Targeting neurodegeneration in Alzheimer's disease using natural products derived from Maya traditional medicine

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ABSTRACT

Alzheimer's disease (AD) is a complex neurodegenerative disorder with limited treatment options. Previous research has shown that metabolism of the platelet activating factor (PAF) family of lipid second messengers is impaired in AD. While PAFs are known to exacerbate glutamate excitotoxicity, signal tau hyperphosphorylation, and mediate amyloid β neurotoxicity, it is not yet clear whether cognitive decline can be ascribed to activation of the G-protein-coupled PAF receptor (PAFR). Here, I assessed whether loss of PAFR would alter Morris water maze performance in the TgCRND8 (Tg) mouse model of AD. I show that learning is impaired in Tg $PAFR^{+/+}$ but not in Tg $PAFR^{-/-}$ mice. Together, these findings suggested that blocking PAFR-mediated glutamate overload or inhibiting PAF-synthesizing enzymes are two relevant therapeutic strategies. As traditional medicine is a major form of health care in regions like Mesoamerica, I conducted an ethnobotanical survey of medicinal plants used by Q'eqchi' Maya healers of southern Belize to treat symptoms relevant to AD. I collected a total of 22 plants, 19 of which were identified to the species level. None of the plant extracts used for symptoms of AD were neurotoxic when tested on cerebellar granule neurons (CGNs). I found that extracts of *Margraviaceae gentlei* and *Gonzalagunia panamensis* protected CGNs from glutamate-induced excitotoxicity*, in vitro,* and *Peperomia hirta* inhibited $sPLA_2$ activity. These results demonstrate a pharmacological basis for Q'eqchi' Maya traditional medicine used to treat symptoms relevant to AD, and highlight several plants with potential for future development into natural products for the treatment of AD.

RÉSUMÉ

La maladie d'Alzheimer (MA) est un désordre neurodégénératif complexe pour lequel il n'existe que peu d'options thérapeutiques. Des recherches antérieures ont démontré que le métabolisme des seconds messagers lipidiques de la famille du facteur d'activation plaquettaire (FAP) est altéré dans la MA. Quoique le FAP est reconnu pour exacerber l'excitotoxicité du glutamate, induire la signalisation de l'hyperphosphorylation de tau et la neurotoxicité de l'amyloïde β, le rôle de son récepteur couplé aux protéines G (RFAP) est, jusqu'à présent, inconnu. Dans ce mémoire, j'ai évalué si l'absence du RFAP pouvait modifier la performance de souris modèle TgCRND8 (Tg) de la MA à l'aide du labyrinthe d'eau de Morris. J'ai démontré que l'apprentissage des souris Tg $PAFR^{+/+}$ est altéré, mais pas celle des Tg $PAFR^{-/-}$. L'ensemble des résultats suggère que la suppression de la surcharge de glutamate signalé par le RFAP ou l'inhibition des enzymes responsables de la synthèse du FAP sont deux stratégies thérapeutiques viables. Puisque la médecine traditionnelle représente une large portion des soins médicaux prodigués dans les régions comme la Mésoamérique, j'ai dirigé une enquête sur les plantes médicinales utilisées par les guérisseurs Maya Q'eqchi' du sud du Bélize pour traiter les symptômes associés à la MA. J'ai collecté un total de 22 plantes, dont 19 ont été identifiés au niveau de l'espèce. Aucune des plantes extraites utilisées pour traiter les symptômes de la MA n'a démontré d'effets neurotoxiques lorsque testée *in vitro* sur des neurones cérébelleux de la couche granulaire (NCG). J'ai démontré que les extraits de *Margraviaceae entlei* et de *Gonzalagunia panamensis* protègent les NCG contre l'excitotoxicité induite par le glutamate alors que ceux de *Peperomia hirta*

inhibent l'activité enzymatique de la $sPLA_2$. Ces résultats démontrent les bases pharmacologiques de la médecine traditionnelle des Maya Q'eqchi' utilisées pour traiter les symptômes associés à la MA. De plus, ils mettent en lumière le fait que plusieurs plantes pourraient être développées en produit naturel pour le traitement de la MA.

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LIST OF ABBREVIATIONS

AAGPC: Alkylacylglycerophosphocholine
Aβ: Amyloid beta
AD: Alzheimer's disease
APP: Amyloid precursor protein
CGN: Cerebellar granule neuron
DIV: Days *in vitro*
DMSO: Dimethyl sulfoxide
Ethd1: Ethidium homodimer-1
LPCAT: *Lyso*phosphatidylcholine acyltransferase
MCI: Mild cognitive impairment
NMDAR: *N*-methyl-D-aspartate receptor
PAF: Platelet activating factor
PAF-AH: PAF acetylhydrolase
PAFR: Platelet activating factor receptor
PLA_2: Phospholipase A_2
QHA: Q'eqchi' Healers Association
SIV: Syndromic Importance Value
$sPLA_2$: Secretory phospholipase A_2
Tg: TgCRND8

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CHAPTER 1

INTRODUCTION

1.1 General introduction

The Maya civilization has existed for thousands of years, with concentrated settlements in Mesoamerica thought to have begun around 2000 BCE (Coe, 2011). Their civilization developed through the Archaic, Preclassic, and Classic period before the so-called “Classic Maya collapse” around 900 CE, and persisted through the difficult Post-Classic period and Spanish conquest to today. Throughout their history, the Maya constructed population centres and monuments, formed complex social and belief systems, and developed economic and trading networks (Coe, 2011). Currently, they are one of the largest indigenous groups in the Americas, with an estimated population of 10 million (Coe, 2011). Their deep spirituality, connection to the land, and skilful use of tropical biodiversity are key elements that have contributed to their success and resilience (Coe, 2011).

Although construction of large ceremonial sites by the Maya has ended, aspects of their culture that continued from the Classic period through to today include calendar and spiritual ceremonies, traditional agriculture, and medicine. Maya medicine has long been neglected by researchers, as compared to Traditional Chinese or Ayurvedic medicine, yet contains a pharmacopoeia of hundreds of species (Amiguet et al., 2005; Ankli et al., 1999; Arnason et al., 1980; Bourbonnais-Spear et al., 2005; Kufer et al., 2005; Michel et al., 2007; Roys, 1931).

Today, like many people around the world, Mesoamericans are facing new modern challenges involving issues of human health. One such challenge is

Alzheimer's disease (AD), a neurological disorder that was declared a global health priority in 2012 (WHO, 2012). Elsewhere, traditional medicine has yielded new pharmacological treatments for AD, such as galantamine, which was developed from a medicinal plant of Europe and Russia (Heinrich and Lee Teoh, 2004). Given the exceptional biodiversity of the Mesoamerican flora (Myers et al., 2000), there is considerable potential for the discovery of new therapeutic leads from nature. In making an informed selection of plants from Mesoamerica for study, perhaps important lessons can be learned from the Maya. Consequently, this thesis examined the potential of botanical medicines used by the Q'eqchi' Maya as sources of extracts and molecules to target some of the underlying mechanisms mediating neurodegeneration in AD.

1.2 Overview of AD

1.2.1 Disease characteristics

AD is a neurodegenerative disease affecting the brain that causes dementia, a syndrome characterized by an impairment in memory and other cognitive functions that interferes with normal daily activities (Alzheimer's Association, 2012; McKhann et al., 2011). AD is the most common cause of dementia, making up over half of all cases (Ferri et al., 2009). There are two main forms of the disease, based on age of onset. Early-onset AD occurs approximately between the ages of 30 to 60 and accounts for less than 10% of all cases (Bekris et al., 2010). Most of these cases are familial, showing some form of family inheritance, with certain incidents being caused by known genetic mutations (Bekris et al., 2010). Late-onset AD is far more

common, responsible for over 90% of all cases, and occurs in people generally over the age of 60 (Bekris et al., 2010). Most of these cases are sporadic, with no known genetic cause, although certain genetic risk factors have been identified (Bekris et al., 2010).

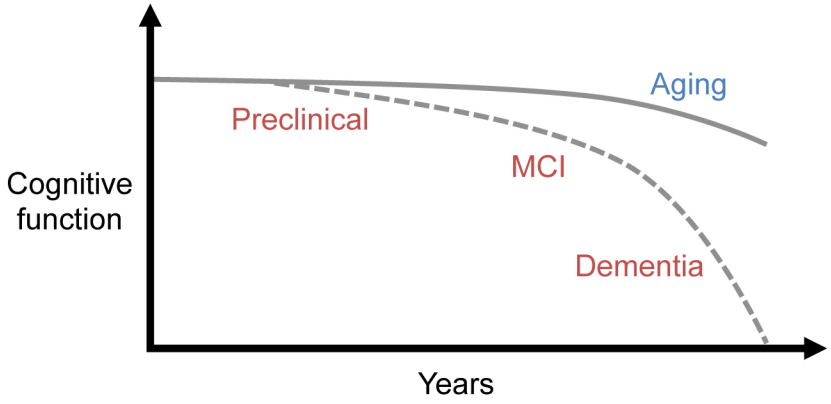
Recent updates made to AD diagnostic guidelines define three main stages of the disease, beginning with preclinical AD, progressing to mild cognitive impairment (MCI) due to AD, and culminating in dementia due to AD (Figure 1.1). Preclinical AD involves measurable changes in biomarker levels before cognitive impairment is detectable (Sperling et al., 2011). While specific criteria for these biomarkers have yet to be established, this stage recognizes that changes in the brain are likely occurring well before the onset of behavioural signs and symptoms (Sperling et al., 2011). When cognitive impairment becomes noticeable and measurable, but does not interfere with daily functioning, patients are considered to have MCI due to AD (Albert et al., 2011). This impairment will progress in many patients, and once it interferes with their ability to function normally, it is considered dementia due to AD (McKhann et al., 2011). The average life expectancy of patients at the time of dementia diagnosis has been shown to range between three to nine years (Fitzpatrick et al., 2005; Walsh et al., 1990; Wolfson et al., 2001).

1.2.2 Prevalence, incidence, and economic impact

In 2010, there were an estimated 35.6 million people with AD in the world, most of whom were living in developing countries (Ferri et al., 2009). The incidence rate was estimated at 7.7 million new cases per year, and there is predicted to be 115.4 million AD patients by the year 2050 (Ferri et al., 2009). The global economic

Figure 1.1. Model of AD progression.

The stages of AD progress from preclinical, to MCI, to dementia as a function of time. Cognitive function declines throughout this progression, with preclinical AD involving measurable changes in biomarker levels before cognitive impairment is detectable, MCI due to AD involving noticeable cognitive deficits that do not interfere with daily functioning, and dementia due to AD involving cognitive impairment that interferes with normal day-to-day function.


Aging
Preclinical
MCI
Dementia
Cognitive function
Years

burden of AD is massive, at an estimated annual expense of $604 billion that results from both direct costs of health care and indirect costs associated with caregiving by families (Wimo and Prince, 2010). The staggering impact of AD has led to it being declared a public health priority by the World Health Organization in 2012 (WHO, 2012).

1.2.3 Risk factors

In a small proportion of familial early-onset AD cases, genetics appear to play a causative role and involve mutations inherited in an autosomal dominant pattern (Bekris et al., 2010). The mutations in these rare cases are directly related to aberrant amyloid β (Aβ) metabolism. Aβ is a protein fragment that has been found to accumulate in the brains of AD patients, and is generated from the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases (Hardy and Selkoe, 2002). These mutations have been localized to the genes for APP, or two components of the γ-secretase complex, presenilin 1 and presenilin 2 (Bekris et al., 2010).

In the majority of AD cases, which involve the late-onset form, the cause or causes of disease development are unknown. Age is the greatest non-genetic risk factor for disease development (Huang and Mucke, 2012). A number of other illnesses, such as diabetes, hypertension, and obesity have been associated with an increased risk of AD, however, concerns of inadequacy of scientific evidence have led to controversy with regards to such risk factors (Daviglus et al., 2011). While there is no known genetic determinant for late-onset AD, there are genetic risk factors (Bekris et al., 2010). The strongest one is the ε4 allele variant of the gene

coding for apolipoprotein E, a lipid transporter (Bertram et al., 2010). People with the ε4 allele are at higher risk of developing AD, yet many carriers maintain normal cognitive function in later life (Huang and Mucke, 2012).

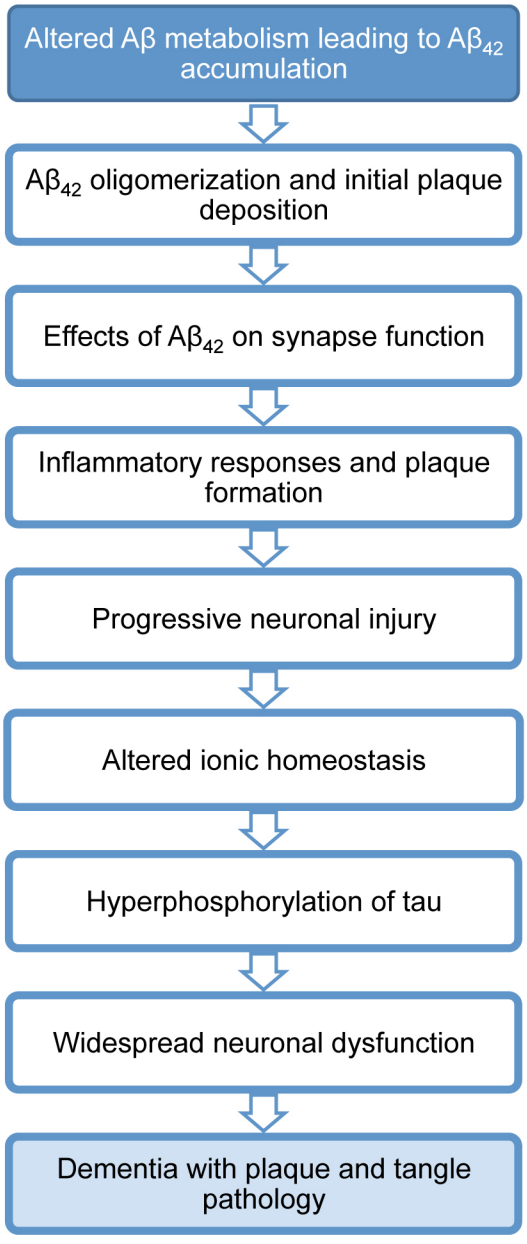
1.3 Molecular pathologies in AD

1.3.1 Plaques and tangles

While the mechanisms underlying AD are still not fully understood, two molecular hallmarks are extracellular plaques composed of Aβ peptide fragments, and intracellular accumulations of neurofibrillary tangles composed of hyperphosphorylated tau protein (Blennow et al., 2006). Aberrant processing of APP, a transmembrane protein of unclear physiological function, through sequential cleavage by β-secretases and γ-secretases generates Aβ monomers, mainly $A\beta_{42}$, which is particularly prone to aggregation into higher-order structures that include oligomers, protofibrils, fibrils, and plaques (Haass and Selkoe, 2007). The “amyloid cascade” hypothesis (Figure 1.2) defines the accumulation and aggregation of $A\beta_{42}$ as an upstream event in AD and the principle cause of the disease (Haass and Selkoe, 2007; Hardy and Selkoe, 2002), with soluble $A\beta_{42}$ oligomers as the major contributor to Aβ toxicity (Walsh and Selkoe, 2007). Further downstream, the hyperphosphorylation of tau, a microtubule-associated protein, leads to microtubule instability and aggregation into toxic neurofibrillary tangles (Figure 1.2) (Hanger et al., 2009). Both plaques and tangles must be present in the brain upon autopsy to confirm a diagnosis of AD (Hyman and Trojanowski, 1997). Yet, despite strong

Figure 1.2. Schematic of amyloid cascade hypothesis.

Aberrant Aβ metabolism that leads to an accumulation of $A\beta_{42}$ is the principle cause of AD, and is upstream of other pathological events that occur throughout disease progression. The cascade leads to AD patients with impaired cognitive function with plaque and tangle brain pathologies.


Altered Aβ metabolism leading to $A\beta_{42}$ accumulation
$A\beta_{42}$ oligomerization and initial plaque deposition
Effects of $A\beta_{42}$ on synapse function
Inflammatory responses and plaque formation
Progressive neuronal injury
Altered ionic homeostasis
Hyperphosphorylation of tau
Widespread neuronal dysfunction
Dementia with plaque and tangle pathology

support for Aβ and tau aggregation as driving pathologies, converging evidence suggests that they likely represent only two of multiple factors necessary for AD development. The existence of cognitively “normal” elderly with significant plaque and tangle deposition (Bennett et al., 2006; Snowdon, 2003) has led to a re-envisionment of the disease, whereby Aβ and tau, together with other metabolic disruptions, contribute to a biologically altered brain predisposed to neurodegeneration (Herrup, 2010). Targeting these metabolic determinants represents a novel, potentially transformative, approach to prevent AD conversion. Thus, while plaques and tangles remain the principal markers of AD, a number of other pathologies have been identified further downstream, including excitotoxicity and aberrant lipid metabolism that represent viable therapeutic options for this intractable disease (Farooqui and Horrocks, 2006; Lipton, 2006).

1.3.2 Excitotoxicity

Excitotoxicity has been well documented in AD, along with a number of other neurological disorders, and involves increased glutamate signalling and *N*-methyl-D-aspartate receptor (NMDAR) overactivation (Dong et al., 2009). Glutamate is an excitatory neurotransmitter that is required for normal brain function. NMDARs are one of three classes of ionotropic glutamate receptors, and considered to be the most permeable to Ca^{2+} (Lipton, 2006). Normally, synaptic glutamate levels are tightly regulated by release and reuptake mechanisms and NMDAR activity is controlled by a voltage-dependent Mg^{2+} block (Lipton, 2006). However, in AD, these regulatory measures can become compromised, leading to NMDAR overactivation and Ca^{2+} overload (Lipton, 2006). Intracellular accumulation of Ca^{2+} can cause

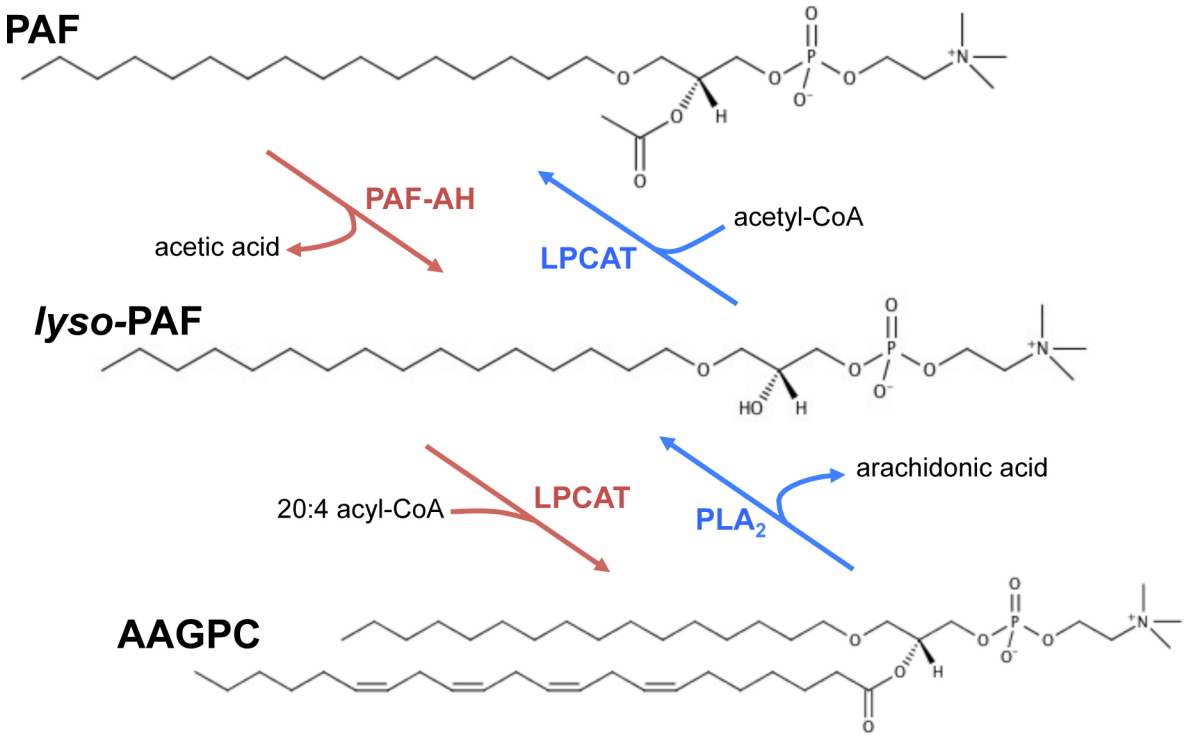
mitochondrial dysfunction and oxidative stress that lead to necrotic and apoptotic death (Dong et al., 2009). Excitotoxic conditions can also activate central nervous system immune cells, microglia, which initiate inflammatory responses that can be toxic to neurons (Kaindl et al., 2012).

1.3.3 Aberrant lipid metabolism

Lipid metabolism has become an area of interest in AD research, as lipid signalling pathways are essential to brain function and an increasing number of them have been found to be dysfunctional in AD (Di Paolo and Kim, 2011; Farooqui and Horrocks, 2006). Recent work from the Bennett laboratory and others has shown that $A\beta_{42}$ disrupts the alkylacylglycerophosphocholine (AAGPC) remodelling pathway or Lands' cycle (Figure 1.3), whereby lipid second messengers known as platelet activating factors (PAFs) accumulate in the brain (Bate et al., 2006; Bate et al., 2004; Ryan et al., 2009). Under normal conditions, PAF synthesis involves the hydrolysis of membrane structural AAGPC lipids by phospholipase A_2 (PLA_2) enzymes, which generates *lyso*-PAFs (Farooqui et al., 2000). These PAF precursors are then either acetylated into PAFs or remodelled back into structural membrane lipids by *lyso*phosphatidylcholine acyltransferases (LPCATs) (Shindou et al., 2009). PAFs can also be remodelled back into *lyso*-PAFs by PAF acetylhydrolases (PAF-AHs). Research on the PLA_2 superfamily, a group of enzymes that were first characterized from snake venom (Dennis et al., 2011), has found a number of members to be upregulated in AD patients and transgenic mouse models. These include the intracellular calcium-dependent cytosolic PLA_2 and the small molecular

Figure 1.3. The AAGPC remodeling pathway (Lands' cycle).

Structural AAGPCs are hydrolysed by PLA_2 enzymes to generate *lyso*-PAFs, which can be acetylated into PAFs, with acetyl-CoA as a donor, or remodelled back into structural membrane lipids, with acyl-CoA as a donor, by LPCATs. PAFs are remodelled back into *lyso*-PAFs by PAF-AHs. Lipid structures were generated using VaLID (Blanchard et al., 2013).


PAF
PAF-AH
acetic acid
LPCAT
acetyl-CoA
lyso-PAF
20:4 acyl-CoA
LPCAT
PLA2
arachidonic acid
AAGPC

weight secretory PLA_2s ($sPLA_2$s) (Chalbot et al., 2009; Moses et al., 2006; Schaeffer et al., 2010) that share a conserved structure and activity to the active component in many snake venoms (Angulo and Lomonte, 2009; Lomonte et al., 2008). Consistent with this upregulation, both *lyso*-PAFs and PAFs are elevated in brain regions of patients and transgenic mice (Ryan et al., 2009). The specific PAF isoform, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PC(*O*-16:0/2:0), and not its *lyso*-PAF precursor, is neurotoxic and signals the hyperphosphorylation of tau at AD-specific phosphoepitopes (Ryan et al., 2009).

PAFs can signal cellular events through activation of the G-protein coupled PAF receptor (PAFR) (Chen et al., 2001; Ishii and Shimizu, 2000) as well as PAFR-independent mechanisms (Brewer et al., 2002; Ryan et al., 2007). In the brain, PAFR has been localized primarily in neurons and microglia, and to a lesser extent in astrocytes (Bennett et al., 1998; Mori et al., 1997). Under normal conditions, PAFR-mediated signaling has been shown to be neuroprotective and important for learning and memory processes (Chen et al., 2001; Izquierdo et al., 1995; Kato et al., 1994). However, when PAF levels become elevated under pathological conditions, like in AD, PAFR signaling cascades can lead to neuronal damage, through mechanisms involving excitotoxicity and microglial activation (Bate et al., 2006; Bellizzi et al., 2005; Bennett et al., 1998). Considering these differential roles under normal and pathological conditions, it remains unclear whether PAFR signaling is beneficial or detrimental to learning and memory in AD.

1.4 Current treatments and limitations

Currently, there is no known treatment to slow or reverse AD. Despite the hypothesized causal role of $A\beta_{42}$ accumulation and aggregation in AD, targeting these events directly has been met with a number of challenges and, as of yet, has not led to effective treatment options (Selkoe, 2011). While research continues to explore this approach, there has been an increased focus on targeting other pathologies further downstream, as avenues for complementary therapeutics (Lane et al., 2012). This approach has been more successful and has led to the development of several therapeutics that temporarily boost cognitive function (Mangialasche et al., 2010). There are currently four certified drugs prescribed in Canada and the United States, which include three acetylcholine esterase inhibitors and one NMDAR antagonist (Mangialasche et al., 2010). The acetylcholine esterase inhibitors increase acetylcholine levels in the brain, as levels of this neurotransmitter are decreased in AD, and the NMDAR antagonist blocks the overactivation of NMDARs that occurs in AD (Mangialasche et al., 2010).

One important consideration for future research and development of new AD therapeutics is that the majority of patients live in developing countries, where pharmaceutical-based medicine may not be accessible or affordable (WHO, 2002a). In these areas, alternative forms of health care are required. Traditional medicine, which is often more available and costs less than pharmaceuticals, represents a valuable option for these patients (WHO, 2002a).

1.5 Traditional medicine as neurodegenerative therapeutics

Many forms of traditional medicine exist around the world, and often involve plant-based treatments as a core component (WHO, 2002a). Plants are well known to possess a large number of secondary metabolites with potent bioactivities (Joyner and Cichewicz, 2011; Newman and Cragg, 2012; Williams et al., 2011). Furthermore, these bioactive molecules often possess unique chemical characteristics that allow them to penetrate the blood-brain barrier to reach the brain, making them suitable for neurodegenerative disorders like AD (Joyner and Cichewicz, 2011). While some forms of traditional medicine have been thoroughly investigated for safety and efficacy, in many cases, there is a lack of knowledge in these regards (WHO, 2002a). In order to help effectively implement traditional medicine into existing health care systems, the World Health Organization has encouraged more scientific studies focused on evaluating these forms of medicine for safety and efficacy (WHO, 2002b).

1.6 Q'eqchi' Maya medicinal plants relevant to AD

Previous ethnobotanical research in Mesoamerica has documented traditional medicinal plants used by a variety of indigenous groups in Belize, Mexico, Guatemala and Honduras (Amiguet et al., 2005; Ankli et al., 1999; Arnason et al., 1980; Comerford, 1996; Giron et al., 1991; Heinrich et al., 1998; Kufer et al., 2005; Lentz, 1993; Michel et al., 2007). The Maya are the largest group in this region and have a rich traditional knowledge, as descendants of the ancient civilization that thrived several millennia ago (Coe, 2011). Past work by the Arnason laboratory with

the Q'eqchi' Maya of southern Belize has shown that traditional healers use a large number of medicinal plants to treat mental health disorders (Amiguet et al., 2005; Bourbonnais-Spear et al., 2005). These studies have focused on neurological disorders including epilepsy and anxiety, and have demonstrated a strong pharmacological basis for many of these medicinal plants (Awad et al., 2009; Bourbonnais-Spear et al., 2005). The plants used for mental health are collected mainly in semi-evergreen tropical forests of southern Belize and adjacent Guatemala. Most plants are herbs, epiphytes and vines rather than trees. High use families include the Acanthaceae, Adiantaceae, Gesneriaceae, Piperaceae, Schizaeaceae, and Lomariopsidaceae. Currently, no studies have focused on Q'eqchi' Maya medicinal plants relevant to AD.

1.7 Hypotheses and research objectives

Due to its implication in pathological conditions, I hypothesized that the loss of PAF-PAFR signaling is beneficial for cognitive performance in the TgCRND8 mouse model, which experiences elevations in specific PAF species as a result of Aβ accumulation. Based on the ethnobotanical data and pharmacological activity of Q'eqchi' Maya medicinal plants used to treat other mental health disorders, I hypothesized that the healers treat symptoms relevant to AD with plants that are pharmacologically active and show preliminary safety and efficacy against AD-relevant pathologies. To test these hypotheses, the objectives of my research were (1) to measure the learning and memory performance of TgCNRD8 mice crossed into a PAFR null mutant background, (2) to interview Q'eqchi' Maya healers about

medicinal plants used to treat symptoms relevant to AD, and (3) to test the traditional botanical medicines for pharmacological activity against two AD targets, excitotoxicity and aberrant lipid metabolism, as a preliminary assessment of safety and efficacy. The loss of PAFR was predicted to prevent spatial learning and memory impairments that have been previously reported in the TgCRND8 model and the ethnobotanical interviews were predicted to yield a list of medicinal plants with *in vitro* bioactivities relevant to AD pharmacological targets.

CHAPTER 2

BEHAVIOURAL ASSESSMENT OF THE ROLE OF PAFR IN A MOUSE MODEL OF AD

2.1 Introduction

AD is currently the most common form of dementia and is characterized by neuronal loss, extracellular deposits of Aβ, and intracellular accumulations of neurofibrillary tangles composed of hyperphosphorylated tau protein (Ittner and Gotz, 2011). The “amyloid cascade” hypothesis defines aberrant Aβ metabolism, resulting in the accumulation and aggregation of Aβ fragments, specifically soluble $A\beta_{42}$ oligomers, as the principle cause of AD (Hardy and Selkoe, 2002). Despite immense efforts, targeting Aβ metabolism directly has faced a number of challenges (Selkoe, 2011), leading to an increased focus on targets further downstream of Aβ biogenesis as potential avenues for complementary therapeutics (Huang and Mucke, 2012).

Lipid metabolism has become an area of interest in AD research, as lipid signalling pathways are essential to brain function and an increasing number of them have been shown to be dysfunctional in AD (Di Paolo and Kim, 2011). Recently, the Bennett laboratory has shown that disruptions in alkylacylglycerophosphocholine metabolism occur in the brains of AD patients, leading to the accumulation of lipid second messengers known as PAFs (Ryan et al., 2009). PAFs can signal cellular events through activation of the G-protein coupled PAFR (Ishii and Shimizu, 2000). Previous studies have demonstrated that under normal physiological conditions, PAF-PAFR signaling is important for learning

and memory processes (Chen et al., 2001; Izquierdo et al., 1995; Kato et al., 1994). However, when PAF levels become elevated under pathological conditions, these signaling cascades can contribute to neuronal damage, through mechanisms involving excitotoxicity and microglial activation (Bate et al., 2006; Bellizzi et al., 2005; Bennett et al., 1998; Ryan et al., 2008). With this complexity, it is unclear whether PAF-PAFR signaling in the brain is beneficial or detrimental to learning and memory in AD.

To address this issue, the objective of the current study was to measure the learning and memory performance of the TgCNRD8 mouse model of AD (Chishti et al., 2001) crossed into a PAFR null mutant background (Ishii et al., 1998). The TgCNRD8 mice express a double mutant form of the human APP and have reported accumulations of discrete PAF species in the brain and impairments in learning and memory (Chishti et al., 2001; Ryan et al., 2009). The effects of the loss of PAFR signal transduction on behavioural indices of cognition under pathological conditions of Aβ accumulation and increased PAF production was assessed using the Morris water maze behaviour test of spatial learning and memory.

2.2 Methodology

2.2.1 Animals

All animal work in this study was approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care. Breeding pairs of $PAFR^{-/-}$ mice in a hybrid C57BL/6 x 129/Ol background (Ishii et al., 1998) were kindly provided by Dr. Takao Shimizu (University of Tokyo, Japan) and were back-bred into a C57BL/6 lineage for 11 generations in the Bennett laboratory. Breeding pairs of TgCRND8 mice (Tg), expressing a double mutant form of the human APP (KM670/671NL+V717F) under the control of the prion protein promoter (Chishti et al., 2001) on a mixed C57BL/6 x C3H background were kindly provided by Dr. Paul Fraser (University of Toronto, Ontario) and back-bred in the Bennett laboratory for 5 generations into a C57BL/6 background. When crossed with $PAFR^{-/-}$ mice, Tg and nonTg mice heterozygous for PAFR were produced. These lines were successively crossed to produce progeny (nonTg $PAFR^{+/+}$ [wild-type], nonTg $PAFR^{-/-}$, Tg $PAFR^{+/+}$, and Tg $PAFR^{-/-}$) on a N5 C57BL/6 x C3H background.

Genotypes were assessed using PCR analysis of ear biopsies, taken at 21 days of age from each animal, and reconfirmed upon sacrifice. Tissue was digested in 100 mM Tris-Cl (pH 8.5), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 10 mg/mL proteinase K solution overnight at 55°C. A solution of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the samples were centrifuged for 10 min at 12,000 g. The supernatant was collected, and the DNA precipitated in isopropanol, followed by a second centrifugation for 10 min at 12,000 g. Pelleted DNA was washed with

70% ethanol and dried at room temperature. Isolated DNA was re-suspended in 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA (pH 8.0) solution in deionized/demineralized water. The DNA was used in three PCR reactions with primer sets (Table 2.1) to test for the presence of the PAFR sequence ($PAFR^{+/+}$), the neomycin targeted disruption sequence ($PAFR^{-/-}$), and the human APP sequence (Tg). For the $PAFR^{+/+}$ and $PAFR^{-/-}$ reactions, 5 µL DNA was added to 16 µL nuclease-free H_2O, 2.5 µL 10X Advantage2 PCR Buffer (Clontech), 0.25 µL 10 mM deoxyribonucleotide triphosphates, 0.5 µL of 200 ng/µL forward primer (IDT), 0.5 µL of 200 ng/µL reverse primer (IDT), and 0.25 µL of 50X Advantage2 DNA Polymerase Mix (Clontech). The reaction was run for 10 min at 95°C, followed by 30 cycles of 20 s at 95°C, 20 s at 65°C, and 50 s at 70°C (Biometra). $PAFR^{+/+}$ mice produce a 289 bp amplicon in the $PAFR^{+/+}$ reaction, and no amplicon in the $PAFR^{-/-}$ reaction. $PAFR^{-/-}$ mice produce a 1.046 kB amplicon in the $PAFR^{-/-}$ reaction, and no amplicon in the $PAFR^{+/+}$ reaction. For the Tg reaction, 4 µL DNA was added to 13.5 µL nuclease-free H_2O, 2.5 µL 10X Advantage2 PCR Buffer (Clontech), 0.5 µL 10 mM deoxyribonucleotide triphosphates, 2 µL of 10 pmol/µL forward primer (IDT), 2 µL of 10 pmol/µL reverse primer (IDT), and 0.5 µL of 50X Advantage2 DNA Polymerase Mix (Clontech). The reaction was run for 3 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 68°C, and 90 s at 72°C, followed by 7 min at 73°C (Biometra). Tg mice produce a 1 kB amplicon.

Genotyped animals were separated from littermates at 2 months of age, and singly housed until the end of behaviour testing. Male animals were tested in the Morris water maze at 5 months of age.

Table 2.1. Primer sequences used for genotyping

Reaction	Primer	Sequence
$PAFR^{+/+}$	Forward	5’-TAT GGC TGA CCT GCT CTT CCT GAT-3’
	Reverse	5’-TAT TGG GCA CTA GGT TGG TGG AGT-3'
$PAFR^{-/-}$	Forward	5'-GGA TGG AAG CCG GTC TTG TC-3'
	Reverse	5’-GGC ACT AGG TTG GTG GAG TC-3'
Tg	Forward	5’-GGC CGC GGA GAA ATG AAG AAA CGC CAA GCG CCG TGA CT-3'
	Reverse	5’-TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A-3’

2.2.2 Morris water maze

The Morris water maze was used to assess learning and memory and was conducted in the Behavioural Core facility at the University of Ottawa. The maze apparatus consisted of a blue plastic pool (Med Associates Inc.) with an internal diameter depth of 127 cm and 42 cm, respectively. The pool was filled with water that was maintained at 21°C (Ranco ETC) and made opaque with white, water-soluble, non-toxic paint (My Scholar's). A hidden platform with a diameter of 10 cm was placed 1 cm below the surface of the water. A black square and cross were placed on the walls surrounding the pool as visual cues for spatial orientation. The experimental room that housed the maze apparatus was kept at a temperature of 21°C and set to a light level of 100 lux (Extech Instruments). White noise emitted at 70 dB (San Diego Instruments) in the experimental room ensured no auditory cues would influence the test.

The test was run for nine consecutive days, between zeitgeber 7.5 and 10.5. One hour before each test, animals were habituated to the experimental room, out of sight from the maze apparatus. The first eight days assessed spatial learning and consisted of 4 trials per day, with a 20-minute inter-trial rest period. The hidden platform remained in the same quadrant of the pool throughout this phase of the test. Mice were placed in the edge of the pool from one of four semi-randomized locations. Each trial lasted a maximum of one minute or until the mouse reached the platform and remained on it for at least five seconds. If the mouse did not reach the platform within one minute, it was directed to it by the experimenter. Animals were removed from the pool after spending 15 seconds on the platform. The ninth day

assessed reference memory and consisted of a probe test, where the platform was removed from the pool and a single trial was run for one minute.

Escape latency, swimming distance, swimming velocity, and percent time spent in the platform quadrant were measured using EthoVision XT 8.0 (Noldus Information Technology). Path efficacy was calculated by dividing the path length swam by the shortest possible path length to the platform, for each trial. Based on the classification system by Brody and Holtzman (2006), search strategy was assigned into three categories: 1) spatial, 2) non-spatial systematic, 3) repetitive looping by assessment of trial video recordings, done by two independent investigators blinded to the identity of the animals (see Appendix 1.1). Animals were classified into a fourth category, floating, if they exhibited a trial escape latency of over 50 s and average velocity below 6 cm/s (Janus, 2004). Animals that exhibited daily averages within this floating category for five or more days out of the spatial learning phase were considered to be floaters, and were excluded from the data analysis.

2.3 Results

To assess the role of PAF activation of PAFR in learning and memory in the context of AD, four strains of mice were tested in the Morris water maze. The Tg PAFR$^{+/+}$ mice, with double mutant human APP expressing PAFR showed impaired learning compared to nonTg PAFR$^{+/+}$ wild-type mice, as indicated by higher escape latencies in the spatial learning phase of the test (Figure 2.1A). Interestingly, the loss of PAFR in the Tg PAFR$^{-/-}$ mice ameliorated learning impairments, as these mice performed comparably to wild-type mice (Figure 2.1A). Reference memory was not impaired in nonTg PAFR$^{-/-}$ mice or in either Tg genotypes, as there was no significant difference from wild-type mice in platform zone bias in the probe test (Figure 2.1B). The learning deficits in Tg PAFR$^{+/+}$ mice were not due to motoric impairments, as these mice swam at a comparable velocity to wild-type mice (Figure 2.1C). Because both Tg PAFR$^{+/+}$ and Tg PAFR$^{-/-}$ mice exhibited slightly higher velocities than wild-type mice, with Tg PAFR$^{-/-}$ speed reaching statistical significance (Figure 2.1C), path efficacy was used as a velocity-independent measure of performance in the maze. The path efficacy analysis was in accordance with escape latencies, as Tg PAFR$^{+/+}$ mice showed reduced path efficacy compared to wild-type mice, while no difference was detected between either Tg PAFR$^{-/-}$ or nonTg PAFR$^{-/-}$ mice and wild-type mice (Figure 2.1D).

An analysis of the search strategies used by the four strains of mice in the Morris water maze revealed differential navigation strategies used over the course of the spatial learning phase (Figure 2.2A,B,C,D). The only group that showed a significant incidence of floating was the nonTg PAFR$^{-/-}$ mice (Figure 2.2E).

Interestingly, only Tg PAFR$^{+/+}$ mice used significantly less spatial strategy than wild-type mice, while both Tg PAFR$^{+/+}$ and, to a lesser degree, Tg PAFR$^{-/-}$ had higher incidences of repetitive looping than wild-type mice (Figure 2.2E).

Figure 2.1. Learning and memory performance in Morris water maze.

Learning and memory of nonTg PAFR$^{+/+}$, nonTg PAFR$^{-/-}$, Tg PAFR$^{+/+}$, and Tg PAFR$^{-/-}$ mice was assessed using the Morris water maze. Measured parameters included A) escape latency [two-way repeated measures ANOVA df [3,33] F=4.2, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), * $p<0.05$], B) average percent time spent in the platform zone on the probe test [one-way ANOVA, df [3,33] F=2.0], C) average swim velocity over the spatial learning phase [one-way ANOVA df [3,33] F=7.8, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), * $p<0.05$], and D) path efficacy [two-way repeated measures ANOVA, df [3,33] F=7.6, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), ** $p<0.01$].

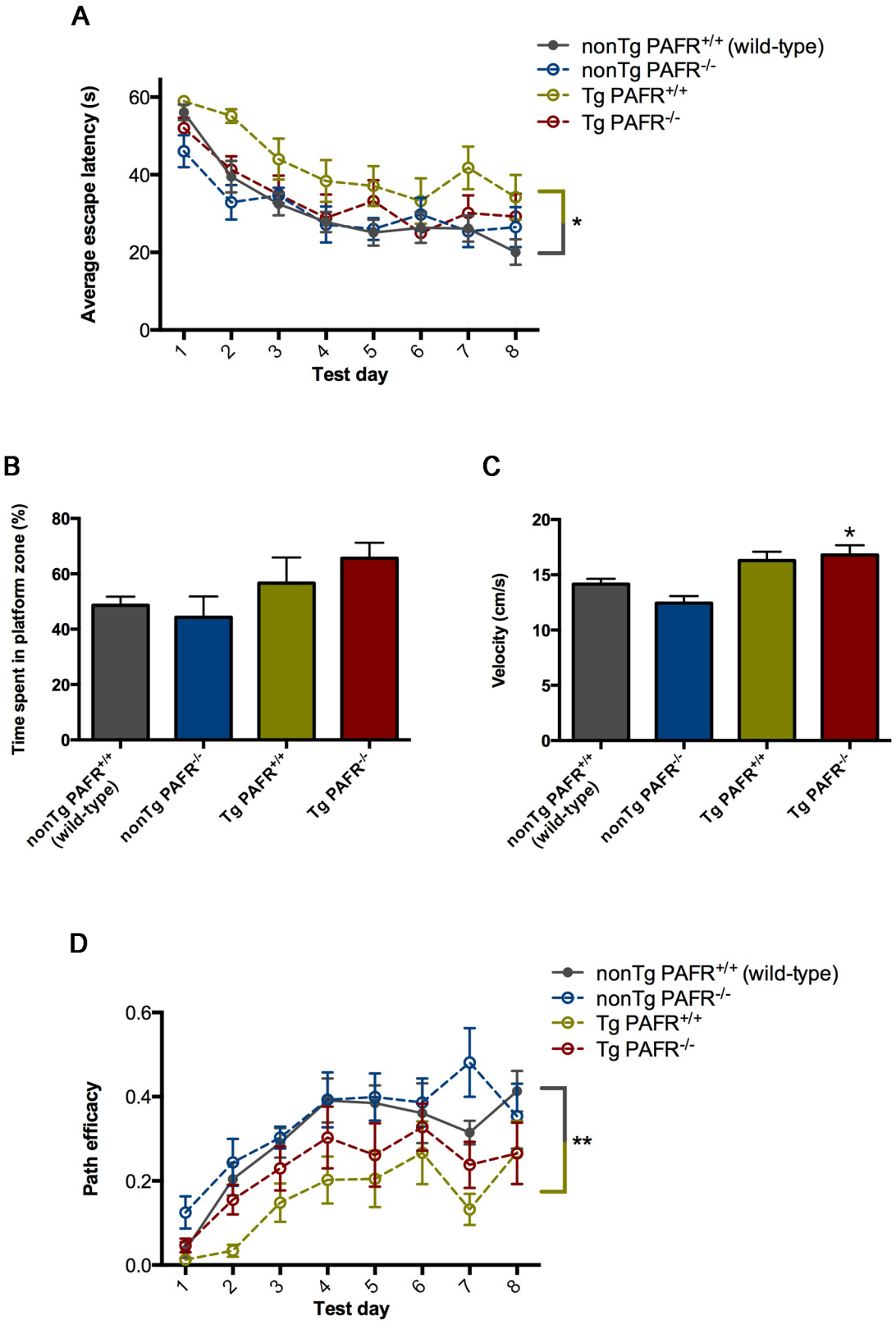
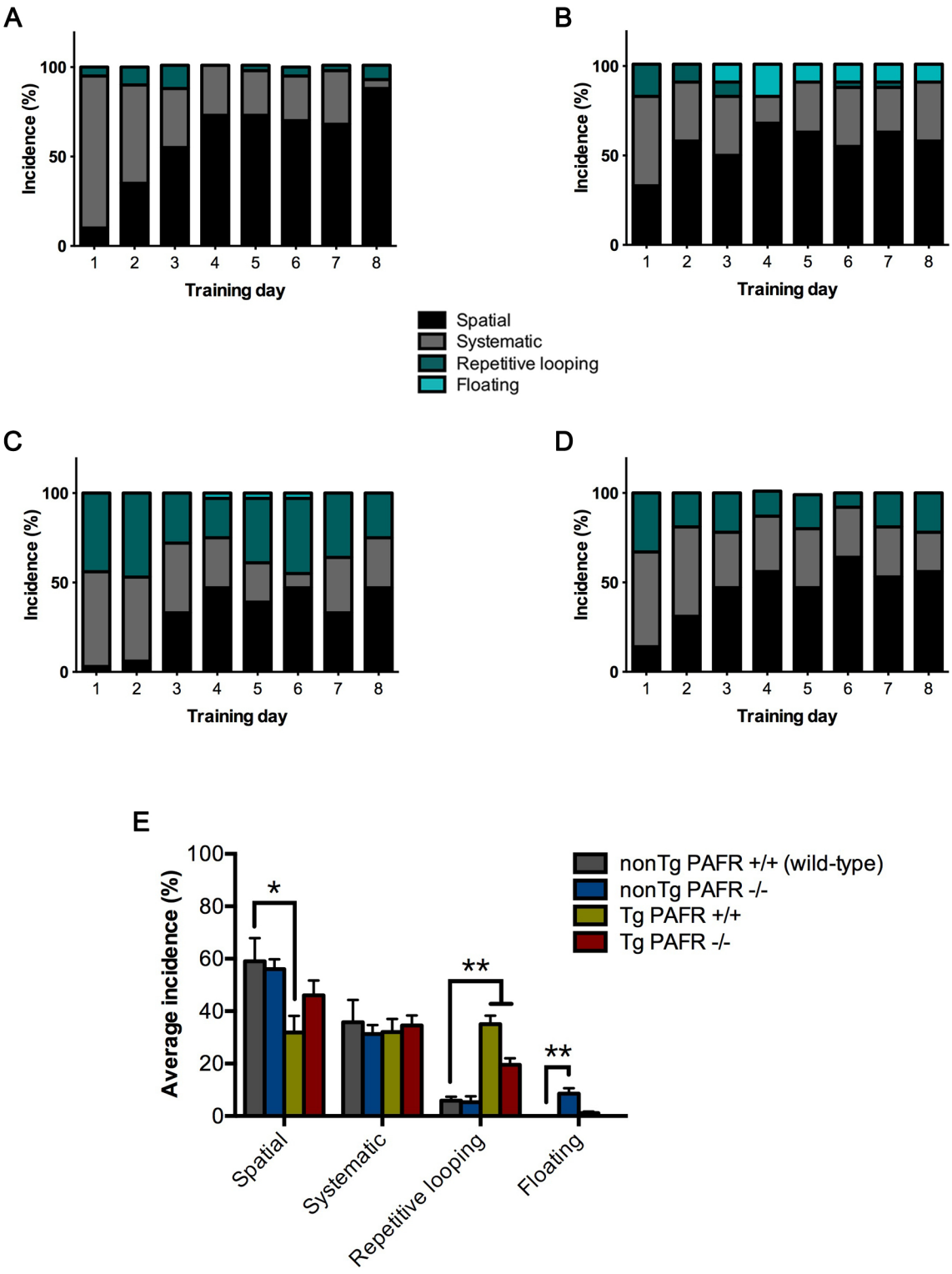

A
nonTg PAFR+/+ (wild-type)
nonTg PAFR-/-
Tg PAFR+/+
Tg PAFR-/-
Average escape latency (s)
60
40
20
0
1
2
3
4
5
6
7
8
Test day
*
B
Time spent in platform zone (%)
80
60
40
20
0
nonTg PAFR+/+ (wild-type)
nonTg PAFR-/-
Tg PAFR+/+
Tg PAFR-/-
C
Velocity (cm/s)
20
15
10
5
0
*
nonTg PAFR+/+ (wild-type)
nonTg PAFR-/-
Tg PAFR+/+
Tg PAFR-/-
D
nonTg PAFR+/+ (wild-type)
nonTg PAFR-/-
Tg PAFR+/+
Tg PAFR-/-
Path efficacy
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
Test day
**

Figure 2.2. Search strategies of mice in the Morris water maze.

Search strategies of nonTg PAFR$^{+/+}$, nonTg PAFR$^{-/-}$, Tg PAFR$^{+/+}$, and Tg PAFR$^{-/-}$ mice were classified into spatial, non-spatial systematic, repetitive looping, and floating. Daily percent incidence of search strategies for the spatial learning phase was determined for A) nonTg PAFR$^{+/+}$, B) nonTg PAFR$^{-/-}$, C) Tg PAFR$^{+/+}$, and D) Tg PAFR$^{-/-}$ mice. E) Average percent incidence of the strategies over the spatial learning phase for the four different genotypes [spatial: one-way ANOVA, df [3,28] F=3.6, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), * $p<0.05$; systematic: one-way ANOVA, df [3,28] F=0.14; repetitive looping: one-way ANOVA, df [3,28] F=32.4, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), ** $p<0.01$; floating: one-way ANOVA, df [3,28] F=14.3, *post-hoc* Dunnett's test comparing each group to control (nonTg PAFR$^{+/+}$), ** $p<0.01$].


A
B
C
D
E
Incidence (%)
Training day
Spatial
Systematic
Repetitive looping
Floating
Average incidence (%)
nonTg PAFR +/+ (wild-type)
nonTg PAFR -/-
Tg PAFR +/+
Tg PAFR -/-
*
**
**
Spatial
Systematic
Repetitive looping
Floating

2.4 Discussion

Here, I show that a loss in PAFR reduces spatial learning impairments in the TgCRND8 mouse model of AD. These learning improvements coincided with a restoration of spatial search strategy comparable to wild-type mice, and a partial reduction in repetitive looping. These behavioural effects were only observed in the context of the aberrant Aβ metabolism present in the TgCNRD8 model, as PAFR null-mutation in nonTg $PAFR^{-/-}$ mice had no impact on learning.

Taken together, these results suggest that inhibiting PAFR signalling under pathogenic conditions of Aβ aggregation and PAF accumulation in the brain is beneficial for learning. This conclusion is consistent with that of Row et al. (2004), whereby mice deficient of PAFR were protected from learning deficits associated with intermittent hypoxia, another form of neurological insult that leads to PAF accumulation in the brain. The protective effect observed in this present study only occurred under pathogenic conditions of predicted PAF elevations, with no measurable learning benefits associated with PAFR knockout in wild-type mice. This is also consistent with Row et al. (2004), as they did not detect any learning differences between normoxic $PAFR^{-/-}$ and $PAFR^{+/+}$ mice. In many ways, these findings are counter-intuitive in that PAFR has been shown previously to contribute to long-term potentiation, a cellular underpinning of learning, albeit with some controversy (Chen et al., 2001; Izquierdo et al., 1995; Kato et al., 1994; Kobayashi et al., 1999; Wieraszko et al., 1993). Interestingly, the Bennett laboratory and others have shown that when specific PAF isoforms are elevated in response to Aβ accumulation, PC(*O*-16:0/2:0) signals neurotoxicity (Bate et al., 2006; Bate et al.,

2004; Bellizzi et al., 2005; Ryan et al., 2009). These findings suggest that mice lacking PAFR retain intact long-term potentiation in the hippocampus, as argued by Kobayashi et al. (1999), likely through PAFR-independent mechanisms, yet are protected from PAFR-dependent impairment elicited by pathogenic concentrations of PC(*O*-16:0/2:0) and likely other PAF species.

Quantitative analysis of search strategies used by Tg mice in the Morris water maze was conducted previously by Janus (2004). Impaired performance coincided with the preferential use of the less efficient repetitive looping strategy over the more efficient spatial strategy exhibited by wild-type mice (Janus, 2004). While the underlying mechanisms leading to higher incidences of repetitive looping in Tg mice remain unknown, my results suggest that a disruption of PAFR can partially restore the ability of impaired animals to use a spatial strategy. One possible explanation for this may involve PAF and glutamate signalling. PAF activation of PAFR signals presynaptic release of glutamate (Clark et al., 1992). Repetitive circling behaviour in rodents can be induced by pharmacological activation of NMDARs, and prevented with an NMDAR antagonist (Antunes Wilhelm et al., 2013; Santamaria and Rios, 1993; Thanos et al., 1992). Arguably, the repetitive looping strategy may be reflective of increased NMDAR activation by elevated glutamate signalling in the Tg mice. Recent published and unpublished work from the Bennett laboratory has shown that PAF levels are elevated in specific brain regions of symptomatic Tg mice, which would be expected to increase glutamate release and NMDAR activity (Ryan et al., 2009). If so, $PAFR^{-/-}$ mice would be protected from this effect. Future work will examine this mechanism in more detail through comparisons of brain PAF levels and markers of excitotoxicity between Tg $PAFR^{+/+}$ and Tg $PAFR^{-/-}$ mice.

While I did not detect any differences in reference memory between any of the genotypes, previous studies have shown memory deficits in the Tg model and mice with disrupted PAFR. Chishti et al. (2001) demonstrated that Tg mice had reduced performance in the probe test of the Morris water maze. It is important to note that the probe test was administered on day five in their maze paradigm, whereas this study administered it on day nine. Together, these findings suggest that reference memory impairments in the Tg model can be overcome with prolonged learning. Izquierdo et al. (1995) showed that administration of a PAFR antagonist to certain regions of the rodent brain resulted in impaired working memory. Similarly, Row et al. (2004) demonstrated impaired working memory in $PAFR^{-/-}$ mice. Work by Saraf et al. (2003) suggests the effects on memory from PAFR disruption is specific to memory recall as opposed to the creation of new memories, as they only detected retrograde amnesia in mice treated with a PAFR antagonist, but not anterograde amnesia. As the maze paradigm used here exposed animals to the learning phase to a greater extent than those used by Row et al. (2004) and Saraf et al. (2003), it suggests that these amnesic effects may be overcome with repeated exposure to the task. Further behavioural work differentiating between short-term and long-term memory, as well as between working and reference memory, will help clarify if such impairments exist in Tg $PAFR^{-/-}$ mice.

In light of the potential amnesic effects of PAFR antagonism, one approach for future therapeutic intervention could focus on the pharmacological modulation of PAF levels. The PLA_2 enzymes, which generate the immediate PAF precursor, *lyso*-PAF, represent potential targets for such modulation. Interestingly, $sPLA_2$ activity

has been shown to be upregulated in the brain and spinal fluid of AD patients (Moses et al., 2006). Inhibiting $sPLA_2$ could effectively reduce PAF levels, restoring normal glutamate signalling and reducing microglial activation (Bate et al., 2006; Bennett et al., 1998), while leaving neuroprotective PAFR pathways intact (Ryan et al., 2007). Another approach would be to target NMDARs to prevent downstream excitotoxicity resulting from the elevated glutamate signalling that occurs in AD, and to which PAFR overactivation likely contributes.

Overall, the behavioural results of this study suggest that PAFR-mediated signalling is, in part, responsible for the learning impairments exhibited in the TgCRND8 model of AD. This supports the pivotal role of lipid metabolism and glutamate signalling in AD, and highlights PAF-generating enzymes and glutamate receptors, including $sPLA_2$s and NMDARs, as potential therapeutic targets that will be explored in Chapters 3 and 4.

CHAPTER 3

ETHNOBOTANY OF Q'EQCHI' MAYA MEDICINAL PLANTS RELEVANT TO AD

3.1 Introduction

With an estimated global prevalence of over 35.6 million people, AD is currently the most common form of dementia (Ferri et al., 2009). Over half of these patients live in developing countries (Ferri et al., 2009), where pharmaceuticals are usually not accessible or affordable (WHO, 2002a). In these areas, traditional medicine is often a more relevant form of health care, due to increased availability and lower costs (WHO, 2002a). Thus, research on traditional medicine helps provide insight into valuable therapeutic options for a large number of AD patients.

Mesoamerica, like many other parts of the world, is currently facing the challenges of AD (Ferri et al., 2009). Given the exceptional biodiversity of this region (Myers et al., 2000), there is considerable potential for the discovery of new therapeutic leads from nature. Interestingly, previous work with the Q'eqchi' Maya of southern Belize has shown that their traditional healers use a large number of plants to treat mental health disorders (Amiguet et al., 2005; Bourbonnais-Spear et al., 2005). While these studies have focused on other neurological conditions including epilepsy and anxiety (Awad et al., 2009; Bourbonnais-Spear et al., 2005), none have examined whether Q'eqchi' Maya healers use plants to treat symptoms relevant to AD.

AD is a complex neurodegenerative disease involving multiple brain pathologies (Huang and Mucke, 2012) that lead patients to exhibit a number of behavioural symptoms (Alzheimer Society, 2013). The objective of this research

project was to document Q'eqchi' Maya botanical medicines relevant to AD. To achieve this, I interviewed healers about their medicinal plants used to treat common symptoms of AD. Furthermore, recent studies have implicated the PLA_2 enzymes in AD and demonstrated an upregulation in $sPLA_2$ activity (Schaeffer et al., 2010). Since $sPLA_2$ is a major active principle in snake venom and medicinal plants for snakebite have been shown to contain $sPLA_2$ inhibitory phytochemicals (Nevalainen et al., 2012; Nunez et al., 2005), I also interviewed the healers about their medicinal plants for treatment of snakebites.

3.2 Methodology

3.2.1 Ethical approval

This ethnobotanical study was part of a collaborative study on traditional medicine under an agreement between the University of Ottawa and the Q'eqchi' Healers Association (QHA), and its parent organization, the Belize Indigenous Training Institute, which is a non-governmental organization in Belize. Prior to fieldwork, the project was approved by the QHA in August 2011 and accepted by the University of Ottawa Research Ethics Board [Approval #H-12-11-03] in February 2012. Plant collection permits were obtained from the Belize Forest Department, Ministry of Natural Resources and the Environment in May 2012 [Ref #CD/60/3/12 (24)].

3.2.2 Participants

Four Q'eqchi' Maya healers from the QHA participated in the study. The healers were recruited and informed consent was obtained according to specific guidelines approved by the University of Ottawa Research Ethics Board. All participants were men, as the QHA is solely composed of male healers. The healers ranged in age from 45-80 and resided in four different villages within the Toledo District of Belize. These participants are well recognized in their communities as traditional healers and together provide a representation of the traditional medicinal knowledge of the Q'eqchi' Maya.

3.2.3 Study site

The ethnobotanical research was conducted during May 2012, in four main areas within the Toledo District of southern Belize (Figure 3.1). Belize has a sub-tropical climate, with a pronounced dry season that lasts from February to May. The southern region of the country receives up to three times more rainfall compared to the Northern region (Beletsky et al., 2005) and supports a semi-evergreen tropical forest. Many of the study areas selected by the healers are preferred collection sites for traditionally used medicinal plants.

3.2.4 Interviews

Each healer was interviewed independently about their traditional medicines used to treat symptoms of AD, as well as their treatments for snakebites. The symptom list for AD was based on the most common behavioural warning signs for the disease (Table 3.1). A partially-scripted, open-ended questionnaire was used to guide the interviews (Appendix 1.2). The interviews were conducted in Q'eqchi' and English, with the aid of translators fluent in both languages.

3.2.5 Plant collections

Botanical collection trips were made with the healers to collect each plant cited in the interviews. Voucher specimens, as well as plant material for laboratory analyses, were collected. The voucher specimens were sent to the New York Botanical Gardens and Universidad Nacional de Costa Rica for taxonomic identification, and deposited in the Belize Forestry and University of Ottawa Herbariums (see Appendix 1.3). The plant material for analyses was bottled

Figure 3.1. Botanical collection sites in the Toledo District of Belize.

Medicinal plants cited in ethnobotanical interviews were collected from four sites in the Toledo District of Belize. The sites included 1) Jalacte, 2) Blue Creek, 3) Big Falls, and 4) Indian Creek. This map was generated using GPS coordinates taken with an Explorist 310 (Magellan) and BaseCamp software (Garmin).

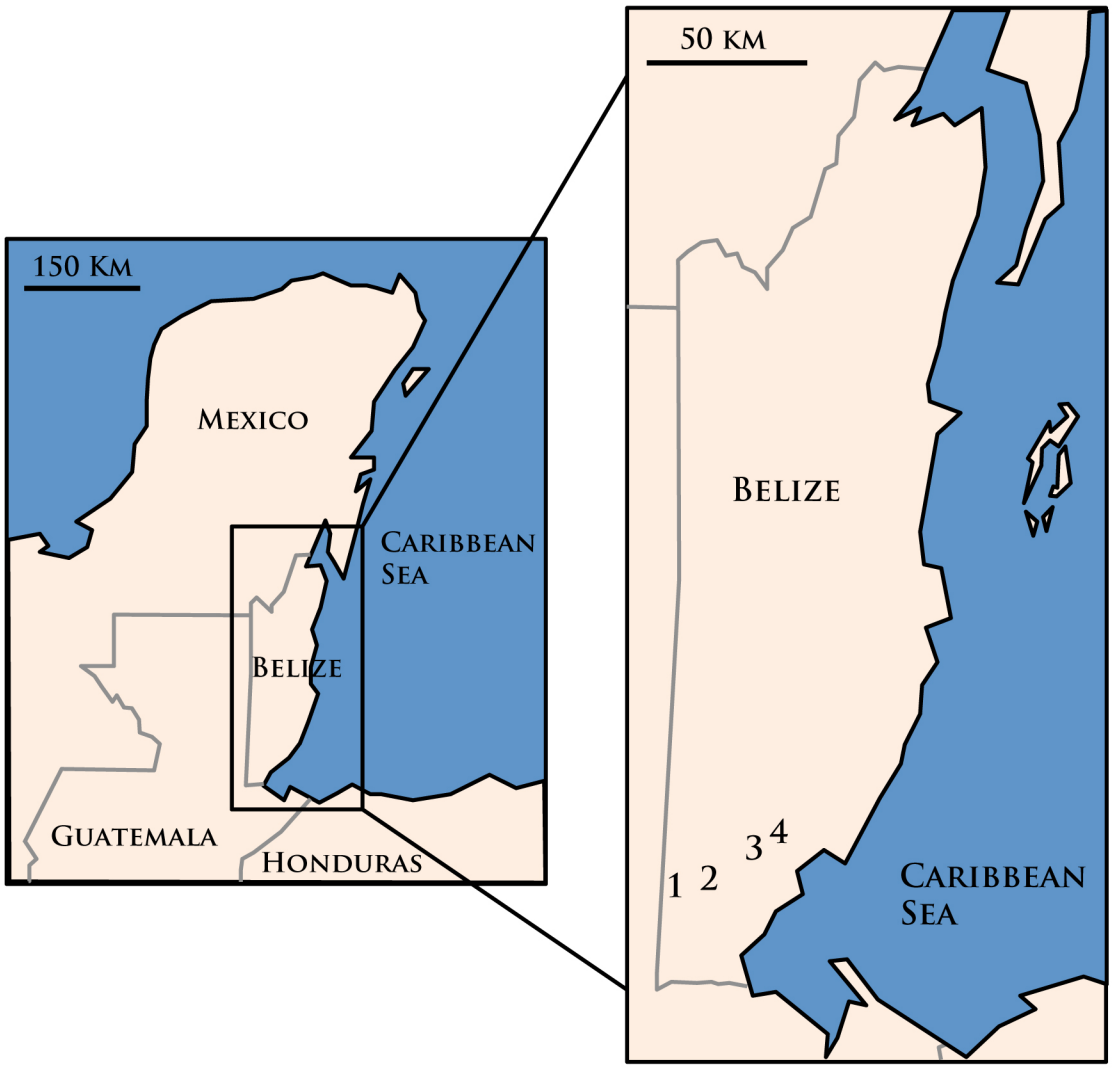

150 Km
Mexico
Caribbean Sea
Belize
Guatemala
Honduras
50 km
Belize
1 2 3 4
Caribbean Sea

Table 3.1. List of AD symptoms included in ethnobotanical interviews with Q'eqchi' Maya healers. Ten common behavioural symptoms of AD were included in the partially-scripted, open-ended interviews to identify Q'eqchi' Maya medicinal plants used to treat symptoms of AD.

Symptoms
Memory loss that affects day-to-day function
Difficulty performing familiar tasks
Problems with language
Disorientation of time and place
Poor or decreased judgment
Difficulty with problem-solving
Misplacing things
Changes in mood and behaviour
Changes in personality
Loss of initiative

individually with approximately 1 mL/g of 70% isopropyl alcohol for the duration of the fieldwork.

3.2.6 Syndromic Importance Value

The Syndromic Importance Value (SIV) was used to quantitatively analyze the ethnobotanical data in order to reveal plant species with a high potential for bioactivity and prioritize future pharmacological investigation. The SIV was developed by Leduc et al. (2006) and incorporates three parameters to rank plants: (1) the number of different symptoms for which the plant is cited, (2) the frequency of citation by the healers, and (3) the relative importance of symptoms for which the plant is cited. The SIV for each plant species was calculated using the following equation according to Leduc et al. (2006):

$$\mathrm{SIV} = \frac{\left[\frac{\sum ws}{S}\right] + \left[\frac{\sum wf}{SF}\right]}{2} = \frac{\sum ws + \left[\frac{\sum wf}{F}\right]}{2S}$$

where *w* is the symptom weight, *s* is the symptom contribution for the species, *S* is the total number of symptoms treated by the healers, *f* is the citation frequency for the species, and *F* is the total number of interviews conducted. The symptom weight, *w*, represents the degree of association of each symptom to ailment of interest. Each *w* is a value between 0 and 1, where a larger value represents a more associated symptom and the $\sum w = 1$. For both the interviews on AD symptoms and snakebites, only symptoms highly associated with these ailments were included in the interviews, thus all were assigned a *w* of equal value. The symptom contribution,

s, is based on a plant species being cited for the particular symptom or not, and is either 1 or 0, respectively. For the AD symptoms interviews, if a plant species was cited for all of symptoms that were treated by the healers, $\sum s = S = 9$ (only 9 out of 10 symptoms included in the interview were treated with at least one plant). For the snakebite interviews, since snakebite is a symptom in itself and was the only one included in the interviews, $\sum s = S = 1$. The citation frequency, *f*, represents the number of times that a plant species is cited for a particular symptom. The maximum $\sum f = SF$, and would be 9 x 4 = 36 for the medicinal plants for AD symptoms and 1 x 4 = 4 for snakebites, if all healers were to cite a plant species for all of the treated symptoms.

3.3 Results

To document the Q'eqchi' Maya medicinal plants relevant to AD, healers were asked in interviews to identify and discuss traditional medicines used to treat symptoms of AD, as well as their treatments for snakebites. Nine out of the ten symptoms of AD (Table 3.1), as well as snakebites, were treated by the Q'eqchi' Maya healers with medicinal plants. Difficulty with problem-solving was the only symptom not treated by the healers with a plant, and thus may not be a culturally relevant symptom for the Maya.

A total of 18 plants used to treat symptoms of AD were collected, 16 of which were identified to the species level (Table 3.2). For the treatment of snakebites, a total of 4 plants were collected, 3 of which were identified to the species level (Table 3.3). These plants were collected with the healers from several locations in the Toledo District, including Jalacte, Blue Creek, Indian Creek, and Big Falls (Figure 3.1).

While the stems, flowers, roots, and/or whole plant may be used, the leaves are the plant organ used most often (Table 3.2, 3.3). All of the plants are prepared by maceration, sometimes without water or in boiling water, but usually in cold water. The treatments are administered orally or used to bathe the full body or the just the head.

Overall, the medicinal plants showed a range of SIVs (Figure 3.2A,B), with *Bolbitis pergamentacea*, *Columnea sulfurea,* and *Mikania leiostachya* identified as the top ranked species for AD symptoms and *Peperomia urocarpa* as the top ranked species for snakebites.

Table 3.2. Medicinal plants used by the Q'eqchi' Maya healers to treat symptoms of AD. Plants are organized by plant family, with botanical names, Q'eqchi' names (when available), plant organs used, preparation methods, and routes of administration included. Of the 18 plants cited in the interviews, only the 16 that could be identified to the species level are shown.

Family	Botanical name	Q'eqchi' Name	Plant organ	Preparation	Administration
Acanthaceae	*Bravaisia grandiflora* Donn.Sm.	*Tzlub che*	Leaves	C	Oral
Acanthaceae	*Justicia pectoralis* Jacq.	*Xu' kuy kok*	Leaves	C	Oral
Acanthaceae	*Odontonema callistachyum* (Cham. & Schltdl.) Kuntze	-	Shoots	C	Oral, Bathe
Adiantaceae	*Adiantum latifolium* Lam.	*Roq tchi quan*	Leaves	C	Oral
Adiantaceae	*Adiantum tetraphyllum* Humb. & Bonpl. ex Willd.	*Caa'nil pim*	Leaves	C	Oral, Bathe
Asteraceae	*Baccharis trinervis* (Lam.) Pers.	*Se'rek pim*	Leaves	C	Oral
Asteraceae	*Mikania leiostachya* Benth.	-	Leaves	C	Oral, Bathe Head
Asteraceae	*Porophyllum ruderale* (Jacq.) Cass.	*So' sol pim*	Leaves	C	Oral, Bathe
Gesneriaceae	*Besleria laxiflora* Benth.	*Xjalom ma' saa'n*	Shoots	C	Oral, Bathe
Gesneriaceae	*Columnea sulfurea* Donn.Sm.	*Ka'ki pim*	Leaves, Flowers, Stems	C	Oral, Bathe, Bathe Head
Gesneriaceae	*Drymonia serrulata* (Jacq.) Mart.	*Bak' nel' pim*	Leaves	C	Oral, Bathe
Lomariopsidaceae	*Bolbitis pergamentacea* (Maxon) Ching	-	Whole plant	C	Oral, Bathe
Marcgraviacieae	*Marcgravia gentlei* Lundell	*Rub'el xsa i'xul*	Leaves, Stems	C	Oral, Bathe
Piperaceae	*Peperomia urocarpa* Fisch. & C.A.Mey.	*Xwa i'xul* or *corazon pim*	Leaves	C	Oral, Bathe
Piperaceae	*Piper schiedeanum* Steud.	*Pu' tuch*	Leaves	C or B	Oral, Bathe
Rubiaceae	*Gonzalagunia panamensis* (Cav.) K.Schum.	*Tzul che*	Leaves	C	Bathe

C – maceration in cold water
B – maceration in boiling water

Table 3.3 Medicinal plants used by the Q'eqchi' Maya healers to treat snakebites. Plants are organized by plant family, with botanical names, Q'eqchi' names, plant organs used, preparation methods, and routes of administration included. Of the 4 plants cited in the interviews, only the 3 that could be identified to the species level are shown.

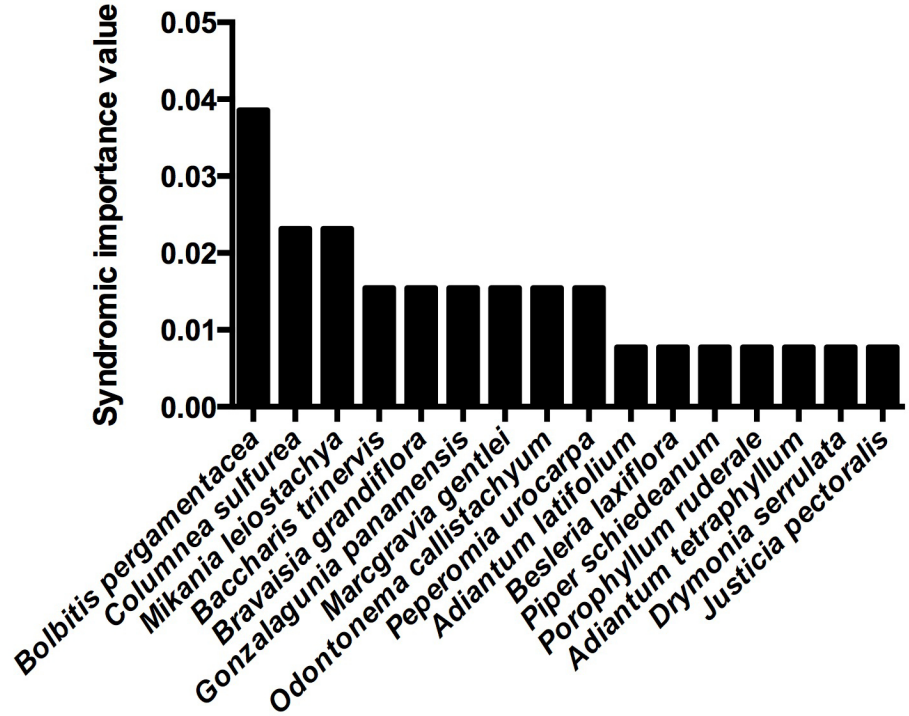
Family	Botanical name	Q'eqchi' Name	Plant organ	Preparation	Administration
Agavaceae	*Sansevieria trifasciata* Prain	*Cu ra riin*	Roots	M	Oral, Topical
Piperaceae	*Peperomia hirta* C. DC.	*Xban' qan chi*	Leaves	C	Oral
Piperaceae	*Peperomia urocarpa* Fisch. & C.A.Mey.	*Xwa i'xul or corazon pim*	Leaves, Stems	M or C	Oral, Bathe, Topical

M – Maceration
C – Maceration in cold water

Figure 3.2. SIVs of Q'eqchi' Maya medicinal plants relevant to dementia.

The SIVs of medicinal plants for A) AD symptoms and B) snakebites. The SIV ranks plants based on three variables, including: (1) the number of different symptoms for which the plant is cited, (2) the frequency of citation by the healers, and (3) the relative importance of symptoms for which the plant is cited.

A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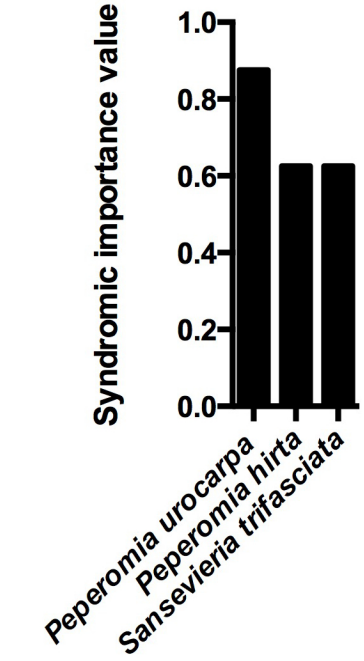
Baccharis trinervis
Bravaisia grandiflora
Gonzalagunia panamensis
Marcgravia gentlei
Odontonema callistachyum
Peperomia urocarpa
Adiantum latifolium
Besleria laxiflora
Piper schiedeanum
Porophyllum ruderale
Adiantum tetraphyllum
Drymonia serrulata
Justicia pectoralis

B


Syndromic importance value
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0.8
0.6
0.4
0.2
0.0
Peperomia urocarpa
Peperomia hirta
Sansevieria trifasciata

3.4 Discussion

In this ethnobotanical study, I show for the first time that the Q'eqchi' Maya healers of southern Belize use a variety of medicinal plants to treat symptoms relevant to AD. While the healers do not recognize the clinical definition of AD, as defined in biomedical nosology, I show that they do treat almost all of the recognized symptoms with a medicinal plant. Previous work has identified snakebite medicinal plants within the Q'eqchi' Maya traditional pharmacopeia (Amiguet et al., 2005), however this is the first study interested in documenting such plants to learn more about their potential to treat $sPLA_2$ upregulation, as it applies to AD.

Earlier ethnobotanical studies in Mesoamerica have documented traditional knowledge from a variety of indigenous groups in Belize, Mexico, Guatemala and Honduras (Amiguet et al., 2005; Ankli et al., 1999; Arnason et al., 1980; Comerford, 1996; Giron et al., 1991; Heinrich et al., 1998; Kufer et al., 2005; Lentz, 1993; Michel et al., 2007). The Maya represent the largest group in this region, and hold extensive traditional knowledge as descendants of the ancient civilization that flourished several millennia ago (Coe, 2011). This, coupled with the extensive biodiversity found in Mesoamerica (Myers et al., 2000), provides a unique environment for ethnobotanical research. Our interest in the Q'eqchi' Maya treatments for AD symptoms stemmed from previous work that documented a large number of plants used by their healers for mental health and neurological pathologies (Amiguet et al., 2005; Bourbonnais-Spear et al., 2005).

A number of plant species identified in our interviews for symptoms of AD belong to the same families as top-ranked plants in a more general survey by

Bourbonnais-Spear et al. (2005) on other neurological ailments. These include Acanthaceae, Adiantaceae, Gesneriaceae, Piperaceae, Schizaeaceae, and Lomariopsidaceae (*B. pergamentacea* has been reclassified from Aspleniaceae to Lomariopsidaceae) (Bourbonnais-Spear et al., 2005). In addition, our study identified several plant species belonging to families that were not top-ranked by Bourbonnais-Spear et al. (2005), including Asteraceae, Marcgraviaceae, Rubiaceae. These findings demonstrate the value of combining general ethnobotanical surveys with more focused surveys on specific ailments when studying a particular traditional medicine.

In the preliminary ethnobotanical survey of Q'eqchi' Maya traditional medicine, Amiguet et al. (2005) documented 20 plant species used for snakebites, a number considerably more than the four documented in our study. This difference is likely attributed to the larger number of healers who were part of the QHA at the time of their study. As with many indigenous communities around the world, the Q'eqchi' are experiencing a decline in traditional practices that parallels the decline in local biodiversity (Alves and Rosa, 2007). The striking differences in the number of healers and plants within the short time frame between these studies highlights the need for conservation of both ecosystems and indigenous cultures.

In an effort to assist the healers in moving their traditional medicine to a more evidence-based system, I describe in the following chapters progress towards evaluating their medicinal plants for safety and efficacy in the context of excitotoxicity and $sPLA_2$ upreguation in AD. The wide variety of species coupled with the range of plant organs used by the healers suggests a large number of potential bioactive principles. The SIV was developed by Leduc et al. (2006) as a quantitative

methodology to rank ethnobotanical data for pharmacological investigation. Little is known on the phytochemistry and bioactivities of the top-ranked plants for symptoms of AD, *B. pergamentacea, C. sulfurea, and M. leiostachya*. A general assessment of neuroprotective activity of these species, along with the other medicinal plants would help in the evaluation of their traditional medicine.

Research on the phytochemistry and bioactivities of the snakebite medicinal plants, *P. urocarpa, Sansevieria trifasciata, and Peperomia hirta* is also limited. The two *Peperomia* species have yet to be characterized phytochemically, or assessed for any biological activity. While certain pure compounds, including steroidal saponins and pregnane glycosides have been isolated from whole plant extracts of *S. trifasciata* (Mimaki et al., 1996, 1997), it is unknown whether they are present in the root, the part of the plant used by the healers. Leaf extracts have been shown to exhibit antipyretic, analgesic, and anti-allergic activities (Anbu et al., 2009; Andhare et al., 2012), but no studies have focused on the activities of root extracts. Future work will aim to assess these snakebite medicines for $sPLA_2$ inhibitory activity.

While the documentation of Q'eqchi' Maya traditional medicine and evaluation of their medicinal plants for safety and efficacy are the primary goals of our collaboration, this work may potentially lead to the identification of novel therapeutic leads for AD. Both the preparation in water and the various routes of administration are appropriate for therapeutics, and the putative bioactivities of the medicines suggest that the active principles are capable of being absorbed, metabolized, and can cross the blood-brain barrier.

Overall, this study highlights the strong medicinal tradition of the Q'eqchi' Maya healers with regards to plants used to treat symptoms relevant to AD.

Continued work towards evaluating these medicinal plants for safety and efficacy will help strengthen the use of Q'eqchi' Maya traditional medicine as a valuable form of health care and may provide AD patients in the region with additional therapeutic options.

CHAPTER 4

IDENTIFICATION OF Q'EQCHI' MAYA MEDICINAL PLANTS WITH NEUROPROTECTIVE AND $sPLA_2$ INHIBITORY ACTIVITY

4.1 Introduction

AD is a complex neurodegenerative disease involving multiple brain pathologies (Huang and Mucke, 2012) that lead to impaired cognitive function in patients. Currently, over half of the estimated 35.6 million patients worldwide live in developing countries where pharmaceuticals that can offer temporary symptomatic relief are usually not accessible or affordable (Ferri et al., 2009; WHO, 2002a). For patients in these areas, traditional medicine is often a more relevant form of health care, with lower costs and increased availability (WHO, 2002a). While the use of some forms of traditional medicine is supported by strong scientific evidence, in many cases, the information on safety and efficacy is inadequate and requires additional scientific evaluation.

Previous ethnobotanical work in Mesoamerica has demonstrated that the Q'eqchi' Maya healers of southern Belize have a strong tradition of treating mental health disorders with medicinal plants (Amiguet et al., 2005; Bourbonnais-Spear et al., 2005). Recently, I documented a number of medicinal plants used to treat symptoms relevant to AD. The primary goal of this work is to assist the healers in moving their traditional medicine towards an evidence-based system. As a continuation of the ethnobotanical research, the objective of the current study was to conduct a pharmacological evaluation of the identified plants against two *in vitro* targets relevant to AD, as a preliminary assessment of safety and efficacy. First, the

medicinal plants used to treat common behavioural symptoms of AD were tested for general toxicity and neuroprotective activity against excitotoxicity. Excitotoxicity is a well-characterized pathological event that occurs in many neurodegenerative diseases, including AD, which involves calcium overload and cellular death as a result of elevated glutamate signalling through NMDARs. Second, the medicinal plants used to treat snakebites were tested for $sPLA_2$ inhibitory activity. The PLA_2 enzymes play an important role in membrane remodelling and generation of lipid signalling molecules in the brain, and recent work has found $sPLA_2$ activity to be upregulated in AD (Schaeffer et al., 2010). The medicinal plants for snakebite were tested against this pathology because $sPLA_2$ is a major active principle in snake venom and medicinal plants for snakebite have been shown to contain $sPLA_2$ inhibitory phytochemicals (Nevalainen et al., 2012; Nunez et al., 2005).

4.2 Methodology

4.2.1 Phytochemical extraction and crude extract preparation

Plant material from medicinal plants was collected with Q'eqchi' Maya healers in Belize, along with voucher specimens for taxonomic identification, which was done at the New York Botanical Gardens and Universidad Nacional de Costa Rica. The material was bottled individually with approximately 1 mL/g of 70% isopropyl alcohol for the duration of fieldwork. Upon return to the laboratory, the plant material was shredded in a blender and extracted with 10 mL/g wet material 80% ethanol, overnight with shaking. The solvent was vacuum filtered with Whatmann No.1 filter paper, and the residue re-extracted with 5 mL/g wet material 80% ethanol, overnight with shaking. The filtrates were combined, roto-evaporated at 40°C, freeze-dried, and stored at -20°C. Crude extracts tested in the cerebellar granule neuron (CGN) and $sPLA_2$ inhibitor bioassays were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a maximum soluble concentration of 10 mg/mL, stored at -20°C, and freeze-thawed a maximum of two times.

4.2.2 CGN culture and treatment

All animal work in this study was approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care. Postnatal day 7-9 C57BL/6 mouse pups were euthanized with 75 µL of 65 mg/mL sodium pentobarbital and their brains collected in 10 cm petri dishes containing ice-cold dissection solution (124 mM NaCl, 5.36 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 14.5 mM D-(+)-glucose, 25 mM HEPES buffer (Sigma-

Aldrich), 3 mg/mL bovine serum albumin (EMD Millipore), pH 7.4). The cerebella were isolated and meninges dissected. The tissue was transferred to 35 mm petri dishes and minced in fresh ice-cold dissection solution. The minced tissue was homogenized in 2 mL of fresh ice-cold dissection solution by gentle trituration with a p1000 micropipette (Eppendorf). The homogenized tissue was centrifuged for 5 minutes at 1000 rpm and the supernatant discarded. Cells were dissociated from the pelleted tissue in 7 mL of dissection solution containing 0.5 mg/mL trypsin (Sigma-Aldrich), shaken gently for 19 min at 37°C. Trypsin was inactivated by the addition of 7 mL of ice-cold dissection solution containing 79 µg/mL chicken egg white trypsin inhibitor (Roche Applied Science) and 240 µg/mL DNAse I (Roche Applied Science). The cells were centrifuged for 5 min at 1000 rpm, and the supernatant discarded. The pelleted cells were re-suspended in 2 mL of dissection solution enriched with 2.3 mM $MgSO_4$, 0.88 mg/mL chicken egg white trypsin inhibitor, and 0.64 mg/mL DNAse I, and triturated with a flame-polished Pasteur pipette. Larger pieces of debris and aggregated cells were allowed to settle for 5 minutes, and the dissociated, single-celled suspension of CGNs was collected. The trituration, settling, and collection steps were repeated in an additional 2 mL of enriched dissection solution, if a tissue precipitate remained. After collection, 0.3 mL of dissection solution enriched with 2.5 mM $MgSO_4$ and 100 µM $CaCl_2$ was added for each 1 mL cell suspension. The CGNs were centrifuged for 5 minutes at 1000 rpm and the supernatant discarded. The pelleted CGNs were re-suspended in media (Eagle's Minimum Essential Medium, 25 mM D-(+)-glucose, 25 mM KCl, 2 mM L-glutamine (Life Technologies), 10% heat-inactivated fetal bovine serum, 100 µg/mL gentamycin (Sigma-Aldrich)) and plated in 24 well plates, coated with 100 ug/mL

poly-D-lysine (Sigma-Aldrich) at a density of 2.5 x 10^5 cells/cm^2 and a volume of 1 mL per well.

4.2.3 CGN bioassay

CGNs were supplemented with 10 μM arabinose cytosine β-D-arabinofuranoside (Sigma-Aldrich) 24 hours after plating to inhibit glial growth. On day *in vitro* (DIV) 7, CGNs were supplemented with 5 mM D-(+)-glucose. In the plant toxicity screen, CGNs were treated on DIV 8 with 10 μg/mL plant extract for 24 hours. In the neuroprotection screen, CGNs were treated on DIV 8 with 100 μM glutamate (Sigma-Aldrich) with or without 1 μL of 10 μg/mL plant extract for 24 hours. The known NMDAR antagonist, MK-801 (Sigma-Aldrich), was used as a positive control to prevent excitotoxicity and tested at a concentration of 10 μM. Glutamate was prepared in sterile water, while plant extracts and MK-801 were prepared in DMSO. For treatments with DMSO as a vehicle, the final concentration was kept at 0.1%. All treatments were done in triplicate. Each plate included DMSO vehicle controls and DMSO with glutamate positive controls, to ensure an excitotoxic response.

4.2.4 Live/Dead assay and fluorescent microscopy

CGN viability was assessed 24 hours after extract/compound treatment using the Live/Dead assay (Life Technologies). Cell media was gently aspirated, and replaced with sterile phosphate-buffered saline containing 0.1 mg/mL Hoechst (Life Technologies), 2.5 μM calcein AM, and 0.75 μM ethidium homodimer-1 (Ethd1). The cells were imaged after a 20 min incubation at room temperature. Each well was

imaged 4 times at 10x magnification under an AxioObserver D1 fluorescent microscope (Zeiss). Manual cell counts were done, with Hoechst+/Calcein+/Ethd1- cells counted as viable.

4.2.5 $sPLA_2$ inhibitor bioassay

A group V $sPLA_2$ inhibitor screening assay kit (Cayman Chemical) was used according to the manufacturer's instructions. Human recombinant group V $sPLA_2$ was prepared in assay buffer (25 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100), and plated in the presence or absence of 45 µg/mL plant extract. The enzymatic reaction was initiated by the addition of the lipid substrate, 1,2-dithio-heptanoylphosphatidylcholine, and incubated at room temperature for 15 minutes. 5,5-Dithiobis-(2-nitrobenzoic acid) was added and the absorbance was read after 1 min at 415 nm (Bio Rad) to detect free thiols released by enzymatic hydrolysis. The known group V $sPLA_2$ inhibitor, LY311727 (Tocris), was used as a positive control and tested at 4.5 µM. For all experiments, plant extracts and LY311727 were prepared in DMSO. All treatments were done in triplicate. Non-enzymatic controls were run for each treatment to account for any effects on absorbance from treatment extracts or compounds and received all of the same reagents, but assay buffer in place of the $sPLA_2$ enzyme.

4.3 Results

The SIV was used to prioritize Q'eqchi' Maya medicinal plants relevant to AD for pharmacological evaluation (Figure 4.1). To assess the plants used to treat symptoms of AD for toxicity and neuroprotective activity against excitotoxicity, plant extracts were tested in a CGN bioassay. DMSO (0.1%) was selected as a vehicle for plant extracts, due to its high solvation properties and acceptable impact on cell viability of CGNs (Figure 4.2). Excitotoxicity was induced with 100 μM glutamate, as detected by a significant reduction in cell viability compared to both untreated and vehicle treated cells, and was blocked entirely with 10 μM MK-801, a known NMDAR antagonist used as a positive control (Figure 4.2). Of the top nine SIV-ranked plants used for symptoms of AD examined in this assay (Figure 4.1), none were toxic to CGNs at 10 μg/mL (Figure 4.3). In the presence of 100 μM glutamate, 10 μg/mL extracts of *Margraviaceae gentlei* and *Gonzalagunia panamensis* protected CGNs from excitotoxicity, as indicated by significantly higher viabilities compared to cells exposed to glutamate alone (Figure 4.4).

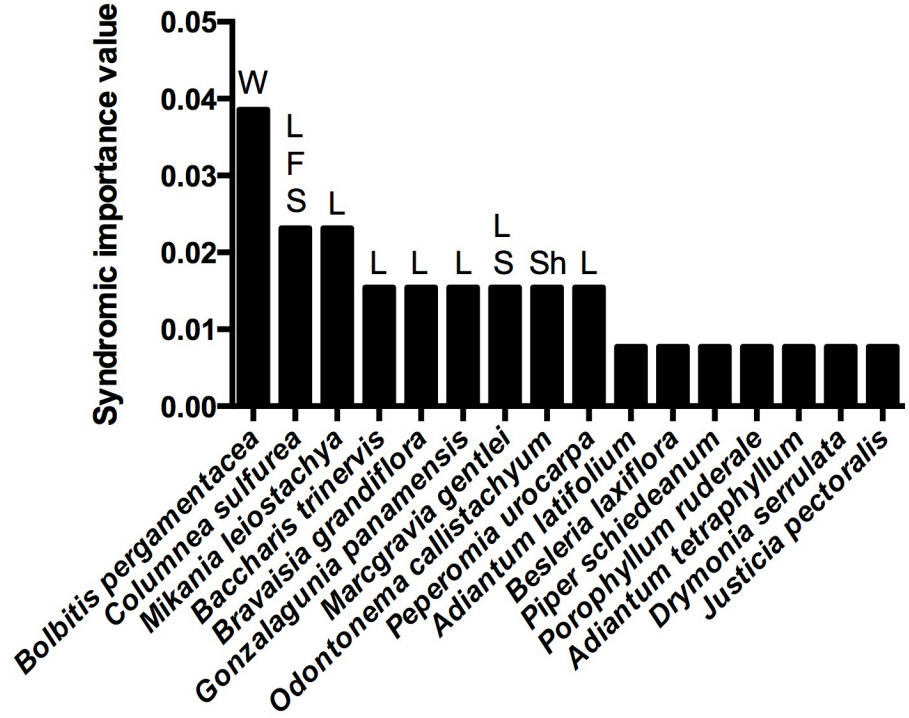
To assess the Q'eqchi' Maya snakebite medicinal plants for *in vitro* $sPLA_2$ inhibitory activity, plant extracts were tested in an $sPLA_2$ enzyme assay. Extracts were tested at the maximum soluble concentration in the DMSO vehicle, 45 μg/mL. While *Peperomia urocarpa* and *Sansevieria trifasciata* did not significantly inhibit $sPLA_2$ activity, *Peperomia hirta* significantly reduced enzyme activity to 67% compared to vehicle control (Figure 4.5A). A known specific inhibitor of $sPLA_2$, LY311727, was used as a positive control and inhibited enzyme activity almost entirely at 4.5 μM (Figure 4.5A). Successive testing of *P. hirta* extract at several

concentrations in the assay showed a linear concentration-dependent inhibition of $sPLA_2$ activity (Figure 4.5B).

Figure 4.1. Prioritization of Q'eqchi' Maya medicinal plants for pharmacological evaluation.

The SIV was used to prioritize medicinal plants for pharmacological evaluation. Plants used to treat A) symptoms of AD and B) snakebite were tested for activity against excitotoxicity and $sPLA_2$ upregulation, respectively. Of the medicinal plants documented, those tested are denoted with a letter corresponding to the plant organ used by the healers and that underwent extraction (L: leaves, R: roots, S: stems Sh: shoots, W: whole plant).

A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Gonzalagunia panamensis
Marcgravia gentlei
Odontonema callistachyum
Peperomia urocarpa
Adiantum latifolium
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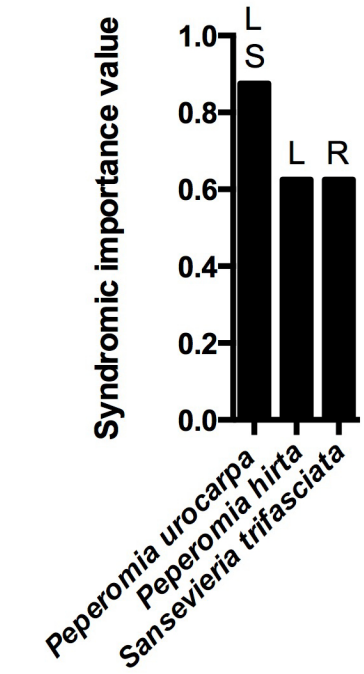
Adiantum tetraphyllum
Drymonia serrulata
Justicia pectoralis

B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0.0
L
S
L
R
Peperomia urocarpa
Peperomia hirta
Sansevieria trifasciata

Figure 4.2. Optimization of CGN bioassay for medicinal plant screening.

Effects of vehicle, glutamate challenge, and NMDAR antagonism were assessed on CGNs directly by Live/Dead assay. Neurons were treated with vehicle (0.1% DMSO), vehicle and 100 μM glutamate (glu), or 10 μM MK-801 and 100 μM glu for 24 hours (filled bars). Hoechst+/Calcein+/Ethd1- cells were imaged using fluorescent microscopy and counted as viable. Data are expressed as mean percent of viable cell counts, normalized to control (untreated CGNs in complete media; unfilled bar) (+SE, n=3 for all treatments). [one-way ANOVA, df [3,8] F=17.9, *post-hoc* Tukey's test, * $p<0.05$, ** $p<0.01$].

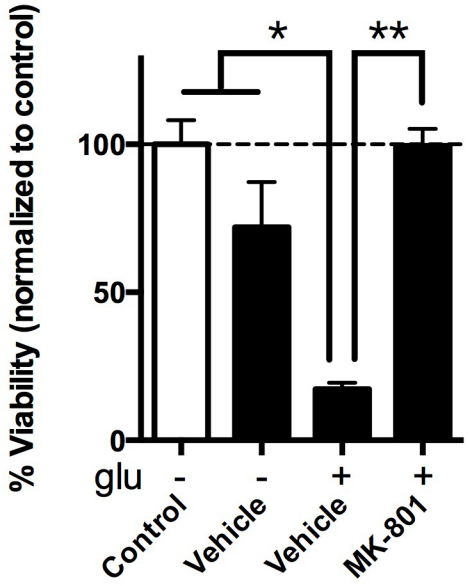

% Viability (normalized to control)
*
**
100
50
0
glu
-
-
+
+
Control
Vehicle
Vehicle
MK-801

Figure 4.3. Toxicity screen of medicinal plants used by Q'eqchi' Maya healers to treat symptoms of AD.

Toxicity of medicinal plants on CGNs was assessed directly by Live/Dead assay. Neurons were treated with plant extracts at a concentration of 10 μg/mL for 24 hours (filled bars). Hoechst+/Calcein+/Ethd1- cells were imaged using fluorescent microscopy and counted as viable. Data are expressed as mean percent viable cell counts, normalized to vehicle-treated cultures (0.1% DMSO; unfilled bar) (+SE, n=6 for vehicle treatments, n=3 for plant extract treatments). [one-way ANOVA, df [9,23] F=0.7].

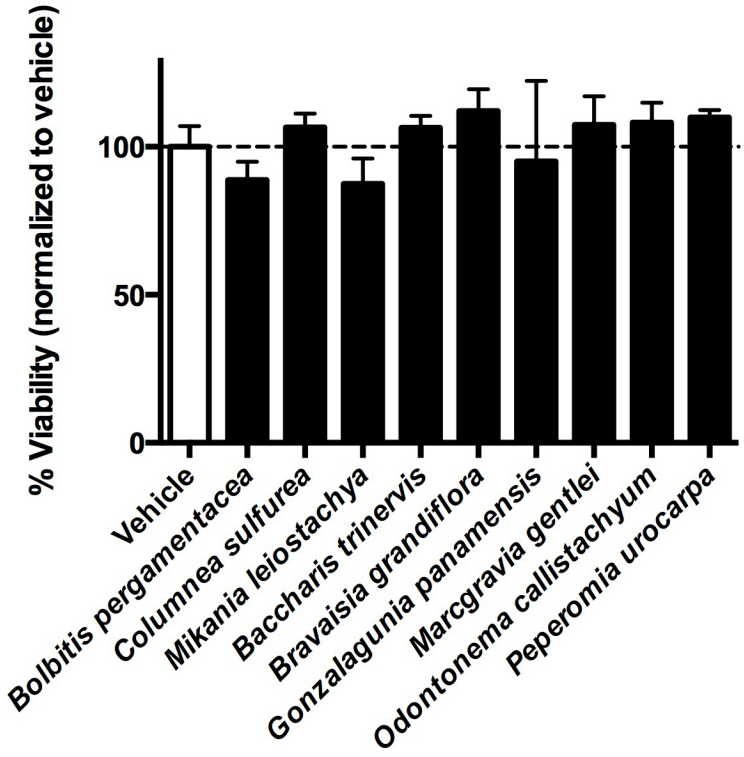

% Viability (normalized to vehicle)
100
50
0
Vehicle
Bolbitis pergamentacea
Columnea sulfurea
Mikania leiostachya
Baccharis trinervis
Bravaisia grandiflora
Gonzalagunia panamensis
Marcgravia gentlei
Odontonema callistachyum
Peperomia urocarpa

Figure 4.4. Neuroprotection screen of medicinal plants used by Q'eqchi' Maya healers to treat symptoms of AD.

Neuroprotective capacity of medicinal plants against excitotoxic challenge on CGNs was assessed directly by Live/Dead assay. Neurons were challenged with 100 μM glutamate (glu) in the presence of vehicle (0.1% DMSO) or plant extracts at a concentration of 10 μg/mL for 24 hours (filled bars). Hoechst+/Calcein+/Ethd1- cells were imaged using fluorescent microscopy and counted as viable. Data are expressed as mean percent viable cell counts, normalized to vehicle-treated cultures (unfilled bar) (+SE, n=9 for vehicle and vehicle with glutamate treatments, n=3 for plant extract treatments). [one-way ANOVA, df [10,34] F=49.2, *post-hoc* Dunnett's test comparing each treatment to vehicle with glutamate, ** $p<0.01$].

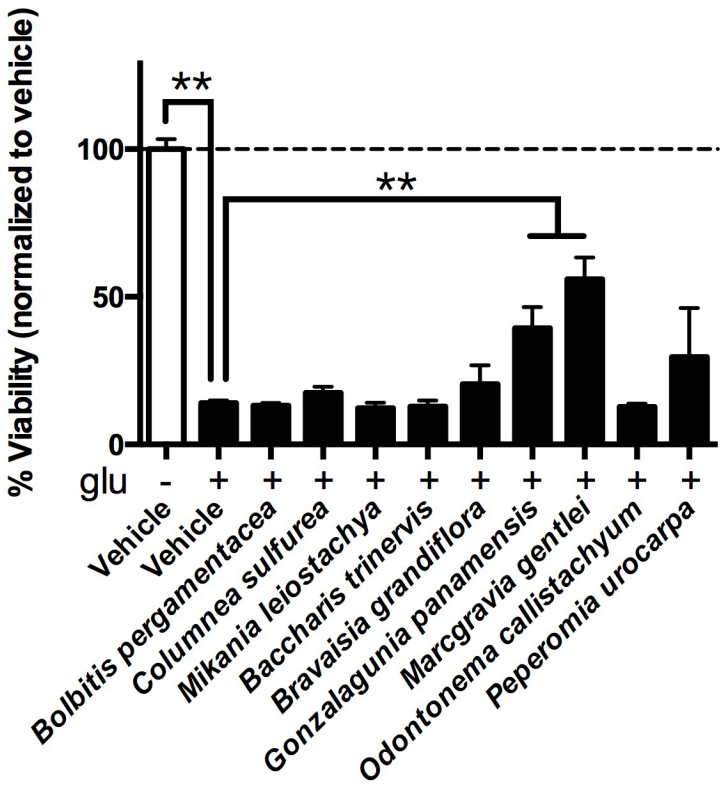
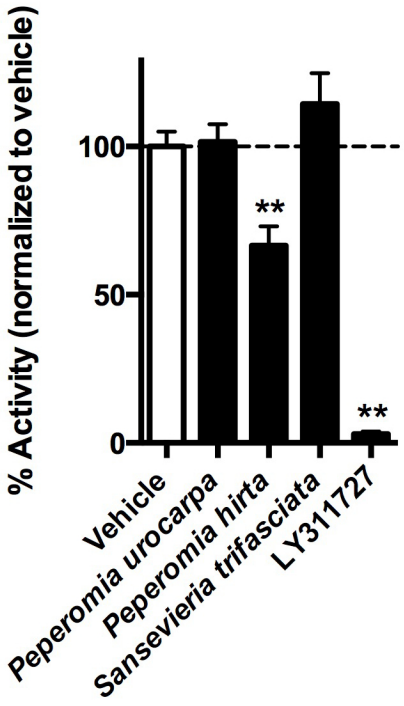

% Viability (normalized to vehicle)
100
50
0
**
**
glu - + + + + + + + + + +
Vehicle
Vehicle
Bolbitis pergamentacea
Columnea sulfurea
Mikania leiostachya
Baccharis trinervis
Bravaisia grandiflora
Gonzalagunia panamensis
Marcgravia gentlei
Odontonema callistachyum
Peperomia urocarpa

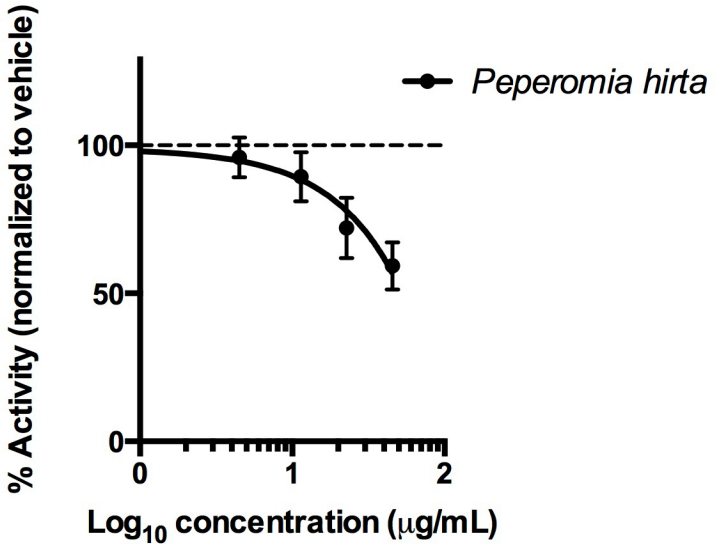
Figure 4.5. $sPLA_2$ inhibitor screen of medicinal plants used by Q'eqchi' Maya healers to treat snakebites.

Human recombinant group V $sPLA_2$ *in vitro* activity was assessed by cleavage of 1,2-dithio-heptanoylphosphatidylcholine as measured by free thiol group detection at 415 nm. A) $sPLA_2$ activity when treated with extracts of snakebite medicinal plants at a concentration of 45 µg/mL and LY311727, a known specific inhibitor of $sPLA_2$ used as a positive control, at a concentration of 4.5 µM (filled bars). Data are expressed as mean percent activities, normalized to vehicle (DMSO; unfilled bar) (+SE, n=6 for vehicle treatments, n=3 for plant extract and LY311727 treatments) [one-way ANOVA, df [4,13] F=44.7, *post-hoc* Dunnett's test comparing each treatment to vehicle, ** $p<0.01$]. B) $sPLA_2$ activity when treated with increasing concentrations of *P. hirta* extract [linear regression analysis, $R^2=0.96$].

A


% Activity (normalized to vehicle)
100
50
0
**
**
Vehicle
Peperomia urocarpa
Peperomia hirta
Sansevieria trifasciata
LY311727

B


% Activity (normalized to vehicle)
Peperomia hirta
100
50
0
0
1
2
Log10 concentration (μg/mL)

4.4 Discussion

Here, I show for the first time that Q'eqchi' Maya medicinal plants relevant to AD are pharmacologically active against two AD pathologies, excitotoxicity and $sPLA_2$ upregulation. None of the plants used for symptoms of AD examined were toxic to CGNs, and extracts of *M. gentlei* and *G. panamensis* protected CGNs from glutamate-induced excitotoxicity. Furthermore, an extract of *P. hirta*, a plant used to treat snakebites, inhibited $sPLA_2$ activity *in vitro.*

To assist the healers in evaluating their traditional medicine for safety and efficacy, medicinal plants relevant to AD were tested for *in vitro* pharmacological activity against two disease pathologies, excitotoxicity and $sPLA_2$ upregulation. The SIV, developed by Leduc et al. (2006), was used as a rapid quantitative method to prioritize their medicinal plants for pharmacological evaluation. This method has been used similarly in other ethnopharmacological studies (de Sousa Araujo et al., 2008; Harbilas et al., 2009; Muhammad et al., 2012), and our results support its usefulness in directing such studies towards biologically active plants.

CGN cultures have been widely used as *in vitro* models to study excitotoxicity, due to their relatively high yield, homogenous neuronal population, and sensitivity to glutamate via NMDAR-dependent mechanisms (Contestabile, 2002). These characteristics were evident upon assay optimization and supported the use of CGNs as a reproducible model for assessment of the medicinal plants for AD symptoms.

The lack of toxicity of the AD symptom medicinal plants on CGNs was not unexpected, considering that the healers use these plants to improve brain health.

Previous studies have shown that certain phytochemicals, including oxyindole and indole alkaloids can be toxic to CGNs at high enough concentrations (Shimada et al., 1999). Based on our results, it is unlikely that these compounds are present in the tested extracts, at least in high abundance.

The neuroprotective effects of *M. gentlei* and *G. panamensis* were also consistent with their traditional use, as excitotoxicity is a well-characterized AD pathology. While their mechanisms of action are currently unknown, one possibility involves NMDAR antagonism, mimicking that of the positive control, MK-801. While this can be evaluated with future receptor binding experiments, alternative mechanisms should also be considered. Although neither species has yet to be phytochemically characterized or examined for neurological activity, close relatives have been evaluated in previous studies and provide insight into possible modes of activity.

Souroubea sympetala, another tropical vine in the Marcgraviaceae family, has been shown to possess anxiolytic properties, with betulinic acid as an active principle (Durst et al., 2009). Interestingly, betulin, an analogue of betulinic acid, is a $GABA_A$ receptor agonist and elicits anticonvulsive effects (Muceniece et al., 2007). If present in *M. gentlei*, betulin activation of the $GABA_A$ receptor in CGNs would hyperpolarize the cells and prevent displacement of the Mg^{2+}-plug from NMDARs, thereby reducing Ca^{2+} influx and downstream excitotoxicity. Future work will aim to characterize the *M. gentlei* extract, with a focus on betulinic acid and derivatives as potential lead compounds, and to evaluate this possible mechanism of action.

Previous work from the Arnason laboratory documented another member of the *Gonzalagunia* genus, *G. rosea*, as a Q'eqchi' traditional medicine for epilepsy

(Awad et al., 2009). Pharmacological investigation of this plant demonstrated potent $GABA_A$ receptor binding (Awad et al., 2009). Bioassay-guided fractionation and phytochemical analysis isolated ethyl caffeate and isopropyl caffeate as two major active principles (Ahmed et al., unpublished data). These two phytochemicals represent candidate lead compounds for future characterization of *G. panamensis* extract.

The upregulation of $sPLA_2$ has been identified as a pathogenic event in AD (rev. by Schaeffer et al. (2010)), and is predicted to underlie, in part, the pathogenic accumulation of PAFs over the course of AD progression. Thus, pharmacological inhibition of this enzyme may be of therapeutic potential. Since a form of $sPLA_2$ is a major active principle in snake venom, I examined Q'eqchi' Maya snakebite medicinal plants for $sPLA_2$ inhibitory activity. The observed inhibition of $sPLA_2$ by *P. hirta* suggests that this extract does contain $sPLA_2$ inhibitory phytochemicals, while the lack of activity of *P. urocarpa* and *S. trifasciata* indicates that alternative modes of action likely exist for these plants. While this is the first documentation of *P. hirta* being used as a traditional medicinal plant for snakebites, other species in the Piperaceae family from this geographic region have been documented previously as snakebite treatments (Coe and Anderson, 2005; Otero et al., 2000). Several of these plants have been investigated for pharmacological activity. The compound 4-nerolidylcatechol was isolated and identified as an active principle responsible for $sPLA_2$ inhibition from *Piper umbellatum* and *Piper peltatum* (Nunez et al., 2005). While these findings provide a lead compound for future analytical work on the extract of *P. hirta*, the lack of activity of its close relative, *P. urocarpa*, suggests that the active principle may be unique *P. hirta*, as opposed to being common across

related species, and might require fractionation and isolation, in order to be identified.

Overall, my findings support a pharmacological basis for Q'eqchi' Maya medicinal plants relevant to AD. Of the plants used for symptoms of AD, *M. gentlei* and *G. panamensis* demonstrated neuroprotective activity against excitotoxicity, a well-characterized pathology in AD. Of the plants used for treatment of snakebite, *P. hirta* demonstrated $sPLA_2$ inhibitory activity. Since the form of $sPLA_2$ in snake venom shares a conserved structure and activity to the human form that is upregulated in AD, this snakebite medicinal plant may also be of therapeutic relevance for AD (Nevalainen et al., 2012). While continued research is required to identify the active principles in these plants and to explore alternative modes of action for the other medicinal plants, this study provides preliminary evidence supporting the safety and efficacy of Q'eqchi' Maya traditional medicine relevant to AD. This is important because traditional medicine is often a more relevant form of health care in developing countries like Belize (WHO, 2002a). In addition to these important contributions to the Q'eqchi' Maya and their local community, future work on the active plants may provide important leads for the future development of novel AD drugs.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary

In this thesis, I investigated a mechanism mediating neurodegeneration in AD and whether natural products derived from Maya traditional medicine could inhibit known pathological processes in AD. My first objective was to determine if PAF activation of PAFR enhanced or impaired cognition, under conditions associated with elevation of PAF in the brain in response to Aβ accumulation. I demonstrated that PAFR-mediated signalling is, in part, responsible for the learning impairments exhibited in the TgCRND8 model of AD. My second objective was to document the traditional medicine used by Q'eqchi' Maya healers of southern Belize relevant to AD. I showed that the healers do recognize and treat multiple symptoms relevant to AD with medicinal plants, and identified 19 of these 22 plants to the species level. My third objective was to evaluate the identified medicinal plants for pharmacological activity against two *in vitro* targets relevant to AD, as a preliminary assessment of safety and efficacy. I found that extracts of *Margraviaceae gentlei* and *Gonzalagunia panamensis* protected CGNs from glutamate-induced excitotoxicity *in vitro,* and that *Peperomia hirta* inhibited $sPLA_2$ activity, which is responsible, in part, for remodelling of alkylacylglycerophosphocholines to the immediate precursors of PAF lipids. Key to proceeding to advanced validation, my study further shows that none of the plant extracts used for symptoms of AD that were examined exhibited any off-target toxicity when tested on CGNs. These findings support my hypotheses that the loss of PAF-PAFR signaling is beneficial for cognitive performance in the TgCRND8

mouse model of AD and that Q'eqchi' Maya healers treat symptoms relevant to AD with medicinal plants that are pharmacologically active against AD-relevant pathologies.

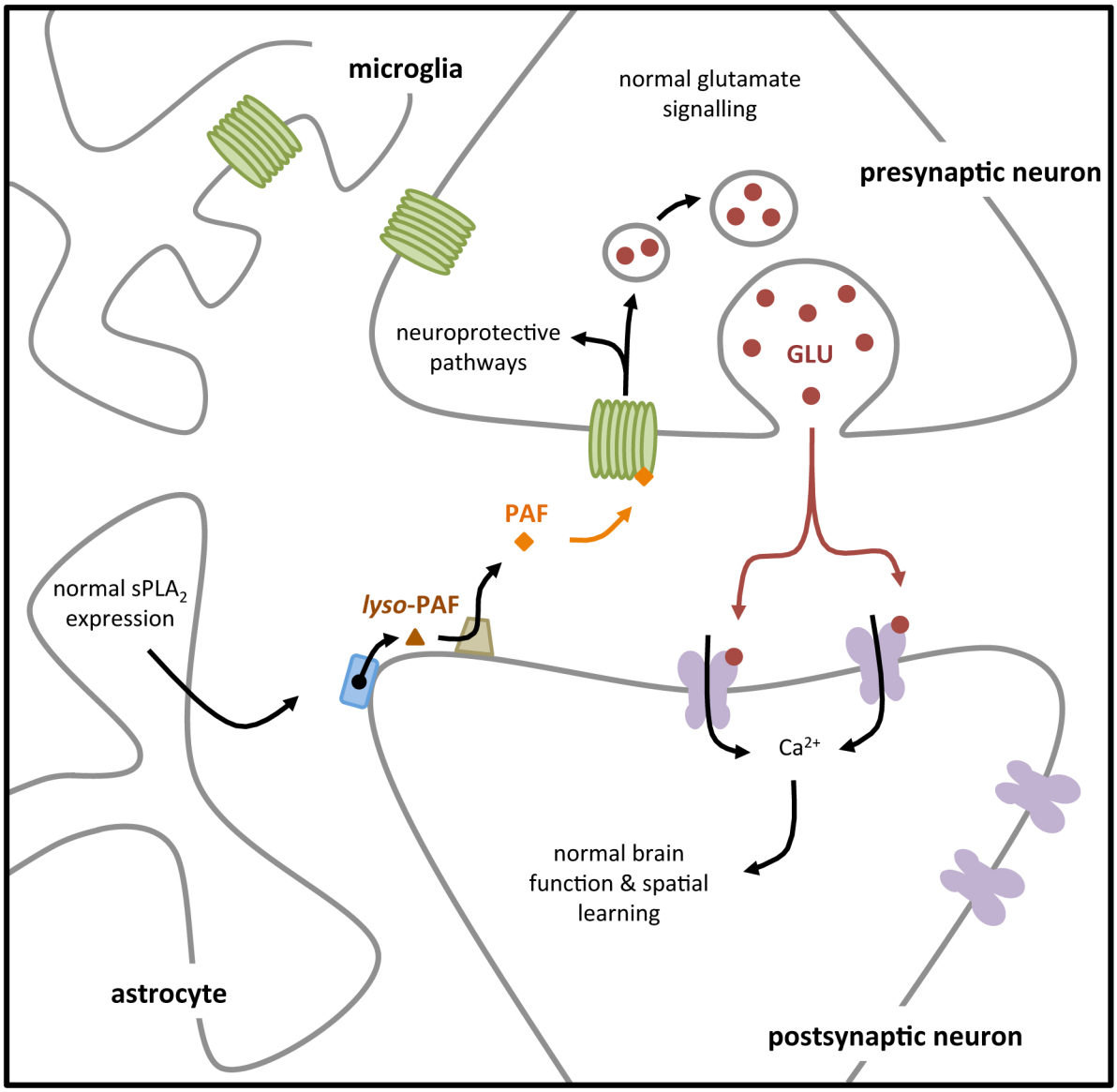
5.2 PAF-PAFR signalling as a therapeutic target in AD

Targeting aberrant Aβ metabolism directly has been met with a number of challenges, leading to an increased focus on other metabolic disruptions further downstream as potential avenues for complementary therapeutics (Huang and Mucke, 2012; Selkoe, 2011). One such target is PAF-PAFR signalling, which is important for normal brain function (Chen et al., 2001; Izquierdo et al., 1995; Kato et al., 1994; Wieraszko et al., 1993) but has been shown to be dysfunctional in AD (Bate et al., 2006; Bate et al., 2004; Ryan et al., 2009). PAFR has been localized primarily in neurons and microglia, and to a lesser extent in astrocytes (Bennett et al., 1998; Mori et al., 1997). PAFs can be synthesized through the Lands' cycle, whereby PLA_2 enzymes hydrolyze structural alkylacylglycerophosphocholine lipids into *lyso*-PAFs, which are then acetylated into PAFs by LPCATs (Nevalainen et al., 2012).

In a healthy brain (Figure 5.1), normal levels of PAF can activate PAFR in neurons, signalling glutamate release from pre-synaptic neurons and stimulating neuroprotective pathways (Clark et al., 1992; Ryan et al., 2007). These processes likely facilitate normal brain function and allow for proper learning and memory, as demonstrated by nonTg $PAFR^{+/+}$ mice. Under the pathological conditions that occur in AD (Figure 5.2), Aβ can activate microglia, which release inflammatory factors

Figure 5.1. Proposed model of normal PAF-PAFR signalling in a healthy brain

Normal levels of PAF, synthesized by Lands' cycle enzymes including $sPLA_2$ and LPCAT, can activate PAFRs in neurons that signal neuroprotective pathways and glutamate release from pre-synaptic neurons. Glutamate binding to NMDARs causes intracellular Ca^{2+} influx, facilitating normal brain function and allowing for proper learning and memory, and a high incidence of spatial search strategy in the Morris water maze.



NMDAR: *N*-Methyl-D-aspartate receptor

PAFR: Platelet activating factor receptor

$sPLA_2$: Secretory phospholipase A_2

LPCAT: *Lyso*phosphatidylcholine acyltransferases

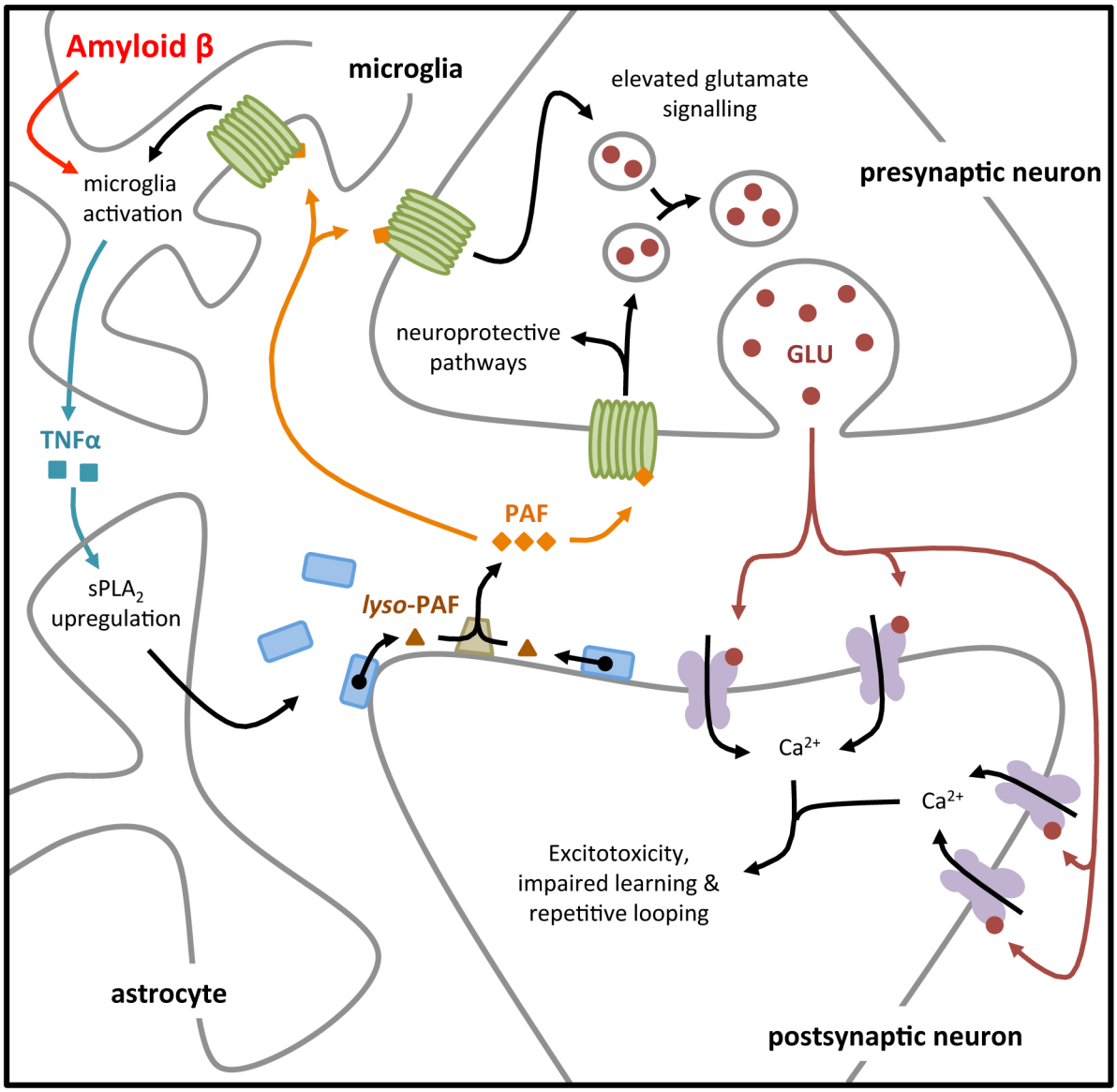
Lyso-PAF: *Lyso*-platelet activating factor

PAF: Platelet activating factor

GLU: Glutamate

Figure 5.2. Proposed model of aberrant PAF-PAFR signalling in an AD brain

Aβ can activate microglia, which release inflammatory factors like tumour necrosis factor α that upregulate Lands' cycle enzymes including $sPLA_2$, and lead to increased PAF synthesis. While normal levels of PAFR activation by PAF signals neuroprotective pathways and normal glutamate release from pre-synaptic neurons, increased PAFR activation causes elevated glutamate release. Overactivation of NMDARs by glutamate causes Ca^{2+} overload, leading to excitotoxicity, impaired learning, and a high incidence of repetitive looping search strategy in the Morris water maze.



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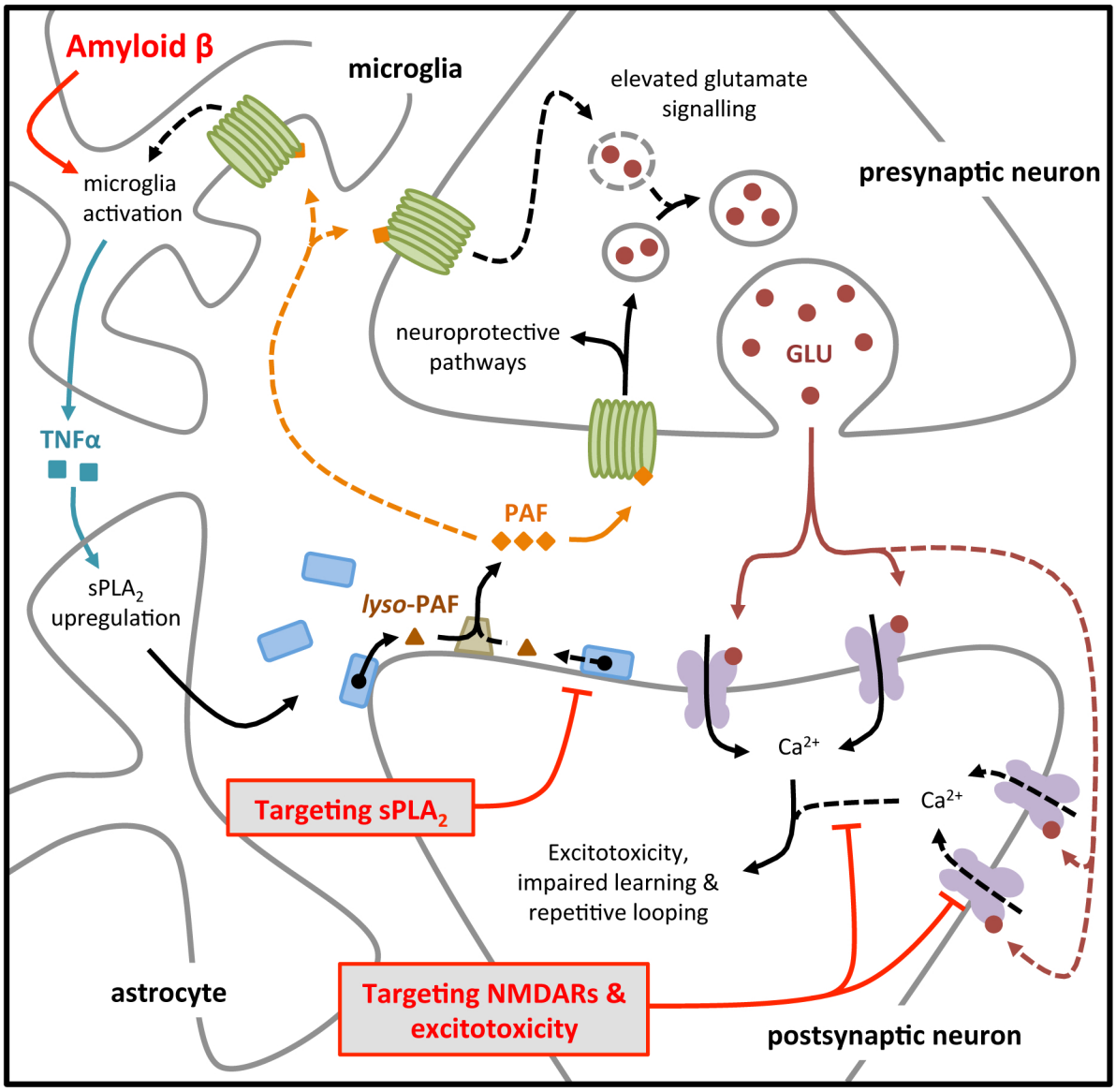
that stimulate PAF synthesis (Bate et al., 2006; Bellizzi et al., 2005; Meda et al., 1995; Thomas et al., 2000). Arguably, elevated PAF would signal further microglial activation and increased glutamate release, leading to excitotoxicity, impaired learning, and a repetitive looping behaviour characteristic of increased NMDAR activity, which was demonstrated by Tg $PAFR^{+/+}$ mice. There are likely alternative PAFR-independent mechanisms that also contribute to learning and memory processes, which is consistent with results from Kobayashi et al. (1999), and could explain the performance of the nonTg $PAFR^{-/-}$ mice that lack the normal PAFR-dependent glutamate release and neuroprotective pathways. The loss of PAFR under conditions of predicted PAF elevation may protect mice from Aβ-induced learning impairments by preventing excess glutamate release and reducing microglial activation. Preliminary evidence for lower glutamate signalling can be found in reductions of repetitive looping in Tg $PAFR^{-/-}$ mice, but needs to be confirmed along with an assessment of brain PAF levels and microglial status.

While loss of PAFR has beneficial effects on learning in a mouse model of AD, therapeutics designed to block PAFR activity may have undesired effects. Memory impairments, particularly involving working memory and memory recall, have been associated with PAFR disruption (Izquierdo et al., 1995; Row et al., 2004; Saraf et al., 2003). The functional/neuroprotective role of PAFR expression demonstrated by Ryan et al. (2007) may be important for these memory processes. As well, blocking PAFR in the periphery could have adverse effects on blood flow and aggregation, due to its role in platelet aggregation (Chignard et al., 1979). An alternative approach that should preserve these PAFR functions would be to target the elevated PAF levels. Since $sPLA_2$ generates the immediate PAF precursor, *lyso*-

PAF, and is also elevated in AD, this enzyme represents a promising target for pharmacological inhibition (Figure 5.3). Another option would be to target the NMDARs and downstream excitotoxicity, a well-established AD pathology, to which PAFR overactivation likely contributes (Figure 5.3). While a synthetic $sPLA_2$ inhibitor was investigated for efficacy against arthritis (Bradley et al., 2005), which involves peripheral $sPLA_2$, little research has been done to assess the therapeutic potential of $sPLA_2$ inhibitors in AD. NMDARs have been recognized as relevant therapeutic targets in AD, and an antagonist, memantine, is currently certified as an AD drug (Lipton, 2006). Future work on the development of pharmaceutical agents targeting these pathologies may lead to the discovery of novel $sPLA_2$ inhibitors and NMDAR antagonists that could complement current therapeutics. While this would benefit a large number of AD patients, it is important to note that the majority of patients currently live in developing countries, where drugs are usually not affordable or accessible (Ferri et al., 2009; WHO, 2002a). For these AD patients, alternative options to conventional pharmaceutical-based medicine must be considered.

Figure 5.3. Therapeutic strategies targeting two pathologies downstream of Aβ biogenesis

Targeting $sPLA_2$ upregulation could restore normal PAF levels, thereby preventing elevated glutamate release and reducing microglial activation. Targeting NMDARs and excitotoxicity could prevent behavioural impairments in learning that result from elevated glutamate signalling. Hashed lines represent inhibited pathways offering therapeutic benefits.



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5.3 Importance of traditional medicine in treating AD

For the majority of AD patients, who live in developing countries (Ferri et al., 2009), traditional medicine is often a more relevant form of health care due to increased availability and lower costs as compared to pharmaceutical drugs (WHO, 2002a). The Q'eqchi' Maya healers of southern Belize have a documented tradition of treating mental health disorders with plants (Amiguet et al., 2005; Bourbonnais-Spear et al., 2005), and this thesis shows that they use medicinal plants to treat symptoms relevant to AD. Many of these species are found in the rainforest and the healers rely on their extensive knowledge of the forest ecology to collect them. Like many indigenous communities around the world, the Q'eqchi' Maya are facing a decline in traditional practices (Alves and Rosa, 2007) and the ethnobotanical documentations provided in this thesis help protect and preserve their traditional knowledge.

In both developed and developing countries, the use of traditional medicine is becoming more popular (WHO, 2002a). While some forms are supported by strong scientific evidence, others lack information on safety and efficacy. In such cases, the proper scientific evaluation of these medicines can help strengthen their use as a valuable form of health care (WHO, 2002b). Assisting the Q'eqchi' Maya in moving their traditional medicine to a more evidence-based system was an important goal of our collaboration. In this preliminary evaluation of their medicinal plants for safety and efficacy, I identified several species with promising bioactivities. *P. hirta*, a plant used to treat snakebites, demonstrated *in vitro* $sPLA_2$ inhibitory activity and may be an effective therapeutic to target $sPLA_2$ upregulation in AD and restore normal PAF

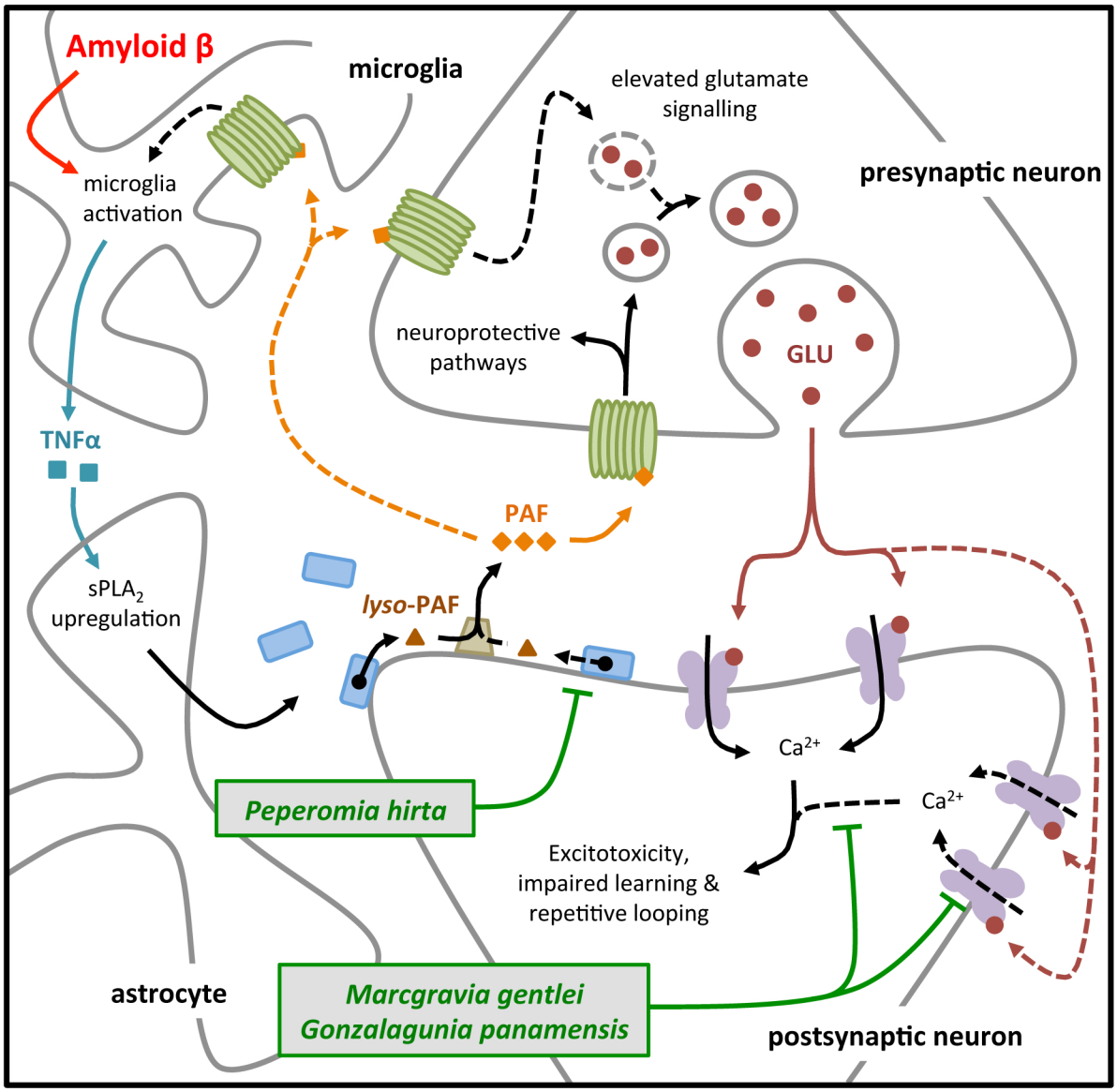
levels in the brain (Figure 5.4). *M. gentlei* and *G. panamensis* were not toxic to neurons and were neuroprotective against excitotoxic challenge. These plants may have potential to target elevated NMDAR activity and excitotoxicity in AD (Figure 5.4). Further work focusing on the identification of the active principles, bioavailability and blood-brain barrier permeability, as well as the safety and efficacy of these plants in animal models, will further support their use as effective health care options. As with all research on traditional knowledge and medicine, close attention must be paid to intellectual property rights of the indigenous communities involved, with consideration towards informed consent, access, and benefit sharing (WHO, 2002b) .

5.4 Medicinal plants as a source of bioactive compounds relevant to AD

Plants have long been considered a major source for drug discovery as they contain a diverse set of metabolites with potent bioactivities (Balunas and Kinghorn, 2005; Butler, 2004). Their distinct chemical characteristics make them particularly suitable for central-nervous system disorders, like AD (Joyner and Cichewicz, 2011). As demonstrated here, and by others (McClatchey et al., 2009), ethnobotany can be a valuable research tool to direct pharmacological studies towards plants of interest, using traditional knowledge as a guide. Traditionally used plants from several cultures have been found to possess pharmacological activities relevant to AD. Several notable examples are *Galanthus* spp. from European and Russian

Figure 5.4. Potential therapeutic mechanisms of Q'eqchi' Maya medicinal plants relevant to AD

Peperomia hirta demonstrated *in vitro* $sPLA_2$ inhibitory activity and may be an effective therapeutic to target $sPLA_2$ upregulation in AD and restore normal PAF levels in the brain. *Marcgravia gentlei* and *Gonzalagunia panamensis* are neuroprotective against excitotoxic challenge and may be effective therapeutics to target NMDAR overactivation and excitotoxicity in AD. Hashed lines represent inhibited pathways offering therapeutic benefits.



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GLU: Glutamate

TNFα: Tumor necrosis factor α

traditional medicine, *Huperzia serrata* and *Gingko biloba* from traditional Chinese medicine, and *Bacopa monnieri* and *Withania somnifera* from Ayurvedic medicine. These plants possess multiple bioactivities, including cholinesterase modulation to boost cholinergic activity, antioxidant effects that protect cells from oxidative stress, anti-inflammatory effects to reduce immune responses, and nootropic effects that enhance cognition (Diamond and Bailey, 2013; Heinrich and Lee Teoh, 2004; Kulkarni and Dhir, 2008; Ma et al., 2007; Rao et al., 2012). Active principles from *Galanthus* spp. and *H. serrata* have been developed into pharmacological treatments, galantamine and huperzine A, which are prescribed for AD in certain countries (Perry and Howes, 2011). *G. biloba*, *B. monnieri,* and *W. somnifera* have been examined in clinical trials and generally demonstrated good safety profiles (Diamond and Bailey, 2013; Kulkarni and Dhir, 2008; Rao et al., 2012). Further studies are needed to evaluate their efficacy as AD therapeutics, since existing evidence is either lacking or complicated by methodological inconsistencies. Working in collaboration with the Q'eqchi' Maya healers of southern Belize, I have demonstrated for the first time that three of their medicinal plants possess bioactivities relevant to AD. These findings place *P. hirta*, *M. gentlei, G. panamensis* alongside the other medicinal plants that are biologically active in the context of AD (Table 5.1). Future studies on these plants should focus on the isolation and identification of active principles, using bioassay-guided fractionation, mass spectrometry and nuclear magnetic resonance analyses. Both extracts and active pure compounds need to be assessed for blood-brain barrier permeability, using cell-based models (Cecchelli et al., 2007) and further evaluated for *in vivo* safety and efficacy in animal models challenged with excitotoxicity and $sPLA_2$ upregulation.

Structural analogs of active compounds can be synthesized and subsequently tested to identify motifs responsible for the effects and to optimize pharmacokinetics for future clinical trials. With continued research on traditional medicine used by indigenous groups like the Q'eqchi' Maya, there is a great potential for the discovery of new therapeutic options for patients with devastating diseases like AD.

Table 5.1. Medicinal plants with biological activity relevant to AD. Previously documented medicinal plants used in different forms of traditional medicine, alongside novel Q'eqchi' Maya medicinal plants identified in this study. Well-characterized bioactivities are listed for each plant, including those demonstrated in this study.

Plant	Traditional uses	Main bioactivities	Reference
Bacopa monnieri (L.) Wettst.	Ayurvedic medicine	Nootropic and antioxidant effects	(Rao et al., 2012)
Galanthus spp.	Traditional medicine from Europe and Russia	Cholinergic modulation	(Heinrich and Lee Teoh, 2004)
Ginkgo biloba L.	Traditional Chinese medicine	Cerebral circulation promotion, antioxidant and anti-inflammatory effects	(Diamond and Bailey, 2013)
Huperzia serrata (Thunb. ex Murray) Trev.	Traditional Chinese medicine	Cholinergic modulation	(Ma et al., 2007)
Withania somnifera (L.) Dunal	Ayurvedic medicine	Antioxidant effects and cholinergic modulation	(Kulkarni and Dhir, 2008)
Peperomia hirta C. DC.	Q'eqchi' Maya traditional medicine	$sPLA_2$ inhibition	This study
Marcgravia gentlei Lundell	Q'eqchi' Maya traditional medicine	Neuroprotection against excitotoxicity	This study
Gonzalagunia panamensis (Cav.) K.Schum.	Q'eqchi' Maya traditional medicine	Neuroprotection against excitotoxicity	This study

CONTRIBUTION OF COLLABORATORS

Chapter 2:

Mark Akins conducted the mouse breeding. Mark Akins and Graeme Taylor performed the mouse genotyping. Matthew W. Granger assisted with the Morris water maze testing.

Chapter 3:

Jonathan Ferrier assisted with fieldwork in Belize. Jonathan Ferrier, Marco O. Rojas, and Brendan W. Walshe-Roussel assisted with plant identification. Frederic Harnois assisted with phytochemical extractions. Hannah Volt, Uroosa Malik, and Coco Donati assisted with plant voucher preparation.

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APPENDIX

1.1 MWM search strategy classification

Spatial strategies:

a) Spatial direct: the mouse swims in one direction to the platform. Exclude any small, tight loops made by the mouse during its initial placement into the maze.

b) Spatial indirect: mouse swims to the platform with a maximum of one complete major loop; small tight loops made by the mouse during its initial placement into the maze are to be excluded. The search should be focused in a maximum of two quadrants with most time spent in the correct quadrant.

c) Focal: correct target quadrant: mouse shows a clear preference or bias for the back right quadrant; at most one complete loop into any incorrect quadrant is allowed not counting where the mouse was released.

Non-spatial systematic strategies:

a) Scanning: mouse searches the interior portion of the tank without spatial bias i.e. shows no preference for any quadrant. The mouse must make at least 2 passes (laps/visual cues) to be considered as scanning.

b) Random: mouse searches the entire tank without bias towards any portion i.e. will search all four quadrants as well as the peripheral section.

c) Focal: incorrect target quadrant: mouse concentrates search in a specific area of the tank that does not contain the platform and then may move on to another area.

Repetitive looping strategies:

a) Chaining: mouse swims in a circular motion along a relatively constant path at a distance greater than 15 cm from the wall for most of the swim path, but not hugging the wall.

b) Peripheral looping: mouse swims around the outer 15 cm of the pool in a “wall-hugging” manner continuously in 3 out of 4 quadrants.

c) Circling: mouse swims constantly in tight circles; circles must be consecutive.

Note: Small, tight circles should be excluded unless the mouse exhibits a clear “circling” strategy.

1.2 Partially-scripted, open-ended questionnaire used to guide ethnobotanical interviews

1. Do you treat [name of ailment/symptom]?
2. If yes, with plants, spiritual ceremonies, or both?
3. Which symptoms do you associate with this ailment/symptom?
4. What causes this ailment/symptom?
5. If treated with plants, which plants do you use for [name of ailment/symptom]?
6. Are they used in combination with other plants?
7. Which part(s) of the plant(s) do you use?
8. Where does the plant grow, and where do you prefer to collect it (e.g. *milpas*, roadside, primary forest, secondary forest)?
9. How do you prepare the remedy (e.g. boiled in water)?
10. How much [plant part(s) used] do you use?
11. How is the remedy administered?
12. How much of the preparation does the patient have to take?
13. How many times a day for how long?
14. Are there restrictions for the patient during the treatment?
15. How many people have you treated with this plant in the past year (average)?
16. Which herbal remedy do you prefer for treating [name of ailment/symptom]?

1.3 University of Ottawa Herbarium voucher numbers

OTT #	Plant
19955	*Odontonema callistachyum* (Cham. & Schltdl.) Kuntze
19956	*Besleria laxiflora* Benth.
19957	*Marcgravia gentlei* Lundell
19958	*Peperomia urocarpa* Fisch. & C.A.Mey.
19959	*Bolbitis pergamentacea* (Maxon) Ching
19960	*Gonzalagunia panamensis* (Cav.) K.Schum.
19961	*Columnea sulfurea* Donn.Sm.
19962	*Lygodium heterodoxum* Kunze
19963	*Porophyllum ruderale* (Jacq.) Cass.
19964	*Bravaisia grandiflora* Donn.Sm.
19965	*Mikania leiostachya* Benth.
19966	*Justicia pectoralis* Jacq.
19967	*Baccharis trinervis* (Lam.) Pers.
19968	*Adiantum latifolium* Lam.
19969	*Peperomia hirta* C. DC.
19970	*Adiantum tetraphyllum* Humb. & Bonpl. ex Willd.
19971	*Drymonia serrulata* (Jacq.) Mart.
19972	*Peperomia urocarpa* Fisch. & C.A.Mey.
19973	*Sansevieria trifasciata* Prain