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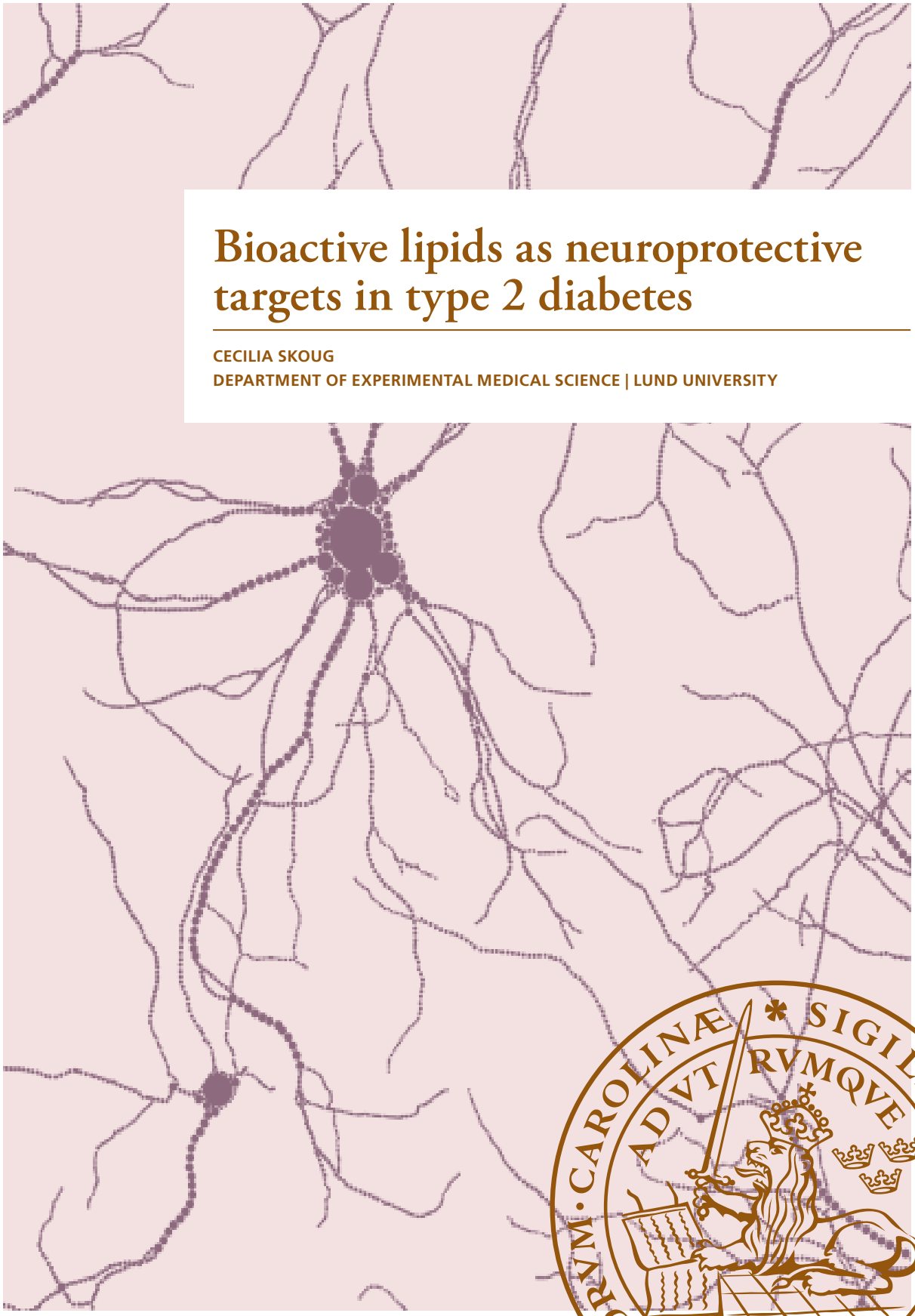

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Cecilia Skoug

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Title and subtitle: Bioactive lipids as neuroprotective targets in type 2 diabetes.

Abstract: Type 2 diabetes (T2D) is a metabolic disease characterized by insulin resistance (cells do not respond to insulin) and hyperglycemia (i.e., high blood glucose levels). One risk factor to develop the pathology is lipid toxicity. Metabolic syndrome components such as obesity and T2D impact the brain through a process that involves synaptic dysfunction. The brain is rich in a variety of lipids, some of which have bioactive roles, such as sphingolipids, endocannabinoids, and eicosanoids. However, the implications of a changed lipidome and the consequences for brain function are not very well explored. The exploration of bioactive lipids and their signaling pathway is emerging as an interest in various dementia research. Metabolism of these lipids and their signaling pathways are putative targets to afford neuroprotection.

This thesis focuses on generation of bioactive lipid metabolism, specifically on one of the major lipases, hormone sensitive lipase (HSL) and also on one specific lipid pathway; the Sphingosine 1-phosphate pathway. Specifically, this thesis investigates the cellular distribution of HSL and S1P receptors in the brain, explore novel roles of brain HSL, and test the ability of S1P receptors to control neuron physiology, and their alterations in models of T2D. Overall, with this work, the aim is at proposing targets for synaptic protection in T2D.

In the brain, which relies mainly on glucose oxidation, we propose that HSL regulates the availability of bioactive lipids for fine tuning of neuronal function. Specifically, we focus on the neurovascular unit and the availability of bioactive lipids regulating vessel constriction/dilation and cerebral blood flow. On the other hand, the changes of brain sphingosine 1-phosphate (S1P) in the brain in diabetes models, as well as its specific receptors and one of the generation enzymes; sphingosine kinase.

These results provide deeper insight in the importance of brain lipid signaling and could in the future serve as a basis for developing a strategy to halt brain dysfunction in metabolic disorders, namely T2D, by modulating lipid metabolism and signaling.

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I dedicate this thesis to my family,
the one I was given and the one I chose.

Table of Contents

Summary

Type 2 diabetes (T2D) is a metabolic disease characterized by insulin resistance (cells do not respond to insulin) and hyperglycemia (i.e., high blood glucose levels). One risk factor to develop the pathology is lipid toxicity. Metabolic syndrome components such as obesity and T2D impact the brain through a process that involves synaptic dysfunction. The brain is rich in a variety of lipids, some of which have bioactive roles, such as sphingolipids, endocannabinoids, and eicosanoids. However, the implications of a changed lipidome and the consequences for brain function are not very well explored. The exploration of bioactive lipids and their signaling pathway is emerging as an interest in various dementia research. Metabolism of these lipids and their signaling pathways are putative targets to afford neuroprotection.

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In the brain, which relies mainly on glucose oxidation, we propose that HSL regulates the availability of bioactive lipids for fine tuning of neuronal function. Specifically, we focus on the neurovascular unit and the availability of bioactive lipids regulating vessel constriction/dilation and cerebral blood flow. On the other hand, the changes of brain sphingosine 1-phosphate (S1P) in the brain in diabetes models, as well as its specific receptors and one of the generation enzymes; sphingosine kinase.

These results provide deeper insight in the importance of brain lipid signaling and could in the future serve as a basis for developing a strategy to halt brain dysfunction in metabolic disorders, namely T2D, by modulating lipid metabolism and signaling.

Sammanfattning

Typ 2 diabetes (T2D) är en metabol sjukdom som karaktäriseras av insulinresistens (celler svarar inte på insulin) och högt blodsocker. En riskfaktor för att utveckla sjukdomen är hög nivå av fettsyror i kroppen. Beståndsdelar av metabola syndrom såsom obesitas och T2D påverkar hjärnan genom olika processer som kan påverka synapsernas funktion. Hjärnan består till stor del av olika typer av fetter, vissa av dem har bioaktiva roller till exempel sfingolipider, endocannbinoider och eicosannoider. Vad som händer när olika typer av fetter i hjärnan förändras är emellertid inte väl utforskat. Bioaktiva fetter, som har möjlighet att påverka olika hjärnceller på olika vis, har blivit ett ämne som väckt mer intresse inom demensforskning. Hur dessa bioaktiva fetter regleras eller signalerar skulle kunna vara mål för behandling, antingen som förebyggande åtgärd eller som lindrande åtgärd.

Denna avhandling fokuserar på bioaktiva lipider. Mer specifikt utforskas enzymet hormonkänsligt lipas (HKL) som reglerar tillgången till nämnda bioaktiva lipider och sfingosin 1 - fosfat (S1P) och dess signalering. Sammantaget, visar denna avhandling resultat gällande hur bioaktiva lipider kan påverka hjärnceller, specifikt neuron, hjärnfunktion och hur dessa påverkas när det sker perifera förändringar i metabolismen. Specifikt behandlar avhandlingen den cellulära distributionen av HKL och S1P-receptorerna i hjärnan, utforskar nya roller för HKL i hjärnan och testar S1P- receptorers möjlighet att påverka hjärnfunktion specifikt i modeller för T2D. Med detta arbete eftersträvas att identifiera mål för att skydda synapser i samband med T2D.

I dessa studier demonstreras att hjärnan, som i huvudsak drivs av glukos-tillgång, också har ett behov av HKL genom att detta reglerar tillgången till bioaktiva fetter för att finreglera hjärnfunktion. Resultaten pekar på att HKL specifikt påverkar den neurovaskulära kopplingen och tillgången till de bioaktiva fetter som reglerar kärlsammandragning och kärlvidgning. Vidare pekar resultaten på förändringar S1P-signaleringen i hjärnan hos diabetesmodeller, framförallt genom en nedreglering av S1P-receptorer

Generellt ger resultaten i denna avhandling en insikt i betydelsen av signaleringen från bioaktiva lipider, vilket i framtiden skulle kunna utnyttjas för att utveckla proaktiva och reaktiva strategier för att bromsa nedsatt hjärnfunktion i samband med T2D.

List of Papers

I. **Skoug C,** Holm C, Duarte JMN (2022) Hormone-sensitive lipase is localized at synapses and is necessary for normal memory functioning in mice. *J Lipid Res 63(5):100195*.

II. **Skoug C,** Holm C, Duarte JMN (2022) Brain perfusion is impaired in both low-fat and high-fat diet-fed male and female hormone-sensitive lipase null mice. [*manuscript in preparation*]

III. **Skoug C**, Martinsson I, Gouras GK, Meissner A, Duarte JMN (2022) Sphingosine 1-phosphate receptors are located in synapses and control spontaneous activity of mouse neurons in culture. *Neurochem Res 47(10): 3114-3125*.

IV. **Skoug C,** Erdogan H, Vanherle L, Vieira JPP, Meissner A, Duarte JMN (2022) Density of sphingosine 1-phosphate receptors is altered in cortical nerve-terminals of type 2 diabetes models. [*manuscript in preparation*]

V. **Skoug C*,** Vanherle L*, Fryklund F, Stenkula K, Meissner A, Duarte JMN (2022) Sphingosine kinase inhibition in mice fed a high fat-diet ameliorates obesity-induced neuroinflammation in the hippocampus. [*manuscript in preparation*]

Published papers outside of the thesis

VI. Duarte, JMN., **Skoug, C**., Silva, HB., Carvalho, RA., Gruetter, R., & Cunha, RA. (2018) Impact of Caffeine Consumption on Type 2 Diabetes-induced Spatial Memory Impairment and Neurochemical Alterations in the Hippocampus. *Front Neurosci, 12,1015*

VII. Mohr, AA., Garcia-Serrano, AM., Viera, JP., **Skoug, C**., Davidsson, H., & Duarte, JMN. (2021) A glucose-stimulated BOLD fMRI study of hypothalamic dysfunction in mice fed a high-fat and high-sucrose diet. *J Cereb Blood Flow Metab.*

VIII. Garcia-Serrano AM. Mohr, AA., Philippe, J., **Skoug, C**., Spégel, P., & Duarte, JMN (2021) Cognitive impairment and Metabolite Profile Alteration in the Hippocampus and Cortex of Male and Female Mice Exposed to a Fat and Sugar-Rich Diet are Normalized by Diet Reversal. *Aging and disease, 13(1)*

IX. Vanherle, L., Lidington, D., Uhl, FE., Steiner, S., Vassallo, S., **Skoug, C.,** Duarte, JMN., Ramu, S., Uller. L., Desjardins, JF., Connelly, KA., Bolz, SS. & Meissner, A., (2022) Restoring heart failure-induced long-term memory impairment by targeting the cystic fibrosis transmembrane regulator. *eBiomedicine*

X. Meissner, A., Garcia-Serrano, A.M., Vanherle, L., Rafiee, Z., Don-Doncow, N., **Skoug, C.,** Larsson, S., Gottschalk, M., Magnusson, M., & Duarte, J.M.N., (2023). Alterations to cerebral perfusion, metabolite profiles, and neuronal morphology in the hippocampus and cortex of male and female mice during chronic exposure to a high-salt diet. J. Mol. Sci

Abbreviations

AA	arachidonic acid
AD	Alzheimer's disease
ATGL	adipose triglyceride lipase
ASL-MRI	arterial spin labelig magnetic resonance imaging
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CB	Cannabinoid receptor
CD	control diet
CE	cholesterol ester
CNS	central nervous system
COX	cyclooxygenase
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DEXA	dual-energy-x-ray
FAAH	fatty acid amide hydrolase
FA	fatty acid
FWHM	full-width half-max
HFD	high-fat diet
HSL	hormone-sensitive lipase
GK	Goto-Kakizaki
GTT	glucose tolerance test
ITT	insulin tolerance test
LD	Lipid droplet
MAG	monoacylglycerol
MAGL	monoacylglycerol lipase
MCI	mild cognitive impairment
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
Sph	Sphingosine
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
SphK	sphingosine kinase
SphK2i	sphingosine kinase 2 inhibitor
TAG	triacylglycerol
T2D	Type 2 Diabetes
TX	thromboxanes
2-AG	2-arachidonoylglycerol

Introduction

Diabetes and brain function

Diabetes mellitus is one of the most prevalent metabolic diseases (Chatterjee et al., 2017; Ng et al., 2017), where type 2 diabetes (T2D) accounts for the majority of diabetes cases and is often linked to obesity. Obesity has been associated with apparent systemic and tissue inflammation (Luft et al., 2013), which further contributes to diabetes complications (King, 2008). The metabolic imbalance that follows type 2 diabetes or metabolic syndrome has also been shown to have a negative effect on brain function, increasing the risk of developing dementia diseases. This negative effect has been proposed to be due to a number of underlying molecular mechanisms, such as neuroinflammation, brain metabolic changes and synaptic dysfunction (Akash et al., 2013; de Bem et al., 2020; Duarte et al., 2018; Garcia-Serrano et al., 2022). Lipid composition and/or the function of bioactive lipids are crucial for adequate brain function.

Lipid toxicity is a known risk factor for T2D, however, the implication of a changed lipidome and the consequences for brain function are not very well explored. It has been reported that dietary intake of certain fatty acids can affect the synthesis of oxylipins and overall lipid profile in brain. (Reemst et al., 2022). Lipid droplets (LDs) have gained increasing attention in the field of neurodegeneration and neuropathology (Farmer et al., 2020). Multiple brain cells contain LDs and are for example present in neurons (Shimabukuro et al., 2016) and astrocytes (Hamilton & Fernandes, 2018; Shimabukuro et al., 2016), and highly abundant in microglia (Marschallinger et al., 2020). The accumulation of lipids in neuropathology is not well studied, although it is one of the hallmarks of Alzheimer's disease (AD) first reported by Alois Alzheimer: "many glia include adipose inclusions" (Alzheimer et al., 1995). The field of lipids is still to be fully explored, with new and more precise tools, lipids are receiving an increased interest both as a biomarker as well as a target for disease control (Markovic et al., 2020).

Brain and lipids

The brain, like most organs, utilizes fatty acids as oxidative substrate (Ebert et al., 2003; Kuge et al., 1995). The brain expresses adipose triglyceride lipase (ATGL) (Etschmaier et al., 2011) and HSL (Haemmerle et al., 2002), which have been suggested to control the release of free fatty acids to be used as energy substrate (Ebert et al., 2003). Brain cells can oxidize alternative substrates under a variety of physiological or pathological conditions, however, glucose is considered to be the obligatory fuel for adequate brain function (Dienel, 2019; Sonnay et al., 2017). Although, lipases in general does not only need to act as to fulfil energetic needs. An example of a lipase with dual action is the monoacylglycerol lipase (MAGL), both with the ability to generate fatty acids as well as the ability to terminate the action of the main endocannabinoid 2-arachidonoylglycerol (2-AG) (Blankman et al., 2007).

The model of lipolysis is understandable based upon lipolysis in adipocytes. Breakdown of triacylglycerols (TAG) in adipose tissue is mediated by ATGL that hydrolyses a fatty acid (FA) from TAG generating diacylglycerols (DAGs). Diacylglycerol lipase (DAGL) and HSL hydrolyses DAG. The latter is regulated by hormones, stimulated by chatecholamines e.g. epinephrine promoting an increase cAMP level. The rise of the cyclic adenosine monophosphate (cAMP) activates protein kinase A (PKA) which in turn phosphorylates HSL causing activation. In opposite fashion, insulin will stimulate phosphodiestareses, accelerating cAMP hydrolysis causing inhibition of HSL. The acylglycerol lipolytic pathway ends with monoglyceride lipase (MAGL) degrading monoacylglycerols (MAGs) (Townsend et al., 2017) (Figure 1).

HSL is highly expressed in adipose tissue, where its main action is to hydrolyse acylglycerols with purpose of energy release. HSL has a preference for DAG hydrolysis, although HSL as a broad-spectrum lipase also have the ability to hydrolyse TAG, MAG, cholesterol ester and retinyl esters (Fredrikson et al., 1981; Raclot et al., 2001). (Edwards & Mohiuddin, 2022; Haemmerle et al., 2002). Besides the abundant expression of HSL in adipose tissue, lower levels have been detected in other tissues including skeletal muscle (Langfort et al., 2003), testis (Vallet-Erdtmann et al., 2004), β-cells of pancreatic islets (Fex et al., 2009) including the brain. Despite the knowledge of HSL being present in the brain (Haemmerle et al., 2002; Hundahl et al., 2021; Skoug, Holm, et al., 2022), its local function has only been limitedly studied.

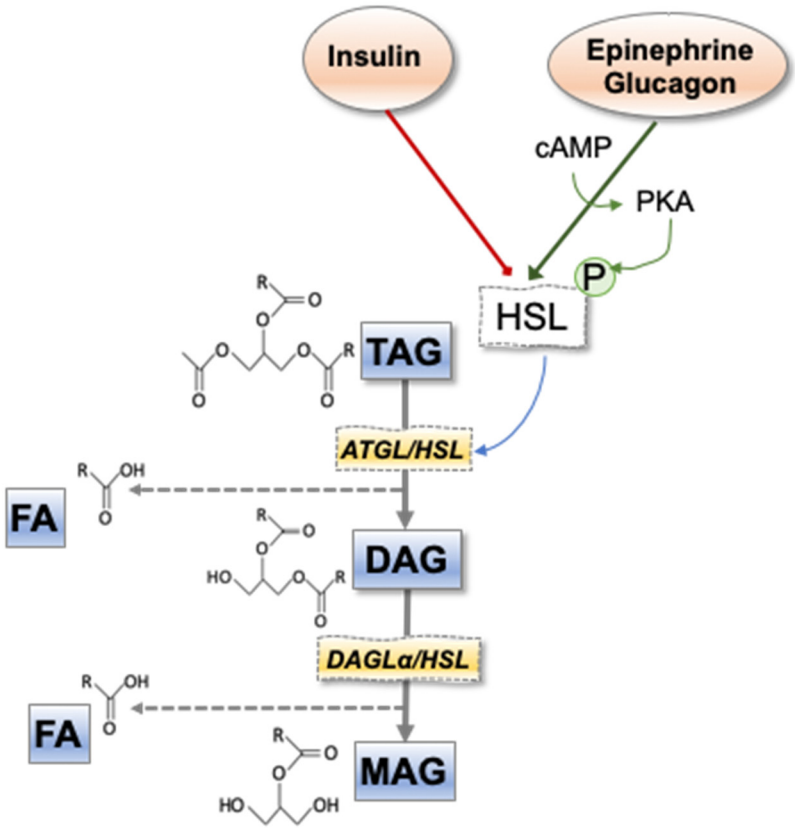

Figure 1. Lipolysis. This describes the metabolic pathway of how tri-/di-/acylglycerols are hydrolyzed into acylglycerol and a free fatty acid. This process of hydrolysis is performed through the activity of lipases.

The other lipase that hydrolyses DAG is more extensively studied in the brain. Presence of DAGLα has been reported in the post-synaptic compartment of the neuron where it is involved in the regulation of endocannabinoids (Gao et al., 2010). Interestingly, DAGLα deletion in mice has also been proposed to disrupt learning and memory due to the depletion of 2-AG and arachidonic acid across the whole brain (Schurman et al., 2019). Lipoprotein lipase, which have been identified in several brain areas has been proposed to hydrolyse very-low-density lipoprotein (VLDL) and TAG, and contributes to regulate energy balance (Cruciani-Guglielmacci & Magnan, 2017; Eckel & Robbins, 1984).

MAGL is known as a key player within of the endocannabinoid system in the brain (Blankman et al., 2007). Together with alpha/beta-Hydrolyse domain containing (ABHD) 12, and ABHD6, it controls about 99% of 2-AG availability in the brain (Savinainen et al., 2012). Each of these enzymes exhibits a distinct subcellular

distribution, suggesting that they regulate distinct pools of 2-AG in the nervous system (Blankman et al., 2007). The other main endocannabinoid anandamide is hydrolysed by fatty acid amide hydrolase (FAAH), which also hydrolyses other bioactive lipids, such as *N*-palmitoylethanolamine (PEA) (Fezza et al., 2008).

Overall, the lipases acting in the brain possess the role of regulating lipid availability.

Bioactive lipids

Bioactive lipids could be described as being lipid messengers, able to act as physiology regulators. Bioactive lipids can be divided into subclasses, fatty acids, glycerolipids, (glycero)phospholipids, sphingolipids and saccharolipids are ones usually considered as subclasses (Park et al., 2021). Their signals contribute to brain function by regulating or interacting in cell development, differentiation and protection. Bioactive lipids also exhibit specific roles in synaptic plasticity, and regulation of mitochondrial function, neurovascular coupling and neuroinflammation (Bazan, 2005). Further, fatty acids are also participating in regulating energy balance via fatty acid sensing neurons (Levin et al., 2011). Synaptic plasticity and neuroinflammation have been connected to T2D-related brain dysfunction but the involvement of lipids in this state needs more investigation.

Endocannabinoids

Endocannabinoids control brain metabolism and synaptic function (Metna-Laurent & Marsicano, 2015) though binding to their receptors. The most well-known endocannabinoids are 2-AG and anademine (AEA)(Lu & Mackie, 2021). Cannabinoid receptor type 1 (CB1) and type 2 (CB2) are G-protein-coupled endocannabinoid receptors which differ in some morphological aspects. Their main difference lies in the fact that CB1 in CNS tissues is mostly found in neurons, while CB2 is more abundant in glia (Ativie et al., 2018; Kano et al., 2009; Miller & Stella, 2008). Furthermore, CB2 has been found to be upregulated in microglial cells in pathological conditions, and has been implicated in the regulation of cytokine release and cell proliferation (Miller & Stella, 2008).

The endocannabinoid 2-AG is synthetized by DAGL α and β at postsynaptic terminals following stimulation by glutamate. 2-AG then released to presynaptic terminals where it stimulates cannabinoid 1 receptors (CB1R), here it can dampen glutamatergic activity (Iannotti et al., 2016). Moreover, CB1R signaling elicits other functions outside of the neuronal active zone, for example inhibiting mitochondrial metabolism in neurons and astrocytes, controlling glycolysis and glycogen

metabolism and gliotransmission (Duarte et al., 2012; Fernandez-Moncada & Marsicano, 2023).

Eicosanoids

Bioactive lipids, such as eicosanoids, are produced from arachidonic acid. Eicosanoids are subdivided in prostaglandins, thromboxanes (TXs) epoxyeicosatrienoic acids (EETs) which are produced via the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) or cytochrome P450-pathway (Andreasson, 2010; Dubois et al., 1998; Harder et al., 1998). Arachidonic acid can either be produced from the breakdown of neutral lipids, such as acylglycerols and CEs, (Konrad et al., 1994) or through release from phospholipid pools (Gao et al., 2010; Rouzer & Marnett, 2011). MAGL is considered a rate-limiting enzyme for free arachidonic acid synthesis in the brain (Nomura et al., 2011).

Eicosanoids are connected to actions regulating or impacting synaptic plasticity and memory formation (Tassoni et al., 2008). Different types of eicosanoids have been proposed to be involved in a number of functions in the CNS for example as pro-inflammatory mediators (Donvito et al., 2018). They have also been described as modulators of cerebral blood flow CBF, participating in the modulation of CBF in response to neuronal demands by acting on the vascular bed (Attwell et al., 2010; Sonnay et al., 2017) which is important for matching nutrient supply from circulation to the demands of neuronal work. Further, prostaglandins have been previously reported to affect cognition, and performance in short-term spatial memory tests, which was improved when inhibiting the prostaglandin E2 (PGE2) receptor (Jiang et al., 2020; Rojas et al., 2016; Yin et al., 2022). Also, PGE2 biosynthesis is associated with several different processes, for example the regulation of synaptic plasticity or the modulation of the pathogenesis of neuroinflammation (Sheppe & Edelmann, 2021; Tassoni et al., 2008).

Sphingolipids

The sphingolipids ceramide-1-phosphate, sphingosine (Sph) and sphingosine1-phosphate (S1P) have been described to regulate proliferation, differentiation, cell growth and inflammation in cells of the CNS (Blaho & Hla, 2014). Ceramide is the more studied in the field of diabetes, accumulation of ceramide have previously been connected to the development of insulin resistance (Boden, 2008) and considered detrimental for cell processes. S1P is produced from ceramide by ceramidase followed by phosphorylation of sphingosine through one of its two kinases, sphingosine kinase 1 or 2 (SphK1/2) (Spiegel & Milstien, 2003) (Hannun & Obeid, 2008). The sphingolipid rheostat which coordinates levels of ceramide ad

S1P is considered important in cell-fate determination due to the opposing signalling pathways of ceramide and S1P (Spiegel & Milstien, 2003).

In steady state, the generation of circulating S1P is mainly mediated via SphK1, while SphK2-mediated S1P production is considered to be localized to intracellular sites and certain tissues such as in the pancreas, liver, and brain (Maceyka et al., 2005). S1P is abundantly produced by erythrocytes and endothelial cells (Kim et al., 2009), however, synthesis in the CNS has also been reported. Namely, astrocytes have been shown to produce S1P upon basic fibroblast growth factor stimulation (Bassi et al., 2006).

S1P in the extracellular space can bind to its five specific G-coupled receptors S1PR1-5, which signal through diverse downstream pathways (Blaho & Hla, 2014).

Sphingosine 1-phosphate receptors

All S1PRs have been identified in at least one cell type of the CNS, such as neuroblasts, neurons, astrocytes, microglia and oligodendrocytes Their subsequent signaling through Gi, Gq or $G_{12/13}$ relays regulation of phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), phospholipases or cAMP (Figure 2). They participate in multiple important functions in CNS development, and further contribute to pathological conditions such as ischemic stroke (Salas-Perdomo et al., 2019), multiple sclerosis (O'Sullivan & Dev, 2017), hearing loss (Herr et al., 2016) and seizures (Choi & Chun, 2013; Scherer et al., 2010).

S1PR1 is the most studied S1P receptor with important roles in angiogenesis and neurogenesis (Mizugishi et al., 2005) and a key function in immunological development as it regulates the amount of circulating immune cells. S1PR2 is a modulator of neuronal excitability during neuronal development (MacLennan et al., 2001) and controls activity of cultured neurons (Skoug, Martinsson, et al., 2022). S1PR2 also plays an essential role in regulating endothelial polarity, which influences blood-brain-barrier permeability and capture of infiltrating lymphocytes as it is connected to regulation of adherent junctions. S1PR3 receptor controls activity of microglia and their participation in neuroinflammation (Gaikwad & Heneka, 2013; Gaire et al., 2018; Riganti et al., 2016), and is highly expressed in astrocytes, where it regulates astrogliosis via activation of the small GTPase RhoA (Dusaban et al., 2017). Until recently, S1PR4 has received little attention in the CNS (Brunkhorst et al., 2014) as the receptor is mainly expressed on immune cells. S1PR5 is considered to be mostly located on oligodendrocyte precursor cells and mature oligodendrocytes, where it contributes to regulate the differentiation process (Blaho & Hla, 2014).

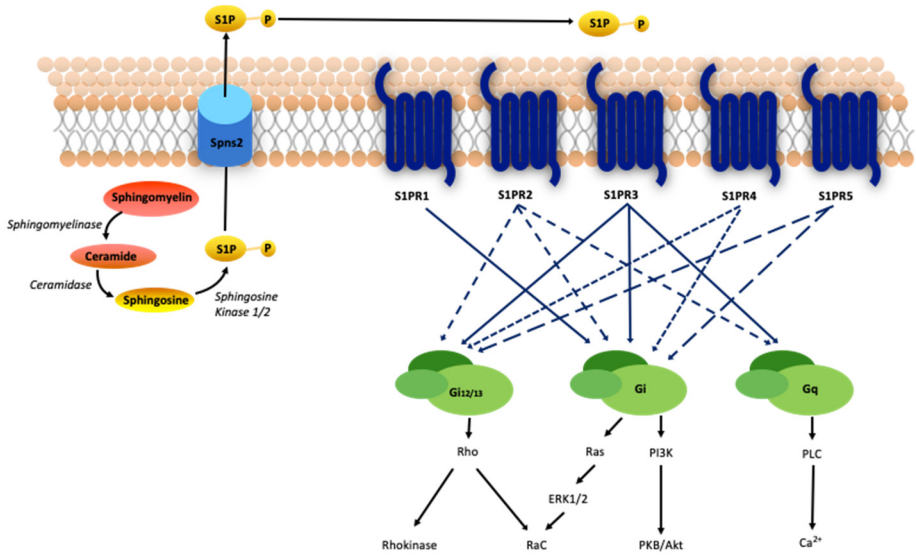

Figure 2 The sphingosine 1-phosphate receptor pathway. S1p is produced from ceramide to sphingosine and is phosphorylated by SphK1/2 to sphingosine 1-phosphate. The S1P transporter Spns2 transports S1P out of the membrane where it can bind to its receptors. The S1PR couples with their G protein which results in inhibition or activation of downstream messengers.

Bioactive lipids in brain pathology

Impairment of lipid metabolism has been proposed in several neurological disorders such as AD, mild cognitive impairment (MCI) and epilepsy (Ajith et al., 2021; Wood, Barnette, et al., 2015). Previous reports indicate increased concentrations of TAGs, DAGs and cholesterol esters (CE) in cortex in individuals with AD or MCI (Akyol et al., 2021; Kalecky et al., 2022; Wood, Barnette, et al., 2015). These changes in lipid pools have been reported already in early pathology of the disease (Wood, Medicherla, et al., 2015).

Sphingolipids play important biological roles, where defects in tis metabolism have been linked to neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Alaamery et al., 2021). Particularly interesting is sphingosine-1-phosphate (S1P) and its signaling axis, which have been implicated in mechanisms of neurodegeneration and neuroinflammation (Hagen et al., 2011; Salas-Perdomo et al., 2019) and thus, have been proposed to offer neuroprotection targets.

S1P receptors have received attention in the field of multiple sclerosis, for which immunomodulation is achieved by the oral drug Fingolimod (FTY720) a S1P receptor modulator that can cross the blood brain barrier, is phosphorylated and

activates S1PR1,3-5 (Chun & Hartung, 2010). This drug has also been studied in the field of dementia and AD.

Similar to the S1P pathway, the endocannabinoid and eicosanoids system has also been suggested as a possible target for treatment of multiple sclerosis (Chiurchiu et al., 2018) or pathologies connected to chronic neuroinflammatory response such as anxiety and depression (Regulska et al., 2021). The endocannabinoids and their receptors have been implicated as targets in several neurological diseases (Cristino et al., 2020) Previous studies show increased levels of endocannabinoids in Parkinson's disease (van der Stelt et al., 2005)and early state of AD (van der Stelt et al., 2006)but deacreased levels in models of Huntington's disease (Bisogno et al., 2008). Further, lipid species downstream from AA such as PGD2, PGE2, 5-HETE and LXA4 are all connected to AD rease of these lipids in the AD brain is a response to the pathology or part of the problem is not determined (Mallick et al., 2021).

Aims of this thesis

The brain contains a large pool of different type of lipids, which have been proposed to have various mechanisms of actions. Although brain expression of bioactive lipids has been reported, its presence in multiple cellular compartments and its role in regulating brain lipid metabolism remains hitherto unexplored. Lipotoxicity occurs in certain neurodegenerative pathologies and in T2D, although alterations of brain lipid metabolism in T2D is not completely understood. The overall aim for this thesis is to investigate brain lipid metabolism and lipid signalling. With purpose of determining whether neuroprotection could eventually be afforded by targeting metabolism and signaling pathways of bioactive lipids that are crucial for adequate brain function.

The specific aim for each paper included in the thesis is to:

I. Define the presence of HSL in neuronal compartments as well as investigating the impact of HSL deletion in on brain function.

II. Explore the impact of HSL deletion on brain vessel density and cerebral blood flow as well if potentiates cerebral effects of high-fat diet exposure.

III. Determine the synaptic distribution of S1PRs and their ability to control neuronal activity

IV. Determine changes of S1P and S1PR density in nerve-enriched terminals in T2D models and models of diet-induced obesity.

V. Investigate whether an inhibitor of the S1P generating enzyme SphK2 ameliorates metabolic syndrome and neuroinflammation in diet-induced obesity.

Methods

In vivo

Below follows a brief description of the methods used in this thesis focusing on the methods I employed myself. The full description of methods can be read in each paper.

Animals (*Paper I-V*)

All animal procedures were approved by the Malmö-Lund Committee for Animal Experiment Ethics and conducted according to EU Directive 2010/63/EU and are reported following the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments, NC3Rsinitiative, UK). In study I & II, $HSL^{-/-}$ mice were used which are generated by targeted disruption of the HSL gene in 129SV-dervied embryonic stem cells and backcrossed in a C57BL/6J background (Mulder et al., 2003). In Study III-V C57Bl6J mice were used and exposed to a high-fat diet at 9 weeks of age. The C57Bl/6 mouse is a well-established inbred mouse model often utilized in HFD-studies due to its predisposition to develop insulin resistance and glucose intolerance (Surwit et al., 1988). In study IV also a lean rat model of type 2 diabetes was used, the Goto-Kakizaki rat which was originally produced by selective breeding of Wistar rats with impairment of glucose tolerance (Goto et al., 1976).

Diet (*Paper II, IV & V*)

The high fat diet and matched control diet used in study II, IV and V is an open-source diet from Research Diets (New, Brunswick, Nj-USA). The high fat diet (D12492) is a lard-base diet with 60% of the kcal from fat with total energy of 5.21 kcal/g. Based on the relative amounts of fat sources; saturated, monosaturated and polyunsaturated fatty acid distribution was estimated to 38%, 48% and 14%. The diet includes 20% kcal from protein and 7% kcal from sucrose and remaining calories from carbohydrates. The control diet (D12450J) was matched in protein, sucrose, and carbohydrate content with a total energy of 3.82 kcal/g with 10% kcal from fat. The relative amount of fat sources was estimated to be 27%, 36% and 38% from saturated, monosaturated and polyunsaturated fatty acids, respectively. In all

studies, food intake was measured by weighing food before and after intake, thus we could follow caloric intake per cage over-time.

Metabolic phenotype

Glucose tolerance test (*Paper II, IV & V*)

Glucose tolerance test (GTT) was performed by intraperitoneal i.p. injection of glucose. Compared to other routes of administrations e.g orally this method is simpler and but lacks physiological similarity, however it is still considered to be robust (Pedro et al., 2020). GTT was performed after 6 hours of fasting, where food was removed from cage and mice were put in new cages to avoid coprophagia before and during the test. A blood sample was collected before each test from *vena saphena*. Blood glucose was measured from tail tip blood with a Breeze glucometer (Bayer, Zürich, Switzerland) to assess basal glycemia. Mice were given 2 g/kg glucose (#G7528, Sigma) i.p. from a 20% (w/v) solution in saline, the injection volume was based on body weight per individual animal which could be skewing the data if there is a big difference of fat mass, as fat mass compared to lean mass is not the main site of glucose utilization. Post injection, blood glucose levels are by determined at 15, 30, 60, 90 and 120 minutes. Commercially available ELISA kit was used to measure plasma insulin (#10-1247-10, Mercodia, Uppsala, Sweden) from plasma derived from the initial blood sample.

Insulin tolerance test (*Paper II & V*)

Insulin tolerance test was performed under non-fasting condition. Carper et al. compared fasting times with insulin tolerance test and concluded that the optimal time of fasting should be 2 hours, as it minimizes metabolic stress ad weight loss (Carper et al., 2020). When looking at the results of this study, only a small difference in the main parameters measured between non-fasting and 2 hour fasting was noticed, therefore, we choose to move on with the non-fasting time point. To avoid coprophagia, mice were also put in new cages in the beginning of the test. Blood glucose was measured from tail tip blood with a Breeze glucometer (Bayer, Zürich, Switzerland) to assess basal glycemia. Mice were given 0.5 U/kg insulin (Apidra, 100U/ml) i.p from a 0.125 U/ml solution in saline. Blood glucose was then determined at 15, 30, 60, 90 and 120 minutes. If blood glucose dropped below 2mmol/L the mice was taken out of study and injected with a glucose solution and monitored until glucose levels were stabilized.

Indirect calorimetry (*Paper II*)

Whole-body energy metabolism was measured using PhenoMaster/LabMaster Homecage system (TSE-systems, Bad Homburg, Germany) operating at controlled temperature of 22°C. Prior to transfer to the cage system, mice were acclimatized to the different type of drinking nozzles in their home cages. The mice were placed individually in each cage for measurement, as a consequence some stress due to sitting alone in a cage could impact the results. Habituation was performed for 24 hours in the metabolic cages and measurements were performed for 24 hours, thus the initial stress of new environment and sitting alone was deduced from the data. Parameters were recorded at a sample interval of 15 minutes.

DEXA (*Paper II & V*)

Dual-energy x-ray absorptiometry (DEXA, Lucar PIXImus2, Lundar corporation, USA) was performed to assess body composition: body fat percentage and lean mass percentage. Mice were anesthetized with Isoflourane and held under anesthesia for a few minutes while performing the scan.

Adipose cell-size distribution (*Paper V*)

Adipocytes derived from epididymal adipose tissue were fixed in an osmium-based solution. Samples were analysed on a Multisizer 4e Coulter Counter (Beckman-Coulter, Brea, USA) using linear bins and (400 bins, bin-size 0.55μm) Multisizer version 3.53. Duplicates or triplicates of total 6,000 cells were counted for each sample.

Behavior

Mice were allowed to acclimatize to the testing room for 1 hour before each experiment, and tests were performed 9:00 to 18:00 for all the tasks. The arenas used were cleaned with 70% ethanol (v/w) between each test to eliminate olfactory clues. All tests were recorded using the AnyMaze software (6.0.1, Stoelting).

Barnes Maze (*Paper I & II*)

Barnes maze tests were conducted under bright light over the platform. Otherwise, room light was adjusted to an illuminance of 15 lx in the test apparatus. A circular Barnes Maze with diameter of 92 cm and 20 holes placed at a height of 90 cm was used to test learning and memory (Attar *et al*., 2003). The target hole had a

removable dark escape box under the maze, and four proximal visual cues were place at 20 cm from the platform. Experiments consisted of habituation, 8-day acquisition (training) and memory retention trial. For the habituation, mice were placed in the escape box for 60 s, and then released in the center of the apparatus, and allow it to explore until re-entering the escape box, or until 5 minutes elapsed. The first acquisition trial was conducted two hours after the habituation and was used to probe short-term memory. Acquisition trials took place in 8 consecutive days, at the same time of the day, in which mice were released in the center of the maze with head pointing in random direction and allowed to explore the maze for 5 minutes. The test ended when mice escaped into the target-hole box. Whenever mice did not find the escape box within 5 minutes, they were gently guided into it. The memory retention trial took place 48 hours after the last acquisition session, under identical conditions but in the absence of any escape box. To eliminate olfactory cues, the surface and the escape box were cleaned with 70% (v/v) ethanol in-between each trial. Measured parameters were path and latency to reach or enter the target hole, and time spent in each quadrant of the maze during retention trial (Harrison et al., 2006).

Y-maze (*Paper I & II*)

Spontaneous alternations were observed in a Y-maze as surrogate of working memory performance (Duarte, Agostinho *et al*, 2012). The Y-maze arms were 30 cm x 15 cm x 5 cm (length x height x wide) and converged to the center in 120° angles. Mice were placed in an arm of the maze and allowed to freely explore for 5 minutes. A complete spontaneous alternation was defined as a successive entrance to each different arm and expressed relative to the total possible alternations in the respective test. The total number of entries was analysed to access locomotor activity and exploratory behavior.

Elevated plus maze (*Paper I & II)*

The Elevated Plus Maze was used to assess anxiety. Each maze arm was 35 cm x 5 cm, and closed arms had 15 cm walls, at a 60 cm height from the floor. The mouse was placed in the maze center facing an open arm and was allowed to freely explore the maze for 5 minutes. Number of entries and time spent in each arm were analysed (Walf *et al*., 2007).

Open field (*Paper I & II*)

Open-field exploration was recorded for 5 minutes in a cubic arena with length of 50 cm by an infrared camera. Arena exploration was analysed for total walk

distance, number of crossings between arena quadrants and number of rearing events, as well as exploration of the arena center at 6 cm from the walls.

Object recognition (*Paper I & II*)

Novel object recognition (NOR) and novel location recognition (NLR) was recorded in the same arena as open-filed measurements. Mice were habituated in the empty arena for 5 minutes prior to test. After 1 hour two identical objects were placed in the arena and the mice were allowed to explore them for five minutes, (Training or familiarization phase). The mice were put back into their home cage for 1 hour (retention phase) and then reintroduced to the arena where one of the objects were either replaced by a different object (NOR) or relocated to a different quadrant of the arena (NLR). Time exploring the different objects was measured in the test to calculate the recognition index (new object / old object*100)

MRI

Arterial spin labeling – MRI (*Paper II*)

Arterial spin labeling magnetic resonance imaging (ASL-MRI) was performed 8weeks after start of diet under isoflurane anesthesia (Vetflurane, Virbac, France) in a 1:1 (v/v) O_2 :N_2O gas mixture, as described by Vanherle et al. (Vanherle et al., 2020). Experiments were performed on a 9.4T Bruker BioSpec AV III (Bruker, Germany) Body temperature was kept at 36-37°C by warm water circulation. Anesthesia was delivered at a rate of 1-2% isoflurane to maintain respiration rate at 70-90 breaths per minute. Breathing rate and body temperature were recorded with SA Instruments (Stony Brook, NY, USA) monitoring system. Regional CBF was analyzed in Fiji (ImageJ,NIH).

Immunolabeling

Total protein extracts (*Paper I, III, IV, V*)

Tissue samples were homogenized with a sonicator probe in lysis buffer (in mmol/L: 150 NaCl, 1 EDTA, 50 tris(hydroxymethyl)aminomethane (Tris)-HCl, 1% (w/v) sodium dodecylsulfate (SDS), pH 8.0) containing protease inhibitors (#11697498001, Roche, Switzerland) and phosphatase inhibitors (#4906837001, Roche, Switzerland). The homogenate was maintained in constant agitation for 2

hours at 4 °C. After centrifugation at 3,000 *g* for 10 minutes at 4 °C to remove major debris, the supernatant was saved.

Preparation of Synaptosomes and synaptic fraction (*Paper I & III*)

Synaptosomal fractionation was modified from (Morato et al., 2017). Briefly, mouse cortex was homogenized in 1 mL of isolation buffer (in mmol/L: 320 sucrose, 0.1 $CaCl_2$, 0.1 $MgCl_2$, pH 7.4) at 4°C in a 5-mL Potter-Elvehjem glass/teflon homogenizer (10 strokes at 700–900 rpm). The resulting homogenate was mixed with 6 mL sucrose (2 mol/L) and 2.5 mL $CaCl_2$ (0.1 mmol/L) in an ultra-clear centrifuge tube (#344059, Beckman Coulter, USA). Then, 2.5 mL of sucrose (1 mol/L) containing 0.1 mM $CaCl_2$ were carefully added on top to form a discontinuous sucrose gradient. All centrifugations were performed in an Optima XL-100K Ultracentrifuge (Beckman Coulter) with SW41Ti swinging bucket rotor (Beckman Coulter). After centrifugation for 3 hours at 100,000 *g*, 4°C, the synaptosomes were collected from the interphase between 1.25 and 1 mol/L sucrose and diluted 10 times in isolation buffer, centrifuged for 30 minutes at 15,000 *g*, 4°C, and the resulting synaptosomal pellet was re-suspended in 1 mL of isolation buffer.

For fractioning synaptosomes, part of each sample was diluted 1:5 in 0.1 mmol/L $CaCl_2$, and an equal volume of solubilization buffer (2% Triton X-100, 40 mmol/L Tris, pH 6.0) was added to the suspension. The suspension was incubated for 30 minutes on ice with constant agitation and the insoluble material (synaptic junctions) was pelleted by centrifugation for 30 minutes at 40,000 *g*, 4°C. The supernatant (extra-synaptic fraction) was concentrated using an Amicon Ultra 15 10K (#UFC901008, Merck Millipore, Ireland) and protein was precipitated with six volumes of acetone at -20 °C and recovered by centrifugation for 30 minutes at 18,000 *g*, -15 °C. The pellet containing synaptic junctions was washed in solubilization buffer at pH 6.0, and then re-suspended in 10 volumes of a second solubilization buffer (1% Triton X-100 and 20 mmol/L Tris, pH 8.0). After incubation under agitation for 30 minutes on ice, the mixture was centrifuged and the supernatant (pre-synaptic fraction) was processed as described for the extra-synaptic fraction, whereas the insoluble pellet corresponds to the post-synaptic fraction. All synaptic fractions were resuspended in 5% SDS with protease inhibitors. Purity of fractions were assessed by immunolabeling with typical markers for the fractions; synaptophysin, SNAP25 and PSD95.

Preparation of Nerve-terminals membranes and total membranes (Paper IV)

Extraction of nerve terminal-enriched membranes (NT) were performed with cortical brain tissue homogenized with a glass/teflon Potter-Elvehjem homogenizer

(12 strokes at 800rmp: rotor head InterMed/STIR20) in Sucrose-HEPES buffer (0.32 M sucrose, 1 mM EDTA,10 mM HEPES, 1 mg/mL BSA, pH 7.4) at 4°C. The homogenate was transferred to 40 mL Beckman centrifugation tubes and 10 mL of buffer added when washing the homogenizer. Homogenates were centrifuged at 3000xg for 10 min at 4°C (Beckman Coulter/Avanti J-20XP). Supernatant were collected and split for the two types of separation. To obtain NT, the supernatant was centrifuged at 14,000 g for 12 min at 4°C. The pellet was resuspended in 1mLof 45% (v/v) Percoll solution (45ml Percoll, 55 ml Krebs-HEPES buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM EDTA, 5 mM glucose, pH 7.4)). Resuspended pellet was centrifuged (Sigma 2-16K/12148-H) at 21,000xg for 2 min at 4°C and top layer consisting of synaptosomes were collected and re-suspended in Krebs-HEPES buffer and centrifuged again. This step was repeated until the Percoll removal. Pellets were re-suspended in Krebs-HEPES solution containing proteinase inhibitors (Roche Diagnostics).

Western blot (*Paper I-V*)

Western blotting was carried out as previously reported (Lizarbe et al., 2018). Briefly, samples were heated for 5 minutes at 95°C in sample buffer (#NP0007, Invitrogen, USA), and then separated on 4-12% Bis-Tris mini gels (#NP0336, Invitrogen, USA), followed by transfer onto nitrocellulose membranes, pore size 0,45 μm (#GE10600002, GE Healthcare, Germany). The membranes were blocked for 60-120 minutes in 5% milk or bovine serum albumin in Tris-buffered saline containing 1% Tween 20 and incubated with primary and secondary antibodies diluted in this blocking solution. Immunoblots were developed with a chemiluminescence kit (#34580, Thermo Scientific, USA) using the Chemidoc XRS+ interfaced to Image Lab 5.2.1 for image analysis (Biorad, Stockholm, Sweden).

Immunostaining (*Paper I, II & III*)

Immunohistochemistry was performed on immunofluorescence (IF) brain slices (paper I), free floating slices (Paper II) or on primary neurons (paper I & III). Briefly, tissue or cells were incubated for 1 hour at room temperature with blocking buffer [PBS containing 5% (v/v) goat serum (#16210-064, Gibco, New Zealand), 1% (w/v) bovine serum albumin and 0.3% (v/v) Triton X-100], followed by incubation with primary antibodies. After washing in PBS, the samples were incubated with AlexaFluor-conjugated secondary antibodies, washed again, mounted for microscopy with ProLong Glass Antifade (#P36980, Invitrogen, USA) or with Flouromount-G with DAPI (Invitrogen by Thermo Fisher, Carlsbad, CA, U.S #00-4959.52).

Microscopy (*Paper I, II, III & V*)

Immunolabeled slices/cells were examined under a Nikon A1RHD confocal microscope with a CFI Apochromat TIRF 100x Oil, NA 1.49 or CFI Plan Apochromat Lambda 20x NA 0.75 (Nikon Instruments, Tokyo, Japan). Images were acquired with NIS-elements (Laboratory Imaging, Nikon), and then processed in ImageJ.

qPCR (*Paper I & V*)

RNA was isolated from cortex and hippocampus from one hemisphere using Trizol (#15596026, Invitrogen, USA), and then 1 µg of total RNA was reverse transcribed with random hexamer primers using the qScript cDNA SuperMix (#95048, Quantabio, England), according to the manufacterers' instructions. The resulting cDNA was used as template for quantitative RT-PCR in triplicates using PerfeCTa SYBRgreen SuperMix (#95054, Quantabio, England) and the primers in table 1. Cycling and detection were carried out using Quantstudio 5 Real-time PCR system (40 cycles of 5 s at 95 °C and 30 s at 60 °C). Primers were optimized prior usage by assessing the optimal annealing temperature. Specificity was monitored using melt-curve validation and confirmation of amplified product by agarose gel electrophoresis. The dynamic range of each PCR assay was determined by constructing a standard curve using serial dilution of cDNA representative of each sample. Samples and standards were run in triplicate. All data were normalized to the expression of 60S ribosomal protein L14 or a combination of L14 and β-actin and analyzed with the comparative threshold cycle method (ΔΔCT).

In vitro

Primary neurons (*Paper I & III*)

Primary embryonic neurons were prepared from the cortices and hippocampi of embryonic day 15-17 WT mouse embryos, as detailed by (Martinsson et al., 2019).Briefly, neurons were dissociated through trypsinization and subsequent trituration in Dulbecco's modified Eagle medium (DMEM, 30243.01#, Cytiva) supplemented with 10% fetal bovine serum (#10100-147, Gibco, Australia), 1% penicillin-streptomycin (#15140122, Thermo fisher) and then placed onto poly-D-lysine coated coverslips. After 3-5 hours, medium was switched to Neurobasal medium supplemented with glutamine (#25030081, Thermo fisher), B27 (#A3582801, Thermo fisher), penicilin-streptomycin. Cells were grown *in vitro* for 19-20 days and then were fixed for 15 minutes in 4% paraformaldehyde and 4%

sucrose at room temperature and stored in phosphate-buffered saline (PBS; in mmol/L: 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 8.1 Na_2HPO_4, pH 7.4) at 4 °C until further analysis.

Live Ca^{2+} imaging (*Paper III*)

Before imaging, primary neurons at 19-20 days in culture were incubated for 30 minutes with 2 µmol/L of the green-fluorescent calcium indicator Fluo-4 (#F14201, Invitrogen, Thermo fisher) prepared in DMSO (0.2% v/v). Live-cell microscopy was performed with a Nikon Eclipse Ti microscope at 10 x with 1.4 NA. Live cell imaging chamber (Okolab) was kept at 5% CO2 and 37 °C. Cells were imaged every 100 ms for a duration of 2 minutes with an iXon Ultra CCD camera (ANDOR Technology).

Regions of interest (ROIs) were defined for the cell body of neurons in each stack of Ca^{2+} images using ImageJ (NIH, Bethesda, MD, USA). Fluorescence intensity over time was extracted, baseline corrected and normalized, and analysed in Peakcaller (Artimovich et al., 2017) running on MATLAB 2019a (MathWorks, Natick, MA-USA). Peak amplitude was calculated as fluorescence variation and normalized to baseline fluorescence (ΔF/F). Spike detection threshold was set to 3% above baseline. Non-responding cells were considered silent neurons and excluded for analysis of frequency. Cells with non-neuronal shape and with full width at half-maximum (FWHM) above 50 ms were not analysed. Cells showing only one spike were excluded from frequency analysis, as their frequency becomes the inverse of the imaging period.

Statistics

Results were analysed with Prism version 9.0.2-9.4.1 depending on time of analysis (GraphPad, San Diego, CA-US). The Kolmogorov-Smirnov test was used for normality testing. Then, results were either analysed with the Mann-Whitney test to compare ranks, or analysed using unpaired, two-tailed Students t-test or ANOVA followed by independent comparisons with the Fisher's least significant difference (LSD) test. Correlation was analysed using Pearson's correlation coefficient. Significance was accepted for $P<0.05$. Dose-dependent effects of S1P were fitted to an inverted logistic sigmoid function to determine the IC50 (concentration that provokes a response halfway between the maximal response and the maximally inhibited response). Partial least-squares discriminant analysis (PLS-DA) was applied on z-scores using MATLAB 2019a (MathWorks, Natick, MA-USA). Results are presented as mean±SD unless otherwise stated. Statistical details of experiments can be found in the figure legends.

Antibodies

Target	Source	RRID
Mouse anti-β-actin	Sigma-Aldrich	AB_262011
Mouse anti-β-tubulin	Abcam	AB_477577
Rabbit anti-GFAP	Abcam	AB_880202
Goat anti-GFAP	Abcam	AB_880202
Chicken anti-HSL	In-house	AB_2892188
Hen anti-rat HSL serum	In-house	AB_2892189
Rabbit anti-HSL	In-house	AB_2892190
Rabbit anti-Iba1	FUJIFILM Wako	AB_839506
Rabbit-anti MAP2 AlexaFlour488-tagged	Abcam	AB_2891057
Chicken anti-MAP2	Abcam	AB_2138153
Mouse anti-NeuN	Abcam	AB_10711040
Mouse anti-PSD95	Millipore	AB_2868387
Rabbit anti-S1PR1	Thermofisher	AB_2184729
Rabbit anti-S1PR2	Origene	AB_1615023
Rabbit anti-S1PR3	Origene	AB_2927737
Rabbit anti-S1PR4	Novus	AB_1503063
Rabbit anti-S1PR5	Novus	AB_2888942
Rabbit anti-syntaxin 1	Sigma-Aldrich	AB_261426
Rabbit anti-syntaxin 4	Abcam	AB_2891056
Rabbit anti-SNAP25	Abcam	AB_10887757
Rabbit anti-synaptophysin	Abcam	AB_2286949
HRP-tagged anti-mouse IgG	Abcam	AB_955439
HRP-tagged anti-rabbit IgG	Abcam	AB_955439
AlexaFlour405-conjugated anti-goat IgG	Invitrogen	AB_2313502
AlexaFlour488-conjugated anti-rabbit IgG	Invitrogen	AB_2556544
AlexaFlour568-conjugated anti-rabbit	Invitrogen	AB_141416
AlexaFlour647-conjugated anti-chicken IgY	Abcam	AB_2921318
Biotinylated Anti-rabbit IgG	Vectorlabs	AB_2313606

Results

HSL in neuronal compartments and impact of HSL on brain function (*Paper I*)

Since there not much known about HSL in the brain the activity and presence of HSL in the different brain areas were investigated. HSL was present and showed activity throughout all the brain regions investigated, without any significant changes comparing the areas (Figure 3C). However, the activity was significantly lower than in adipose tissue, as to be expected.

When immunoblotting for HSL in fractionated synaptosomes, the lipase was clearly enriched in synaptosomes compared to the total extract of cells with a tendency for increased expression in the post-synaptic compartment of the neuron (Figure 3A-B). The presence of HSL in neurons could be noted when immunoblotted in cortex, as cells positive for neuronal marker NeuN were stained for HSL, but some NeuN- cells could also be noted to be HSL+, indicating that HSL is possibly present also in other cell types of the CNS (Figure 3D).

To investigate the impact of HSL on brain function, behavioral tests were utilized in a global $HSL^{-/-}$ mice model. The results from the Barnes maze showed no differences in the learning capacity between the $HSL^{-/-}$ mice and their wildtype litter mate controls, with an impact in both short-term and long-term memory. Using the Y-maze, $HSL^{-/-}$ mice showed similarly impaired function, here in spatial working memory. No differences were noted in the open field test and elevated plus maze, investigating locomotor activity and anxiety-like behavior.

The same cohort was investigated *ex vivo* concerning gene expression and lipid profile in the cortex and hippocampus, both areas which are highly involved in memory formation and retention. The lipid profiles from the two brain areas revealed a shift in lipids levels, with emphasis on eicosanoids, some of which have been connected to neuroinflammation as well as regulation of CBF.

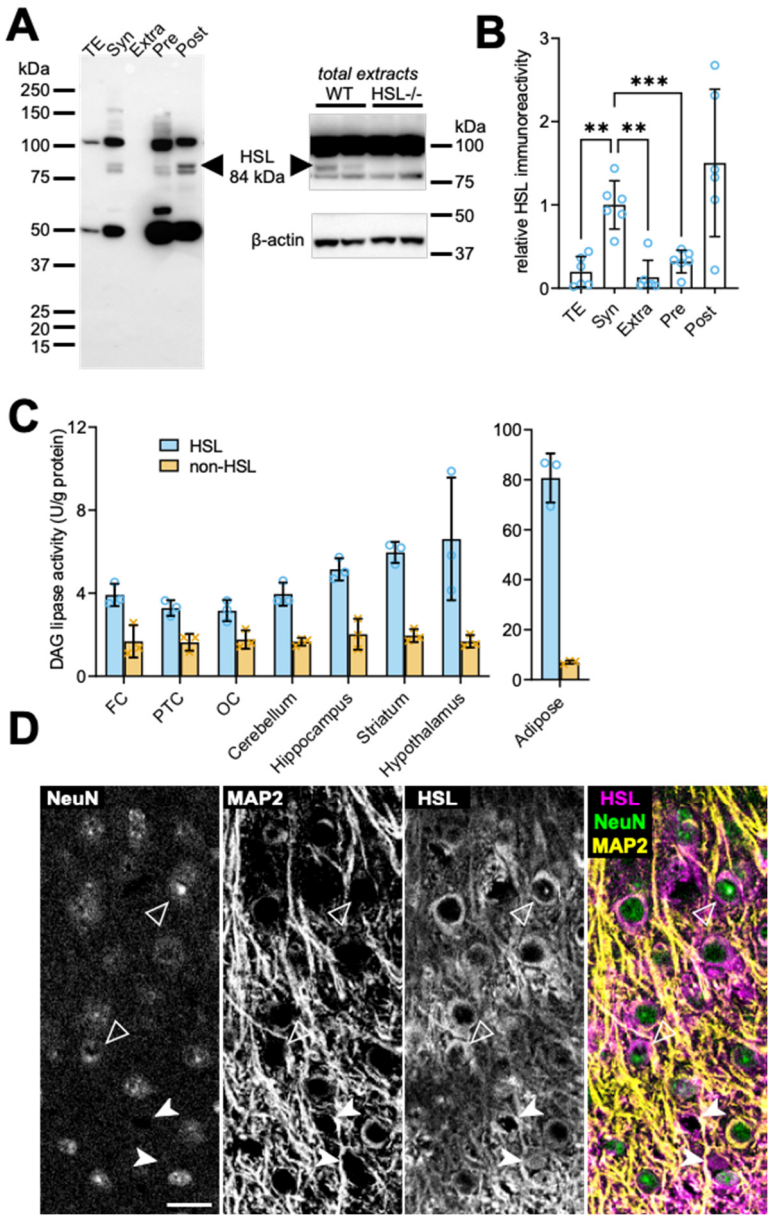

Figure 3 HSL in the CNS. A) Relative to total protein extracts (TE), synaptosomes, presynaptic and postsynaptic fractions show immunoreactivity against HSL B) Quantitative analysis of Western blot immunoreactivity suggests presynaptic and mostly postsynaptic HSL enrichment. C) HSL-specific (blue circles) and nonspecific (orange crosses) DAG lipase activity in brain areas and adipose tissue. D) Representative fluorescence micrographs of the mouse brain cortex immunolabeled for HSL (magenta), NeuN (green), and MAP2 (yellow).

Similarly, expression of genes connected to neuroinflammation was elevated, accompanied by a shift of gene expression of genes regulating lipid and glucose metabolism. When investigating levels of synaptic proteins, no changes were found.

We concluded from this study that HSL is indeed present and active throughout the brain, with specific enrichment in synapses. Lack of HSL in a global knock-out model presents as impaired memory without the synaptic degradation, however, a shift in gene expression and bioactive lipids. We hypothesize from this study that HSL could possibly regulate the pool of bioactive lipids, with specific actions of the regulating the neurovascular compartment.

Impact of HSL on cerebral blood flow and vessel density (*Paper II*)

As the study from Paper I was performed in an aged cohort of $HSL^{-/-}$ mice, a younger cohort of the same genotype was exposed to the same memory tests. Interestingly, the memory impairment changes that were noted in the aged cohort were not observed in the young cohort of mice.

Therefore, when further investigating the implication on CBF and brain vessel density in the same model, exposure of high-fat diet was applied to conclude whether the lack of HSL effects brain function only when combined with a metabolic stressor. This study included homozygous and heterozygous HSL knock-out mice of HSL as well as their wild-type littermates. The metabolic phenotype of all groups was tested using insulin- and glucose tolerance tests, DEXA scan for body composition as well as metabolic cages measuring indirect calorimetry. The outcome of these tests showed a similar metabolic phenotype as previously reported, with $HSL^{-/-}$ mice showing reduced insulin sensitivity even without the exposure of HFD, although less affected in the glucose tolerance test. The $HSL^{-/-}$ mice were also less affected by the HFD considering weight gain and body fat percentage. The indirect calorimetry did not show differences considering energy expenditure, O_2 consumption and CO_2 production. However, the respiratory exchange rate was significantly increased in the $HSL^{-/-}$ mice on control diet, and nearly normalized when exposed to the HFD.

CBF was investigated using arterial spin labeling MRI under resting conditions. Interestingly the exposure of HFD only resulted in a reduction of blood flow in the hippocampus of wild-type mice. However, both diet groups of the $HSL^{-/-}$ mice showed reduced CBF measured in cortex (Figure 4A). Similarly, the $HSL^{-/-}$ mice on CD also presented with a reduced CBF in the hippocampus (Figure 4C).

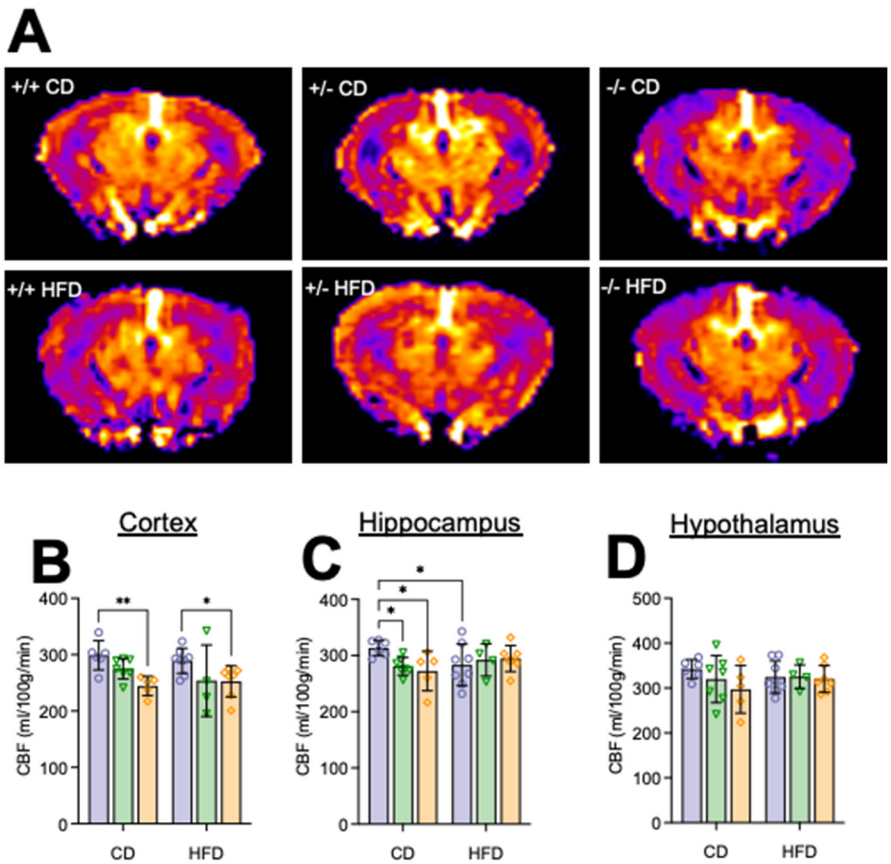

Figure 4 Reduced cerebral blood flow I cortex and hippocampus of HSL-/- mice. (A) Cerebral blood flow (CBF) maps measured by ASL-MRI for HSL $^{+/+}$, $^{+/-}$, $^{-/-}$ mice exposed to HFD or CD (B) Mean cortical (C) hippocampal (D) and hypothalamic CBF after 8 weeks of diet exposure.

Due to the results of reduced CBF in cortex, vessel density in the cortex, hippocampus and hypothalamus were measured. Similarly, to the results of CBF, vessel density was reduced in the cortex (Figure 5B). Also, a tendency of reduction in vessel density in the dentate gyrus and cornu ammonis 1 of the hippocampus could be noted (Figure 5B).

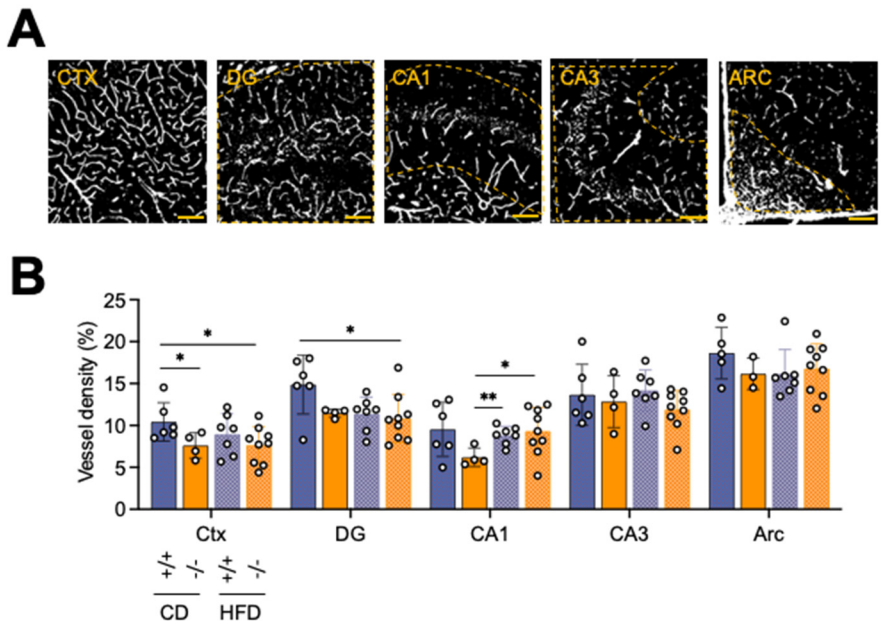

Figure 5. Decrease of vessel density in cortex of HSL$^{-/-}$ mice. (A) Confocal images showing vessel density (Lectin) in the five brain areas investigated, cortex (ctx), dentate gyrus (DG), cornu ammounis 1 (CA) cornu ammonius 3 (CA3), arcuate nucleus (ARC), (B) mean vessel density in the five different areas of HSL$^{+/+}$ and HSL$^{-/-}$ mice exposed to CD or HFD.

Assessing memory, anxiety-like behavior, and locomotor activity in a battery of behavior test, no genotype difference could be noted when assessing spatial and contextual memory in novel recognition test. The HSL$^{-/-}$ showed an increased locomotor activity in the open-field without any other signs of anxiety-like behavior assessed on parameters such as time spent in center and frequency of rearing's. This was not confirmed in the elevated plus maze.

Overall, this study shows that the young HSL$^{-/-}$ mice do not show memory impairment however. However, as hypothesized a clear reduction of CBF in the cortex and hippocampus in HSL$^{-/-}$ was observed, similarly a reduction in vessel density in these areas.

Synaptic distribution of S1PR and ability to control neuronal activity (*Paper III*)

S1P is a regulator for a plethora of functions throughout the body. In the CNS, S1P has been described to act as a neuromodulator, mainly as a ligand to its five different receptors. In this study we set out to investigate the cellular distribution the five different S1P receptors in the neuronal compartment as well as investigating the impact of these on neuronal activity measured via Ca^{2+} imaging.

By enriching crude synaptosomes from cortical tissue of wild-type mice and fractionating these into extra-, pre- and postsynaptic compartment and immunoblotting for the different receptors, the cellular distribution of the receptors in neurons could be assessed. This revealed that the S1PR1 and S1PR3 were enriched in the extrasynaptic compartments of the neurons, while the S1PR2 and S1PR4 were enriched in the active zone of the pre-synaptic compartments of the neurons. Only the S1PR2 and S1PR3 were found in the post-synaptic compartment.

Exposing primary neurons to S1P revealed that S1P inhibits spontaneous neuronal activity in a dose-dependent fashion. Applying specific agonists for the different receptors revealed that only S1PR1, S1PR2 and S1PR4 agonist affected spontaneous activity in any of the parameters measured: fractions of responding neurons, Ca^{2+} spike frequency, amplitude or area of peak (Figure 6).

Taken together, this study points to the interesting finding that S1PR2 and S1PR4 are both present in the pre-synaptic zone of the neurons and both control spontaneous activity by inhibition.

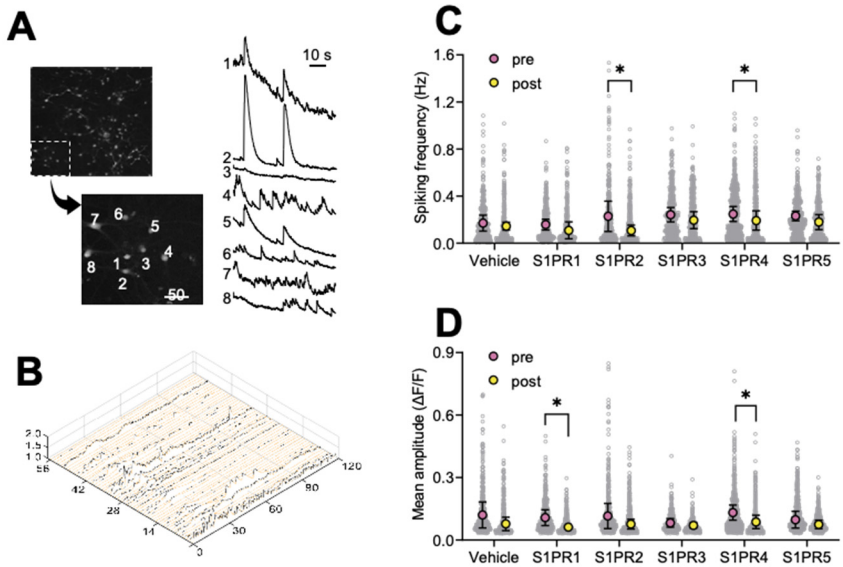

Figure 6 Effects of S1PR activation on spontaneous activity of primary neurons. A) Example of raw fluorescence Ca^{2+} signals traced with Flou-4. B) Typical traces in neurons C) Changes in frequency D) Changes in amplitude.

Changes of S1P and S1PR in T2D and pre-diabetic models (*Paper IV*)

Considering the results from study III investigating the S1PR location in neuronal compartments and their ability to modulate spontaneous neuron activity we set to investigate the density of the receptors in nerve-enriched terminals in models of obesity/pre-diabetes and a lean model of T2D.

The Goto-Kakizaki rat is a well-studied model of T2D. Nerve-enriched terminals from the cortex were obtained through by percoll gradient and the density of S1PR1-4 was assessed in the model. In the GK rat model the immunoreactivity of three out of the four receptors were decreased compared to wistar rats, with exception for the S1PR3. Two of which, S1PR2 and S1PR4 we described in study III to be enriched in the active pre-synaptic zone and which elicited an inhibitory effect when stimulated with an agonist. S1PR5 was not investigated in this study due to its low expression in neurons, as well as the lack of respons when stimulated with agonist in primary neurons.

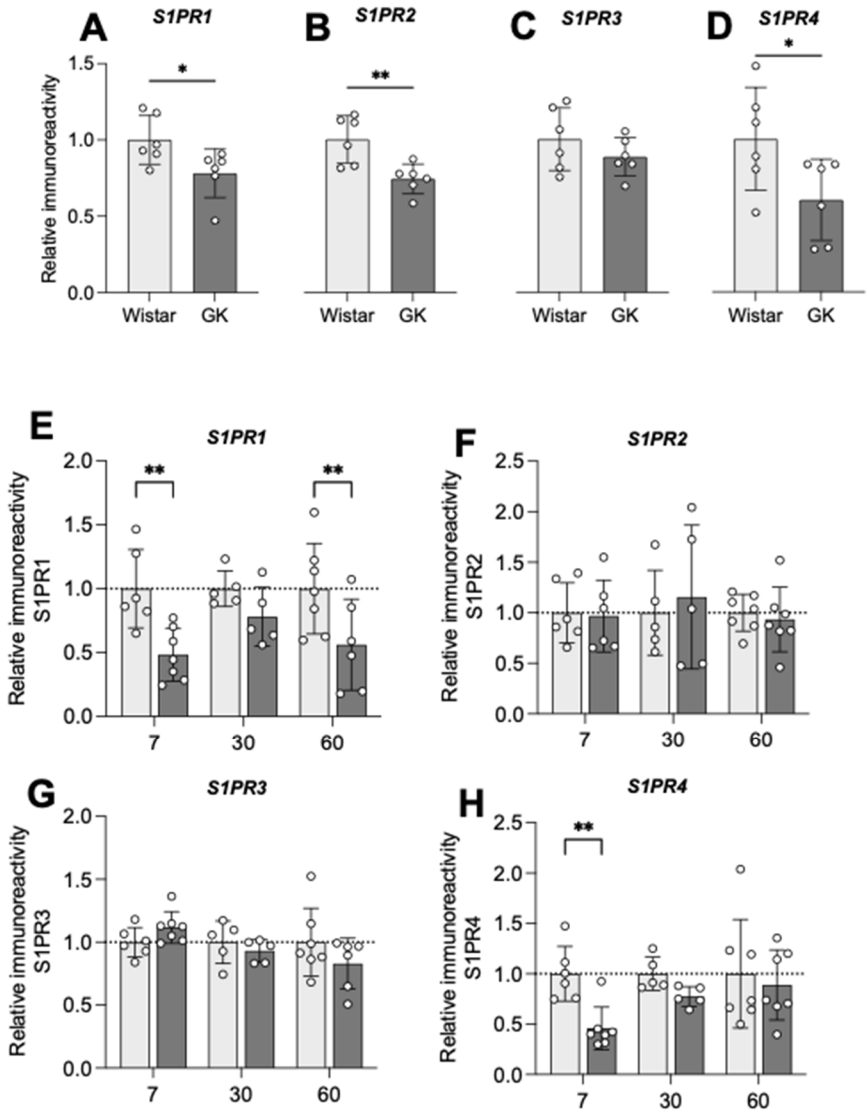

Figure 7. Levels of S1PR in cortex of diabetes models. A) Western blot shows a decrease of S1PR1 (B) S1PR2 (C) but no change in S1PR3 in GK rats compared to wistar. E) THE S1PR4 show a similar decrease as the first two receptors. Exposing mice to 60% HFD for 7, 30 or 60 days decreased the S1PR1 in cortical nerve-terminals with diet. F) S1PR2 was not decreased (G) neither S1PR3. However (H) S1PR4 showed a decrease in the acute phase of HFD at 7 days.

To investigate the density of S1PR during the development of pre-diabetes, C57Bl/6J mice were exposed to HFD for 7, 30 and 60 days. During this period of time, weight was increasing together with glucose intolerance as measured by area-under the curve from glucose tolerance test. Nerve enriched terminals were

extracted from cortex, from which density of the S1PR were assessed. The S1PR1 was throughout the diet period decreased, significantly so in the 7- and 60- day timepoint. No changes were observed concerning the S1PR2 and the S1PR3. For the S1PR4 only an acute decrease of immunoreactivity compared to wistar was noted in the 7-day timepoint.

Changes of plasma S1P has previously been reported in obese animal models as well as in an obese human cohort. Here we measured S1P levels in both brain and plasma using mass spectrometry. The GK rats did not show any difference in brain S1P concentration, while there was a diet-effect with the mice exposed to HFD with a significant increase in the 30-day timepoint. This effect was similar when measuring in the plasma. Although no clear correlation could be noted for neither brain nor plasma S1P when correlating to different factors such as glycemia or glucose tolerance measurement.

In this study we conclude that S1P signaling through its receptors is altered in synapses of insulin- resistance and diet-induced obesity models, suggesting a role of S1P signaling in T2D-associated synaptic dysfunction.

Changes of S1P in HFD-induced obesity model and the impact of SphK2 inhibitor (*Paper V*)

Considering the increased interest of the S1P signaling system in diabetes we investigated in this study the role of S1P and one of its two generating enzymes, sphingosine kinase 2 (Sphk2). This enzyme is less studied but has also been proposed to have a role in age-induced obesity. Although here we hypothesize that diet-induced obesity affected by pharmacologically inhibition of SphK2 can reverse the HFD-induced phenotype. Mice were subjected to an HFD exposure for 9 weeks, where the last two weeks an SphK2-specific inhibitor was administered.

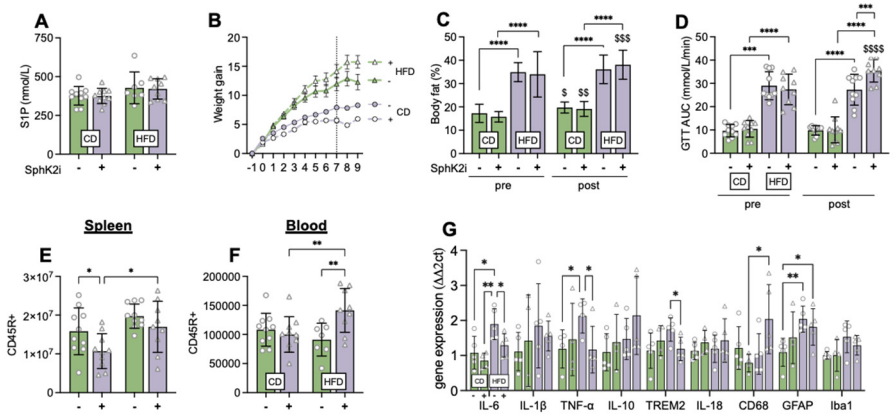

Figure 8. SphK2 inhibition does not ameliorate HFD associated phenotype, but does alter inflammatory profile. A) S1P levels in fed plasma samples measured at last week before endpoint by mass spectrometry B) Weight gain from week -1 to endpoint monitored. C) Fat percentage as per measured by DEXA pre- to post-treatment. D) Caloric intake pre-post injection start. E) Splenic CD45R+ cells (F) CD45R+ cells (G) Relative gene expression of hippocampal fractions measured with qPCR

As previous reports present results of plasma S1P increase in obese animal models and obese human cohorts, S1P was measured in plasma. There was no significant increase comparing the groups, although a slight diet effect of the S1P plasma concentration, measured with mass spectrometry.

Regarding the metabolic changes, the SphK2 inhibitor treatment has no effect on neither glucose nor insulin tolerance. There was a slight effect of body weight increase in the HFD exposed group with inhibitor and the opposite action in the CD exposed group. This was also noted when measuring adipose cell size distribution as the CD exposed mice treated with SphK2 inhibitor elicited smaller adipocytes than their vehicle controls, suggesting decreased adipocyte maturation.

Considering that systemic inflammation has previously been reported to develop in diabetes and diabetes-associated comorbidities, peripheral- and neuroinflammation was investigated. Mice receiving HFD and the inhibitor presented with a higher frequency of circulating leucocytes compared to the vehicle treated HFD exposed group. Although, interestingly, the HFD exposed vehicle group showed hippocampal neuroinflammation, which was ameliorated by SphK2 inhibitor treatment.

Overall, this study does not show any beneficial systemic effects of inhibiting SpHK2 in a state of metabolic stress, although interestingly, it shows signs of dampening diet-induced hippocampal neuroinflammation.

Discussion and future perspectives

It is unknown which abnormalities in lipid metabolism are the main contributor to neurodegenerative disease and how to target these pathways. In this thesis we have worked with identifying bioactive lipid metabolism upstream from a lipase perspective as well as specific bioactive lipid signaling pathway (i.e., the S1P pathway).

Lipid metabolism and lipid reprogramming has been emerging in the glioma field, as a significant lipid shift could be noted concerning for example DAG, TAG, CE, S1P and PGEs. All of these are lipids considered promoting carcinogenic effects (Abdul Rashid et al., 2022). In the first two papers of this thesis, we have investigated lipids shifts, specifically concerning bioactive lipids, in a global knock-out model of HSL. HSL has previously been discussed as a target of prevention or treatment or type 2 diabetes, as partial inhibition of HSL have been suggested to improve insulin sensitivity (Althaher, 2022). The impact of HFD exposure to the brain lipid profile of $HSL^{-/-}$ mice is still to be determined.

We have verified the presence and activity of HSL in the brain, although mainly investigating neurons and synapses of neurons. However, we also showed results of HSL presence in NeuN- cells. These could be microglia cells when comparing to previous published data concerning mouse brain single cell proteomics (Sharma et al., 2015), and considering LDs are highly abundant in microglia (Marschallinger et al., 2020), the presence of HSL would be reasonable. The role of HSL in microglia and whether HSL in microglia would be translocating to the lipid droplet would still need to be concluded. For a future perspective, when studying HSL in the brain to determine the specific role of HSL, a cell-specific neuronal or microglial knock-out would be interesting. Hundahl et al used a hypothalamic cell-type specific knock-out model of HSL, and showed HSL to be involved in appetite regulation (Hundahl et al., 2021). It would be interesting whether a cortical neuronal knock-out model of HSL would show similar results as the global knock-out with changed gene expression, lipid profile and CBF as outcomes.

To assess whether HSL could be activated in a similar way as in adipose tissue, we treated primary murine neurons with an adenylyl cyclase inhibitor, Forskolin (10µM), in the presence of IBMX (100µM), a non-selective phosphodiesterase inhibitor. Preliminary results from Western blot experiments show that HSL is phosphorylated at the Ser563, Ser660 and Ser565 at different degrees when subjected to stimulation, Su et al 2003 suggested that Ser-563, 565 and 660 all need to be phosphorylated in order for HSL to translocate and execute its functions in vivo (Su et al., 2003). An option for future studies of specific mechanisms at cellular level would then be to stimulate primary cells from HSL knock-out animals with Forskolin and IBMX to verify cellular roles of HSL. Further, available selective HSL inhibitors could also be applied and give important insights the actions of HSL in the brain.

In paper III-V, we have explored the role of S1P, its receptors and one of its

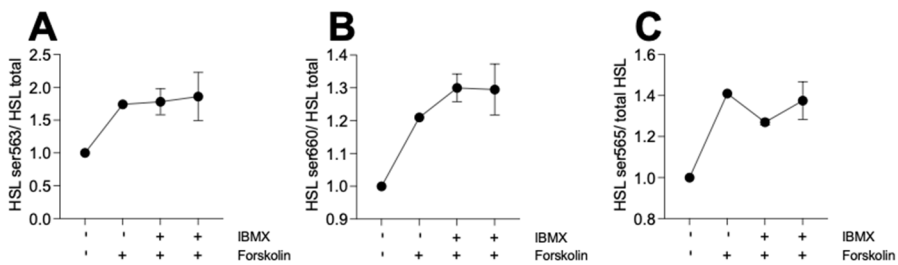

Figure 9. Results of primary neuron stimulation with IBM and Forskolin (30 or 60min) on relative immunoreactivity at three different phosphorylation sites of HSL. A) ser563, B) ser660 C)ser565. All data is relative to total HSL immunoreactivity.

generating enzymes, SphK2, in the brain of control animals and in animal models of diabetes. To summarize the findings, we investigated the localization of the S1PRs in the neuronal synapses and their ability to regulate spontaneous neuronal activity, here we found S1PR2 and S1PR4 to be especially interesting, due to their presence in the pre-synaptic active zone as well as the dampening effect of neuronal activity when stimulated with their specific agonists. We investigated the same receptors in cortical synapses of diabetic models and identified again S1PR1, 2 and 4 as interesting due to decrease of protein levels. Finally, we investigated whether SphK2 could be targeted to benefit the progression of disease by targeting inflammatory pathway. Here, we could see that in HFD-exposed animals, an inhibition of SphK2 was not beneficial except for when it comes to neuroinflammation, while the CD exposed animals treated with the inhibitor showed decent effects. To further verify our findings, especially concerning the role of SphK2 in neuroinflammation a more detailed analysis would be necessary, preferably through e.g., microglia morphology analysis or gliosis assessment.

In two of the studies, we measured S1P in HFD exposed mice. Previously it has been reported that plasma S1P is increased in obese mice and humans, measured with ELISA. However, with our studies of HFD-exposed mice we could see a diet effect of the plasma S1P however not such a clear increase, this could possibly be due to the animal model and the genetic drift depending on vendors (Siersbaek et al., 2020). However, to establish the role of S1P in diabetes and/or obesity and whether S1P is detrimental or preventing disease progression, this should be assessed. Also, the interplay between peripheral S1P and brain S1P would be interesting to further explore. Plasma S1P has previously been described to be decreased in plasma of human cohorts of neurodegenerative diseases, such as Lewy body dementia, AD, and idiopathic Parkinson's disease (Oizumi et al., 2022). Though this needs further validation to know if the changes can be observed in cerebrospinal fluid or in the brain tissue. Considering the possible endogenous production of S1P in the CNS.

Lipids and lipid pathways could be a target for treatment options in several ways. Firstly, as an adjuvant for drugs to be able to be absorbed, due to many drug targets being lipophilic as its origin, however, this subject has not been addressed in this thesis. The second option is to target lipid metabolism in acylglycerol lipolysis, for example to target a lipase. DAGLα has been suggested as a target to downstream affect the endocannabinoid system to prevent headaches, alcohol consumption in abuse disorder and food intake to prevent obesity (Levine et al., 2020; Perez-Morales et al., 2014; Winters et al., 2021). HSL inhibition has previously been suggested as a treatment option for T2D as haploinsufficiency of HSL and treatment with an HSL inhibitor improve insulin sensitivity (Girousse et al., 2013). In this thesis, we have focused on HSL, in the brain and on how a complete HSL activity affects brain function. However, how a partial inhibition of HSL would affect brain function and whether this would be beneficial is yet to be determined. Considering that both the haplodeficient and homodeficient animals of HSL displayed reduced cerebral blood flow. It has also been suggested in opposite from the inhibitors that a specific activator of HSL could be beneficial to treat T2D and dyslipidemia to regulate systemic lipid and glucose homeostasis (Albert et al., 2014) although no specific activator of HSL has yet been discovered (Lan et al., 2019). A third option is to target the bioactive lipids and their receptors specifically. Here there are more options such as a 20-HETE agonist and inhibitors (Williams et al., 2010), PGE_2 inhibitors (Jiang et al., 2020), the endocannabinoid system (Tudorancea et al., 2022) and S1P system (Jung et al., 2022; Squillace et al., 2022). Of course, the challenge is, as with many druggable targets, that the targets often are present and acting in several different tissues where it can be beneficial to manipulate the target in one but not the other. In this thesis we have wanted to aim the light towards bioactive lipids as target for neuroprotection and synaptic function in brain dysfunction connected to T2D, as a start to map out potential targets. Here we can conclude that HSL is present in the synapse and disruption of it leads to brain dysfunction, whether

activation of HSL could be a future option is to be determined. For the S1P system in connection to synaptic dysfunction we want to highlight two of the receptors $S1PR_2$ and $S1PR_4$ both present in the presynaptic active zone, able to modify neuronal activity and $S1PR_4$ is outside of the fingolimod pathway perhaps making it a specific druggable target, although more research is needed to determine the importance of the S1PR2 receptor for synaptic function.

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