Is Sphingosine 1-Phosphate (S1P) Produced by Sphingosine Kinase 2 (SphK2); Important for Learning and Memory?

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By

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Dedicated to

My beloved parents, my strength and inspiration,

Dr. C.M. Shafique and Mrs. Tabassum Shafique

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Adeena Shafique

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LIST OF ACRONYMS

AD	Alzheimer's Disease
CB	Cheeseboard Paradigm
CNS	Central Nervous System
CS	Conditioned Stimulus
EPM	Elevated Plus Maze
FC	Fear Conditioning
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
S1P	Sphingosine 1-Phosphate
SL	Sphingolipids
SphK1	Sphingosine Kinase 1
SphK2	Sphingosine Kinase 2
SphK2-/-	Sphingosine Kinase 2 Knock-Out Mice
US	Unconditioned Stimulus
WT	Wild-type

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Abstract

Sphingosine 1-Phosphate (S1P) is a potent lipid-derived signaling molecule that signals through its own family of five G-protein coupled receptors, promoting neurotransmission and neuroprotection. Recent research has demonstrated a loss of S1P in individuals with pre-clinical and clinical Alzheimer 's disease (AD), particularly in brain regions that are heavily affected by the disease. The loss of S1P was attributed to loss of its biosynthetic enzyme, sphingosine kinase 2 (SphK2). Given that loss of S1P in the hippocampus and temporal cortex tracks closely with neuronal atrophy in AD brains, we hypothesized that loss of SphK2 in mice may produce deficits in spatial, recognition, and associative learning and memory. These aspects of memory were tested using the elevated plus maze, cheeseboard paradigm and fear conditioning tests in SphK2-/- and Wild-type (WT) mice. The findings of this study are not indicative of deficits in learning and memory but indicate an anxiety phenotype in the SphK2-/- mice as indicated by the behavioral tests. The total S1P content was markedly lowered in the brains of SphK2-/- mice as compared to the WT mice.

Keywords

Sphingosine 1-Phosphate; S1P; Sphingosine Kinase; Learning; Memory; Alzheimer's Disease; Elevated Plus Maze; Cheeseboard Paradigm; Fear Conditioning

Chapter 1: Introduction

1.1 Sphingosine 1-Phosphate- an enigma in the brain

Sphingosine 1- Phosphate (S1P), an important sphingolipid, has been recognized as a critical bioactive signaling metabolite with highest concentrations in the brain (Edsall and Spiegel, 1999). Sphingolipid metabolism is central to normal neurological functioning as impaired sphingolipid metabolism results in numerous neurological disorders (Muhle *et al.,* 2013). Most important of these lipids, notably sphingosine, S1P and ceramide are vital in regulating various cellular signaling pathways (Hannun and Obeid, 2008).

Ever since the discovery of S1P more than two decades ago; tremendous progress has been made to comprehend its mechanism of action and role in health and disease. S1P is a degradation product and functional antithesis of ceramide. Ceramide is hydrolysed by ceramidases to sphingosine, which in turn is phosphorylated to S1P or converted back to ceramide via a salvage pathway (van Echten-Deckert and Herget, 2006). Sphingosine is phosphorylated to S1P by two kinases, sphingosine kinase 1 and 2 (SphK1 and SphK2) (Kohama *et al.,* 1998; Liu *et al.,* 2000). The two SphKs and the enzymes that catalyze its degradation; S1P lyase, two S1P phosphatases and three lipid phosphate phosphatases tightly regulate S1P levels (Brindley, 2004). The S1P that is formed can then act intracellularly as well as extracellularly. Secreted S1P acts through five G-protein coupled receptors, $S1P_{1\text{-}5}$ (Maceyka *et al.,* 2012) via either an autocrine or a paracrine fashion in a variety of physiological processes. S1P is vital for neural tube and vascular system development during embryogenesis (Mizugishi *et al.,* 2005) and has been shown to protect cultured cortical neurons against Aβ toxicity, further potentiating its protective role in the brain (Malaplate-Armand *et al.,* 2006). It also promotes glutamate secretion via $S1P_3$ mediated signaling from hippocampal neurons thereby validating its role in memory consolidation (Kajimoto *et al.,* 2007; Kanno *et al.,* 2010).

The recent explosion of interest in S1P and its signaling system is attributed to FDA approval of the drug Fingolimod (FTY720) for the treatment of relapsing multiple sclerosis (Mandala *et al.,* 2002; Scott, 2011). FTY720 is a synthetic analogue of sphingosine and is phosphorylated *in vivo* by SphK2 to its S1P mimetic, FTY720-phosphate. Phosphorylated FTY720 is an agonist of S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$) (Mandala *et al.,* 2002; Brinkmann *et al.,* 2002). FTY720 can cross the

blood-brain barrier and accumulate in the CNS. FTY720-phosphate like S1P also protects against Aβ toxicity (Doi *et al.,* 2013). In agreement to this study, rats were administered with Aβ peptides in another study and were protected against hippocampal neuronal loss upon FTY720 treatment (Asle-Rousta *et al.,* 2013). Accordingly FTY720 treatment is reported to enhance fear extinction memory (Hait *et al.,* 2014; Efstathopoulos *et al.,* 2015) and suppress anxiety (di Nuzzo *et al.,* 2015) in mice. These effects are postulated to occur through nuclear-localised SphK2 and suppression of histone deacetylases (Hait *et al.,* 2014; di Nuzzo *et al.,* 2015) but could also be related to enhanced neurogenesis (Efstathopoulos *et al.,* 2015; di Nuzzo *et al.,* 2015).

The two sphingosine kinases have tissue specific expression. SphK1 is mainly localized to the cytosol whereas SphK2 has nuclear expression. SphK2 is involved in regulating gene transcription in the nucleus where it produces S1P that inhibits histone deacytylases thereby increasing histone acetylation (Hait *et al.,* 2009). The expression of the two SphKs in the brain has been debatable with contradictory reports as to which isoform has predominant expression. One study reported regulation of glutamate secretion in embryonic rat hippocampal neurons by SphK1 (Kajimoto *et al.,* 2007). Another study has reported that SphK1 is the dominant isoform in the mouse brain (Fukuda *et al.,* 2003). On the other hand, there are studies that have reported SphK2 as the dominant isoform in human as well as rodent brain (Katsel *et al.,* 2007; Blondeau *et al.,* 2007).

Recently loss of S1P in the hippocampus and associated temporal grey matter of subjects with pre-clinical Alzheimer's Disease (AD) pathology has been demonstrated (Couttas *et al.,* 2014), highlighting the importance of determining whether S1P does indeed play a significant role in learning and memory. We therefore sought to confirm in this study whether the absence of SphK2 produces deficits in spatial reference memory, or fear memory acquisition and extinction.

1.2 Problem Statement

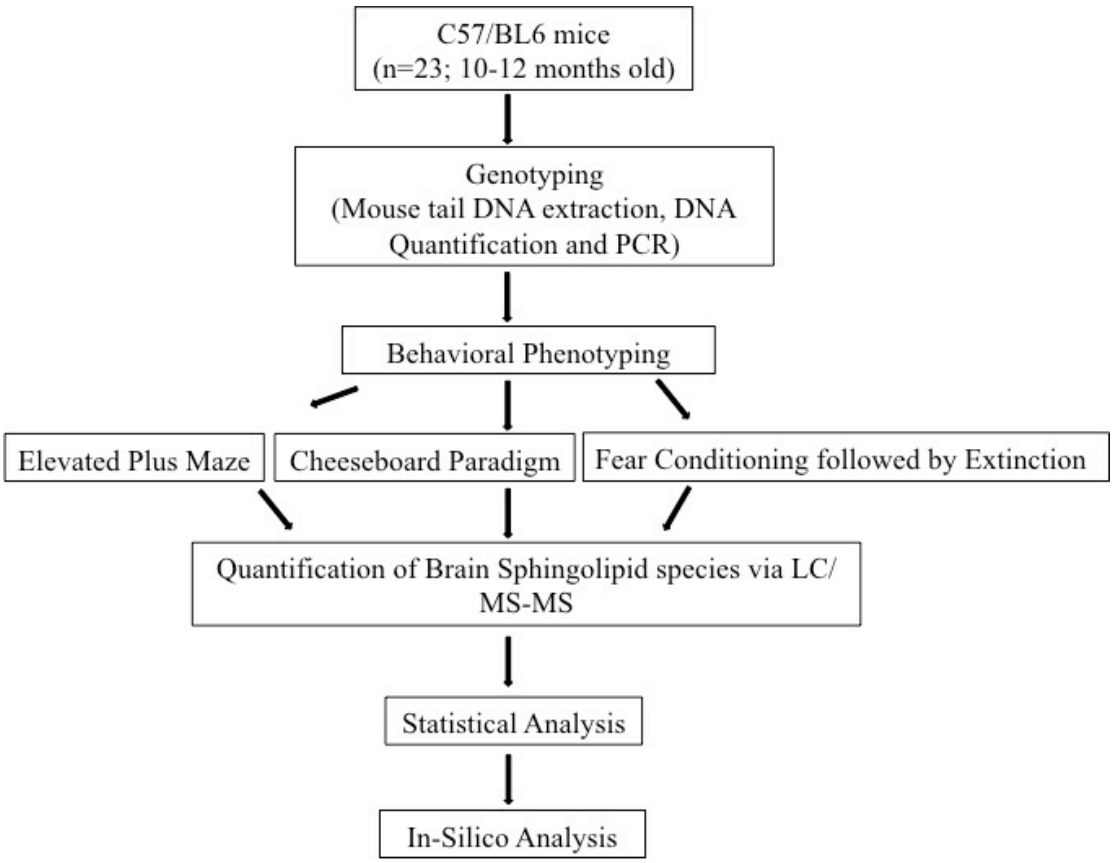
Worldwide there are 47.5 million people with dementia and 7.7 million cases are being diagnosed every year (Alzheimer's Association, 2016). The total number of people with dementia is projected to triple by the year 2050 (Alzheimer's Association, 2016). AD, the major form of dementia is a public health concern of colossal magnitude. Currently there are no treatments to cure AD and palliative methods offer

a very bleak prognosis to this mentally-crippling disease. Pakistan being a lower-middle income country has an underrated record of AD patients, mostly because it is assumed to be an unavoidable consequence of old age. Our population needs to know the difference between dementia, which is not necessarily associated with significant morbidity and mortality, and AD, which is based on a definite underlying pathology, making it susceptible to manipulation by therapeutics to prevent and possibly treat this debilitating neurodegenerative condition. Besides supportive methods, there is no permanent treatment for AD. There is a dire need to improve our comprehension of the disease's pathology to introduce better therapeutic strategies to combat and reduce the global financial burden of medical, social and informal care of this mentally-debilitating condition. It has only been recently reported that S1P levels are lowered in patients of AD (Couttas *et al.,* 2014). This raises the possibility that manipulating S1P or its receptors could lead to novel therapeutic approaches for AD. Further studies exploring the molecular mechanisms of defective S1P signaling are clearly required to better understand its role in AD pathology. The findings of this research could have vast implications in the comprehension of the pathology, treatment and prevention of AD. It can lead to a better understanding of the aberrant S1P signaling pathways involved in this condition and possibly guide the use of target-specific therapeutics to arrest or reverse the progression of the disease.

1.3 Research Objectives:

- To determine whether loss of S1P in the brain produces deficits in spatial, recognition, and associative memory and learning in mice.
- To determine whether SphK1 compensates levels of S1P in brains of SphK2-/- mice.

1.4 Experimental Design



Chapter 2: Literature Review

2.1 Alzheimer's Disease - A Brief Introduction

Alzheimer's Disease (AD), a mentally crippling and fatal neurodegenerative disease, is the most common form of dementia (Kalaria *et al.,* 2008; Forette and Boller, 1991). Several hypotheses have been postulated to explain the causes of AD but the actual etiology of the disease remains elusive (Karran *et al.,* 2011; Francis *et al.,* 1999; Maccioni *et al.,* 2010). Currently, AD affects 25-30 million people worldwide, with the number of patients diagnosed with AD projected to double in the next ten years (Thiess and Bleiler, 2011; Prince *et al.,* 2013) and quadruple by 2050 if disease progression is left untreated (Brookmeyer *et al.,* 2007). AD is the sixth leading cause of mortality in the United States. The chief risk factor associated with AD is ageing as dementia strikes people over the age of 60 (Prince *et al.,* 2013). The majority of patients have late-onset AD whereas early-onset AD accounts for less than 1% of all the AD cases (Brouwers *et al.,* 2008). This rare form of AD can affect people who are genetically predisposed to the disease. Affected individuals may carry mutations in the gene that codes for amyloid precursor protein (APP) or mutations in either the presenelin 1 or presenilin 2 gene, which codes for part of the enzyme responsible for the production of amyloid β (Revest, 1998).

The major pathological hallmarks of AD are the accumulation of extracellular, senile plaques of amyloid β (Aβ) peptides (Braak *et al.,* 1989; Glenner, 2012) and intracellular neurofibrillary tangles (NFTs) (Grundke-Iqbal, 1986; Braak *et al.,* 2006). Aβ peptides are produced by the cleavage of amyloid precursor protein (APP) whereas NFTs are formed by aggregation of hyperphosphorylated paired helical filaments of the protein Tau.

AD is characterized clinically by progressive memory loss (Braak and Braak, 1988; Butter *et al.,* 1988), cognitive decline (Sperling *et al.,* 2011), aberrant behavior (Lyketsos *et al.,* 2000), and anosognosia (Agnew and Morris, 1998; Kalbe *et al.,* 2005; Orfei *et al.,* 2010); and pathologically by neuronal cell death (Braak and Braak, 1988), loss of synapses (DeKosky and Scheff, 1990), loss of functional cholinergic neurons (Whitehouse *et al.,* 1981; Coyle *et al.,* 1983) and astrogliosis (Diedrich *et al.,* 1987).

2.2 Current Treatments for AD

Current treatments available for AD offer a bleak prognosis to the patients and are only palliative in nature. Four commercially available drugs to treat dementia include the cholinesterase inhibitors donepezil, rivastigmine and galantamine, and the glutamate antagonist memantine. Most of the recent treatment strategies have been directed at anti-amyloid approaches with unsuccessful results. Trials including monoclonal antibodies namely bapineuzumab and solanezumab against Aβ peptides did not yield positive clinical results (Salloway *et al.,* 2014; Doody *et al.,* 2014). These discouraging results demonstrate a need for more research into the molecular basis for AD. Besides anti-amyloid trials, tau-modulating therapies are also under clinical investigation. A 5-HT_6 antagonist, idalopirdine used in combination with donepezil showed significant cognitive benefit for the treatment of mild-to-moderate AD (Atri and Colding-Jorgensen, 2014). Other therapeutic strategies include targeting insulin signalling (Claxton et al., 2015), deep brain stimulation (Lyketsos *et al.,* 2012) and using medical foods like Axona (aimed at supplying energy to neurons) (Sharma *et al.,* 2014) and Souvenaid (enhances synaptic function) (Olde *et al.,* 2015).

2.3 Lipids and AD

Brain, after adipose tissue is the most lipid rich organ of the body. Most important lipid species in the brain include cholesterol, phospholipids and sphingolipids. Lipids are significant for exerting crucial structural and physiological roles in the central nervous system (CNS) (Adibhatla and Hatcher, 2007). Lipids are essential structural components of cell membranes where they are responsible for maintaining the integrity of membrane bilayer and modulating cell fluidity. Lipids are also essential components of the myelin sheath, providing the hydrophobic barrier that maintains fast propagation of electrochemical gradients along axons (Lane and Farlow, 2005). Aberrant lipid metabolism can result in homeostatic dysregulation and neurodegeneration (Abad-Rodriguez *et al.,* 2004).

The link between AD and lipids was first established after the discovery of the major genetic risk factor for AD, the ε4 allele of the *APOE* gene, which encodes the lipid transport protein apolipoprotein E (apoE) (Corder *et al.,* 1993; Bertram and Tanzy, 2008). ApoE mediates the transport of cholesterol, phospholipids and sulfatides in the brain and is known to mediate lipid metabolism (Han et al., 2003). Although the role of apoE is not clear in AD pathogenesis, it is suggested to play a role in Aβ deposition

in the brain (Holtzman et al., 2000). Interestingly, in 1906, Alois Alzheimer also observed a third pathological hallmark of the disease: the presence of lipid filled granules in the cell bodies of neurons (Alzheimer, 1911). This hallmark has only recently gained attention of the scientific world owing to technical advances in the study of lipid vacuoles. Indeed, AD brains are composed of abnormally elevated levels of lipoid granules (Foley, 2010).

Lipids also modulate amyloid β and tau pathologies. The processing of APP to form insoluble Aβ peptides occurs in cholesterol and sphingolipid rich microdomains known as 'lipid rafts'. This processing is predominantly amyloidogenic, catalyzed by β-site APP cleavage enzyme 1 (BACE1 or β-secretase), as ample amounts of the enzyme are localized to lipid rafts (Ehehalt *et al.,* 2003). Outside the rafts APP processing largely occurs via the non-amyloidogenic α-secretase pathway. Studies have demonstrated that lipids modulate the activity of BACE1 in lipid rafts (Riddell *et al.,* 2001; Ehehalt *et al.,* 2003). Depleting cholesterol results in reduced association of BACE1 with lipid rafts, which in turn decreases the amyloidogenic processing of APP (Hattori et al., 2006). On the contrary, APP processing by BACE1 is strongly enhanced by increasing cholesterol levels (Vetrivel *et al.,* 2010). These studies collectively indicate that appropriate levels of BACE1 in lipid rafts are important for the production of amyloid β. An important subgroup of lipids, sphingolipids which is significantly dysregulated in AD will now be reviewed.

2.4 Sphingolipids

Sphingolipids (SL), a diverse class of signaling and structural lipids, represent one-third of the total lipid content in eukaryotic cell membranes and are found in high abundance in the CNS. SL consist of a fatty acid chain amide linked to a sphingoid base backbone, which is usually sphingosine in mammals (Bartke and Hannun, 2009). Functional diversity among SL arises from variation in the fatty acid chain length, hydroxylation and saturation of sphingosine and fatty acid chains (Merrill, 2011). Some of these lipids including sphingomyelin, ceramide, dihydroceramide and their derivatives play crucial roles in the CNS. Sphingomyelin (SM) is a major component of myelin sheath of neurons and lipid rafts of cell membranes. Many neurological disorders associated with disruptions in action potentials are due to degradation of sphingomyelin. Altered levels of SM also lead to some irreversible neurological disorders (Muhle *et al.,* 2013). Ceramide, the precursor of sphingosine is an important

sphingolipid associated with apoptosis and regulation of neurotransmitter release (Hannun and Obeid, 1995; Bartke and Hannun, 2009). Studies have shown that ceramide and sphingosine levels are enhanced in the brains of AD patients (He et al, 2010). On the other hand, sphingomyelin and sphingosine 1-phosphate (S1P) levels are greatly reduced in AD brains. The enzymes that regulate sphingomyelin levels are sphingomyelin synthase and sphingomyelinases. One possible cause for elevation of ceramide in AD is due to increased activity of acidic sphingomyelinase (He *et al.,* 2010). S1P production is illustrated in figure 1 (Fig. 1).

Cumulatively, these studies suggest that perturbations of sphingolipid metabolism have implications in memory, learning and AD pathology. However, the focus of this review will now be on sphingosine 1-phosphate.

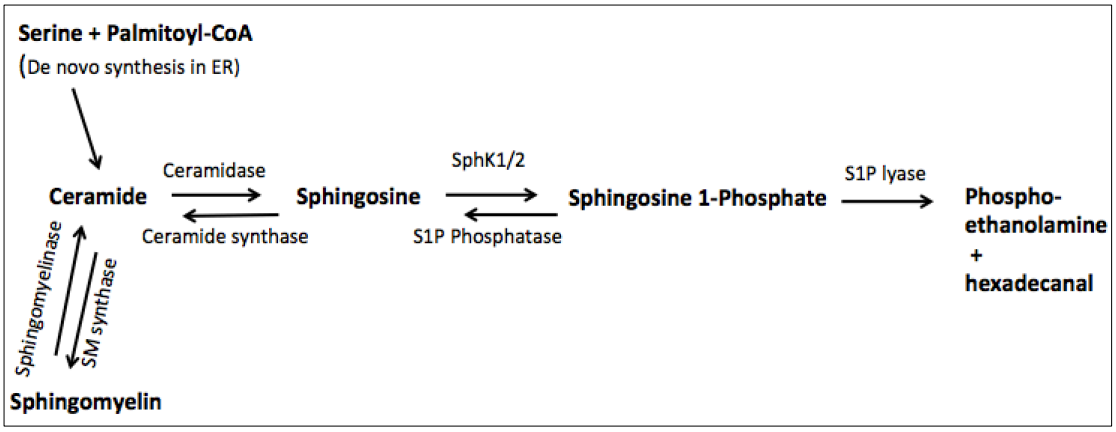


Figure 1: Formation of Sphingosine 1-Phosphate. Ceramide is the precursor lipid of sphingosine which is then phosphorylated by either of the two kinases, SphK1 or SphK2 to produce S1P. ER: Endoplasmic reticulum.

2.5 Sphingosine 1-Phosphate (S1P)

Sphingosine 1-phosphate is a potent bioactive signaling metabolite crucial for numerous physiological processes such as endothelial barrier integrity, lymphocyte trafficking, neurotransmitter release, cell growth and survival (Maceyka *et al.,* 2012). S1P is formed by the phosphorylation of sphingosine, the backbone of sphingolipids in mammalian cells [refer to a diagram here]. Phosphorylation of S1P is mediated by two isoforms of the enzyme sphingosine kinase; sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) (Liu *et al.,* 2012; Maceyka *et al.,* 2012). The levels of S1P are strictly regulated by the two sphingosine kinases, as well as the enzymes that catalyze its degradation, S1P lyase and S1P phosphatases (Maceyka *et al.,* 2012).

Unlike SphK1 which is localized exclusively in the cytosol, SphK2 is found mainly in the nucleus. S1P may function either as an intracellular signaling molecule or as an extracellular ligand. A family of five G-protein coupled receptors, $S1P_{1-5}$, mediates the extracellular functions of S1P. S1P receptors are widely expressed throughout the CNS, both in neurons and glial cells (Dev *et al.,* 2008). $S1P_{1-3}$ are highly expressed in neurons and microglia. Astrocytes predominantly express $S1P_1$ and $S1P_3$. Mature oligodendrocytes abundantly express $S1P_5$ in addition to $S1P_1$, $S1P_2$ and $S1P_3$ (Novgorodov *et al.,* 2007). Binding of S1P to any of its receptors activates G-proteins, which then leads to a cascade of intracellular events ultimately regulating many physiological processes (O'Sullivan and Dev, 2013) (Fig. 2).

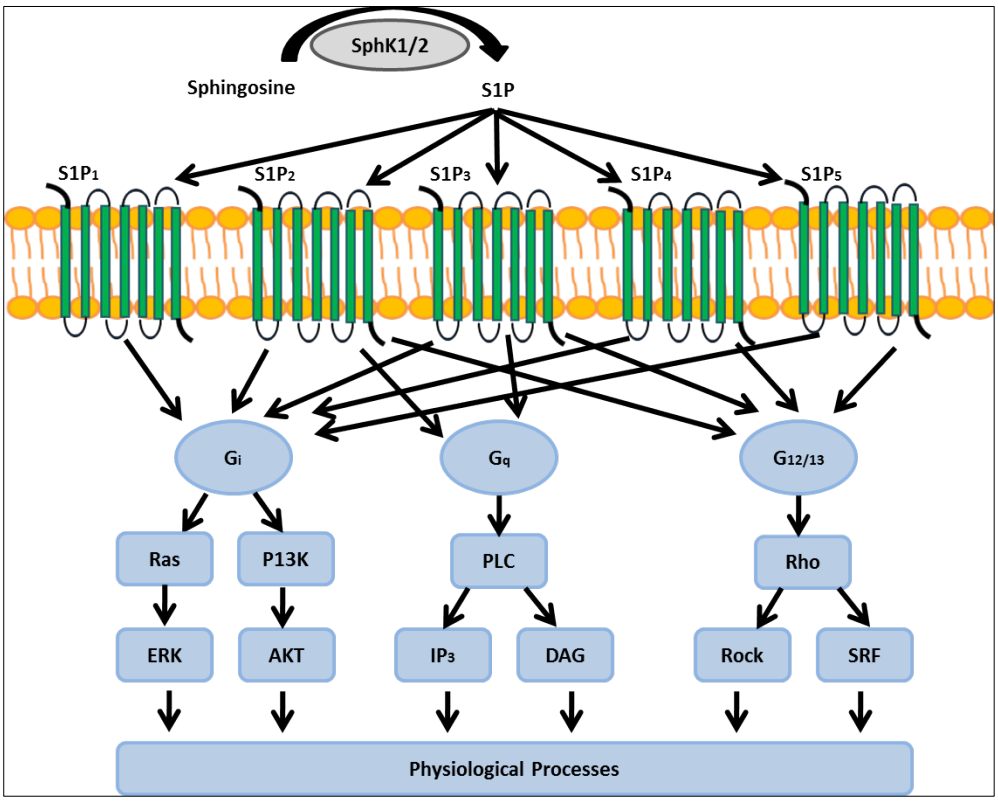


Figure 2: Signaling pathways activated by S1P and its receptors. DAG: Diacylglycerol; ERK: extracellular regulated kinase; IP3: Inositol triphosphate; PI3K: Phosphatidylinositol 3' kinase; Rock: Rho-associated protein kinase; S1P1–5: S1P receptors 1 to 5; SPHK1: Sphingosine kinase 1; SPHK2: Sphingosine kinase 2; SRF: Serum response factor. *Modified from (Don et al., 2014).*

2.5.1 Physiological functions of S1P

S1P is the functional antithesis of ceramide; wherein S1P is anti-apoptotic and ceramide is pro-apoptotic. S1P is known to promote cell growth, stimulate cell proliferation, differentiation and survival via signal transduction through various

different pathways (Olivera et al., 1999). S1P is known to induce proliferation of neural precursor cells (NPCs) via S1PRs (Kimura *et al.,* 2008). A study employing SphK knockout mice showed mice lacked complete neural tube development during embryogenesis, implicating a role for S1P in normal growth and development (Mizugishi *et al.,* 2005). The role of S1P in neurogenesis has been demonstrated by using cell lines such as PC12, where S1P promoted survival of cultured neurons (Milstien *et al.,* 2007). S1P has also been implemented in inducing cell proliferation in rat hippocampal NPCs (Choi and Chun, 2013). In another study, S1P conferred neuroprotection against cerebral ischemia in mice (Zhou *et al.,* 2010). In support of this study, sphingosine kinase 2 KO mice suffered from neurological damage following cerebral ischemia (Pfeilschifter *et al.,* 2011).

By utilizing primary neurons and neuronal cell lines in vitro it has been shown that S1P signaling regulates important neuronal functions. S1P signaling regulates neuronal survival/death, neuronal excitability, neuronal cell migration, synapse formation and synaptic neurotransmission. S1P signaling in astrocytes (the main type of glial cells in the CNS) has been shown to influence astrogliosis in many disease models (Choi *et al.,* 2011). Particularly, $S1P_1$ and $S1P_3$ are the receptors predominantly implicated in disease conditions. S1P signaling is also important for microglial activation.

S1P signaling system has gained immense attention in recent years especially following FDA approval of S1P analog drug FTY720 (Fingolimod) for the treatment of relapsing multiple sclerosis (Brinkmann *et al.,* 2010). FTY720 has been shown to cross the blood-brain barrier (Foster *et al.,* 2012) and increase levels of brain-derived neurotrophic factor (BDNF) via MAPK pathway in mouse models of Rett syndrome lacking *Mecp2* gene, indicating a neuroprotective role of FTY720 (Deogracias *et al.,* 2012). In another study, primary human astrocytes were stimulated with fingolimod to analyze its neuroprotective function. This study reported induction of neurotrophic factors namely leukemia inhibitory factor (LIF), interleukin 11 (IL11) and heparin-binding EGF-like growth factor (HBEGF) and also inhibition of inflammatory cytokines in astrocytes. They also reported similar neuroprotective effects of endogenous S1P in astrocytes (Hoffmann *et al.,* 2015).

Recently, fingolimod's functions were analyzed in terms of neuronal gene regulation in mouse neuronal cultures. It was reported that fingolimod enhanced neurite growth

in primary neurons. In CNS neurons, fingolimod upregulated genes including *cFos, FosB, Egr1 and Egr2* (Anastasiadou, S., and Knöll, 2016). This study validates a neuroprotective role for FTY720 and S1P.

2.5.2 Role of S1P in memory formation and cognition

Evidence supports an important role of S1P in memory formation and cognition. S1P has been shown to modulate neuronal excitability by activating release of glutamate in primary hippocampal mossy fibre neurons (Kajimoto *et al.,* 2007). Activation of hippocampal mossy fibre neurons is linked to short-term memory formation. S1P mediated glutamate secretion, from pre-synaptic hippocampal neurons, is essential for spatial memory (Kanno *et al.,* 2010). SphK1 KO mice that had reduced levels of S1P performed poorly in cognitive tests compared to their wild-type littermates. Adding exogenous S1P to hippocampal neurons of these mice increased excitatory post synaptic potentials (Kanno *et al.,* 2010). More recently it was shown that S1P levels are significantly reduced over the course of AD (Couttas *et al.,* 2014). The loss of S1P was attributed to the reduced activity of sphingosine kinase 2. It is also important to note that loss of S1P was concurrent with brain tau pathology, suggesting a direct relationship between tau pathology and S1P depletion. Reduced expression of sphingosine kinase 1 and increased sphingosine 1-phosphate lyase was also recently reported in AD brains, which would be expected to result in decreased levels of S1P (Ceccom *et al.,* 2014). These results implicate a strong role of S1P in AD pathogenesis.

2.5.3 Effect of S1P via SphhK2 on histone acetylation

Studies have sought to determine the nuclear role of SphK2 owing to its predominant nuclear localization (Ding *et al.,* 2007). Hait et al., (Hait *et al.,* 2009) determined the association of SphK2 with histone proteins. It was observed that histone 3 (H3) was specifically associated with SphK2 and not with SphK1 in nuclear extracts of MCF7 breast cancer cells. Acetylation of lysine residues in histone proteins is maintained by a delicate equilibrium between histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Clayton *et al.,* 2006; Yang and Seto, 2008). S1P was found to inhibit the catalytic activity of endogenous as well as recombinant HDAC1 and HDAC2 (Hait *et al.,* 2009). Consequently, overexpression of SphK2 enhanced acetylation of lysine 9 of H3 (H3-K9) and lysine 5 of H4 (H4-K5). On the contrary, catalytically inactive SphK2 or active SphK1 did not affect the acetylation status of

these residues. This further confirmed the specificity of SphK2 for these histone proteins in the nucleus. Interestingly, levels of S1P were reduced in the nucleus upon depletion of SphK2 by small interfering RNA against SphK2. This in turn reduced acetylation of H3-K9 and H4-K5. These effects were not observed upon depletion of SphK1. Moreover, exogenously added S1P reversed the effects on these histones (Hait *et al.,* 2009).

This finding is important as HDACs are implicated in the pathology of numerous human diseases including neurodegenerative diseases, cancer and cardiovascular diseases (Haberland *et al.,* 2009; Kazantsev and Thompson, 2008). HDACs have also been known to suppress the expression of genes related to memory formation and learning (Alarcon *et al.,* 2004; Levenson *et al.,* 2004). In this regard HDAC inhibitors have been implicated in enhancing memory formation (Vecsey *et al.,* 2007). A study by Guan et al., demonstrated that HDAC2 specifically inhibits H4-k5 acetylation in hippocampal neurons. This negatively regulates genes associated with memory formation and synaptic plasticity (Guan *et al.,* 2009). Another study confirmed the increase of HDAC2 in 5XFAD mouse model of AD (Gräff *et al.,* 2012).

Chapter 3: Materials and Methods

3.1 Animals

Male C57BL6 (10-12-months-old) mice were acclimated for 2 weeks prior to experimentation. SphK2 Knock-Out (KO) mice were developed as previously described (Mizugishi *et al.,* 2005), maintained at Australian BioResources, Mossvale, and transferred to the animal holding facility at the Neuroscience Research Australia (NeuRA, Sydney) for testing. The mice were C57BL6 genetic background. Mice were pair-housed in Polysulfone cages (1144B: Tecniplast, Rydalmere, Australia) with minimal environmental enrichment in the form of a red, transparent, polycarbonate igloo (certified polycarbonate mouse igloo: Bioserv, Frenchtown, USA) and tissues for nesting material (Kimwipes®, Kimberley-Clark, Australia). Mice were maintained on a 12/12 h light/dark cycle and had access to animal chow and water *ad libitu* except during the Cheeseboard experiment. Animals were divided into two cohorts for testing based on their age so that all animals were 10-12 months old during the tests. Animal experiments were approved by the University of *New South Wales Animal Care* and *Ethics Committee* (ACEC) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2 Chemicals

All chemicals used for biochemical analyses were procured from Sigma (Sigma Companies Group Pty Ltd, Castle Hill, Australia) and/or MERCK (Merck, Darmstadt, Germany).

3.3 Genotyping

Genotyping for *SphK2* alleles was done by PCR analysis as previously described (Mizugishi *et al.,* 2005). Genomic DNA after weaning by tail biopsy was used as the template (40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min) with primers P3 (5′-GCACCCAGTGTGAATCGAGC-3′), P4 (5′-TCTGGAGACGGGCTGCTTT A-3′), and P5 (5′-CGCTATCAGGACATAGCGTT-3′). Expected product sizes for the wild-type and targeted alleles were 680 and 310 bp, respectively.

3.4 Behavioural Phenotyping

Mice were tested in a battery of behavioural (memory and cognitive) tasks at NeuRA. All experimental devices and objects were thoroughly cleaned with 70% ethanol in between experimental trials.

3.4.1 Elevated Plus Maze

Elevated Plus Maze (EPM) is a widely used test for anxiety, which represents a natural conflict between the animal's tendency to explore a novel environment and its preference to avoid a brightly lit, open area (Montgomery and Monkman, 1955). The apparatus used in the current study was a ''+''-shaped maze which consisted of two enclosed arms and two open arms; all arms extending from a central platform. The maze was raised at a height of 70 cm from the ground (Fig. 3). Each animal was placed on the central platform facing one enclosed arm and allowed to explore the maze for 5 min. A camera was mounted above the EPM to record the frequencies and latencies of the four behaviours: stretch-attend, head-dipping, grooming and rearing as well as frequency of entries into, time spent and distance travelled in the open and enclosed arms using Any-Maze™ software. After each trial the maze was thoroughly cleaned with 70% ethanol.

Figure 3: Elevated Plus Maze Apparatus. The apparatus used in the current study was a ''+''-shaped maze which consisted of two enclosed arms and two open arms; all arms extending from a central platform. The maze was raised at a height of 70 cm from the ground. Each animal was placed on the central platform facing one enclosed arm and allowed to explore the maze for 5 min.

3.4.2 Cheeseboard Paradigm

In cheeseboard (CB) test, mice were trained over a stretch of days to find a food reward; spatial reference memory was indexed by a decreased latency to find the reward over days. The CB was a grey painted circular wooden board 1.1 m in diameter, raised 60 cm above the floor (Fig. 4). There were 32 bottle caps (3.1

cm diameter, 1.3 cm deep) evenly spaced out across the board (spaced in a radial pattern with 8 lines of 4 wells each radiating from the centre area; each well was 5 cm from the next well and the last well was 10 cm from the edge of the board). One of the caps contained the food reward (100 µl sweetened condensed milk; diluted 1:4 with water) and all of the remaining caps were lightly brushed with the milk. External cues were located around the CB. A camera was mounted above the CB to measure distance travelled and velocity as well as time spent in CB zones using Any-Maze™ software. Latency to find the target was measured using a stopwatch. During habituation (three days to the blank side of the CB) three 2 min trials were conducted each day with a 10 min Inter Trial Interval (ITI). Mice were food-restricted for 1 day prior to habituation and fed for 1-2 h per day during the testing. Mice were kept at 85–90% of their pre-test body weight throughout the experiment.

Spatial Reference Memory Acquisition: Mice were trained over 8 days (three trials per day with a 10 min ITI) to locate the food reward. The location of the target well was kept constant for each mouse between trials and across days; the target well location was different for each mouse and was counterbalanced across genotypes. If the target well was not located within 2 min, mice were placed next to the target well and allowed to consume the food reward. A probe trial was conducted on day 9, where no wells were baited and mice were given 2 min to explore the board freely. On the probe trial, the board was divided into 8 zones corresponding to each line of 4 caps (the line of wells was in the centre of the zone), as well as a centre zone (40 cm diameter); the time spent in each zone (%time) was measured using Any-Maze™. Data presented for 'zone time' excludes the time spent in the centre zone. During days 10-13, mice went through reversal training where the food reward was now located in the exact opposite direction on the board. A probe trial was conducted on day 14.

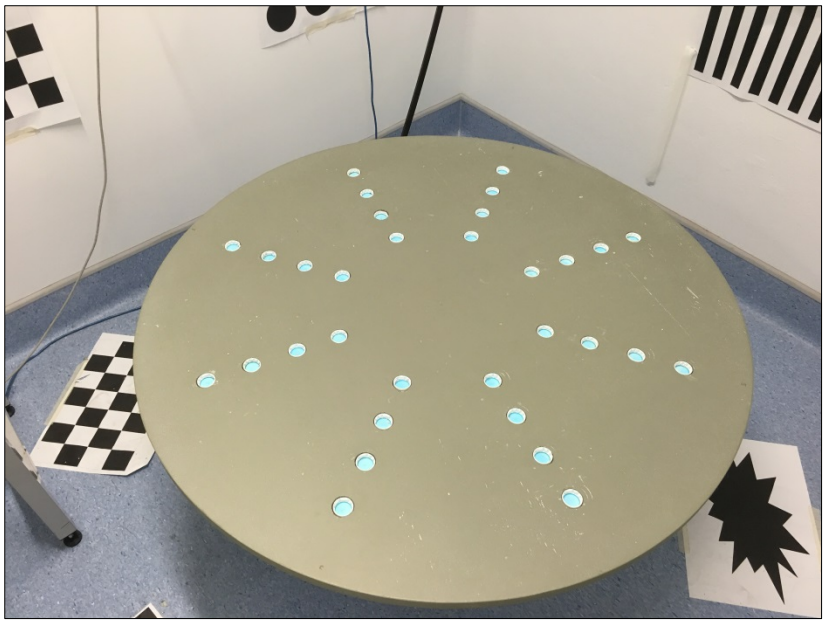


Figure 4: Cheeseboard Apparatus. The CB was a grey painted circular wooden board 1.1 m in diameter, raised 60 cm above the floor. There were 32 bottle caps (3.1 cm diameter, 1.3 cm deep) evenly spaced out across the board (spaced in a radial pattern with 8 lines of 4 wells each radiating from the centre area; each well was 5 cm from the next well and the last well was 10 cm from the edge of the board). External cues were located around the CB.

3.4.3 Fear Conditioning followed by Extinction

The present fear conditioning followed by extinction task was conducted over five days (24 h ITI). On day 1 (conditioning), animals were placed in the test chamber (Model H10-11R-TC: Coulbourn Instruments, Whitehall, USA) for a total of 7 min. After 120 s (pre-conditioned stimulus (CS)/baseline) an 80 dB conditioned stimulus (CS) was then presented for 30 s with a co-terminating 0.4mA 2 s foot shock (unconditioned stimulus (US)) twice with an inter-pairing interval of 120 s. The test concluded 120 s later. On day 2 (cue test) animals were placed in an altered context (i.e. grid floor replaced by a flat plastic floor, clear Perspex walls replaced with pink acrylic panels) for 9 min. After 120 s (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 120 s without the CS. On day 3 (context test), the animals were returned to the apparatus for 7 min. Days 4 and 5 were the same as day 3. During days 3-5, no CS or US was provided. There was a 68 dB white noise background for all tests. The protocol is outlined in Fig. 5. Time spent *freezing* and distance travelled were measured using Any-Maze™ software (Any-Maze™ *freezing* parameters: freezing on: 3, freezing off: 13), where *freezing* was

defined as complete behavioural immobility except for natural respiratory motions (Stiedl and Spiess, 1997).

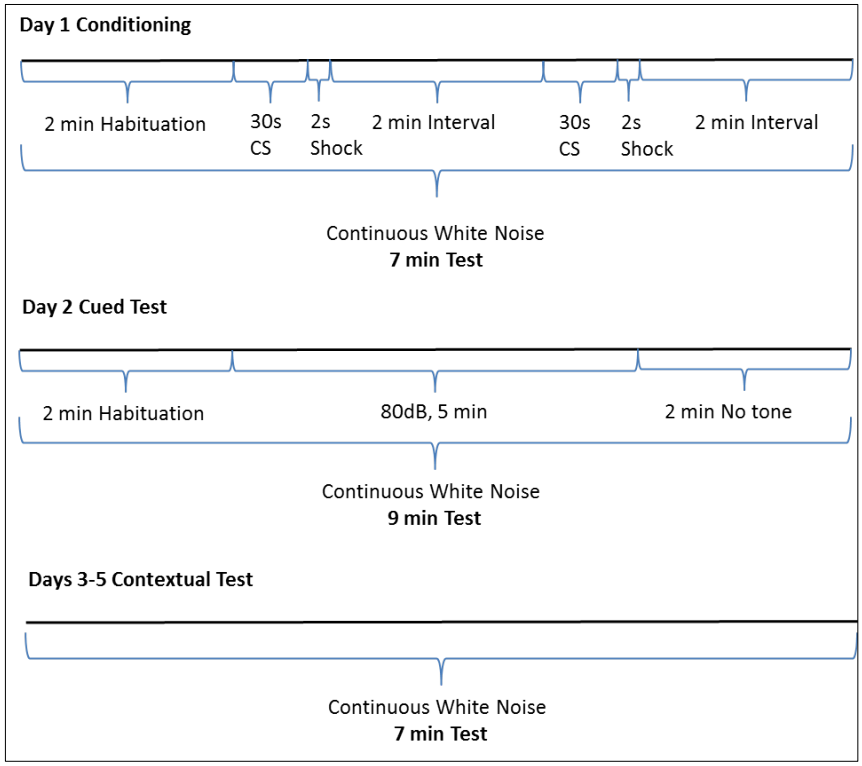


Figure 5: Fear Conditioning and Extinction Protocol. Freezing measured by Any-Maze™ software.

3.5 Mice Euthanasia

Mice were euthanized using carbon-dioxide and regionally dissected brain regions (hippocampus and cerebellum) were stored at -80°C until further use.

3.6 Lipid extraction, analysis and quantification by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Lipids were extracted from 10–15 mg of frozen brain tissue, as described previously (Wong et al., 2012). Lipid extracts were reconstituted in 400 µl of HPLC mobile phase (1 mM ammonium formate in 80% methanol/ 20% MilliQ water, containing 0.2% formic acid) and stored at −20°C until LC-MS/MS analysis. Quantification of extracted lipids was performed on a ThermoFisher Scientific Quantum Access triple quadrupole mass spectrometer equipped with an Accela UPLC and a 3 × 150 mm Agilent XDB-C8 column (5 µm pore size), as described previously (Wong et al., 2012). Peaks were integrated using Xcalibur software (ThermoFisher Scientific). Analytical (HPLC) grade solvents were purchased from Merck. All lipid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama).

3.7 Statistical Analysis

Two-way analysis of variance (ANOVA) was used to analyse behavioural parameters for main effects of genotype. For CB probe trials single sample t-tests were used to assess whether the % time spent in the target zone was greater than chance (12.5 %). Differences were regarded as significant if $p < 0.05$. Data are shown as means ± standard error of means (SEM). Analyses were conducted using GraphPad Prism software (version 7).

3.8 In silico analysis of functional association network of SphK2

To investigate functional association network of SphK2 with related proteins, its respective UniProtKB accession number was submitted in STRING 8.3 database (http://string-db.org/). The resulting algorithm was used to assess interactions of SphK2 with other enzymes.

Chapter 4: Results

4.1 Genotyping of WT and SphK2-/- mice

Of the 25 DNA samples, 11 were successfully genotyped as WTs at 680bp and 12 SphK2-/- at 310bp (Fig. 6).

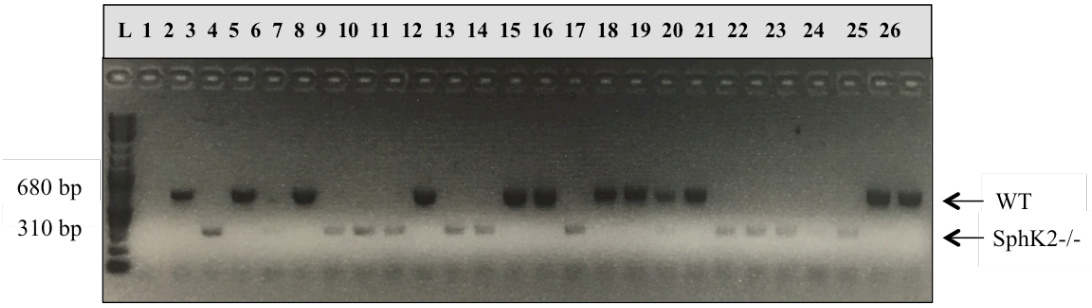


Figure 6: Mouse-tail genotyping on 2% agarose gel. L= 10 kb ladder; Lane 1= negative control; Lanes 2-26=sample DNA. (N= 11 WTs and 12 SphK2-/-).

4.2 Anxiety related parameters tested in the Elevated Plus Maze

Table 1 presents the dataset for all the behaviours measured in the EPM. Among the main exploratory behaviours (grooming, rearing, head dipping and stretch-attend posture), frequency of grooming was significantly elevated by 2.5 fold in SphK2-/- compared to the WT mice (Fig. 7a). Other exploratory behaviours (rearing, head-dipping, stretch-attend posture and distance travelled on the EPM) were similar between the two genotypes. SphK2-/- mice also spent significantly increased time freezing compared to their WT littermates (Fig. 7b). Total number of entries in the centre of the EPM was significantly lower for the SphK2-/- mice compared to the WT mice (Fig 7c). Number of entries in both open and closed arms appeared lower for the SphK2-/- mice compared to the WT mice but was not statistically significant. Time spent in closed arms trended higher in the SphK2-/- mice whereas time spent in the centre and open arms trended higher in the WT mice but it wasn't statistically different between the two genotypes.

	WT (n=11)	**SphK2-/- (n=12)**	
Time Spent (s)	*Mean ± SEM*	*Mean ± SEM*	*p VALUE*
Grooming	18.60±3.83	47.41±8.99	0.0095
Rearing	11.55±*3.06*	8.76±*1.67*	0.423
Head Dipping	7.682 ± 0.7005	7.425 ± 0.764	0.81
Stretch-attend	34.91 ± 2.686	32.46 ± 3.654	0.6002
Freezing	47.85 ± 6.606,	91.59 ± 10.77	0.003
Centre	55.35 ± 4.811	43.46 ± 4.275	0.077
Closed Arms	198.2 ± 6.192	216.3 ± 7.209	0.0732
Open Arms	38.12 ± 4.628	33.54 ± 5.643	0.54
Total Distance Travelled (m)	18.92 ± 2.474	15.02 ± 2.773	0.31
No. of entries			
Centre	38.09 ± 4.770	24.92 ± 3.959	0.0443
Closed Arms	26.18 ± 2.572	18.92 ± 2.690	0.0654
Open Arms	21.00 ± 3.861	13.50 ± 3.615	0.1705

Table 1: Dataset for the EPM Test Measures. All behaviors tested in the EPM are shown. Data are presented as mean ± SEM. *P* value is by two-tailed Student's t-test. Significantly altered behaviors are time spent grooming, time spent freezing and number of entries in the center with $p < 0.05$.

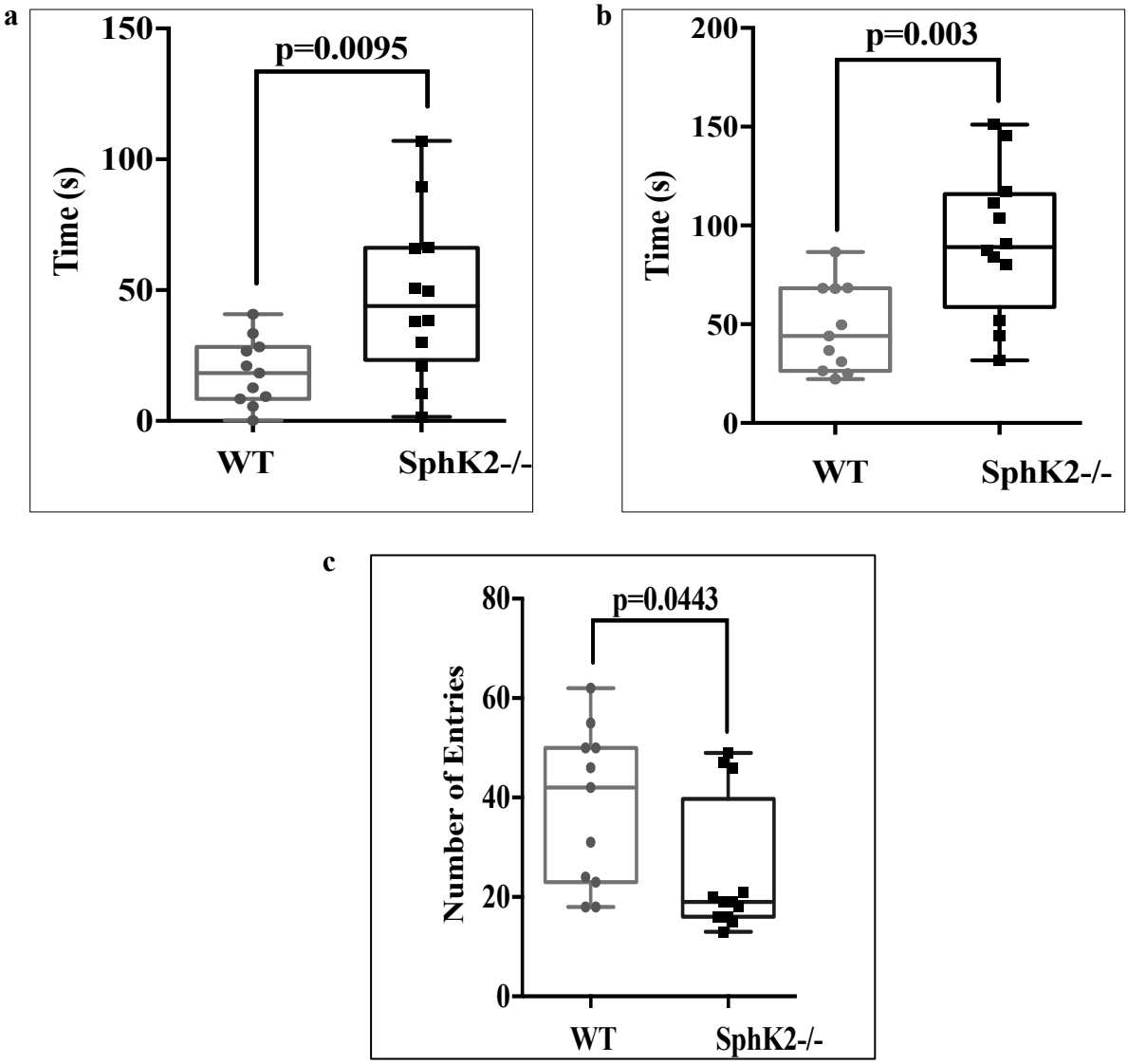


Figure 7(a-c): Significantly altered behaviors in the EPM. (a) Significant difference in time spent grooming between the two genotypes (as analyzed by Student's t test) is indicated by p value=0.0095. SphK2-/- mice spent more time grooming compared to the WT mice. (b) Significant difference in time spent freezing between the two genotypes (as analyzed by Student's t test) is indicated by p value=0.003. SphK2-/- mice spent more time freezing compared to the WT mice. (c) Significant difference in the number of entries in the Centre of the EPM between the two genotypes (as analyzed by Student's t test) is indicated by p value=0.0443. All mice were given 5 min to explore the maze. WT mice entered the center of the EPM more than the SphK2-/- mice. Data are presented as mean ± SEM.

4.3 Spatial Learning and Memory in the Cheeseboard

In the CB paradigm, WT and SphK2-/- mice found the food reward faster as training proceeded (Fig. 8a). 2way ANOVA revealed a significant effect of 'day' for the latency to find the food reward ($p<0.0001$) whereas genotype had no effect ($p=0.42$).

After 8 days of training, a probe trial was conducted to test spatial reference memory. Although WT mice showed a greater preference for the exploration of the target zone than expected by chance (WT: 20.4% vs 12.5%, P = 0.041 in a paired t-test; SphK2: 16.3% vs 12.5%, P = 0.11>), but the difference was not significant between the two genotypes (p=0.42 by unpaired student's t test). Mice were then trained in reversal training where the food reward was now located on the precise opposite side of the CB (Fig. 8b). Again, latency to find the food reward was influenced by day and not genotype (p=0.0003; p=0.08 respectively, 2way ANOVA) (Fig. 8c). After 4 days of reversal training, a probe trial was conducted. Contrary to the original probe trial, SphK2-/- mice but not WT showed a greater preference for the target zone than expected by chance (WT: 17.8%, P = 0.080; SphK2-/-: 22.0%, P = 0.016). Moreover, there was no significant difference between the two strains (p=0.42 by student's t test) (Fig. 8d).

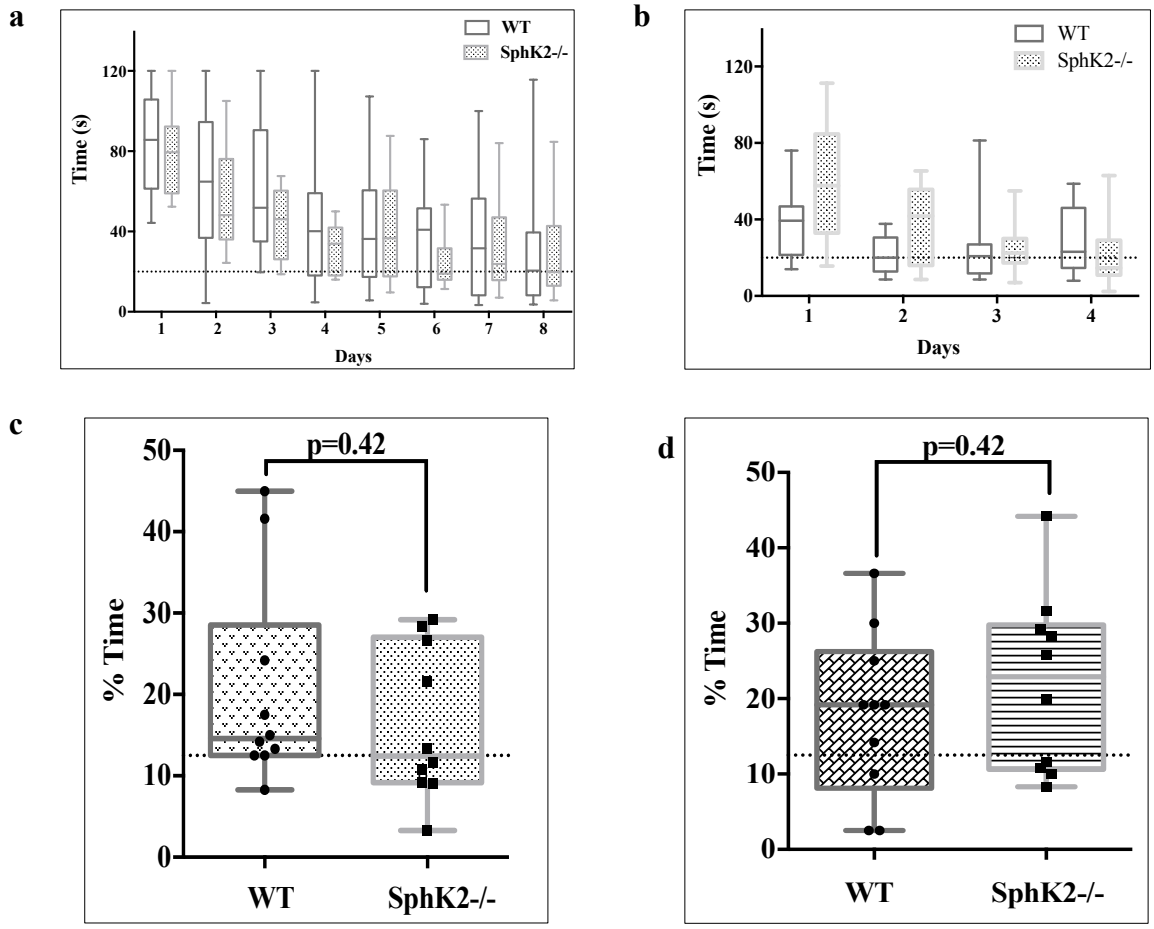


Figure 8 (a-d): Spatial learning and memory in the cheeseboard. (a) Latency (s) to find the food reward (averaged across 3 trials per day) during training. Time spent in the target zone according to chance (=20s) is marked with a dotted line; (b) Latency (s) to find the food reward (averaged across 3 trials per day) during reversal training. Time spent in the target zone according to chance (=20s) is marked with a dotted line; (c) Percentage time (%) spent in the target zone of the CB (i.e. in close proximity to the well containing food reward) during the 2 min probe trial; (d) Percentage time (%) spent in the target zone of the CB during the 2 min reversal probe trial. Time spent in the target zone according to chance (=12.5%) is marked with a dotted line. Data presented for zone time does not include the time spent in the centre zone. Data for SphK2-/- and WT mice are shown as mean ± SEM.

4.4 Fear Conditioning followed by Extinction

WT and SphK2-/- mice responded to the electric foot-shock (FS) delivered during Conditioning (Fig. 9). Both genotypes displayed elevated freezing during the first 2 min on Context Day (re-exposure to the shock cage; day 3) compared with the first 2 min of pre-conditioning (Day 1) indicating that all mice had learnt the association

between the context and the FS. Upon repeated re-exposure to the context on days 4-5 (fear extinction trials with 24 h interval) without receiving the foot-shock again, SphK2-/- mice showed significantly increased freezing compared to the WT mice. There was no evidence of extinction of the fear memory (Fig. 9). However, SphK2-/- mice showed elevated freezing compared to the WT mice (as in the EPM).

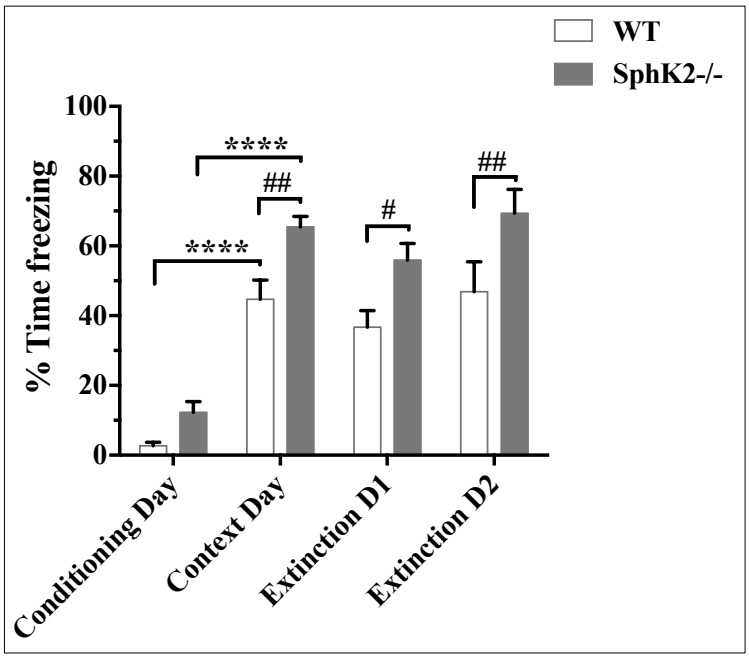


Figure 9: Fear Conditioning- Context Test. % Time spent freezing during fear conditioning (day 1), context (day 3) and extinction (days 4 and 5) trials. Both genotypes learnt the association between the CS and FS (****, $p < 0.0001$ compared to context test day). There is no evidence of extinction of the fear memory however difference between the two genotypes is significant (#, $p < 0.05$ SphK2-/- versus WT; ##, $p < 0.01$ SphK2-/- versus WT). P values are analyzed by 2way ANOVA. Data are presented as mean ± SEM.

In the cue test (Day 2), there was a significant difference in percentage time freezing between the two genotypes before the cue was presented ($p=0.01$ by 2way ANOVA) whereas there was no significant difference during and after the cue (Fig. 10).

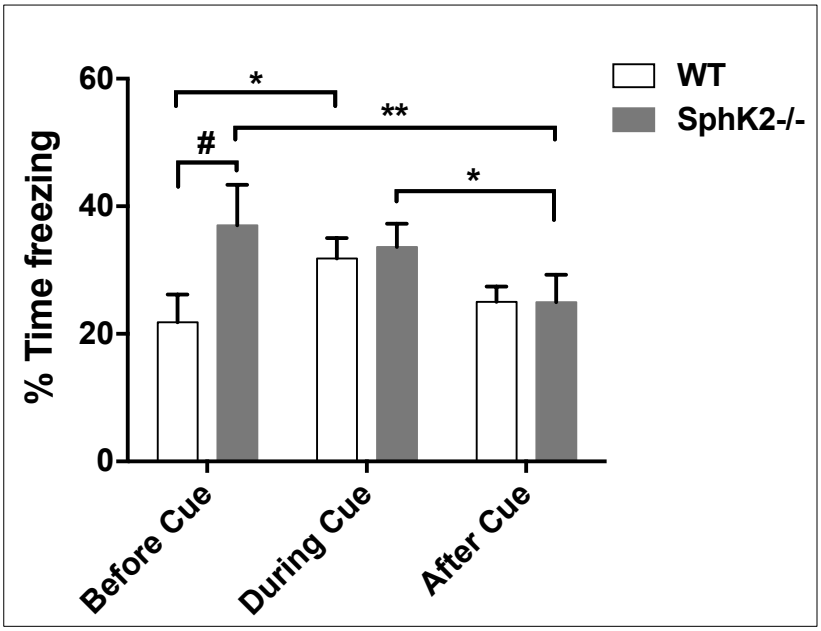


Figure 10: Fear Response to the Cue. Time spent freezing (%) in the cue test. Mice were exposed to the auditory cue in a different contextual environment. Graph shows percentage of total time spent freezing before, during, and after the auditory cue P value analysed by 2way ANOVA revealed significant difference between genotypes before the cue (#, p=0.01). Data are presented as mean ± SEM.

4.5 Quantification of Brain Sphingolipids

LC-MS/MS was used to analyse changes to sphingosine, dihydrosphingosine, S1P and dihydro-S1P. Tissue samples were derived from hippocampus and cerebellum of the WT and SphK2-/- mice. S1P levels in the hippocampus and cerebellum of SphK2-/- mice were reduced by 86% and 90%, respectively, compared to the WT mice ($P < 0.0001$ and $P = 0.0037$ respectively) (Fig. 11a). The closely related lipid dihydro-S1P was reduced by 84% ($P = 0.0212$) in hippocampus and 91% ($P = 0.0008$) in cerebellum (Fig. 11b). Levels of the substrates for SphK2, sphingosine and dihydrosphingosine, increased 1.6-fold ($P = 0.0022$) and 2.9-fold ($P = 0.0005$), respectively, in the hippocampus (Fig. 11c). There was no statistically significant increase in sphingosine or dihydrosphingosine in the cerebellum, although mean dihydrosphingosine levels increased 1.5-fold (Fig. 11d).

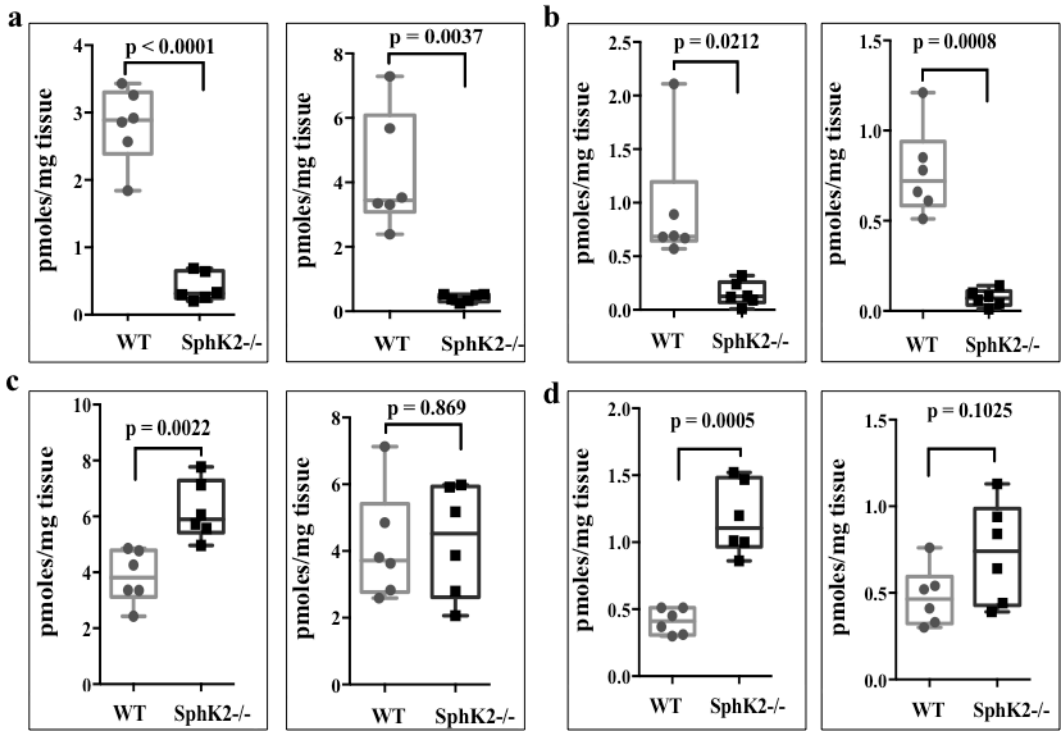


Figure 11: Brain levels of S1P and dihydro-S1P remarkably low in SphK2-/- mice. (a) S1P and (b) dihydro-S1P content is heavily reduced in hippocampus and cerebellum of SphK2-/- mice compared to WT mice; (c) Levels of sphingosine and (d) dihydrosphingosine increased in hippocampus of SphK2-/- mice compared to WT mice with no significant change in the cerebellum. Data are presented as mean ± SEM.

4.6 Functional Association Network of SphK2

In silico analysis of SphK2 revealed its interaction with several other enzymes involved in sphingolipid metabolism. A total of 10 interacting partners of SphK2 were revealed. Strongest interactions of SphK2 were found with S1P phosphatase 1 and S1P phosphatase 2 (SGPP1 and SGPP2) and S1P lyase (SGPL1). Ceramide synthases were also revealed as interacting protein partners of SphK2.

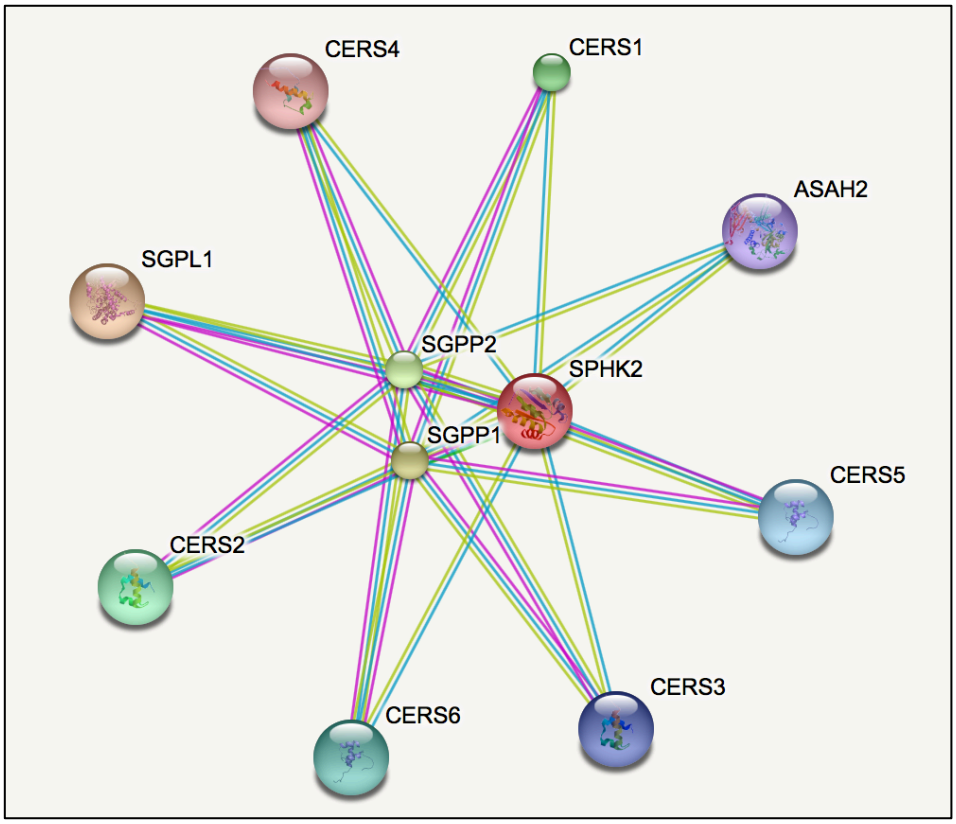


Figure 12: Functional association network of identified proteins. High confidence protein-protein interaction network of identified proteins derived from the STRING database (http://string-db.org). Each protein is represented as a node with edged interactions.

Chapter 5: Discussion

The present study has conducted several behavioral tests to assess learning and memory patterns of SphK2-/- mice. In the fear-conditioning test, increased time spent freezing on day 3, when the mice were placed back into the shock cage, indicated that both strains had learned the association between the context and the FS. On subsequent days, freezing time was reduced, suggestive of fear extinction, but this was only statistically significant for SphK2-/- mice on the third day as compared to the first day of re-exposure to the context. SphK2-/- mice spent more time freezing during pre-conditioning, on context test day, and on both subsequent days testing for fear extinction. The association between the aural cue and freezing time, tested on day 2, was less robust and statistically significant only in the WT mice. The difference between the genotypes likely resulted from the increased time that SphK2-/- mice spent freezing prior to the cue, which supports the conclusion of a generalized anxiety deficit in SphK2-/- when exposed to a new environment. In contrast to previously published results, our findings are not indicative of a specific deficit in fear extinction in SphK2-/- mice (Hait *et al.,* 2014).

Mice were extensively analyzed for anxiety-related parameters in the EPM, which is a widely used test of anxiety (Rodgers and Dalvi, 1997). Further supporting the conclusion of a general anxiety phenotype, SphK2-/- mice spent significantly more time freezing in the EPM. SphK2-/- mice made significantly less entries into the center of the maze compared to the WT mice. Moreover, they made fewer entries and spent less time in the open arms compared to the WT mice, however, this difference was not statistically significant. SphK2-/- mice spent significantly more time grooming in the maze, which is why they made fewer entries into the center and open arms. These behaviors support our conclusion of a generalized anxiety phenotype displayed by the SphK2-/- mice compared to the WT mice consistent with previously described results (Rodgers and Dalvi, 1997).

The CB paradigm was employed as a less stressful dry-land alternative to the Morris Water Maze (MWM) test (Llano Lopez *et al.,* 2010). A previous study used MWM and reported a spatial memory deficit in SphK2-/- mice (Hait *et al.,* 2014). Test animals in our study exhibited no impairments in the spatial reference memory. Our contradicting results could potentially be due to the general anxiety that SphK2-/- mice display when placed in a novel environment. Further studies that utilize

behavioral tests unraveling S1P's role in memory and learning are required.

In light of the behavioral phenotyping results, functional association network analysis of SphK2 was performed to better comprehend the impact of interacting protein partners on SphK2. This in-silico analysis revealed associations of SphK2 with ceramide synthases, sphingosine lyase and phosphatases. Sphingolipid signaling molecules; S1P and ceramide form an intricately regulated 'rheostat' where these substrates are interconvertible via action of their specific enzymes (Cuvillier *et al.,* 1996). This functional rheostat is altered in disease states. Studies have reported altered sphingolipid metabolism in neurodegenerative diseases including but not limited to AD. Role of sphingolipids in major anxiety related disorders and depression has been investigated by manipulating the relevant enzymes. Anxiety related disorders and depression have been associated with increased levels of ceramides in the brain. One study used an animal model of anxiety and reported association of anxiety with increased levels of galactosylceramide concentrations in the amygdala, a major region of brain implicated in anxiety disorders (Ono *et al.,* 2008). In another study, pharmacological intervention decreased levels of ceramides in the hippocampus of mouse models of anxiety further validating the role of increased ceramides in anxiety (Gulbins *et al.,* 2013).

The remarkably low S1P levels in the brains of SphK2-/- mice observed in the present study indicate that SphK2 and not SphK1 is the dominant isoform in the brain that synthesizes S1P. This is consistent with previous findings that reported significantly reduced levels of S1P in the spinal cord of mice deficient in SphK2 (Canlas *et al.,* 2015). Accumulation of sphingosine and dihydrosphingosine in the hippocampus of SphK2-/- indicate substrate accumulation in the absence of SphK2. In the cerebellum, sphingosine is more efficiently reutilized for the synthesis of more complex sphingolipids (termed the "salvage" or sphingolipid recycling pathway) and therefore does not accumulate (van Echten-Deckert and Herget, 2006). S1P has been shown to stimulate glutamate release from CA3 hippocampal neurons via $S1P_3$ signaling thereby inducing long term potentiation (LTP) (Kanno *et al.,* 2010). In a previously reported study, LTP was significantly altered in SphK1-/- mice suggesting that SphK1 is required for S1P generation in hippocampus. This is in contrast to our findings, as SphK1 did not compensate hippocampal S1P content in our mice models. Higher SphK2 activity compared to SphK1 in mouse and human brain extracts were also

reported previously that supports the findings of the present study (Blondeau *et al.,* 2007; Couttas *et al.,* 2014).

Cumulatively, these studies have strengthened our conclusion of a generalized anxiety phenotype in the SphK2-/- cohort and warranted further studies imploring the involvement of S1P in AD pathology.

Chapter 6: Conclusion

In the present study, SphK2-/- mice spent significantly more time freezing in the EPM and fear conditioning experiments, supporting the conclusion of a general anxiety phenotype. SphK2-/- mice did not exhibit any deficit in spatial reference memory in the cheeseboard paradigm. S1P levels in the brains of SphK2-/- mice were markedly lower than their WT littermates, indicating that SphK2, and not SphK1, is the dominant source of S1P synthesis in the brain. We infer from functional association network analysis of SphK2 that knocking out SphK2 disturbed the functional rheostat between S1P and ceramides and made the SphK2-/- mice more prone to anxiety.

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