

The Role of the Glycerophosphocholine Remodelling in Alzheimer's Disease

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“Be the change that you wish to see in the world.”

Ghandi

Dedicated to those dear to me.

ABSTRACT

Advances in high performance liquid chromatography-electrospray ionization-mass spectrometry made in proteomics and now applied to the emerging field of lipidomics has enabled the identification of lipid composition at the molecular level. These improvements have given fresh impetus to lipid research. Modulating lipid compositions has been suggested to represent a novel therapeutic target for intervention in Alzheimer's disease. A better understanding of how metabolic alterations in the lipid landscape alter Alzheimer's disease prognosis is required to realize this promise. To achieve this goal, further methodological improvement in lipidomic data acquisition and analysis are required as are comprehensive comparative analyses of lipid metabolism at the systems level in clinical samples and mouse models of human neurodegenerative disease. In this thesis, I present two new lipidomic bioinformatic tools Retention Time Standardization and Registration (RTStaR) and Visualization and Phospholipid Identification (VaLID) designed to facilitate analysis of high performance liquid chromatography-electrospray ionization-mass spectrometry lipidomic data. Using these tools and methodologies, I then comparatively profiled the glycerophosphocholine lipidome in the plasma of young adults, cognitively normal elderly with vascular impairment, mild cognitive impairment and late-onset Alzheimer's disease patients and the entorhinal-hippocampal circuit of late-onset Alzheimer's disease patients, TgCRND8 human amyloid beta precursor protein transgenic mice (Alzheimer's disease mouse model), and across the lifespan of NonTg female littermates. Systems-level analyses identified aberrant glycerophosphocholine metabolic pathways systemically perturbed by age, disease, and amyloid beta biogenesis resulting in the regionally-specific accumulation of critical platelet-activating factor and, to a lesser

extent, the lysoglycerophosphocholine, metabolites in brain that could be, in part, predicted by changes in plasma. Finally, using proteomic approaches I identified additional changes in lipid metabolic pathways associated with phenocopy in the TgCRND8 mouse model of Alzheimer's disease.

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LIST OF ABBREVIATIONS

Δ	denotes the position of the double bonds counting from the carboxyl carbon
2D	two-dimensional
3D	three-dimensional
AA	arachidonic acid
AAPC	alkylacylglycerophosphocholine
Aβ	amyloid beta peptide
Aβ₄₀	amyloid beta peptide of 40 residues
Aβ₄₂	amyloid beta peptide of 42 residues
Ab	antibody
ACN	acetonitrile
AICD	amyloid beta precursor protein intracellular C-terminal domain
AD	Alzheimer's disease
ANOVA	analysis of variance
APAF	acyl-platelet-activating factor
ApoE	apolipoprotein E
APP	amyloid beta precursor protein
BBB	blood-brain barrier
CAD	collision gas
Cd	calibrator dataset
CDK5	cyclin-dependent kinase 5
CDP-DG	cytidine 5'-diphosphate 1,2-diacyl- <i>sn</i> -glycerols
CE	collision energy
CIHR	Canadian institutes of health research
CoA	coenzyme A
cPLA₂	cytosolic phospholipase A ₂
CT83	amyloid beta precursor protein 83-residue C-terminal fragment
CT99	amyloid beta precursor protein 99-residue C-terminal fragment
CTPNL	Canadian institutes of health research training program in neurodegenerative lipidomics
CUR	curtain gas
CXP	collision cell exit potential
DAPC	diacylglycerophosphocholine
DG	1,2-diacyl- <i>sn</i> -glycerol
DGy	dentate gyrus
DHA	docosahexaenoic acid
DHAP	dihydroxyacetone phosphate
DP	declustering potential
DPYSL	dihydropyrimidinase-related protein
E	unsaturation in the <i>trans</i> configuration
EC	entorhinal cortex
Ed	experimental dataset

EP	entrance potential
EOAD	early-onset Alzheimer's disease
ER	endoplasmic reticulum
ESI	electrospray ionization
FA	formic acid
FPC	formylglycerophosphocholine
FDR	false discovery rate
FRSQ	"Fonds de recherche en santé du Québec"
glycerol-3-P	<i>sn</i> -glycerol-3-phosphate
GP	glycerophospholipids
GS1	ion source gas 1
GS2	turbo gas
GSK3β	glycogen synthase kinase 3 beta
H	hippocampus
hAPP	human amyloid beta precursor protein
Hv	heavy isotopic dimethyl labelling
HPLC	high performance liquid chromatography
HSFO	Heart and stroke foundation of Ontario
ID	internal diameter
iPLA₂	calcium-independent phospholipase A ₂
iPLA₂-S	short isoform of the calcium-independent phospholipase A ₂
iPLA₂-L	long isoform of the calcium-independent phospholipase A ₂
IR	internal reference
IS	ion spray voltage
Lg	light isotopic dimethyl labelling
LC	long fatty chain moieties (20 and more carbons)
LCh	liquid chromatography
LIS1	lissencephaly-1 homolog
LOAD	late-onset Alzheimer's disease
locFDR	local false discovery rate
LPAF	lysoplatelet-activating factor
LPC	lysoglycerophosphocholine
LPCAT	lysophosphatidylcholine acyltransferase
LPPAF	lysoplasmenyl platelet-activating factor
lysoPA	lysoglycerophosphate
m/z	mass to charge ratio
M	molecular mass of an analyte
MAD	median absolute deviation
Md	medium isotopic dimethyl labelling
MALDI	matrix-assisted laser desorption ionization
MAP1a	microtubule-associated protein 1A
MAP1b	microtubule-associated protein 1B
MAP2	microtubule-associated protein 2
MBP	myelin basic protein
MC	medium fatty chain moieties (16-19 carbons)
MCI	mild cognitive impairment
MMSE	mini mental state exam

MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MUFA	monounsaturated fatty acid chain
n	denotes the position of the first double bond counting from the CH ₃ terminal end
ND	not determined
NFT	neurofibrillary tangles
NonTg	non transgenic
OIC	outlier identification and correction
OMHF	Ontario mental health foundation
P3	amyloid beta precursor protein fragment of 3 kDa
PA	glycerophosphates
PAF	platelet-activating factor
PAFAH	platelet-activating factor acetylhydrolase
PAFR	platelet-activating factor receptor
PA_N	normalized peak area under the curve
PAPC	plasmacylglycerophosphocholine
PA_S	peak area under the curve (intensity)
PBS	phosphate buffered saline
PC	glycerophosphocholines
PE	glycerophosphoethanolamines
PG	glycerophosphoglycerols
PGP	glycerophosphoglycerolphosphates
PI	glycerophosphoinositols
PICALM	phosphatidylinositol-binding clathrin assembly protein
PIP	glycerophosphoinositols monophosphate
PIP₂	glycerophosphoinositols bisphosphate
PIP₃	glycerophosphoinositols triphosphate
PIP_X	glycerophosphoinositols phosphate
PIS	precursor ion scan
PLA₂	phospholipase A ₂
PPA	glyceropyrophosphates
PPAF	plasmacyl platelet-activating factor
PS	glycerophosphoserines
PS-1/PS-2	presenilin protein 1 and 2
PSEN	presenilin gene
PUFA	polyunsaturated fatty acid
Q	quadrupole
R	fatty chain moieties
RIPA	radioimmunoprecipitation assay
R_m	resolution
ROUT	robust regression and outlier removal
RT	retention time
RTStaR	retention time standardization and registration
sAPPα	secreted amyloid beta precursor protein alpha
sAPPβ	secreted amyloid beta precursor protein beta

SC	short fatty chain moieties (12-15 carbons)
SCX	strong cation-exchange monolithic column
SEM	standard error of the mean
SD	standard deviation
SFA	saturated fatty acid chain
SHANK3	SRC homology 3 domain and multiple ankyrin repeat domains protein 3
<i>sn</i>	stereospecific numbering
sPAFAH	secreted platelet-activating factor acetylhydrolase
SYS	Saguenay youth study
TFA	trifluoroacetic acid
Ti⁴⁺-IMAC	titanium-immobilized metal ion affinity chromatography
Tg	transgenic
TWW	tissue wet weight
VaLID	Visualization and Phospholipid Identification
$\bar{X}$	mean
Z	unsaturation in the <i>cis</i> configuration

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CHAPTER 1. MEMBRANE METABOLISM AND ALZHEIMER'S DISEASE (AD)

1.1 “Re-imagining” the Amyloid Hypothesis

1.1.1 AD is a global pandemic

AD is primarily a disease of the elderly (Fjell et al., 2014). The prevalence of AD is 0.2 % in persons between 55 and 64 years of age, 0.9 % in persons between 65 and 74 years of age, 7.4 % in persons between 75 and 84 years of age, and 26.8 % in persons 85 years of age and older, representing a mean prevalence of 4.5 % (Ott et al., 1995). The World Health Organization predicts that the number of affected people will rise dramatically from 25 million to 80 million worldwide by 2050 given our aging population and the increased life expectancy (World Health Organization, 2012). These numbers do not even consider family members indirectly affected by the disease through care-giving. Given that AD accounts for 50 to 70 % of all cases of dementia (Blennow et al., 2006, World Health Organization, 2012), the devastating effects of dementia on both the patient and his or her family cannot be underestimated. The progressive, chronic nature of this neurodegenerative disorder is exceedingly demoralizing on care-givers, requiring tremendous and burdensome day-to-day care.

Two forms of AD exist: early-onset AD (EOAD) and late-onset AD (LOAD). EOAD accounts for less than 0.1 % of AD cases (Harvey et al., 2003). All EOAD and the majority of LOAD cases are “familial” in that heritability accounts for 60-80 % of cases (Gatz et al., 2006). Heritable genetic determinants have, however, only been definitively established for EOAD. All of the patients diagnosed with EOAD to date present with

autosomal dominant mutations in the amyloid beta precursor protein (APP) or the presenilin (PSEN) 1 or 2 genes (Blennow et al., 2006, Mucke, 2009). These mutations increase propensity of APP cleavage to toxic amyloid beta (A β) peptides by the γ -secretase complex (of which the presenilin-1 and -2 proteins (PS-1 and PS-2) are the catalytic subunits) and reduce the age of AD onset to below 65 years of age (Blennow et al., 2006). EOAD genetics are at the core of “amyloid cascade hypothesis” of AD wherein aberrant A β synthesis is considered the cause of AD described in detail below.

The majority of AD patients, however, suffer from LOAD. The heritable cause of LOAD (also known as sporadic AD) has yet to be determined. Cases are understood to be the consequence of complex interactions between different genetic and non-genetic risk factors. The strongest genetic risk association is carrier-status for apolipoprotein E (ApoE) ϵ 4 allele. Carriers are definitively at increased risk of LOAD. A single copy is associated with a four-fold increase in LOAD risk; two copies enhance risk by twelve-fold (Spinney, 2014). Close to 60 % of all LOAD patients are ApoE ϵ 4 carriers. Furthermore, genetic risk factors for LOAD have been associated with sortilin-related receptor (Rogaeva et al., 2007), clusterin (Harold et al., 2009, Lambert et al., 2009, Jun et al., 2010), bridging integrator 1 (Seshadri et al., 2010), adenosine triphosphate-binding cassette sub-family A member 7 (Corder et al., 1993, Hollingworth et al., 2011), and phosphatidylinositol-binding clathrin assembly protein (PICALM) (Harold et al., 2009, Jun et al., 2010) gene variants. Intriguingly, even if these gene products act on distinct biochemical pathways, they each share in common the modulation of some aspects of neural glycerophospholipids (GP) metabolism with the ApoE risk allele (Strittmatter et al., 1993). Non-genetic risks can be subdivided into environmental, lifestyle, and comorbid diseases. Environmental risk factors include pesticide exposure and head injury (Blennow et al., 2006, Mucke, 2009,

Campdelacreu, 2012). AD lifestyle risk factors have been suggested to include an unhealthy diet, low physical exercise, low social engagement, low mental activity, and low levels of education (Blennow et al., 2006, Mucke, 2009, Campdelacreu, 2012, Cummings et al., 2015). Comorbid diseases such as diabetes mellitus, coronary heart disease, atherosclerosis, hypertension, hypercholesterolaemia, and obesity further contribute to cerebrovascular pathology, one of the strongest co-morbid associations with AD dementia (Snowdon, 1997, Blennow et al., 2006, Hassing et al., 2009). Cerebrovascular pathology is defined, post-mortem, by the presence of lacunar infarcts (both small and large), microbleeds, atherosclerosis as well as cerebral amyloid angiopathy (Honjo et al., 2012). These pathologies in living patients are hypothesized to disturb blood flow and disrupt the blood-brain barrier (BBB) increasing intracerebral exposure to toxic mediators (Mawuenyega et al., 2010, Honjo et al., 2012). Cerebral amyloid angiopathy, in particular, is co-morbid with AD dementia in 83 % of all cases (Ellis et al., 1996).

Despite these strong correlations with risk, the greatest non-genetic risk factor for AD remains age. Several hypotheses have been put forward to explain how aging enhances AD risk. As reviewed by Mucke (2009), it has been proposed that (1) Aging enhances the prevalence of the comorbid pathologies described above. (2) Young brains are better equipped to deal with cellular insults, notably with the downstream effect of APP, PSEN1, and PSEN2 mutations. (3) Neuroinflammation, the activation of macrophages, microglia, and astrocytes, associated with AD phenoconversion (Herrup, 2010) is increased over the course of aging (Mucke, 2009). In this thesis, we will examine whether neural lipid metabolism is also altered over the course of aging.

1.1.2 Pathological hallmarks of AD

In 1906, Dr. Alois Alzheimer described the now canonical pathological hallmarks of AD, neurofibrillary tangles (NFT) and amyloid plaques (Alzheimer, 1907, Alzheimer et al., 1995, Blennow et al., 2006). Both pathologies are formed by the aggregation of endogenous proteins, tau and A β respectively (Hardy and Selkoe, 2002, Palop and Mucke, 2010, Benilova et al., 2012). NFTs are intracellular filamentous aggregates of hyperphosphorylated microtubule-associated protein tau. Tau promotes microtubule assembly and stability, and is itself tightly regulated by phosphorylation on multiple sites. Hyperphosphorylated tau tends to dissociate from the microtubules resulting in instability and impaired axonal transport (Blennow et al., 2006). Labile hyperphosphorylated tau can self-assemble into paired helical filaments that aggregate as NFTs (Figure 1-1) (Cowan and Mudher, 2013). Interestingly, the degree of abnormal tau deposition is highly correlated with the severity of AD progression (Bierer et al., 1995).

The second defining pathology is the extracellular deposition of amyloid plaques in brain parenchyma. Amyloid plaques are composed of A β peptides, the most toxic being the 42 residue peptide (A β ₄₂) (Teplow, 1998, Selkoe, 2002, Bernstein et al., 2005). A β peptides are generated by the sequential cleavage of APP. APP is a ubiquitous type I integral membrane protein that is highly expressed in neurons. The APP gene is located on chromosome 21 (Hardy and Allsop, 1991, Selkoe, 1991). Three major isoforms are obtained by alternative splicing generating isoforms of 695, 751 and 770 amino acids with APP695 being neuron-specific. Aberrant processing, described below, generates A β

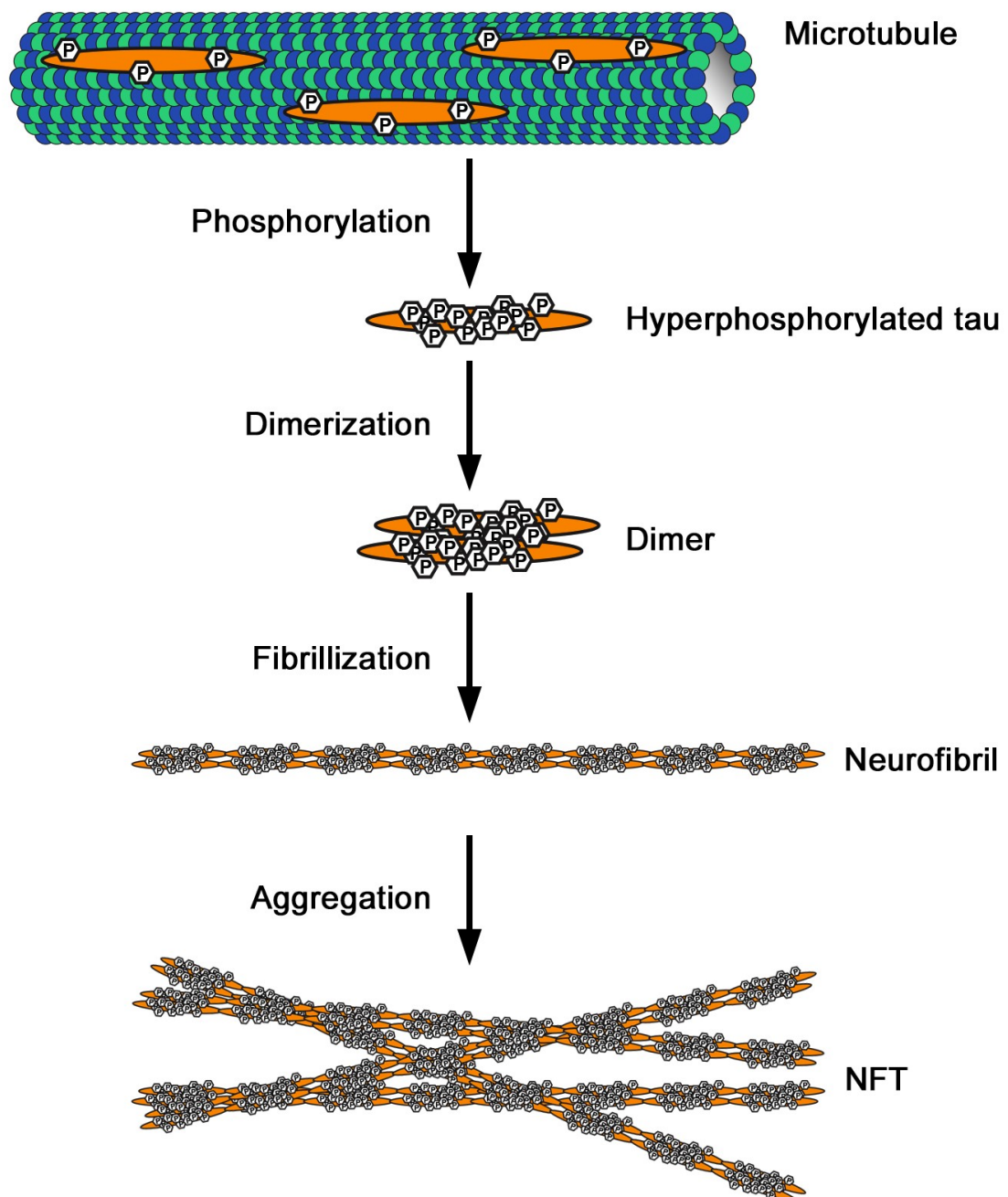


Figure 1-1

Figure 1-1 NFT Formation

Simplified schematic depicting physiologically phosphorylated microtubule-associated protein tau associating with and stabilizing microtubules. Pathophysiologically hyperphosphorylated tau dissociates from the microtubule, dimerizes, fibrillizes, and finally precipitates inside the neurons as NFT.

peptides of various lengths, ranging from 39 to 43 residues (Prelli et al., 1988, Teplow, 1998). A β ₄₀ and A β ₄₂ are the most abundant forms detected *in vivo* (Teplow, 1998, Bernstein et al., 2005).

The amyloidogenic pathway constitutively generates A β through sequential proteolytic cleavage of APP by β -secretase and γ -secretase (Blennow et al., 2006, C. Crdenas-Aguayo et al., 2014) (Figure 1-2). The γ -secretase complex, which is also regulated by PICALM, identified as an AD risk factor, is a membrane enzymatic protein complex formed by the PS-1 or PS-2, nicastrin, anterior pharynx defective 1, and presenilin enhancer 2. The catalytic subunits, PS-1 and -2, cleave the carboxyl terminal of APP directly in the lipid bilayer, where the first 28 amino acids of the 39 to 43 residues A β region are extracellular and the remaining 11 to 15 are embedded in the membrane (Selkoe, 1991). Thus, variation in the length of A β is due to the γ -secretase cleavages at different positions of APP. Abnormal accumulation of A β peptides, initially generated in α -helical conformation, will self-assemble following conversion to a β -sheet conformation leading to dimerization, oligomerization, protofibril aggregation of toxic oligomers, fibril formation and finally deposition in amyloid plaques (Teplow, 1998, Nag et al., 2011). Under normal physiological conditions, the balance between the A β production, degradation (mediated by the insulin-degrading enzyme, neprilysin, and an endothelin-converting enzyme), and clearance is tightly regulated (Blennow et al., 2006, Shankar and Walsh, 2009). In AD, this balance is altered such that A β accumulates (Hardy and Selkoe, 2002, Selkoe, 2002). By contrast, the non-amyloidogenic pathway precludes A β production because α -secretase cleaves APP in the middle of the A β region (Mattson, 2004) (Figure 1-2).

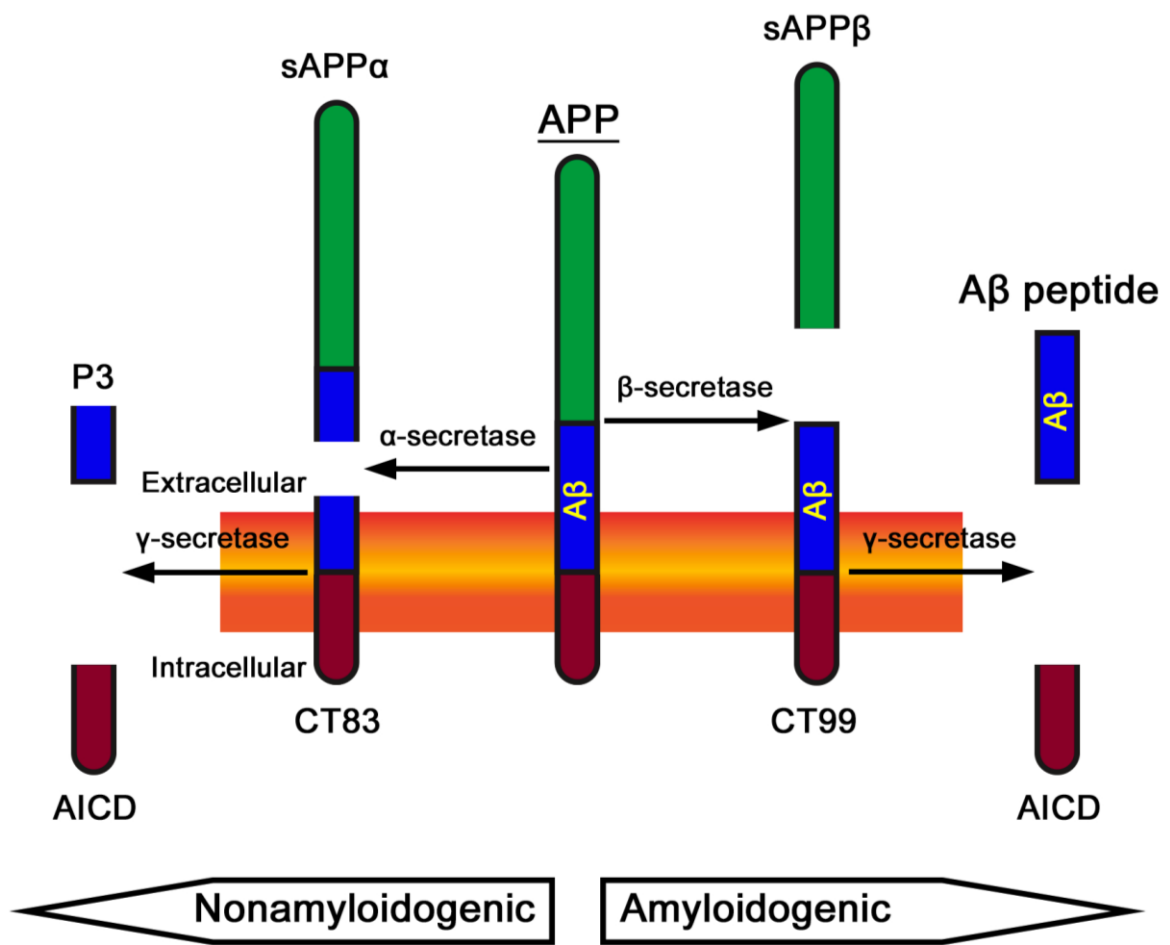


Figure 1-2

Figure 1-2 APP Processing

Simplified schematic depicting the processing of the APP protein through either the nonamyloidogenic or amyloidogenic pathway. The nonamyloidogenic pathway precludes the formation of A β peptide, because α -secretase cleaves within the A β domain of the APP to form the secreted N-terminal APP α (sAPP α) fragment and the 83-residue C-terminal fragment (CT83). The latter fragment is further processed by the γ -secretase to release the 3 kDa protein (P3) and the APP intracellular C-terminal domain (AICD) fragment. The A β fragment is produced by the amyloidogenic pathway through sequential cleavage of APP by the β - and γ -secretases. First, the β -secretase cleavage produces the secreted N-terminal APP β (sAPP β) fragment and the 99-residue C-terminal fragment (CT99). Second, the A β and the AICD fragment are released by the γ -secretase from the CT99. Adapted from Crdenas-Aguayo et al. (2014).

The presence of NFTs and amyloid plaques is not, however, sufficient for a diagnosis of AD although it has been suggested that detection of these pathologies in cognitively normal elderly is evidence of preclinical disease. These pathologies must be accompanied by a progressive decline in cognition, notably memory (Jack et al., 2013). There is a long latency for disease conversion in that A β accumulation is detected years even decades prior to evidence of cognitive decline (preclinical disease) and/or some persons can resist the neurodegenerative effects of the defining AD pathologies (Jack et al., 2013). For example, A β plaques and NFT can be found in cognitively normal elderly (Snowdon, 2003, Fjell et al., 2010, Morris et al., 2010). Moreover, reduced levels of A β in cerebrospinal fluid, a biomarker of central fibrillary A β_{42} deposits (Strozyk et al., 2003) are detected in pre-symptomatic patients (Buchhave et al., 2012). Phenocconversion from this preclinical condition to a clinical AD state requires manifestation of learning and memory impairments as well as cognitive deficits in executive function (reasoning, judgment, and problem solving), language (word-finding), and visuospatial presentation (object agnosia, impaired face recognition) (Larson et al., 1992, McKhann et al., 2011).

1.1.3 Amyloid cascade hypothesis

The amyloid cascade hypothesis has been the defining causal hypothesis for AD for the past two decades (Hardy and Allsop, 1991, Selkoe, 1991, Hardy and Higgins, 1992, Herrup, 2010, Swerdlow et al., 2010, Herrup et al., 2013, McGeer and McGeer, 2013). This hypothesis postulates that AD is caused by the progressive accumulation of A β peptides, due to an imbalance in A β production and A β clearance. A β accumulation leads to its self-assembly, oligomerization, and deposition as diffuse and dense-core plaques (Figure 1-3), triggers subtle synaptic deficits as soluble A β oligomers, activates chronic

inflammatory responses (microglial and astrocytic), signals neuritic dysfunction, and alters a myriad of kinase and phosphatase activities including those leading to NFT development (Hardy and Allsop, 1991, Selkoe, 1991, Hardy and Higgins, 1992, Hardy and Selkoe, 2002).

Certainly, converging genetic evidence provides strong support for this hypothesis. First, all the mutations in genes responsible for the EOAD enhance the amyloidogenic production of A β peptides (Mucke, 2009). The EOAD autosomal dominant mutations in APP, PSEN1, and PSEN2 favour either the production of A β through the amyloidogenic processing pathway or its propensity to self-aggregate resulting in its accumulation (Wisniewski et al., 1991, Citron et al., 1992, Cai et al., 1993, Suzuki et al., 1994). Second, patients with Down syndrome (trisomy 21) and hereditary cerebral haemorrhage with amyloidosis of the Dutch type present with AD symptoms earlier than LOAD patients. In Down syndrome patients, the triplication of chromosome 21 leads to increased production of APP that accelerates the assembly of toxic A β peptides (Hardy and Allsop, 1991, Hardy and Selkoe, 2002). In hereditary cerebral haemorrhage with amyloidosis of the Dutch type, a point mutation in the APP gene favours A β precipitation associated with cerebrovascular dementia (Levy et al., 1990, Van Broeckhoven et al., 1990). Third, overexpressing human APP with EOAD mutations in transgenic mouse models results in A β deposition (Bilkei-Gorzo, 2014). Plaque load is reduced when mice are crossed with ApoE null-mutants, a risk factor for AD implicating lipid metabolism in the acceleration of A β processing (Bales et al., 1997). Finally, perhaps the strongest evidence that A β production is required for AD comes from recent evidence that mutations in the APP gene that render the protein resistant to β -secretase cleavage is sufficient to protect carriers from both AD and age-related dementia (Jonsson et al., 2012).

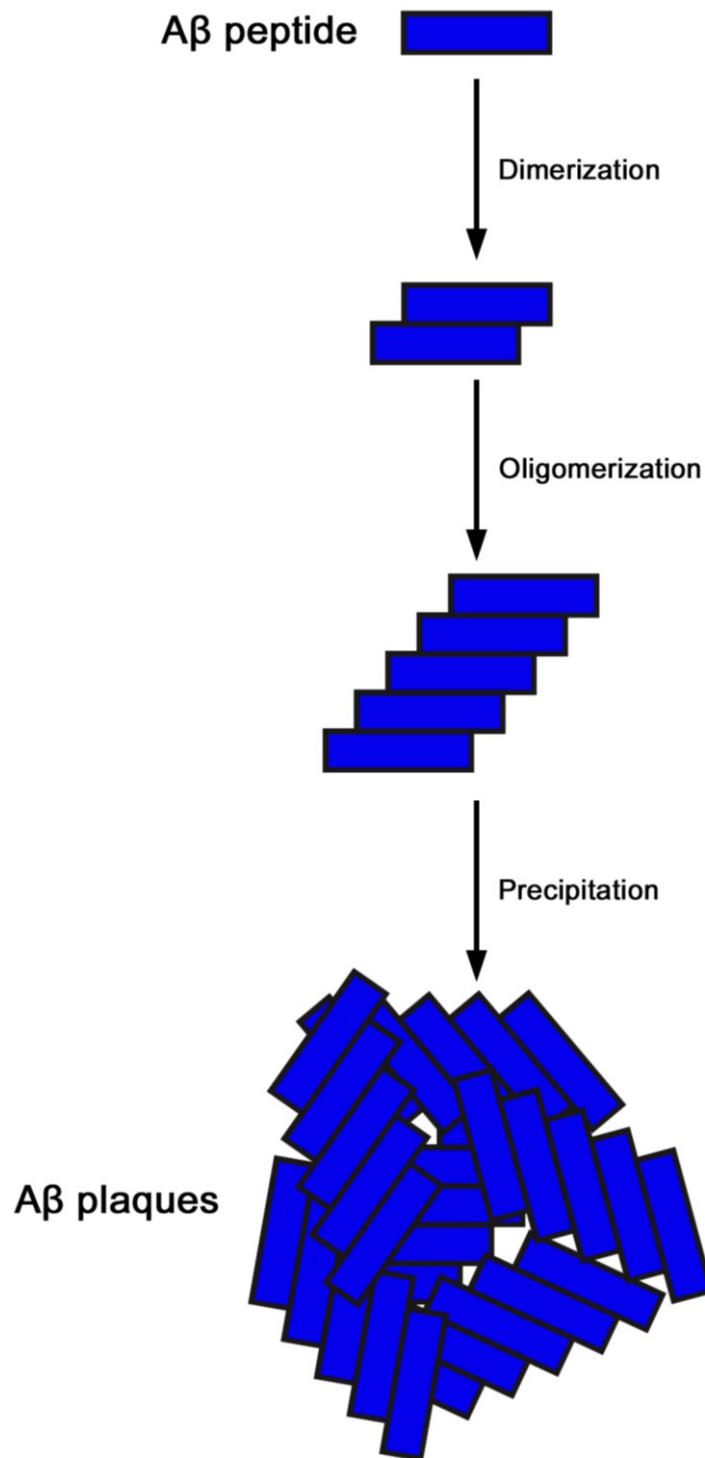


Figure 1-3

Figure 1-3 A β Plaque Formation

Simplified schematic depicting the dimerization, oligomerization, and precipitation of accumulating A β peptides, notably A β_{42} , as extracellular A β plaques.

A β assembly appears to be upstream of tau pathology in that tau pathology does not, in and of itself, cause aberrant A β assembly yet A β can increase aberrant tau processing. For example, autosomal dominant mutations in the tau gene causing frontotemporal dementia with parkinsonism result in NFT assembly (Hutton et al., 1998, Poorkaj et al., 1998, Spillantini et al., 1998). In this dementia, amyloid plaques are undetectable (Spillantini et al., 1998). However, in humanized transgenic and knock-in mouse models expressing human mutant tau, NFT deposition can be increased by concomitant overexpression of human mutant APP (Lewis et al., 2001). In this same mouse model, soluble A β_{40} and A β_{42} concentration and A β plaque loads were comparable in presence or absence of human mutant tau suggesting that A β may accelerate tau pathology but tau pathology does not directly impact on A β accumulation (Lewis et al., 2001, Hardy and Selkoe, 2002). In collaboration with other members of our laboratory, I have also shown that A β can signal tau hyperphosphorylation in human neurons on AD-associated epitopes, a crucial step in the formation of NFT (Ryan et al., 2009a). A β_{42} signals the intraneuronal accumulation of platelet activating factor lipid metabolites, specifically a species with a hexadecyl alkyl-linked fatty acid at the stereospecific numbering (*sn*)-1 position, through an endoplasmic reticulum (ER) stress pathway (Ryan et al., 2009a). These disruptions in lipid in alkylacylglycerophosphocholine metabolism elicited activation of cyclin-dependent kinase 5 (CDK5) that resulted in aberrant tau phosphorylation were shown to enhance AD pathology (Ryan et al., 2009a). Other signalling evidence suggests that tau is downstream A β , since tau acts as a modulator of A β toxicity both *in vitro* (Rapoport et al., 2002) and *in vivo* (Roberson et al., 2007).

A β peptides are neurotoxic, with soluble oligomeric assemblies of A β_{42} the most damaging of species (Lambert et al., 1998, Walsh et al., 2002, Lesne et al., 2006, Shankar

et al., 2008). A β toxicity starts at the synapses where the presence of A β_{42} peptides have been shown to inhibit long-term potentiation and facilitate long-term depression (Lambert et al., 1998, Walsh et al., 2002, Wang et al., 2005, Shankar et al., 2008). Synaptotoxic mechanisms following A β exposure include a reduction in synaptic density that is associated with progressive cognitive impairment (Terry et al., 1991, Shankar et al., 2008). Decreasing A β burden reduces AD-like behavioural impairments in rodent models. A β immunization by acute or chronic vaccination lowers A β load (Schenk et al., 1999) and leads to memory improvements in APP transgenic mice (Janus et al., 2000, Morgan et al., 2000, Dodart et al., 2002, Kotilinek et al., 2002, Yu et al., 2014b). Other A β burden reduction strategies that ameliorate memory deficits in animal models include the genetic ablation (Ohno et al., 2004, Kimura et al., 2010) or pharmacological inhibition (Fukumoto et al., 2010, Zhu et al., 2010, Chang et al., 2011) of β -secretase, the activation of phagocytosis by the Toll-like receptor 9 activation (Scholtzova et al., 2009), and the lipid-centric destabilization of A β aggregates by the intraperitoneal administration of liposomes that cross the BBB (Balducci et al., 2014).

1.1.4 A β is only one of the molecular determinants of AD

The primacy of the amyloid hypothesis as the sole cause of AD has, however, been recently challenged. There is growing consensus that A β accumulation is likely not the only pathology necessary for AD phenocconversion. It is now clear that not all persons with a high burden of amyloid and NFT pathology present with any of the cognitive impairments normally associated with AD (Crystal et al., 1988, Katzman et al., 1988, Mochizuki et al., 1996, Troncoso et al., 1996, Price and Morris, 1999, Knopman et al., 2003). Moreover, the clinical relevance of the neurotoxicity hypothesis has been

challenged in that A β is neurotoxic *in vitro* at micromolar concentrations (Ryan et al., 2008), but A β levels are now known to range *in vivo* from 1 to 15 picomole per gram, estimated at approximately 1-15 nM in AD brain (Mintun et al., 1984, Kies et al., 2004, McGeer and McGeer, 2013). Thus, the extensive synaptic loss and cell death observed in LOAD (Davies et al., 1987, Masliah et al., 1991) may not be entirely the direct consequence of A β neurotoxicity.

1.1.5 AD hypothesis: reborn from its ashes

The AD field is in the process of “re-imagining” the AD hypothesis (Herrup, 2010, Swerdlow et al., 2010, Herrup et al., 2013, McGeer and McGeer, 2013). Here, A β ’s role has been relegated from the primary cause of AD to a requisite toxic factor but only one of multiple converging metabolic pathways necessary for conversion from pre-symptomatic to symptomatic AD (Figure 1-4). In Herrup’s age-dependent hypothesis, healthy aging is distinguished from the pathological aging associated with AD (Herrup, 2010). In the first step, the thin line between normal age-associated changes in cognition and pathological AD is crossed by acute injury. These injuries could include a cerebrovascular event, a major illness or stress, or an infection. Critical injuries trigger a neuroinflammatory cascade. If this neuroinflammation does not resolve, it accelerates, via positive feedback, the ongoing amyloid deposition cycle, thereby amplifying A β accumulation that in turn sustains the neuroinflammation. This self-perpetuating cycle both accelerates aberrant A β processing and maintains a chronic neuroinflammatory state, is considered the second step in promoting AD conversion. The third and last step, is the induction of a metabolic “change of state”, an all-encompassing shift in normal brain metabolism priming the brain to adopt a pathological state. Herrup (2010) defines this change of state as a key shift in metabolism

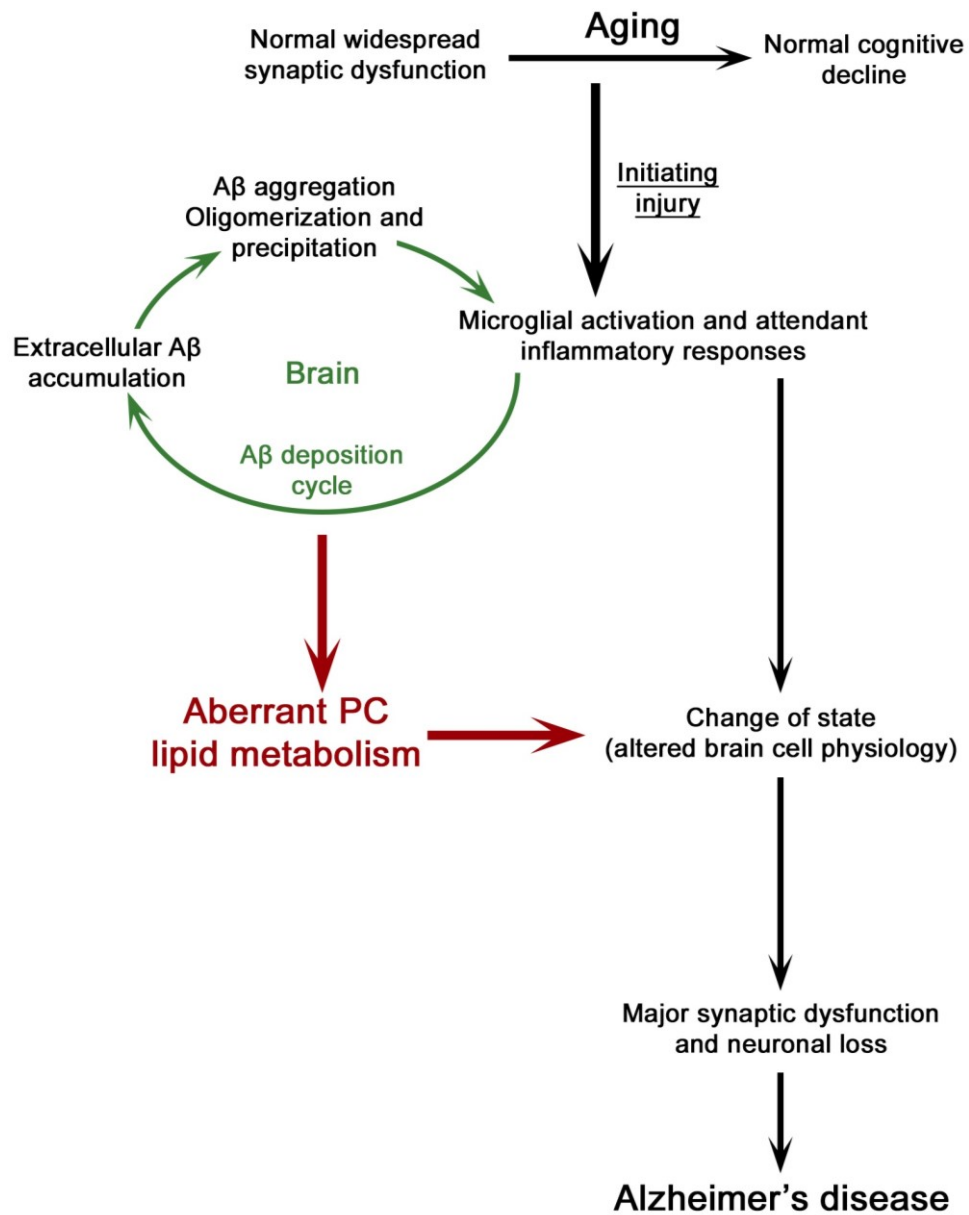


Figure 1-4

Figure 1-4 “Re-Imagining” AD

An alternative to the amyloid hypothesis was advanced by Karl Herrup in 2010 wherein the amyloid cascade represents one of several prerequisites for AD phenoconversion enhanced by age. With advance in age, there is normal cognitive decline and increased A β deposition. This decline can progress to AD if the A β deposition cycle is further reinforced by chronic neuroinflammation induced by acute injury. If this neuroinflammatory response is sustained chronically in aging individuals, as a result of induction of a vicious feedback loop associating the A β deposition cycle with activation of brain-resident and peripheral inflammatory cells infiltrating the BBB, then a change of state can occur whereby the brain's own metabolic processes signal multiple neurodegenerative processes required for AD phenoconversion. This change of state is the root cause of the major synaptic dysfunction underlying AD. Schematic is modified from Herrup (2010).

“that results in a ‘new normal’ biology, primed toward neurodegeneration and dementia”. Together, this “new normal” (pathological) metabolism progressively leads the brain toward the neurodegeneration, synaptic dysfunction and neuronal death, responsible for AD memory and cognitive deficits (Figure 1-4) (Herrup, 2010). Although not explicitly addressed by Herrup (2010), I argue, in this thesis, that defining changes in lipid metabolism represent one of the critical changes required for this dramatic change of state and, thus, for the conversion from pre-symptomatic to symptomatic AD.

1.2 Do Impairments in Neural Membrane Lipid Metabolism Signal AD Conversion?

1.2.1 Lipids are essential for brain function

Lipids constitute half of the dry weight of the human brain (Piomelli et al., 2007) which is the second most lipid-abundant organ after adipose tissue (Adibhatla et al., 2006). Lipid preponderance in the brain makes it an attractive subject for a metabolic regulator of brain function. Furthermore, lipids play a role in learning and memory (Kato et al., 1994, Yaguchi et al., 2009b, a, Kofeler et al., 2010). A potential link between lipid dysregulation and this AD change of state is also strengthened by known associations between aberrant lipid metabolism in other brain injuries, diseases, and neurodegenerative conditions including bipolar disorders, Niemann-Pick disease, Parkinson’s disease, stroke, schizophrenia, and traumatic brain (Adibhatla and Hatcher, 2008).

Why are lipids so important? Life as we know it is impossible without lipids. Lipids self-assemble to form biological membranes that compartmentalize tissues and cells. Disruption of membrane integrity results in the loss of energy, ions, and proteins essential to life (Klein, 2000). GP are the major component in eukaryotic membranes accounting for

approximately 50 % of all membrane lipids (Farooqui, 2007). GP species are defined by their structures. Each GP contains a central glycerol backbone linked to three defining subgroups: a phospho-headgroup and two fatty chain moieties (Bou Khalil et al., 2010, Shevchenko and Simons, 2010). The phospho-headgroups in the *sn*-3 position of the glycerol backbone define the GP class (Fahy et al., 2005, Fahy et al., 2009):

- (1) glycerophosphocholines (PC),
- (2) glycerophosphoethanolamines (PE),
- (3) glycerophosphoserines (PS),
- (4) glycerophosphoglycerols (PG),
- (5) glycerophosphoglycerophosphates (PGP),
- (6) glycerophosphoinositols (PI),
- (7) glycerophosphoinositol monophosphates (PIP),
- (8) glycerophosphoinositol bisphosphates (PIP₂),
- (9) glycerophosphoinositol trisphosphates (PIP₃),
- (10) glycerophosphates (PA),
- (11) glyceropyrophosphates (PPA), and
- (12) CDP-glycerols (CDP-DG).

The *sn*-1 and *sn*-2 position of the glycerol backbone are linked either to an hydroxyl group or a carbon chain of varying lengths. Species identity is further defined by the linkage of these carbon chains, ester (acyl chain), ether (alkyl chain), or vinyl ether (alkenyl chain), to the backbone, the length of the carbon chain length generally from 12 to 24 carbons in structural membrane lipids with 1-10 carbons (or an hydroxyl group) defining biologically active membrane metabolites; and the number of unsaturations within these carbon chains commonly up to six unsaturations. To date, at least 57 different carbon chains have been

identified in the mammalian tissues, including 27 common (Table 1-1) (Kishimoto et al., 1973, Rezanka, 1989, Soderberg et al., 1991, Johnson et al., 1992, Couture and Hulbert, 1995, Vance and Vance, 2008, Fonteh et al., 2014). Permutations of all these “building blocks” and linkages to the backbone generates an impressive diversity of GPs. To date, the mammalian curated GP lipidome contain 9387 different species and the PC family 1863 species (Fahy et al., 2007b, Fahy et al., 2009, Fahy et al., 2011) although exact numbers are predicted to be higher once full biological lipidomes have been elucidated (Shevchenko and Simons, 2010, Wenk, 2010, Laaksonen and Ekroos, 2011). Nomenclature has recently been standardized by the LIPID MAPS consortium (Figure 1-5) (Fahy et al., 2005, Fahy et al., 2009, Fahy et al., 2011).

PC is the major GP in the brain, representing 40-50 % of the total GP membrane content (Gunstone et al., 2007, van Meer et al., 2008). PC species are divided into six naturally occurring subfamilies (Duclos, 1993, Fahy et al., 2009, Fahy et al., 2011):

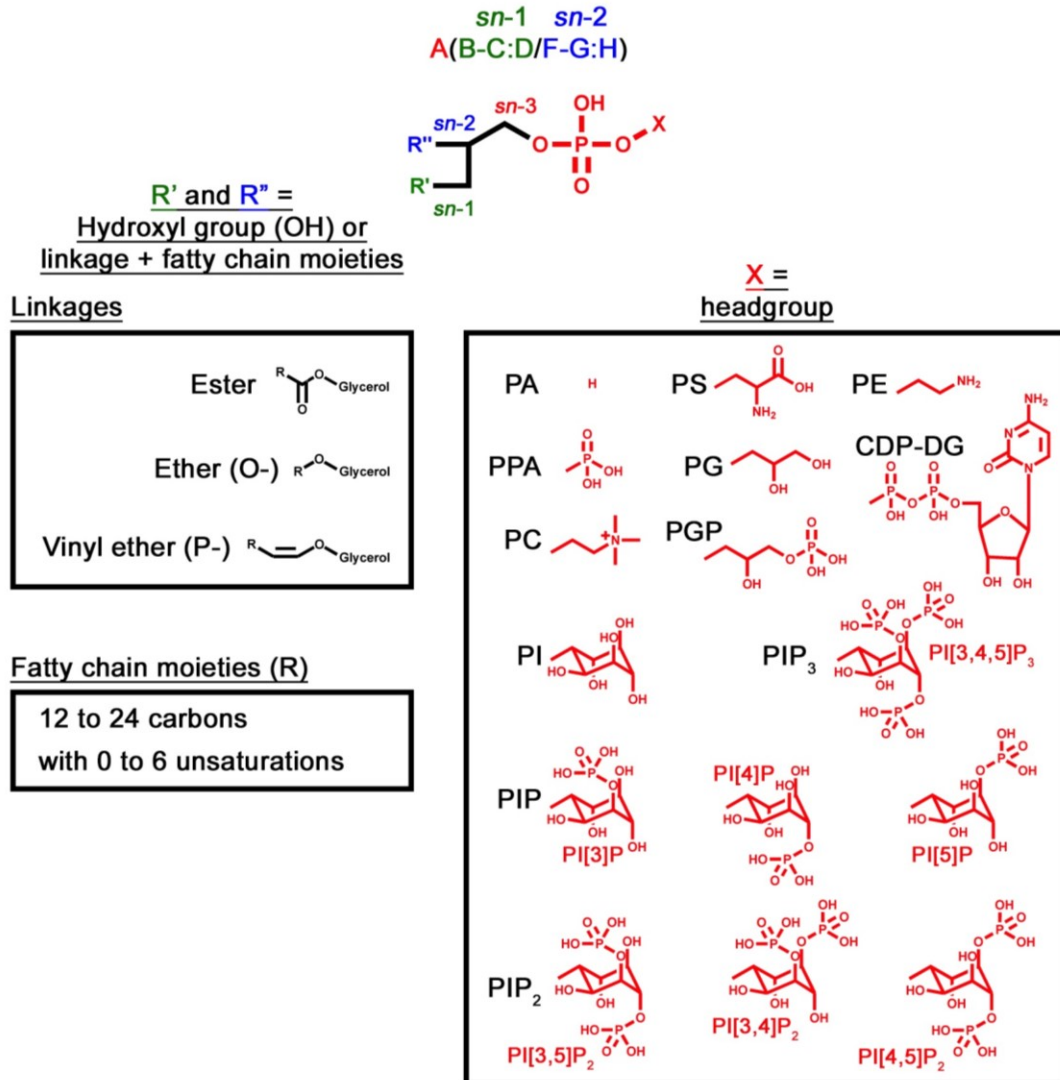
- (1) 1,2-diacylPC,
- (2) 1-alkyl-2-acylPC,
- (3) 1-alkenyl-2-acylPC,
- (4) monoacylPC,
- (5) monoalkylPC, and
- (6) monoalkenylPC.

1,2-diacylPC (diacylglycerophosphocholine: DAPC), 1-alkyl-2-acylPC (alkylacylglycerophosphocholine: AAPC), and 1-alkenyl-2-acylPC (plasmenylacylglycerophosphocholine: PAPC) are membrane precursors that can be metabolized into potent metabolites: 1-acyl-2-acetylPC (acyl-platelet-activating factor: APAF), 1-alkyl-2-acetylPC (platelet-activating factor: PAF), and 1-alkenyl-2-acetylPC

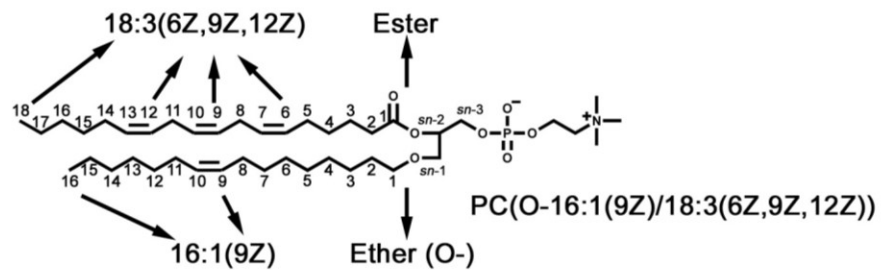
Table 1-1 Common and Rare Fatty Acids in Mammalian Cells

Fatty acids	#	Rarity	Abbreviation	Number of carbon	Unsaturation	Double bond positions
Saturated fatty acid (SFA)	1	Rare	C10:0	10	0	
	2	Rare	C11:0	11	0	
	3	Common	C12:0	12	0	
	4	Common	C14:0	14	0	
	5	Rare	C15:0	15	0	
	6	Common	C16:0	16	0	
	7	Rare	C17:0	17	0	
	8	Common	C18:0	18	0	
	9	Rare	C19:0	19	0	
	10	Common	C20:0	20	0	
	11	Common	C22:0	22	0	
	12	Common	C24:0	24	0	
	13	Rare	C25:0	25	0	
	14	Rare	C26:0	26	0	
	15	Rare	C28:0	28	0	
Monounsaturated fatty acid (MUFA)	16	Rare	C14:1	14	1	ND
	17	Rare	C15:1n-7	15	1	Δ8
	18	Common	C16:1n-7	16	1	Δ9
	19	Rare	C16:1n-9	16	1	Δ7
	20	Common	C16:1n-10	16	1	Δ6
	21	Rare	C17:1n-7	17	1	Δ10
	22	Rare	C17:1n-8	17	1	Δ9
	23	Common	C18:1n-7	18	1	Δ11
	24	Common	C18:1n-9	18	1	Δ9
	25	Rare	C19:1n-8	19	1	Δ11
	26	Rare	C19:1n-10	19	1	Δ9
	27	Common	C20:1n-7	20	1	Δ13
	28	Common	C20:1n-9	20	1	Δ11
	29	Rare	C21:1n-8	21	1	Δ13
	30	Rare	C22:1n-7	22	1	Δ15
	31	Common	C22:1n-9	22	1	Δ13
	32	Rare	C22:1n-11	22	1	Δ11
	33	Rare	C23:1n-7	23	1	Δ16
	34	Rare	C23:1n-8	23	1	Δ17
	35	Rare	C24:1n-7	24	1	Δ17
	36	Common	C24:1n-9	24	1	Δ15
	37	Rare	C25:1n-7	25	1	Δ18
	38	Rare	C25:1n-9	25	1	Δ16
	39	Rare	C26:1n-7	26	1	Δ19
	40	Rare	C26:1n-9	26	1	Δ17
	41	Rare	C27:1n-7	27	1	Δ20
	42	Rare	C28:1n-7	28	1	Δ21
	43	Rare	C28:1n-9	28	1	Δ19
Polyunsaturated fatty acid (PUFA)	44	Common	C18:2n-6	18	2	Δ9,12
	45	Common	C18:3n-3	18	3	Δ9,12,15
	46	Common	C18:3n-6	18	3	Δ6,9,12
	47	Common	C18:4n-3	18	4	Δ6,9,12,15
	48	Rare	C20:2n-6	20	2	Δ11,14
	49	Common	C20:3n-6	20	3	Δ8,11,14
	50	Common	C20:3n-9	20	3	Δ5,8,11
	51	Common	C20:4n-6	20	4	Δ5,8,11,14
	52	Common	C20:5n-3	20	5	Δ5,8,11,14,17
	53	Common	C22:4n-6	22	4	Δ7,10,13,16
	54	Common	C22:5n-3	22	5	Δ7,10,13,16,19
	55	Common	C22:5n-6	22	5	Δ4,7,10,13,16
	56	Common	C22:6n-3	22	6	Δ4,7,10,13,16,19
	57	Rare	C24:2	24	2	ND

The n and Δ symbol denote the position of the double bonds. The n nomenclature considers only the first double bond counting from the terminal CH₃ end. The Δ nomenclature begins carbon count with the carboxyl carbon. All unsaturations are in the *cis* (Z) configuration (Kishimoto et al., 1973, Rezanka, 1989, Soderberg et al., 1991, Johnson et al., 1992, Couture and Hulbert, 1995, Vance and Vance, 2008, Fonteh et al., 2014). ND: not determined.



Example 1



Example 2

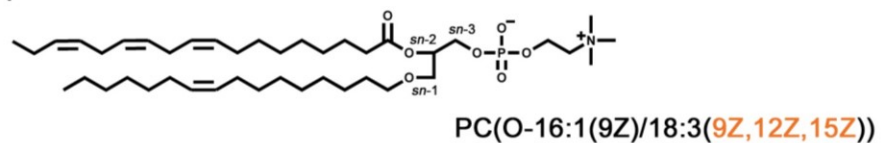


Figure 1-5

Figure 1-5 The Standardized GP Nomenclature Established by LIPID MAPS

GP species can be identified by their A(B-C:D/F-G:H) structure, where A denotes the GP headgroup; B, C, and D, the fatty chain moieties in *sn*-1; and F, G, and H, the fatty chain moieties in *sn*-2 position of the glycerol backbone. B and F describe the linkage by which the fatty chain moieties are linked to the glycerol backbone, i.e., ester, ether (O-), or vinyl ether (P-). C and G denote the number of carbons and, D and H, the number of unsaturation present in each fatty chain moieties. The addition of the position and relative orientation of the double bonds [i.e. *cis* (Z) and *trans* (E)] is added in parenthesis after the number of unsaturation completes the full identification of a specific species. This nomenclature is able to convey defining structural elements of even the most closely related isomeric and isobaric species. As an example, PC(O-16:1(9Z)/18:3(9Z,12Z,15Z)) refers to a GP with a phosphocholine headgroup at the *sn*-3 position, a fatty alkyl carbon chain linked by an ether linkage at the *sn*-1 position that contains 16 carbons with one Z unsaturation between the 9th and 10th carbon, and a fatty acyl chain moiety (ester linkage) at the *sn*-2 that contains 18 carbons with three Z unsaturations between the 9th and 10th, 12th and 13th, and 15th and 16th carbon, respectively. In contrast, PC(O-16:1(9Z)/18:3(6Z,9Z,12Z)) is an anatomically similar species that differs only by the position of its double bonds between the 6th and 7th, 9th and 10th, and 12th and 13th carbon.

(plasmenyl platelet-activating factor: PPAF) as well as their immediate precursors and metabolites, monoacylPC (lysoglycerophosphocholine: LPC), monoalkylPC (lysoplatelet-activating factor: LPAF), and monoalkenylPC (lysoplasmenyl platelet-activating factor: LPPAF).

1.2.2 *De novo* and remodelling pathways: production of bioactive GP metabolites

The brain is a largely autonomous organ that fulfills its own lipid needs by lipid synthesis (Edmond, 2001). All cell types synthesize lipids; however, glial cells are the major source of lipids in the brain (Jones et al., 2010, Camargo et al., 2012). GP metabolic pathways are summarized in Figure 1-6 (Vance and Vance, 2008). The production of GP species requires fatty acids as “building blocks”. While major non-essential fatty acids (palmitic C16:0, stearic C18:0, and oleic C18:1) are synthesized in the brain, the essential fatty acids (alpha-linolenic acid C18:3n-3 and linoleic acid C18:2n-6) cannot be produced by mammalian cells. Thus, the essential fatty acids as well as their polyunsaturated fatty acids metabolites must be imported across the BBB (Edmond, 2001, Farooqui et al., 2007b, Camargo et al., 2012, Nguyen et al., 2014). In order to be linked to the glycerol backbone, these fatty acids need to be activated (Figure 1-7B) (Vance and Vance, 2008). Fatty acid-coenzyme A (CoA) synthetase is the enzyme responsible for the activation of free fatty acid through their conversion to acyl-CoA. This reaction occurs in different organelles, including the peroxisome, ER, mitochondria, and mitochondria-associated membranes (Vance and Vance, 2008, Watkins and Ellis, 2012). The production of ether lipid prerequisites, an additional step that converts the acyl-CoA to a fatty alcohol chain by the fatty acyl-CoA reductase, occurs exclusively in the peroxisome (Figure 1-7B). General

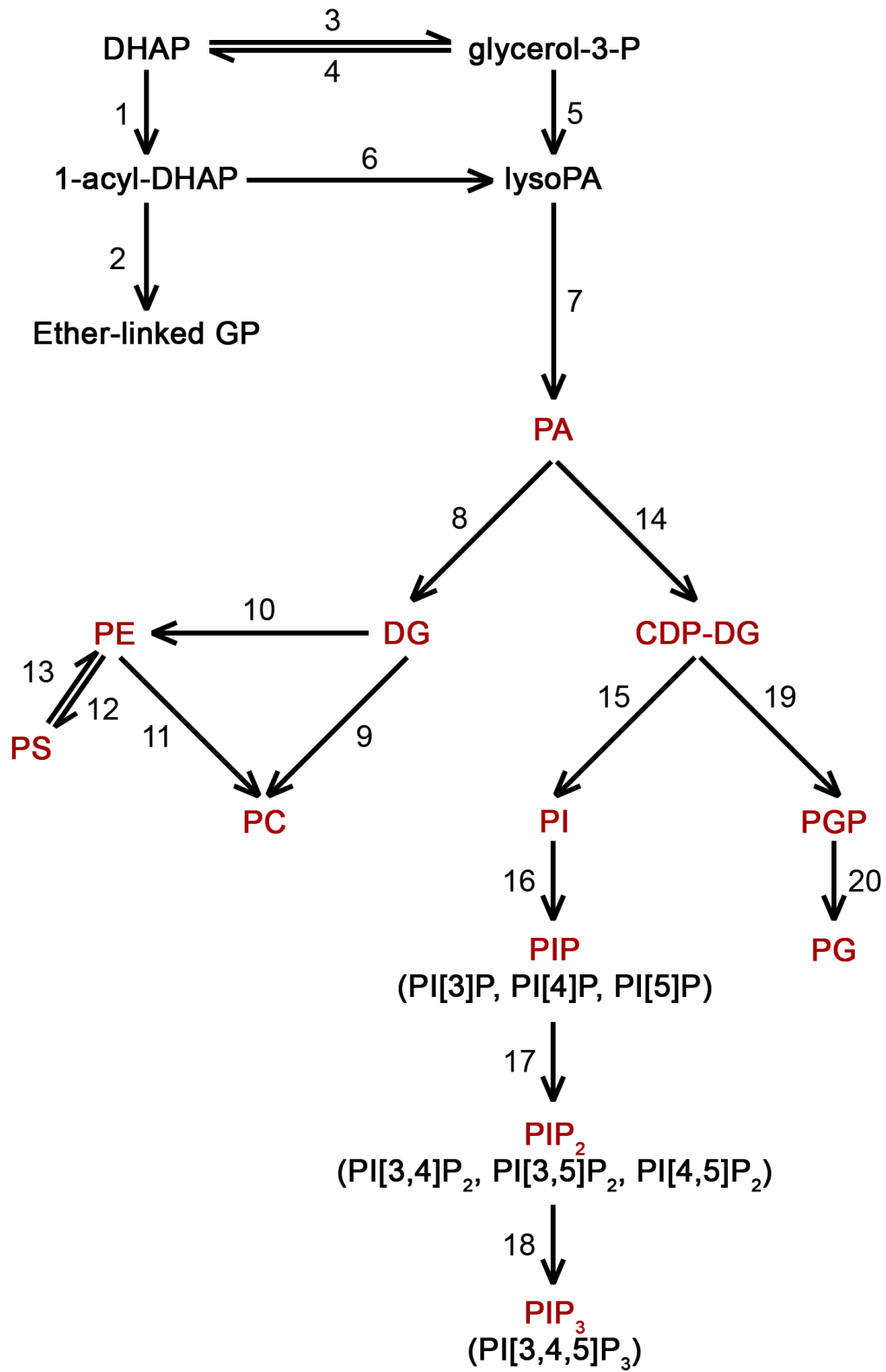
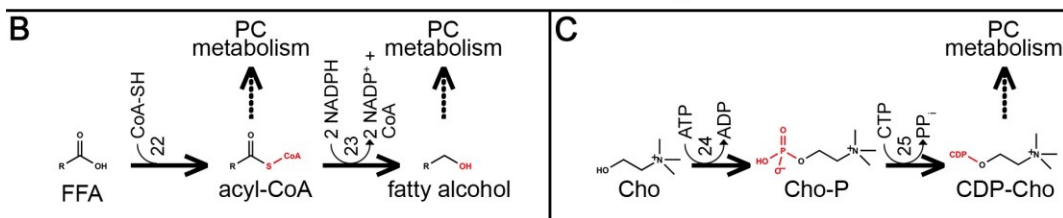


Figure 1-6

Figure 1-6 GP *de novo* Synthesis

The overall *de novo* synthesis of GP is initiated by the conversion of dihydroxyacetone phosphate (DHAP), a product from the glycolysis metabolic pathway, to either *sn*-glycerol-3-phosphate (glycerol-3-P) or 1-acyl-DHAP. The first reaction is catalyzed by the glycerol-3-P dehydrogenase in mitochondria. The second reaction, catalyzed by the DHAP acyltransferase occurs in the peroxisome. Glycerol-3-P and 1-acyl-DHAP can be converted to lysoglycerophosphate (lysoPA, 1-acyl-glycerol-3-P or 1-acylPA) by glycerol-3-P acyltransferase in mitochondria or ER and 1-acyl/alkyl DHAP reductase in peroxisomes, respectively (Vance and Vance, 2008). In addition, 1-acyl-DHAP is the first precursor, in the synthesis of ether-linked GP. 1-acyl-*sn*-glycerol-3-P acyltransferase adds a fatty chain moiety to lysoPA to form PA (1,2-diacyl-glycerol-3-P or 1,2-diacylPA) in either the ER or mitochondria. PA is central to the GP synthesis because it gives rise, through two distinct pathways, to all of the GP. PA species are transformed to 1,2-diacyl-*sn*-glycerol (DG) by the phosphatidic acid phosphatase in the ER. DG is the precursor of PC, PE, and PS species. If PA is converted to CDP-DG by the CDP-DG synthase in the ER and mitochondria, it give rises to the PI, PIP, PIP₂, PIP₃, PGP, and PG species (Vance and Vance, 2008). (1) DHAP acyltransferase, (2) 1-alkyl DHAP synthase, (3) cytosolic NAD⁺ glycerol-3-P dehydrogenase, (4) mitochondrial FAD glycerol-3-P dehydrogenase, (5) glycerol-3-P acyltransferase, (6) 1-acyl/alkyl DHAP reductase, (7) 1-acyl-glycerol-3-P acyltransferase, (8) PA phosphatase, (9) CDP-choline:1,2-diacylglycerol cholinephosphotransferase, (10) CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase, (11) PE N-methyltransferase, (12) PS synthase, (13) PS decarboxylase, (14) CDP-DG synthase, (15) PI synthase, (16) PI kinase, (17) PIP kinase, (18) PIP₂ kinase, (19) PGP synthase, and (20) PGP phosphatase.

[illegible]

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Figure 1-7 PC Metabolism

PC homeostasis is a highly dynamic and interactive process, tightly regulated by anabolism (*de novo*, Kennedy pathway), catabolism, and remodelling through reacylation/deacylation cycle (remodelling, Land's cycle). In addition to PC species with ester-linked hydrocarbon chains presented in Figure 1-6, formation of the ether and vinyl ether linkages begins with the reduction by the acyl-CoA reductase of the acyl-CoA into a fatty alcohol. This alcohol replaces the acyl chain in 1-acyl-DHAP to produce 1-alkyl-DHAP by the 1-alkyl DHAP synthase. The latter is reduced to 1-alkyl-glycerol-3-P (1-alkylPA), acylated to 1-alkyl-2-acyl-glycerol-3-P (1-alkyl-2-acylPA), and subsequently, dephosphorylated to 1-alkyl-2-acyl-glycerol by the 1-acyl/alkyl DHAP reductase, 1-alkyl-glycerol-3-P acyltransferase, and PA phosphatase, respectively. Similarly to the DG, 1-alkyl-2-acyl-glycerol is the precursor of PC, PE, and PS lipids. The vinyl ether linkage in *sn*-1 is formed from the 1-alkyl-2-acylPE. 1-alkyl-2-acylPE is synthesized either directly from the addition of the PE headgroup to the 1-alkyl-2-acyl-glycerol by the cytidine 5'-diphosphate-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase or the exchange of the headgroup of the 1-alkyl-2-acylPC by the cytidine 5'-diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase. The 1-alkyl-2-acylPE desaturase add a double bond at the first carbon to produce the vinyl ether linkage and the 1-alkenyl-2-acylPE. Vinyl ether containing-PE species can then be converted to PC via based-exchange enzymes or via hydrolysis by phospholipase D followed by the addition of the PC headgroup by the cytidine 5'-diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase. Remodelling occurs when lipids are hydrolyzed by a phospholipase and re-acylated by an acyltransferase. The majority of PC remodelling is due to the phospholipase A₂ (PLA₂) enzymes (Richmond and Smith, 2011). PLA₂ hydrolyzes PC species into a free fatty acid and LPC, LPAF, or LPPAF depending on their membrane precursor. They can either be converted back to the membrane precursor by the addition of a long chain fatty acid or to APAF, PAF, or PPAF by the addition of an acetyl-CoA. The lysophosphatidylcholine acyltransferase (LPCAT) family of enzymes catalyzes both of these reactions. R is used to define all the possible fatty chain moieties (Table 1-1). (1) cytosolic NAD⁺ glycerol-3-P dehydrogenase, (2) glycerol-3-P acyltransferase, (3) 1-acyl-glycerol-3-P acyltransferase, (4) PA phosphatase, (5) cytidine 5'-diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase, (6) phospholipase A₂, (7) LPC acyltransferase 1/2, (8) PAF acetylhydrolase (PAFAH), (9) LPC acyltransferase 1/2/3/4, (10) phospholipase C, (11) diacylglycerol kinase, (12) PLA₂, (13) phospholipase A₁, (14) mitochondrial flavin adenine dinucleotide glycerol-3-P dehydrogenase, (15) DHAP acyltransferase, (16) 1-alkyl DHAP synthase, (17) 1-acyl/alkyl DHAP reductase, (18) 1-alkyl glycerol-3-P acyltransferase, (19) cytidine 5'-diphosphate-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase, (20) 1-alkyl-2-acyl PE desaturase, (21) phospholipase C, (22) fatty acid-CoA synthetase, (23) fatty acyl-CoA reductase, (24) choline kinase, (25) cytidine triphosphate:phosphocholine cytidyltransferase.

principles of GP synthesis are summarized in Figure 1-6 (Vance and Vance, 2008, Richmond and Smith, 2011). Specifics of *de novo* synthesis (Kennedy pathway) and PC remodelling (reacylation/deacylation cycle or Land's cycle) are summarized in Figure 1-7 (Vance and Vance, 2008, Richmond and Smith, 2011).

1.2.3 Lipid metabolism as a new risk factor for AD

The role of lipids in AD is genetically supported. Genome wide association studies have identified variants in adenosine triphosphate-binding cassette subfamily-A7, bridging integrator 1, apolipoprotein J, low-density lipoprotein receptor, and PICALM in addition to the well-known ApoE gene as conferring AD risk (Jones et al., 2010, Hollingworth et al., 2011, Olgiati et al., 2011, Lambert et al., 2013). Among them, the APOE ϵ 4 allele (Corder et al., 1993, Blacker et al., 1997), bridging integrator 1, and PICALM variants are linked to an earlier age of AD onset in LOAD (Naj et al., 2014).

Adenosine triphosphate-binding cassette subfamily-A7 is a member of the adenosine triphosphate-binding cassette family that is highly expressed in the brain. This family is involved in the translocation of specific substrates across the membranes. More specifically, the adenosine triphosphate-binding cassette subfamily-A7 protein interacts with apolipoproteins, including ApoE, and promotes microglial and neuronal efflux of PC and sphingomyelin, but not cholesterol (Wang et al., 2003, Kim et al., 2006).

The ApoE protein is the main lipid carrier in the brain (Jones et al., 2010). ApoE and apolipoprotein J proteins are also implicated in A β clearance in brain as well as cholesterol and GP transport (Gong et al., 2002, Jones et al., 2010, Spinney, 2014, Yu et al., 2014a). The ApoE ϵ 4 allele, which increases AD risk, is a less efficient lipid carrier than the common ϵ 3 (Gong et al., 2002).

Bridging integrator 1 and PICALM proteins are involved in regulating receptor mediated endocytosis by which lipoproteins are internalized by cells. This internalization is also regulated by the interaction of ApoE and apolipoprotein J with their receptors, including the low-density lipoprotein receptor encoded by low-density lipoprotein receptor (Vitali et al., 2014).

1.2.4 GP levels are altered in AD

Why do changes in lipid metabolism enhance AD risk? A change in GP metabolism has been linked to neurodegenerative disease since the 1960s. Oxygen deprivation was shown 50 years ago to increase the hydrolysis of the structural membrane PCs in brain slices (Webster and Thompson, 1965), verified five years later *in vivo* in mouse brain (Bazan, 1970). Importantly, studies have also demonstrated GP alterations in post-mortem AD brain. Overall levels of PE are reduced in hippocampus and cortex with plasmalogen-PEs, a subclass of PE, specifically targeted in cortex (Soderberg et al., 1992, Ginsberg et al., 1995, Wells et al., 1995). Other studies have reported a decrease in PI and an increase in PS (Stokes and Hawthorne, 1987, Wells et al., 1995). Interestingly, PC and PE metabolism occurs prior to onset of dementia (Pettegrew et al., 1995) and correlate with severity (Pettegrew et al., 1988a, Pettegrew et al., 1988b) as well as psychotic symptoms (Sweet et al., 2002). These results suggest that disruption of neuronal membrane integrity could be at the very heart of AD. Critical species have only begun to be identified.

1.2.5 Pathological membrane remodelling is associated with AD conversion

Readouts of PC metabolites are more sensitive than that of structural precursors. Even if a majority of studies has shown that overall PC levels in post-mortem AD brains are constant (Klein, 2000), the breakdown of structural membrane PCs is occurring as

shown by the increase in *sn*-glycerol-3-phosphocholine metabolites in both post mortem tissue (Barany et al., 1985, Pettegrew et al., 1988b, Blusztajn et al., 1990, Nitsch et al., 1992) and cerebrospinal fluid of AD patients (Walter et al., 2004). Both synthesis and remodelling of PC are significantly altered in post-mortem AD brain. The enzymatic activities of the rate-limiting cytidine triphosphate:phosphocholine cytidyltransferase involved in the *de novo* synthesis and the LPCAT involved in the remodelling pathway are significantly increased (Ross et al., 1998). Under normal physiological conditions, LPCAT1 has a reported substrate affinity for 6:0-16:0-, 18:2-, 18:3-CoA and LPCAT2 20:4-CoA (Shindou and Shimizu, 2009). We have shown that the presence of A β can alter this substrate specificity of LPCAT1/2 favouring 2:0-CoA over longer fatty acyl-CoAs. This change in substrate specificity promotes formation of the biologically potent PAFs specifically with alkyl-linked 16 carbon chains at the *sn*-1 position (Ryan et al., 2009a).

PAFs signal through both receptor-dependant and receptor-independent pathways (Xu et al., 2013). Only one platelet-activating factor receptor (PAFR) has been identified (Honda et al., 1991). PAFs are implicated in many functions in the brain including modulation of calcium and neuropeptides level, transcription of genes, long-term potentiation, neuroinflammation, and neurodegeneration (Farooqui et al., 2000, 2007b). Moreover, PAFs have been identified as key mediators of neurotoxicity *in vivo* in other brain diseases including ischemia, seizure, and acquired immune deficiency syndrome dementia and *in vitro* mediating A β -induced toxicity (Perry et al., 1998, Bazan et al., 2002, Farooqui and Horrocks, 2006, Ryan et al., 2009a). Our laboratory has shown that A β neurotoxicity is mediated, in part, by the intraneuronal accumulation of PC(O-16:0/2:0) *in vitro* and *in vivo* in both the temporal cortex of TgCRND8 mouse model of AD and the posterior-entorhinal cortex of AD patients post mortem. As indicated above, we have

found that A β , through PC(O-16:0/2:0), signals the hyperphosphorylation of tau by CDK5 on the AD-specific epitope AT8 (Ser202/Thr205) (Ryan et al., 2009a).

A β is a critical regulator of the remodelling pathway. Calcium-dependent cytosolic PLA₂ (cPLA₂) protein expression is increased in AD cortex (Stephenson et al., 1996) with A β eliciting the phosphorylation, activation, and translocation of the cPLA₂ to the nuclear envelope and ER (Lehtonen et al., 1996, Kriem et al., 2005, Sanchez-Mejia et al., 2008a). Enhanced cPLA₂ activation is, in turn, responsible for the extensive A β -dependent neuronal death *in vitro* (Kriem et al., 2005). cPLA₂ preferentially hydrolyzes arachidonic acid (AA, C20:4n-6) from the *sn*-2 position of PCs. AA participates in signalling A β -induced neuronal death through its conversion to eicosanoids by cyclooxygenase 2 (Kriem et al., 2005). Importantly, genetic ablation of cPLA₂ in the human APP AD mouse model restores not only the life expectancy of these mice, but also prevents learning and memory impairment (Sanchez-Mejia et al., 2008a). We and others have shown that cPLA₂ activation generates not only AA but also PC(O-16:0/0:0) remodelled to PC(O-16:0/2:0) as well as other 1-radyl-2-LPC (radyl denotes either an acyl, alkyl, or alkenyl fatty chain moiety) among other LPCs (Shimizu et al., 2006, Ryan et al., 2009a). LPC receptors include GPR4 and G2A (Meyer zu Heringdorf and Jakobs, 2007, Sun et al., 2009). Like PAFs, LPC signalling is implicated in a plethora of functions including calcium homeostasis and calcium-dependent functions, adhesion, migration, proliferation, survival, and inflammation (Meyer zu Heringdorf and Jakobs, 2007). Like AA and PAF, LPC is neurotoxic *in vitro* (Sun et al., 2009). Furthermore, LPC species specifically increase the hydrophobicity of A β ₄₂ and promote its oligomerization (Sheikh and Nagai, 2011) potentiating the neurotoxicity of A β ₄₂ *in vitro* (Sheikh et al., 2015).

1.3 Neurolipidomic: Cataloguing Membrane Diversity in the Central Nervous System

Neurolipidomics refers to the study of lipid composition, regulation, protein interaction, metabolism, and signalling in the central nervous system (Spener et al., 2003, Wenk, 2005, Bennett et al., 2013). It is primarily dependent upon mass spectrometry (MS) technologies.

1.3.1 Neurolipidome profiling: methodological considerations

MS is extensively used for the analysis of lipids. The first structural elucidation of an unknown lipid by MS was reported in 1969 (Granstrom and Samuelsson, 1969) and MS still remains the best technique to identify and quantify individual lipids contained in complex mixtures.

MS instruments measure the mass to charge ratio (m/z) of an analyte. This occurs in three stages: an ion source transfers the analytes to the gas phase and charges the analytes, a mass analyzer separates the analytes by their m/z , and a detector measure the abundance of ionic analytes in accordance to their specific m/z . To prevent the collision of analytes with other gas-phase molecule and insure their free movement into the mass spectrometer, the instrument is operated under vacuum (Dass, 2000). Critically, mass spectrometers can only handle charged analytes in the gas phase (Dass, 2000). The transfer to the gas phase is relatively easy for volatile or heat-volatile compounds, however, the majority of lipids are non-volatile and decompose upon heating (Murphy and Gaskell, 2011). As well, the analytes need to have (or need to acquire) a charge during the transfer to the gas phase. Thus, only molecular structures that can acquire or lose charges in the form of protons, or other ions can be assessed. In MS, the ionization method refers to the

combination of two distinct processes: ionization and desorption/desolvation. The transfer of intact larger/non-volatile molecules to the gas phase became possible with the development of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn et al., 1989). In both techniques, the transfer of the analytes to the gas phase is achieved with a minimal transfer of energy to the analytes thus preventing their degradation.

ESI (Figure 1-8) has been the ionization technique most readily applicable to lipidomics as it is compatible with high performance liquid chromatography (HPLC) separation and direct infusion of lipids. GP ionization efficiency is dependent on lipid class. PC, PE, and PS are more readily analyzed in positive ion mode; PG and PI are more efficient in negative ion mode (Hummel et al., 2011). Two main MS methodologies are used in GP lipidomics: direct infusion ESI-MS and HPLC-ESI-MS. The lipid cocktail can be either directly infused in the mass spectrometer through the ESI, a technique named direct infusion (ESI-MS or shotgun lipidomic), or separated by HPLC prior to ESI, a technique named HPLC-ESI-MS (Bou Khalil et al., 2010, Xu et al., 2013). ESI-MS is a simple, and reproducible, method to analyze and quantify complex lipid mixtures. ESI-MS challenges include ion suppression and complex resolution of isomeric and isobaric species (Cajka and Fiehn, 2014, Li et al., 2014). Ion suppression occurs at the time of injection into the MS and causes the reduction of the intensity of a single or multiple analytes. This phenomenon happens when one of two co-eluting analytes affects the ionization of the other leading to its inaccurate quantification.

These challenges are mitigated by the addition of the HPLC separation prior to the MS (HPLC-ESI-MS). Chromatographic separation uses two phases: one mobile and one stationary. In HPLC, the stationary phase is the surface of beads packed in a column. The

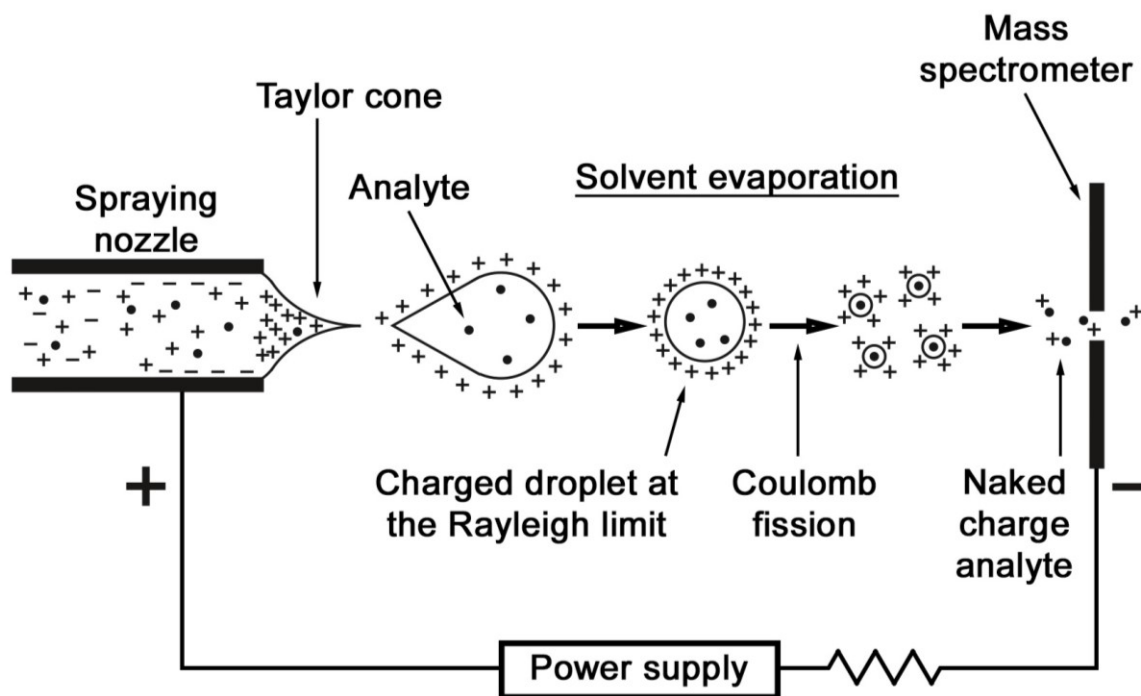


Figure 1-8

Figure 1-8 ESI Process in Positive Ion Mode

Typically, in ESI, a solution containing the analytes is pushed through a needle. A higher electric potential is applied at the tip of the needle while the entrance of the MS is maintained at a lower potential or ground thus producing an electric field. Typically, for the analysis of positively charged analytes a positive potential is applied to the needle, whereas a negative potential is applied to the needle for negatively charged analytes. In positive ion mode, the positive ions (and their surrounding solvent molecules) move due to the electric field towards the mass spectrometer while the negative ions move towards the emitter. The accumulation of positive charges in solution at the tip deforms the flow and cause the formation of a Taylor cone (Taylor, 1964). The instability of the cone, in addition to the electric field, induces the release of a fine liquid jet that explodes into positively charged droplets, dispersed as far as possible from each other due to Coulombic repulsion to minimize the charge density. While the charged droplets continue their travel toward the MS, the solvent evaporates. With evaporation, there is an increase of the surface charge density. Once the surface tension is exceeded by the Coulombic repulsion Rayleigh limit (Rayleigh, 2009), the droplet explodes into smaller droplets (i.e., increase in total surface area). This process continues until the ionic analytes are fully or partly desolvated, or the droplet reaches the entrance of the MS (Piomelli, 2005, Banerjee and Mazumdar, 2012). The electrospray process is not driven by the analytes, but by the higher concentration of the other ions present in the solution. For example, in positive ion mode, a volatile acid is often added to the solution. The analytes are either already charged in solution or acquire charge during the electrospray process. The presence of charge on the analyte is essential to control the analyte movement in the gas phase and its detection (Dass, 2000). Ionization efficiency can be improved by protonation/deprotonation or by addition of an adduct ions: NH_4^+ , Na^+ , Li^+ , K^+ , Cl^- , etc (Bou Khalil et al., 2010, Cajka and Fiehn, 2014). For example, PC species can be protonated in solution containing 0.1 % formic acid (FA) (Ryan et al., 2009a). This addition affects its mass (M) by adding the atomic weight of hydrogen (H^+), i.e. $[\text{M}+\text{H}]^+$. Adapted from (Banerjee and Mazumdar, 2012).

mobile phase is pushed through the column and its composition changes over time. HPLC separation can be performed using normal-phase and reverse-phase. In normal-phase chromatography, the mobile phase is hydrophobic and the stationary phase is hydrophilic. The analytes partition between these two phases. The more polar compounds will stay longer in the stationary hydrophilic phase, thus the less polar compounds will elute first. The normal-phase HPLC separates the lipid species based on their headgroup polarity, i.e., by their GP subclass as examples $PE > PI > PS \gg PC$ (Pulfer and Murphy, 2003) and $PG > PE \gg PC \gg PI \geq PS > PA$ (Shui et al., 2010). In reverse phase, the mobile phase is hydrophilic and the stationary phase is hydrophobic. Reverse-phase HPLC separates the lipid species based on the hydrophobicity of their fatty chain moieties. The hydrophobicity of a lipid species is increased with longer hydrocarbon chains and fewer unsaturations. Moreover, the linkage of the fatty chain moieties also affects its hydrophobicity. The ether linkage is more hydrophobic than the ester linkage which is more hydrophobic than the vinyl ether linkage (Houjou et al., 2007, Bou Khalil et al., 2010). Chromatographic separation decreases the complexity of the lipid mixture by eluting the species at different retention times (RT) (Bou Khalil et al., 2010, Xu et al., 2013). To increase reproducibility, HPLC is automated and coupled to the mass spectrometer. Two-dimensional (2D) HPLC of lipids can be achieved by coupling normal-phase HPLC followed by reverse-phase HPLC. Although 2D HPLC provides better separation, it is more time-consuming (Cajka and Fiehn, 2014). This combination is a laborious process, which may result in sample loss (Xu et al., 2013). However, 2D HPLC is especially useful for the comprehensive characterization of the lipidome (Cajka and Fiehn, 2014).

Three primary MS strategies are employed in HPLC-ESI-MS and ESI-MS lipidomic approaches: high resolution MS, tandem MS (MS/MS), and multidimensional

MS (Xu et al., 2013). High resolution MS in lipidomics mainly exploits the high mass resolution and accuracy of time-of-flight mass analyzers (Han et al., 2012, Xu et al., 2013, Cajka and Fiehn, 2014). Time-of-flight mass spectrometers can differentiate species with closely related m/z as long as there is a measurable mass difference (Han et al., 2012, Xu et al., 2013, Cajka and Fiehn, 2014), for example PC(O-18:1/18:2) $[M+H]^+$ 770.6063 m/z and PC(18:3/17:0) $[M+H]^+$ 770.5700 m/z (Blanchard et al., 2013b). Isomeric lipid species, however, have identical exact mass. They are defined by their identical atomic composition, similar structure, but different atomic arrangement (i.e., same headgroup, *sn*-1 and *sn*-2 linkage). As an example, PC(16:0/18:1(9E)), PC(16:0/18:1(9Z)), PC(16:0/18:1(11Z)), PC(18:1(11Z)/16:0) and PC(22:1(11Z)/12:0) are isomeric species since they share the molecular formula $C_{42}H_{82}NO_8P$, the PC headgroup and their ester linkages (Fahy et al., 2009, Fahy et al., 2011). To differentiate these isomeric species MS/MS strategy can be employed.

MS/MS strategies can be achieved with multiple mass analyzers such as the triple quadrupole MS. Triple quadrupole MS contains three quadrupoles mass analyzers linearly aligned in front of the detector. The second quadrupole is used as a collision cell. The first and third quadrupole mass analyzers can be used to separate lipid species based on their m/z . The possibility to achieve MS/MS strategy compensates for the low resolution of the quadrupole mass analyzer. Without high resolution MS, if PC(O-18:1/18:2) $[M+H]^+$ 770.6063 m/z and PC(18:3/17:0) $[M+H]^+$ 770.5700 were detected, both would be observed at 770.6 m/z and considered isobaric species, i.e. sharing the same m/z within the tolerance of the MS instrument. MS/MS strategies include product ion scan, precursor ion scan (PIS), neutral loss scan, and selected reaction monitoring/multiple reaction monitoring (MRM) (Li et al., 2014). MS/MS monitors the fragmentation of a lipid to acquire

additional information about its structural identity (Xu et al., 2013). This fragmentation occurs when a lipid collides with neutral gas molecules and some of its covalent bonds break. This technique is named collision-induced dissociation. The fragmentation pattern of a species depends on two parameters: its molecular structure and the collision energy (Han and Gross, 2005). As an example, the PIS is frequently used to detect the 184 m/z ion, a characteristic fragment of the PC headgroup, to identify a PC-containing species (Bou Khalil et al., 2010, Xu et al., 2013).

Multidimensional MS combines different MS/MS strategies in order to characterize the “building blocks” –the headgroup and fatty chain moieties– of a species (Xu et al., 2013). Multidimensional MS can be achieved with a hybrid instrument such as the ion trap triple quadrupole. The ion trap stores ions with specific m/z for further analysis. For example, the PC headgroup of a species is analyzed by PIS while its counter fragment is stored in the ion trap. This fragment is released afterward to acquire additional structural information, such as the composition of its fatty chain moieties in neutral loss scan (Xu et al., 2013).

1.3.2 Bioinformatic roadblocks to realizing the potential of a neurolipidomic approach

The pace of MS development in lipidomics has not been matched by the pace of its bioinformatic support. The lack of bioinformatic tools represents a major roadblock to comprehensive lipid profiling. As reviewed by Niemela et al. (2009), four main challenges have been identified in lipidomic bioinformatics: (1) data acquisition and lipid identification, (2) statistical analysis, (3) pathway analysis, and (4) lipid modelling. Here, I

focus on challenges primarily relevant to HPLC-ESI-MS lipidomics, the MS methodology used in this thesis.

In HPLC-ESI-MS, data acquisition refers to the initial step of capturing empirical instrumental data. Existing MS software record the m/z and intensity (i.e., peak height, area, full-width/half maximum values, etc.) in addition to the RT (Yetukuri et al., 2007). Data acquisition is the first step in transforming the pertinent structural data into a lipidomic dataset (Niemela et al., 2009). In recent years, software programs have been developed or adapted to facilitate this acquisition including MZmine and LipidXplorer (Pluskal et al., 2010, Brennan et al., 2012). The identity of the lipid species is then curated using lipid identification databases. These databases predict species identity based on their m/z . Databases for lipid identification include LIPID MAPS, LipidBank, and LipidBlast (Yasugi and Watanabe, 2002, Fahy et al., 2005, Fahy et al., 2009, Fahy et al., 2011, Kind et al., 2013). LIPID MAPS and LipidBank databases contain the m/z of curated lipid species. LIPID MAPS and LipidBlast databases enclose the m/z of fragments of species from MS/MS experimental (LIPID MAPS, LipidBlast) or theoretical (LipidBlast) databases (Yasugi and Watanabe, 2002, Fahy et al., 2005, Fahy et al., 2009, Fahy et al., 2011, Kind et al., 2013).

Following data capture, registration of MS spectra across runs is required – a process of lipid identification also called correspondence. Correspondence is the process of matching the same species across multiples MS runs (Smith et al., 2015). In HPLC-ESI-MS, lipid species will exhibit characteristic diagnostics include peak shape, intensity, m/z , and RT. RT is more informative than intensity and peak shape since they are altered according to experimental conditions including disease signalling. m/z is constant but resolution dependent on the type of mass spectrometer employed as reviewed above. RT

is, however, difficult to reproduce. Even using identical chromatographic methods, small run-to-run variations in flow rate, composition of the mobile phase, temperature, pH, packing of chromatographic columns etc., can cause substantial variations in analyte RT (Smith et al., 2015). These variations in RT make species correspondence difficult requiring bioinformatic tools. As reviewed by Smith et al., 2015, over 50 algorithms have been developed to align lipidomic spectra across runs using at least two characteristic parameters (peak shape, intensity, m/z, and/or RT). Smith et al. (2015) critically questions the reproducibility of many of these algorithms due to the lack of details provided with respect to the mathematical process of establishing the correspondence, little verification of accuracy within or between methods, length of time to process dataset due to algorithmic complexity, or simply algorithmic solutions without the means to implement these programs (i.e., no underlying software).

Statistical analysis faces challenges associated with standardization of the medium- to large-scale datasets generated by lipidomic approaches (Niemela et al., 2009). Quantification can be either relative or absolute (Xu et al., 2013). Discovery-driven lipidomic approaches use mainly relative quantification. Thus, quantification is based on intensity, i.e., the spectral count of the MS in counts per second. Peak intensities must first be corrected for the extraction efficiency and the amount of tissue or fluid assessed and then, normalized to internal standards spiked at time of MS to correct for run to run technological variation (Cajka and Fiehn, 2014). Thus, extraction efficiency is corrected using a fixed amount of lipid standards added at the time of extraction, the amount of tissue is corrected using either its wet weight or protein concentration (Xu et al., 2013), and technological normalization is achieved using the standards spiked at time of MS. These corrected intensities can be used for relative quantification. Relative quantification can be

expressed in mole units, but relative to a given species within the same family in order to approximate lipid quantity. Thus, relative quantification is corrected and transformed with a standard from the same subclass of the lipid of interest, i.e. D₄-PC(O-16:0/0:0) is used to express the relative abundance of PC(O-19:0/0:0). For absolute quantification, species intensities must be converted into absolute quantity using a standard curve. The best practices for absolute quantification use a standard curve built from a quantitative MS-grade (> 99 %) isotopologue (usually ¹³C) of the species of interest (Griffiths and Wang, 2009, Xu et al., 2013). The standard species and its endogenous counterpart must be analyzed in the same run. It is mandatory to use a standard with an identical structure to the endogenous species to conserve the same ionization and fragmentation efficiency (Xu et al., 2013). While the fragmentation efficiency is fully dependant on the structural identity of the species, the ionization efficiency is similar within a lipid subclass such as the PC species (Han, 2007). Importantly, only absolute quantification corrects for these unique species characteristics and thus, only absolute quantitation can compare quantity of different species in a study. If the spectrum is too complex to add an isotopologue (or an isotopologue of the species of interest is unavailable), absolute quantification can be obtained by the standard addition method. This method consists of adding increasing amount of the analyte of interest the lipid cocktail matrix. A standard curve is built from the data acquired from these runs in order to calculate the absolute amount of the endogenous species (Liebisch et al., 2002, Whitehead et al., 2007). The prerequisite of having a standard for each endogenous species to measure their absolute quantification inevitably limits its practical use (Xu et al., 2013). Absolute quantification may not be achievable due to the lack of commercially available lipid standards, the complexity of the spectrum precluding specific standard addition, and the practical concern of labour

intensiveness. Moreover, while simple in concept, practical application of data normalization handling unwanted sample-to-sample (and run-to-run) variation, notably when applied to multisite or longitudinal studies requires considerable planning (Livera et al., 2015).

Whether absolute or relative quantification, relationships between lipid species can be established by cluster analysis, performed for example using Cluster 3.0 (de Hoon et al., 2004) and visualized through heatmaps using TreeView (Saldanha, 2004). The integrity of these analyses, however, depends on the assumptions of the normalization practices employed as reviewed in Livera et al. (2015). By grouping the species that are behaving similarly in terms of lipid species quantification alterations, clustering helps in finding patterns relevant to data interpretation yet this method requires that the normalization has not artificially correlated normalized data with the behaviour of the internal standards (Livera et al., 2015). Then, the user compares the changes of interest in specific species quantification in diverse samples and conditions using standard statistical techniques such as regression analyses, Student's t-tests or multivariate analysis of variance (ANOVA). These analyses must be followed by a false discovery rate (FDR) correction for multiple comparisons (Benjamini and Hochberg, 1995, 2000, Benjamini et al., 2006). These methods can be handled by standard statistical software (i.e., GraphPad Prism, IBM SPSS) yet the size of the datasets remain a challenge with respect to maximum sample sizes handled by biological software and number of covariates. Certainly, recent statistic advances, notably for exploring lipidomes for biomarkers, considering methods of normalization, validation of supervised and unsupervised approaches, and the types of “unwanted” variation are emerging that is allowing multisite and longitudinal studies to be replicated (Mapstone et al., 2014, Livera et al., 2015).

Once the species that are significantly altered are identified, pathway analysis facilitates the interpretation of these changes at different levels including biochemical, signalling, and regulatory pathways (Niemela et al., 2009). Pathways analysis tools in lipidomics are almost inexistent. For example, VANTED (Junker et al., 2006) is a promising tool to build such pathways, but it uses the KEGG database (Kanehisa, 2002). This database contains precious information about the lipid metabolic pathways and the enzymes involved. These pathways are built with lipid subclass information and lack the crucial data on enzymatic specificity for a given chain length or unsaturation that will dictate the fate of the lipid at the species level.

Lipid modelling is essential to understand how these changes affect the physicochemical proprieties which underlie the enzymes/receptors distribution and activity (Niemela et al., 2009, Sadiq et al., 2013). The lipid composition affects the thermodynamic state of the membrane which influences its permeability of small molecules and lateral diffusion (Niemela et al., 2009). There are few membrane computational modelling tools. CHARMM-GUI Membrane Builder (Jo et al., 2009), GROMACS (Hess et al., 2008) and LipidWrapper (Durrant and Amaro, 2014) represent three modelling and simulation packages. However, the protein-membrane simulations performed using these tools have not reached the complexity of biological membranes. For example, membrane simulations are classically using only few components of the membrane, i.e. only PC species or PC species with cholesterol, often without taking into consideration the asymmetry in the composition of the two leaflets of the membrane (Sadiq et al., 2013). Taken together, these bioinformatic challenges clearly indicate that the computational capacity of lipidomic is in early days.

1.4 Rationale, Hypothesis, and Objectives

In summary, lipids are unique signalling molecules with metabolic deficits in phospholipid and cholesterol processing at the molecular level linked to AD risk. Here, I hypothesize that aberrant PC metabolism, elicited in part by A β ₄₂ and altered by age, compromises neuronal function. Specifically, I hypothesize that the accumulation of discrete PC metabolites generated through the Land's cycle remodelling pathway signals AD pathology. To test this hypothesis, I applied complementary bioinformatic, lipidomic, and proteomic approaches to address three specific aims:

- #1 Improve bioinformatic capacity to register HPLC-ESI-MS spectra across MS runs, conditions, and datasets and predict PC identities within these datasets.
- #2 Determine the effects of aging and aberrant APP processing on the plasma and entorhinal-hippocampal lipidomes *in vivo* using the TgCRND8 mouse model of AD and clinical young adults, cognitively normal elderly with vascular impairment, mild cognitive impairment (MCI) and LOAD plasma and tissue samples.
- #3 Identify lipid-relevant pathways altered in TgCRND8 mice at phenoconversion using an unbiased phosphoproteomic approach to map the post-translationally modified proteome of pre-symptomatic and symptomatic TgCRND8 mice.

CHAPTER 2. RETENTION TIME STANDARDIZATION AND REGISTRATION (RTStaR): A SIMPLE ALGORITHM THAT MATCHES CORRESPONDING AND IDENTIFIES UNIQUE SPECIES IN HPLC-ESI-MS LIPIDOMIC DATASETS

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2.1 Objective of this Study

In collecting the longitudinal data required for an assessment of aging on the murine lipidome (Chapter 4), I recognized that available software to register rapidly and reproducibly large numbers of HPLC-ESI-MS lipidomic spectra were limited and that existing tools were inapplicable to the HPLC-ESI-MS methodologies we were employing. To address this need, part of my thesis was dedicated to participating in developing the necessary bioinformatic tools for lipidome analysis. The objective of this study was to develop an easy to use algorithm capable of facilitating the identification of both corresponding and unique lipid species across multiple MS runs, biological replicates,

organisms, and sample origin in HPLC-ESI-MS datasets. **This tool was submitted for publication at the time of thesis submission.**

2.2 Statement of Author Contributions

APB with SALB wrote, designed, and validated the algorithm, and the RTStaR workbook templates. ZP, TP, and SALB collected the human datasets. HX, YW, APB, and SALB performed the MS experiments and analyzed the data with advice from DF in the uOttawa Proteomics Core Facility and India Taylor Lipidomic Research Platform. APB, MG, and GPT analyzed the validation datasets using different HPLC methods. SF developed and hosts the online resource page as part of the Carleton Immersive Media Studio. APB with SALB wrote the manuscript with input with from all authors.

2.3 Summary

Motivation: Bioinformatic tools capable of registering, rapidly and reproducibly, large numbers of high performance HPLC-ESI-MS lipidomic spectra are limited. We provide here a freely available Retention Time Standardization and Registration (RTStaR) algorithm that aligns the HPLC-ESI-MS spectra of biological replicates within a single dataset and then compares this alignment to the RTStaR-standardized RT of multiple datasets. This two-step calibration matches corresponding and identifies unique lipid molecular species in different lipidomes. RTStaR was developed using a population-based study of 1001 human serum samples composed of 71 distinct glycerophosphocholine metabolites aligning a total of 68,572 analytes across subjects. Platform and matrix independence were validated using different MS instruments, liquid chromatography (LCh) methodologies, and glycerophospholipidomes. We show that RTStaR can reliably align multiple HPLC-ESI-MS spectra in a single study and can register unique and corresponding lipid features in lipidomes of different sample origins or organisms as long as profiles are captured using the same method and spectra meet the defined criteria for registering different system-level variations. The complete algorithm is packaged in two modular ExcelTM workbook templates for easy implementation. **Availability:** RTStaR can be downloaded from the India Taylor Lipidomics Research Platform (ITLipidomics) website <http://www.neurolipidomics.ca/rtstar/rtstar.html>. **Contacts:** Technical support is provided through lipawrd@uottawa.ca. **Supplementary Information:** Supplementary data are at *Bioinformatics* online and <http://www.neurolipidomics.ca/rtstar/rtstar.html>.

2.4 Introduction

Lipid diversity is phenomenal. Mammalian cells are estimated to have 9,000 to 100,000 unique lipid species (Shevchenko and Simons, 2010). This liberal range reflects how little we know about lipid composition in biological life. Lipid structures are primarily assembled from five atoms: hydrogen, carbon, oxygen, nitrogen, and phosphate. Species diversification results from subtle structural differences. For example, GP with identical atomic composition can exhibit dramatically different biological activities depending upon the stereochemical nature of their hydrocarbon chain length, degree of unsaturation and linkages to the glycerol backbone (Bennett et al., 2013). Mapping cellular lipids is challenging.

Lipidomics refers to the systems-level qualitative and quantitative analyses of lipid diversity, lipid interactions, and lipid regulation (Brown, 2012). Advances in HPLC-ESI-MS methodologies have enabled the medium- to large-scale profiling of lipid composition (Brown and Murphy, 2009). These profiles are revealing intriguing metabolic changes associated with aging, sex, and neurodegenerative diseases (Rapple et al., 2009, Ryan et al., 2009a, Mapstone et al., 2014). Complete organismal, cellular, and subcellular organelle lipidomes, however, have yet to be realized.

One of the obstacles facing lipidomics is our limited bioinformatic capacity to match corresponding and identify unique lipid species across lipidomes. It remains exceedingly labour-intensive for different laboratories, and even within targeted lipidomic experiments from the same laboratory, to register, rapidly and reproducibly, large numbers of MS spectra. Despite application of identical chromatographic methods, small run-to-run variations in flow rate, composition of the mobile phase, temperature, pH, packing of

chromatographic columns etc., can cause substantial variations in RT (Smith et al., 2015). Easily implemented algorithms capable of standardizing RTs across HPLC-ESI-MS datasets would facilitate this process. Here, we describe development and validation of the Retention Time Standardization and Registration (RTStaR) algorithm. RTStaR statistically verifies its alignment of species RTs across multiple MS spectra representing a single dataset. The calibrated dataset can then be used as reference to standardize corresponding and identify unique analytes across multiple lipidomes. The complete algorithm is packaged in two modular ExcelTM templates for easy use and is available online at: <http://www.neurolipidomics.ca/rtstar/rtstar.html>.

2.5 Methods

2.5.1 Datasets

Three PC metabolite datasets composed of 1061 different MS spectra were used to develop and validate RTStaR. These datasets were: (1) a population-based study of circulating PC metabolites in human serum consisting of 1001 participants, (2) a genotype and intervention comparison study of PC metabolites in the hippocampus of 30 wild-type (NonTg) and TgCRND8 (Tg) littermates, a mouse model of AD (Chishti et al., 2001, Wang et al., 2013), and (3) a comparative biomarker study of PC metabolites in the plasma of these same 30 animals. Accuracy was assessed using a dataset composed of 20 lipid standards (Avanti Polar Lipid and Cayman Chemical). To examine platform independence, RTStaR was further challenged with datasets profiled using different LCh methodologies and MS instruments. These additional lipidomes examined PC metabolism in: (1) a longitudinal study from birth to elderly in the parietal-temporal cortex of 50 NonTg and Tg littermates, (2) a genetic ablation study in the parietal-temporal cortex of 49 PAFR wild-

type (NonTgPAFR^{+/+}), null mutant (NonTgPAFR^{-/-}), TgPAFR^{+/+} and TgPAFR^{-/-} littermates, and (3) a post-mortem study profiling the composition of PC metabolites in the parietal-temporal cortex of 10 AD and age-matched human controls.

2.5.2 Sample collection and lipid extraction

Human blood was collected in silicone-coated gold BD Hemogard™ tubes (#367986, BD, ON, Canada) from 1001 male and female adolescents, 12 to 18 years of age, enrolled in the SYS, a cross-sectional study of cardiometabolic and brain health (Paus et al., 2015). Consent was obtained in strict accordance with the research ethics committees of the Chicoutimi Hospital (Chicoutimi, QC, Canada) and the Hospital for Sick Children (Toronto, ON, Canada). After clotting, serum was separated by centrifugation at 1300 x g for 15 min at room temperature and stored in 1 ml aliquots -80 °C. Parietal-temporal cortex of n = 5 patients suffering from AD and n = 5 age-matched controls were obtained from the Douglas Hospital Research Centre Brain Bank (Montreal, QC, Canada) and lipids were extracted as described (Ryan et al., 2009a).

Hippocampi were dissected from 30 six month old female NonTg and TgCRND8 N5 C57BL/6 x C3H/He mice. Blood from these animals was collected in heparin-coated tubes (#022379216, Eppendorf, ON, Canada) and plasma was prepared by collecting the supernatant following centrifugation at 1500 x g for 15 min at 4 °C. Experimental animals received daily dietary supplementation of 75 mg/kg body weight/day omega-3 free fatty acids (40/20 EE 1000 mg fish oil capsules, Ocean Nutrition, NS, Canada) or 75 mg/kg body weight/day omega-6 and omega-9 free fatty acids (1000 mg corn/soybean oil capsules, Ocean Nutrition, NS, Canada) emulsified in vehicle (25 % sweetened condensed milk) for four months. Mice receiving vehicle daily served as controls. In addition,

parietal-temporal cortices were dissected from 50 female N5 NonTg and TgCRND8 littermates at two, four, six, eight, twelve or eighteen months of age and 49 male and female N5 NonTgPAFR^{+/+}, NonTgPAFR^{-/-}, TgCRND8/PAFR^{+/+}, and TgCRND8/PAFR^{-/-} littermates between two and eight months of age. Dissections were performed as previously described (Wang et al., 2013). Tissue was flash frozen in liquid nitrogen and stored at -80 °C until extraction. Experiments were approved by the Animal Care Committee of the University of Ottawa and performed in strict accordance with the ethical guidelines for experimentation of the Canadian Council for Animal Care.

Lipids were extracted from serum, plasma, hippocampi, and parietal-temporal cortex using a modified Bligh and Dyer method (Bligh and Dyer, 1959), adapted to enrich not only for acyl-linked but also for alkyl- and alkenyl-linked PC metabolites as we have described (Xu et al., 2013).

2.5.3 Internal reference (IR) species and lipid standards dataset

Up to six IRs, PC(13:0/0:0) #855476, PC(12:0/13:0) #LM-1000, D₄-PC(O-16:0/0:0) #360906, D₄-PC(O-16:0/2:0) #360900, D₄-PC(O-18:0/0:0) #10010228, and D₄-PC(O-18:0/2:0) #10010229, spanning a m/z range of 454.3 to 636.5, were added to each sample. PC(13:0/0:0), PC(12:0/13:0) and the standards were from Avanti Polar Lipid (AL, USA). Deuterated species and PC(O-16:0/O-2:0) (#78858-42-1) were from Cayman Chemical (MI, USA). The non-naturally occurring PC(13:0/0:0) and PC(12:0/13:0) were added at 187.3 ng and 500 ng per sample respectively at time of extraction. Deuterated species were isotopologues of four naturally occurring PC metabolites were added at time of HPLC-ESI-MS as a mixture containing 1.25 ng of D₄-PC(O-16:0/2:0), 1.25 ng of D₄-PC(O-18:0/2:0), 2.5 ng of D₄-PC(O-16:0/0:0) and 2.5 ng of D₄-PC(O-18:0/0:0). In the standards dataset, 1.4

μM PC(13:0/0:0), 2.0 μM PC(14:0/0:0) (#110684), 2.0 μM PC(15:0/0:0) (#855576), 2.0 μM PC(16:0/0:0) (#110685), 2.0 μM PC(17:0/0:0) (#110686), 2.0 μM PC(18:0/0:0) (#110687), 2.0 μM PC(22:0/0:0) (#855779), 2.0 μM PC(17:1/0:0) (#110905), 2.0 μM PC(18:1/0:0) (#110688), 2.0 μM PC(P-16:0/0:0) (#110693), 2.0 μM PC(P-18:0/0:0) (#110694), 3.1 μM PC(O-16:0/2:0) (#110853), 3.1 μM PC(O-17:0/2:0) (#110626), 3.1 μM PC(O-18:0/2:0) (#110627), 2.0 μM PC(O-16:0/4:0) (#878115), 2.0 μM PC(O-18:0/4:0) (#878116), 2.0 μM PC(O-16:0/O-2:0) and 1 μM PC(12:0/13:0) were analyzed.

2.5.4 HPLC-ESI-MS

In the development and validation datasets, lipids were prepared for HPLC-ESI-MS as follows: 5 μl of lipid extract (or 5 μl of lipid standards) and 2.5 μl of the isotopologue IR master mix were added to 15.75 μl of H_2O (#9831-03, JT Baker, NJ, USA) with 0.1 % (v/v) FA (#14265, Sigma-Aldrich, ON, Canada). Gradient chromatographic separation was performed on a 10 cm $\times$ 75 μm (internal diameter: ID) analytical column packed with ReproSil-Pur 120 C₄, 5 μm , 120 Å beads (#r15.4e, Dr. Maisch GmbH, Germany) using an Agilent 1100 HPLC System (Agilent Technologies, CA, USA). The injection volume was 3 μl . The mobile phase consisted of solvent A (H_2O) and B (5:2, acetonitrile (ACN):isopropanol, v/v) (#A416-4, Fisher Scientific, ON, Canada, #9829-03C, JT Baker, respectively) both containing 0.1 % (v/v) FA and 10 mM ammonium acetate (#2145, OmniPur, ID, USA). The sample was loaded for 15 min in 5 % solvent B. Gradient elution was performed with a linear increase of solvent B from 5 to 35 % in one minute and from 35 to 100 % in 14 min. One hundred percent solvent B was maintained for an additional 14 min before the column was equilibrated back to 5 % B for 15 min. The eluent was nanoelectrosprayed into a QTRAP 5500 (AB SCIEX, MA, USA) through an emitter

column (#FS360-50-15-N-20, PicoFrit, New Objective, MA, USA) and analyzed in MRM mode. The parent ion m/z were set in quadrupole (Q) 1, keeping the Q3 mass analyzer set to 184 m/z identifying the product ion that defines PC. MS parameters were as follows: ion spray voltage (IS), 3500 V, ion source gas 1 (GS1), 10 psi; turbo gas (GS2), 0 psi, curtain gas (CUR), 20 psi; collision gas (CAD), 10 psi, declustering potential (DP), 100 V, entrance potential (EP), 10 V, collision cell exit potential (CXP), 9 V, collision energy (CE), 47 eV, and scan time, 1.98 sec/cycle. High purity nitrogen (#UN1977, Boc Gases, ON, Canada) was used for CUR, GS1, and CAD. Data were acquired using AB SCIEX Analyst v1.5.1 and analyzed using MultiQuant v2.0.2.

Three additional datasets were acquired on a QTRAP 2000 using a different LCh method. Gradient chromatographic separation was performed using an Agilent 1100 HPLC System on a 5 cm $\times$ 200 μ m (ID) analytical column packed with Magic C₁₈ AQ 100A 5U 5 μ m resin (#PM5/61100/00, Michrom BioResources, CA, USA) coupled to a 5 cm packed analytical emitter column (#PF360-75-10-N-5, PicoFrit, New Objective) packed with the same beads. The injection volume was 8 μ l. The mobile phase consisted of H₂O containing 0.1 % FA (solvent A) and ACN (#A416-4, Fisher Scientific, ON, Canada) containing 0.1 % FA (solvent B). Gradient elution was performed with a linear increase of solvent B from 5 to 30 % in 2 min, 30 to 60 % in 7 min, 60 to 80 % in 33 min, and 80 to 95 % in 4 min. The analytes were nanoelectrosprayed at 3500 volts into a QTRAP 2000 and analyzed in PIS mode for the detection of PCs. CE was set to 40 eV. Data acquisition (m/z and RT) was performed and analyzed using Analyst v1.4.2.

2.5.5 Statistics

Statistical analyses were performed using GraphPad Prism 6.0a and IBM SPSS v22.

2.6 Algorithm

The RTStaR algorithm was designed to (a) verify user alignment of the RTs of lipid species from run-to-run within a single dataset/lipidome, (b) standardize RT alignments across multiple runs between datasets/lipidomes, (c) predict the empirical RTs of known lipid species in any given spectra, and (d) register corresponding and unique lipids across datasets/lipidomes. Users can also use it to build their own HPLC-ESI-MS analyte database specific to their particular LCh methodology. RTStaR is based on four assumptions: (1) a specific lipid species will *theoretically* have the same RT in every HPLC-ESI-MS spectra if separated using the same LCh method; (2) the RT of a peak is measured at the point of maximal intensity; (3) isobaric lipids can only be distinguished if the difference in RT between peaks is measurable; and (4) empirical RT differences between runs resulting from *system-level variations* can be corrected using a monotonic function whereas *component-level variations* cannot. Following Smith et al. (2015), we define system-level variations as run-to-run differences that affect the chromatography of *all* species in a given run. These differences can result from sample extraction and preparation by multiple researchers, replacement of connection tubing between runs, fluctuations in separation column temperature, decline in column performance over time, column-to-column variations upon replacement, etc., (Smith et al., 2015). Conversely, component-level variations are alterations that affect discontinuous chromatographic events (Smith et al., 2015). These changes can result from transient column clogging, transient changes in flow rate, random fluctuations in ionization efficiency, periodic ion suppression, etc. (Smith et al., 2015).

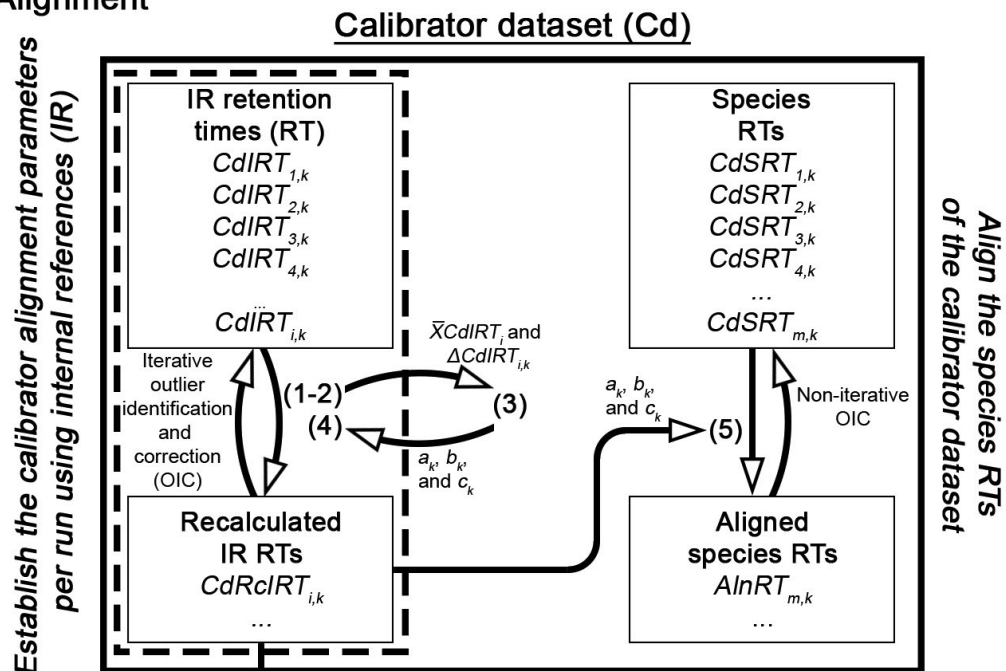
2.6.1 HPLC-ESI-MS quality control criteria

As with any bioinformatic application, spectra must meet best-practice HPLC-ESI-MS quality control criteria prior to submission to RTStaR. First, accurate peak identification requires an adequate signal-to-noise ratio of, at least, ten-fold (Kim et al., 2007). Second, peak shape must be relatively symmetrical and smooth to establish reproducibly the point of maximum intensity defining the RT (Barwick et al., 2006). Third, a sufficient number of IRs must be added either at time of sample extraction, time of HPLC-ESI-MS injection, or both. This number is defined empirically by the span (in minutes) of the RTs of all species of interest present in a given lipidome. We show below (Results) that IR RTs should be relatively equally distributed across the range of target RTs in both calibrator and experimental datasets and that optimal alignment is achieved when runs contain (a) approximately one IR per minute of RT and (b) the first and last IR RT flank the RTs of all of the endogenous species of interest.

2.6.2 Registering the RT of each lipid species in HPLC-ESI-MS spectra

To align spectra, RTStaR begins with a user-defined calibrator dataset that (a) estimates the average system-level variation associated with the user's particular LCh methodology and (b) calculates the theoretical RT for every species present in a given lipidome (Figure 2-1A). Next, the algorithm standardizes other datasets collected using identical LCh methods to this calibrator dataset (Figure 2-1B). Here, datasets are defined as a single study made up of a series of biological replicates, each represented by a unique MS run. A dataset can include both control and experimental groups. Our alignment and standardization pipeline is presented in Figure 2-1.

A - Alignment



B - Standardization

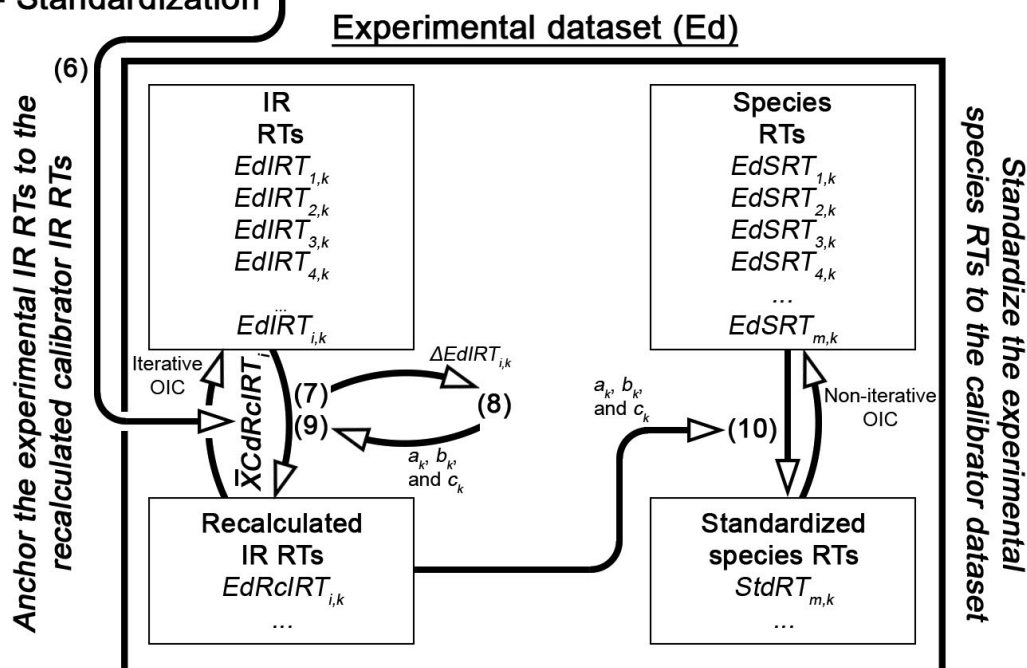


Figure 2-1

Figure 2-1 Schematic of the RTStaR Spectral Alignment, Standardization, and Registration Process

Diagram depicts the iterative algorithmic pipeline through which the RTs of species in multiple experimental datasets are registered to a single calibrator dataset. This process enables users to match corresponding lipid species within and between studies. Details are described in Section 2.6. Formulas (indicated by brackets) are defined in Section 2.6.4. The alignment verification protocols defined in (A) are packaged in RTStaR_Calibrate_v1 workbook. The standardization protocols defined in (B) and the registration protocols are packaged in RTStaR_Register_v1 workbook.

2.6.3 Defining the calibrator dataset

To determine the theoretical RT of each species in a given dataset, the user defines this dataset as the calibrator dataset. Different LCh methods require different reference datasets. This reference (calibrator) dataset is used to (a) quantify and correct the degree of system-level variation in every run and (b) identify runs in which component-level variation (outliers) precludes alignment. The user first identifies a dataset or a subseries of MS spectra that meet two basic criteria (Results):

- 1) The dataset [after alignment and outlier removal (Figure 2-1A)] must be composed of a minimum of 10 independent MS runs.
- 2) Analytes must be present in at least 66 % of all spectra after alignment and outlier exclusion for that particular species to be analyzed. RTStaR_Calibrate automatically removes species that do not meet the 66 % criterion from the calibration list.

2.6.4 Aligning the calibrator dataset

The IRs added to span the RT region of interest in the calibrator dataset are assessed first (Figure 2-1A, left panel). The number of IRs required must be pre-determined by the user. Our laboratory uses a targeted lipidomics discovery approach to establish this number for each new lipidome. We analyze a small cohort (e.g., $n = 2-5$) of new organisms and/or new sample origins in PIS mode, profiling the lipidome of interest (i.e., PC, PS, PE, etc.). The number of peaks (assuming the best-practice quality control criteria defined above), their m/z , the number of isobaric peaks, and the RT span of the target m/z range are recorded. Using these data, the number of IRs required to span the RT range of interest (approximately one IR per RT min in the LCh methodologies tested) can be calculated. Lipid abundances are subsequently determined in MRM by including all the m/z transitions

necessary to profile the complete lipidome of interest and the required IRs. Once MRM data for an entire calibrator study is collected, the theoretical IR RTs in the dataset is then established by calculating their respective means ($\bar{X}$) (Figure 2-1A, left panel).

$$\bar{X}CdIRT_i = \frac{1}{k} \sum_{k=1}^n CdIRT_{i,k} \quad (1)$$

where $\bar{X}CdIRT_i$ denotes the RT mean of i -th IR in all the runs of the calibrator dataset and $CdIRT_{i,k}$, the empirical RT of the i -th IR in the k -th run. i includes both the number of IRs added at time of extraction and the number of IRs added at time of MS.

Although m/z does not need to be considered when choosing the IRs for RT alignment, best practice routinely includes IR species from different lipid subfamilies (i.e., PC with hydrocarbons chains of different lengths, linkages, or degrees of unsaturation). This choice enables the same IRs to be used after RTStaR alignment for subsequent normalization and species abundance calculations.

Next, the system-level variation from the theoretical mean for each IR in every run is calculated as the distance (in minutes) from the $\bar{X}CdIRT_i$.

$$\Delta CdIRT_{i,k} = CdIRT_{i,k} - \bar{X}CdIRT_i \quad (2)$$

where $\Delta CdIRT_{i,k}$ is the distance of the i -th IR species in the k -th run from the mean RT of all runs.

The calibrator equation for each run is defined as:

$$\begin{array}{cc}
 x & y \\
 CdIRT_{i,k} & \Delta CdIRT_{i,k} \\
 \dots & \dots \\
 CdIRT_{n,k} & \Delta CdIRT_{n,k}
 \end{array}$$

$$y_k = a_k x^2 + b_k x + c_k \quad (3)$$

where a_k , b_k , and c_k are the specific parameters of a run calculated by fitting a quadratic regression that relates all of the empirical IR RTs to their respective distances from their theoretical means.

These specific parameters are used to recalculate the aligned RT of each IRs.

$$CdRcIRT_{i,k} = CdIRT_{i,k} - (a_k (CdIRT_{i,k})^2 + b_k (CdIRT_{i,k}) + c_k) \quad (4)$$

where $CdRcIRT_{i,k}$ denotes the recalculated (aligned) RT of the i -th IR in the k -th run.

These recalculated IR values, $CdRcIRT_{i,k}$, are used to assess the degree of component-level variation in each spectrum. RTStaR cannot align runs with extreme component-level variations. To identify these runs, the recalculated IRs are analyzed for outliers using the Robust regression and Outlier removal (ROUT) method (Motulsky and Brown, 2006). q values are set to 1 %. The empirical RT values for each outlier species are then re-examined by the operator. If any data acquisition errors (i.e., human error inputting the wrong RT value) are detected, the values are corrected and the IR RTs are iteratively recalculated using Equation (1 to 4). If no human errors are detected, the $CdRcIRT_{i,k}$ is considered a *bona fide* outlier. In this case, the component-level variation in

that particular run is said to exceed the capacity of RTStaR for alignment. The entire run is discarded from the calibrator dataset and the average IR RTs ($\bar{X}CdIRT_i$) are recalculated. The process is reiterated until no IR RT outliers are detected. Thus, manual and component-level errors are easily flagged by this “go-no-go” outlier statistic and quickly corrected or excluded. The final calibrator dataset must contain a minimum of 10 runs after outlier runs are discarded.

Once the IR RT run-dependent parameters a_k , b_k , and c_k are obtained through Equation (3), the RTs of each species ($CdSRT_{m,k}$) are then standardized by adapting Equation (4) (Figure 2-1A, right panel).

$$AlnRT_{m,k} = CdSRT_{m,k} - (a_k (CdSRT_{m,k})^2 + b_k (CdSRT_{m,k}) + c_k) \quad (5)$$

where $AlnRT_{m,k}$ denotes the aligned (theoretical) RT and $CdSRT_{m,k}$, the empirical RT of the m -th species in the k -th run.

Data acquisition errors are monitored through a non-iterative outlier identification process. At this stage, only outlier *species* (not *runs*) are discarded as the IRs (and thus spectra) have already met criteria. Following a single outlier exclusion assessment using the ROUT method (Motulsky and Brown, 2006), we apply a second statistical criteria, the Median Absolute Deviation (MAD) method (Leys et al., 2013), to assure that the elution time differences (i.e., component-level variations) between $AlnRT_{m,k}$ fall within three MAD. Species with $\log_{10}$ -transformed standard deviations (SD) over this threshold are excluded. Finally, any aligned species that is not represented in 66 % of all runs is discarded from the entire dataset. This criterion ensures that there are sufficient replicates to calculate the theoretical aligned RT $AlnRT_{m,k}$. Here, the user is cautioned that if their

calibrator dataset is composed of equal numbers of control and treatment/disease replicates with treatment/disease anticipated to deplete levels of particular analytes below limits of their particular methodology or introduce novel species into a lipidome, they may wish to consider registering experimental to control data using RTStaR_Register as these species will not meet the 66 % criteria necessary to establish baseline calibration.

2.6.5 Using the calibrator dataset to identify corresponding lipids across multiple experimental datasets

It is possible to match corresponding and identify unique lipid species in two or more datasets by standardizing their RTs to that of the calibrator dataset (Figure 2-1B). Each dataset must share the same LCh method and, ideally, the same IRs although we show below that omission of some IRs can be tolerated (see Results). Critically, *only species that elute within the range of the IRs held in common between datasets can be effectively registered.*

In the standardization algorithm (Figure 2-1B, left panel), the experimental IR RTs ($EdIRT_{i,k}$) shared between the calibrator and experimental datasets are recalculated (i.e., standardized to the calibrator dataset). This process accounts for system-level variation in each experimental run. First, the means of all of the recalculated calibrator IR RTs are determined. These values act as anchor points for the RT standardization of the experimental runs.

$$\bar{XCdRcIRT}_i = \frac{1}{k} \sum_{i,k=1}^n CdRcIRT_{i,k} \quad (6)$$

where $\bar{XCdRcIRT}_i$ is the mean of the recalculated RT of the i -th IR in the k -th run of the calibrator dataset.

Next, the system-level variation of every IR RT in the experimental dataset is calculated (Figure 2-1B, left panel). Here, RTStaR standardizes one experimental dataset at a time.

$$\Delta EdIRT_{i,k} = EdIRT_{i,k} - \bar{X}CdRcIRT_i \quad (7)$$

where $\Delta EdIRT_{i,k}$ is the distance (in minutes) of the i -th IR species in the k -th run from the mean recalculated RT of all runs in the calibrator dataset.

The run-dependent standardized equations are defined as:

$$\begin{array}{cc} x & y \\ EdIRT_{i,k} & \Delta EdIRT_{i,k} \\ \dots & \dots \\ EdIRT_{n,k} & \Delta EdIRT_{n,k} \end{array}$$

$$y_k = a_k x^2 + b_k x + c_k \quad (8)$$

where a_k , b_k , and c_k are the specific parameters of a run calculated by fitting a quadratic regression that relates all of the empirical IR RTs of the experimental dataset to their respective distances from the recalculated mean RTs of the IRs in the calibrator dataset.

These specific parameters (a_k , b_k , and c_k) are used to recalculate the standardized RT of each $EdIRT_{i,k}$.

$$EdRcIRT_{i,k} = EdIRT_{i,k} - (a_k (EdIRT_{i,k})^2 + b_k (EdIRT_{i,k}) + c_k) \quad (9)$$

where $EdRcIRT_{i,k}$ denotes the recalculated (standardized) RT of the i -th IR in the k -th run in the experimental dataset.

These recalculated IR values $EdRcIRT_{i,k}$ are used to control the quality of the experimental dataset LCh using a process analogous to that described above for the calibrator dataset. The $EdRcIRT_{i,k}$ values are analyzed for outliers using the ROUT method with a q set to 1 %. Any $EdIRT_{i,k}$ value identified as an outlier following recalculation as a result of data acquisition errors are corrected and the $EdRcIRT_{i,k}$ are recalculated using Equations 7 to 9. If the RT of any IR in a run is a *bona fide* outlier, the entire run is discarded as described above.

As indicated in Results, not all datasets can be used to calibrate different experimental lipidomes. In cases where the system-level variations different between datasets exceed the capacity of RTStaR to register spectra, the RTStaR algorithm is unable to match corresponding or identify unique species accurately. To verify whether the chosen calibrator can register a given experimental dataset, the user first verifies whether the algorithm accurately matches all of the IRs held in common by both datasets. If experimental IRs are not matched, a different calibrator dataset must be used to align that particular lipidome. This go-no-go criteria is embedded in RTStaR_Register and can be simply established by assessing the Lin's concordance correlation coefficient between $EdRcIRT_{i,k}$ and its respective $\bar{X}CdRcIRT_i$. If the lowest Lin's concordance correlation coefficient in a given run equal to or greater than 0.9995, all IR RTs will be registered and accuracy for correspondence will be 95% or greater (Results).

Once the registration passes these IR criteria, the experimental species RT ($EdSRT_{i,k}$) are then standardized by adapting Equation 10.

$$StdRT_{m,k} = EdSRT_{m,k} - (a_k (EdSRT_{m,k})^2 + b_k (EdSRT_{m,k}) + c_k) \quad (10)$$

where $StdRT_{m,k}$ denotes the standardized (registered) RT and $EdSRT_{m,k}$, the empirical RT of the m -th species in the k -th run.

Again, data acquisition errors are monitored through outlier identification as described above. Outlier species that cannot be registered in a particular run are flagged and discarded. This process is repeated for each experimental dataset under investigation and corresponding (or novel) lipids present are identified across multiple lipidomes.

2.7 Results

2.7.1 Validating RTStaR alignment of a population-based calibrator dataset

2.7.1.1 The RTs of 71 unique endogenous metabolites and six IRs in 1001 MS run can be accurately aligned using RTStaR

The RTStaR algorithm is packaged as two user-friendly ExcelTM workbooks: RTStaR_Calibrate_v1.xlsx and RTStaR_Register_v1.xlsx (Supplemental Data). In addition to empty templates, workbooks that include the serum and plasma datasets and following RTStaR analysis are also available as supplemental information (Supplemental Data).

We used a population-based dataset of PC metabolites extracted from serum of 1001 male and female adolescents representing 84475 analytes with unique or isobaric m/z and 6006 IRs across all runs to establish the calibrator dataset requirements. In this study, chromatographic methodology remained constant but multiple sources of system-level variations could be attributed to the following practical concerns: (1) samples were extracted by eight different investigators; (2) biological replicates were run by three different operators over a period of eight months; (3) MS was run 24 hours a day, 7 days a

week until maintenance was required to introduce changes in column performance with repeated usage, etc.; (4) heating, ventilation and air conditioning system cooling the room was turned off for a 24 h period twice during the 1001 runs (affecting 24 runs) to model extreme increases and decreases in ambient temperature; (5) packed chromatographic columns were changed six times over the course of the full study; (6) LCh capillaries, restriction columns, and electrospray emitters were changed as needed when operators detected component-level variations that necessitated re-running samples (i.e., tubing clogging, random pressure fluctuations etc.); and (7) the nanosource was cleaned once.

Theoretically, lipid species separated by the same chromatography should always elute with identical RTs. Practically, RTs vary dramatically between runs (Figure 2-2A-C). Despite the fact that all of the 1001 spectra submitted to RTStaR met the standard operating MS quality control criteria, we found that maximal variations in the six IR RTs ranged from 6.16 min for PC(13:0/0:0) to 9.42 min for PC(12:0/13:0) (Figure 2-2A, white bars). To address this problem, we applied Equations 1-4 (Figure 2-1A), packaged in the first worksheet of RTStaR_Calibrate (1. Align the Calibrator IRs). After alignment, the maximal RT variation ranged from 0.03 min for PC(13:0/0:0) to 0.13 min for D₄-PC(O-18:0/2:0) (Figure 2-2A, blue bars). Alignment decreased the average minimum to maximum variation by approximately 116-fold across all IRs. Importantly, alignment did not affect the theoretical RT; fold change in the average RT was 0.99-fold (i.e., was equivalent). Only the variance around these mean values was reduced, i.e., the mean SD decreased by approximately 85-fold across IRs and runs (Figure 2-2A, compare error bars for white and blue symbols).

Next, we used cumulative frequency graphs to assess the distribution of the IR RTs across runs (Figure 2-2B). Prior to alignment, bi- and tri-modal distributions were clearly

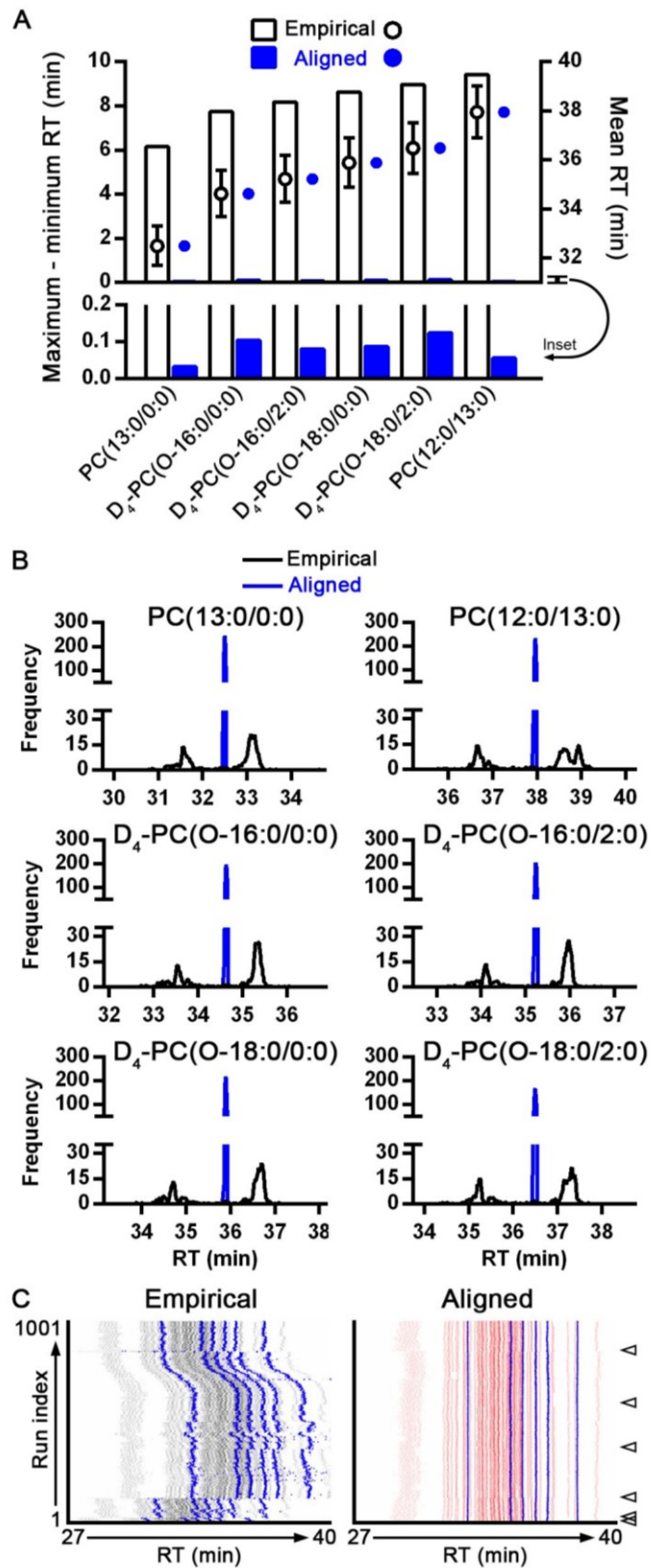


Figure 2-2

Figure 2-2 RTStaR Alignment of the Calibrator Dataset

(A) Alignment reduced maximum to minimum run-to-run variation in IR RTs (compare white and blue bars, left Y-axis) and their SDs around the theoretical IR RT (compare white and blue symbols represent average IR RT $\pm$ SD, right Y-axis). In the lower panel (inset), the left Y-axis is expanded between 0 and 0.2 min to display the markedly reduced maximum to minimum run-to-run variation after calibration. (B) Before alignment, bi-modal, and sometimes tri-modal, RT distributions were detected, indicative of different system-level variations in groups of runs (black lines). After alignment, these differences were effectively resolved, transforming five of six IR RT datasets into Gaussian distributions (blue lines). The exception was the D₄-PC(O-16:0/2:0) RT distribution, transformed from a multi-modal to single distribution but with a significant skew ($p < 0.0001$). Statistics were D'Agostino-Pearson omnibus normality tests with p set to 0.05. (C) Alignment effectively corrected the system-level variations of endogenous species (grey, empirical; red, aligned) that fell within the range of IR RTs (blue). Less effective alignment was noted for species that eluted outside of the IR RT range (see also Figure 2-4). Empirical run-to-run variations associated not only with column changes (indicated by arrowheads) but also with other unidentified sources were corrected effectively (compare vertical endogenous species alignments between left [grey, empirical] and right [red, aligned] panels) as well as IR alignment (blue).

observed, likely indicative of different system-level variations in groups of runs. RTStaR calibration transformed the majority of the multi-modal distributions (five of six IRs) into Gaussian distributions assessed by the D'Agostino-Pearson omnibus normality test (Figure 2-2B).

Finally, the effectiveness of the RTStaR alignment algorithm was quantified (Figure 2-2C). We applied Equation 5 (Figure 2-1A), packaged in the second worksheet of RTStaR_Calibrate (2. Align Calibrator Species). Descriptive statistics of results are presented in the third worksheet of the sample datasets provided (3. Calibrator Results, Supplemental Data). Major shifts in empirical RTs of both the endogenous species (grey) and IRs (blue) over the 1001 runs were clearly observed (Figure 2-2C, left panel). Many of the major variations corresponded to timing of column replacement (Figure 2-2C, arrowheads). These shifts were effectively corrected following RTStaR alignment (Figure 2-2C, right panel). Unexplained system-level aberrancies were also aligned, notably unknown differences that manifested as random spectral-wide RT shifts in single run flanked by adjacent runs with near identical chromatography separations (Figure 2-2C). Finally, we observed that the alignment of endogenous species with RTs that fell out of the IR RT range was less accurate than the alignment of species with RTs eluting within the IR RT span (Figure 2-2C).

2.7.1.2 RTStaR can align isobaric species eluting in close proximity

Minor changes in PC metabolite structures generate numerous isobaric species that share the same m/z yet elute with proximal (but quantifiably different) RTs (Figure 2-3A). In our validation dataset, the 85 endogenous and 6 IR species detected represented 49 distinct m/z . Seventy-three species in each run were isobaric; 40 species were isobaric

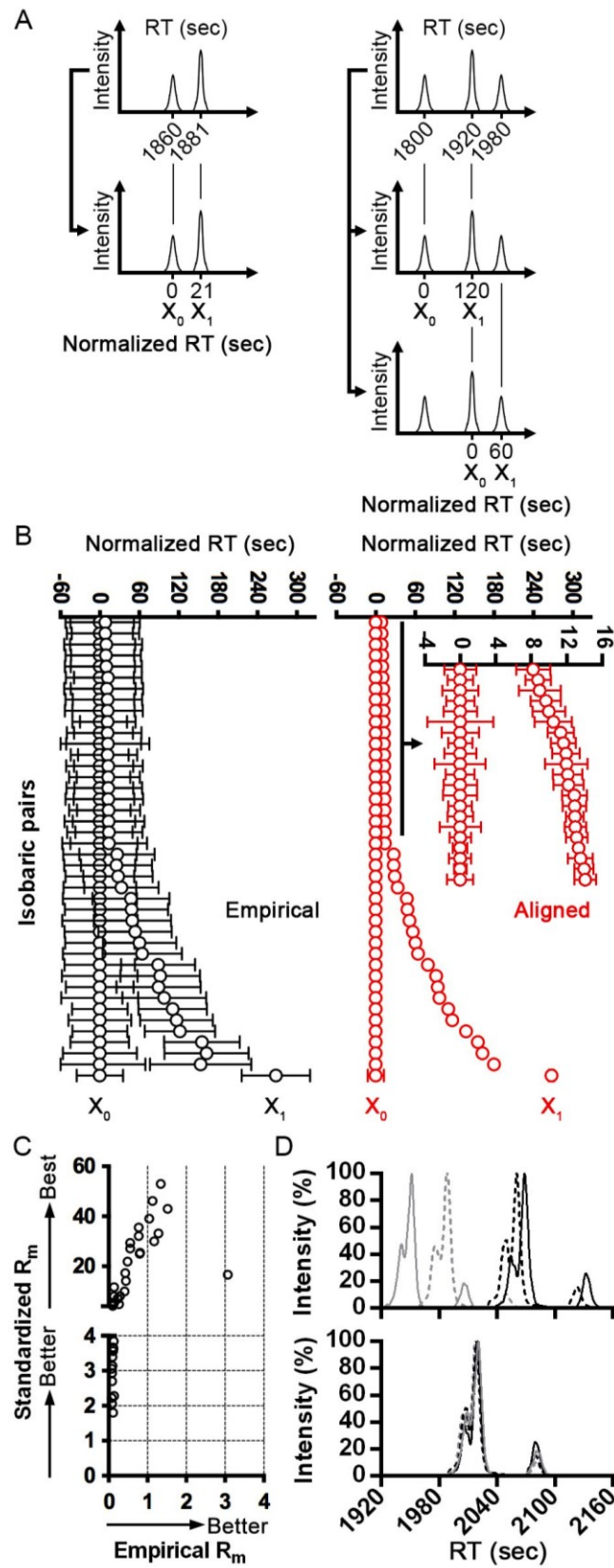


Figure 2-3

Figure 2-3 RTStaR Alignment can Effectively Identify Corresponding Isobaric Species Eluting in Close Proximity

(A) Schematic of proximal isobaric pairs (left panel) and triplets (right panel). To assess effectiveness of alignment, the empirical and aligned RTs were normalized setting the species eluting first (X_0) to 0 sec and calculating the RT of its downstream neighbour (X_1) relative to X_0 in sec. X_0 and X_1 were calculated for each isobaric species. (B) Normalized RTs pre- (left panel) and post- (right panel) RTStaR alignment. Data represent the mean normalized RT for each isobaric X_0 and X_1 pair $\pm$ SD. RTStaR reduced the variance between runs. Each species was clearly distinguished without overlap of SD after alignment. (C) Resolution (R_m) was calculated as described in Section 2.7.1.2 (D'Andrea et al., 2007). A higher R_m is indicative of a better resolution of isobaric species. Because RTStaR alignment does not change the theoretical mean RT, increases in R_m can only be attributed to a decrease in variance after alignment. R_m was markedly improved by RTStaR alignment. (D) Visualization of the effectiveness of this alignment on raw spectral data. The extracted-ion chromatogram from four samples each containing three isobaric species (482.4 m/z) were aligned using RTStaR. Individual runs are indicated by black, black-hashed, grey, and grey-hashed lines respectively. The top panel depicts overlay of the empirical chromatograms. Bottom panel depicts overlay of the chromatograms following alignment of each cycle time read.

pairs; 33 species were isobaric triplets. To establish the resolution required to match corresponding isobaric species, the distances separating these isobaric species were quantified in every run. Distances were defined as the peak-to-peak (RT) elution time in sec. For each species, we normalized both the empirical RTs ($CdSRT_{m,k}$) and the aligned RTs ($AlnRT_{m,k}$) setting the isobaric species eluting first to 0. When more than two isobaric species were detected at a given m/z, once the first distance was established, X_0 was set to the RT of the second species and X_1 was set to the RT of the third (Figure 2-3A, right panel).

Empirical RT

$$X_0 = CdSRT_{X_0,k} - \bar{X}CdSRT_{X_0}$$

$$X_1 = CdSRT_{X_1,k} - \bar{X}CdSRT_{X_0}$$

Align RT

$$X_0 = AlnRT_{X_0,k} - \bar{X}AlnRT_{X_0}$$

$$X_1 = AlnRT_{X_1,k} - \bar{X}AlnRT_{X_0}$$

where X_0 denotes the isobaric species eluting first and X_1 its closet subsequent neighbour (Figure 2-3A).

Empirical RTs between isobaric species clearly overlapped from run to run, precluding unambiguous assignation of the majority of species (Figure 2-3B, left panel). Only 8 isobaric species, eluting at least 111 sec apart, could be matched in the empirical data (Figure 2-3B, left panel). By contrast, all of the isobaric species could be distinguished following RTStaR alignment, i.e., no overlap in SD was observed (Figure 2-

3B, right panel and inset). Together, these data demonstrate that RTStaR alignment sufficiently decreased the variance around the theoretical mean such that RTs did not overlap, allowing even the closest isobaric species in our datasets to be distinguished.

To further quantify this marked improvement in discrimination, a R_m equation was applied, modified from D'Andrea et al., 2007:

$$R_m = \frac{(X_1 - X_0)}{(SD_0 + SD_1)}$$

where the numerator is the distance of each downstream isobaric species X_1 from its upstream X_0 and the denominator is the sum of the SD around each normalized X_0 and X_1 RT (Figure 2-3C). RTStaR alignment improved the mean resolution by 33-fold (Figure 2-3C).

Finally, the effect of this registration was graphically depicted by overlaying 4 representative extracted ion chromatograms pre- (top panel) and post- (bottom panel) RTStaR alignment (Figure 2-3D). Unambiguous resolution of a proximal isobaric triplet was evident after algorithmic correction of each cycle time read (Figure 2-3D).

2.7.2 Datasets composed of distinct lipid metabolites can be compared following RTStaR standardization and registration

A useful advance in chromatographic analysis would be to simply yet unambiguously (a) match corresponding lipid species across multiple datasets and (b) identify novel species specific to a given lipidome. RTStaR addresses the problem of lipid correspondence by standardizing the IRs of different experimental datasets to a single calibrator dataset and then registering these standardized datasets to each other. To validate the algorithm, we first anchored the IR RTs of two different profiling studies (mouse

NonTg and Tg hippocampal and plasma PC metabolite lipidomes) to the recalculated calibrator IR RTs ($\bar{X}CdRcIRT_i$) of the human serum calibrator dataset using Equations 6-9 (Figure 2-1B). These equations are packaged in the first worksheet of RTStaR_Register (1. Standardize Experimental IRs). These three studies were chosen to challenge RTStaR with diverse experimental effects predicted to influence matrix composition (i.e., sex, treatment, genotype, animal species, tissue, fluids, etc.) All experimental datasets contained the same six IRs.

2.7.2.1 The RTs of the IRs and endogenous analytes in two different datasets can be standardized to the calibrator dataset

Each experimental dataset was standardized separately to the calibrator dataset. The mean of each recalculated calibrator IR RT ($\bar{X}CdRcIRT_i$) was determined (Equation 6, Figure 2-1B). The relationship between these calibrator anchor points and the IR RTs in experimental run was then established. These parameters were used to standardize the IR RTs across datasets ($EdIRT_{i,k}$), verify the quality of the spectra, and exclude runs that could not be standardized due to extreme component-level variation (Equation 7-9, Figure 2-1B). In both experimental datasets, no outliers were detected in the IR RTs; no run was excluded. Figure 2-4A presents the results of the IR RT standardization. Note the near perfect registration of the experimental IRs in both datasets following RTStaR standardization. Deviation between empirical IR RT ($\bar{X}EdIRT_i$) and the $\bar{X}CdRcIRT_i$ was reduced by 96.6 % in mouse hippocampal and 99.5 % in mouse plasma following standardization ($EdRcIRT_{i,k}$) (Figure 2-4A). Moreover, RTStaR standardization transformed all of the IR RT frequency distributions in both experimental datasets into Gaussian distributions (D'Agostino-Pearson omnibus normality tests with p set to 0.05).

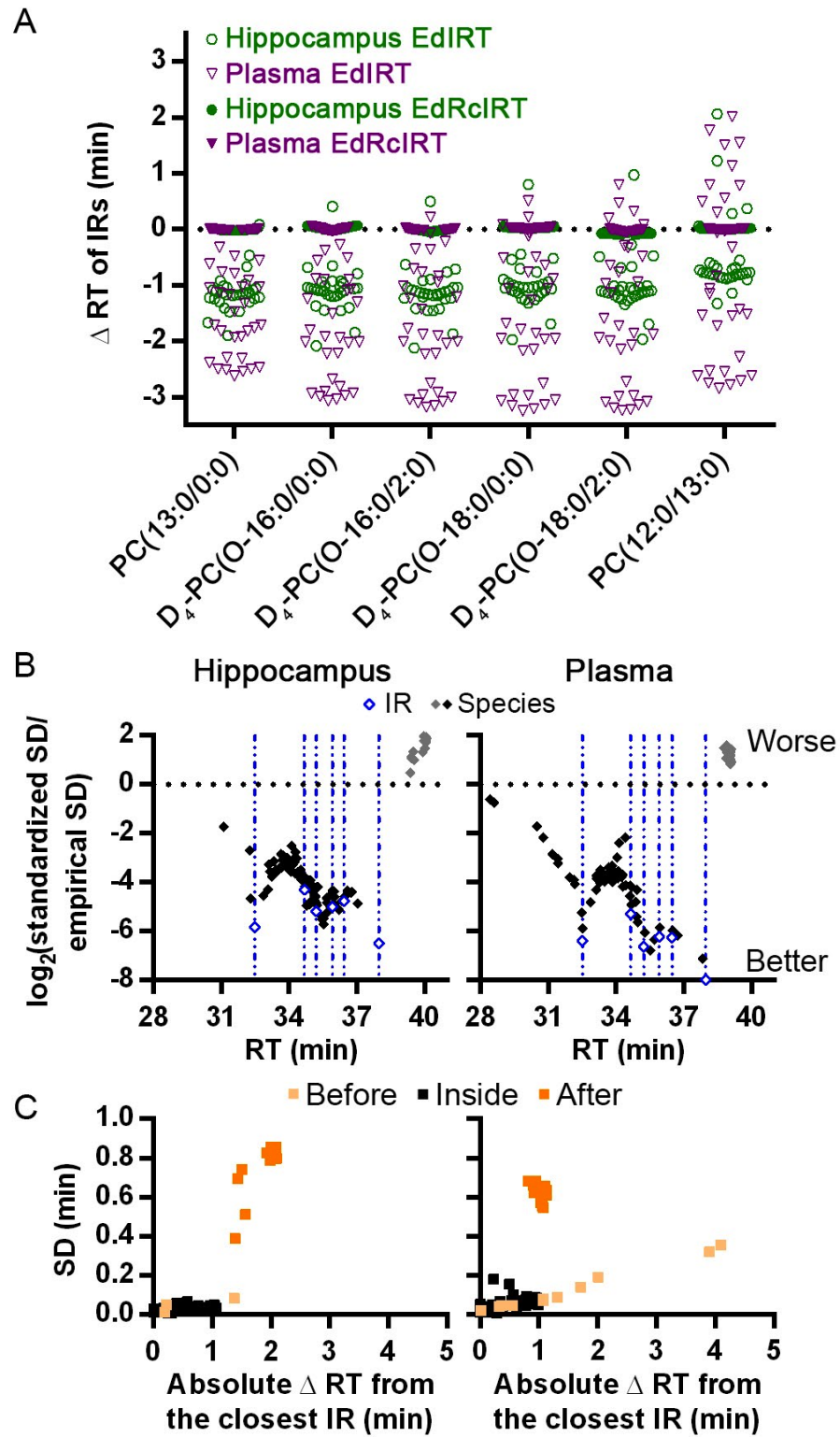


Figure 2-4

Figure 2-4 RTs from Multiple Datasets can be Standardized to the Calibrator Dataset

(A) The IR RTs in two distinct lipidomes, a murine plasma and a murine hippocampus PC metabolite datasets, were standardized to the $\bar{X}CdRcIRT_i$ of the calibrator dataset. Open symbols represent the difference in min between the empirical $EdIRT_{i,k}$ of each experimental run and the appropriate $\bar{X}CdRcIRT_i$ of the calibrator dataset. Closed symbols represent this difference after standardization. Note the near perfect registration of the $EdRcIRT_{i,k}$ in both experimental datasets with the calibrator dataset following RTStaR standardization. (B) RTStaR standardization improved the registration of $StdRT_{m,k}$ (black symbols) that eluted within the range of the $EdRcIRT_{i,k}$ (blue symbols, blue lines). RTStaR was less effective at correcting system-level variance that eluted before the initial IR and could not correct the system-level variance for species that eluted after the terminal IR. In these species, SD around the theoretical mean increased following RTStaR standardization (grey symbols). (C) The reduction in variance was clearly dependent upon the distance to the closest IR anchor point (black symbols, inside). Species eluting before the first IR $\bar{X}EdRcIRT_i$ (tangerine symbols, before) were less affected than species than eluted after the last IR $\bar{X}EdRcIRT_i$ (orange symbols, after).

Compared to the calibrator dataset, the mouse hippocampal and plasma datasets displayed Lin's concordance correlation coefficients that ranged between 0.9995 to 0.9999 and 0.9998 to 0.9999 across all runs respectively. Statistics are presented in sample workbook RTStaR_Register_SerumCd_vs_PlasmaEd.xlsx, '1. Standardize Experimental IRs', "Descriptive Statistics" (Supplemental Data).

The experimental species RTs were then standardized ($StdRT_{m,k}$) using Equation 10 packaged in the second worksheet of RTStaR_Register (2. Standardize Exp Species). Results are found in RTStaR_Register (3. Calibrator Results). In the mouse hippocampal dataset, 35 of 2863 empirical species RT ($EdSRT_{m,k}$) were identified as *bona fide* outliers (data not shown). In the mouse plasma dataset, 35 of 2248 empirical species RT ($EdSRT_{m,k}$) were identified and removed as outliers in RTStaR_Register (3. Calibrator Results). Following outlier identification and standardization, a marked reduction in variance was observed in 85.6 % and 74.4 % of the PC metabolites detected in the murine hippocampus and plasma, respectively (Figure 2-4B, black symbols). Species alignment, however, *deteriorated* in 14.4 % and 25.6 % of the hippocampal and plasma lipids respectively, meaning that RTStaR standardization *increased* SD around the respective means of these particular lipids. In each case, affected species eluted with a $StdRT_{m,k}$ that fell outside of the range of the recalculated calibrator IR RTs ($EdRcIRT_{i,k}$, Figure 2-4B, outside). To explore this deviation in more detail, we compared the mean distance that each PC metabolite eluted from its closet IR anchor point to the SD of their $StdRT_{m,k}$ (Figure 2-4C). Clearly, when the $StdRT_{m,k}$ fell within the range of IR RT span, RTStaR alignment reduced the variance across runs (Figure 2-4B,C, inside), however if the $StdRT_{m,k}$ was not flanked by an IR anchor point then RTStaR standardization failed

(Figure 2-4B,C, outside). Species that eluted after the last IR were the most affected (Figure 2-4B,C, after). Collectively, these data show that RTStaR can effectively register multiple datasets only if the RTs of target species fall within the RT range of the IR anchor points. Lipids that elute outside of this range are not accurately calibrated.

2.7.2.2 Reverse calculating (i.e., predicting) the empirical RTs facilitates analysis of target analytes and correct peak picking

A useful feature of the RTStaR algorithm is the capacity to predict the theoretical empirical IR RT ($TheoEmpRT_{i,k}$) of all species found in the calibrator dataset in any experimental dataset. This feature allows the user to scan rapidly a new dataset for corresponding lipids that share m/z with the calibrator dataset. This feature is packaged in the seventh worksheet of RTStaR_Register workbook (7. Predict Empirical RTs). These prediction equations can also be used to control the quality of the experimental data. Using this worksheet, if a species is identified as an outlier according to RTStaR_Register '3. Standardize Exp Species', the user can be provided with the predicted empirical RT by querying the run specific value reported in RTStaR_Register (7. Predict Empirical RTs). The original spectra can then be quickly assessed to determine (a) if the peak in question in fact exhibits this RT and thus error was due to data input, (b) whether component-level variation is the cause for the extreme shift in RT and the species must be excluded from that run, or (c) whether the analyte in fact represent a isobaric lipid unique to the new dataset.

To obtain the predicted $EdIRT_{i,k}$ and $EdSRT_{m,k}$ values, RTStaR uses the aligned RTs of the calibrator dataset ($AlnRT_{m,k}$):

$$\bar{X}AlnRT_m = \frac{1}{k} \sum_{i,k=1}^n AlnRT_{m,k} \quad (11)$$

where $\bar{X}AlnRT_m$ is the mean of the aligned RT of the m -th species in the calibrator dataset.

As every run must be standardized individually, only the IR empirical RTs of each runs of interest need to be acquired and processed through Equations 7-9 as described in Section 2.6.5 to obtain the run parameters a_k , b_k , and c_k . $\bar{X}AlnRT_m$ is then used to calculate the theoretical RT of that species in a given run.

$$\frac{(b_k - 1) + \sqrt{(b_k - 1)^2 - 4(a_k)(c_k + \bar{X}AlnRT_m)}}{2(-a_k)} = TheoEmpRT_{m,k} \quad (12)$$

where a_k , b_k , and c_k are obtained from Equations 7-9 and $TheoEmpRT_{m,k}$ is the theoretical empirical RT of the m -th species in the k -th run. We show below that the accuracy of this prediction requires that the calibrator and experimental dataset IRs display a Lin's concordance correlation coefficient of 0.9995 or greater.

We assessed the effectiveness of this standardization in percent ($E\%$) by adapting the equations developed by (Lantos et al., 2000):

$$E\% = 100 - \left[ABS \left(\frac{EdIRT_{m,k} - TheoRT_{m,k}}{EdIRT_{m,k}} \right) \right] 100 \quad (13)$$

For the analytes in hippocampal dataset standardized to the human serum calibrator dataset, the average $E\%$ per species after outlier removal ranged between 99.6 % and 100.0 %. The mean percent effectiveness for the 47 species was 99.9 %.

2.7.2.3 RTStaR can match corresponding isobaric species across datasets

Next, we asked whether RTStaR can match corresponding and identify new isobaric species across multiple datasets. We first standardized the lipid species present in murine

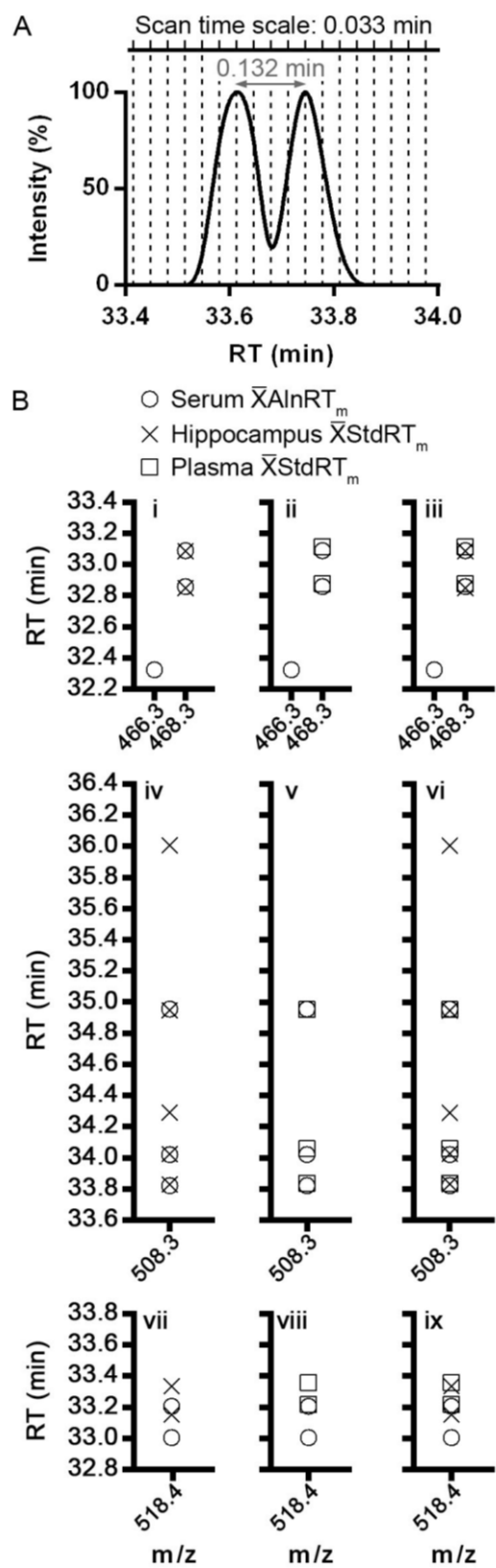


Figure 2-5

Figure 2-5 RTStaR can Register RTs Across Multiple Datasets, Match Corresponding Isobaric Peaks, and Identify Novel Analytes

(A) Schematic of an extracted ion chromatogram depicting two isobaric species and the dependence of resolving these species on MS cycle time. (B) Registration of endogenous species in the human serum, murine hippocampal, and murine plasma datasets after RTStaR alignment and standardization. Data represent $\bar{X}AlnRT_{m,k}$ and $\bar{X}StdRT_{m,k}$ values for lipids with m/z of 466.3, 468.3, 508.3, and 518.4 in human serum (calibrator dataset), murine hippocampal, and murine plasma datasets (experimental datasets).

hippocampus and plasma experimental datasets to the human serum calibrator dataset (Figure 2-5). We then registered all three datasets (Figure 2-5B). In total, 54,161 corresponding features were registered across all of the spectra in the three datasets. The murine hippocampus dataset contained 79 species in each run eluting within the range of the first and last IR RT. Of these 79 species, 66 shared m/z with species in the calibrator dataset. Thirteen species were unique. Of the 66 species potentially held in common with the human serum calibrator dataset, 31 distinct isobaric lipids were represented. In murine plasma, 47 species eluted within the RT range of the IRs. These 47 species shared 22 common m/z with lipids in the calibrator dataset.

As indicated in Section 2.6, isobaric lipids can only be distinguished if the distance between their peaks is measurable. As shown in Figure 2-3, we were able to resolve all of the isobaric peaks present in the calibrator dataset. However, peak to peak resolution is dependent not only on the quality of chromatography but also the MS cycle time thus capacity to match proximal isobaric species between different datasets is finite (Figure 2-5A). Peak to peak distance must exceed machine cycle time specifications by a factor of three for isobaric species to be quantifiably distinguished as separate peaks. For example, the MS cycle time of the MRM methodology we used to analyze the human serum, murine hippocampus, and murine plasma datasets was 0.033 min (Figure 2-5A). Thus, the smallest distance between isobaric lipids distinguishable under optimal chromatographic conditions would be 0.099 min or three cycle reads. Practically, peaks must be separated by a distance that exceeds four cycle reads (i.e., > 0.132 min in this particular methodology) considering full-width half-maximum peak values of even the best LCh separation (Figure 2-5A). In Figure 2-3B, we found that that the most proximal isobaric pair in the calibrator dataset, separated by 8.2 sec or 0.137 min, could be accurately aligned and both species statistically

separated. Thus, we show empirically that a lipid species present in either the calibrator or experimental dataset can be accurately registered (and thus matched or identified as a unique species) if (a) the difference between their mean standardized RT ($\bar{X}StdRT_{m,k}$) RT in the calibrator dataset and their mean aligned ($\bar{X}AlnRT_{m,k}$) in the experimental dataset is less than twice the cycle time of the particular MS methodology (i.e., ± 0.066 sec in this example) and (b) their m/z is within the MS tolerance (± 1 m/z) (i.e., they share the same m/z). Based on these results, we built these criteria into RTStaR for any user-inputted cycle time or MS tolerance (see RTStaR_Register (5. Identify Corresponding Lipids). The algorithm now automatically excludes isobaric alignments and correspondences when the $\log_{10}$ transformed SD is greater than three MAD (see RTStaR_Register (3. Standardize Exp Species and 4. Experimental Results)).

We show, in the mouse hippocampal dataset, 47 of its 66 PC metabolites sharing m/z with analytes present in human serum were matches. The remaining 19 species were unique to the hippocampus despite sharing m/z with proximal species in the calibrator dataset. Out of 47 species in the mouse plasma dataset, 37 were matched to the serum calibrator dataset. Ten were specific to murine plasma. Moreover, when we compared the $\bar{X}AlnRT_{m,k}$ and $\bar{X}StdRT_{m,k}$ values of all three datasets, we found that we could distinguish between corresponding species common to all three datasets, corresponding lipids found in two of three datasets, and lipids unique to a single dataset. Figure 2-5B (panels i-iii) shows the simplest example wherein the human serum dataset contained a unique species at 466.3 m/z not present in either the mouse hippocampal or plasma datasets. All three datasets shared two isobaric species at 468.3 m/z registered with near perfect alignment after RTStaR standardization (Figure 2-5B, panel iii). A more complicated example is presented

in Figure 2-5B (panels iv-vi). The human serum dataset contained three isobaric species with an m/z of 508.3; the murine hippocampal dataset contained five species; the murine plasma dataset contained three species. After standardization, the first, second, and fourth species of the hippocampus dataset matched to the three species found in both the human serum and mouse plasma dataset (Figure 2-5B, panels iv-vi). The most complex example is presented Figure 2-5B (panels vii-ix) wherein novel species eluting in close proximity to corresponding lipids were distinguished. Here, two isobaric species were detected with an m/z of 518.4 in all three datasets. After RTStaR standardization and registration, these species were resolved as three distinct species. The first eluting species was specific to the serum dataset (33.00 ± 0.03 min) (Figure 2-5B, panel ix) and while the last was specific to both mouse datasets (hippocampus 33.33 ± 0.04 min and plasma 33.36 ± 0.06 min) (Figure 2-5B, panel ix). The middle species was match between the murine hippocampus (33.15 ± 0.03 min), the murine plasma (33.22 ± 0.05 min) and the human serum dataset (33.21 ± 0.03 min) (Figure 2-5B, panels vii-ix). The log transformed SD for these correspondences was less than three MAD and differences between alignments (thus different species) were greater than three MAD. These data demonstrate the capacity of RTStaR to differentiate distinct isobaric doublets between multiple datasets despite their close proximities.

2.7.3 Defining the requirements of a calibrator dataset: Not all datasets can be calibrator datasets

The calibrator dataset used to validate RTStaR was composed of 1001 runs. To establish the minimum number of runs required, we repeated the calibration using 10 or 30 runs randomly chosen from the larger population-based dataset. These minimal calibrator datasets were capable of standardizing the mouse plasma dataset with the same results as

the population-level calibration when the reproducibility of the alignment of each $EdRcIRT_{i,k}$ with its respective $\bar{X}CdRcIRT_i$ equalled or exceeded a Lin's concordance correlation coefficient of 0.9995. Based on these results, we established that a calibrator dataset must be composed of a minimum of 10 independent MS runs, after alignment and outlier removal to be effective.

Next, we asked how many IRs must be held in common between a calibrator dataset and an experimental dataset for registration to occur. Using the human serum dataset and its six IRs as the calibrator dataset, we sequentially removed IRs from the murine hippocampal dataset and assessed impact on its registration. With all six IRs present in both datasets, 47 PC metabolites in murine hippocampus could be matched with species in human serum (Figure 2-6A-C, triangles). As expected based on our empirical data (Figure 2-4), when the initial IR with the smallest RT [PC(13:0/0:0)] was excluded, lipid species in the experimental dataset that eluted before this RT could not be matched (Figure 2-6A, open circle). We then assessed the loss of two consecutive IRs in the middle of the RT range, D₄-PC(O-16:0/2:0) and D₄-PC(O-18:0/0:0). All of the 47 species were correctly registered, but the average absolute RT distance deteriorated by 9.2 %, from 0.025 to 0.027 min (Figure 2-6B, open circles). When three consecutive IRs were removed towards the end of the elution range, discarding D₄-PC(O-18:0/0:0), D₄-PC(O-18:0/2:0) and PC(12:0/13:0) (Figure 2-6C, open circles), we were unable to register species that eluted after the last IR shared by both datasets. Moreover, registration of the species that remained within the range of common anchor points range deteriorated by 144.3 % with average absolute distance from their calibrator counterparts increasing from 0.025 to 0.060 min. These data demonstrate RTStaR alignment, standardization, and registration function

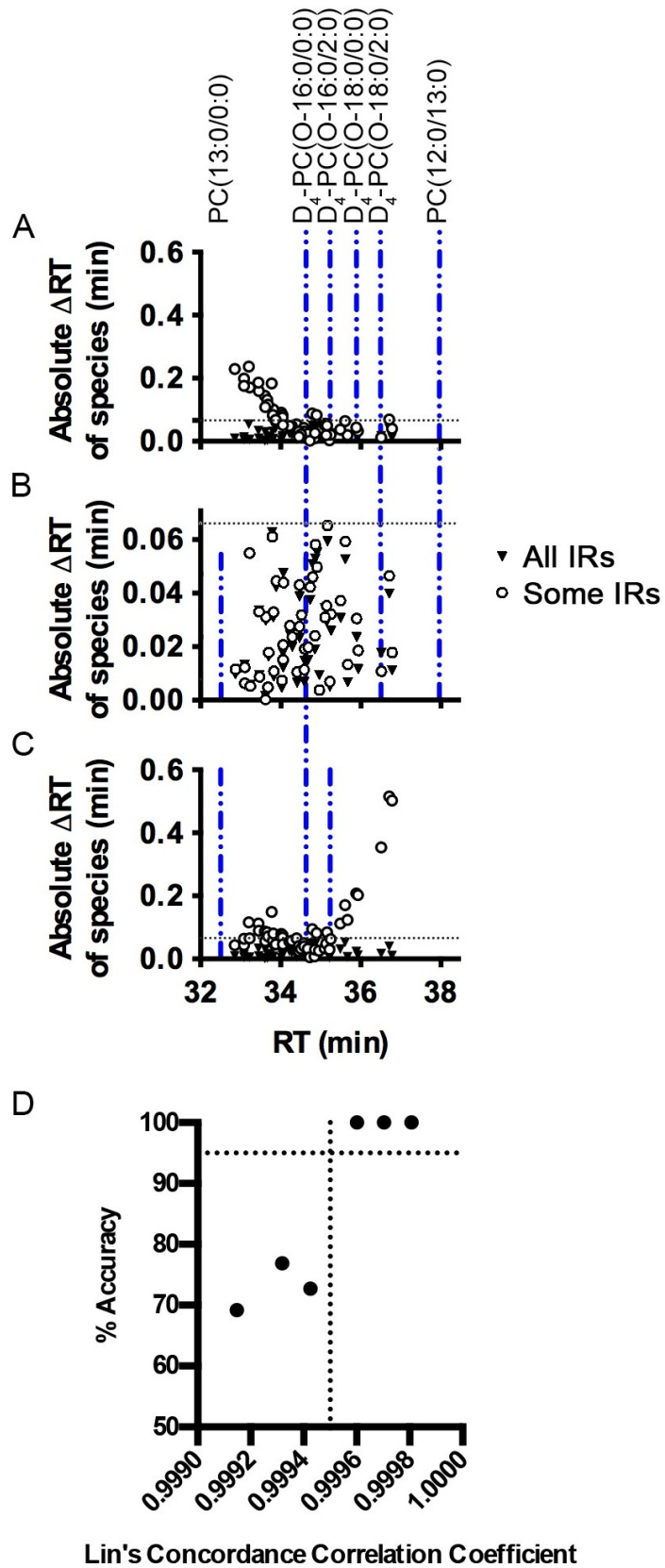


Figure 2-6

Figure 2-6 RTStaR Functions Optimally When the Calibrator and Experimental Datasets Share the Same IRs

(A) Lipid species in the experimental dataset that elute before the first IR held in common between calibrator and experimental datasets cannot be matched to the corresponding lipids present in calibrator dataset. (B) Experimental species that fall within the elution range of the RTs held in common with the calibrator dataset can be registered effectively but with less precision than when all IRs are held in common. (C) Lipids that elute after the terminal IR RT shared by both calibrator and experimental datasets cannot be effectively registered. (D) Accuracy of calibration is dependent on the degree of concordance between the $EdRcIRT_{i,k}$ dataset with its respective $\bar{X}CdRcIRT_i$. We compared capacity of a dataset composed of 22 standards (Avanti Polar Lipid and Cayman Chemical) to calibrate the human serum, murine plasma, and murine hippocampal datasets and vice versa. The number of endogenous species matching these standards present in each biological lipidomes was verified by addition of exogenous standards. We found that any dataset with a Lin's concordance correlation coefficient of 0.9995 between calibrator $\bar{X}CdRcIRT_i$ and experimental $EdRcIRT_{i,k}$ effectively registered all of the endogenous species correctly with an accuracy of 100 %. Any value below this concordance, failed to reproducibly match corresponding species with an accuracy of greater than 95 %.

optimally when the calibrator and the experimental datasets share the same IRs. Where IRs differ, only species that fall within the elution range of the RTs held in common can be registered effectively with resolution dependent on the number of IRs present.

Finally, we quantified the reproducibility and the accuracy of RTStaR alignments using different calibrator datasets. We compared inter-calibrator agreement between a dataset composed of 22 lipid standards both calibrating and being calibrated by the human serum dataset (1001 runs), the murine hippocampal dataset (30 runs), and the murine plasma dataset (30 runs). All of the lipids added to the lipid standard dataset were isobaric with endogenous species present in all three lipidomes. By addition of standards to each matrix, we verified that human serum contained 13 of the 20 standards; murine plasma contained 11 of the 20 standards; murine hippocampus contained 13 of the 20 standards. Each lipidome contained at least one corresponding species that differed from one of the other three biological lipidomes. Accuracy of correspondence with these validated results was 100 % when the lowest degree of agreement between $EdRcIRT_{i,k}$ with its respective $\bar{X}CdRcIRT_i$ was equal to or greater than 0.9995 (Lin's concordance correlation coefficient) (Figure 2-6D). Thus, RTStaR requires that (a) a calibrator dataset is composed of a minimum of 10 runs in which each analyte represented in at least 66 % of each spectra, (b) only species that fall between the RTs of calibrator datasets can effectively registered, and (c) the minimal $EdRcIRT_{i,k}$ concordance with calibrator $\bar{X}CdRcIRT_i$ must be equal to or exceed a Lin's concordance correlation coefficient of 0.9995 for correspondence accuracy to be 95 % or greater.

2.7.4 RTStaR functions independently of LCh and MS methodology as long as datasets being aligned share the same methods

Lastly, we tested RTStaR's platform independence with respect to LCh and MS. We used RTStaR to align human and murine parietal-temporal cortex datasets (a) longitudinally assessing PC metabolite lipidomes across the lifespan N5 NonTg and TgCRND8 mice, (b) in NonTgPAFR^{+/+}, NonTgPAFR^{-/-}, TgPAFR^{+/+} and TgPAFR^{-/-} littermates, (c) in post-mortem human brain of AD and age-matched human controls. Data were collected using a different LCh protocol on a QTRAP 2000 at a cycle time of 0.028 min with five IRs held in common. RTStaR_Register was capable of aligning datasets as long as the $EdRcIRT_{i,k}$ with its respective $\bar{X}CdRcIRT_i$ was equal to or greater than 0.9995 (Lin's concordance correlation coefficient).

2.8 Discussion

Comparative lipidomics lacks sufficient bioinformatic capacity to register rapidly and reproducibly corresponding analytes across biological replicates and unambiguously identify lipid species unique to a given organism or sample origin in HPLC-ESI-MS datasets. To begin to address this bioinformatic need, we developed the RTStaR algorithm. RTStaR combines warp functions used to estimate the system-level variations particular to a given HPLC-ESI-MS methodology in a calibrator dataset with direct match correspondence functions used to register multiple datasets applying the same methodology to this comparator. We show here that (a) the calibrator dataset must be composed of a minimum of 10 independent MS runs; (b) analytes must be present in at least 66 % of all runs after alignment and outlier exclusion; (c) the Lin's concordance correlation coefficient between $EdRcIRT_{i,k}$ and its respective $\bar{X}CdRcIRT_i$ must be equal to or greater than

0.9995 in each MS spectra being registered for a dataset to be used as a calibrator dataset and; (d) sufficient IRs must be introduced in both calibrator and experimental datasets to flank RT range of interest (approximately one IR RT per min). When these criteria are met, RTStaR aligns a given dataset and registers this calibrator dataset with other lipidomes with a correspondence accuracy of 95 % or higher.

2.9 Conclusion

We show here that RTStaR can align over 68572 lipids in 1001 human serum samples, standardize 3708 lipids in 30 murine plasma samples and 30 murine hippocampal PC metabolite lipidomes to the calibrator dataset, and register all three lipidomes to each other. The algorithm was then challenged with datasets collected using other HPLC-ESI-MS methodologies, registering PC metabolites detected post-mortem in the temporal-parietal cortex of AD and age-matched controls with those identified in a murine life-span study of PC metabolism in the parietal-temporal cortex in a mouse model of AD. Together, these data show that HPLC-ESI-MS datasets can be accurately and rapidly registered using RTStaR if users compare lipidomes generated using the same methodologies. The complete algorithm is packaged in two modular ExcelTM workbook templates for easy implementation. RTStaR is freely available and can be downloaded from the India Taylor Lipidomics Research Platform (ITLipidomics) website <http://www.neurolipidomics.com/resourcesLIPIDS.html>.

CHAPTER 3. VISUALIZATION AND PHOSPHOLIPID IDENTIFICATION (VaLID): AN ONLINE INTEGRATED SEARCH ENGINE CAPABLE OF IDENTIFYING AND VISUALIZING GP WITH GIVEN MASS

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3.1 Objective of this Study

In analyzing the neural lipidomes generated in Chapter 4, I found that many of the m/z of the phospholipids present in the profiles were not represented in even the most comprehensive non-propriety species identification resources developed by the LIPID MAPS consortium (<http://www.lipidmaps.org/>) and LipidBank (<http://lipidbank.jp/>) when queried between 2008 and 2015. Thus, the objective of this study was to assist researchers in predicting the identity of novel lipid species in their MS spectra. To achieve this objective, in collaboration with my colleague Graeme McDowell, we applied an *in silico* approach to return all theoretically possible combinations of straight-chained phospholipids, considering multiple user-defined MS conditions in an online search engine (<http://neurolipidomics.ca/>). **This study was published as two separate papers:**

Blanchard AP[†], McDowell GS[†], Valenzuela N[†], Xu H, Gelbard S, Bertrand M, Slater GW, Figeys D, Fai S & Bennett SAL (2013). Visualization and Phospholipid

Identification (VaLID): online integrated search engine capable of identifying and visualizing GP with given mass. *Bioinformatics* 29, 284-285.

McDowell GS[†], **Blanchard AP[†]**, Taylor GP, Figeys D, Fai S & Bennett SAL (2014). Predicting glycerophosphoinositol identities in lipidomic datasets using VaLID (Visualization and Phospholipid Identification)-an online bioinformatic search engine. *Biomed Res Int* 2014, 818670. [†]Joint first authors

3.2 Statement of Author Contributions

These studies were the result of the close collaborative work of four different laboratories under SALB's direction. APB was the study coordinator for VaLID 1.0.0 and joint first authors with GSVM and NV. For VaLID 2.0.0, APB and GSVM were joint coordinators and joint first authors. APB developed the computational databases through *in silico* calculation of predicted mass for each species. GSVM wrote the code, designed the graphical databases, the automated drawing tools, and integrated the modules. The Maya three-dimensional (3D) representations of the curated species were built by NV. MB developed the high resolution skeletal representations. The graphical user interface was designed by APB, GSVM and SG; html coded by SG; and updated by GSVM and APB. HX provided critical theoretical computational input throughout VaLID 1.0.0 development and performed final beta testing, GPT developed the PI metabolism figure.

3.3 Summary

The capacity to predict and visualize all theoretically possible GP molecular identities present in lipidomic datasets is a time-consuming and labour-intensive process. To address this issue, VaLID was developed. VaLID is a web-based application that links a user-friendly search engine, GP databases, and multiple visualization features that identify all theoretically possible phospholipids for any mass to charge value under a variety of MS conditions. The VaLID databases contain the exact and average mass of more than 1,473,168 individual species from 12 GP classes: PA, PPA, PC, PE, PG, PGP, PI, PIP, PIP₂, PIP₃, PS, CDP-DG. Furthermore, VaLID's graphical algorithms can generate the skeletal representations for every identity (i.e., every double bond, every double bond position etc.,). A subset of species curated by the CTPNL team is provided as an array of high-resolution structures. VaLID is freely available and responds to all users through the CTPNL resources web site at (<http://neurolipidomics.ca/>).

3.4 Introduction

The emerging field of lipidomics seeks to answer two seemingly simple questions: How many lipid species are there? What effect does lipid diversity have on cellular function? To address these questions, a lipidomic approach includes the comprehensive assessment of cellular, regional, and systemic lipid homeostasis. This assessment expands beyond lipid profiling to include the transcriptomes and proteomes of lipid metabolic enzymes and transporters, as well as that of the protein targets that affect downstream lipid signalling (Bennett et al., 2013). Lipidomic analyses also encompass an unbiased mechanistic assessment of lipid function ranging from the physicochemical basis of lipid behaviour to lipid-protein and lipid-lipid interactions triggered by intrinsic and extrinsic

stimuli (Bennett et al., 2013). The first step, however, lies in identifying the molecular identities of the lipid constituents in different membrane compartments.

Technological advances in ESI and MALDI MS, ESI-coupled to HPLC, developed in proteomics and now applied to field of lipidomics allow lipid diversity and membrane composition to be quantified at the molecular level (Piomelli et al., 2007, Brown and Murphy, 2009, Bou Khalil et al., 2010, Xu et al., 2013). Thousands of unique lipid species across the six major lipid structural categories in mammalian cells (fatty acyls, glycerolipids, GPs, sphingolipids, sterol lipids, and prenol lipids) and two lipid categories synthesized by other organisms (saccharolipids and polyketides) can now be identified using HPLC-ESI-MS and, in some cases, MALDI-MS imaging (Piomelli et al., 2007, Han et al., 2012, Bennett et al., 2013). Yet, with these successes come new challenges. Turning raw MS spectral data into annotated lipidomic datasets is a time-consuming, labour-intensive, and highly inefficient process. Predicting identities of “new” species, not previously curated, is exceedingly difficult. Lipidomic investigations lack essential bioinformatic tools capable of enabling automated data processing and exploiting the rich compositional data present in MS lipid spectra.

The critical first step is to unambiguously assign molecular identities from the MS structural information present in large lipidomic datasets (Niemela et al., 2009). Where genomics and proteomics capitalize on sequence-based signatures, lipids lack such easily definable molecular fingerprints. Identities must be reconstructed by analysis of (a) lipid m/z following “soft” ionization ESI and MALDI techniques and (b) defining fragmentation patterns obtained after collision-induced dissociation in various MS modes (Xu et al., 2013). Once these molecular identities are predicted, further information about stereospecificity of critical species can then be assessed (e.g., by MS/MS, analysis of lyso-

form fragment ions, product ion spectral evaluation) (Whitehead et al., 2007, Smith et al., 2008, Hou et al., 2011, Tang et al., 2011). For example, membrane GP are derivatives of glycerol-3-P with (a) an acyl, an alkyl (ether-linked plasmanyl), or an alkenyl (alkyl-1'-enyl, vinyl ether-linked plasmenyl) carbon chain at the *sn*-1 position, (b) a long-chain fatty acid usually esterified to the *sn*-2 position, and (c) a polar headgroup composed of a nitrogenous base, a glycerol, or an inositol unit modifying the phosphate group at the *sn*-3 position. The polar headgroup defines membership in one of 20 different GP classes (e.g., PA, PPA, PC, PE, PG, PGP, PI, PIP, PIP₂, PIP₃, PS, CDP-DG) (Fahy et al., 2011). Molecular species are further distinguished by individual combinations of carbon residues (chain length and degree of unsaturation) and the nature of each *sn*-1 or *sn*-2 chemical linkage (acyl, alkyl, or alkenyl) to the glycerol backbone. PI(18:0/22:6), for example, defines a lipid with a phosphoinositol polar headgroup, a fully saturated 18 carbon chain (referred to as :0) ester-linked at the *sn*-1 position, and a 22 carbon chain which is characterized by six unsaturations (indicated by :6) ester-linked at the *sn*-2 position (Figure 3-1). Immediate PI metabolites (PIs phosphate: PIP_x) are then produced by carbon-specific phosphorylation of the PI headgroup with unique fatty acyl, alkyl, and/or alkenyl *sn*-1 and *sn*-2 chains (Figure 3-1 and 3-2). Among others, the tight regulation of PI metabolism and its critical impact on cellular function clearly underlines the importance of these compositional changes (Figure 3-1). Yet, to date, biological significance of the astonishing number of potentially unique GP is unknown. This is primarily due to the challenges associated with unambiguous compositional identification of GPs in biological membranes (Igbavboa et al., 2002, Osawa et al., 2008, Axelsen and Murphy, 2010, Sharman et al., 2010, Chan et al., 2012, Bennett et al., 2013).

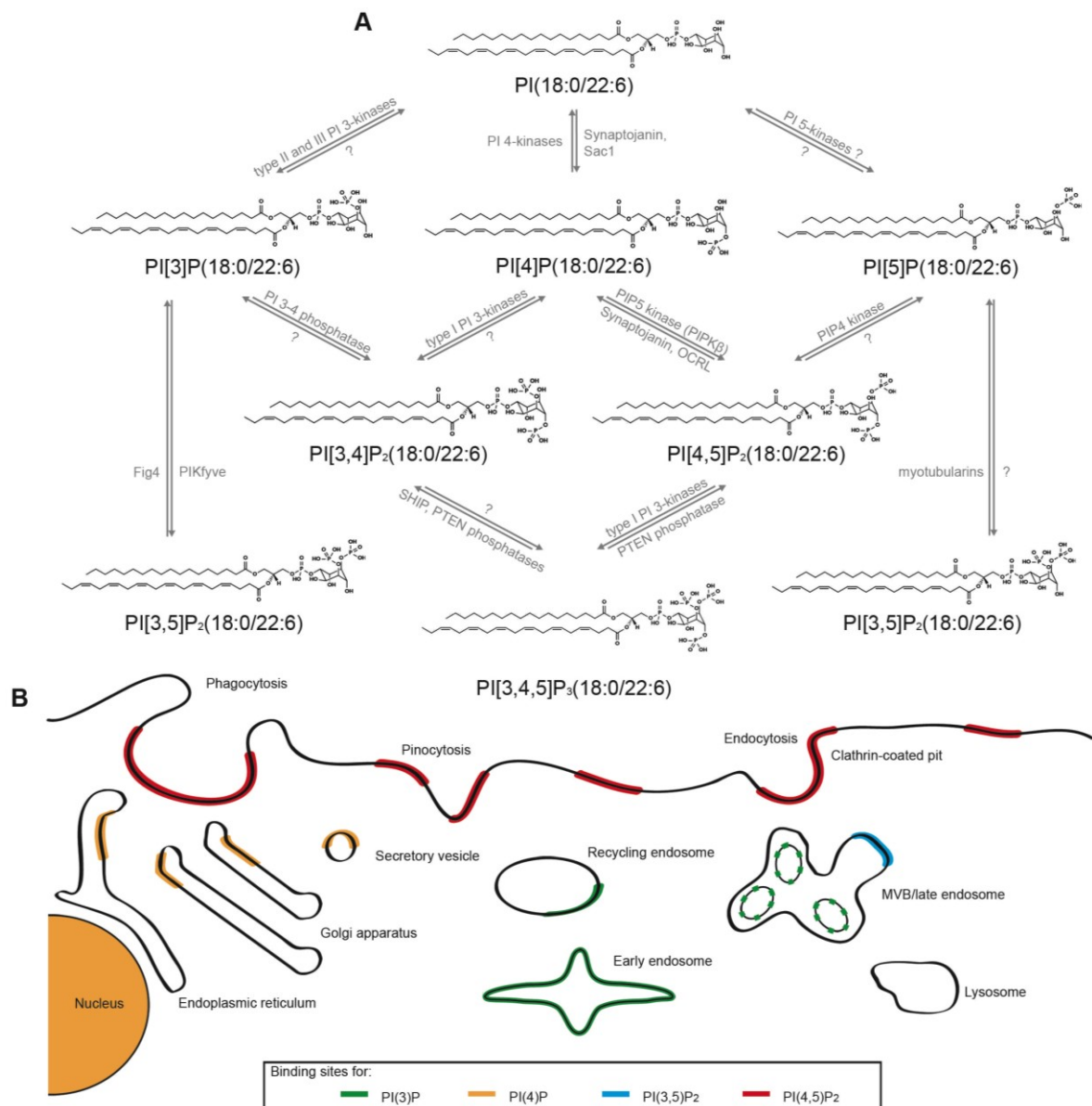


Figure 3-1

Figure 3-1 PI Metabolism to PIP_x

(A) Metabolism of membrane PIs to bioactive PIP_x metabolites. The molecular identity of each species, defined by carbon chain length and linkage to the GP backbone, is predicted to affect signalling specificity in addition to known effects of PI headgroup phosphorylation. (B) Phosphorylation of PIP_x species regulates the different localization of PI-binding proteins and targets them to specific organelles (i.e. lipid-protein interaction). Phosphorylation status and carbon chain length dictates localization and likely restricts functions. Together, structural PIs and their PIP_x metabolites regulate vesicular fusion, exocytosis, and endocytosis as reviewed in (and adapted from) (Le Roy and Wrana, 2005) and (Skwarek and Boulianne, 2009).

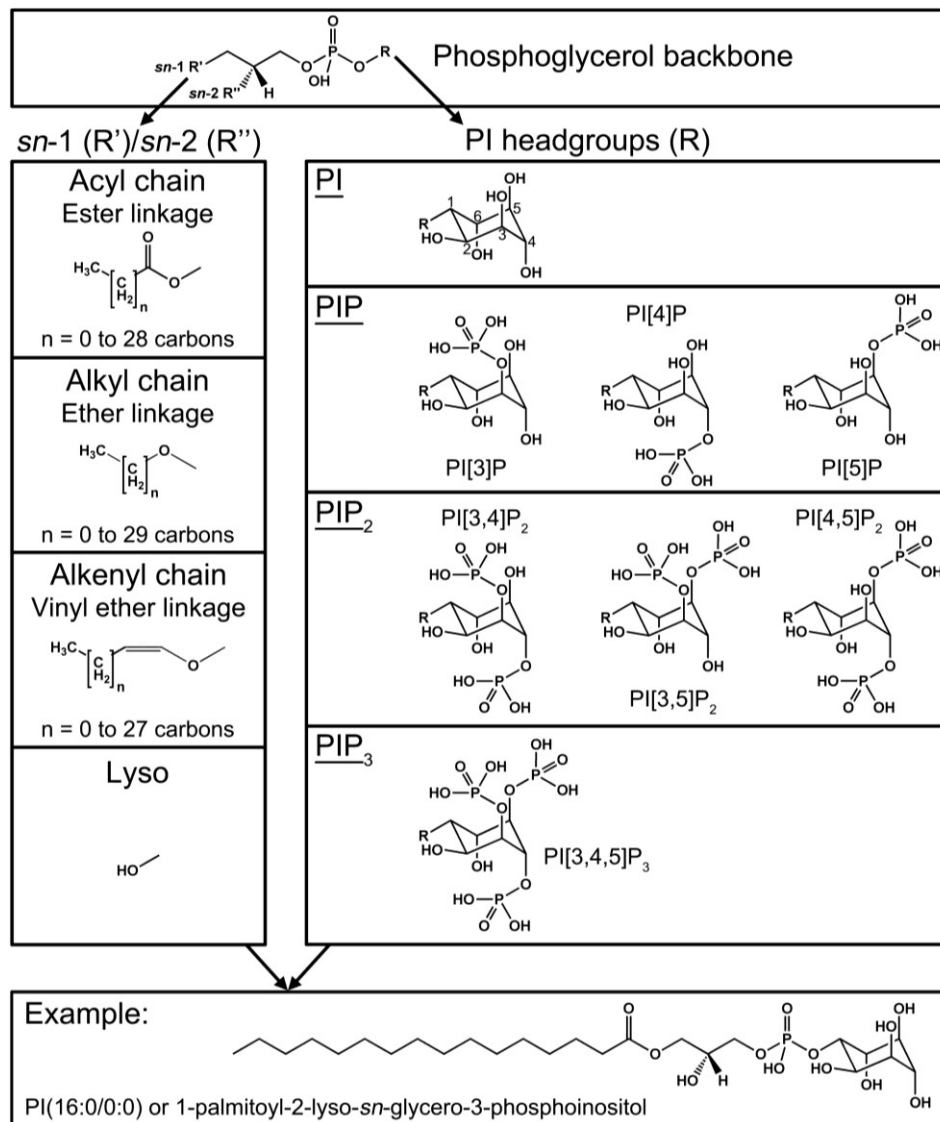


Figure 3-2

Figure 3-2 Component and Composite Structural PI and PIP_x Features Used to Calculate Masses

Exact and average masses for all theoretically possible PI and PIP_x species were calculated from the masses of every component possibility: (top panel) the phosphoglycerol backbone, (left panel) *sn*-1 and *sn*-2 hydroxyl residues (lyso-lipids) and *sn*-1 and *sn*-2 fatty chains ranging from 1 to 30 carbons with up to six unsaturations, considering ester, ether, or vinyl ether linkages to the phosphoglycerol backbone, and (right panel) PI polar headgroup and all biologically relevant phosphorylation possibilities. The bottom panel provides one composite PI example.

Key advances in lipidomic bioinformatics have been led by the LIPID MAPS consortium both in the development of online spectral databases and the reorganization of lipid class ontologies (Fahy et al., 2007a, Fahy et al., 2011). These toolsets and classification systems have recently been complemented by the *in silico* generation of a searchable library of all theoretically possible MS/MS lipid spectra in different ionization modes (LipidBlast) (Kind et al., 2013). Such fundamental toolkits are supported by a growing compendium of targeted spectral tools, reviewed in Fahy et al., 2007, Bou Khalil et al., 2010, Xu et al., 2013. Few existing bioinformatic resources, however, provide necessary information on all potential acyl chain inversions (e.g., *sn*-1 vs. *sn*-2), critical GP linkages that define lipid function, or theoretically possible double bond positions for every possible species. To address this need, we have developed Visualization and Phospholipid Identification (VaLID) – a web-based application linking a user-friendly on-line search engine, structural composition database, and multiple visualization features – capable of providing users with all theoretically possible GP calculated from any m/z under a variety of MS conditions including mass accuracy, adduct, and GP subclass. Its exhaustive GP database contains more than 1,473,168 unique PA, PPA, PC, PE, PG, PGP, PI, PIP_x, PS, and CDP-DG species (Table 3-1). VaLID is freely available for commercial and non-commercial use at <http://neurolipidomics.ca>. User support is provided through the lipawrd@uottawa.ca e-mail.

3.5 Experimental Procedures

3.5.1 GP compositional database

VaLID's underlying database contains exact and average masses of all theoretically possible PA, PPA, PC, PE, PG, PGP, PI, PIP_x, PS, and CDP-DG calculated *in silico* from

Table 3-1 Total Number of Species from Each Subclass that is Included in VaLID

Phospholipid subclass	LIPIDMAPS classification	Abbreviation	Number of species*
Glycerophosphates	GP10	PA	92073
Glyceropyrophosphates	GP11	PPA	92073
Glycerophosphocholines	GP01	PC	92073
Glycerophosphoethanolamines	GP02	PE	92073
Glycerophosphoglycerols	GP04	PG	92073
Glycerophosphoglycerophosphates	GP05	PGP	92073
Glycerophosphoinositols	GP06-09	PI, PIP _x	736584
Glycerophosphoserines	GP03	PS	92073
Cytidine 5'-diphosphate glycerols	GP13	CDP-DG	92073
Total			1473168

The calculated number of species does not include lipids formed by changing the position of the double bond beyond those represented in VaLID's structural models. Each lipid m/z has been calculated for exact and average masses and can be searched using even and odd carbon chains with mass tolerance ranging from ± 0.0001 to ± 2 and MS ion modes $[M + H]^+$, $[M + K]^+$, $[M + Li]^+$, $[M + Na]^+$, $[M - H]^-$, or $[M \text{ (Neutral)}]$.

corresponding atomic masses (De Laeter et al., 2003). Component structural masses were first established for: (a) the glycerol backbone, (b) GP polar headgroups, (c) *sn*-1 and *sn*-2 hydroxyl residues (lyso-lipids), (d) *sn*-1 and *sn*-2 fatty chains ranging from 1 to 30 carbons with up to six unsaturations, considering (e) ester, ether or vinyl ether linkages to the phosphoglyceride backbone. Figure 3-2 depicts an example for the PI and PIP_x headgroups. Composite masses were then calculated for every theoretically possible combination. Thus, the underlying database includes all GP, as well as every acyl, alkyl, and alkenyl variant, for every carbon chain and double bond position, of all GP headgroup including mono- (PIP), bi- (PIP₂), and tri- (PIP₃) phosphorylated PI modified on the hydroxyl group of carbon 3, 4, and/or 5.

3.5.2 Programming language and packages

VaLID was developed using Oracle's Java programming language version 6 and external Java libraries from JExcelApi Structures are displayed within the program by ChemAxon's Marvin View 5.5.1.0 software (<http://www.chemaxon.com>). The code was written using the IDE Eclipse Kepler and packaged using the Fat Jar Eclipse version 0.0.31 plugin. VaLID is a web-based Java applet, and thus requires that Java be both installed and enabled on a user's web browser.

3.5.3 Search engine features

The VaLID search engine predicts lipid identity taking into consideration user-specific MS conditions (Figure 3-3). Users choose exact or average mass before inputting their *m/z* of interest. Simple pull-down menus restrict carbon chain lengths and linkages within GP classes while considering appropriate ion type. Predictions appear in two panels: (i) *Possible Lipids Include* and (ii) *Possible Isomeric Lipids Include*. The first panel

returns lipid identities with arbitrarily assigned *sn*-1 and *sn*-2 carbon chains. The second panel lists corresponding isomers. The list position is ordered by the lipid subclass (polar headgroup) and sorted by ascending (i) *m/z*, (ii) number of carbons (*sn*-1 chain), (iii) degree of unsaturation (*sn*-1 chain), (iv) number of carbons (*sn*-2 chain), and (v) degree of unsaturation (*sn*-2 chain). Nomenclature adheres to the LIPID MAPS classification system (Fahy et al., 2011). To assist in decision-making, a “best guess” feature is available whereby lipids in blue are considered “most likely” based on the prevalence of constituent fatty acids in mammalian cells (Miyazaki and Ntambi, 2008). Lipids in red indicate that (i) the species is part of our CTPNL curated database of neural lipids for which (ii) we have generated multiple high-resolution representations. Lipids in black represent theoretically possible combinations.

3.5.4 GP structural visualization

To display all theoretically possible GP conformations, users can highlight the lipid of interest and click on the “Display All” button. Structures have been restricted to display only *Z* double bond separated by a minimum of two carbons. To achieve this goal, the basic structure of the GP backbone including their headgroup was created manually and the atom placement corrected mathematically to match known structures. Slight adjustments to atom placement were further made to improve visibility. The locations of each atom in the headgroup were then established on a Cartesian plane and coded into the software. The automated drawing feature was integrated into the database and search functions, allowing GP to be visualized on demand. Using JExcelApi, VaLID calculates where each atom of the fatty chain should be in 2D-space and generates 2D representations displayed using ChemAxon's Marvin View. This enables the user to toggle through structural views and

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VaLID: Visualization and Phospholipid Identification
a glycerophospholipid m/z prediction database
Developed by Graeme S.V. McDowell, Alexandre P. Blanchard and Nico Valenzuela
Version 2.0.0

Exact Mass
☐ Average Mass

Ionic Mass (m/z):

Chain Lengths:

Mass Tolerance (± m/z):

Lipid Subclass:

Fatty Chain Linkage:

Ion:

Possible Lipids Include:		Possible Isomeric Lipids Include:	
PI(3,4)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801	PI(3,4)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801
PI(3,5)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801	PI(3,5)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801
PI(4,5)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801	PI(4,5)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801
PI(3)P(P-10:3/6:2)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-10:4/6:2)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(P-12:4/4:1)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-12:5/4:1)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(P-14:5/2:0)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-14:6/2:0)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(16:6/0:0)	Exact Mass [M+H] ⁺ - 641.1764	PI(3)P(0:0/16:6)	Exact Mass [M+H] ⁺ - 641.1764
PI(3)P(O-2:0/14:6)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(P-4:0/12:5)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-4:1/12:5)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(P-6:1/10:4)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-6:2/10:4)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(P-8:2/8:3)	Exact Mass [M+H] ⁺ - 641.1764		
PI(3)P(O-8:3/8:3)	Exact Mass [M+H] ⁺ - 641.1764		
PI(5)P(P-10:3/6:2)	Exact Mass [M+H] ⁺ - 641.1764		
PI(5)P(O-10:4/6:2)	Exact Mass [M+H] ⁺ - 641.1764		
PI(5)P(P-12:4/4:1)	Exact Mass [M+H] ⁺ - 641.1764		
PI(5)P(O-12:5/4:1)	Exact Mass [M+H] ⁺ - 641.1764		
PI(5)P(P-14:5/2:0)	Exact Mass [M+H] ⁺ - 641.1764		

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Please enable popups to access the structural databases and the help icons. Thank you.
The first search time may take up to 90 sec, depending upon the connection speed, and search options selected. Subsequent searches will be much faster.

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Figure 3-3

Figure 3-3 VaLID 2.0.0 Interface

The new PI and PIP_x search options are shown for the VaLID interface's drop down menu. Each member of the PI family can be searched individually, as well as in various combinations. PIP_x refers to phosphoinositol mono-, bi- or tri-phosphate, and can be searched with, or without, PIs. The graphical interface may differ slightly between web browsers.

save each species in a variety of formats. Chemical structures are drawn in accordance to the standards developed by the LIPID MAPS consortium (Fahy et al., 2011). The “Best Prediction” button displays only those lipid species found in VaLID’s *Predicted to be Common* database. The “Structural Representations” button displays lipids that are part of VaLID’s curated *Structural Representations* database identified in neural tissue by CTPNL researchers. These species can be viewed in high-resolution as (i) 2D skeletal models, (ii) 3D “ball and stick” models, (iii) space filling models, or (iv) rendered “VaLID view” models. “VaLID view” models were assembled in ChemDraw3D®, translated into a 3D model where each atom was marked by an x, y and z coordinate, and exported into Autodesk® Maya® v2012. Rigid and dynamic models were derived using Maya® nParticles, converted into smooth polygonal meshes. These meshes were directed to the original x, y, and z coordinates and imported as points in space to recapitulate the original molecular structure in an abstracted, organic form. Resulting VaLID view models are available for download as rigid polygons.

3.6 Results and Discussion

3.6.1 VaLID can predict and display any GP species identified under multiple MS conditions

Since its inception, VaLID was designed to be a comprehensive GP database linking a convenient search engine with visualization features for identification and dissemination of large-scale lipidomic datasets. VaLID has the capacity to (a) predict identities of GP species present in MS spectra from m/z under user-defined MS conditions and (b) automatically visualize every theoretically possible molecular species for each GP family at given m/z (Figure 3-3). Its database contains 12 GP classes including the PI

family and their metabolites, the number of possible structural and biochemical combinations results in colossal structural diversity, more than 1,473,168 unique structures (Table 3-1).

At first release VaLID 1.0.0 (Blanchard et al., 2013b) did not include the PI superfamily due to difficulties associated with computing and drawing this extensive superfamily. VaLID 2.0.0 (McDowell et al., 2014a) added this feature. To accelerate searches in the PI family, users can restrict searches to subclasses (PI, PIP, PIP₂, PIP₃) or sub-subclasses (PI, PI[3]P, PI[4]P, PI[5]P, PI[3,4]P₂, PI[3,5]P₂, PI[4,5]P₂, PI[3,4,5]P₃). For example, if the option PI[3,4]P₂ is chosen, all molecular species with an inositol backbone phosphorylated only at the 3rd and 4th carbon positions will be provided and VaLID will not return any related PI[3,5]P₂ or PI[4,5]P₂ species. The “PI + PIP_x” option restricts searches to the entire PI superfamily excluding other GP families. The “All without the PIP_x” option returns the entire GP in the database including PI structural precursors with the exception of PIP_x metabolites. Finally, the “All” option returns results from every headgroup. When more than one headgroup is being searched, the program will let the user know how many headgroups have been loaded, and how many are remaining to be loaded.

With respect to the visualization features for PIP or PIP₂, the program will draw the phosphate groups on the inositol ring in the locations that the user specified from the dropdown menu for lipid species selected. As with the other subclasses, choosing the “Display All” button will draw all the theoretically possible structures associated with the selected lipid name. Potential variants in degrees of unsaturation are drawn sequentially in every location along the fatty acid chain, separated by at least two carbons, and in Z configuration. If the selected lipid meets criteria for the “Best Prediction,” selecting this option will return only the lipids in VaLID’s “Predicted to be Common” database. These

species are categorized based on the relative abundance of prevalent fatty acid chains in mammalian cells (Miyazaki and Ntambi, 2008).

3.6.2 A search and visualization example from the PI and PIP_x family

A search example is shown in Figure 3-3, where the user seeks to identify a PI or PIP_x species with even carbon fatty chains that has an exact m/z of 642 from their MS spectra. The user has defined the MS spectrum ion mode to $[M+H]^+$ as well as the mass tolerance to ± 1 amu. From approximately 1,500,000 possible GPs, VaLID has predicted 102 identities under these conditions. While 49 of these species belong to the PI, the PIP_x contains 53 species which are divided into 45, 6, and 2 species that respectively belong to the PIP, PIP₂, and PIP₃. Providing all the theoretically possible species accelerates and facilitates the identification of an unknown species because this list largely reduces the number of possibilities for users to validate. In this example, species PI[4,5]P₂(10:4/0:0) has been selected by the user to visualize all theoretical possibilities for this entry (Figure 3-4).

3.6.3 Curating the central nervous system lipidome: theoretically possible species are not all biologically relevant

With increasing MS sensitivity, many species not previously considered to be biologically relevant are detected, hence it is impossible to determine with confidence which species are present when researchers are in this “discovery mode” without showing all the theoretical possible species. As aforementioned, a single species in the PI or PIP_x family has more than one hundred possibilities. Its identification is crucial to understand its biological role, but in a large-scale study which contains a few hundred species, this identification must be fast, at least, for the majority of species, until users decide to “drill

down” to mechanistic validation. Most common, well-curated, species should be considered first as they likely represent the most abundant species in any spectra. In the previous example, VaLID has predicted three species PI(P-22:0/0:0), PI(O-22:1/0:0), and PI(0:0/O-22:0) which facilitates its final identification. A deductive and eliminative process is required to validate the most biologically possible species. In this example, the use of a more precise and accurate MS spectrum and a search on a curated database (Table 3-2) would have helped to greatly reduce the number of possibilities. Thereafter, researchers should focus first on the most common toward the least common species starting with even carbon fatty chains ranging from 12 to 24 with a lower number of unsaturation ester linked GP.

3.7 Conclusion

VaLID is a web-based application linking a convenient search engine, exhaustive GP database, and multiple visualization features for identification and dissemination of large-scale lipidomic datasets. VaLID returns all theoretically possible species based on m/z and user-defined MS conditions from 12 GP families. The user is cautioned that VaLID includes lipids (and isomeric bond configurations) that may not be biologically relevant. Investigators are encouraged to mine these lists for species most relevant to their specific biological system for subsequent validation that would include multidimensional MS and spiking of known standards (where available) or in-house synthesis. To assist in decision-making, a “best guess” feature is available in VaLID to focus on lipids predicted to be common based on the prevalence of the fatty acid chains in mammalian cells. Every

CIHR Training Program
Neurodegenerative Lipidomics

VaLID: Visualization and Phospholipid Identification

a glycerophospholipid m/z prediction database

Developed by Graeme S.V. McDowell, Alexandre P. Blanchard and Nico Valenzuela
Version 2.0.0

Search Parameters:

☒ Exact Mass
☐ Average Mass

Ionic Mass (m/z):

Chain Lengths:

Mass Tolerance (± m/z):

Lipid Subclass:

Fatty Chain Linkage:

Ion:

Possible Lipids Include:

PI(3,4)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801
PI(3,5)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801
PI(4,5)P ₂ (10:4/0:0)	Exact Mass [M+H] ⁺ - 641.0801

Possible Isomeric Lipids Include:

PI(3,4)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801
PI(3,5)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801
PI(4,5)P ₂ (0:0/10:4)	Exact Mass [M+H] ⁺ - 641.0801

Possible Lipid Structures Include:

Table	1	2	3
PI(3)P(O-10:4/6:2)			
PI(3)P(O-12:4/4:4)			
PI(3)P(O-12:5/4:4)			
PI(3)P(O-14:5/2:0)			
PI(3)P(O-14:6/2:0)			
PI(3)P(16:6/0:0)			
PI(3)P(O-2:0/14:4)			
PI(3)P(O-4:0/12:4)			
PI(3)P(O-4:1/12:4)			
PI(3)P(O-6:1/10:4)			
PI(3)P(O-6:2/10:4)			
PI(3)P(O-8:2/8:3)			
PI(3)P(O-8:3/8:3)			
PI(4)P(O-10:3/6:2)			
PI(4)P(O-10:4/6:2)			
PI(4)P(O-12:4/4:4)			
PI(4)P(O-12:5/4:4)			
PI(4)P(O-14:5/2:0)			

GETTING STARTED | **FAQs** | **LIPID MODELS** | **USER GUIDE**

Please enable popups to access the structural databases and the help icons. Thank you.
The first search time may take up to 90 sec, depending upon the connection speed, and search options selected. Subsequent searches will be faster.

University of Ottawa - Faculty of Medicine: Biochemistry, Microbiology, and Immunology, Faculty of Science: Biology - Sunnybrook Health Sciences
Carleton University - Faculty of Engineering and Design: Architectural and Urbanism - University of Toronto - Centre for Research in Food Safety and Food Quality

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Figure 3-4

Figure 3-4 Automated Drawing Feature of VaLID 2.0.0

An example of a search button, returning all possible PI and PIP_x lipids with m/z of 642 (exact mass with a user-defined tolerance of 1 amu), restricted to displaying even carbon chains only, and selecting [M + H]⁺ ion mode in MS (back panel). The user then selected PI[4,5]P₂(10:4/0:0) and its *sn*-1/*sn*-2 chain inversion species and pressed the “Display All” button. The window labelled “Possible Lipid Structures Include” displays a table containing the possible structures for this lipid, with the restrictions as laid out in the user manual (inset). These drawings can be easily exported for use in publication figures as described in the user manual. The graphical interface may differ slightly between web browsers.

Table 3-2 m/z and Spectral Databases and Prediction Engines for Lipid Identification

Tool	Availability	References
lipID	email to: mass-spec@fz-borstel.de	(Hubner et al., 2009)
LIPID MAPS Structure Database (LMSD)	http://www.lipidmaps.org/data/structure/	(Fahy et al., 2011)
Lipid Mass Spectrum Analysis (LIMSA)	http://www.helsinki.fi/science/lipids/ software.html	(Haimi et al., 2009)
Lipid Qualitative/ Quantitative Analysis (LipidQA)	http://msr.dom.wustl.edu/research/ Downloadable_Software.htm	(Song et al., 2007)
LipidBank	http://lipidbank.jp/	(Watanabe et al., 2000)
LipidomeDB Data Calculation Environment (DCE)	http://lipidome.bcf.ku.edu:9000/ Lipidomics	(Zhou et al., 2011)
LipidXplorer	https://wiki.mpi-cbg.de/wiki/lipidx/ index.php/Main_Page	(Brennan et al., 2012)
multiWayCCA	http://research.ics.aalto.fi/mi/software/ multiWayCCA/	(Huopaniemi et al., 2010)

theoretical conformation (in *Z* configuration) for each species can be viewed in 2D and 3D. Curated species can also be downloaded in multiple high-resolution representations for further visualization and model production.

CHAPTER 4. DISRUPTIONS IN LAND'S CYCLE

REMODELLING OF STRUCTURAL PC ARE A DEFINING AD

PATHOLOGY

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4.1 Objective of this Study

Over the course of this thesis, changes in plasma PC compositions were reported to predict phenoconversion from preclinical AD to either MCI or LOAD (Mapstone et al., 2014). Using the bioinformatic tools generated in Chapters 2 and 3, I applied an unbiased lipidomic approach to identify aberrant pathways and metabolic networks disrupted in MCI and LOAD brain that were reflected in changes in plasma PC composition. The overarching objective was to determine how circulating phospholipid compositions reflect AD neuropathology.

4.2 Statement of the Author Contributions

This study was the result of the close collaborative work of six different laboratories under SALB's direction. APB and LAS designed the experiments with JW, ZP, DF, KLL, SEB, HX, and SALB. APB did all the mice work at the exception of the immunofluorescence performed by AP and the reproductive status analysis by SS. LAS and HX extracted and acquired the human data from brain and plasma collected by GM, KLL, BL, SEB, ZP, and SALB. Data were analyzed by APB assisted by HX under the direction of DF and SALB.

4.3 Summary

Changes in the plasma PC lipidome are reported to precede AD phenocopy; however, how circulating GP compositions reflect AD neuropathology is uncertain. Here, we used a targeted lipidomics approach to profile PC metabolism in plasma of young adults, cognitively normal elderly with vascular impairment, MCI and LOAD. Plasma PC metabolite lipidomes were compared to entorhinal-hippocampal lipidomes of LOAD patients, TgCRND8 human amyloid beta precursor protein (hAPP) transgenic mice, and across the lifespan of wild-type female littermates. Systems-level analyses identified PC metabolic pathways perturbed by age, disease, and A β biogenesis. Disruptions in Lands' cycle remodelling of DAPCs, AAPCs, PAPCs were associated with age and disease. Regional alterations in the Kennedy pathway of PC biosynthesis were indicated by changes in PC compositions in LOAD plasma and posterior/temporal cortex but not hippocampus. Finally, changes in entorhinal-hippocampal PC metabolites in TgCRND8 mice linked A β exposure in early life to changes in mono- (MUFA) and polyunsaturated fatty acid (PUFA) biosynthesis that elevated specific compositions of LPCs, LPAFs, and PAFs. Taken together, these data identify a convergence of PC metabolic impairments, associated with age, disease, and A β exposure, that contribute to LOAD phenocopy.

4.4 Introduction

Intraneuronal accumulation of NFT composed of hyperphosphorylated tau and aberrant processing of the APP to toxic A β fragments are primary AD determinants. The most damaging of these fragments is A β_{42} . Accumulation is gradual with assembly of soluble A β_{42} oligomers impairing synaptic function and signalling synaptotoxicity (Cleary et al., 2005, Palop and Mucke, 2010, Benilova et al., 2012). The “amyloid hypothesis”

defines these events as the root cause of AD (Hardy and Selkoe, 2002, Palop and Mucke, 2010, Benilova et al., 2012) yet aberrant $A\beta_{42}$ assemblies, at LOAD levels, can also be found in cognitively normal elderly (Snowdon, 2003, Fjell et al., 2010, Morris et al., 2010). Further, reductions in cerebrospinal fluid $A\beta_{42}$, a biomarker of fibrillary $A\beta_{42}$ deposits in brain (Strozyk et al., 2003), are detected up to a decade before MCI or LOAD diagnosis (Buchhave et al., 2012). Thus, it is likely that other metabolic determinants, in addition to tau misprocessing and $A\beta_{42}$ biogenesis, are required to trigger cognitive decline in preclinical adults (Herrup, 2010, Kuller and Lopez, 2011, Nelson et al., 2011).

Age remains the primary risk factor for AD (Fjell et al., 2014); disease incidence increases dramatically after 60 years of age (Kawas et al., 2000). Herrup (2010) has proposed a model in which normal aging, vascular trauma, and chronic neuroinflammation renders the elderly brain vulnerable to the amyloid cascade. Here, any injury that triggers chronic neuroinflammatory responses in the presence of $A\beta_{42}$ can precipitate a metabolic “change in state”. The elderly brain, weakened by normal metabolic decline and comorbid cardiovascular and cerebrovascular pathologies, is considered to be more vulnerable than younger brain to these injuries (Herrup, 2010). This “change of state” is envisioned as a convergence of critical metabolic dysfunctions that impair cognitive function and underlie phenoconversion (Herrup, 2010). Alternatively, age-related risk of AD may not reflect normal age-related decline but rather the emergence of pathological metabolic changes, initiated by $A\beta_{42}$ earlier in life. Here, LOAD is considered to be a pathological form of aging (Nelson et al., 2011) whereby sustained exposure to $A\beta_{42}$ in young adults triggers a slow, catastrophic, neurodegenerative metabolic cascade propagated independently of the amyloid cascade. This model predicts that LOAD phenoconversion will not be delayed by efforts to reduce cerebral amyloid load in elderly but will require strategic targeting of

multiple A β ₄₂-initiated changes in metabolism that converge to the level of systems-wide network failure at phenoconversion (Fortea et al., 2011, Shim and Morris, 2011, Sperling et al., 2011). Clearly, identifying both the normal and pathological metabolic determinants of LOAD transition will assist in developing adjuvant disease-modifying therapeutic agents beyond A β ₄₂.

Aberrant phospholipid membrane metabolism is associated with LOAD risk. Decreases in plasma DAPCs, LPCs, and AAPCs, are detected in cognitively normal elderly 2-3 yrs prior to phenoconversion (Mapstone et al., 2014). These decreases are maintained in plasma of both MCI and LOAD patients (Oresic et al., 2011, Mapstone et al., 2014, Whiley et al., 2014). By contrast, in superior temporal and inferior parietal cortices, overall increases in choline- and ethanolamine-containing phospholipid metabolites in LOAD patients are associated with more severe cognitive impairment, a faster rate of deterioration, and an increased incidence of AD-psychosis (Klein, 2000, Sweet et al., 2002). *In vivo* and *in vitro*, accumulation of PAF, AAPC metabolites, and release of AA from their AAPC precursors have been shown to signal tau hyperphosphorylation at AD-relevant sites and enhance neurotoxicity elicited by soluble oligomeric A β ₄₂ (Sweet et al., 2002, Kriem et al., 2005, Sanchez-Mejia et al., 2008b, Ryan et al., 2009b). These toxic metabolites are produced at the expense of regenerating structural membranes due to increased activity of the group IV isoform of PLA₂, cPLA₂ (Kriem et al., 2005, Sanchez-Mejia et al., 2008b) and a A β ₄₂-dependent shift in LPCAT substrate preference from fatty acyl-CoAs to acetyl-CoA (Ryan et al., 2009b). In hAPP models, inhibition or genetic ablation of phospholipase D₂ (Oliveira et al., 2010) or cPLA₂ (Sanchez-Mejia et al., 2008b, Qu et al., 2013) confers synaptic protection and rescues memory deficits. Moreover, pharmacological inhibition of PC(O-16:0/2:0) PAF signalling reduces the rate of tau hyperphosphorylation and protects

human neurons from A β ₄₂ toxicity (Ryan et al., 2009b). Thus, while PC metabolism may be altered in LOAD brain, a link between decreases in PC plasma lipidomes and increases in regional brain metabolites has yet to be elucidated.

To address this question, we used an unbiased lipidomics approach to comparatively profile PC metabolism in plasma, hippocampus, and posterior temporal/entorhinal cortex of young and old cognitively normal adults, elderly MCI patients, and following LOAD phenoconversion. To identify neural PC metabolic pathways altered over the course of normal aging and perturbed by A β ₄₂, we compared PC compositions in the entorhinal-hippocampal circuit of hAPP TgCRND8 mice before and after behavioural phenoconversion with age-associated changes over the course of the murine lifespan. This substrate-product network analysis revealed age-, disease-, and A β -dependent changes in the Kennedy pathway of PC biosynthesis, the Land's cycle remodelling of membrane PCs, and the biosynthesis of MUFAs and PUFAs that altered specific PC fatty acyl, alkyl, and alkenyl chain compositions associated with LOAD progression.

4.5 Methods

4.5.1 Tissues

A total of 50 female NonTg and Tg mice with a N5 C57BL/6 x C3H/HE genetic background were used for the lipidomic profiling. Cohorts were lethally anesthetized with 65 mg/ml euthanyl (#1EUS001, Bimeda-MTC Animal Health Ins., ON, Canada) and decapitated at two, four, six, eight, twelve or eighteen months of age (n = 5 per group). For the study requirement, only the NonTg mice were collected at the two last time points. Prior to sacrifice, animals were assessed for reproductive status as we have described

previously (McLean et al., 2012). Parietal-temporal cortex (bregma -1.3 to -3.8 mm), including the somatosensory, auditory, perirhinal, entorhinal and piriform cortices, was quickly dissected and immediately flash-frozen in liquid nitrogen. Additional animals at 4 and 12 months of age (n = 7-8 per group) were transcardially perfused with 10 mM phosphate buffered saline (PBS; 10 mM phosphate pH 7.2, 154 mM NaCl [#SPD307, #SPM306, and #SOD001, BioShop, ON, Canada]) followed by 4 % paraformaldehyde (#F1635, Sigma-Aldrich, ON, Canada) in 10 mM PBS. Brains were removed, post-fixed for 24 h, and cryoprotected in 20 % sucrose (20 % (w/v) sucrose [#SUC507.5, BioShop], 0.001 % (w/v) sodium azide (NaN_3) [#S2002, Sigma-Aldrich], and 10 mM PBS). Serial 30 μm sagittal cryosections (Leica Microsystems, Wetzlar, Germany) were cut in a 1:10 series through one hemisphere of the brain, and either slide mounted immediately (#12-550-15, Superfrost/Plus, Fisher Scientific, ON, Canada) or stored in 0.1 M PBS with 0.1 % NaN_3 at 4 °C until processed for immunofluorescence. Life-span was further established in cohorts followed for up to 24 months or the duration of their natural life. All animal manipulations were approved by the Animal Care Committee of the University of Ottawa and performed in strict accordance with the ethical guidelines for experimentation established by the Canadian Council for Animal Care.

4.5.2 Immunofluorescence

Frozen cryosections were rehydrated in 10 mM PBS and permeabilized for one hour in 0.3 % Triton X-100 (#T8787, Sigma-Aldrich) in 10 mM PBS prior to an overnight incubation with primary antibody (Ab) (Table 4-1) at 4 °C. Following washes with 10 mM PBS sections were incubated with secondary Ab (Table 4-1) for one hour at room temperature, and finally washed with 10 mM PBS prior to being coverslipped in ProLong

Gold antifade reagent (#P36930, Invitrogen, ON, Canada). All Ab were diluted in Ab buffer (3 % bovine serum albumin [#BP1600-100, Fisher Scientific] and 0.3 % Triton X-100 in 10 mM PBS).

4.5.3 Microscopic analysis

Confocal laser scanning microscopy, Leica TCS Sp5 (Leica Microsystems), was used to identify pSer505-cPLA₂ α -positive cells, co-labelled with antigenic lineage markers within the temporal cortex. pSer505-cPLA₂ α -positive cells were identified and then assessed for the presence of the lineage marker of interest in z-stacks captured with Leica LAS AF software v2.7.3.9723 using an HCX PL APO CS 63X 1.4 oil objective with a pinhole size of one Airy unit (or equal optical slices for multiple fluorophores). We considered antigenic lineage markers colocalized with pSer505-cPLA₂ α -labelled cells only if all labels extended from top to bottom of the z-stack.

4.5.4 Immunoblotting

Dissected parietal-temporal cortex were homogenized in Urea-Tris (8 M Urea, 50 mM Tris pH 8.2, 65 mM DTT [#U5128, #T1503, and #43815, Sigma-Aldrich], EDTA-free protease inhibitor and phosphatase inhibitor cocktail tablets [#04693124001 and #04906837001, Roche, QC, Canada]) and sonicated (#Sonicator3000, QSonica, CT, USA) three times at 12 W for 20 s with 30 s intermission on ice. The samples were passed through a 26-gauge needle (#309625, BD Biosciences, ON, Canada) eight times, rested on ice for 20 min, and centrifuged at 22,000 g for 15 min. The protein concentration of the supernatant was assessed using the Bio-Rad DC protein assay kit (#500-0112, Bio-Rad, ON, Canada). 30 μ g of proteins per well were resolved on NuPAGE 4–12% SDS-PAGE

Table 4-1 Primary and Secondary Ab Used for Immunofluorescence

Ab	Company or kindly gift by	Cat. number	Primary Ab concentration	Secondary Ab concentration
Primary Ab				
α -p-cPLA2 (pSer505)	Abcam	53105	1:100	
Secondary Ab				
Alex 488 α -rabbit IgG	Invitrogen	A-11070		1:800

gels (#NP0335, Life Technologies, ON, Canada), following their denaturation using the NuPAGE lithium dodecyl sulphate sample buffer and reducing agent (#NP0007, and #NP0009, Life Technologies) in accordance to the manufacturer's guidelines. Gels were transferred onto nitrocellulose membranes (#66485, Pall Life Sciences, ON, Canada), and blocked for more than 30 min with PBS-T (10 mM phosphate pH 7.2, 154 mM NaCl [#SPD307, #SPM306, and #SOD001, BioShop, ON, Canada], 0.1 % (v/v) Tween 20 [#P1379, Sigma-Aldrich]) containing 5 % (w/v) non-fat milk (Carnation, ON, Canada). Membranes were rinsed twice in PBS-T prior to overnight incubation at 4 °C with the primary Ab (Table 4-2) in PBS-T with 3 % (w/v) non-fat milk. Membranes were rinsed twice 10 min in PBS-T. Horseradish peroxidase-conjugated α -mouse or α -rabbit secondary Ab in 3 % (w/v) non-fat milk PBS-T were incubated with the membranes for one hour at room temperature. Membranes were washed four times 10 min in PBS-T prior to a 5 min incubation with Immobilon Western Chemiluminescent HRP Substrate (#WBKLS0500, Millipore), and chemiluminescence was detected on film (#1788207, Carestream Health, NY, USA or #E3218, Danville Scientific, NJ, USA). Membranes were stripped using 1 X ReBlot plus solution (#2504, Millipore) according to the manufacturer's guidelines, and were blocked and reprobed to detect another protein of a different size. All membranes were finally probed with α -actin as the loading control. Relative protein expression was quantified by densitometry using the image processing program ImageJ v1.48 National Institutes of Health. Protein quantifications are presented relative to their actin loading control abundance.

Table 4-2 Primary and Secondary Ab Used for Western blots

Ab	Company or kindly gift by	Cat. number	Primary Ab concentration	Secondary Ab concentration
Primary Ab				
α -cPLA2	Millipore	05-1406	1:1,000	1:5,000
α -iPLA2	Upstate	07-169	1:100	1:2,000
α -LPCAT1	Sigma-Aldrich	HPA012501	1:300	1:10,000
α -LPCAT2	Sigma-Aldrich	HPA007891	1:500	1:10,000
α -PAFAH1B1	Chemicon	AB5413	1:1,000	1:10,000
α -PAFAH1B2	Dr. Arai		1:700	1:10,000
α -PAFAH1B3	Dr. Arai		1:700	1:10,000
α -PAFAH II	Dr. Arai		1:500	1:10,000
α -sPAFAH	Cayman Chemical	160603	1:500	1:10,000
α -MBP	Sigma-Aldrich	M3821	1:4,000	1:10,000
α -GFAP	Sigma-Aldrich	G9269	1:20,000	1:10,000
α - β III-tubulin	Sigma-Aldrich	T8660	1:5,000	1:10,000
α -actin	Cedarlane	CLT9001	1:30,000	1:10,000
Secondary Ab				
HRP α -mouse	Rockland	610-1319-0500		1:5,000 - 1:10,000
HRP α -rabbit	GE Healthcare	NA934V		1:2,000- 1:10,000

4.5.5 Lipid extraction

Lipid extractions were performed using a modified Bligh and Dyer method as we have described previously (Xu et al., 2013). Briefly, dissected tissue was homogenized in methanol (#A412P-4, Fisher Scientific) acidified with 2 % acid acetic (#351271-212, Fisher Scientific) using a Tissue-Tearor (#985370-395, Biospec Products, OK, USA) for 30 seconds. PC(13:0/0:0) (187.5 ng; #LM-1600, Avanti Polar Lipid, AL, USA) was added as an internal standard at the time of extraction. Chloroform (#C298-500, Fisher Scientific) and 0.1 M sodium acetate (#SAA304, BioShop, ON, Canada) were added at a final ratio of 0.8/1/0.95;v/v/v of acidified methanol/chloroform/0.1 M sodium acetate. The solution was mixed and centrifuged at 800 x g for two minutes at 4 °C. The organic phase was collected. Residual aqueous phases were back-extracted three times with 0.8/1/0.5;v/v/v acidified methanol/chloroform/0.1 M sodium acetate. The organic phases from all four extractions were combined and evaporated at room temperature under a constant stream of nitrogen gas. Lipid extracts were suspended in 300 µl of anhydrous ethanol (#P016EAAN, GreenField Ethanol, ON, Canada) and stored as 50 µl aliquots in amber vials, filled with nitrogen gas at -80 °C. Total PC content was assessed biochemically using the PC assay kit (#10009926, Cayman Chemical, MI, USA) as described by the manufacturer.

4.5.6 HPLC-ESI-MS/MS

Eight µl of a 23.25 µl mixture consisting of 15.75 µl of 0.1 % (v/v) FA (#TSBAK983403, JT Baker, NJ, USA), 5 µl lipid extract, 2.5 µl deuterated standards [1.25 ng of D₄-PC(O-16:0/2:0) #360900, 1.25 ng of D₄-PC(O-18:0/2:0) #10010229, 2.5 ng of D₄-PC(O-16:0/0:0) #360906 and 2.5 ng of D₄-PC(O-18:0/0:0) #1010228)] were analysed by HPLC-ESI-MS/MS. All internal standards were from Cayman Chemical. For the mice PC

metabolite profiling, chromatography was performed using an Agilent 1100 Series HPLC System (Agilent Technologies, CA, USA) with a 10 cm $\times$ 200 μ m ID trap column packed with Magic C18 AQ 100A 5U 5- μ m resin (#PM5/61100/00, Michrom BioResources, CA, USA). Following a five minute wash with 95 % (v/v) 0.1% FA and 5 % (v/v) ACN + 0.1 % (v/v) FA (#TSBAK983403, JT Baker), lipids were separated with a gradient increase of ACN + 0.1 % FA from 5 to 30 % in two minutes, 30 to 60 % in seven minutes, 60 to 80 % in 33 minutes, and 80 to 95 % in four minutes. The eluent was nanoelectrosprayed from a packed analytical emitter column (#PF360-50-10-N-5, PicoFrit 5 cm $\times$ 75 μ m ID $\times$ 10 μ m tip; New Objective, MA, USA) into a QTRAP 2000 (Applied Biosystem, CA, USA) by applying 3500 volts. To target PC metabolites, PIS for the 184 m/z fragment ion diagnostic of the PC headgroup was performed for species with m/z between 450 to 600 m/z. CE was set to 40 eV. Data acquisition and analysis were performed using AB SCIEX Analyst v1.4.2. For the human structural PC and PC metabolite profiling, three μ l of the lipid mixture was loaded by an Agilent 1100 HPLC System (Agilent Technologies, CA, USA) on a 10 cm $\times$ 75 μ m (ID) analytical column packed with ReproSil-Pur 120 C4, 5 μ m, 120 Å beads (#r15.4e, Dr. Maisch GmbH, Germany). The chromatography was performed using solvent A (H₂O) and B (5:2, ACN:isopropanol, v/v) (#A416-4, Fisher Scientific, ON, Canada, #9829-03C, JT Baker, respectively) both containing 0.1 % (v/v) FA and 10 mM ammonium acetate (#2145, OmniPur, ID, USA). Following the loading of the sample for 15 min in 5 % solvent B, the gradient chromatographic separation was performed with a linear increase of solvent B from 5 to 35 % (1 min) and from 35 to 100 % (14 min). Then, solvent B was maintained at 100 % (14 min) and the column was equilibrated with 5 % B (15 min). The eluent was nanoelectrosprayed through an emitter column (#FS360-50-15-N-20, PicoFrit, New Objective, MA, USA). The lipid mixture was analyzed in MRM mode

where Q3 mass analyzer was set to 184 m/z to identify the PC headgroup product ion. The MS is a QTRAP 5500 (AB SCIEX, MA, USA) using high purity nitrogen (#UN1977, Boc Gases, ON, Canada) and set at: IS, 3500 V; GS1, 10 psi; GS2, 0 psi; CUR 20 psi; CAD, 10 psi; DP, 100 V; EP, 10 V; CXP, 9 V; CE, 47 eV; and scan time, 1.98 sec/cycle. Data acquisition and analysis were performed using AB SCIEX Analyst v1.5.1 and MultiQuant v2.0.2, respectively. For each species, the peak intensity (PA_S) in counts per second, representing the area under the curve were normalized (PA_N) to the PA_S of the synthetic internal standard ($PA_{PC(13:0/0:0)}$) and the tissue wet weight (TWW) in milligrams and multiplied by 10^6 . Chromatographic alignment was achieved using RTStaR (Blanchard et al., 2015). One specific eight months NonTgPAFR^{+/+} mice was excluded since it failed to be aligned. Species structural identities were predicted using the online bioinformatic tool VaLID v3.2.0 (<http://www.neurolipidomics.ca/valid.html>) (Blanchard et al., 2013a, McDowell et al., 2014a, McDowell et al., 2014b) and, where indicated, subsequently validated by addition of known standards at time or by tandem MS as we have described previously (Whitehead et al., 2007, Smith et al., 2008).

4.5.7 Statistical analyses

For clustering analysis, the PA_N of each species were $\log_2$ transformed, median centered, and hierarchically clustered using Spearman rank correlation and average linkage analysis in Cluster 3.0. Clusters were visualized in TreeView v1.1.6.r4. For analysis of lipid abundance, data were expressed as $\log_2(PA_N)$ and analyzed by Student's t-test with two-tailed distribution and two-sample unequal variance, one-way ANOVA followed by Dunnett's or Tukey post hoc tests, or two-way ANOVA followed by Holm-Sidak post hoc test. Analyses were corrected for multiple comparisons using the two-stage sharpened FDR

method (Benjamini et al., 2006, Pike, 2011). The FDR cut-off was set to have less than 0.5 false positive. Only changes that met $p < 0.05$, pass the FDR cut-off and equal or greater than 1.3-fold change were considered biologically relevant and, thus significant. Data were graphed using relative fold change $\pm$ standard error of the mean (SEM). For regression analyses, linear regression was performed followed by Pearson correlation analysis. Statistics were performed using Excel 2010, and GraphPad Prism v6.0.

4.6 Results

4.6.1 Age- and disease-associated changes in the plasma PC lipidome

A total of 71 subjects were included from the Saguenay Youth Study (SYS), a cross-sectional of study of cardiometabolic and brain health (n=20 young adults) (Paus et al., 2015), the Coronary Artery Disease Intervention Study, an ongoing assessment study of cognitive deficits and depression over the course of cardiac rehabilitation (n=17 cognitively normal elderly) (Mazereeuw et al., 2014, Mazereeuw et al., 2015), and the Sunnybrook Dementia Study, an ongoing longitudinal study of dementia risk and intervention (n=17 MCI and n=17 LOAD patients) (Ramirez et al., 2014, Intzandt et al., 2015, Ramirez et al., 2015). Inclusion criteria for young adults, MCI, and LOAD subjects have been described in detail in (Mazereeuw et al., 2014, Mazereeuw et al., 2015, Paus et al., 2015). It must be emphasized that our control group differs from the majority of human lipidomic studies exploring circulating lipid LOAD biomarkers (Mapstone et al., 2014, Whiley et al., 2014). Because cohort studies are moving towards examining cardiovascular health, cerebrovascular disease and LOAD as comorbid pathologies (Black and Iadecola, 2009, Knopman, 2012, Portet et al., 2012, de la Torre, 2014), we choose cognitively normal elderly with cardiovascular impairments as our age- and sex-matched controls. This cohort

considered the potential impact of vascular insufficiency on circulating and brain PC metabolism not associated with MCI or LOAD. Recruitment and patient characteristics are as we have previously published (Mazereeuw et al., 2014, Mazereeuw et al., 2015). Plasma collection and neuropsychological testing occurred at least 10 weeks post-intervention to ensure cardiovascular stability. Patients profiled in this study were followed for a period of three years to ensure none of the subject analyzed phenoconverted to either MCI or LOAD.

Plasma phospholipids were extracted using a modified Bligh and Dyer method adapted to obtain not only DAPCs and their LPC metabolites but also oxidized PCs, AAPCs, PAPCs, and their downstream metabolites (Whitehead et al., 2007, Ryan et al., 2009b, Xu et al., 2013). All detectable PC compositions were profiled using HPLC-ESI-MS in PIS mode (Whitehead et al., 2007, Ryan et al., 2009b). We identified 175 PC species in human plasma (Bennett et al., 2013); 58 % were structural membrane lipids; 42 % were PC metabolites (Figure 4-1A). Metabolites were made up of four subgroups (1) DAPC, (2) AAPC, (3) PAPC, and (4) non-enzymatically oxidatively modified metabolites. The DAPC metabolites, LPC and APAFs, represented the majority of species detected followed by the AAPC metabolites, LPAFs and PAFs. Fewer LPPAFs, PAPC metabolites, and formylglycerophosphocholine (FPCs), non-enzymatically oxidative products of DAPCs were detected (Figure 4-1A).

PC abundance was quantified by HPLC-ESI-MS in MRM mode. A significant increase in overall PC levels, both structural PCs and PC metabolites, was evident in LOAD plasma compared to elderly controls (Figure 4-1B). These elevations in circulating structural PCs ($r^2 = 0.17$, $p = 0.004$) and their metabolites ($r^2 = 0.20$, $p = 0.002$) in elderly control, MCI, and LOAD patients linearly correlated with severity of diagnosis (Figure 4-1B). PC metabolomes were also affected by age/vascular impairment. Metabolite levels

were significantly higher in elderly controls with vascular impairment compared to young adults (Figure 4-1B). Age-dependent increases were the result of elevations in circulating APAF, LPPAF, and LPC levels (Figure 4-1C). Disease-dependent changes were restricted to circulating levels of LPCs, PAFs, and LPAFs. Plasma abundance of disease-associated PC metabolites linearly increased in MCI and LOAD patients (Figure 4-1C, LPC: $r^2 = 0.16$, $p = 0.005$; PAF: $r^2 = 0.12$, $p = 0.012$; LPAF $r^2 = 0.12$, $p = 0.019$). Non-enzymatic oxidation of DAPCs, assessed by changes in FPC concentrations, was not altered by age or by disease (Figure 4-1C).

Metabolite identities were validated by addition of known standards; PC structural identities were assigned using ValID v3.2.0 (Blanchard et al., 2013a, McDowell et al., 2014a, McDowell et al., 2014b) and the LIPID MAPS Structural Database (Sud et al., 2007). Critical compositions in plasma were defined as species that exhibited a significant change of at least two-fold in either MCI or LOAD relative to elderly controls with vascular impairment (Figure 4-1D). Twenty species met these criteria. Ten metabolites strongly associated with cognitive impairment as assessed on the mini mental state exam (MMSE) (Figure 4-1D). Higher plasma abundance in MCI and/or LOAD negatively correlated with poorer cognitive performance.

4.6.2 Land's cycle remodelling of PAFs and LPAFs is regionally disrupted in the LOAD brain

PC levels are primarily regulated by *de novo* PC synthesis through the Kennedy pathway and remodelling through the Land's cycle. Plasma compositional changes suggested that both Kennedy and Land's pathways were altered by age and AD. To

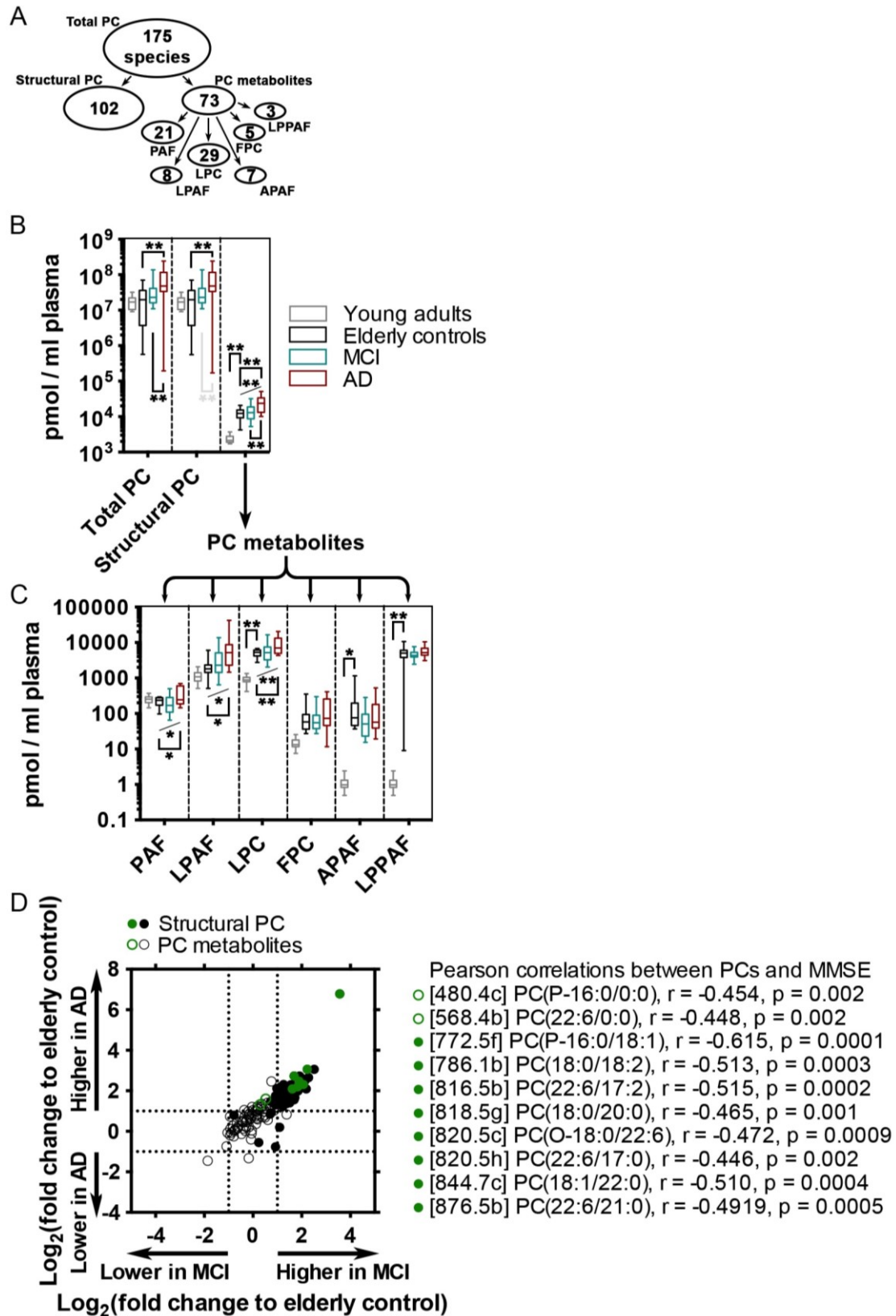


Figure 4-1

Figure 4-1 The Plasma PC Lipidome as Altered by Age and Disease

(A) Number of PC species profiled in the circulation divided into their PC family and subfamily. (B) Overall concentration in circulation of total PC, structural PC, and PC metabolites divided by young adult, elderly controls, MCI and AD. These three groups are modified in AD, but only PC metabolites concentration was raised during aging. (C) The overall concentration of the PC metabolites as divided by their subfamily. The observed alterations are different between aging and AD. (D) Log₂ relative abundance of the individual species compared to the elderly control. Species altered significantly by at least two-fold in MCI and/or AD were correlated to the MMSE score. Eight structural PC and two PC metabolites were significantly correlated. Statistics were one-way ANOVA with FDR correction followed by a Tukey's (B,C) or a Post hoc test for linear trend (B,C). Pearson correlation analysis (D). * $p < 0.05$ and ** $p < 0.01$.

determine whether these circulating changes reflected comparable synthetic or remodelling impairments in brain, we quantified total PC content and profiled the PC metabolome in the entorhinal-hippocampal circuit of five cognitively normal elderly and five LOAD patients post-mortem. Our control group had a history of cerebrovascular impairment but normal age-standardized cognition and no neurological disease at time of death. We focused on the posterior/entorhinal cortex and hippocampus as encoding (and retrieval) of episodic learning and memory requires a functional entorhinal-hippocampal circuit (Levy et al., 2004, Eichenbaum and Lipton, 2008). Input to the hippocampus is, in part, defined by the excitatory afferents of stellate neurons in layer II of the entorhinal cortex synapsing on granule cells in the dentate gyrus (DGy) of the hippocampal formation and by entorhinal layer III afferents synapsing primarily on CA1 and CA3 hippocampal pyramidal cells (van Groen et al., 2003, van Strien et al., 2009).

Lipids were extracted from layers II and III of the entorhinal cortex and from the DGy and CA3 of the hippocampus (Figure 4-2A). At time of autopsy, two tissue blocks comprising the entorhinal cortex and hippocampus were collected from the same patient. Tissue was flash-frozen (anterior block) for subsequent microdissection of target brain regions or formalin-fixed (posterior block) allowing for both lipidomic profiling and blinded clinical analysis of A β and tau pathologies. One control subject was excluded following assessment of clinical pathology due to A β plaque load, distribution, and characteristics comparable to LOAD patients despite diagnostic report of normal age-standardized cognition at time of death.

Regional- and disease-dependent differences in tissue PC content were quantified biochemically. Total PC abundance was significantly higher in the entorhinal cortex compared to hippocampus of elderly controls (Figure 4-2B). Overall PC levels were

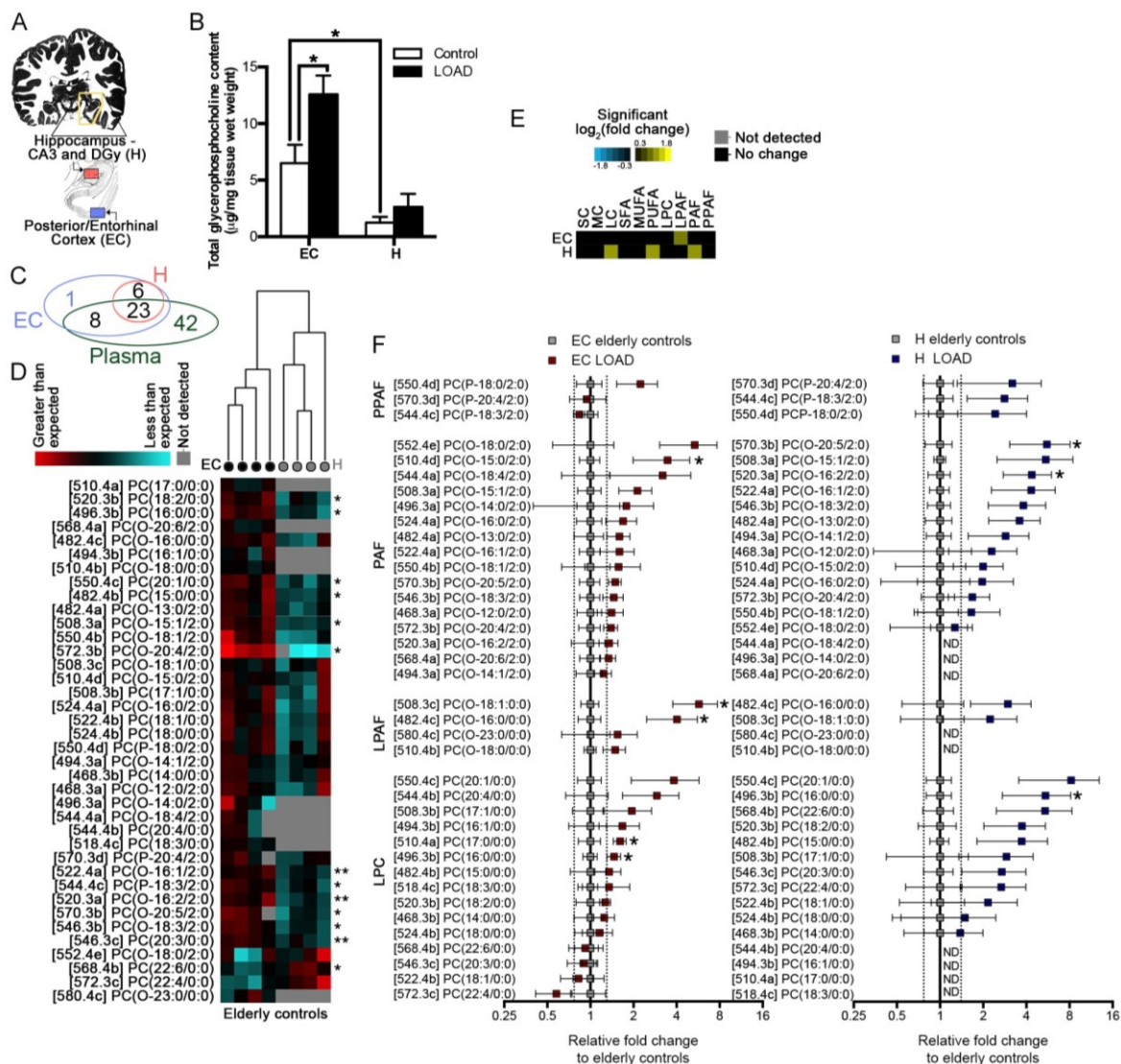


Figure 4-2

Figure 4-2 PC Land's cycle remodelling is altered in LOAD

(A) Schematic of the human brain regions dissected. (B) Total PC level content was measured in the posterior/entorhinal cortex (EC) and in the hippocampus (H) in LOAD. (C) The PC metabolites were profiled in the plasma, the hippocampus, and the posterior/entorhinal cortex. Many PC metabolites were found in two or three of the tissues/fluids, but some were specific. (D) Individual PC metabolites were profiled in human posterior/entorhinal cortex and hippocampus in order to further characterise the enrichment of the cortex. The relative quantification of all metabolites clustered the lipids by brain region. (E) A statistical heat map showing the significant changes between LOAD and elderly control expressed as a ratio that occurred in defined groups of PC metabolites in specific brain regions. Data is expressed as ratio of LOAD over elderly control. SC: short fatty chain moieties (12-15 carbons), MC: medium fatty chain moieties (16-19 carbons), LC: long fatty chain moieties (20 and more carbons), SFA: saturated fatty acid chain, MUFA: monounsaturated fatty acid chain, PUFA: polyunsaturated fatty acid chain (F) Relative fold change of individual PC metabolites as divided by brain region and PC metabolites subfamily. Regional specificity of alteration of the Land's cycle disruption in LOAD is shown by the different species altered. Statistics were Student's t-tests with FDR correction. * $p < 0.05$ and ** $p < 0.01$.

elevated LOAD entorhinal cortex but not hippocampus, consistent with an increase in *de novo* synthesis via the Kennedy pathway (Figure 4-2B). Taken together, these data suggested that alterations in Kennedy synthesis of PCs were regionally specific at end-stage of LOAD.

Land's cycle PC metabolism was assessed by HPLC-ESI-MS in PIS mode. The PC metabolite lipidome in the entorhinal-hippocampal circuit was regionally specific yet shared features with the circulating lipidome. Thirty-eight PC metabolites were detected in the entorhinal-hippocampal circuit. Twenty-nine were present in plasma. Seven PC metabolites were only detected in brain (Figure 4-2C). Three PPAF species, PC(P-18:0/2:0), PC(P-20:4/2:0), and PC(P-18:3/2:0), were found in the entorhinal-hippocampal circuit, although multiple LPPAFs, the immediate precursors and metabolites of PPAFs in the Land's cycle, were present in plasma (Figure 4-1C). Two PAFs, PC(O-15:0/2:0) and PC(O-18:1/2:0), and two LPAFs, PC(O-18:1/0:0) and PC(O-23:0/0:0), were detected in brain not plasma. PC composition also distinguished entorhinal cortex from hippocampus. Nine LPC, LPAF, and PAF species in the layer II and III of the entorhinal cortex were absent or below our limits of detection in the DGy and CA3 of the hippocampus (Figure 4-2C,F). Thirteen of the twenty-nine species, common to both regions, were significantly more abundant in the entorhinal cortex than hippocampus (Figure 4-2D). One LPC, PC(22:6/0:0), defined by docosahexaenoate (C22:6) at the *sn*-1 position, was enriched in the hippocampus compared to entorhinal cortex (Figure 4-2D).

LOAD-specific disruptions in AAPC metabolism and *sn*-1 fatty acyl, alkyl, and alkenyl compositions were evident in both entorhinal cortex and hippocampus. LPAF levels were statistically elevated in the entorhinal cortex. In hippocampus, PAF levels increased, specifically PAFs with long polyunsaturated *sn*-1 alkyl chains (Figure 4-2E,F).

Interestingly, none of the metabolites found to associate with cognitive impairment in plasma (Figure 4-1D) were significantly elevated in the LOAD entorhinal-cortex at time of death (Figure 4-1D, 2F) suggesting that plasma changes reflected differences in the metabolism of PC families but did not mirror compositional changes at the molecular level.

4.6.3 Chronic A β exposure is associated with compositional changes in LPC, PAF, and PPAF metabolites in young mice consistent with alterations in MUFA and PUFA biosynthesis

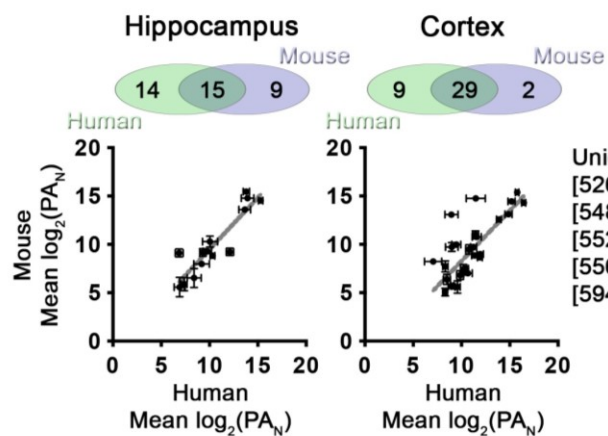
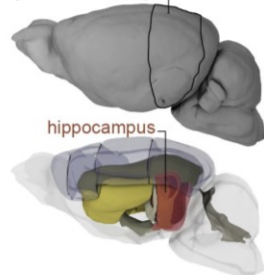
To identify critical PC metabolic disruptions initiated by the amyloid cascade independently of normal aging, we used the early-onset TgCRND8 mouse model (Chishti et al., 2001). TgCRND8 mice express hAPP with double KM670/671NL+V717F Swedish and Indiana familial AD mutations under control of the Syrian Hamster prion promoter (Chishti et al., 2001). This line models aggressive early-onset A β biogenesis. When backcrossed for five generations to a C57BL/6 background, A β plaques are first detected in Tg posterior-temporal cortex and hippocampus at two months of age (Wang et al., 2013, Granger et al., 2015). The A β_{42} /A β_{40} ratio exceeds unity by four months of age (Chishti et al., 2001, Wang et al., 2013, Granger et al., 2015). Plaque burden and A β_{42} levels reach LOAD levels by six months of age (Wang et al., 2013, Granger et al., 2015). Tg females manifest learning and memory deficits between 5.5 and six months of age (Wang et al., 2013, Granger et al., 2015) (see also Chapter 5).

To identify differences in human and murine brain lipidomes, we compared the entorhinal-hippocampal PC metabolite composition in elderly human and NonTg mouse brain (Figure 4-3). Abundances in human and mice were strongly correlated in both hippocampus ($r = 0.903$, $p < 0.0001$) and cortex ($r=0.754$, $p < 0.0001$). Composition varied

with fewer PC metabolites detected in mice than humans. Thirty-one PC metabolites were quantified in murine parietal-temporal cortex, comprising the human equivalent of the posterior/entorhinal cortex. Thirty-eight PC metabolites were detected in human posterior/entorhinal cortex. Twenty-four species were detected in murine hippocampus compared to 29 species in human hippocampus (Figure 4-3, Figure 4-4A). Eight species were unique to humans; five species were unique to mice (Figure 4-3).

Next, we compared PC metabolism in the entorhinal-hippocampal circuit of Tg and NonTg female littermates. Lipidomes were compared when Tg mice were presymptomatic (two months of age), following sustained A β biogenesis (four months of age), and after behavioural phenoconversion (six months of age). No differences were detected between NonTg and Tg mice at two months of age (Figure 4-4A). Progressive A β accumulation did not impair DAPC, AAPC, or PAPC metabolism at the systems level; overall abundances of LPCs, PAFs or LPAFs in parietal-temporal cortex and hippocampus were not altered in Tg mice at any time point (Figure 4-4A,B). Specific compositions of eight PC metabolites were affected (Figure 4-4B). Elevated levels of LPCs and PAFs defined by *sn*-1 chains containing polyunsaturated hexadecadienoate (C16:2), lineolate (C18:2), eicosadienoate (C20:2) docosaheptaenoate (DHA, C22:6) or monounsaturated palmitoleate (C16:1) were present in the Tg entorhinal-hippocampal circuit at four and six months of age (Figure 4-4C). In Tg mice, levels of these metabolites remained constant over time whereas, in NonTg mice, abundance decreased (Figure 4-4C). These specific patterns were not consistent with an impairment of Land's cycle remodelling but rather suggestive of a change in MUFA and PUFA biosynthesis in response to accumulating A β , specifically an increase in stearoyl-CoA desaturase and Δ 6 desaturase (fatty acid desaturase 2) activities as

Parietal-temporal cortex
(including motor, somatosensory,
auditory, perirhinal, entorhinal,
and piriform cortices)



Unique to mouse:
[526.8a] PC(O-20:6/0:0)
[548.4c] PC(20:2/0:0)
[552.4f] PC(20:0/0:0)
[556.4a] PC(O-22:5/0:0)
[594.3a] PC(O-24:0/0:0)

Unique to human:
[482.4b] PC(15:0/0:0)
[494.3a] PC(O-14:1/2:0)
[510.4d] PC(O-15:0/2:0)
[518.4c] PC(18:3/0:0)
[544.4c] PC(P-18:3/2:0)
[550.4d] PC(P-18:0/2:0)
[572.3c] PC(22:4/0:0)
[580.4c] PC(O-23:0/0:0)

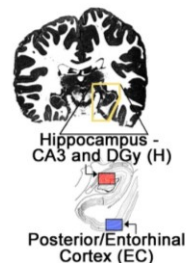


Figure 4-3

Figure 4-3 The Mouse Brain PC Metabolome Mimics The Human Brain

Mouse and human PC metabolites were profiled in the hippocampus and cortex. The schematic human and mouse brains show the respective dissection areas. Many species were shared between these two mammalian species both in the hippocampus and the cortex as shown by the Venn diagram. The correlation between the relative quantification of these shared PC metabolites in both brain regions can be clearly seen. Statistics were Pearson correlation.

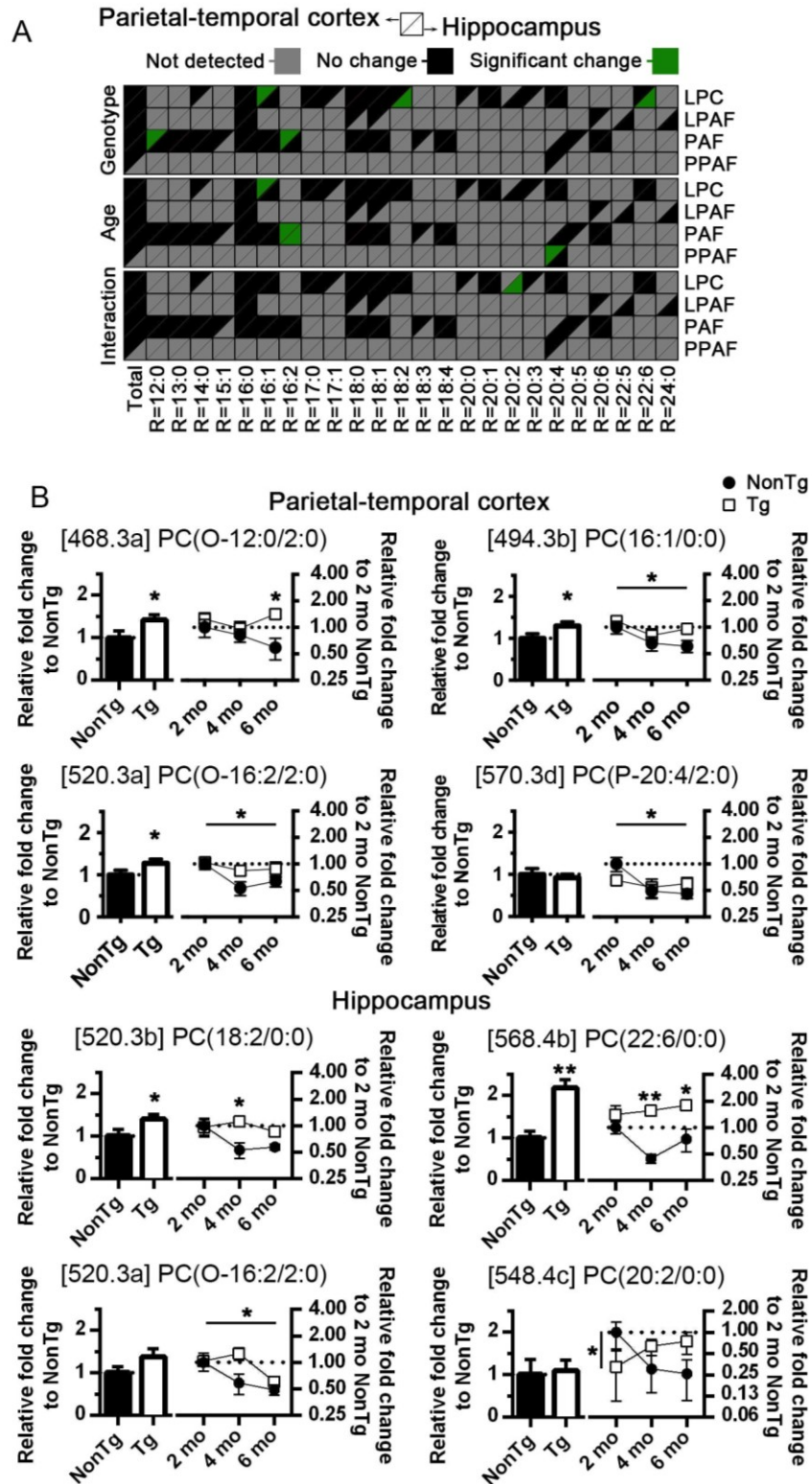


Figure 4-4

Figure 4-4 Chronic A β Exposure Modulates the Alteration of the PC Land's Cycle

Remodelling

(A) The PC metabolite alterations in the parietal-temporal cortex and hippocampus of young (two, four, and six month old) TgCRND8 AD mice are shown by statistical heat maps. Significant changes are clearly region specific. (B) The significant individual alterations caused by chronic accumulation of A β are shown collapsed by genotype or individually grouped by age. Statistics were one-way ANOVA with FDR correction. * $p < 0.05$ and ** $p < 0.01$.

have been previously reported in LOAD temporal cortex and hippocampus (Nakada et al., 1990, Astarita et al., 2011). Compositional changes also implicated increases in elongase activity, required for conversion of dietary 9,12-hexadecadienoic acid (16:2n-4) present in mouse chow to 11,14-octadecadienoic acid (18:2n-4) (Nakano et al., 1999) (Figure 4-2C). These A β -dependent metabolic disruptions in Tg females were further supported by the interaction between age and genotype apparent in PC(20:2/0:0) levels and consistent with an increase in elongase of very-long-chain fatty acids 5 activity in Tg hippocampus. Finally, behavioural phenoconversion at six month old Tg females was associated with an increase in the abundance of the PAF, PC(O-12:0/2:0) in the parietal-temporal cortex (Figure 4-4C).

4.6.4 Metabolic changes in the Land's cycle over the course of normal aging

To assess age-dependent changes in neural membrane metabolism associated with risk of LOAD phenoconversion, we comparatively examined the PC metabolite lipidome in the parietal-temporal cortex of NonTg female mice at mature, middle-aged, and elderly human age equivalents (Figure 4-5A). Age equivalents were defined by calculating 10% estrous acyclicity, 50% estrous acyclicity, and 50% survivorship ratios as previously described (Flurkey et al., 2007). A marked compositional change in the PC metabolome was observed when animals transitioned from human middle-aged to elderly age equivalents. A clear separation of the majority of 12-18 month old mice from all other age groups was evident following cluster analysis (Figure 4-5B). Forty-four percent of the PC metabolite landscape was significantly altered. This change primarily increased the abundance of the majority of PAF lipids (Figure 4-5C,D). Overall PAF levels rose by 1.5-

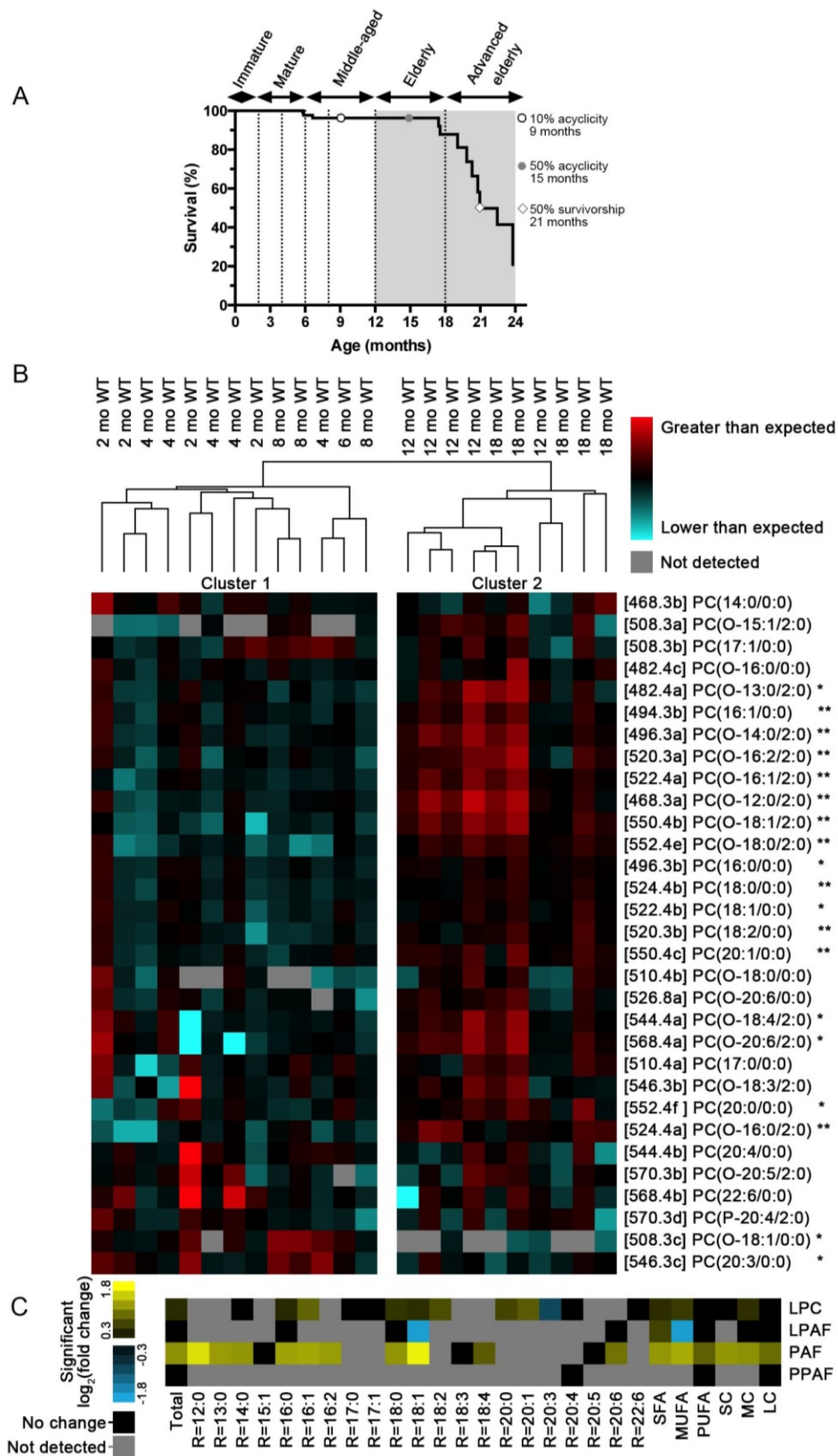


Figure 4-5

Figure 4-5 PC Remodelling is Enhanced with Advancing Age in the Mouse Brain

(A) Survival curve of our NonTg mice (C57BL/6 X C3H background) depicted in correlation to human age equivalencies (Flurkey et al., 2007). (B) The relative quantification of all species profiled in the parietal-temporal cortex of various mice clustered by age. A clear division is observed into two groups: young (two, four, six, and eight month old) and elderly mice (twelve and eighteen month old) suggesting the importance of aging on the PC remodelling. (C) A statistical heat map summarizing the alteration of the individual PC metabolites, total PC metabolites based on subfamily, and based on chain length or unsaturation within specific subfamilies. PAF, and to a lesser extent LPC, were the most affected subfamilies in aging. R: fatty chain moieties. Statistics were Student's t-tests with FDR correction. * $p < 0.05$ and ** $p < 0.01$.

fold in elderly compared to mature and middle-aged females (Figure 4-5D). PAFs enriched in elderly females were defined by short (12-15 carbons) or medium (16-19 carbons) *sn*-1 fatty alkyl chains, composed primarily of SFA or MUFA (Figure 4-5D). Accumulation was accompanied by marked depletions in the levels of the LPAF PC(O-18:1/0:0), the immediate precursor of PAF PC(O-18:1/2:0), consistent with an increase in Land's cycle remodelling of O-18:1 LPAF to O-18:1 PAF in elderly mice. By contrast, PPAF levels were not altered (Figure 4-5C,D). Thirty-seven percent of all LPC species significantly changed; overall LPC abundance was significantly increased (Figure 4-5C,D).

4.6.5 Accumulation of PAF metabolites in elderly murine parietal-temporal cortex is not the result of reproductive senescence

In establishing human lifespan equivalents (Figure 4-4A), we found that 50 % of NonTg females were acyclic by 15 months of age. Acyclicity indicates the onset of reproductive senescence, the human equivalent of menopause. In the cohort used for lipidomic assessment, four of the ten elderly females were reproductively senescent at time of analysis (Figure 4-6A). To establish whether PC metabolite composition in elderly females was dependent hormonal status, we compared the PC metabolome in elderly reproductive competent females with that of elderly reproductive senescent females (Figure 4-6B). The human equivalent of menopause was not responsible for the dramatic accumulation of PC metabolites in parietal-temporal cortex of elderly mice. Conversely, acyclicity was associated with a significant decrease in levels of PAF PC(O-16:0/2:0) and, although not significant, its immediate LPAF metabolite and precursor, PC(O-16:0/0:0) (Figure 4-6B).

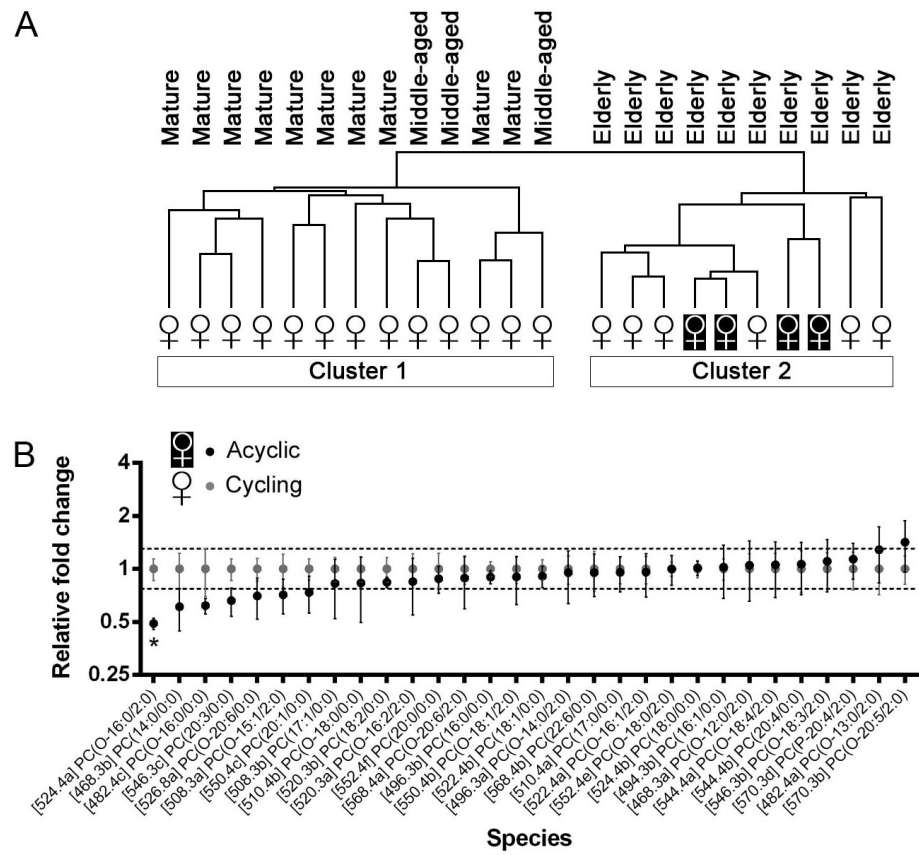


Figure 4-6

Figure 4-6 Reproductive Senescence is not Responsible for the Major Shift in PC

Remodelling

(A) Cyclic and acyclic mice are depicted based on the aging cluster tree produced in Figure 4-5B to verify that the enhancement of PC metabolism seen between 8 and 12 months of age was not due to reproductive senescence. (B) Comparison of the level of PC metabolites between cyclic and acyclic elderly mice. Only one PC(O-16:0/2:0) was altered by the reproductive senescence. Statistics were Student's t-tests with FDR correction. * $p < 0.05$ and ** $p < 0.01$.

4.6.6 Age-dependent changes in Land's cycle enzymes

To identify age-dependent enzymatic changes in Land's cycle metabolism, we identified the enzymes responsible for PC remodelling in parietal-temporal cortex and assessed age-dependent changes in levels and activity (Figure 4-7A). Hydrolysis of the *sn*-2 ester bonds of DAPCs, AAPCs, and PAPCs by PLA₂ enzymes generates LPCs, LPAFs, and LPPAFs respectively (Figure 4-7A). We detected both the short (iPLA₂-S) and long (iPLA₂-L) isoforms of the calcium-independent Group VIA PLA₂ (iPLA₂) β in mature and elderly parietal-temporal cortex (Figure 4-7B). In addition, cPLA₂ α (Group IVA) in both its inactive (unphosphorylated) and active (phosphorylated) forms but not cPLA₂ β (Group IVB) were seen in mature and elderly mice (Figure 4-7B). We did not assess Group IIA sPLA₂ activity due to an inbred frame-shift mutation in C57BL/6 mice that results in loss of functional activity (Kennedy et al., 1995). Levels of active phosphorylated cPLA₂ α were elevated in elderly mice assessed by Western analysis (Figure 4-7B). Although densitometric changes did not reach statistical significance (Figure 4-7B), activated pSer505-cPLA₂ α was clearly absent from the medial parietal association cortex of mature yet present in elderly (Figure 4-7B).

LPCs, LPAFs, and LPPAFs can be reacylated in brain by two by acyl coA-dependent LPCAT1 and 2 regenerating structural membrane PCs using acyl-CoA as a substrate, or bioactive APAFs, PAFs, PPAFs using acetyl-CoA as substrate (Figure 4-7A). Both LPCAT1 and 2 were detected in parietal-temporal cortex of mature and elderly mice (Figure 4-7C). PAF lipids are themselves remodelled, and thereby inactivated, by three different PAFAH. Cytosolic PAFAH1b is a G-protein-like trimer composed of two 29 kDa α_1 and 30 kDa α_2 catalytic subunits, either as homodimers or heterodimers, that complex with a non-catalytic regulatory β subunit, lissencephaly-1 homolog (LIS1) (Bonin et al.,

2004). Expression is specific to neurons and neuronal precursor cells in brain (Bonin et al., 2004). All three subunits were detected in parietal-temporal cortex with increased protein expression, although not significant, of the α_1 catalytic subunit evident in elderly animals (Figure 4-7D). PAFAHII is a cytosolic 40 kDa monomer expressed by neurons (Umemura et al., 2007). No age-dependent changes were detected in protein levels (Figure 4-7D). Secreted PAFAH (sPAFAH, Group VII PLA₂) is a 45 kDa monomer secreted by astrocytes in mouse brain (Bachoo et al., 2004). A marked age-dependent decrease in protein levels was detected (Figure 4-7D). Taken together, these data implicate an age-associated activation of cPLA₂ α and a decrease in the levels of sPAFAH in elderly mice in the accumulation of PAFs and LPC, notably species upregulated in response to A β *in vivo* in younger Tg littermates [PC(O-12:0/2:0), PC(O-16:2/2:0), PC(16:1/0:0), PC(18:2/0:0)].

4.7 Discussion

Here, we show that changes in plasma PC metabolism associated with cognitive decline in MCI and LOAD patients correspond with the age-, disease- and A β -dependent alterations in PC metabolism in the entorhinal-hippocampal circuit of human LOAD and murine brain. Our unbiased lipidomic approach identified ten significant elevations in plasma PC abundance that associated with severity of cognitive impairment assessed using the MMSE. Increases in PC compositions in LOAD plasma, indicative of changes in the Kennedy pathway of PC biosynthesis, were reiterated in posterior/entorhinal cortex, but not hippocampus of LOAD patients analysed post-mortem.

Disease-associated network-level changes in Kennedy PC biosynthesis were identified based on the composition of significantly elevated PC species in plasma, notably the incorporation of DHA (C22:6) into the *sn*-2 position. It has been repeatedly

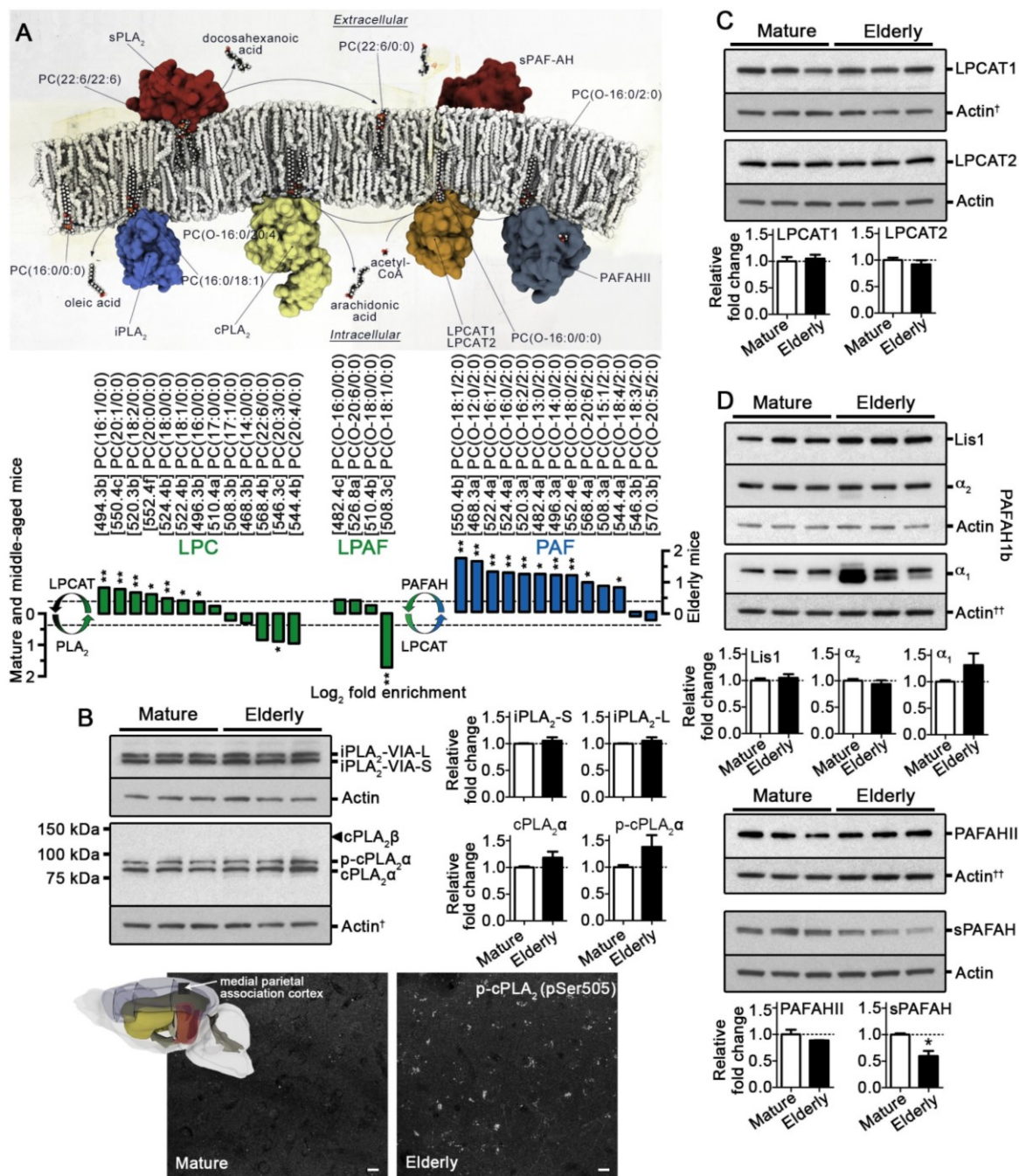


Figure 4-7

Figure 4-7 Enzymes cPLA₂ and sPAFAH are Involved in the Impairment of the Land's Cycle Remodelling in Aging

(A) Individual PC metabolites' subfamilies in aging are shown in the context of the PC remodelling Land's cycle. (B) cPLA₂ and iPLA₂ protein expression was quantified by Western blot. Phosphorylation of cPLA₂ was assessed by immunofluorescence. The increased phosphorylation of the cPLA₂ in elderly mice was indicative of its activation. (C) Quantification of LPCAT 1 and 2 protein expression was quantified by Western blot. (D) Quantification of the three PAF degrading enzymes: PAFAH1b complex, PAFAHII, and sPAFAH by Western blot. The protein expression of the sPAFAH was significantly reduced as shown by relative fold change. Statistics were Student's t-tests, and lipid significance was corrected by FDR. * $p < 0.05$ and ** $p < 0.01$.

demonstrated that DHA concentrations decrease in AD brain, liver, and AD risk models (Farooqui et al., 2007a, Lukiw and Bazan, 2008, Pomponi and Bria, 2008, Pauwels et al., 2009, Astarita et al., 2010, Seshadri et al., 2010, Bennett et al., 2013). LPC is the preferred carrier form of DHA into brain, with esterified LPC passage through the blood-brain barrier greater than that of unesterified DHA (Bernoud et al., 1999, Lagarde et al., 2001, Nguyen et al., 2014). In addition to being a source of DHA on *sn*-1 remodelling, PC(22:6/0:0) also exerts powerful anti-inflammatory activities (Huang et al., 2010). Cognitive decline, measured using the MMSE, was strongly negatively correlated with elevated levels not only of PC(22:6/0:0) but also one of its immediate yet rare precursors in normal controls, PC(22:6/17:2). The source of this rare precursor in plasma of LOAD and MCI may be mobilization from visceral and abdominal fat shown to be one of the few tissues that incorporate 17:2 fatty acid through intake of dairy products and sea fish (Hellmuth et al., 2013).

Disruptions in Land's cycle remodelling of DAPCs, AAPCs, and PAPCs were associated with age and disease. These changes were inferred by compositional changes in LPC, LPAFs, and PAFs in plasma, entorhinal-hippocampal circuit of LOAD patients post-mortem and at transition from middle-age to elderly human age equivalents over the murine lifespan. We found that age enhances risk of PAF accumulation through an age-dependent activation of cPLA₂ and a decrease in sPAFAH and that these changes in female mice are not the result of reproductive senescence. Moreover, critical changes in PAF species, notably PC(O-12:0/2:0) and PC(O-16:2/2:0) were accelerated by early exposure to accumulating A β , independent of age, in TgCRND8 mice. These data are consistent with previous evidence that A β ₄₂ activates cPLA₂ (Lee et al., 2011). LPCAT activity has also been shown to increase in AD (Ross et al., 1998), notably in the posterior-temporal

entorhinal cortex, a region characterized by the earliest tau pathology (Bierer et al., 1995). We have shown that in the presence of A β ₄₂, LPCATs substrate preference for acyl-CoA shifts to acetyl-CoA converting LPAFs to PAFs and not back to AAPC structural lipids (Ryan et al., 2009b).

Finally, compositional changes in LPCs, LPAFs, and PAFs in the entorhinal-hippocampal circuit of TgCRND8 mice link A β exposure in early life to changes in MUFA and polyunsaturated fatty acid PUFA biosynthesis. Incorporation of dietary C16:2 fatty acids present in laboratory rat chow into phospholipids can be elongated to C18:2 fatty acids further processed to C20:4 and C22:6 whose composition is altered in TgCRND8 mice. Changes are consistent with previously reported increases in stearyl-CoA desaturase and Δ 6 desaturase (fatty acid desaturase 2) activities in LOAD temporal cortex and hippocampus (Nakada et al., 1990, Astarita et al., 2011).

Despite this evidence for multiple changes in phospholipid metabolism in clinical and experimental samples, our data differ from recent reports that plasma PC compositions decline in MCI and LOAD patients (Mapstone et al., 2014, Whiley et al., 2014). Before addressing this disconnect, we have identified a major difference between our studies and these reports. We addressed the issue of comorbid vascular impairment in MCI and LOAD by comparing our patient populations to that of cognitively normal controls with vascular impairment. Our intent was to identify changes in PC content that associated with risk of LOAD phenoconversion independently of vascular risk. While our data are consistent with changes in brain of LOAD patients and experimental mouse models (Sweet et al., 2002, Kriem et al., 2005, Sanchez-Mejia et al., 2008b, Ryan et al., 2009b), they differ from reported changes in plasma. Thus, prior to publication, I intend to compare our profiles to those obtained from cognitively normal elderly without vascular impairment and from

patients with dementia with Lewy Bodies. This comparison will allow us to distinguish definitively between normal aging, changes in PC metabolism associated with vascular insufficiency, and MCI/LOAD-specific changes with respect to neurodegenerative disease and thus address whether differences in neurodegenerative lipidomes can be addressed by issues of comorbidity in circulating read-outs of defining systemic metabolic impairments.

4.8 Conclusion

Taken together, these data identify a convergence of PC metabolic impairments, associated with age, disease, and A β exposure, that contribute to LOAD phenoconversion and may identify contribution of comorbid pathologies.

CHAPTER 5. PHOSPHOPROTEOME ANALYSIS OF AN EARLY-ONSET MOUSE MODEL (TgCRND8) OF AD REVEALS TEMPORAL CHANGES IN NEURONAL AND GLIA SIGNALLING PATHWAYS

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5.1 Objective of this Study

The objective of this study was to profile the phosphoproteome in pre-symptomatic and symptomatic TgCRND8 mice and further to identify changes in lipid metabolic pathways associated with phenoconversion. **This study was published as:**

Wang F[†], **Blanchard AP[†]**, Elisma F, Granger M, Xu H, Bennett SAL, Figeys D & Zou H (2013). Phosphoproteome analysis of an early-onset mouse model (TgCRND8) of Alzheimer's disease reveals temporal changes in neuronal and glia signalling pathways. *Proteomics* 13, 1292-1305. [†]Joint first authors

5.2 Statement of the Author Contributions

This study was the result of the close collaborative work of three different laboratories from two countries under DF's, SALB's, and HZ's direction. APB and FW were the study coordinators and joint first authors. APB and FW designed the experiments with SALB, DF, HX, and HZ. MG contributed to the behavioural design and performed all of the behavioural studies. APB and HX collected the mice using the TgCRND8 mouse model adapted in SALB's laboratory, prepared the tissues, and immunoblotted the proteins. SALB performed the A β plaque quantitation. FW enriched the phosphopeptides and with APB analyzed this mixture by HPLC-ESI-MS. Data were analyzed by APB assisted by FE and FW under the direction of DF, SALB, and HZ.

5.3 Summary

Sustained exposure to soluble A β ₄₂ oligomers is predicted to impair synaptic function in the hippocampal-entorhinal circuit, signalling synaptic loss, and precipitating cognitive impairment in AD. Regional changes in overall patterns of protein phosphorylation are likely crucial to promote the transition from a pre-symptomatic to a symptomatic state in response to accumulating A β ₄₂. Here, we used unbiased proteomic approaches to compare the phosphoproteome of pre-symptomatic and symptomatic TgCRND8 mice and identify network disruptions in signalling pathways implicated in the manifestation of behavioural indices of learning and memory impairment. Phosphopeptide enrichment with triple isotopic dimethylation labelling combined with online multidimensional separation and MS was used to profile phosphoproteome changes in two- and six-month old TgCRND8 mice and congenic littermate controls. We identified 1026 phosphopeptides representing 1168 phosphorylation sites from 476 unique proteins. Of these, 595 phosphopeptides from 293 unique proteins were reliably quantified and 139 phosphopeptides were found to change significantly in the hippocampus of TgCRND8 mice following conversion from a pre-symptomatic to a symptomatic state.

5.4 Introduction

Two central pathological events define AD: (i) the intraneuronal accumulation of NFT composed of hyperphosphorylated tau, and (ii) the aberrant processing of the APP to different toxic assemblies of A β peptides (Goedert and Spillantini, 2006). The most damaging of these peptide assemblies are soluble oligomeric A β ₄₂ (Cleary et al., 2005). Genomic evidence confirms that mutations in either the APP gene or the presenilin genes accelerate A β ₄₂ biosynthesis resulting in the early-onset familial AD (Cleary et al., 2005).

Sustained exposure to soluble A β ₄₂ oligomers is predicted to impair synaptic function in the hippocampal-entorhinal circuit, signalling synaptic loss, and precipitating cognitive impairment (Selkoe, 2002, Bernstein et al., 2005, Cleary et al., 2005, Nimmrich and Ebert, 2009). Despite these strong proofs that A β ₄₂ accumulation is a driving pathology in AD, underlying disruptions in signal transduction pathways have only begun to be elucidated.

Regional changes in overall patterns of protein phosphorylation are likely crucial to promote the transition from a pre-symptomatic to a symptomatic state in response to accumulating A β ₄₂. These changes would contribute to disruption of multiple signalling pathways and, in part, contribute to the “change of state” postulated to be necessary for cognitive decline (Herrup, 2010). The hyperphosphorylation of tau represents a primary example of “phospho-pathology” wherein progressive alterations in phosphorylation status alter the capacity of tau to stabilize microtubules thereby impairing axonal transport (Reddy, 2011). Certainly, the severity of AD dementia is highly correlated with the degree of abnormal tau phospho-processing in the hippocampal-entorhinal circuit associated with progressive aggregation and deposition of NFT (Bierer et al., 1995). Significant alterations in the phosphoproteome status have already been reported in post-mortem hippocampus of AD patients relative to age-matched controls (Di Domenico et al., 2011). It is not known whether these changes are a late-stage event manifested following catastrophic memory failure and neuronal loss, or whether key phospho-signalling pathways are disrupted upon transition from a pre-symptomatic to symptomatic disease state in response to accumulating A β ₄₂.

To address this question, we applied unbiased phosphoproteomic approaches to study a rapid onset mouse model of AD and to identify network disruptions in phosphorylation-dependent signalling pathways exhibited upon transition from a pre-

symptomatic to a symptomatic state. The TgCRND8 mouse model expresses the human APP695 gene with both familial Swedish and Indiana AD mutations under the control of the prion protein promoter (Janus et al., 2000, Chishti et al., 2001, Van Vickle et al., 2007). This model is widely used for large-scale “omics studies” of AD associated with A β ₄₂ biogenesis (Phinney et al., 2003, Wong et al., 2004, Supnet et al., 2006, Van Vickle et al., 2007). Here, we show that the TgCRND8 hippocampal phosphoproteome is significantly altered between two and six months of age. Briefly, we applied phosphopeptide enrichment with triple isotopic dimethylation labelling combined with online multidimensional separation and MS-to-profile phosphoproteome changes in hippocampi of a pre-symptomatic (two month old) and a symptomatic (six month old) Tg mice relative to NonTg littermates. A total of 1026 phosphopeptides, representing 1168 phosphorylation sites from 476 unique proteins, were identified. Of these, 595 phosphopeptides from 293 unique proteins were quantified and 139 phosphopeptides were found to change significantly in the hippocampi of TgCRND8 mice compared to controls between two and six months of age. Network alterations in identified phospho-signalling pathways within the hippocampus are discussed with respect to TgCRND8 transition from a pre-symptomatic to a symptomatic state.

5.5 Methods

5.5.1 Animals and tissues

TgCRND8 mice on a mixed C57BL/6 X C3H hybrid background were kindly provided by Dr. Fraser (University of Toronto) and were backcrossed for five generations (N5) to a C57BL/6 lineage in the Bennett laboratory. C57BL/6Cr1 mice were obtained from Charles Rivers Laboratories (Senneville, Canada). Heterozygote Tg and congenic

NonTg littermates were maintained by breeding NonTg females with Tg males. The TgCRND8 line expresses a double mutant form (KM670/671NL+V717F) of the human APP gene under the control of the prion protein promoter (Chishti et al., 2001). Previous studies have shown that pathogenic increases in the ratio of A β ₄₂/A β ₄₀ in a mixed C57BL/6 X C3H hybrid background are first detected at 2.5 months of age and behavioural learning and memory impairment are detected between three and four months of age (Chishti et al., 2001). Here, a total of 62 female N5 Tg (n = 26) and NonTg (n = 36) littermates was analyzed. For genotyping, genomic deoxyribonucleic acid was isolated from a biopsied tail and PCR amplification was 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 terminating after the last cycle with 7 min at 73 °C. Primers were as follows: 5'-GGC CGC GGA GAA ATG AAG AAA CGC CAA GCG CCG TGA CT-3' (forward) and 5'- TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A-3' (reverse). Tg mice produce a 1 kB amplicon. To verify template integrity, all animals were genotyped for the PAFR. PCR amplification was 10 min at 95 °C followed by 30 cycles of 20 s at 94 °C, 20 s at 65 °C, and 50 s at 70 °C. Primers were as follows: 5'-TAT GGC TGA CCT GCT CTT CCT GAT-3' (forward) and 5'- TAT TGG GCA CTA GGT TGG TGG AGT-3' (reverse). Tg and NonTg mice produce a 289-kB amplicon. Behavioural assessments were performed on n = 5 Tg and n = 12 NonTg mice at two months of age and on n = 5 Tg and n = 8 NonTg mice at six months of age. An additional n = 3 mice per genotype and time point were used to quantify A β plaque load. For Western analyses, n = 2 mice/genotype were analyzed at two, four, and six months of age. For proteomic assessments, the hippocampi (including dorsal and ventral regions) of two females per genotype and time point were dissected, immediately flash-frozen in liquid nitrogen, and stored at -80 °C until protein extraction. All animal manipulations were performed in strict

accordance with the ethical guidelines for experimentation established by the Canadian Council for Animal Care and with the approval of the University of Ottawa Animal Care Committee for the ethical treatment of experimental animals.

5.5.2 Behavioural assessment

Learning and memory was assessed in the Morris Water Maze (Noldus Information Technology). The apparatus consisted of a blue plastic pool measuring 134.5 cm in diameter and 53.3 cm deep with floor insert. The pool was filled with water rendered opaque with white water-soluble nontoxic paint to a depth of 1 cm above the 10 cm diameter white escape platform located in the back-right quadrant. Water temperature was maintained at 21 °C. Visual cues consisted of a square and an “X” located within visual range of the swimming mice at the front (opposite entry) and left walls of the test room (2.98 m × 3.97 m × 2.62 m), respectively. Mice were habituated to the room 1 h prior to testing. The room was lit with overhead white light of 100 lux. Constant white noise at 70 dB was provided during acclimatization and testing. Each mouse completed four trials per day. Each trial lasted until the animal found the platform or for a maximum of 60 s. Mice that failed to find the platform within this time period were guided to the platform by the experimenter and then removed from the pool. Each trial was randomized to one of four possible starting locations. Mice were run in cohorts of 6 to 12 with 20 min between each trial. Performance was recorded with a video camera mounted directly above the pool and data were acquired and analyzed with Ethovision v7 software (Noldus Information Technology, Netherlands). Data were analyzed by repeated measures (test day) two-way ANOVA (test day and genotype) as indicated followed by Holm–Sidak's multiple

comparisons test maintaining family-wise error at either 0.05 or 0.01. Analyses were performed using Prism 6.0a (GraphPad).

5.5.3 A β plaques quantitation

Plaque number was determined as described previously (McLaurin et al., 2006). Animals were euthanized by lethal injection of sodium pentobarbital and transcardially perfused with 10 mM PBS (10 mM sodium phosphate and 154 mM NaCl) followed by 3.7 % paraformaldehyde in 10 mM PBS. Brains were removed and post-fixed for 24 h in this same solution. Brains were switched to 10 mM PBS, paraffin-embedded, and serially sectioned by the uOttawa Pathology Core Facility. Every tenth section in a series of sagittal 10 μ m serial sections through one paraffin-embedded hemisphere from start of hippocampus to midline was analyzed. Deparaffinized, hydrated sections were incubated in 10 % FA, pH 1.6–2.0 for 10 min, washed repeatedly in PBS, and incubated overnight with mouse anti-4G8 (Covance SIG-39220 mouse at 1:500) in Ab buffer (3 % bovine serum albumin, 0.3 % Triton X-100 in 10 mM PBS). Anti-4G8 recognizes both murine and human A β . Secondary Ab was biotin-labelled goat anti-mouse IgG (1:300, Sigma B9904) detected with extravidin-horseradish peroxidase (1:20 Sigma, E2886) using 3,3'-diaminobenzidine tetrahydrochloride tablets as substrate (Sigma D5905). Images were captured on a Leica DMXAR2 microscope and converted to binary images for quantification of plaque number and hippocampal area using the OpenLab v5.08 Advanced Measurements module. Plaques were defined as immunoreactive, compacted, and spherical aggregates greater than 10 μ m in diameter. Data were expressed as mean $\pm$ SEM per section and were analyzed two-way ANOVA (age and genotype) by two-way ANOVA

followed by Holm–Sidak's multiple comparisons test maintaining family-wise error at either 0.05 or 0.01. Analyses were performed using Prism 6.0a (GraphPad).

5.5.4 Immunoblotting

Total proteins were extracted from a whole mouse hippocampus homogenized in radioimmunoprecipitation assay (RIPA) buffer (1 % Nonidet P40 substitute, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 300 µg/ml aprotinin, and 100 µg/ml phenylmethylsulfonyl fluoride in 10 mM phosphate-buffered saline [10 mM phosphate, 154 mM NaCl]) using a Tissue-Tearor (Biospec Products). The protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad 500–0112) in accordance with the manufacturer's guidelines. Protein samples of 15 µg were incubated for 10 min at 70 °C in NuPAGE lithium dodecyl sulphate sample buffer and resolved on a NuPAGE 4–12 % SDS–PAGE gel (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (Pall Life Sciences 66485) and blocked for 30 min in 5 % non-fat milk in TBS-T (50 mM Tris base, 150 mM NaCl, 0.1 % Tween 20). Western blot analysis was performed by an overnight probe at 4 °C with α -myelin basic protein (MBP) (Sigma M3821 rabbit at 1:4000), α -APP (4G8 Covance SIG-39220 mouse at 1:1000), or α -actin (Cederlane CLT9001 mouse at 1:15 000). Secondary Ab, horseradish peroxidase-conjugated α -mouse (Rockland; 610–1319-0500 at 1:10 000) or α -rabbit (GE Healthcare; NA934V at 1:10 000), were incubated for 1 h at room temperature. The signals were revealed by chemiluminescence using Immobilon Western (Millipore WBKLS0500) and the protein expression was quantified by ImageJ analysis software (v1.45 National Institutes of Health) based on densitometry.

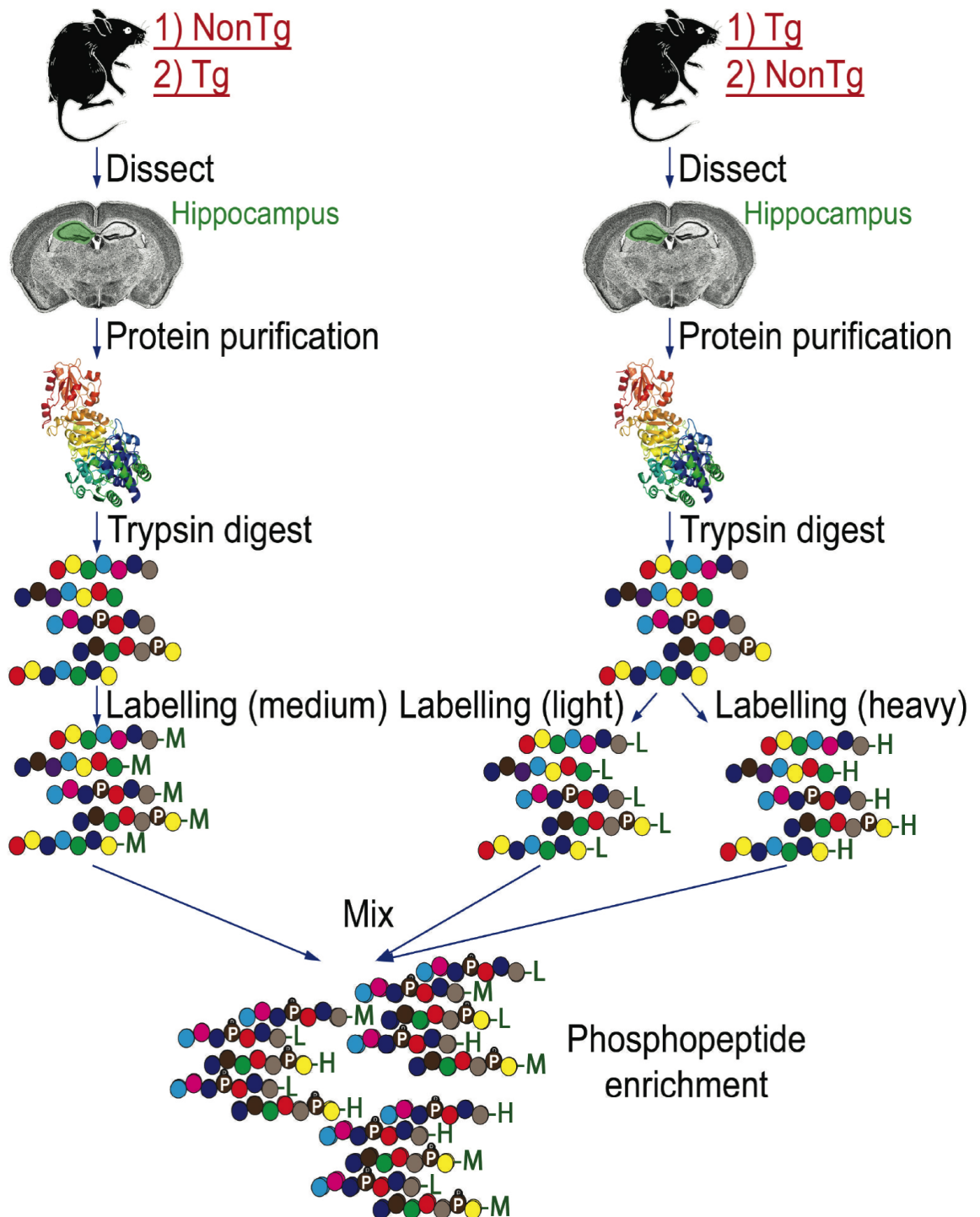


Figure 5-1

**Figure 5-1 Schematic of the Processing of the Tg and NonTg Hippocampal Samples
for Labelling and Enrichment of Phosphopeptides**

5.5.5 Protein extraction and sample preparation for MS

A schematic of the processing of Tg and NonTg hippocampi for labelling and enrichment of phosphopeptides is presented in Figure 5-1. The hippocampal formation from one cerebral hemisphere per animal was finely minced, and incubated in 200 μ l extraction buffer (8 M Urea, 50 mM Tris pH 8.2, 65 mM DTT, EDTA-free protease inhibitor, and phosphatase inhibitor cocktail tablets [Roche]). The tissue was sonicated at 24 W for 20 s with 30 s intermission on ice. Following a 20 min incubation on ice and a centrifugation at $22,000 \times g$ for 15 min, the supernatant was subjected to a chloroform–methanol precipitation (Wessel and Flugge, 1984) and protein redissolved into 1 ml 8 M Urea, 50 mM Tris pH 8.2 buffer. The protein concentration was measured using the DC Protein Assay (Bio-Rad) as per manufacturer's protocol. The proteins were reduced using 5 mM DTT (Sigma) at 60 °C for 1 h and alkylated with 10 mM iodoacetamide (Sigma) in the dark at room temperature for 40 min. The solution was diluted to 1 M urea by the addition of 50 mM Tris pH 8.2 buffer, digested overnight with 1:50 w/w sequence-grade trypsin (Promega), and the enzymatic reaction was stopped by the addition of 0.25 % trifluoroacetic acid (TFA). The peptidic solution was loaded onto a prewashed [(i) 1 ml ACN; (ii) 1 ml 0.1 % FA aqueous solution], and pre-equilibrated (1 mL 50 mM sodium phosphate pH 7.5) 200 mg tC18 cartridge (Waters). To label proteins (Table 5-1), 5 ml isotopic labelling reagents containing (i) 0.2 % CH₂O and 30 mM NaBH₃CN (light [Lg] form labelling); or (ii) 0.2 % CD₂O and 30 mM NaBH₃CN (medium [Md] form labelling); or (iii) 0.2 % ¹³CD₂O and 30 mM NaBD₃CN (heavy [Hv] form labelling) were added (Boersema et al., 2009). After a wash with 1 mL 0.1 % FA aqueous solution, the isotopic-labelled peptides were eluted from the tC18 cartridge using 1 mL 80 % ACN. The eluted peptides were lyophilized to powder and stored at -80 °C.

Table 5-1 The Labelling Reagents Combination, Labelling Group, and Mass Shift for Each Isotopic Labelling

Label	Lg	Md	Hv
Formaldehyde	CH ₂ O	CD ₂ O	¹³ CD ₂ O
Cyanoborohydride	NaBH ₃ CN	NaBH ₃ CN	NaBD ₃ CN
Labelling group	2CH ₃	2CHD ₂	2 ¹³ CD ₃
Mass shift	+28.0313	+32.0564	+36.0757

5.5.6 Phosphopeptide enrichment

The procedures for preparation of the titanium-immobilized metal ion affinity chromatography (Ti^{4+} -IMAC) monodisperse microspheres were as described previously (Yu et al., 2009). The Ti^{4+} -IMAC microspheres were well dispersed into 80 % ACN, 6 % TFA aqueous solution at a concentration of 10 mg/ml. For comparative analyses, the same amount of isotopic dimethyl-labelled samples were dissolved into 0.1 % FA aqueous solution and mixed. Then, the labelled peptides mixture was incubated with ten times Ti^{4+} -IMAC microspheres (w/w). Following 30 min of incubation at 4 °C, the Ti^{4+} -IMAC microspheres with the adsorbed phosphopeptides were collected by centrifugation at $22,000 \times g$ for 15 min. The microspheres were then successively washed with 500 μl of an aqueous solution of 50 % ACN, 6 % TFA, and 200 mM NaCl, and then with 500 μl of an aqueous solution of 30 % ACN and 0.1 % TFA to remove nonspecific adsorption. Finally, 500 μl $\text{NH}_3\text{-H}_2\text{O}$ (12.5 %) was used to elute the enriched phosphopeptides from the microspheres by stirring for 15 min at 4 °C and sonicating for 15 min at 4 °C. After centrifugation at $22,000 \times g$ for 15 min, the phosphopeptide-containing supernatant was collected and lyophilized under vacuum at 45 °C for 15 min.

5.5.7 Online multidimensional separation and Ultra-HPLC-ESI-MS/MS Detection

A vent sample injection system was established for this study (Figure 5-2). A 14 cm $\times$ 200 μm ID biphasic trap column with a 7-cm-long phosphate strong cation-exchange (SCX) monolith column adjacent to a 7 cm-long C18 packing material (C18 AQ, 5 μm , 300 Å, Dr. Maisch GmbH, Germany) was used for sample injection. A 75 μm ID tip column (New Objective Inc.) packed with an 18 cm-long C18 material (C18 AQ, 3 μm , 120

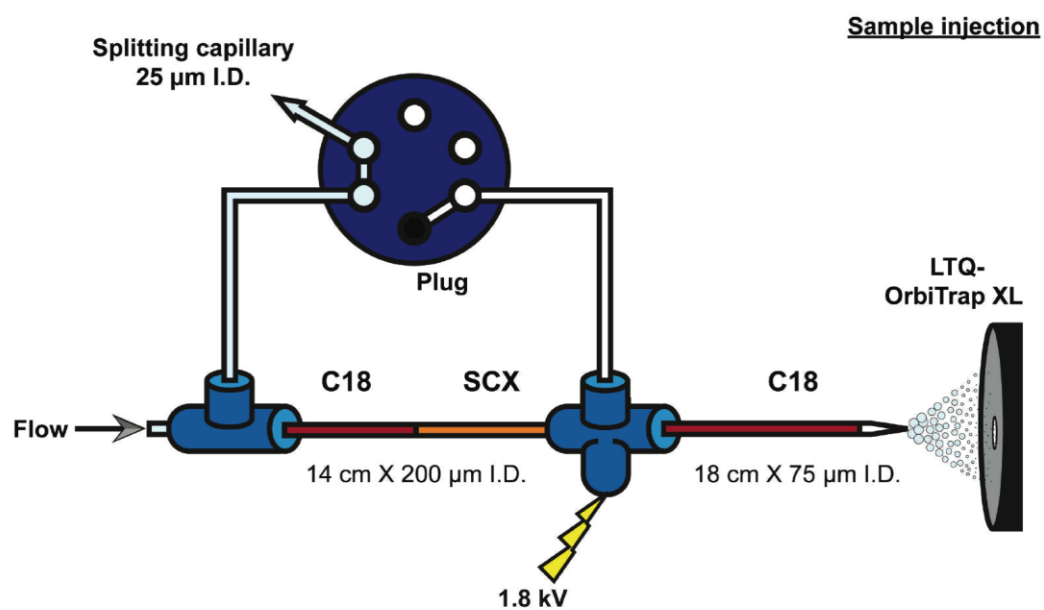
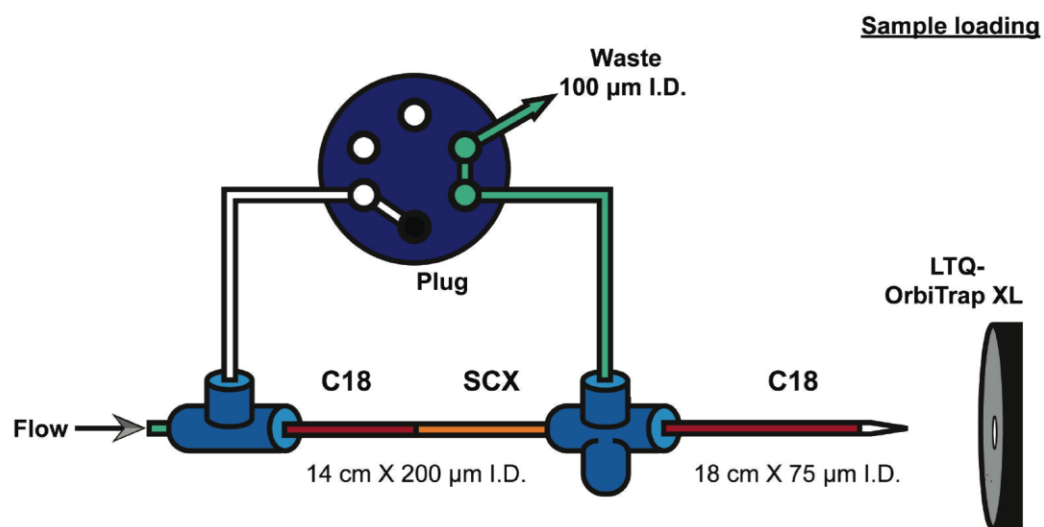


Figure 5-2

Figure 5-2 Schematic Diagram of the Vent Sample Injection System

Å, Dr. Maisch GmbH) was used for peptides separation (Wang et al., 2007a, Wang et al., 2010). The sample was injected into the trap column with a flow rate of 10 μ l/min for 8 min, and the separation flow rate was adjusted to about 300 nl/min. After sample loading onto the biphasic trap column, the peptides were separated using a reverse phase binary gradient and were detected by MS. 0.1 % FA aqueous solution (solvent A) and 0.1 % FA ACN (solvent B) were used as mobile phases for reverse phase gradient separation. The reverse phase binary gradient was set as follows: for the first 5 min, the mobile phase was ramped from 1 to 5 % solvent B; for the next 140 min, the mobile phase was ramped from 5 to 30 % solvent B, and finally for the next 5 min, the mobile phase was ramped from 30 to 90 % solvent B and maintained at 90 % solvent B for the next 8 min. The system was re-equilibrated using 100 % of solvent A for 12 min. Peptide fractions were transferred from the SCX monolith column to the separation column by NH_4AC (pH 2.7) salt step elutions. The NH_4AC (pH 2.7) salt steps were as follows: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 80, 100, and 1000 mM. Each salt step lasted 8 min at a flow rate of 300 nl/min, and was followed by reverse phase binary gradient separation and MS/MS detection.

MS detection was performed using an LTQ-OrbiTrap XL (Thermo, San Jose, CA, USA). The temperature of the ion-transfer capillary was 200 $^{\circ}\text{C}$, the electrospray voltage was +1.8 kV, and the normalized collision energy was 35 %. One microscan was set for each MS and MS/MS scan. The MS full scans were acquired in centroid mode from m/z 400 to 2000 in the OrbiTrap with a resolution of 60,000, and the MS/MS scans were acquired in the LTQ linear ion-trap. All MS and MS/MS spectra were acquired in the data-dependent analysis mode, in which the ten most intense ions within an MS scan were selected for MS/MS scan by CID. The target ion setting was $5e^5$ and $3e^4$, with a maximum fill time of 500 and 100 ms, for the OrbiTrap and LTQ scans, respectively. The dynamic

exclusion function was as follows: repeat count 2, repeat duration 30 s, and exclusion duration 90 s.

5.5.8 Data analysis

The *.raw files obtained from the LTQ-Orbitrap XL were processed by MaxQuant (<http://maxquant.org/>, version 1.1.1.36) (Cox and Mann, 2008) to identify and quantify the phosphopeptides. The International Protein Index (IPI) mouse database (v3.52, 55303 entries) was used for database searching and the IPI was converted to RefSeq. Static modification was set as carbamidomethyl on cysteine residues with +57.0215 Da. Variable modifications were set as oxidation on methionine residues with +15.9949 Da; amino termini and lysine residues with +28.0313, +32.0564, and +36.0757 Da, respectively, for Lg, Md, and Hv isotopic dimethyl labelling and phosphorylation on serine, threonine, and tyrosine with +79.9663 Da. Peptides were searched under the conditions of full tryptic cleavage with up to two missed cleavage sites. The other parameters were used as the default setting of the MaxQuant software (Cox and Mann, 2008). Both the protein and peptides identification false-positive rates were less than 5 %. The data have been expressed for each individual mass spectral analysis as a $\log_2$ normalized ratio Tg/NonTg. Only the phosphopeptides that had at least two ratios at two months and two ratios at six months were considered for further analysis. The statistical significances of the phosphopeptide alteration were determined by a two-tailed distribution with two-sample unequal variance Student's t-test on the $\log_2$ ratio (Tg/NonTg). The *p*-values were then used to calculate the asymmetric local false discovery rate (locFDR) (Bickel, 2013) by the binomial-based estimator method for each phosphopeptides (Hong et al., 2009). A locFDR threshold of 20 % (97 % of which have a *p*-value less than 0.05) was used as a cut-off to

identify phosphopeptides abundance altered over time in Tg mice relative to age-matched NonTg littermates. Molecular functions and protein networks were analyzed by Ingenuity Systems Pathway Analysis software (IPA version 8.8, Ingenuity Systems Inc).

5.6 Results

5.6.1 The reproducibility of the online multidimensional HPLC-ESI-MS/MS system for quantitative phosphoproteome analysis

One challenge in phosphoproteomics is the replicate analysis of the phosphopeptides. In phosphoproteomics, a single phosphopeptide is identified per phosphorylation site in contrast to proteomics, which relies on multiple peptides to identify a protein. This complicates the interpretation of repeat analyses as the same phosphopeptide must be identified in each replicate sample to enable quantification. We recently described a pseudo triplex stable isotope dimethyl labelling approach that enables two simultaneous repeat analyses in a single experiment (Song et al., 2011). Here, we applied this approach to phosphopeptide quantification from brain tissue, specifically murine hippocampus. We first tested the performance of the pseudo triplex stable isotope dimethyl labelling for analysis of complex brain samples using protein samples extracted from a NonTg cerebrum to establish reproducibility. Briefly, three 200 µg-tryptic digests of the same protein sample were labelled with Lg, Md, and Hv isotopic dimethyl groups and mixed together to obtain the standard test sample. Then, the phosphopeptides were enriched and analyzed. The majority of the replicate labelling ratios from identical samples fell between -0.75 and 0.75 ($\log_2$ ratio) with less than 0.1 % of the peptides quantified falling outside this limit (Figure 5-3). These results indicate that the quantification

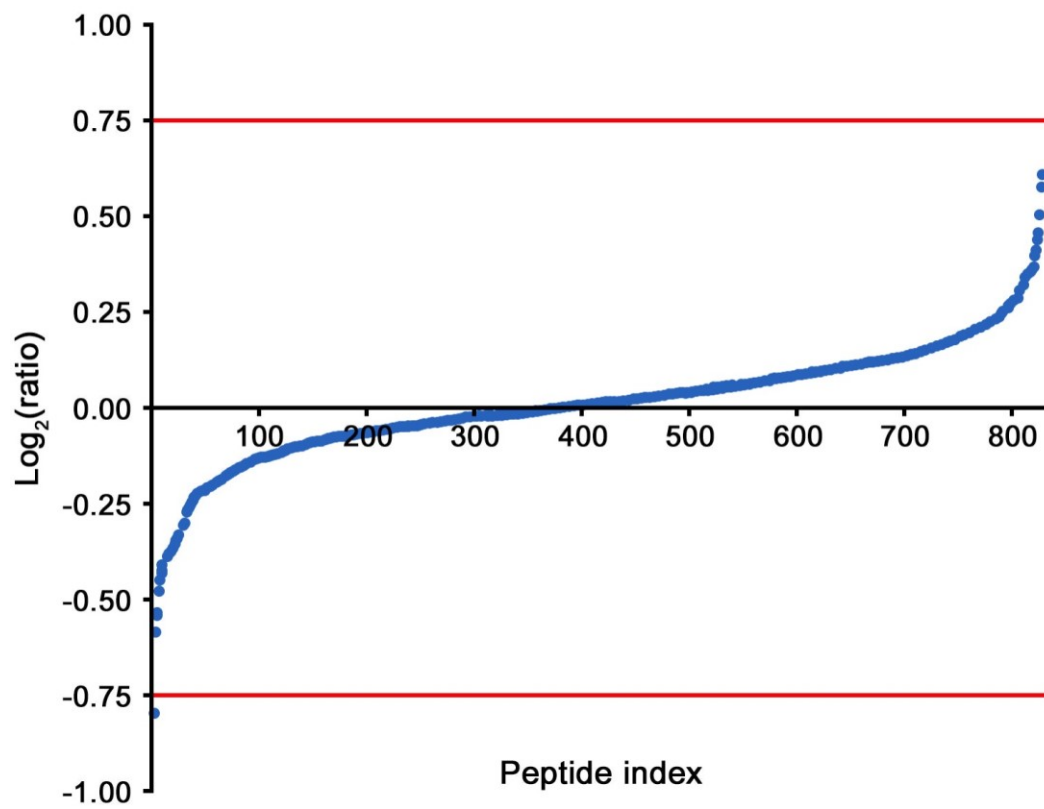


Figure 5-3

**Figure 5-3 Phosphopeptide $\text{Log}_2(\text{Ratio})$ Distribution Quantified by Pseudo Triplex
Stable Isotope Dimethyl Labelling With a Coefficient of Variation $\leq 40\%$ Filtering**

The sample labelling amounts ratio is (Lg/Md/Hv) = (1/1/1), and 200 μg of each labelled sample was used for phosphopeptides enrichment.

accuracy of the pseudo triplex stable isotope dimethyl labelling approach is adequate for the study of complex phospho-samples derived from mouse brain.

5.6.2 Conversion from a pre-symptomatic to a symptomatic state in the TgCRND8 mouse model is associated with the widespread deposition of amyloid plaques in the hippocampus

A β plaque load was established in Tg and NonTg mice at two and six months of age and behavioural indices of cognition were assessed in the Morris Water Maze at these same time points. Learning and memory impairment in TgCRND8 mice have previously been reported when pathogenic increases in the A β ₄₂ to A β ₄₀ ratio are detected and A β oligomers aggregate into plaques within the hippocampus (Chishti et al., 2001). In mixed hybrid C57BL/6 X C3H F1 TgCRND8 mice, this conversion takes place between eight and sixteen weeks of age with a mean age of onset detected at approximately 11 weeks of age (Chishti et al., 2001). There is, however, considerable variation between animals likely due, in part, to the different genetic backgrounds of different lines (Chishti et al., 2001, Glazner et al., 2010). To ensure genetic reproducibility in this study, we backbred C57BL/6 X C3H Tg animals for five generations into a C57BL/6 lineage followed by 10 filial-generation interbreedings. Elevated human APP protein levels in our N5 Tg mice were demonstrated by Western analysis at two, four, and six months of age. We used the 4G8 Ab to detect both murine and human protein and thereby demonstrate extent of ectopic expression over physiological levels (Figure 5-4A). A β plaque number was quantitated using the same Ab. Significant increases in A β plaque number were detected in six month old Tg animals relative to either age-matched NonTg controls or two month old Tg animals (two-way ANOVA: genotype main effect $F(1,8) = 31.89$; age main effect $F(1,8) = 36.52$;

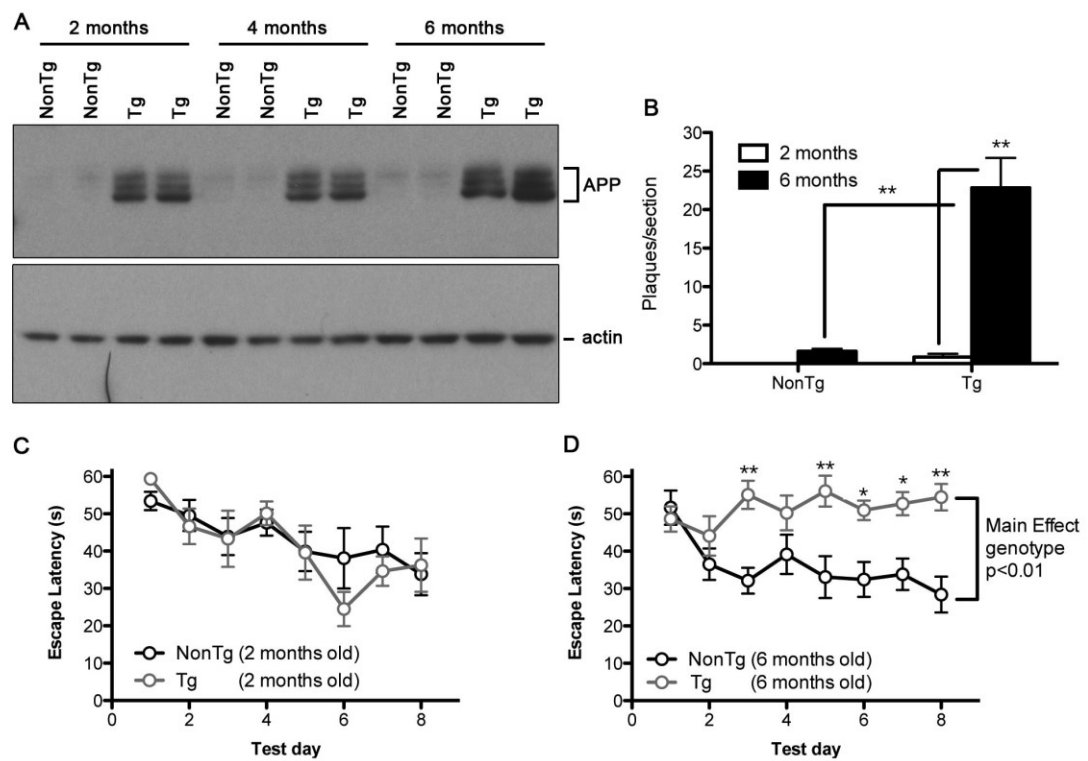


Figure 5-4

Figure 5-4 Behavioural Impairment in Learning and Memory is Associated with a Significant Increase in A β Plaque Number and is Indicative of Conversion from a Presymptomatic to a Symptomatic State in Tg Mice

(A) Western analysis of APP using the 4G8 Ab that detects both murine and human protein. Transgene protein expression is markedly elevated and remains comparable between two and six months of age in Tg mice. Blots were stripped and reprobed for total MBP and thus share the same actin loading control (see Figure 5-8). (B) Aggregation of 4G8-immunoreactive A β into compacted and spherical plaque greater than 10 μ m in diameter was quantified in $n = 3$ animals per genotype and condition. A significant increase in the plaque number was evident at six months of age. Statistics were two-way ANOVA as reported in Section Discussion followed by Holm–Sidak's tests corrected for multiple comparisons, ** $p < 0.01$. (C) Learning and memory as assessed using the Morris Water Maze was comparable between Tg and NonTg mice at two months of age. Animals learned to locate the platform and escape the maze at equal rates. (D) Significant impairment in learning and memory was evident by six months of age in Tg mice. Statistics were repeated measures (test day) two-way ANOVA (test day and genotype) followed by Holm–Sidak's tests corrected for multiple comparisons to identify significant genotype X test day interactions. * $p < 0.05$, ** $p < 0.01$.

genotype X age interaction $F(1,8) = 27.26$, Figure 5-4B). This aggregation corresponded with transition from a pre-symptomatic to a symptomatic state with respect to behavioural indices of learning and memory in the Morris Water Maze. In this paradigm, mice are required to learn to use minimal external visual cues to find a hidden escape platform submerged in a water pool over an eight day test period consisting of four training trials per day. At two months of age, performance of NonTg and Tg mice was comparable (Figure 5-4C). Mice learned to find the escape platform at the same rate exhibiting comparable escape latency over each of the test days. At six months of age, a significant main effect of genotype ($F(1,11) = 15.44$ $p < 0.01$ repeated measures two-way ANOVA) and a significant interaction between genotype and test day ($F(7,77) = 2.91$, $p < 0.01$) was detected (Figure 5-4D). Tg mice clearly exhibited marked deficiencies in their ability to acquire the task and find the escape platform (Figure 5-4C). Swimming velocity was comparable between genotypes indicating impairment of learning and memory and not motoric impairment (data not shown). Taken together, these data demonstrate that N5 C57BL/6 X C3HF10 Tg mice have converted from a pre-symptomatic phenotype to a symptomatic phenotype by six months of age.

5.6.3 Dynamic changes in the phosphoproteome of TgCRND8 upon conversion from a pre-symptomatic to symptomatic state

For phosphoproteomic assessment, two pairs of TgCRND8 and congenic NonTg controls were sacrificed at two and six months of age. Their hippocampi were carefully excised and the hippocampal proteins were extracted and labelled using the pseudo triplex stable isotope dimethyl labelling approach (Song et al., 2011). Two labelling assignments were performed per analysis. For the first analysis in both two and six month old samples,

identical amounts (300 µg) of the two NonTg control samples were labelled with Lg and Hv dimethyl group, respectively. The Tg comparator mouse sample (300 µg) was labelled with Md dimethyl group. Therefore, this procedure enables two replicate quantification comparisons of Tg and NonTg samples in just a single experiment (Md/Lg and Md/Hv). In contrast, for the second biological replicate analysis, equal concentrations of the two independent Tg samples (per time point) were labelled with Lg and Hv dimethyl group, respectively; and the NonTg mouse control comparator was labelled with the Md dimethyl group. For each analysis, the three isotopic-labelled samples were mixed and the phosphopeptides were enriched by Ti^{4+} -IMAC followed with nanoflow HPLC-ESI-MS/MS analysis.

We identified 1026 phosphopeptides representing 1168 phosphorylation sites (83 % pS, 15 % pT, and 2 % pY) from 476 unique proteins. Of the 1168 phosphorylation sites, 82 % were assigned to a protein kinase according to the PHOSIDA database (Gnad et al., 2011) (Figure 5-5), whereas 18 % had unknown phosphorylation motifs. Our quantitative analysis using the MaxQuant software (Cox and Mann, 2008), to extract intensities for the Lg, Md, and Hv trios from each replicates and time points, revealed that 595 phosphopeptides from 293 unique proteins could be confidently quantified. Of these, significant changes in the ratios of 139 phosphopeptides were detected in the hippocampus of the Tg mice compared to the congenic NonTg control mice upon conversion to a symptomatic state (i.e. two versus six months of age). Two axis hierarchical clustering, based on the city-block distance with an average linkage analysis, of the significant phosphopeptide changes grouped the results based on age (Figure 5-6) whereas clustering performed on the whole phosphoproteomic dataset failed to separate the animal based on age (data not shown). Taken together, these data further identify the subset of phospho-

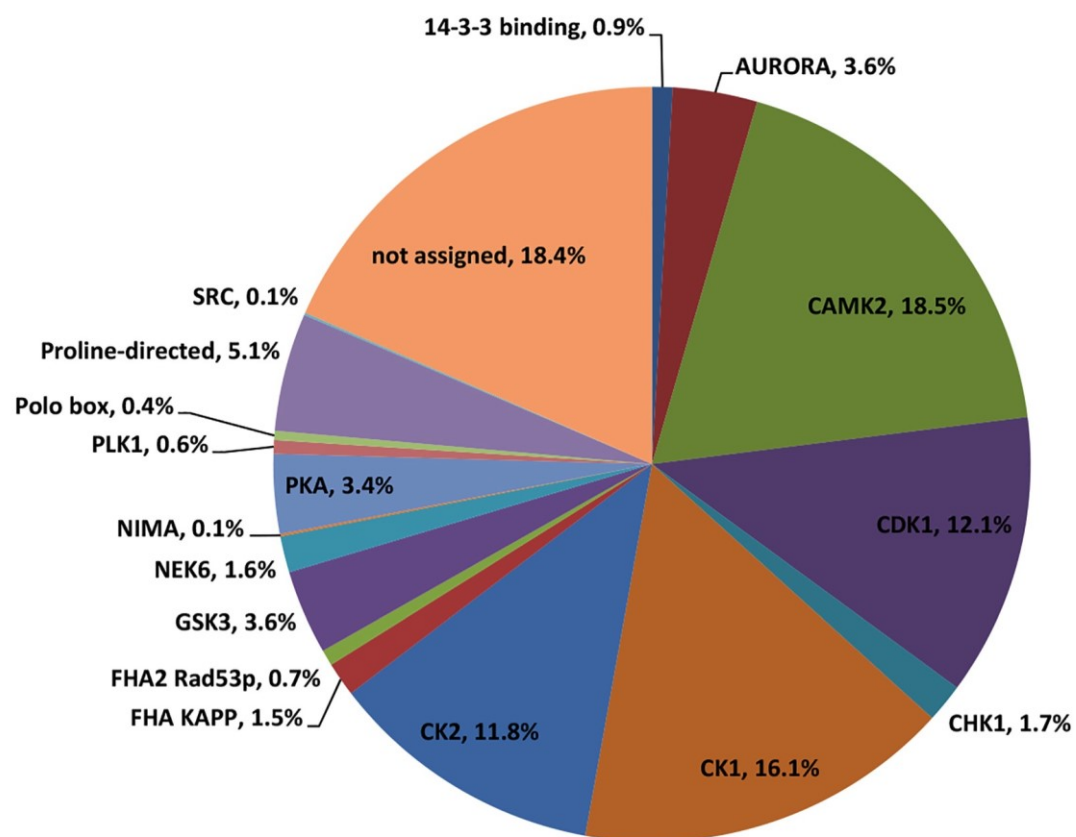


Figure 5-5

Figure 5-5 Distribution of Protein Kinases Predicted to be Responsible for the Phosphorylation in Global Phosphopeptides



Figure 5-6 Cluster Analysis of Significant Changes in Relative Phosphopeptide Abundance $\text{Log}_2(\text{Tg}/\text{NonTg})$ at Two (Presymptomatic) and Six (Symptomatic) Months of Age

Increase in phosphorylation is indicated in red, decrease in blue, and not detected in grey.

signalling targets altered following symptomatic onset in Tg mice. Bioinformatics analysis of the corresponding protein functions for this phosphopeptide subset revealed that the top five functions are (i) neurological disease, (ii) cellular assembly and organization, (iii) cellular function and maintenance, (iv) cellular movement, and (v) nervous system development and function.

5.6.4 Network analysis

Protein interaction network analysis for direct protein–protein interactions among the 92 phosphoproteins dynamically regulated in Tg mice between two and six months of age was performed using Ingenuity Systems Pathway Analysis software. We identified a subset of 36 phosphoproteins that directly interact and whose phosphorylation status is consistently altered upon conversion from a pre-symptomatic to a symptomatic state (Figure 5-7). This protein interaction network points implicates phosphorylation changes in proteins involved in synaptic function and in cytoskeletal maintenance.

5.7 Discussion

Our phosphoproteome analysis of an early-onset mouse model (TgCRND8) of AD reveals specific temporal changes in the phosphoproteome involved in neuronal and glia signalling pathways. We identified 1026 phosphopeptides representing 1168 phosphopeptides of which 595 phosphopeptides were confidently quantified. Of these, 139 phosphopeptides were found to change in the hippocampus of the Tg mice compared to congenic NonTg control mice over time. This subgroup of phosphopeptides clearly separates the animal according to their symptomatic states (Figure 5-6). Moreover, some of these phosphorylated proteins are known to directly interact (Figure 5-7).

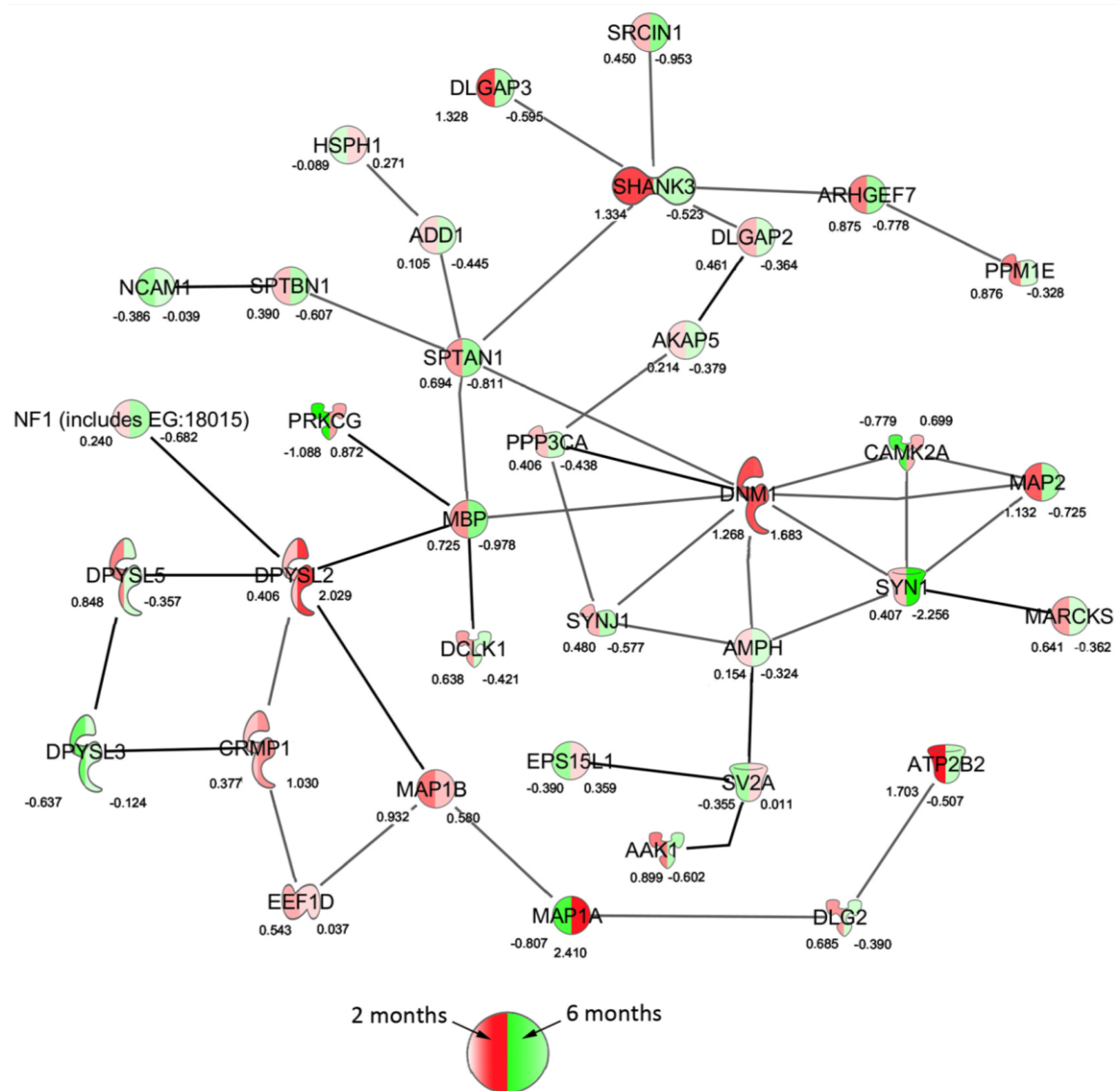


Figure 5-7

Figure 5-7 Direct Protein–Protein Interactions Between the 92 Phosphoproteins whose Phosphorylation Status Changes Between Two and Six Months of Age in TgCRND8 Mouse Model Versus Congenic Littermates

Interaction networks were identified by Ingenuity Pathway Analysis software using curated protein–protein interactions obtained from public databases. Green represents proteins whose average phosphostatus decreases, whereas red represents increases in phosphorylation upon conversion from a presymptomatic to symptomatic state.

The clustering analysis and protein interaction network implicates phosphorylation changes in proteins involved in synaptic function and in cytoskeletal maintenance. For example, phosphorylation of the protein SRC homology 3 domain and multiple ankyrin repeat domains protein 3 (SHANK3), which is a large scaffold postsynaptic density protein implicated in dendritic spine and synapse formation, at S856 (TKpSVGEDEKSLASLLEGR) was shown to be significantly downregulated 3.6-fold in Tg mice between two and six months of age but not in NonTg controls over time. A decrease in the level of SHANK3 protein was recently shown to correlate with the progressive accumulation of A β oligomers in APP Tg mice (Verpelli et al., 2011). Here, we add to this finding by demonstrating that this decrease is likely also accompanied by a reduction in phosphorylation. Moreover, the SHANK3 complex is important for proper synaptic function and is organized around a Zn²⁺ ion (Grabruker et al., 2011). A β is known to affect assembly of the SHANK3 scaffolding platform through the sequestration of Zn²⁺ ions associated with a decrease in hippocampal synapse density (Grabruker et al., 2011). Here, we show that known interactors of SHANK3 such as the protein products of Rho guanine nucleotide exchange factor 7, and discs, large homolog-associated protein such as isoforms 2 and 3 show similar regulation trends in phosphoregulation. Thus, a known signalling network implicated in AD synaptic dysfunction is disrupted not only at the transcriptional/translation level (Grabruker et al., 2011) but also at the post-translational level. Clearly, it will be essential to explore the functional significance of SHANK3 phosphorylation and its decrease during the transition from a pre-symptomatic to a symptomatic disease state in AD.

Our unbiased phosphoproteome analysis also reveals differential regulation of many proteins involved in cytoskeletal functions in symptomatic Tg mice. In particular, the dihydropyrimidinase-related proteins DPYSL1–5 (also named CRMP1–5) are known to

form homo-/hetero-tetramers and to mediate Semaphorin3A and neurotrophin signalling leading to cytoskeletal rearrangement (Yamashita and Goshima, 2012). Although their phosphorylation has been previously reported, the change in levels of phosphorylation found here during the symptomatic progression in a mouse model of A β deposition is novel (Table 5-2). Interestingly, it was recently demonstrated that the phosphorylation of DPYSL1 and DPYSL2 at a position S522 by the CDK5 are involved in axonal guidance and spine development and that the loss of S522 phosphorylation leads to abnormal dendritic patterning (Yamashita et al., 2012). Phosphorylation at S522 in DPYSL2 has been shown to decrease its binding affinity to tubulin and Numb, as well as facilitate its phosphorylation by glycogen synthase kinase-3 beta (GSK3 β) (Nishimura et al., 2003, Uchida et al., 2005, Cole et al., 2006). DPYSL2 is hyperphosphorylated in the human brain and some animal models of AD at both GSK3 β (T514) and CDK5 (S522) consensus sites (Cole et al., 2007). However, the phosphorylation of DPYSL3 at T509, by GSK3 β has not been found to increase in human AD at post-mortem analysis (Cole et al., 2007). Here, we also observed an increase in the ratio (Tg/NonTg) of CDK5-dependent phosphorylation of DPYSL1 (two-fold) and DPYSL2 (four-fold) but not DPYSL3 (not significant) at a position S522 upon conversion from the pre-symptomatic to the symptomatic stage. Our observed changes in phosphorylation at S522 are in agreement with what has been previously observed in human post-mortem tissues (Cole et al., 2007). In contrast, the changes in phosphorylation that are dependent on GSK3 β on DPYSL1 (S518, two-fold decrease), DPYSL5 (T514, two-fold decrease), and DPYSL2 and 5 (T509, no changes) were not detected or, surprisingly, changed in the opposite direction compared to what has been previously reported in human. Further, we observed an increase in phosphorylation at T524 (increase) of DPYSL3 in symptomatic Tg mice. The phosphorylation of DPYSL3 at

Table 5-2 Phosphopeptides Observed for the DPYSL Protein Family

Gene names	Modified sequence	Position	Two months	Six months	Signi- ficant
Dpysl1	HAAPAPpSAK	S518	0.4 ± 0.3	-0.88 ± 0.05	*
Dpysl1	pSSPSKHQPPPIR	S522	0.1 ± 0.2	1.0 ± 0.1	*
Dpysl1	pSIPHITSDR	S8	0.38 ± 0.04	-0.2 ± 0.1	*
Dpysl2	TVTPASpSAKpTSPAK	S518, T521	0.5 ± 0.1	-0.4 ± 0.4	
Dpysl2	pTSPAKQQAPPVR	T521	0.0 ± 0.4	2.0 ± 0.1	*
Dpysl2	TpSPAKQQAPPVR	S522	0.0 ± 0.4	2.0 ± 0.1	*
Dpysl2	NLHQpSGFSLSGAQIDNIPR	S537	0.4 ± 0.2	-0.5 ± 0.1	*
Dpysl2	TVpTPASSAK	T514	0.16 ± 0.09	-0.10 ± 0.06	*
Dpysl2	NLHQSGFSLpSGAQIDNIPR	S542	-0.1 ± 0.6	-1.08 ± 0.05	
Dpysl2	TVTPApSSAK	T517	0.1 ± 0.6	0.2 ± 0.2	
Dpysl2	GLYDGPVCEVSVpTPK	T509	0.4 ± 0.1	0.20 ± 0.03	
Dpysl2	pTVTPApSpSAKTSPAK	S517,S518	0.0 ± 0.6	-0.5 ± 0.5	
Dpysl3	GpSPTRPNPPVR	S522	-0.5 ± 0.1	-0.2 ± 0.1	
Dpysl3	GSPpTRPNPPVR	T524	-0.6 ± 0.1	-0.12 ± 0.10	*
Dpysl5	EMGTPLADpTPTRPVTR	T514	0.85 ± 0.05	-0.36 ± 0.04	*
Dpysl5	EM(ox)GpTPLADTPTRPVTR	T509	-0.9 ± 0.9	0.14 ± 0.01	

* indicates a *p*-value lower than 0.05 and a locFDR lower than 20%.

T524 has been previously postulated based on MALDI data (Zhou et al., 2008). Finally, we report a significant decrease in a new phosphorylation site at position S8 of the N-terminal of DPYSL1 in Tg compared to NonTg mice at both two and six months of age. Taken together, these data suggest that, in the TgCRND8 AD mouse model, changes in CDK5-dependent phosphorylation of DPYSL proteins recapitulate those detected in post-mortem human tissue whereas the GSK3 β -dependent events are not consistently reproduced. It is tempting to speculate that this disconnect between mouse and human data may, in part, contribute to the lack of cytoskeletal rearrangement in the TgCRND8 mouse model upon conversion to a symptomatic state and it would be interesting to establish whether older animals, representative of late-stage AD, would eventually exhibit this stepwise progression in dysfunctional phospho-signalling.

A similar intermediate pattern was detected with respect to phosphorylation and interaction between DPYSL2 and MAP1b (Good et al., 2004). Both DPYSL1 and DPYSL2 have been shown to interact with MAP1b in a GSK3 β -dependent manner (Lin et al., 2011). Interestingly, both DPYSL proteins and MAP1b are part of the semaphorin 3A signalling network and are phosphorylated by both CDK5 and GSK-3 β . We observed numerous phosphorylated peptides on MAP1b; however, only one was altered over time in Tg mice compared to NonTg controls (TIKpTPEDGGYTCEITEK) with over a two-fold decrease (casein kinase 2 site). A five-fold increase in the relative abundance of phosphorylated microtubule-associated protein 1A (MAP1a) (EpTSPTRGEPVPAWEGK; predicted PKA site) and a two-fold increase in both the GSK3 β (ARVDHGAEIITQpSPSR) and casein kinase 1 sites (VDHGAEIITQSPpSR) on microtubule-associated protein 2 (MAP2) were also detected upon symptomatic conversion in Tg mice. Again, the majority of GSK3 β phosphorylation sites on MAP1a, microtubule-associated protein 1B (MAP1b),

and MAP2 detected in both Tg and NonTg mice at two and six months of age did not change in this study which may, in part, also underlie why neuronal cytoskeletal integrity is not compromised in TgCRND8 mice exhibiting both significant A β plaque load and behavioural indices of learning and memory.

Changes in glia phospho-networks were also detected. A decrease in the abundance of MBP phosphorylated on two different sites was found without a change in total MBP levels (Figure 5-8). One of the primary functions of MBP is to participate in the adhesion of myelin by binding negatively charged lipids at the membrane on the cytosolic membrane leaflet of oligodendrocytes (Boggs et al., 2006). In this configuration, MBP can also act as scaffold for other molecules including SRC homology 3 domain-containing proteins (Polverini et al., 2008) and actin (Boggs et al., 2011). Interestingly, phosphorylation of MBP reduces its ability to attach actin to membrane microdomains enriched in negatively charged lipids as would be predicted in lipid rafts (Boggs et al., 2011). Thus, the decrease in MBP phosphorylation in symptomatic Tg mice may be indicative of a compensatory attempt to increase MBP scaffolding of actin and other molecules to the negatively charged lipids at the membrane, potentially within raft domains.

Finally, differences and similarities in post-translational murine tau modification were detected following symptomatic conversion in TgCRND8 mice compared to clinical AD. Hyperphosphorylated tau, aggregating into fibrils, protofibrils, and finally intraneuronal NFT is a hallmark pathology of AD. In this study, we quantified 13 phosphopeptides (Table 5-3) in murine tau. To assess relevancy to clinical AD, we aligned these phospho-amino acid sequences to that of the 441 amino acid isoform of human tau (NP_005901.2). Two phosphopeptides represented sites not conserved in the human sequence: GAAPSPAQK and TTPpSPKTPPGSGEPPKSGER. Ten tau

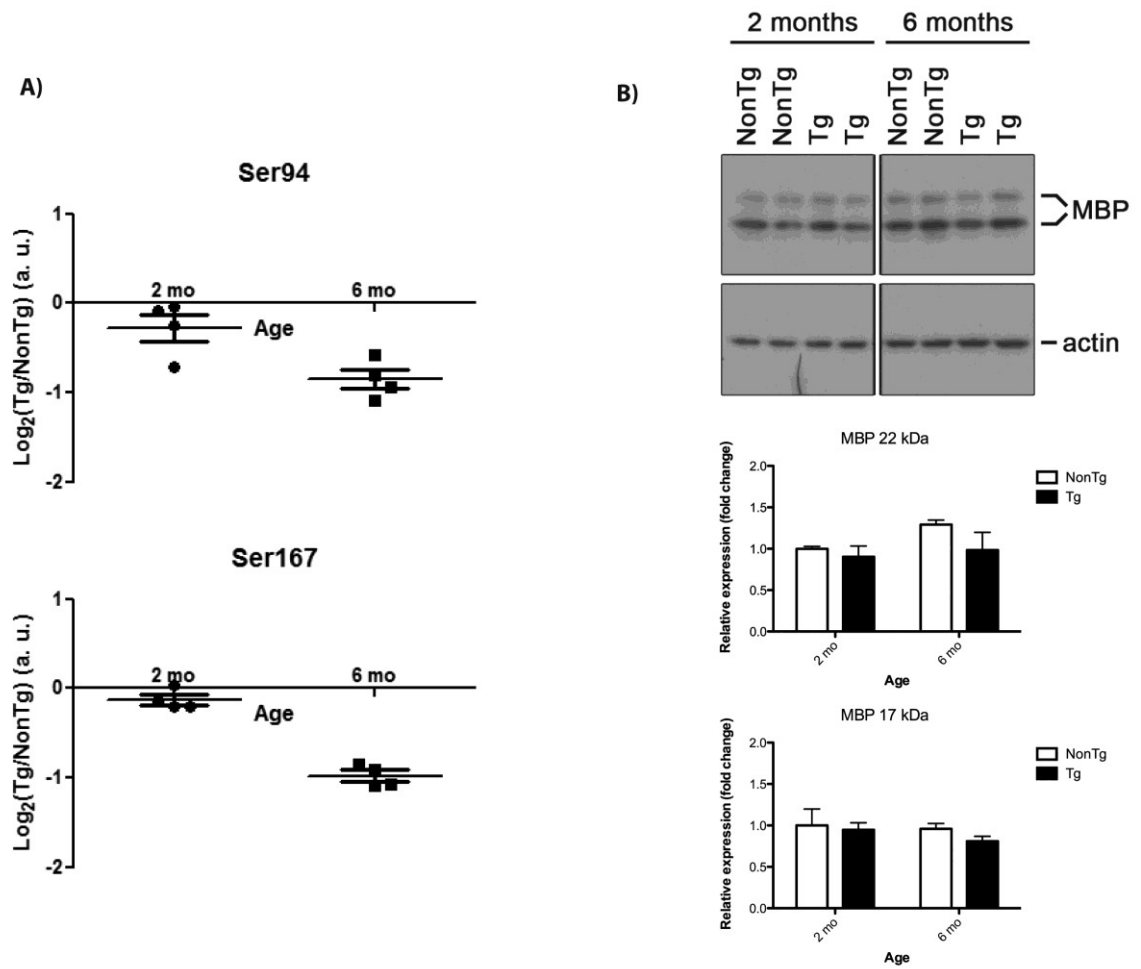


Figure 5-8

**Figure 5-8 Decreases in Phosphorylation of MBP Phosphorylation at S94, and S167
Without Alteration in Total Protein Levels are Detected in Symptomatic Tg Mice**

(A) Significant changes in phosphorylation of MBP and (B) Western blot analysis of two isoforms of MBP. Blots reacted for APP were stripped and reprobed for total MBP and thus share the same actin loading control (see Figure 5-4).

Table 5-3 Phosphopeptides Observed for the Tau Protein

Modified sequence	Position in the mouse canonical sequence (NP001033 698.1 /733 aa)	Position in the human canonical sequence (NP058519. 3 /758 aa)	Position in the human consensus sequence in the field (NP005901.2 /441 aa)	Two months	Six months
SGYSpSPGSPGTPGSR	Ser491	Ser516	Ser199	-0.2 ± 0.1	0.5 ± 0.1
SGYSSPGpSPGTPGSR	Ser494	Ser519	Ser202	0.0 ± 0.1	0.4 ± 0.1
TPpSLPTPPTREPK	Ser506	Ser531	Ser214	0.3 ± 0.1	0.4 ± 0.1
TPPKpSPSASK	Ser527	Ser552	Ser235	1.1 ± 0.1	-0.7 ± 0.1*
TPPKSPpSASK	Ser529	Ser554	Ser237	0.4 ± 0.1	-0.3 ± 0.2
TDHGAEIVYKpSPVVSGDpTSP	Ser688/ Thr695	Ser713/ Thr720	Ser396/ Thr403	0.2 ± 0.1	-0.1 ± 0.1
SPVVpSGDTSPR	Ser692	Ser717	Ser400	-0.4 ± 0.1	0.8 ± 0.1*
SPVVSGDTpSPR	Ser696	Ser721	Ser404	0.0 ± 0.1	0.3 ± 0.1
TTPSPKpTPPGSGEPPK	Thr473	Thr498	Thr181	-0.1 ± 0.1	1.1 ± 0.1*
VAVVRpTPPKSPSASK	Thr523	Thr548	Thr231	-0.1 ± 0.1	0.1 ± 0.1
SPVVSGDpTSPR	Thr695	Thr720	Thr403	-0.1 ± 0.1	0.3 ± 0.1
GAApSPAQK	Ser451	---	---	1.5 ± 0.7	0.1 ± 0.3
TTPpSPKTPPGSGEPPKSGER	Ser470	---	---	-0.1 ± 0.1	0.8 ± 0.2

* indicates a *p*-value lower than 0.05 and a locFDR lower than 20%.

phosphopeptides were found to be singly phosphorylated and one was shown to be doubly phosphorylated (S396 and T403). Of these, a two-fold increase in the abundance of tau phosphopeptides phosphorylated at T181 and S400, and a 3.4-fold decrease in abundance of phosphopeptides phosphorylated at S235 were detected upon conversion to a symptomatic state in Tg mice compared to NonTg control. Human tau fibrillization is precipitated by phosphorylation at T231, S262, T181, S199, S202, T212, S396, T403, and S404 (Wang et al., 2007b). We did not detect T212 and S262 phosphoresidues in Tg mice. Moreover, the abundance of protein phosphorylation at S199, S202, T231, T403, and S404 and the doubly phosphorylated S396 and T403 phosphorylation were not altered by age or genotype. Only T181 showed an increased phosphorylation in the TgCRND8 mouse model exhibiting impairment in behavioural indices of learning and memory. CDK5 has been shown to phosphorylate tau at S202, S235, and S404 (Illenberger et al., 1998). Phosphorylation by CDK5 at S235 primes tau for phosphorylation by GSK3 β at T231; phosphorylation by CDK5 at S404 primes tau for sequential phosphorylation by GSK3 β at S396 and S400 (Li et al., 2006). Surprisingly, we found that, in symptomatic TgCRND8 mice, phosphorylation at S235 was reduced between two and six months suggesting that CDK5 is not priming tau for phosphorylation by GSK3 β at T231 (wherein no change in phosphorylation was detected in this study). In contrast, GSK3 β -dependent phosphorylation of tau at S400 (but not S396) was observed to increase between two and six months without a concomitant phospho-priming by CDK5 at S404. These data highlight a primary difference between the TgCRND8 mouse model of AD and the human condition. Moreover, hyperphosphorylation at T181, S202, and T231 has been shown to occur early in the formation of NFT whereas hyperphosphorylation at S396 and S404 occur later in the aggregation process (Noble et al., 2003). Here, only phosphorylation at T181 of

tau increased upon conversion to a symptomatic state. These differences hint at underlying mechanisms through which tau hyperphosphorylation but not aggregate assembly into NFT is observed in the TgCRND8 mice (Chishti et al., 2001, Ryan et al., 2008).

5.8 Conclusion

In summary, our study of the hippocampal phosphoproteome in pre-symptomatic and symptomatic TgCRND8 mice, normalized-to-age-dependent changes in control animals, identifies network-wide changes in protein phosphorylation associated with A β accumulation, extracellular deposition, and learning and memory impairment. Targets include phospho-dependent pathways linked to AD as well as differences between murine and human conversion to a symptomatic state that may underlie differences between murine models of A β deposition and the human AD condition.

CHAPTER 6. GENERAL DISCUSSION

This thesis addressed the hypothesis that a dysregulation of PC metabolism elicited by A β ₄₂ and exacerbated by age is, in part, required for AD conversion. To test this hypothesis, two new bioinformatic tools were developed to facilitate this investigation: RTStaR (described in Chapter 2) and VaLID (described in Chapter 3). RTStaR addresses the problem of aligning multiple spectra collected using the same HPLC-ESI-MS methodologies over extended periods of time and from different tissues; VaLID assists in the identification of species following alignment. Using these tools, PC metabolites were profiled in the plasma of young adults, cognitively normal elderly with vascular impairment, MCI and LOAD patients. They were also profiled in the entorhinal-hippocampal circuit of post-mortem AD patients, TgCRND8 mice, and over the course of the murine lifespan (described in Chapter 4). Because a neurolipidomic approach encompasses not only the comprehensive profiling of lipid composition in the CNS, but also a detailed examination of the enzymes and effector protein responsible for compositional changes and downstream signalling, I sought to provide further mechanistic insight using an unbiased phosphoproteomic approach to identify signalling pathways and potential protein effectors of lipid changes associated with AD phenoconversion (described in Chapter 5). Taken together, these studies provide converging evidence that aberrant PC remodelling, notably the accumulation of PC metabolites through impairment of the Land's cycle, is part of AD pathology.

6.1 RTStaR and VaLID: New Bioinformatic Lipidomic Tools

Neurolipidomes are composed of multiple samples that are analyzed individually by HPLC-ESI-MS (Figure 6-1) (or other lipidomic MS methodologies). Aligning the same

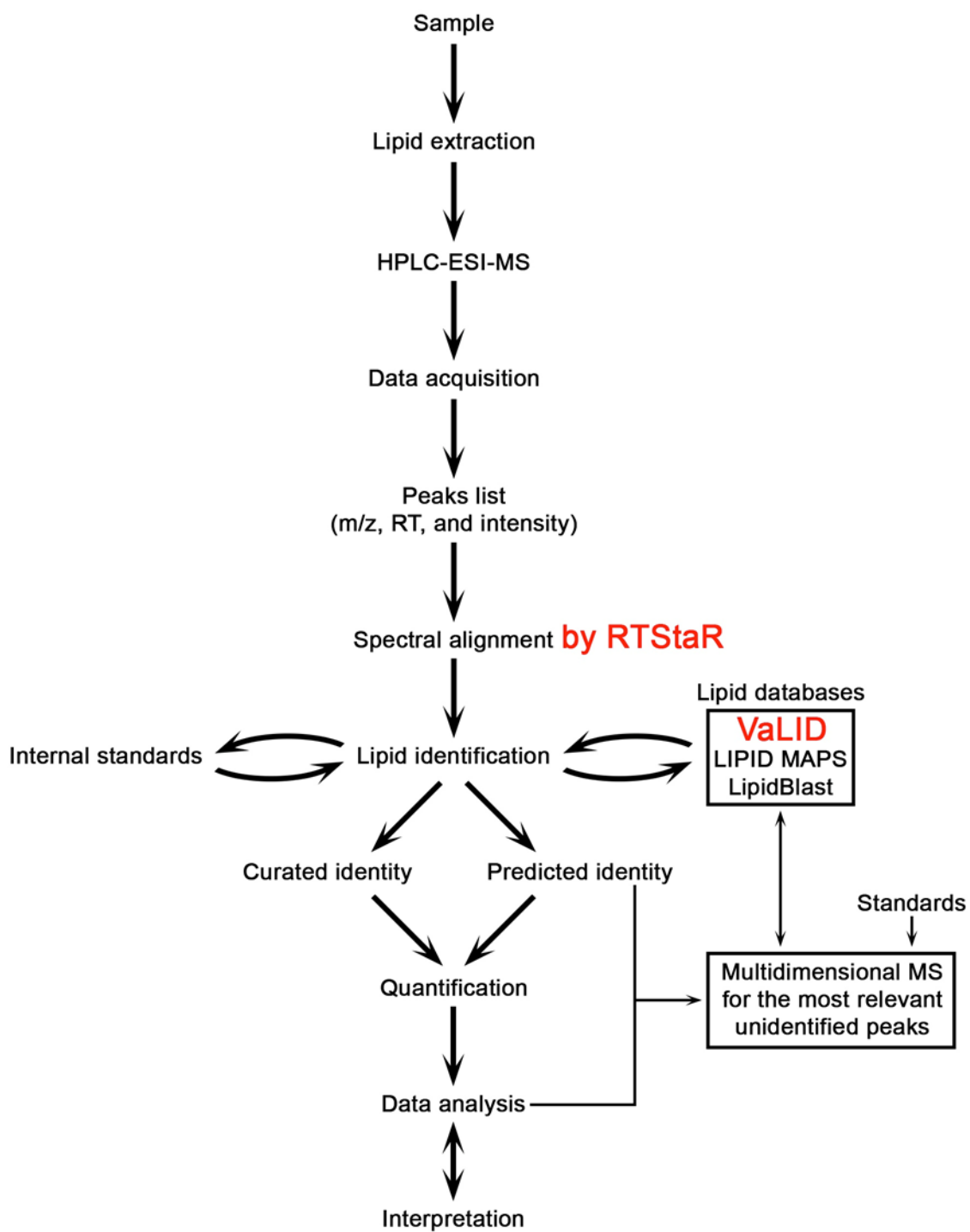


Figure 6-1

Figure 6-1 Lipidomic Analysis Pipeline

The two tools developed, RTStaR and VaLID, are shown in the context of the complete lipidomic pipeline from the sample preparation to the interpretation of the data. RTStaR plays a key role in lipidomics since it brings an objective alignment to the species, increasing reproducibility and facilitating the identification and quantification processes. VaLID is one of few databases that facilitate lipid structural identification. Adapted from Yetukuri et al. (2007).

species across MS runs is essential to accurately quantify their abundance. To address issues of run to run variations in chromatography, I designed the RTStaR algorithm (Figure 6-1) packaged in two Excel workbooks that aligned and standardized the RTs across lipidomes. Placed in context with the literature, RTStaR is one of approximately 50 algorithms developed to address issues in correspondence with HPLC-ESI-MS over the course of this thesis [for a recent review see (Smith et al., 2015)]. The majority of the algorithms, including RTStaR, are warping functions (Smith et al., 2015). Warping functions distort RTs to compensate for system-level variations between runs thereby aligning RTs of identical species across runs allowing for their direct correspondence. Alternative approaches attempt to match directly analytes using other characteristics, including the charge state and isotopic envelope (Smith et al., 2015). GPs are difficult to analyze in this fashion. For example, direct correspondence cannot be achieved with PC species since they have similar charge states and isotopic envelopes. Hence, warping functions are more appropriate.

A weakness and a strength of RTStaR, is the use of exogenous standards as anchor points to estimate the system-level variation between runs. In a dense spectrum, it is admittedly challenging to include additional species yet this practice will enable more precise normalization in subsequent quantification steps. This feature is a common warping challenge. IRs have been successfully used not only by RTStaR, but also by the *Chrompare* algorithm (Frenzel et al., 2003). While *Chrompare* uses an arbitrary five internal standards, RTStaR requires approximately one standard per minute of RT. Thus, RTStaR is flexible since it can use different standards in accordance with laboratory practice, in contrast to *Chrompare* that relies on fixed standards. Accuracy of both RTStaR and *Chrompare* is limited by the range of the standards applied (Frenzel et al., 2003). The

use of exogenous standards avoids anchoring the spectrum to species that are assumed to be the same, as is applied in the RANSAC aligner (Pluskal et al., 2010). Moreover, RTStaR can be accomplished in one step in contrast to PEPpeR and SuperHirn which applies a rough alignment based on pattern recognition and, then a fine alignment, based on the species identified. As a result, PEPpeR and SuperHirn alignment requires intensive computational power and time (Jaffe et al., 2006, Mueller et al., 2007). As an example, PEPpeR must be run on a computer cluster (Jaffe et al., 2006). RTStaR, however, can be handled by any personal computer equipped with Excel with alignment of more than a 1000 runs achieved within an hour. It is applicable to targeted lipidomics as it does not require a high-precision MS as do other methods (Anderle et al., 2004, Silva et al., 2005). The Excel workbooks can be easily modified to be adapted to the specific needs of each laboratory. In contrast to SuperHirn, RTStaR users do not have to optimize parameters (Mueller et al., 2007). Furthermore, like IDEAL-Q (Tsou et al., 2010), RTStaR can be used to predict the RT of target species through comparison with a calibrator dataset.

There is room for further improvement. Practically, RTStaR has not been coded for automatic input directly from MS analysis packages thus data must be imputed by the user. In addition, it suffers from the necessity of relying on external statistical software for the ROUT outlier identification test. Moreover, RTStaR is unable to match the species assessed using different HPLC-ESI-MS methodologies although this deficit is inherent to all correspondence algorithms developed to date. This is expected since changing the chromatographic methods, i.e., beads, solvent composition and gradient, temperature, and pressure, will change the not only elution time of target lipids and but also can alter the elution order of isobaric species (Smith et al., 2015).

A second challenge faced over the time-span of this thesis was a lack of curated databases composed of neural GP. Thus, my PC neurolipidome profiling identified many species with m/z that were absent in other databases including LIPID MAPS. To address this need, I applied an *in silico* approach and developed VaLID (Figure 6-1), a search engine linked with a comprehensive GP database that contained all the theoretically possible species. VaLID complements LIPID MAPS, the lipidomic gold standard database, as it includes species not yet profiled by the consortium. VaLID and LIPID MAPS use different strategies to identify lipid species. LIPID MAPS offers fewer, but *bona fide* species limiting the number of possibilities that users must validate. VaLID employs an *in silico* approach to return all possible species. VaLID's exhaustive GP database contains more than 1,473,168 species, while other databases such as LipidBANK (Yasugi and Watanabe, 2002) and LIPID MAPS (Fahy et al., 2005, Fahy et al., 2009, Fahy et al., 2011) contain 310 and 9,387 species as of August 2015. The LipidBANK and LIPID MAPS databases focus mainly on curated lipid species from mammalian cells. For example, LIPID MAPS primarily contains lipids that have been profiled from the mouse macrophage cell line RAW 264.7 (Fahy et al., 2005, Fahy et al., 2009). However, lipid composition varies in different cells and tissues leading to an astounding diversity that cannot be represented by the profiling of one cell type (Gottfried, 1967). VaLID returns GP species possibilities for any m/z from any tissue, fluid, or cell. To limit choices, VaLID also highlights curated species from our team in red or indicates the most biologically abundant predicted species based on prevalence of hydrocarbon chains in food sources in blue. Like the LIPID MAPS search engine, a VaLID search can be restricted to a specific headgroup, i.e., PC. Two types of databases further assist identification: those built from pure chemical standards, including HMDB (Wishart et al., 2009), LIPID MAPS (Schmelzer et

al., 2007), MassBank (Horai et al., 2010), Metlin (Smith et al., 2005), and the US National Institutes of Standards and Technology (<http://chemdata.nist.gov/mass-spc/msms-search/>) and those built from chemical prediction also generated *in silico* such as LipidBlast (Kind et al., 2013) and Lipid-Pro (Ahmed et al., 2014).

6.2 Altered PC Metabolism is a Defining AD pathology

6.2.1 Altered PC remodelling is part of the AD “change of state”

PC metabolism is clearly altered in AD as shown here in the plasma, hippocampus, and posterior temporal/entorhinal cortex. Plasma changes have previously been reported in the circulation (Schaefer et al., 2006, Mapstone et al., 2014, Whiley et al., 2014). In total, we detected 175 PC species in the circulation: 102 structural PC and 73 PC metabolites. As previously shown by Mapstone et al. (2014), we show that structural PC and PC metabolites correlate with memory deficits; however, our study shows that plasma PC and their PC metabolites species are increased in AD. These results contrast that of Mapstone et al. (2014) which report the decrease of 10 structural PC and PC metabolites in the MCI/AD group and from Whiley et al. (2014) which demonstrate the decrease of three structural PC. As discussed in Chapter 4, these differences may reflect the differences between our control groups. Unlike previous studies, all our control patients share vascular pathologies with our MCI and LOAD patients, a comorbid pathology for AD (Justin et al., 2013).

Unlike previous studies, our lipidomic capacity affords greater coverage of PC metabolites than previously published results. Our study profiled 73 PC metabolites which were divided into multiple PC metabolites subfamilies. Of these species, 10 LPC species were held in common with Mapstone et al. (2014): [468.3b] PC(14:0/0:0), [496.3b]

PC(16:0/0:0), [494.3b] PC(16:1/0:0), [510.4a] PC(17:0/0:0), [524.4b] PC(18:0/0:0), [522.4b] PC(18:1/0:0), [520.3b] PC(18:2/0:0), [546.3c] PC(20:3/0:0), [544.4b] PC(20:4/0:0), and [608.5c] PC(24:0/0:0). This difference in the number of PC metabolites detected between these profiles is dictated by the methodology. (1) Our lipid extraction protocol had been optimized for the PC metabolites recovery (Xu et al., 2013). (2) Mapstone et al. (2014) used a kit that is designed to profile only 14 LPC metabolites and no other PC metabolites subfamilies. Of the LPCs common to both studies, seven were not altered over the course of phenoconversion (Mapstone et al., 2014) or in MCI and LOAD patients here. We found that three species, reported in both studies [468.3b] PC(14:0/0:0), [494.3b] PC(16:1/0:0), and [522.4b] PC(18:1/0:0) significantly increased in LOAD and MCI. The last species will be taken as an example to discuss the problem of quantification and interpretation of lipidomic profiles collected using different methodologies. We report an increase in [522.4b] PC(18:1/0:0) from 776 to 1458 nM between controls with vascular impairment and LOAD patients while Mapstone et al. (2014) demonstrated a decrease of approximately 47 to 41 nM in the plasma of their MCI/AD group. Two inconsistencies occur: the quantification of the same species gives fundamentally different concentrations and the changes observed are in opposite directions.

Quantification is affected by the methodology. Mapstone et al. (2014)'s extraction protocol is optimized to extract a broad range of biological molecules including lipids. This extraction is achieved in a less hydrophobic milieu than ours, thus it will lead to a lower recovery of phospholipids. A lower recovery decreases the amount of species quantified by MS and thus affects the final concentration after normalization. This quantification is also affected by the normalization methodologies. Our quantification was first corrected for MS response across runs and, then adjusted to account for extraction

efficiency. pmol/mg quantitation was established relative to signal obtained using the exogenous standard PC(13:0/0:0). The use of a different species from the same PC subfamily will give similar, but not identical ionization efficiencies per pmol, leading to what is called absolute quantification per standard equivalent. Like us, Mapstone et al. (2014) normalized peak areas to isotope-labelled exogenous standards. But, they did not provide the identity of these standards nor is there any report of how they accounted for variations in extraction efficiency and MS reproducibility beyond a statement indicated that the average percent coefficient of variation in quality control runs of the same sample averaged between 8 and 15%. This does not, in any way, discount the calculations made in either studies, but they are simply highlighted in this thesis to emphasize the need to standardize quantification methods or, at very least, provide a detailed description of the procedures employed to allow for comparative data analysis.

The disconnect in the direction of response (increase vs decrease) is more complicated. First, as aforementioned, the simplest explanation lies in the difference between our controls. Cognitively normal elderly without clinical vascular impairment may present plasma profiles significantly higher than our controls and our MCI and LOAD patients. Thus, our data may reflect differences in LOAD independent of vascular disease while other published profiles may reflect both comorbid and LOAD-specific changes. We are currently testing this hypothesis directly. Second, Mapstone et al. (2014) combined patients who phenoconverted to MCI and LOAD. We treated both groups separately. Third, Mapstone et al. (2014) exploited the high resolution mass accuracy of time-of-flight-MS to identify lipid species. This powerful tool, however, does not distinguish between isomeric species. Using our targeted lipidomic approach, we distinguished between two isobaric lipids PC(O-16:2/2:0) and PC(18:2/0:0), necessarily reported as a single species

using only high resolution mass accuracy of time-of-flight-MS. The combined impact of PC(18:2/0:0) and PC(O-16:2/2:0), each with different disease-dependent responses in our hands, likely affects total quantification. In consequence, their reduction could be mainly due to the reduction of PC(O-16:2/2:0) in the MCI group, notably if the MCI patient number was higher than LOAD in the published study. Clearly, these issues will be resolved once our analysis of additional control groups is completed.

6.2.2 An increase in Land's cycle PC metabolism in AD is linked to aging and A β

Here we show that aging results in a general increase of PC metabolites in the circulation and in the brain due to enhanced PC remodelling. These findings are consistent with previously reported data (Pettegrew et al., 1990, Klein, 2000, Rappley et al., 2009). All the LPC species detected by Rappley et al. (2009) in mice were found in our study. In their study, PC(16:0/0:0), PC(20:4/0:0), and PC(22:6/0:0) were not altered. PC(18:0/0:0) was decreased while PC(18:1/0:0) and PC(20:1/0:0) were increased (Rappley et al., 2009). Our study demonstrated the same differences over aging with one exception, PC(18:0/0:0). We find PC(18:0/0:0) significantly increases over the course of aging. This discrepancy is likely explained by the regional differences in the PC landscape reported. While Rappley et al. (2009) pooled brain regions (cortex, hippocampus, striatum, and cerebellum) of mice, we specifically assessed the parietal-temporal cortex. We have shown in humans that the entorhinal-hippocampal circuit exhibits regionally distinct lipid profiles.

We specifically linked PAF changes to risk of LOAD associated with aging. These changes were associated with increases in cPLA₂ activity and decreases in levels sPAFAH isoform consistent with a specific impairment of AAPC (PAF) metabolism. Our study is the first, to my knowledge, to follow the change of PC metabolism throughout aging

progression. These data show that the main alterations in the PAF and LPC families are occurring between 8 and 12 months of age. The human age equivalencies correspond approximately to 45 to 57 years old (Fox, 2007). Interestingly, this timeline matches the predicted preliminary metabolism alteration occurring 20-30 years before AD onset (Davies et al., 1987, Blennow et al., 2006). We further find that A β accumulation converges on these age-associated changes in PC metabolism through the remodelling pathway (Kriem et al., 2005, Ryan et al., 2008, Sanchez-Mejia et al., 2008a). A β accumulation activates cPLA₂ and alters the substrate specificity of the LPCAT favouring the formation of PAF species (Kriem et al., 2005, Ryan et al., 2008, Sanchez-Mejia et al., 2008a). Our compositional changes are consistent with these metabolic impairments.

6.2.3 AD altered protein phosphorylation level is a quantifiable metabolic “change of state”

This thesis also demonstrated that the hippocampal phosphoproteome of the TgCRND8 mouse model of AD is altered, as has been shown in AD substantia nigra and frontal cortex by Zahid et al. (2012) and in AD hippocampus by Di Domenico et al. (2011). Our study reports that 85 out of 293 (29 %) phosphoproteins were altered in TgCRND8 hippocampus after phenoconversion while 9 out of 72 (13 %) (Zahid et al., 2012) and 13 out of an unknown number of total phosphoproteins changed in AD hippocampus analyzed post-mortem compared to elderly controls (Di Domenico et al., 2011). Fewer phosphoproteins in the Zahid et al. (2012) study could be explained by the difference in their definition of significant phosphoproteins. This definition included a fold change cut-off in order to ensure biological relevance. This cut-off is generally set between 1.3 to 2 fold (Mann and Kelleher, 2008). While we used a 1.5 fold, Zahid et al. (2012) use a much

more stringent cut-off of 2 fold (Zahid et al., 2012). As discussed in Chapter 5, we identified both AD-relevant changes in tau phospho-epitopes that corresponded with human consensus sequences, although no relevant changes in phosphorylation of PC remodelling enzymes, a novel phosphorylation linked to binding of negatively charged lipids to the myelin sheath, were identified.

6.2.4 Summary: AD mechanistic insight

Taken together, this thesis identifies changes in PC metabolism and protein phosphorylation associated with AD pathology. Clearly, additional work is required to address the differences between the plasma lipidomic data reported here and those previously published (Mapstone et al., 2014, Whiley et al., 2014). Ongoing work is currently being performed to profile cognitively normal elderly without cardiovascular or cerebrovascular complications to determine if the increases in PC composition seen here in MCI and LOAD circulation in fact reflect an overall reduction in PC abundance associated with vascular insufficiency when compared to elderly controls without vascular complications. In addition, we are profiling the plasma of patients diagnosed with dementia with Lewy Bodies to determine whether the changes reported here are specific to LOAD or common across different neurodegenerative diseases.

There is considerable excitement surrounding the idea that lipid subfamilies and particular lipid compositions may participate in signalling AD pathology. Lipids have, for too long, been considered as unitary entities, wherein PC families, for example, are represented by single, most generic, species. For example, we and others have shown that closely related lipid species differing by only two carbons have different signalling and biological effects (Callender et al., 2007, Ryan et al., 2008). Elucidation of specific and

family-wide signalling pathways is still in the early days and little is known about the biological effects of many of the metabolites identified here.

We showed here that, both PAF and LPC metabolites accumulate in plasma and entorhinal-cortex in both clinical and experimental samples and, taken together, that elevations associate with disease, age, and early A β exposure. A model is presented in Figure 6-2 that hypothesizes how PAFs and LPCs at the molecular level may contribute to AD pathology considering their crucial roles in inflammation including the recruitment of microglia, in A β oligomerization and toxicity, in neurotransmission, in regulation of blood vessel and blood brain permeability, and finally, in cytoskeletal integrity and cellular morphology (Cabellos et al., 1992, Aihara et al., 2000, Qiao et al., 2006, Lu et al., 2007, Moriguchi et al., 2010, Fang et al., 2011, Semini et al., 2011, Sheikh and Nagai, 2011, Fang et al., 2014, Yuan et al., 2014, Sheikh et al., 2015). For example, PAF activates JNK (Cuschieri et al., 2005). JNK phosphorylates the scaffolding protein SHANK3 (Kunde et al., 2013) shown, in part, to regulate cellular morphology (Cochoy et al., 2015). Intraneuronal accumulation of PC(O-16:0/2:0) activates GSK3 β through an ER-stress-calpain cascade (Ryan et al., 2009a). DPYSL2 has been suggested to be phosphorylated by GSK3 β (Rigbolt et al., 2011). We show here that when PAF levels are elevated at phenoconversion the abundance of phospho-DPYSL2 increases possibly as a result of these changes in PAF abundance. These changes may underlie the change in cellular morphology observed in other cell systems in response to sustained exposure of different PAF family members (Semini et al., 2011). The changes reported here in Land's cycle metabolism with age are predicted to exacerbate this vicious cycle resulting in further

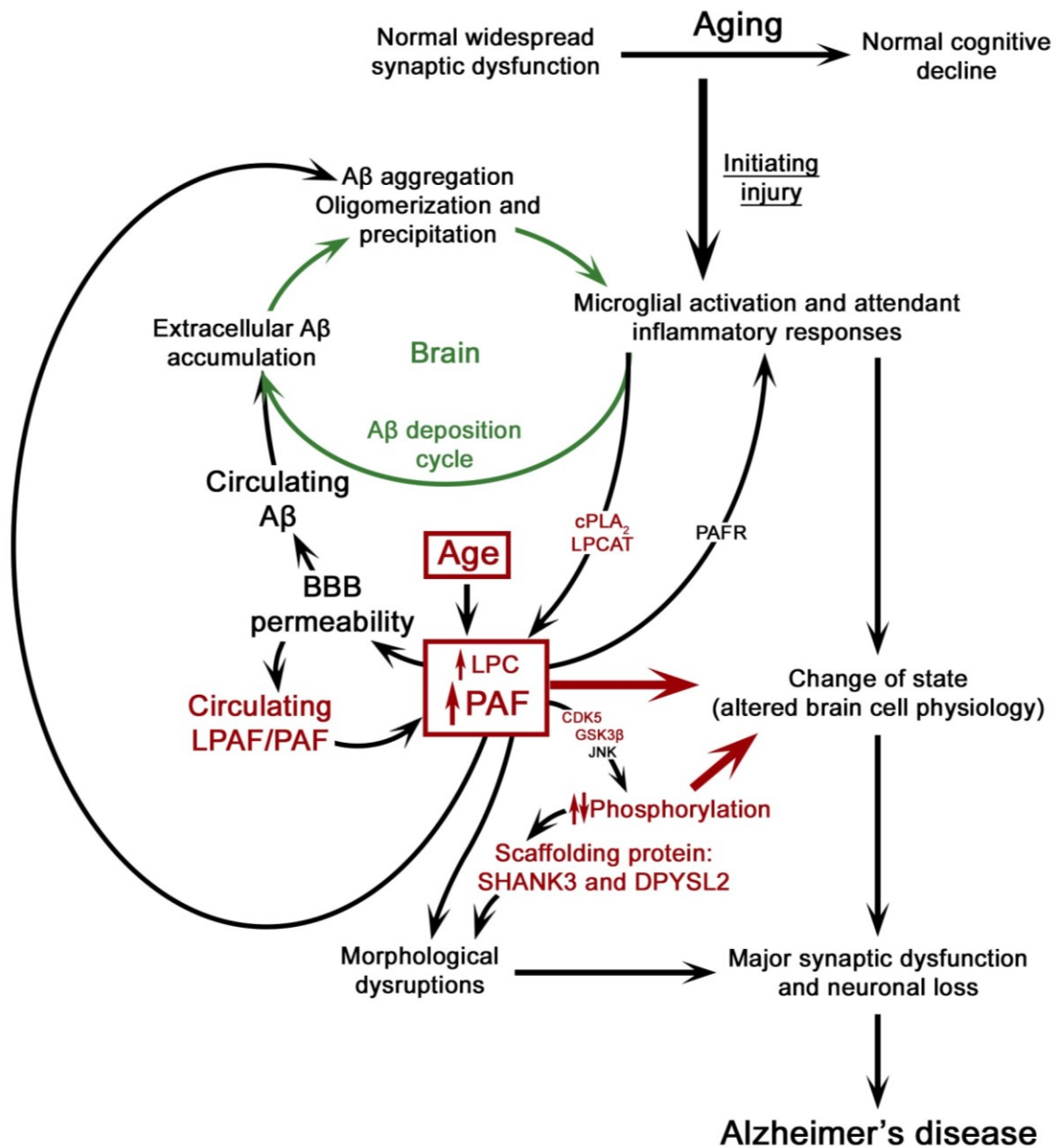


Figure 6-2

Figure 6-2 Central Role Played by PAF and LPC in the Underlying AD Signalling

Increase in PAF and LPC level take place during aging and is exacerbated by A β accumulation. Their increased level in AD could put in place deleterious feedback loops with inflammation and A β deposition cycle. In addition, they could disrupt the neuronal morphology and neurotransmission. The propose role of PC metabolites have been embedded in Herrup (2010)'s age-dependent AD hypothesis.

accumulation of PAFs and LPCs (Szabo et al., 1993, Mori et al., 1996, Aihara et al., 2000, Meyer zu Heringdorf and Jakobs, 2007) that propagate these injuries and arguably play a role in precipitating phenoconversion. Intervening in these converging metabolic cascades represents a testable strategy to delay, or perhaps one day halt, AD at its preclinical stage.

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APPENDIX 1: VaLID 2.0.0 User Guide

To help the reader, VaLID user guide has been attached to this thesis.

VaLID: Visualization and Phospholipid Identification a glycerophospholipid m/z-based prediction database

User guide
Version 2.0.0

Developed by:

Graeme S.V. McDowell, Alexandre P. Blanchard, and Nico Valenzuela as part of the Canadian Institute of Health Research (CIHR) Training Program in Neurodegenerative Lipidomics.

Citations:

Blanchard AP, McDowell GSV, Valenzuela N, Xu H, Gelbard S, Bertrand M, Slater GW, Figeys D, Fai S, Bennett SAL (2013) Visualization and Phospholipid Identification (VaLID): An online integrated search engine capable of identifying and visualizing glycerophospholipids with given mass. *Bioinformatics*, 29: 284-285

And/or:

McDowell GSV, Blanchard AP, Taylor GP, Figeys D, Fai S, Bennett SAL (2013) Predicting glycerophosphoinositol identities in lipidomic datasets using VaLID (Visualization and Phospholipid Identification) – an online bioinformatic search engine. Submitted to *BioMed Research International*

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Online at:

<https://www.med.uottawa.ca/lipidomics/resources.html>

For tech and resource support:

Please email: lipawrd@uottawa.ca



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I. What is VaLID?

“Visualization and Phospholipid Identification” (VaLID) is a web-based application linking a convenient search engine, a phospholipid database, and multiple visualization features for the identification and dissemination of large-scale lipidomic datasets. Given the still developing nature of the lipidomic field, many mass to charge (m/z) values are not yet represented in open-access web-based engines. To address this need, VaLID returns all theoretically possible species based on user-defined m/z and MS conditions in addition to highlighting species identified by members of the Canadian Institutes of Health Research (CIHR) Training Program in Neurodegenerative Lipidomics (CTPNL) as of high interest to the neuroscience field. The intent is to complement existing curated databases by enabling users to predict the identity of “new”, unrepresented, species detected in their particular mass spectrometry (MS) datasets and thus proceed more rapidly to validation. While useful for lipid discovery, the user is cautioned that VaLID includes lipids (and isomeric bond configurations) that may not be biologically relevant. Investigators are encouraged to mine the presented possibilities for species most relevant to their specific biological system for subsequent validation. To assist in decision-making, a “best guess” feature is available whereby lipids listed in blue are predicted to be the most likely based on the prevalence of constituent fatty acid chains in mammalian cells (Miyazaki and Ntambi, 2008). Every theoretical conformation (in *cis* configuration) for each species can be viewed in 2D and 3D. In this version, curated species detected in brain tissue by members of the Canadian Institutes of Health Research (CIHR) Training Program in Neurodegenerative Lipidomics (CTPNL) can also be downloaded in multiple high-resolution representations for further visualization and model production.

In version 2.0.0, the VaLID search engine encompasses:

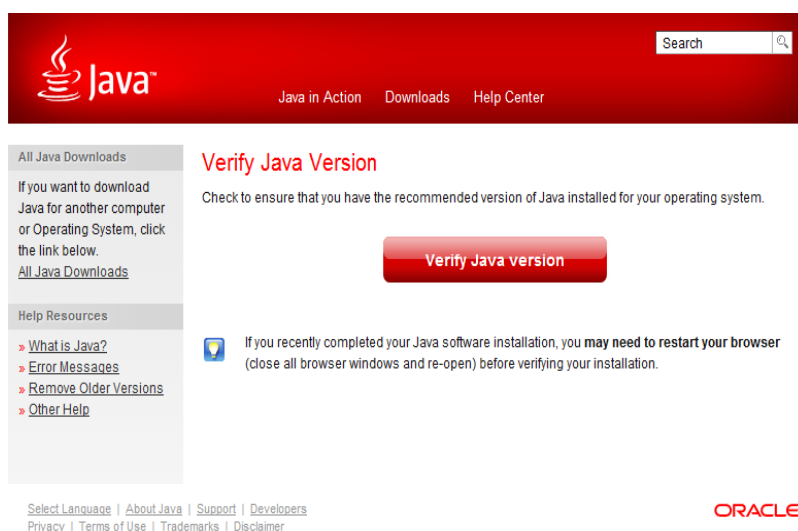
PA [GP10]: glycerophosphates
PPA [GP11]: glyceropyrophosphates
PC [GP01]: glycerophosphocholines
PE [GP02]: glycerophosphoethanolamines
PG [GP04]: glycerophosphoglycerols
PGP [GP05]: glycerophosphoglycerophosphates
PI and PIP_x [GP06-09]: glycerophosphoinositols and glycerophosphoinositol mono-, bis- and tris-phosphates
PS [GP03]: glycerophosphoserines
CDP-DG [GP13]: cytidine 5'-diphosphate glycerols

VaLID follows the LIPID MAPS classification and nomenclature system in lipid naming (Fahy et al., 2011).

II. Using VaLID

1. VaLID requirements

- 1.1. An Internet connection is required.
- 1.2. VaLID is accessible at <https://www.med.uOttawa.ca/lipidomics/resources.html>
- 1.3. VaLID 2.0.0 has been tested to work with Java™ v7 on the following web browsers:
 - 1.3.1. Safari: v6.1 - OS X
 - 1.3.2. Opera: v17.0.1241.53- Windows
 - 1.3.3. Firefox: v25.0 - Windows; v25.0 - OS X
 - 1.3.4. Internet Explorer: v10.0.9200.16721 - Windows
 - 1.3.5. Chrome: 30.0.1599.101 m - Windows
- 1.4. The help feature and structure drawing features of VaLID require that the user enable pop-up windows for the site.
- 1.5. Before starting VaLID, verify that Java™ is installed and up to date on your computer. Java is a programming language required for VaLID to function and it is free to download. Go to <http://java.com/en/download/installed.jsp> and click on “Verify Java version.”



- 1.6. If you do not have Java™ installed, follow the instructions on the Java website to install. Alternatively;

For Microsoft Windows, the latest Java version can be found at:
<http://java.com/en/download/manual.jsp>.

For Apple OS X, Java can be updated through Software Update (for more information concerning Software Update, go to: <http://support.apple.com>).

2. Running VaLID

2.1. You will be asked to accept the security certificate for our lipid database. This message will not appear again once you have accepted the certificate.

CIHR Training Program
Neurodegenerative Lipidomics



VaLID: Visualization and Phospholipid Identification

a glycerophospholipid m/z prediction database

Developed by Graeme S.V. McDowell, Alexandre P. Blanchard and Nico Valenzuela

Version 1.0.1

Security Warning

Do you want to run this application?

 Publisher: UNKNOWN
Location: http://134.117.226.70:8888

Running applications by UNKNOWN publishers will be blocked in a future release because it is potentially unsafe and a security risk.

Risk: This application will run with unrestricted access which may put your computer and personal information at risk. The information provided is unreliable or unknown so it is recommended not to run this application unless you are familiar with its source
[More Information](#)

Select the box below, then click Run to start the application

☐ I accept the risk and want to run this application.

Run

Cancel

GETTING STARTED

FAQs

LIPID MODELS

USER GUIDE

CITE US

CONTACT US

Please enable popups to access the structural databases and the help icons. Thank you.
The first search time may take up to 30 sec. depending upon the connection speed, and search options selected. Subsequent searches will be much faster.

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Carleton University - Faculty of Engineering and Design: Azrieli School of Architecture and Urbanism - University of Toronto - Centre for Research in Neurodegenerative Disease

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In some browsers, VaLID will not start until you click once in the center of the browser window to initiate Java. Once properly loaded, this window will appear:

CIHR Training Program
Neurodegenerative Lipidomics

VaLID: Visualization and Phospholipid Identification

a glycerophospholipid m/z prediction database

Developed by Graeme S.V. McDowell and Alexandre P. Blanchard and Nico Valenzuela

Version 0.9.0

☒ Exact Mass

☐ Average Mass

Ionic Mass (m/z):

Ionic Mass

Chain Lengths:

Even Chains

Mass Tolerance ($\pm$ m/z):

1.0

Lipid Subclass:

PC

Fatty Chain Linkage:

All

Ion:

$[M+H]^+$

Search

Cancel

? Display All

? Best Prediction

? Structural Representations

Possible Lipids Include:

Possible Isomeric Lipids Include:

GETTING STARTED

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Please enable popups to access the structural databases and the help icons. Thank you.
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Carleton University - Faculty of Engineering and Design: Azrieli School of Architecture and Urbanism - University of Toronto - Centre for Research in Neurodegenerative Disease

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3. Searching VaLID's glycerophospholipid database

3.1. Choose Exact Mass or Average Mass

3.2. Input the m/z of your lipid of interest into the "Ionic Mass" field.

3.3. Use the drop-down menus to select:

3.3.1. Chain Lengths: Even Chain, Odd Chain or All

3.3.2. Mass Tolerance: ± 0.0001 to ± 2

3.3.3. Lipid Subclass: PA, PPA, PC, PE, PG, PGP, PI and PIP_x (PI[3]P, PI[4]P, PI[5]P, PI[3,4]P₂, PI[3,5]P₂, PI[4,5]P₂, and PI[3,4,5]P₃), PS and CDP-DG.

3.3.4. Fatty Chain Linkage: All, Alkenyl-Acyl, Alkyl-Acyl, Diacyl, Dialkyl, or Mono-Acyl, Alkyl and Alkenyl.

3.3.5. Lipid ions: [M+H]⁺, [M+K]⁺, [M+Li]⁺, [M+Na]⁺, [M-H]⁻ or [M(Neutral)].

Note: The glycerophospholipid database contains phospholipids with fatty chains from 0 to 30 carbons and up to 6 cis unsaturations. The differential combinations of these individual fatty acyl chains at the *sn*-1 and *sn*-2 positions with ester, ether, or vinyl ether linkages lead to the formation of the majority of biologically relevant lipids.

3.4. When all of the search options are selected based on your MS conditions, click on the "Search" button or press the "Enter" key on the keyboard.

Note: The program currently does not support parallel searches of multiple lipid species in different tabs of the same web browser.

3.5. Initial searches may take longer than a minute, depending upon the connection speed, and search options selected. Why? When a new search is started the program loads all the lipids with the mass you are searching for into memory, as well as lipids with mass ratios 25 m/z above and below the target mass. This cached buffer of lipids with 50 m/z of your target range allows for subsequent searches close to the original search to occur much faster than if the program reloaded the database each time.

- 3.6. The possible lipids based on the search criteria will appear in two panels, (i) “Possible Lipids Include” and (ii) “Possible Isomeric Lipids Include.”

VaLID: Visualization and Phospholipid Identification
a glycerophospholipid m/z prediction database
Developed by Graeme S.V. McDowell and Alexandre P. Blanchard and Nico Valenzuela
Version 0.9.0

CIHR Training Program
Neurodegenerative Lipidomics

☒ Exact Mass
☐ Average Mass

Ionic Mass (m/z):

Chain Lengths: Even Chains

Mass Tolerance ($\pm$ m/z): 0.50

Lipid Subclass: PC

Fatty Chain Linkage: All

Ion: [M+H]⁺

Search
Cancel

? Display All
? Best Prediction
? Structural Representations

Possible Lipids Include:		Possible Isomeric Lipids Include:	
PC(10:4/8:3)	Exact Mass [M+H] ⁺ - 524.2418	PC(8:3/10:4)	Exact Mass [M+H] ⁺ - 524.2418
PC(12:5/6:2)	Exact Mass [M+H] ⁺ - 524.2418	PC(6:2/12:5)	Exact Mass [M+H] ⁺ - 524.2418
PC(14:6/4:1)	Exact Mass [M+H] ⁺ - 524.2418	PC(4:1/14:6)	Exact Mass [M+H] ⁺ - 524.2418
PC(O-10:4/O-10:3)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-10:3/O-10:4)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-12:4/O-8:3)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-8:3/O-12:4)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-12:5/O-8:2)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-8:2/O-12:5)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-14:5/O-6:2)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-6:2/O-14:5)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-14:6/O-6:1)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-6:1/O-14:6)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-16:6/O-4:1)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-4:1/O-16:6)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-2:0/16:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-4:0/14:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-6:0/12:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-8:0/10:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-10:0/8:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-12:0/6:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-14:0/4:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-16:0/2:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(18:0/0:0)	Exact Mass [M+H] ⁺ - 524.3718	PC(0:0/18:0)	Exact Mass [M+H] ⁺ - 524.3718

GETTING STARTED
FAQs
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Please enable popups to access the structural databases and the help icons. Thank you.
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 Carleton University - Faculty of Engineering and Design; Azrieli School of Architecture and Urbanism - University of Toronto - Centre for Research in Neurodegenerative Disease
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- 3.7. The left panel (Possible Lipids Include) shows lipid predictions with different chains at *sn*-1 and *sn*-2 positions of the glycerol backbone while the right panel (Possible Isomeric Lipids Include) shows corresponding isomers with the same chains but at different positions on the glycerol backbone. Lipid location in these lists is not sorted by biological significance but grouped according to lipid subclass (polar head group) and ascending (i) m/z, (ii) number of carbons (*sn*-1 chain), (iii) degree of unsaturation (*sn*-1 chain), (iv) number of carbons (*sn*-2 chain), and (v) degree of unsaturation (*sn*-2 chain).

☒ Exact Mass
☐ Average Mass

Ionic Mass (m/z):

Chain Lengths:

Mass Tolerance ($\pm$ m/z):

Lipid Subclass:

Fatty Chain Linkage:

Ion:

?

?

?

Possible Lipids Include:		Possible Isomeric Lipids Include:	
PC(10:4/8:3)	Exact Mass [M+H] ⁺ - 524.2418	PC(8:3/10:4)	Exact Mass [M+H] ⁺ - 524.2418
PC(12:5/6:2)	Exact Mass [M+H] ⁺ - 524.2418	PC(6:2/12:5)	Exact Mass [M+H] ⁺ - 524.2418
PC(14:6/4:1)	Exact Mass [M+H] ⁺ - 524.2418	PC(4:1/14:6)	Exact Mass [M+H] ⁺ - 524.2418
PC(O-10:4/O-10:3)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-10:3/O-10:4)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-12:4/O-8:3)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-8:3/O-12:4)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-12:5/O-8:2)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-8:2/O-12:5)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-14:5/O-6:2)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-6:2/O-14:5)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-14:6/O-6:1)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-6:1/O-14:6)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-16:6/O-4:1)	Exact Mass [M+H] ⁺ - 524.3138	PC(O-4:1/O-16:6)	Exact Mass [M+H] ⁺ - 524.3138
PC(O-2:0/16:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-4:0/14:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-6:0/12:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-8:0/10:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-10:0/8:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-12:0/6:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-14:0/4:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(O-16:0/2:0)	Exact Mass [M+H] ⁺ - 524.3718		
PC(18:0/0:0)	Exact Mass [M+H] ⁺ - 524.3718	PC(0:0/18:0)	Exact Mass [M+H] ⁺ - 524.3718

GETTING STARTED	FAQs	CITE US	CONTACT US	LIPID MODELS
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Please enable popups to access the structural databases and the help icons. Thank you.

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Carleton University - Faculty of Engineering and Design: Azrieli School of Architecture and Urbanism - University of Toronto - Centre for Research in Neurodegenerative Disease

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3.8. Each lipid entry is selectable (click once to highlight a given species). Name and m/z value can be copied by pressing “CTRL + C” and pasted by “CTRL + V”.

4. Displaying glycerophospholipid structures

4.1. All possible lipid structures can be viewed in a new pop-up window. Make sure that pop-up windows are allowed on your web browser. The text colour helps the user to restrict the number of structures presented:

4.1.1. Lipids in black represent theoretically possible combinations.

4.1.2. Lipids in blue are predicted to be the most likely based on prevalence of fatty acid chains in mammalian cells (Miyazaki and Ntambi, 2008).

4.1.3. Lipids in red indicate that (i) the lipid is part of our CTPNL curated database of lipids detected by MS methodologies in brain tissue or neural cells (neurons, astrocytes, or oligodendrocytes including precursor populations) and (ii) we have generated multiple high-resolution representations for further visualization and model production.

4.2. To view all possible lipid structures, click once on the lipid of interest. Then click:

4.2.1. The “Display All” button to display all theoretically possible lipid species in 2D. All entries in the database can be visualized in this mode.

Note: Displayed species contain only cis double bonds separated by a minimum of 2 carbons.

Possible Lipid Structures Include			
Table			
1	2	3	4
PC(18:1[22]/16:0)	PC(16:0/18:1[22])	PC(18:1[22]/16:0)	PC(16:0/18:1[22])
5	6	7	8
PC(18:1[42]/16:0)	PC(16:0/18:1[42])	PC(18:1[52]/16:0)	PC(16:0/18:1[52])
9	10	11	12
PC(18:1[62]/16:0)	PC(16:0/18:1[62])	PC(18:1[72]/16:0)	PC(16:0/18:1[72])
13	14	15	16
PC(18:1[82]/16:0)	PC(16:0/18:1[82])	PC(18:1[92]/16:0)	PC(16:0/18:1[92])
17	18	19	20
PC(18:1[102]/16:0)	PC(16:0/18:1[102])	PC(18:1[112]/16:0)	PC(16:0/18:1[112])

4.2.2. The “Best Prediction” button displays only lipid species that are predicted to be the best guess. This function works only with lipid entries in blue font.

Note: Lipids in VaLID’s “Predicted to be Common” database were identified based on the relative abundance of certain fatty acid chains in mammalian cells (Miyazaki and Ntambi, 2008). If both of the fatty acid chains at the *sn-1* and *sn-2* positions or if a single chain in a lysophospholipid are considered common, the lipid is included in the Predicted Common database and appears in blue.

Possible Lipid Structures Include			
Table			
1	2	3	4
PC(18:1[22]/16:0)	PC(16:0/18:1[22])	PC(18:1[22]/16:0)	PC(16:0/18:1[22])

- ValID: Visualization and Phospholipid Identification**
 a glycerophospholipid m/z prediction database
 Developed by Graeme S.V. McDowell and Alexandre P. Blanchet
 Version 0.1

PC(16:0/18:1)

Skeletal	Ball and stick	Space filling	ValID view
			
  	  	  	  

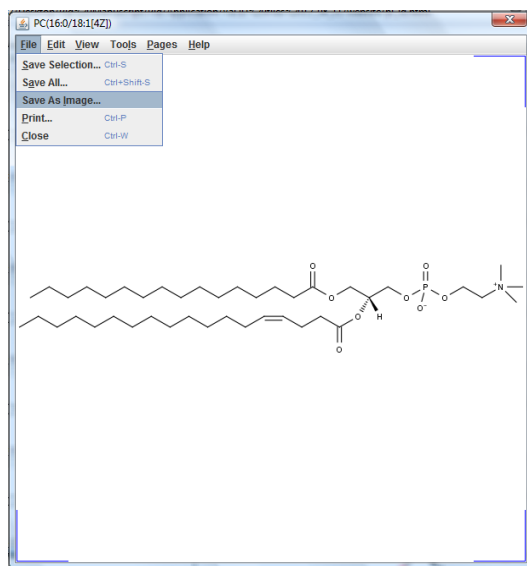
Click on the representations for high-resolution versions

5.1. To save a lipid structure from the “Display All” or “Best Prediction” windows, double-click on a structure of interest in the pop-up window displaying all possible configurations. A new window will open with the selected lipid structure.

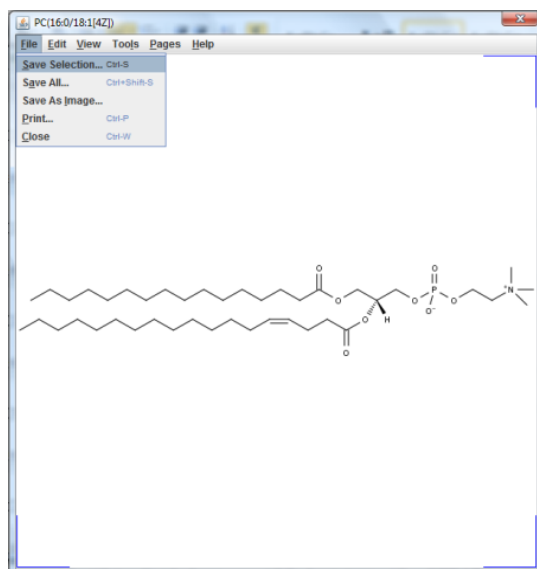
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5.1.1. The image can now be saved in low resolution in two different file types:

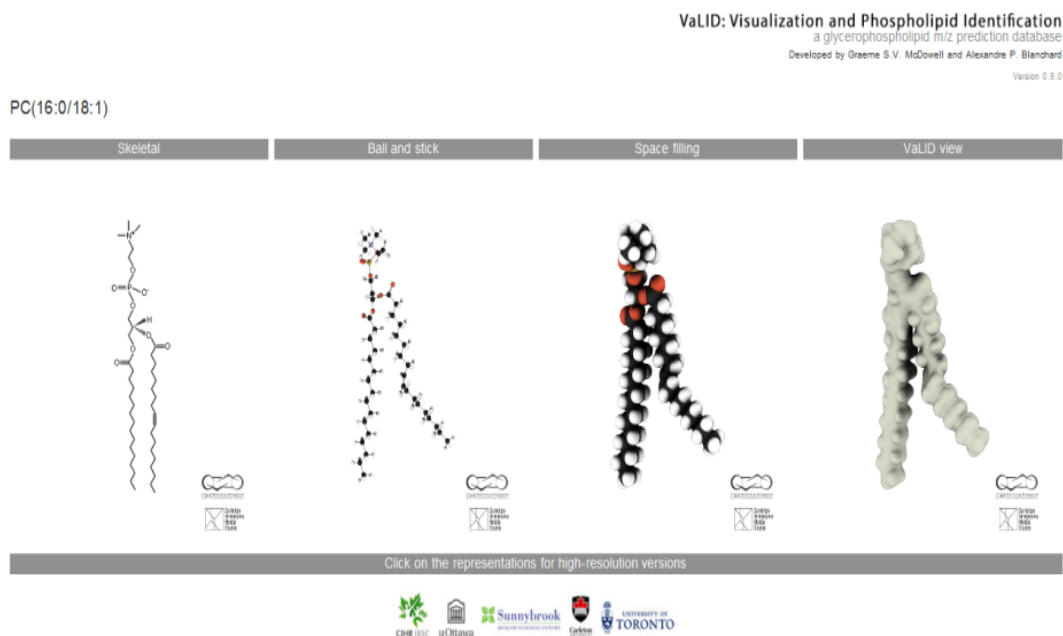
5.1.1.1. As an image file, go to the “File” menu, select “Save As Image.” You will have a choice of the following formats: joint photographic experts group (.jpg, .jpeg), portable document format (.pdf), portable network graphics (.png), portable pixmap (.ppm), scalable vector graphics (.svg, .svgz), Windows BMP (.bmp) and Windows enhanced metafiles (.emf).



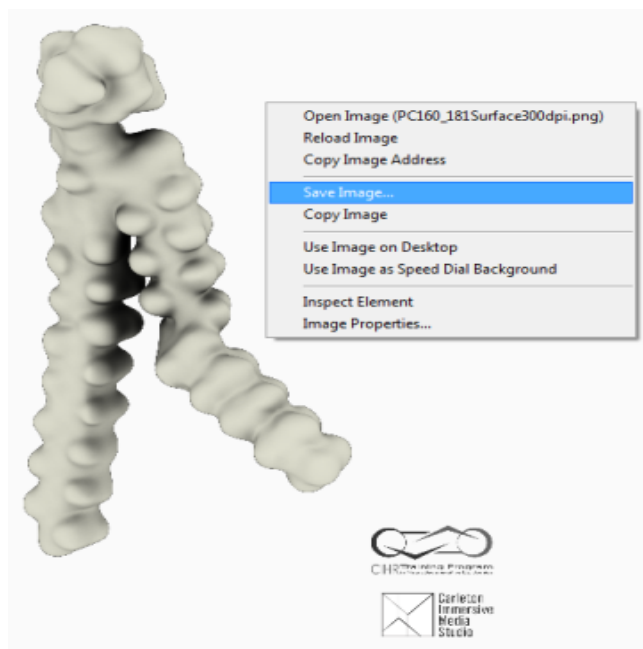
5.1.1.2. As a chemical structure information file that is compatible with ChemDraw and Marvin, go to the “File” menu and choose “Save Selection.” A save dialogue box will appear, allowing you to save the file on your hard drive. You can save the file as one of many file formats, including ChemAxon Marvin (.mrv), MDL Mol file (.mol) and CDX file (.cdx, suitable for ChemDraw) formats.



- 5.1.2. To save the high quality (300 dpi) lipid structures present in our “Structural Representation” databases, click on the model of interest: (i) 2D skeletal model, (ii) 3D “Ball and stick” model, (iii) “Space filling” model, or (iv) our own rendered “VaLID view” model.



- 5.2. A new window will open in the web browser with the name of the species you have selected. Right click (or double click) on the image and click on “Save Image” or choose “Save As” from the browser menu.



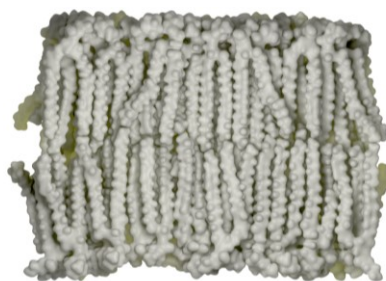
6. Exiting the application

- 6.1. To close the application, navigate away from the page.
- 6.2. To free up all resources the application has used, quit/close your browser. By closing your browser, you close Java and any system resources used by the application.

7. Supplementary information

7.1. Coding:

- 7.1.1. The numerical database is contained in Excel™ files.
- 7.1.2. The search engine and applet are coded in Java™.
- 7.1.3. The Excel files are read with JExcelApi.
- 7.1.4. The 2D structures are visualized with ChemAxon's Marvin View plugin.
- 7.1.5. The structural representations have been created with ChemDraw® and Autodesk® Maya® of curated lipid species identified by our CTPNL researchers by MS methodologies in neural tissue. The models have been developed by CTPNL researchers for purposes of representation and dissemination of simulation readouts. Of these models, VaLID view representations can be used as components of comprehensive membrane structures. Each lipid is first assembled in ChemDraw 3D. Structures drawn in 2D with ChemDraw's drawing tools are automatically translated into a 3D model in an adjacent panel where each atom of the visualized molecule are marked by an x, y and z coordinate. These points are then exported into the 3D modeling and animation software package, Autodesk Maya v2012. Rigid and dynamic VaLID view models are derived using Maya's nParticles tools. Typically, these tools are used in the animation of dynamic systems such as fire, smoke and water. nParticles are easily converted into smooth and organic polygonal meshes. By directing these particles to the x, y, and z coordinates originally generated in ChemDraw 3D and imported in Maya as points in space, a mesh that recapitulates the original molecular structures in an abstracted, organic, form. Here, VaLID view models are available for download as rigid polygon models however in Maya, we have fitted each species between each atom with a rig of movable joints, a process typically used by graphic artists to animate human or animal characters. By creating a skeleton embedded within the mesh model, we have enabled each of these structures to move in a 'biological' or dynamic way. Joint attributes are modified to restrict or limit movement (i.e., in the case of a double bond) and can be reassembled into membrane form.



III. Citing VaLID in a scientific paper

1. How do I cite use of VaLID in publications?

1.1. When citing predictions performed using the program, use this example as a guide:

Glycerophospholipid identities were predicted based on m/z and appropriate ion type taking into consideration the mass tolerance of the QTRAP 5500 [replace with name of appropriate mass spectrometer] using VaLID version 2.0.0 (Blanchard *et al.*, 2013).

1.2. When citing use of structural images, use this example as a guide: “Structural images were obtained from the VaLID structural database version 2.0.0 (Blanchard *et al.*, 2013).”

2. Citation information:

Blanchard AP, McDowell GSV, Valenzuela N, Xu H, Gelbard S, Bertrand M, Slater GW, Figeys D, Fai S, Bennett SAL (2013) Visualization and Phospholipid Identification (VaLID): An online integrated search engine capable of identifying and visualizing glycerophospholipids with given mass. *Bioinformatics*, 29: 284-285

And/or:

McDowell GSV, Blanchard AP, Taylor GP, Figeys D, Fai S, Bennett SAL (2013) Predicting glycerophosphoinositol identities in lipidomic datasets using VaLID (Visualization and Phospholipid Identification) – an online bioinformatic search engine. Submitted to *BioMed Research International*

IV. References

- Fahy E, Cotter D, Sud M, Subramaniam S (2011) Lipid classification, structures and tools. *Biochim Biophys Acta* 1811:637-647.
- Miyazaki M, Ntambi JM (2008) Fatty acid desaturation and chain elongation in mammals. In: *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance DE, Vance JE, eds), pp 191-211. Oxford: Elsevier.