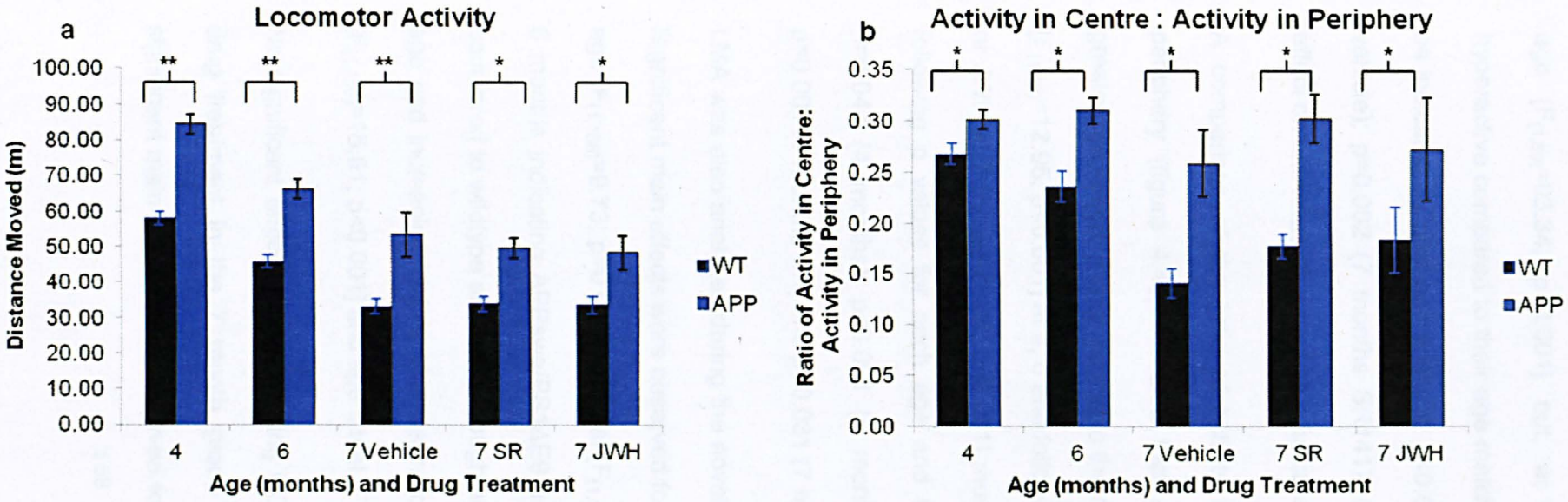

Figure 4.4 (data presented as mean ± sem): Locomotor activity and ratio of activity in the centre:activity in the periphery. (a) Total distance travelled in 30 minutes by wildtype (WT) and APPswe/PS1ΔE9 (APP) animals at 4 (n=35 WT;39 APP), 6 (n=33 WT; 38APP) and following treatment with either vehicle, SR141716A (SR) or JWH133 (JWH) at 7 (n=10) months of age. Pairwise comparisons: **p<0.001; *p<0.01. **(b)** Ratio of activity levels in the centre of the arena to those in the periphery during the 30 minute testing session for wildtype (WT) and APPswe/PS1ΔE9 (APP) animals at 4 (n=35 WT;39 APP), 6 (n=33 WT; 38APP) and following treatment with either vehicle, SR141716A (SR) or JWH133 (JWH) at 7 (n=10) months of age. Pairwise comparisons: *p<0.05.

age [$F_{(1,59)}$=83.34; p<0.001] but, at all ages, APPswe/PS1ΔE9 were hyperactive compared to their age-matched wildtype littermates (figure 4.4a) as indicated by post-hoc analysis: p<0.001 (4 months, 6 months, 7 months vehicle); p=0.002 (7 months SR141716); p=0.008 (7 months JWH). No effect on LMA was observed as a function of drug treatment.

A comparison of the ratio of activity in the centre of the arena to the periphery (figure 4.4b) revealed that APPswe/PS1ΔE9 animals spent a greater proportion of time moving in the centre compared to wildtype animals [$F_{(1,59)}$=12.95; p<0.001] at 4, 6 and following treatment with either vehicle, SR or JWH at 7 [$F_{(1,55)}$=24.86; p<0.001] months. Post hoc analysis yielded the following p values for each age and treatment condition (vs WT mice): p=0.042 (4 months); p<0.001 (6 months); p=0.004 (7 months vehicle); p=0.001 (7 months SR) and p=0.031 (7 months JWH).

LMA was also analysed during the novel object recognition trials (table 4.1). Significant main effects were observed for genotype [$F_{(1,138)}$=49.21; p<0.001], age [$F_{(1,138)}$=9.73; p=0.002] and trial [$F_{(1,136)}$=67.86; p<0.001] between 4 and 6 months indicating APPswe/PS1ΔE9 animals are significantly hyperactive compared to wildtype animals although activity levels decline as a function of age and increasing trial number as indicated by significant genotype x trial [$F_{(1,136)}$=15.51; p<0.001] and age x trial [$F_{(1,136)}$=10.61; p=0.001]interactions.

No significant effects on LMA during NOR were observed as a function of drug treatment in the 7 month group although in line with the above, significant main effects were observed for genotype [$F_{(1,54)}$=10.11; p=0.002],

with APPswe/PS1ΔE9 mice being hyperactive and trial [$F_{(1,54)}$=4.60; p=0.036] with locomotor activity increasing in the novel object trial compared to the acquisition. In addition a significant genotype x trial interaction [$F_{(1,54)}$=5.33; p<0.025] was also observed indicating the increase in locomotor activity between trials was greater for wildtype animals compared to APPswe/PS1ΔE9 (table 4.1).

4.4.3 Novel Object Recognition

All animals spent significantly more time exploring the novel object than the familiar at 4, 6 [$F_{(1,137)}$=17.79; p<0.001] and 7 months [$F_{(1,55)}$=20.37; p<0.001]. A significant main effect of age [$F_{(1,137)}$=7.13; p=0.009] on object exploration times was also observed between 4 and 6 months reflecting the reduction in exploration times with age (table 4.1). No effects of drug treatment on exploration times were observed in the 7 month group but a just significant main effect of genotype [$F_{(1,55)}$=4.19; p=0.046] was observed which reflected the higher object exploration times displayed by APPswe/PS1ΔE9 compared to wildtype animals.

Total exploration times (table 4.1) were significantly higher in the novel object test trial compared to the acquisition trial at 4, 6 [$F_{(1,140)}$=43.35; p<0.001] and 7 [$F_{(1,54)}$=14.39; p<0.001] months. Exploration times decreased significantly with age between 4 and 6 months [$F_{(1,140)}$=13.10; p<0.001]. A significant genotype x trial interaction [$F_{(1,54)}$=4.35; p=0.042] was observed at 7 months but not between 4 and 6 months. No significant effects of drug treatment on total exploration times were observed.

					Vehicle		SR141716A		JWH133	
Distance Moved (m)	**4M WT**	**4M APP**	**6M WT**	**6M APP**	**7M WT**	**7M APP**	**7M WT**	**7M APP**	**7M WT**	**7M APP**
Acquisition	24.69±1.08	32.19±0.96	21.10±0.80	28.03±1.09	21.66±2.37	27.10±2.32	19.91±1.37	24.93±1.08	21.67±0.99	25.96±1.95
Pairwise comparisons	P<0.001		P<0.001		P=0.02		P=0.02		NS	
Novel Object	22.00±0.91	26.41±0.80	20.70±0.61	26.03±1.43	23.41±1.88	28.32±3.92	22.30±1.14	25.86±1.10	23.66±1.10	27.09±1.78
Pairwise comparisons	P<0.001		P=0.002		NS		NS		NS	
Object Exploration Time (s)										
Acquisition	28.93±3.12	37.35±3.35	19.01±2.26	25.83±2.79	13.66±4.65	13.10±3.24	18.10±4.24	17.93±1.95	11.58±3.09	13.43±3.26
Novel Object	26.90±3.03	23.77±2.02	18.53±2.59	18.72±1.68	11.47±3.58	14.94±4.29	11.12±1.56	15.65±1.88	10.48±2.17	15.08±1.81
Total Exploration Time (s)										
Acquisition	28.93±3.12	37.35±3.35	19.01±2.26	25.83±2.79	13.66±4.65	13.10±3.24	18.10±4.24	17.93±1.95	11.58±3.09	13.43±3.26
Novel Object	43.15±3.96	43.70±3.31	32.76±3.11	34.27±2.95	16.82±4.00	25.47±8.05	17.98±2.96	26.31±3.37	18.94±3.12	25.20±3.21
Preference Index (%) Time										
Novel Object	60.60±3.06	56.43±2.68	54.78±4.45	54.98±2.72	63.75±6.99	63.15±3.87	65.77±4.52	59.58±4.43	62.48±5.34	61.54±2.88
One sample t-test (vs 50%)	P=0.001	P=0.022	NS (P=0.291)	NS (P=0.076)	NS (P=0.085)	P=0.007	P=0.006	NS (P=0.056)	0.044	P=0.004
Pairwise comparisons	NS		NS		NS		NS		NS	

Table 4.1 (data presented as mean ± sem): Performance in the Novel Object Recognition test for drug naive 4 (n=35 WT;39 APP) and 6 (n=33 WT; 38APP) month animals, and for 7 month old animals (n=10) following treatment with either vehicle, SR141716A (SR) or JWH133 (JWH). Distance moved in 10 minutes per trial. Object exploration times in the acquisition and test trials. Total exploration times during each 10 minute trial. Preference index calculated as a percentage using the following formula: novel object exploration time / total exploration time x100%. One sample t-test was used to compare preference index values to those achievable by chance (50%).

No significant differences in preference index were observed at 4, 6 or 7 months (table 4.1). Preference index was significantly above chance for 4 month, 7 month vehicle and JWH treated APPswe/PS1ΔE9 and 7 month JWH133 and SR141716 treated wildtype animals.

4.4.5 Contextual Fear Conditioning

All animals were able to learn the contextual fear (CF) as indicated by immobility increasing significantly [$F_{(9,504)}$=181.93; p<0.001] with time (figure 4.5 a-c). APPswe/PS1ΔE9 mice appeared to learn the task at a significantly slower rate than wildtype animals [$F_{(1,56)}$=17.99; p<0.001] as indicated by a significant genotype x time interaction [$F_{(9,504)}$=2.30; p=0.015]. Post-hoc analysis indicated that this was due to the APPswe/PS1ΔE9 mice in the vehicle-treated (p=0.003; figure 4.5a) and JWH133-treated conditions (p=0.007; figure 4.5c) learning CF at a slower rate.

No significant differences between wildtype and APPswe/PS1ΔE9 animals were observed in the retention of contextual fear memory (figure 4.5d). However, a significant main effect of drug was observed [$F_{(2,55)}$=3.33; p=0.043] which post-hoc analysis indicated was due to JWH133-treated APPswe/PS1ΔE9 animals displaying significantly higher immobility levels than those treated with vehicle (p=0.014).

A significant main effect of drug was observed in the extinction trial [$F_{(2,55)}$=7.45; p=0.001] independent of genotype. Post-hoc analysis indicated this also to be due to immobility differences between vehicle-treated and JWH133-treated APPswe/PS1ΔE9 mice (p=0.004).

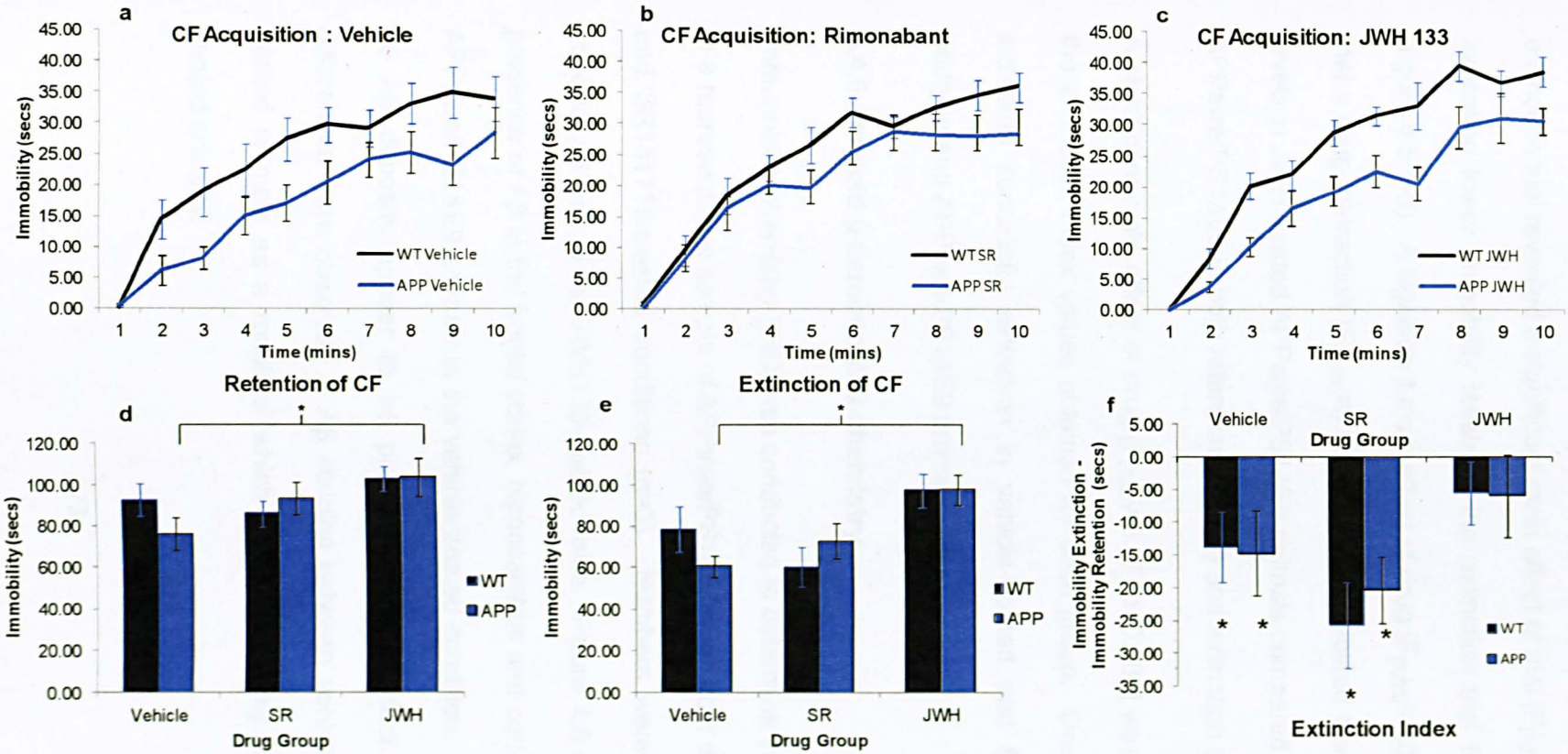

Figure 4.5 (data presented as mean ± SEM): Contextual Fear Memory. Immobility levels displayed by wildtype and APPswe/PS1ΔE9 animals (n=9-11) treated with vehicle, SR141716A (SR) or JWH133 (JWH) in the contextual fear memory task. (a-c) Wildtype and APPswe/PS1ΔE9 animals acquired contextual fear although vehicle (a) and JWH (c) treated APPswe/PS1ΔE9 animals learned at a slower rate than SR treated (b) JWH treated APPswe/PS1ΔE9 displayed significantly higher immobility levels than vehcile treated in the CF retention (d) and extinction trials (e). Post hoc *p≤ 0.01. Vehicle and SR treated animals displayed significantly lower immobility levels in the extinction trial (e) than the retention (d). (e) vehicle and SR treated wildtype and APPswe/PS1ΔE9 animals displayed successful extinction. One sample t-test *p<0.05 compared to 0 (no extinction).

A comparison of immobility levels in the retention trial to those in the extinction trial revealed a significant main effect of trial [$F_{(1,55)}$=35.21; p<0.001] indicating lower immobility levels in the extinction trial than the retention (figure 4.5 c-d). A significant main effect of drug [$F_{(2,55)}$=6.03; p=0.004] and a trial x drug interaction [$F_{(2,55)}$=3.66; p=0.032] indicated the higher immobility levels in JWH treated APPswe/PS1ΔE9 animals compared to vehicle treated APPswe/PS1ΔE9 in both retention (p=0.02) and extinction trials (p=0.002).

A significant main effect of drug [$F_{(2,56)}$=3.67; p=0.032] was also observed in the extinction index values obtained for each group. One sample t-testing indicated successful extinction in vehicle-treated and SR141716-treated wildtype and APPswe/PS1ΔE9 animals only.

4.4.6 Amyloid β immunohistochemistry

Immunohistochemistry (IHC) was conducted to determine the presence of Aβ in a representative sample of APPswe/PS1ΔE9 mice from the vehicle-treated and SR141716-treated conditions (n=3). Numbers were not sufficient to conduct Aβ IHC in the JWH133-treated mice. Figure 4.6 demonstrates the presence of Aβ in the frontal cortex, hippocampus and cortex of 7 month old APPswe/PS1ΔE9 animals in the vehicle-treated condition. However, almost no Aβ deposits appear to be present in the striatum. No qualitative differences were observed in Aβ staining between vehicle and SR141716 treated animals as a result of which images are only shown for vehicle treated animals.

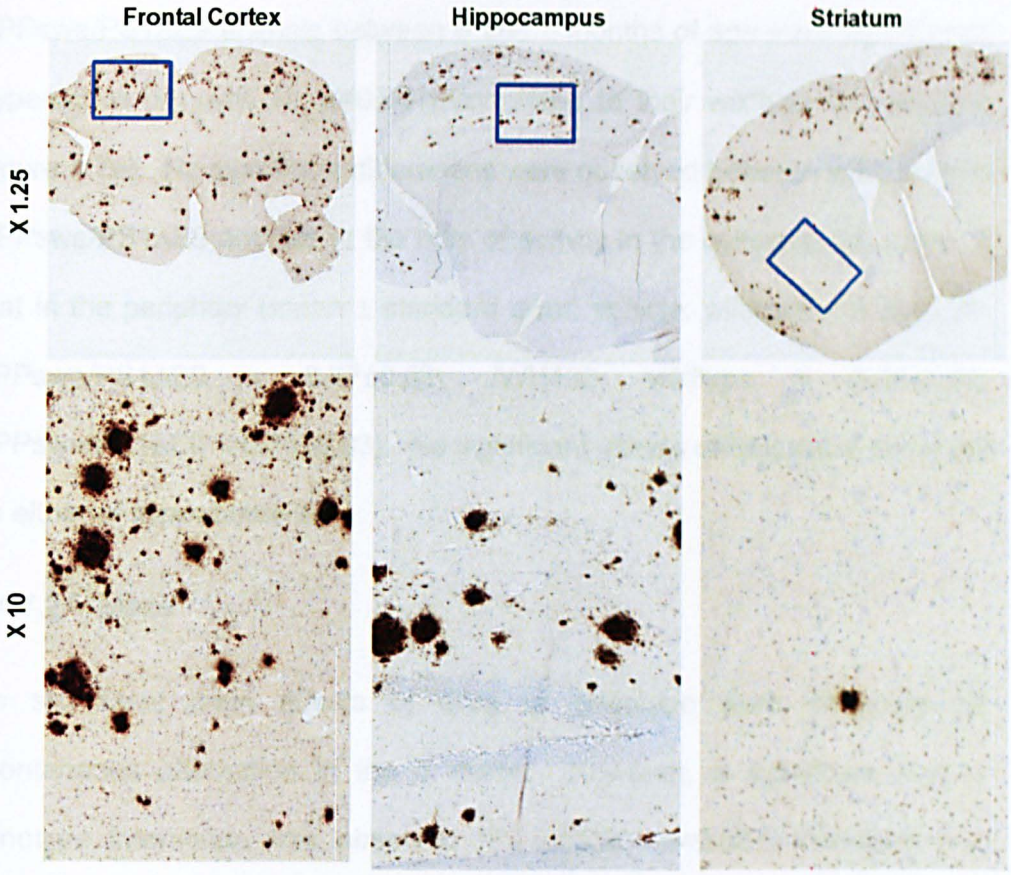

Figure 4.6: Representative samples of amyloid β staining in the frontal cortex, hippocampus and striatum of vehicle treated APPswe/PS1ΔE9 mice at 7 months of age. Areas selected in low power images indicate region of high power image.

4.4.7 Acute JWH133 Treatment

In order to ensure that the absence of a drug effect in the LMA and spontaneous alternation in the Y maze was not due to the development of tolerance in JWH treated animals a small study examining the effects of acute administration of JWH133 in these animals was conducted.

4.4.7.1 Locomotor Activity

APPswe/PS1ΔE9 animals between 6 and 7 months of age were significantly hyperactive [$F_{(1,11)}$=5.32; p=0.041] compared to their wildtype counterparts (figure 4.7a). No significant differences were observed between wildtype and APPswe/PS1ΔE9 animals in the ratio of activity in the centre of the arena to that in the periphery (mean ± standard error, vehicle: wildtype = 0.23±0.02, APPswe/PS1ΔE9 = 0.27±0.02; JWH133: wildtype = 0.24±0.02, APPswe/PS1ΔE9 = 0.25±0.03). No significant effects of drug were observed on either of these behaviours.

4.4.7.2 Y Maze

No significant main effects of drug or genotype were observed for spontaneous alternation in the Y maze. However, a significant drug x genotype interaction was observed [$F_{(1,10)}$=5.80; p=0.037] indicating that APPswe/PS1ΔE9 animals exhibited greater spontaneous alternation following JWH133 treatment whilst wildtype animals display reduced levels (figure 4.7b). Post-hoc analysis yielded an almost significant (p=0.052) difference between vehicle and JWH133-treated APPswe/PS1ΔE9 animals. All animals displayed alternation rates significantly above chance (50%) levels (p<0.05 wildtype vehicle; p<0.01 APPswe/PS1ΔE9 vehicle and JWH; wildtype JWH).

No significant differences were observed in number of arm entries made by either wildtype or APPswe/PS1ΔE9 animals as a function of vehicle or JWH133 treatment (mean ± standard error vehicle: wildtype = 19±3.92,

APPswe/PS1ΔE9 = 23±2.96; JWH133: wildtype = 20.50±1.48, APPswe/PS1ΔE9 = 21.50±3.05).

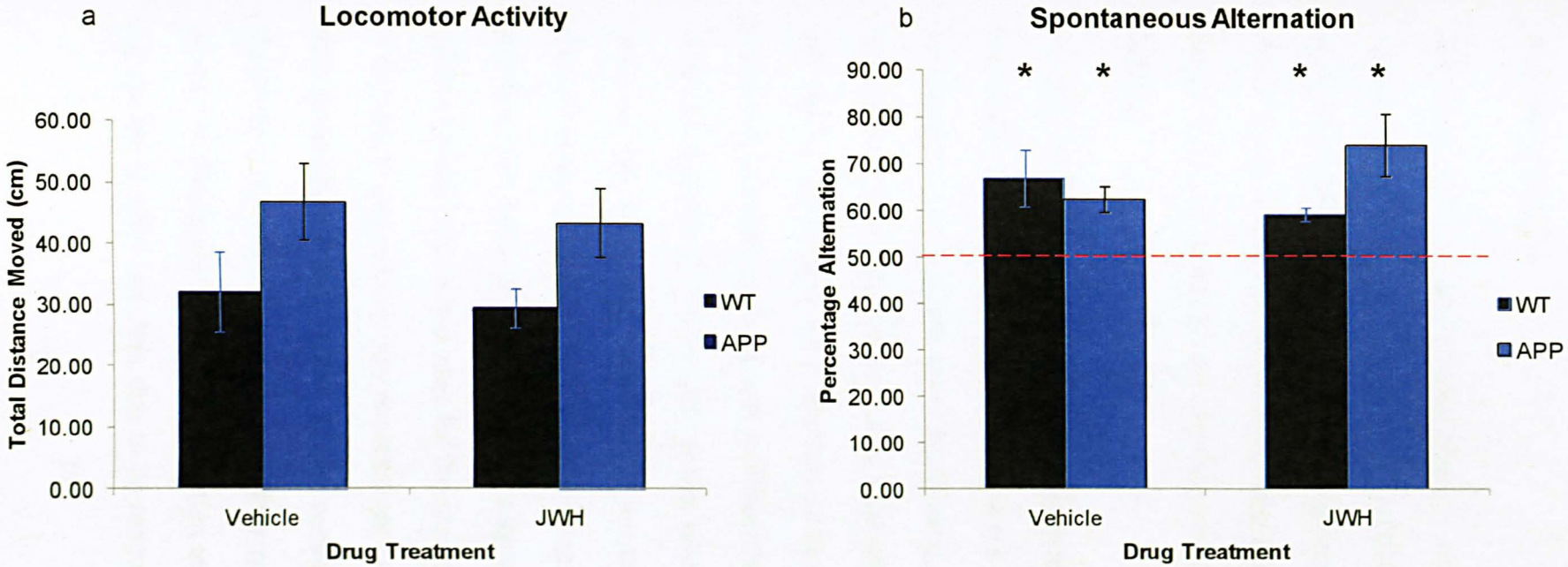

Figure 4.7 (data presented as mean ± sem): Locomotor activity and spontaneous alternation in the Y Maze following acute administration of JWH133 (JWH). (a) Total distance travelled in 30 minutes by wildtype (WT; n=5) and APPswe/PS1ΔE9 (APP; n=8) animals aged 6-7 months. (b) Percentage alternation in the Y maze for WT and APP animals. All animals alternated significantly above chance * $p<0.05$.

4.5 Discussion

The main aim of this chapter was to assess the effects of modifying components of the endocannabinoid system on the behaviour of wildtype and APPswe/PS1ΔE9 animals at 7 months of age. An additional aim was to observe the behaviour of these animals between 4 and 6 months of age when levels of amyloid beta are known to increase (Garcia-Alloza et al. 2006).

Female APPswe/PS1ΔE9 mice were hyperactive compared to their wildtype counterparts at all ages. Hyperactivity is not related to an anxiogenic trait as APPswe/PS1ΔE9 animals spent significantly more time in the centre (i.e do not display thigmotaxis) of the arena than wildtype animals. Interestingly, no differences were observed in the ratio of activity in the centre:activity in the periphery between wildtype and APPswe/PS1ΔE9 animals following acute JWH133 treatment. This is due to the wildtype animals displaying higher ratios in the acute trial compared to the chronic whilst APPswe/PS1ΔE9 show equivalent ratios in both treatment regimes. This may be due to the fact that LMA trials were only conducted for 15 minutes in the acute study in contrast to the 30 minutes used for the chronic study. The natural tendency of animals to explore any new environment they are placed in would result in both genotypes spending time in the centre of the arena as part of this exploratory process. However, once they have become habituated to the arena it is reasonable for animals to return to the periphery which is usually considered a 'safer' location due to its reduced exposure. This process is

more likely to be observed in longer trial durations which allow time for animals to become habituated in opposition to shorter trials which may not. Using a trial duration of 15 minutes would suggest that wildtype animals have not become habituated to the arena and thus there is no difference in the ratios between wildtype and APPswe/PS1ΔE9 animals. However, with a longer trial time as in the chronic study the wildtype animals may have become habituated and thus lose the exploratory drive to remain the more exposed central area. However, as the APPswe/PS1ΔE9 display elements of behavioural disinhibition, they do not follow the expected pattern and thus the difference between the genotype is revealed.

In line with their hyperactive behaviour, APPswe/PS1ΔE9 animals also showed higher object exploration times in the novel object recognition paradigm as well as a greater number of arm entries in the Y maze spontaneous alternation task. Pharmacological intervention of the endocannabinoid system by means of CB1 receptor blockade using the CB1 selective antagonist SR141716 or stimulation of CB2 receptors using JWH133 had no influence on activity levels or exploration times. These results would indicate that activity levels are not influenced by CB1 activity which is somewhat in contrast to previous studies conducted in this group which demonstrated that chronic SR141716 administration increased locomotor activity in rats (ElBatsh et al. 2012). This may be due to species differences between rats and mice or may be related to differences in dose, as the study by ElBatsh et al. (2012) used a dose of 5mg/kg whereas the present study used 3mg/kg. Alternatively, it may be related to the method of

administration, as ElBatsh et al. used intraperitoneal injections which would have resulted in fluctuating plasma levels of SR141716 in contrast to the consistent levels presumably maintained by steady state infusion by osmotic minipump (although no plasma or brain levels were measured). Additionally, the use of i.p injections in long term studies is likely to result in animals experiencing chronic stress which could potentially influence the results obtained. Similarly, chronic and acute JWH133 administration had no influence on locomotor activity levels, in contrast to work by Xi et al. (2011) which demonstrated that JWH133 reduced hyperactivity induced by cocaine in wildtype and CB1 knockout animals. However, the dose of JWH133 used by Xi et al. to obtain these effects was very high (20mg/kg) compared to the relatively low dose of 1.2mg/kg used in this study.

In contrast to previous work by Bonardi et al. (2011) in these animals, APPswe/PS1ΔE9 showed significantly lower alternation rates compared to wildtype animals at 4 months whilst no differences in alternation rate were observed at 6 and 7 months. All animals alternated above chance (50%) levels indicating that spatial memory is intact in these animals. Curiously, a significant genotype x age interaction indicated that spontaneous alternation increased with age in APPswe/PS1ΔE9 mice whilst simultaneously decreasing with age in wildtype animals between 4 and 6 months. This may be due to the chronic nature of the study and may indicate impaired recall in APPswe/PS1ΔE9 animals. As both wildtype and APPswe/PS1ΔE9 animals undertook the spontaneous alternation task at 4 months, it is possible that, when exposed to it again at 6 months, wildtype animals habituated to the

apparatus quickly due to having been exposed to it previously, whilst APPswe/PS1ΔE9 animals may not have habituated as quickly despite previous exposure. This may manifest in spontaneous alternation being reduced in wildtype animals whilst increasing in APPswe/PS1ΔE9.

Chronic administration of SR141716A or JWH133 had no effect on spontaneous alternation rates, although there appeared to be a trend towards JWH133 administration increasing alternation rates relative to the vehicle treated condition. This might reach significance with greater experimental numbers. Support for this concept was provided by the observed increase in spontaneous alternation in APPswe/PS1ΔE9 animals following acute administration of JWH133 (1.2mg/kg i.p). In contrast to the trend observed in the chronic study, wildtype animals did not show increased rates of alternation following acute JWH133 administration. However, as wildtype animal numbers were low (n=5) in the acute study, it is possible that a larger sample size could reveal an increase in alternation.

No differences were observed in the object preference index between wildtype and APPswe/PS1ΔE9 animals at any age, indicating that both genotypes were able to discriminate between the novel and familiar objects to an equal extent. However, in contrast to 4 month old animals, 6 month old animals from both genotypes did not display a preference index significantly different from chance, thus suggesting that they may not be able to distinguish between the novel and familiar objects. However, as no differences were detected in preference index by means of a two way

ANOVA it is more likely that the lack of a significant difference from chance levels is due to large variations in the performance of individual animals in the wildtype and APPswe/PS1ΔE9 groups at 6 months of age.

Chronic administration of vehicle, SR141716 or JWH133 had no influence on the novel object preference index at 7 months, although vehicle-treated wildtype and SR141716-treated APPswe/PS1ΔE9 animals did not show a preference index significantly different from chance. However, in both cases p values obtained were almost significant and, therefore, the lack of statistical significance is most likely due to a high individual variation in preference index values of animals in those groups.

Total exploration times were significantly higher in the novel object test trial compared to the acquisition trial suggesting animals had greater motivation to explore in a trial with 2 objects than 1. As with other aspects of the novel object paradigm, no drug effects on behaviour were observed.

The results discussed above lead to three possible conclusions. First, spatial memory and object recognition memory are not impaired in APPswe/PS1ΔE9 animals between 4 and 7 months. Support for this concept is provided by a number of studies. Bonardi et al. (2011) observed no deficits in recognition memory indices in 4 month old female APPswe/PS1ΔE9 mice compared to wildtype. Savonenko et al. (2005) demonstrated intact spatial memory in APPswe/PS1ΔE9 at 6 months using the Morris water maze. Lalonde et al. (2004) and Park et al. (2010) also

found no deficits in spontaneous alternation behaviour in APPswe/PS1ΔE9 animals at 7 and 8 months respectively.

Second, no drug effects could be identified in the chronic treatment condition due to wildtype and APPswe/PS1ΔE9 animals performing at equivalent levels, thereby precluding the presence of a deficit. Drug effects are unlikely to manifest in the absence of memory impairment as demonstrated by Mazzalo et al. (2003) who reported that administration of SR141716 alone had no effect on the memory performance of mice. However, a drug and genotype interaction was observed for the performance of JWH133 treated animals in the spontaneous alternation task, indicating further refinements of tests and increasing experimental numbers might reveal a more distinct effect. Based on this, it is possible that the constant infusion of drugs for 28 days in the chronic study results in desensitisation, thus masking any beneficial effects. However, as this suggestion does not account for the main conclusion of these tests, which is the absence of an impairment for the drugs to act upon, desensitisation is unlikely to explain the observations made.

Third, spontaneous alternation and novel object recognition paradigms are not sensitive enough to detect the presence of subtle cognitive impairments which may or may not be present in these animals at the ages tested. Additionally, although novel object recognition is thought to be a hippocampal-dependant task, some studies have shown that, when the learning and test contexts are the same, as in this case, object recognition

memory is unaffected by hippocampal inactivation (Oliveira et al. 2010; O'Brien et al. 2006). Furthermore, with respect to the spontaneous alternation task, Gerlai, (1998) indicated the behaviour of an animal in this task is influenced by its strain, with some mouse strains, such as the C57BL/6 performing better than others. Additionally, it is thought animals bred on a mixed background show more impairments than those on a pure background (Marie C Pardon, personal communication). Hence as APPswe/PS1ΔE9 animals used in this study are bred on a pure C57 background this observation may explain why these animals display no impairment in this task. Based on these considerations, the lack of a prominent drug effect does not indicate that the treatment is not effective, but rather that the tests may not be sensitive enough to distinguish any effects.

In contrast to the above tests, significant drug effects were observed in the CFC paradigm. As stated in Chapter 1, endocannabinoid signalling is a prerequisite for the successful extinction of aversive memories (Marsciano et al. 2002). In addition, an early impairment in contextual fear extinction has previously been reported in these mice (Bonardi et al. 2011) and is thought to represent a neural correlate of the deficits in cognitive flexibility associated with the preliminary stages of human AD (Alberts, 1996). These two factors indicate that the CFC paradigm may provide the necessary sensitivity to discern any beneficial effects of drug treatment which may be present. With respect to this assertion, 7 month old vehicle controls and JWH133-treated APPswe/PS1ΔE9 animals were able to learn the contextual fear (CF) responses, albeit at a slower rate than their wildtype counterparts. However,

this learning impairment was not observed in SR141716-treated APPswe/PS1ΔE9 animals which learned at an equivalent rate to their wildtype littermates, thus indicating that CB1 antagonism is able to reverse or counteract this effect, possibly by blocking CB1 mediated inhibition of neurotransmitter (e.g ACh) release in the prefrontal cortex. This is in line with previous studies which demonstrated that SR141716 is able to counteract the negative effects of central Aβ administration (Mazzola et al. 2003) and Δ9 tetrahydrocannabinol (Wise et al. 2009). In contrast, other studies have demonstrated the beneficial effects of enhancing central endocannabinoid transmission in AD (van der Stelt et al. 2006).

All animals were able to recall CF indicating the absence of memory impairments. Indeed, JWH133-treated APPswe/PS1ΔE9 animals displayed significantly better recall than those administered vehicle. Similarly, in the extinction trial, JWH133 administration resulted in significantly higher immobility levels in APPswe/PS1ΔE9 mice compared to vehicle treatment, despite the reduced learning. The main effect of drug was due to JWH133-treated animals being unable to extinguish CF memory. The effect was independent of genotype as illustrated by it being present in both wildtype and APPswe/PS1ΔE9 animals. This is surprising as JWH133 is a highly selective CB2 receptor agonist and CB2 has not previously been associated with the extinction of aversive memories. Indeed, the existence of CB2 receptors on neurons is contentious (Lanciego et al. 2011) although support for the presence of the receptors in the CNS is increasing (den Boon et al. 2012; Lanciego et al. 2011; Gong et al. 2006; Onaivi, 2006). In relation to

neurodegeneration, many of the reported beneficial effects of JWH133 are related to its anti-inflammatory actions through the stimulation of CB2 on microglia (Ramirez et al. 2005; 2012; Aso et al. 2012). It is unlikely that the deficit in CF extinction observed in this study originates from an anti-inflammatory mechanism as it was also evident in wildtype animals which are unlikely to exhibit neuroinflammation at the same levels as APPswe/PS1ΔE9 mice. One possibility could be that JWH133 results in a robust memory trace which is resistant to the modification necessary to promote extinction over short time intervals such as 24 hours. This could be tested by increasing the amount of time over which extinction lakes place from 24 hours to 48/72. Should it be the case that the memory is particularly robust, it is possible JWH133 treated animals require multiple extinction sessions prior to being able to successfully extinguish CF. Further work is necessary to elucidate the signalling mechanisms responsible for the observed effects, but these results provide further tentative support for the existence of functional CB2 receptors on CNS neurons.

Alternatively, the JWH133 effects may be due to indirect effects on neurons as a result of triggering CB2 receptors in microglia and astrocytes and thereby triggering a rise in intracellular Ca^{2+}. This in turn may result in the release of glutamate subsequently able to activate neuronal NMDA receptors and thereby potentially initiating the process of LTP. Furthermore, it has recently been demonstrated that LTP can be inhibited by preventing the release of the NMDA receptor co-agonist D-Serine from astrocytes (Henneberger et al. 2010). Hence, JWH133 mediated astrocytic CB2

activation may promote the release of D-Serine thereby increasing the likelihood of LTP initiation and possibly forming a more durable memory trace.

In contrast to JWH133, both wildtype and APPswe/PS1ΔE9 animals treated with SR141716 were able to successfully extinguish CF memory. This is also surprising, as previous reports in the literature indicate that administration of the antagonist results in impairments in CF extinction (Marsciano et al. 2002; Pamplona et al. 2008; Niyuhire et al. 2007). However, the protocols used in these latter studies differed from that used in the present study. Marsciano et al. (2002) used auditory fear conditioning; Pamplona et al. 2008 used contextual fear conditioning but did not administer SR141716 until 24 hours after CF acquisition, and subsequently tested for extinction 7 days later; Niyuhire et al. (2007) also administered SR141716 24 hours after CF acquisition and the animals were subsequently assessed for extinction 30 minutes later. However, in the present study, CF extinction was assessed 7 days after the removal of the osmotic minipumps (figure 4.1) by which time it is possible that the effects of chronic CB1 antagonist infusion would have begun to dissipate as a consequence of which animals were able to display normal extinction behaviour.

Both vehicle-treated wildtype and APPswe/PS1ΔE9 animals were able to successfully extinguish contextual fear as indicated by an extinction index significantly different from 0. This is in contrast to what would be expected for APPswe/PS1ΔE9 mice based on previous observations (Bonardi et al.

2011) in that they might be expected to be unable to successfully extinguish CF. The successful extinction observed in these animals may be related to a number of factors. It is possible that the repeated exposure to the behavioural experiments may act as a mild form of environmental enrichment. Environmental enrichment in the form of enriched cages has previously been shown to alleviate cognitive impairments in APPswe/PS1ΔE9 mice (Jankowsky et al. 2005). Alternatively, it is possible that the vehicle itself may have had some beneficial effect on the performance of APPswe/PS1ΔE9 in the CFC paradigm. However, this is unlikely as the beneficial effects of vehicle would be expected in wildtype animals also which would be expected to manifest as a more negative extinction index value compared to that shown by the APPswe/PS1ΔE9 mice which was not the case. Interestingly, a deficit in extinction observed in TASTPM animals (Pardon et al. 2009) has been rescued when animals receive injections (Marie C Pardon, personal communication). Based on this, it is possible the act of surgery itself is having a beneficial effect on the behavioural performance of animals. Immunohistochemical analysis confirmed the presence of Aβ in these animals, thereby supporting their validity as a model of AD. However, very little Aβ deposition was observed in the striatum of vehicle-treated APPswe/PS1ΔE9 animals in this study, although substantial amounts were found in cortical and hippocampal regions. It is possible that substantial Aβ deposits do not form in the striatum, in which earlier studies have detected lowered 2AG levels and is therefore an area of particular interest in this study, until later in the course of the disease. This is

supported by Perez et al. (2005) and Richner et al. (2009) who also reported very little Aβ deposition in the striatum at 6 months but substantial levels at 12 months. Additionally, it should be noted that the lack of Aβ deposition in the striatum at 7 months does not indicate a lack of pathological processes in this region. As can be seen, extensive Aβ deposition is observed in the frontal cortex of APPswe/PS1ΔE9 which is connected to the striatum via the frontostriatal loop thought to be involved in cognitive flexibility (Clarke et al. 2008; Devan et al. 2011).

4.6 Conclusion

The results presented in this chapter demonstrate, for the first time in a transgenic mouse model of AD, that chronic administration of a cannabinoid CB1 antagonist via osmotic minipump is able to alleviate impairments in learning as predicted. It has also been demonstrated, for the first time, that JWH133 a CB2 receptor agonist prevented the extinction of CF memories in wildtype and APPswe/PS1ΔE9 animals, implying for the first time, a role for the CB2 receptor in this process, and providing support for its presence on neurons.

Chapter 5

General Discussion

5.1 General Discussion

The main aim of this project was to determine the role of the endocannabinoid system (ECS) in an Alzheimer's disease (AD) model. A number of *in vitro* and *invivo* studies over the years have indicated that modification of the ECS may prove beneficial in a number of neurodegenerative diseases including AD. However, at the point in time at which this project was initiated, none of these studies had investigated ECS function in a transgenic model of AD.

Prior to the commencement of a full scale study, the performance of wildtype and APPswe/PS1ΔE9 animals in a locomotor activity task and contextual fear paradigm was observed at 4 and 5.5 months to establish their suitability for studying AD-related memory impairments. The results obtained from this initial analysis confirmed that APPswe/PS1ΔE9 animals represented a valid model for studying AD-like deficits and that 4 months was a suitable starting point for future studies.

Once suitability had been determined, the first full scale study examined the behaviour of pharmacologically naive male 4, 6 or 8 month old wildtype and APPswe/PS1ΔE9 animals in a battery of behavioural tests followed by post-mortem measurement of regional concentrations of endocannabinoids and cannabinoid receptor coupling. The results of this study revealed that APPswe/PS1ΔE9 were hyperactive compared to their wildtype counterparts and displayed deficits in behavioural flexibility. The results also indicated that spatial memory was intact in these animals whilst indicating possible

perturbations in recognition memory. AEA, OEA and PEA levels in the hippocampus and frontal cortex increased with age in all animals whilst no changes were observed in 2AG levels. AEA and OEA levels also increased with age in the striatum whilst 2AG was lower in APPswe/PS1ΔE9 animals compared to wildtype. Cannabinoid receptor-effector coupling was higher in the frontal cortex and striatum of APPswe/PS1ΔE9 animals compared to wildtype whilst no differences were observed in the hippocampus. Additionally, lipid markers of inflammation and oxidative stress were raised in the striatum of both wildtype and APPswe/PS1ΔE9 with age.

Based on these observations, it was reasonable to conclude that alterations in the ECS were playing a role in the AD-like cognitive impairments. Following on from these observations, another study was conducted in 4, 6, and 7 month old female wildtype and APPswe/PS1ΔE9 animals. The aims of this study were twofold: to observe changes in the behavioural performance of animals between 4 and 6 months and to observe the influence of 28 days administration of the CB1 antagonist SR141716A or CB2 agonist JWH133 on behaviour. The results of this study demonstrated that, in common with their male counterparts used in the previous study, APPswe/PS1ΔE9 females were also hyperactive and displayed greater exploratory activity. Additionally, spatial and recognition memory appeared to be intact in these animals between 4 and 7 months of age. Prominent Aβ deposition was observed in the frontal cortex and hippocampus, but not in the striatum, of the APPswe/PS1ΔE9 animals showing that the model did display the prime marker of AD-like pathology. The administration of neither SR141716A nor

JWH133 had any effect on any of the above parameters. In contrast, vehicle and JWH133 treated APPswe/PS1ΔE9 animals learned the contextual fear (CF) conditioning paradigm at a slower rate than their wildtype counterparts. This impairment in learning was absent in SR141716A treated animals. All animals were able to recall CF but JWH133 treated animals showed greater recall than vehicle or SR141716A treated mice. Whilst vehicle and SR141716A treated animals were able to extinguish CF, JWH133 treated animals were not.

Taken together, these studies indicate that levels of ECs and related molecules increase with age. Alterations in the ECS do take place in the presence of AD-like pathology and altering signalling by means of CB1 receptor blockade can reverse some of the impairments in learning and may, therefore, be of potential clinical beneficial in AD. Additionally, promoting signalling through the CB2 pathway can result either in enhanced recall or impaired cognitive flexibility. Distinguishing which of these two processes are occurring will provide a clearer answer in relation to whether CB2 stimulation has potential as a treatment for AD-related memory impairments.

5.2 Future Directions and Points to Consider

In order to fully appreciate the results of the drug intervention study it will be necessary to assess levels of neuroinflammation in wildtype and APPswe/PS1ΔE9 animals treated with cannabinoid receptor ligands. This is particularly important in relation to the effects of stimulating the CB2 receptor which is known to play a central role in anti-inflammatory processes. This

will be achieved using immunohistochemistry (IHC) with an antibody directed against the ionized calcium binding adaptor molecule 1 (IBA1) located on microglia, the major central cell type expressing CB2 receptors. IHC will also be employed in determining CB1 expression levels in wildtype and APPswe/PS1ΔE9 treated with cannabinoids to determine whether drug intervention resulted in changes in CB receptor levels in response to chronic deactivation (CB1) and stimulation (CB2). These studies are still ongoing and complete data sets were not available for inclusion in the thesis.

Further work which is not within the scope of the present project but would be beneficial includes determining the impact of drug interventions on neurogenesis in these animals. This could be done by administering a marker of proliferation such as bromodeoxyuridine (BrdU) to a subset of animals prior to schedule 1 killing followed by IHC using an anti-BrdU antibody.

It would also be beneficial to assess, immunohistochemically, the levels of enzymes involved in the synthesis and degradation of ECs, to determine whether AD pathology or drug intervention results in an alteration in their levels.

As stated in Chapter 1, in addition to stimulating CB1 and CB2, ECs are also able to act on PPAR and TRPV1 receptors. Thus, to fully determine which receptors are responsible for the behavioural effects observed, it will be necessary to attain an accurate indication of PPAR and TRPV1 expression before and after drug treatment. Alternatively, a separate study could be

undertaken whereby animals are administered vehicle, SR141716A or JWH133 but are also simultaneously administered either a PPAR or TRPV1 antagonist. Furthermore, as both CB1 and CB2 receptors are capable of forming hetrodimers it would be beneficial to determine whether this is occurring in the wildtype and APPswe/PS1ΔE9 animals used in these studies and, if so, what impact these associations have on the cognitive status of these animals.

As stated in Chapter 3, the hyperactivity observed in APPswe/PS1ΔE9 mice may be as a consequence of the reduced 2AG levels in the striatum of these animals. To further elucidate whether this is the case, preventing 2AG catabolism using a MAGL inhibitor such as JZL184 may prove instructive. The behavioural outcome of this intervention would be of particular interest as the lack of Aβ pathology observed in the striatum of APPswe/PS1ΔE9 animals suggests that hyperactivity is not related to Aβ deposition. However, the absence of hyperactivity in the wildtype littermates indicates that whilst it may not be directly due to the presence of Aβ, the hyperactive behaviour is connected to the expression of the APP/PS1 genes and probably with Aβ deposition in related brain regions. Thus, elucidating the pathway responsible may provide some insights into mechanisms not previously associated with AD.

The benefits of lipidomic analysis were discussed in Chapter 3 and future work employing this strategy is likely to yield dividends. This can be observed using the example of the raised levels of inflammatory markers and

disturbances in lipid metabolism highlighted by this study which correspond well with the findings of recent genetic studies attributing over 50% of the risk for developing AD to genes related to lipid transport and inflammation. Because lipidomic analysis requires no prior knowledge of disease mechanism it is not constrained by pre-conceived ideas relating to which factors might have a greater impact in a given disease process. As an example, it has been contended by many that fixation on the amyloid cascade hypothesis has prevented alternative mechanisms being explored and, thus, is partially responsible for the lack of progress in the search for a cure. The use of lipidomics may reveal pathways and mechanisms not previously associated with AD and, as a consequence, suggest alternative therapeutic courses, thereby possibly accelerating the process of generating effective treatments for AD. An extension of this analytical approach would be to employ MALDI mass spectrometry imaging (MSI) to study the lipid expression pattern in different brain regions (Gagnon et al., 2012). Hence, future studies employing MS techniques to further elaborate the results obtained in this study are highly recommended.

Finally, one factor outside the scope of this project but of importance is the effect of stress. Stress has been shown to increase the risk of developing AD as well as exacerbating Aβ pathology, and has also been linked to impairments in cognition during normal ageing (Pardon et al. 2008; Li et al. 2010). Stress results in the activation of the hypothalamic-pituitary-adrenal axis (HPA) leading to the release of glucocorticoid hormones which self-regulate through negative feedback shown to be mediated in part by 2AG

(Evanson et al. 2010). Additionally, chronic glucocorticoid treatment results in a decrease in CB1 receptor density whilst acute exposure has been shown to reduce AEA levels in the hippocampus (Hill et al. 2008). Studies have also shown that acute stress results in decreased AEA levels which are thought to be involved in activation of the HPA axis, whilst concurrently increasing 2AG mobilisation to terminate the stress response (Hill et al. 2010).

It is likely that the procedures used in the course of this project prompted some level of stress response in the animals and possible activation of the HPA axis. As all animals underwent all procedures, they were all exposed equally to stressors, and it is thus unlikely that this factor would have had too great an influence on the data. However, given the influence of stress on EC levels it would be beneficial in future studies to obtain an indication of the baseline levels of stress experienced by animals at different ages and in response to different behavioural experiments. The results obtained should then be cross-referenced with EC levels in these animals in order to determine whether any caveats are likely to exist in other studies employing these animals.

5.3 Conclusion

This study demonstrates that, as predicted, CB1 receptor blockade may be beneficial in alleviating some aspects of AD-related memory impairments. However, whilst CB1 receptor blockade may be beneficial acutely in AD, it could not be considered to be an effective treatment alone. Due to the

complex multidimensional aetiology of AD, any effective treatment approach is likely to involve a combination of agents acting on different systems, one of which may be the ECS.

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Appendix

High in 6 month APPswe/PS1ΔE9		
PCA Rank	Common Name	Adduct MW (Da) [Matching HMDB MW]
11	Pyrogallol	149.020905 [126.031693]
11	1,3,5-trihydroxybenzene	149.020905 [126.031693]
11	1,2,3-trihydroxybenzene	149.020905 [126.031693]
11	Hydroxymethylfurfural	149.020905 [126.031693]
11	2-Oxo-4-methylthiobutanoic acid	149.020688 [148.019409]
14	Pyrogallol	190.047455 [126.031693]
14	1,3,5-trihydroxybenzene	190.047455 [126.031693]
14	1,2,3-trihydroxybenzene	190.047455 [126.031693]
14	Hydroxymethylfurfural	190.047455 [126.031693]
14	Isopyridoxal	190.047455 [167.058243]
14	Pyridoxal	190.047455 [167.058243]
14	Beta-Glycerophosphoric acid	190.047501 [172.013672]
14	Glycerol 3-phosphate	190.047501 [172.013672]
14	Kynurenic acid	190.049866 [189.042587]
16	19-Oxo-deoxycorticosterone	325.180359 [344.198761]
16	11-Dehydrocorticosterone	325.180359 [344.198761]
19	9'-Carboxy-alpha-chromanol	407.303009 [389.269196]
3	Coprocholic acid	473.323761 [450.334634]
3	3a,7a,12a-Trihydroxy-5b-cholestanoic acid	473.323761 [450.334634]
15	CE(19:0) Cholesteryl ester	708.665283 [666.631470]
41	DG(22:5(7Z,10Z,13Z,16Z,19Z)/20:0/0:0)	737.548035 [698.584900]
41	DG(22:5(4Z,7Z,10Z,13Z,16Z)/20:0/0:0)	737.548035 [698.584900]
41	DG(22:4(7Z,10Z,13Z,16Z)/20:1(11Z)/0:0)	737.548035 [698.584900]
41	DG(22:2(13Z,16Z)/20:3(8Z,11Z,14Z)/0:0)	737.548035 [698.584900]
41	DG(22:2(13Z,16Z)/20:3(5Z,8Z,11Z)/0:0)	737.548035 [698.584900]
41	DG(22:1(13Z)/20:4(8Z,11Z,14Z,17Z)/0:0)	737.548035 [698.584900]
41	DG(22:1(13Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	737.548035 [698.584900]
41	DG(22:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	737.548035 [698.584900]
41	DG(20:5(5Z,8Z,11Z,14Z,17Z)/22:0/0:0)	737.548035 [698.584900]
41	DG(20:4(8Z,11Z,14Z,17Z)/22:1(13Z)/0:0)	737.548035 [698.584900]
41	DG(20:4(5Z,8Z,11Z,14Z)/22:1(13Z)/0:0)	737.548035 [698.584900]
41	DG(20:3(8Z,11Z,14Z)/22:2(13Z,16Z)/0:0)	737.548035 [698.584900]
41	DG(20:3(5Z,8Z,11Z)/22:2(13Z,16Z)/0:0)	737.548035 [698.584900]
41	DG(20:1(11Z)/22:4(7Z,10Z,13Z,16Z)/0:0)	737.548035 [698.584900]
41	DG(20:0/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)	737.548035 [698.584900]
41	DG(20:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	737.548035 [698.584900]
41	DG(18:4(6Z,9Z,12Z,15Z)/24:1(15Z)/0:0)	737.548035 [698.584900]
41	DG(24:1(15Z)/18:4(6Z,9Z,12Z,15Z)/0:0)	737.548035 [698.584900]
46	TG(16:0/18:0/18:0)[iso3]	885.788147 [862.798950]
46	TG(16:0/16:0/20:0)[iso3]	885.788147 [862.798950]
46	TG(18:1(9Z)/18:1(11Z)/18:1(9Z))[iso3]	885.790527 [884.783264]
46	TG(18:1(9Z)/16:0/20:2(11Z,14Z))[iso6]	885.790527 [884.783264]
46	TG(18:1(11Z)/18:0/18:2(9Z,12Z))[iso6]	885.790527 [884.783264]
46	TG(18:1(9Z)/18:1(9Z)/18:1(9Z))[iso]	885.790527 [884.783264]
46	TG(16:1(9Z)/18:2(9Z,12Z)/20:0)[iso6]	885.790527 [884.783264]
46	TG(16:1(9Z)/18:1(9Z)/20:1(11Z))[iso6]	885.790527 [884.783264]
46	TG(18:0/18:1(9Z)/18:2(9Z,12Z))[iso6]	885.790527 [884.783264]
46	TG(16:0/18:2(9Z,12Z)/20:1(11Z))[iso6]	885.790527 [884.783264]

High in 6 month wildtype		
PCA Rank	Common Name	Adduct MW (Da) [Error (Matching HMDB MW)]
26	Sphingosine	322.271667 [299.262440]
26	Palmitoylethanolamide	322.271667 [299.262440]
26	3-Dehydrosphinganine	322.271667 [299.262440]
26	Linoleic acid	322.274048 [280.240234]
26	10E,12Z-Octadecadienoic acid	322.274048 [280.240234]
26	9E,11E-Octadecadienoic acid	322.274048 [280.240234]
26	Bovinic acid	322.274048 [280.240234]
26	Linoelaidic acid	322.274048 [280.240234]
26	Arachidonic acid	322.274048 [304.240234]
26	Cis-8,11,14,17-Eicosatetraenoic acid	322.274048 [304.240234]
26	Alpha-Linolenoyl ethanolamide	322.274048 [321.288785]
24	Stearic acid	348.287292 [284.271515]
24	N-Oleoylethanolamine	348.287292 [325.298065]
24	8,11,14-Eicosatrienoic acid	348.289703 [306.255890]
24	5,8,11-Eicosatrienoic acid	348.289703 [306.255890]
24	4,7,10,13,16-Docosapentaenoic acid	348.289703 [330.255890]
24	Docosapentaenoic acid (22n-6)	348.289703 [330.255890]
24	Docosapentaenoic acid	348.289703 [330.255890]
24	Anandamide	348.289703 [347.282440]
24	O-Arachidonoyl Ethanolamine	348.289703 [347.282440]
27	Stearoylethanolamide	350.302948 [327.313721]
27	N,N-Dimethylsphingosine	350.302948 [327.313721]
27	Eicosadienoic acid	350.305328 [308.271515]
27	Adrenic acid (prostacyclin inhibitor	350.305328 [332.271515]
27	Dihomo-gamma-Linolenoyl ethanolamide	350.305328 [349.298065]
33	3L,7D,11D-Phytanic acid	376.318604 [312.302826]
33	3D,7D,11D-Phytanic acid	376.318604 [312.302826]
33	Arachidic acid	376.318604 [312.302826]
33	Phytanic acid	376.318604 [312.302826]
33	Docosatrienoic acid	376.320984 [334.287170]
33	Tetracosapentaenoic acid (24:5n-3)	376.320984 [356.287170]
33	Tetracosapentaenoic acid (24:5n-6)	376.320984 [356.287170]
33	Adrenoyl ethanolamide	376.320984 [375.313721]
15	Lithocholic acid	394.331573 [376.297760]
15	Isolithocholic acid	394.331573 [376.297760]
15	Isoallolithocholic acid	394.331573 [376.297760]
15	Allolithocholic acid	394.331573 [376.297760]
15	7a-Hydroxy-5b-cholanic acid	394.331573 [376.297760]
15	12b-Hydroxy-5b-cholanoic acid	394.331573 [376.297760]
46	DG(24:0/16:0/0:0)	698.665710 [680.631897]
46	DG(22:0/18:0/0:0)	698.665710 [680.631897]
46	DG(20:0/20:0/0:0)	698.665710 [680.631897]
46	DG(18:0/22:0/0:0)	698.665710 [680.631897]
46	DG(16:0/24:0/0:0)	698.665710 [680.631897]

High in 8 month APPswe/PS1ΔE9		
PCA Rank	Common Name	Adduct MW (Da) HMDB MW)
24	10,11-dihydro-20-trihydroxy-leukotriene B4	427.256430 [386.227670]
24	10,11-dihydro-20-trihydroxy-leukotriene B4	427.256430 [386.227670]
	Capsianoside I	661.342957 [660.335683]
25	S-(PGA1)-glutathione	661.347656 [643.313043]
25	S-(9-hydroxy-PGA1)-glutathione	661.347656 [643.313043]
25	S-(11-hydroxy-9-deoxy-delta12-PGD2)-glutathione	661.347656 [643.313043]
6	DG(22:5(7Z,10Z,13Z,16Z,19Z)/20:0/0:0)	737.548035 [698.584800]
6	DG(22:5(4Z,7Z,10Z,13Z,16Z)/20:0/0:0)	737.548035 [698.584800]
6	DG(22:4(7Z,10Z,13Z,16Z)/20:1(11Z)/0:0)	737.548035 [698.584800]
6	DG(22:2(13Z,16Z)/20:3(8Z,11Z,14Z)/0:0)	737.548035 [698.584800]
6	DG(22:2(13Z,16Z)/20:3(5Z,8Z,11Z)/0:0)	737.548035 [698.584800]
6	DG(22:1(13Z)/20:4(8Z,11Z,14Z,17Z)/0:0)	737.548035 [698.584800]
6	DG(22:1(13Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	737.548035 [698.584800]
6	DG(22:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	737.548035 [698.584800]
6	DG(20:5(5Z,8Z,11Z,14Z,17Z)/22:0/0:0)	737.548035 [698.584800]
6	DG(20:4(8Z,11Z,14Z,17Z)/22:1(13Z)/0:0)	737.548035 [698.584800]
6	DG(20:4(5Z,8Z,11Z,14Z)/22:1(13Z)/0:0)	737.548035 [698.584800]
6	DG(20:3(8Z,11Z,14Z)/22:2(13Z,16Z)/0:0)	737.548035 [698.584800]
6	DG(20:3(5Z,8Z,11Z)/22:2(13Z,16Z)/0:0)	737.548035 [698.584800]
6	DG(20:1(11Z)/22:4(7Z,10Z,13Z,16Z)/0:0)	737.548035 [698.584800]
6	DG(20:0/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)	737.548035 [698.584800]
6	DG(20:0/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	737.548035 [698.584800]
6	DG(18:4(6Z,9Z,12Z,15Z)/24:1(15Z)/0:0)	737.548035 [698.584800]
6	DG(24:1(15Z)/18:4(6Z,9Z,12Z,15Z)/0:0)	737.548035 [698.584800]

High in 8 month wildtype		
PCA Rank	Common Name	Adduct MW (Da) (Matching HMDB MW)
11	Eicosapentaenoyl Ethanolamide	384.229950 [345.266785]
23	2-Arachidonylglycerol	401.266235 [378.277008]
23	MG(20:4(8Z,11Z,14Z,17Z)/0:0/0:0)	401.266235 [378.277008]
23	MG(20:4(5Z,8Z,11Z,14Z)/0:0/0:0)	401.266235 [378.277008]
23	MG(0:0/20:4(8Z,11Z,14Z,17Z)/0:0)	401.266235 [378.277008]
23	Canrenone	401.269196 [340.203857]
10	4alpha-formyl-5alpha-cholesta-8-en-3beta-ol	475.415131 [414.349792]
10	(22Alpha)-hydroxy-campest-4-en-3-1	475.415131 [414.349792]
8	MG(24:0/0:0/0:0)	506.417969 [442.402222]
8	MG(0:0/24:0/0:0)	506.417969 [442.402222]