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2013

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Citation for published version (APA):

Voss, U. (2013). *Adapt, Survive or Die - Metabolic Imbalances and the Enteric Nervous System*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Department of Experimental Medical Science, Lund University.

Total number of authors:

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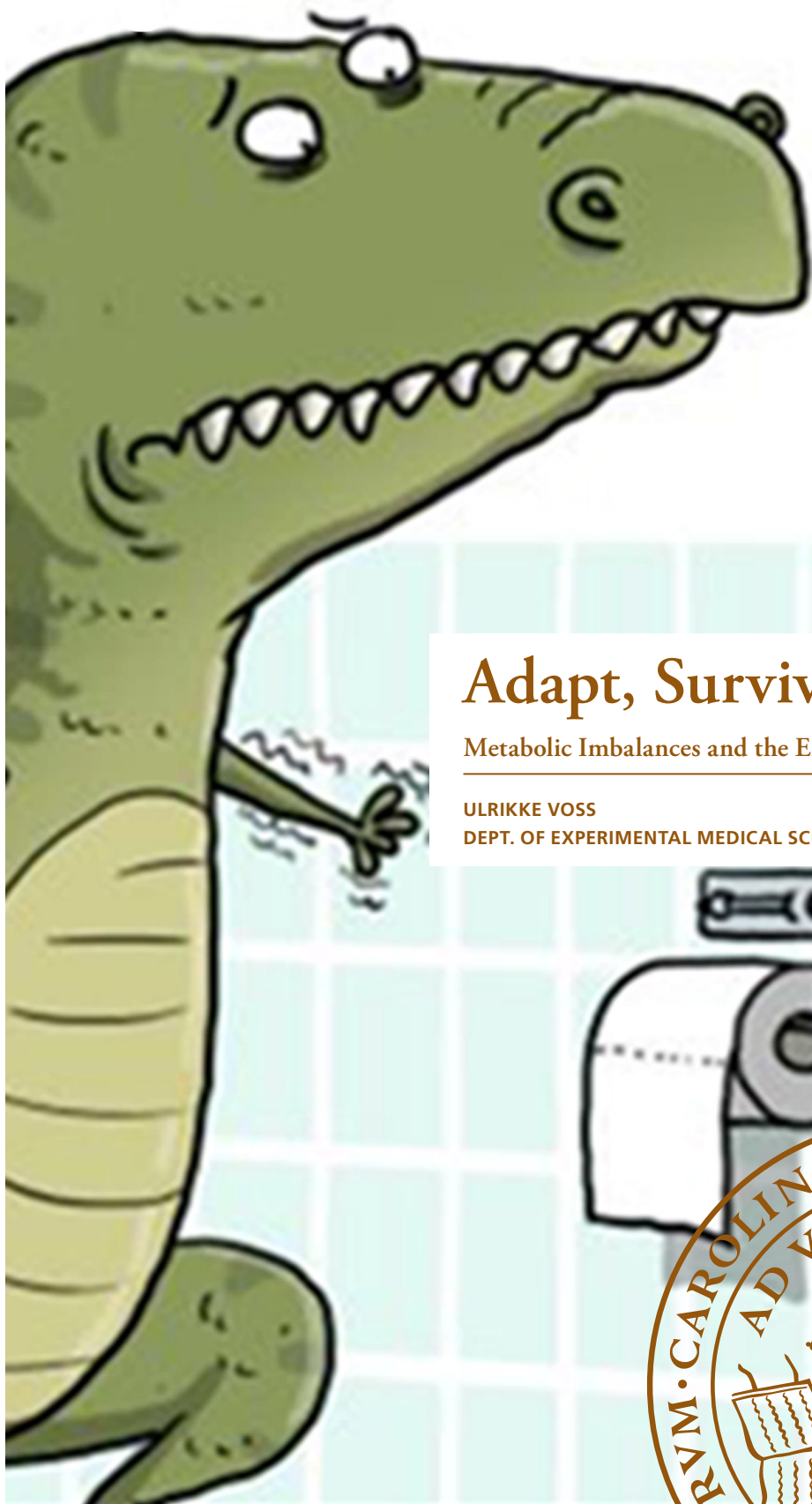
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Adapt, Survive or Die

Metabolic Imbalances and the Enteric Nervous System

ULRIKKE VOSS

DEPT. OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY



Adapt, Survive or Die

Metabolic Imbalances and
the Enteric Nervous System

Ulrikke Voss



LUND
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DOCTORAL DISSERTATION

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To be defended at Belfrage Hall BMC, D15, Lund on Friday the 20th of December
2013 at 2pm.

Faculty opponent

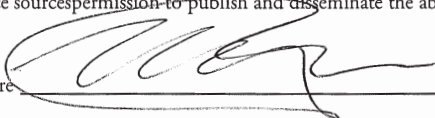
Professor Lars Fändriks

Göteborgs Universitet

Organization LUND UNIVERSITY Faculty of Medicine, Experimental Medical Science Neurogastroenterology Author(s): Ulrikke Voss	Document name : DOCTORAL DISSERTATION Date of issue: 27 th of November 2013 Sponsoring organization
Title and subtitle: Adapt, Survive or Die – Metabolic Imbalances and the Enteric Nervous System	
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Key words: enteric nervous system, high fat diet, neuropathy, neuroplasticity, metabolic imbalance	
Classification system and/or index terms (if any)	
Supplementary bibliographical information	Language: English
ISSN and key title: 1652-8220	ISBN: 978-91-87651-22-9
Recipient’s notes	Number of pages Price Security classification

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Date 14 November, 2013

Adapt, Survive or Die

Metabolic Imbalances and
the Enteric Nervous System

Ulrikke Voss



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ISBN 978-87651-22-9

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2013



Abstract

In this thesis the questions “do enteric neurons adapt to survive in conditions of obesity/type 2-diabetes (T2D) related metabolic imbalances? Or do they die?” are asked. Obese and T2D patients have high rates of gastrointestinal (GI) symptoms. The GI tract comprises the body’s largest surface to the outside environment; it performs diverse and complex roles in an ever changing external environment. It has evolved into a fine tuned sensory organ, with a complex network of sensory-, taste-, baro-, mechano- and pathogen recognizing receptors (PRR). The presence of PRRs allows the GI tract to mediate and modulate immune responses, in response to both pathogens and toxins present in our microbiota. The microbiota is shaped by our diet, as well as our genetics. The fluid adaptation and control of the GI tract is mediated by the enteric nervous system (ENS). ENS is an extensive interconnected network of neurons and glia cells, controlling intestinal motility, secretion and blood flow. It harbors a neurotransmitter variety and receptor diversity enabling interactions with neurons, immune, endocrine and intestinal cells as well as with luminal factors.

Results show that mice fed a high fat diet (HFD) for 6 months have a significant loss of enteric neurons. To evaluate metabolic factors known to be altered in the obese/T2D-condition (ODC), we used isolated enteric neurons. We noted that palmitic acid (PA), a lipid known to be increased in ODC caused a significant loss of enteric neurons, through mechanisms involving deranged energy metabolism and the purinergic P2Y₁₃ receptor. In ODC an increased permeability of the intestinal barrier causes increased translocation of lipopolysaccharide (LPS) to the circulation and initiation of immune responses. We found that exposing cultured enteric neurons to LPS caused neuronal loss through activation of AMP activated protein kinase. Glucagon-like peptide (GLP) 1 and 2 are involved in satiety, insulin release and intestinal barrier function. Both GLPs are reduced in ODC. We showed a great neuroprotective potential of the hormones. Based on these findings, HFD-induced enteric neuronal loss is suggested to be a culmination of several factors, including increased PA- and LPS-exposures and a decreased GLP level, leading to dysregulation and altered function of enteric neurons.

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals

- I. *Glucagon-like peptides 1 and 2 and vasoactive intestinal peptide are neuroprotective on cultured and mast cell co-cultured rat myenteric neurons.* Voss U, Sand E, Hellström PM, Ekblad E. **BMC Gastroenterology** 2012 Apr 1; 12:30
- II. *Enteric Neuropathy can be induced by high fat diet in vivo and palmitic acid exposure in vitro.* Voss U, Sand E, Olde B, Ekblad E. **PLOS ONE**, In press. 2013
- III. *The enteric nervous system of P2Y₁₃ receptor null mice is resistant against high fat diet- and palmitic acid-induced neuronal loss.* Voss U, Foldschak Turesson M, Robaye B, Boeynaems JM, Olde B, Erlinge D, Ekblad E. Submitted Nov. 2013
- IV. *Lipopolysaccharide-induced loss of cultured rat myenteric neurons - role of AMP activated protein kinase.* Voss, U, Ekblad E. Submittet Nov. 2013

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Additional peer-reviewed papers, not included in the thesis

- *N-cadherin is dispensable for pancreas development but required for beta-cell granule turnover.* Johansson JK, Voss U, Kesavan G, Kostetskii I, Wierup N, Radice GL, Semb H. **Genesis** 2010 Jun; 48(6):374-81
- *Distribution of melatonin receptors in murine pancreatic islets.* Nagorny CL, Sathanoori R, Voss U, Mulder H, Wierup N. **J. Pineal. Res.** 2011 May;50(4):412-7.
- *Nesfatin-1 stimulates glucagon and insulin secretion and beta cell NUCB2 is reduced in human type 2 diabetic subjects.* Riva M, Nitert MD, Voss U, Sathanoori R, Lindqvist A, Ling C, Wierup N. **Cell and Tissue Res.** 2011 Dec;346(3):393-405
- *High glucose and free fatty acids induce beta cell apoptosis via autocrine effects of ADP acting on the P2Y(13) receptor.* Tan C, Voss U, Svensson S, Erlinge D, Olde B. **Purinergic Signal.** 2013 Mar;9(1):67-79.
- *Gonadotropin-releasing hormone analog buserelin causes neuronal loss in rat gastrointestinal tract.* Sand E, Voss U, Hammar O, Alm R, Nordin Fredrikson G, Ohlsson B, Ekblad E. **Cell and Tissue Res.** 2013 Mar;351(3):521-34.
- *Enhanced β -cell function and anti-inflammatory effect after chronic treatment with the dipeptidyl peptidase 4 inhibitor vildagliptin in advanced age diet induced obesity mouse model.* Omar B.A, Vikman J, Winzell M.S, Voss U, Ekblad E, Foley J.E, Ahrén B. **Diabetologica** 2013 Aug;56(8):1752-60

Abbreviations

4-PBA	4-phenyl-butyric-acid
ABC	avidin-biotin complex
AC	adenylate cyclase
Ach	acetylcholine
ADP	adenosine diphosphate
ALA	α lipoic acid
AMP	adenosine monophosphate
AMPK	adenosine monophosphate protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BrdU	5-bromo-2'-deoxyuridine
CACT	carnitine acylcarnitine translocase
CaMKK	Ca ²⁺ /calmodulin dependent kinase kinase
CART	cocaine and amphetamine regulated transcript
CCK	cholecystokinin
CNS	central nervous system
CPT1	carnitine palmitoyl transferase I
CPT2	carnitine palmitoyl transferase II
CREB	cAMP response element-binding protein
DAB	3,3'-diaminobenzidine
DAG	diacylglycerol
EdnrB	endothelin receptor type B
EdU	5-ethyl-2'-deoxyuridine
ENCC	enteric neural crest cells
ENS	enteric nervous system
ER	endoplasmatic reticulum
ET-3	endothelin-3
FA-CoA	acyl co-enzyme A

FFA	free fatty acid
FGID	functional gastrointestinal diseases
G	glia
G3P	glycerol 3-phosphate
GDNF	glia derived neurotrophic factor
GI	gastrointestinal
GIRK	G protein-activated inwardly rectifying potassium channels
GL	glycerolipid
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
HDL	high density lipoprotein
HFD	high fat diet
HuC/HuD	human neuronal protein
IBS	irritable bowel syndrome
ICC	interstitial cells of Cajal
IL-1	interleukin-1
IL-6	interleukin-6
LGI4	leucine rich repeat LGI member 4
LKB	liver kinase B
L-NAME	N-nitro-L-arginine methyl ester
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
MAG	monoacylglycerol
MMC	migrating motor complex
N	neurons
NANC	non-noradrenergic, non-cholinergic
ND	normal diet
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PA	palmitic acid
PAA	phosphatidic acid
PAC ₁	PACAP type 1 receptor
PACAP	pituitary adenylate cyclase-activating peptide
PDH	pyruvate dehydrogenase complex

PDK1	phosphoinositide dependent kinase
PGC-1	peroxisome proliferator-activated receptor γ cofactor 1
PGE ₂	prostaglandin E ₂
PGP9.5	human gene product 9.5
Pi3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PL	phospholipids
PLC	phospholipase C
PRR	pathogen recognizing receptors
ROS	reactive oxygen species
SIRT1	sirtuin 1
SMC	smooth muscle cells
SPT	serine palmitoyl transferase
T2D	type 2-diabetes
TAG	triacylglycerol
TAK1	transforming growth factor β activated kinase
TLR4	toll-like receptor 4
TNF α	tumor necrosis factor α
TRAM	TRIF-related adaptor molecule
TRIF	toll-interleukin 1 receptor domain containing adaptor inducing interferon- β
TRPV1	vanilloid receptor 1
VIP	vasoactive intestinal peptide
VLDL	very low density lipoprotein
VPAC	VIP receptor

Populærvidenskabelig sammenfatning

Gennem hele min barndom hang denne plakat på døren til mit klasselokale på folkeskolen. Den hed: Du bli'r, hvad du spiser. På plakaten løber en række madvarer om kap, og selvfølgelig er det rugbrødet, mælken og guleroden der vinder, mens is, slik og pommers fritter kommer på sidstepladsen. Plakaten skulle få børn til at ville vælge sunde madvarer, for hvem ville tabe kapløbet.



Er du, hvad du spiser?

Vi er, hvad vi spiser på grund af vores fordøjelsessystem. Fordøjelsen starter i munden, når vi tygger maden og slutter når vi går på toilettet og har afføring. Undervejs igennem tarmen nedbrydes maden til dens enkelte bestanddele, som kan optages og bruges til energi og opbygning af kroppens celler. Tarmen frigiver også en række appetitregulerende hormoner. Mave-tarmkanalen er opbygget som et rør bestående af flere lag, der er optimeret til at optage næring. Inderst er mukosaen, udenom submukosaen, dernæst et todelt muskellag og yderst et bindevævslag.

Mave-tarmkanelen udgør den største overflade mellem os og det eksterne miljø. Hos voksne er tarmen ca. 9 meter lang og hvis man spredte den ud til et fladt lag ville den fylde ca. 400 m², og den udfylder en mangeartet og vigtig rolle i et miljø som ofte ændre sig. Tarmen er et fintunet sensorisk organ, der er udstyret med en række receptorer, der kan reagere på stræk, tryk, smag og på skadelige patogener. At tarmen er udstyret med disse receptorer, giver mening idet tarmen er hjem for vores mikrobiota bestående af over 100 millioner mikroorganismer, det vil sige langt flere end vi har celler i kroppen. Og mellem dem og os er tarmvæggen som sørger for, at vi optager næring fra føden. Langt de fleste mikroorganismer er gode og hjælper med fordøjelsen og styrker immunforsvaret. Det vi spiser påvirker i høj grad hvilken type mikroorganismer vi har i tarmen. Udover hjælp til fordøjelsen, indikerer flere nye

studier, at de også er i stand til at påvirke os og vores mentale tilstand. Så vi er både det vi spiser OG de mikroorganismer vi har i tarmen.

Hvem har kontrollen?

Mikroorganismer spiller en stor rolle i vores tarm, men vi er også udstyret med et enterisk nervesystem (ENS) på ca. 500 millioner nerveceller, der sidder langs hele tarmen i et kompleks netværk og kontrollerer tarmbevægelser, udskillelse af elektrolytter og vand samt blodomløb. ENS er inddelt i to sammenvævede nerveretværk. Et myentetisk, der sidder mellem de to muskellag og hovedsaglig kontrollerer tarmbevægelser, og et submukosalt, der sidder i submukosaen, og kontrollerer udskillelse. ENS har nerveender ud i andre væv, såsom bugspytkirtel, galdegange og rygmarven. Sensoriske nerveender i mukosaen reagerer på tarmens indhold og sender besked til motorneuroner via interneuroner, der løber i en enten oral eller anal retning eller i tarmens omkreds. Mellem hvert måltid udfører tarmen et rengøringsprogram, der sikrer at der ikke sidder madrester eller sker en ophobning af bakterier. Afhængig af madens bestanddele iværksætter ENS forskellige bevægelser, enten blandede, korte eller lange peristaltiske bevægelser og sender vand og elektrolytter ud i tarmen, hvilket sikrer korrekt konsistens og næringsoptag. Ligeledes tilpasser ENS de immunologiske svar til patogener i tarmen; både via øget udskillelse og peristaltik og frigivelse af neurotransmittere, som aktiverer immunceller og forbedrer tarmbarrieren.

Hvad hvis vi mister kontrollen?

Vi kender det alle sammen godt. Ondt i maven, oppustet, forstoppelse eller diarre er desværre hverdag for en stor del af befolkningen. Kroniske mave-tarmsygdomme dækker over en bred vifte af symptomer, som både kan være den primære sygdom eller være en komplikation i forbindelse med andre sygdomme, som for eksempel diabetes og Parkinson. Overvægtige mennesker og type 2-diabetes (T2D) patienter har flere mave-tarmproblemer end resten af befolkningen. Symptomerne varierer fra patient til patient og dækker hele mave-tarmkanalen, fra gastroparese og hypersensitivitet til diarre og forstoppelse. Ændringer i ENS kan ligge til grund for mange af de symptomer som mave-tarmpatienter oplever. Langt de fleste mave-tarmsygdomme er relaterede til både strukturelle og funktionelle forandringer i ENS som for eksempel øget eller nedsat nervefibertæthed og ændret frigivelse og sensitivitet af neurotransmittere. Da de fleste underliggende mekanismer for udviklingen af mave-tarmsygdomme stadig er ukendte stillede vi os følgende spørgsmål i denne

afhandling: Hvordan reagere ENS på overvægt og overvægt-induceret T2D-lignende metaboliske forandringer?

Effekten af high-fat-diet

På trods af genernes rolle i udvikling af T2D, spiller livsstil en endnu større rolle, idet 70 % af patienter der udvikler T2D er overvægtige. Vi udnyttede dette faktum i vores forsøg og brugte en såkaldt diet-induced-obesity model, hvor mus blev fodret med enten almindelig foder eller foder med højt indhold af mættet fedt. Efter 6 måneder undersøgte vi, hvorledes det havde påvirket tarmen og ENS. Resultaterne fra high-fat-diet (HFD) forsøgene viste, at indtag af en diæt med meget mættet fedt førte til et markant tab af enteriske nerver. Idet HFD ikke blot påvirker ENS, men hele dyret, undersøgte vi udvalgte faktorer i isolerede nerver. Glucagon-like peptide (GLP) 1 og 2 er to hormoner der udskilles i tarmen. GLP-1 påvirker appetitregulering og er vigtig for frigivelse af insulin. GLP-2 er vigtig for opretholdelse af tarmvæggen. Begge hormoner forekommer i lavere koncentrationer hos overvægtige. Vi viste, at disse hormoner beskytter isolerede nerver fra ENS både generelt og mod et inflammatorisk mastcelle-induceret nervetab. Når tarmvæggen udsættes for HFD øges gennemtrængeligheden og flere patogener kan derfor komme ind i kroppen gennem tarmen hvorved der iværksættes en immunologisk proces. Både overvægtige og T2D-patienter har øgede niveauer af lipopolysaccharider (LPS) fra bakterier i blodet. Vi viste, at LPS forårsager nervetab via aktivering af en intercellulær signaleringsvej, der involverer enzymet AMP aktiveret kinase (AMPK). Da overvægtige og T2D-patienter har øgede mængder af frit cirkulerende fedt i blodbanen undersøgte vi også om det mættede fedt påvirkede nervecellerne. Her kunne vi vise, at palmitinsyre (en mættet fedtsyre) forårsagede nervetab via formationen af energi-intermediater, som ledte til cellulært stress.

Puriner har en prominent rolle i biologiens verden. Livets byggesten DNA og RNA består af puriner og cellers vigtigste energikilde ATP er også en purin. ATP fungerer også som en neurotransmitter i ENS, og kan binde til en række receptorer. Vi undersøgte derfor om purinreceptoren P2Y₁₃, der påvirker transporten af fedt var involveret i det HFD og palmitinsyre forårsagede nervetab. Vi viste, at nervetabet udeblev i mus, der genetisk havde fået fjernet P2Y₁₃ receptoren, efter et 6 måneder på HFD. Det samme resultat viste sig, når vi behandlede nerver fra disse dyr med palmitinsyre.

Samlet viser vores resultater, at overvægt og T2D-relaterede metaboliske forandringer påvirker ENS og forårsager et neuralt tab. Dette tab sker sandsynligvis via de akkumulerende negative påvirkninger som øget fedt, inflammatorisk belastning og nedsatte GLP-niveauer betyder.

Populärvetenskaplig sammanfattning

I min barndom hängde denna affisch på dörren till mitt klassrum i grundskolan. Titeln var: Du är vad du äter. På affischen springer olika matvaror i kapp och det är självklart rågbrödet, mjölken och moroten som vinner, medan glass, godis och pommes frites kommer sists. Affischen skulle få barn att vilja välja sunda matvaror, för vem vill förlora loppet.



Är du, vad du äter?

Vi är vad vi äter på grund av vårt matsmältningssystem. Matsmältningen startar i munnen när vi tuggar maten och slutar när vi går på toaletten. På väg genom tarmen bryts maten ner till enkla beståndsdelar som kroppen kan ta upp och använda som energi eller för att bygga nya celler. Tarmen frisätter också olika aptitreglerande hormoner. Mag-tarmkanalen är konstruerad som ett rör bestående av flera skikt, optimerade för att ta upp näring. Innerst finns mukosan, utanför den submukosan, sen ett tvådelat muskelskikt och ytterst ett bindvävsskikt.

Mag-tarmkanalen utgör den största ytan hos oss i direkt förbindelse med den yttre miljön. Hos vuxna är tarmen ca 9 meter lång och om man sprider ut den i ett plant skikt skulle dess yta uppta ca 400 m². Den har en komplex och viktig roll i en miljö som ofta förändras. Tarmen är ett finstämt sensoriskt organ utrustat med en stor uppsättning receptorer som kan reagera på sträckning, tryck, smak och skadliga ämnen. Att tarmen är utrustad med alla dessa receptorer är ändamålsenligt då tarmen är hem för vår tarmflora (mikrobiota) som består av mer än 100 miljarder mikroorganismer; långt fler än vi har celler i kroppen. Mellan dem och oss finns tarmväggen som sörjer för näringsupptaget från födan. Den stora majoriteten av mikroorganismer är bra och hjälper till med matsmältningen och stärker immunförsvaret. Vad vi äter påverkar i hög grad viken typ av mikroorganismer vi har

i tarmen. Förutom att hjälpa till med matsmältningen kan dessa också påverka vårt mentala tillstånd, visar flera nya studier. Så vi är både vad vi äter OCH de mikroorganismer vi har i tarmen .

Vem har kontrollen?

Mikroorganismerna spelar en viktig roll i vår tarm, men vi är också utrustade med ett enteriskt nervsystem (ENS) bestående av ca 500 miljoner nervceller. Dessa sitter längs hela tarmen i ett komplext nätverk och kontrollerar tarmrörelser, utsöndring av elektrolyter och vatten samt blodcirkulationen. ENS är indelat i två sammanvävda nätverk av nerver, ett myententeisk placerat mellan de två muskelskikten detta kontrollerar huvudsakligen tarmrörelserna och ett submukosalt som sitter i submukosan och kontrollerar tarmsaft utsöndringen. ENS har nervterminaler ut till andra vävnader, såsom bukspottskörtel, gallgångar och ryggmärg. Sensoriska nervändar i slemhinnan reagerar på tarminnehållet och skickar meddelande till motoriska nervceller via interneuron, vars förlopp är i oral eller anal riktning eller utmed tarmens omkrets. Mellan varje måltid utför tarmen ett rengöringsprogram vilket ser till att matrester inte ligger kvar eller att det sker en ansamling av bakterier. Beroende på matens beståndsdelar sätter ENS i gång olika rörelser, antingen blandande, korta eller långa peristaltiska rörelser samt skickar vatten och elektrolyter ut i tarmen, vilket säkerställer korrekt konsistens och näringsupptag. Likaså anpassar ENS det immunologiska svaret mot patogener i tarmlumen, genom ökad sekretion och peristaltik och frisättning av signalsubstanser som aktiverar immunceller och förbättrar tarmbarriären.

Vad händer om vi tappar kontrollen?

Vi känner alla igen det. Ont i magen, uppblåst, förstoppad eller dräppad av diarre är tyvärr vardag för en stor del av befolkningen. Kroniska mag-tarmbesvär täcker ett brett spektrum av symptom som kan vara både primära eller en komplikation till andra sjukdomar, såsom diabetes och Parkinsons sjukdom. Överviktiga och typ 2-diabetiker (T2D) har mer mag-tarmproblem än resten av befolkningen. Symptomen varierar från patient till patient och kan uppstå varhelst i mag-tarmkanalen, från gastropares och hypersensitivitet till diarré och förstoppning. Förändringar i ENS kan ligga bakom många av de symptom som mag-tarmpatienter upplever. De allra flesta av mag-tarmsjukdomarna är relaterade till både strukturella och funktionella förändringar i ENS, såsom ökad eller minskad nervfibertäthet och ändrad frisättning och känslighet för neurotransmittorer. Då de flesta underliggande mekanismer bakom

utvecklingen av mag-tarmsjukdomar fortfarande är okända frågade vi oss följande frågor i denna avhandling: Hur reagerar ENS vid övervikt och T2D liknande metabola störningar?

Effekten av en high-fat-diet

Trots den roll som generna spelar i utvecklingen av T2D, spelar livsstilen en än större roll; där 70 % av de patienter som utvecklar T2D är överviktiga. Vi utnyttjade detta faktum i våra studier och använde en så kallad diet-induced-obesity model, där möss gavs antingen ett vanligt foder eller ett foder med högt innehåll av mättat fett. Efter 6 månader undersökte vi huruvida det hade påverkat tarmen och ENS. Resultaten från high-fat-diet (HFD) försöken visade att intag av en diet med mycket mättat fett leder till en betydande förlust av enteriska nerver. Då HFD inte bara påverkar ENS, utan hela djuret, undersökte vi specifika faktorer i en försöksmodell för isolerade nerver. Glucagon-like peptide (GLP) 1 och 2 är två hormoner som utsöndras i tarmen. GLP-1 påverkar aptitreglering och är viktig för frisättningen av insulin. GLP-2 är viktig för upprätthållandet av tarmväggen. Båda hormonerna förekommer i lägre koncentrationer hos överviktiga individer. Vi har visat att dessa hormoner skyddar isolerade nerver från ENS både generellt och mot en mastcells inducerad nervcells förlust. När tarmväggen utsätts för HFD ökas permeabiliteten (genomsläppligheten) och fler patogener kan därför komma in i kroppen genom tarmen varvid en immunologisk process sätts igång. Både överviktiga individer och T2D patienter har ökade nivåer av lipopolysaccharider (LPS) från bakterier i blodet. Vi har visat att LPS förorsakar nervcells förlust via en aktivering av en intracellulär signaleringsväg, som involverar enzymet AMP aktiverat kinase (AMPK). Då överviktiga och T2D patienter har ökade mängder av fria fettsyror i blodet undersökte vi också om denna typ av fett påverkar nervcellerna. Palmitinsyra (en mättat fettsyra) visade sig orsaka nervcells förlust via bildandet av energi-intermediärer, som ledde till intracellulär stress.

Puriner har en framträdande roll biologin. Livets byggstenar DNA och RNA består av puriner och cellens viktigaste energikälla ATP är också en purin. ATP fungerar även som en neurotransmittor i ENS och kan binda till en rad olika receptorer. Vi undersökte därför om purinreceptorn P2Y₁₃, som påverkar transporten av fett var involverad i nervcells förlusten som förorsakades av HFD och palmitinsyra. Vi kunde visa att nervförlusten efter 6 månader på HFD, uteblev i möss som genetiskt saknade P2Y₁₃ receptorn. Samma resultat erhöles när vi behandlade nerver från dessa möss med palmitinsyra.

Sammantaget visar våra resultat att övervikt och T2D relaterade metabola förändringar påverkar ENS och orsakar nervcells förlust. Denna förlust sker sannolikt genom de ackumulerade negativa effekter som ökad mängd fett, inflammatorisk belastning och minskade GLP-nivåer innebär.

General introduction

We can all relate to gastrointestinal (GI) symptoms, the uncomfortable feeling of having a belly ache or being bloated, nauseous and how little we can accomplish when we are sick. Even if it is only for a short while it affects how we act and think, and we look forward till it is over.

For a large part of the population GI symptoms is more than a temporary inconvenience, it is a chronic condition.[1] Functional gastrointestinal diseases (FGID) can either be a primary condition or a complication to other diseases like diabetes and Parkinson.[2-4] FGID is an umbrella for a range of diseases including irritable bowel syndrome (IBS). FGID are all characterized by chronic symptoms attributable to the upper or lower GI tract. [5, 6] FGID as well as other GI diseases affect patient's quality of life. The emotional stress due to lack of control over symptoms and lack of disease validation often leads to depression and anxiety disorders.[1, 7-10]

GI diseases also burden society, though difficult to estimate; in 2000 the American Gastroenterological Association estimated the direct cost of IBS to be 1.7 billion dollars while the indirect costs reached 20.2 billion dollars in the United States. Considering the range of GI diseases this cost is likely to be much higher.[1] The reason the cost are so high, not just for society but also for the patients, is that underlying mechanisms causing FGID are unknown. This leads to treatment of symptoms rather than a treatment directed against the disease or a cure.[11]

Due to the complexity in symptom severity, environmental, physiological and psychological factors and the lack of biomarkers, patients and physicians often face tremendous challenges handling the diseases.

Background

The gastrointestinal tract

The GI tract is the body's largest surface to the outside environment. In the adults it is about 9 meters long and has a surface area upward of 400m².^[12] It performs diverse and complex roles in an ever changing external environment. It has to allow for nutrients and electrolytes to be absorbed as well as be a barrier against pathogens and toxins. To achieve this amazing feat the GI tract has evolved into a fine tuned sensory organ. A complex network of sensory-, taste-, baro- and mechanoreceptors as well as pathogen recognizing receptors (PRR) are involved in fluid adaptation and control of the GI tract.^[13] The presence of PRR allows the GI tract to play an important role in modulating and mediating immune responses.^[12]

The digestive tract extends from the oral cavity to the anus. It is modified along its length to optimize digestion of food while maintaining a similar morphological organization. It can roughly be described as a tube whose wall consists of four concentric layers:

1. The innermost layer surrounding the lumen is the mucosa, which is separated into three sublayers; the wet epithelial lining in contact with the lumen with secretory and absorptive functions, the lamina propria containing lymphatic and vasculature and the muscularis mucosa a thin layer of smooth muscle cells responsible for mucosa motility.
2. The submucosa is a layer consisting of connective tissue that harbors lymphatics, vasculature and, in most parts of the GI tract the submucous ganglia of the enteric nervous system (ENS).
3. The muscularis externa is divided into sub-layers. The circular and longitudinal smooth muscle layers wrap around the tube in tight and loose helices, moving and mixing luminal content along the GI tract. Between the two muscle layers are blood vessels and the myenteric ganglia of the ENS present.
4. The outermost layer is the serosa, or in some regions adventitia.

The enteric nervous system

The ENS comprises the largest subdivision of the peripheral and autonomic nervous systems. It is an extensive interconnected network of neurons and glia cells. The human ENS consists of about 500 million neurons, a number suggested equal to that of the spinal cord.[14]

Development of ENS

The ENS arises from neuroectoderm derived enteric neural crest cells (ENCC) that during embryologic development migrate and populate the gut. This means that the ENS has a different embryonic origin compared to the rest of the gut that develops from the endoderm and splanchnic mesoderm.[15-17] In man the colonization of the gut by ENCC takes about 3 weeks, from gestational week 4-7. The majority of ENCC that give rise to the ENS is derived from vagal neural crest. Sacral neural crest cells account for a minor population of neurons in the distal gut and migrate along axons of extrinsic neurons.[15] Several important coordinated events have to take place for the successful ENS development. The ENCC have to migrate into and along the developing gut, they have to proliferate and differentiate, form ganglia, mediate axon path-finding and synaptogenesis.[15, 17] For this to occur, several signaling and effector molecules need to be present and act concomitantly.[18] Migration of ENCC starts in the foregut and continues caudally. During gut development the mid small intestine is juxtaposed to the proximal colon and a small population of ENCC takes a shortcut giving rise to the colonic ENS in trans-mesenteric colonization.[15, 19]

Signaling pathways involved in ENS development

One of the important signaling pathways determining proliferation, migration and differentiation in the ENS is the glia derived neurotrophic factor (GDNF)/RET system. GDNF produced by the gut mesenchyme acts as a chemoattractant for ENCC in a temporal and foregut-to-cecum manner, pulling ENCC migration with it.[15] GDNF binds to RET, a tyrosine kinase receptor on ENCC, known to promote survival, proliferation, differentiation and migration.[20] The importance of the GDNF-RET signaling pathway in ENS development is highlighted in genetic knock out models where ENS is absent distal to the stomach. Another important signaling system controlling ENS development is endothelin-3 (ET-3)/(endothelin receptor type B (EdnrB)). Like GDNF/RET ET-3 is expressed in the gut mesenchyme and the EdnrB receptor on ENCC. However unlike the GDNF/RET system ET-3/EdnrB have an inhibitory role on ENCC differentiation. Genetic knock out of ET-3/EdnrB causes lack of ENS in the distal gut.[15, 21]

ENCC cells provide the origin for both enteric neurons and enteric glia cells. The factors determining if ENCC continues to proliferate or exit the cell cycle and differentiate into either neurons or glia is complex and not fully understood. One signal that has been shown to be important for glia differentiation is leucine rich repeat LGI member 4 (LGI4), secreted both by ENCC and glia. Genetic modulations of *lgi4* or its receptor cause reduction in glia number and alter ENS structure. Some ENCC early on express neuronal markers such as human neuronal protein (HuC/HuD) or human gene product 9.5 (PGP9.5), however they remain in cell cycle and migration still occur. When ENCC expressing neuronal markers exit cell cycle they differentiate into diverse ENS neuronal subtypes. Neurochemical phenotypes are even suggested to depend on the embryonic time in which the neuronal marker-expressing ENCC leaves the cell cycle.[15, 18, 21]

Organization

In most parts of the digestive tract ENS is organized into two major ganglionated plexa. The myenteric ganglia situated between the muscular layers of muscularis externa and the submucous ganglia located in the submucosa, with a dense network of nerve terminals innervating mucosa, muscle cells, vessels and other ganglia.[22-25] ENS also possesses intestinofugal neuronal projections that terminate in pancreas, biliary tract and sympathetic ganglia.[26] The neuronal complexity of ENS is high and it constitutes an integrated system that entails ENS reflexes. Such reflexes often involve extrinsic neuronal inputs and modulations.[14, 25, 27]

Parts of the GI tract are highly controlled by extrinsic neurons through vagal and spinal innervations terminating within the GI wall. Extrinsic nerve endings terminate within musculature, interstitial cells of Cajal (ICC), mucosal epithelium, ganglia and vasculature. Extrinsic neurons form intramural collateral branches innervating vasculature and ganglia.[14, 25]The diverse location of extrinsic neurons allows for tight monitoring of GI homeostasis and sensation.[13, 14, 25, 27]

Neuronal sub-types

Several types of neurons are present in the ENS, capable of modulating intrinsic intestinal reflexes.[14, 25, 28] Neurons can be subdivided based on morphology using the Dogiel classification, electrochemical properties or function. Based on function three types of neurons exist. Sensory neurons able to detect mechanical changes and luminal factors, interneurons running in oral, anal or circumferential direction, and motor and secretomotor neurons terminating by circular, longitudinal muscles or glandular cells.[29, 30] Intestinofugal projections entail both motor/secretomotor neurons as well as sensory neurons depending on target organ.[26] Based on

electrochemical properties neurons in the ENS are able to convey fast and slow neurotransmission. Fast neurotransmission within the ENS is mainly excitatory. Neurons responding with fast neurotransmission are referred to as synaptic or S type. Slow neurotransmission can be either excitatory or inhibitory and neurons responding with slow neurotransmission are referred to as having prolonged afterhyperpolarization or AH type. Slow excitatory transmission in the ENS is an important signaling mechanism to modulate and direct transmission. Slow excitatory transmission act as a primer for fast excitatory transmission, as well as modulating membrane conductance depending on neuronal type.[25, 29, 31, 32]

Neurotransmitters

Excitatory transmitters in the ENS include acetylcholine, serotonin, substance P, neuropeptide Y, neurokinin A and galanin. Inhibitory transmission is mainly mediated by vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), nitric oxide (NO) and purines.[25, 29, 33] During inhibitory hyperpolarization excitatory neurotransmission is inhibited. Neurons in ENS, while having preferred neurotransmitter content, often co-express secondary or tertiary neurotransmitters. These are able to modulate the primary transmitter response.[29, 33] ENS displays a high number and high diversity of neurotransmitters similar to central nervous system (CNS). Allowing also signaling with endocrine cells, immune cells as well as nutrients or luminal factors.

VIP, NO and adenosine triphosphate (ATP) are classic inhibitory neurotransmitters present in non-noradrenergic, non-cholinergic (NANC) neurons throughout the gut.[34] VIP and ATP are released from secretory vesicles, while NO is a gaseous neurotransmitter produced on demand by neuronal NO synthase (nNOS).[34, 35] These transmitters mediate e.g. muscle relaxation and vaso-dilator responses. They are often co-expressed and co-released to optimize the inhibitory response.[34]

Function

Motility

Four main types of motility are present and controlled by the ENS in the GI tract; the interdigestive migrating motor complex (MMC), the post-meal mixing, propulsion and neurally programmed musculomotor quiescence. MMC acts as an intestinal cleaning program that between meals and during fasting, causes anally directed contractions of varying intensity. This ensures that no intestinal debris or bacterial overgrowth compromise the intestinal barrier.[25, 36] After ingesting a meal an intestinal mixing program is initiated. The volume and nutritional content of the

meal determines the duration, e.g. stimulation on the mucosa changes the motility pattern from a propulsive to a mixing state. Propulsion of the chyme is mediated through peristaltic movements. Two types of peristaltic movements occur in the intestine, one that during digestion propagate short distances in an oral to anal manner and the power propulsion. The power propulsion is a strong peristaltic movement that travels long intestinal distances. The power propulsion is effective in moving content in an anal direction. In certain conditions this movement can give rise to abdominal cramps, pain and rapid movements as present in diarrhea.[25, 29, 37, 38] The neuronal mechanism underlying peristalsis in the GI tract includes muscular relaxation in front of the chyme and a contraction behind. The model suggests that sensory receptors in the mucosa signal interneurons in myenteric ganglia that run in anal or oral direction. Interneurons running in an anal direction signal motor neurons in the circular and longitudinal muscle layers causing a relaxation in front on the mucosal stimuli. Interneurons running in an oral direction signal motor neurons in the circular and longitudinal muscle layer, causing a contraction behind the mucosal stimuli. Thereby moving the chyme forward in an oral to anal direction.[29, 38, 39] The motor neurons terminating by the neuromuscular junction relay their signal transduction via the pacemaker cells ICC's slow wave activity.[40, 41]

Secretion and blood flow

Water and electrolyte secretion into the lumen is mediated through enteric reflexes involving both myenteric and submucous ganglia. Chloride is the main electrolyte pulling water across the intestinal barrier into the lumen.[30, 42] Serotonin and distention sensitive neurons are important triggers behind the initiation. Serotonin is released from enteroendocrine cells by a variety of signals such as mechanical stimulation, stretch and pH. Serotonin acts via serotonin receptors on sensory neurons originating in submucous and/or myenteric ganglia. Secretion due to distension is mediated mainly by submucous sensory reflexes.[30] Toxin mediated secretion is conveyed by both submucous and myenteric sensory reflexes, leading to integration of both secretion and peristalsis.[14, 43] Activation of sensory neurons in the submucosal secretion arch leads to activation of secretomotor neurons releasing VIP, ATP or acetylcholine, in close proximity to the stimulation site. Activation of the myenteric secretion arch involves activation of myenteric interneurons and a longer reflex arch causing secretion further from the mucosal stimulation site.[30, 42, 44] Electrolytes and water are pulled from the interstitial fluid and vaso-dilation is necessary to keep up secretion. ENS is able to modulate blood flow by perivascular secretomotor neurons causing either vasoconstriction or -dilation.[14, 29]

The modulators

The ENS is a key element in the seamless and smooth control and modulation of the GI tract. It adapts to microenvironmental and homeostatic changes by altering neuronal signaling and activity patterns. In the following sections neuronal modulators relating to data and results obtained in the thesis will be described.

Hormones

Several hormones are able to modulate enteric neuronal responses, either direct or through sympathetic and/or central neurons.[45, 46] The hormones glucagon-like peptide (GLP) 1 and 2 are members of the secretin/glucagon superfamily, like VIP. They are released from enteroendocrine L-cells present in the proximal small intestine, but with the highest density in ileum and colon.[47] GLP-1 and 2 are released in a 1:1 manner in response to nutrients, especially glucose and lipids are potent stimulators. [47, 48] GLP-1 is an incretin hormone causing increased insulin and decreased glucagon secretion after a meal.[47, 49] This however, is not the only effect of GLP-1; others include increased satiety, slowed gastric emptying and reduced gut motility, to mention a few.[50, 51] GLP-2 is increasingly recognized for its intestinotrophic effects on intestinal growth, barrier permeability, nutrient absorption and anti-inflammatory potential.[47, 52, 53] Both GLPs are able to interact with enteric neurons and modulate enteric responses. The inhibitory effect of GLP-1 on GI motility is suggested to be mediated by enteric NO release.[50, 54, 55] GLP-2 anti-inflammatory effects are believed to be through GLP-2 receptors situated on enteric neurons causing up-regulation and increased release of VIP.[53, 56, 57] The potential for these hormones to regulate intestinal barrier function, enteric motor activity, appetite and insulin secretion make them intriguing targets in pathological states where intestine and homeostasis is compromised.

Pathogens and the immune response

The intestinal barrier plays an important role in maintaining homeostasis between the microbiome and the host; altered membrane permeability can initiate immune responses.[58, 59] The innate immune system comprises a conserved range of PRRs that are activated by compounds abundantly and uniquely expressed on pathogens. Upon activation complex effector mechanisms are initiated until the adaptive immune system is altered and initiates a more direct response.[60] The importance of this is evident considering the GI tract is inhabited by at least 100 trillion microorganisms from the entire phylogenetic tree including *vira*.[61] Their genetic

pool make up the human microbiome and about 7000 strains are identified to date.[62] Microbiota is no longer considered free-riders, sifting free nutrients from their host. They play an important role in the development and modulation of the innate and adapted immune response.[61-63] The microbiota composition varies between individuals, it is shaped by not only the diet but also the genetics of the host, creating an individual microbiome.[61, 64] The contribution of the microbiota in modulating the immune system is highlighted in disease states, such as obesity, type 2-diabetes (T2D) and autoimmune diseases.[45, 61-64] Besides the modulatory effects on the immune system, the microbiome affects the nervous system and is suggested to modulate not only intestinal neurons but even cognitive behaviors. [45, 61, 65, 66]

Liposaccharide (LPS) is an abundant membrane component of gram negative bacteria, able to activate the innate immune response. The PRR specific for LPS is toll like receptor 4 (TLR4). Trace amounts of LPS is able to activate TLR4 leading to the production of several pro-inflammatory mediators such as tumor necrosis factor α (TNF α), interleukin-1 (IL-1) and -6. For LPS to elicit a full TLR4 response, a receptor complex consisting of a TLR4 dimer, CD14 and MD-2 need to be present. TLR4 signal transduction response is dependent on the presence of intracellular adapter proteins like MyD88 and toll-interleukin 1 receptor domain containing adaptor inducing inteferon- β (TRIF). The adapter proteins can elicit different immune responses.[60, 67-69]

Energy metabolites

Lipids

Neuronal energy supply is mainly dependent on glucose and lactate; lipids constitute a limited source.[70] Cells utilize lipids for energy through beta oxidation. This process involves lipids to be transported into the mitochondrion or peroxisome through the carnitine shuttle situated in mitochondrial or peroxisomal membranes. The carnitine shuttle is a three protein complex consisting of carnitine palmitoyl transferase I (CPT1), carnitine acylcarnitine translocase (CACT) and carnitine palmitoyl transferase II (CPTII). In neurons CPT1c is the main isoform. However this expression is localized to endoplasmatic reticulum (ER) and not to mitochondria or peroxisoms.[71, 72] Besides providing cellular energy, lipids play a significant role as structural components and metabolic signals. Cellular lipid cycling through the glycerolipid (GL) or in most cases triacylglycerol TAG/free fatty acid (FFA) cycle and its accompanying smaller cycles provide the cell with a frame work to synthesize GL from FFA and FFA from GL. The intermediates generated provide the cell with signal molecules controlling biological functions like diacylglycerol (DAG) and phosphatidic acid (PAA). Both DAG and PAA are also able to participate in the

formation of phospholipids (PL). Many cells have the ability to store excess lipids as TAG in lipid droplets formed in the ER. The formation of TAG is through lipogenesis, initiated by the synthesis of lysophosphatidic acid (LPA) from acyl-coenzyme A (FA-CoA) and glycerol 3-phosphate (G3P). LPA is further acylated to PAA, which is further dephosphorylated to DAG that through another acylation event becomes TAG. Cells are able through lipolysis, to break down TAG to FFA. This is initiated by lipases and the formation of DAG, which can be further hydrolyzed to monoacylglycerol (MAG) and FFA. The individual steps in the cycle are shown in figure 1.[73-75]

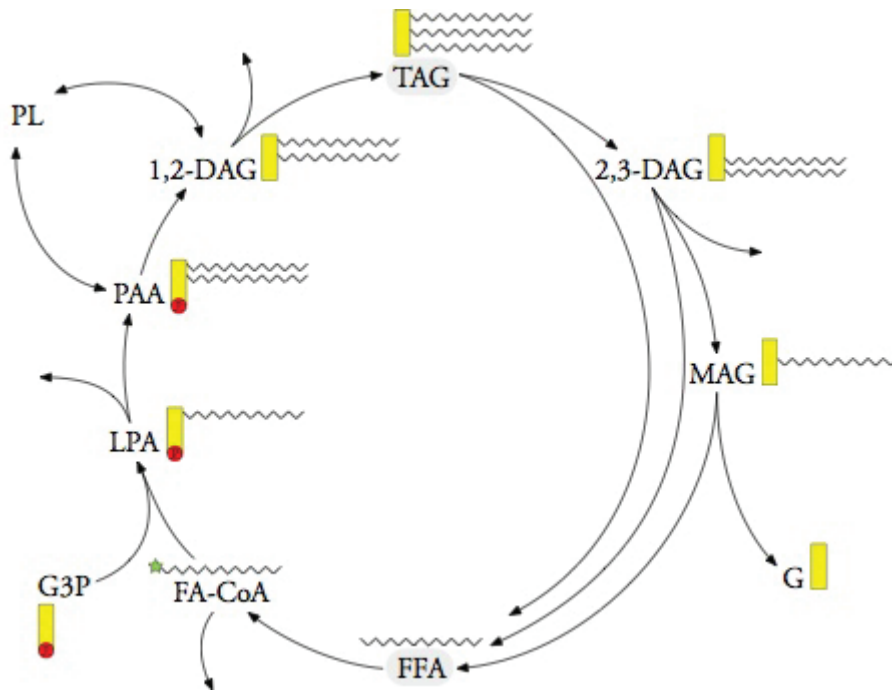


Figure 1 GL or TAG/FFA cycle adapted and modified from [73]. For details regarding the individual steps see preceding section. Arrows pointing away from the cycle denote the ability of the cycle intermediates to participate in signaling cascades or formation of structural elements.

Adenosine mono-phosphate protein kinase

Adenosine monophosphate (AMP) protein kinase (AMPK) is an important intracellular regulator, coordinating metabolic pathways to meet cellular energy demands. Thereby ensuring that intracellular ATP levels are maintained for optimal cellular metabolism. AMPK is an energy sensing complex able to modulate cellular energy metabolism both short and long term. In the short term AMPK senses the

energy level (AMP:ATP ratio) in the cell and initiates processes that favors ATP production such as beta oxidation, glucose uptake and glycolysis. Long term regulation is through transcriptional regulation of genes involved in energy metabolism, as well as interactions with hormones regulating nutritional intake and appetite. [76-79]

AMPK is a heterotrimeric complex with a catalytic α ($\alpha 1$, $\alpha 2$) unit and two regulatory β ($\beta 1$, $\beta 2$) γ ($\gamma 1$, $\gamma 2$, $\gamma 3$) subunits.[76] In central neurons $\alpha 1$: $\beta 2$: $\gamma 1$ are the main isoforms of AMPK.[80] Phosphorylation of threonine 172 is vital for AMPK activity. Binding of AMP to the regulatory site increases AMPK activity, by inducing a conformational change that inhibits dephosphorylation and cause allosteric activation. Phosphorylation of AMPK can be by the conserved liver kinase B (LKB), Ca^{2+} /calmodulin dependent kinase kinase (CaMKK) and, the lesser described, transforming growth factor β activated kinase (TAK1).[76, 81] Transcriptional targets of AMPK activation include peroxisome proliferator-activated receptor γ cofactor 1 (PGC-1), sirtuin 1 (SIRT1) and cAMP response element-binding protein (CREB).[76, 79]

Purines

Purines have a prominent position in biology as the building blocks of life, DNA and RNA are purines. ATP is also a purine and constitutes the key energy source in cells.

In the 1970's professor G. Burnstock presented evidence that ATP could act as a sole or co-neurotransmitter in NANC neurons in the gut.[82, 83] The ATP concentration in secretory vesicles is in the range of 1-100mM and circulating levels of ATP is about 5mM.[35, 84, 85] Once released ATP either binds to purine receptors selective for ATP or is rapidly broken down to ADP, AMP or adenine. The enzymes responsible for the breakdown of ATP are called ectonucleotidases. Their activity is vital in shaping purinergic signaling, as the ATP metabolites produced (ADP, AMP and adenine) all are active ligands, fine-tuning the purinergic response.[85-87] Purinergic receptors are divided into two main types: P1 receptors selective for adenine and P2 receptors selective for ATP or ADP. The P2 receptors have been further subdivided into P2X, ionotropic and P2Y, G-protein coupled receptors. P2X receptors are all activated by ATP.[84-86] P2Y₁₃ is the purinergic receptor focused on in the thesis. It is activated by ADP and couples primarily to G_{i/o}. G_{i/o} and its $\beta\gamma$ subunit can convey a range of intracellular signals, such as inhibition of adenylate cyclase (AC) and subsequent reduction in protein kinase A (PKA) signaling. In addition it is also able to convey intracellular signaling through activation of phosphoinositide 3-kinase (Pi3-K,PKB activation) and/or phospholipase C (PLC, PKC activation).[86] In neurons P2Y₁₃ activation has been suggested to couple to G protein-activated inwardly rectifying potassium channels (GIRK) suggesting that

P2Y₁₃ receptor activation hampers neuronal depolarization and action potential propagation.[86]

Neuroplasticity and neuropathies

Adverse symptoms in the GI tract include chronic pain, visceral hypersensitivity, bloating, nausea, constipation and diarrhea. Neuronal plasticity, the ability to rewire connections or adapt to the microenvironment, is an increasingly recognized feature of the ENS.[88, 89] The majority of GI disorders display elements of both structural and functional neuroplasticity. In the ENS, neuroplasticity includes altered innervation density such as sprouting, neuronal and ganglionic hyper- or hypotrophy. Also changes in chemical coding, neurotransmitter release patterns, altered neuronal sensitivity and activation of glia are part of plasticity-induced changes observed in the ENS.[31, 88, 90]

Functional adaptations

GI neuropathies can either be the primary disease as seen in many patients suffering FGID, but they can also be secondary. The mechanisms leading to neuronal damage or dysfunction is far from understood, and is probably disease dependent.[2, 91]

The conceptual model of FGID involves a range of factors that, like many other diseases affect the outcome. A person's genetics and life experiences can render them more or less susceptible to environmental, physiological and psychological alterations.[8, 92] Physiological changes in the gut like altered motility, hypersensation, inflammation and microbiota overgrowth indicate onset of FGID. Though underlying causes for FGID are unknown several pathological observations have been attributed to them. Immune and inflammatory modulations are becoming a recognized feature of many of the FGIDs, observed by elements of inflammatory infiltrates in and around enteric ganglia and intestinal wall.[91, 93] Inflammatory cells release cyto- and chemokines altering the microenvironment faced by enteric neurons and glia. Inflammation is associated with neuronal chemical coding alterations, degeneration and functional impairment.[2, 93-95] One of the best described FGID is IBS. Patients with IBS display mast cell infiltrations in mucosa and in close proximity to enteric neurons and fibers. Intestines from these patients also display increased barrier permeability and reduced levels of anti-inflammatory modulators. Hyperinnervation of substance P and serotonin positive nerve fibers have been observed in GI tract of IBS patients.[96, 97] Taken together the noted immune

responses and/or neuroplastic alterations cause altered gut motility, secretion and sensitivity.

Obesity and type 2-diabetes

Overweight and obesity is associated with a high risk of developing T2D, about 70% of T2D is caused by overweight. Genes are increasing being recognized for their role in disease susceptibility, however lifestyle still play a prime role.[98] Overweight and obesity is related to an increased risk for developing a range of conditions. Besides T2D are cardiovascular diseases, rheumatoid, GI diseases as well as others increased in obesity. Obesity, like T2D is associated with insulin resistance. However where obese patient are normoglycemic the onset of hyperglycemia marks the transition to T2D. Insulin resistance and hyperglycemia cause adverse metabolic profiles placing an unhealthy strain on the body.[99, 100] Vascular damage and autonomic neuropathy are common complications of T2D.[101, 102] Patients suffering from diabetes have more GI symptoms than the healthy population.[103, 104] Their symptoms cover the entire scale suggesting diabetes to affect all parts of the GI tract.[2, 105] One of the best described neuropathies is diabetes-induced gastroparesis.[106, 107] Diabetes-induced gastroparesis display loss of nitrergic neurons and ICC in the stomach. Loss of nitrergic neurons and ICC are also reported in other parts of the GI tract.[105] Hypoinnervation of VIP and nitrergic neurons and hyperinnervation by excitatory neurons have been reported in the small intestine of diabetic patients.[105]

Aims and hypotheses

The general aims of this thesis were to study the enteric nervous system and the effects metabolic imbalances have on neuronal survival. Further to investigate receptors and signaling cascades involved in mediating the protective or harmful effects of metabolic imbalance.

The main hypotheses of the four studies are as follows:

- In paper I the hypothesis that members of the sekretin/glucagon superfamily have neuroprotective effects on enteric neurons, is investigated.
- In paper I it is further hypothesized that receptors for GLP-1 and/or VIP situated on enteric neurons are involved in the protective effects of the peptides.
- In paper II and III the hypothesis that gastrointestinal symptoms observed in obese and T2D originate in high fat diet (HFD)-induced alterations of the ENS is investigated.
- In paper II and III it is further hypothesized that fatty acids, the purinergic receptor P2Y₁₃ and metabolic energy pathways are at the root of HFD-induced neuronal loss.
- In paper IV the hypothesis that exposures of the enterotoxin LPS, causes enteric neuronal loss, and that intracellular energy related processes and VIP release are involved, is investigated.

Methodology

The aim of this chapter is to give an overview of the different techniques and methodological approaches used in the thesis. Discussions and reflections of the chosen techniques are also included in this chapter. Details about specific material and methods are given in paper I-IV.

Feeding experiments

The animals used in the feeding experiments (paper II and III) were served either a normal diet (ND) or HFD for an extended time period. Diet-induced-obesity is a standard protocol when investigating effects of metabolic imbalances indicative of metabolic syndrome and T2D. The protocol involves groups of mice either continuing on standard chow (Lactamin R36) or changing to a purified diet containing high levels of fat (Research diets RD12451). A range of confounding factors has to be considered when performing diet experiments. Changing one diet component automatically changes the composition of other nutritional factors. The fat source of the ND is soybeans, while fat in the HFD originates from both soybeans and lard. This affects the content of saturated, mono-unsaturated and poly-unsaturated lipid and their relative ratios. Another confounding factor in the diet experiments is the differences between chow or plant based diet and a purified diet. Where chow contains a range of i.e. carbohydrates, purified diets only contain one carbohydrate, sucrose. Even the fiber type and content may affect nutritional and overweight status of animals in the experiments. These are factors that need to be kept in mind when analyzing the *in vivo* data.

Genetic knock out of the P2Y₁₃ receptor in mice

The use of genetically modified mice has become a standard model in today's biomedical research. The field has greatly expanded with new and improved models. Conditional and cell type specific genetic modifications are now readily available. In

this thesis mice genetically modified to lack the P2Y₁₃ receptor were used. The protocol used to obtain the genotype is a non-conditional removal, rendering the receptor absent throughout embryonic development as well as postnatally. The phenotype and subsequent results have to be seen in the light of possible compensatory mechanisms altering the observed phenotypical expression in the genetically modified mice compared to naïve or non-genetically modified mice. However these data still provides valuable insight to the role of the P2Y₁₃ receptor in the enteric nervous system.

Primary cell cultures

The use of primary cell cultures have its advantages and disadvantages compared to stem cell or blastoma derived cell lines. The primary cell cultures used in this thesis takes advantage of the intestinal morphology and position of the myenteric ganglia, allowing cellular isolation without penetrating the intestinal barrier thereby avoiding contamination of luminal content. The isolation procedure involves mechanical and enzymatic separation of the isolated tissues and results in a single cell suspension consisting on myenteric neurons, glia and intestinal smooth muscle cells. Dissociation of cells causes axotomy of neurons and rounding of all cells and hence disruption of cell to cell contact. However this also allows for multi-well seeding and screening of several substances and pharmacological interventions in the same animal. Isolating primary cells implies removal from their original environment and placement in an artificial one, which provides cells with buffer solution and nutrient cocktail for optimized survival. Foetal bovine serum provides additional factors needed for cellular growth and survival. The factors present in the serum may slightly vary depending on e.g. the health status and filtration protocol used. This may affect the cultures. The change in micro-environment from *in vivo* to *in vitro* affects cellular organization, stimulation and protein expression patterns. These limitations have to be kept in mind when using primary cell cultures. However the benefits of using non-immortalized adult cells outweigh the disadvantages and provide results that are a step closer to the *in vivo* situation. With optimas internal controls, primary cultures of enteric neurons provide a powerful tool in investigating neuronal plasticity.

In cultures derived from rat (paper I, II, IV) the protocol allows for a relatively large quantities of glia and smooth muscle cells to be seeded in wells, creating a dense mat for the neurons to settle and sprout. In cultures derived from mice (paper III) the protocol has been optimized to reduce the number of smooth muscle cells.

Isolation of peritoneal mast cells (paper I) make use of the fact that laboratory animals are inbred, and cultures are devoid of immune cells minimizing rejection of foreign cells. Thereby allowing cultures to establish prior to mast cell challenge and though

not used in this thesis opens possibilities for combinations of genetically modified cross testing. An aspect to keep in mind when using primary mast cells is the “per animal yield” and health status of the animal from where the cells are isolated.

Pharmacological treatments

Investigations of receptors involved (paper I and III) and/or underlying cellular mechanisms (paper II and IV) have been accomplished *in vitro* using pharmacological interventions. Pharmacological agents are useful tools elucidating the mechanism behind phenomenological observations. However the use of pharmacological interventions provides its own challenge. Agonist and antagonist selectivity, affinity and mode of action all affect the results. For all pharmacological agents used in the thesis, individual dose response curves, in regards to neuronal survival have been performed based on concentrations previous used by others. However no pharmacological cross-interactions have been tested in the thesis.

Proliferation

Unlike adult neurons, glia and intestinal smooth muscle cells in the primary cultures have the ability to proliferate. The effect micro-environmental and pharmacological treatments have on the rate of proliferation of cells in cultures, was in paper II estimated using the EdU click-IT technique. This takes advantage of the ability of the thymidine analog EdU (5-ethynyl-2'-deoxyuridine) to be incorporated during DNA synthesis. EdU can be detected in the copper(I) catalyzed azide-alkyne cycloaddition reaction, avoiding the use of denaturation steps associated with the BrdU (5-bromo-2'-deoxyuridine) detection technique. The EdU click-IT kit used in paper II was co-stained with Hoechst to visualize nuclei. Hoechst is a fluorescent bis-benzimide dye that binds to A-T rich regions in the minor groove of DNA. Hoechst staining also allows for detection of chromatin condensation which was utilized in paper II. Under the assumption that neurons do not proliferate, chromatin condensation was used as a measure of neuronal stress and eminent loss.

Immunocyto- and histochemistry

Visualization and quantification of tissues or cultures are essential in understanding both functional and morphological alterations between the “normal” and the altered or pathologic situation. To prevent autolysis and structural decomposition cells need to be fixated. This process involves cross-linking of proteins and hampering of enzymatic degradation and removal of water to further denaturate cellular proteins. Throughout the thesis cells and tissues have been fixated in Stefanini’s solution, the combined presence of picric acid and formaldehyde allow for quick and efficient fixation of cells and preservation of antigenicity. To optimize antibody penetration fixed cultures and tissues were frozen and thawed prior to staining.

Toluidine blue staining was used in paper I, II and III to visualize mast cells and general tissue morphology. Toluidine blue is an acidophilic metachromatic dye, meaning it stains acidic tissue components such as sulfates and nucleic acids. Metachromasia is attributed to stacking of dyes (di- to polymers) to areas in proteins with high density of anionic groups, causing a so called hypsochromic shift and a change in emitted color from blue to red. In mast cells glycosaminoglycans such as heparin and histamin cause metachromasia characterized by a purple color. [108]

In paper II and III BODIPY 493/503 staining was used to visualize lipid droplet accumulation in cultures and tissues. BODIPY (boron-dipyrromethene) fluorophores are versatile due to their high fluorescent yield, solubility and intense absorption profile.[109] The BODIPY493/503 dye is a nonpolar derivative that specifically stains neutral lipids, with a precision surpassing oil red O and Nile red. [110, 111]

The majority of results in the thesis have been accumulated with the use of immunocytochemistry. Primary antibodies directed against particular molecular or protein targets were visualized using indirect detection techniques. Both monoclonal and polyclonal antibodies were used. Monoclonal antibodies are produced from one immune cell using hybridoma cell lines. This gives rise to immunoglobins recognizing a single epitope. Polyclonal antibodies arise from several immune cells and represent a collection of immunoglobins recognizing several epitopes. All antibodies have been tested for cross-reactivity in the lab using, when possible, absorption controls. If antigens for such controls were unavailable positive tissue controls as well as omission controls were performed.

Detection of primary antibodies have been achieved with a range of techniques, the main method being Dy-light® or Alexa Fluor® fluorophore conjugated secondary antibodies raised against the primary immunoglobulin. Both fluorophores exhibit enhanced photo-stability and brighter emission over standard dyes with similar emission properties. In paper II and III the avidin-biotin complex (ABC) is used together with 3,3'-diaminobenzidine (DAB) substrate reaction to detect biotin

labeled primary antibodies in tissues. In paper III an Alexa Fluor® conjugated streptavidin is used to detect biotin labeled primary antibodies.

Statistics

Data presented throughout this thesis are in mean and standard error of mean (SEM). Statistical analyses on tissues from *in vivo* experiments are done using two way analysis of variance (ANOVA) followed by Bonferoni post hoc test. The reason for using a two way ANOVA is the presence of more than one independent factor for each tissue, such as diet and genotype. Within each of the factors several observations exists. Bonferoni test was chosen to correct for multiple comparisons as the number of comparisons for each experiment are low and towards a defined control.

For *in vitro* experiments one way ANOVA followed by Dunnet's post hoc test against control have been performed. The reason for using one way ANOVA is that samples are always compared to either control run in parallel or as compared to total number of one cell type. Dunnet's test was chosen to correct for multiple comparisons towards a defined control.

Results and discussion

In this chapter individual findings and discussions from the papers included in this thesis is presented.

Glucagon-like peptide 1 and 2 and vasoactive intestinal peptide are neuroprotective on cultured and mast cell co-cultured rat myenteric neurons (paper I)

GI symptoms are prevalent in many conditions such as T2D and intestinal disorders.[103, 104] Intestinal L-cells react to luminal content with release of GLP-1 and 2. GLP-1 has received much attention due to its incretin and satiety effects.[47, 112] GLP-2, released together with GLP-1 in a 1:1 manner, has shown beneficial effects in the intestine, reducing inflammation and improving barrier function.[47, 113, 114] VIP has neuroprotective,[115] and immuno-supportive properties.[68, 116] Paper I explores the effects on neuronal survival of GLP-1 and 2 and VIP on cultured enteric neurons and the possible receptors involved.

Neuroprotective effects of GLP-1, 2 and VIP

All peptides tested enhanced survival of cultured myenteric neurons. All peptides were also found to protect against mast cell induced neuronal loss.

GLP-1 and VIP receptors involved in the neuroprotection

Receptor antagonists selective for GLP-1 or VIP receptors were used to test receptor interactions. GLP-1 mediated myenteric neuroprotection, with or without mast cell challenge, was blocked with any of the two receptor antagonists while GLP-2 mediated protection was not. VIP mediated myenteric neuroprotection, with or without mast cell challenge, could only be blocked by the selective VIP receptor antagonist.

Discussion and conclusions

That VIP is neuroprotective on cultured and mast cell co-cultured myenteric neurons has previously been shown.[115, 117] The VIP receptor antagonist used is a broad spectrum antagonist selective for VIP (VPAC) and PACAP type 1 (PAC₁) receptors.[118] Present results suggest that it also recognizes the “VIP specific receptor” present on enteric neurons.[119] The finding that GLP-1-induced neuroprotection could be reversed using the VIP receptor antagonist was unexpected. Suggesting that GLP-1 receptors located on VIP containing neurons release VIP upon activation thereby mediating neuroprotection. GLP-2 receptor activation and consequent VIP release have previously been highlighted in several models of intestinal inflammation and gastric relaxation.[53, 57] GLP-2 is further shown to regulate the expression of VIP in enteric neurons.[120, 121] However in the current model GLP-2 mediated neuroprotection was not mediated through VIP release or VIP receptor activation.

Collectively, the results highlight the positive effects GLP-1, 2 and VIP have on survival of enteric neurons. Therapies using these peptides may help alleviate some of the enteric neuropathies present in patients.

Enteric Neuropathy can be induced by high fat diet in vivo and palmitic acid exposure in vitro (paper II)

GI symptoms are prevalent in overweight and T2D.[103, 104] Overweight is the largest risk factor in developing T2D and patients often present with peripheral neuropathies.[122, 123] Neuropathy in ENS has been scarcely studied in diet-induced-obesity models or models of T2D. Metabolomic profiles of plasma lipid levels of overweight and type 2-diabetic patients reveal elevated circulating levels of the saturated fatty acid palmitic acid (PA) compared to lean controls.[124, 125] Paper II investigated the effect long term intake of a HFD has on ENS and the effects of lipid exposures on cultured myenteric neurons.

HFD induces enteric neuronal loss

After 6 months on either HFD or ND, the numbers of myenteric neurons in ileum and colon were reduced in response to HFD. In ileum mucosal thinning was observed in HFD fed mice, otherwise no intestinal structural changes were observed. Bodipy® staining revealed a lipid droplet accumulation in muscularis propria in mice on HFD.

In vitro lipid exposure

Exposing primary cultures of intestinal smooth muscle cells, glia and myenteric neurons from rat small intestine to PA caused a significant and concentration dependent loss of neurons and smooth muscle cells. Glia did not alter their survival rate but changed their morphology from a stellate to a rounded shape. PA also caused a concentration dependent increase in neuronal chromatin condensation. Oleic acid exposure of cultures caused an improved survival of neurons, but a loss of smooth muscle cells. Glia remained unchanged. Both lipids *per se* caused reduced cellular proliferation, which could be reversed by co-exposure of both lipids. Co-exposure of both lipids led to improved survival of smooth muscle cells and an intramuscular lipid droplet accumulation.

Pharmacological interventions

Possible mechanisms behind PA-induced neuronal loss were investigated using selected agonists and antagonists of key metabolic pathways described in table 1. PA-induced neuronal loss could be prevented by inhibition of CPT1 and supplementation with α lipoic acid (ALA) or L-carnitine. None of these pharmacological manipulations had any effect on smooth muscle cell survival, as noted in table 1.

Table 1 Brief overview of pharmacological substances, their targets and effects on PA-induced neuronal and smooth muscle cell losses as well as on PA-exposed glia. To facilitate comparisons, the effect of PA treatment in highlighted in bold.

Agents / Function	Effects on PA exposed cultures		
	N	SMC	G
PA / saturated fatty acid	-	-	+
Etomoxir / CPT1 inhibitor, inhibit beta oxidation	+	-	-
L-Carnitine / Acyl and acetyl carrier	+	-	-
ALA / Enzymatic co-factor, antioxidant	+	-	-
Myriocin / SPT inhibitor, inhibits ceramide formation	-	-	-
Catalase / Antioxidative enzyme	-	-	-
L-NAME / NO synthetase inhibitor	-	-	-
4-PBA / Chemical chaperone	-	-	-
Ketamine / NMDA receptor inhibitor	-	-	-

N, neurons; SMC, smooth muscle cells; G, glia; SPT, serine palmitoyl transferase; L-NAME, N-nitro-L-arginine methyl ester; 4PBA, 4-phenyl-butyric-acid. + denotes rescue, - denotes no changes in survival as compared to PA treatment

Discussion and conclusions

Long term intake of HFD causes loss of myenteric neurons in ileum and colon, this effect can be mimicked by PA *in vitro*. The mechanism behind the neuronal loss is multifaceted but is suggested to involve L-carnitine depletion and palmitoylcarnitine formation. Culminating in deranged energy metabolism, reduced acetylcholine synthesis, ER membrane deterioration and oxidative stress. A suggested model is presented in figure 2. The lipid-induced loss of smooth muscle is suggested to be due to an unbalanced lipid metabolism, as co-exposure of both lipids induced survival and extensive intramuscular droplet formation. The latter likely serving as a lipid reservoir, halting lipid metabolism.

Collectively, the data shows that long term intake of HFD is detrimental to myenteric neurons and suggests PA as a crucial factor in mediating this effect.

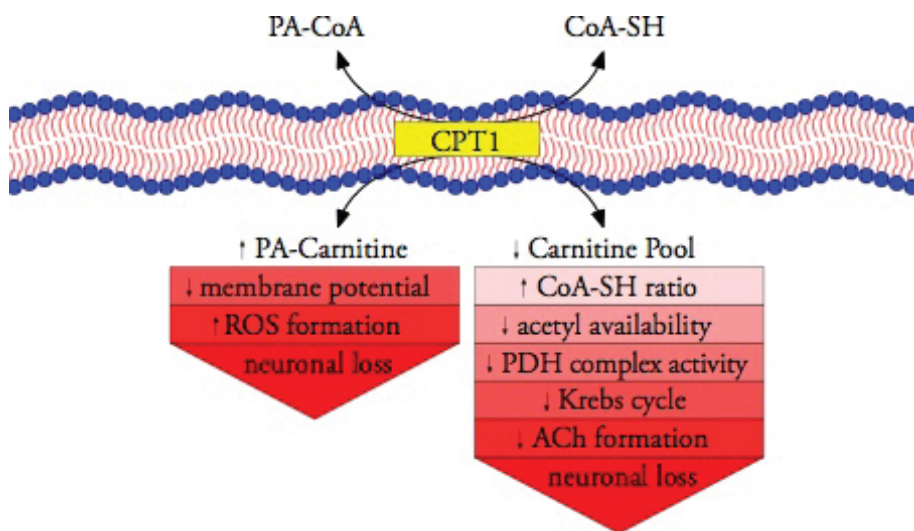


Figure 2 Suggested model for the metabolic energy derangements increased PA exposure causes on cultured enteric neurons. The formation of the metabolically active palmitoylcarnitine and resulting depletion of the carnitine pool, causes strain on neurons. ROS, reactive oxygen species; PDH, pyruvate dehydrogenase complex; Ach, acetylcholine

The enteric nervous system of P2Y₁₃ receptor null mice is resistant against high fat diet- and palmitic acid-induced neuronal loss (paper III)

Knowledge on purinergic signaling has evolved extensively over the last 40 years. ATP was the first described purinergic signaling molecule. In ENS ATP is suggested to be a co-transmitter of NO and VIP in the inhibitory motor response.[83] In addition to motility purines and their receptors are involved in intestinal secretion.[126] P2Y₁₃ is an adenosine diphosphate (ADP) activated G protein coupled receptor that has been highlighted in lipid metabolism,[127-129] inflammatory pain and axonal elongation.[130, 131] Paper III investigated if P2Y₁₃ is involved in HFD- and PA-induced neuronal loss and if neuronal expression of VIP and nNOS is altered.

HFD causes no neuronal loss in P2Y₁₃^{-/-} mice

P2Y₁₃^{+/+} and P2Y₁₃^{-/-} littermates fed either ND or HFD for 6 months were studied. The previously described HFD-induced neuronal loss was confirmed in P2Y₁₃^{+/+} mice. In contrast P2Y₁₃^{-/-} mice had no HFD-induced loss. Notable was that P2Y₁₃^{-/-} mice, in colon displayed increased numbers of myenteric neurons and reduced number of VIP expressing submucous neurons, irrespective of diet. No change in the relative numbers of neurons expressing VIP and nNOS was observed in response to HFD. Regardless of genotype HFD caused a thinning of the ileal mucosa.

Palmitic acid causes no loss of cultured P2Y₁₃^{-/-} neurons

Enteric neuronal cultures derived from ND fed P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice were used *in vitro*. PA-induced neuronal loss was evident in P2Y₁₃^{+/+} derived cultures. Such loss was prevented by the addition of the selective P2Y₁₃ receptor antagonist MRS2211. Cultures derived from P2Y₁₃^{-/-} mice were resistant to PA treatment. In cultures the relative number of VIP expressing neurons was similar regardless of genotype. At the highest concentration of PA tested both genotypes displayed a reduced number of VIP expressing neurons. This could in P2Y₁₃^{+/+} derived cultures not be reversed by the addition of MRS2211. The P2Y₁₃ receptor agonist 2meSADP was applied to investigate possible effects of P2Y₁₃ receptor activation. Surprisingly the agonist did no induce any change in neuronal survival regardless of if derived from P2Y₁₃^{+/+} or P2Y₁₃^{-/-} mice.

Discussion and conclusions

Mice lacking the P2Y₁₃ receptor were resistant to HFD-induced neuronal loss *in vivo* and to PA-induced neuronal loss *in vitro*. Previous studies suggest the PA-induced loss partly to be mediated through L-carnitine depletion and palmitoylcarnitine formation. Paper III further suggests activation of P2Y₁₃ receptors in mediating the response. This since cultured neurons treated with P2Y₁₃ antagonist and neurons derived from mice lacking this receptor are resistant to PA-induced loss. The mechanisms behind are enigmatic, as exposure of the selective agonist had no effect on neuronal survival. Figure 3 outline possible downstream pathways possibly involved in mediating the P2Y₁₃ response. Complex links are suggested to exist between P2Y₁₃ receptor activation and intracellular lipid- and energy metabolism and/or neuronal trans-membrane lipid transport.

Collectively, the data highlight the need of more investigations to understand the complex role of purinergic signaling in ENS. Also the roles purinergic signaling plays in neuronal and intestinal homeostasis and maintenance need further investigations. The data place the P2Y₁₃ receptor in a central position in mediating HFD- and lipid-induced neuropathies, probably present in many obese and type 2-diabetic patients.

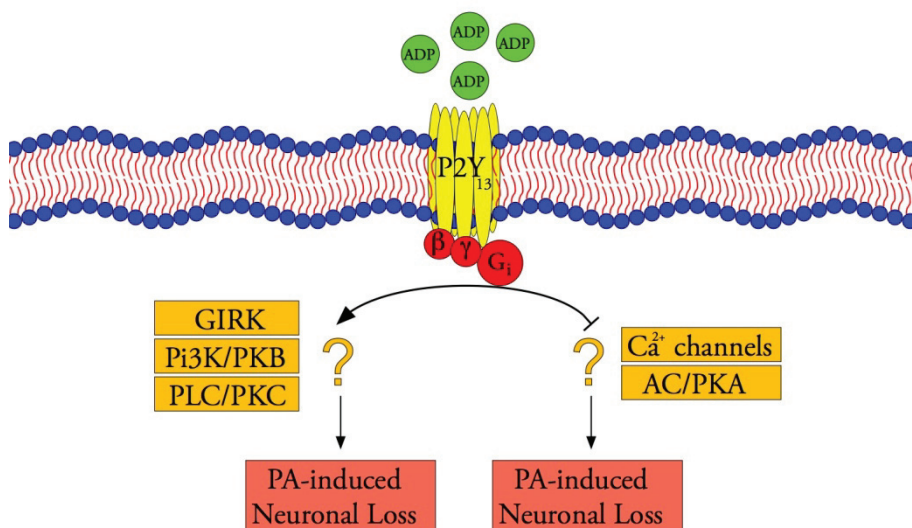


Figure 3 Possible downstream pathways involved in P2Y₁₃ receptor mediated responses. P2Y₁₃ null mice or inhibition of the receptor leads to protection against HFD- and PA-induced neuronal loss..P2Y₁₃ receptor activation has the possibility to initialize a range of downstream pathways.

Lipopolysaccharide-induced loss of cultured rat myenteric neurons - role of AMP activated kinase (paper IV)

The GI tract is the body's largest surface to the outside environment. It is vital for nutrient uptake and contains the human microbiome.[62, 132] A compromised intestinal barrier function leads to increased plasma levels of LPS.[58, 133-135] LPS is a membrane component of gram negative bacteria known to bind to TLR4 and mediate an inflammatory response.[134, 136] Plasma LPS levels are elevated in many diseases including overweight and T2D.[58, 135] Paper IV investigates underlying mechanisms causing the previously described LPS-induced loss of cultured myenteric neurons.

LPS exposure and AMPK activation both cause neuronal loss

Both LPS exposure and AMPK activation induce neuronal loss of cultured myenteric neurons. Two AMPK activators with different molecular targets, AICAR and A-769662, were used and both induced loss of cultured myenteric neurons from rat small intestine. LPS and AICAR both increased the relative numbers of cultured neurons expressing VIP.

AMPK inhibition prevents LPS induced neuronal loss

The selective AMPK antagonist compound C prevented both LPS and AICAR induced losses of cultured myenteric neurons. It also reduced the LPS- and AICAR-induced increase in relative numbers of neurons expressing VIP to control levels. Compound C exposure *per se* displayed a biphasic dose-response curve; low concentrations resulted in neuroprotection and high concentrations in neuronal loss. The concentration used in combination with LPS and AMPK agonists did not affect neuronal survival or relative number of neurons expressing VIP. Compound C did not reverse the selective AMPK activator A-769662 induced neuronal loss.

Discussion and conclusions

LPS is previously shown to induce enteric neuronal loss *in vitro*, and TLR4 receptors are identified on a subgroup of myenteric neurons.[137, 138] Exogenous VIP was in those studies able to protect against LPS-induced neuronal loss.[138] Current results suggest AMPK activation to mediate LPS-induced neuronal loss. *In vitro* LPS has, in low concentrations, been shown to promote enteric neuronal survival in

prenatals.[139] TLR4 may directly modulate ENS, as mice with low TLR4 activity or inhibited down-stream signaling display delayed colonic transit and reduced numbers of nitrergic neurons.[139, 140] In the healthy gut, intestinal permeability and LPS levels are low; under such conditions LPS may positively regulate enteric neuronal responses. In conditions where the intestinal permeability is altered LPS levels are increased, causing a dysregulation of the normal response leading to neuronal loss. The increase in relative neuronal VIP expression may in such cases be a part of the neuron's protective response, as VIP has been ascribed roles in neuroprotection and immuno- and tight junction modulation.[68, 115, 141, 142]

Collectively, the data show that LPS induces enteric neuronal loss through a mechanism involving AMPK activation and up-regulation of VIP expression. Figure 4 outline the current working model. The purposes of the responses triggered by LPS are suggested to be activation of the neurons adaptive innate-immune response to commensal intestinal bacteria and neuroprotection.

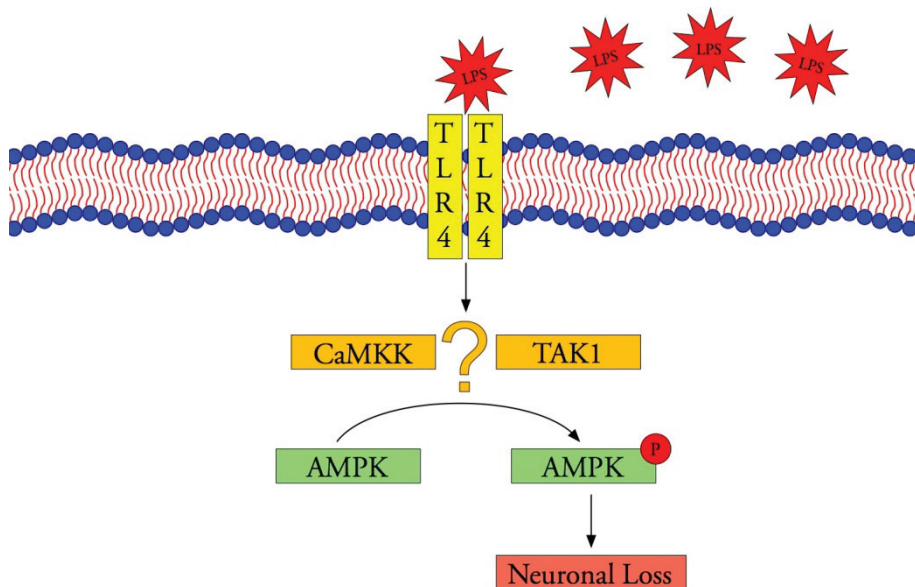


Figure 4 Overview of possible downstream targets of TLR4, causing AMPK activation.

General discussion

Do enteric neurons adapt to survive in conditions of metabolic imbalance or do they die? These were the questions asked and explored in this thesis. Metabolic imbalances selected relate to obesity and obesity-induced T2D. GI dysmotility and dysfunction are part of many GI diseases and are common complications of obesity and T2D. Enteric neuropathy probably plays a significant role in the development of symptoms experienced by patients. ENS has significant impact on gut maintenance, motility, secretion and intestinal blood flow. Imbalances in neuronal reflex activity affect the entire gut homeostasis.

From a GI point of view lipids are a double edged sword, on one side mediating satiety through release of gut hormones like cocaine and amphetamine regulated transcript (CART), cholecystokinin (CCK) and GLP-1.[143, 144] On the other hand long term intake of a HFD decreases lipid sensitivity in the digestive tract, causing increased food intake.[144, 145] Interestingly obesity and obesity-induced diabetes have lower plasma concentrations of GLP-1.[145, 146] As GLP-1 and 2 are released in a 1:1 manner, it can be predicted that GLP-2 is reduced as well. Both GLP-1 and 2 interact with the ENS, GLP-1 slow gastric motility through NO release and GLP-2 mediate cytoprotective effects via VIP up-regulation and release.[50, 53-57] In paper I a strong neuroprotective potential of GLP-1 and 2 was shown, the protective potential persisted even when challenged by mast cells. Unpublished data show that the neuroprotective potential of GLP-1 and 2 does not extend to PA-induced loss.

Increased intake of lipids has been suggested to push the microbiome towards an obesity-associated composition, with enrichments of LPS producing bacteria.[147] LPS translocation from the gut lumen to the circulation is increased in obesity and T2D.[148] LPS, through binding to its receptor TLR4, initiates an immune response and releases pro-inflammatory cytokines.[58, 147, 149] Targeted deletion of TLR4 in ENS leads to reduced motility and loss of NO producing neurons, suggesting LPS is able to directly modulate enteric motility.[139, 140] In paper IV LPS is shown to mediate neuronal loss through AMPK activation and increase the number of VIP expressing neurons. Neuropeptides released from ENS are able to modulate the immune response in the gut in a way that goes beyond secretion and power peristalsis. VIP not only fights pathogens by promoting secretion it also has anti-inflammatory properties. Its anti-inflammatory effects are mediated via different mechanisms including down regulation of pro-inflammatory cytokines, down regulation of TLR4

receptor complex and reducing intestinal barrier permeability. [116, 141, 150] In paper IV LPS and AMPK cause an up-regulation of VIP expressing neurons and paper I showed GLP-1, but not GLP-2, mediated neuroprotection to be mediated through VIP receptor activation. Interestingly mice fed HFD or P2Y₁₃ null mice did not show altered numbers of VIP containing neurons. Unpublished *in vitro* results even show PA to reduce the relative number of VIP-containing neurons while GLP-1 or 2 does not to alter VIP expression.

Ingested fat is packed into chylomicrons and transported via the lymph, from the intestine to the liver. Chylomicrons and very low density lipoproteins (VLDL) deliver lipids to target tissues for storage and energy use. The lipids are taken up by cells; however this is not 100%, the spillover of FFA represent one fraction of circulating FFA.[101] Both obesity and T2D have altered lipid metabolism causing dyslipidemia. Increased circulating levels of FFA are a recognized feature of obesity and obesity-induced T2D due to increased release from adipose tissue and decreased clearance from plasma.[101, 124, 125] In paper II and III HFD is shown to cause loss of myenteric neurons in ileum and colon and thinning of ileal mucosa. The neuronal loss was mimicked by PA *in vitro*. The underlying mechanisms are suggested to involve a deranged energy metabolism and the purinergic receptor P2Y₁₃.

Purines were first established as neurotransmitters in NANC neurons in the gut, and ATP is an important inhibitory neurotransmitter. Purinergic signaling is in the gut involved in mechanosensory transduction, chloride, potassium, mucous and acid secretion as well as glia-neuron communication.[42, 126, 151] With its complex receptor network and diverse roles in gut homeostasis it is not hard to imagine an imbalanced purine response to cause intestinal dysfunction. Purinergic signaling is implicated in mediating intestinal inflammation, pain and delay of colonic transit in postoperative ileus.[126, 152, 153]The potential of purinergic signaling is underlined in paper III, where P2Y₁₃ receptor inhibition show a remarkable protective potential on HFD- and PA-induced neuronal losses.

Results from the diet-induced-obesity and obesity related metabolic imbalances investigated in the papers suggest GI symptoms to originate in a metabolic “trifecta of bad”. Findings from the *in vivo* model not only reveal the effect of lipids on ENS, but also the effects HFD have on the entire body, including HFD-induced alterations in hormone profiles, barrier permeability and lipid metabolism. Prolonged intake of HFD reduces the circulating levels of GLP-1 and 2. This may reduce the beneficial effect of GLP hormones on satiety, mucosal growth, permeability as well as on ENS protection and motility. HFD was shown to reduce ileal mucosa height; this may be an effect of reduced GLP-2 levels and lipid exposure.[154] Prolonged intake of HFD may also push the microbiome in an LPS producing direction. With HFD not increasing VIP levels while supposedly causing a decrease in GLP-2 levels, the barrier may not withstand the increased LPS load, resulting in an increased translocation of

LPS and initiation of an immune response. LPS-induced immune response on ENS includes activation of AMPK and an up-regulation of neurons expressing VIP. In addition to the neuroprotection elicited by VIP its up-regulation may be an attempt to improve permeability and luminal secretion, to disrupt the harmful cycle initiated. AMPK is situated at metabolic crossroads and is able to mediate a range of positive effects in metabolically challenged tissues. The AMPK activation duration, however seems to be essential in keeping metabolic balance and neuronal survival.[155, 156] In this respect it is noticeable that a common side effect of metformin, a T2D drug and an AMPK activator, is GI symptoms such as diarrhea and abdominal pain.[157, 158] Prolonged intake of HFD, besides causing hormone and barrier alterations, increases levels of circulating FFA. It is debated if the increased plasma levels of FFA in obesity and T2D are a culprit in disease development, as lipids per kg adipose tissues does not correlate to insulin resistance.[159] However metabolomic profiling of serum from obese and T2D patients and from animal models show PA levels to be increased compared to lean or controls.[124, 125] PA induces neuronal loss and reduces VIP and nNOS levels *in vitro*. It is intriguing to speculate that it might not be the actual concentration of circulating FFA but the composition of FFA that causes the adverse effects in states of hyperlipidemia. The role of P2Y₁₃ needs to be further investigated; however ATP release in the intestine is likely altered in the obese or type 2-diabetic state as has been hinted at in type 1-diabetes.[160, 161] This alteration may play an important role in mediating pain and pathological responses.

Collectively the neuronal loss observed in HFD may be a culmination of increased PA- and LPS-exposure and decreased GLP levels, all causing dysregulation of enteric neurons and eminent dysfunction. In patients each component can be differently represented, thereby explaining various degrees of dysfunction and symptoms.

Conclusions

Collectively results presented in this thesis suggest that

- Long term feeding with HFD causes a significant loss of myenteric neurons in ileum and colon
- PA exposure *in vitro* mimics the HFD-induced neuronal loss. PA-induced neuropathy is caused by a multifaceted derangement of energy metabolism. The formation of palmitoylcarnitine and depletion of the carnitine pool appear to be central.
- GLP-1 and 2 are neuroprotective *in vitro*, also when neuronal survival is challenged by mast cells. Neuroprotective effects of GLP-1 but not GLP-2 are suggested to be mediated through VIP receptor activation.
- LPS exposure causes enteric neuronal loss *in vitro*, through activation of AMPK
- Purinergic signaling through P2Y₁₃ receptors has emerged as an interesting target, involved in mediating HFD- and PA-induced neuronal loss.
- The HFD-induced neuronal loss is suggested to be a culmination of a range of metabolic imbalances including, increased circulating levels of PA, reduced GLP mediated neuroprotection and increased LPS translocation. Culminating in enteric neuronal loss, dysregulation and eminent dysfunction,

Future perspectives

After several investigations to answer the question “do enteric neurons adapt to survive in conditions of metabolic imbalance or do they die?” we are only a little closer to the answer. Though results suggest metabolic imbalance cause a multifaceted strain on the neurons, leading to neuronal loss, the questions remain. The time course, underlying signals and how different factors interact leading to neuronal loss are unanswered. Future studies will aim to answer some of these questions.

HFD-induced neuronal loss

To further the investigations into HFD and enteric neuronal loss, an improved characterization of the model should be established. Functional studies investigating motility, as well as serum analyses for LPS and GLP levels would be an initial step. Characterization of neuronal subpopulations and neuroplastic alterations should also be further explored. Besides the diet-induced-obesity model used in the thesis several well described animal models of T2D are available, where animals display other aspects of the disease. Mice in our studies did not become hyperglycemic or insulin resistant (unpublished observations). Exploring the effects progression from a normo- to a hyperglycemic and insulin resistant state has on ENS would be of interest, to elucidate if there are differences between obesity-dependent and -independent type 2-diabetic changes.

Further characterizations of the role of various lipids in the induction of neuronal loss will also be interesting to further explore. Exposing neurons *in vitro* to lipid-profiles resembling that of serum in various metabolic diseases would be a first step, to investigate the levels and compositions of FFA harmful to enteric neurons.

The connection and interplay between LPS and AMPK is also an interesting avenue to further investigate with identification of upstream activation kinases as the first step. Initial data inhibiting CaMKK suggests LPS not to induce AMPK activation via CaMKK. Next target will be TAK1 known to be a downstream kinase of TLR4 activation and upstream of AMPK. Testing if metformin mimics the AMPK

activators and causes enteric neuronal loss would be interesting to investigate *in vitro* and *in vivo*.

P2Y₁₃ in the ENS

The intriguing findings that P2Y₁₃ null mice or addition of a P2Y₁₃ antagonist renders enteric neurons resistant to PA-induced neuronal loss call for further investigations. Studies pinpointing neuronal subpopulations expressing P2Y₁₃ receptors as well as general intestinal distribution and localization of the receptor need to be performed. Functional studies investigating if P2Y₁₃ activation alters gut motility would be interesting as well. As receptor activation studies using the agonist 2meSADP have yielded little response on neuronal loss, further studies investigating activation and possible links to PA-induced neuronal loss needs to be performed. It would be interesting to test if P2Y₁₃ receptors are involved or connected to other pathways like those leading to LPS- or mast cell-induced neuronal loss.

PKC ϵ a possible link between metabolic imbalance-induced neuronal loss and P2Y₁₃

An interesting and not previously described avenue to explore is the involvement of PKC in particular PKC ϵ , in the HFD- and P2Y₁₃ receptor activation-induced effects on ENS. PKC ϵ belongs to the PKC superfamily. PKC ϵ is dependent on DAG for activation, however it has been suggested that Pi3K indirectly through phosphoinositide dependent kinase (PDK1) is able to activate PKC ϵ . [162] The reason for the interest is that several PKC inhibitors indicate PKC ϵ activation to mediate both LPS- and PA-induced neuronal loss (unpublished data). Unpublished data further suggests that Pi3K is involved in LPS-induced neuronal loss. This since inhibition of Pi3K protects against both LPS- and AMPK activation-induced neuronal loss. The effect of Pi3K on PA-induced neuronal loss has not been tested, yet.

PKC ϵ is expressed in e.g. CNS, liver, beta cells and enteric neurons. [163-166] In central neurons PKC ϵ activation is suggested to mediate neurite outgrowth, to modulate long term potentiation and inhibit sodium currents. [162, 164] PKC ϵ is also involved in mediating pain from peripheral sensory neurons, and modulates pain sensation through phosphorylation of vanilloid receptor 1 (TRPV1). [164] It is suggested that the transition from acute to chronic pain induced by prostaglandin E₂ (PGE₂) is by switching from G_s/PKA to G_{i/o}/PKC ϵ signaling. [167] A similar switch to

G coupled PKC signaling has been suggested in opioid induced hyperalgesia.[168] Also the P2Y₁₃ receptor has been implicated in inflammatory and neuropathic pain, P2Y₁₃ receptor mRNA increases 3-10 day after inflammatory and neuronal injurious events in spinal cord neurons and microglia.[130, 169] Further in response to peripheral nerve injury, P2Y₁₃ inhibition suppresses neuropathic pain.[169]

An interesting link between PKC ϵ and inflammatory responses is the findings that PKC ϵ activation enhances LPS induced inflammation. PKC ϵ is an upstream kinase of the TLR4 adaptor molecule TRIF-related adaptor molecule (TRAM).[162, 170] It could be speculated that LPS-induced enteric neuronal loss utilizes PKC ϵ to modulate the TLR4 response. In this regard it would be of interest to test if P2Y₁₃ inhibition protects neurons against LPS-induced loss.

In mouse embryonic fibroblast and HepG2 cells PA has been shown to induce autophagy in a PKC dependent manner.[171]. It is suggested that PA exposure increases DAG content and PKC signaling, as the enzyme converting DAG to TAG prefer 18:1 fatty acids over 16:0 fatty acids. Inhibition of PKC blocks PA-induced autophagy.[171, 172] It remains to be investigated if this happens in neurons and if PKC ϵ is involved. PKC ϵ has also been implicated in beta cell lipotoxicity. Genetic knock out or inhibition of PKC ϵ prevents HFD-induced glucose intolerance, through increased insulin release, decreased hepatic clearance and increased incorporation of PA into TAG.[163, 173-175] An interesting connection to P2Y₁₃ is the role the receptor plays in insulin release and lipid metabolism. Inhibition of P2Y₁₃, like PKC ϵ , causes increased insulin secretion.[176] Reverse cholesterol transport and high density lipoprotein (HDL) internalization are reduced by P2Y₁₃ inhibition.[127, 128] Though PKC ϵ has not been directly investigated several data suggest a role for PKC activation in the expression and translocation of the lipid transporter CD36.[177]

Though no clear link has been established between PKC ϵ and the P2Y₁₃ receptor several suggestive findings elude to possible interactions in LPS- and PA-induced neuronal losses. A possible role of PKC ϵ as a key mediator in metabolically induced enteric neuronal plasticity or neuropathy is thus highly suspected.

Acknowledgements

I wish to thank all the people who have been involved in shaping the path that has led to here. However I doubt I am able to remember, let alone recognize all the little and big contributions. So as they say, “no one mentioned, no one forgotten”.

To begin with I have to thank my supervisor for introducing me to the **most amazing research field in the world!** I also have to thank her for her patience with my at times somewhat impulsive ideas. I am sure it has been a battle to keep everything somewhat on track. But what can you do, there are so many ideas out there that often seem more exciting than accumulating “n’s”.

I have to thank my co-supervisors, collaborators and members of the lab. Without your help this thesis would never have been completed. Also an extra thanks to my lab members, and the “Friday-breakfast-club” for making going to work enjoyable every day and extra special on Fridays. I have enjoyed going to work not only because I like my research, but because B11 and C11 are filled with wonderful and knowledgeable people who make smiling easy.

I have to send my gratitude to Lund University for supporting student influence at all levels. It has given me the opportunity to get to know the people leading the university and especially the Faculty of Medicine. I have enjoyed every minute and people have smiled back at me, I hope because they liked what I said. However there is a chance my Danish-Swedish mix may have prompted a “just smile and wave,” response. I also have to thank the members of MDR, not just for the work they have done but also for eating my meeting popcorn with a smile several years in a row while other student councils have enjoyed lunches and dinners.

I have to thank all the people who have made sure cake and goodies have been available at meetings, seminars and afternoon tea. They have made sure I never had to settle with just one piece and at times even providing a box so I could stock up for later.

I have to thank all of my friends old and new for being a colorful and exciting collection of people that make everything just a little better and brighter. I value all the fun, serious, crazy and dangerous moments we have had. Even the toe-curling ones, where I have woken up hoping it was just dream.

I have to thank my family, no words truly describe how much they all mean to me. They have shaped, helped and supported me. Made me laugh and picked me up when I was sad. They have given me the sense of right and wrong and to always believe the best in people. I will have them in my heart, always. Last but absolutely not least I have to thank my husband. He has supported my dreams without judgment and encourages me to be the best version of me that I can. He pulls me away from the hustle and bustle of life into the wild to recharge and enjoy the wonders of nature. Something I am truly grateful for.

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