The chemokine CCL5, a key regulator of neuroinflammation and type 2 diabetes associated with diet-induced obesity

Katharina Stobbe

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UNIVERSITÉ CÔTE D'AZUR

ÉCOLE DOCTORALE
SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT

La chimiokine CCL5, un régulateur clé de la neuroinflammation et du diabète de type 2 associé à l'obésité nutritionnelle

Katharina STOBBE

Institut de Pharmacologie Moléculaire et Cellulaire

Présentée en vue de l'obtention du grade de docteur en Sciences de la Vie et de la Santé
d'Université Côte d'Azur

Dirigée par : Dr Carole Rovère

Soutenue le : 31 Octobre 2019

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UNIVERSITÉ CÔTE D'AZUR ACADÉMIE D'EXCELLENCE COMPLEXITÉ ET DIVERSITÉ DU VIVANT

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UNIVERSITÉ CÔTE D'AZUR ACADÉMIE D'EXCELLENCE ESPACE, ENVIRONNEMENT, RISQUES ET RÉSILIENCE

La chimiokine CCL5, un régulateur clé de la neuroinflammation et du diabète de type 2 associé à l'obésité nutritionnelle

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Résumé

Le mode de vie occidental favorise le développement de l'obésité et du diabète de type 2. Le lien entre l'obésité et le diabète de type 2 est bien établi au niveau épidémiologique. Toutefois, ce lien reste encore mal défini au niveau étiologique. Les marqueurs du diabète de type 2, tels que l'hyperglycémie et la résistance à l'insuline et à la leptine, peuvent résulter d'un état pro-inflammatoire chronique du tissu adipeux, associé à une sécrétion de cytokines (IL-1β, IL-6, TNF-α) et chimiokines (RANTES/CCL5, MCP-1/CCL2) diabétogènes. Ces médiateurs pro-inflammatoires sécrétés seraient les promoteurs de l'inflammation systémique et de la neuroinflammation dans l'hypothalamus, région importante du cerveau, qui contient des réseaux neuronaux impliqués dans le contrôle du métabolisme énergétique et du comportement alimentaire.

Nous nous sommes intéressés à l'impact de régimes riches en lipides sur le développement de l'obésité nutritionnelle et les paramètres métaboliques associés, d'autre part sur la réponse inflammatoire dans l'hypothalamus et le tissu adipeux. Nous nous sommes focalisés sur le rôle de la chimiokine CCL5 et de son récepteur CCR5. Notre hypothèse est que la chimiokine CCL5 régule l'activité des neurones de l'hypothalamus impliqués dans le contrôle de l'homéostasie énergétique et de l'homéostasie glucidique. Nous proposons d'étudier le rôle de la chimiokine CCL5 dans le contrôle de la balance énergétique, l'obésité, le diabète de type 2 et la neuropathie périphérique diabétique, une des complications diabétiques les plus fréquentes, affectant jusqu'à la moitié des patients atteints de diabète de type 2.

Dans ma thèse, nous avons testé les effets à long terme (16 semaines) du régime hyperlipidique apportant 40% de lipides, comparé au régime standard (contenant 5% de lipides), sur le développement de l'obésité chez les souris invalidées pour le gène de CCL5 (souris CCL5$^{-/-}$) ou le gène de son récepteur CCR5 (CCR5$^{-/-}$) en comparaison aux souris sauvages (contrôles). Dans un premier temps, nous avons confirmé la présence de CCL5 et de son récepteur CCR5 dans l'hypothalamus par hybridation *in situ* (technologie RNAScope®) chez les souris contrôles. Nous avons alors montré que les souris CCL5$^{-/-}$ et CCR5$^{-/-}$ semblent plus résistantes à l'obésité et avoir une homéostasie du glucose moins perturbée que les souris sauvages. Pour évaluer l'implication du CCL5 dans la douleur neuropathique associée au diabète, la sensibilité à la douleur thermique des souris CCL5$^{-/-}$ et CCR5$^{-/-}$ a été mesurée chez chaque groupe de souris. Les souris CCL5$^{-/-}$ sous régime hyperlipidique semblent moins sensibles à la douleur thermique que les souris contrôles. De plus, les souris CCL5$^{-/-}$ semblent présenter une expression génique des neuropeptides hypothalamiques modifiée par rapport aux souris contrôles.

Nos résultats suggèrent d'une part que l'absence de CCL5 et de son récepteur CCR5 protège contre le développement de l'obésité et le diabète de type 2, d'autre part que l'absence du CCL5 abolit la sensibilité accrue à la douleur thermique observée sous régime hyperlipidique. Ainsi, la cascade de signalisation CCL5/CCR5 pourrait représenter une nouvelle cible thérapeutique pour lutter contre l'obésité.

Mots clés : Obésité, Comportement alimentaire, Homéostasie glucidique, Régimes hyperlipidiques, Inflammation, Cytokine, Chimiokine, Signalisation de CCL5, Hypothalamus, Neuropeptides.

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Abstract

Obesity is defined by the excessive accumulation of body fat and accompanied by chronic low-grade inflammation of peripheral metabolic tissues, especially of adipose tissue. Adipocytes secrete inflammatory mediators such as cytokines and chemokines, which can act at the cerebral level and modulate neuronal activity. The hypothalamus is an important region of the brain, which contains neural networks involved in the control of energy metabolism and feeding behavior. Emerging evidence indicates that inflammation occurs also at the level of the hypothalamus. Our recent results showed that the chemokines can be involved in the deregulation of energy homeostasis.
CCL5 is a chemoattractant cytokine well known for its role in cerebral and peripheral inflammation. Together with one of its cognate receptors, CCR5, it also contributes to neural function and diseases such as obesity, type 2 diabetes and neuropathic pain.
We were interested in the inflammatory response of the hypothalamus and different adipose tissues to high-fat diet and its role in the development of diet-induced obesity. In particular, we are focusing on the role of the previously identified chemokine CCL5 and its receptor CCR5, in the central inflammation associated with the deregulation of energy metabolism and the pathogenesis of obesity.
In this study, we tested the long-term effects of an obesogenic high-fat or standard diet on the development of obesity in adult $CCL5^{-/-}$, $CCR5^{-/-}$ and wild-type mice. After 16 weeks of feeding, animals were sacrificed and peripheral and cerebral tissues collected. Metabolic parameters, locomotor activity, expression levels of pro-inflammatory mediators and peptides involved in feeding behavior were measured. We discovered that both $CCL5^{-/-}$ and $CCR5^{-/-}$ mice seem to be protected from weight gain and the associated impairment of glucose metabolism compared to WT mice. To evaluate the implication of CCL5 in neuropathic pain associated with diabetes, thermal pain sensitivity of $CCL5^{-/-}$ and $CCR5^{-/-}$ mice was measured in both conditions. Remarkably, in high fat diet condition, $CCL5^{-/-}$ mice displayed higher tolerance to heat pain compared to control mice.
Furthermore, $CCL5^{-/-}$ mice show a different expression pattern of inflammatory markers and hypothalamic neuropeptides compared to control mice. In addition, we used RNA *in situ* hybridization (RNAScope® Technology) to verify the cellular localization of CCL5 and its receptor CCR5 in the hypothalamus of wild-type mice.

Our results indicate that the absence of CCL5 and its receptor CCR5 protects against the development of obesity and type 2 diabetes and the absence of CCL5 abolishes the increased thermal pain sensitivity observed under high fat diet challenge. Thus, the CCL5 signaling cascade could represent a new putative target for the development of therapeutic strategies.

Keywords: Obesity, Feeding behavior, Glucose homeostasis, High fat diet, Inflammation, Cytokine, Chemokine, CCL5 signaling, Hypothalamus, Neuropeptides.

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Specialty: Cellular and Molecular Interactions

Presented by

Katharina Stobbe

THE CHEMOKINE CCL5, A KEY REGULATOR OF NEUROINFLAMMATION AND TYPE 2 DIABETES ASSOCIATED WITH DIET-INDUCED OBESITY

Thesis directed by Dr Carole Rovere

Defended on the 31 October 2019

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List of Publications

Original articles

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2016 Le Thuc O., Cansell C., Bourourou M., Denis R.G.P., **Stobbe K.**, Devaux N., Guyon A., Cazareth J., Heurteaux C., Rostène W., Luquet S., Blondeau N., Nahon JL., Rovère C. Central CCL2 signaling onto MCH neurons mediates metabolic and behavioral adaptation to inflammation. ***EMBO Rep.*** Oct 12. pii: e201541499. **IF 2015/16: 7,739**

Reviews

2019 Le Thuc O., **Stobbe K.**, Rovère C. Inflammation hypothalamique et deregulation de la balance énergétique : rôle des chimiokines. ***La lettre des Neurosciences*** 56, 20-23.

2017 Le Thuc O., **Stobbe K.**, Cansell C., Nahon JL., Blondeau N., Rovère C. Hypothalamic Inflammation and Energy Balance Disruptions: Spotlight on Chemokines. ***Front. Endocrinol.*** doi.org/10.3389/fendo.2017.00197. **IF 2017: 3,675**

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Participation in National and International Conferences

Oral communications

July 2019	**Oral communication** at the 7th International Mediterranean Neuroscience Conference in Marrakech, Morocco.
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May 2018	**Oral communication** and **prize for best oral communication** at the JEDN 2018, Nice, France.
September 2017	**Oral communication** at the 42nd conference of the SNE, Dijon, France.

Poster presentations

July 2018	**Poster presentation** at the 9th International Congress of Neuroendocrinology (ICN), Toronto, Canada.
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	Member of the young ambassador's committee for the organization of the 9th ICN.
May 2017	**Poster presentation** at the Keystone Symposium Z5 "Neuronal Control of Appetite, Metabolism and Weight", Copenhagen, Denmark.
November 2016	**Poster presentation** and **best poster prize** at the LABEX SIGNALIFE Student Conference/Retreat, Nice, France.
October 2016	**Poster presentation** and **best poster prize** at the 41st Conference of the SNE, Corte, France.

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Abbreviations

2-DG	2-deoxy-D-glucose
3V	third ventricle
5-HT	serotonin or, 5-hydroxytryptamine
7TM	seven transmembrane domain
ACTH	adrenocorticotropic hormone
ACKR1	"atypical" chemokine receptor 1
AD	Alzheimer's disease
AGE	advanced glycation end products
AgRP	agouti-related peptide
AMPK	5' adenosine monophosphate-activated protein kinase
pAMPK	phosphorylated AMPK
ANS	autonomic nervous system
ARC	arcuate nucleus
ASICs	acid-sensing ion channels
AT	adipose tissue
ATP	adenosine triphosphate
BAT	brown adipose tissue
BBB	blood-brain-barrier
BNDF	brain-derived neurotrophic factor
BMI	body mass index
BW	body weight
CART	cocaine- and amphetamine-regulated transcript
CCBP-2	chemokine-binding protein 2
CCK	cholecystonkinin
cCKRs	"conventional" chemokine receptors
CCL5	CC-chemokine ligand 5; RANTES, "Regulated upon activation, normal T-cell expressed and secreted"
CGI-58	comparative gene identification-58 protein
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRH	corticotrophin-releasing hormone
CTLA-4	cytotoxic T lymphocyte-associated antigen-4 gene
DAG	diacylglycerol
DARC	"duffy antigen receptor for chemokines" also known under the acronym "atypical chemokine receptor 1" (ACKR1)
DCs	dendritic cells
DHA	docosahexaenoic acid
DIO	diet-induced obesity
DM	diabetes mellitus
DMN	dorsomedial nucleus
DN	diabetic neuropathy
DNL	de novo lipogenesis
DPN	length-dependent diabetic polyneuropathy/distal symmetric polyneuropathy/diabetic polyneuropathy
DPP4	dipeptidyl peptidase 4
DRG	dorsal root ganglia
EAE	experimental autoimmune encephalomyelitis
EE	energy expenditure
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERa	estrogen receptor alpha
FFAs	free fatty acids
FAs	fatty acids
FOXO1	factor Forkhead box O1
GABA	γ-aminobutyric acid
GAD6	glutamate decarboxylase
GAG	glycosaminoglycans
GDM	gestational diabetes mellitus
GFAP	glial fibrillary acidic protein
GHSR	growth hormone secretagogue receptor
GIP	gastric inhibitory polypeptide
GIT	gastrointestinal tract
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter

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GS	glycogen synthase
GPCR	G protein-coupled receptor
GPR103	G protein-coupled receptor 103
GPR75	G protein-coupled receptor 75
GRK	G protein-coupled receptor kinase
GTT	glucose tolerance test
iGTT	intraperitoneal GTT
oGTT	oral GTT
HbA1c	increased glycated hemoglobin A1c
HFD	high fat diet
HPA	hypothalamic-pituitary-adrenal axis
HPT	hypothalamic-pituitary-thyroid axis
IA-2	insulinoma-associated protein 2
IASP	International Association for the Study of Pain
Iba1	ionized calcium binding adaptor molecule 1
ICV	Intracerebroventricular
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IFN	interferons
IFN-γ	interferon γ
IGT	impaired glucose tolerance
IGF	insulin-like growth factor
IKKβ	inhibitor of κB kinase β
IL	interleukin
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IP	intraperitoneal
IR	Insulin receptor
IRS	insulin receptor substrate
IRTK	insulin receptor tyrosine kinase
ITT	insulin tolerance test
JAK	janus kinase
JNK	c-Jun N-terminal kinase
K_{ATP}	ATP-dependent potassium channels
KO	knockout
LDL	low-density lipoproteins
LH	lateral hypothalamus
LPS	lipopolysaccharide
β-LPH	β-lipotropin
LOX1	Lectin-like oxidized LDL receptor 1
MAPK	mitogen-activated protein kinase
MBH	mediobasal hypothalamus
MCH	melanin-concentrating hormone
MCR	melanocortin receptor
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
MCHR1	melanin-concentrating hormone receptor 1
ME	median eminence
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	multiple sclerosis
α-MSH	α-melanocyte-stimulating hormone
mTOR	mammalian target of rapamycin
NASH	non-alcoholic liver steatosis
NAFLD	non-alcoholic fatty liver disease
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NKT	natural killer T cells
NO	nitric oxide
NOD	non-obese diabetic (mouse model)
NPY	neuropeptide Y
NTS	nucleus tractus solitaries; nucleus of the solitary tract
ob/ob	leptin-deficient mice
PAI-1	plasminogen activator 1
PBN	parabrachial nucleus
PC	pro-hormone convertases
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMN	polymorphonuclear neutrophils
PNS	peripheral nervous system
PNL	partial sciatic nerve ligation

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POMC	proopiomelanocortin
PP	pancreatic polypeptide
PPARγ	peroxisome proliferator-activated receptor γ
PUFA	polyunsaturated fatty acid
PVN	paraventricular nucleus
PYY	peptide YY
RANTES	**R**egulated upon **A**ctivation **N**ormal **T** cells **E**xpressed and Secreted
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SAA	serum amyloid A
SD	standard diet
SDF-1	stromal cell-derived factor 1
SFAs	saturated fatty acids
SF-1	steroidogenic factor-1
SNS	sympathetic nervous system
SOCS3	suppressor of cytokine signaling-3
SREBP1	sterol regulatory element-binding protein
STATs	signal transducer and activator of transcription
STAT3	signal transducer and activator of transcription-3
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TG	triglycerides
TGF-a	tumor growth factor-a
TGF-β	tumor growth factor β
TLR4	toll-like receptor 4
TRH	thyrotropin-releasing hormone
TRPA1	transient receptor potential cation channel subfamily A member 1
TRPM8	transient receptor potential cation channel subfamily M member 8
TRPV1	transient receptor potential cation channel subfamily V member 1
UCP-1	uncoupling protein-1
UPR	unfolded protein response
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
WAT	white adipose tissue
WHO	World Health Organization
WT	wild-type
ZDF	Zucker diabetic fatty rat
ZNT8	zinc transporter 8

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Introduction

1. Hypothalamus and the Regulation of Energy Balance

Energy, in the form of adenosine triphosphate (ATP), is the life force for every existing organism, whether they are unicellular or multicellular organisms. Different from autotrophs, humans and animals are heterotrophs that require external organic sources for energy production which they acquire through nutrition. Nutrition is mainly based on four macromolecules that can be divided into proteins, lipids, carbohydrates and nucleic acids and constitute sources of energy for cellular processes. While it seems that the decision to eat, what, when and how much to eat is made deliberately by humans, it is yet a highly complex and regulated process with the aim to maintain energy balance, and controlled by different areas of the brain, most notably and famous of which is the hypothalamus (Morton, Meek and Schwartz, 2014). The brain is the center of regulation of many processes including energy balance. The brain maintains energy balance by adjusting energy intake to match energy output, through regulating food intake and energy expenditure (EE). EE can be subdivided into basal metabolic rate, thermogenesis and locomotor activity. To be able to do this complex task, it is dependent on both peripheral and central signals that keeps it informed about the nutritional or energy state of the body, previous experiences, taste, emotions associated with food and other factors that are encoded as memories (Fig. 1). The brain is constantly monitoring the energetic state and receives various information via different sensors, integrates them, compares it to the target set point and with previous experiences and elicits a response that results in adaptive behavior with the aim to keep the body in homeostasis (Fig. 1). In this sense, by adjusting feeding behavior and EE, the brain can maintain the body weight (BW) of an organism within a set range. Because of the dependence on external sources of energy, it is not surprising that the energy balance regulatory system consists of redundant pathways that assures

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energy intake without fail (Schwartz *et al.*, 2000; Zheng and Berthoud, 2008; Yuan, Xiong and Guan, 2013; Morton, Meek and Schwartz, 2014). The brain receives information about nutrient availability and stored energy in form of humoral, mechanical stimuli and neuronal signals from innervations of different organs via the enteric and autonomous nervous system (Fig. 1).

Ghrelin, a gut hormone, is the only known orexigenic or "hunger" signal that is secreted by the empty stomach to stimulate feeding in the central nervous system (CNS) (Fig. 1). It is secreted often in anticipation of food, with the highest increase in plasma levels detectable right before meals and decreases when nutrition reaches the duodenum (Zheng and Berthoud, 2008).

In contrast, leptin is a satiety-inducing hormone or one among many adipokines released by white adipose tissue (WAT) in proportion to the state of adiposity, and thus informs the brain about the amount of long-term energy stores secured in the body (Fig. 1). However, although its serum level is dependent on fat mass, plasma leptin levels can vary during fasting and feeding. While fasting transiently but rapidly suppresses leptin, feeding rapidly increases leptin levels (Zheng and Berthoud, 2008). Leptin binds to leptin receptor that is expressed on neurons in the hypothalamus, brain endothelial cells, astrocytes and tanycytes. Leptin exerts its anorexigenic effect by activating anorexigenic-, and inhibiting orexigenic neurons in the hypothalamus to regulate energy balance as well as systemic lipid and glucose metabolism (Freire-Regatillo *et al.*, 2017).

Another anorexigenic hormone that induces satiety and a decrease in food intake is insulin, a hormone secreted by the endocrine pancreas in response to increases in blood glucose levels. Together with the incretins that are secreted by enteroendocrine cells and induce the secretion of insulin by the pancreas in proportion to incoming glucose, it maintains stable glucose levels and leads to glucose uptake by different metabolic tissues. In the brain, insulin binds to insulin receptor (IR) on neurons, astrocytes and endothelial cells to

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induce different effects including the increase in EE, a decrease in food intake and energy storage as well as the regulation of systemic glucose levels (Freire-Regatillo *et al.*, 2017).

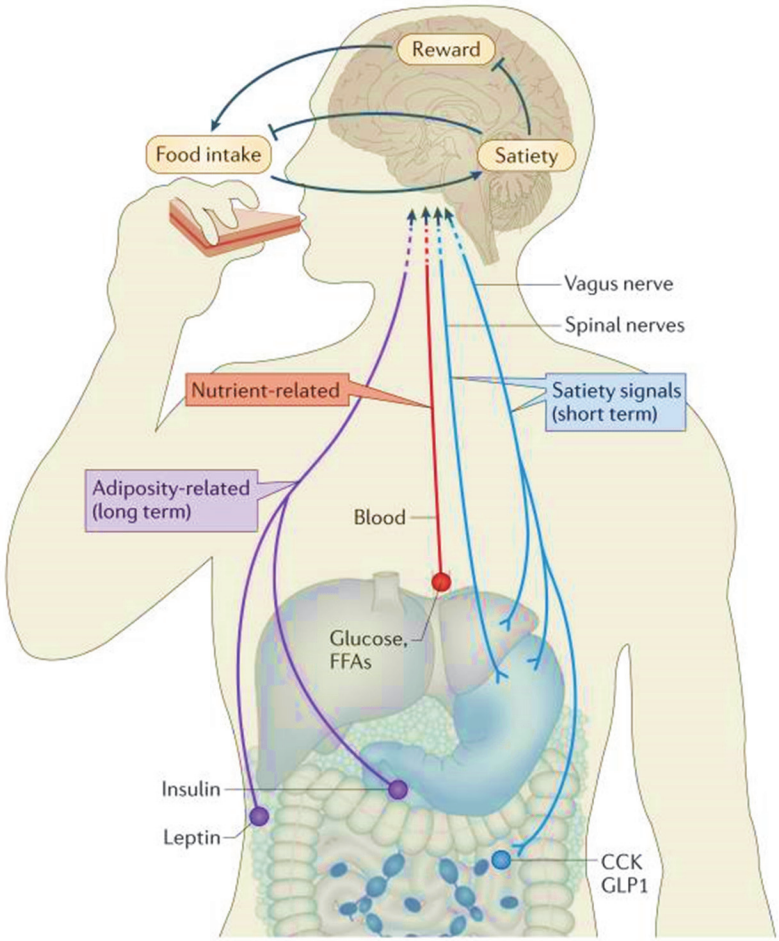

Figure 1: Energy balance regulation by the CNS.
(Adapted from (Morton, Meek and Schwartz, 2014)).

Furthermore, the stomach can transmit signals about incoming fats and proteins via the release of cholecystokinin (CCK) and peptide YY (PYY). In addition to that, it informs the brain about incoming food via receptors stimulated by mechanical distention of the stomach and transmitting the signal via vagal afferents. Glucose sensing is mediated by taste receptors in the mouth, incretins and 5-hydroxytryptamine (5-HT or serotonin) released by enteroendocrine cells in the intestines. While some of these signals act on vagal sensory neurons that innervate the portal vein and liver, that can sense the incretin glucagon-like peptide-1 (GLP-1) and circulating glucose levels, to

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transmit information to the brain, others can act directly in the brain (Zheng and Berthoud, 2008). Such sensors and receptors for nutrition-related molecules can also be found in several brain areas. The brain is protected by a blood-brain barrier (BBB) that limits the access to the brain. However, specific transport systems are in place to enable the passage of nutrients and important metabolic signals (Zheng and Berthoud, 2008). In addition to that, the brain contains a few areas, the so-called circumventricular organs, that are characterized by increased permeability and fenestrated capillaries, and include the median eminence (ME) in the hypothalamus and the area postrema in the medulla oblongata (Ganong, 2000). Feeding is regulated by three interacting brain systems in the brain, two of which are responsible for sensing and integrating physiological signals and circulating humoral signals from the periphery (hypothalamus), and neuronal signals from nerves innervating the gut (brainstem) and thereby control satiety, while the third neuronal circuit, which receives inputs from the other two systems, controls motivation for food foraging and feeding (Myers and Olson, 2014).

1.1. The Hypothalamus

The hypothalamus is one of the most important regions of the brain that participates in the control of energy metabolism and feeding regulation by controlling adaptive behaviours like food intake or suppression and EE as well as adipose tissue (AT) function, in response to circulating nutritional and hormonal cues from the periphery (Leibowitz and Wortley, 2004; Berthoud and Morrison, 2008).

Being composed of highly heterogenic groups of neurons and glial cells such as tanycytes in the ME, astrocytes, microglia cells and some macrophages, it constitutes a highly interactive and complex neural-glia network (Leibowitz and Wortley, 2004; Morton, Meek and Schwartz, 2014; Freire-Regatillo *et al.*, 2017). With its placement around the 3V, it is well positioned for its task in regulating energy balance as it is in contact with the cerebrospinal fluid of the 3V and fenestrated microvessels in the ME where specialized neurons and

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glia cells can sense different nutrients, hormones and other important factors from the peripheral circulation and respond rapidly to metabolic changes and nutrient flux (Murphy and Bloom, 2006; Morton, Meek and Schwartz, 2014). In addition to these humoral factors it receives also neuronal input from other brain regions and via the vagal afferents that inform the brain about the nutritional state of the body, which is processed by the brai, which then elicits an adaptive response as behavioral, autonomic and endocrine output through changes in neuronal modulation (Berthoud and Morrison, 2008).

The hypothalamus consists, beside a circumventricular organ at the base of the 3V termed the ME, of several nuclei, including the arcuate nucleus (ARC), ventromedial hypothalamic nucleus (VMH), paraventricular nucleus (PVN), the dorsomedial nucleus (DMN) and the lateral hypothalamus (LH) (Fig. 2) (Berthoud and Morrison, 2008; Roh, Song and Kim, 2016).

1.1.1. The Arcuate Nucleus

The ARC is one of the nuclei in the ventral part of the mediobasal hypothalamus (MBH) and lies adjacent to each side of the 3V. It contains mainly two populations of neurons, which co-express either the orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY), or the anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Fig. 2).

These two neural populations express leptin and IRs and thus, can receive peripheral signals like leptin, insulin and ghrelin as well as nutrients and transmit the information to second order neurons in the PVN and LH to regulate food intake and EE. They also send projections to other nulcei of the hypothalamus like the VMH and the perifornical area. AgRP/NPY neurons secrete both γ-aminobutyric acid (GABA) as well as the orexigenic neuroendocrine peptides AgRP and NPY to stimulate food intake and reduce EE (Schwartz *et al.*, 2000).

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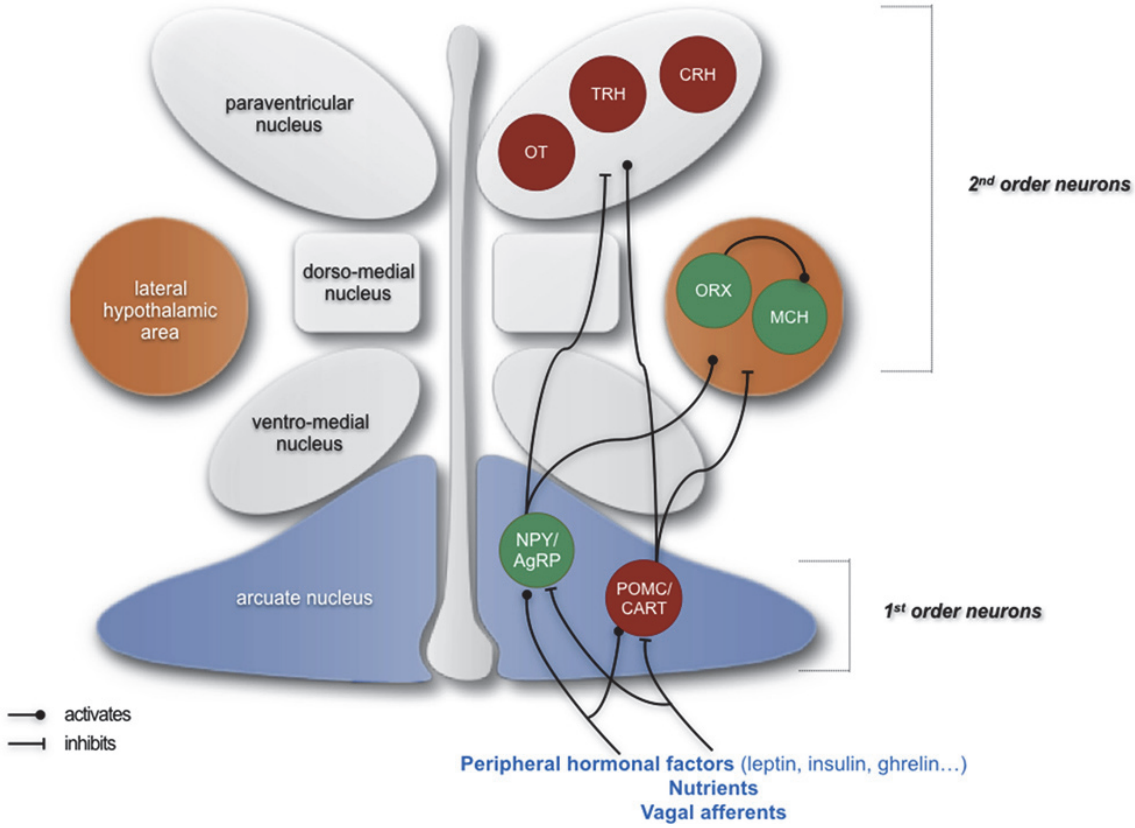

Figure 2: Hypothalamic control of food intake behavior.
(Adapted from (Le Thuc et al., 2017)).

In contrast to that, POMC neurons produce and secrete the neuroendocrine peptide called α-melanocyte-stimulating hormone (α-MSH), that is derived from the peptide POMC, and acts on second order neurons via melanocortin receptors (MCRs), to reduce food intake and induce EE (Morton, Meek and Schwartz, 2014). Interestingly, both AgRP and α-MSH are competing for the same receptor, called melanocortin 4 receptor (MC4R), yet induce opposing effects by binding to the receptor on second order neurons (Spiegelman and Flier, 2001). Furthermore, ARC neurons have direct and rapid access to blood-borne and peripheral signals, as it is a vascularized area with fenestrated vessels forming the BBB, allowing the passage of hormones and other proteins or nutrients such as free fatty acids (FFA) (Tran *et al.*, 2016). It has been shown that acute injection of palmitate-rich milk fat and 4 weeks of HFD leads to raised and accumulated FFAs and palmitate in the former case in the hypothalamus (Valdearcos *et al.*, 2014; Tran *et al.*, 2016).

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NPY and AgRP, peptides co-expressed in neurons of the ARC, are both very similar in their functions and effects, even though they bind to different receptors. NPY exerts its function by binding to different receptor subtypes, namely Y1R, Y5R, and potentially also Y2R, while AgRP belongs to the central melanocortin system and is an antagonist for the MC3R and MC4R receptors (Leibowitz and Wortley, 2004).

These neurons are highly responsive to states of energy deficiencies or increased metabolic demands, such as food deprivation, increased exercise, cold, and pregnancy. Thus during these states, the expression and synthesis of both peptides is increased and mediate the stimulation of food intake to meet the energy demands of the body (Hahn *et al.*, 1998; Chen *et al.*, 1999; Mizuno and Mobbs, 1999). Food deprivation or negative energy balance is mediated by the stomach-derived hormone ghrelin, which can bind the growth hormone secretagogue receptor (GHSR) on NPY/AgRP neurons to increase the expression of both peptides, leading to increased food intake. Experiments using either central or peripheral ghrelin administration in rodents found that it stimulates food intake and c-fos expression (marker for neuronal acitivty) in neurons of the PVN and leads to increased weight gain and fat mass through reduction in lipid utilization (Leibowitz and Wortley, 2004).

Furthermore, NPY and AgRP are also stimulated by the adrenal steroid called corticosterone, which exerts its effect through glucocorticoid type II receptors to stimulate the expression and production of both peptides and ultimately leading to an increase in food intake (Hisano *et al.*, 1988; Stanley *et al.*, 1989; Akabayashi *et al.*, 1994; Tempel and Leibowitz, 1994; Arvaniti, Huang and Richard, 2001; Savontaus, Conwell and Wardlaw, 2002; Makimura *et al.*, 2003). Interestingly, this steroid is implicated in circadian rhythm and thus rises at the onset of the nocturnal feeding period in rodents, but also during energy deficiency, stimulating NPY and AgRP expression (Akabayashi *et al.*, 1994; Tempel and Leibowitz, 1994; Xin-Yun *et al.*, 2002; Leibowitz and Wortley, 2004).

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NPY and AgRP are stimulated during states of negative energy balance, when ghrelin and corticosterone are increased and insulin and leptin, hormones correlated with adiposity and energy abundance, are low. In contrast, states of positive energy balance are characterized by low ghrelin and high levels of leptin and insulin. These hormones, in contrast to ghrelin and corticosterone, suppress the expression of NPY and AgRP peptides and inhibit food intake. The effect of these hormones was demonstrated by central injections of leptin and insulin, which decreased NPY and AgRP expression and synthesis, and resulted in a reduction of not only food intake but also meal size and weight gain, while at the same time increasing EE and sympathetic nervous system (SNS) activity (Leibowitz and Wortley, 2004). In accordance with this, the ablation of insulin and leptin in animals renders them hyperphagic and obese. Moreover, mice with genetic forms of obesity like ob/ob mice that lack leptin have strongly activated NPY/AgRP neurons as well as hyperphagia (Schwartz *et al.*, 1996). It thus seems like

NPY/AgRP neurons are inhibited by leptin and insulin through binding to their respective receptors, which are expressed by those neurons.

Another interesting property of NPY/AgRP neurons was revealed in a series of experiments, which showed that NPY and AgRP respond to dietary nutrients, in particular to glucose levels. For example, both NPY and AgRP were decreased upon glucose injection, but increased when glucose levels returned to baseline. However, these peptides do not change to Intralipid-induced increases in circulating lipids. Similarly, pharmacological inhibiting glucose utilization with 2-deoxy-D-glucose (2-DG) increased the expression of both peptides. Strikingly, in rats with access to a low-energy diet high in carbohydrates, NPY and AgRP expression was consistently higher than when they were given a diet high in fat (Leibowitz and Wortley, 2004). Consistent with this, studies performing central injections of NPY or AgRP report increases in levels of corticosterone, food intake, a preferred intake of carbohydrates to fats, reduced EE and thermogenesis, increased lipogenesis and hence elevated body fat accumulation. Interestingly, the administration of both

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peptides stimulates food intake, the effect of AgRP seems to last longer than NPY (Leibowitz and Wortley, 2004).

NPY/AgRP neurons that also express GABA can also interact directly with POMC neurons through local axon collaterals and inhibit their neuronal activity (Berthoud and Morrison, 2008).

Studies have identified NPY and AgRP as primary inducers of food intake, because central injection of either of those peptides led to significant increases in food intake, while acute ablation of these peptides in adult rodents induced significant weight loss and hyperphagia. Interestingly, constitutive genetic deletion of either peptide has only a limited effect on weight regulation or leptin sensitivity, most likely due to compensation for their loss by other neuronal networks or pathways during early development (Gropp *et al.*, 2005; Luquet *et al.*, 2005; Berthoud and Morrison, 2008).

It seems that both peptides are redundant in that they act on parallel neuronal circuits inducing the same effect on feeding behavior in particular inducing feeding in situations of food or energy scarcity (Leibowitz and Wortley, 2004).

CART and POMC belong to the peptide family known as the melanocortins that mediate the anorexigenic neuronal response to decrease food intake and increase EE. The expression of these neuropeptides increases in response to rising adiposity signals stimulating these neurons (Singla, 2010).

POMC/CART neurons that are situated in the ARC represent likewise first-order neurons, which respond to blood-borne metabolic signals. There is a debate however, whether POMC/CART neurons are directly stimulated by metabolic signals or indirectly regulated through NPY/AgRP neurons via their inhibitory axonal collaterals (Berthoud and Morrison, 2008).

It seems that POMC/CART neurons express, similar to their neighboring neurons, leptin and IRs and thus can be directly regulated through these metabolic signals. Studies have shown that central leptin administration

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stimulates POMC expression and c-fos expression, while POMC expression is markedly reduced in mice with leptin-deficient (ob/ob) mice that develop obesity and hyperphagia, but become normal upon administration of leptin (Leibowitz and Wortley, 2004). Interestingly, while leptin has a stimulatory effect on POMC, insulin, serotonin and ghrelin lead to inhibition. Studies suggest that this might be due to increased inhibition by GABA-expressing axonal collaterals from NPY/AgRP neurons (Leibowitz and Wortley, 2004). Different from NPY/AgRP, POMC does not seem to be affected by dietary intake or components. CART, however, which is co-localized with POMC neurons increases with HFD feeding (Leibowitz and Wortley, 2004).

In addition to that, they appear to be regulated by 5-HT through their 5HT-2C receptor to mediate their anorexigenic effect (Heisler *et al.*, 2002). Similar to NPY/AgRP neurons, POMC/CART neurons target second-order neurons in different hypothalamic nuclei, such as the PVN, LH and VMH as well as autonomic preganglionic neurons in the brain stem and spinal cord (Roh, Song and Kim, 2016).

Apart from the ARC, POMC is expressed in many tissues such as the nucleus tractus solitarius (NTS) in the CNS but also outside the brain such as in the pituitary, the adrenal gland and the intestine among others. It is a polypeptide that upon cleavage by pro-hormone convertases (PC) gives rise to several different peptides of the melanocortin family, defined by which type of PC the tissue produces (Harno *et al.*, 2018). In the pituitary, the expression of PC1/3 but not PC2 results in the sequential cleavage of POMC to yield the peptides adrenocorticotropic hormone (ACTH) and β-lipotropin (β-LPH). In the hypothalamus however, PC1/3 and PC2 are expressed, that further process the resulting ACTH and β-LPH into α-, β- and γ-MSH (melanocyte-stimulating hormone) and β- endorphins.

α-MSH induces a reduction in food intake and increase in EE through binding to melanocortin receptors 3 and 4 (MC3R & MC4R) on neurons in the PVN

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and LH of the hypothalamus and the NTS in the brainstem (Coll *et al.*, 2004; Harno *et al.*, 2018).

The importance of MC4R in the regulation of feeding behavior and BW was shown in mice harboring a deletion in this gene because they develop obesity due to hyperphagia and reduced EE (Roh, Song and Kim, 2016). Similarly, mutations in this gene lead to severe obesity with early-onset in humans and account for about 6% of obesity cases (Tao, 2005). Furthermore, mice with deletions in the gene coding for POMC, develop similar to MC4R or leptin receptor deletion, hyperphagia and obesity, while central leptin injection has a reduced effect, when the melanocortin signaling is blocked in neurons (Morton *et al.*, 2006).

Interestingly, POMC neurons mediate also the stimulating effect of leptin on locomotor activity and can thereby modulate EE.

CART is one of the anorexigenic neuropeptides abundantly expressed in the ARC and thus an important regulator of food intake and BW. In addition to that, CART is expressed also in the PVN, DMN, LH of the hypothalamus, but also many different neural circuits outside the hypothalamus as well as the adrenal and pituitary glands (Leibowitz and Wortley, 2004; Lau and Herzog, 2014). Although no receptor has been identified that binds CART, it has been implicated in many different functions apart from energy balance such as reward and addiction, the stress response as well as psychostimulant and neuroendocrine functions (Lau and Herzog, 2014).

The expression of CART in hypothalamic neurons has been found to be regulated by several hormones like leptin, insulin and also by glucocorticoids and food deprivation (Leibowitz and Wortley, 2004).

While leptin administration, hyperleptinemia and chronic exposure to cold stimulate the expression of CART, leptin deficiency such as in ob/ob mice, reduces it. Similarly, CART expression falls in the ARC in states of food deprivation, which is also associated with decreases in serum leptin concentrations. It was suggested that CART might mediate SNS activation by

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leptin (Leibowitz and Wortley, 2004). Interestingly, injecting one of the peptides encoded by the gene for CART into the PVN or ARC stimulated the expression of uncoupling protein-1 (UCP-1) revealing its implication in the regulation of EE by modulating thermogenesis. Furthermore, CART has been shown the be part of the regulation of the HPA axis in experiments where application of hypothalamic explants induced the production and secretion of corticotrophin-releasing and thyrotropin-releasing hormone (CRH and TRH)(Lau and Herzog, 2014). Intracerebroventricular (ICV) injection of CART revealed some contradicting results. While some studies reveal decreases in food intake and weight loss as well as inhibition of gastric acid secretion, gastric emptying and increased circulating FFAs after ICV injections, others injecting directly into the ARC report increases in food intake. Inhibiting central CART via antibodies in turn augments nocturnal food intake (Leibowitz and Wortley, 2004).

In a few interesting studies, it was demonstrated that CART expression is sensitive to nutritional lipids. Studies have shown not only that CART expression is increased in the ARC and PVN in HFD-fed and obese rats, compared to low-diet-fed controls, but also increased CART expression in Intralipid administered animals, which raises blood triglycerides (TG) without changing leptin. Furthermore, HFD-feeding in CART-deficient mice leads to hyperphagia, increased weight gain, as well as fat mass, while SD-fed mice exhibit a normal BW. The increase in CART after HFD might be a compensatory mechanisms by the body to sense and react to high energy and dissipate excess energy by increasing EE (Leibowitz and Wortley, 2004).

1.1.2. The Ventromedial Hypothalamic Nucleus

The VMH of the hypothalamus that is situated just above the ARC is known as the satiety center of the hypothalamus because studies stimulating the VMH showed a decrease in food intake, while bilateral lesions in the VMH results in obesity and hyperphagia but also hyperglycemia (Shimizu *et al.*, 1987; Singla, 2010). It reciprocally connects with the ARC and sends further projections to

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other areas such as the DMN, LH and areas of the brain stem (Roh, Song and Kim, 2016). Similar to neurons of the ARC, subsets of cells in the VMH also express receptors for leptin and insulin and thus are implicated not only in energy metabolism regulation but also in glucose homeostasis (Fei *et al.*, 1997; Gonzàlez, Reimann and Burdakov, 2009). The VMH contains distinct populations of neurons, that can express apart from leptin receptor and IR also brain-derived neurotrophic factor (BNDF), estrogen receptor alpha (ERa), and steroidogenic factor-1 (SF-1) (Fei *et al.*, 1997; B. Xu *et al.*, 2003; Gonzàlez, Reimann and Burdakov, 2009; Choi *et al.*, 2013). Leptin administration in the VMH was shown to stimulate glucose uptake in peripheral tissues like skeletal muscle, heart and brown adipose tissue (BAT) via the SNS (Haque *et al.*, 1999; Minokoshi, Haque and Shimazu, 1999; Toda *et al.*, 2009). Interestingly, glucose-sensing neurons in the VMH are activated by both low and high glucose and impairing IR signaling in the VMH in fasting or hypoglycemic state stimulates glucagon secretion (Paranjape *et al.*, 2010, 2011). Moreover, studies have shown that the VMH regulates BW and energy homeostasis via controlling the autonomic nervous system (ANS) (Choi *et al.*, 2013).

1.1.3. The Paraventricular Nucleus

The PVN is another nucleus of the dorsorostral part of the hypothalamus situated beside the 3V and contains many different neurons that are implicated in neuroendocrine, autonomic and behavioral functions (Richard and Timofeeva, 2009). It receives many neuronal projections such as from the brainstem, limbic system and the ARC and integrates visceral information about nutrition, emotional and reward-related signals as well as energy balance related signals to exert adaptive responses by sending projections towards different brain areas. For example, It harbors MC4R-expressing secondary neurons that receive terminals from both NPY/AgRP and POMC/CART neurons from the ARC and thus can mediate effects on appetite and thermogenesis (Richard and Timofeeva, 2009; Roh, Song and Kim, 2016). As such it mediates the net catabolic effect of these neurons. However, it can also control fatty acid (FA) oxidation and lipolysis in

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peripheral metabolic tissues via sympathetic efferents (Roh, Song and Kim, 2016).

Apart from that, it contains other neurons of the parvocellular neurosecretory system, such as CRH, and TRH as well as the magnocellular neurosecretory system including vasopressin and oxytocin expressing neurons among others. It thus can also mediate different effects via the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-thyroid axis (HPT) axis and is involved in regulating blood pressure, water balance and lactation (Berthoud and Morrison, 2008; Richard and Timofeeva, 2009).

The PVN receives many inputs from various areas including the ARC and has many downstream connections, for example it can regulate neuroendocrine functions via the hypothalamic pituitary axis or control the ANS (Berthoud and Morrison, 2008). Its role in energy balance has been shown in animals harboring lesions in the PVN or mutations in the sim1 gene that is a transcription factor, important for the correct development of the PVN leading to obesity and hyperphagia.

1.1.4. The Lateral Hypothalamus

Different from the VMH, the LH is known as the hunger center of the brain, because stimulation of the LH mediates feeding, while lesions leading to ablation of neurons within the LH have the opposite effect (hypophagia and weight loss) (Singla, 2010; Roh, Song and Kim, 2016).

The second-order neurons of the LH, which receive direct inputs from ARC neurons, express the neuropeptides orexin/hypocretin (ORX), melanin-concentrating hormone (MCH) (Figure 2) and 26RFa among others (Chartrel *et al.*, 2003, 2016; Leibowitz and Wortley, 2004; Berthoud and Morrison, 2008). Interestingly, 26RFa has been identified as a ligand for the orphan G protein coupled receptor (GPCR) GPR103 and its expression was found in neurons of the ARC, LH and VMH. Furthermore, this neuropeptide has been shown to act as an incretin but also as an important regulator of food intake and energy balance in the hypothalamus (Prévost *et al.*, 2015; Chartrel *et al.*, 2016).

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Additionally, neurons of the LH receive input from areas other than the ARC, namely areas transmitting sensory information like the insular and olfactory cortex as well as areas that integrate reward, motivation but also learning and memory (orbitofrontal cortex, nucleus accumbens, amygdala and ventral tegmental area). Furthermore, LH neurons are target for ascending neurons from brain stem areas such as the NTS, parabrachial nucleus (PBN) and locus coeruleus, which transmit vagal and visceral sensory information from the periphery (Berthoud and Morrison, 2008).

The information that LH neurons receive from the various areas mentioned above is processed, integrated and further transmitted to different areas throughout the brain from the cortex to the spinal cord affecting many different neural networks to elicit the appropriate action (Berthoud and Morrison, 2008).

Orexin is important in the regulation of glucose sensing and sleep-wake cycles and mutations of its receptor can lead to narcolepsy (Roh, Song and Kim, 2016).

Pharmacological and genetic studies have implicated MCH in development of obesity and the regulation of feeding behavior and identified it as a potent orexigenic peptide, mainly expressed in neurons of the posterior LH and zona incerta and projecting to different areas in the brain and hence controlling many different processes (Leibowitz and Wortley, 2004). It can bind the seven transmembrane (7TM) receptor melanin-concentrating hormone receptor 1 (MCHR1), which is widely expressed not only expressed in the ARC and VMH of the hypothalamus but also abundantly expressed in areas outside the hypothalamus that are related to olfactory and reward-related functions (Leibowitz and Wortley, 2004).

1.1.5. The Dorsomedial Nucleus

The DMN is interconnected with the ARC as it receives high amounts of terminals of NPY and a-MSH containing neurons and participates in the regulation of food intake as lesions in the DMN lead to hyperphagia and

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obesity (Roh, Song and Kim, 2016). In addition to that, the DMN plays an important role in thermogenesis and thus contributes to EE. Downregulation of NPY neurons in the DMN has been shown to increase BAT activity and so-called browning of WAT, which is the development of brown-like adipocytes in WAT. This is further accompanied by higher rates of thermogenesis and EE. Knockdown of NPY in DMN also ameliorates high fat diet (HFD)-induced obesity and glucose metabolism (Bi, 2013).

The hypothalamus thus contains different nuclei with distinct neurons that in concert adapt to peripheral signals informing the brain about nutritional and energy state, integrate these with information from other areas of the brain and elicit a behavioral response via the ANS or SNS or HPA to control food intake and EE in an attempt to maintain energy balance.

However, areas outside the hypothalamus such as the limbic system, the ventral tegmental area (VTA), NTS and PBN, are crucial in the regulation of energy balance and feeding behavior.

1.1.6. The Caudal Brainstem

The caudal brainstem contains important nuclei such as nucleus accumbens or raphe nucleus that are in contact with the hypothalamus and the periphery and together with the hypothalamus it plays an important role in homeostatic function. Furthermore, some appreciation has to be given to other areas in the cortex and the limbic system which play important roles, too, especially in the integration of past experience, emotions, reward and hedonic function as well as social and environmental information into the already complex system of energy balance regulation (Berthoud and Morrison, 2008).

Strikingly, a recent study has identified another region of the brain, that is implicated in the control of feeding, namely the PBN.

In an interesting study using an optogenetic approach, they identified so called caltinonin gene-related peptide (CGRP) neurons, that receive inputs

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from AgRP neurons from the ARC of the hypothalamus, which when activated suppress feeding. They further found that the functions of these neurons seem to regulate meal duration (Wu, Clark and Palmiter, 2012; Roman, Derkach and Palmiter, 2016).

An important region for regulation of food intake, the NTS, receives signals from the gastrointestinal tract (GIT) about satiety by sensing the gut released hormone CCK or via the vagus nerve, which represents the major neuronal connection between the brain and the periphery and thus plays a big role in the regulation of many different processes (Ellacott, Halatchev and Cone, 2006; Roh, Song and Kim, 2016). Cutting this connection decreases meal size and duration. The NTS is also connected with the PVN of the hypothalamus and contains NPY, POMC as well as GLP-1 neurons that make the NTS another metabolic sensing area of the brain. Similarly, to the hypothalamus the NTS is in close proximity to another circumventricular area called the area postrema, which places it anatomically strategically for metabolite and signal sensing. POMC neurons in the NTS sense apart from CCK also leptin as indicated by upregulation of a leptin receptor marker called signal transducer and activator of transcription-3 (STAT3) (Ellacott, Halatchev and Cone, 2006).

1.2. Brain Regulation of Energy Expenditure

Leptin stimulation of hypothalamic POMC neurons as well as orexin-A from LH neurons both stimulate locomotor activity, while suprachiasmatic nucleus released tumor growth factor-a (TGF-a) inhibits locomotor activity via hypothalamic subparaventricular zone neurons in a circadian manner (Roh, Song and Kim, 2016).

BAT thermogenesis is regulated via SNS by the brain by inducing norepinephrine release, which stimulate β3-adrenergic receptors in BAT and inguinal fat pad cells. This triggers signaling pathways that lead to the increase in UCP-1 expression in BAT mitochondria.

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Especially hypothalamic areas such as the preoptic area, VMH and ARC play an important role in thermogenic regulation by influencing the SNS according to the sensation of body temperature (Roh, Song and Kim, 2016). While the preoptic area is involved in the control of body temperature, the VMH seems to regulate BAT activity.

ARC secreted a-MSH has a stimulatory role on BAT activity and sympathoexcitatory neurons of the DMN play a part in the regulation of thermogenic activity (Roh, Song and Kim, 2016). Several metabolites contribute to the central regulation of BAT activity. For example, leptin, MC3/4R agonist, glucagon and GLP-1 can stimulate BAT activity when infused into the brain, while insulin has a dual role. At high concentrations, insulin seems to increase nerve activity in the BAT while low doses have the opposite effect.

Interestingly, BAT thermogenesis affects BW and fat mass, as it uses metabolic substrates like glucose and FAs as substrates and thereby dissipates excess energy after consumption of high amounts of calories or upon exposure to cold temperature to maintain body temperature (Roh, Song and Kim, 2016). Interestingly, interleukin-6 (IL-6), which is secreted by skeletal muscle upon contraction during exercise, can cross the BBB and has been reported to stimulate EE in the brain (Roh, Song and Kim, 2016).

2. Obesity

2.1. Definition, Facts and Generalities

The physiological ability of vertebrates and humans to store energy as food has become one of the major burdens of the century for the human population. Obesity is a disorder that has become a huge economic and public health burden of global epidemic proportions in both developing and developed countries. It is affecting around 13% off the population worldwide, corresponding to over 650 million obese individuals. In addition to that, of the whole world population 1.9 billion adults (39%) were overweight in 2016. With 41 million children under 5 years old being overweight or obese, childhood

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obesity constitutes one of the major health challenges of the 21st century (WHO, 2018). With the recent change in lifestyle to a more sedentary life and unhealthy food choices, as well as the growing population of older people, the numbers are bound to keep increasing dramatically. With around 2.8 million deaths it is at the fifth position for being the major and leading risk for deaths globally ('WHO | Global health risks', 2015).

Obesity is a metabolic disease defined as an excess accumulation of energy in form of body fat in AT resulting in a risk to health (WHO - Obesity) and mainly assessed by the body mass index (BMI). The BMI is the BW in kilograms divided by the height in meters squared (Fig. 3). BMI thus classifies the state of the body as normal weight (BMI=18.5-24.9), between 25 and 29.9 as overweight, obesity by a BMI of ≥30, and morbid obese with a BMI of 40 or higher (Fig. 3).

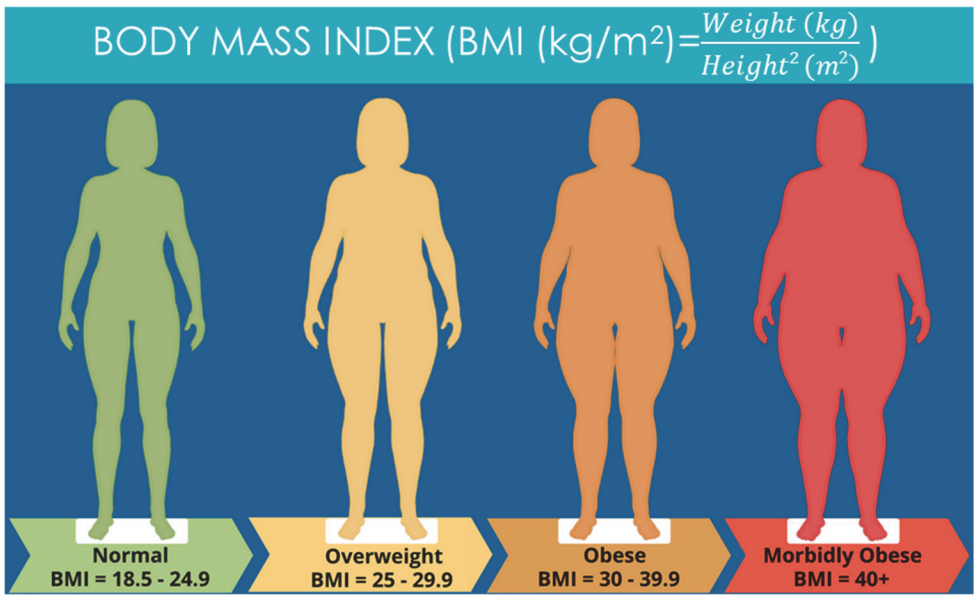

Figure 3: Body mass index.
Calculation of BMI to classify the state of obesity. (Adapted from Mexico Bariatric Center, BMI chart).

However, as useful the BMI might be, it is but an estimate of body fat and health risk and has its limitations as it does not take into account the specific body composition such as muscles, body fat mass and distribution, lean mass,

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bone density and other factors and thus cannot accurately assess the state of the body.

Obesity is part of the metabolic syndrome, which is defined as a cluster of both chronic and complex disorders, that have in common at least one feature of metabolic deterioration (Hotamisligil, 2006). Others that are often associated with obesity are hyperglycemia, dyslipidemia, hypertension (Oussaada *et al.*, 2019). Metabolic syndrome is associated with an increased risk for insulin resistance, fatty liver disease, type 2 diabetes mellitus (T2DM), atherosclerosis, cardiovascular diseases but also with dementia, airway disease and cancer (Hotamisligil, 2006; Oussaada *et al.*, 2019).

Obese patients and mouse models of obesity often have increased serum levels of FFAs, leptin (hyperleptinemia), indicative of leptin resistance, and develop insulin resistance with hyperinsulinemia and glucose intolerance (Klok, Jakobsdottir and Drent, 2007; Dalmas *et al.*, 2011; Adamska *et al.*, 2015; González-Muniesa *et al.*, 2017). Interestingly, leptin and insulin resistance can also be seen at the central level (Clegg *et al.*, 2011; Faulconbridge and Hayes, 2011; Cheng, Yu, Szabo, *et al.*, 2015; Tran *et al.*, 2016). A few mechanisms have been proposed to explain leptin resistance: a reduced transport of leptin through the BBB as well as an impairment in the central leptin signaling pathway (El-Haschimi *et al.*, 2000).

Obesity is a complex disorder and often caused by an interaction of various factors that can be genetic, environmental and psychosocial factors. Apart from monogenic forms of obesity, which are relatively rare and account for about only 2-3% of cases, obesity can be also caused by certain medication, depression, hormones and a disequilibrium between energy intake and EE, but also be due to a specific unhealthy diet (González-Muniesa *et al.*, 2017).

2.2. Genetic Causes of Obesity

Although genetic causes of obesity are accounting only for a small part of cases of obesity and cannot account for the tremendous rise of obesity prevalence in recent years, there are several cases of monogenic forms of

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obesity. One of the most famous and most studied forms of obesity are obesity induced by mutations in genes coding for both leptin and its receptor.

Leptin, a hormone or adipokine secreted by adipocytes of the AT has an important role in energy homeostasis. It is secreted in proportion to body fat mass and informs the brain about the energy state of the body. If leptin is missing due to a mutation in the gene for leptin, as was well researched in a model of obese mice (ob/ob), mice become hyperphagic and obese even on a normal diet (Zhang *et al.*, 1994). Such mutations in the gene for leptin were also found in children, who had no detectable serum levels of leptin and suffered from congenital early-onset obesity (Montague *et al.*, 1997; Gibson *et al.*, 2004). This autosomal recessive form of the disorder is heritable and leads to extreme forms of early-onset obesity, which produces a dysfunctional form of the protein leptin, that is either secreted in low amounts or fails to be secretion due to proteasomal degradation (Montague *et al.*, 1997; Rau *et al.*, 1999). Leptin deficiency is also associated with other endocrine abnormalities like hypogonadotrophic hypogonadism in addition to alterations in the SNS as well as high infection rates, which are attributable to a lack of T cells but without impacts on EE (Sadaf Farooqi *et al.*, 2002; Dubern and Clement, 2012). Other than leptin deficiency, which can be treated via injections of leptin to provide the body with the deficient protein, another monogenic form of obesity has no treatment options so far and is attributable to a loss of function mutation in the leptin receptor gene (db/db) (Tartaglia *et al.*, 1995; Chua *et al.*, 1996; Anderson, Hill and Hasty, 2012). The leptin receptor belongs to the family of cytokine receptors and the gene coding for the receptor seems to be inherited in an autosomal recessive manner causing human leptin receptor deficiency due to a mutation in this gene. Human leptin receptor deficiency leads similar to leptin deficiency to extreme obesity associated with hyperphagia but different from (ob/ob) to diabetes. Several models for this pathology have been produced in both mice (db/db mice) and rats (*fatty* Zucker rat). A deficiency in the leptin receptor similarly affects leptin signaling through phosphatidylinositol 3-kinase

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(PI3K) and STAT3, in particularly in the hypothalamus, where the receptor is expressed in normal patients or animals and affects appetite and feeding behavior (Chua *et al.*, 1996; Bates and Myers, 2003).

Another form of monogenic obesity is due to an autosomal recessive inherited loss of function mutation in the gene encoding POMC, a neuropeptide of the melanocortin system in the hypothalamus responsible for regulating energy balance by suppressing food intake. This mutation leads to a POMC deficiency, found in a few human subjects, leading to severe and early-onset obesity, adrenal insufficiency, and red hair pigmentation and similar to leptin and its receptor deficiency, it is associated with hyperphagia (Krude *et al.*, 1998, 2003; Buono, 2005). Interestingly, POMC-expressing neurons in the ARC of the hypothalamus are a target of leptin, which upon binding induce POMC synthesis (Coll *et al.*, 2004). POMC is expressed in many tissue such as the ARC of the hypothalamus and the NTS in the CNS but also outside such as in the pituitary, the adrenal gland and the intestine among others. It is a polypeptide that upon cleavage by PC gives rise to several different peptides, defined by which type of PC the tissue produces (Harno *et al.*, 2018). In the pituitary, the expression of PC1/3 but not PC2 results in the sequential cleavage of POMC to yield the peptides ACTH and β-lipotropin (β-LPH). In the hypothalamus however, PC1/3 and PC2 are expressed, that further processes the resulting ACTH and β-LPH into α-, β- and γ- MSH (melanocyte-stimulating hormone) and β- endorphins.

α-MSH induces a reduction in food intake and increase in EE through binding to MC3R & MC4R on neurons in the PVN and LH of the hypothalamus and the NTS in the brainstem (Coll *et al.*, 2004; Harno *et al.*, 2018). POMC deficient humans and mice thus develop severe cases of obesity but also other deficiencies related to the other peptides produced from the POMC precursor peptide, including pale skin color and red hair pigmentation and low cortisol levels (Krude *et al.*, 1998, 2003; Buono, 2005; Farooqi *et al.*, 2006).

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Similar to POMC deficiency, autosomal recessive forms of obesity can be induced by mutations in the gene for carboxypeptidase E (CPE) as was demonstrated in a *fat/fat* mouse model, that lead to less severe forms of obesity with later onset, compared to leptin or leptin receptor deficient forms of obesity. Interestingly, this enzyme is also responsible for the processing of prohormones including proinsulin in the pancreas and thus show an interesting genetic link between obesity and diabetes (Naggert *et al.*, 1995). In a similar example, a mutation in the PC1 gene that exerts its effect upstream of CPE caused obesity with elevated proinsulin levels and POMC levels with similar phenotype to the *fat* mouse in two human patients (Jackson *et al.*, 1997, 2003).

The most common form of monogenic human form of obesity is attributable to mutations in the gene for the MC4R. While deletion of murine MC4R leads to hyperphagia and reduced EE and ultimately obesity, human MC4R mutation is responsible for ~6% of severe and early-onset obesity cases and inherited in a co-dominant manner (Tao, 2005; Chung, 2012).

Furthermore, obesity can also appear as a consequence of other syndromes caused by mutations in several genes and include for example, Prader-Willi Syndrome and Cohen Syndrome among others.

Other factors that can lead to obesity are certain medications, depression and other environmental factors. One of the environmental causes of obesity and also one of the most common causes is a sedentary lifestyle and poor nutrition, especially a nutrition high in fat, leading to the dysfunction of energy balance regulation.

2.3. Obesity and Inflammation

It is characterized by a state of chronic low-grade systemic inflammation, the secretion of inflammatory cytokines and chemokines and an infiltration of immune cells including macrophages and T cells (Gregor and Hotamisligil, 2011).

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2.3.1. Inflammation

The classical inflammatory response of the body´s immune system is a necessary and beneficial process to recognize and eliminate sources of infection or inflammation such as exogenous intruders like bacteria or other pathogens. However, inflammation can also take place in cases such as tissue injury to promote wound healing. In any case, these inflammatory processes are usually acute, and once the pathogenic intruder or wound has been successfully eliminated or healed, the inflammation subsides. The classical characteristics of inflammation are redness, swelling, heat and pain. Furthermore, classical inflammation is also associated with an increased metabolic rate (Medzhitov, 2008).

Inflammation mainly involves cells of the immune system that can be broadly subdivided and into lymphocytes and myeloid cell and functionally into pro- and anti-inflammatory immune cells (Lee and Lee, 2014). Lymphocytes can be further classified into T, B, natural killer (NK), and natural killer T (NKT) cells, which belong to the adaptive immune system, while myeloid cells belong to the innate immune system and include macrophages, dendritic cells (DCs), mast cells, neutrophils, basophils and eosinophils. However, myeloid cells contribute to the adaptive immune system, too, for example as antigen presenting cells that activate lymphocytes, or through the production of cytokines and chemokines to help the development and activation of T and B cells and attract them to the target sites (Lee and Lee, 2014).

However, when the inflammation does not resolve, it can become of chronic nature and caused by an excess in triggers of inflammation, for example as seen in the case of obesity, where a metabolic trigger such as an excess consumption of nutrients or metabolic surplus is thought to be the cause of inflammatory processes. Moreover, it is suggested that metabolic cells such as adipocytes sustain the insult by the excess of nutrients and respond with initiating inflammatory processes, leading to a disturbed metabolic homeostasis.

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Different from classical infection or inflammatory response, chronic inflammation often found in obesity but also other diseases, is although significant, rather modest and thus often called "low-grade" inflammation and can be local in certain tissues.

This inflammatory state, characterized by a chronic low-grade inflammation in metabolic tissues such as pancreas, muscles, AT but also liver and brain and often found in obesity, is triggered by metabolic stimuli like excess energy and nutrients and was termed "metaflammation". It is not an inflammation in the traditional meaning of the word, as none of the classical features of inflammation are present, although it does involve similar actors or inflammatory signaling pathways as does the classical inflammation (Hotamisligil, 2006). Hence, in this manuscript inflammation will be referred to the sub-class of metabolic inflammation. Chronic inflammation or meaflammation can further be linked to insulin resistance and metabolic dysfunction (Gregor and Hotamisligil, 2011).

2.3.2. Peripheral Inflammation in Obesity

Obesity is as mentioned previously associated with a cluster of disorders commonly referred to as metabolic syndrome. The disorders included in this syndrome all have in common the occurrence of chronic, low-grade inflammation that seems to be the cause of these disorders. Long-term overconsumption of HFD leads to an overpowering of the storage capacity of ATs and lipid/FA accumulation in metabolic tissues such as skeletal muscle, liver, pancreas, brain and gut and can result not only in lipotoxicity but also induce inflammation in these tissues including AT.

AT was long thought to have the sole purpose of energy storage of lipids as triacylglycerols in the body. However, since the discovery of leptin, a hormone secreted by WAT in proportion to the energy stored in the tissue, AT is viewed rather as the largest endocrine organ (Calder *et al.*, 2011; Coelho, Oliveira and Fernandes, 2013). As an endocrine gland it is capable of synthesizing and releasing many bioactive compounds including hormones, cytokines and

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chemokines collectively referred to as adipokines. These adipokines like leptin have important regulatory functions like whole body metabolism and appetite control but also in immune function and others (Coelho, Oliveira and Fernandes, 2013). AT can be broadly subdivided into two types, BAT and WAT. The former has a lower storage capacity and different from WAT contains abundant mitochondria and more but smaller lipid vacuoles. Although BAT is more abundant in infants, adults also have BAT, which is located in supraclavicular and interscapular regions, around organs like kidneys, heart, pancreas but also aorta and the trachea (Santhanam *et al.*, 2015; Prodhomme *et al.*, 2018). BAT contributes to energy balance regulation through its participation in nonshivering thermogenesis and diet-induced thermogenesis. It thereby contributes to EE and contains many mitochondria due to the high energy demand of these processes. (Bartelt *et al.*, 2011; Saely, Geiger and Drexel, 2011). A cold temperature setting induces the increased uptake of FAs and their oxidation for heat production (Ertunc and Hotamisligil, 2016). BAT is highly innervated by sympathetic efferent fibers that link it to the hypothalamus and enables tight regulation by the brain (Hankir, 2018). In that manner, a cold temperature or energy surplus, sensed by afferent nerves of the hypothalamus lead to noradrenaline secretion via sympathetic efferent nerves that in turn activate BAT to produce heat and increase the corporal temperature (Hankir, 2018). Interestingly, while WAT is positively correlated with BMI, the inverse is true for BAT (Saito *et al.*, 2009). Moreover, BAT was implicated in the control of vascular lipoprotein homeostasis through TG-rich lipoprotein uptake from the circulation (Bartelt *et al.*, 2011). Furthermore, obese patients often have increased serum concentrations of leptin and decreased adiponectin, which is associated with higher adiposity (Huber *et al.*, 2008).

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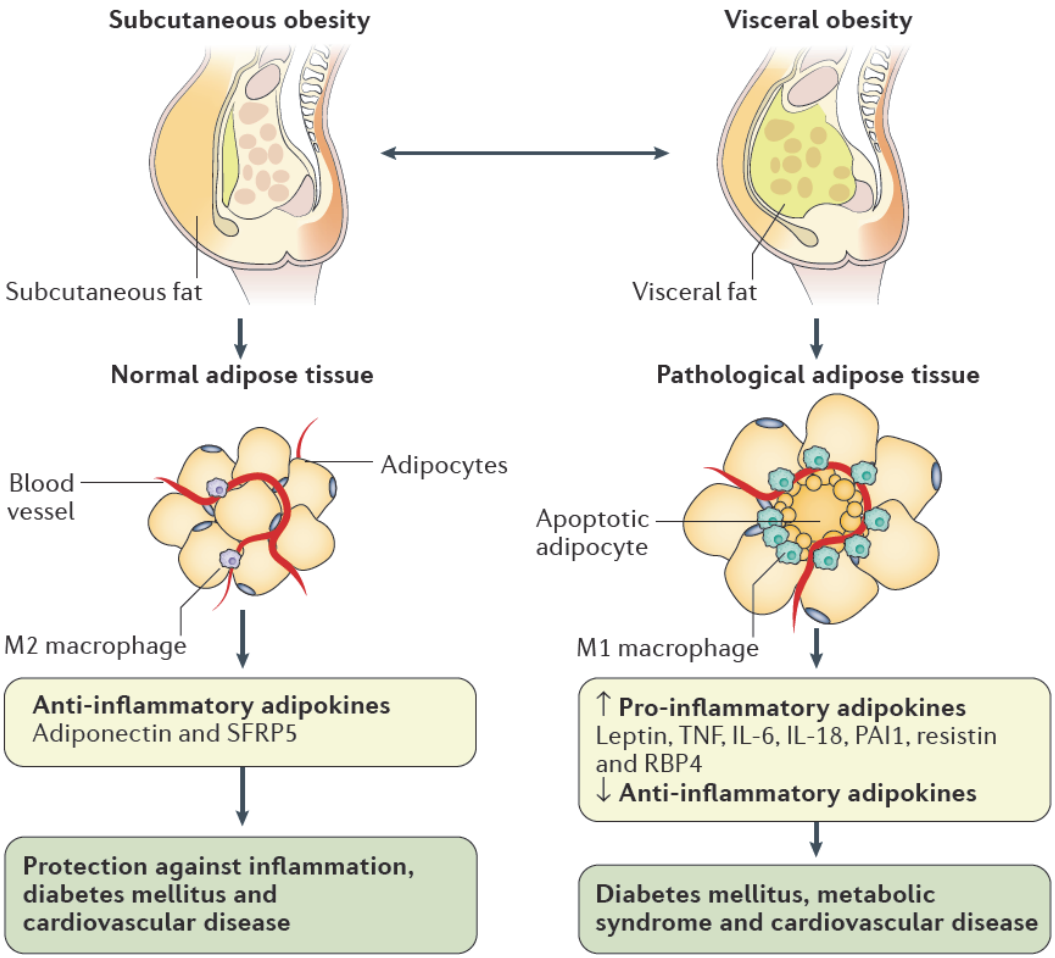

Figure 4: HFD-induced alterations in WAT associated with obesity and insulin resistance.
(Adapted from (González-Muniesa et al., 2017)).

Depending on their location in the body, WAT can further be classified into subcutaneous and visceral AT, which in turn consists of intra-abdominal, perirenal and pericardial AT (Fig. 4)(González-Muniesa *et al.*, 2017). WAT is composed of primarily adipocytes, a cell serving as a storage unit of energy as triacylglycerol, but contains also other cells like fibroblasts, mast cells, granulocytes, lymphocytes, macrophages, endothelial cells, pericytes, and precursors of adipocytes, and are commonly referred to as stroma-vascular fraction (Coelho, Oliveira and Fernandes, 2013). FAs are stored in white adipocyte lipid droplets as esterified and compartmentalized FAs. Although AT is the primary storage unit for lipids and FAs, this form of lipid storage can occur in all cells of higher organisms (Ertunc and Hotamisligil, 2016).

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When the body is in a situation of increased energy demand, adipocytes can liberate free FAs from lipid droplets via lipolysis and release them into the circulation to be accessible by other tissues for mitochondrial FA oxidation (Ertunc and Hotamisligil, 2016).

When the body undergoes metabolic stress like an excess consumption of energy dense food, AT can undergo changes like hypertrophy or hyperplasia, where adipocytes either increase in size or in number, in adaptation for the increased energy storage requirements of incoming lipids (Fig. 4). However, due to limited capacity of AT, chronic overnutrition might cause the saturated and hypertrophied adipocytes to rupture and cause inflammation and phagocytosis by macrophages. In addition, the saturation of AT capacity causes ectopic lipid accumulation in other undesired locations such as the liver, muscle, pancreas, heart, BAT, kidneys and the brain (Fig. 5) (Posey *et al.*, 2009; Guebre-Egziabher *et al.*, 2013; González-Muniesa *et al.*, 2017). This excess accumulation of intra-abdominal visceral fat during the development of diet-induced obesity (DIO) can be measured via waist circumference and used as a clinical determinant of obesity. Interestingly, while increased abdominal fat is associated with metabolic syndrome and inflammation, subcutaneous fat is less associated with metabolic dysfunction, insulin resistance and inflammation (Fig. 4) (Koster *et al.*, 2010; Esser *et al.*, 2013).

This increase in AT as well as ectopic lipid accumulation gives rise to chronic low-grade inflammation, upregulation of inflammatory gene expression and acute phase proteins, infiltration of macrophages and production of cytokines and chemokines, which in turn can attract other immune cells to infiltrate the tissue (Fig. 5)(Gregor and Hotamisligil, 2011; Ertunc and Hotamisligil, 2016). AT is an endocrine organ that secretes adipokines including hormones like leptin, adiponectin, visfatin, and omentin; acute phase proteins like plasminogen activator inhibitor 1 (PAI-1), lipocalin and serum amyloid A (SAA) among others. Adipokines include also cytokines like interleukin-1 (IL-1β) and its receptor antagonist IL-1Ra, IL-6 IL-10, IL-7 and tumor necrosis factor-α (TNF-α); chemokines like interleukin-8 (IL-8), CCL2, 3, 4, and

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CCL5 but also growth factors like tumor growth factor (TGF)-β as well as components of the complement system (Calder *et al.*, 2011). Obesity and increased visceral adiposity is associated with increases in leptin secretion and leptin resistance, while adiponectin is negatively correlated with obesity and adiposity (Calder *et al.*, 2011; Jung and Choi, 2014). Furthermore, while leptin is considered to be pro-inflammatory and secreted like SAA in proportion to AT size, adiponectin appears to be anti-inflammatory (Calder *et al.*, 2011).

In the context of HFD-induced obesity, many studies have shown that cytokines and chemokines, in particular TNF-α, IL-1β, IL-6, IL-10, CCL2 and CCL5 are not only upregulated both systemically and in AT and other tissues but play important roles in metabolic dysfunction and insulin resistance (Calder *et al.*, 2011; Gregor and Hotamisligil, 2011).

Cytokines are glycoproteins, which are produced and secreted on demand by many different cell types in response to different triggers like infection, injury but also other signals like cellular stress and metabolic stress. They play important roles in different functions but are most famous for their role in the immune response and as part of the innate immune response can activate other immune cells via specific cytokine receptors on the membrane of target cells. Similarly, they are implicated in the differentiation of lymphocytes and hematopoiesis among other functions. Cytokines include, TNF-α, interleukins (IL), chemokines, and interferons (IFN). Cytokines can be further classified into functional groups depending on their effect, such as pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory cytokines (IL-4, IL-10 and IL-13).

Cytokine and chemokine levels were found to be higher in serum of obese patients and mice. For instance, one study found elevated serum levels of C-reactive protein in addition to IL-6 in obese patients compared to lean controls as well as upregulated macrophage markers in not only visceral but

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also SC AT, which is indicative of increased immune cell infiltration (Huber *et al.*, 2008).

Several important studies have demonstrated not only an increase in the infiltration of macrophages into the expanding AT in both obese mice and humans (Weisberg *et al.*, 2003). In addition to that, it was also shown that macrophages shift their phenotype from an anti-inflammatory M2 profile ("classically activated") to pro-inflammatory M1 type ("alternatively activated") in obesity development (Lumeng, Bodzin and Saltiel, 2007; Kitade *et al.*, 2012; Chylikova *et al.*, 2018). Chemokines like CCL2 and CCL5 have been shown to participate in the recruitment of immune cells and the polarization of macrophages to M1 types in AT in response to HFD (Kanda *et al.*, 2006; Wu *et al.*, 2007; Keophiphath *et al.*, 2010; Kitade *et al.*, 2012). These macrophages can recruit other immune cells to the AT through the secretion of cytokines and chemokines as has been shown previously (Chawla, Nguyen and Goh, 2011). In fact, other immune cells like T cells have been shown to play an important role in the contribution of chronic low-grade inflammation in AT but also other tissues in obesity. Interestingly, infiltration of T cells has recently been reported as important factor in the regulation of macrophage trafficking and polarization in AT in the context of DIO, as TH1 $CD4^+$ and $CD8^+$ T cell count was raised in AT of obese rodents and humans (Nishimura *et al.*, 2009; Strissel *et al.*, 2010). (Wu *et al.*, 2007; Feuerer *et al.*, 2009; Nishimura *et al.*, 2009; Calder *et al.*, 2011; Esser *et al.*, 2013). Some studies found not only infiltration by T cells into AT in an obesity context, but also that T cell infiltration might precede recruitment of macrophages (Wu *et al.*, 2007; Kintscher *et al.*, 2008; Rausch *et al.*, 2008).

Furthermore, AT macrophages can maintain and further contribute to the obesity associated low grade-inflammation through the secretion of cytokines and chemokines (Curat *et al.*, 2006).

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Interestingly, weight loss interventions such as exercise, diet or surgery decreases recruitment of macrophages and pro-inflammatory markers in AT and plasma of obese patients (Cancello *et al.*, 2005; Bruun *et al.*, 2006).

Obesity is associated with other impairments of AT like increased lipolysis and decreased lipogenesis, which adds to the lipotoxicity of lipds (Gregor and Hotamisligil, 2011; Gao *et al.*, 2015; Ertunc and Hotamisligil, 2016).

Interestingly, TNF-a treatment of adipocytes induces lipolysis, increasing the amount of FFAs, and inhibits the differentiation of pre-adipocytes into mature adipocytes (Kawakami *et al.*, 1987; Pape and Kim, 1988; Feingold *et al.*, 1992). The latter effect might be mediated by peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor of the nuclear hormone receptor family, which plays a crucial role in adipogenesis, differentiation, AT function and gene expression and is downregulated by inflammation in AT (Guilherme *et al.*, 2008). PPARγ can be activated by molecules like FAs and eicosanoids, that can act as ligands, and is further implicated in peroxisome proliferation, lipid metabolism in neurons but also in insulin sensitivity in different tissues, energy balance regulation and feeding behavior (Issemann and Green, 1990; Dreyer *et al.*, 1993; Lu *et al.*, 2011; Ryan *et al.*, 2011; Garretson *et al.*, 2015).

Interestingly, neuron-specific knockout (KO) of PPARγ in a DIO mouse model resulted in reduced food intake and increased EE and ultimately in BW loss in HFD-fed KO mice compared to controls. Furthermore, this study showed that PPARγ signaling is required for the insulin sensitizing effect of thioazolidinediones (Lu *et al.*, 2011).

Experimental models of obesity and diabetes demonstrated increased TNF-a expression and accumulation of inflammatory cells in muscle tissue and showed a link between elevated circulating levels of FAs and TG with muscle insulin resistance (Ertunc and Hotamisligil, 2016).

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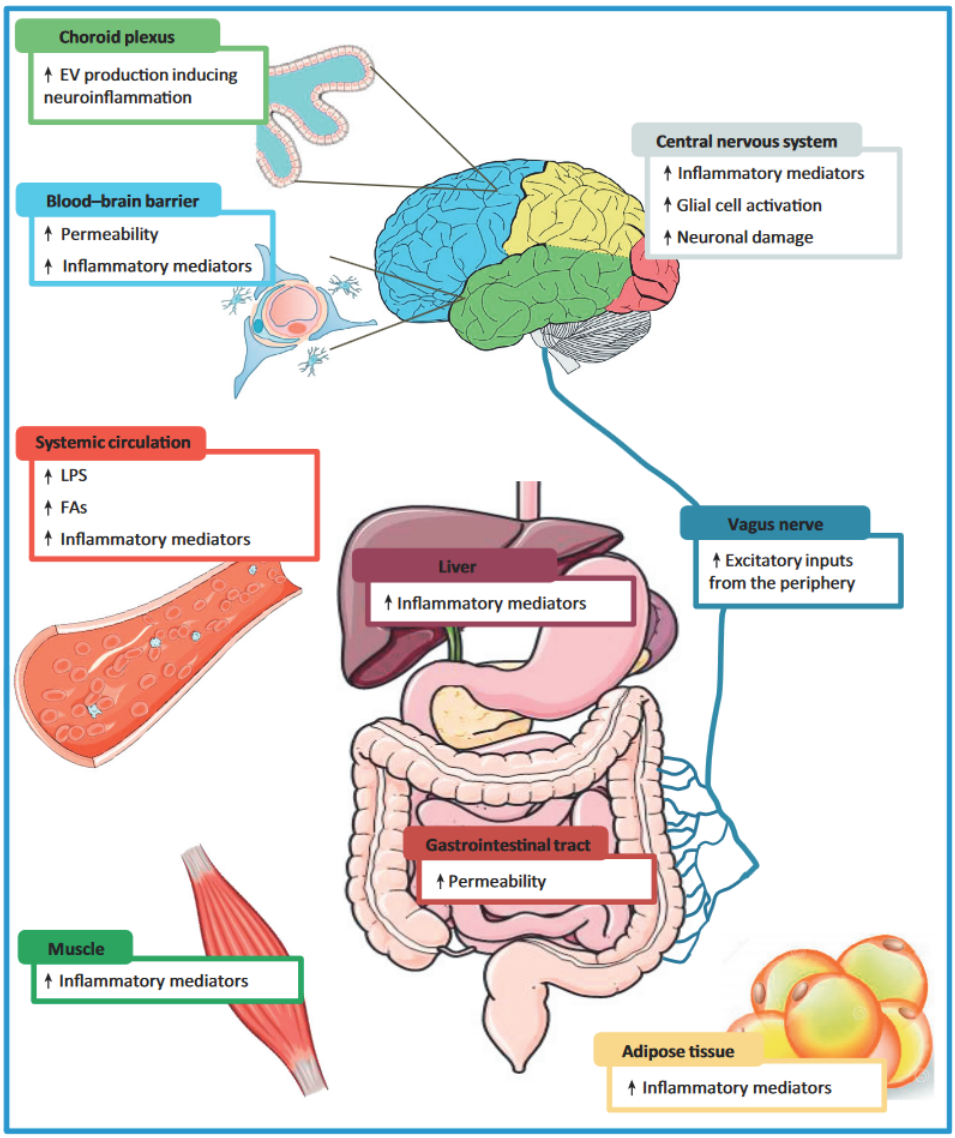

Figure 5: Inflammatory effects of HFD on different tissues in DIO.
HFD-induced development of obesity and metabolic syndrome involves many different tissues and players. Chronic HFD feeding in DIO leads to excess lipid accumulation in AT and eventually ectopic lipid accumulation in other tissues, once the capacity of AT is reached and increases serum levels of FFAs. Excess of lipid accumulation impairs the proper neutralization and storage of lipid metabolites and results in lipotoxicity. This in turn leads to infiltration of immune cells into AT as well as other tissues, a change in phenotype from a pro-inflammatory to anti-inflammatory state and induces the increased production of inflammatory mediators. HFD induces an increase in the permeability of intestines and changes in microbiota leading to gut dysbiosis, and the increased release of lipopolysaccharide (LPS) by gut bacteria resulting in endotoxemia and further potentiation of the inflammatory response. Furthermore, the BBB increases its permeability, increasing the flux of nutrients into the brain as well as the infiltration of immune cells. The increased accumulation of lipids in the hypothalamus induces neuroinflammation, the increase in immune mediators as well as the activation of glia cells. Hypothalamic inflammation can ultimately result in changes in neuronal activity, as well as neuronal damage or apoptosis. (Adapted from (Guillemot-Legris and Muccioli, 2017)).

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2.3.3. Central Inflammation in Obesity

Many studies have shown that HFD induces chronic but low-grade central inflammation in the hypothalamus but also in other extra-hypothalamic areas such as the hippocampus, cortex, brainstem or amygdala (Guillemot-Legris and Muccioli, 2017). It has been documented that hypothalamic inflammation occurs as early as 1 to 3 days after HFD consumption and is characterized by an upregulation of pro-inflammatory cytokine expression such as IL-1β and inhibitor of nuclear factor kappa-B kinase, but also a marker for neuronal injury, namely heat shock protein 72 (Thaler *et al.*, 2012). This early, acute but transient immune response is followed by reactive gliosis, which is referring to the change in microglial and astrocyte activity, which then recedes in an adaptive response to avoid neuronal injury. However, with long-term exposure to HFD reinstates the inflammatory response, which becomes chronic (Thaler *et al.*, 2012; Berkseth *et al.*, 2014). Recent results confirm the occurrence of hypothalamic gliosis also in human obese subjects via magnetic resonance imaging and link it with insulin resistance (Schur *et al.*, 2015). Other studies have found not only hypothalamic inflammation in DIO rodent models, but also signs of apoptosis in the ARC and the LH and more specifically in neurons like AgRP and POMC of the ARC, which seemed to be dependent on diet composition more than caloric intake (Moraes *et al.*, 2009). Interestingly, the same study identified that the presence of toll-like receptor 4 (TLR4) seemed to be protective in the context of HFD-induced neuronal apoptosis and suggested a dual-function of TLR4 in hypothalamic inflammation.

Interestingly, another study demonstrated the importance of inhibitor of κB kinase β (IKKβ)/ nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway activation and endoplasmic reticulum (ER) stress in HFD or overnutrition-induced obesity and associated leptin and insulin resistance (Zhang *et al.*, 2008). Forced activation of IKKβ/NF-κB impaired hypothalamic insulin and leptin signaling, they showed that inhibition thereof, including specific inhibition in AgRP neuron, protects against glucose

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intolerance and obesity. Authors suggested that this effect might be mediated through suppressor of cytokine signaling-3 (SOCS3), a common inhibitor of both insulin and leptin signaling pathways (Zhang *et al.*, 2008). Other inflammatory pathways found to be activated in DIO-associated hypothalamic inflammation are IKKβ/NF-κB and mitogen-activated protein kinase (MAPK) pathways as well as c-jun N-terminal kinase (JNK) signaling (Milanski *et al.*, 2009; Benzler *et al.*, 2013, 2015; Rahman *et al.*, 2018). NF-κB is a key mediator of cellular immune and inflammatory responses and mediates the expression of pro-inflammatory genes as a transcription factor. MAPK kinases are upstream of JNKs and induce their activation via phosphorylation in response to stress signals like nutritional components, hyperglycemia, ER stress or oxidative stress, resulting in the production of cytokines. JNKs are a lso inflammatory signaling molecules than can affect many metabolic processes because they can modulate the activity of many nuclear transcription factors like c-Jun, activator protein, forkhead box protein O4, nuclear hormone receptors and activating transcription factor 2 among others (Rahman *et al.*, 2018).

The implication of inflammatory pathways in hypothalamic inflammation and its contribution to DIO was shown for example in a study that inhibited specifically IKKβ in the hypothalamus and observed a restoration of both leptin and insulin sensitivity of obese rodents (Zhang *et al.*, 2008). TNF-a has been shown to induce the expression of other cytokines like IL-1β, IL-6 and anti-inflammatory molecules like IL-10 and SOCS3, as well as activate signal transduction pathways like JNK and NF-κB, and modulate the expression of neuropeptides involved in the regulation of energy balance and feeding regulation in the hypothalamus of rats (Amaral *et al.*, 2006; Romanatto *et al.*, 2007). In addition to that blocking either TLR4 or TNF-a reduces hypothalamic inflammation as well as its resistance to leptin and restores hepatic glucose production (Milanski *et al.*, 2012). Interestingly, also the inhibition of neuronal IKKβ/NF-κB signaling in ARC neurons reduces BW and fat gain in response to HFD feeding and increased expression of EE (Benzler *et al.*, 2015). Another

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study demonstrated that inhibiting TLR4 signaling pathway in the hypothalamus reduces hypothalamic inflammation, including IKKβ and improves insulin and leptin resistance as well as BW gain associated with HFD (Kleinridders *et al.*, 2009).

Chemokines are important participants in the hypothalamic inflammation associated in DIO. For example, the chemokine fractalkine (CX3CL1) was found to be involved early on in the hypothalamic inflammation in HFD-fed mice (Morari *et al.*, 2014). Its expression was induced early in neurons of HFD-fed mic, while its inhibition decreased hypothalamic inflammation and the hypothalamic infiltration of bone marrow-derived monocytes.

It is still uncertain though what initiates that first inflammatory response in the hypothalamus. Several theories have been explored in an attempt to explain the as of yet uncovered mechanism.

Oxidative stress is one of the factors that was suggested to be an initiator of inflammatory events and metabolic abnormalities associated with DIO. It has been reported that oxidative stress and the production of reactive oxygen species (ROS) are upregulated in metabolic tissues in the periphery of DIO mice well before metabolic impairments such as insulin resistance become apparent (Matsuzawa-Nagata *et al.*, 2008). Similarly, hypothalamic neurons can be effected particularly by ROS as the brain consumes 15-20% of the total energy generated by the body (Garbarino *et al.*, 2015). Mitochondria use glucose or lipids and their metabolites for energy production in form of ATP, which as a side product yields ROS. Neurons in particular have many mitochondria due to their high demand in energy, and are thus more susceptible to ROS. Furthermore the brain has a high proportion of ω-3 polyunsaturated FAs (PUFA) containing phospholipids, which are highly susceptible to lipid peroxidation compared to less saturated lipids (Garbarino *et al.*, 2015).

Some studies implicate ER stress and oxidative stress as an important component in the initiation of HFD-induced hypothalamic inflammation

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(Özcan *et al.*, 2004; Zhang *et al.*, 2008; Milanski *et al.*, 2009). A chronic excess of nutrients can lead to cellular stress like ER or oxidative stress, for example through an increase in mitochondrial ATP production, which in turn increases the production of ROS, for example during mitochondrial oxidation of FFAs (Tran *et al.*, 2016). ROS can lead to protein misfolding and thereby add more stress to the ER, which might activate the unfolded protein response (UPR) (Özcan *et al.*, 2004; Tran *et al.*, 2016).

Another interesting factor that was linked to hypothalamic dysfunction in the context of DIO is mitochondrial abnormalities and changes. It has been found that changes in the number of mitochondria, their size and the number of contacts they have with ER seems in AgRP and POMC neurons play a role in the development of DIO (Dietrich, Liu and Horvath, 2013; Schneeberger *et al.*, 2013). Furthermore, it was shown that chronic microglial activation associated with DIO leads to secretion of TNF-a, which in turn induces mitochondrial stress in POMC neurons and thus contributes to obesity (Yi *et al.*, 2017).

Interestingly, studies have found that acute hypertriglyceridemia as well as increased glucose increases mitochondrial respiration and transiently elevates ROS production in the hypothalamus without inducing any cytotoxic effects and rather play a part in hypothalamic nutrient signaling (Leloup *et al.*, 2006; Benani *et al.*, 2007). Systemic overload of lipids has not only been shown to promote the activity of hypothalamic neurons and alter their expression of neuropeptides but also hepatic insulin sensitivity. Moreover, it was shown that ICV injections of long-chain FAs has an inhibitory effect on food intake and whole body glucose production while promoting energy storage (Obici, Feng, Morgan, *et al.*, 2002; Obici and Rossetti, 2003; Obici *et al.*, 2003). The study by Benani and colleagues further extended these results by demonstrating that hypothalamic ROS production is required for the reduction of food intake induced by hypertriglyceridemia, while fasting abolished the hypertriglyceridemia-induced ROS production (Benani *et al.*, 2007). The authors suggest that mitochondrial ROS production might thus be a physiological part of nutrient sensing in hypothalamic neurons (Leloup *et al.*,

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2006; Benani *et al.*, 2007). It is thus not difficult to imagine that chronic overconsumption of nutrients such as glucose and lipids, could lead to and overproduction of ROS, and overload the cells antioxidant capacities and thus lead to damage induced by ROS, which might affect hypothalamic neurons regulating energy balance in the ARC.

Another interesting study investigated the time course of events in the hypothalamus like inflammation, ER stress and mitochondrial changes associated with HFD. The first event they observed in mice on a HFD after 3 hours was an increase in the hypothalamic expression of the chemokine fractalkine, followed by an inflammatory response characterized by an increase in cytokine expression. Subsequent to these events, they found increases in an ER chaperone after 6 hours although ER stress was not detected until 3 days after HFD. One day after HFD started, reductions in markers for mitochondrial fusion and tethering to ER were found, which however increased days after HFD consumption began (Carraro *et al.*, 2018). These experiments show that inflammation seems to be the earliest event after HFD consumption and might lead to other cellular event that could be damaging to the cell if HFD consumption persists. However, all events seem to have an important part to play in the development of hypothalamic dysfunction leading up to the development of DIO and associated metabolic impairments. Some studies have also investigated autophagy in this context. Autophagy is a cellular mechanism that helps maintain homeostasis through the elimination of dysfunctional organelles or proteins but also lipids and can have pathological role during metabolic stress (Kim and Lee, 2014; Rahman *et al.*, 2018). Some studies found that HFD consumption can lead to defects in autophagy mechanisms in cells of the hypothalamus, which was associated with hypothalamic inflammation and a change in BW gain different susceptibility to DIO depending on the cell type that is affected (Kaushik *et al.*, 2011, 2012; Meng and Cai, 2011; Portovedo *et al.*, 2015). It was further shown that inflammatory mediator production in part by microglia could decrease autophagy in neurons while chronic HFD seemed to increase

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autophagy and decrease the number of hypothalamic POMC neurons (Alirezaei *et al.*, 2008; Thaler *et al.*, 2012).

Firstly, it was suggested that elevated circulating FFAs, in particular saturated FAs (SFA), or certain lipids accumulate in the hypothalamus upon HFD consumption and could themselves induce the inflammatory response (Posey *et al.*, 2009). One of the hypothesis stated that hypothalamic neurons themselves could start the inflammatory process in response to sensing elevated FFAs (Tran *et al.*, 2016). Neurons of the hypothalamus can sense FFAs through either expression of TLR4, or FFA metabolism-dependent mechanisms to initiate inflammation (Tran *et al.*, 2016). Interestingly, a study has shown that inhibiting TLR4 or TNF-a via antibodies, pharmacologically or via mutation, abrogated hypothalamic inflammation as well as improved hypothalamic leptin resistance and protected not only from obesity but also abolished signs of liver steatosis and hyperglycemia (Milanski *et al.*, 2009, 2012).

One study found that specific FFAs such as long-chain palmitoyl and stearoyl-CoA but not oleoyl-CoA are found accumulated in the hypothalamus in response to HFD and induce hypothalamic inflammation, leptin and insulin resistance (Posey *et al.*, 2009). Similarly, ICV injection of FFAs such as palmitate induced the upregulation of pro-inflammatory cytokines like TNF-a, IL-1β, and IL-6 and activated inflammatory pathways such as JNK, NF-κB and IKKβ in the hypothalamus of rodents. These events resulted in hypothalamic leptin and insulin resistance (Arruda *et al.*, 2011; Cheng, Yu, Szabo, *et al.*, 2015). Central inhibition of IKKB in HFD-fed rats was associated with reduced food intake and improved hypothalamic insulin sensitivity (Posey *et al.*, 2009).

Interestingly, another study found that not HFD specifically, but rather overconsumption of single nutrients can induce the activation of inflammatory pathways like IKKβ/NF-κB in the hypothalamus as demonstrated in a model of hyperphagic obese mice on a normal diet, and ICV infusion of glucose or oleic acid into the 3V (Zhang *et al.*, 2008). In contrast to other studies, this study also showed that the hypothalamic activation of IKKβ/NF-κB

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pathway is independent of cytokine upregulation but rather induced via elevated ER stress. Apart from neurons, FAs and their metabolites can induce inflammatory responses in non-neuronal cells like microglia and astrocytes and resident macrophages (Lee *et al.*, 2003; Anderson, Hill and Hasty, 2012; Valdearcos *et al.*, 2014). Studies have shown that unlike unsaturated, SFAs like palmitic acid, lauric acid and stearic acid can induce inflammatory responses in cultured astrocytes from rat brains, such as the release of cytokines like TNF-a and IL-6 (Gupta *et al.*, 2012). However, there seem to be some variations, as another study in mice, did not find inflammatory responses in astrocytes, while another study confirmed no responses for astrocytes but found that microglia became "activated" in mice after palmitic acid treatment. Moreover, it has been found that microglia and resident macrophages react to palmitic and stearic acid with activation of pro-inflammatory signaling pathways dependent on TLR and lead to impairments in insulin signaling by phosphorylation of protein kinase C (PKC), Akt and insulin receptor substrate (IRS) 1 (Lee *et al.*, 2003; Benoit *et al.*, 2009; Anderson, Hill and Hasty, 2012; Valdearcos *et al.*, 2014). Interestingly, intracellular accumulation of FFA in macrophages can result in activation of inflammatory pathways independent of TLR, which mediate ER stress and ultimately lead to apoptosis. Furthermore, the polarization of macrophages to a pro-inflammatory state (M1), as was found in infiltrated macrophages in AT of obese mice and humans, renders them more susceptible to ER stress (Anderson, Hill and Hasty, 2012; Kitade *et al.*, 2012; Chylikova *et al.*, 2018).

Fitting to these results studies have suggested that a high ratio of ω-6 to ω-3 ratio is unfavorable and increases the risk of developing obesity (Simopoulos, 2016). While ω-3 PUFAs are considered to have anti-inflammatory properties, the opposite seems to be true for SFAs and ω-6, which are pro-inflammatory (Simopoulos, 2008, 2016; Margioris, 2009).

One study reported that SFAs and polyunsaturated ω-3 FAs both had a reciprocal modulating effect on the TLRs and its downstream signaling pathways. While the SFAs like lauric acid induced TLR4 activation, the PUFA

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docosahexaenoic acid (DHA) inhibited Akt, a downstream signaling component of TLR4, induced by lipopolysachharide (LPS) or lauric acid and suppressed TLR4-dependent NF-κB activation (Lee *et al.*, 2003).

Data has previously shown that the ICV injection of arachidonic acid, an ω-6 PUFA) induces hypothalamic leptin resistance and impairs its downstream signaling regulating hepatic glucose and lipid metabolism (Cheng, Yu, Zhang, *et al.*, 2015).

Moreover, another theory suggests that diet-induced inflammation might be due to HFD causing a shift in the gut microbiota (Everard *et al.*, 2011). Changes in the composition of gut microbiota populations can in turn cause an increased permeability of the intestines, which enables facilitated entry of bacterial endotoxins like LPS into the blood stream leading to endotoxemia. Endotoxemia was shown to activate the innate immune system in both humans and rodents in response to HFD (Fig. 5) (Cani *et al.*, 2007, 2008; Pendyala, Walker and Holt, 2012).

2.3.4. Hypothalamic Gliosis

Apart from neurons the brain contains also glia cells that comprise about 50% of the total cells of the brain and can be broadly divided into astrocytes, microglia, tanycytes and oligodendrocytes (Azevedo *et al.*, 2009). Glia cells usually are reported as having a supporting function to neurons, regulate neuronal activity and play a critical role in regulating nutrient uptake and metabolism. In addition to that, microglia are often seen as the immune cells of the CNS (Morari *et al.*, 2014; Le Foll, 2019).

The HFD-induced inflammation is also associated with hypothalamic reactive gliosis – the change in glial activity such as astrocytes and microglia into a "reactive phenotype" characterized by a change in morphology, proliferation and an altered gene expression profile (Horvath et al., 2010). This reactive state is characterized by the upregulation of cell specific structural proteins such as glial fibrillary acidic protein (GFAP) for astrocytes and ionized calcium binding adaptor molecule 1 (Iba1) for microglia. Furthermore, they

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produce cytokines such as TNF-a, IL-1β and IL-6 (De Souza *et al.*, 2005) but also chemokines like CXCL1 by microglia (Morari *et al.*, 2014).

3. Glucose Homeostasis

Glucose belongs to the carbohydrates, which we take up with our nutrition, and is the major source of energy to sustain the functioning of the human organism. While other tissues or cells can use other metabolites like FAs, glucose is the main source of energy for the brain. It is of major importance that glucose levels remain stable throughout day and night, independent of activity, food intake or times of fasting. If plasma glucose levels get too low this can be dangerous, is referred to hypoglycemia, and can lead to apart from cognitive dysfunction, seizures, myocardial infarction, and retinal cell death to coma and death (Kalra *et al.*, 2013). Equally important, hyperglycemia, a condition of chronic elevation of plasma glucose levels can have damaging effects on the functioning of the organism, as seen in diseases such as diabetes, obesity and cardiovascular disease among others. In healthy individuals, glucose is maintained between 4 to 7mM. To remain in this range glucose metabolism is tightly regulated by different mechanisms including gluconeogenesis (Fig. 6) in the liver, glucose absorption from food in the intestine and glucose uptake and utilization by peripheral tissues (Saltiel and Kahn, 2001).

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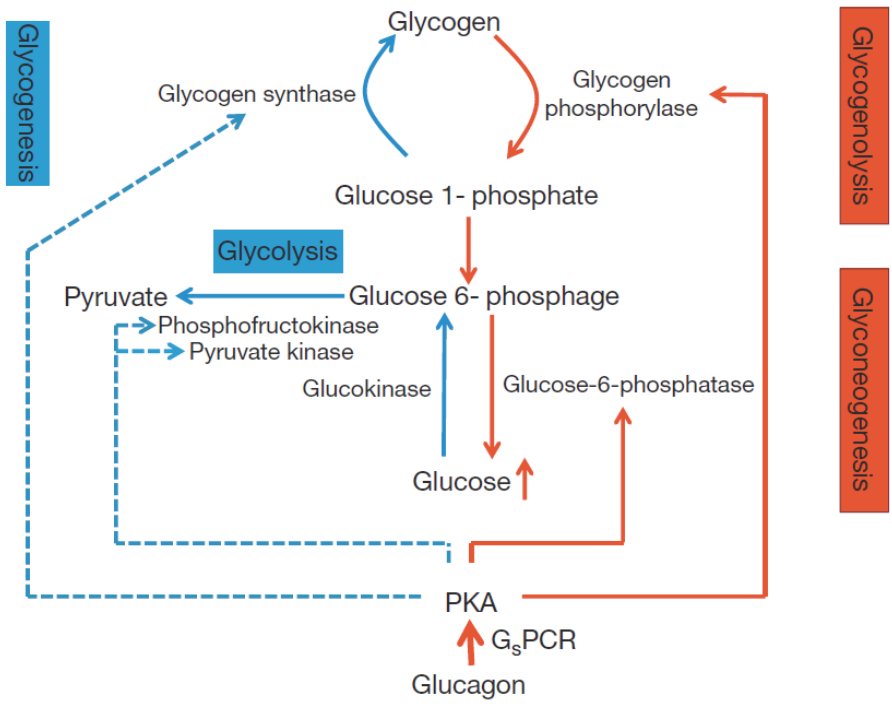

Figure 6: Glucose metabolism: Glucogenolysis and gluconeogenesis. (Adapted from (Shi and Liu, 2014)).

3.1. Central Regulation of Glucose Homeostasis

Glucose is the major source of energy for the brain and can enter the CNS via specific transporters like glucose transporter (GLUT) 1 in the BBB and is regulated based on the energy demand of the brain. GLUT1 is expressed throughout the CNS by astrocytes and endothelial cells of the BBB as well as in tanycytes. Circulating glucose is then taken up by perivascular astrocytes either via GLUT2 or passively through gap junctions and used for glycolysis or glycogenesis or could also represent a signal to inform glucose-sensing neurons about energy availability. Interestingly, several hormones have been reported to regulate glucose uptake in astrocytes, such as an increasing uptake via leptin and insulin-dependent pathway and a ghrelin-induced decrease in glucose uptake (García-Cáceres *et al.*, 2016; Freire-Regatillo *et al.*, 2017). Alternatively, glucose can alternatively be metabolized to lactate or derived from glycogen metabolism (Fig. 6). Lactate can then be secreted into the extracellular space for neurons to use as energy (Freire-Regatillo *et al.*, 2017). Some studies suggest that lactate provided by astrocytes is the primary and preferred source of energy for neurons and seems to be

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neuroprotective in cases of hypoglycemia (Wyss *et al.*, 2011). Furthermore, astrocytes store glucose in form of glycogen, which is converted to lactate via glycogenolysis for situations of shortage of nutrients or increased neuronal activity (Fig. 6). Interestingly, several hormones have been reported in increasing glycogen production in astrocytes, like insulin, insulin-like growth factor (IGF)-1 and leptin, while ghrelin seems to induce glycogenolysis in neurons of the hypothalamus (Fuente-Martín *et al.*, 2016; Freire-Regatillo *et al.*, 2017). In cases of prolonged fasting or when glycogen stores are exhausted, astrocytes can take up FAs as alternative source of energy, which undergo β-oxidation in mitochondria FAs into ketone bodies like β-hydroxybutyrate. These are either used themselves or released for neurons or other glia cells to use as energy.

Moreover, special glia cells lining the basal wall of the 3V in the hypothalamus, which are called tanycytes, are in close contact with capillaries and have been reported to sense and metabolize glucose and express GLUT2 (Elizondo-Vega *et al.*, 2015; Barahona *et al.*, 2018). Interestingly, other glucose transporters like GLUT1, 3 and 5 have been found on astrocytes, neurons and microglia, respectively (Pomytkin *et al.*, 2018).

Central glucose sensing is attributed to two types of neurons. Firstly, there are neurons that are excited when glucose levels increase in the extracellular fluid and are mostly found in hypothalamic nuclei like VMH, Arc and PVN. Secondly, neurons that are inhibited by declining extracellular levels, are located in the LH, ARC and the PVN. Interestingly, the dorsal vagal complex consisting of the NTS, area postrema and the dorsal motor nucleus of the vagus contain both type of glucose-sensing neurons (Roh, Song and Kim, 2016).

3.2. Pancreatic Regulation of Glucose Homeostasis

The pancreas is a complex organ that is subdivided into four continuous parts, a hook-shaped head around the superior mesenteric vein, a neck, body and tail (Fig. 7). It is composed of a system of ducts connecting exocrine glands

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that regulate digestion by secreting digestive enzymes via a duct, which is fused at the distal end with the common bile duct, into the duodenum (Fig. 7, lower panel). Additionally, it contains endocrine glands called islets of Langerhans, which are involved in controlling nutrient metabolism such as glucose, protein and fat metabolism by secreting hormones into the circulation via the portal vein (Cade and Hanison, 2017). Around 1-2 million ovoid-shaped islets are interspersed throughout the pancreas between acini of a healthy human. The pancreas is located in between the duodenum underneath the stomach and connected via the vena cava to the liver and via ducts to the intestine as well as to lymphatic vessels (Fig. 7) (Shi and Liu, 2014; Cade and Hanison, 2017). It is regulated in part by the brain through dense innervations by sympathetic fibres and parasympathetic fibers as well as neurons expressing neuropeptides, which stimulate the release of both amines and peptides. While sympathetic fibers signal through the splanchnic nerves, the parasympathetic fibers transmit information via the vagus nerve to the brain.

The pancreas is subdivided into two functionally distinct glands, the exocrine pancreatic gland and the endocrine pancreatic gland.

The exocrine pancreas represents 99% of the pancreas and constitutes a large ductal system interconnecting a large amount of acini that form lobules of 1-10 mm size and connected by interlobular pancreatic duct. One acinus consists of around 40 pyramid-shaped acinar cells, collage, fibroblast and a tissue surround the acinus including capillaries. Acinar cells contain zymogen granules containing proenzymes, necessary for digestion such as trypsin, chymotrypsin, lipase, amylase and elastase (Shi and Liu, 2014).

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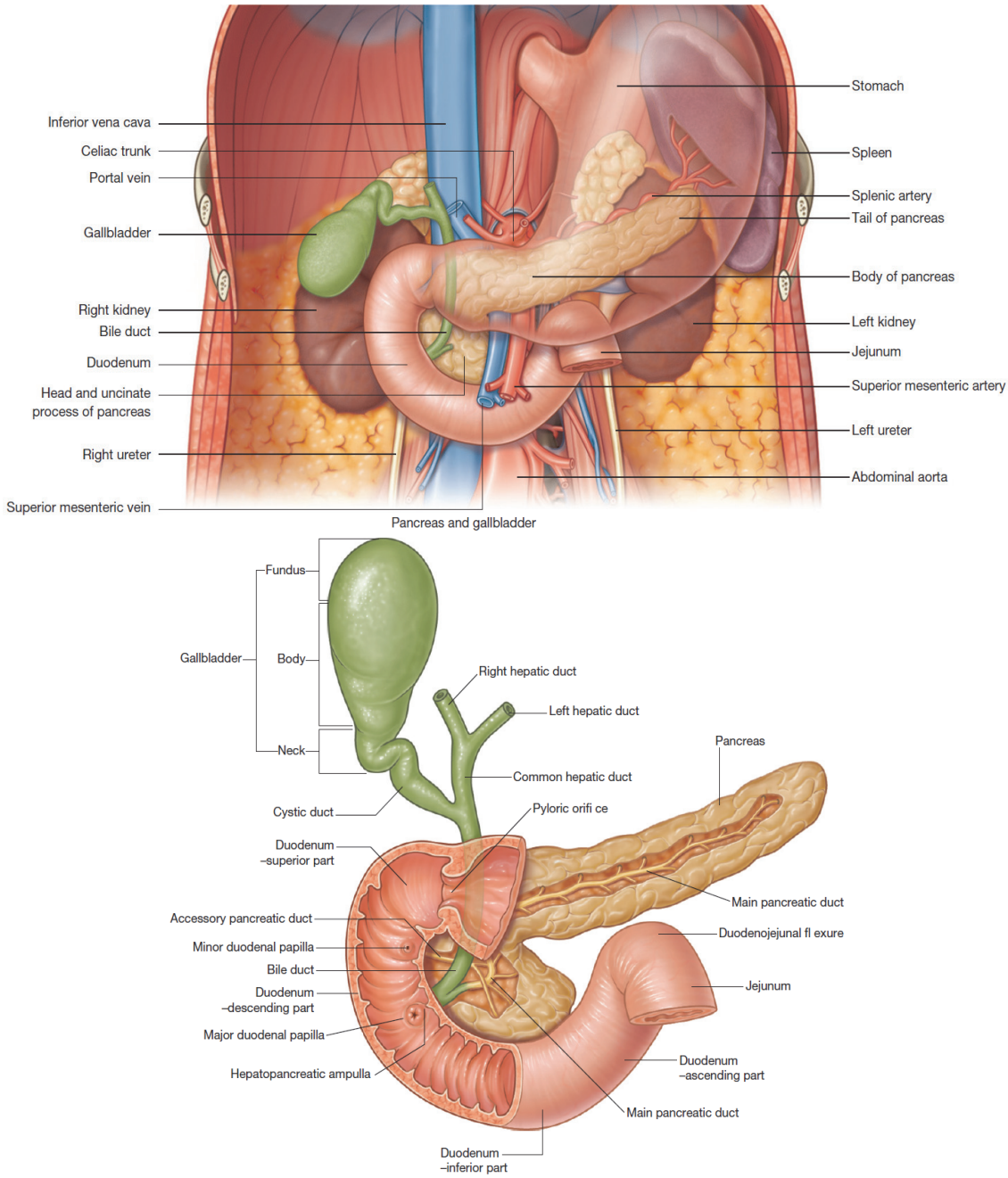

Figure 7: Anatomical location of the human pancreas.
(Adapted from (Shi and Liu, 2014)).

The endocrine pancreas consists mainly of islets of Langerhans scattered across the pancreas and represents 1% of the whole pancreas. The human pancreatic islets range in size between 50 to 250 µm and are made up of 30% glucagon-producing alpha cells; ~60% insulin-producing Beta cells; 10% somatostatin-producing Delta cells and 1% pancreatic polypeptide-producing PP cells (also known as F cells) (Cabrera *et al.*, 2006; Shi and Liu, 2014). Furthermore, the islets of Langerhans contain ghrelin-producing epsilon

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cells, which are primarily found during gestational development and can give rise to alpha cells and PP cells, but rarely to beta cells in the adult pancreatic islets (Arnes *et al.*, 2012). The pancreas is well irrigated as it contains 10% of the pancreatic blood supply, which allows for the direct access of hormones to blood vessels, which drains into the hepatic portal vein before entering the systemic circulation. Secretion of hormones is tightly regulated through humoral paracrine and neural communication, which is facilitated by an innervation of islets by both sympathetic and parasympathetic nerve fibers. Furthemore, those innervations control not only blood flow of surrounding vessels but also increase cholinergic and adrenergic stimulation to increase or inhibit insulin secretion (Cade and Hanison, 2017).

The pancreas is continuously secreting small amount of insulin, which passes the liver before acting on other organs and tissue, and is secreting a larger amount upon carbohydrate stimulation after a meal (Katsarou *et al.*, 2017)

3.2.1. Glucagon

Glucagon, a hormone derived from proglucagon precursor by enzymatic cleavage by PC2, is composed of 29 amino acids, produced, and secreted from a-cells of the pancreatic islets in response to declining blood glucose levels. This happens during fasting state as well as during aerobic exercise, but has also been shown to be induced by increased levels of amino acids and the intestinal peptides oxyntomodulin and gastric inhibitory polypeptide (GIP). Interestingly, oxyntomodulin, like another peptide that has an inhibitory effect on glucagon, such as the intestinal incretin GLP-1, are likewise derived from the proglucagon peptide in the intestine through alternative cleavage by PC1/3 among other enzymes (references in Albrechtsen et al., 2016).

Furthermore, glucagon secretion is also regulated through paracrine actions (intra-islet factors), such as somatostatin, which inhibits glucagon.

It has been shown to not only be a powerful stimulator of insulin secretion, but is highly implicated in glucose metabolism regulation through regulating hepatic glucose production. In a fasting state, when glucose concentration in

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the blood is low, glucagon stimulates the hepatic production of glucose, termed gluconeogenesis and the biochemical breakdown of glycogen to yield glucose to raise blood glucose levels and keep them stable, and thereby regulates blood glucose homeostasis (Wewer Albrechtsen *et al.*, 2016). The subsequent rise in blood glucose levels, in addition to a stimulatory effect of glucagon on insulin secretion, maintains the balance of euglycemia in healthy individuals. It regulates blood glucose homeostasis, by increasing glucose production, termed gluconeogenesis in the liver, as well as glycogenolysis, the biochemical breakdown of glycogen to yield glucose in a fasting state (Albrechtsen et al 2016).

3.2.2. Somatostatin

Somatostatin is a peptide hormone and not only produced in pancreatic δ-cells but also in neurons of the hypothalamus and other endocrine tissues like the GIT. It inhibits both a - and β-cell activity by binding to one of five inhibitory GPCRs called somatostatin receptors 1-5 and thus reduces the synthesis and release of insulin, glucagon and pancreatic polypeptide (PP) through paracrine action. In addition, somatostatin acts as a hormone by acting on the anterior pituitary and GIT to inhibit growth hormone and GIT hormone activity and decrease gut motility, gastric acid, gastrin secretion and also pancreatic exocrine secretion (Shi and Liu, 2014; Cade and Hanison, 2017). Somatostatin is secreted by macronutrients like glucose, amino acids, and fat-derived ketone bodies, but also by hormones like CCK, gastrin, secretin and insulin in hyperglycemic states to prevent hypoglycemia as well as by glucagon to regulate its own secretion (Shi and Liu, 2014).

3.2.3. PP Peptide

PP, a hormone produced and secreted by pancreatic PP or F cells in fasting and hypoglycemic conditions or after protein meals, it has an inhibitory action on the secretion of insulin and somatostatin. It also reduces gallbladder contractility, gastrointestinal motility, gastrin acid and pancreatic enzyme secretion (Shi and Liu, 2014; Cade and Hanison, 2017).

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3.2.4. Insulin

Insulin is an anabolic hormone produced and secreted in a constant fashion and increased in response to elevated plasma glucose levels by pancreatic β-cells. It is stored as hexameric insulin/Zn2+ crystals in secretory granules in β cells (Fig. 8) that fuse with the plasma membrane upon glucose stimulation (Plum, Schubert and Brüning, 2005; Pomytkin *et al.*, 2018). Insulin is a very important hormone for the regulation of carbohydrate, protein and lipid metabolism. Postprandially, insulin secretion happens in two phases. During the first phase, which lasts about 10 min, the preformed insulin stored in vesicles is released by stimulation of ATP-sensitive K^+ (K_{ATP}) channels upon glucose absorption that induces changes in the ratio of ATP/ADP and cell depolarization activating Ca^{2+} channels. The entry of calcium then induces exocytosis of insulin granules. The second phase is much longer, can take up to an hour because insulin is newly synthetized, and released along with some remaining preformed insulin (Shi and Liu, 2014). Interestingly, other nutrients like amino acids and FFAs as well as hormones and neurotransmitters are also able to trigger or potentiate insulin release but most likely through different mechanisms that lead to changes in the electrical activity of β-cells. The latter are glucose dependent and involve the activation of the parasympathetic nervous system. For example at the level of oral taste buds, that signal glucose to the brainstem; or through vagal afferent neurons connected to the portal vein of the liver that signal to the brainstem and hypothalamus; and by direct glucose-sensing by neurons in the hypothalamus and respond by regulating β-cell activity (Shi and Liu, 2014).

3.2.4.1. Insulin Receptor Expression

Insulin can bind to IR, of which two isoforms are known until now, known as the short isoform IR-A and the long isoform IR-B. They are expressed either in cells of the brain or in adult peripheral tissues, respectively. Interestingly, neurons express exclusively IR-A, which binds, in addition to high affinity binding to insulin, IGF-2 that can activate it even at low concentrations (Pomytkin *et al.*, 2018). Astrocytes however seem to express both isoforms of IR. IR is expressed

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in several regions of the brain and the highest expression is found in the olfactory bulb, the hypothalamus, cerebral cortex, cerebellum and hippocampus (Plum, Schubert and Brüning, 2005; Pomytkin *et al.*, 2018). Interestingly, IGF-2, which can bind also other receptors IGF-1R and IGF-2R, is also widely expressed throughout the brain like the hippocampus, is upregulated in situations of cellular stress such as acute hypoxia, chronic stress, cerebral ischemia and exposure to toxicity. IGF-2 seems to confer neuroprotection in these stressful scenarios (Plum, Schubert and Brüning, 2005; Pomytkin *et al.*, 2018).

IRs are heterotetramers consisting of two α-subunits and two β-subunits, which are linked via disulfide bonds. While the extracellular α-subunits are responsible for ligand binding, the intracellular part of the transmembrane domain of the β-subunits are the sites of tyrosine kinase activity.

Additionally, it is also expressed in axons and especially in nodes of Ranvier in sensory neurons in dorsal root ganglia (DRG)(Feldman *et al.*, 2019).

3.2.4.2. Peripheral Insulin Action

It is a major regulator of glucose homeostasis and metabolism through a multitude of effects. Firstly, when plasma glucose levels rise postprandially, insulin is secreted from pancreatic β cell granules into the circulation, enters the liver via the portal vein in a pulsatile manner and is cleared there by 50% via degradation, before it gets to the heart and is distributed to the periphery via arterial circulation (Fig. 8B and C) (Tokarz, MacDonald and Klip, 2018). Insulin then can pass through the liver a second time via arterial circulation to regulate metabolic function. In the liver, the interaction of insulin with the IR activates the insulin receptor tyrosine kinase (IRTK) which induces the phosphorylation of IRS 1 or 2 that act through Akt to activate glycogen synthase (GS) to convert glucose to the storage form glycogen (Fig. 9). Likewise, the increased intracellular amount of glucose inhibits glucogenolysis and is processed via glycolysis and used for de novo lipogenesis (DNL). This process is facilitated by sterol regulatory element-binding protein (SREBP1),

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whose expression is upregulated through insulin signaling (Fig. 9). Furthermore, Akt phosphorylation inhibits the transcription factor called factor Forkhead box O1 (FOXO1) and thereby downregulates the transcription of enzymes needed for gluconeogenesis (Samuel and Shulman, 2016). In addition to that, the liver takes up plasma FAs, which are absorbed after food intake or from chylomicron remnants that are taken up from the circulation and uses them for lipogenesis (Fig. 8C).

The microvasculature and arterial circulation transmits insulin to muscle cells, AT and the brain (Fig. 8C). In skeletal muscles and AT, activation of insulin signaling leads to a translocation of vesicle-stored glucose transporters (GLUT: GLUT4 in AT and muscles; GLUT2 in liver, kidney, intestine, pancreas and neuronal and glial cells of the CNS (Thorens, 2015)) via the canonical Insulin signaling pathway to PI3K and Akt and downstream factors, to the plasma membrane to enable glucose uptake (Fig. 8-9) (Samuel and Shulman, 2016; Tokarz, MacDonald and Klip, 2018). Additionally, insulin signaling also activates GS to promote glycogen synthesis. However, glucose is not the only energy source of skeletal muscle as it also takes up FAs for oxidation.

AT is the primary location for energy storage in form of TG. Similar to skeletal muscles, insulin action in AT leads to increased glucose uptake via GLUT4 and insulin signaling via IRS and Akt inhibit lipolysis. However, glucose uptake and utilization is lower in AT then in muscles or brain and is converted to glyceride as a form of storage of excess glucose. Once insulin exerted its action in the target tissues, it is slowly internalized and degraded in the lysosome.

Finally, potentially remaining insulin is then degraded by the kidney within 30 min of release by the pancreas (Fig. 8) (Tokarz, MacDonald and Klip, 2018).

Insulin can also interact with endothelial cells of the vasculature to induce vasodilation to facilitate better substrate distribution via IR signaling which phosphorylates IRS2 and in turn activates Akt, which phosphorylates endothelial nitric oxide (NO) synthase (eNOS) to produce NO. NO then acts on the smooth muscle cell layer surrounding the blood vessels to induce

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vasorelaxation via cyclic guanosine monophosphate (Tokarz, MacDonald and Klip, 2018).

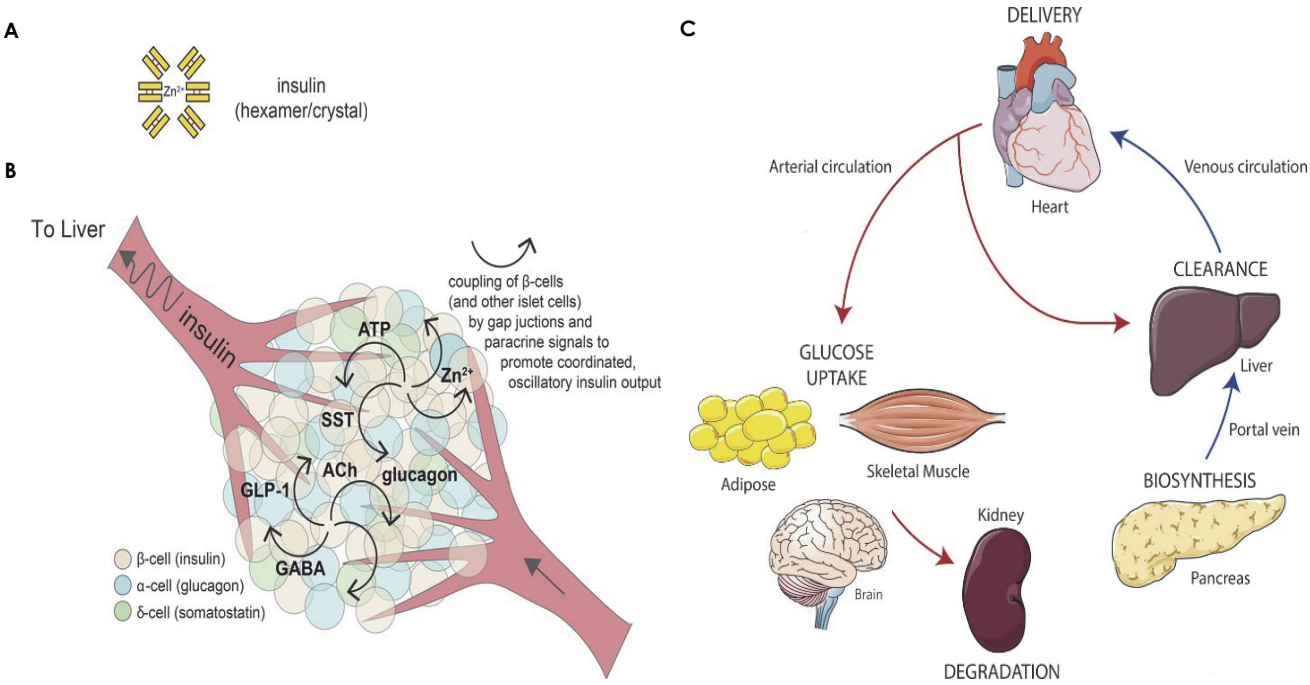

Figure 8: Insulin action in the whole body.
Ach, Aceytlcholine; SST, somatostatin; (Adapted from (Tokarz, MacDonald and Klip, 2018)).

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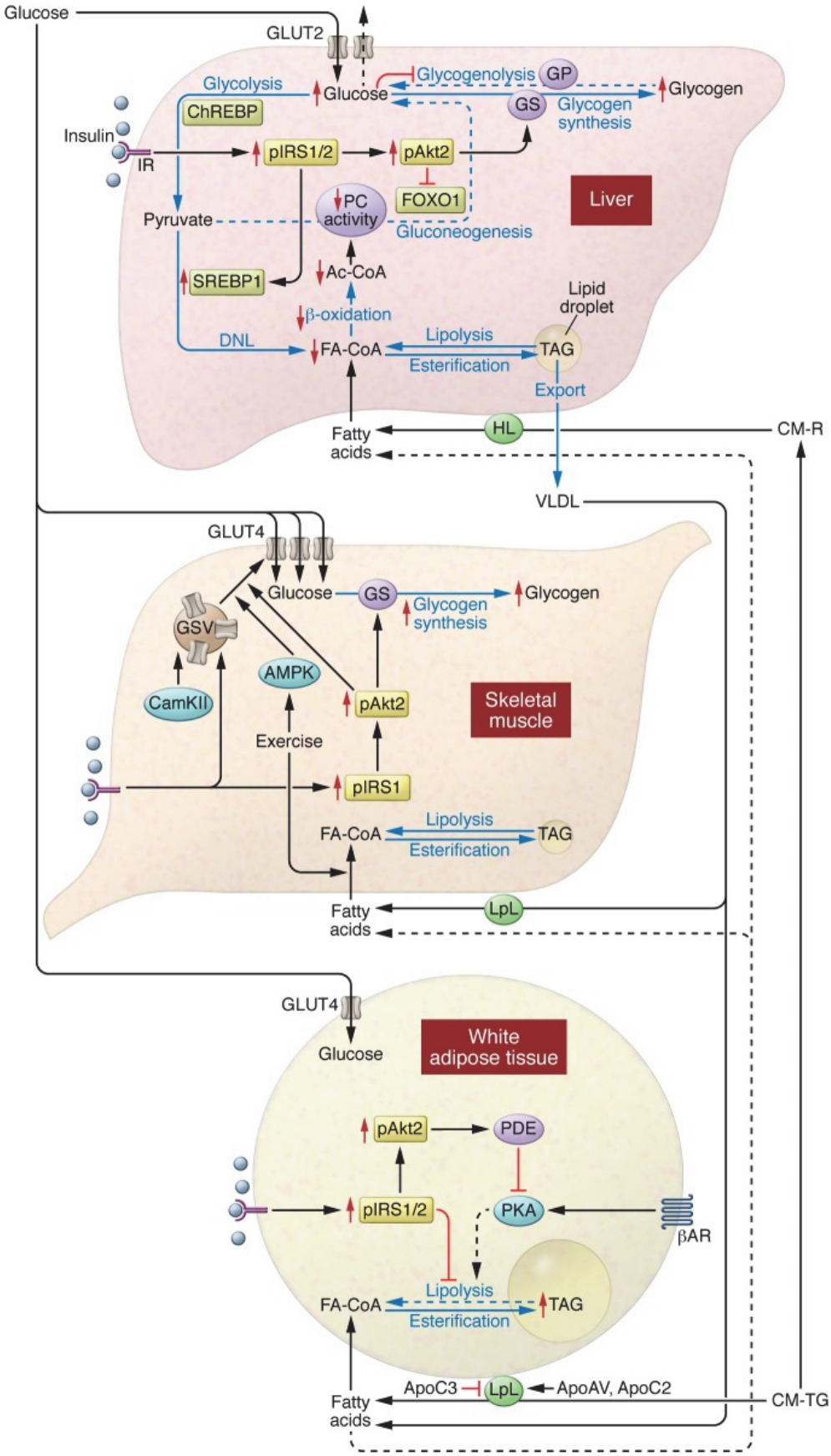

Figure 9: Insulin action in peripheral metabolic organs.
(Adapted from (Samuel and Shulman, 2016)).

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3.2.4.3. Central Insulin Action

Insulin exerts its effect not only in the periphery but has also important functions in the brain, such as in regulating glucose metabolism, food intake, sympathetic activity, neuroprotection and synaptic plasticity, reproductive endocrinology as well as hepatic gluconeogenesis inhibition (Plum, Schubert and Brüning, 2005; Pomytkin *et al.*, 2018).

It uses receptor-mediated transport to transfer across the BBB, which is altered under certain conditions. For example, while fasting, obesity aging and certain medication decrease the transport of insulin, it is increased in some models of diabetes mellitus and in the neonatal period (Plum, Schubert and Brüning, 2005).

Central insulin administration inhibits food intake, reduces of BW, while inhibition of insulin signaling increases food intake and BW and results in insulin resistance. Furthermore, ICV infusion of insulin decreases expression of orexigenic neuropeptide NPY in the ARC, upregulates α-MSH and consequently increases the anorexigenic expression of CRH in the PVN (Plum, Schubert and Brüning, 2005).

Interestingly, insulin shares a signaling pathway with leptin via PI3K signaling and both activate hypothalamic K_{ATP} channels in this way to hyperpolarize and inhibit glucose-sensitive neurons and thereby modulate glucose metabolism and potentially food consumption and BW (Fig. 10) (Plum, Schubert and Brüning, 2005). Insulin was further implicated in the modulation of reward pathways as it was shown to directly activate neurons of the mesolimbic dopamine-mediated pathway and might thus affect motivation to eat or its hedonic attributes (Sipols *et al.*, 2000).

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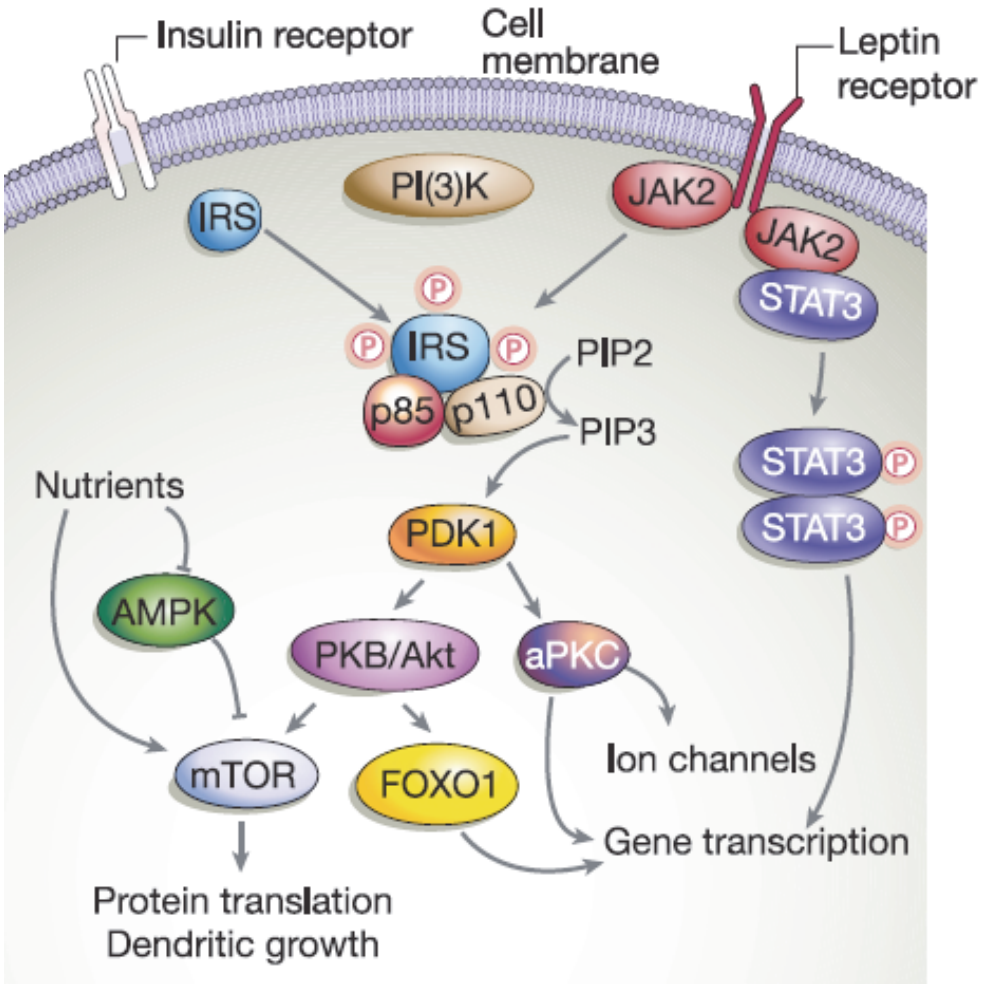

Figure 10: Insulin and leptin signaling pathway in the brain.
(Adapted from (Morton et al., 2006)).

For example, it was shown that ICV infusion of insulin into the 3V suppressed hepatic glucose production, while central administration of insulin signaling blockers in presence of insulin blocked this insulin effect on hepatic glucose production (Obici, Feng, Karkanias, *et al.*, 2002; Obici, Zhang, *et al.*, 2002). Similarly, overexpression of actors of the insulin-signaling pathway like IRS2 and Akt in the hypothalamus promoted glucose-decreasing effects in diabetic rats (Gelling *et al.*, 2006). Interestingly, IR decrease in the hypothalamus (mostly observed in the ARC) was associated with a rapid onset hyperphagia and increase in fat mass (Obici, Feng, Karkanias, *et al.*, 2002). The central effects of insulin seem to be mediated through an K_{ATP} sensitive channel in hypothalamic neurons as well as via vagal efferent fibers, which constitute the connection of the brain-liver axis (Spanswick *et al.*, 2000; Roh, Song and Kim, 2016). Moreover, ICV administration of insulin leads to an increase in

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hepatic IL-6 expression, which in turn is associated with activation of STAT3 signaling. This results in inhibition of gluconeogenic gene expression via FOXO1 blocking by STAT3 in the liver.

The IR is expressed more specifically on MCH neurons in the LH of the hypothalamus as well as on NPY/AgRP- and POMC-expressing neurons of the ARC (Obici, Zhang, *et al.*, 2002; Hausen *et al.*, 2016). Previous studies have shown that impairment of IR or IRS activity in the hypothalamus through different methods can lead to insulin resistance and glucose intolerance (Withers *et al.*, 1998; Obici, Feng, Karkanias, *et al.*, 2002; Kubota *et al.*, 2004; Lin *et al.*, 2004; Chou *et al.*, 2016).

3.2.5. Gut Hormones: Incretins

Incretins are intestinal-derived humoral or neural factors capable of potentiating insulin secretion from β-cells after oral food ingestion. Two well-known incretins are GLP-1 and GIP. Both GLP-1 and GIP are released by enteroendocrine cells in the small intestine within minutes upon food ingestion and increase glucose-dependent insulin secretion (Nauck *et al.*, 1993). Incretins are responsible for about 50-70% of insulin secretion after glucose-containing food consumption (Baggio and Drucker, 2007).

GLP-1 is another hormone involved in glucose homeostasis and is produced from the proglucagon gene by alternative posttranslational processing and secreted by L-cells in the GIT in a postprandial manner but also in neurons of the NTS. It is both decreasing glucagon secretion and stimulating the second phase of insulin secretion as well as enhancing β-cell growth and survival (Faulconbridge and Hayes, 2011; Shi and Liu, 2014). Furthermore it was shown to suppress food intake and delay gastric emptying (Faulconbridge and Hayes, 2011; Pais *et al.*, 2014). GLP-1 signal through the GPCR GLP-1R to exert it's action, which is expressed throughout the brain including the hypothalamus and brainstem, and on vagal afferent neurons in the GIT, portal vein, liver and in pancreatic islet cells (Baggio and Drucker, 2007; Faulconbridge and Hayes, 2011).

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GIP, a 42 amino acid peptide, is produced in cells of the stomach and K cells of the duodenum, but was found to be expressed in the CNS, too (Drucker, 2006). In addition to that, GIP acts on AT and bones to promote energy storage and bone formation, respectively (Baggio and Drucker, 2007). GIP is secreted upon glucose or fat ingestion and like GLP-1 acts as an incretin to potentiate glucose-stimulated insulin secretion. GIP signals through the GIP receptor, which is also a GPCR and expressed in the pancreas, stomach, small intestine, AT and some parts of the brain among other tissues. Interestingly, GIP is also implicated in AT function and the control of lipid metabolism by stimulating FA synthesis, re-esterification and insulin-induced incorporation into TG as well as decreasing lipolysis upon glucagon stimulation and thus plays a role in the development of obesity. Both incretins are degraded and thus regulated by dipeptidyl peptidase 4 (DPP4) (Baggio and Drucker, 2007). Thus, both present attractive potential therapeutical targets for diabetes mellitus.

3.3. Diabetes Mellitus (DM)

DM is an umbrella term for metabolic disorders that are united by a common characteristic: hyperglycemia. Interestingly, in 2000 it was reported that about 150 million people have diabetes and it was estimated that by 2025, 300 million people would be affected. However, only 17 years later the numbers exceeded any estimate and have been more than doubled, as in 2017 the International Diabetes Federation (IDF) reported already about 425 million adults to be diabetic worldwide, which underestimates the actual number due to a large number of asymptomatic and yet undiagnosed patients (*World Health Organization. (2019)*; Zimmet, Alberti and Shaw, 2001; International Diabetes Federation, 2017). While the population increased by ~16% in the 17 years, the cases of diabetes augmented by staggering ~183% in this time frame and thus are most likely attributed less to a rise in population size but more to changes in lifestyle and environmental factors and maybe advances in diagnostic methods.

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In addition to that, 1.1 million children between the age of 14 and 19 are considered to have T1DM (International Diabetes Federation, 2017). Apart from the two major forms of DM, type 1 diabetes mellitus (T1DM) and T2DM there are various other forms such as gestational diabetes mellitus (GDM) and others.

While all forms of diabetes share similar characteristics like dysfunction and destruction of β-cells of pancreatic islets, which results in either a dysfunction of insulin secretion (T1DM) or insulin sensitivity (T2DM and other forms) or both and ultimately leading to hyperglycemia, the mechanism leading up to it and the age of onset differ between types of diabetes. Due to the different pathogenesis mechanisms of different forms of obesity, (they respond each to different treatments)a different treatment has to be adapted for each form individually (International Diabetes Federation, 2017).

As DM is a complex and heterogeneous group of metabolic disorders, many factors play into the etiology of the diseases such as genetic predisposition, epigenetic processes, auto-immunity, inflammation and environmental factors. Furthermore, other factors such as certain medication or chemicals and virus infections can induce or exacerbate diabetes and its symptoms (Forrest, Menser and Burgess, 1971; Karjalainen *et al.*, 1988; Pandit *et al.*, 1993; Esposti, Ngo and Myers, 1996).

Although many advances were made in the understanding of diabetes, a lot of knowledge is still missing to decipher the exact mechanism and develop effective cures or treatment options (International Diabetes Federation, 2017).

Independent of type, apart from hyperglycemia, DM is accompanied by symptoms such as excessive urine production, compensatory thirst and fluid intake, blurred vision as well as lack of energy, tiredness, unexplained weight loss and abnormal modifications in energy metabolism including carbohydrate, lipid and protein metabolism. The latter is most likely due to the anabolic actions of insulin action, which are impaired in DM and thus can't

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induce the adequate response in metabolic target tissues (Lin and Sun, 2010; Kharroubi and Darwish, 2015; International Diabetes Federation, 2017).

The World Health Organization (WHO) has established different diagnostic criteria to diagnose patients with diabetes or a pre-condition (impaired fasting glucose (IFG) or glucose tolerance (IGT)) that can lead to diabetes if not properly treated through medication or altering unhealthy lifestyle factors (Fig. 11) (*World Health Organization, 2019*; International Diabetes Federation, 2017).

DIABETES should be diagnosed if **ONE OR MORE** of the following criteria are met	**IMPAIRED GLUCOSE TORELANCE** (IGT) should be diagnosed if **BOTH** of the following criteria are met	**IMPAIRED FASTING GLUCOSE** (IFG) should be diagnosed if **BOTH** of the following criteria are met
Fasting plasma glucose ≥7.0 mmol/L (126 mg/dL)	Fasting plasma glucose <7.0 mmol/L (126 mg/dL)	Fasting plasma glucose 6.1-6.9 mmol/L (110 to 125 mg/ dL)
or	and	and
Two-hour plasma glucose ≥11.1 mmol/L (200 mg/dL) following a 75g oral glucose load	Two-hour plasma glucose ≥7.8 <11.1mmol/L (≥140 to <200 mg/dL) following a 75g oral glucose load	Two-hour plasma glucose <7.8mmol/L (140mg/dL) following a 75g oral glucose load
or		
A random glucose > 11.1 mmol/L (200 mg/ dL) or HbA1c ≥ 48 mmol/mol (equivalent to 6.5%)		

Figure 11 : Diagnostic Criteria of DM.
The WHO has established diagnostic criteria for diabetes mellitus. Patients have to be presents with at least one of the criteria described in the first column to be diagnosed with DM. IGT, impaired glucose tolerance; IFG, impaired fasting glucose. (Adapted from IDF Diabetes Atlas, Eight Edition, 2017.)

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3.3.1. Type 1 Diabetes Mellitus (T1DM)

T1DM, previously known as juvenile diabetes, but with the increasing prevalence of T1DM with adult onset, this term is not justified anymore, is the least frequent form of DM with a prevalence of around 10% worldwide. However, compared to T2DM the onset of disease is in younger patients. While it is a chronic autoimmune form of the disease, causing the production of autoantibodies that destroy β-cells of the endocrine pancreas and result in insulin deficiency and eventually in hyperglycemia, a subtype with less than 10% of T1DM cases shows no signs of autoimmunity and the onset seems rather idiopathic (Paschou *et al.*, 2018). Several factors such as genetic, environmental and immunological factors can play into the pathogenesis of the disease. The major genetic alleles that are responsible for 15% of the genetic predisposition of T1DM are with 40% the gene region for major histocompatibility complex (MHC), also called human leucocyte antigen, polymorphisms in the insulin gene (ins-VNTR, IDDM 2) and cytotoxic T lymphocyte-associated antigen-4 gene (CTLA-4) (Paschou *et al.*, 2018).

The environmental factors that represent a trigger for pathogenesis of T1DM include viruses, toxins and nutrients.

The autoantibodies produced by lymphocytes, which most likely escape the selection process of T cells and B cells during their production and maturation in thymus and bone marrow, respectively, occur early in the pathogenesis of patients with T1DM, when they are still asymptomatic and euglycemic (normal concentration of blood glucose). The most common autoantibodies associated with T1DM pathogenesis are antibodies targeting insulin, 65kDa glutamic acid decarboxylase/glutamate decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZNT8) and can serve as biomarkers for diagnosis of the development of T1DM (Katsarou *et al.*, 2017). Interestingly, some autoantibodies like GAD65 and for insulin usually appear earlier, while the others tend to be detected later on during disease

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progression. They are used to separate the pathogenesis of the disorder into three different stages (Fig. 12).

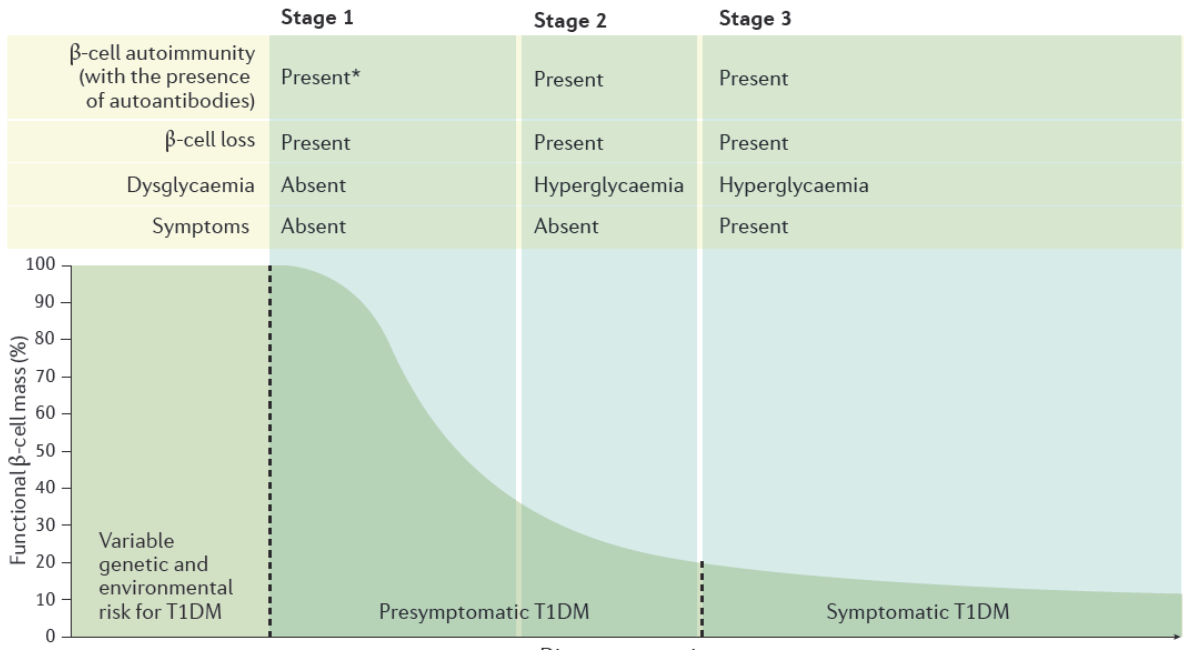

Figure 12: Different stages of T1DM.
(Adapted from (Katsarou et al., 2017)).

This form of diabetes that is predominantly found in children and adolescents, is characterized by autoantibodies, β-cell destruction and an absence of insulin secretion (Kharroubi and Darwish, 2015). Unfortunately, the mechanism leading to β-cell destruction and disease initiation as well as the role of the autoantibodies is not deciphered yet.

However, the theory suggests that the first stage of T1DM is characterized by a detection of serum autoantibodies, most commonly in early stages they are targeting GAD65 and insulin. However, patients in stage 1 are pre-symptomatic and have still normal glucose levels. Autoantibodies are often found months or years before β-cell damage occurs in stage 1. It was suggested that the cognate interaction between T and B cells might induce the production of autoantibodies against islet cell antigens, that might lead to β-cell damage (Fig. 13)(Wong, 2014). Interestingly, certain autoantibody occurrence is associated with patients having certain haplotypes in the gene

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for human leukocyte antigen. The progression to stage 2 is characterized by a progressive destruction of pancreatic insulin-secreting β-cells that leads to hyperglycemia, although patients still remain asymptomatic. It is assumed that β-cell destruction is mediated through immune cell infiltration and action of T cells and B cells that induce an inflammatory reaction that can result in insulitis or pre-insulitis (Fig. 13) (Kharroubi and Darwish, 2015; Katsarou *et al.*, 2017). Recent evidence suggests that T cells preferably recognize β-cell peptides with post-translational modifications which are believed to be caused by cellular stress and thus lead to loss of tolerance to β-cell autoantigens (Yang *et al.*, 2013; Van Lummel *et al.*, 2014). Interestingly, non-obese diabetic (NOD) mouse models of diabetes show no autoantibody production, which might be due to differences in the immune system and pathogenesis compared to humans. However, NOD mice display inflammation in and around the islets in the pancreas, known as insulitis and peri-insulitis, respectively. This occurs before mice present themselves with hyperglycemia and is associated with an infiltration of immune cells into pancreatic islets. Furthermore, it was reported that β-cells of NOD mice also show perturbation in ER UPR that were linked to β-cell destruction and T1DM phenotype (Engin *et al.*, 2013; Magnuson *et al.*, 2015).

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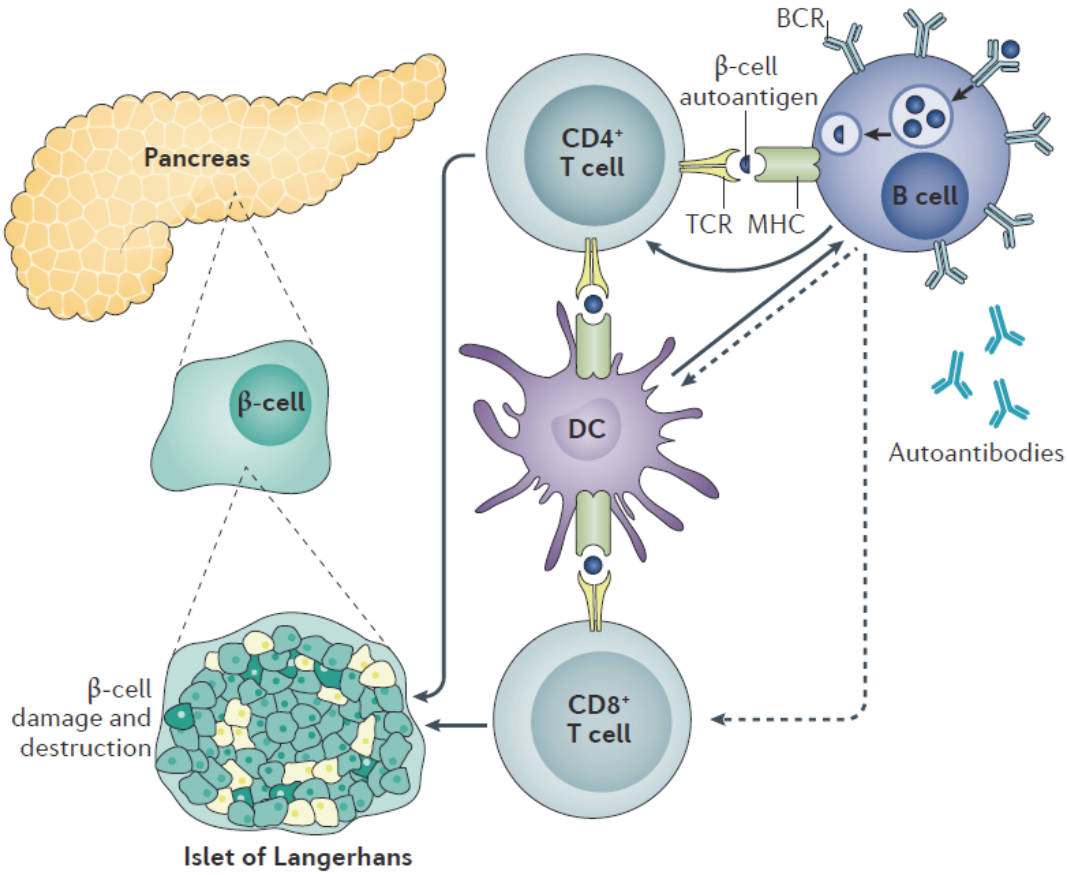

Figure 13: Pathogenesis of T1DM.
T1DM is an autoimmune-mediated disease that is initiated upon antigen-presentation of DC and activated B cells that interact with and activate CD4+ and CD8+ T cells. Furthermore, contact with β-cell autoantigens drives the production of autoantibodies against islet peptides. Activated T cells can infiltrate the islets and lead to loss of β-cells due to damage and destruction. (Adapted from (Katsarou et al., 2017)).

Upon diagnosis, careful blood-glucose monitoring is required in addition to subcutaneous administration of insulin with the aim to achieve close to normal physiological glycemia, while avoiding hyperglycemia, hypoglycemia and ketoacidosis. Ketoacidosis occurs in some diabetic patients as a metabolic decompensation, when the insulin deficit leads to an increase in serum glycogen levels and a decrease of glucose utilization by metabolic tissues and once the liver glycogen stores are depleted, the body turns to ketones as alternative fuel derived from fat (Fedorovich, Voronina and Waseem, 2018). Ketone bodies produced by lipid degradation accumulate in the blood as ketone production is faster than its utilization and eventually overpowers the kidneys buffer capacity of the pH leading to metabolic

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acidosis, which in turn can lead to neuronal damage and coma or even death, if not treated (Fedorovich, Voronina and Waseem, 2018). Rigorous blood-glucose was shown to reduce HbA1c levels and reduce both micro- and microvascular complications in T1DM patients (Nathan, 2014). Hypoglycemia, like hyperglycemia can have a negative impact on health, as frequent hypoglycemia after insulin injections can result in a shortage of glucose by the CNS and peripheral nervous system (PNS) and neuronal cell death (Auer *et al.*, 1984; Mohseni, 2014; Auer, 2018). However, it also leads to fear and anxiety, which in turn affects glucose regulation and reduces the quality of life of not only the patient but also caregivers and family of the patient (Katsarou *et al.*, 2017).

3.3.2. Type 2 Diabetes Mellitus (T2DM)

T2DM is the most common form that represents more than 90% of all diabetes cases (International Diabetes Federation, 2017) and occurs mostly in adults. In fact, incidence of T2DM increases with age and with the onset of hyperglycemia. Because it is closely linked with obesity, which is one of the most common risk factors leading to T2DM, I will be focusing on T2DM in this project. Along with obesity is has become a major global public health issue (DeFronzo *et al.*, 2015). Other major risk factors for developing diabetes are age, family history, unhealthy lifestyle like poor diet and physical inactivity, smoking, increased adiposity and a history of GDM or IFG or IGT, which is indicative of a prediabetic state that, if not reversed through medication or lifestyle changes can progress into T2DM. T2DM occurs slightly more frequent in males than females as well as adults compared to children and adolescent. However, with the increase in obese cases and unhealthy sedentary lifestyle changes and increasingly poor diet, among younger adults and children, the incidence of T2DM also increases in this population (International Diabetes Federation, 2017). Similar to T1DM it is characterized by hyperglycemia, a progressive deficiency in insulin secretion of β-cells but also insulin resistance, which occurs in metabolic tissues such as skeletal muscle, liver and AT and worsens the hyperglycemia (DeFronzo *et al.*, 2015). Due to

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the ineffectiveness of insulin, the body tries to compensate the rising glucose levels with a higher insulin production and secretion (hyperinsulinemia), but over times this overpowers the pancreas and becomes inadequate in light of increasing deficiency of β-cells and peripheral insulin resistance (International Diabetes Federation, 2017).

3.3.2.1. Different Stages of T2DM

Diabetes pathogenesis and development can be subdivided into different stages as proposed by Weir and Bonner-Weir in 2004 (Weir and Bonner-Weir, 2004), or simply into prediabetes and diabetes.

The latter distinguishes diabetes from a prediabetic state, which is characterized by increased or impaired IFG, IGT (Fig. 11) or increased glycated hemoglobin A1c (HbA1c) levels (5.7-6.4%), which are increased or impaired compared to normal individuals but below clinical diagnostic values, and presents a high risk of progression to diabetes (Abdul-Ghani, Tripathy and DeFronzo, 2006; DeFronzo *et al.*, 2015). While, IGF is characterized by hepatic insulin resistance and impaired early or first-phase insulin secretion, people with IGT have rather muscular insulin resistance and impaired post-prandial late or second-phase insulin secretion (Abdul-Ghani, Tripathy and DeFronzo, 2006; DeFronzo *et al.*, 2015).

The first sign of prediabetes tends to be a decline in β-cell function, which can begin as early as 12 years before diagnosis of diabetes and gets worse throughout the progression of disease and in combination with hyperglycemia and the associated glucose toxicity (Fig. 14) (Fonseca, 2009). This might be due to a higher intake in carbohydrates and fats that lead to higher fat accumulation and increased glucose and FFA levels that puts pressure on β-cells, muscles and liver, that leads to local insulin resistance, while inducing increased glucose production in liver (Fonseca, 2009). At the same time, β-cells try to compensate for hyperglycemia with increase in cell mass (hypertrophy) and insulin secretion, which leads to further deterioration

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of β-cells over time and causes progression to T2DM. Interestingly, β-cells dysfunction in pancreatic islets leads to apoptosis of β-cells.

The initial decline in β-cell function is presented as insulin resistance, which increases over time. However, in the beginning, β-cells respond to that with an increase in insulin production and secretion (hyperinsulinemia) to compensate for insulin resistance to keep blood glucose levels normal (Fig. 14).

There are two adaptations that islets adopt in the face of insulin resistance: They adapt by increasing insulin secretion for each β-cell and/or increase in β-cell mass to increase constant output of insulin (Fonseca, 2009). This is often the case in obese patients, that maintain normal glycemia but present with increased β-cell mass as well as insulin secretion, that does not decrease to baseline levels between meals (Fonseca, 2009).

Interestingly, some obese individuals with prediabetes never progress to diabetes, as their β-cells are able continue to function and maintain normal glucose control while compensating for the insulin resistance with insulin secretion (Fonseca, 2009).

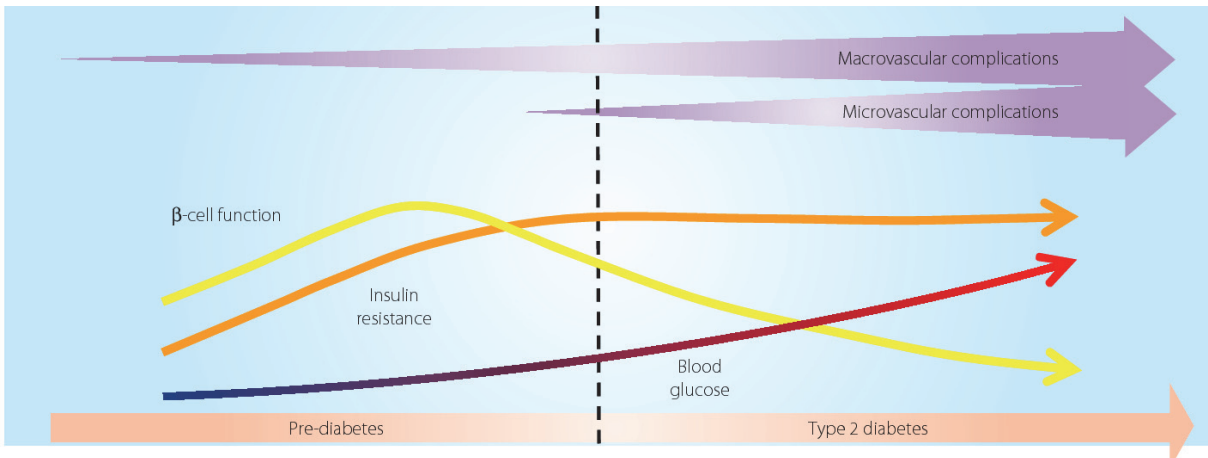

Figure 14: Different stages of T2DM development.
(Adapted from (Xie, Chan and Ma, 2018)).

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3.3.2.2. Pathophysiology

The hallmarks of T2DM are hyperglycemia, β-cell loss and failure to secrete insulin as well as insulin resistance and high levels of blood FFAs. Insulin resistance is due to an inefficiency or defective and delayed insulin action or response. In other words, the target cells that express the IR will no longer respond to insulin, because the signal transduction is impaired and thus the target cells fails to take up glucose (Lin and Sun, 2010).

There are several theories about the mechanisms behind these characteristics in T2DM, including inflammation, lipotoxicity, glucotoxicity, mitochondrial dysfunction and ER stress (Fig. 15). One of the proposed mechanisms is local inflammation–induced β-cell dysfunction. Similar to obese patients, a key aspect in the inflammation leading to insulin resistance is infiltration of AT by pro-inflammatory macrophages and T cells (Lumeng and Saltiel, 2011).

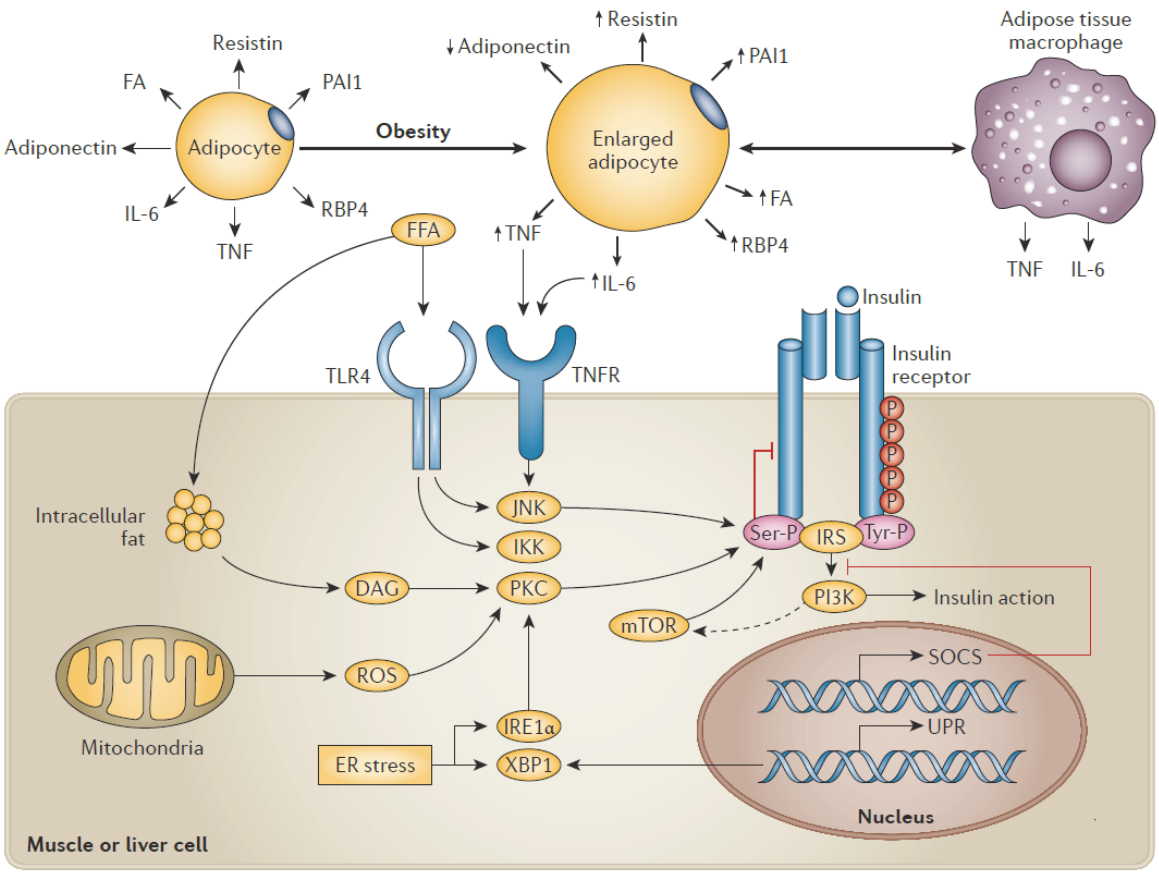

Figure 15: Mechanism behind insulin resistance in T2DM.
(Adpated from (DeFronzo et al., 2015)).

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Pro-inflammatory cytokines and chemokines have been implicated in insulin resistance, because the downstream pathways activated by them such as IKKβ, JNK1 and p38 MAPK can contribute to insulin resistance by either blocking IRS proteins through inducing the production of SOCSs or through phosphorylation of IRS proteins (Fig. 15)(DeFronzo *et al.*, 2015). Furthermore, pharmacological and genetic models of NF-κB pathway invalidation as well as blocking TNF activity lead to an amelioration of insulin sensitivity in mice (Uysal, Wiesbrock and Hotamisligil, 1998; Arkan *et al.*, 2005).

Moreover, increased FAs can activate TLR4, which induces IKK and JNK activity to stimulate the biosynthesis of the sphingolipid ceramide and thereby lead to a phosphorylation of IRS serine residues and inhibit IRS tyrosine phosphorylation and ultimately contribute to impaired insulin sensitivity and signaling (Fig. 15-16) (Shi *et al.*, 2006; Chavez and Summers, 2012). This is supported by data showing that TLR4 deficient mice are resistant to DIO and insulin resistance (Tsukumo *et al.*, 2007).

Another theory is based on hyperlipidemia induced lipotoxicity (Cnop, 2008; Giacca *et al.*, 2011; Xiao, Giacca and Lewis, 2011).

Several tissues, such as muscle, liver and others reflect the pathogenesis and metabolic abnormalities seen in T2DM. According to one of the hypotheses, adiposity and associated lipotoxicity is crucial for the development of insulin resistance and T2DM. Obesity or adiposity is closely linked with the occurrence of T2DM and is a major risk for T2DM. The increased adiposity is reflected by an excessive storage of fat in visceral AT leading to metabolic abnormalities. When the capacity of AT storages is surpassed, lipids accumulate in ectopic sites, such as muscle, liver and heart among others, which contributes to insulin resistance as well as other metabolic comorbidities such as hepatic steatosis or cardiovascular disorders. Studies report that ectopic lipid accumulation in muscles precedes hyperglycemia and liver insulin resistance and leads to increased fat oxidation, citrate production and induces

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alterations in insulin-stimulated glucose metabolism such as glucose transport impairment (Fig. 16)(DeFronzo *et al.*, 2015; Samuel and Shulman, 2016).

This can be due to abnormalities in mitochondrial oxidative and phosphorylation activity as offspring of T2DM in a study that were healthy, young and lean, but presented with insulin-resistance, had reduced muscle mitochondrial oxidative-phosphorylation activity as well as reduced mitochondrial density (Petersen *et al.*, 2004; Befroy *et al.*, 2007). Previous studies showed that lipid infusion in rodents and healthy humans, as well as HFD feeding in rodents induced increased muscle diacylglycerol (DAG) content. This in turn activated PKCθ, which inhibits insulin signaling by preventing IRS-1 phosphorylation by IRTK and resulted in muscle insulin resistance (Dresner *et al.*, 1999; Yu *et al.*, 2002; Szendroedi *et al.*, 2014). Further support for the important role of muscle insulin resistance in the pathophysiology of T2DM and other metabolic disorders was provided by studies looking at transgenic mice with muscle-specific deletions of IRTK or GLUT4, which results in selective insulin resistance in muscles (Fig. 16). Those mouse models developed diabetes-like phenotypes with secondary glucose toxicity induced impairment in glucose uptake in AT and hepatic glucose production (Kim *et al.*, 2001) as well as hepatic steatosis and increased adiposity due to decreased uptake of glucose by muscles and redistribution of glucose to other tissues (Kim *et al.*, 2000; Petersen *et al.*, 2007).

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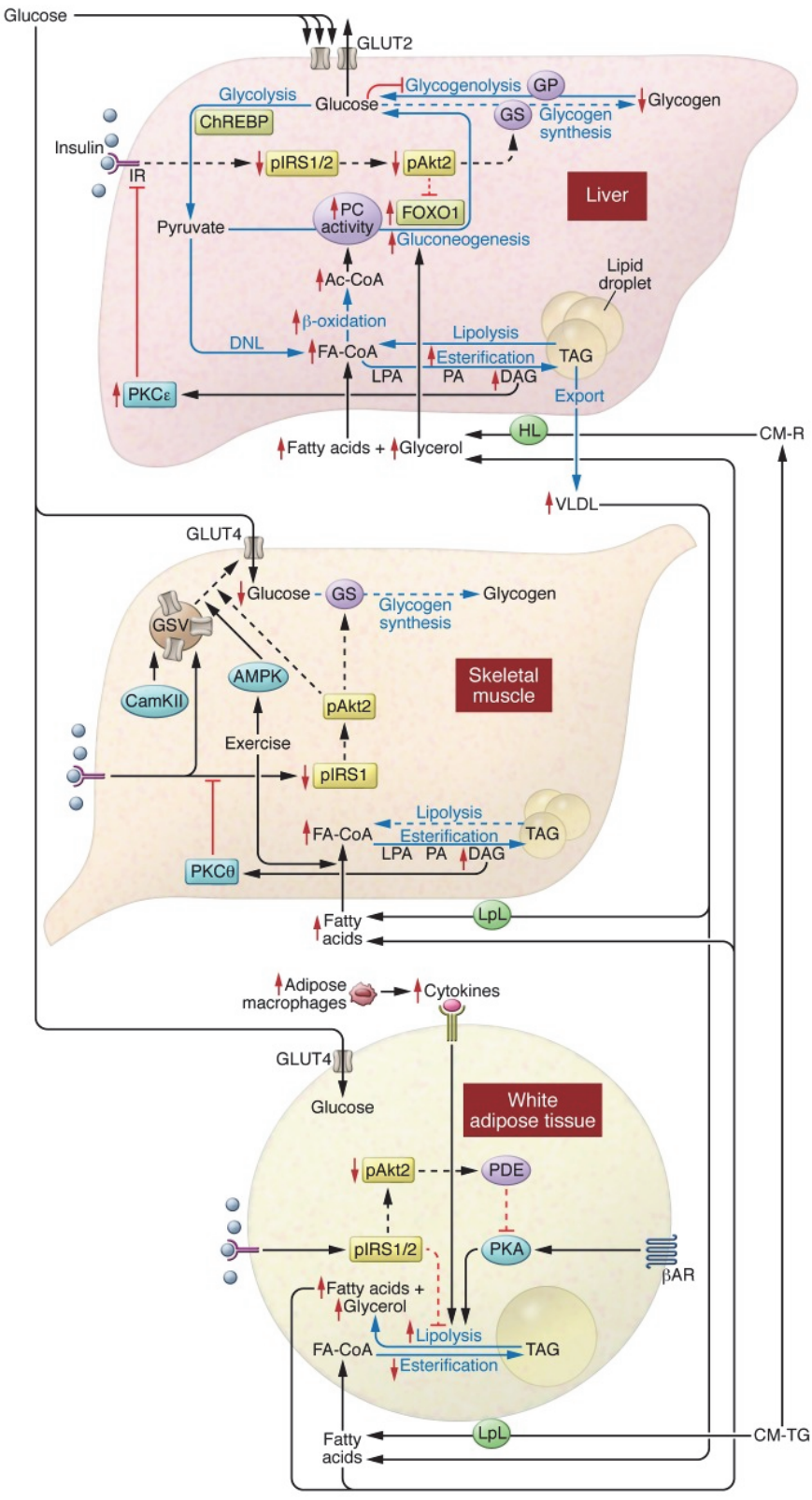

Figure 16: Mechanism of peripheral insulin resistance in metabolic organs. Inflammation and insulin resistance in WAT leads to infiltration by ATM through chemotactic attraction by chemokines leads to inflammation through cytokine production. Cytokines and impaired insulin action then increase lipolysis and release of FAs. Ectopic lipid accumulation and delivery of FAs in skeletal muscle leads to impaired glucose transport into muscle cells through increases in DAG. This in turn activates PKCθ and leads to insulin signaling impairment and ultimately decreased glucose uptake. Glucose is diverted to the liver and together with the increased flux of FAs from WAT increases both hepatic lipid synthesis through TG production. In addition, it raises levels of acetyl-CoA, which drive gluconeogenesis through activation of PC activity and glycerol and ultimately increase hepatic glucose production. This induces hyperglycemia. Hepatic increase in DAG activates PKCε, which inhibits insulin signaling via IRS serine phosphorylation and decrease in glycogen synthesis. (Adapted from (Samuel and Shulman, 2016)).

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A recent study by Perry et al. in 2015 described another interesting mechanism involving the interaction between WAT and liver to induce hyperglycemia in an attempt to explain how hyperglycemia comes about in T2DM.

Using an in vivo metabolomics approach, they found that WAT lipolysis increases hepatic acetyl CoA concentration by increasing the flux of pyruvate carboxylase (Fig. 16). They thus show that hepatic acetyl CoA is a key regulator mediating insulin effects in the liver, and that insulin can suppress hepatic glucose production via inhibiting lipolysis in WAT and is responsible for mediating the inflammation-induced insulin resistance in the liver of T2DM patients (Perry *et al.*, 2015). In other words, in T2DM insulin loses its ability to inhibit lipolysis in WAT, which mediates hepatic glucose production and thereby leads to hyperglycemia. Interestingly, they found that WAT lipolysis was induced by local IL-6 increase in WAT macrophages in HFD fed rats, which was suggested to be mediated by WAT comparative gene identification-58 protein (CGI-58) (Perry *et al.*, 2015).

The other suggested mechanism trying to explain the defects observed in T2DM and associated complications are based on hyperglycemia-induced glucotoxicity (Dubois *et al.*, 2007; Wallace, Whelan and Brennan, 2013; Luo *et al.*, 2016). Chronically elevated glucose levels are toxic and increase the activation of certain pathways, which lead to toxicity and the dysfunction or destruction of different cells.

Under normal physiological conditions, glucose is usually processed by cells via glycolysis and the TCA cycle, however under pathological elevated glucose conditions, pathways with low affinity for glucose, which are usually dormant, are activated. The pathways activated or upregulated by hyperglycemic stress, also known as glucotoxicity, and carbon stress include the polyol pathway, the PKC activation pathway, the hexosamine pathway, the advanced glycation end products (AGE) pathway, and the enediol

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pathway, all of which ultimately end in oxidative stress (Fig. 17) (Bensellam, Laybutt and Jonas, 2012; Luo *et al.*, 2016).

Several in vitro experiments showed that β-cell cultures exposed to chronic high glucose medium presented with defects such as decreased levels of insulin mRNA, insulin content and insulin release, which were reversed in a time-dependent fashion when culture medium was switched back to low glucose-containing medium (Robertson *et al.*, 1992; Gleason *et al.*, 2017). It was shown that glucotoxicity seems to act through oxidative stress, because when β-cell lines were again cultured in high glucose-containing medium but supplemented with antioxidants, β-cells were protected from apoptosis and other toxic effects of chronic high glucose exposure (Kaneto *et al.*, 1996; Poitout, Olson and Robertson, 1996). In fact, due to a very low amount of antioxidant enzymes, β-cells appear to be more sensitive to oxidative stress compared to other tissues (Grankvist, Marklund and Täljedal, 1981; Tiedge *et al.*, 1997). Similarly, the overexpression of antioxidant enzyme, glutathione peroxidase, that catabolizes hydrogen peroxide and lipid peroxides to oxygen and water, protected β-cells from ribose-induced damage (Tanaka *et al.*, 2002). Later, studies confirmed these results in in vivo studies using *Zucker diabetic fatty* (ZDF) rats and *db/db mice*, which harbor a mutation in the leptin receptor gene and develop both obesity and diabetes. These rodent models were treated with antioxidants, which protected them from elevated ROS and hyperglycemia, compared to placebo-treated mice that had elevated circulating levels of oxidative stress markers (Kaneto *et al.*, 1999; Tanaka *et al.*, 1999).

Glucotoxicity leads to β-cells becoming sensitized to the increased presence of hyperglycemia, which depletes β-cells of their insulin secretory granules thereby decreasing the secretable amount of insulin in response to glucose (Fonseca, 2009).

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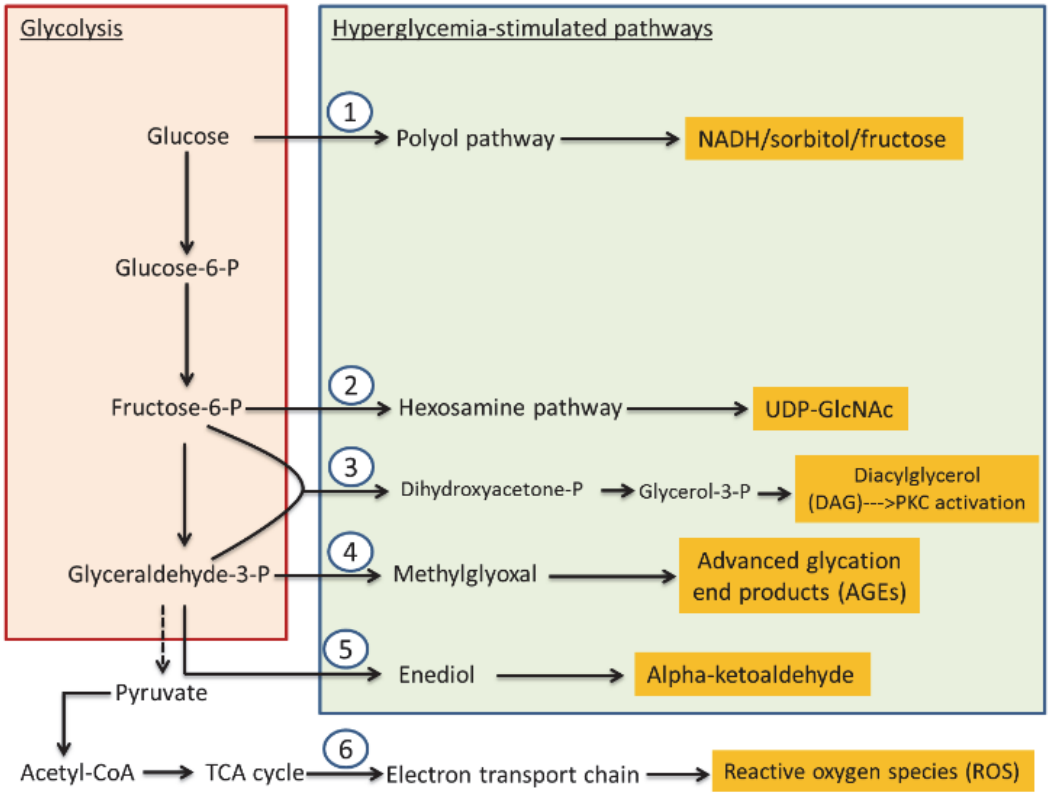

Figure 17 : Hyperglycemia-induced glucotoxicity pathways.
(Adapted from (Luo *et al.*, 2016)).

It is believed that loss of β-cell can be attributed to apoptosis and dysregulated autophagy (Marchetti *et al.*, 2007; Marchetti and Masini, 2009).

β-cell dysfunction is associated with ER stress that might be caused by elevated glucose concentrations (Marchetti *et al.*, 2007) and lipotoxicity (Cnop, 2008; Giacca *et al.*, 2011; Xiao, Giacca and Lewis, 2011). Cultures of isolated islets from diabetes patients showed impaired secretion of insulin in response to glucose as well as a higher threshold for glucose levels to induce insulin secretion. Interestingly, other studies confirmed these results when they showed that prolonged exposure of high levels of glucose of FFAs, especially palmitate, induced similar disturbances in insulin secretion in islets of non-diabetic persons (Igoillo-Esteve *et al.*, 2010; Xiao, Giacca and Lewis, 2011). This shows evidence that lipotoxicity and toxicity of high glucose play important parts in the development of islet dysfunction and T2DM.

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Other studies found strong associations between excessive consumption of sugar and fat, whereas others found relations between fat intake and T2DM and some did not find any association (Peterson *et al.*, 1986)

Diabetes can often lead to the development of several complications or comorbidities, affecting multiple organs and tissues, such as eyes, kidnes, heart and nerves. They can be subdivided into macrovascular complications such as, coronary disease, stroke and neuropathy (Katsarou *et al.*, 2017) and microvascular complications like retinopathy (Harris *et al.*, 1992) and nephropathy, among others. The risk of developing complications increases with earlier age of onset, delayed diagnosis and the length of duration of T2DM and T1DM but also with certain genes and mutations (Kharroubi and Darwish, 2015). Other severe complications include blindness, renal failure and slow wound healing (Lin and Sun, 2010). These complications are strongly associated with hyperglycemia and are more likely to occur if hyperglycemia is not tightly controlled (Stratton, 2000).

One of the most common comorbidity of diabetes is diabetic neuropathy (DN). The rate of incidence of this type of neuropathy among diabetic patients varies and can range between 50% and 90%.

4. Neuropathic Pain

Neuropathy commonly refers to diseases, injury or dysfunction of nerves and can affect either the CNS such as cranial nerves; the ANS, namely nerves belonging to the involuntary nervous system. Then there is focal neuropathy, which refers to neuropathy restricted to one body part or nerve or group of nerves and peripheral neuropathy, which affects nerves of the peripheral nervous system innervating the extremities.

The International Association for the Study of Pain (IASP) has defined neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system" (Jensen *et al.*, 2011; International Association for the Study of Pain, 2017).

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The somatosensory system is a system of interconnected neurons of the periphery and CNS and in close contact with the motor system to receive and respond to different stimuli. The somatosensory system can perceive different forms of stimuli, apart from the 5 senses like olfaction, gustatory, vision, hearing and touch, it is also responsible for the perception of pain, temperature, movement, vibration and the position of the body or body parts, termed proprioception. To be able to perceive stimuli, somatosensory nerves or DRG are connected to peripheral tissues like skin, muscles, joints and facia and contain different receptors at their nerve endings that detect specific stimuli and transduce this information into electrical stimulation that is transmitted to and through the dorsal horn of the spinal cord and to the brain. In the area of the face, the stimulus is detected by nerves called trigeminal nerve ganglia and transmitted directly to the brain. There are different fibers and different receptors that are specific for certain stimuli. For example, there are thermoreceptor containing nerves responsible for detection of different temperature ranges; mechanoreceptors that detect mechanical stimuli such as pressure; chemoreceptors that perceive different molecules such as CO_2, O^2 or pH; prurireceptors that detect itchy sensations and nociceptors that can perceive noxious stimuli like pain (Colloca *et al.*, 2017).

Pain is a protective mechanism of the organism in order to protect from potential or further damage or injury and encourages a behavior in aid of wound healing and escaping the dangerous situation that could possible inflict injury.

Nociceptive pain is a protective mechanism that is transmitted through nociceptors, also known as sensory nerve fibers, which are activated by painful stimuli in order to protect us from potential or further tissue injury (Basbaum *et al.*, 2009; Fernandes *et al.*, 2018). Tissue damage or injury of peripheral nerves, DRG or parts of the CNS can cause hypersensitivity and ultimately neuropathic pain. Nociceptors can be subdivided into two major classes, the first being myelinated Aδ afferent sensory nerve fibers with a medium diameter, transduce acute, well-localized fast pain. This class

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includes also Aβ afferent fibers that have a fast conducting speed due to their large diameter and myelination and are activated by mechanical stimuli, such as light touch. The second class of afferent nociceptor fibers consist of C-fibers, small diameter and unmyelinated fibers that respond to poorly localized and slow pain (Basbaum *et al.*, 2009). Aδ-fibers respond to both mechanical and chemical stimuli, but dependent on the subtype they can also be activated by lower or higher temperature thresholds. Similarly, C-fibers are also heterogeneous and polymodal, and can be further subdivided into subgroups, for example into peptidergic C-fibers, which release neurotransmitters and non-peptidergic C-fibers that express different receptors including GPCRs. There are many more subpopulations of C-fibers, and depending on the population, they convey either heat and mechanical stimuli or chemical stimuli such as itch-producing pruritogens, whereas others mediate non-noxious stimuli like "pleasant touch" or cool temperatures. But they do not respond to heat and mechanical stimuli and thus are not regarded as nociceptors (Basbaum *et al.*, 2009).

Different channels and receptors are present on free nerve endings of different fibers adding to the complexity and providing another potential classification method. These channels include the transient receptor potential cation channel subfamily V Member 1 (TRPV1), which is sensitive to heat, the transient receptor potential cation channel subfamily M member 8 (TRPM8) channel that conveys cold stimuli, acid-sensing ion channels (ASICs), and channels that respond to chemical irritants like the transient receptor potential cation channel subfamily A member 1 (TRPA1) (Basbaum *et al.*, 2009).

The most common cause of neuropathy worldwide is DM and increases with poor intervention and blood glucose control (Said, 2007).

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4.1. Diabetic Neuropathy (DN)

DN is a heterogeneous and complex disorder, which can be a result of nerve injury or damage, secondary to stroke or autoimmune disorders but often occurs as a comorbidity of metabolic disorders such as diabetes. In fact, diabetes is the most common cause of neuropathy worldwide, with an estimated 60% of diabetic patients that will develop peripheral DN and more than $10 billion annual costs of DN alone in the United States (O'brien, Sakowski and Feldman, 2014; Feldman *et al.*, 2019). While, Type 1 and T2DM patients have similar symptoms, they seem to be caused by different pathophysiological mechanisms.

Furthermore, the incidence of neuropathy is higher in patients of T2DM than T1DM , whereas the prevalence is similar and increases with duration of disease and age (Feldman *et al.*, 2019). Components of metabolic syndrome like obesity, hypertriglyceridemia, hyperglycemia, hypertension, dyslipidemia and prediabetes seem to increase the risk of developing DN and might play a role in the pathogenesis (Callaghan *et al.*, 2012).

Unfortunately, apart from tight control of glycemia, there seems to be no effective treatment for DN. While glucose control works well for T1DM patients to prevent neuropathy complications, it is not as successful for T2DM patients (Callaghan *et al.*, 2012; O'brien, Sakowski and Feldman, 2014). Paradoxically, glucose control can reduce the risk for neuropathies, but the treatment for diabetes and hyperglycemia can also cause neuropathies (Callaghan *et al.*, 2012). With the progression of neuropathy and implication of more nerve fibers, it will result in hyperalgesia and ultimately the complete loss of sensation (Callaghan *et al.*, 2012). Furthermore, DPN is associated with impaired wound healing and together with the loss of sensation, it can lead to ulcers and the common comorbidity known as "diabetic foot" that might result in amputation (O'brien, Sakowski and Feldman, 2014). In fact, diabetes is the major cause for lower extremity amputations, which is in turn associated

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with a 30% risk of death in diabetic patients with neuropathy (Callaghan *et al.*, 2012; O'brien, Sakowski and Feldman, 2014).

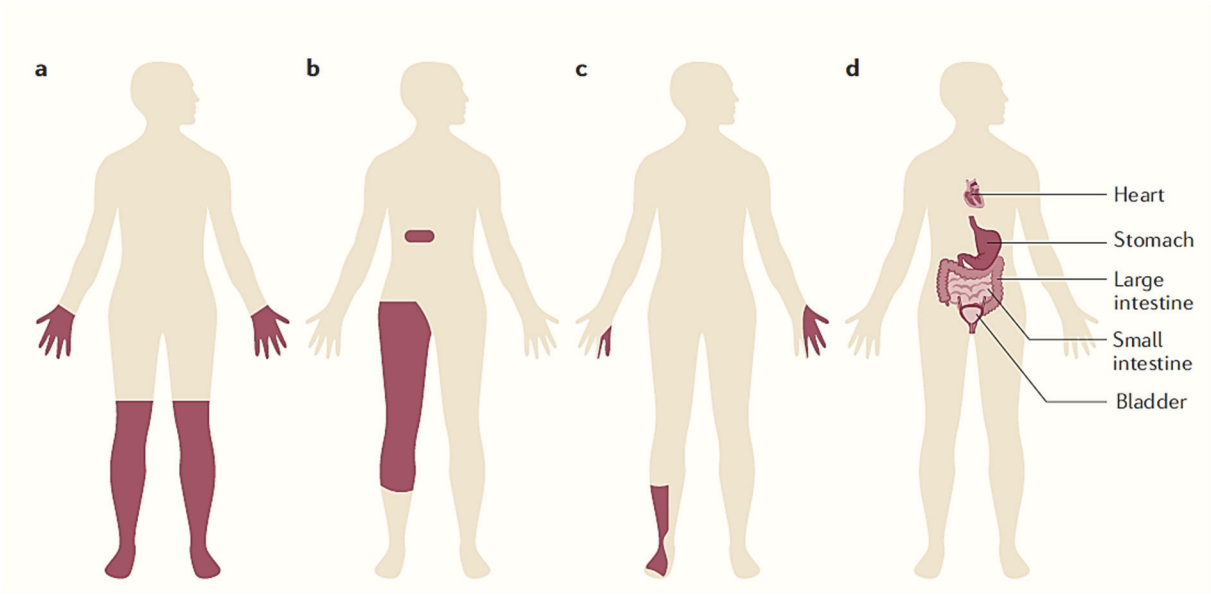

Figure 18: Different types and patterns of DN.
Diabetic patients can develop different patterns of neuropathy subdivided into (**a**) distal symmetric polyneuropathy, small-fibre-predominant neuropathy or treatment-induced neuropathy; (**b**) radiculoplexopathy or radiculopathy; (**c**) mononeuropathy; (**d**) autonomic neuropathy or treatment-induced neuropathy. The most common type is DSP, which has a similar distribution pattern as small-fibre-predominant neuropathy. (Adapted from (Feldman et al., 2019)).

The most common neuropathy seen in diabetes is diabetic polyneuropathy or length-dependent diabetic polyneuropathy (DPN), defined as the symmetric loss of nerve function of long-distant nerves innervating the outermost limbs such as toes of the feet and fingers of the hands and progressing from distal to proximal parts of the extremities (Fig. 18a). This dysfunction or degeneration of peripheral nerves leads to symptoms such as weakness, tingling, pain and eventual loss of sensation (O'brien, Sakowski and Feldman, 2014). Symptoms depend largely on which nerves and nerve fibers are affected and is not always accompanied by pain. Clinical manifestations of DN can include numbness, tingling, weakness, that spreads in a symmetrical fashion from distal to proximal parts of the body according to the nerve dysfunction pattern as described above, and is often referred to as " stocking and glove distribution" (Callaghan *et al.*, 2012). The pattern of symptoms can be described by the underlying pathological pattern that usually starts with the

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degeneration of the small unmyelinated C-fibers, which have a small diameter and thus belong to the slow conducting nerve fibers and are responsible for transduction of temperature and pain. This first step leads to symptoms of hyperalgesia and allodynia. Whereas hyperalgesia is referred to increased sensitivity to painful stimuli, allodynia is when nonpainful stimuli is perceived as painful (Callaghan *et al.*, 2012). DPN then progresses involving the larger diameter and myelinated nerve fibers leading to loss of ankle reflexes, sensory ataxia and decreased proprioception, which is the awareness of body position and sense of movement (Tesfaye *et al.*, 2010). These negative impacts of this disabling disease can lead to secondary effects, such as injuries from falls, due to balance problems, affect patients negatively both physically and mentally, and reduce quality of life (Callaghan *et al.*, 2012).

Loss of C-fibers along with their sensation, which paradoxically can lead to development of painful neuropathy with painful sensation despite the loss of sensation. This is likely due to a different mechanism of detecting different sensory and noxious painful stimuli.

Different fibers exist, that are important in sensory sensation including myelinated A-fibers, and unmyelinated C-fibers.

Once, polyneuropathy is established, it is irreversible and at best remains stable or worsens slowly over time with treatment of diabetes (Said, 2007).

4.2. Pathophysiological Mechanisms

Despite a great effort in research, the underlying mechanism is still unclear. However, it was identified that several factors may play a major role in the development of DN like hyperglycemia, visceral adiposity and associated dyslipidemia, impaired insulin signaling and sensitivity of neurons and chronic inflammation associated with obesity (Callaghan *et al.*, 2012). Apart from those, other factors such as macro- and microvascular dysfunction in nerve and endoneurial perfusion as well as hypertension are potential contributors to DN.

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Excess and prolonged hyperglycemia can saturate the traditional metabolic pathways and increase the processing of glucose through alternative pathways that can result in metabolites, which if in abundance, can create cellular damage. These pathways are the polyol-, the hexosamine pathway, and the overload of mitochondrial oxidative phosphorylation that leads to the production of ROS (Fig. 19). Excess of glucose can also result in the increased generation of AGEs, which form reactive attachments to proteins, lipids and nucleic acids and thereby impair their function and cellular maintenance.

Dyslipidemia is a very common comorbidity of T2DM that could lead to DN via several mechanisms. Increased FFAs can either cause direct damage to glia cells like Schwann cells or induce systemic inflammation by stimulating the secretion of cytokines in AT and immune cells. Moreover, the modification of lipoproteins like low-density lipoproteins (LDL) via either oxidation or glycation can trigger signaling cascades through several receptors such as oxLDL receptor Lectin-like oxidized LDL receptor 1 (LOX1), TLR4 and the receptor for advanced glycation end products (RAGE) to induce oxidative stress and inflammatory processes (Callaghan *et al.*, 2012). Similarly, the oxidation of cholesterol by oxysterols might induce apoptosis in neurons and thereby potentially contribute to DN (Callaghan *et al.*, 2012).

While insulin signaling has been implicated as a neurotrophic factor, impaired insulin signaling such as IR or insulin deficiency has been suggested to play a contributing role to DN through inhibition of the PI3K/Akt pathway in neurons and thereby disrupting mitochondrial function and inducing oxidative stress (Fig. 19) (Callaghan *et al.*, 2012).

Most of these pathways end up inducing oxidative stress through mitochondrial dysfunction, ER stress or inflammatory pathways that result in cellular and DNA damage as well as apoptosis (Callaghan *et al.*, 2012).

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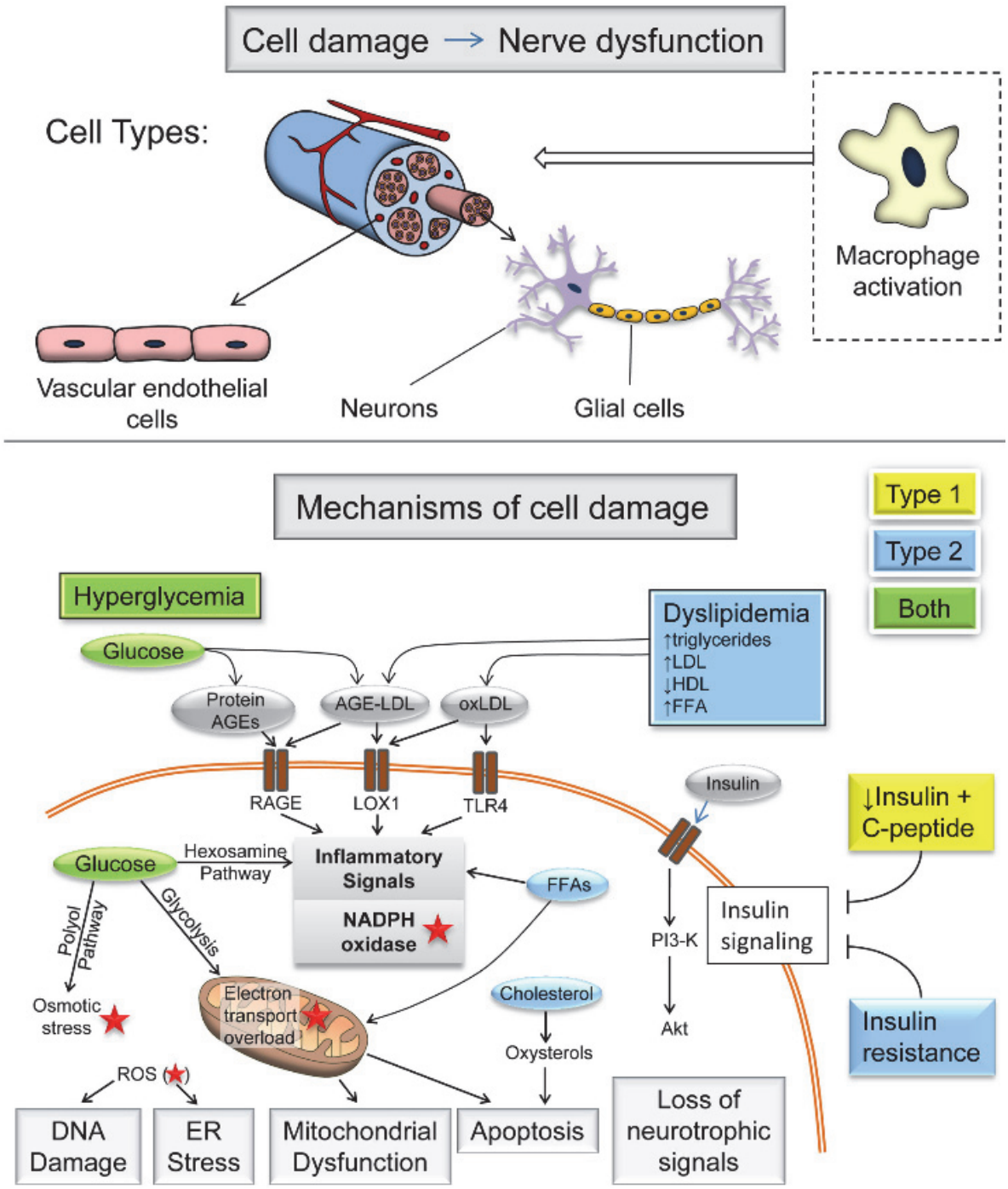

Figure 19: Mechanism behind the Pathophysiology of DN.
The upper panel indicates the cell types affected by the mechanisms leading to cell damage and ultimately to DN. These are vascular endothelial cells, neurons as well as glial cells such as Schwann cells. The lower panel shows the different mechanisms suggested to contribute to the development of DN. In yellow are the mechanisms involved in DN in T1DM, in blue are presented the mechanisms involved in T2DM associated DN and in green mechanisms that can apply to both conditions. Abbreviations: AGE, advanced glycation end products; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acids; ROS, reactive oxygen species (red star); ER, endoplasmic reticulum; PI3K, phosphatidylinositol 3-kinase. (Adapted from (Callaghan et al., 2012)).

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5. Chemokines

Chemokines were first discovered in 1987, when the cytokine called IL-8 was identified and demonstrated chemotactic ability for neutrophils. It was later called CXCL8 and considered a chemokine in 1992 (Yoshimura *et al.*, 1987; Rostène, Kitabgi and Parsadaniantz, 2007). Chemokines represent since a subgroup within the large family of cytokines as cyto*kines* with *chemo*tactic ability and have grown in number as more than 50 chemokines and around 23 chemokine receptors have been identified since (Xue *et al.*, 2019). They are small heparin-binding proteins ranging from 8 to 14 kDa with up to four conserved cysteine residues that are linked by disulfide bonds, giving rise to the characteristic structure called the "chemokine fold" (Bajetto *et al.*, 2002; Legler and Thelen, 2016). Chemokines are structurally classified into four subgroups based on the number and position of their first N-terminal cysteine residues: the α-chemokines or CXC group, whose cysteine group is separated by one amino acid, β-chemokines or CC group with adjacent cysteines, γ-chemokines or C group and the δ-chemokines or CX3C group (Bajetto *et al.*, 2002; Rostène, Kitabgi and Parsadaniantz, 2007).

The α-chemokines and β-chemokines are the largest group of chemokines and are similar in that they both contain four cysteine groups, with the former having an amino acid separating the first cysteine residues, while the latter has two adjacent cysteines. Members of the CXC group can chemoattract neutrophils, NK cells and B and T lymphocytes, while the CC group attracts basophils, eosinophils, DCs, macrophages, monocytes, NK cells and T lymphocytes, but not or only barely neutrophils (Mantovani, 1999; Rostène, Kitabgi and Parsadaniantz, 2007).

Unlike the other groups γ-chemokines possess only two of the four conserved cysteine residues and one disulfide bond. They consist only of two members, called XCL1 or lymphotactin-α and XCL2 or lymphotactin-β and attract T lymphocytes (Bajetto *et al.*, 2002; Rostène, Kitabgi and Parsadaniantz, 2007;

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Legler and Thelen, 2016). These two chemokines bind, according to current knowledge, specifically to XCR1 receptor.

The δ or CX3C group is at present represented by only one chemokine called fractalkine or CX3CL1 and has a characteristic structure, whose N-terminal cysteines are separated by three variable amino acids, in addition to a transmembrane region and a mucin-like domain. It exists in both a secreted soluble form, that is chemoattractive for T lymphocytes, NK cells and monocytes but not neutrophils, and as a membrane-anchored state on activated primary endothelial cells, where it facilitates adhesion of leukocytes (Bazan *et al.*, 1997; Mantovani, 1999; Rostène, Kitabgi and Parsadaniantz, 2007).

5.1. Structure

Chemokines can be active as monomers as well as homodimers, heterodimers or other forms of higher-order aggregates which can include either one or more chemokine types but also be more complicated by interaction with other molecules such as glycosaminoglycans (GAG) and thereby adding to the complexity of the system of chemokine interaction (Miller and Mayo, 2017; Hughes and Nibbs, 2018).

Monomeric forms of chemokines are fully active, so dimeric or other forms of oligomerization, often supported by heparin sulfate or other sulfated sugars, could elicit different responses within the same receptor as shown for some chemokines. Furthermore, oligomerization especially with other chemokines might enable synergistic effects that have other or more enhanced effects, such as in case of inflammation or infection when many different chemokines are secreted simultaneously (Legler and Thelen, 2016).

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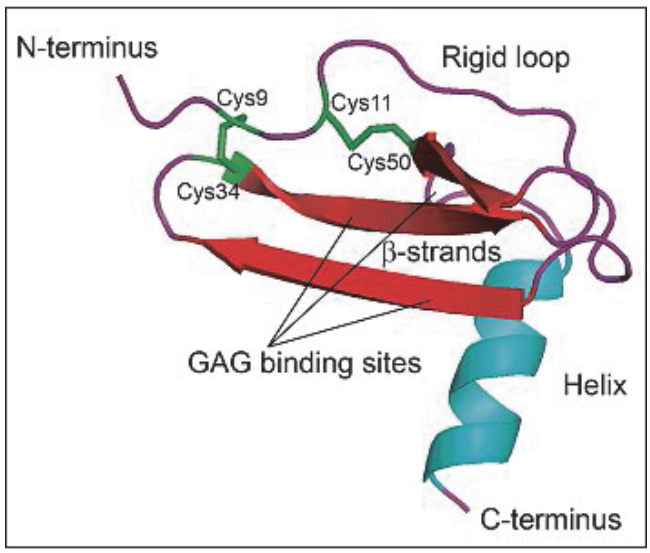

Figure 20: Chemokine structure and characteristic features exemplified by monomeric human CXCL12.
X-ray structure. (Adapted from (Legler and Thelen, 2016)).

Despite differences in their sequence identity, all chemokines have a common secondary or tertiary structure, which is given by their cysteine residues and disulfide bonds, and share the resulting chemokine fold (Bachelerie *et al.*, 2013). A chemokine monomer is usually composed of a flexible but unstructured N-terminus followed by one or more cysteine residues, continued by a long rigid loop and ending in a central three antiparallel-stranded β-sheet, which contains cysteines that are connected to the N-terminal through disulfide bridges. The construct ends with a helical C-terminus overlying the sheets and a short unfolded end (Fig. 20) (Bachelerie *et al.*, 2013; Réaux-Le Goazigo *et al.*, 2013; Legler and Thelen, 2016; Hughes and Nibbs, 2018).

The functionally most relevant domains for chemokines seem to be the N-terminus including the rigid loop, which are important for receptor binding, and GAG binding domains within the β-sheets or in the C-terminus (Clark-Lewis *et al.*, 1995; Rossi and Zlotnik, 2000; Rostène, Guyon, *et al.*, 2011).

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5.2. Chemokine Receptors

Chemokines bind and activate heptahelical GPCRs with 7TM domains and thereby mediate their hallmark function to chemoattract leukocytes and polymorphonuclear neutrophils (PMN) (Baggiolini, 1998; Moser *et al.*, 2004). These receptors are funtionally coupled to heterotrimeric and pertussis-toxin sensitive Gai proteins (Hughes and Nibbs, 2018).

Despite their redundancy in their interaction with promiscuous receptors, chemokines usually associate with receptors of the same class and thus the receptors match the nomenclature of chemokines they bind, followed by the letter R designating "receptor" and a number (Mantovani, 1999; Bajetto *et al.*, 2002). Only two monogamous chemokine-receptor interaction exist so far: CXCL12/ stromal cell-derived factor 1 (SDF-1) with CXCR4 and CX3CL1/fraktalkine with CX3CR1(Bajetto *et al.*, 2002).

So far five CXC (CXCR1-5), eight CC (CCR1-8), one of CX3C (CX3CR1) and C (XCR1) receptors have been described (Mantovani, 1999; Rostène, Guyon, *et al.*, 2011).

Most chemokine receptors share the following common features: a relatively short, extracellular N-terminus connected with four extracellular loops, that each contain a cysteine residue, in addition to a conserved sequence of amino acids (DRYLAIVHA) in the second intracellular loop, seven TM domains and ending in a C-terminus (Rajagopalan and Rajarathnam, 2006; Réaux-Le Goazigo *et al.*, 2013). The C-terminus contains multiple serine and threonine residues that are targets of phosphorylation during receptor-ligand interaction.

Apart from the above-mentioned "conventional" chemokine receptors (cCKRs) chemokines are also able to bind so called atypical chemokine receptors (ACKRs), of which only 4 are known so far: ACKR1 or Duffy antigen receptor for chemokines (DARK), ACKR2 or D6 or chemokine-binding protein 2 (CCBP2), ACKR3 or CXCR7 and ACKR4 or CCRL1 (Hughes and Nibbs, 2018).

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Although atypical chemokine receptors are similar in structure to cCKRs, they are not coupled to signaling pathways due to modifications in or lack of a part of the conserved region (DRYLAIVHA) in the second intracellular loop that is important for coupling with the G protein (Zlotnik and Yoshie, 2012). Thus, instead of exerting the characteristic effects of chemokines such as their chemotaxis for leukocytes, they have rather been reported in chemokine scavenging or as decoy/interceptor receptor and thereby keep chemokine levels low or preventing them to bind to other receptors (Zlotnik and Yoshie, 2012).

5.3. Ligand-Receptor Interaction and Signaling

It was reported that chemokines interact with their receptors mainly with their N-terminus and a region within their exposed loop of the backbone that extends beyond the second cysteine (Baggiolini, 1998).

In general, structure-function studies have suggested the two-step model for chemokine ligand-receptor binding and activation. Simplified, this model states that the first contact between chemokine and its receptor is established between the rigid loop of the ligand and the N-terminus of the cognate receptor, which acts as chemokine recognition site and determines the affinity and specificity of binding (Kleist *et al.*, 2016; Legler and Thelen, 2016). Subsequently, the N-terminus of the chemokine is inserted into the activation pocket of transmembrane helices of the receptor and induces a conformational change and activation of the latter (Crump *et al.*, 1997; Legler and Thelen, 2016). This then leads to the signal transduction via the heterotrimeric coupled G protein to exert its manifold actions via different secondary pathways.

5.4. Function

Chemokines exert their function through interaction with their receptors and target cells that express those receptors. Each of these interactions is redundant, because several chemokines can bind several chemokine receptors, and vice versa. The same is true for their target cells, although

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chemokines are diverse in which cells they attract, many chemokines can target similar immune cells, and one cell can express and secrete several chemokines (Mantovani, 1999).

Apart from the structural categorization, chemokines can be further discriminated based on their functional classification in "inflammatory" or inducible, "homeostatic" or constitutive, or in "dual-function" for a few chemokines (Rajagopalan and Rajarathnam, 2006). Chemokines in the inflammatory category are principally induced and produced during inflammatory conditions and function, mainly in the recruitment of immune cells to target locations upon injury or infection (Zlotnik and Yoshie, 2012). For example, chemokines such as CCL3-5 or CCL2 can act on their respective receptors CCR1/CCR5 and CCR2, to regulate the cytolytic activity of NK cells as well as bactericidal protease release upon infection with a pathogen (Maghazachi, 2010; López-Cotarelo *et al.*, 2017). Interestingly, chemokines can be induced by pro-inflammatory cytokines such as IL-6, IL-1 and TNF-a (Kalinkovich, Weisman and Bentwich, 1999). Different from that, homeostatic chemokines are rather expressed constitutively in different tissues and aid in immune cells circulation through tissues for immune surveillance and under physiological conditions. For example, CXCL12 acts through CXCR4 in a homeostatic context to regulate hematopoiesis (Moser *et al.*, 2004). Chemokines in the dual-function category can be both homeostatic and inflammatory (Mantovani, 1999; Zlotnik and Yoshie, 2012). Dual-function chemokines can be act in both homeostatic and inflammatory conditions like CCL5 that is acting both as a chemoattractant to recruit macrophages to the site of inflammation for example in obesity, but also can play a role in a homeostatic context by controlling glucose uptake in T cells (Keophiphath *et al.*, 2010; Chan *et al.*, 2012).

The chemokine system is tightly regulated and destabilization of this system can have detrimental effects and result in potential pathological conditions such as development of allergies, autoimmunity, inflammatory diseases and chronic inflammatory conditions and cancer progression and metastasis

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(Rajagopalan and Rajarathnam, 2006; Sahin, Trautwein and Wasmuth, 2010; Roh and Seki, 2018).

Chemokines are most famous for their functions in cell migration especially in the pathological context of infection, injury or inflammation, where they regulate leukocyte recruitment and their trafficking to the source of the inflammatory stimuli. In general, invading pathogens or other inflammatory stimuli lead to a local secretion of chemokines, forming a chemokine gradient. Circulating leukocytes can then migrate along this gradient to the source of the gradient and exert a localized and specific effect to contain the infection or inflammation, or promote wound healing and repair. In addition to that, many different cells and tissues throughout the body can express chemokines and their cognate receptor and depending on which cell or tissue they act on it can result in various other responses (Stellato *et al.*, 2001; Cinamon *et al.*, 2008; Griffin *et al.*, 2010; Beck *et al.*, 2014; Kulkarni, Pathak and Lal, 2017). This includes but is not limited to proliferation, activation and differentiation, extracellular matrix remodeling (Baggiolini, 1998; Moser *et al.*, 2004). Additionally, some studies report a chemorepulsive effect (also called chemofugetaxis) in that some cells can move against the chemokine gradient (Tharp *et al.*, 2006).

However, chemokines can act also under physiological conditions, such as chemokines belonging to the homeostatic functional group. They are constitutively expressed in different tissues and participate in normal immune surveillance, and cell adhesion, especially in the context of transcellular migration or diapedesis via the interaction with GAG (Cinamon *et al.*, 2008).

A few other studies have even reported direct microbicidal activity by some chemokines such as CXCL14, CXCL17 and CCL28 (Poznansky *et al.*, 2000, 2002; Hieshima *et al.*, 2003; Ogilvie *et al.*, 2003; Vianello *et al.*, 2005; Tharp *et al.*, 2006; Maerki *et al.*, 2009; Burkhardt *et al.*, 2012).

Some chemokines and chemokine receptors are even indispensable for life, such as CXCL12 and the receptors CXCR4 and ACKR3 because they play

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crucial roles in the development of various organs and cells including the heart, brain, hematopoietic system and vascular system (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998; Finke *et al.*, 2002; Sierro *et al.*, 2007; Valentin, Haas and Gilmour, 2007; Klein *et al.*, 2014).

Moreover, chemokines play important roles in angiogenesis (Dimberg, 2010), tumor metastasis (Müller *et al.*, 2001) and thereby can contribute to cancer or in some cases have anti-tumor effects (Keeley, Mehrad and Strieter, 2010, 2011; Rezaeeyan *et al.*, 2018).

Besides their well-known role in the immune system, an accumulating body of research seems to increasingly suggest that chemokines might play an important role in the CNS (Bajetto *et al.*, 2002; Rostène, Dansereau, *et al.*, 2011; Réaux-Le Goazigo *et al.*, 2013).

5.1. Chemokines Function in the CNS

Several different in vivo and in vitro studies have indicated that chemokines and their receptors are expressed in the CNS, not only in pathological conditions but also in a constitutive manner, which will be described more in the following sections.

For chemokine receptors studies reported the expression of all CXC type receptors, all CC type receptors apart from CCR6 and CCR7, and even CX3CR1 in the CNS. Moreover, many studies have described the expression of the respective chemokine ligands, too. It was reported that chemokines and chemokine receptors are expressed by nearly all cell types in the CNS, including different glia cells like astrocytes, oligodendrocytes, microglia but also neurons and endothelial cells (reviewed in (Dorf *et al.*, 2000)). Apart from constitutive expression chemokines have not only been found to play important roles in many inflammatory diseases of the periphery but many studies found their implication in diseases of the CNS, such as experimental autoimmune encephalomyelitis (EAE), Alzheimer's disease (AD), multiple sclerosis (MS), acquired immune deficiency syndrome dementia complex and many other inflammatory brain conditions. Other than that, chemokines were

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also found to be expressed in normal healthy brains. For example, studies have found that fetal human and murine cells express chemokine receptors CCR5, CCR3 and CXCR4 (Klein *et al.*, 1999; Croitoru-Lamoury *et al.*, 2003).

Chemokine receptors (CXCR4/CCR5) have been found to be expressed in neurons and many other chemokine receptors also in glia cells such as microglia and astrocytes (Dorf *et al.*, 2000; Banisadr *et al.*, 2002; Westmoreland *et al.*, 2002).

Another three studies used immunofluorescence and confocal microscopy to detect chemokine expression in primary cultures of human embryonic neurons, microglia and astrocytes. They found functional CCR5 and CXCR4 expression and transcript synthesis in cell body and processes of embryonic neurons, while embryonic astrocytes and microglia expressed in addition to CCR5 and CXCR4 also CCR3. While microglia were the only cell that responded to eotaxin or CCL5, a CCR3 agonist, with modulated calcium response, neurons and astrocytes had increased intracellular Ca^{2+} levels only upon stimulation with the respective agonist of CCR5 (CCL5 and MIP-1β) and CXCR4 (SDF-1α) (Hegg *et al.*, 2000; Boutet, Salim, Leclerc, *et al.*, 2001; Boutet, Salim, Taoufik, *et al.*, 2001). Moreover, in 2005, Omari and colleagues found chemokine receptors CXCR1-3 expressed on oligodendrocytes in the adult human CNS (Omari *et al.*, 2005). Chemokines were not only expressed but also involved in brain development. For example, CXCR4 is not only a co-receptor for viral entry of HIV-1, but also was found to be involved in neuronal cell migration and patterning during cerebellar development (Zou *et al.*, 1998; Tran and Miller, 2005).

Chemokine (BNDF and SDF-1) signaling through CXCR4 and CCL5 signaling was shown to be neuroprotective and promoting neuronal survival (Bachis, Major and Mocchetti, 2003; Imitola *et al.*, 2004; Bachis and Mocchetti, 2005; Nosheny, Mocchetti and Bachis, 2005; Kaul *et al.*, 2007).

Interestingly, chemokines have been also been implicated in the regulation of energy balance. For example, the chemokine CCL2 has been recently shown

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to mediate the LPS-induced anorexic effect in the hypothalamus by modulating the activity of MCH neurons and thereby leading to weight loss (Le Thuc *et al.*, 2016).

5.2. Role of Chemokines in Obesity and Comorbidities

Chronic Inflammation is a common factor, and plays an important role in the etiology of several diseases such as obesity and associated comorbidities such as type 2 diabetes and neuropathic pain, metabolic syndrome, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) and liver steatosis (Esser *et al.*, 2014).

Although a multifactorial condition, obesity is often caused by environmental factors such as a diet rich in saturated fats and sugars in addition to a sedentary life-style. Although the exact mechanism behind the etiology of obesity is not quite clear, several studies have identified some characteristics of this pathology.

Obesity is a complex condition caused by interaction of different factors and is often associated with chronic low-grade inflammation and a secretion of pro-inflammatory cytokines and chemokines. The cytokines most often associated and studied in relation to obesity are IL-1β, IL-6 and TNF-a (Hotamisligil, Shargill and Spiegelman, 1993; Uysal *et al.*, 1997; Spranger *et al.*, 2003; Kristiansen and Mandrup-Poulsen, 2005).

In addition to that, activation of pathways including JNK, IKKβ/NF-κB as well as mammalian target of rapamycin (mTOR)/S6 kinase, that were found to affect insulin action in both adipocytes and hepatocytes, seem to be inherent to obesity-associated inflammatory processes (Ota, 2013).

Despite the fact that chronic inflammation in obesity is multifactorial and involves many chemokines, chemokines like CCL2, CCL5 and CCL3/MIP-1a among others, have been particularly implicated in obesity. For example, they are expressed in WAT of obese mice and with their chemotactic ability it is not a surprise that chemokines have been found to play a major role in

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macrophage recruitment and contribution to insulin resistance in obesity (H. Xu *et al.*, 2003; Sartipy and Loskutoff, 2003; Kanda *et al.*, 2006). CCL2 and its receptor CCR2 in particular are highly increased in obese AT and the overexpression of CCL2 leads to an elevated increase in macrophage recruitment of AT macrophages and ultimately insulin resistance (Kanda *et al.*, 2006). In support of this, CCR2 genetic deficiency protected partially from glucose intolerance, and AT macrophage infiltration as well as inflammation, when mice were fed a HFD for an extended period of time (Weisberg *et al.*, 2006; Lumeng, Bodzin and Saltiel, 2007; Gutierrez *et al.*, 2011). Contrary to that, deficiency of the chemokine CCL3 seemed to have no ameliorating effect on macrophage or T cell recruitment to AT in DIO mice (Kennedy *et al.*, 2012). However, the study demonstrated that CCL3-deficient bone marrow had an effect on plasma cholesterol, TG concentrations and reduced AT mass, plasma insulin and leptin levels, compared to bone marrow transplants from either C57BL/6 or $CCL2^{-/-}$ mice. This indicates that the effects are specifically mediated by bone marrow-derived CCL3-deficient cells and that CCL3 has a potential role in lipid metabolism in mice fed a hyperlipidemic diet (Kennedy *et al.*, 2012).

Several studies implicated a number of chemokines also in the pathogenesis of type 1 and 2 diabetes and showed their upregulation in human and murine pancreatic tissue (Saurer *et al.*, 2000; C. Herder *et al.*, 2006; Marselli *et al.*, 2008; Planas *et al.*, 2010; Sarkar *et al.*, 2012; Esser *et al.*, 2014; Moretto *et al.*, 2017).

5.2.1. CCL5/RANTES

First identified by Schall in 1988 the chemokine (C-C motif) ligand 5 or also known as **R**egulated upon **A**ctivation **N**ormal **T** cells **E**xpressed and **S**ecreted (RANTES), CCL5 is encoded by a gene on the q-arm of chromosome 17 in humans (4 exons) (Donlon *et al.*, 1990) and on 11 (3 exons) in mice. The gene product of human CCL5 was predicted to be 10 kDa in size, that upon cleavage results in an 8 kDa sized peptide, with 68 amino acid residues,

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among which are 4 cysteines. Schall and colleagues have not found any sites for N-linked glycosylation, nor a transmembrane domain in the positively charged, basic and mature protein (Schall *et al.*, 1988).

CCL5 belongs to the group of inflammatory β-chemokines (Levy, 2009) and exerts its effects through 7TM GPCRs including CCR1, CCR3 and CCR5 (Raport *et al.*, 1996) .

In addition, a few studies have found that apart from its cognate chemokine receptors CCL5 can bind also ACKRs such as CCBP2, DARK (Horuk, 2015) as well as the orphan GPCR 75 (GPR75) (Ignatov *et al.*, 2006).

Characterisation of T cell-derived human CCL5 showed that although CCL5 was constitutively expressed at basal levels, after cellular activation, expression reaches high levels only 5 to 7 days after stimulation and decreases dramatically 6 to 24 hours after, before returning to basal levels within 3 days (Schall *et al.*, 1988). Contrary to initial belief, CCL5 is inducible in many different cell types, including macrophages, T lymphocytes and human Th1 clones but only minimally by Th2 cells (Schrum *et al.*, 1996). Furthermore, it is secreted by fibroblasts, thrombin-stimulated platelets, endothelial cells, mesangial cells, TNF-α or IL-1β-stimulated renal tubular epithelial cells, macrophages, eosinophils, AT (Skurk *et al.*, 2009).

CCL5 is a pleiotropic chemokine that exerts chemotactic activity for monocytes, CD4 T cells (Schall *et al.*, 1990) and is also chemotactic and activating for eosinophils (Alam *et al.*, 1993) in addition to modulating histamine release from basophils (degranulation) (Alam *et al.*, 1992; Kuna *et al.*, 1992).

Many studies reported CCL5 to be involved in the pathophysiology of many inflammatory diseases, or conditions characterized by a chronic inflammatory state such as, atherosclerosis (Braunersreuther *et al.*, 2007; Jones, Maguire and Davenport, 2011), liver disease (Pérez-Martínez *et al.*, 2014; Mohs *et al.*, 2017), rheumatoid arthritis (Rathanaswami *et al.*, 1993) but also neurodegenerative diseases like AD (Xia *et al.*, 1998), MS (Trebst *et al.*, 2001; Mori *et al.*, 2016), EAE

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and cancer (Conti and DiGioacchino, 2001; Vaday *et al.*, 2006; Soria and Ben-Baruch, 2008; Réaux-Le Goazigo *et al.*, 2013; Weitzenfeld and Ben-Baruch, 2014; D'Esposito *et al.*, 2016).

5.2.1.1. Expression

It was found to be upregulated in psoriatic lesions and secreted in epidermal keratinocytes upon stimulation by a combination of TNF-a and interferon-γ (IFN-γ) and production is inhibited by an active vitamin D3 analogue (Fukuoka *et al.*, 1998; Warhurst, Hopkins and Warhurst, 2010). This indicates that CCL5 signaling is regulated downstream of TNF-a pathway in synergy with other cytokines. Interestingly, CCL5 is expressed also in adipocytes and murine AT stromal/vascular cells of mice and humans and CCL5 mRNA levels are negatively correlated with murine AT adiponectin (Wu *et al.*, 2007).

Adiponectin-deficient mice on a HFD had upregulation of CCL5 mRNA levels in AT compared to WT mice (Wu *et al.*, 2007).

Interestingly, expression of the mRNA of CCL5 was also found in small intestine, with highest expression in duodenum, jejunum and ileum of mice (Shang *et al.*, 2009).

Apart from peripheral expression, CCL5 was found to be expressed in and secreted by astrocytes (and secreted by astrocytes) (Chou *et al.*, 2008), microglia (Hu *et al.*, 1999; Avdoshina *et al.*, 2010) and neurons in the CNS (Lanfranco *et al.*, 2018). Interestingly, CCL5 was expressed, and induced differentiation in an age-dependent manner, in human fetal astrocytes and seems to have an important role in the ontogenetic development of the CNS and PNS (Bolin *et al.*, 1998; Bakhiet *et al.*, 2001).

Additional support for a central role of CCL5 was provided in a murine Huntington's disease (HD) model, in which CCL5 promoted cortical neurite outgrowth and neuronal activity. They also confirmed previous results showing that astrocytes express and secrete CCL5, which is impaired in HD (Chou *et al.*, 2008; Park *et al.*, 2009). Interestingly, HD is associated with metabolic

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impairment, such as insulin and glucose regulation impairments (Hurlbert *et al.*, 1999; Lalić *et al.*, 2008).

Researcher have also found that CCL5 among other chemokines is a downstream factor of hepatocyte growth factor in promoting axon extension (Bhardwaj *et al.*, 2013).

CCL5 is not only expressed in embryonic DRG cells, Schwann cells and spinal cord, but was also found to elicit neural migration in DRG sensory neurons, that differentiate into NPY and CGRP expressing cells, which are associated with the phenotype of thermoceptive and nociceptive DRG neurons, that transmit pain and temperature sensation (Bolin *et al.*, 1998). Furthermore, CCL5 elicits a transient Ca^{2+} mobilization in embryonic DRG cells through coupling to a GPCR (Bolin *et al.*, 1998).

5.2.1.2. Receptor Interaction and Synergistic Effects With Other Chemokines

Induced by TNF-α, IL-1β and IFN-γ and when TNF-α or IL-1β receptor are inhibited, CCL5 induction is inhibited (Pattison, Nelson and Krensky, 1995; Fukuoka *et al.*, 1998). Additionally, corticosteroids are able to inhibit the transcription of CCL5 in fibroblasts (Pattison, Nelson and Krensky, 1995). Furthermore, IL-4 and IL-13 inhibit CCL5 expression and secretion in endothelial cells (Marfaing-Koka *et al.*, 1995). The ability of microglia cells to produce CCL5 can be inhibited by IL-10 and TGF-β, which shows that also other cytokines can regulate CCL5 at least in microglia cells (Hu *et al.*, 1999). This study found also that chemokine binding protein M3, which interacts with many members of all chemokine families and blocks their activity, binds with high affinity to CCL5 among others.

CCL5 expression and secretion was inducible in a time-dependent manner by opiates, and morphine in astrocytes and LPS seemed to induce an increased production of CCL5 in microglia cells *in vitro* (Avdoshina *et al.*, 2010).

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Furthermore, CCL5 has been shown to act as a substrate for DPP4, which appears to cleave the N-terminal serine and proline residues in CCL5. It might thereby modulate its activity, by affecting its binding affinity to chemokine receptors or by potentially decreasing the bio-availability of CCL5 (Schols *et al.*, 1998; Oravecz *et al.*, 2002; Liu *et al.*, 2013).

In support of its potential role in neuromodulation and neurotransmission studies have found that increases in Intracellular Ca^{2+} induced by CCL5 have been also demonstrated in spinal cord synaptosomes. Additionally, studies found CCL5 to be involved in the control of glutamate release, with opposing effects depending on which receptor is activated (Meucci *et al.*, 1998; Klein *et al.*, 1999; Musante *et al.*, 2008; Di Prisco *et al.*, 2012).

Studies indicate that CCL5 might regulate glucose uptake and 5' adenosine monophosphate-activated protein kinase (AMPK)-a signaling in T cells through activation of the mTOR pathway in an attempt to provide enough energy for chemotaxis (Chan *et al.*, 2012). Interestingly, a recent study implicated CCL5-CCR5 signaling in the hypothalamic regulation of insulin signaling (Chou *et al.*, 2016). In a study, administration of CCL5 (50ng/ml) induced rapid calcium influx from the extracellular environment through CCR3 activation in human fetal microglia cultures (Hegg *et al.*, 2000) and potentially through CCR5, as it is expressed in microglia, too (He *et al.*, 1997; Albright *et al.*, 1999).

Apart from identifying CCR5 expression specifically in neurons of the ARC and CCL5 immunoreactivity in both ARC and VMH of the hypothalamus, they found increased phosphorylated AMPKa (pAMPKa) immunoreactivity in the ARC of $CCR5^{-/-}$ mice compared to control mice. Furthermore, whereas pAMPKa was increased in fasted conditions in WT mice, CCR5 and CCL5 deficient mice showed elevated pAMPKa in both fed and fasted conditions (Chou *et al.*, 2016). Interestingly, qPCR analysis of hypothalamic neuropeptide expression indicated an upregulation of both orexigenic (NPY and AgRP) and anorexigenic (POMC) neuropeptides in KO mice for CCL5 and its receptors

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compared to WT controls fed a regular diet. In agreement with this, primary hypothalamic neuron cultures identified CCR5 expression in $POMC^+$ neurons. These results suggest a potential role of CCL5 signaling in the regulation of energy balance and feeding regulation as proposed by the authors. According to the authors, both, CCL5 and CCR5 deficient mice revealed an impaired insulin response, which was supported by downregulation of genes associated with energy and insulin signaling regulation in KO mice.

CCL5 seems to have different ways of eliciting its function and through different pathways, depending on its concentration and state. Apart from chemotaxis, it was found to activate different types of cells including T cells, monocytes and neutrophils (Appay *et al.*, 1999, 2000). CCL5 is capable of aggregation, and depending on its oligomerized forms as a dimeric, trimeric or a monomer it can have different effects. For example, it was reported that activation of immune cells requires CCL5 to be in aggregated form, as non-aggregating variants had less or no effect on leukocyte activation (Appay *et al.*, 1999). Cell-surface GAG seem to have a promoting effect on the oligomerization ability of CCL5 and other chemokines and thus are important for leukocyte activation, while soluble GAG seem to have a more inhibiting effect on chemokine action (Hoogewerf *et al.*, 1997; Kuschen *et al.*, 1999). Similarly, the concentration of chemokines has an impact on their function. At high micromolar concentrations, CCL5 can activate leukocytes through a GPCR independent manner (protein tyrosine kinase-mediated pathway). The activation of this pathway is believed to elicit cell activation and cytokine release or cell proliferation via induction of a prolonged calcium influx, hyperphosphorylation (Appay *et al.*, 1999, 2000; Bacon, 2004). In contrast to this, CCL5 seems to act in a manner characteristic for chemokines at nanomolar concentrations to induce chemotaxis and receptor polarization by activating GPCRs and generating a transient calcium influx (Nieto *et al.*, 1997).

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5.2.2. Receptors of CCL5

5.2.2.1. C-C Motif Chemokine Receptor 1 (CCR1)

CCR1 is a typical 7TM GPCR and expressed on immune cells like monocytes, memory T cells, basophils and DCs (Neote *et al.*, 1993; Bachelerie *et al.*, 2013). In addition, it seems to be also expressed on intestinal eosinophils and macrophages (Shang *et al.*, 2009), lung airway smooth muscle cells, murine neutrophils, endothelial cells and different myeloma cells (Horuk, 2001).

Moreover, it seems to be expressed in cells of the CNS such as astrocytes and hippocampal neurons, in intestinal cell lines, GLP-1 secreting enteroendocrine L-cells and other cells of murine small intestine (Meucci *et al.*, 1998; Dorf *et al.*, 2000; Pais *et al.*, 2014).

CCR1 is like many chemokine receptors promiscuous because it can bind to several chemokines including CCL3, CCL5 and with lower affinity to CCL6, CCL9, CCL15 and CCL23 but does not seem to have a selective ligand so far (Neote *et al.*, 1993; Bachelerie *et al.*, 2013). Surprisingly, it was reported, that although CCL5 binds with lower affinity to CCR1 compared to other ligands, its ability to induce a calcium signal was much more efficient than chemokines that bind with higher affinity to the receptor (CCL2 and human CCL4) (Neote *et al.*, 1993). CCR1 signaling follows the classical chemokine pathway and induces inhibition of adenylyl cyclase and activation of phospholipase C (PLC), PKC, calcium flux and phospholipase A2 via G proteins belonging to the Gi/G0 class (Bachelerie *et al.*, 2013).

CCR1 invalidation studies implicated the receptor in the pathophysiology of MS and organ transplant rejection, protection from secondary pulmonary inflammation and injury in an acute pancreatitis model (Gerard *et al.*, 1997).

5.2.2.2. C-C Motif Chemokine Receptor 3 (CCR3)

Belonging to the CC group of chemokine receptors, CCR3 is expressed on intestinal eosinophils and contrary to the expression of CCR5, it is highly expressed in $CD4^+$ Th2 cells but undetected in Th1 cells (Loetscher *et al.*, 1998;

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Shang *et al.*, 2009). Furthermore, expression was found in the CNS, more specifically in astrocytes (Dorf *et al.*, 2000) and microglia (He *et al.*, 1997; Ghorpade *et al.*, 1998; Hegg *et al.*, 2000) and subpopulations of neurons (Klein *et al.*, 1999).

CCR3 has several ligands including the selective agonist eotaxin (CCL11), but can be also activated by CCL5 and CCL7, CCL8, CCL13, CCL15, CCL24, CCL26 and CCL28 (Heath *et al.*, 1997; Albright *et al.*, 1999; Alexander, Mathie and Peters, 2011)

A study reported that signaling through CCR3 can be induced by binding of its ligands CCL5 (or CCL11) and evokes a rapid influx of extracellular calcium in 26% of microglial cells (Hegg *et al.*, 2000).

5.2.2.3. G Protein-coupled Receptor 75 (GPR75)

First identified by Tarttelin et al. in 1999 as a novel human orphan GPCR, GPR75 is a 540 amino-acid sequence protein that belongs to the Gqa family of G proteins. It was reported to be mainly expressed in the CNS, the retina and vascular endothelial cells, but not in immune organs contrary to chemokine receptors (Tarttelin *et al.*, 1999; Sauer *et al.*, 2001; Garcia *et al.*, 2017). So far, only few ligands have been identified that bind GPR75, namely, the eicosanoid 20-hydroxyeicosatetraenoic acid, CCL3 and CCL5 (Ignatov *et al.*, 2006; Garcia *et al.*, 2017). It is quite surprising that CCL5 is an agonist for GPR75, seeing as it shares only 12-16% amino acid identity with chemokine receptors. The highest homology of GPR75 was found with neuropeptide Y receptor. Apart from the basic 7TM structure, GPR75 differs from chemokine receptors in that it has a longer C-terminal tail, a lack of the characteristic "aspartate-arginine-tyrosine" motif in the transmembrane region and signaling is unimpaired by pertussis toxin (Ignatov *et al.*, 2006; Liu *et al.*, 2013; Garcia *et al.*, 2017).

It has been reported, that CCL5 signaling through endogenously expressed GPR75 in hippocampal cell lines protected Aβ-induced neuron death by activating signaling pathway PLC/PI3K/Akt/MAPK (Fig. 21)(Ignatov *et al.*,

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2006). A recent study has further demonstrated the constitutive expression of GPR75 in islets of murine and human pancreas, where it was involved in CCL5 mediated calcium modulation and related insulin secretion (Liu *et al.*, 2013).

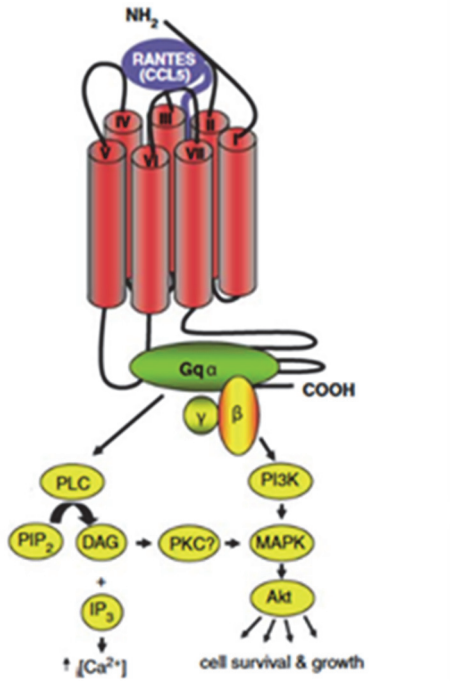

Figure 21 : The signaling cascade of CCL5 coupling to GPR75.
(Adapted from (Pease, 2006)).

5.2.2.4. Duffy Antigen Receptor For Chemokines (DARC)

Apart from the most common receptors CCL5 binds to, it was shown that it can also bind to a promiscuous and atypical and non-signaling chemokine receptor, called DARC (Horuk, 2015). DARC consists of 338 amino acids and with its 7TM domains, it belongs to the GPCR family. However, it has only less than 20% amino acid identity with chemokine receptors belonging to the CXC or CC family. Apart from erythrocytes and some B and T cells, the receptor is also found in many tissues like heart, lung, thymus, spleen, kidney and pancreas, which might be due to its expression in endothelial cells lining post-capillary venules. It is postulated that expression of DARC on erythrocytes might attenuate the inflammatory chemokine activity in the blood by internalization of the bound ligand, or similar to binding to GAG it might keep them cleared from circulation. Furthermore, it was found that chemokine

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binding to DARC on erythrocytes protects from malaria-causing parasite *Plasmodium vivax* by blocking their binding to and infection of erythrocytes (Chitnis *et al.*, 1996). Endothelial cell expression of DARC might further provide a path for attachment to establish a chemokine gradient, attract leukocytes and potentially induce diapedesis or transport chemokines across endothelial cells (Horuk *et al.*, 1996; Baggiolini, Dewald and Moser, 1997; Horuk, 2015). Interestingly, DARC is also expressed in the brain, more specifically in Purkinje cells of the cerebellum, which opens up the possibility of DARC having a role in modulating neuronal activity stimulated by chemokines. CCL5 appears to bind to DARC receptor on human and mouse erythrocytes with a high affinity (K_D) of 4.8 nM and 8.3 nM respectively (Horuk *et al.*, 1996).

5.2.2.5. Chemokine-binding Protein 2 (CCBP2)

CCBP2, also called ACKR2 or D6 is an atypical 7TM chemokine receptor and different from other chemokine receptors, it does not transduce ligand binding into an intracellular signal. This is most likely due to changes in the characteristic chemokine motif "D**R**YL**A**IV" to "D**K**YL**E**IV" and the lack of coupling to G proteins (Nibbs *et al.*, 1997; Hughes and Nibbs, 2018) Therefore it is often referred to as decoy receptor that ia able to bind promiscuously to most members of the inflammatory β-chemokine family, which has 12 identified ligands so far (Bonecchi *et al.*, 2004). Several tissues express D6 receptor including skin, gut and lung tissues but mostly lymphatic endothelial cells (Nibbs *et al.*, 2001).

Similar to DARK, it seems to act on chemokines by internalizing them through a β-arrestin dependent way and degrading them upon binding and thereby potentially playing a role in the homeostasis of circulating chemokine levels and their clearance from inflamed tissues (Nibbs, Graham and Rot, 2003; Galliera *et al.*, 2004).

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5.2.2.6. C-C Motif Chemokine Receptor 5 (CCR5)

CCR5 is like the other above mentioned chemokine receptors a 7TM GPCR (Fig. 22) and well known for its role in the immune system but also as primary co-receptor facilitating the cell entry for HIV (Sorce, Myburgh and Krause, 2011).

Most studies report that apart from CCL5, CCR5 binds to other chemokines such as macrophage inflammatory proteins (MIP-1a or CCL3) and MIP-1β (CCL4) with high affinity (Raport *et al.*, 1996; Blanpain *et al.*, 1999; Waller *et al.*, 2018).

However, it was reported that it seems to bind also MCP-2 (CCL8), eotaxin (CCL11), HCC-1 (CCL14a), HCC-4 (CCL16) and that CCL7 (MCP-3) can act as an antagonist on CCR5 (Blanpain *et al.*, 1999; Alexander, Mathie and Peters, 2011).

It is expressed on many immune cells such as $CD4^+$ Th1 cells (high expression) and almost undetectable in Th2 cells (Loetscher *et al.*, 1998), but is found on macrophages and DCs (Waller *et al.*, 2018). CCR5 mRNA expression was also found on intestinal T cells and macrophages (Shang *et al.*, 2009).

Interestingly, several studies have reported the constitutive expression of CCR5 in the CNS (Sorce, Myburgh and Krause, 2011). For example, CCR5 expression was found on microglia (He *et al.*, 1997; Ghorpade *et al.*, 1998; Albright *et al.*, 1999; Waller *et al.*, 2018), astrocytes and neurons in the CNS (Meucci *et al.*, 1998; Klein *et al.*, 1999; Avdoshina *et al.*, 2011). Moreover, when microglia were stimulated with CCL3 and CCL5, it lead to a response in intracellular calcium levels through either CCR3 or CCR5, which has higher expression levels in microglia than CCR3 (Albright *et al.*, 1999).

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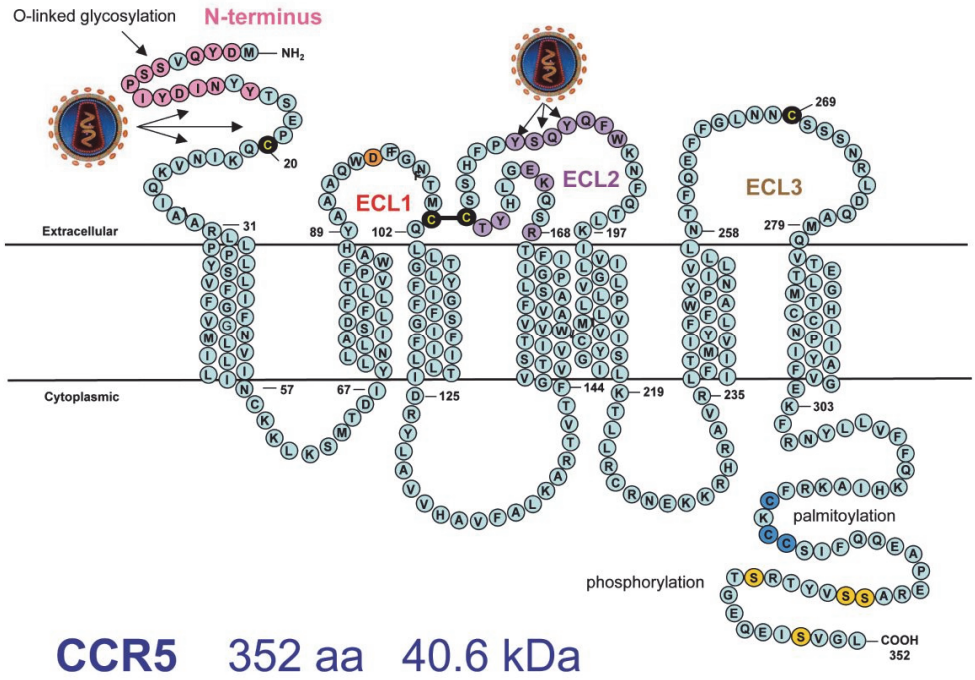

Figure 22 : Structure of CCR5 7TM chemokine receptor.
The structure of CCR5 includes different sites of regulation and HIV binding sites. ECL: Extracellular loop. (Adapted from Lapalco, C. 2011).

Heterozygous mice for BNDF have increased expression levels of CCR5 mRNA in the hippocampus, striatum and cerebral cortex. This means that BDNF seems to be able to negatively regulate and reduce CCR5 expression in the brain and that CCR5 is indeed expressed in parts of the brain (Ahmed *et al.*, 2008). Another study reinforces the idea that CCL5 through CCR5 signaling is involved in brain development as they found CCR5 expression and production in human embryonic neurons, located inside the neuronal cell body and processes, and upon stimulation evoked an increased intracellular Ca^{2+} response (Boutet, Salim, Leclerc, *et al.*, 2001). CCR5 was localized in neuronal cell body and processes, especially in Purkinje cells of the cerebellum and pyramidal neurons of the hippocampus, as well as subpopulations of striatal and cortical neurons (Avdoshina *et al.*, 2011). Other findings confirmed and added to these results by showing CCR5 expression in subpopulations of cortical neurons of human and macaque brain, in fetal neurons and astrocyte culture cells (Klein *et al.*, 1999; Westmoreland *et al.*, 2002).

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Although CCL5-CCR5 signaling seems to play an important role in development, it is intriguing that KO models or humans with a deletion in the gene for CCR5 do not have any particular or obvious phenotype, apart from partial impairments in immune function in mice. Homozygous $CCR5^{-/-}$ in humans even established protection from HIV-1 infection and progression. But it was reported that homozygous individuals also are more prone to other diseases, such as listeria infection or West Nile virus and with more serious outcomes than people without the mutation (Alkhatib, 2009; Sorce, Myburgh and Krause, 2011). In this study we were particularly interested in CCL5 and CCR5 singling and will thus focus more in detail on this chemokine and receptor.

5.2.2.6.1. Signaling

GPCRs such as CCR5 mediate their effects through heterotrimeric G proteins. It has been reported that CCR5 can signal through all G protein subunits such as $G\alpha_i$ and βγ dimers to induce several different effectors (Fig. 23). CCR5 activation by its ligands leads to signal transduction via three members of the MAPK family, namely ERK1/2, p38 and stress-activated protein kinase/JNK. However, CCR5 can also signal through adenylyl cyclase or PLCβ leading to intracellular calcium influx and formation of inositol-1,4,5-trisphosphate (IP_3) (Fig. 23)(Oppermann, 2004; Wu and Yoder, 2009). Firstly, signal transduction via the $G\alpha_i$ subunit usually results in adenylyl cyclase inhibition, followed by a decline in intracellular cAMP levels. Secondly, signaling through the $G_{q\alpha}$ subunit of the G protein can activate PLC leading to a transient increase in intracellular calcium, via DAG and IP_3 production followed by increased PKC activity (Fig. 23)(Réaux-Le Goazigo *et al.*, 2013). Signaling by interaction with the $G_{\beta\gamma}$ subunit leads to the release of the dimeric subunit and the activation of PLCβ, which in turn activates PKCε. Chemokine receptors can act also via more distant signaling pathways such as the MAPK pathway and other pathways including janus kinases (JAK), STATs and NF-κB pathways resulting in a plethora of effects (Réaux-Le Goazigo *et al.*, 2013).

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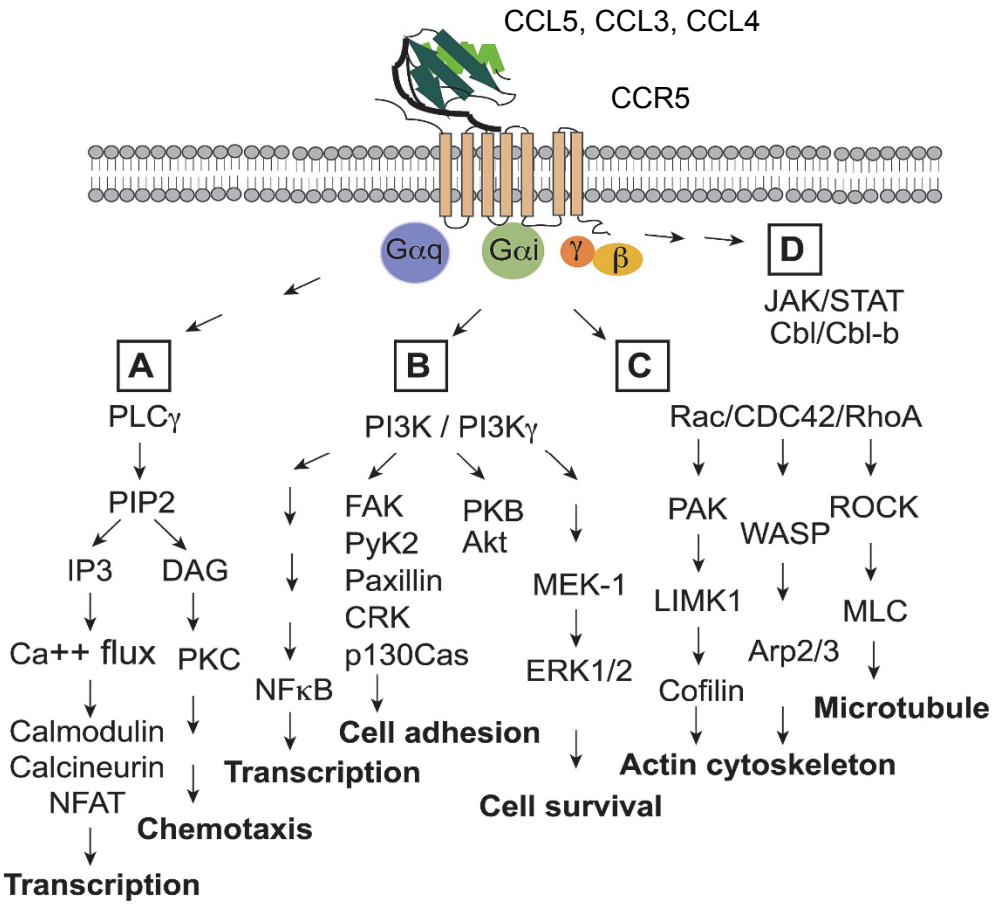

Figure 23: Signal transduction Pathways of CCR5.
(Adapted from (Wu and Yoder, 2009)).

5.2.2.6.2. Regulation

Chemokine receptors like CCR5 can be regulated by different mechanisms, including endocytosis, palmitoylation, arrestin interaction, receptor oligomerization and phosphorylation, which modulate receptor function and cell surface expression (reviewed in (Oppermann, 2004)).

β-Arrestins are known for their role as GPCR signaling regulators, which can be involved in either initiation or termination of cellular responses induced by ligand binding to C-terminal serine phosphorylation sites. They are involved in receptor desensitization, clathrin-mediated receptor internalization, but also in signal transduction and scaffolding protein that can mediate a second signaling response through the recruitment of different kinases such as MAPK, src family tyrosine kinases, ERK1/2 or JNK3 (Oppermann, 2004; Lopalco, 2011).

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Endocytosis or internalization of receptors can regulate their activity and expression level. Firstly, after ligand-mediated signal transduction, CCR5 can be internalized via interaction with β-arrestins, which act as adaptor proteins to initiate endocytosis by linking the phosphorylated receptor to clathrins of clathrin-coated pits and the AP-2 adaptor complex. Binding to the phosphorylated receptor β-arrestins uncouples the receptor from G proteins to prevent their binding and activation. Other clathrin- or β-arrestin-independent endocytosis pathways have been reported involving cholesterol-enriched raft microdomains which engage with palmitoylated C-terminal cysteine domains of the receptor. After internalization, CCR5 receptors accumulate in perinuclear endosomes, where they can be recycled and sent back to the membrane in a dephosphorylated state, or be degraded (Oppermann, 2004).

Furthermore, phosphorylation of C-terminal serine residues of chemokine receptors is performed by GPCR kinases (GRK) or PKC, which belong to the family of serine threonine protein kinases and mediate ligand-induced receptor phosphorylation and homologous desensitization. The latter describes the refractory period of an activated receptor unable to get stimulated again for a short period of time (Oppermann, 2004). In addition to that, CCR5 is capable of oligomerization into homo- or heterooligomers, which can be modulated by its agonists and alter signaling properties. This only adds to the already complex promiscuous and redundant chemokine system, whose exact mechanisms of signaling and regulation have not been sufficiently defined yet.

5.2.3. Role of CCL5/CCR5 in Peripheral Inflammation in Obesity

As mentioned before, CCL5 and its receptors, have a crucial role in the immune system including leukocyte trafficking and attraction to sites of injury, infection and inflammation, adhesion among others. Obesity is a condition defined by excessive accumulative of body fat in AT and its expansion, which is associated with lipotoxicity and secretion of cytokines and chemokines. This

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in turn leads to infiltration of AT and other organs by macrophages, monocytes and other immune cells, which further the inflammatory state and lead to chronic and systemic inflammation, hyperglycemia, impaired glucose metabolism and ultimately to dysfunction of pancreatic β-cells and insulin resistance among other comorbidities.

CCL5 has been repeatedly reported in studies that provide evidence for its role in obesity. An important correlation between obesity and CCL5 signaling was made when studies reported increased serum levels of CCL5 in obese humans and mice (Keophiphath *et al.*, 2010; Abubaker *et al.*, 2013; Baturcam *et al.*, 2014; Montecucco *et al.*, 2015; Hu *et al.*, 2018).

In contrast to this, Hu et al have found levels of serum levels of CCL5 to be decreased in HFD condition compared to control diet condition in a mouse model of transgenic adenocarcinoma of mouse prostate, that mimics prostate cancer progression associated with obesity (Hu *et al.*, 2018).

In collaboration with our team, Dumas and colleagues performed a study in which they followed up obese patients before and after they underwent bariatric surgery. It was demonstrated that among a panel of cytokines and chemokines CCL5 was increased in obese human patients compared to healthy control patients and seemed to correlate with the weight of the patients, because three months after Roux-en-Y gastric bypass, CCL5 levels dropped and so did the obese patients weight (Dalmas *et al.*, 2011). These results have been confirmed a few years later, when Montecucco and colleagues observed elevated circulating and SC AT CCL5 levels in morbid obese patients with higher insulin levels compared to control patients and one year after gastric bypass surgery CCL5 levels dropped in circulation and SC AT (Montecucco *et al.*, 2015). Other studies have found increased CCR5 expression in obese mice and human patients (Wu *et al.*, 2007; Kitade *et al.*, 2012).

Studies indicate the constitutive expression of CCL5-binding chemokine receptors including CCR1 and CCR5 on the cell-surface of human pre-

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adipocytes and mature adipocytes. Furthermore, CCR5 expression was found to be increased upon differentiation of pre-adipocytes into mature adipocytes, while CCR1 levels remained constant (Gerhardt *et al.*, 2001). The study further showed that apart from chemokines CCL2 and IL-8 also CCL3/MIP-1a (CCR5 ligand) were produced in pre-adipocytes and decreased expression in mature adipocytes, but could get upregulated through stimulation with TNF-a. Interestingly, stimulation of adipocytes by the above mentioned chemokines also induced TNF-a, which shows a reciprocal regulation between chemokines and cytokines (Gerhardt *et al.*, 2001). Intriguingly, the study found that mature adipocytes had a great reduction in the quantity of TG and PPARβ expression and tyrosine phosphorylation of proteins (one of the pathways activated by chemokine receptor stimulation) upon stimulation with the above-mentioned chemokines. In addition, long term stimulation of adipocytes with CCL3 significantly increased leptin concentrations starting after 8 days, whereas acute stimulation with chemokines lead to an increasing effect on leptin concentrations in the medium by all above chemokines after 6 hours, which disappeared for IL-8 but were still increased for CCL3 and CCL2 24 hours post-stimulation. The increase in leptin secretion seemed to be induced by post-transcriptional processes (Gerhardt *et al.*, 2001). It would be interesting to repeat the study with a bigger panel of chemokines in particular including CCL5.

Other studies that have linked CCL5/CCR5 to obesity and AT inflammation showed that CCL5 and CCR5 are upregulated in WAT of obese humans (Wu *et al.*, 2007; Keophiphath *et al.*, 2010), which was confirmed by other studies in both genetic and DIO mouse models (Huber *et al.*, 2008; Kitade *et al.*, 2012).

In addition, gene expression studies have found an increase in several chemokines such as CCL2, CCL3 and CCL5 among others and chemokine receptors including CCR1-3 and CCR5 in not only visceral AT, but also in SC AT of obese and morbidly obese patients (Huber *et al.*, 2008; Duffaut *et al.*, 2009; Keophiphath *et al.*, 2010; Montecucco *et al.*, 2015). CCR1 was found to be

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the most expressed among them in monocytes, AT macrophages and visceral WAT (Keophiphath *et al.*, 2010).

Interestingly, one of the studies found that CCL5 was secreted at higher levels in visceral AT compared to SC AT. Furthermore, the expression of CCL5 was in particular high in WAT macrophages and correlated positively with macrophage marker expression such as CD11b and CD3 and also with TNF and IL-6 expression levels, that are markers associated with M1 macrophages, but not with M2 macrophage markers (CD206 or AMAC-1) (Keophiphath *et al.*, 2010). Additionally, CCL5 seems to trigger adhesion of blood monocytes to AT endothelial cells and their transmigration through endothelial cells in human WAT (Keophiphath *et al.*, 2010). CCL5 also protected AT macrophages from apoptosis, induced by free cholesterol accumulation, via activating Akt/Erk pathways. Interestingly, M2 macrophages were more sensitive to protection from free cholesterol-induced apoptosis by CCL5 than M1 macrophages, while in vitro M1 macrophages were found to secrete higher levels of CCL5 compared to M2 macrophages (Keophiphath *et al.*, 2010).

Kitade and colleagues further show not only that both CCR5 and its ligands (CCL3, 4 and 5) are highly elevated in WAT of both genetic and DIO obese mouse models, but also an upregulation of CCR5, specifically on AT macrophages in the DIO model. Interestingly, they also found a phenotypic shift of macrophages from the "classically activated" pro-inflammatory M1 polarization state to a "alternatively activated" non-inflammatory activation state that usually predominates in lean mice compared to the M1 state of obese mice. M1 AT macrophages are associated with increased pro-inflammatory cytokine expression and secretion like TNF-a and IL-6. CCR5 deficiency and the shift to M2 macrophages resulted in an attenuated inflammatory response and improval of impaired glucose tolerance and insulin sensitivity in DIO (Kitade *et al.*, 2012).

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When characterizing the metabolic state of the KO animals versus WT mice, they found no difference in BW or food intake, but an improvement of diabetes and liver steatosis associated with obesity. Furthermore, $CCR5^{-/-}$ presented themselves with a marked reduction in infiltrated macrophages, that expressed less TNF-a and iNOS, and reduced serum levels of TG and TNF-a in HFD condition and lower FFAs in normal diet condition (Kitade *et al.*, 2012).

Adiponectin-deficient mice on a HFD had upregulation of CCL5 mRNA levels in AT compared to WT mice (Wu *et al.*, 2007). Similarly, CCL5 levels were lowered in WAT of a mouse model deficient for phosphatidylethanolamine N-methyltransferase (molecule involved in adipocyte differentiation) and resistant to DIO (Gao *et al.*, 2015).

However, although some studies report beneficial findings of CCR5 signaling in obesity, there is some controversy as other studies report conflicting results (Kitade *et al.*, 2012; Kennedy *et al.*, 2013; Chou *et al.*, 2016).

For instance, Kennedy and colleagues presented quite contrasting results. In fact, the only results that both studies seem to have in common is a similar diet and the genotype of the mice and the result showing a reduction in $CD11c^{+}$ M1-macrophages in WAT of $CCR5^{-/-}$ DIO mice (Kennedy *et al.*, 2013). Different from the study of Kitade and colleagues, CCR5 did not seem to play a major but rather minor role in immune cell infiltration and inflammation. They demonstrated that CCR5 deficient mice had even an increase in T cells infiltration in WAT compared to WT mice fed a HFD and no reduction in macrophages as reported by Kitade et al. (Kitade *et al.*, 2012; Kennedy *et al.*, 2013). Another contrasting result they found was that $CCR5^{-/-}$ mice became more glucose intolerant with reduced insulin-induced phosphorylated Akt and IRβ (in AT and muscle) compared to WT mice, but had equal levels of adiposity, blood glucose and plasma insulin (Kennedy *et al.*, 2013).

Surprisingly, in 2018 a study by Kim et al in 2018 implicated CCL5 in lipid metabolism. They found that CCL5 among other chemokines was produced

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in human SC fat upon UV exposure and downregulated several lipogenic enzymes via CCR5, which led to impaired TG synthesis (E. J. Kim *et al.*, 2018).

Although some of the above mentioned studies, have not found any effect on food intake in CCR5$^{-/-}$ mice on SD or HFD, there are some that report an effect of CCL5 on modulation of food intake, suggesting a neuroendocrine role in the brain, as well as changes in body temperature and water intake (Tavares and Miñano, 2000, 2004). All these processes are regulated by the brain and provide evidence in support of a central role for CCL5.

Intriguingly, CCL5 was found to be a potent pyrogen and inducing a dose-dependent fever in rats, when injected into the preoptic area of the anterior hypothalamic containing thermosensitive-cells (Tavares and Miñano, 2000). This response seemed to be mediated specifically through CCR5 signaling, when activated by injected CCL5 and not other ligands like CCL4 (Tavares and Miñano, 2004).

It was previously discovered that cytokines like TNF-a (Plata-Salamán, Oomura and Kai, 1988; Schreyer, Chua and Leboeuf, 1998; Romanatto *et al.*, 2007), IL-1β (Plata-Salamán, 1991), and IFN (Plata-Salaman, 1992) can induce, similar to bacterial endotoxins (Plata-Salamán and Borkoski, 1993), a reduction in food intake and play an important role in inflammation in DIO. Likewise, a few studies show that chemokines might have a modulatory role on neural networks that regulate food intake as well (Plata-Salaman and Borkoski, 1993, 1994; Le Thuc *et al.*, 2016). As already mentioned above, cytokines like TNF-a, IFN-γ and IL-1β have been shown to induce the production of chemokines. Thus, it makes sense that chemokines could mediate the effects of cytokines on feeding behavior and be a modulator bridging the gap between cytokine and neuronal communication. Interestingly, CCL5 has been shown to modulate a short-term reduction in food intake, 2 hours after intracerebral infusion into the 3V at 20ng/rat but not at higher concentrations (Plata-Salaman and Borkoski, 1994).

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Another study was looking at the effects of chemokines in the context of HIV-induced wasting syndrome and performed ICV injections of HIV120IIIB or various chemokines including CCL5, or a combination of HIV120IIIB and either one of the chemokines. They found that HIV120IIIB induced a reduction in food and water intake over the course of 5 days with daily injections, as well as a reduction in BW after the first day. Although they found no effect on food or water intake nor BW for CCL5 alone, CCL5 injection in combination with HIV120IIIB seemed to from the effect of HIV120IIIB on all three factors (Guzmán *et al.*, 2006). The difference in effect of CCL5 on food intake might be due to differences in time of measurement or different concentration of injections. The other study found only an effect of CCL5 after 2 hours but not after, whereas in this study the food intake was measured only every 24h for 5 days.

Interestingly, CCL5 seemed to not only to protect form HIV120IIIB-induced motor impairment, but even improved the rat's ability on the rota rod (Guzmán *et al.*, 2006).

Another interesting study, performed an oral lipid tolerance test in 100 healthy subjects and found that while other chemokines' blood concentrations like CCL2 and IP-10 (CXCL10) were decreased, CCL5 levels increased after TG administration (Schmid *et al.*, 2016).

Ueba et al found in a cross-sectional study of human patients, that elevated serum CCL5 levels were positively associated with metabolic syndrome and correlated with hypertension and glucose intolerance. Furthermore, age and IL-6 were one of the most significant predictors of CCL5 levels (Ueba *et al.*, 2014). Another study investigating sex differences between middle-aged female and male mice, points to an interesting increase in CCL5 levels in AT, accompanied by a higher AT mass and higher levels of pro-inflammatory T cells of middle-aged female mice as opposed to males (Ahnstedt *et al.*, 2018).

Urinary CCL5 was increased in both hypertensive obese and obese children compared to control patients (Hacıhamdioğlu *et al.*, 2015). Interestingly, this

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study found that anti-hypertensive treatment reduced levels of CCL5, but also TG, LDL cholesterol levels and improved insulin sensitivity among other effects.

Another interesting study, confirmed previous findings of CCL5 being increased in non-diabetic obese patients, along with TG and leptin and a reduction in resistin in serum, along with TNF-a and IL-6 in AT (Baturcam *et al.*, 2014). However, apart from CCL5 and IP-10 no other elevated inflammatory markers among cytokines and chemokines were found. Circulating CCL5 seemed to correlate negatively with IL-1ra and positively with IP-10 (Baturcam *et al.*, 2014). Surprisingly, while CCR5 mRNA expression was also upregulated in AT in obese compared to lean patients, peripheral blood mononuclear cells demonstrated a lower expression of CCR5.

Additionally, this team investigated the effects of physical exercise intervention for 3 months on CCL5 and other factors in the circulation in a previous study and on CCL5 and CCR5 levels in AT in this study. Interestingly, while they found physical intervention to decrease circulating inflammatory markers like TNF-a and IL-6, no difference was found in CCL5 levels (Abubaker *et al.*, 2013). However, both CCL5 and CCR5 levels decreased after physical exercise in AT of obese patients, along with other pro-inflammatory factors (Baturcam *et al.*, 2014). Contrasting these result another study looked at the effects of either caloric restriction or exercise while remaining on a HFD on metabolic and inflammatory parameters. They study reported that after a HFD for 16 weeks, swimming exercise and caloric restriction had different results and showed that, although both interventions improved BW and AT weight in addition to circulating values of TNF-a and IL-1β, exercise surprisingly increased BAT weight and serum IL-6 as well as CCL5 levels. Contrary to other studies, HFD-fed mice without any intervention had no significant difference in CCL5 levels compared to control diet fed mice and had lower levels than mice in the exercise group (Wasinski *et al.*, 2013). The increase in CCL5 and IL-6 levels was accompanied by an increase in NK cell numbers in AT in exercise conditions, while HFD condition without intervention had increased expression

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of $CD4^+$ and $CD8^+$ cells that was reduced in intervention groups with higher decline in the caloric restriction group for the latter (Wasinski *et al.*, 2013).

Another interesting study looked at the effects of different dietary protein sources in a HFD in obese nondiabetic patients on postprandial inflammatory markers and found an interesting but different effect of proteins on CCL2 and CCL5 blood values. While CCL2 levels decreased post-prandially, after all four protein diets supplemented with either cod protein, whey protein, gluten or casein, CCL5 levels increased for all diets. Interestingly, the smallest overall increase in CCL5 levels resulted after a diet supplemented with whey protein, while the same diet induced the smallest decrease in CCL2 levels (Holmer-Jensen *et al.*, 2011). The study further established a positive correlation between CCL2 levels and postprandial insulin, and a negative correlation between CCL5 levels and postprandial insulin. Intriguingly, whey protein has been associated with reductions in postprandial lipedema (Mortensen *et al.*, 2009; Pal, Ellis and Ho, 2010) in other studies, which has not been confirmed by the above-mentioned study.

5.2.4. Role of CCL5/CCR5 Signaling in Insulin Signaling and Diabetes

There is quite some controversy of results implicating both CCL2/CCR2 and CCL5/CCR5 signaling in obesity-associated T2DM. A great amount of studies suggested that inflammation in obese mice represents the missing link between development of obesity and T2DM.

For instance, a study in male mice fed either a standard or HFD, or either of both supplemented with a dual antagonist of both CCR2 and CCR5, found that the antagonist protected mice fed a HFD not only from BW gain and increase of adipocyte size of WAT, but also improved insulin resistance and glucose tolerance (Huh *et al.*, 2018). This was accompanied by reduction in ATM and $CD8^+$ T cell infiltration and pro-inflammatory cytokines and shifted macrophage polarization towards M2 macrophages within WAT of HFD-fed mice with antagonist (Huh *et al.*, 2018). Although impressive in showing the implication of both chemokine receptors in the etiology of obesity and

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diabetes, this study cannot distinguish between the distinct roles of both chemokines and receptors in the pathological processes. Fortunately, other studies helped to shed more light into this issue.

For example, a study showed that CCR5 and its ligands were upregulated in the adipocyte and macrophage fraction in WAT of obese mice and serum CCL5 is increased in obese humans and mice. Additionally, Kitade and colleagues showed that $CCR5^{-/-}$ mice have a better glucose tolerance and insulin sensitivity including a lower plasma insulin concentration in fed state compared to HFD fed controls. Hence, CCR5 deficiency seems to protect from insulin resistance and diabetes associated with HFD. Moreover, bone transplanted mice from $CCR5^{-/-}$ on HFD were equally protected from hyperinsulinemia and glucose intolerance (Kitade *et al.*, 2012).

Contrasting these results, Kennedy et al. found that HFD feeding of $CCR5^{-/-}$ resulted in greater, systemic impairment of glucose tolerance and reduced insulin-induced phosphorylation of Akt and IRβ in AT and muscle than WT mice (Kennedy *et al.*, 2013).

Interestingly, in a diabetes type 1 model, different chemokines including CCL5 were induced by separate or a combined administration of the cytokines IL-1β, TNF-a and IFN-γ in islet cells of human pancreata donors. CCL5 had a more than 30-fold upregulation after treatment of islet cells with the cytokine cocktail. Notably, while those cytokines usually signal through the NF-κB signaling pathway as well as through engagement with pro-inflammatory iNOS and immunomodulatory factors, the upregulation of chemokine mRNA observed in this study in human islet cells was not due to NF-κB signaling but dependent on iNOS signaling (Sarkar *et al.*, 2012). Immunohistochemical and RT-PCR analysis revealed that CCL5 was not expressed in pancreata of healthy donors or mice, but, while not being the most abundant chemokine, it was expressed in islet and a-cells of pancreas samples of different models of type 1 diabetes in mice and diabetic human patients (Sarkar *et al.*, 2012).

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Further evidence implicating CCL5 in T2DM was provided by a study showing higher CCL5 serum levels in type 2 diabetic patients and further identified strong correlation between CCL5 levels and insulin resistance as determined by the Ohkura index as well as 1,5-anhydro-D-glucitol serum levels (Dworacka *et al.*, 2014). The latter appears to be an indicator of metabolic control and reflect short-term hyperglycaemic episodes.

The potential involvement of CCL5 signaling through CCR5 was further demonstrated by a study showing CCL5 expression and production by β cells of pancreatic islets in NOD and control mice and high levels of CCR5 by most peripheral T cells and B cells (Carvalho-Pinto *et al.*, 2004). NOD mice are often used as mouse model for diabetes research as they spontaneously develop type 1 diabetes, which shares many features with human diabetes. Neutralizing CCR5 via antibodies in the NOD mouse model of diabetes, did not amend insulitis development but prevented from progression of insulitis to β-cell destruction and diabetes (Carvalho-Pinto *et al.*, 2004).

Supporting this, the islet-protecting role of CCR5 was also shown in islet allografts into CCR5 deficient mice and control mice that developed chemically induced diabetes. Islet allografts survival in $CCR5^{-/-}$ recipients was much more prolonged compared to $CCR5^{+/+}$ mice (Abdi *et al.*, 2002). It appears that cytokine-induced CCL5 production in islet cells leads to chemotaxis of lymphocytes and their activation via CCR5 signaling. This leads to Th1 cell polarization and destruction of islet cells (Carvalho-Pinto *et al.*, 2004). It seems that the cytokine IFN-γ might be responsible for the progression of insulitis to diabetes, as its expression is associated with lymphocyte infiltration of islets. However, the NOD mouse model of diabetes indicates that CCR5 is less responsible for lymphocyte invasion of islets, but more for their invasiveness and deleterious actions. Other chemokines also secreted by pancreatic islet cells seem to be responsible for the initial stages of T cell infiltration leading to insulitis (Frigerio *et al.*, 2002; Carvalho-Pinto *et al.*, 2004). These results were confirmed and extended by another study implicating the chemokines CCL3 and 4, in particular the CCL3 to CCL4 ratio in the

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development of diabetes and the progression of insulitis to destructive diabetes (Cameron *et al.*, 2000). Interestingly, these chemokines can act like CCL5 through CCR5. Moreover, IL-4 administration to NOD mice was shown to protect from diabetes through promoting a Th2-like cytokine environment by diminishing pancreatic CCL3 expression, while increasing CCL4 expression. Likewise, IL-4 treatment downregulated CCR5 expression, but not other chemokine receptors like CCR1, 2 , 3 and others, in splenic T cells and islets (Cameron *et al.*, 2000). In fact, this is interesting in particular in the context that CCR5 expression is associated with Th1-type immune responses, whose activation in turn is believed to mediate β-cell destruction (Bonecchi R *et al.*, 1998; Loetscher *et al.*, 1998; Cameron *et al.*, 2000). Recent studies have described the constitutive expression of CCL5 in pancreatic islets of both mice and humans (Liu *et al.*, 2013) and expression of CCL5 in islets of NOD mice and human patients with type 1 diabetes (Bouma *et al.*, 2005; Sarkar *et al.*, 2012).

Data in support of the assumption that CCL5 has a critical role in diabetes have shown that plasma CCL5 is increased in human type 1 and type 2 diabetes (Nomura *et al.*, 2000; Pfleger *et al.*, 2008; Keophiphath *et al.*, 2010). In addition to that, patients with T2DM have reduced levels of GLP-1 (Nauck *et al.*, 1986; Toft-Nielsen *et al.*, 2001), which is an incretin that amplifes insulin secretion among other effects (Drucker, 2006; Campbell and Drucker, 2013).

Recently, CCR1, a receptor for CCL5 was found to be expressed in enteroendocrine and GLP-1 positive cells in murine small intestine and in a human intestinal enteroendocrine cell line (Pais *et al.*, 2014). In addition to that, CCL5 inhibited glucose stimulated GLP-1 and GLP-2 secretion both *in vitro* and *in vivo,* while the effect was prevented with Met-RANTES, an antagonist for CCL5 receptors. The inhibitory effect of CCL5 on GLP-1 secretion appeared to be mediated through a decrease in intracellular cAMP and PKA activity (Pais *et al.*, 2014). The study further showed that CCL5 diminished transport activity of rheogenic sodium-coupled glucose

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transporter, which seems to mediate glucose-dependent GLP-1 secretion by enteroendocrine cells (Pais *et al.*, 2014).

Further evidence was provided by a study demonstrating the constitutive expression and production of CCL5 by glucagon expressing pancreatic cells in mice, and in both human glucagon producing α and insulin producing β cells (Liu *et al.*, 2013).

Recently, it was shown that CCL5 can bind and signal through a newly discovered orphan GPCR called GPR75 (Ignatov *et al.*, 2006). While the above mentioned study confirmed the expression of CCR1 in nondiabetic human islets, and CCR3 and CCR5 in healthy mouse pancreatic islets, they also demonstrated the expression of GPR75 in α and β cells of both mice and human islets and at higher levels than the other receptors of CCL5 (Liu *et al.*, 2013). The study further demonstrates an alternative physiological role of CCL5 in pancreatic β cell function. They showed that CCL5 seems to stimulate insulin secretion in mouse and human β cells through GPR75 activation, which in turn appears to act by coupling to Gq proteins to induce an extracellular calcium influx and PLC activation (Liu *et al.*, 2013). Lastly, Liu and colleagues have found that *in vivo* peripheral co-administration of CCL5 during a glucose tolerance test (GTT) and insulin tolerance test (ITT), improved glucose tolerance and increased insulin secretion in both lean and insulin-resistant ob/ob mice, but did not alter insulin sensitivity (Liu *et al.*, 2013).

Interestingly, insulin was reported to have anti-inflammatory effects. Insulin infusion of obese patients suffering from type 2 diabetes for 4 hours significantly reduced plasma concentrations of chemokines including CCL3 and CCL5 as well as the expression of CCL4 and CCL5 and chemokine receptors such as CCR2 and CCR5 in mononuclear cells (Ghanim *et al.*, 2010). Should CCL5 signaling really be involved in insulin secretion as suggested in the data presented so far, it would be tempting to assume that the suppressive effect of insulin on chemokines could be part of a regulatory feedback loop.

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In a Finnish diabetes prevention study, Herder et al. found a positive correlation between elevated CCL5 levels and the development of T2DM. It seems that CCL5 concentrations were indicative of high-risk patients that are more likely to become diabetic (Christian Herder *et al.*, 2006). These results were confirmed in a second population-based study, in which systemic CCL5 levels were higher and correlated in both patients with impaired glucose and T2DM (Herder *et al.*, 2005).

Important evidence for a role of CCL5-CCR5 signaling in hypothalamic insulin regulation was shown by a recent study, which not only confirmed CCR5 expression in the ARC of the hypothalamus, most likely in POMC-expressing neurons as shown by immunostaining of primary hypothalamic neurons, but also that CCR5 seems to associate with IR in the hypothalamus. Furthermore, they established that CCR5 and CCL5 deficient mice or mice treated with ^{Met}CCL5, an antagonist for CCR5, are insulin resistant and glucose intolerant on a normal diet (Chou *et al.*, 2016). On the cellular level, this was likely due to modification in insulin signaling pathways in the hypothalamus, such as raised AMPKa and phosphorylation of IRS-1, which is indicative of a reduced activation of insulin signaling and CCL5 can induce GLUT4 translocation in hypothalamic neurons (Chou *et al.*, 2016).

5.2.5. Role of CCL5/CCR5 Signaling in Neuropathic Pain

Some opiate abusers tend to also be infected with HIV and have higher probability of acquiring neurological complications such as ADC reviewed in (Anthony, 2008). It seems that the neuronal degeneration caused by HIV is modulated by opiates. HIV-1 infection is facilitated by chemokine co-receptors such as CCR5 and CXCR4 and thus it seems possible that opiates might act through chemokine receptors to modulate the effects on neurons, especially since CCL5 was shown to prevent neurotoxic effect of a HIV envelope glycoprotein called gp120IIIB. This neuroprotective effect was blocked in CCL5-immunoneutralized cerebellar cultures of neurons and glia cells that subsequently were treated with morphine and gp120 (Avdoshina *et*

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al., 2010). This study seems to indicate that morphine might have a neuroprotective effect against gp120Bal toxicity, which is mediated by CCL5 released by astrocytes.

Interestingly, a study found that morphine induced CCL5 secretion from astrocytes in primary cultures in a time-dependent manner (Avdoshina *et al.*, 2010). Furthermore, CCR5 expression and secretion was increased in the immune system and astrocytes by morphine and a μ-opioid receptor agonist treatment (Suzuki *et al.*, 2002; Steele, Henderson and Rogers, 2003).

Lack of CCL5 in a chronic neuropathic pain model of a partial sciatic nerve ligation (PNL) model was investigated using $CCL5^{-/-}$ mice (Liou *et al.*, 2012). They found less hypersensitive behaviour in $CCL5^{-/-}$ mice compared to controls, which was accompanied by diminished macrophage infiltration of damaged nerves and pro-inflammatory cytokines while anti-inflammatory proteins such as IL-4 and IL-10 were increased in CCL5 deficient mice but not in controls. Interestingly, significant differences in levels of opioid peptides (ekephalins, dynorphins and β-endorphins) have been found between KO and control mice (Liou *et al.*, 2012). These results are among the first to implicate CCL5 and CCR5 in regulating pathways of hyperalgesia in neuropathic pain associated with inflammation and authors suggest that decreased hyperalgesia might be due to a decreased macrophage infiltration and activity in the injured nerve.

5.2.6. Role of CCL5 Signaling in NASH/NAFLSD

CCL5 has been shown to participate in diseases that display chronic inflammation, some of which are discussed here. One other example of the important role of CCL5 in another chronic inflammatory disease associated with obesity is NASH/NAFLSD and liver fibrosis.

CCR5 expression was found to be increased in liver tissue of obese mice and humans that are associated with hepatic steatosis and NASH (Wu *et al.*, 2007; Huber *et al.*, 2008; Bertola *et al.*, 2010; Kitade *et al.*, 2012). However, apart from a decreased inflammatory phenotype in WAT and decreased diabetic

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phenotype on HFD, CCR5$^{-/-}$ mice had also reduced liver steatosis, hepatic TG and lipogenic mRNA expression, after 15 weeks on a HFD diet (Kitade *et al.*, 2012). Interestingly, bone marrow transplant from CCR5$^{-/-}$ to WT mice could ameliorate also the DIO-induced liver steatosis, which seems to be mediated by myeloid specific CCR5 signaling. (Kitade *et al.*, 2012).

5.2.7. Role of CCL5/CCR5 Signaling in HIV

CCR5 acts as primary HIV-1 co-receptor to facilitate entry of HIV into cells expressing CCR5. For example, M-tropic HIV (macrophage trophic HIV preferentially uses CCR5 as co-receptor for cell entry, whereas T cell (T-) tropic HIV preferentially uses CXCR4 chemokine receptor for entry. However, dual tropism for some strains of HIV have been also observed, meaning they can use either of the receptors for viral entry (Waller *et al.*, 2018). HIV tropism for CCR5-expressing cells happens usually early during infection and can transit tropism to CXCR4 or can even develop a dual tropism for both Chemokine-expressing cells later on during infection.

Surprisingly, some studies report that although CCR5 facilitates HIV-1 infection, its chemokine ligands CCL5, MIP-1a and MIP-1β seemed in contrary to act as suppressive factors for HIV infection by blocking viral infection of cells, or by delaying of disease progression (Cocchi *et al.*, 1995). However, these results seem to be controversial as other studies have either confirmed (Zanussi *et al.*, 1996; Mackewicz *et al.*, 1997; Berger, Murphy and Farber, 1999; Polo *et al.*, 1999; Levy, 2003) or contradicted these data and even found that these ligands rather increase HIV infection (Schmidtmayerova, Sherry and Bukrinsky, 1996; Kelly *et al.*, 1998; Kinter *et al.*, 1998; Ye *et al.*, 2004).

It is not surprising that mutations in CCR5 genes have been observed in the human populations that have a resistance for HIV-1 infection and delays AIDS progression of infected patients.

A polymorphism found in the gene for CCR5 is a 32 base pair deletion in the coding domain, leading to a frame shift mutation (CCR5Δ32), that results in a truncated protein that no longer is expressed at the cell surface and thus

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prevents HIV-1 infection by M-tropic or dual tropic strains (Samson *et al.*, 1996). Whereas rare individuals homozygous for the CCR5Δ32 allele seem to be resistant to M-tropic strains of HIV-1 despite repeated exposure, people who are heterozygous seem to have a partial protection from HIV-1 infection, slower disease progression and better survival compared to patients with a normal CCR5 allele (Mellors *et al.*, 1996). A cohort frequency of HIV-infected Caucasian patients presented with a 32% lower frequency of heterozygous and no homozygous alleles than the general population (Mellors *et al.*, 1996; Samson *et al.*, 1996). People being heterozygous for the polymorphous allele are quite common compared to its homozygous allele frequency and was reported to be ~20% in some populations (Landau *et al.*, 1996). With the highest prevalence of the allele in European people and a frequency of 1-3% in the Caucasian population, homozygous CCR5Δ32 allele was not found so far in the population of African or Asian origin (Huang *et al.*, 1996; Samson *et al.*, 1996).

In a famous case known as "Berlin patient", a patient called Timothy Ray Brown was HIV positive, before developing acute myeloid leukemia and ultimately undergoing hematopoietic stem cell transplantation from a donor homozygous for CCR5 the CCR5Δ32 mutation. Despite the discontinuation of antiretroviral treatment, the patient's viral load remained undetectable for 20 months in both lymphocytes and serum after the transplant (Hütter *et al.*, 2009). The patient still remains HIV-free, while a second patient, who similar to Timothy was HIV positive and undergoing alleiogenic hematopoietic stem cell transplant for Hodgin's lymphoma from a CCR5-deficient donor. Similar to the first patient to undergo this procedure also this patient virus free in the most recent follow-up 18 months after stopping his antiretroviral medication (Gupta *et al.*, 2019). These successful treatments, including with the presented studies show how CCR5 and its ligands could represent a potential therapeutic target for HIV treatment. Indeed one drug named maraviroc (Pfitzer) that acts as a CCR5 antagonist, was FDA approved in 2007, whereas some other

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candidates are still in different stages of clinical trials ((Melica *et al.*, 2010; Tiraboschi *et al.*, 2010; Sorce, Myburgh and Krause, 2011).

Interestingly, although the presence of CCR5 and CXCR4 was found to facilitate viral entry to Immune cells in the periphery, a study found that astrocytes and microglia express both co-receptors CCR5 and CXCR4, and are equally susceptible to viral HIV-1 infection. Since chemokine co-receptors are expressed in the CNS, it makes sense that HIV-1 infection affects also the CNS and causes several neuropathological and behavioral disorders (Liu *et al.*, 2004).

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Objectives

In summary, the here presented data show that many of the conditions mentioned above such as AD, PD, Huntington's disease, HIV-associated metabolic changes and T2DM have an inflammatory component in common, as well as energy metabolism and glucose impairment with insulin resistance. Cytokines and chemokines are very important in the regulation of immune system responses in many conditions such as the ones mentioned above and have been implicated manifold in functions of the CNS and in the periphery.

A certain chemokine, CCL5 seems to share a special connection, as CCL5 signaling through its cognate receptor CCR5 is not only Implicated in all diseases mentioned above in particular in obesity and diabetes, but also seems to be expressed in the CNS and was suggested to have a neuromodulatory function and effects on insulin regulation. Moreover, CCL5 and its receptor represent potential and interesting therapeutic candidates for various conditions.

However, the mechanisms behind CCL5-CCR5 signaling in the central regulation of energy metabolism, insulin regulation and glucose homeostasis remain unclear and need further investigation.

For this reason, the present study is divided into two parts that focus on:

1. Developing a mouse model of DIO to test the effect of different lipid nature and ω6/ω3 ratio on the development of DIO and associated comorbidities such as diabetes and neuropathic pain.

2. Investigate the role of CCL5/CCR5 in the central regulation of energy balance:
    a. Does CCL5 signaling have an effect on neuropeptides involved in the regulation of energy balance?

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b. Do ICV CCL5 and ^{Met}CCL5 (CCL5 antagonist) injections have an effect on food intake, blood glucose and insulin regulation?
c. Are CCL5 and its receptor CCR5 expressed in the hypothalamus, in particular in the ARC?

3. Identify the role of CCL5/CCR5 in the development and maintenance of DIO and associated comorbidities including T2DM and neuropathic pain using the mouse model of DIO identified in part one of the study.

    a. Characterize the inflammatory and metabolic phenotype of CCL5$^{-/-}$ mice on a standard diet (SD) and high-fat diet (HFD)
    b. Characterize the inflammatory and metabolic phenotype of CCR5$^{-/-}$ mice on a standard diet (SD) and high-fat diet (HFD)

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Experimental Strategy

In the first part of this study, our aim was to establish a mouse model of DIO by testing different HFDs with different lipid nature and ω6/ω3 ratio on the development of DIO and associated comorbidities. We used 4 weeks old weight-matched male mice, which were were given either a SD, a HFD-B with 40% of animal-derived lipids based on butter, HFD-C with 20% vegetal lipids (ω6/ω3=2.3), HFD-SC with 20% of vegetal lipids (ω6/ω3=7.3) or HFD-SN with 20% vegetal lipids (ω6/ω3=17.3) (Table 1) and measured their development of obesity and monitored glucose metabolism as well as inflammatory markers in the hypothalamus after 12 weeks of experimental protocol (Fig. 24).

	STANDARD (SD)	BUTTER (HFD-B)	RAPESEED (HFD-C)	SOJA/CORN (HFD-SC)	SUNFLOWER (HFD-SN)
Energie (kcal/g)	3.4	5.3	4.4	4.5	4.5
Proteins (%)	25.2	20.0	23.0	18.2	20.1
Carbohydrates (%)	61.3	36.7	43.0	46.8	46.8
Lipids (%)	5.1	36.0	20.0	20.0	20.0
Details of lipid composition of HFD		Anhydrous butter 33.3 % Soja oil 2.5%	Rapeseed oil 20%	Anhydrous butter 6.1% Soja oil 1.3% Crisco 6.0% Lard 6.0% Corn oil 0.5% Cholesterol 0.15%	Anhydrous butter 5.1% Crisco 5.0% Lard 5.0% Sunflower oil 4.77% Cholesterol 0.15%
Energy provided by lipids (%)	13.5	58.8	40.5	40.3	40.3
ω6/ω3 ratio	6.5	8.0	2.3	7.3	17.3

Table 1: Composition of diets used for the first part of this study.

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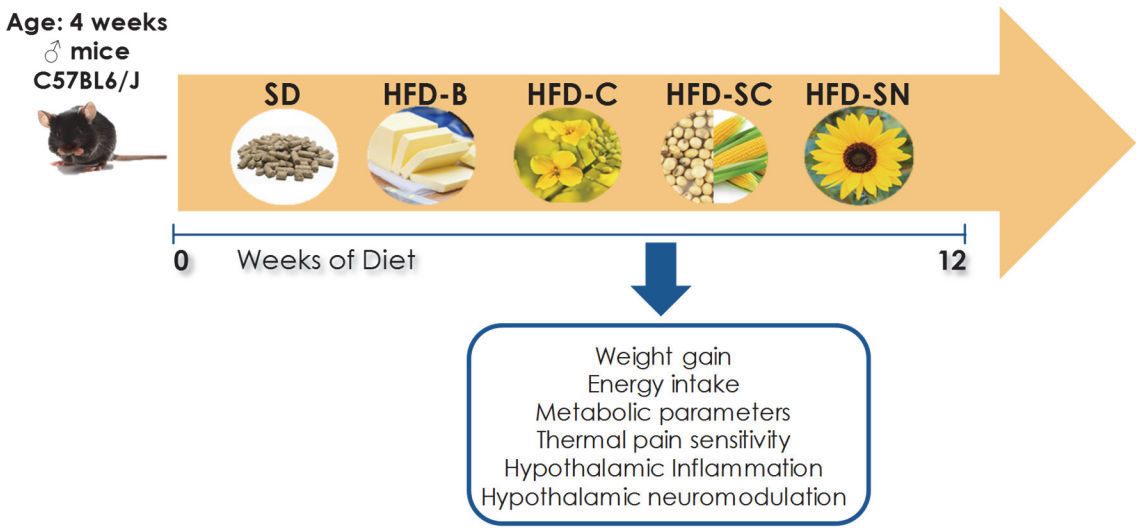

Figure 24: Schematic representation of our experimental approach for the first experiment.

In the second part of our project, our aim was to study the effect of CCL5-CCR5 signaling in the regulation of energy balance in the context of diet-induced obesity and its associated comorbidities such as diabetes and neuropathic pain.

To study the role of CCL5 and its receptor CCR5 in the regulation of energy balance and in DIO, we decided to use a loss of function approach by employing a mouse model of DIO as depicted in figure 25 with mice deficient for CCL5 and CCR5 as compared to WT littermates. We decided to use the most obesogenic diet identified in the first part of our experiment (HFD-B which will be called HFD in the second part of the study) to assess the effects of CCL5 and CCR5 deficiency in KO mice in a HFD context. We assessed the development of obesity and T2DM as well as neuropathic pain by measuring food intake, body weight and other metabolic parameters at 8 and 16 weeks of diet as representative time points of obesity installation and maintenance. At the end of the experiment, we sacrificed mice and took different blood and tissue samples to be able to assess the modulation of peptides, and inflammatory markers.

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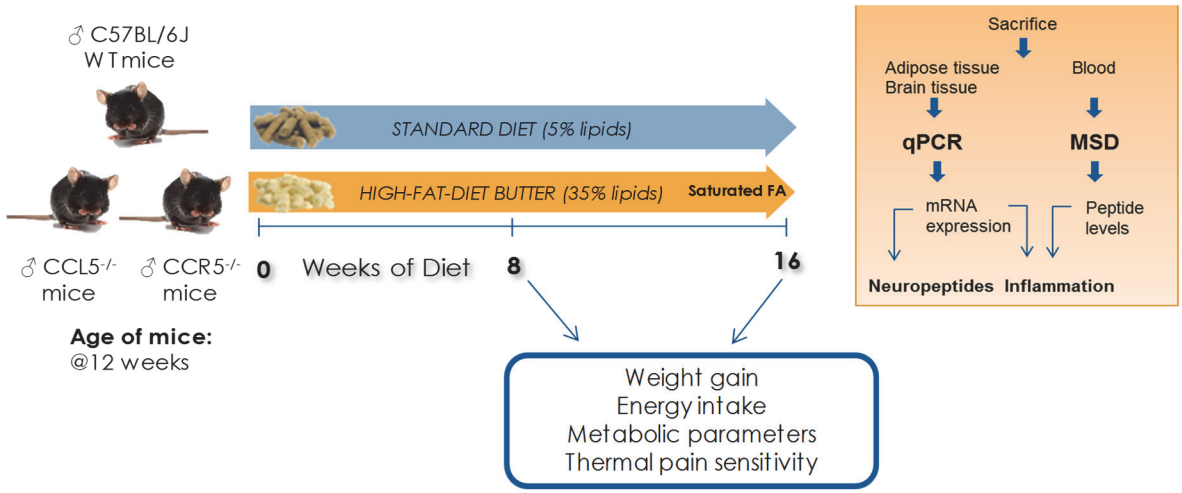

Figure 25: Schematic representation of the experimental approach employed in this study.
In this study, we used a adult transgenic mice harboring a deletion of the chemokine CCL5 or its receptor CCR5 in the context of DIO and surveilled several parameters during the course of 16 weeks of diet.

Furthemore, to assess the role of CCL5/CCL5 signaling in a physiological context in the regulation of energy balance we decided to use an immunohistochemical/in situ hybridization approach to verify the expression of CCL5 and CCR5 in hypothalamic nuclei. In addition to that, we employed stereotaxic surgery to implant cannula and repeatedly inject CCL5 and its antagonist ^{Met}CCL5 ICV to assess its role on BW and food intake regulation in a physiological context (Fig. 26 and 27 in Materials & Methods).

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Materials and Methods

1. Animal Procedures

8-week-old C57Bl/6J male mice (20-25g) used in this study were from Janvier Labs, France. CCL5$^{-/-}$ (CCL5 KO; B6.129P2-*Ccl5*^{tm1Hso}/J) and CCR5$^{-/-}$ (CCR5 KO; B6.129P2-*Ccr5*^{tm1Kuz}/J) mice were originally from the Jackson Laboratory, USA. Animals were housed in an SPF facility in medium-size (up to 5 animals/cage) or large cages (up to 10 animals/cage) filled with wooden bedding, one plastic house and nesting material for enrichment. All CCL5-WT/CCR5-WT and CCL5-KO/CCR5-KO male mice used in this study were littermates born to heterozygous females. Pups were weaned at 28 days of age, identified by ear tags, and genotyped by PCR as described by Jackson Laboratory.

Pups from various litters were then randomly grouped according to their genotype in cages and had ad libitum access to water and standard chow (SD; 3 395 kcal/kg of which 25.2% are from proteins, 13.5% fat and 61.3% from carbohydrates; Safe #A03) (Table 1).

Animals were housed in a temperature (21-23°C) and hygrometry (70-80%)-controlled room with a 12-h light/12-h inversed dark cycle (lights on at 8 pm). All the described diet experiments were performed on male animals and started at 4 or 12 weeks of age.

For experiments involving treatment, animals from the same genotype were randomly assigned to experimental groups.

All of the protocols were carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Nice-French Riviera, registered number 04042.02).

2. Blood and Tissue Collection

At the end of the experimental protocol, mice were anesthetized via inhalation of 3% isofluarane and blood was withdrawn from retro-orbital sinuses using heparinized Pasteur pipettes (heparin 50 mg/mL, Sigma) from

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animals in non-fasting or fasting conditions as stated. Blood samples were collected in 1.1mL Z-Gel Micro tubes (Sarstedt) and centrifuges for 10 min at 4°C, at 10000 rpm. The supernatant was recovered in Eppendorf tubes and stored at -80°C. In nonfasting conditions, animals were fed ad libitum and in fasting conditions, animals were fasted overnight for 6 or 16 hr. Furthermore, brain, dorsal root ganglia, spinal cord and AT were collected, frozen on dry ice and stored at -80°C until further processing. For GTT serum was collected from tubes (Sarstedt, France) after centrifugation at 3000 rpm for 10 min.

3. Diets

Mice were food deprived 1h-2h prior the onset of the dark period to synchronize groups. For the first part of our study, mice were administered one of four HFDs (custom diets by Safe diets, France) or SD according to table 1 at the onset of the dark cycle for 12 weeks. For the second experiment of our study, at the beginning of the dark cycle (T=0h) either standard diet (SD; 3 395 kcal/kg of which 25.2% are from proteins, 13.5% fat and 61.3% from carbohydrates; Safe #A03) or high-fat diet (HFD; 5 283 kcal/kg; 20.0% from proteins, 58.8% from fat and 36.7% from carbohydrates, enriched in anhydrous butter 33.3% + soybean oil 2.5%; Safe #U8978P V0019) (Table 2) were delivered for 16 weeks. Food intake was measured and changed every second day. At the end of the experimental procedure, mice were sacrifized and brain, AT as well as blood samples were taken.

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	Standard Diet SAFE A03	High Fat Diet HF260 (HFD)
Energy (Kcal/g)	3.4	5.3
Energy provided by lipids (%)	13.5	58.8
Lipids (%)	5.1	36.0
Ratio ω-6/ ω-3	6.5	8.0
Lipid composition (% in diet)		
Palmitic acid C16:0	1%	11%
Stearic acid C 18:0	0.005%	4%
Oleic acid C 18:1	0.01%	7%
Linoleic acid C18:2	3%	2%
Arachidonic acid C20:4	0.01%	0.036%
Proteins (%)	25.2	20.0
Carbohydrates (%)	61.3	36.7

Table 2: Composition of the diets used in the second part of the experiment.

4. Glucose Tolerance Test (GTT)/Insulin Tolerance Test (ITT)

To measure the capacity to regulate glucose metabolism and insulin sensitivity a GTT and ITT were performed according to the IMPReSS guidelines (International Mouse Phenotyping Resource of Standardized Screens) and in agreement with (Heikkinen *et al.*, 2007) and (Ayala *et al.*, 2010). Mice were starved overnight (16h) or 6h respectively and distributed into single cage housing for the duration of the test. Mice were left 30 min for habituation at red light conditions, before basal glycaemia values were established by measuring the blood of tail vein using a glucose meter (Bayer Contour® XT and test strips (Contour® Next test strips). Glucose (1.5 g/kg, Sigma-Aldrich) or human insulin (0.5 U/kg, Humalog, Lilly) diluted in NaCl 0.9% was injected intraperitoneal (ip) as per recommended procedures and blood glucose was measured again 15, 30, 45, 60, 90 and 120 min post-injection.

To further, characterize glucose metabolism and the capacity of HFD-fed mice to secrete insulin we measured glucose-stimulated insulin secretion. This

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was done during a GTT as described above, but in addition to measuring blood glucose, blood samples of ~20µL were collected from tail vein after incision at the tip of the tail at basal conditions "T0" and 15, 30 and 60min after glucose injection. Blood was collected using Microvette® CB 300 Z tubes (Sarstedt) and kept on ice until centrifuged was done at 4°C for 10 min at 3000 rpm after the test. Plasma supernatant was recuperated and kept at -80°C until ELISA for insulin testing was performed.

5. Microcomputed Tomography Analysis

AT quantification of anesthetized mice was carried out using a SkyScan-1178 X-ray micro-computed tomography analysis and performed as previously described (Beranger *et al.*, 2014).

Briefly, mice were scanned using the following parameters: 104 µm of voxel size, 49 kV, 0.5 mm thick aluminum filter, 0.9° of rotation step. Total AT volume was determined between the cervical vertebra 1 (C1) and the thoracic vertebra 13 (T13), whereas intra-abdominal and subcutaneous AT area was measured on one section at the lumbar 5 (L5) level. AT area and volume were analyzed using NRecon and CTAn software (Bruker microCT, Kontich, Belgium).

5.1. Adipose Tissue Histology

Tissues were collected, fixed for 24 hours in 4% paraformaldehyde, paraffin-embedded, sliced into 5-µm thick sections using a microtome RM 2255 (Leica) and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through alcohol, and washed in phosphate-buffered saline. Hematoxylin-eosin–stained sections were imaged with the Vectra Polaris slide scanner using 20X NA 0.45 objective and visualized with Phenochart software. Automated analysis of adipocytes surface was performed using an in-house ImageJ software macro (F. Brau, imaging and cytometry platform).

5.2. Plasma Triglyceride (TG) and Glycerol Contents

TG plasma contents and glycerol plasma contents were immediately measured with a serum TG kit (T2449; Sigma-Aldrich France), and free glycerol

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reagent (F6428; Sigma-Aldrich), according to the manufacturer's instructions and as described in (Pisani *et al.*, 2016). We used 50µl of plasma and 200µl of reagent.

6. RNA Isolation and Quantitative qPCR

Total RNA from frozen hypothalamus or AT was isolated using Fast Prep apparatus (Q-Biogene, France) as we previously described (Le Thuc *et al.*, 2016). First-strand cDNAs were synthesized from 1-2 µg of total RNA with 200U of SuperScript III reverse transcriptase (SuperScriptIII, Invitrogen, France) in the appropriate buffer in the presence of 25 ng/µl oligo-dT primers, 0.5 mM desoxyribonucleotide triphosphate mix, 5 mM dithiothreitol, 40U RNAsin (Promega, France). The reaction was incubated 5 min at 25 °C, then 50 min at 50 °C then inactivated 15 min at 70 °C.

Real-time quantitative PCR was performed using the SYBR® Green method (Roche France) with the LightCycler 480 sequence detector system (Roche Diagnostics France) for amplification of mouse IL-1β (Interleukin 1 beta), IL-6 (Interleukin 6), TNF-a (Tumor necrosis factor alpha), CCL2, CCL5 (Chemokine (C-C motif) ligand 2-5), Iba1 (Ionized calcium Binding Adaptor molecule 1), GFAP (Glial Fibrillary Acidic Protein), MCH (Melanin-Concentrating Hormone), ORX (OReXine), POMC (Pro-OpioMelanoCortin), CART (Cocaine- and Amphetamine-Regulated Transcript), NPY (Neuropeptide Y), AgRP (Agouti-Related Peptide), IRS1 (Insulin Receptor Substrate 1), PPARγ (Peroxisome Proliferator-Activated Receptor gamma) and GAPDH (GlycerAldehyde Phosphate DesHydrogenase) mRNA. GAPDH was used as housekeeping gene for normalization with the $2^{\Delta\Delta}$CT method. Primers (detailed in Table 3) were purchased from Eurogentec (France).

Gene	sequence 5'-3'	concentration
mIL-1β-F	TGGTGTGTGACGTTCCCATT	
mIL-1β-R	CGACAGCACGAGGCTTTTTT	600 nM
mIL6-F	CCCAATTTCCAATGCTCTCC	
mIL6-R	TGAATTGGATGGTCTTGGTCC	600 nM

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mTNF-a-F	TGTACCAGGCTGTCGCTACA	
mTNF-a-R	AGGGCAATTACAGTCACGGC	600 nM
mCCL2-F	CCAACTCTCACTGAAGCCAGC	
mCCL2-R	CAGGCCCAGAAGCATGACA	300 nM
mCCL5-F	ACACCACTCCCTGCTGCTTT	
mCCL5-R	AAATACTCCTTGACGTGGGCA	600 nM
mIba1-F	GGATTTGCAGGGAGGAAAA	
mIba1-R	TGGGATCATCGAATTG	600 nM
mGFAP-F	TCGACATCGCCACCTACAG	
mGFAP-R	GTCTGTACAGGAATGGTGATGC	600 nM
mMCH-F	GAAGGAGAGATTTTGACATGCTCA	
mMCH-R	CCAGCAGGTATCAGACTTGCC	600 nM
mORX-F	GCCGTCTCTACGAACTGTTGC	
mORX-R	CGCTTTCCCAGAGTCAGGATA	600 nM
mPOMC-F	AGTGCCAGGACCTCACCA	
mPOMC-R	CAGCGAGAGGTCGAGTTTG	600 nM
mCART-F	CGAGAAGAAGTACGGCCAAG	
mCART-R	CTGGCCCCTTTCCTCACT	600 nM
mNPY-F	CCGCTCTGCGACACTACAT	
mNPY-R	TGTCTCAGGGCTGGATCTCT	600 nM
mAgRP-F	CCCAGAGTTCCCAGGTCTAAGTCT	
mAgRP-R	CACCTCCGCCAAAGCTTCT	300 nM
mIRS1-F	CTATGCCAGCATCAGCTTCC	
mIRS1-R	TTGCTGAGGTCATTTAGGTCTTC	500 nM
mPPARγ-F	GGGGGTGATATGTTTGAACTTG	
mPPARγ-R	GAAAGACAACGGACAAATCACC	500 nM
mGAPDH-F	GAACATCATCCCTGCATCC	
mGAPDH-R	CCAGTGAGCTTCCCGTTCA	300 nM

Table 3: Primers used for real time PCR.
Forward and reverse primers were designed using Primer Express software. Genes were analyzed with a SYBR® Green I system.

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7. Cytokines, Chemokines and Hormones Quantification

A V-Plex multiplex assay (Meso Scale Discovery, USA) and mouse ELISA kits for CCL5 (RayBiotech, France), leptin (AssayPro, France) were used to measure respectively the plasma levels of inflammatory mediator and leptin, according to the manufacturer's protocols. Plasma insulin concentrations were determined using the Insulin AlphaLisa detection kit (cat. number AL204C) from Perkin Elmer.

8. Immunohistochemical Analysis

Brains were harvested from mice perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) and post-fixed in the same fixative overnight 4°C. Thirty µm thick brain coronal sections were cut on a vibratome (HM 650V, Microm, Germany), blocked for 1h with 3% normal goat serum in PBS containing 0.1% Triton X-100 and incubated with primary antibodies overnight at 4°C. Primary anti-rabbit antibodies were against Iba1 (1:500, CP290A, B, Biocare Medical, USA) and GFAP (1:300, Z0334, Dako, Danemark). Primary anti-rabbit antibodies were against Iba1 (1:500, CP290A, B, Biocare Medical, USA) and GFAP (1:300, Z0334, Dako, Danemark). The immunoreactivity of the receptors of CCL5, namely CCR1, CCR3 and CCR5 were tested with the following antibodies: anti-CCR1 Pa1-41062 (Invitrogen - Thermofisher Scientific, France; tested with dilutions: 1/200, 1/3000 and 1/9000), anti-CCR1 (CAT#: TA336235 - lot010509-03, OriGene, France, tested at 1/200, 1/500 and 1/1000), anti-CCR3 (ab36827, Abcam, France tested at 1/1000), anti-CCR5 (17833 lotK1317, Santa Cruz Biotech, USA tested at 1/300, 1/500, 1/1000, 1/3000 and 1/9000).

Adequate AlexaFluor conjugated secondary antibodies were used for immunofluorescence microscopy. Sections were mounted in Vectashield solution (H-1000, Vector Laboratories). Images were acquired with confocal laser-scanning microscope (TCS SP5, Leica Biosystems, Germany).

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9. Single-molecule RNA *In Situ* Hybridization

RNA *in situ* hybridization experiments were performed using the RNAScope® technology, which has been previously described (Wang *et al.*, 2012). Paired double-Z oligonucleotide probes were designed against target RNA using custom software. The following probes were used:

- RNAScope® Probe- Mm-Ccl5, cat.no. 469601, NM_013653.3, 11pairs, nt 4-527.
- RNAScope® Probe- Mm-Ccr5-C3, cat.no. 438651-C3, NM_009917.5, 20pairs, nt 1337-2336.
-RNAScope® Probe- Mm-Aif1-C2, cat.no. 319141-C2, _NM_019467.2, 18 pairs, nt 31-866.

The RNAScope® Multiplex Fluo kit v2 (cat. no. 323100) (Advanced Cell Diagnostics, Newark, CA) was used according to the manufacturer's instructions. Perfused frozen mouse brains were cut into sections of 10 µm thickness and prepared according to manufacturer's recommendations. Each sample was quality controlled for RNA integrity with a mix of probes specific to the housekeeping genes PPIB, POLR2A and UBC (RNAScope® 3-plex Positive Control Probe – Mm, cat.no. 320881). Negative control background staining was evaluated using a probe specific to the bacterial dapB gene (RNAScope® 3-plex Negative Control Probe – Mm, cat.no. 320871). Probes were visualized by conjugation to Opal™ flourescent dyes (Opal™ 570, 650 and 520) according to manufacturer's recommendations. Fluorescent images were acquired using a confocal microscope (SP5) using a 40x objective.

10. Stereotaxic Cannula Guide Placement

ICV injections were performed via guide cannula (26G PED 4 mm, 4 mm length below pedestal PHYMEP, France) into the right lateral ventricle of male C57BL6/J mice purchased from Jackson Laboratory in France. To place guide cannula mice were anesthetized by ip injection of a ketamine-xylazine mix (80mg/kg - 12 mg/kg) in addition to topical lidocaine application. Mice were implanted unilaterally with a 26 stainless steel gauge guide cannula (Plastics

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One Inc, Roanoke, Virginia, USA) using a Kopf stereotaxic instrument (David Kopf Instruments, Tujunga, USA) to allow icv injection. Unilateral implantation was made into the right lateral ventricle (stereotaxic coordinates relative to Bregma: X: +1 mm; Y: −0.34 mm; Z: −2.5 mm below the surface of the skull (Fig. 26) according to "The Mouse Brain in Stereotaxic Coordinates", Second Edition, by Paxinos & Franklin.

The injection site was drilled open and the cannula guide was inserted according to the stereotactic coordinates into the brain. Dental cement mix (Dentalon® plus powder and liquid, PHYMEP, France) was used to maintain cannula guide anchored to a stainless steel screw (1.6mm length screw for mice, PHYMEP, France), which was fixed contralaterally to the skull, left to dry, before being sutured using coated Vicryl™ surgical suture (6-0, Polyglactin 910, ref: V302H, Ethicon) and applying betadine on the closed scalp. The cannula guide was closed with a small cap (4mm PED for 26G guide, no projection, PHYMEP, France). At the end of surgical procedures, mice received 1 mg/kg ip injection of atipamezole, 5 mg/kg subcutaneously injected ketoprofen and were left to recover for 2 weeks housed in pairs, while being handled, accustomed to the placement of the 26-gauge internal cannula and their BW monitored daily.

10.1. Composition of Solutions for ICV Infusion

The aCSF is composed of 100 mL of physiological stock (119.0 mM NaCl, 2.5 mM KCl, 1.25 mM $NaH2PO_4$*H2O, 1.3 mM $MgSO_4$*$7H_2O$ and 2.5 mM $CaCl_2$*$2H_2O$), 100 mL of Bicarbonate stock (26 mM $NaHCO_3$) 1.98 g Glucose and was buffered with 95% O_2 and 5% CO_2 before being stored frozen at -20°C.

Recombinant murine RANTES/CCL5, a 7.8kDa protein in powdered form, was purchased from Peprotech, France and reconstituted and diluted in aCSF (as described above) to achieve a final concentration of 10 ng, 50 ng and 100 ng per mouse per day for the intial experiment and 0.15 µg/µL for the second experiment and stored frozen at -20°C until injection.

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^{Met}RANTES human trifluoroacetate salt stock (50µg) purchased from Bachem AG, Switzerland was diluted in aCSF to a final concentration of 0.15 µg/µL and stored frozen at -20°C until infusion (Proudfoot *et al.*, 1996). ^{Met}RANTES will be referred to as ^{Met}CCL5 in the manuscript.

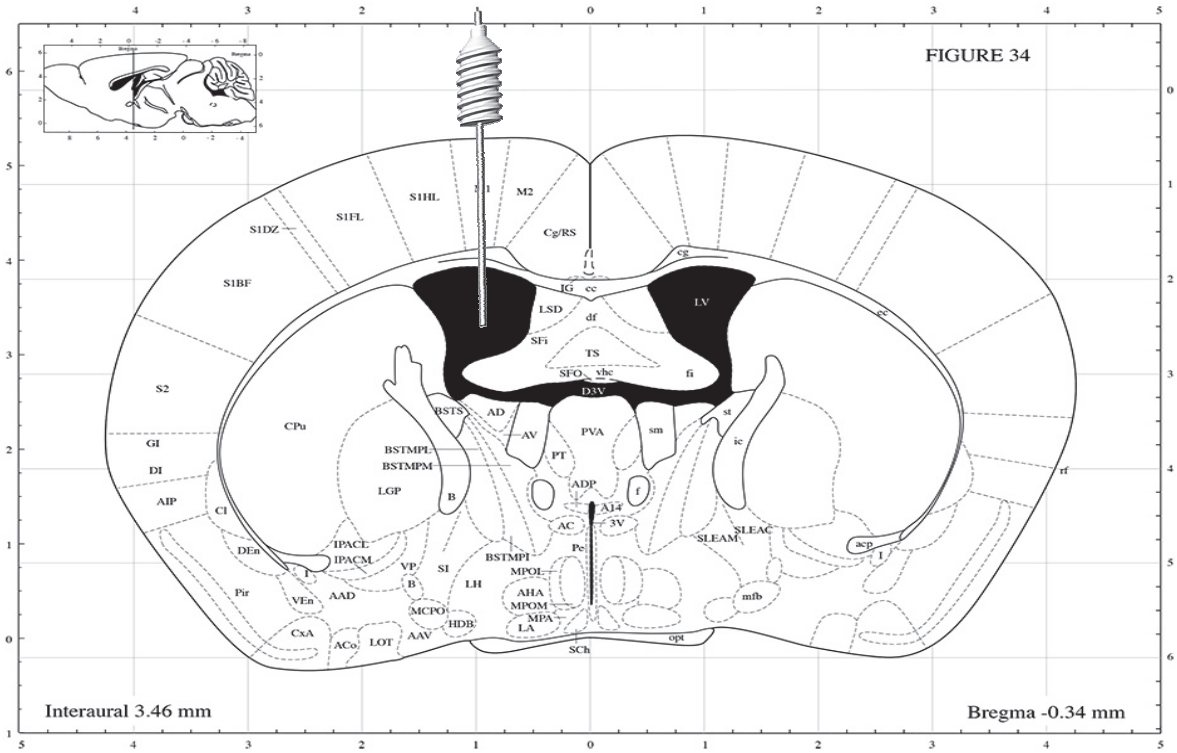

Figure 26: Schematic representation of unilateral cannula placement in the lateral ventricle.
(Adapted from "The Mouse Brain in Stereotaxic Coordinates", Second Edition, by Paxinos & Franklin.)

10.2. Chronic ICV Infusions

After two weeks of recuperation and handling of mice, including the removal and reattachment of the dummy cap for the guide cannula and verifying that guide cannula is not blocked, mice were habituated for injections.

For the first experiment of repeated injections of CCL5 with different concentrations, a single aCSF infusion was performed to habituate mice before starting the actual experiments. For the second experiment of repeated injections of aCSF, CCL5 and antagonist ^{Met}CCL5, mice were infused every second day using a Hamilton syringe (1701 SN 10µL, 22G/30mm/PS3; PHYMEP, France), which was placed into an adaptor.

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Then 2 µL of artificial cerebrospinal fluid (aCSF) were infused at a rate of 0.5 µL/min via an internal injector (33 GA, 4 mm PED, with 1 mm projection, PHYMEP, France) connected to the syringe through a polyethylene tube (0.023"X.0.038"X0.0075", PHYMEP, France). Once mice started to recover from the initial weight loss from aCSF infusions, they were randomly assigned to groups and infused every second day with 2 µL of either aCSF, ^{Met}CCL5 (0.15 µg/µL) or CCL5 (0.15 µg/µL) and BW and food intake were measured before and 2h and 24h after infusion. For the second experiment, Hargreaves' thermal pain sensitivity, GTT, ITT and was tested at 3 and 4 weeks after initiation of injections as depicted in Fig. 27.

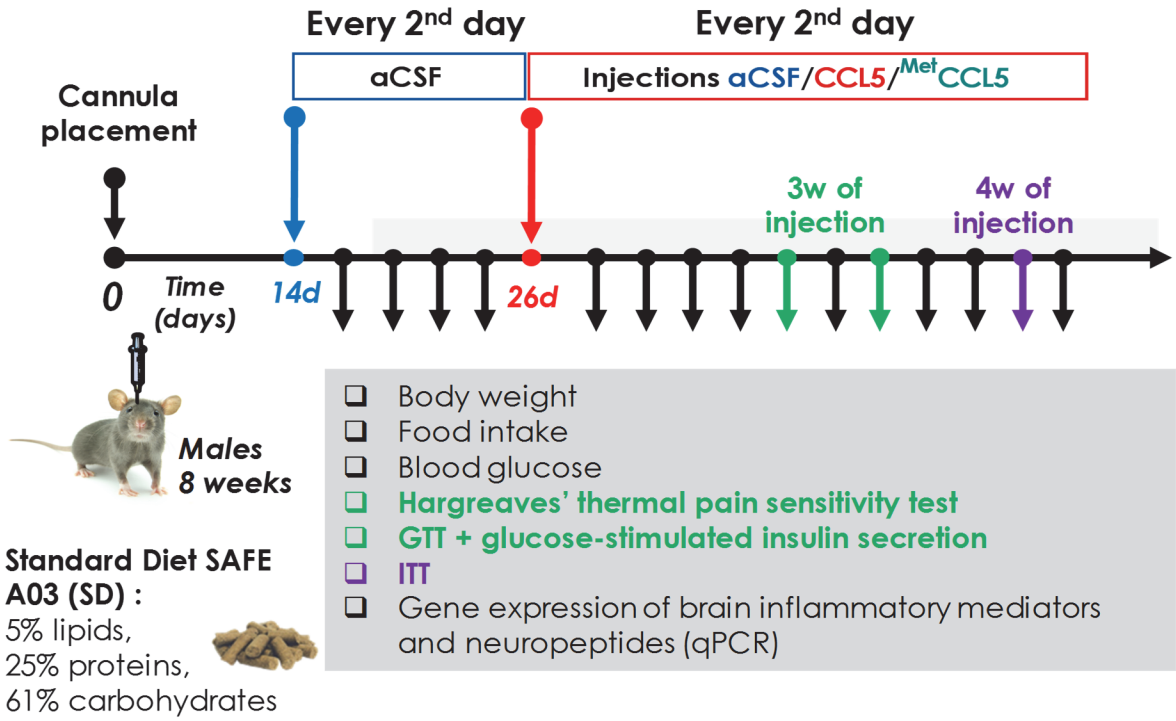

Figure 27: Protocol of chronic injections.
Abbreviations: w: weeks after the start of different injections (red arrow). d: days; aCSF: artificial cerebrospinal fluid; GTT: glucose tolerance test; ITT: insulin tolerance test.

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11. Hargreaves' Test of Thermal Pain Sensitivity

The thermal heat pain thresholds were measured with the plantar Hargreaves' radiant heat test (Analgesia Meter, Bioseb). Mice were placed individually in elevated observation chambers (8 x 8 cm) with translucent glass at the bottom, where they were acclimated for at least 20 min before measures. A radiant infrared heat source was then focused under the animal's hind paw and maintained until the mice retrieved its paw. Reaction scores, paw withdrawal thresholds (PWT), were the results of two measures on both hind paws, with a minimum of 5 min interval between measures.

12. Statistical Analysis.

Displayed values are means ± SD. The ROUT method (Robust regression and Outlier removal) was used to identify outliers with a Q coefficient equal to 1%. Comparisons between groups were carried out using a non-parametric Mann-Whitney test for single comparison and a Kruskal-Wallis test with Dunn's correction or two-way ANOVA for multiple comparisons and interaction. A P-value of ≤ 0.05 was considered statistically significant. All tests were performed using GraphPad Prism 7.02 and Microsoft Office Excel. Numbers of animals and cells are given in the legends.

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Results

1. The Effects of Lipid Nature on the Development of Obesity

In the first part of the project, we focused on establishing a mouse model of DIO to test the effect of different long-term HFDs based on different lipid nature on the development of obesity and the associated comorbidities such as diabetes and neuropathic pain. The following part will depict the results obtained of feeding young 4 weeks old C56BL6/J mice (WT) with different HFD based on either soja/corn (HFD-SC), sunflower (HFD-SN), rapeseed oil (HFD-C) or butter (HFD-B) and compared to SD-fed mice and characterizing their phenotype during the development of obesity for 12 weeks. Additionally, the different lipid composition of the HFDs resulted in a different ω6/ω3 ratio. We wanted to know whether the ratio of ω6/ω3 in the HFDs had a different impact on obesity development and inflammatory state of mice.

1.1. The Effect of Lipid Nature on the Development of Obesity and Food Intake

Feeding age- and weight-matched mice with different HFDs with different ω6/ω3 ratio for 12 weeks of time lead to a different development of obesity between the different HFDs. The diet with the highest percentage of lipids (35%) based on butter (HFD-B) as a lipid source, is as expected, the most obesogenic diet among the HFDs and leads to a rapid weight gain and development of obesity, starting to be significantly different from SD fed mice at 3 weeks of diet (Fig. 28A). Compared to that, all other HFDs with 20% lipids independent of lipid source showed the same rate of weight gain, which was markedly reduced compared to HFD-B fed mice. They developed obesity only slow and were significantly different form SD-fed mice only starting from 8 weeks of diet for HFD-SC (ω6/ω3=7.3), 9 weeks for HFD-C (ω6/ω3 =2.3) and 10 weeks for HFD-SN (ω6/ω3=17.3)(Fig 28A). We did not find any significant difference between HFDs with 20% lipids and different ω6/ω3 ratios. We also

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measured food intake every second day and found, that although HFD-fed mice, independent on source of lipids or ω6/ω3 ratio, consumed on average less grams of food per mice, that there was no significant difference between kcal intake inbetween diets, and it remained stable over time (not shown). It thus seems that a different ratio of ω6/ω3 does not seem to affect BW gain or food intake differently, especially seeing that HFD-B has a lower ω6/ω3 ratio with 8.0 compared to HFD-SN, which has lower amounts of lipids but a ratio of 17.3.

Our measurement of serum leptin concentrations at 12 weeks of diet confirmed the trend seen in BW gain, as HFD-B had the highest concentration of Leptin at 12 weeks of diet compared to SD diet (Fig. 28C). We did not observe any significant difference for any other HFDs with 20% of lipids, but the data suggests a tendency for an increase in leptin concentration according to the ω6/ω3 ratio, as we see the highest increase in leptin with HFD-SN, which has the highest ω6/ω3 ratio of 17.3 at 12 weeks of diet. This is followed by an increase in serum leptin levels in mice fed HFD-SC (ω6/ω3=7.3), which in turn is higher than HFD-C (ω6/ω3=2.3), that resembles the closest to the leptin concentrations of SD-fed mice (Fig. 28C).

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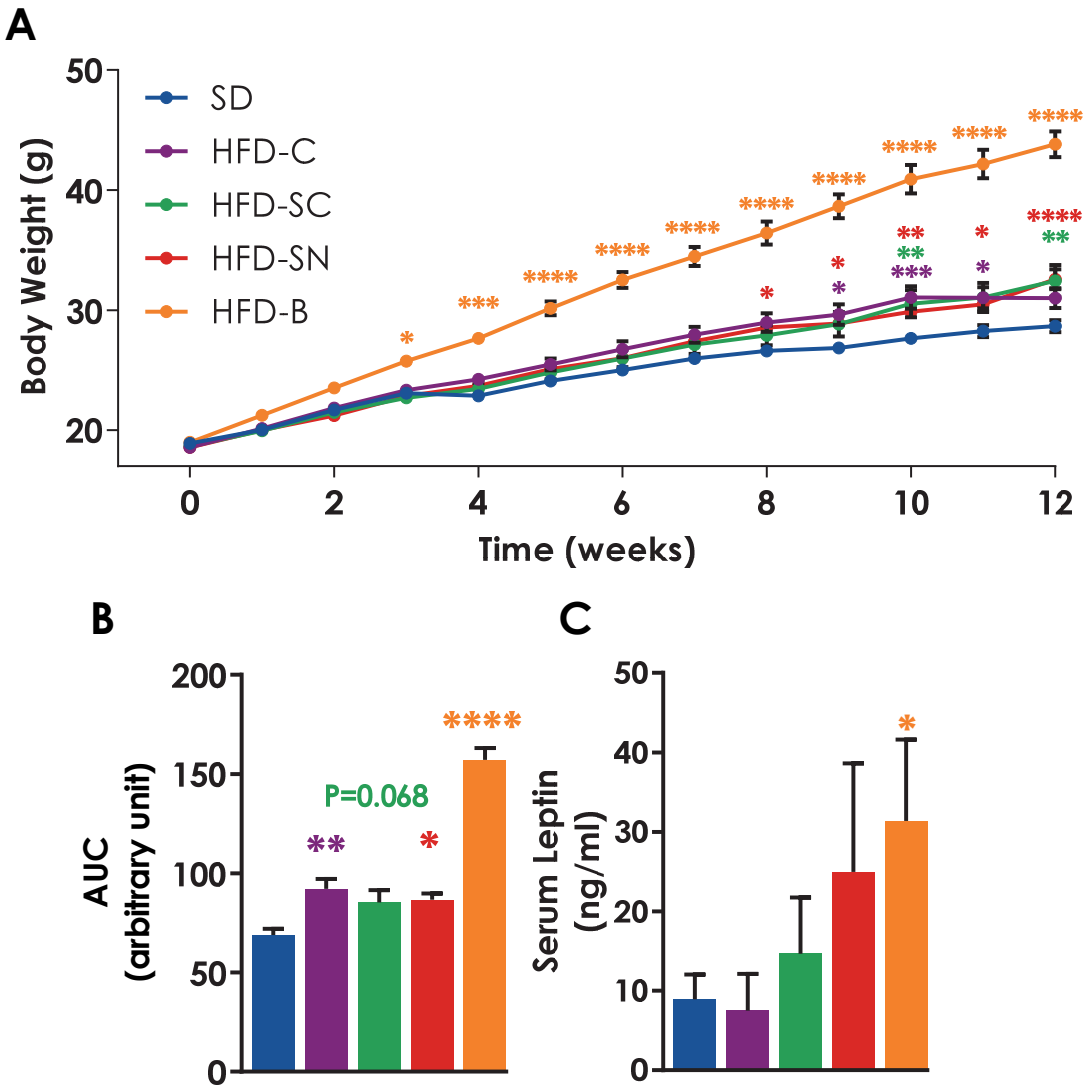

Figure 28: The effects of lipid nature on body weight gain and leptinemia.

A: Evolution of average BW during 12 weeks of experimental protocol of DIO containing different lipids. **B**: Quantification of (**A**) as area under the curve - AUC. **C**: Serum leptin concentration measured by ELISA after 12 weeks of different HFD. Data are reported as the means ±SEM (n=10/group for A and B; n=5-6/group for C). Two-way ANOVA or one-way ANOVA followed by Dunnett's *post hoc* test or Mann Whitney test. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, all *vs* SD; ns: not significant.

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1.2. The Effects of Lipid Nature on Hypothalamic Neuropeptide Expression

Although, we have no perceived any differences in kcal intake between mice fed different diets, we still were interested in determining, whether the consumption of HFDs based on different lipid nature and ω6/ω3 ratio could distingly affect the expression of hypothalamic neuropeptides, which are implicated in the control of food intake and energy balance.

We thus performed qPCR to quantify the relative expression of hypothalamic neuropeptides and found that mice fed a HFD-B had significant downregulation of anorexigenic POMC mRNA, as well as an upregulation of orexigenic neuropeptides NPY and MCH mRNA in the hypothalamus but no differences in other neuropeptides tested (Fig 29A-F). Interestingly, we did not find any significant changes in neuropeptide expression between HFDs based 20% of lipids from a plant source and different ω6/ω3 ratio, indicating that ω6/ω3 ratio seems to have no effect on neuropeptide expression in the hypothalamus after 12 weeks of diet. Our results indicate that a HFD based on butter had an effect on neuropeptide expression. However, we cannot infer from the data obtained, whether the effect is due to higher lipid content in HFD-B as opposed to all other diets or due to the nature of lipids, which are retrieved from animal products.

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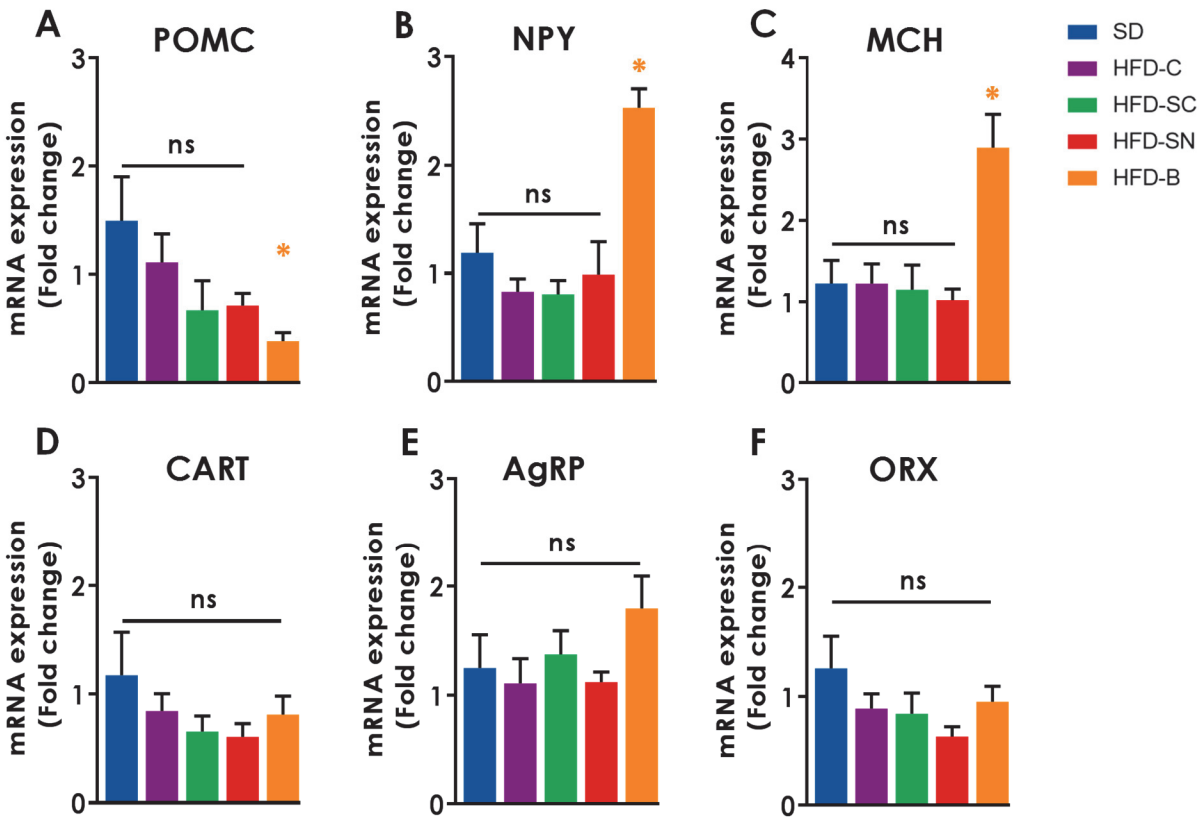

Figure 29: The effects of lipid nature on hypothalamic neuropeptide expression.
Real-time PCR quantification of relative mRNA expression of hypothalamic neuropeptides such as anorexigenic neuropeptides POMC (**A**) and CART (**D**), and orexigenic neuropeptides NPY (**B**), AgRP (**E**), MCH (**C**) and ORX (**F**) in WT mice after 12 weeks of SD or one of four HFDs containing different lipids. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control group. Values are the mean ± SEM (n=7 mice/ group). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test; P<0.05 *, P<0.01 **, P<0.001 ***, all *vs* SD; ns: not significant.

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1.3. The Effects of Lipid Nature on Glucose Homeostasis

Due to the close association between obesity and diabetes, we decided to test whether the weight gain observed in mice on a HFD is accompanied by dysfunctions in glucose metabolism. We performed an ipGTT and ITT at 12 weeks of diet and found that our results reflect the changes in BW we showed earlier (Fig. 28). Mice fed a HFD-B showed perturbations in glucose tolerance) after 12 weeks of diet compared to SD-fed mice (Fig. 30A-C, D). When comparing diets based on vegetal sources of lipids with different ω6/ω3 ratio, we did not find any difference between diets. None of the HFDs containing 20% of lipids showed a measurable impairment in glucose tolerance irrespective of lipid sourse, when compaired to SD (Fig.30A-B). In accordance with these results, we observed a slight increase, although not significant, of fasting blood glucose levels for HFD-B and HFD-C at 8 weeks of diet and a significant increase in fasted blood glucose levels of mice fed a HFD-B after 12 weeks of diet (Fig. 30D). Concurrently, when we performed an ITT at 12 weeks of diet, we found that mice on a HFD-B were significantly resistant from SD-fed mice, while mice fed one of the other vegetal lipid based diets, showed no impairment in insulin sensitivity (Fig. 30C).

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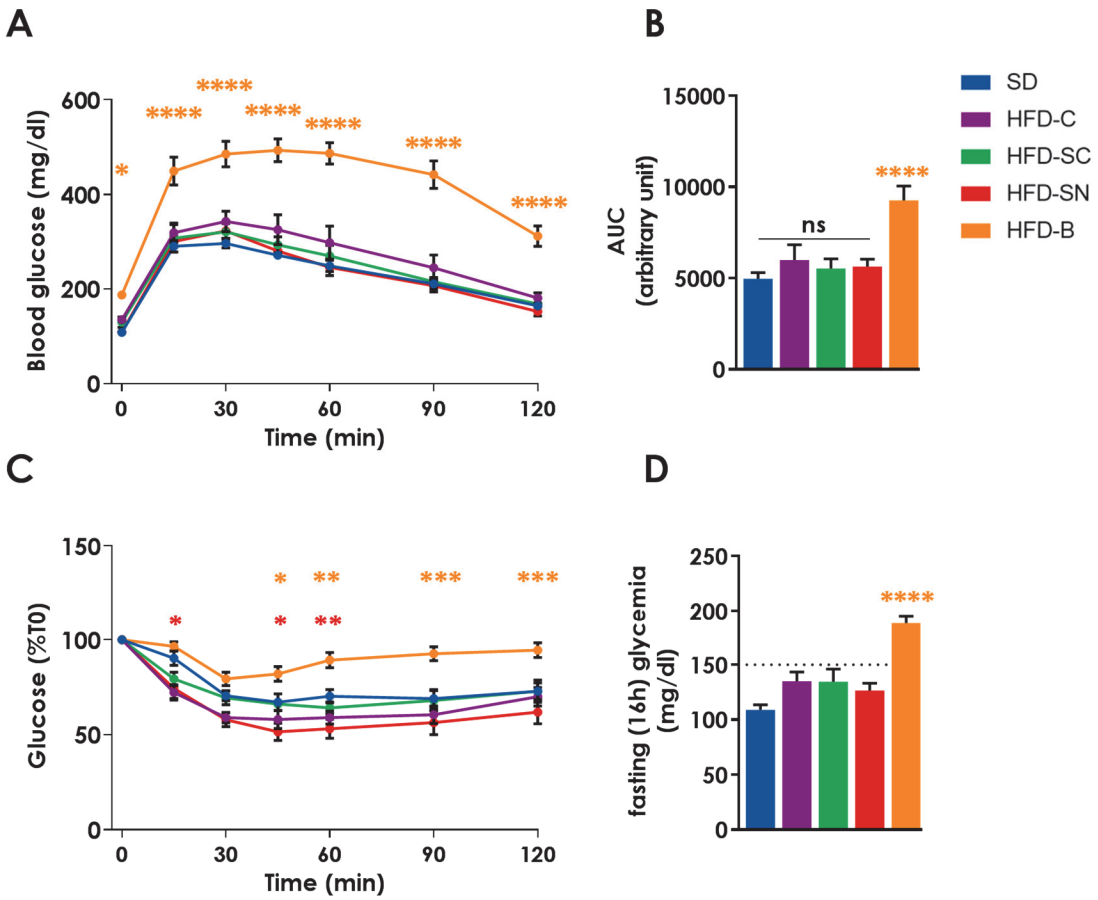

Figure 30: The effects of lipid nature on glucose homeostasis.
Effect of different lipid nature on **A**: ipGTT after 12 weeks of diet and **B**: quantification of (**A**) represented as area under the curve (AUC). **C:** Effect of different lipid nature on insulin sensitivity after 12 weeks of diet. **D**: Basal blood glucose levels after 16h of fasting after 12 weeks of diet. Dashed line indicates the threshold for diabetic levels of blood glucose at 150 mg/dL. Values are means ± SEM (n=10/group). Statistical significance was determined with two-way ANOVA or one-way ANOVA with Dunnett's post hoc test or Kruskal-Wallis test with Dunn's *post hoc* test. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, all *vs* SD; ns: not significant.

1.4. The Effect of Lipid Nature on HFD-Induced Central Inflammation

After 12 weeks of HFD treatment, we collected the hypothalamus of all mice and quantified the mRNA expression of inflammatory markers relative to SD-fed mice. Our data indicates that mice fed a HFD-B, based on animal-derived lipids, had higher expression levels of inflammatory cytokines such as TNF-a and IL-6 as well as upregulaton of glial cell markers Iba1 and GFAP compared to the SD control group (Fig. 31A-G). Among the HFDs with

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vegetal-derived lipids we found increased upregulation of pro-inflammatory cytokines only in the hypothalamus of HFD-SN fed mice, indicating that a HFD with lower lipid content but unfavorable ω6/ω3 ratio of 17,3 equally is able to induce inflammation (Fig. 31A-G). In addition to that, HFD-SN induced also an upregulation of iba1 expression but not GFAP. Interestingly, HFD-SC induced an upregulation of both iba1 and GFAP expression without any increase in pro-inflammatory markers in the hypothalamus. Strikingly, only HFD-B induced hypothalamic upregulation of the chemokine CCL5 (Fig. 31D).

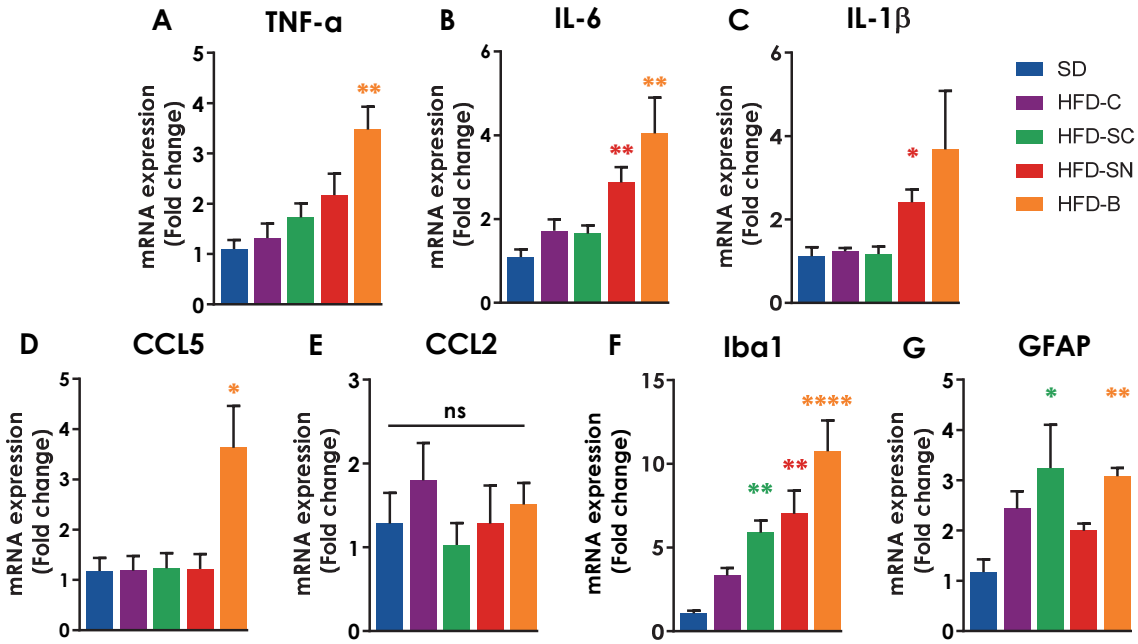

Figure 31: The effect of lipid nature and composition on hypothalamic inflammation and gliosis.

Real-time PCR quantification of hypothalamic mRNA expression of inflammatory cytokines (**A**) TNF-α, IL-6 (**B**), and IL-1β (**C**), chemokines, CCL5 (**D**) and CCL2 (**E**); and glia cell markers (**F**) for Iba1 positive microglia and (**G**) for GFAP positive astrocytes after 12 weeks of diet in WT on either SD or one of four HFDs based on different lipids. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control group. Values are represented as means ± SEM (n=6-7 mice/group). Statistical significance was determined with Kruskal-Wallis test with Dunn's post hoc test. P<0.05 *****, P<0.01 ******, P<0.001, *******, P<0.0001 ********. All vs SD; ns: not significant.

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1.5. The Effects of Lipid Nature on Thermal Pain Sensitivity

Because T2DM is associated with many macro- and microvascular complications, one of which is neuropathic pain, we were interested to assess, whether HFDs with different nature of lipids have a different propensity to reduce the pain threshold called hyperalgesia. Thus, in collaboration with J. Noël in the team of E. Lingueglia at IPMC, we performed a Hargreaves' thermal pain test after 12 weeks of diet and found that only HFD-B the most obesogenic diet that rendered mice diabetic also reduced the threshold for thermal pain sensitivity compared to mice fed a SD (Fig. 32).

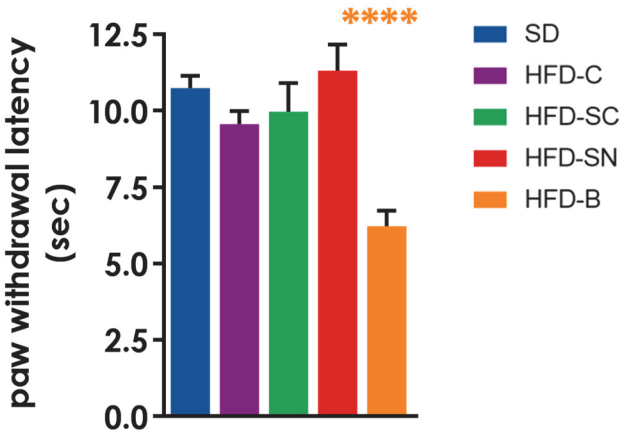

Figure 32: The effect of lipid nature and composition on thermal pain sensitivity at 12w of diet.
Estimation of of thermal pain sensitivity by paw withdrawal latency in seconds as determined with Hargreaves' test in WT mice fed a SD or one of four HFDs based on different lipid nature. Values are represented as means ± SEM (n=9-15mice/group). Statistical significance was determined with Kruskal-Wallis test with Dunn's post hoc test. (n=9-15 mice); P<0.05 *, P<0.01 **, P<0.001, ***, P<0.0001 ****, all vs SD; ns: not significant.

In summary, the first part of our study focused on testing four diets high in fat with different lipid nature and composition on the development of obesity and associated complications such as diabetes, neuroinflammation, alteration of hypothalamic neurons and neuropathic pain. We have identified that HFD-B seem to be the most obesogenic diet, that induces both hypothalamic inflammation as well as modulation of the expression of hypothamamic neuropeptides. In addition to that, it was the only diet that

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after 12 weeks of diet rendered mice diabetic and more sensitive to thermal pain. For these reasons, we focused on HFD-B in the second part of our experiment to investigate the effects of CCL5 and CCR5 in an obese context. In the following section "HFD-B" will be referred to simply as "HFD".

2. Role of Chemokine CCL5 in the Development of Obesity

Previously, we have identified that a HFD and based on lipids obtained from an animal source such as butter is the most obesogenic diet and induces a rapid development of obesity, with both peripheral and central inflammation. We have also shown that CCL5 is one of the inflammatory markers that is persistently increased in the serum of obese mice and as previously reported in obese human patients (Dalmas *et al.*, 2011). This part of the study thus investigated the role chemokine CCL5 plays in the development and maintenance of obesity. To investigate this, we employed our previously developed mouse model of DIO and applied it to our KO model of the chemokine CCL5. In other words, to identify the effects of chemokine CCL5 on DIO, we gave either SD or HFD to either wildtype littermates (WT) or CCL5 KO mice ($CCL5^{-/-}$) and characterized several physiological and metabolic parameters. The following section of this manuscript will develop the results we have obtained.

2.1. Chemokine CCL5 Deficiency Reduces HFD-Induced BW Gain and Food Intake.

To elucidate the role of CCL5 in DIO we compared age-matched WT and $CCL5^{-/-}$ mice that were started on either a SD or a HFD at an adult age of ~3 months, maintained on the diet for 16 weeks and measured different physiological and metabolic parameters.

We have observed no difference in BW gain in $CCL5^{-/-}$ mice compared to WT when fed a SD. However, as expected we have confirmed previous findings of our team and literature that when WT mice were fed a HFD, they rapidly gain weight and develop obesity with significant changes in BW starting from week three (Fig. 33A). Interestingly, when we compare $CCL5^{-/-}$ with WT mice on a HFD, we see a significant reduction in the BW gain in $CCL5^{-/-}$ mice

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starting from three weeks of HFD with a milder and delayed development of obesity (Fig. 33A). This finding is equally represented as area under the curve (AUC) with an average reduction of BW in CCL5$^{-/-}$ as opposed to WT mice on a HFD with no difference in SD-fed mice in fig. 33B.

Interestingly, despite the difference in BW gain, we have not found any difference in serum leptin levels between HFD fed CCL5$^{-/-}$ and WT mice (Fig. 33F), while the leptin level increases in the serum of HFD WT mice compared to SD mice in proportion to BW as described in literature (Ahrén *et al.*, 1997)

Next, we wanted to find out what might cause the observed reduction in BW gain and focused on food intake. When we looked at food intake, we observed that CCL5$^{-/-}$ mice eat on average less grams of food compared to WT mice independent on diet. Surprisingly, although CCL5$^{-/-}$ mice on a SD do not differ in BW from their controls, they eat significantly less food (Fig 33C). As expected, both genotypes consumed on average significantly less grams of food compared to their controls on a SD (Fig. 33C).

However, there was no difference in the average kcal consumption between WT mice or CCL5$^{-/-}$ mice independent of diet. However, although CCL5$^{-/-}$ mice on a SD still showed a reduced kcal intake, it was no longer significantly different from HFD fed CCL5$^{-/-}$ mice, who showed a significant reduction in average consumed kcal compared to their HFD-fed controls (Fig. 33D). Interestingly, mice on a HFD drank less water than SD-fed mice, and CCL5$^{-/-}$ mice on a SD drank significantly less water than WT controls.

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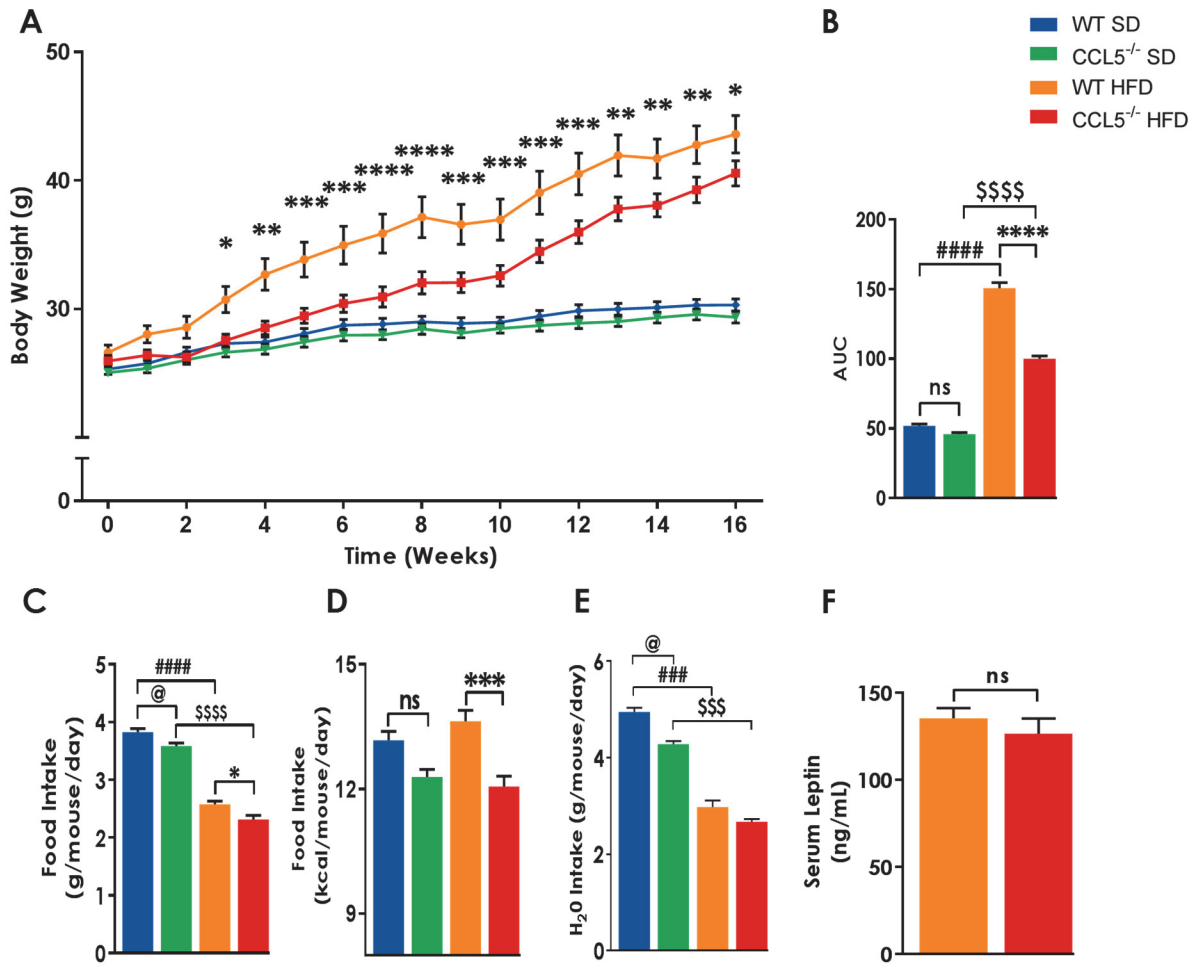

Figure 33: Effect of CCL5 deficiency on DIO development.

A: Evolution of average BW at 16 weeks of experimental protocol of DIO. **B**: Quantification of (A) as area under the curve - AUC. **C**: Average food consumption during 16 weeks represented as grams per mouse per day. **D**: Average food consumption during 16 weeks represented as kcal per mouse per day. **E:** Average H_2O consumption during 16 weeks in grams per mouse per day. **F**: Serum leptin concentration measured by ELISA after 16 weeks of HFD. Data are reported as the means ±SEM (n=14-25/group for A and B)(n=2-3 experiments/group for C-E)(n=8-9/group for F). Two-way ANOVA followed by Tukey *post hoc* test, one-way ANOVA or Mann Whitney Test for comparisons between two groups. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, WT HFD *vs* CCL5$^{-/-}$ HFD; #, WT SD *vs* WT HFD; $, CCL5$^{-/-}$ SD *vs* HFD; @, WT SD *vs* CCL5$^{-/-}$ SD; ns: not significant.

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2.2. Chemokine CCL5 Has a Neuromodulatory Effect on Hypothalamic Neurons

Due to the difference observed in food intake between genotypes, and because we are also interested in the role of CCL5 in the regulation of energy balance within the CNS, we next used realtime qPCR to quantify and compare the relative mRNA expression of both anorexigenic and orexigenic neuropeptides in hypothalamic samples of CCL5$^{-/-}$ and WT mice after 16 weeks of SD or HFD feeding. First, we wanted to know whether HFD could alter the neuropeptide expression within a genotype, so we normalized the mRNA expression of each HFD-fed group to their proper SD control group as you can see in Fig. 34. Then, because we wanted also to find out whether CCL5 deficiency has a neuromodulatroy effect in physiological conditions on a SD or in pathological conditions on a HFD, we also normalized the mRNA expression of the neuropeptides by the WT SD or HFD condition, respectively to compare between genotypes (Fig. 35).

We found that diet does not seem to change the expression of orexigenic neuropeptides of the hypothalamus in WT or CCL5 deficient mice after 16 weeks as there was no significant difference between HFD and SD fed mice (Fig. 34). Interestingly, there was a tendency for increased mRNA expression in orexigenic NPY in WT HFD mice compared to their SD fed controls (P=0.072) and a decrease in AgRP in CCL5$^{-/-}$ mice on a HFD compared to their SD controls (P=0.077) (Fig. 34C and D). However, we found a significant increase in both HFD fed groups in the mRNA expression of anorexigenic neuropeptide CART relative to their SD controls (Fig 34B). When comparing genotypes within the same diet, we have found a slight but significant increase in MCH and a 2-fold increase in ORX expression in SD-fed CCL5$^{-/-}$ mice compared to their WT controls (Fig. 35E and F).

Interestingly, when comparing between HFD-fed mice, we found a significant decline in orexigenic NPY mRNA expression in the hypothalami of CCL5$^{-/-}$

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relative to WT mice but no difference in any of the other neuropeptides (Fig. 35C).

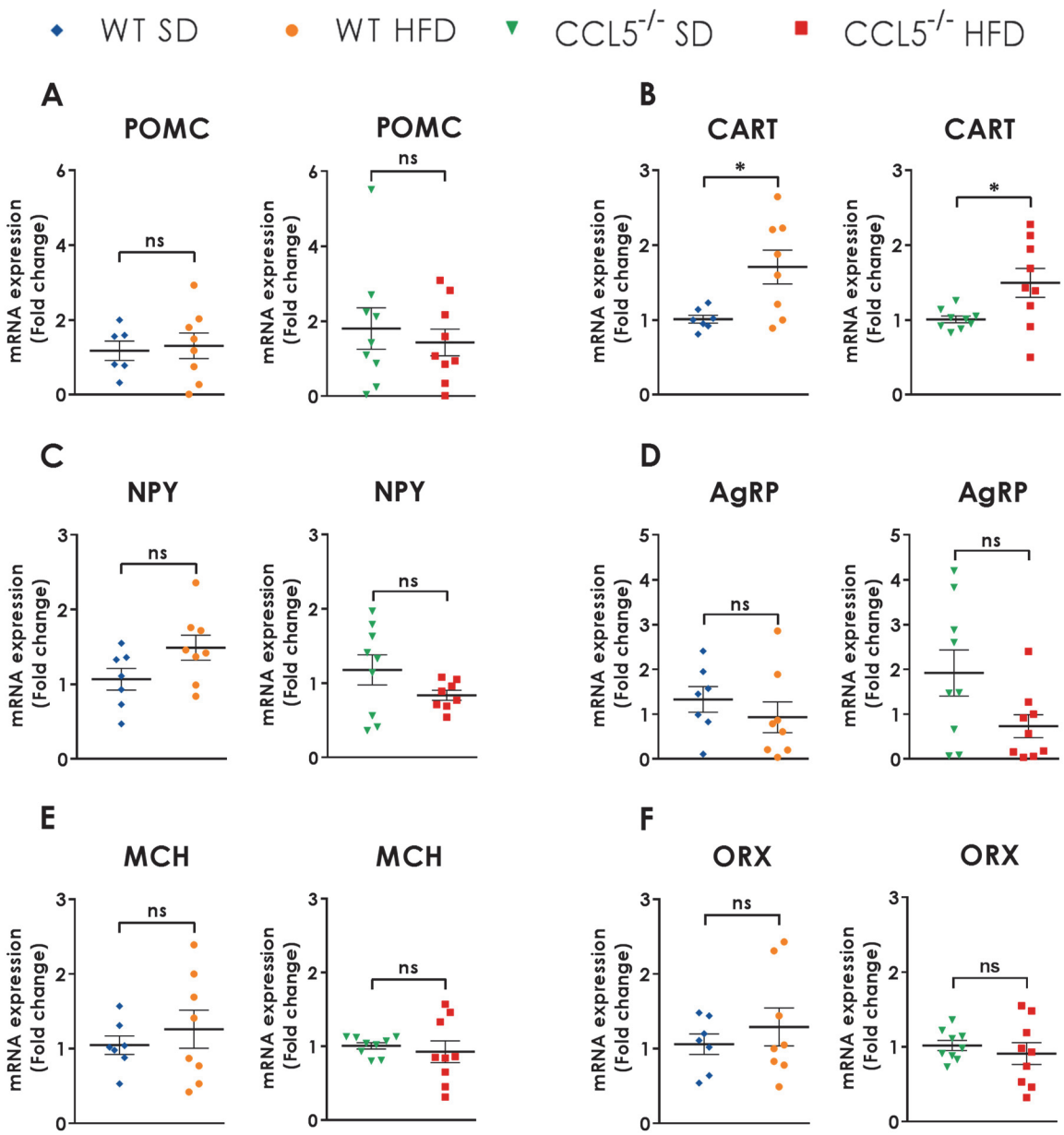

Figure 34: Effect of CCL5 deficiency on hypothalamic neuropeptide expression compared between diets.

Real-time PCR quantification of relative mRNA expression of hypothalamic neuropeptides such as anorexigenic neuropeptides POMC (**A**) and CART (**B**), and orexigenic neuropeptides NPY (**C**), AgRP (**D**), MCH (**E**) and ORX (**F**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are the mean ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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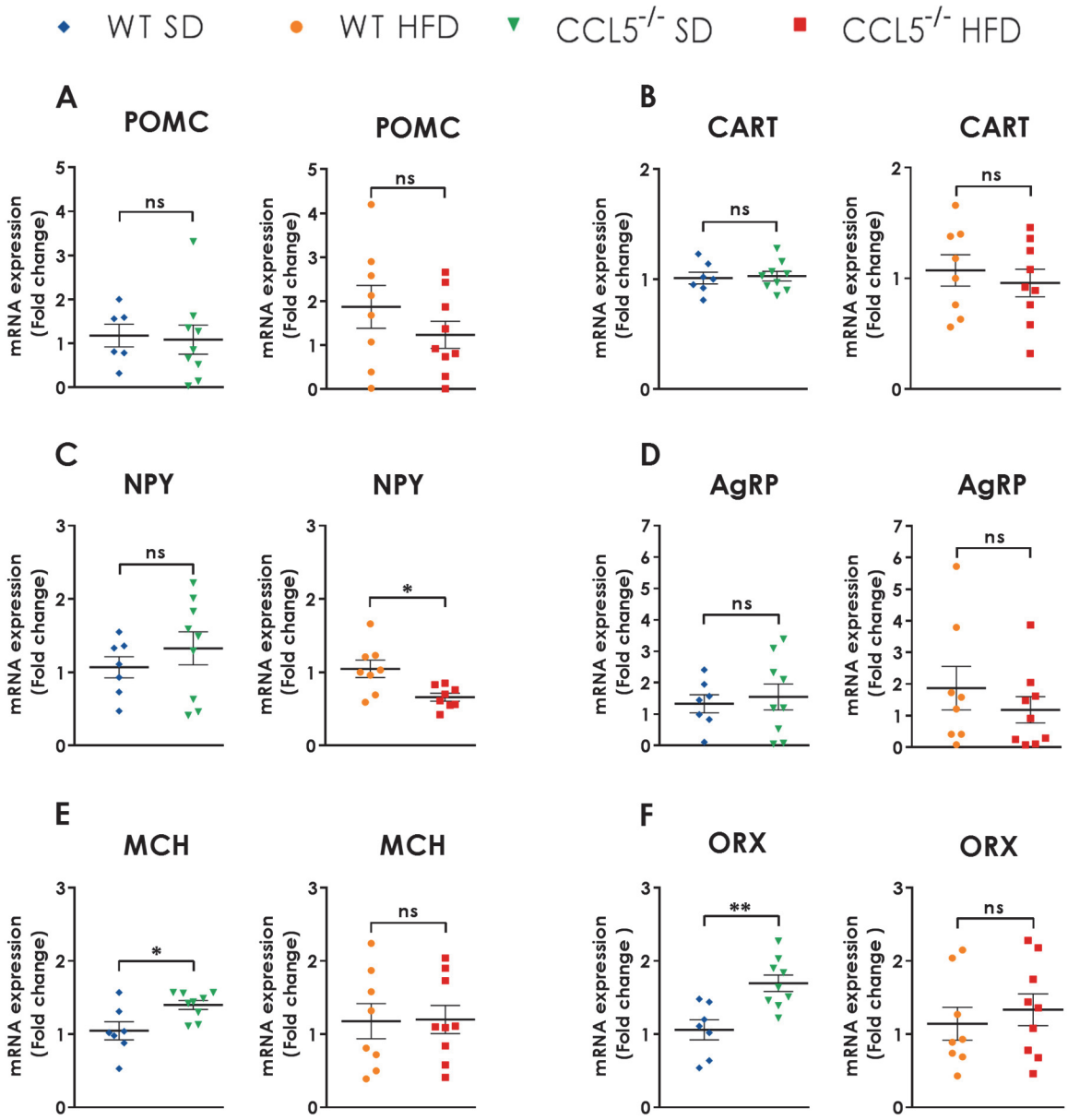

Figure 35: Effect of CCL5 deficiency on hypothalamic neuropeptide expression compared between genotypes.

Real-time PCR quantification of mRNA expression of hypothalamic neuropeptides such as anorexigenic neuropeptides POMC (**A**) and CART (**B**), and orexigenic neuropeptides NPY (**C**), AgRP (**D**), MCH (**E**) and ORX (**F**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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2.3. Chemokine CCL5 Deficiency Improves Glucose Metabolism in Obese Mice

Because obesity is associated with many other comorbidities, of which a common one is diabetes, we wanted to further investigate if CCL5 deficiency is not only reducing weight gain but also might impact the impaired glucose metabolism seen in obese mice and humans as mentioned earlier. We performed an IP injected GTT at 8 and 16 weeks of diet and found that WT mice on a HFD showed signs of diabetes already at 8 weeks of diet compared to WT mice on SD (Fig. 36A and C-D). Diabetes in mice has been previously defined by basal blood glucose levels of 150mg/dL, which was the case for the HFD-fed WT mice at 8 weeks and 16 weeks of diet after both 16h and 6h of fasting (Sullivan *et al.*, 2007)(Fig. 36D-E and G-H). The GTT of HFD-fed WT mice showed an impaired glucose-stimulated response at both 8 and 16 weeks of diet compared to SD fed control mice (Fig. 36A and B). While there seems to be no difference in glucose metabolism between $CCL5^{-/-}$ and WT mice on a SD diet, $CCL5^{-/-}$ mice on a HFD are significantly more tolerant to glucose with lower fasting blood glucose levels (Fig. 36D) compared to HFD-fed WT controls and show no significant difference to $CCL5^{-/-}$ mice on a SD after 8 weeks of diet (Fig. 36A and C). Although HFD-fed $CCL5^{-/-}$ mice are still significantly different from their WT mice controls at 16 weeks of diet (Fig. 36A), there is no longer a difference in glucose tolerance and fasting glucose levels between CCL5 deficient and WT mice on a HFD at 16 weeks of diet (Fig. 36B, F-H). Furthermore, $CCL5^{-/-}$ mice displayed no difference in insulin sensitivity at 8 and 16 weeks to WT mice on a SD (Fig. 36I and J). While WT mice presented with insulin resistance at both 8 and 16 weeks of HFD diet, $CCL5^{-/-}$ deficiency seemed to prevent this impairment significantly at both time points, despite being equally glucose intolerance at 16 weeks as WT controls (Fig. 36B, I and J). Interestingly, $CCL5^{-/-}$ mice on a HFD were more sensitive to insulin than WT controls, despite showing less glucose-stimulated insulin secretion compared to WT controls on a HFD, which display hyperinsulinemia upon glucose stimulation but no significant difference at baseline (Fig 36J-L).

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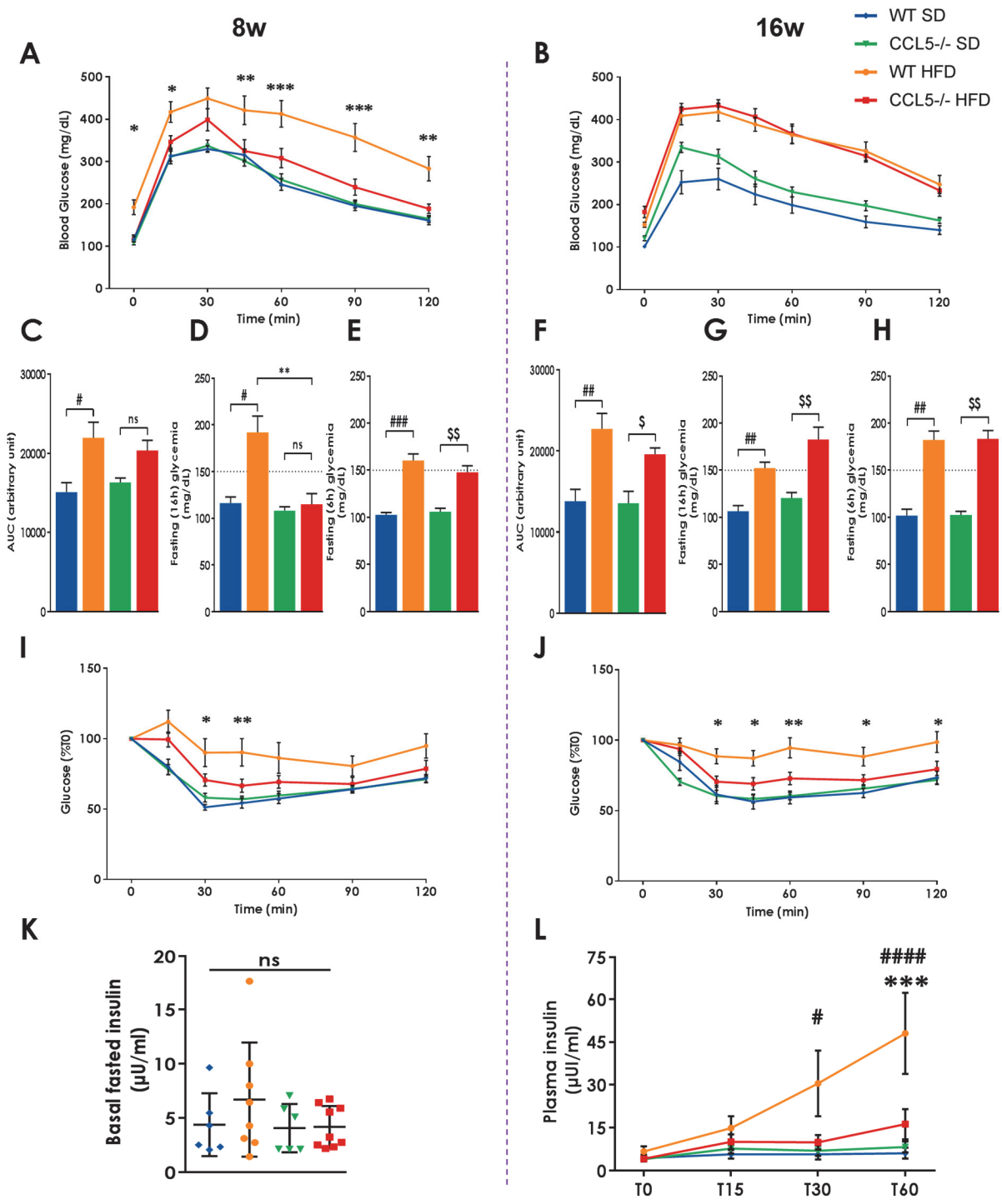

Figure 36: Glucose homeostasis perturbations in WT and CCL5$^{-/-}$ mice after 8 and 16 weeks of SD or HFD.

Effect of genetic CCL5 deletion on **A**: IP GTT after 8 weeks of diet and **B**: 16 weeks of diet. **C** and **F**: Quantification of (**A**) and (**B**) represented as area under the curve (AUC). **D**: and **G**: Basal blood glucose levels after 16h of fasting after 8 and 16 weeks of diet, respectively. **E**: and **H**: Basal blood glucose levels after 6h of fasting after 8 and 16 weeks, respectively. **I** and **J**: Effect of CCL5 deficiency on insulin tolerance in mice fed a SD or HFD for 8 weeks (**I**) and 16 weeks (**J**). K: Basal fasted insulin levels at 16 weeks of diet measured via ELISA and **L**: acute glucose-stimulated insulin secretion

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measured via ELISA at 16 weeks of diet. Dashed line indicates the threshold for diabetic levels of blood glucose at 150 mg/dL. Values are means ± SEM (n=8-10/group for A-J and n=6-9 for G+H). Statistical significance was determined with two-way ANOVA with Tukey's post hoc test or Kruskal-Wallis test with Dunn's post hoc test. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, WT HFD vs CCL5$^{-/-}$ HFD; #, WT SD vs WT HFD; $, CCL5-/- SD vs HFD. ns, not significant; 8w: 8 weeks of diet; 16w: 16 weeks of diet.

We further wanted to know if insulin signaling was altered at the level of the brain, so we compared the mRNA expression of IRS1 and as shown in Fig. 37A HFD reduced the mRNA levels significantly in WT mice compared to their SD controls but not in CCL5$^{-/-}$ mice (Fig. 37A-B, E-F). Furthermore, we tested PPARγ expression levels in the hypothalamus, as it was reported to be a determinant of insulin sensitivity (Lu *et al.*, 2011). We did not find any difference for hypothalamic PPARγ mRNA expression levels between WT mice on different diets, but CCL5$^{-/-}$ mice expressed more PPARγ mRNA in the hypothalamus relative to their WT controls and independent of diet (Fig. 37G-H).

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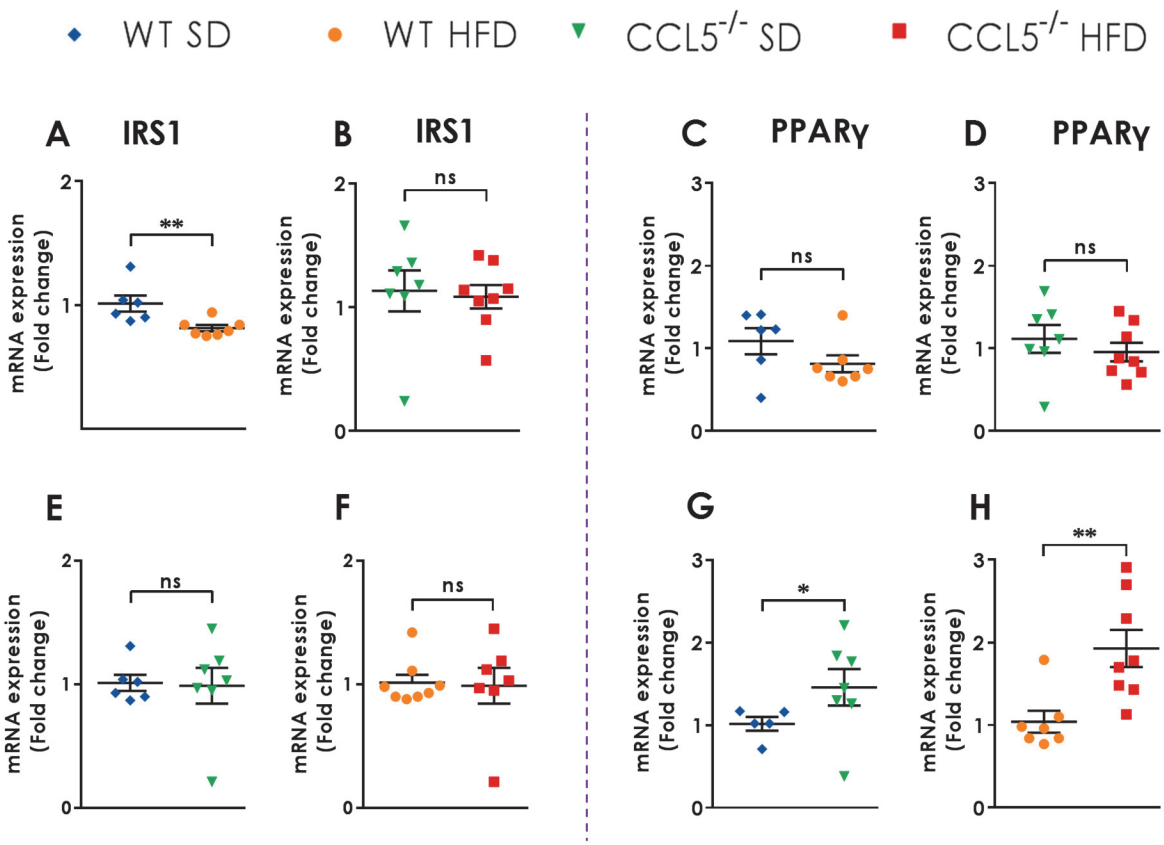

Figure 37: Effect of CCL5 deficiency on hypothalamic insulin signaling.
A and **B:** Effect of 16 weeks of diet on hypothalamic mRNA expression of IRS1 in WT (**A**) and CCL5$^{-/-}$ (**B**) mice. **C** and **D:** Effect of 16 weeks of SD or HFD on hypothalamic mRNA expression of PPARγ in WT (**C**) and CCL5$^{-/-}$ (**D**) mice. **E** and **F:** Effect of CCL5 deficiency on hypothalamic IRS1 expression between genotypes at 16 weeks of SD (**E**) and HFD (**F**) diet. **G** and **H:** Effect of CCL5 deficiency on hypothalamic PPARγ expression between genotypes at 16 weeks of SD (**G**) and HFD (**H**) diet. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to respective controls. Values are represented as means ± SEM (n=6-9 mice/ group). Statistical significance was determined using Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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2.4. The Effect of CCL5 on Central Inflammation and Hypothalamic Gliosis

To characterize the HFD-induced phenotype of CCL5$^{-/-}$ and WT mice further, we wanted to know if they are less obese due to less chronic low-grade inflammation that accompanies obesity. First, we determined the hypothalamic mRNA expression levels of inflammatory cytokines and chemokines. We found as expected a significant increase in inflammatory cytokines TNF-a and IL-6 and an increase in CCL5 levels although not significant in HFD-fed WT mice as opposed to their SD controls (Fig. 38A-B). Different from our expectations, we found a similar significant increase in HFD-fed CCL5$^{-/-}$ mice compared to their controls for TNF-a and a slight but not significant increase in IL-6 expression levels relative to their SD controls (Fig. 38A-B). We did not find any difference in expression levels of inflammatory markers between genotypes independent of diet (Fig. 39A-D).

Obesity is not only associated with central low-grade inflammation but several studies have also identified gliosis in the hypothalamus of obese mice. Hence, we wanted to find out whether CCL5 might act on glia cells and whether the lack of it has a protective effect on HFD-induced hypothalamic gliosis. We therefore examined the mRNA expression of the common astrocyte marker GFAP and microglial marker Iba1 in the hypothalamus after 16 weeks of diet and found that surprisingly WT mice on a HFD had no significant upregulation of both glial markers, although there was a tendency of increase for GFAP, although not significant (Fig. 40A and C). We have not found any HFD-induced upregulation in CCL5$^{-/-}$ mice, neither (Fig. 40B and D). Interestingly, we found that compared to HFD-fed WT mice, CCL5$^{-/-}$ had reduced expression of GFAP, which indeed might indicate that CCL5 could act through GFAP^{+} cells such as astrocytes.

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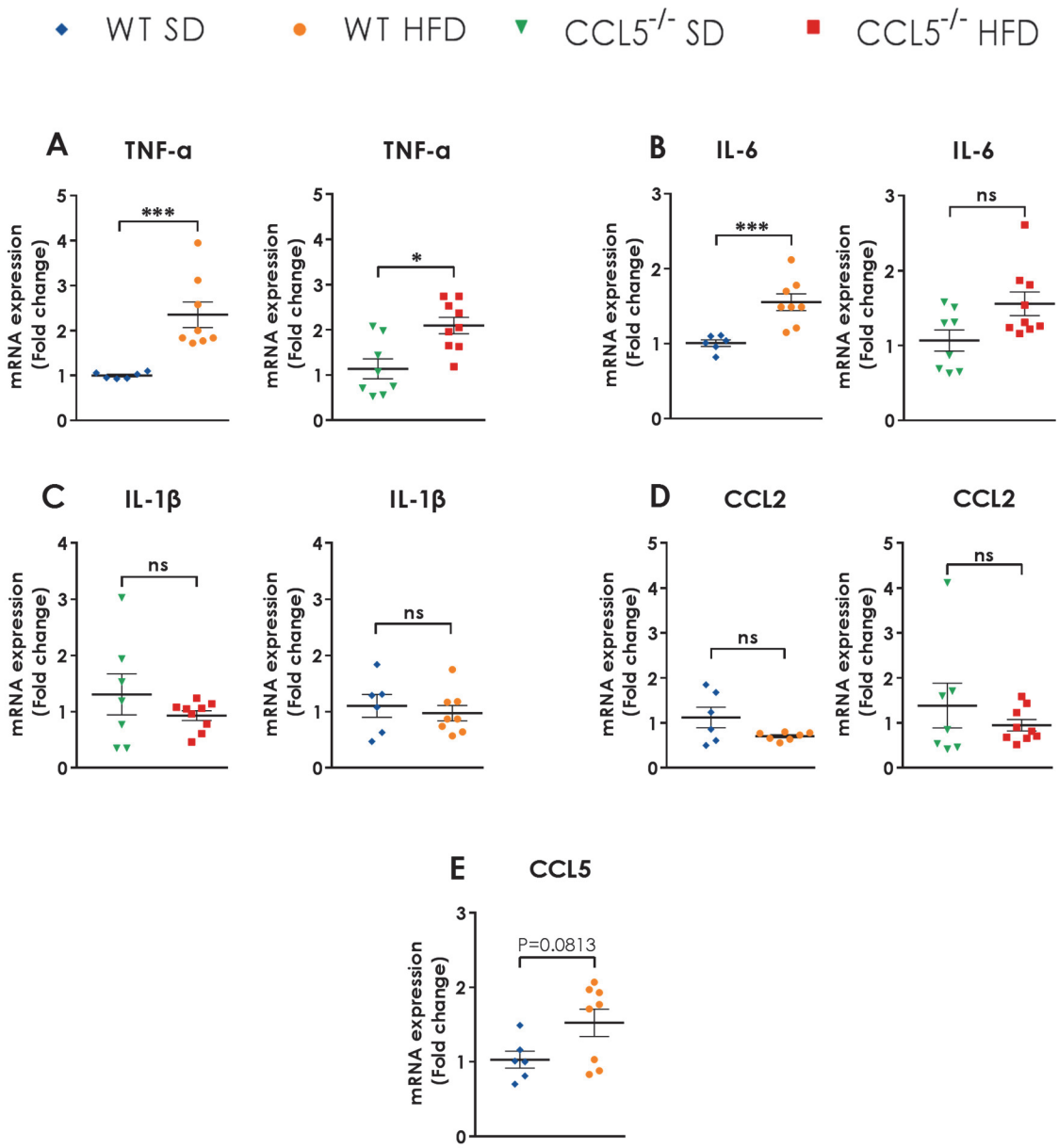

Figure 38: Effect of CCL5 deficiency on hypothalamic neuroinflammation compared between diets.

Real-time PCR quantification of hypothalamic mRNA expression of inflammatory markers TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines, CCL2 (**D**) and CCL5 (**E**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are represented as means ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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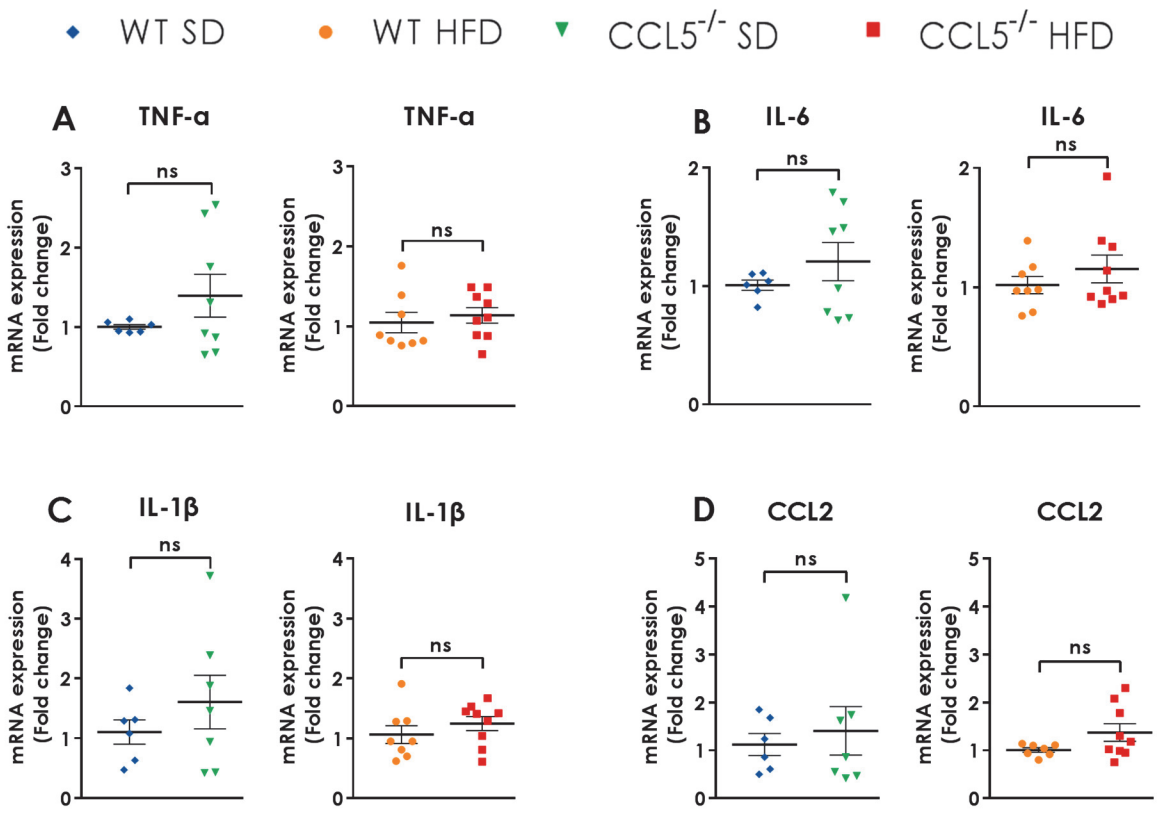

Figure 39: Effect of CCL5 deficiency on hypothalamic neuroinflammation compared between genotypes.

Real-time PCR quantification of hypothalamic mRNA expression of inflammatory markers such as the cytokines: TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokine CCL2 (**D**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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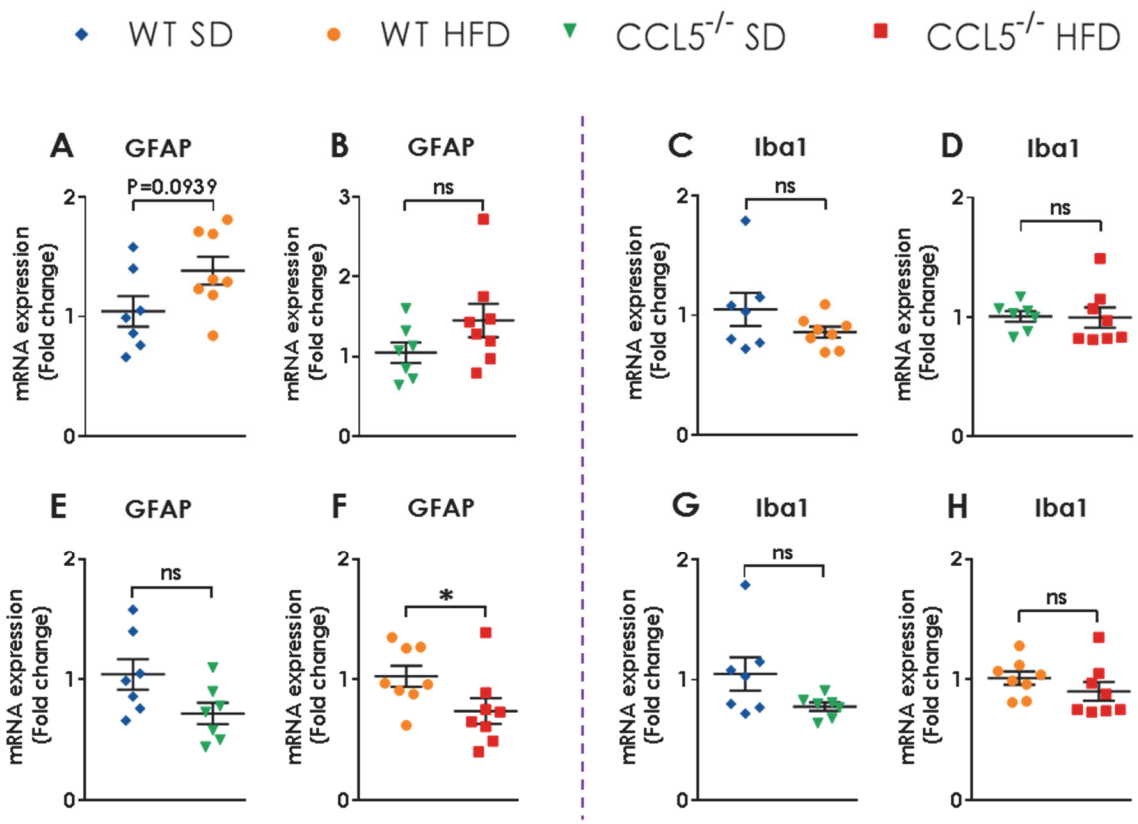

Figure 40: Effect of CCL5 deficiency on DIO associated hypothalamic gliosis.
Effect of 16 weeks of SD or HFD diet on hypothalamic mRNA expression of glial markers quantified by qPCR. **A** and **B**: Relative hypothalamic expression of astrocyte marker GFAP in WT (**A**) and CCL5$^{-/-}$ (**B**) mice and comparing between mice fed a SD (**E**) and HFD (**F**) diet. **C** and **D**: Relative hypothalamic expression of microglial marker Iba1 in WT (**C**) and CCL5$^{-/-}$ (**D**) mice and comparing between mice fed a SD (**G**) and HFD (**H**) diet for 16 weeks. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to respective controls. Values are represented as means ± SEM (n=7-8 mice/ group). Statistical significance was determined using Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant; GFAP: glial fibrillary acidic protein; Iba1: ionized calcium binding adaptor molecule 1.

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2.5. The Effect of CCL5 on Peripheral and Systemic Inflammation

At the peripheral level of AT, HFD induced a marked increase in mRNA expression levels of cytokines IL-1β, IL-6 and chemokines CCL2 and CCL5 in perigonadal (PG) AT of WT mice after 16 weeks of diet compared to their SD-fed controls (Fig. 41A-E). Similarly, HFD seemed to induce an increased expression of cytokines like IL-1β and a slight increase in IL-6 but not in TNF-a and no significant increase in CCL2 in $CCL5^{-/-}$ mice compared to SD-fed controls (Fig. 41A-F). Interestingly, when compared between genotypes, we found a significant decrease in TNF-a levels in $CCL5^{-/-}$ mice on a HFD, although when fed a SD they are significantly increased in compared to WT controls with the respective diet (Fig. 42A).

However, systemically we have not found any significant increase in inflammatory markers apart from CCL5 in sera of HFD-fed WT mice compared to SD-fed mice (Fig. 43A-G). Interestingly, although TNF-a mRNA levels are increased in PG of $CCL5^{-/-}$ mice on a SD diet, their serum levels of TNF-a are significantly reduced compared to WT controls. Similarly, $CCL5^{-/-}$ mice show a decline in IL-10 in HFD- and SD-fed mice, although only significant for the former, relative to respective controls (Fig. 43E). Furthermore, serum levels of CCL2 were significantly increased upon HFD feeding in CCL5 deficient mice compared to SD-fed control (Fig. 43F).

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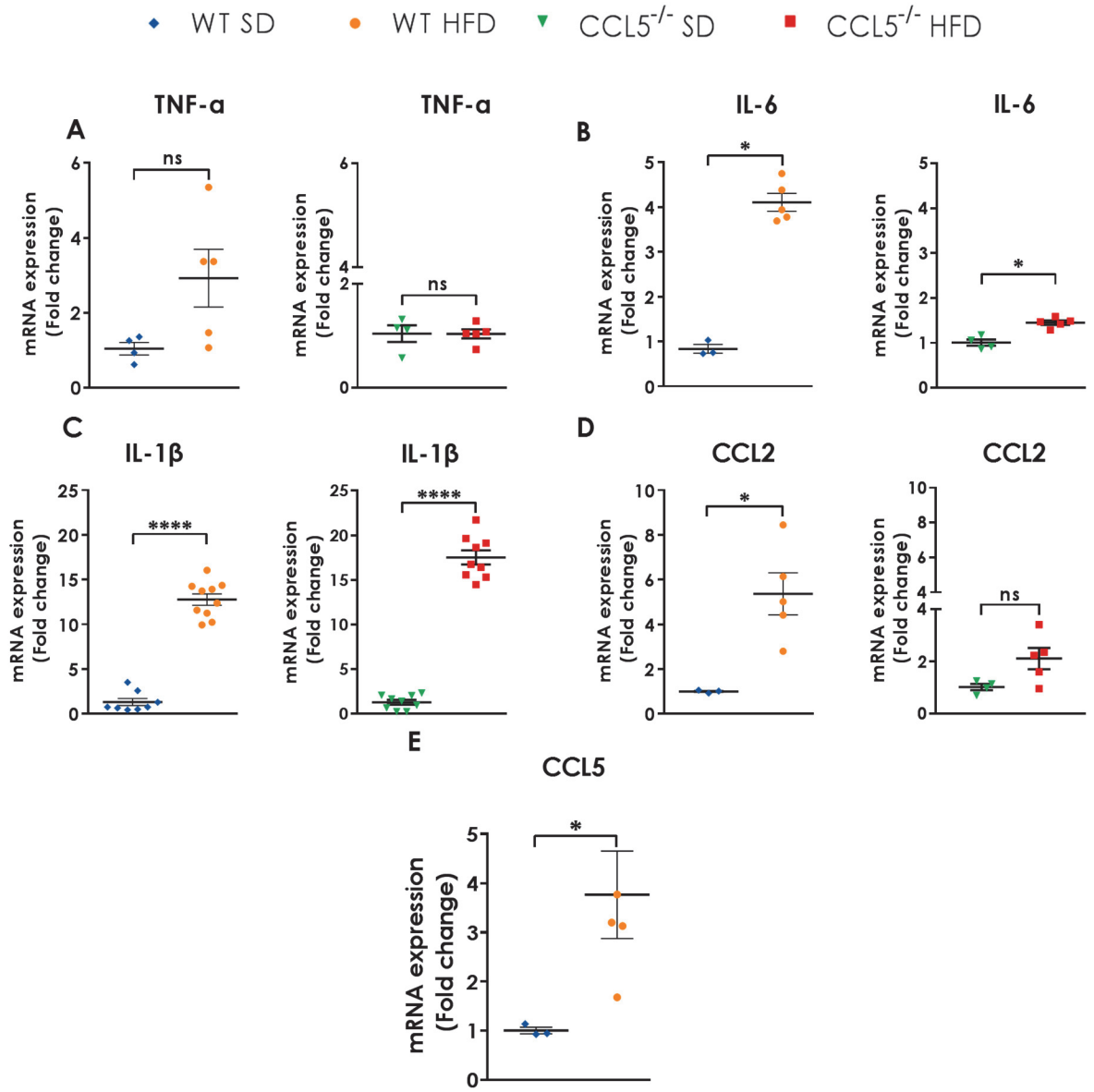

Figure 41: Effect of CCL5 deficiency on peripheral inflammation compared between diets.

Real-time PCR quantification of mRNA expression of inflammatory markers TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines, CCL2 (**D**) and CCL5 (**E**) after 16 weeks of diet in perigonadal AT of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=3-10 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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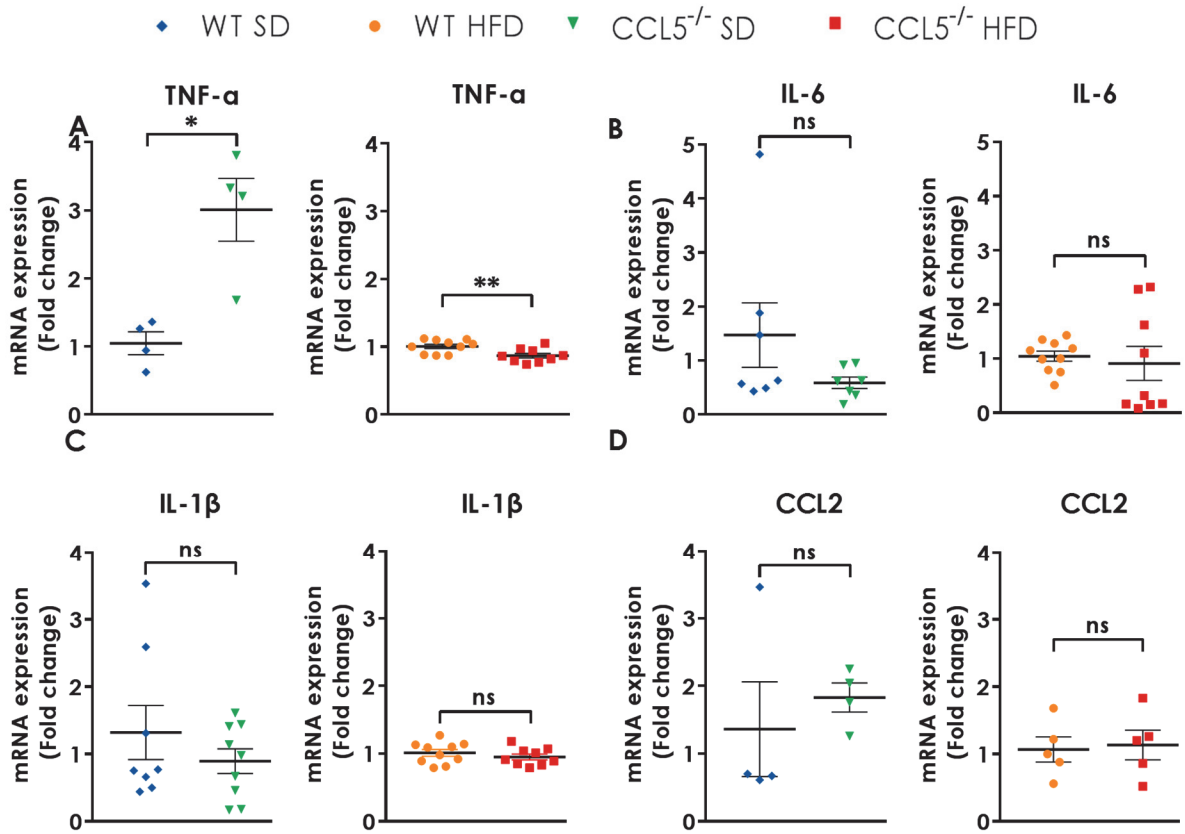

Figure 42: Effect of CCL5 deficiency on peripheral inflammation compared between genotypes.

Real-time PCR quantification of mRNA expression of inflammatory markers such as the cytokines: TNF-a (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokine CCL2 (**D**) after 16 weeks of diet in perigonadal AT of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are represented as means ± SEM (n=3-10 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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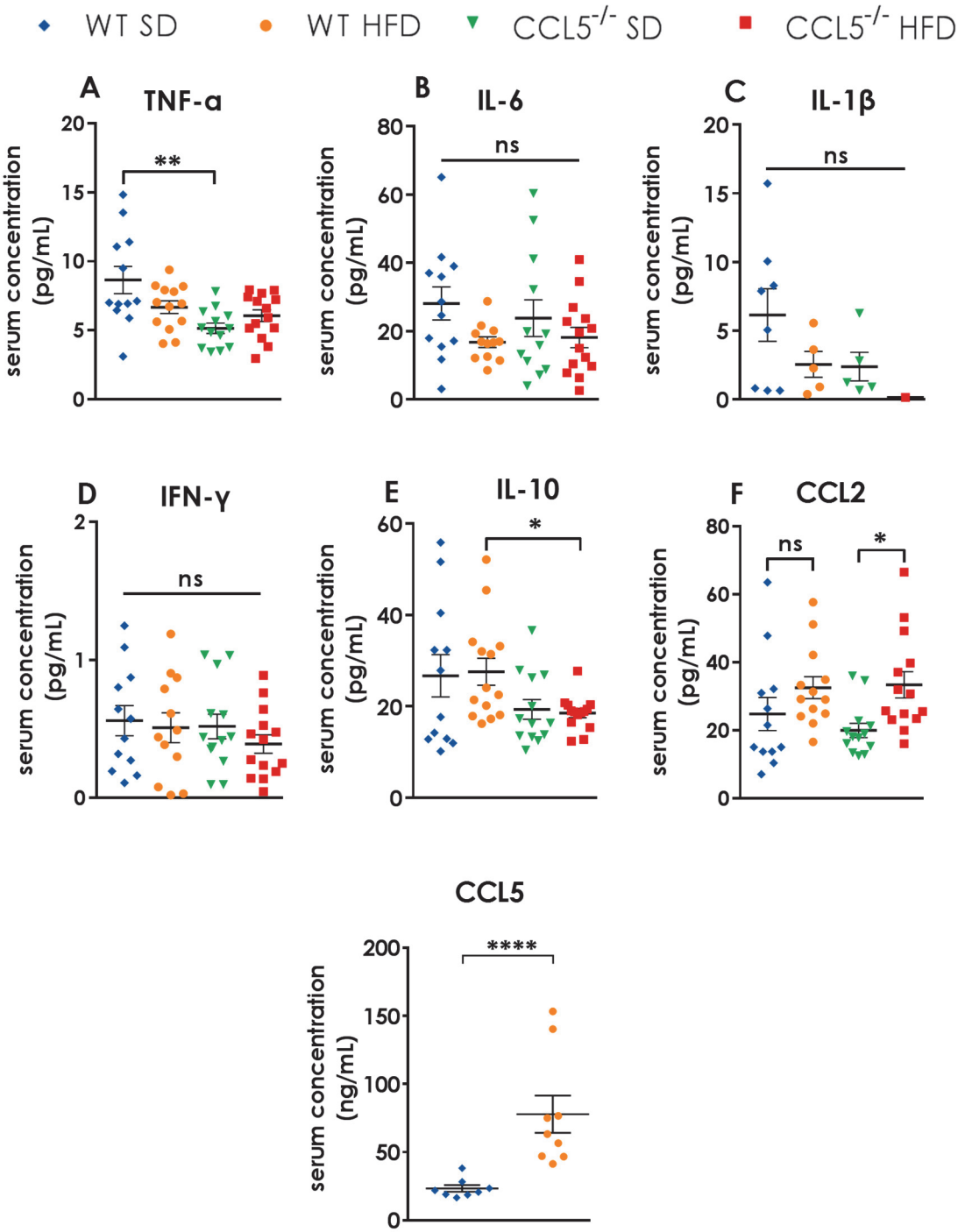

Figure 43: Effect of CCL5 deficiency on systemic peripheral inflammation.
Serum levels of pro-inflammatory cytokines TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), IFN-γ (**D**), anti-inflammatory cytokine IL-10 (**E**) and the chemokines, CCL2 (**F**) and CCL5 (**G**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. Serum CCL5 (**G**) concentration quantified via ELISA in DIO WT vs control mice. Serum CCL5 was measured for CCL5$^{-/-}$ mice on both diets as well, but were beyond or at the limit of detection and therefore not shown. Values are represented as means ± SEM (n=12-14 mice/ group; for IL-1: n=1-8, for CCL5: n=8-9). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test or Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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2.6. CCL5 Deficiency Partly Protects from HFD-Induced Increase in Neuropathic Pain Sensitivity

A very common comorbidity of obesity and diabetes is neuropathic pain. As a model of neuropathic pain, we chose the Hargreaves' test of thermal pain sensitivity, to test whether CCL5 might have an impact on HFD-induced neuropathic pain sensitivity. At 16 weeks of diet, WT mice on a HFD show, as anticipated, a significant reduction in the duration until paw withdrawal upon presentation of a heat source to their hind paw compared to SD-fed control mice. This is indicative of a higher pain sensitivity in HFD-fed mice (Fig. 44). Compared to that, CCL5$^{-/-}$ mice show no significant difference in thermal pain sensitivity compared to their SD-fed control group (Fig. 44).

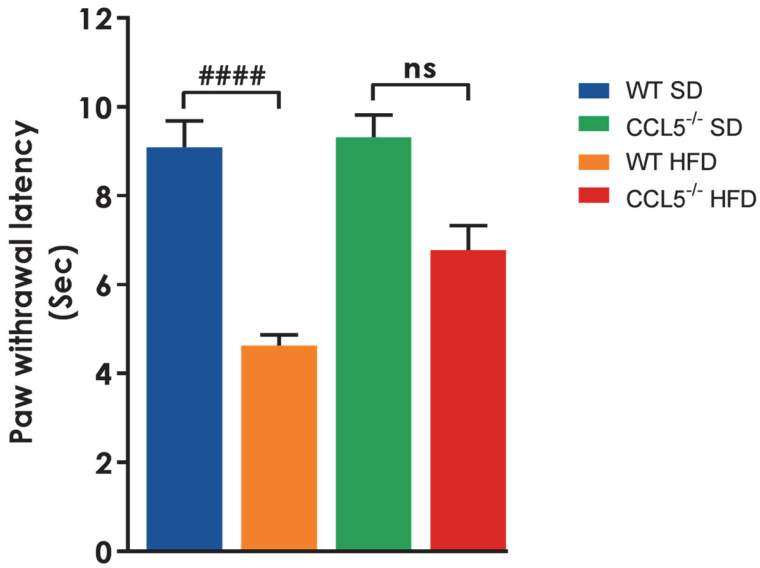

Figure 44: Effect of CCL5 deficiency on thermal pain sensitivity in DIO mice.
Estimation of mechanical perception of thermal pain sensitivity by paw withdrawal latency in seconds as determined with Hargreaves' test in WT and CCL5$^{-/-}$ mice on either a SD or HFD for 16 weeks. Values are represented as means ± SEM (n=12-21/ group). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test; P<0.05 **#**, P<0.01 **##**, P<0.001, **###**, P<0.0001 **####**; ns: not significant.

We then wanted to know whether the difference in pain sensitivity could be due to a difference inflammation. We thus determined the expression levels of inflammatory markers in samples of DRG and spinal cord samples of mice after 16 weeks of SD or HFD. We did not find any significant increase in

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inflammatory marker expression in DRG nor any difference in inflammatory marker expression between CCL5$^{-/-}$ and WT mice independent of diet (Fig. 45 and 46). Interestingly, we found no significant increase in spinal cords of HFD-fed WT mice neither, apart from a slight increase in IL-6, although not significant (Fig. 47).

However, against our expectations, when comparing inflammatory cytokine and chemokine expression in spinal cords between CCL5$^{-/-}$ and WT mice, we found significantly higher levels of TNF-α, IL-1β, and CCL2 in HFD-fed CCL5$^{-/-}$ mice and significantly increased TNF-α and IL-6 levels in SD-fed CCL5 deficient mice (Fig. 48A-D).

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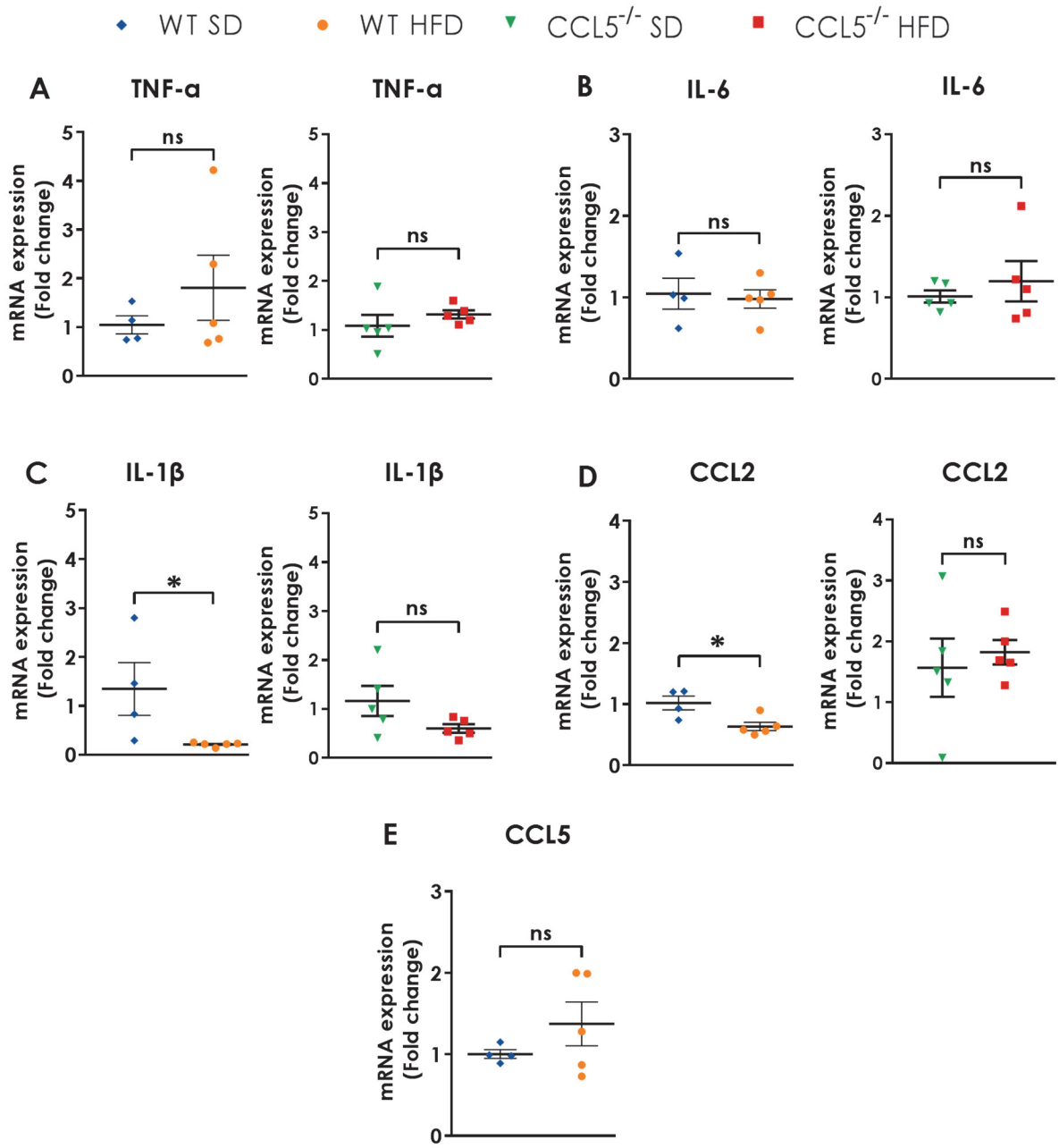

Figure 45: Effect of CCL5 deficiency on inflammatory marker expression in DRG compared between diets.

Real-time PCR quantification of mRNA expression of inflammatory markers TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines, CCL2 (**D**) and CCL5 (**E**) after 16 weeks of diet in DRG of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=4-5 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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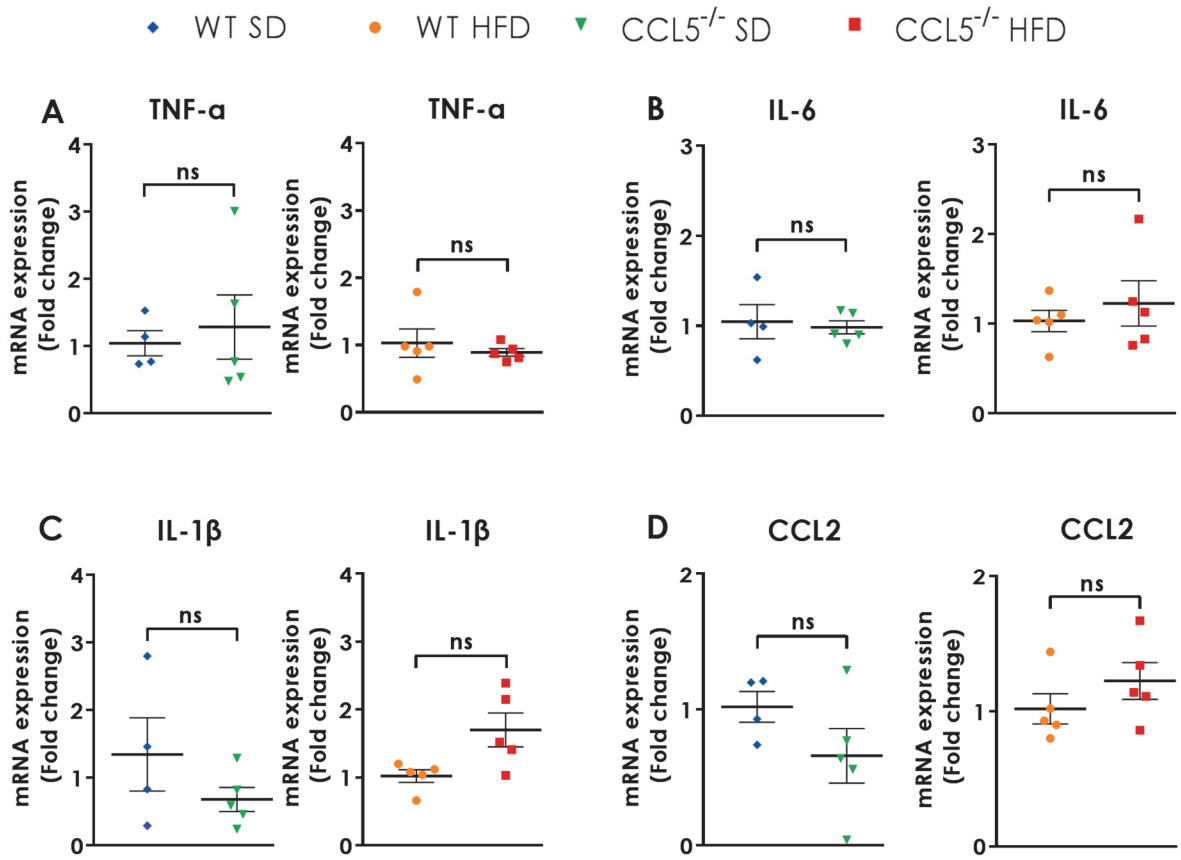

Figure 46: Effect of CCL5 deficiency on inflammatory marker expression in DRG compared between genotypes.

Real-time PCR quantification of mRNA expression of inflammatory markers such as the cytokines: TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokine CCL2 (**D**) after 16 weeks of diet in DRG of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are represented as means ± SEM (n=4-5 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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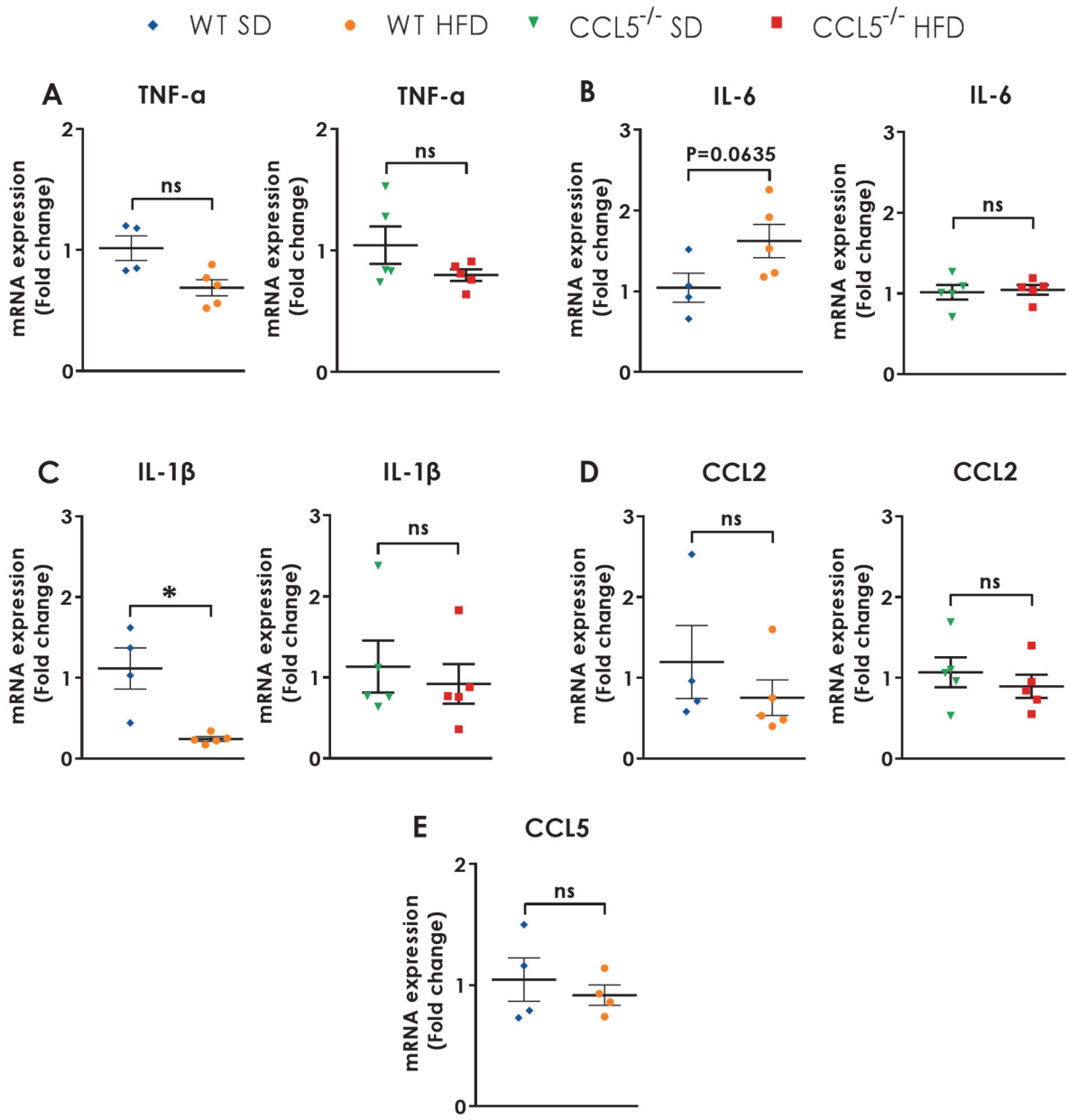

Figure 47: Effect of CCL5 deficiency on inflammatory marker expression in spinal cords compared between diets.

Real-time PCR quantification of mRNA expression of inflammatory markers TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines, CCL2 (**D**) and CCL5 (**E**) after 16 weeks of diet in spinal cords of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=4-5 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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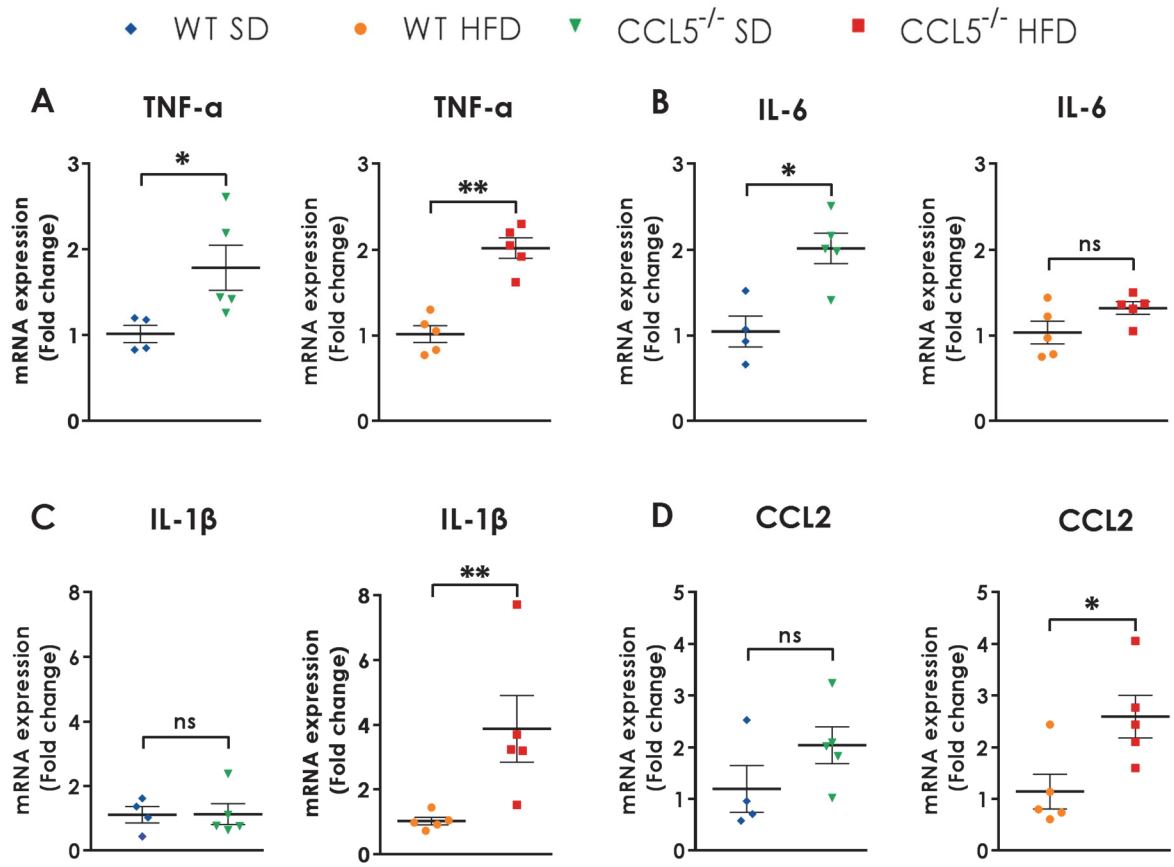

Figure 48: Inflammatory marker expression in spinal cords of DIO WT and CCL5$^{-/-}$ mice compared between genotypes.

Real-time PCR quantification of mRNA expression of inflammatory markers such as the cytokines: TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokine CCL2 (**D**) after 16 weeks of diet in spinal cords of WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are represented as means ± SEM (n=4-5 mice/ group). Mann-Whitney Test; $P<0.05$ *, $P<0.01$ **, $P<0.001$ ***; ns: not significant.

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2.7. Hypothalamic Expression of CCL5 and CCR5

We have so far confirmed that CCL5 indeed plays a role in the development and maintenance of obesity and diabetes. In addition to that, we showed some evidence, that CCL5 might have a central effect on neuropeptides. As previously mentioned, there are also already many reports about the expression of CCL5 and CCR5 in the context of obesity though mostly in the periphery. We wanted to confirm the role of CCL5 in the CNS and further identify, whether the effect might be mediated by one of its cognate receptor CCR5. This is why we used RNAScope® technology to verify the central expression of CCL5 and CCR5 mRNA in the brain, more specifically in the hypothalamus of WT mice on a long term diet with SD or HFD. Firstly, we have identified the expression of CCL5 and CCR5 in SD-fed adult WT mice in the several nuclei of the hypothalamus. As depicted in figure 49 the upper panel shows the expression of CCR5 mRNA (red) and CCL5 mRNA (green) in the LH, where important neurons are located that regulate energy balance and food intake (Fig. 49). The outermost panel on the right, shows a magnification of the white square in the merged picture in the left site of the figure. The middle panel of the figure shows the expression of both molecules also in the ventromedial hypothalamus. The lower panel reveals that WT mice on SD diet also constitutively express the chemokine CCL5 and its receptor also in the ARC that lies directly lateral to the 3V (Fig. 49). We have also noticed expression in the median eminence and the PVN of the hypothalamus as well as in extra hypothalamic sections. In these pictures, we observed that CCR5 mRNA is quite abundantly expressed but localized only to a few cells, whereas CCL5 seems to be expressed more diffusely in many cells but at low amounts. We can further see that although there are cells co-expressing both ligand and receptor, such as in the LH (yellow dots), they are not often co-localized in the same cell, but rather in neighboring cells or even further apart. We further noticed, that while, CCR5 seems to have a perinuclear expression pattern, CCL5 seems to be rather expressed within the cytoplasm.

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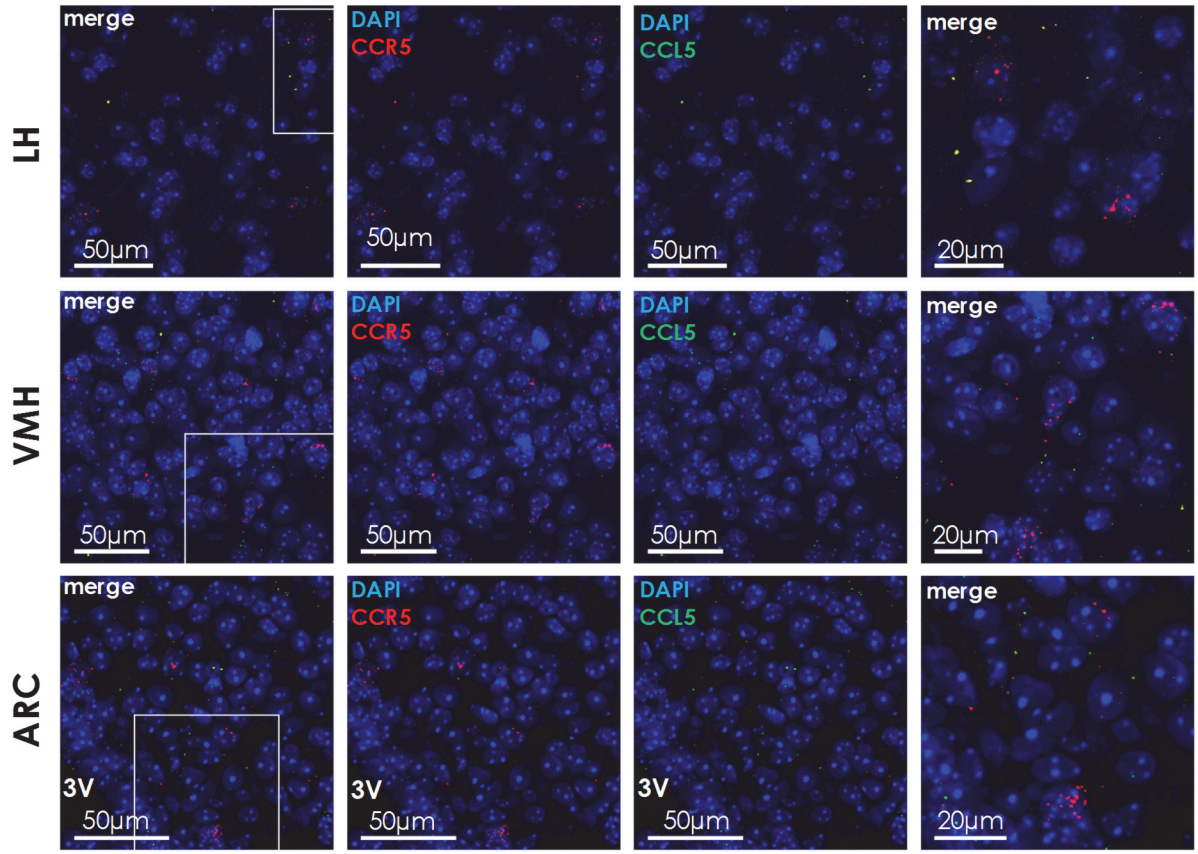

Figure 49: Hypothalamic expression of CCL5 and receptor CCR5 in SD-fed WT mice. Representation of Immunohistochemical brain sections of WT mice on a SD diet stained with RNAScope® technology using a sonde labeling CCL5 (green) and CCR5 (red) in the LH, VMH and ARC. The outermost panel on the right represents the enlarged section labeled with a white square in the merged picture on the left. Scale bar indicating either 50µm or 20µm as depicted in the images. LH: lateral hypothalamus; VMH: ventromedial hypothalamus; ARC: arcuate nucleus; 3V: 3rd ventricle.

We also investigated whether we see a difference in the expression pattern between WT mice, which were fed a different long-term diet of either SD or HFD. Figure 50 displays images of the ARC nucleus section of the brain laterally to the 3V, with the brain sections in the SD condition being on the left and the HFD-fed mouse brain sections on the right hand side. Interestingly, it seems like that under HFD condition a greater number of cells are expressing both CCL5 (green, upper panel Fig. 50) and its receptor CCR5 (red, lower panel Fig. 50). In addition, it even seems like the expression of CCL5 and CCR5 are upregulated in the cells. Note also the number of cells in the lower

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corner of the HFD condition, that CCL5 has a perinuclear expression and is co-expressed with its receptor CCR5 in the outer corner of the ME.

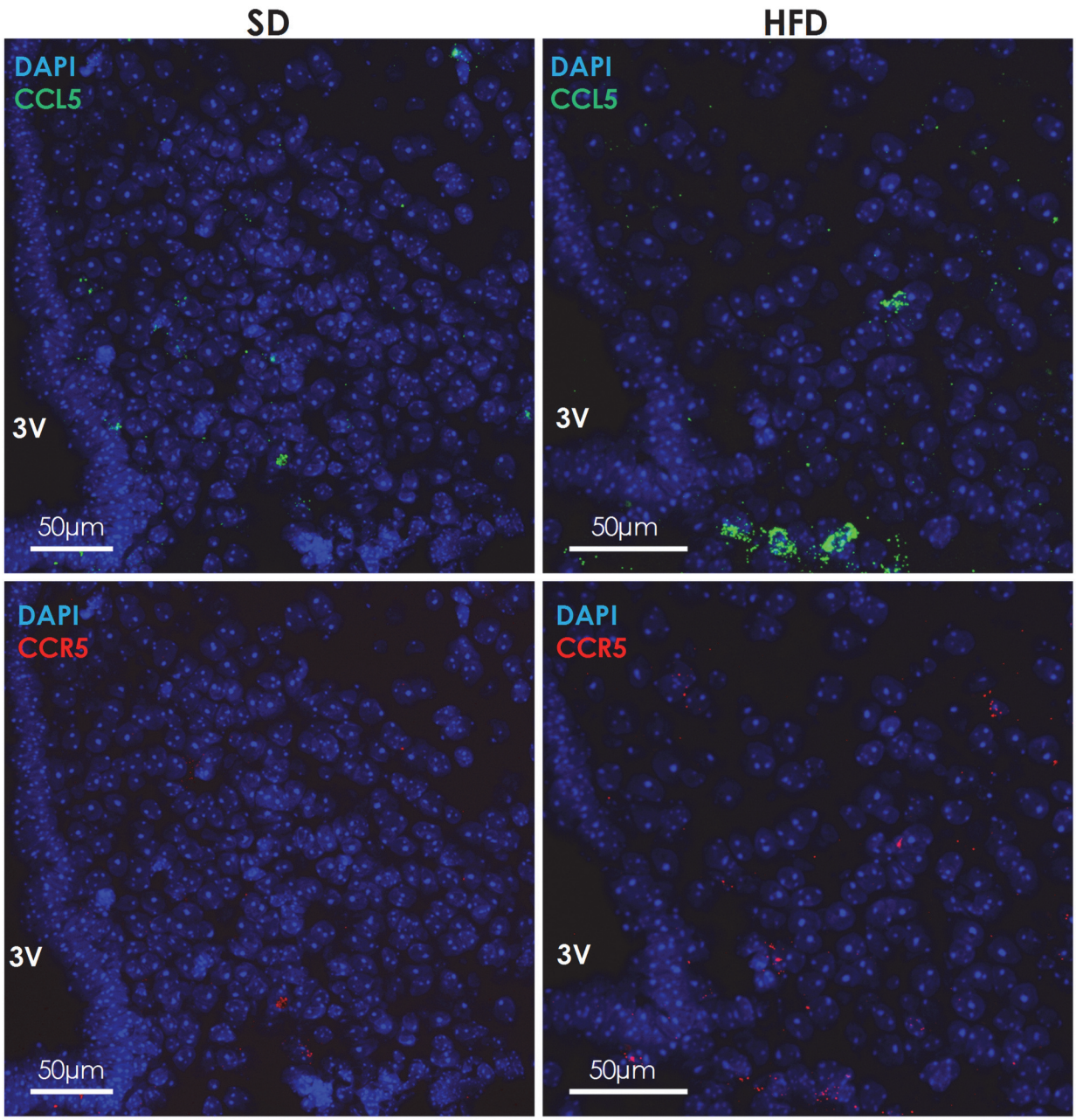

Figure 50: Hypothalamic expression of CCL5 and receptor CCR5 in SD vs HFD-fed WT mice.
RNAScope® staining of immunohistochemical brains sections of WT mice on a SD vs HFD showing CCL5 mRNA fluorescent staining (green) and CCR5 mRNA (red). Scale bar indicating 50µm. 3V: 3rd ventricle.

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3. Role of Chemokine Receptor CCR5 in the Development of Obesity

We have seen in the previous section that CCL5 indeed seems to play a role in the development and the maintenance of obesity. We further established that together with its cognate chemokine receptor CCR5, it is expressed in hypothalamic nuclei controlling energy balance and feeding regulation. Based on these findings, we decided to also elucidate the role of CCR5 in the development of DIO and characterize the phenotype of CCR5 deficient mice as opposed to WT mice.

3.1. Chemokine Receptor CCR5 Deficiency Partly Protects Against HFD-Induced Development of Obesity.

To establish the role of the cognate chemokine receptor CCR5 in the role of DIO we performed the same set of experiments as discussed above for CCL5. We have fed CCR5 deficient and WT mice with either SD or HFD for 16 weeks and found no difference in weight gain between $CCR5^{-/-}$ and WT mice when fed a SD diet. However, we have seen a similar trend in significantly reduced BW gain in $CCR5^{-/-}$ mice as opposed to WT mice on when fed a HFD (Fig. 51A-B). Interestingly, different from the results seen earlier with $CCL5^{-/-}$ mice, $CCR5^{-/-}$ mice on did not consume less HFD than WT controls (Fig. 51C-D). In fact, we observed no difference in food intake neither in grams not kcal between genotypes. As expected, both HFD-fed groups consumed significantly less grams compared to their SD controls but the same amount of kcals (Fig. 51C-D). Similarly, we observed the same trend for water intake as for food intake as HFD consuming groups drank less water compared to their SD controls, without any difference between genotypes (Fig. 51E).

Although we did not see any difference in food intake, but observed a hypothalamic expression of CCR5 in the ARC of the hypothalamus of WT mice, we wanted to see whether the lack of CCR5 has a modulatory effect on neuropeptide expression.

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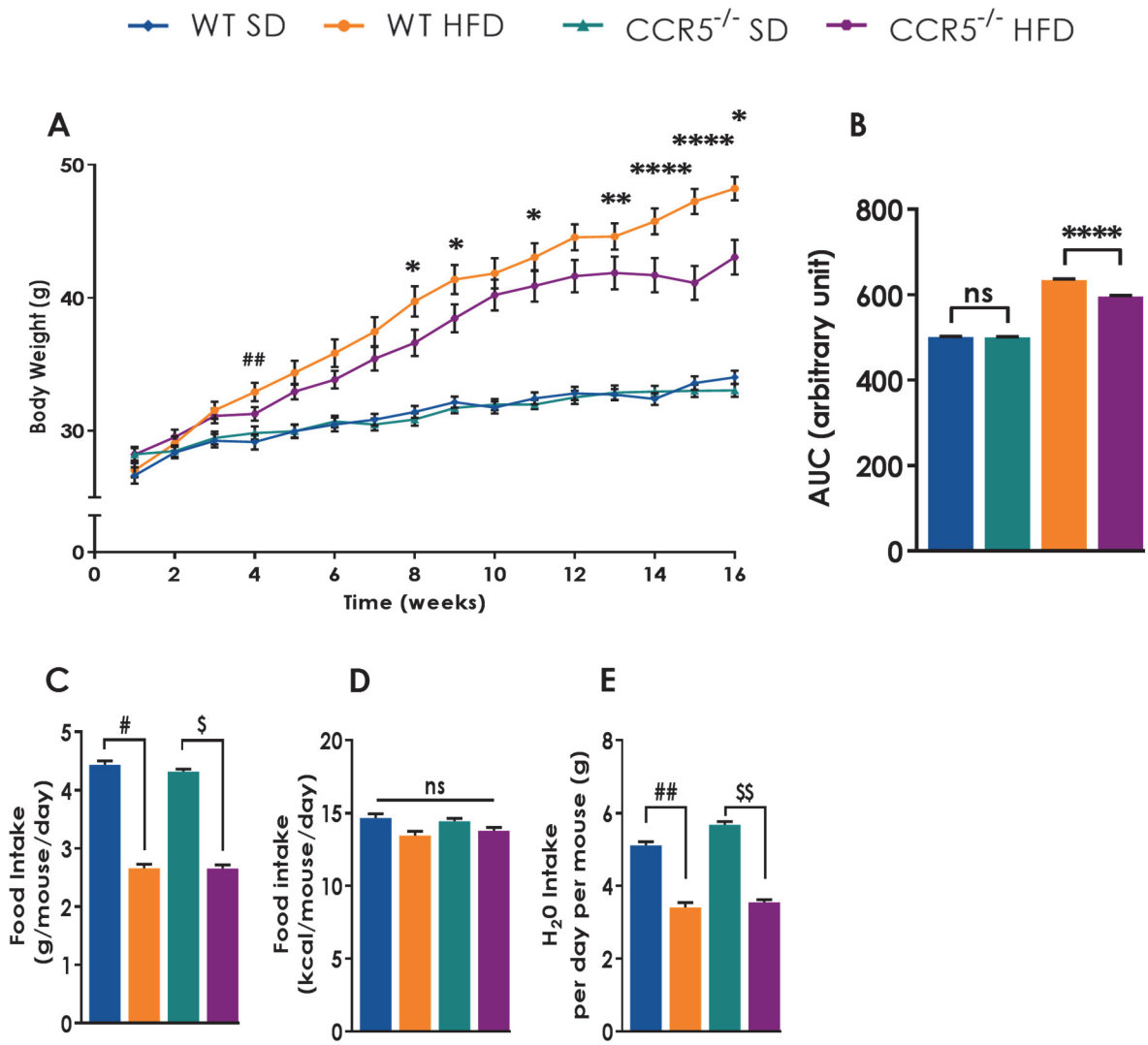

Figure 51: Effect of CCR5 deficiency on DIO development.

A: Evolution of average BW at 16 weeks of experimental protocol of DIO. **B**: Quantification of (A) as area under the curve - AUC. **C**: Average food consumption during 16 weeks represented as grams per mouse per day. **D**: Average food consumption during 16 weeks represented as kcal per mouse per day. **E:** Average H_2O consumption during 16 weeks in grams per mouse per day. **F**: Serum leptin concentration measured by ELISA after 16 weeks of HFD. Data are reported as the means ± SEM (n=13-16/group for A and B; n=2 experiments/group for C-E). Two-way ANOVA followed by Tukey *post hoc* test, one-way ANOVA or Mann Whitney Test for comparisons between two groups. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, WT HFD *vs* CCL5$^{-/-}$ HFD; #, WT SD *vs* WT HFD; $, CCL5$^{-/-}$ SD *vs* HFD; ns: not significant.

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3.2. Chemokine Receptor CCR5 Has a Neuromodulatory Effect on Hypothalamic Neurons

As for CCL5, we have also found a difference in neuropeptide expression for CCR5 deficient mice. Although we did not observe any difference in neuropeptide expression in mice fed a SD diet, we did find a significant downregulation of NPY expression in $CCR5^{-/-}$ mice on a HFD compared to both their SD control and their WT control on a HFD (Fig. 52B and Fig 53B). In addition to that, we identified further downregulation in both anorexigenic POMC and orexigenic MCH and AgRP in $CCR5^{-/-}$ mice fed a HFD compared to their WT controls.

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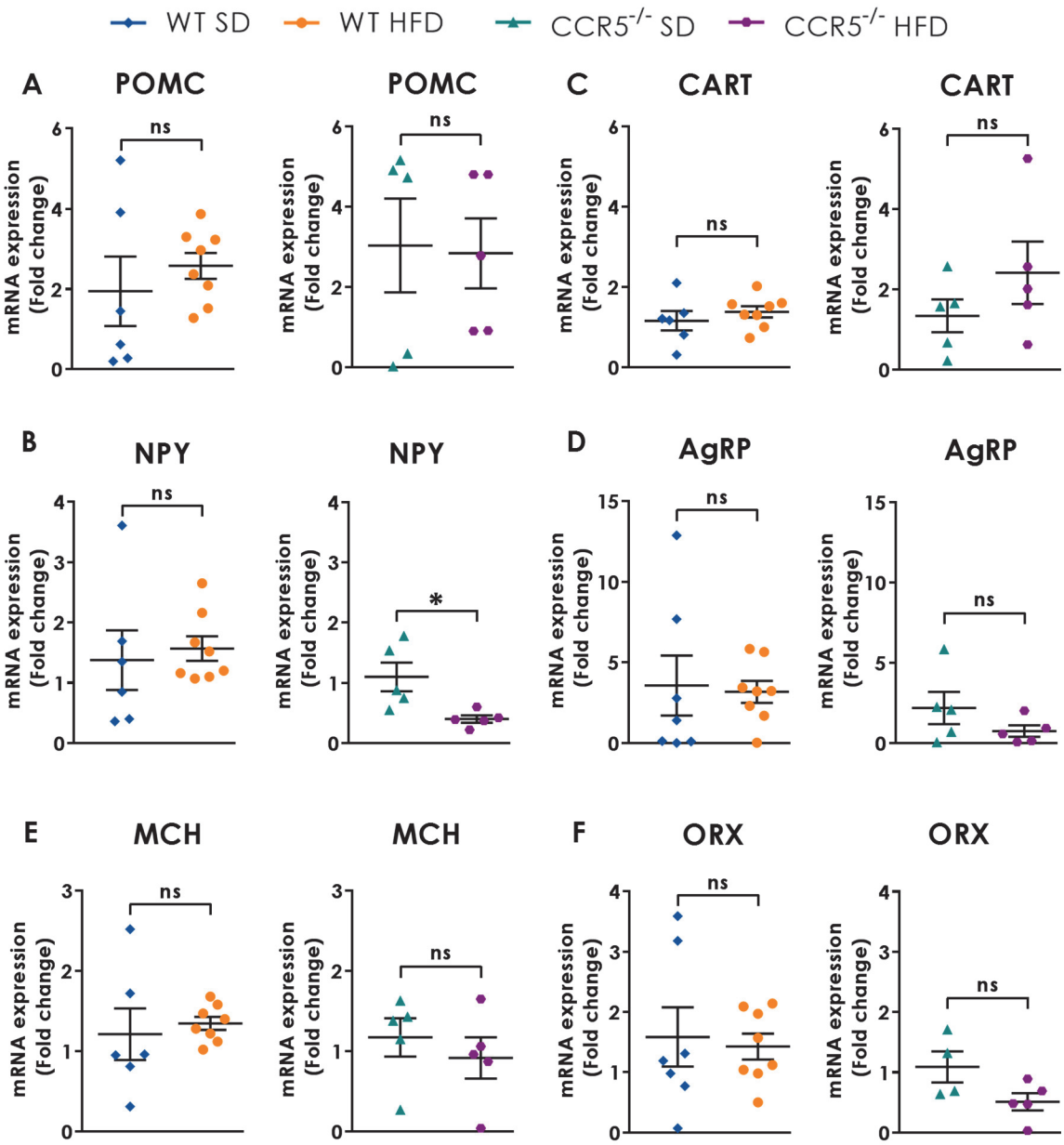

Figure 52: CCR5 deficiency has a neuromodulatory effect on hypothalamic neurons. Real-time PCR quantification of relative mRNA expression of hypothalamic neuropeptides such as anorexigenic neuropeptides POMC (**A**) and CART (**B**), and orexigenic neuropeptides NPY (**C**), AgRP (**D**), MCH (**E**) and ORX (**F**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are the mean ± SEM (n=5-8 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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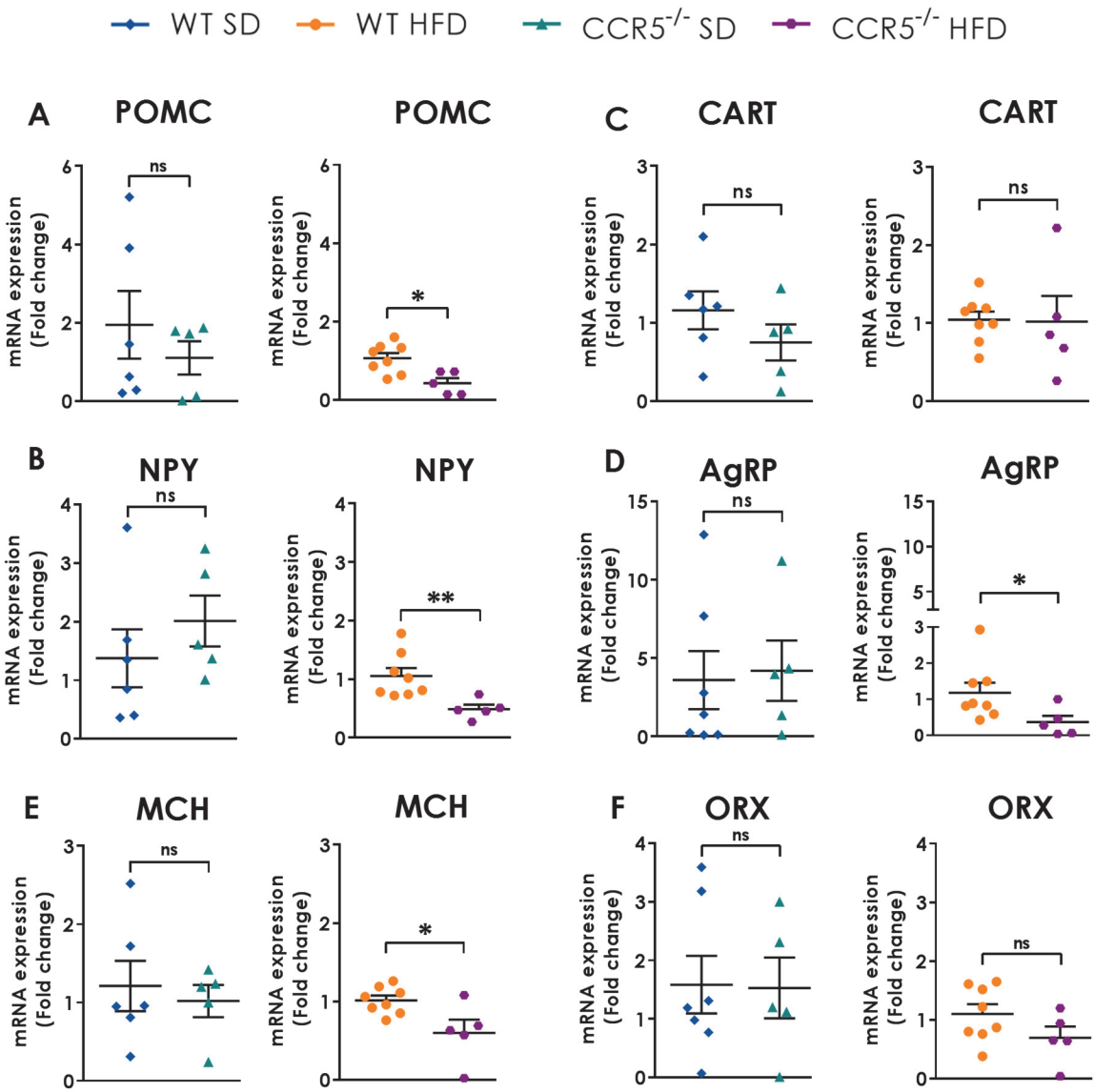

Figure 53: CCR5 deficiency has a neuromodulatory effect on hypothalamic neurons. Real-time PCR quantification of mRNA expression of hypothalamic neuropeptides such as anorexigenic neuropeptides POMC (**A**) and CART (**B**), and orexigenic neuropeptides NPY (**C**), AgRP (**D**), MCH (**E**) and ORX (**F**) after 16 weeks of diet in WT and CCL5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=5-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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3.3. CCR5 Affects Glucose Metabolism in Obese Mice

We tested glucose tolerance of WT and CCR5$^{-/-}$ mice after 8 and 16 weeks of diet by IP injection of 1.5mg/kg of glucose. While we found no difference in fasting glucose levels or glucose tolerance in both genotypes on a SD diet (Fig. 54A-H), CCR5$^{-/-}$ mice fed a HFD were significantly more glucose tolerant than WT mice on a HFD and had significantly reduced basal glucose levels after 16h of fasting after 8 weeks of diet (Fig. 54A-D). However, shorter fasting times revealed a significantly higher blood glucose levels in not only WT but also CCR5$^{-/-}$ mice on a HFD after 8 weeks of diet but not at 16 weeks of diet (Fig 54E and H). Interestingly, while the blood glucose levels of HFD-fed WT mice were not only significantly higher than SD-fed mice, but mostly above the diabetic threshold of 150 mg/dL, CCR5$^{-/-}$ mice had fasting blood glucose levels that remained below that threshold throughout testing (Fig. 54D-E, G-H). At 16 weeks of diet, HFD-fed CCR5$^{-/-}$ mice showed no difference in glucose tolerance compared to the HFD-fed WT control group (Fig. 54B and F). Regarding insulin resistance, we have not found any difference between genotypes of neither SD-fed nor HFD-fed mice. Although there was no difference between HFD-fed groups at 8 weeks of diet, the WT groups on a HFD was significantly less sensitive to insulin compared to their WT control on a SD, while the HFD-fed CCR5$^{-/-}$ group was not (Fig. 54I). Furthermore, we determined glucose-stimulated plasma insulin for SD and HFD groups. Unfortunately, we did not get enough data points for the SD control groups at basal levels due to limits of detection and hemolysis of plasma collected and thus could not determine the potential differences in basal plasma insulin levels (Fig. 54K). However, we could observe that glucose-stimulated insulin secretion was similar in SD-fed groups between genotypes (Fig. 54L). Moreover, HFD-fed WT mice seemed to have significantly elevated insulin levels throughout the 60 min of testing compared to SD-fed controlled controls, indicative of hyperinsulinemia (Fig. 54L). Compared to their SD controls, CCR5$^{-/-}$ mice secreted significantly more insulin 15 min after IP glucose injection (1.5mg/kg), which decreased to levels not significant to

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control mice at time points T30 and T60 (Fig. 54L). We found that plasma levels were significantly lower 60 min after glucose injection in CCR5$^{-/-}$ compared to WT mice on HFD (Fig. 54L).

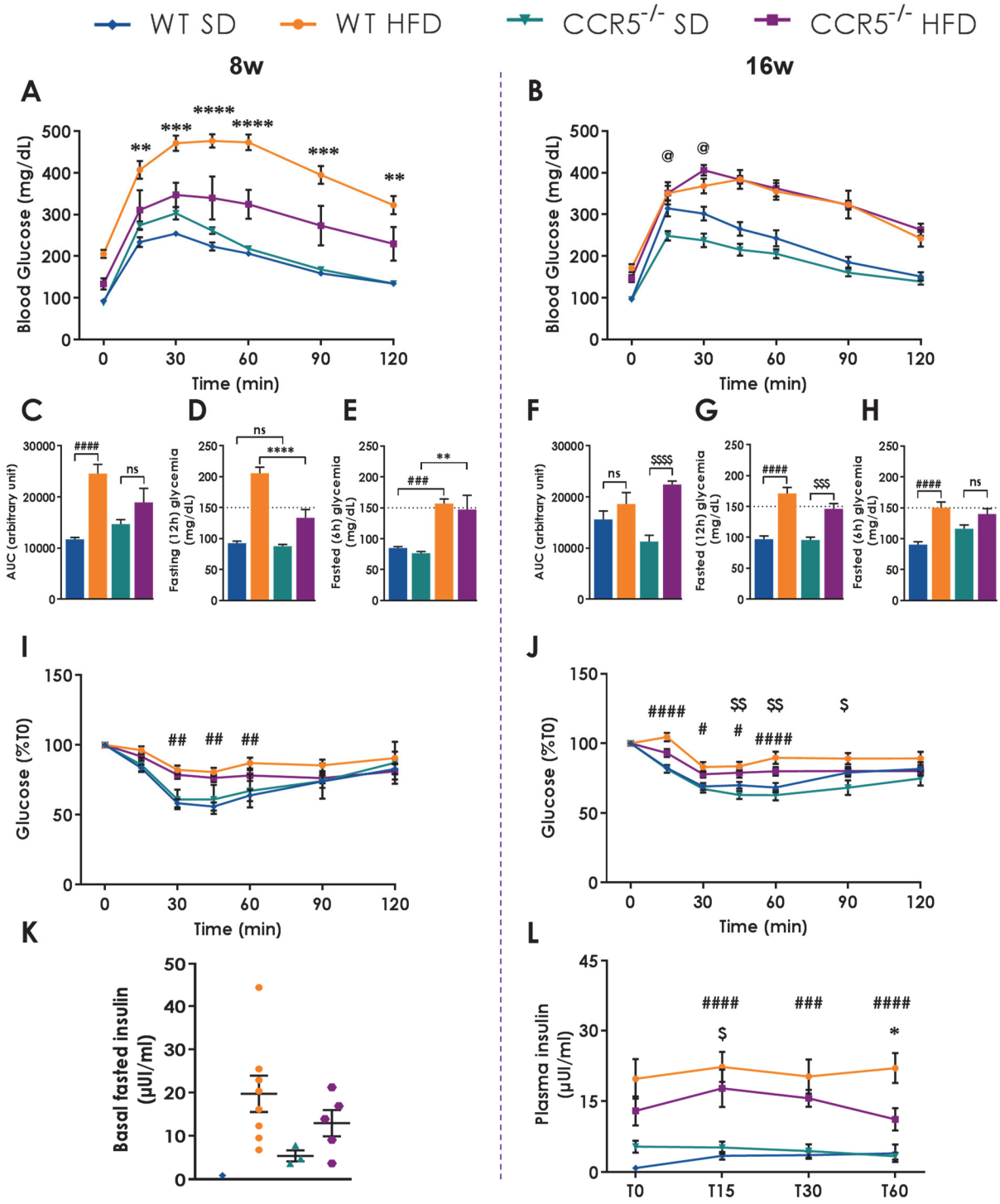

Figure 54: Glucose homeostasis perturbations in WT and CCR5$^{-/-}$ mice after 8 and 16 weeks of SD or HFD.

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Effect of genetic CCR5 deletion on **A**: IP GTT after 8 weeks of diet and **B**: 16 weeks of diet. **C** and **F**: Quantification of (A) and (B) represented as area under the curve (AUC). **D**: and **G**: Basal blood glucose levels after 16h of fasting after 8 and 16 weeks of diet, respectively. **E**: and **H**: Basal blood glucose levels after 6h of fasting after 8 and 16 weeks, respectively. **I** and **J:** Effect of CCR5 deficiency on insulin tolerance in mice fed a SD or HFD for 8 weeks (**I**) and 16 weeks (**J**). **K**: Basal fasted insulin levels at 16 weeks of diet measured via ELISA at 16 weeks of diet and **L**: acute glucose-stimulated insulin secretion measured via ELISA at 16 weeks of diet. Dashed line indicates the threshold for diabetic levels of blood glucose at 150 mg/dL. Values are means ± SEM (n=5-8/group for A+C-E+I and n=8-9 for B+F-H+J; n= 1-8 for K+L). Statistical significance was determined with two-way ANOVA with Tukey's *post hoc* test or Kruskal-Wallis test with Dunn's *post hoc* test. * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, WT HFD *vs* $CCR5^{-/-}$ HFD; #, WT SD *vs* WT HFD; $, $CCR5^{-/-}$ SD *vs* HFD; @, WT SD *vs* $CCR5^{-/-}$ SD. ns, not significant. Dashed line indicates the threshold for diabetic levels of blood glucose at 150 mg/dL; ns: not significant; 8w: 8 weeks of diet; 16w: 16 weeks of diet.

At the level of the hypothalamus, we found that HFD induced a significant decrease in IRS1 mRNA expression after 16 weeks of diet compared to SD controls in WT mice but not in $CCR5^{-/-}$ mice on a HFD (Fig. 55A-B). In fact, when comparing genotypes on the same diet, we found that HFD-fed $CCR5^{-/-}$ mice expressed significantly more IRS1 mRNA than WT mice (Fig. 55F).

Interestingly, $CCR5^{-/-}$ mice expressed also more PPARγ mRNA in the hypothalamus compared to WT mice when fed a HFD (Fig. 55H) but not on a SD (Fig. 55G). We did not find any difference in mRNA expression between SD and HFD-fed mice when comparing within genotypes (Fig. 55C-D).

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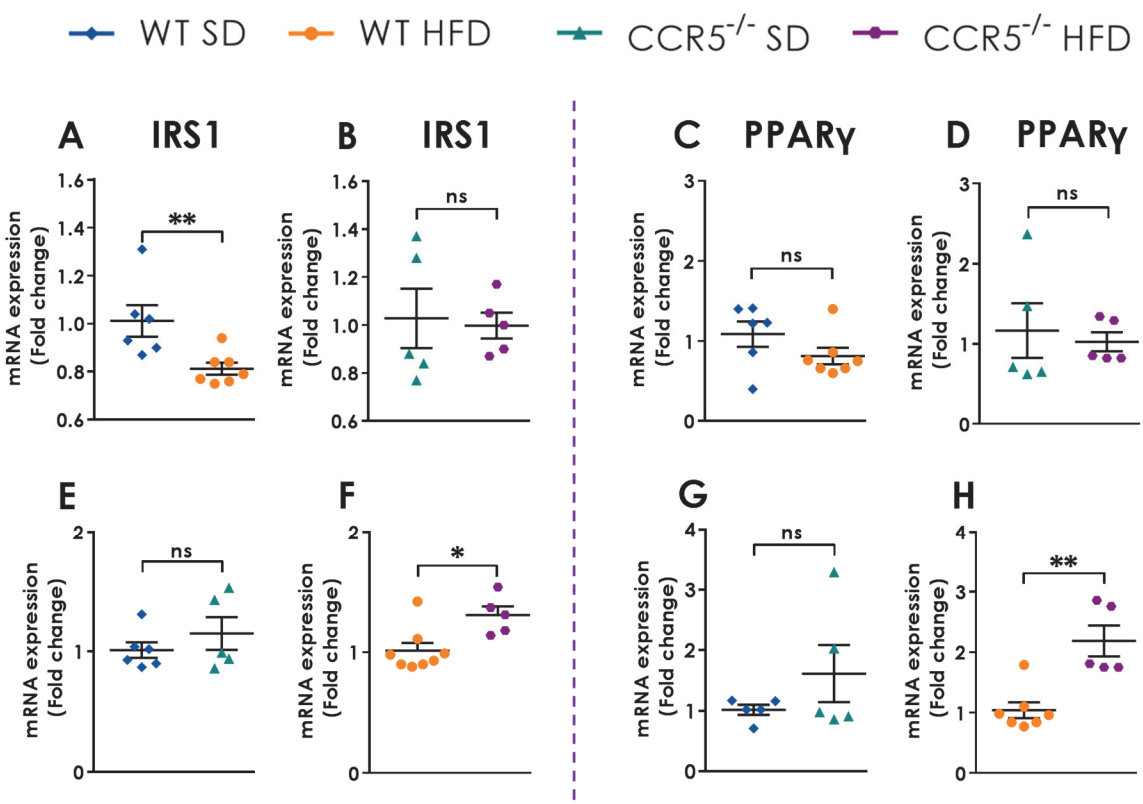

Figure 55: Effect of CCR5 deficiency on hypothalamic insulin signaling.
A and **B:** Effect of 16 weeks of diet on hypothalamic mRNA expression of IRS1 in WT (**A**) and CCR5$^{-/-}$ (**B**) mice. **C** and **D:** Effect of 16 weeks of SD or HFD on hypothalamic mRNA expression of PPARγ in WT (**C**) and CCR5$^{-/-}$ (**D**) mice. **E** and **F:** Effect of CCR5 deficiency on hypothalamic IRS1 expression between genotypes at 16 weeks of SD (**E**) and HFD (**F**) diet. **G** and **H:** Effect of CCR5 deficiency on hypothalamic PPARγ expression between genotypes at 16 weeks of SD (**G**) and HFD (**H**) diet. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to respective controls. Values are represented as means ± SEM (n=5-8 mice/ group). Statistical significance was determined using Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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3.4. The Effect of CCR5 on Central Inflammation and Hypothalamic Gliosis

At the end of the 16 week diet protocol we examined the hypothalamus of CCR5$^{-/-}$ and WT mice for the expression of inflammatory cytokine and chemokine expression by qPCR. However, we have not found any difference induced by the HFD in WT mice compared to their SD controls and only a small reduction in mRNA levels of IL-6 and CCL2 (P=0.056 and P=0.087), although not significant in HFD-fed CCR5$^{-/-}$ mice compared to their SD control (Fig 56B and D). Interestingly, when compared to their respective diet control, CCR5$^{-/-}$ mice expressed more TNF-a when on a SD diet and significantly less IL-1β in the HFD group (Fig. 57A and C). A small reduction in mRNA expression levels of IL-6 was also observed in the hypothalamus of HFD-fed CCR5$^{-/-}$ mice compared to WT mice, although not significant (P=0.065; Fig. 57B).

We also looked at hypothalamic gliosis at 16 weeks of diet, but apart from a slight but not significant increase in GFAP in WT mice compared to their SD controls (P=0.094), we did not found significant differences in GFAP and Iba1 mRNA expression (Fig 58A-H).

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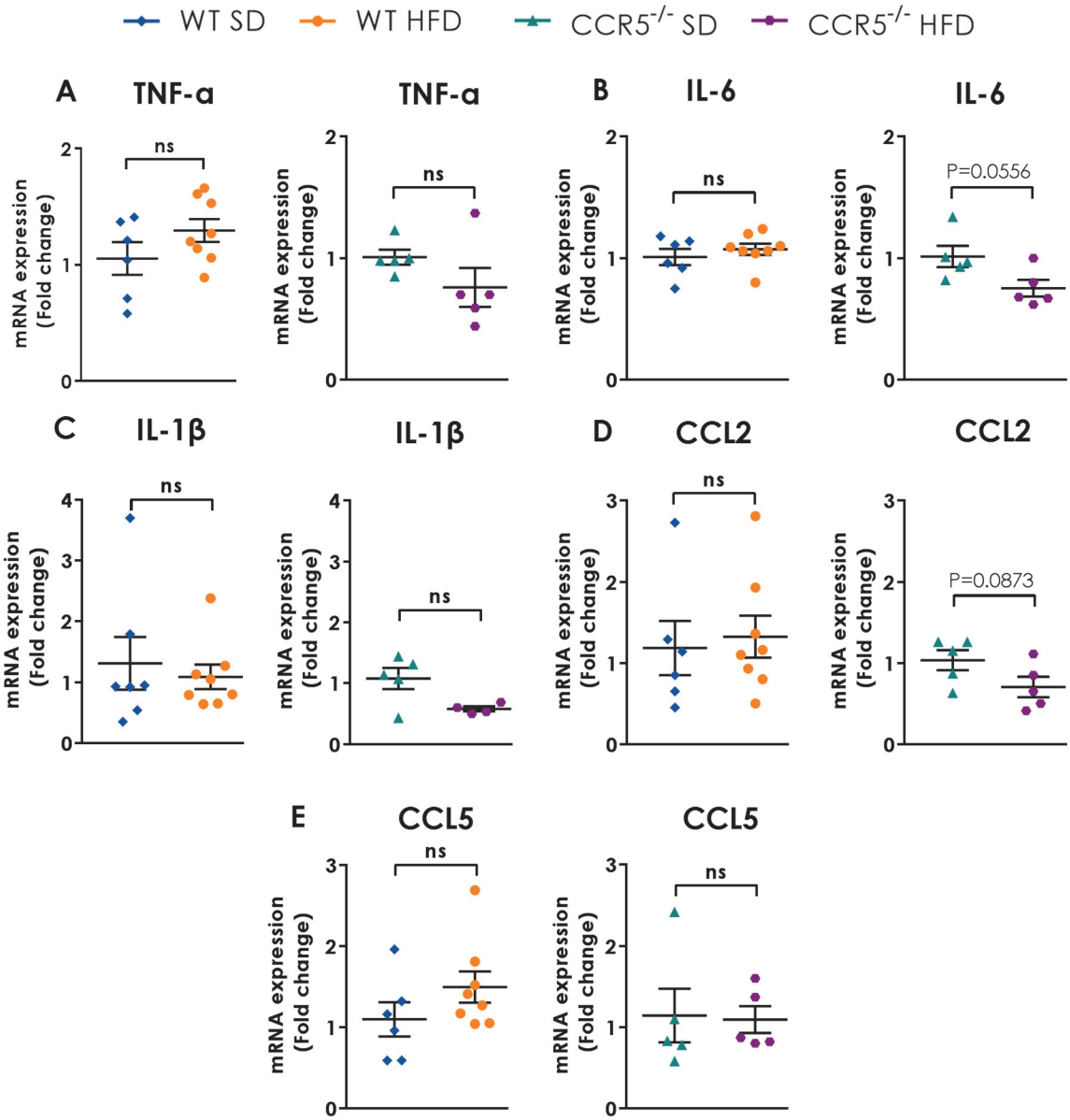

Figure 56: Effect of CCR5 deficiency on hypothalamic neuroinflammation compared between diets.

Real-time PCR quantification of hypothalamic mRNA expression of inflammatory markers TNF-a (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines, CCL2 (**D**) and CCR5 (**E**) after 16 weeks of diet in WT and CCR5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to SD-fed control groups. Values are represented as means ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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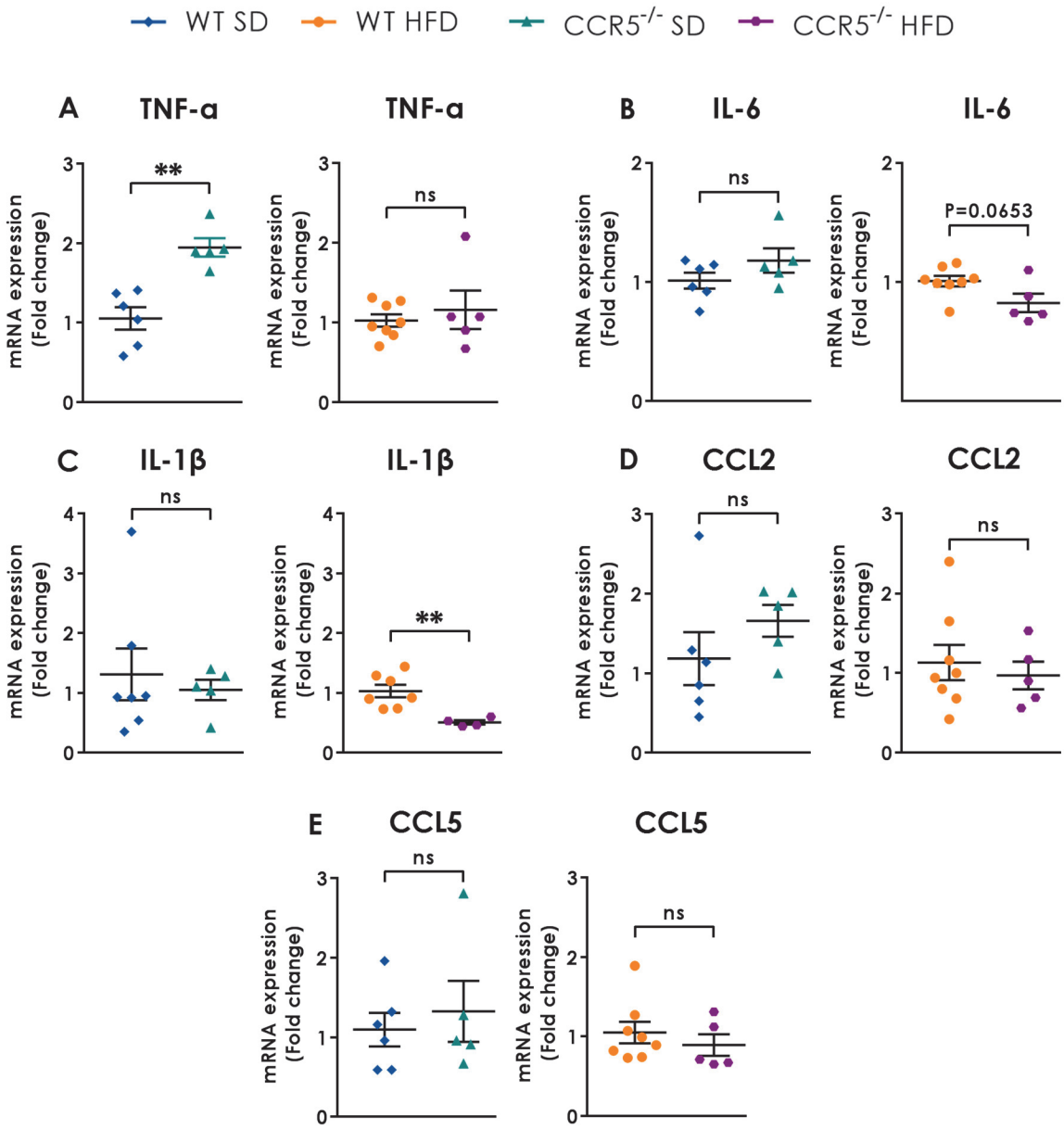

Figure 57: Effect of CCR5 deficiency on hypothalamic neuroinflammation compared between genotypes.

Real-time PCR quantification of hypothalamic mRNA expression of inflammatory markers such as the cytokines: TNF-a (**A**), IL-6 (**B**), and IL-1β (**C**), and the chemokines CCL2 (**D**) CCR5 (**E**) after 16 weeks of diet in WT and CCR5$^{-/-}$ mice on either SD or HFD. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to WT control groups. Values are represented as means ± SEM (n=6-9 mice/ group). Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant.

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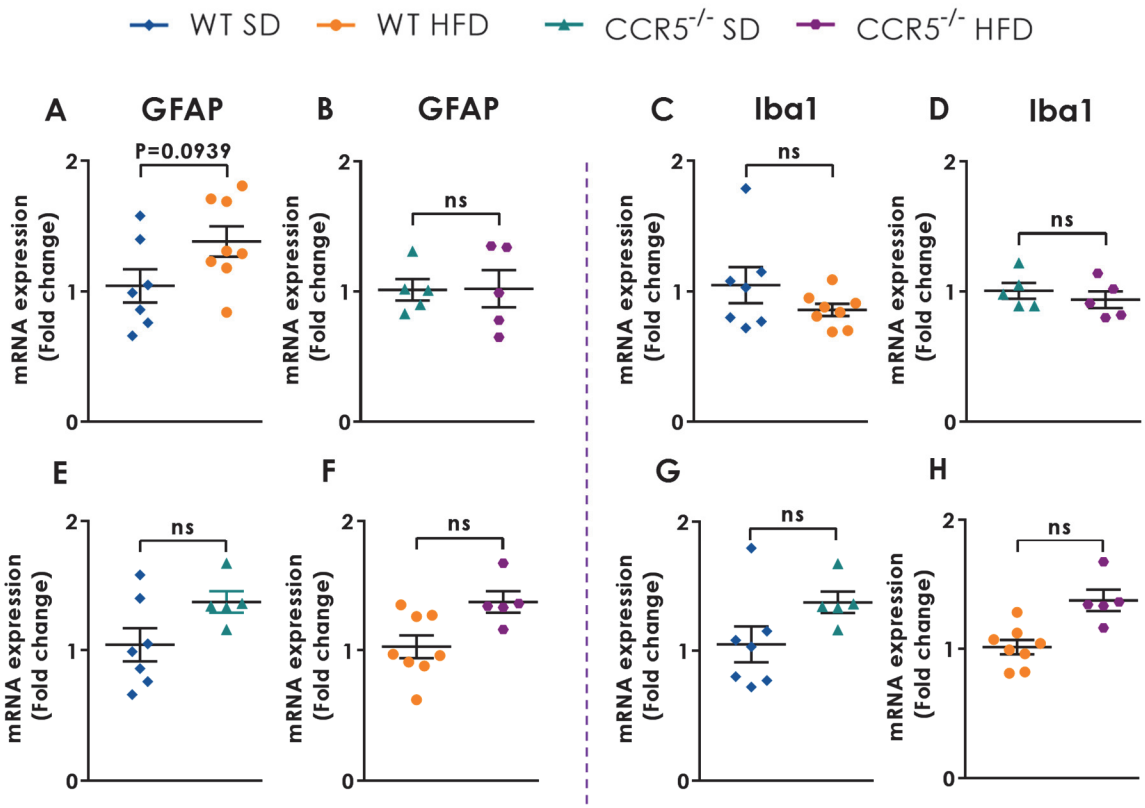

Figure 58: Effect of CCR5 deficiency on DIO associated hypothalamic gliosis.
Effect of 16 weeks of SD or HFD diet on hypothalamic mRNA expression of glial markers quantified by qPCR. **A** and **B**: Relative hypothalamic expression of astrocyte marker GFAP in WT (**A**) and CCR5$^{-/-}$ (**B**) mice and comparing between mice fed a SD (**E**) and HFD (**F**) diet. **C** and **D**: Relative hypothalamic expression of microglial marker Iba1 in WT (**C**) and CCR5$^{-/-}$ (**D**) mice and comparing between mice fed a SD (**G**) and HFD (**H**) diet for 16 weeks. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to respective controls. Values are represented as means ± SEM (n=5-8 mice/ group). Statistical significance was determined using Mann-Whitney Test; P<0.05 *, P<0.01 **, P<0.001 ***; ns: not significant; GFAP: glial fibrillary acidic protein; Iba1: ionized calcium binding adaptor molecule 1.

3.5. The Effect of CCR5 Deficiency on Systemic Inflammation

Among a panel of pro- and anti-inflammatory cytokines and chemokines tested, HFD-fed WT and CCR5$^{-/-}$ mice showed both a significant increase in serum levels of TNF-α (Fig. 59A). Apart from that, they showed a different inflammatory pattern. Obese WT mice had also significantly increased levels of anti-inflammatory IL-10 compared to their SD-fed controls (Fig. 59E).

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Interestingly, CCR5$^{-/-}$ mice on a SD had significantly higher concentrations of pro-inflammatory cytokines IL-1β and IFN-γ, compared to WT control mice (Fig. 59C-D). Both mice fed a HFD had significantly higher serum levels of TNF-a levels compared to SD fed mice, but were no different between genotypes (Fig. 59A and J).

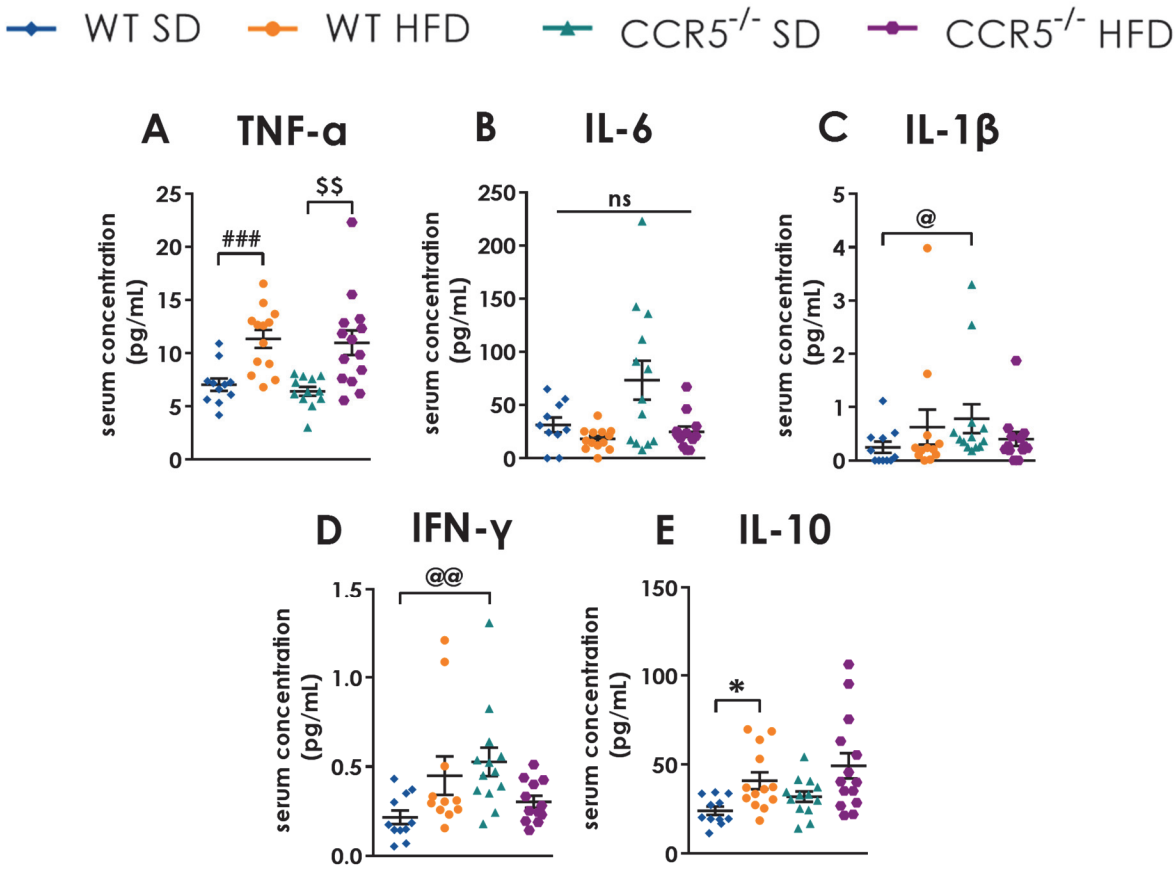

Figure 59: Effect of CCR5 deficiency on systemic peripheral inflammation.
Serum levels of pro-inflammatory cytokines TNF-a (**A**), IL-6 (**B**), IL-1β (**C**), IFN-γ (**D**) and anti-inflammatory cytokine IL-10 (**E**) after 16 weeks of diet in WT and CCR5$^{-/-}$ mice on either SD or HFD. Values are represented as means ± SEM (n=12-14 mice/ group; for IL-1β: n=1-8, for CCR5: n=8-9). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test; * P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****, WT HFD *vs* CCR5$^{-/-}$ HFD; #, WT SD *vs* WT HFD; $, CCR5$^{-/-}$ SD *vs* HFD; @, WT SD *vs* CCR5$^{-/-}$ SD; ns: not significant.

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3.6. The Effect of CCR5 Deficiency on Diabetes-Associated Neuropathic Pain

At the end of the DIO protocol, at 16 weeks of diet, we performed a Hargreaves' test to investigate if CCR5$^{-/-}$ mice showed altered sensitivity to thermal pain sensitivity, which is common after prolonged HFD in mouse models of obesity and diabetes. Within our expectations, we documented a significant decrease in WT HFD paw withdrawal latency upon stimulation with an infrared heat source, which is indicative of an increased sensitivity to thermal pain. In addition to that, the lack of CCR5 did not significantly increase the thermal pain sensitivity threshold compared to WT mice in SD condition. Similarly, under HFD condition CCR5$^{-/-}$ mice remained within normal range of thermal pain sensitivity and where not significantly different from their SD control group (Fig. 60).

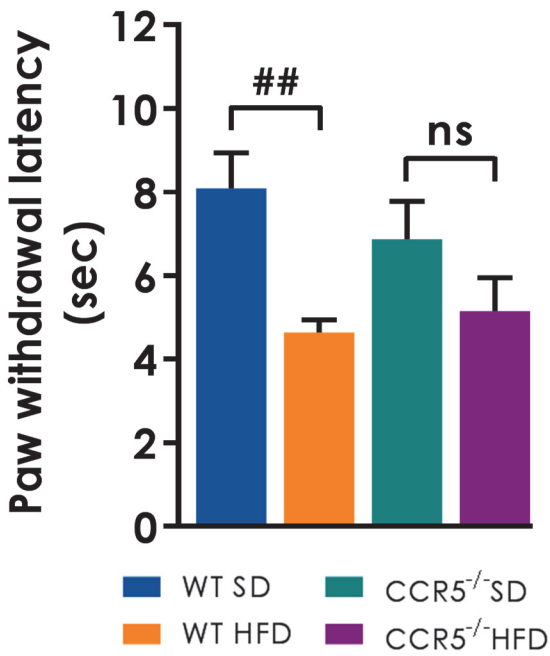

Figure 60: Effect of CCR5 deficiency on thermal pain sensitivity in DIO mice.
Estimation of mechanical perception of thermal pain sensitivity by paw withdrawal latency in seconds as determined with Hargreaves' test in WT and CCR5$^{-/-}$ mice on either a SD or HFD for 16 weeks. Values are represented as means ± SEM (n=10-14/group). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test; P<0.05 **#**, P<0.01 **##**, P<0.001, **###**, P<0.0001 **####**. WT SD *vs* WT HFD; ns: not significant.

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3.7. The Effect of CCL5 and CCR5 on Subcutaneous AT Morphology

Due to common reports implicating CCL5 and CCR5 in AT infiltration and function, in particular in the context of obesity, we decided to take a closer look at SC AT morphology. Hence, we stained fixed sections of SC AT of WT, $CCL5^{-/-}$ and $CCR5^{-/-}$ mice, which were fed either a SD or HFD for 16 weeks, with H&E and quantified the morphological distribution of SC adipocyte size. As depicted in figure 61, all three groups on a HFD have on average larger adipocytes compared to their respective SD-fed controls. Interestingly, although SD-fed mice do not differ in the average size of their SC adipocytes along genotypes, we found a different frequency distribution between those groups (Fig. 61B-C). According to Fig. 61B it seems, that WT mice on a SD have a higher frequency (~25.5%) of very small adipocytes in the range of 1500µm², while adipocyte area of $CCL5^{-/-}$ mice have the highest frequency (20.3%) at around 3500µm² while $CCR5^{-/-}$ mice have the highest frequency of adipocytes with 18.4% around 2500µm² (Fig. 61B). The frequency of adipocytes of both KO mice is therefore more skewed to the right compared to WT mice on a SD. WT mice under HFD condition have very little adipocytes in the small range and have the highest frequency of adipocytes in the range of 7000µm² (19.4%), while HFD-fed $CCL5^{-/-}$ mice share the most abundant cell size of 7000µm² but have a higher frequency (26.6%) of those compared to WT HFD-fed mice. CCR5 deficient mice on a HFD seem to have a higher number of even larger adipocytes, as they have around 22.7% of adipocytes in the range of 9000µm². Interestingly though, WT mice on a HFD have a wider range of adipocyte sizes with the largest adipocytes reaching an area of around 21000µm² (0.971%), while the size of adipocytes of CCL5 and CCR5 deficient mice on a HFD seem to stop at around 13000µm² (1.3%) for the former and 14000µm² (4%) for the latter.

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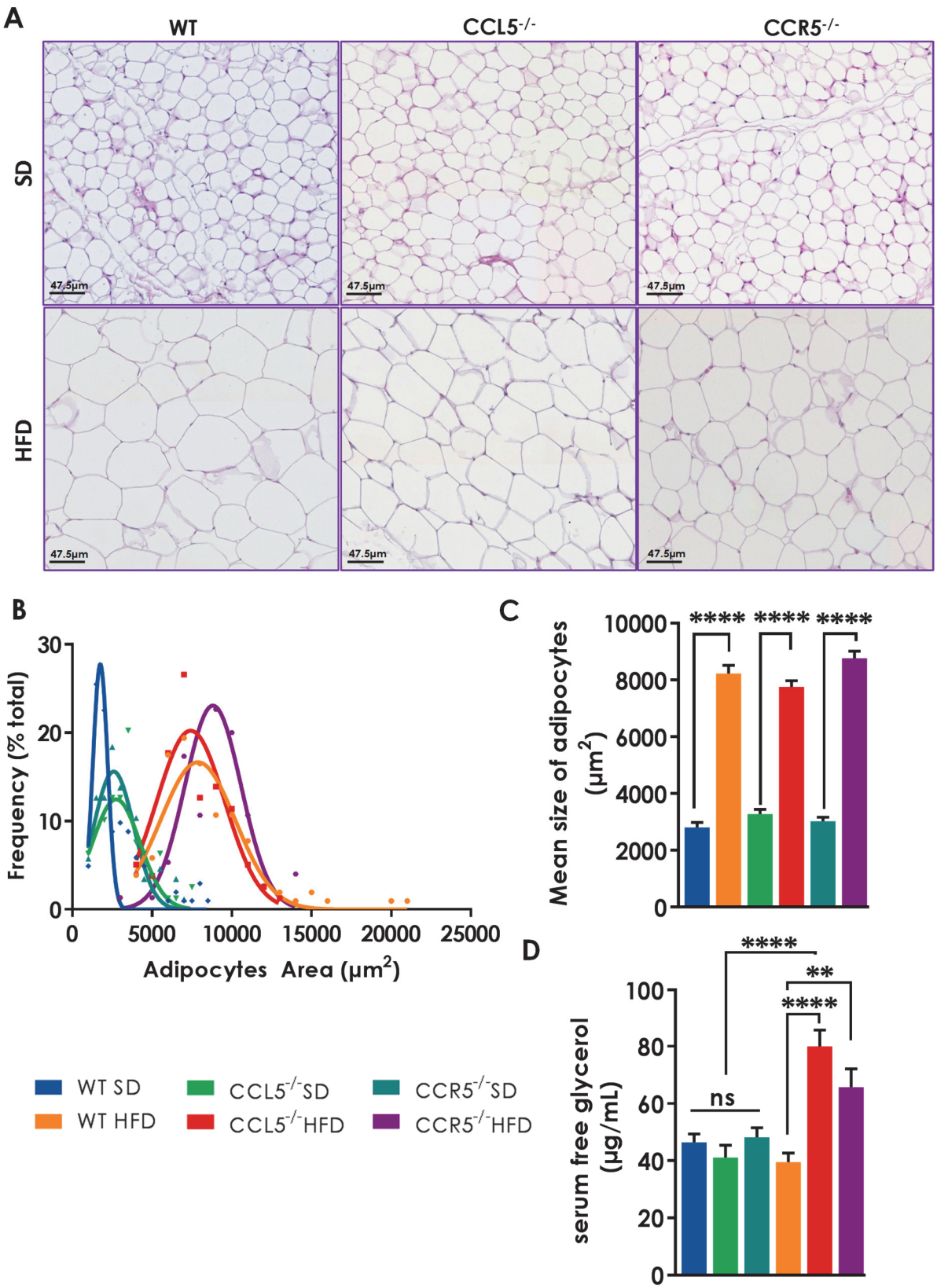

Figure 61: Effect of CCL5 and CCR5 deficiency on AT morphology and lipolysis. H&E staining of representative sections of subcutaneous AT (**A**) and their quantification in percent frequency of adipocyte area. Upper panel showing the subcutaneous AT of WT, CCL5$^{-/-}$, CCR5$^{-/-}$ under SD condition and lower panel

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containing images of the respective HFD condition; (**B**) frequency distribution in percent of mean adipocyte area of different genotypes after 16 weeks of SD or HFD; (**C**) Quantification of mean adipocyte size of WT, CCL5$^{-/-}$, CCR5$^{-/-}$ after 16 weeks of SD or HFD condition. **D**: Effect of CCL5 and CCR5 deficiency on systemic lipolysis activity measures represented as free glycerol (µg/mL) in serum of mice on an experimental protocol of DIO. Values are represented as means ± SEM (n=1-9 images per animal and n=13-16 animals/group for B and C; n=13-17/group for D). Statistical significance was determined using Kruskal-Wallis test with Dunn's *post hoc* test and two-way ANOVA with Tukey's *post hoc* test; P<0.05 *****, P<0.01 ******, P<0.001, *******, P<0.0001 ********. WT SD *vs* WT HFD; ns: not significant.

Furthermore, we tested the amount of free serum glycerol, indicative of the rate of lipolysis in mice of different genotypes under both diet conditions. Interestingly we found, as depicted in Fig. 61D, that while mice on a SD, were not different in their levels of glycerol, mice deficient in CCL5 and CCR5 on a HFD had significantly higher amounts of free glycerol and therefore seem to have higher rates of lipolysis compared to WT control mice (Fig. 61D). The highest concentration of free glycerol was observed in CCL5$^{-/-}$ mice under HFD condition (Fig. 61D).

3.8. The Effect of Chronic ICV Infusions of Different Concentrations of CCL5

To further characterize and confirm the results we have seen in previous section, most notably on BW gain, we have performed experiments, where we implanted a guide cannula into the right lateral ventricle of mice and chronically injected different concentrations of CCL5 (10ng, 50ng and 100ng) and aCSF as control. We then determined the effect of the injections on BW, food intake and a panel of inflammatory marker and neuropeptide expression in the hypothalamus. Figure 62A and B display the effects of different concentrations of CCL5 on BW as opposed to aCSF injections. We have found that even though we performed a single aCSF injection to all mice prior to the initiation of different injections, that mice lost BW and recuperated only about a week after initiation of the injections, independent on the agent we injected (Fig. 62A). Furthermore, we observed that injecting recombinant CCL5 at concentrations of 50ng and 100ng but not 10 ng induced a more prominent loss of BW compared to aCSF vehicle injection

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(Fig. 62A and B). However, only the difference seen between injections of 100ng CCL5 and aCSF was significant. After the first injection all mice eventually recuperated without any significant difference between different concentrations or agents injected and most interestingly, despite the continuous chronic injection of each agent every second day. The result indicates that chronic injections under SD conditions lose their effect after the first initial injection. We examined also the effect of injections of food intake every day, but although there seems to be a tendency of reduction in food intake when injecting 100ng of CCL5, although the effect is not significant (P= 0.067) and thus remains inconclusive (Fig. 62C). Furthermore, we quantified the hypothalamic mRNA expression of inflammatory markers and neuropeptides after chronic injections of different concentrations of CCL5 and found a significant increase in Iba1 expression with chronic injections of 10ng CCL5 compared to aCSF injected mice (Fig. 62C). Although there were some variations for mRNA expression of hypothalamic GFAP, it remained not significant. When we quantified the mRNA expression of neuropeptides, we again so several variations in expression and tendencies that remained at the limit of significance. For example, we found that CCL5 injections of 10, 50 and 100ng of CCL5 increased the expression of AgRP mRNA (P=0.067, P=0.065 and P=0.093 respectively). In addition to AgRP, we found a significant increase in POMC mRNA expression after 50ng injection of CCL5 and a tendency for increase in 100ng injected mice (P=0.058) (Fig. 62C).

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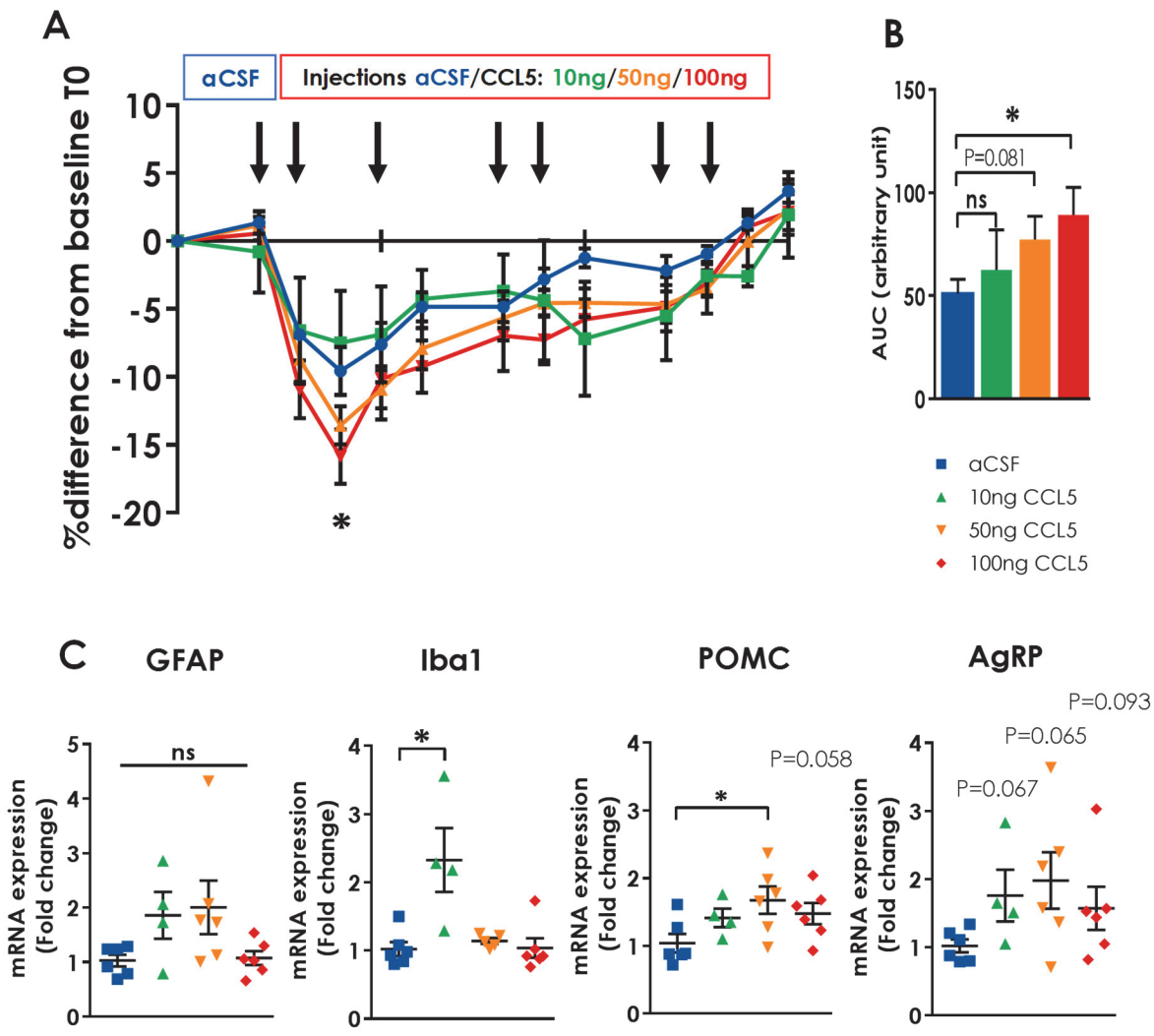

Figure 62: Effect of ICV administration of different concentrations of CCL5 on changes in BW.

A: Graph depicting the % difference in BW relative to baseline before aCSF injection. Arrows indicate injections via implanted cannula of either aCSF, or recombinant CCL5 (10ng, 50ng, 100ng). First arrow indicated the first aCSF injection to all mice for habituation. **B**: Quantification of (**A**) as area under the curve (AUC). Values are means ± SEM (n=4-6/group). **C**: Quantification of hypothalamic mRNA expression relative to aCSF injected mice. All mRNA species were quantified relative to Gapdh housekeeping gene expression by ΔCT method and presented as fold change relative to respective controls. Statistical significance was determined using two-way ANOVA with Dunn's *post hoc* test and two-way ANOVA with Dunnet's *post hoc* test and Unpaired t-test with Welch's correction; P<0.05 *, P<0.01 **, P<0.001, ***, P<0.0001 ****. All vs aCSF; ns: not significant.

In a second series of experiments, we wanted to confirm the effect of CCL5 injection on the reduction of BW and food intake and additionally examine whether CCL5 injection also has an impact on glucose homeostasis and thermal pain sensitivity as it was suggested by our data discussed above. In

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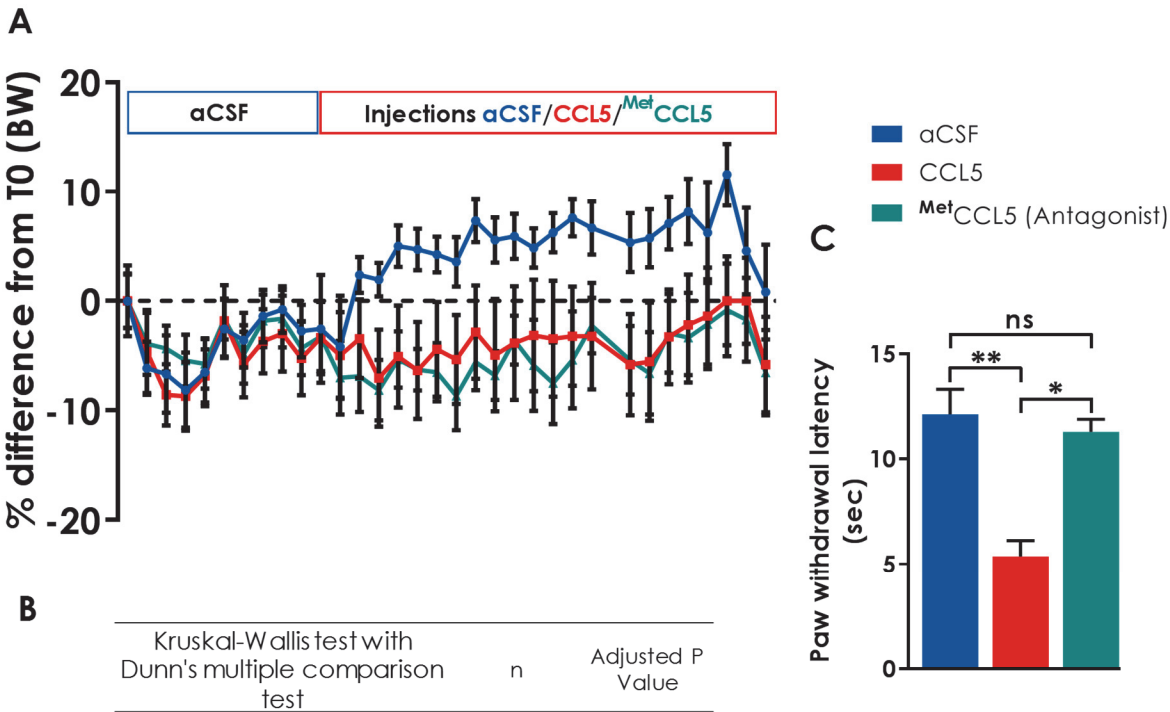
addition to the chronic injection of 0.3ng of CCL5 over the course of 4 weeks, we injected also 0.3ng of the antagonist called ^{Met}CCL5 every second day, after one week of aCSF injections for habituation (Fig. 63). The results indicate that chronic injections of CCL5 induce a significant reduction of BW as indicated in figure 63A and B. Interestingly, injection of the antagonist had a similar effect on the reduction of BW compared to aCSF as CCL5 injection. The effect on food intake was again inconclusive, although it suggests that CCL5 induces a reduction in food intake within 24h, which disappears after 48h.



B

Kruskal-Wallis test with Dunn's multiple comparison test	n	Adjusted P Value
aCSF vs. CCL5	5-9	<0.0001
aCSF vs. ^{Met}CCL5 (Antagonist)	5-8	<0.0001
CCL5 vs. ^{Met}CCL5 (Antagonist)	5-9	>0.9999

Figure 63: Effect of ICV CCL5 and antagonist ^{Met}CCL5 administration on changes in BW, food intake and thermal pain sensitivity.
Effect of ICV administration of aCSF, CCL5 (0.3µg) and ^{Met}CCR5 (0.3µg) on **A:** BW gain, **B:** Table representing statistical significance for (A). **C**: Hargreaves' thermal pain sensitivity test. Statistical significance was determined with Kruskal-Wallis test with Dunn's *post hoc* test. P<0.05 *, P<0.01 **, P<0.001, ***, P<0.0001 ****. All vs aCSF; ns: not significant.

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Next, after 3 weeks of chronic aCSF, CCL5 and ^{Met}CCL5 injection, we performed a Hargreaves' test to see whether injection of CCL5 induces an increased sensitivity of thermal pain under SD. As expected, our results show that CCL5 injection induces a significant reduction in the threshold for pwl compared to both aCSF and ^{Met}CCL5 injection, which did not show the same effect (Fig. 63C). In fact, ^{Met}CCL5 injection showed no difference in pwl compared to aCSF injections and was significantly higher compared to CCL5 injected mice. At about 4 weeks after starting repeated injections every second day, we performed a GTT, and ITT and found no difference between basal fasting glucose levels, glucose tolerance or insulin sensitivity compared to aCSF injected mice (Fig. 64A-F). Although we observed a slight increase in insulin sensitivity of ^{Met}CCL5 injected mice compared to either aCSF or CCL5 injected mice, this difference was not significant (Fig. 64D). When we analyzed the serum insulin and glucose-stimulated insulin secretion, we did not find any significant difference, although it seemed like CCL5 injected mice had lower basal insulin (non-fasted condition) compared to aCSF and ^{Met}CCL5 injected mice, but slightly more insulin upon glucose-stimulation at 30 and 60 min after glucose stimulation (Fig. 64E and G).

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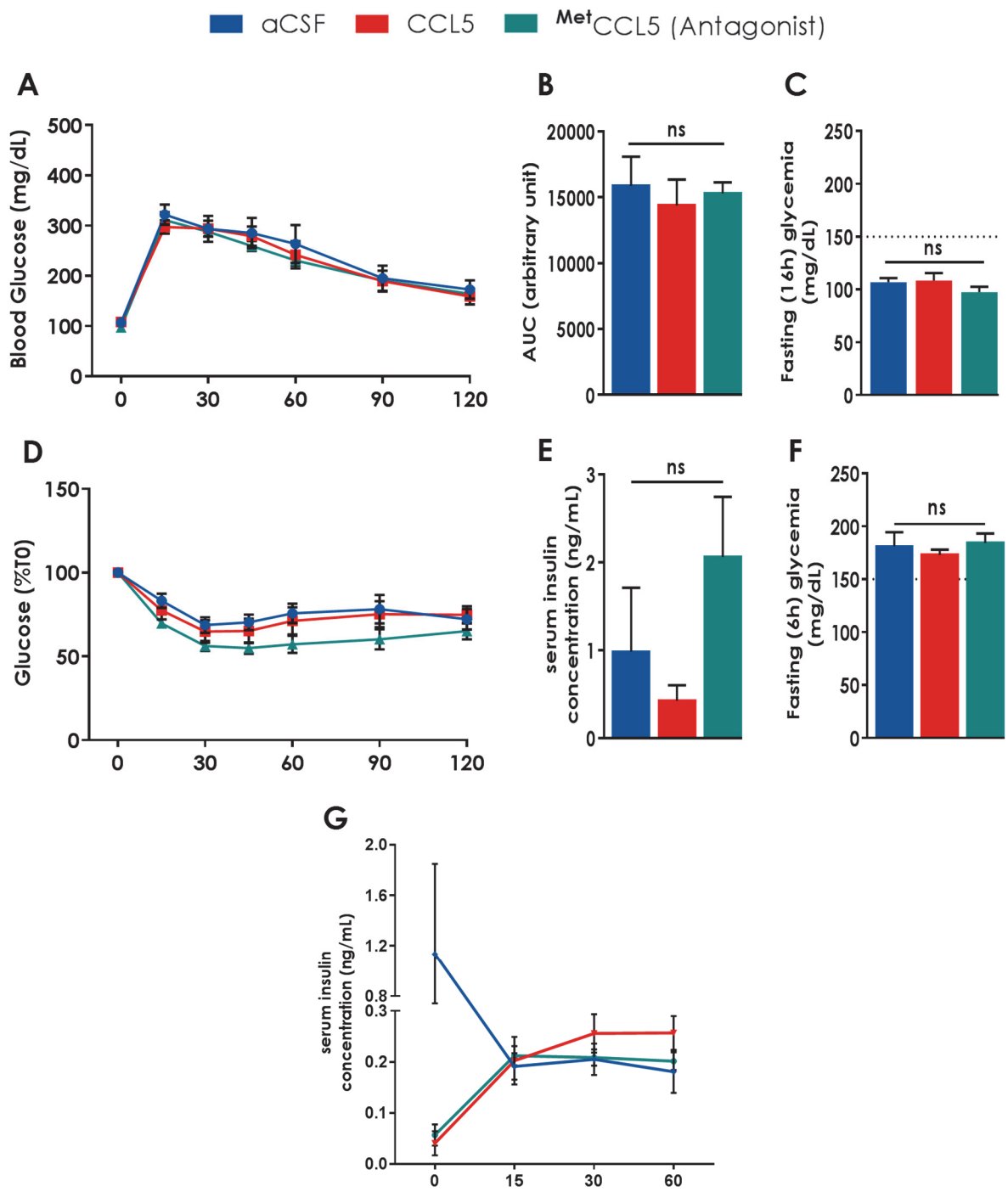

Figure 64: Effect of ICV CCL5 and antagonist administration on glucose homeostasis. Effect of ICV administration of aCSF, CCL5 (0.15µg/µL) and ^{Met}CCL5 (0.15µg/µL) on **A:** GTT, **B:** Quantification of (A) as AUC, **C**: Basal blood glucose levels after 16h of fasting, **D**: ITT, **E**: Plasma insulin concentration measured by ELISA, **F:** Basal blood glucose levels after 6h of fasting, and **G:** Acute glucose-stimulated insulin secretion test. Data are represented as means ± SEM (n=5-7/group). Statistical significance was determined with Friedman's test or Kruskal-Wallis test with Dunn's *post hoc* test. Dashed line indicates the threshold for diabetic levels of blood glucose at 150 mg/dL. ns: not significant.

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Discussion

DIO is one of the most common causes of obesity and associated not only with comorbidities like diabetes and neuropathic pain among others, but also with metabolic disorders and chronic low-grade inflammation. This inflammation affects several metabolic tissues in the periphery but also is very well present in the CNS especially the hypothalamus. Furthermore, many studies have shown the link between inflammation, insulin resistance, obesity and HFD consumption. Several studies have described HFD-induced upregulation of pro-inflammatory cytokines and chemokines as well as reactive gliosis, well before the first signs of obesity appear as well as after long-term HFD consumption (J. P. Thaler *et al.*, 2012; Kälin *et al.*, 2015). Although there are many theories to what might cause the intial inflammatory response, some studies have shown evidence that lipids and FFAs themselves induce the activation of inflammatory pathways such as NF-κB or IKKβ through TLR4 (Holland *et al.*, 2011). This inflammatory process in the hypothalamus leads to disruption of the energy balance and might thus lead to hypothalamic resistances to leptin and insulin (Clegg *et al.*, 2011; Holland *et al.*, 2011). The disruption of energy balance regulation and resistance to peripheral hormones alters feeding behavior and EE, which ultimately will precipitate the development of obesity and diabetes among other consequences (De Souza *et al.*, 2005; Kleinridders *et al.*, 2009; Arruda *et al.*, 2011).

1. The effect of lipid nature on diet-induced obesity

Several animal studies as well as a few human studies point towards a protective role of ω3 in the development of DIO (Buckley and Howe, 2010; Cintra *et al.*, 2012; Oliveira *et al.*, 2015). It has been reported that a lower ω6/ω3 ratio or higher intake of ω3 PUFA might be beneficial, because while SFAs and ω6 are pro-inflammatory, ω3 FAs seem to have anti-inflammatory

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and serum lipid lowering effects (Kelley, 2001; Lee *et al.*, 2001; Cleland, James and Proudman, 2003; Gupta *et al.*, 2012; Yan *et al.*, 2013; Leslie *et al.*, 2015).

In the first part of this study, we sought to establish a mouse model of DIO and to investigate whether a HFD based on different lipid nature and ω6/ω3 ratio has different effects on the development of DIO and associated comorbidities. In line with the literature, our data show that the HFD with a high amount of saturated fat derived from animals (HFD-B) was the most obesogenic diet and rendered mice obese, diabetic and more sensitive to thermal pain as well as upregulated inflammatory markers in the hypothalamus after 12 weeks of diet (Maric, Woodside and Luheshi, 2014).

Although all HFDs based on vegetal lipid sources showed a milder development of DIO, we did not find any difference in BW between diets with same amount of lipids but different ω6/ω3 ratio. Similarly, we have not found any effects between ω6/ω3 ratios on the development of diabetes. Based on our results it seems that the amount of lipids in a HFD is more important in the development of obesity and diabetes, as well as hypothalamic inflammation and ultimately the deregulation of energy balance. To be able to answer the question of whether ω6/ω3 ratio and lipid source is crucial in the development of DIO and associated metabolic syndrome, our model was insufficient and it would require a higher contant of the respective lipids, similar to the HFD-B that contains 40% of lipids.

Interestingly, although there was no difference in BW gain between HFDs with vegetal sources of fat, we found that HFD-SN with the highest and most harmful ratio of ω6/ω3 did upregulate inflammatory markers and glial cell markers in the hypothalamus, which is in line with the literature. Thus it seems like, although ω6/ω3 ratio does not seem to play a major role in body weight gain, it might be responsible for the inflammatory response associated with obesity. It is possible that due to the lower amount of lipids, glucose metabolism impairment and body weight gain might have developed later on if the diet was to be continued longer than 12 weeks for the HFD-SN.

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In line with other studies the diets with lower ω6/ω3 ratio did no show any upregulation of inflammatory markers in the hypothalamus, unlike the diet based on butter and HFD-SN with an elevated ω6/ω3 ratio (Milanski *et al.*, 2009; Cintra *et al.*, 2012; Oliveira *et al.*, 2015). Interestingly, other studies report, that supplementing their HFD with ω9 or ω3 FAs ameliorated both hypothalamic inflammation as well as hypothalamic leptin and insulin resistance but also whole body insulin resistance. In addition to that, they found that icv injection of either ω9 or ω3 FAs in HFD-fed mice also reversed neuropeptide expression of POMC and CART. This is similar to our results as non of the diets with beneficial ω6/ω3 ratio had upregulated POMC or CART expression unlike the diet high in saturated FAs (HFD-B) (Cintra *et al.*, 2012).

However, our model was sufficient to develop a model of DIO with diabetes and thermal pain sensitivity to test the effects of the chemokine CCL5 in the second part of the study in this setting.

2. The role of chemokine CCL5 in the development of diet-induced obesity

Chemokines like CCL2 and CCL5 and their receptors have been linked to the development of obesity and peripheral insulin resistance as well as the infiltration of immune cells into AT as was described for obesity. Although chemokines and chemokine receptors are known for their role in the peripheral immune system, their constitutive expression in the CNS has been reported and many authors suggest that they might have a physiological role in the CNS (Kitabgi, Mélik-Parsadaniantz and Rostène, 2006; Rostène, Guyon, *et al.*, 2011; Réaux-Le Goazigo *et al.*, 2013). Previous work has shown some first results on CCL5 and CCR5 KO models in SD and HFD conditions, while studying different aspects and showing conflicting results. Kitade and colleagues have shown that young $CCR5^{-/-}$ mice are seemingly protected from DIO-induced effects on AT infiltration, inflammation and glucose metabolism impairment. In contrast to this, other studies have shown rather contrasting data of impairments in insulin signaling and glucose metabolism, systemically and in peripheral tissues like AT and muscle but also in the hypothalamus (Kitade *et al.*, 2012; Kennedy *et al.*, 2013; Chou *et al.*, 2016).

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In this study, we wanted to shed light on the conflicting results so far in the literature and further add to the existing results in the literature of the role of CCL5 not only in the periphery but also in the brain in the context of energy balance and DIO. Thus, using a genetic invalidation model as well as some pharmacological tools, we attempted to provide the missing link between (neuro) inflammation, energy balance regulation and obesity and its comorbidities by studying the role of CCL5 and its receptor CCR5 in the development of obesity, diabetes and neuropathic pain.

First, we confirmed that serum CCL5 is increased in HFD-induced DIO in adult WT mice after 16 weeks of diet, as was previously reported in both obese humans and mice (Wu *et al.*, 2007; Dalmas *et al.*, 2011; Hu *et al.*, 2018). Furthermore, we have shown that HFD induced a rapid development of DIO, in WT mice on the chosen obesogenic diet based on butter with 35% fat.

We found, using a KO model in a DIO context, that both CCL5 and CCR5 seem to be involved in the pathogenesis of obesity and associated comorbidities like diabetes and neuropathic pain. It seems that the lack of either CCL5 or CCR5 protects from DIO associated weight gain, neuropathic pain and delays the glucose impairment and insulin resistance associated with obesity and the metabolic syndrome. This seems to indicate that CCL5 might exert its function in energy and glucose metabolism via its cognate receptor CCR5. Furthermore, we observed that both CCL5 and CCR5 are not only expressed in the ARC and ME of the hypothalamus, but also seem to have a neuromodulatory role, as seen by the change in neuropeptide expression for example with NPY. Our data suggest that CCL5 might have an effect on BW in a situation of high lipid intake possibly mediated through the modulation of neuronal activity in the hypothalamus and affecting feeding behavior either directly, or indirectly by affecting glucose metabolism and/or insulin signaling. CCL5 seems to have an endocrine function as suggested by many studies in the literature, a function however, that is not evident in KO mice under physiological conditions as they have a rather normal phenotype. Only subtle changes are visible under physiological conditions, such as

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differences in neuropeptide expression levels at the mRNA level, which are not enough to cause a distinct phenotype. The effect of CCL5 and its receptor CCR5 is more prominent however in pathophysiological stress conditions like a metabolic challenge of long-term lipid excess.

3. CCL5 and CCR5 mRNA expression in subpopulation of cells in the nuclei of the hypothalamus

Although several studies have reported the expression of chemokines and their receptors in the CNS including CCL5, we have not found any convincing results among them that show the expression of this chemokine in the hypothalamus. Some studies have reported the expression of CCL5 and CCR5 in human fetal astrocytes and microglia in primary cultures, while other report their expression in neurons (He *et al.*, 1997; Hu *et al.*, 1999; Bakhiet *et al.*, 2001; Westmoreland *et al.*, 2002; Avdoshina *et al.*, 2010; Sorce, Myburgh and Krause, 2011). Hence, we wanted to confirm whether CCL5 is expressed in the region of energy balance and feeding regulation. We have shown that the mRNA of CCL5 and its receptor CCR5 is indeed expressed in a few cells in regions of the hypothalamus, including the ARC, LH, VMH and cells of the ME but also outside the hypothalamus. The perinuclear expression pattern of CCR5 mRNA might indicate its localization in ER that surrounds the nucleus. CCL5 mRNA expression seems to be more dispersed and difficult to assign to certain DAPI-stained nuclei, which might suggest a cytoplasmic location, possibly even in axons of neurons. However, these are only speculations, which require further experiments.

These results are in line with the literature as one study used RT-PCR to examine RNA expression in different parts of the brain and found CCR5 and CXCR4 mRNA to be expressed in the hypothalamus among other regions of the brain (Bajetto *et al.*, 1999). Furthermore, another study demonstrated the expression of CCR5 in the mouse brain in areas such as cerebellar and cerebral cortex, striatum and hippocampus. Interestingly, they reported that the majority of CCR5 seems to colocalize with neuronal marker NeuN and mainly in the cytoplasm, neuronal perikaryon and processes (Avdoshina *et al.*,

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2011). However, they have not reported anything about the expression or lack thereof in the hypothalamus.

A few recent studies have confirmed some of our data. First, one study reports having tested different antibodies and encountered trouble with them due to unspecific binding. Hence, they opted similar to us, for fluorescence *in situ* hybridization via RNAScope® technology to assess the expression pattern of CCL5 mRNA in adult rat brain. The study has shown that CCL5 mRNA is expressed throughout the brain with highest expression occurring in major fiber tracts. However, CCL5 probe fluorescent signals were found in other areas of the brain including the hypothalamus and the VTA, confirming previous and our reports (Lanfranco *et al.*, 2018). Unfortunately, the study does not specify, nor show the exact location of fluorescent signals in different nuclei of the hypothalamus. In contrast to our results, they show a nuclear localization of CCL5 mRNA, while our results show expression mostly around DAPI-stained nuclei and only a few colocalized with DAPI. However, they found, as did we, that CCL5 is expressed only by subpopulations of cells. Interestingly, the study reported positive staining for CCL5 mRNA in the region of the periaqueductal grey, an area that has been implicated in pain transduction, which will be discussed further below.

Moreover, the study performed colocalization with markers of astrocytes (GFAP), microglia (Iba1), neurons (NeuN) and oligodendrocytes (Nkx2.2) and observed that while CCL5 mRNA expression was found in all types of cells, it seems to be expressed more in neurons than other cell types, at least in the VTA. Furthermore, they found that only a subset of neurons expressed CCL5, some of which have been identified to be a subpopulation of dopaminergic neurons as indicated by tyrosine hydroxylase (TH) (Lanfranco *et al.*, 2018).

The differences in our and their result might be due to differences in protocol despite using the same technique as they used free-floating sections in 20µM brain section while we used fixed-frozen sections of 10µM thickness, both of which have different pretreatment protocols for the RNAScope® procedure.

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For example, the protocol of the study as reported did not include a protease step while ours did. This might also explain why our combining of RNAScope® technology with immunohistochemistry was not successful, while the study had positive outcomes. Furthermore, species-specific differences between the expression of CCL5 mRNA between mice and rats are also likely.

Another study reported the specific expression of CCR5 exclusively in the ARC of the hypothalamus but not in the VMH or other hypothalamic nuclei, while they demonstrated immunoreactivity of antibodies with CCL5 in both the ARC and VMH of the hypothalamus suggesting an autocrine loop in the ARC (Chou *et al.*, 2016). This is in part matching our results, as we equally found expression of both CCL5 and CCR5 in the ARC, but different from this study and rather in agreement with the study by Lanfranco and colleagues, we found both ligand and receptor to be expressed throughout the brain and not only specific to the ARC and VMH. Furthermore, we attempted to visualize all three receptors of CCL5 (CCR1, CCR3 and CCR5) using the same and other antibodies as in the study and did not manage to replicate the staining achieved seemingly without any kind of amplification or other additional steps apart from a standard immunofluorescence staining protocol.

Furthermore, the authors report that CCR5 is expressed in POMC and MP2 (neuronal marker used in the study) positive and POMC negative neurons in *in vitro* primary hypothalamic neuron cultures (Chou *et al.*, 2016).

Our results seem to indicate that long-term consumption of HFD might increase the expression of both the ligand CCL5 and its receptor CCR5 in the hypothalamus of HFD-fed WT mice compared to SD controls. It appears to be the case due to both an increased expression of ligand and receptor mRNA in the same cells, as well as increased number of cells expression both CCL5 and CCR5. However, these first observations have to be confirmed with quantification, and further experiments could be done to further quantify the mRNA expression of different receptors in the hypothalamus of HFD-fed WT mice compared to SD-fed mice via quantitative RT-PCR. Due to the limitation

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of our experiment, we cannot make any assumption about whether the seemingly increased mRNA levels translate to protein levels. However, this result shows the need for further experiments, investigating protein levels via ELISA/MSD or Western blot in hypothalamic tissue samples or primary cultures of HFD and SD-fed mice. In this way, one could use specific culture conditions of FACS sorted cells to test the protein levels of CCL5 and various receptors in specific cultures of neurons, microglia or astrocytes. Although we have attempted to visualize the expression of CCR1, CCR3 and CCR5 in brain samples of WT mice at the protein level via different receptor-specific antibodies with different protocols, we have not managed to make the antibodies work. Further optimization might be needed or other antibodies have to be tested with appropriate controls from other tissues to visualize protein expression of CCL5 and its receptors. Similarly, we attempted to combine RNAScope® technology with immunoflurescent-based antibodies to colocalize CCR5 and CCL5 mRNA expression with specific cell-markers of neurons (NeuN), microglia (Iba1) and astrocytes (GFAP). However, the only antibody that worked was GFAP. The specific protocol for RNAScope® might be problematic for combinations as the protocol for fixed-frozen sections as we have used includes a proteinase step, which might degrade the epitopes of proteins of interest, and thus are no longer detectable by the antibodies tested. One might, try to optimize by adjusting the protease treatment time, although the result is no longer guaranteed by RNAScope® in case of deviations of the protocol.

4. The effect of CCL5/CCR5 signaling on HFD-induced body weight gain and feeding behaviour

Our results demonstrated a partial protection of HFD-induced BW gain in $CCL5^{-/-}$ and $CCR5^{-/-}$ mice compared to WT mice, which suggests that CCL5 has an impact on BW regulation mediated by CCR5 signaling. This difference in BW gain cannot be attributed to changes in leptin, as leptin levels were not significantly different. These results, although not directly comparable, are in line with results of Kitade and colleagues, who showed no difference in leptin

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mRNA expression in WAT of CCR5 deficient mice on a HFD compared to WT controls (Kitade *et al.*, 2012). This indicates that, according to our data, CCL5 has no visible impact on peripheral leptin secretion of ATs and suggests that $CCL5^{-/-}$ and WT mice on a HFD have similar amounts of WAT, since leptin levels are usually proportional to fat mass. The difference in BW was highest around 8 weeks of diet and decreased towards 16 weeks of diet. However, serum leptin levels were obtained at the end of the experimental protocol, when the difference in BW between genotypes was although significant, much less pronounced, which could explain the similar levels of leptin. Further experiments are necessary to examine serum leptin levels at ≤8 weeks of diet to see whether CCL5/CCR5 signaling might be implicated in leptin signaling. Another possibility for the difference in BW could be that CCL5 is involved in hypothalamic leptin signaling and thus $CCL5^{-/-}$ and $CCR5^{-/-}$ mice could be protected from hypothalamic leptin resistance and/or impaired leptin uptake, associated with DIO. It is intriguing to speculate, that $CCL5^{-/-}$ mice might be protected from HFD-induced leptin resistance or impaired leptin uptake into the CNS and thus reduce their food intake, while WT mice are resistant and ingest the same kcal as control mice. Additional experiments are necessary to investigate hypothalamic leptin resistance in $CCL5^{-/-}$ and $CCR5^{-/-}$ mice in order to assess these possibilities. However, both our data and the literature are not suggestive of this possibility and far from enough to conclude anything in this regard.

Regarding CCL5 and CCR5 KO mice there is quite some controversy in the literature. All previous studies so far showed no difference in BW gain or food intake between genotypes on either a SD or HFD (for studies that used HFD) nor any difference in leptin mRNA expression in the AT (only (Kitade *et al.*, 2012) tested for leptin expression) of $CCR5^{-/-}$ mice or $CCL5^{-/-}$ (Chou *et al.*, 2016) on a SD diet. This is in part comparable to our result as in SD condition, we have not found any difference between genotypes for BW neither, and no difference in food intake for CCR5 deficient mice on either diet. However, in contrast to aforementioned studies, CCL5 deficient mice on a SD diet had

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no difference in BW but showed a slight reduction in average food intake over 16 weeks of diet. So far and to the best of our knowledge, no study has yet examined the effects of CCL5 deletion in HFD-induced obesity. We showed that on average CCL5$^{-/-}$ mice consumed less g and kcal of HFD and gain strikingly less BW compared to WT controls. The reduction in food intake in HFD-fed mice might explain the reduction in BW we see for CCL5$^{-/-}$ mice. Interestingly, our data indicate that the difference in BW between CCL5$^{-/-}$ and WT mice on a HFD is greater than for CCR5$^{-/-}$ mice. This is an interesting result, especially since CCR5$^{-/-}$ mice show no effect on feeding behavior. This might suggest that the difference in BW gain between HFD-fed CCL5$^{-/-}$ and WT mice can be explained only in part by the difference in feeding behavior. Furthermore, the reduction in food intake that we observed for CCL5$^{-/-}$ mice might be mediated by one of the other receptors of CCL5. It would be interesting to perform similar DIO protocols for CCR1 and CCR3 deficient mice, to elucidate which receptor is responsible for the effect on feeding.

5. Hypothalamic neuromodulation by CCL5/CCR5 signaling

Our data on the effect of CCL5 and CCR5 deficiency on hypothalamic neuropeptide expression indicates that CCL5 might exert a neuromodulatory effect on hypothalamic neurons via direct or indirect manner, quite possible through CCR5 signaling. For example, we found a consistent reduction in orexigenic NPY mRNA expression for HFD-fed CCL5$^{-/-}$ and CCR5$^{-/-}$ mice. This fits with the reduction in food intake observed for HFD-fed CCL5$^{-/-}$ mice, but is conflicting with the only study so far, that has observed neuropeptide expression in CCL5$^{-/-}$ and CCR5$^{-/-}$ mice and showed an upregulation of hypothalamic NPY, AgRP and POMC mRNA expression in both KO mice compared to WT mice. However, the study by Chou and colleagues, has measured neuropeptide expression only in SD-fed mice. While, we saw an upregulation of hypothalamic orexigenic neuropeptide expression, it was for MCH and ORX in SD-fed CCL5$^{-/-}$ mice and no changes for CCR5$^{-/-}$ mice. These differences, including the contrasting results on feeding behavior in CCL5$^{-/-}$ mice could be due to differences in housing conditions of animal facilities,

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such as humidity but also in sacrificing protocols (whether mice were fed or fasted), which can affect feeding behavior and neuropeptide expression. In any case, both their and our results have in common that CCL5 and CCR5 deficiency has an effect on hypothalamic mRNA expression of neuropeptides that are implicated in energy, glucose metabolism and feeding regulation. In line with this, we found a marginal increase in AgRP expression and a significant increase in POMC expression after ICV infusion of CCL5, which provides further evidence for the neuromodulatory effect of CCL5. However, our cannula implantation experiment showed surprisingly and against our expectations that ICV CCL5 infusion seems to reduce BW in a dose-dependent manner in SD condition. Although only the highest dose (100ng) was significant while the lower dose of 50ng was only marginally increased, which could be explained with a reduction in food intake although our data on food intake is not conclusive (not shown). This result is contradicting the result we found in our HFD study in CCL5 and CCR5 deficient mice. If CCL5 or CCR5 deficiency leads to protection from HFD-induced weight gain, then the chronic infusion of CCL5 into the brain of WT mice should increase food intake and BW over time. However, this seems to not be true. Due to technical difficulties, we had only a small number of mice left for experimentation, as mice started to lose the guide cannula after around 2 weeks, indicating that long-term experimentation with guide cannula implantation into the brain might be difficult. We therefore decided to repeat the experiment and optimize the technique, for example by using a different dental cement for better adhesion and inject apart from CCL5, the antagonist ^{Met}CCL5. Furthermore, we observed that handling of mice during infusion as well as frequent infusion is stressful for mice and might lead to a decrease in BW. Thus, we increased handling and habituated mice for aCSF infusion, before we administered CCL5 and antagonist. However, when we repeated the experiment, we observed a similar effect as in the first experiment. Chronic (every 2nd day) infusion over several weeks of CCL5 (0.3ng) and ^{Met}CCL5 (0.3ng) both reduced the BW of mice on a SD, which is against our expectation. This confirmed the results of CCL5 infusion into brains of mice in

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reducing the weight gain; however, we expected that the antagonist would have the opposite effect. Although these results are contradicting our HFD studies in KO mice, these results are in line with other studies that have previously performed ICV injections of CCL5 and found that injection of 20ng CCL5 reduced short-term food intake in rats (Plata-Salaman and Borkoski, 1994). In another study, daily administration of CCL5 (0.92nM) for 5 days had no impact on food intake in rats, but when combined with the neurotoxic HIV glycoprotein 120 IIIB (HIVgp120IIIB), prevented the HIVgp120IIIB-induced reduction in food intake (Guzmán *et al.*, 2006). This study shows, different from our results and the previously mentioned study that CCL5 might have a role in food intake modulation, but only in a pathological context. To further speculate, that might explain why we did not observe an increase in food intake, as CCL5 might not induce an effect on its own but its effect might rather be evident in a pathological context such as chronic HFD. Another possibility is that CCL5 has a different concentration-depending effect. Since we do not know the exact concentration of CCL5 in the hypothalamus or aCSF of obese mice and it is technically difficult to find out, as very sensitive tests are required since inflammation associated with obesity is a low-grade inflammation, it is hard to know which concentration should be used to achieve an effect. Maybe the concentrations we used in chronic injections (between 10ng and 300ng) might be too high, and could instead mediate an acute inflammation. An acute inflammation could rather reduce food intake as was demonstrated in a previous study by our team, where acute ICV injections of CCL2 reduced BW and food intake in mice, mimicking the effects of LPS-induced inflammation (Le Thuc *et al.*, 2016). The same could be true in this experiment. While the concentration of CCL5 might be too high to mimic the chronic low-grade inflammation, and instead induces an acute inflammatory response, the concentration of antagonist used might impair the effect of endogenous CCL5, which in physiological concentrations is present but not abundant, and induce the actual expected effect of a reduction in BW as seen in our KO mice. However, this experiment is only preliminary and although it seems to suggest that CCL5 has a dual role

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depending on the context, further experiments and replications are necessary with higher number of animals to explain this phenomenon.

The difference in BW could be explained by the difference in food intake we observed between genotypes on a HFD, at least for CCL5 deficient mice. While CCL5 deficient mice consumed on average less kcals compared to their HFD-fed WT control, CCR5 deficient mice did not show any difference in food consumption. Thus, the question remains about the reason for the difference in BW. The other obvious possibility would be a difference in EE such as locomotor activity. Maybe CCR5 mice exert more activity and thus burn more calories, which translates into less BW. Another possibility would be that their basal metabolic rate, also a component of EE, is higher. Unfortunately, we do not have the equipment to measure basal metabolic rate. These questions have thus to be answered in additional future experiments with metabolic cages, that are able to measure the respiratory echange ratio (VO2/CO2) and give indirect indications on metabolism.

In addition to that, the change in NPY neuropeptide expression we saw in both CCL5 and CCR5 deficient mice during HFD challenge, suggests that CCL5 exerts its effect by binding to CCR5 and modulates either directly or indirectly NPY or MCH neuronal activity.

In support of its role as neuromodulator, more evidence has been shown in studies indicating that CCL5 can modulate the release of glutamate from both astrocytes and neurons (Musante *et al.*, 2008; Pittaluga, 2017).

We cannot exclude the possibility that microglia or astrocytes, who have been reported to express CCR5 receptor and secrete or produce CCL5 (Avdoshina *et al.*, 2010; Škuljec *et al.*, 2011; Sorce, Myburgh and Krause, 2011; Lanfranco *et al.*, 2018) to be the mediator between CCL5 signaling and modulation of neuronal activity. For example, studies report that direct activation of GFAP-expressing cells through calcium signaling pathways in the ARC leads to increases in food intake, while disruption of calcium signaling in GFAP positive cells results in diminished feeding behavior through the

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interaction with AgRP/NPY and POMC neurons (Chen *et al.*, 2016). This study indicates that glia might be more than just a support for neurons and have roles to play in the regulation of energy balance. Further experiments are required to shed light on this thematic.

6. The effect of CCL5/CCR5 signaling on glucose homeostasis

Intriguingly, our results have shown that both CCL5 and CCR5 deficiency seems to delay the HFD-induced glucose metabolism impairment and insulin resistance, as evidenced by a protection from glucose intolerance and insulin resistance in $CCL5^{-/-}$ and $CCR5^{-/-}$ mice at 8 weeks of diet. This is accompanied by a reduced overnight fasting blood glucose at 8 weeks of diet in both mice deficient of either CCL5 or CCR5 in HFD condition. Surprisingly, blood glucose levels are significantly increased in $CCL5^{-/-}$ and $CCR5^{-/-}$ HFD-fed mice after a short fasting period (6h) at 8 weeks of diet, but different from WT controls, they stay below the diagnostic margin of 150mg/dL for diabetes. In a previous study, authors argued that overnight fasting renders mice catabolic, where they lose a great percentage of their BW, which could improve the outcome of GTT testing at least in $CCR5^{-/-}$ mice because their lean body mass is correlated with glucose intolerance (Kennedy *et al.*, 2013). It is unclear so far if the same correlation is true for $CCL5^{-/-}$ mice on a HFD. In any case, despite elevated fasting blood glucose levels compared to their SD control, $CCL5^{-/-}$ mice had a more favorable outcome in ITT after a 6h fast both at 8 weeks and 16 weeks of diet compared to HFD-fed WT mice. Similarly, we found that $CCR5^{-/-}$ mice, although not significantly different from their HFD controls, had no significant difference in their insulin tolerance compared to their SD control group. This suggests that although glucose metabolism might be slightly impaired, they still are able to compensate for this impairment, due to mechanisms that remain unclear and remain in a non-diabetic state different from their WT controls on HFD. It might be possible that a CCL5/CCR5 dependent mechanism might be involved the regulation of hepatic glucose production to compensate for short periods of stress like fasting. CCR5 mRNA expression is expressed and upregulated not only in AT but also in liver cells in

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DIO mice (Kitade *et al.*, 2012). Furthermore, CCL5 and CCR5 have been implicated in the early development of HFD-induced hepatic steatosis and liver fibrosis. Hepatic stellate cells as well as other hepatic cell types can secrete CCL5 and thus contribute to a pro-inflammatory state and steatosis via CCR5 in healthy hepatocytes (Li *et al.*, 2017; B.-M. Kim *et al.*, 2018). This mechanism that induces hepatic steatosis, which is associated with obesity could also have an effect on hepatic glucose metabolism.

It might be possible that CCL5-CCR5 dependent signaling mechanisms might be involved in the hypothalamic regulation of hepatic glucose metabolism, for example by inhibiting glucose production. Thus, when CCL5/CCR5 is missing, hepatic glucose production "overcompensates" the hypoglycemic state of fasting in an already metabolically stressed environment. This compensation might be lost during prolonged fasting.

Previous studies have reported confounding results regarding the glucose tolerance of $CCR5^{-/-}$ and $CCL5^{-/-}$ on SD and HFD. Kitade and colleagues report an improved glucose tolerance and insulin sensitivity despite reduced insulin secretion after 16 weeks of HFD, which is similar to our results. Although our results show a similar protection of $CCR5^{-/-}$ mice from glucose intolerance in HFD fed mouse, there are some differences. Contrary to their data, we did not see any difference in glucose tolerance or insulin sensitivity in SD-fed $CCR5^{-/-}$ and WT mice. Furthermore, the improvements they observe are after 16 weeks of diet, whereas our resistance to impairment is only transient and visible at 8 weeks of diet, but no longer present later on, suggesting that the improvement they see is stronger and long lasting. Interestingly, they show similar to us a decrease in glucose-stimulated insulin secretion between HFD-fed genotypes, in particular, which is significantly different 60 min after glucose-stimulation. These differences could be due to variations in diet fat content, source and composition. Factors such as lipid nature (saturated vs unsaturated FAs) and ratio of ω-6/ω-3 have proven to be important factors in the pathogenesis of DIO and insulin resistance due to their different capacity to induce inflammation and lipotoxicity (Simopoulos, 2008, 2016; Gupta *et al.*,

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2012; Yan *et al.*, 2013). Another factor that might lead to variations is the age of diet initiation. While the authors used adolescent mice of 5 weeks, we used 12 weeks old mice. However, in some preliminary age-dependent experiments we found no differences between WT of $CCL5^{-/-}$ mice when initiating HFD exposure at an age of 4 weeks (not shown).

Furthermore, another study used 10 week old mice to start a HFD and reported the opposite effect, namely that $CCR5^{-/-}$ have a greater HFD-induced impairment in glucose tolerance compared to WT mice after 16 weeks of diet (Kitade *et al.*, 2012; Kennedy *et al.*, 2013).

Similarly, Chou and colleagues reported that genetic ablation of CCR5 or CCL5 as well as chronic ICV (3V) infusion of the antagonist ^{Met}CCL5 (7 to 14 days) rendered mice intolerant to glucose and insulin resistant compared to either WT mice or aCSF infusion (Chou *et al.*, 2016). However, they assessed glucose tolerance and insulin sensitivity only under SD condition (Chou *et al.*, 2016). This is in contrast to our results as we did not observe any impairment or difference in glucose tolerance or insulin resistance in SD-fed mice between $CCL5^{-/-}$, $CCR5^{-/-}$ or ^{Met}CCL5 ICV infused mice. These results are conflicting with both our study and the one reported before, even though they used older mice and had a HFD, with a similar kcal value as ours. In contrast, the former study had a different GTT protocol, with a shorter fasting period (5h) and glucose injections according to lean mass, while we fasted our mice overnight, so did the other study, and injected according to BW. Adding to further differences, the study by Chou and colleagues performed an oral GTT (oGTT), as opposed to the IP injected GTT (iGTT) that we, and the aforementioned study, used. While the iGTT is dependent on two factors, pancreatic insulin secretion and insulin sensitivity, it does not account for the incretin-induced potentiation of insulin secretion. Incretins are hormones that are secreted when nutrients pass through the gastrointestinal tract, where glucose is detected and leads to incretin release, which in turn enhances the insulin secretion by the pancreas. Thus, an oGTT provides information of whether an impairment could take place at the level of incretin signaling.

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Interestingly, the study by Chou and colleagues tested glucose tolerance in WT mice after infusion (ICV into 3V) of only the antagonist ^{Met}CCL5 but not CCL5, which blocked CCL5 signaling and rendered mice glucose intolerant and insulin resistant on a SD. A distinct study has reported that CCL5 can impair insulin secretion in mice by reducing the glucose-stimulated secretion of incretins such as GLP-1 and 2 in the intestines (Pais *et al.*, 2014). However, it is unlikely that ^{Met}CCL5 infusion would affect peripheral CCL5 action, but rather induce local effects in the hypothalamus. It is unclear yet, if CCL5 can regulate incretin function via efferent nerve fibers from the hypothalamus. Because this is unlikely, for our purposes an iGTT was sufficient.

In our study, we performed a similar experiment, where we implanted guide cannula into the lateral ventricle of WT mice to infuse every second day either the control solution (aCSF), ^{Met}CCL5, or CCL5 and tested 14-21d after, glucose tolerance via iGTT and insulin sensitivity via ITT. Our results differ from the previously reports, as we found no difference in glucose tolerance nor in insulin sensitivity between conditions. There are variations in experimental approaches that could explain the differences seen in our results compared to the other study. First, we used an infusion technique via implanted guide cannula to administer every second day the same antagonist as in the study, however at higher concentrations (300ng as opposed to 10ng/mL in the literature) while the authors of the other study used a more expensive osmotic pump to continuously infuse the molecule. While the osmotic pump is a great technique to continuously infuse a product, not like our choice to inject only every second day, the method has a disadvantage (Tanabe *et al.*, 1997; Dorf *et al.*, 2000; van der Meer *et al.*, 2000). Recombinant proteins often have a short half-life, which is why the supplier recommends the fresh preparation of the protein before usage. Osmotic pumps however are filled before implantation and the solution containing the protein are subjected to the temperature of the animal, which could affect the stability and half-life of the protein leading to different effects. Second, the difference in concentration of protein injected could lead to different effects. Inflammation was

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previously shown to affect energy metabolism differently depending on whether it is acute and high-grade or chronic and low grade. For example, while the chemokine CCL2 is well known for its role in the chronic low-grade associated with DIO, weight gain and insulin resistance, a study has shown, that when injected ICV acutely and at high concentrations, CCL2 induces anorectic effects leading to weight loss (Le Thuc *et al.*, 2016). This suggests that chemokines can have different concentration-dependent functions and might explain why we found no difference in glucose tolerance and insulin resistance after long-term ICV infusion of antagonist or CCL5. Third, we injected into the lateral ventricle while the study by Chou *et al* injected into the 3V, which is more specifically targeting the hypothalamus, even though it cannot be avoided that the product is diffused into other regions. However, we chose deliberately to inject a higher dose to compensate for injecting into the lateral ventricle, knowing that it will be diluted in the CSF of the mouse and the actual dose that might finally reach the hypothalamus is much lower. The lateral ventricle is also more accessible and inserting a cannula into the lateral ventricle instead of all the way to the ventral part of the brain could avoid excessive damage to the brain. However, an advantage of the Alzet pump is that handling of mice is not necessary for infusion and thus reduces the confounding factor of stress on the animal that can affect glycemia and food intake. Due to the redundancy of the chemokine system as mentioned previously, it might be possible that the effect of the antagonist was compensated by one of the other receptors or chemokines, that have been shown to be expressed in the CNS as well (Tanabe *et al.*, 1997; Dorf *et al.*, 2000; van der Meer *et al.*, 2000). Furthermore, the concentration of CCL5 and antagonist might not have been the right one to see the expected effect, or the preparation of the protein not adequate for this application. Injecting CCL5 in mice, that already have basal levels of the chemokine but not adapted numbers of receptors to accommodate the higher concentration of ligand, might thus be lost if CCL5 does not have a positive feedback effect to increase the numbers of the receptors. In addition to that, as described in the introduction, certain scavenger receptors exist that might play a role in

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decreasing the concentration of chemokines in the circulation. Similar mechanisms might be in place in the CNS, to avoid potential adverse effects due to prolonged exposure to high concentrations of chemokines. Glia cells such as microglia could take that role and take up and degrade excess amount of chemokines. Both hypoglycemia and hyperglycemia can be detrimental to health, thus evolution could have favored a highly regulated and redundant mechanism for insulin signaling and secretion to avoid situations such as hypoglycemia, and thus might require more than one chemokine to cause deregulation. Our result does not seem to suggest that centrally administered CCL5 has an impact on peripheral glucose metabolism, at least not in SD condition. However, these results represent only preliminary data, which have to be verified in additional experiments with further optimization and more animals. In fact, it might be possible that the effect of CCL5 or the antagonist will only be evident in a pathological context. Our data is suggestive of this, as CCL5 or CCR5 deficient mice show no difference in glucose tolerance or insulin resistance under SD condition but differences in HFD condition. Hence, it would be interesting to perform cannula-mediated ICV infusion of CCL5 and antagonist in obese mice and CCL5 deficient mice to test whether they could reverse the phenotype observed in this study in WT mice (induce weight loss upon antagonist ^{Met}CCL5 administration) and CCL5$^{-/-}$ mice (induce weight gain upon CCL5 administration). A few selective antagonist have been reported for receptors of CCL5, such as maraviroc for CCR5, CCX354-C for CCR1 and GW766994 for CCR3, which should be tested to identify the specific effects of each receptor via ICV infusion (Tak *et al.*, 2013; Marciniak *et al.*, 2015; Laudati *et al.*, 2017; Zhu *et al.*, 2017).

As mentioned above, other factors causing the differences in our data compared to literature in regard to glucose metabolism in HFD and SD-fed KO or WT mice, could be diet composition, type and amount of different lipids. Nevertheless, in spite of the differences between studies, it seems like CCL5-CCR5 signaling plays an important role in central and peripheral insulin

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signaling, especially in the context of DIO and the associated metabolic syndrome. Other studies are in line with the observed effect of our study, as it was reported that CCL5 reduces the glucose-stimulated secretion of incretins, such as GLP-1 and 2 in L-cells of the intestine via CCR1 signaling and thereby impairs insulin secretion (Pais *et al.*, 2014). Another study demonstrated that CCL5 can act as an agonist for another receptor called GPR75, which is expressed in addition to CCL5 in human and mouse pancreatic islet cells and can signal via Gq and PKC to elevate intracellular calcium and lead to insulin secretion. Interestingly, in addition to GPR75 and CCL5, healthy human and mouse islets expressed also other CCL5 receptors such as CCR1 for the former and CCR3 and CCR5 for the latter (Liu *et al.*, 2013). Furthermore, CCR5 expression in the pancreas was reduced by administration of anti-inflammatory cytokine IL-4. In addition to this, the temporal expression of chemokine receptors like CCR5 in pancreatic islets was associated with the progression to insulitis and T1DM in mouse models of T1DM (Cameron *et al.*, 2000).

In line with this, our results further show a reduction of IRS1 mRNA in response to HFD in obese and diabetic WT mice compared to their SD controls, but no difference between diets in $CCL5^{-/-}$ and $CCR5^{-/-}$ mice, and a significant increase for $CCR5^{-/-}$ mice on a HFD compared to their WT controls on a HFD. Similarly, hypothalamic PPARγ mRNA expression was increased in $CCL5^{-/-}$ mice independent of diet, and in $CCR5^{-/-}$ on HFD, compared to their respective WT control. While insulin is an important regulator of whole body glucose metabolism and inducing the uptake of glucose into different tissues, insulin action in the brain has two effects. First, it reduces food intake and BW when injected ICV in primates or administered intranasally in humans (Woods *et al.*, 1979; Benedict *et al.*, 2008). Second, it suppresses endogenous hepatic glucose production and improves peripheral glucose metabolism (Carey, Kehlenbrink and Hawkins, 2013; Kimura *et al.*, 2016). Brain-specific deletion of IR, as well as in hypothalamic neurons induces hyperphagia,

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hypertriglyceridemia, insulin resistance and enhanced DIO in rodents (Bruning *et al.*, 2000; Obici, Feng, Karkanias, *et al.*, 2002).

IRS is an important regulator of insulin signaling downstream of the IR. Insulin binding to IR induces autophosphorylation of the IR, thereby activating it and leading to the phosphorylation of IRS1. Depending on the phosphorylation of IRS1, it can either positively or negatively affect insulin signaling via PI3K/PIP3/Akt pathway. Impairments at the IR or IRS1 in the hypothalamus can lead to insulin resistance or glucose intolerance (Kubota *et al.*, 2004; Lin *et al.*, 2004; Chou *et al.*, 2016). Modulations at IRS1 thus might indicate that CCL5 can contribute to the insulin signaling regulation in the hypothalamus. Indeed a study has shown that CCR5 seems to associate with the IR in the hypothalamus of mice and thereby modulate pathways implicated in GLUT4 translocation and affect pathways including IRS via S6 kinase and AMPK, a transporter for glucose uptake (Chou *et al.*, 2016). In line with this, CCL5 has previously been reported to regulate cellular metabolism and glucose uptake via CCR5 activation and AMPK signaling in a PI3K/mTOR-dependent manner in T cells (Chan *et al.*, 2012). It was shown that CCL5 treatment can both increase metabolites such as glucose-6-phosphate and activate Akt, which in turn lead to an increase in GLUT1 expression in breast cancer cell lines and an increase in glucose uptake, glycolysis and ATP production in a CCR5 and mTOR-dependent way (Gao, Rahbar and Fish, 2016). Moreover, CCL5 treatment changed cellular metabolic activity to a state of anabolic metabolism with decreases in FA levels while increasing β-oxidation and the metabolite acetyl CoA, and downstream ketone bodies (Chan *et al.*, 2012; Chou *et al.*, 2016; Gao, Rahbar and Fish, 2016). All these studies show that CCL5 has a role in cellular metabolism and affects pathways that coincide with or can affect actors of the insulin signaling pathway. Thus, with the evidence presented including our observation of the modulation of IRS, it is not surprising that CCL5 can affect insulin signaling, most likely via CCR5 in hypothalamic cells. Interestingly, IR is expressed on hypothalamic neurons such as POMC/CART and NPY/AgRP producing neurons. It is intriguing to

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speculate that CCL5 could modulate insulin signaling due to its ability to modulate the expression of neuropeptides like NPY, as suggested by our data.

PPARγ is another factor that is seemingly affected by CCL5 or CCR5 deletion, under normal conditions for the former and HFD condition for the latter. PPARγ is a transcription factor, whose expression has been reported in particular in AT but also in kidneys, intestine, skeletal muscle, liver, macrophages and the brain and represents an attractive therapeutic target for metabolic syndrome (Dreyer *et al.*, 1993; Lu *et al.*, 2011; Xu *et al.*, 2017). In fact, it has been implicated as key regulator in adipogenesis, lipogenesis, glucose and lipid homeostasis, insulin sensitivity and inflammation as well as hypothalamic energy balance and food intake regulation (Stienstra *et al.*, 2007; Ryan *et al.*, 2011; Janani and Ranjitha Kumari, 2015). Interestingly, PPARγ expression was found in AgRP/NPY and POMC/CART expressing neuronal cell lines, and treatment with agonist led to excitement of AgRP and inhibition of POMC neuronal activity (Diano *et al.*, 2011). Moreover, while food deprivation in rodents increased hypothalamic AgRP and PPARγ expression, ICV or ip injection of the antagonist of PPARγ had a similar effect in rodents, namely increasing both AgRP and NPY expression and food intake. In line with this, antagonist injection, prevented the food deprivation-induced effect on hypothalamic neurons and food intake, without affecting ghrelin (Ryan *et al.*, 2011; Garretson *et al.*, 2015).

Activated by dietary lipids and their metabolites, this nuclear receptor induces the transcription of genes important for lipid and glucose metabolism (Ryan *et al.*, 2011). In WAT it is key in the regulation of adipogenesis, and upon activation promotes lipid storage (Xu *et al.*, 2017). It is an attractive potential target for metabolic syndrome, because it was found to be targeted by insulin-sensitizing drugs, used for diabetes treatment. It thus could play an important role in insulin sensitivity/insulin resistance, however it has two interesting side-effects confirming the results discussed herein: increase in subcutaneous WAT and increased food intake (Garretson *et al.*, 2015; Xu *et*

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al., 2017). Another interesting study used neuron-specific PPARγ deletion and found that HFD feeding in those mice led to decreased food intake, increased EE and resulted in reduced weight gain (Lu *et al.*, 2011). The authors thus concluded that HFD-induced weight gain is in part due to PPARγ signaling in neurons to both increase food intake and limit thermogenesis. This is in opposition however, with our finding that PPARγ expression is increased in the hypothalamus of HFD-fed $CCL5^{-/-}$ and $CCR5^{-/-}$ mice, which present with a decreased obese phenotype compared to WT mice that have lower PPARγ expression in the hypothalamus.

It might suggest though that the consumption of HFD can induce PPARγ activation. This in turn might be impaired in WT HFD-fed mice due to chronic exposure to HFD, while the lack of CCL5/CCR5 signaling in KO mice could prevent this disturbance in PPARγ signaling either upstream or downstream and thus avert the increases in food intake and adipogenesis. Another possibility is that PPARγ increase might be a compensatory reaction to a decrease in food intake in $CCL5^{-/-}$ mice compared to WT mice. Other studies reported that another partial agonist of PPARγ (DBZ), without the side-effects associated with the other compound, could prevent both LPS-stimulated inflammation and lipid accumulation in macrophages (Xie *et al.*, 2011). Other studies however, as mentioned previously in the introduction, suggested that PPARγ mediated the lipolysis inducing effect of TNF-a on adipocytes. These studies show that PPARγ might provide a link between inflammation and changes in metabolism.

Another study in agreement with our results, using the same partial agonist for PPARγ, showed that daily gavage of DBZ protected against HFD-induced DIO and associated metabolic impairments such as insulin resistance, while also stimulating BAT browning and maintaining the intestinal barrier integrity (Xu *et al.*, 2017). Interestingly, it was reported that constitutive activation of PPARγ in adipocytes leads to improved glucose tolerance and inflammatory profiles in DIO mice, without causing weight gain (Sugii *et al.*, 2009). In addition to that, other studies have reported anti-inflammatory properties for PPARγ, which

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might reduce pro-inflammatory cytokines production via interaction with NF-κB in monocytes (Jiang, Ting and Seed, 1998).

In summary, these results in addition to our data show that CCL5 and its receptors play an important role in insulin signaling, possibly by modulating IRS1 and PPARγ expression in hypothalamic neurons like NPY and indirectly or directly affect food intake and other components, leading to changes in BW and cellular metabolism of macronutrients. In the context of HFD-induced obesity, lipids could chronically activate PPARγ and thereby contribute to hypothalamic dysfunction like leptin resistance and insulin resistance as well as deregulation of food intake and energy balance. At the same time, it could increase adipogenesis in the periphery in an attempt to store the incoming excess of lipids, thereby saturating the limited capacities of AT, leading to inflammation and the development of metabolic syndrome. It seems like the link between PPARγ and CCL5 signaling could be found in macrophages, as they both express CCL5 receptors and PPARγ, and both seem to be important in the regulation of cellular macromolecule metabolism. Although there are some parallels between CCL5 and PPARγ signaling, and both seem to play important roles in DIO and associated insulin resistance, their potential interaction and the mechanisms behind it remain unclear and require further experiments to shed some light into the potential link between these two molecules.

Similarly, other chemokines like CCL2 and its receptor CCR2 have been shown to ameliorate obesity-induced insulin resistance if deleted or inhibited through genetic or pharmacological means or worsen it if overexpressed. These effects on insulin sensitivity seem to be linked to the ability of CCL2 to recruit macrophages into AT in obesity (Kamei *et al.*, 2006; Kanda *et al.*, 2006; Tamura *et al.*, 2008, 2010). However, for CCL2 there are also conflicting results in the literature. This indicates that the chemokine family forms a complex network of signaling molecules that interact with each other as well as other signaling molecules to exert their multifactorial functions that are far from limited to immune function. This complexity requires more sophisticated

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approaches that take into account the multiple interactions of chemokines to delineate the mechanism behind such a multilayered pathology as obesity. The redundancy of the chemokine system such as the fact that chemokines can bind several receptors, which in turn can bind several ligands suggests that chemokines are far from having a single function as chemoattractants and invites the possibility of different receptors mediating different functions. Nevertheless, this redundancy also permits the compensation of ligands or receptors if one is lacking, thereby potentially leading to confounding results or incomplete reversal of phenotypes in such studies. This might explain the confounding results found in different studies.

One of the remaining mysteries behind DIO pathogenesis is the fact that some individuals seem to be more resistant to the development of DIO, while others are more prone to it. In an attempt to solve part of this mystery, one group used a specific model of rats, that are bred into resistant and DIO prone animals. They found that these groups seem indistinguishable from each other in their phenotype on a SD diet but differed in their synaptic arrangement at the level of hypothalamic neuronal input of POMC and NPY neurons. They found that before rats are exposed to HFD that POMC and NPY neurons differ in their number of inhibitory and stimulatory inputs, which might make them more or less susceptible to DIO. Interestingly, they found that HFD induced a loss of synaptic inputs on POMC and NPY neurons, which was either inhibitory or stimulatory, respectively. Furthermore, these lost inputs on neuronal perikarya where replaced by astrocytic processes, which might impair peripheral signals from reaching these neurons and thereby contribute to a resistance to peripheral satiety or hunger signals like leptin, ghrelin or insulin (Horvath *et al.*, 2010). This might be one of several reasons for the variations of results demonstrated by different studies, like our results in glucose tolerance from the ones of other studies. Studies from different countries often have inbred colonies of mice, which despite being the same strain of mice, could accumulate subtle but important changes and thus

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cause differences in their susceptibility to DIO between groups of mice from different laboratories.

7. The role of CCL5/CCR5 signaling in HFD-induced changes in adipose tissue

Interestingly, DIO induces many changes in AT, like hypertrophic adipocytes and adipogenesis, in an attempt to store the increasing influx of lipids from HFD but also inflammatory changes, like infiltration of immune cells into AT, their activation and polarization to a pro-inflammatory state and production of pro-inflammatory mediators. These changes become evident in an increased amount of AT but also morphological changes of adipocyte number and size. We wanted to know whether the reduction in BW in HFD-fed CCL5 and CCR5 deficient mice is reflected in their WAT. Immunohistological assessment of subcutaneous AT revealed that CCL5$^{-/-}$ and CCR5$^{-/-}$ mice have a slightly shifted frequency distribution of adipocytes from many very small adipocytes to less but slightly bigger adipocytes in SD conditions, which is not significant though, when comparing average adipocyte size between genotypes. This finding was different from expectations, as we expected to find smaller adipocytes in CCL5$^{-/-}$ and CCR5$^{-/-}$ mice. It suggests that CCL5/CCR5 signaling might play a role in the regulation of some of these changes that translate into a shift in morphology. These changes could include adipogenesis, lipid uptake and storage, or lipolysis. Similarly, small differences are visible between KO mice and WT mice on a HFD. Histological images as well as the frequency distribution seem to indicate that CCL5$^{-/-}$ mice have more adipocytes that are slightly smaller than WT mice, whereas CCR5$^{-/-}$ mice have more adipocytes that are slightly bigger than WT mice. However, both KO mice seem to have a few smaller adipocytes as wells as a lower maximum size of adipocytes than WT mice on a HFD. These changes are interesting, although not significant. However, we found a significant increase in free serum glycerol in both HFD-fed CCL5$^{-/-}$ and CCR5$^{-/-}$ mice compared to WT mice on a HFD, which is indicative of increased lipolysis in KO mice. These results could be due to the higher expression of PPARγ mRNA that is known for its role in adipogenesis and could lead to morphological

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changes through lipolytic activity. This result is surprising as no changes in average adipocyte size were detected between HFD-fed animals, indicating that all of them have increased adipocytes due to the higher load of lipids in their diet. In support of our results, previous studies reported no difference in adipocyte size between HFD-fed CCR5$^{-/-}$ and WT mice (Kitade *et al.*, 2012).

8. The role of CCL5/CCR5 signaling in HFD-induced inflammation

The HFD-induced changes in AT of obese mice are associated with infiltration and accumulation of macrophages and T cells in AT as wells as upregulation of cytokines, most notably, TNF-a, IL-1β and IL-6 as well as chemokines like CCL2, CCL3 and CCL5 and their receptors such as CCR2 and CCR5. The inflammation is not limited to AT, but can be found systemically, as increased immune factors in serum of obese humans and mice, as well as other metabolic tissues and the hypothalamus. Due to the pro-inflammatory nature of CCL5 and its well-known contribution to AT inflammation, we expected a reduced inflammatory profile in AT, serum and hypothalamus of CCL5$^{-/-}$ and CCR5$^{-/-}$ mice after HFD, compared to WT mice. In support of previous reports, we found a HFD-induced upregulation of inflammatory markers like IL-6 and IL-1β and CCL5 as well as CCL2 in PG AT, but surprisingly, they were not different between WT and CCL5$^{-/-}$ mice, apart from a reduction in TNF-a in CCL5$^{-/-}$ mice on a HFD. We have not found any HFD-induced increase in inflammatory markers apart from CCL2 in CCL5$^{-/-}$ mice and a trend for increase in WT mice, although not significant. Similar to reports in literature, serum CCL5 was increased in HFD-fed WT mice compared to their SD control.

Surprisingly, CCL5$^{-/-}$ mice showed no differences in serum levels of cytokines compared to WT mice, but an increase in the chemokine CCL2 compared to their SD control. Although not significant, we saw the same tendency in WT mice on a HFD. Furthermore, IL-10 an anti-inflammatory cytokine is significantly reduced in CCL5$^{-/-}$ mice on a HFD. This suggests that CCL5 could be involved in the anti-inflammatory feedback response to inflammation, which is reduced in these mice due to CCL5 deficiency.

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Surprisingly, we found a different inflammatory profile for CCR5$^{-/-}$ mice in serum, as we have not determined the AT inflammation for those mice yet. In this group of mice, we found an increase in serum TNF-a levels for HFD-fed mice, without any difference between genotypes.

Contrary to our expectations, our results indicate, that despite a decrease in TNF-a levels in HFD-fed CCL5$^{-/-}$ mice, long-term HFD feeding induces inflammation in PG AT but not in serum of both WT and CCL5$^{-/-}$ mice. In agreement with the literature and previous findings of our team, we found increases in serum levels of CCL5, confirming its role in DIO associated inflammation. Previous studies found that CCL5 mRNA expression in WAT correlated positively with other cytokines like TNF-a and IL-6 in human obese patients (Keophiphath *et al.*, 2010). Our results are in part contradicting these results, as the lack of CCL5$^{-/-}$ did not prevent the increase of IL-6, but only of TNF-a.

This indicates that at least in mouse WAT, IL-6 increase as well as other cytokine regulation might be independent of CCL5 signaling. While we have not determined the inflammatory state of CCR5$^{-/-}$ WAT yet, our results are in part fitting to reports of other studies, who found a decrease in TNF-a levels of HFD-fed CCR5$^{-/-}$ mice compared to WT mice in WAT but no difference between IL-6 (Kitade *et al.*, 2012). This is similar to the results observed in our CCL5$^{-/-}$ mice. However, another study reported no changes in inflammatory markers in AT of HFD-fed WT and CCR5$^{-/-}$ mice. While we have not determined the phenotype of immune cells and their infiltration into the WAT of our mice, conflicting reports exist on infiltration of AT macrophages in CCR5$^{-/-}$ mice. While one study reports no reduction in the infiltration of macrophages into AT of CCR5$^{-/-}$ mice on a HFD, another study found that lacking CCR5 on a HFD led to a reduced infiltration of macrophages into AT compared to WT mice (Kitade *et al.*, 2012; Kennedy *et al.*, 2013). However, results are more consisting in regard to the reduction in pro-inflammatory macrophages. Moreover, one of the studies has reported apart from a reduction in macrophages on HFD, a reduction in different macrophage markers as well as TNF-a levels in WAT of

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HFD-fed WT mice that received a bone-marrow transplant from CCR5$^{-/-}$ mice, which was associated with improved glucose tolerance (Kitade *et al.*, 2012). This indicates that infiltration of CCR5-expressing macrophages is an important contributor to AT inflammation and glucose intolerance in DIO.

Similarly, in line with previous reports, we found upregulation of inflammatory cytokines in the hypothalamus of HFD-fed mice compared to SD controls, but no difference between CCL5$^{-/-}$ and WT mice. However, results were a little bit different for hypothalamic cytokine and chemokine expression for CCR5$^{-/-}$ mice. We found a decrease in IL-1β expression as well as a marginal but not significant decrease in IL-6 mRNA levels in the hypothalamus of CCR5$^{-/-}$ after 16 weeks of HFD compared to WT mice. Due to the implication of glia cells in hypothalamic inflammation and the association with hypothalamic gliosis, we tested the expression of the commonly used markers of astrocytes (GFAP) and microglia (Iba1) after 16 weeks of HFD in CCL5$^{-/-}$ and CCR5$^{-/-}$ mice. Interestingly, although we have not seen a significant increase in glial marker expression in hypothalami of HFD-fed mice but only a marginal and not significant increase in GFAP in HFD-fed WT mice, we found a significant reduction in GFAP expression in HFD-fed CCL5$^{-/-}$ mice compared with WT controls, but not in CCR5$^{-/-}$ mice. This result suggests that CCL5 might affect GFAP expressing glia cells, but this effect might be a CCR5-independent, although astrocytes have been reported to express CCR5 (Sorce, Myburgh and Krause, 2011). Adding to these results, chronic ICV injections of CCL5 induced an upregulation of Iba1 in our experiment. Although it is a preliminary result with low number of animals, due to technical difficulties in the cannula implantation technique, is suggests that CCL5 might have a modulatory role on glia cells such as astrocytes and microglia. In support of this, astrocytes, neurons and microglia have been reported to express and release CCL5 but also receptors of CCL5, suggesting an autocrine or paracrine signaling mechanism (Guillemin *et al.*, 2003; Škuljec *et al.*, 2011; Sorce, Myburgh and Krause, 2011; Louboutin and Strayer, 2013; Lanfranco *et al.*, 2018). Furthermore, a study found that CCL5 stimulated both chemotaxis and

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increased NO secretion while decreasing IL-10 in activated microglia (Škuljec *et al.*, 2011).

So far, to the best of our knowledge, no study has tested hypothalamic inflammation or gliosis in CCR5$^{-/-}$ or CCL5$^{-/-}$ mice after long-term HFD feeding. However, in a previous study, our team (Cansell et al., 2019; see annex), investigated the post-prandial effects of HFD on the hypothalamus and found that lipid-rich diet evokes post-prandial inflammatory and neuromodulatory responses involving both astrocytes and microglia. Interestingly, upregulation of GFAP-expressing cells was found as early as 1h after HFD exposure, which coincides with upregulation of hypothalamic CCL5 expression. This study indicates that HFD can induce inflammatory changes only hours after diet ingestion and implicates CCL5 in this response. It further suggests that the inflammatory response observed, might be part of the physiological regulation of energy balance. Our results suggest that the expression of cytokines and chemokines are dependent on a multifactorial and complex regulation, which cannot be simply explained by one factor deletion. However, judging from the results presented herein and the reports of the literature in regards to HFD-induced inflammatory processes as well as the in part conflicting data on CCR5$^{-/-}$ mice, it seems that inflammatory cytokine expression is either upstream of chemokine release or in part independent of chemokine action. Reports from the literature as well as unpublished results of our team (not shown), suggest that HFD induces early inflammation in the hypothalamus, which precedes the development of obesity and associated changes in AT and insulin sensitivity (Thaler *et al.*, 2012). Although it is still under debate, whether hypothalamic inflammation is induced locally or is initiated by infiltrating immune cells, some studies report a HFD-induced 30% increase in the recruitment of peripheral immune cells into the hypothalamus, the majority of which have a phenotype similar to microglia/macrophages (Buckman *et al.*, 2014). Hypothalamic inflammation in DIO is apart from its early onset characterized by similar events as peripheral metabolic tissues, namely accumulation of lipids in the hypothalamus, expression of pro-

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inflammatory cytokines and chemokines, infiltration of immune cells, change into a pro-inflammatory phenotype of immune cells and in the case of the hypothalamus of astrocytes and microglia as well as an upregulation of their characteristic proteins GFAP and Iba1, resistance to hormones (leptin and insulin) and some studies also suggest apoptosis via ROS production, mitochondrial abnormalities and ER stress.

Our data indicate that CCL5 and CCR5$^{-/-}$ deficiency does attenuate and delay the development of obesity and insulin resistance, but still shows some cytokine upregulation, in the hypothalamus, AT or circulating levels, which seem to not be consistent. Furthermore, CCL5/CCR5 deficient mice have no significant differences in WAT size and only little effects on the frequency distribution of adipocyte size but significantly increases lipolysis. One possibility for the observed effect is, that the difference in BW is not attributable to the size of adipocytes but could be due to a difference in total amount in AT. Although not shown here, our data indeed shows a reduction in total AT volume in WAT of CCR5$^{-/-}$ mice on HFD compared to WT mice, but no difference between CCL5$^{-/-}$ mice and WT mice after 16 weeks on HFD. The difference in AT volume seems to be mainly due to a decrease In SC AT in CCR5$^{-/-}$ mice. This finding seems to be in line with the increase in lipolysis found for CCR5$^{-/-}$ mice. However, the question remains as to what happens with the released FFAs. An increase in the release of FFAs can cause ectopic lipid accumulation and is associated with insulin resistance due to lipotoxicity. Although both KO mice had improved glucose tolerance and insulin sensitivity, this difference was after around 8 weeks of diet, whereas the body composition X-ray and lipolysis test was performed only at the end of the experimental protocol. After 16 weeks of diet both CCL5$^{-/-}$ and CCR5$^{-/-}$ mice showed no difference in glucose tolerance to HFD-fed WT. Furthermore, CCL5$^{-/-}$ mice although still significant were much closer in BW to WT mice, which might explain why we did not find any significant difference in AT volume for CCL5$^{-/-}$ mice. Although further experiments are necessary, it seems that lipolysis might be in part responsible for the decrease in SC AT and might

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explain the development of glucose intolerance for both CCL5$^{-/-}$ and CCR5$^{-/-}$ mice. The question remains to why this difference develops only later on. As mentioned before, CCL5 signaling was implicated in cellular metabolism in CCR5-expressing T cells and breast cancer cell lines, where it seemed to regulate glucose uptake and lipid metabolism. Another study reported that CCL5 contributes to GLUT4 translocation in the hypothalamus, a protein that is involved in glucose uptake as well. Since AT and other metabolic organs express and secrete CCR5 and CCL5, it could be possible that CCL5 signaling might be involved in lipid metabolism, especially seeing as macrophages also express CCR5 and have been reported to accumulate lipids, too. In any case, further experiments have to be performed to investigate the body composition at an earlier time in KO mice to see, whether differences in adiposity and adipocyte morphology can be found, while differences in BW are highest. Another possibility might be that the difference in BW could be due to an increase in basal metabolic rate, thermogenesis or locomotor activity and thereby contribute to a reduction in lipid excess and protecting from lipotoxicity-induced glucose metabolism impairment. This could be a possibility concerning the implication of CCL5/CCR5 signaling in lipid metabolism and oxidation. Interestingly, some studies have found that NPY-expressing neurons in the DMN of the hypothalamus are implicated in regulating not only food intake but also EE like locomotor and BAT thermogenic activity. These studies demonstrated that knockdown of NPY in neurons of the DMN resulted in a decrease in BW combined with browning of WAT mediated by the SNS that innervates AT. This was further accompanied by increased thermogenesis in BAT and locomotor activity and associated with improved glucose homeostasis and insulin sensitivity (Chao *et al.*, 2011; Bi, 2013). Our results suggest that CCL5/CCR5 might have a modulatory role on NPY neurons and are expressed in different nuclei of the hypothalamus. Thus, it is intriguing to speculate that CCL5/CCR5 might be implicated in this function of NPY circuitry and could modulate thermogenesis or locomotor activity. This possibility has to be verified in additional experiments.

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Furthermore, although surprising and against our hypothesis, the upregulation of some cytokines and chemokines still observed in CCL5$^{-/-}$ and CCR5$^{-/-}$ mice is not necessarily indicative of a lack of effect on inflammation in both hypothalamus or peripheral organs. As suggested previously, lipotoxicity leads to inflammation, initially in the hypothalamus and if not resolved, later on also in peripheral metabolic tissues. Lipids have the capacity to activate different inflammatory pathways like TLR or IKKB, and activation of immune cells, which leads to the upregulation of cytokines and chemokines. First, the inflammatory response is a complex response including several different cells and mediators. Chemokines are only one of those mediators and although important, one chemokines might not be able to resolve the inflammatory response completely. Second, studies have shown that cytokines like TNF-α, IL-1β and IFN-γ, can induce the expression of chemokines like CCL5 (Sarkar *et al.*, 2012). These results suggest that chemokines could be induced secondary to cytokine production and thus rather act as a mediator between cytokines and the downstream inflammatory response leading to alterations in the function of various tissues. This is in line with the results shown in the literature that the deletion of chemokines reduces the infiltration of immune cells and leads to a shift in their phenotype to a less activated or anti-inflammatory phenotype. As chemokines are well known for their chemoattractive role, it makes sense that CCL5 might mediate the inflammatory response by recruiting immune cells like macrophages, monocytes, and T cells and activate them in the periphery but also in the brain. In the brain, glia cells that express chemokine receptors including CCR3 and CCR5 might thus be part of the inflammatory response either upstream or downstream of chemokine action, but as they have been shown to also secrete CCL5, it might also be an autocrine feedback loop, that potentiates the inflammatory response. This is in line with our results that seem to indicate that ICV infusion of CCL5 or deletion of CCL5 modulates the expression of GFAP during a HFD challenge. Thus, it is still possible that HFD can still induce an initial inflammatory response both in the brain and AT, but that this response is not potentiated and with the lack of one of the downstream mediators like CCL5/CCR5, the attenuated

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inflammatory response might thus not translate into a disrupted metabolism as seen in obese WT mice. Indeed, as mentioned previously, the lack of one actor of the inflammatory response might, help understand the implication of this specific actor and might explain the partial amelioration of the phenotype but is not sufficient for a full recovery of the healthy phenotype. This is especially the case since the chemokine system is such a redundant system with many overlaps in signaling pathways, ligands and receptors. Thus, experiments have to be more sophisticated and adapted to this complex process of inflammatory regulation in order to answer the remaining questions.

9. The role of CCL5/CCR5 signaling in thermal pain sensitivity

Lastly, apart from their role in immune function and as potential neuromodulators, chemokines and their receptors such as CCL5, CX3CL1, CCL3, CCR2 among others have also been implicated in hyperalgesia, although the mechanism behind their ability to modulate pain responses is still unknown (Zhang *et al.*, 2004; Staniland *et al.*, 2010; Lee *et al.*, 2013). Chemokine receptor expression has been reported on primary sensory neurons and their activation by chemokines associated with hyperalgesia (Lee *et al.*, 2013).

Several studies have investigated and associated CCL5 and CCR5 with neuropathic pain in different contexts and phenotypes. Previous reports showed that CCR5 has a possible association with the μ-opioid receptor and thereby can modulate nociceptive pain responses.

In collaboration with J. Noël in the team of E. Lingueglia at IPMC, who specialize in the study of pain, we performed experiments to assess another comorbidity in obese and diabetic patients, namely the occurrence of neuropathic pain. Their team has observed in many experiments that HFD feeding in mice induces neuropathic pain after about 8 weeks of HFD, which becomes evident and measurable in a thermal pain sensitivity test called Hargreaves' Test. We employed this test to confirm the increased pain

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sensitivity to thermal pain in HFD fed WT mice and tested whether the lack of CCL5 and CCR5 might be implicated in the HFD-induced increase in the sensitivity to pain. Indeed, we found that HFD-fed WT mice become more sensitive to thermal pain after 16 weeks of diet, whereas both $CCL5^{-/-}$ and $CCR5^{-/-}$ mice show an amelioration of this hypersensitivity. We further tested whether CCL5 and antagonist ^{Met}CCL5 ICV infusion in SD-fed mice could alter the sensitivity to thermal pain. In line with the genetic deletion experiment, we found that CCL5 infusion increased thermal pain sensitivity, while ^{Met}CCL5 infused mice showed no difference in their thermal pain sensitivity to aCSF infused mice. Although, this is a preliminary result with low number of mice and has to be repeated together with our genetic deletion study, our results show that CCL5 and CCR5 seem to have a role in HFD-induced thermal hyperalgesia. We further examined the expression of inflammatory factors in the spinal cord and dorsal root ganglia of $CCL5^{-/-}$ and $CCR5^{-/-}$ mice under HFD and SD conditions to answer whether the alteration of thermal pain sensitivity due to CCL5 and CCR5 might be due to HFD-induced inflammation. However, against our expectations we have not found any HFD-induced upregulation in pro-inflammatory markers in DRG of WT nor $CCL5^{-/-}$ mice. Similarly, we did not find inflammatory marker expression in the spinal cords of obese WT mice, but an increase in expression of pro-inflammatory markers like IL-1β, CCL2 and TNF-a in the spinal cords of HFD-fed $CCL5^{-/-}$ mice and TNF-a in $CCL5^{-/-}$ mice on a SD. This result is rather surprising and counter-intuitive, and requires further testing to be able to explain this phenomenon, as the number of mice is low and represents the results of only one independent experiment. The increased inflammatory response could be explained as mentioned above in that CCL5 is a downstream mediator of the inflammatory response that induces the recruitment of immune cells and thereby potentiates the inflammation as well as mediates the dysfunctions induced by inflammation. Thus, one of the limitation of our study is that we have not tested the infiltration of immune cells and glia cells as well as their change into an activation state in DRG of mice. However, a study has shown similar results to ours, although the experimental model differed. The study used instead of DIO

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a model of PNL to induce inflammation and neuropathic pain in the injured site in both WT and CCL5$^{-/-}$ mice. In line with our results the authors report an attenuated response to thermal pain but also mechanical pain in CCL5$^{-/-}$ compared to WT mice (Liou *et al.*, 2012) Furthermore, they report different from our results that CCL5 deficiency decreased both recruitment of immune cells as well as pro-inflammatory cytokine and increased anti-inflammatory marker production in the sciatic nerves. This was accompanied by a decrease in the mRNA expression of endogenous opioid peptides. In line with our result, another study performed CCL5 administration into the periaqueductal gray (PAG), a critical area for mediating the analgesic effect of opioids, of the brain in rats. They found that this reduced the effect of an anti-nociceptive agent (DAMGO), that acts as agonist of μ-opioid receptor and rendered rats more sensitive to painful stimuli (Szabo *et al.*, 2002; Lee *et al.*, 2013). This study in line with our cannula experiment suggests that the effect of CCL5 could act at the levels of the brain and not only in the periphery and further indicates that the mechanisms behind the effect of CCL5/CCR5 on pain sensitivity might be related to sensitization/desensitization of opioid receptors like the μ-opioid receptor. Further evidence for this hypothesis was provided by a study, demonstrating that opioid receptors are expressed in several regions of the CNS such as cerebral cortex, hippocampus, locus coeruleus, dorsal root ganglia, striatum and the PAG. They further report that opioid receptors can interact and form heterodimers with chemokine receptors, resulting in desensitization or other effects on both receptors through binding of one of the ligands (Chen *et al.*, 2004).

In partial agreement with our results another study showed that CCR5 deletion in mice lowered nociceptive pain response, and found enhanced expression of μ-opioid receptor in PAG of CCR5$^{-/-}$ mouse brains compared to WT control (Lee *et al.*, 2013). However, different from our experiments they have not found any difference in thermal pain sensitivity between WT and CCR5$^{-/-}$ mice but rather in chemical- and inflammatory pain (Lee *et al.*, 2013). Ip administration of the antagonist ^{Met}CCL5, in an inflammatory pain model of

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nerve damage attenuated the hypersensitivity to pain after PNL, which was concurrent with decreased infiltration of immune cells, inflammatory marker expression and opioid agonists like enkephalins and endorphins (Liou *et al.*, 2012). These results provided by our study and the literature strongly suggest the involvement of CCL5 in different forms of neuropathic pain sensitivity. Furthermore, it seems like inflammatory pain is reduced by CCL5 or CCR5 deficiency, as it reduces infiltration of immune cells to the site of damage and the lack of inflammatory response potentiation might diminish hyperalgesia, possibly via interaction with opioid receptors. Furthermore, the evidence provided indicates that the effect of CCL5 might not only be in the periphery but also can act on analgesia pathways at the level of the brain. Our study has added to our knowledge the first evidence of the importance of CCL5/CCR5 signaling in the modulation of HFD-induced hyperalgesia associated with obesity and diabetes.

Based on our results and previous studies we hypothesize that CCL5 can act via CCR5 but also other receptors in nuclei of the hypothalamus to affect the activity of neurons such as NPY-expressing neurons either by directly modulating their activity via chemokine receptors or indirectly through modulating glia cell activation. Moreover, CCL5 seems to act on IR expressing neurons in the hypothalamus to regulate glucose homeostasis and insulin sensitivity. CCL5/CCR5 signaling might thus affect food intake either through the modulation of neuropeptide expression in neural networks important for energy balance and feeding regulation or indirectly by affecting insulin and glucose homeostasis. Furthermore, is seems to act in the periphery in immune cells, AT, pancreas and intestine to regulate cellular metabolism, immune function and insulin secretion via incretins. The multitude of different results seems to suggest that chemokines such as CCL5 have different functions based on their concentration, for example in acute and chronic inflammation chemokines seem to have potentially opposing effects. Furthermore, their function might be different depending on tissue, cell and receptor interaction.

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Previous results have shown impaired T cell and macrophage infiltration into the CNS of hepatitis virus-infected mice that were depleted of CCL5 (Lane *et al.*, 2000). Similarly, CCL5 could be involved in the sustaining or mediation of hypothalamic inflammation as a downstream mediator, induced by cytokines, and recruiting blood-borne T cells and macrophages into the CNS. In this way, it could contribute to hypothalamic inflammation, worsening it or rendering it chronic as infiltrated cells can in turn induce cytokine and chemokine production. Depending on the intensity of the stimulus, CCL5 signaling could mediate acute or chronic inflammation. Similar to the controversial and dual effect observed for CCL2 in both acute inflammation, where it induces fever and weight loss, but also is important for the development of obesity, CCL5 signaling could induce different effects with different outcomes, such as weight loss in acute hypothalamic inflammation and weight gain in chronic inflammation. In particular, one could imagine different outcomes for different CCL5-receptor interactions. It would be interesting to investigate the effects of CCL5 signaling in different KO models for each receptor or even a double KO of two of the receptors of CCL5 to pinpoint the specific effect of each receptor of CCL5. To circumvent the problem of receptor or ligand promiscuity, one could even try to combine receptor or ligand double knockouts with antibody-mediated inhibition of other ligands for the same receptor and vice versa as CCR5 binds to other chemokines apart from CCL5. In any case, this would require more complex and time-consuming but very intriguing experiments.

In summary, our study has taken a broad approach to investigate different aspects of the role of chemokine CCL5 and its receptor in the context of HFD-induced obesity and associated pathologies like diabetes and neuropathic pain. We have contributed to advancing the knowledge on the multiple functions of chemokines by showing that CCL5 and CCR5 are both important actors in HFD-induced obesity and associated metabolic syndrome and can act at different locations to contribute to weight gain, insulin resistance, thermal pain sensitivity and modulation of neuronal activity to affect energy

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balance regulation. We thus have provided evidence confirming that chemokines can indeed have a multitude of functions apart from their role in immune system and that the redundancy of the chemokine system and the complexity of the immune system requires more sophisticated experiments than just one factor elimination to unravel the exact mechanism behind chemokine action.

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Conclusion & Perspectives

The present study aimed at elucidating the role of chemokines in the CNS. In particular, we were interested in the role of chemokines in the regulation of energy balance and their contribution to its deregulation as is the case in modern disorders like obesity. Several studies have suggested that chemokines are more than just recruiters of leukocytes in the immune system and might be actors in the physiology of brain function. We have taken a global approach in an attempt to characterize the phenotype associated with mice deficient in CCL5 or CCR5 by investigating different aspects of the complex disorder obesity. We have studied both HFD-induced inflammation in the periphery as well as in the hypothalamus and looked at the modulation of neuropeptides in the hypothalamus by diet and by CCL5 deficiency. We assessed central expression of CCL5 and CCR5 as well as glucose metabolism and HFD-induced neuropathic pain, associated with diabetes. Herein we have found that both CCL5 and CCR5 are expressed in hypothalamic nuclei such as the ARC, LH, ME among others in adult mice under SD conditions and their expression seems to be upregulated in HFD condition, although this has to be confirmed. Furthermore, we showed that although deleting either CCL5 or CCR5 did not necessarily change the expression of cytokines it yet greatly improved the HFD-induced obesity phenotype by reducing weight gain and delaying the glucose intolerance and insulin resistance associated with DIO. In accordance with this, we see also differences at the level of the brain in the expression of genes important for lipid metabolism and insulin signaling and sensitivity. Lastly, we have seen also improvements in another aspect associated with obesity – neuropathic pain. This suggests that although inflammation is crucial for the development of obesity as shown in the literature, chemokines are crucial as a secondary mediator after the initial inflammatory insult, which might contribute by recruiting blood-borne immune cells and/or activate microglia and astrocytes and thereby potentiating the inflammation. Studies have previously shown that CCL5 induces a pro-

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inflammatory state in microglia and can be secreted by astrocytes and microglia. This is confirmed by our preliminary results showing upregulation of microglia markers after CCL5 injections and in long-term HFD condition by a decrease in astrocyte marker expression in CCL5 deficient mice. To confirm these results, it would be interesting to verify whether CCL5 and CCR5 deficient mice prevent the infiltration of peripheral and local immune cells and their activation including glia cells such as microglia and astrocytes via immunohistochemistry using specific markers for each marker. Furthermore, it would be interesting to inject CCL5 and the antagonist ^{Met}CCL5 in CCL5$^{-/-}$, CCR5$^{-/-}$ and WT mice on a HFD to see whether it would reverse the phenotype seen in our study. Likewise, because ^{Met}CCL5 antagonizes the effect of CCL5 on all the receptors, our results should be completed with experiments using specific antagonists for each receptor. Further experiments to complete our results by studying earlier time points in the development of obesity are necessary, as hypothalamic inflammation occurs early in the development of obesity. Our data should be supplemented with tests of serum leptin levels in CCR5 mice as well as testing whether CCL5 and CCR5 deficient mice are leptin resistant.

Our results further indicate that CCL5 might act on orexigenic neurons to affect food intake, which seems to be CCR5 independent and rather involving one of the other receptors that CCL5 might bind to. Thus it would be interesting, to perform similar experiments with mice deficient in the other receptors for CCL5, namely CCR1 and CCR3 in a HFD context to identify, which receptor mediates the effects of CCL5 on food intake, BW and glucose metabolism regulation. While our results indicate that CCL5 deficiency seems to improve glucose tolerance and insulin resistance in a HFD context, other studies suggest that CCL5 receptors may be associated with the IR in the hypothalamus and thereby affect insulin signaling directly. However, our results are not conclusive if glucose metabolism is improved due to a potential attenuation of downstream inflammation or due to effects on insulin-expressing cells such as NPY/AgRP and/or POMC/CART neurons as is

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indicative with our results. It would be indeed important to confirm our results with Alzet pumps, which provide a more consistent infusion of product while reducing confounding factors such as stress.

In summary, we conclude that chemokines like CCL5 are important actors in the deregulation of energy balance, whether it is in an acute way as we have shown with CCL2, which mediates the LPS-induced anorexic effect in the hypothalamus (see annex, (Le Thuc *et al.*, 2016)), or in the chronic low-grade inflammation as seen with obesity. Furthermore, other authors suggest that chemokines might have a role as neuromodulators in a physiological context. Our results are consistent with this hypothesis as well as another article that we have submitted recently, which I have contributed to, too (Cansell et al., 2019; see Annex). It seems fitting to speculate that chemokines like CCL5 can act as neuromodulator in physiological conditions either by acting directly on neurons or indirectly through chemokine receptors expressed on astrocytes and microglia, or in a pathological context by potentiating the inflammatory response in the hypothalamus or peripheral tissues by recruiting other immune cells (Fig. 65).

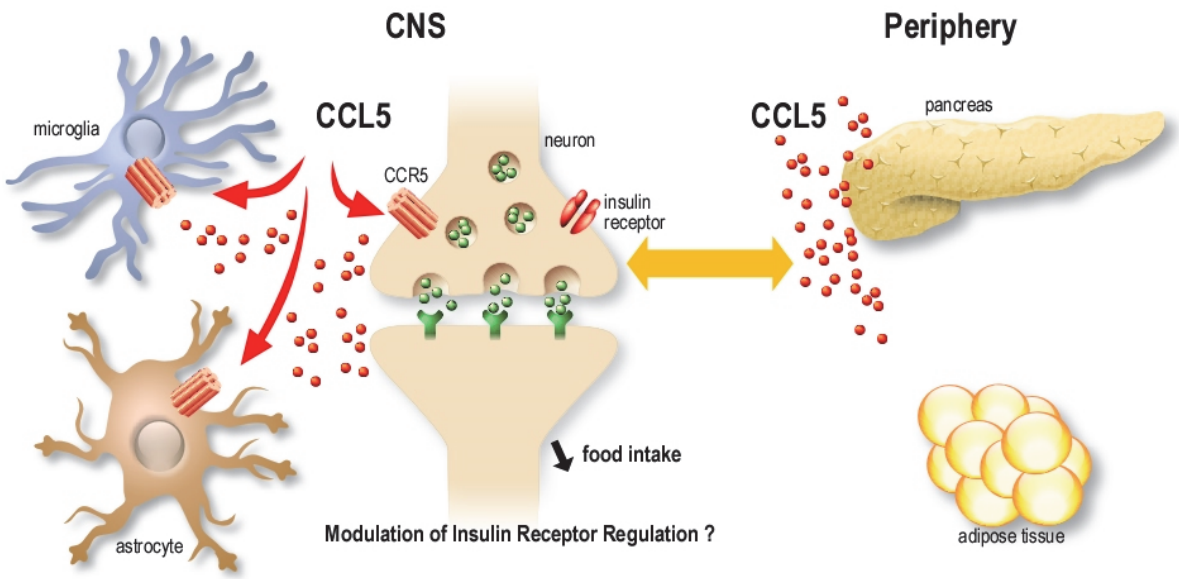

Figure 65: Proposed mechanism of action of CCL5 in the regulation of energy balance in the hypothalamus.

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Annexes

During the course of my thesis, I had the opportunity to assist and participate in several national and international conferences and present the results of my project in poster and oral communications as depicted in the following pages. At some of these events, I had the opportunity to be awarded the best poster or oral communication prize. Furthermore, I had the possibility to participate in the organization of the International Neuroscience Conference and as an invited chair.

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Original articles

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Post-prandial hypothalamic inflammation displays an exacerbated response to a single high-fat meal and involves both GFAP-positive and microglial cells

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Abbreviations: ACN, Acetonitrile; AgRP, Agouti-Related Peptide; ARC, Arcuate nucleus; CART, Cocaine- and Amphetamine-Regulated Transcript; CCL, Chemokine (C-C motif) ligand; ChCl3, Chloroform; CNO, N-Oxide Clozapine; CSF1R, Colony Stimulating Factor 1 Receptor; DREADD, Designer Receptors Exclusively Activated by Designer Drugs; GAPDH, GlycerAldehyde Phosphate DeHydrogenase; GFAP, Glial Fibrillary Acidic Protein; HFD, High-Fat Diet; HT, Hypothalamus; Iba1, Ionized Calcium Binding Adaptor Molecule 1; IL, Interleukin; INF, Interferon; IPA, Isopropanol; MBH, Medial Basal Hypothalamus; MCH, Melanin-Concentrating Hormone; MeOH, Methanol; NPY, NeuropeptideY; ORX, Orexine; PBS, Phosphate Buffer Saline; POMC, Pro-OpioMelanocortin; SD, Standard Diet; TG, Triglyceride; TNF, Tumor Necrosis Factor.

Abstract

Obesity and metabolic syndromes are concomitant with a state of chronic systemic inflammation. In humans, obesity was associated with brain inflammation and glial cell proliferation. Moreover, recent studies in rodents have shown that proliferation of microglia and astrocytes occurs within the first 24 hours of high-fat diet (HFD) consumption, well before the onset of obesity. This proliferation was mainly observed in the hypothalamus, a crucial structure for controlling the adaptive metabolic and behavioral responses that regulate body weight. Therefore, in this study, we sought to understand the cell-based mechanisms involved in the post-prandial hypothalamic inflammatory response to a standard diet (SD) and HFD. We investigated the effects of short-term SD and HFD feeding within 1, 3 and 6 hours on cellular and molecular hypothalamic remodeling in mice. A short 1-hour HFD exposure increased gene expression of an astrocyte marker (GFAP) in the hypothalamus, whereas expression of the microglial marker (Iba1) only increased after 3 hours of HFD exposure. Morphological modifications of microglial cells were observed in the arcuate nucleus of the hypothalamus after 6 hours of HFD consumption. This remodeling was associated with higher expression of pro-inflammatory genes and differential activation of hypothalamic neuropeptides involved in energy balance regulation in HFD-fed mice compared to SD fed-mice. The use of DREADD technology and PLX5622 to modulate GFAP-positive or microglial cells activity, respectively, suggested the involvement of both glial cell types in hypothalamic post-prandial inflammation, but in a different time frame and with a diet specificity. These results in adult mice demonstrate, for the first time, that the increase of nutritional lipid content in diet promotes hypothalamic post-prandial inflammation, which is known to be involved in the central regulation of energy balance, and that this response involves hypothalamic GFAP-positive glial cells and microglia.

Keywords: Energy Balance, Lipids, Obesity, Inflammation, Hypothalamus, Microglia, Astrocyte, Cytokines, Chemokines, Neuropeptides.

1. Introduction

Obesity is a major public health problem that has been highlighted by the World Health Organization. In 2016, almost 40% of the worldwide population was overweight and 13% was obese (http://www.who.int/en/). The main driving force of this body weight disturbance is an energy balance dysregulation, due to kilocalorie consumption overriding energy expenditure (Gao and Horvath, 2008). Therefore, chronic exposure to the Western diet rich in saturated fat (commonly known as the high-fat diet (HFD)) is now recognized as a significant contributor to the development of obesity and its associated comorbidities, such as insulin resistance, type 2 diabetes mellitus and other chronic illnesses such as cardiovascular disease or cancer (Hotamisligil, 2006).

Excessive intake of dietary fat induces chronic low-grade inflammation in many metabolic organs including the pancreas, adipose tissue, muscle and liver, as well as in the brain (Lumeng and Saltiel, 2011). HFD consumption increases the expression of inflammatory mediators (De Souza et al., 2005; Thaler and Schwartz, 2010), especially the pro-inflammatory cytokines Interleukin-1 beta (IL-1β) , Tumor Necrosis Factor alpha (TNF-α) and Interleukin 6 (IL-6), and disrupts insulin and leptin signaling in murine and primate hypothalamus (HT), a brain area involved in energy balance regulation (Arruda et al., 2011; Grayson et al., 2010; Milanski et al., 2009; Zhang et al., 2008). HFD-induced inflammatory modifications of brain signals also converge to alter levels of hypothalamic neuropeptides, suggesting a new concept where hypothalamic neuropeptides act as a link between central inflammation, dysregulation of feeding behavior and energy homeostasis, and indicating that these peptides could be potential therapeutic targets for the treatment of obesity (Timper and Bruning, 2017).

Since 2012, several studies have shown that HFD consumption induces astrogliosis and microgliosis in the arcuate nucleus (ARC) of the HT (Andre et al., 2017; Balland and Cowley, 2017; Baufeld et al., 2016; Gao et al., 2014; Guillemot-Legris et al., 2016; Kim et al., 2019; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014). The microglia activation that is induced by lipids and connects dietary fat and brain inflammatory signaling is begging to be seen as the

missing link between the hypothalamic response to HFD and susceptibility to obesity (Andre et al., 2017; Kim et al., 2019; Valdearcos et al., 2017; Valdearcos et al., 2014). Nevertheless, it has recently been shown that HFD-induced inflammation in astrocytes may have a protective effect against the development of obesity (Buckman et al., 2015). Interestingly, hypothalamic inflammation has been described very early in response to HFD, before the onset of obesity (Balland and Cowley, 2017; Buckman et al., 2015; Kim et al., 2019; Thaler et al., 2012; Waise et al., 2015). Exposure to HFD for a 24h period increases gene expression of inflammatory astrocyte and microglia markers as well as astrogliosis in the HT (Buckman et al., 2015; Dalvi et al., 2017; Thaler et al., 2012). Microgliosis has also been found in the ARC after a 72-hour exposure to HFD (Thaler et al., 2012). These findings tempt us and others to speculate that hypothalamic inflammation may not be limited to chronic exposure to dietary fat, but may occur after consumption of a single meal (Cani et al., 2007; Emerson et al., 2017; Ghanim et al., 2009; Herieka and Erridge, 2014; Kelly et al., 2012; Khor et al., 2014). Therefore, we investigated cellular and molecular hypothalamic remodeling in mice that were exposed for the first time to a HFD for short periods of time (1h, 3h or 6h) and we compared it to mice exposed to standard diet (SD).

2. Materials and methods

2.1. Animals.

8-week-old C57Bl/6J male mice (20-25 g, Janvier Labs, France) and 9-10 week-old CX3CR1eGFP/eGFP male mice (Jung et al., 2000) were housed in a room maintained at 22+/-1°C with a 12h light/12h reversed dark cycle and were acclimatized for 2-3 weeks before experiments were performed. Animals had access to water and standard diet *ad libitum* (SD; 3395 kcal/kg with 25.2% from proteins, 13.5% fat and 61.3% from carbohydrates; Safe #A03). All protocols were carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Nice-French Riviera, registered number 04042.02; University Paris Descartes registered number CEEA34.EA.027.11 and CEEA16-032; APAFIS#14072-2018030213588970 v6). The heterozygous CX3CR1eGFP/+ mice used for electrophysiology were obtained by crossing CX3CR1eGFP/eGFP (Jung et al., 2000) with C57BL/6J (Janvier Labs, France) wild-type mice.

2.2. Short-term high-fat feeding studies.

Mice were food-deprived for 2h prior the onset of the dark cycle to synchronize groups. At the beginning of the dark cycle (T=0h) mice were fed either standard diet (SD; 3395 kcal/kg with 25.2% from proteins, 13.5% fat and 61.3% from carbohydrates; Safe #A03) or high-fat diet (HFD; 4494.5 kcal/kg with 16.1% from proteins, 40.9% from fat and 43% from carbohydrates; Safe #U8954P V0100). One hour, 3h, and 6h after food exposure, food intake was measured and brain and blood samples were collected. Microglia were depleted by administering the Colony Stimulating Factor 1 Receptor (CSF1R) inhibitor PLX5622 (Plexxikon, USA), formulated in AIN76A (3827.7 kcal/kg with 18.2% from proteins, 12.6% fat and 69.2% from carbohydrates; Research Diet, USA) at a dose of 1.2 g/kg for 2 weeks. Clozapine N-oxide (CNO; Sigma, France) was prepared in water at 1 mg/kg.

2.3. Stereotactic virus injections.

Mice were anesthetized by intraperitoneal (i.p.) injection of a ketamine-xylazine mix (80 mg/kg - 12 mg/kg). They were then placed on a stereotaxic frame. AAV8/GFAP-HA-hM4G(Gi)-mCitrine (Translational Vector Core, France) virus was injected bilaterally into the medio basal HT (MBH). Stereotactic coordinates relative to bregma were: x: ±0.3 mm; y: -1.5 mm; z: +6 mm. Injections were applied at a rate of 0.5 µL/min for 1 min per side. At the end of the surgical procedures, mice received 1 mg/kg i.p. atipamezole and 5 mg/kg subcutaneous (s.c.) ketoprofen.

2.4. RNA isolation and quantitative PCR.

Total RNA from HT was isolated using Fast Prep apparatus (Q-Biogene, France) as previously described (Le Thuc et al., 2016). First-strand cDNAs were synthesized from 2 µg of total RNA with 200 U of SuperScript III reverse transcriptase (SuperScriptIII, Invitrogen, France) in the appropriate buffer in the presence of 25 ng/µL oligo-dT primers, 0.5 mM desoxyribonucleotide triphosphate mix, 5 mM dithiothreitol, 40 U RNAsin (Promega, France). The reaction was incubated 5 min at 25 °C, then 50 min at 50 °C then inactivated for 15 min at 70 °C. Real-time PCR was performed for amplification of mouse IL-1β, IL-6, TNF-α, CCL2, CCL5 (Chemokine (C-C motif) ligand 2-5), Iba1 (Ionized calcium Binding Adaptor molecule 1), GFAP (Glial Fibrillary Acidic Protein), MCH (Melanin-Concentrating Hormone), ORX (OReXine), POMC (Pro-OpioMelanoCortin), CART (Cocaine- and Amphetamine-Regulated Transcript), NPY (Neuropeptide Y), AgRP (Agouti-Related Peptide) and GAPDH (GlycerAldehyde Phosphate DesHydrogenase) mRNA. GAPDH was used as housekeeping gene for normalization. Primers (detailed in Supplementary data, Supplementary Table 1) were purchased from Eurogentec (France).

2.5. Cytokine and chemokine quantification.

A V-Plex multiplex assay (Meso Scale Discovery, USA) and a mouse CCL5 ELISA Ready-SET-Go (eBiosciences, France) were used to measure the levels of inflammatory mediators in mice serum according to the manufacturer's protocol.

2.6. Immunohistochemical analysis.

Brains were harvested from mice perfused with 4% paraformaldehyde in phosphate buffer saline (PBS) and post-fixed in the same fixative overnight at 4°C.

Brain coronal sections (30 µm) were cut on a vibratome, blocked for 1h with 3% normal goat serum in PBS containing 0.1% Triton X-100 and incubated with primary antibodies overnight at 4°C. Primary anti-rabbit antibodies were against Iba1 (1:500, CP290A, B, Biocare Medical, USA) and GFAP (1:300, Z0334, Dako, Danemark). Adequate Alexa Fluor ® 488conjugated secondary antibodies were used for immunofluorescence microscopy. Sections at -1.70 mm relative to Bregma were mounted in Vectashield solution (H-1000, Vector Laboratories). 3D mosaics of 1024x1024 images were acquired with a TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Nanterre, France) through a 40X/1.4 Oil immersion objective for GFAP staining and 63X/1.4 Oil immersion objective for Iba1 staining, with a z-step of 2µm. GFAP, Iba1 and soma size measurements were done on ImageJ (Schneider et al., 2012) on maximal intensity projections of these z-stacks of images. After the z-projection, a Region Of Interest (ROI) corresponding to the ARC of the hypothalamus was manually drawn on these images. GFAP and Iba1 staining areas were selected and measured (in μm^2) in these ROIs by intensity thresholding and measurement above this threshold. The same process was applied to measure the soma size of microglial cells.

2.7. *Acute HT slices preparation for microglia recordings.*

Male mice aged between 9 and 10 weeks were anesthetized with isoflurane before the brain was harvested and coronal slices through the HT were cut using a Leica VT1200 vibratome in an oxygenated (5% CO_2 and 95% O_2) ice-cold protective extracellular solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 NaHCO3, 20 HEPES, 2 thiourea, 25 D-glucose, 5 sodium ascorbate, 3 sodium pyruvate, 10 $MgCl_2$, and 0.5 $CaCl_2$ (pH 7.3, 320 mOsm). Slices were then incubated in the same protective extracellular solution for 7 min at 34°C and then incubated for 30 min in artificial cerebro-spinal fluid (aCSF; pH 7.3, 310 mOsm) at 34°C containing (in mM): 135 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 D-glucose, 1 sodium pyruvate, 1 $MgCl_2$, and 2 $CaCl_2$. The slices were then maintained at room temperature (RT, 22°C) for 0.5–5 h in the regular oxygenated aCSF. After incubation, individual slices selected for best visibility of the ARC were transferred to a recording chamber on the stage of an Olympus microscope (BX50WI) with a 40x water immersion objective, equipped with a CCD camera (Hamamatsu ORCA2-AG, France). Slices were constantly perfused at RT with oxygenated aCSF (5 mL/min).

2.8. *Electrophysiological recordings.*

Visually-identified eGFP-expressing microglial cells, located at least 50 µm below the slice surface, were patched in whole-cell configuration in the ARC. Micropipettes (6 to 7 MV) were filled with a solution containing (in mM): 132 K-gluconate, 11 HEPES, 0.1 EGTA, 4 $MgCl_2$ (pH 7.35, osmolarity 300 mOsm). All potential values given in the text were corrected for a junction potential of 10 mV. Voltage-clamp recordings were performed using an Axopatch 200B (Molecular Devices, USA). Series resistance (Rs) as well as the cell membrane resistance (Rm), capacitance (Cm) were determined from the current response to a 10 ms depolarizing pulse of 10 mV that was performed at the beginning of each recording. These currents were filtered at 10 kHz and collected using PClamp 10 (Molecular Devices, USA) at a frequency of 100 kHz. To measure the I/V relationship, we used hyperpolarizing and depolarizing steps (from –150 to +40 mV for 50 ms), and currents were low-pass filtered at 5 kHz and collected at a frequency of 20 kHz. Currents were analyzed off line using Clampfit 10.7 (Molecular Devices, USA). To measure capacitance, currents were first fitted with a double exponential function in order to determine the time constant tau (τweighted) defined as τweighted = (A1*τ1+A2*τ2)/(A1+A2) and then capacitance was calculated as Cm=(τweighted(Rm+Rs))/((Rm*Rs)).

2.9. *LPS mass quantitation by LC-MSMS.*

Lipopolysaccharide (LPS) mass concentration was determined by direct quantitation of 3-hydroxytetradecanoic acid (or 3 hM) by LC/MS/MS as previously described by Pais de Barros et al. (Pais de Barros et al., 2015). Quantitative LPS analysis using HPLC/MS/MS was performed as well as the lysate assay of its combination with the limulus amebocyte according to Pais de Barros et al. with modifications. Plasma (ca 50 µL) was mixed with saline (up to 100 µL), 4 pmol of internal standard (3-hydroxytridecanoic acid, 1 pmol/µL in ethanol) and finally hydrolyzed with 300 µL of 8 M HCl for 3h at 90°C. Total fatty acids were extracted with 600 µL distilled water and 5 mL ethyl acetate/hexane (3:2 v/v). After recovery and vacuum evaporation of the organic upper phase, fatty acids were dissolved in 200 µL ethanol and transferred into 200 µL micro-inserts for LC vials. After evaporation of ethanol under vacuum, samples were finally dissolved in 50 µL ethanol for LC/MS/MS analysis (injection volume 2 µL). Fatty acid separation was performed on an Infinity 1260 HPLC binary system (Agilent Technologies, France)

equipped with a ZORBAX SB-C18 C18 50 x 2.1 mm 1.8 µm (Agilent Technologies, France) maintained at 45°C with a binary set of eluents (eluent A: 1 M ammonium formate/formic acid/water 5/1/996 v/v/v and eluent B: 1 M ammonium formate/formic acid/water/acetonitrile 5/1/44/950 v/v/v) at a flow rate of 0.4 mL/min. An 8 min eluent gradient was established as follows: from 0 to 0.5 min, the mobile phase composition was maintained at 45% B; then the proportion of B was increased linearly up to 100% over 2.5 min and maintained for 5 additional minutes. The column was equilibrated with 45% B for 5 min after each sample injection. MS/MS analysis was performed on a 6490 triple quadruple mass spectrometer (Agilent Technologies, France) equipped with a JetStream-Ion funnel ESI source in the negative mode (gas temperature 290°C, gas flow 19 L/min, nebulizer 20 psi, sheath gas temperature 175°C, sheath gas flow 12 L/min, capillary 2000 V, V charging 2000 V). Nitrogen was used as the collision gas. The mass spectrometer was set up in the selected reaction monitoring (SRM) mode for the quantification of selected ions as follows: for 3-hydroxytetradecanoic acid, precursor ion 243.2 Da, product ion 59 Da, fragmentor 380 V, collision cell 12 V, cell acceleration 2 V; for 3-hydroxytridecanoic acid, precursor ion 229.2Da, product ion 59 Da, fragmentor 380 V, collision cell 12 V, cell acceleration 2 V.

2.10. *Triglyceride analysis.*

The detailed protocol is provided in the Supplementary data, Supplementary Materials and Methods. Briefly, lipids from mouse serum were extracted according to a modified Bligh and Dyer protocol with a mixture of methanol/chloroform (2:1). The lipid extract was then separated on a C18 column in an appropriate gradient. Mass spectrometry data were acquired with a Q-exactive mass spectrometer (ThermoFisher, France) operating in data dependent MS/MS mode (dd-MS2). Finally, lipids were identified using LipidSearch software v4.1.16 (ThermoFisher, France) in product search mode.

2.11. *Statistical analysis.*

Displayed values are means ± SD. To test if the data set was well-modeled, a Kolmogorov-Smirnov normality test was conducted (with Dallal-Wilkinson-Lillie for P value). The ROUT method (robust regression and outlier removal) was used to identify outliers with a Q coefficient equal to 1%. Variance equality was tested using a

F-test. If samples fulfilled normal distribution and variance equality criteria, comparisons between groups were carried out using an unpaired t test for single comparison and a two-way ANOVA for multiple comparisons and interaction. If samples did not follow a normal distribution or had different variances, comparisons between groups were carried out using a non-parametric Mann-Whitney U test for single comparison and non-parametric Kruskal-Wallis test with Dunn's correction for multiple comparisons. When appropriate, comparison with the theoretical mean control (1 or 100%) was done using a non-parametric Wilcoxon signed rank test. A p-value of 0.05 was considered statistically significant. All tests were performed using GraphPad Prism 7.02 and Microsoft Office Excel. Numbers of animals and cells are given in the legends.

3. Results

3.1. *A single high-fat meal increases serum levels of saturated triglycerides and endotoxins, but not those of cytokines and chemokines.*

Obesity and metabolic syndromes are concomitant with a state of chronic systemic inflammation (Lumeng and Saltiel, 2011) and the degree to which triglycerides (TG) increase after HFD consumption correlates with the probability of developing metabolic syndrome (Herieka and Erridge, 2014; Nogaroto et al., 2015). Because the main difference between SD and HFD is lipid content, we measured TG serum levels after short exposure to the diet. By mass spectrometry, we found that HFD consumption did not change the total TG serum levels (Fig. 1A) but led to increase the percentage of TGs containing only saturated fatty acids (Fig. 1B) compared to SD-fed mice. Previous studies have linked HFD consumption to post-prandial systemic inflammation (Cani et al., 2007; Emerson et al., 2017; Ghanim et al., 2009; Herieka and Erridge, 2014; Kelly et al., 2012; Khor et al., 2014). Therefore, we measured the serum levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα, pro-inflammatory chemokines CCL2 and CCL5 (Chemokine (C-C motif) Ligand 2-5), as well as endotoxin (lipopolysaccharide, 3OH C14:0) after SD and HFD short-term exposure (1h, 3h and 6h). Interestingly, HFD consumption did not increase pro-inflammatory cytokine or chemokine serum levels compared to SD-fed mice over the 3 time points (Fig. 1C-G). However, 3h and 6h of HFD consumption increased endotoxin serum levels compared to SD-fed mice (Fig. 1H). As anticipated (Buckman et al., 2015), an increase in kilocalories consumed was noticeable in mice fed for 6h with HFD (SD)-fed mice (Supplementary Fig. 1A), while the total quantity in grams of ingested food was similar between groups (Supplementary Fig. 1B). Altogether, these observations prove that short-term HFD exposure does not affect systemic levels of inflammatory markers, but increases endotoxin concentrations and the proportion of saturated TGs in the serum compared to SD-fed mice.

3.2. *A single high-fat meal induces an exacerbated inflammatory-like gene response in the hypothalamus.*

Given the lack of a peripheral inflammatory response in HFD-fed mice, we subsequently investigated the impact of a HFD on hypothalamic inflammatory markers. Consistent with previous studies (Andre et al., 2017; Baufeld et al., 2016; Buckman et al., 2015; Gao et al., 2017; Gao et al., 2014; Guillemot-Legris et al., 2016; Nadjar et al., 2017; Thaler and Schwartz, 2010; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014) we found that gene expression of the pro-inflammatory cytokine genes IL1β, IL6 and TNFα, as well as chemokine genes CCL2 and CCL5 was significantly increased in HFD-fed mice compared to SD-fed mice (Fig. 2A-E). The novelty of our study relies on the demonstration that an inflammatory-like gene response in the HT is physiologically induced by SD and arises within the first hours of food consumption. Moreover, this inflammatory-like gene response exhibits a diet specific profile defined by cytokine-/chemokine-type, amplitude and time course of the response, and is exacerbated in HFD-fed mice. In fact, the increase in IL-1β and TNFα gene expression appeared after 1h of food exposure (Fig. 2A and C) with a greater increase in HFD-fed mice for IL-1β (Fig. 2A). Moreover, the increase in CCL2 and CCL5 gene expression also appeared within 1h of food exposure, but was observed exclusively in HFD-fed mice (Fig. 2D and E). After 3h of food exposure, IL-1β, IL-6, TNFα and CCL2 gene expressions were increased only in HFD-fed mice (Fig. 2A- D). Finally, after 6h of food exposure, pro-inflammatory cytokine and chemokine gene expression in HFD-fed mice tend to return to baseline. Taken together, these results indicate that short term HFD exposure induces a specific and exacerbated hypothalamic inflammatory-like gene response, different from the one observed in SD-fed mice.

3.3. *A single high-fat meal induces a specific acute neuropeptide gene expression profile in the hypothalamus*

Given the acute and specific response in hypothalamic inflammatory-like gene expression of HFD-fed mice, we subsequently investigated the impact of a single high fat meal on hypothalamic neuropeptide genes. We examined changes in hypothalamic neuropeptide gene expression involved in energy balance regulation in

the HT after 1h, 3h and 6h exposure to SD and HFD (Gao and Horvath, 2008). Food exposure induces changes in both SD- and HFD- fed mice. However, hypothalamic gene expression levels of orexigenic peptides MCH, AgRP and ORX (Fig. 3C, D and F) and anorexigenic peptide CART (Fig. 3E) were higher in HFD-fed mice compare to SD-fed mice while NPY levels remained equivalent in the 2 groups (Fig. 3A). In contrast, a decrease in anorexigenic peptide POMC gene expression, was observed at 1h only in HFD-fed mice (Fig. 3B). Interestingly, the time course of those changes was very specific for each diet. For example, SD-fed mice presented a transient and modest increase in MCH gene expression after 3h of food exposure compared to basal level (Fig. 3C) while it increased within the first hour of food exposure in HFD-fed mice and remained high for 6h (Fig. 3C). AgRP and ORX gene expression levels increased within the first hours of food exposure in both diet groups, with an exacerbated response in HFD-fed mice that lasted for 6h (Fig. 3D and F), unlike the increase in CART gene expression, which did not occur until 3h of food exposure and was only observed in HFD-fed mice (Fig. 3E). Taken together, our results show that short term HFD exposure is sufficient to specifically modulate hypothalamic neuropeptide gene expression involved in energy balance regulation, differently from what is observed in SD-fed mice.

3.4. *A single high-fat meal induces an acute increase of GFAP gene expression in the hypothalamus.*

Hypothalamic inflammation after medium-term (1 to 10 days) and long-term (2 to 32 weeks) HFD exposure is associated with astrogliosis (Balland and Cowley, 2017; Baufeld et al., 2016; Buckman et al., 2015; Thaler et al., 2012). It has yet to be determined whether the same is true at earlier time points. Given that our results showed an exacerbated hypothalamic inflammatory-like gene response in HFD-fed mice, we tested whether short-term exposure (within 1h, 3h and 6h) to HFD might affect astrocytes in the HT. To this end, we used the astrocyte marker GFAP for genetic and histological analysis. Surprisingly, 1h of food exposure increased GFAP gene expression in the HT of HFD-fed mice but not in SD-fed mice (Fig. 4A). Moreover, the increase in GFAP gene expression appeared to be transient as expression levels returned to baseline at 3h and 6h (Fig. 4A). We then tested if the increase in GFAP gene expression was associated with changes in astrocyte remodeling in the ARC, as described previously for medium term and long term HFD

exposure (Balland and Cowley, 2017; Buckman et al., 2015; Thaler et al., 2012). SD and HFD-fed mice presented similar GFAP staining in the ARC, as shown in the photomicrographs displayed in Fig. 4C and corroborated by fluorescence quantification in the ARC of mice fed for 1h, 3h and 6h (Fig. 4B). These findings demonstrate that short term HFD exposure, specifically and transiently, upregulates hypothalamic GFAP gene expression.

3.5. A single high-fat meal induces acute microglial remodeling in the arcuate nucleus of the hypothalamus.

In addition to astrogliosis, hypothalamic inflammation after medium-term (3 to 7 days) and long-term (2 to 20 weeks) HFD exposure is also associated with microgliosis (Andre et al., 2017; Baufeld et al., 2016; Dorfman et al., 2017; Gao et al., 2017; Gao et al., 2014; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014). As for astrocytes, we tested whether short-term exposure (1h, 3h and 6h) to HFD might affect microglial cells in the HT. HFD exposure increased microglial marker Iba1 gene expression in the HT at 3h and 6h compared to SD-fed mice (Fig. 5A). We then tested whether Iba1 gene upregulation impacted Iba1 protein levels in the ARC, as previously described for medium term and long term HFD exposure (Andre et al., 2017; Baufeld et al., 2016; Dorfman et al., 2017; Gao et al., 2017; Gao et al., 2014; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014). No change in Iba1 immunofluorescence staining was observed (Fig. 5B and D). We also considered whether Iba1 gene expression modification might be associated with a change in microglial activity. Using the patch clamp technique, membrane properties of microglial cells were determined in 3h HFD-fed mice. We did not observe changes in the current-voltage relationship (Fig. 5E), but a significant increase in the cell capacitance in HFD-fed mice (Fig. 5F). We therefore looked more closely into cell morphology. While the histological approach may have had insufficient resolution to detect changes in soma size after 3h of HFD exposure (Fig. 5C and D), we did observe an increase in soma size in 6h HFD-fed mice (Fig. 5C), which is probably linked to the change observed in cell capacitance (Fig. 5F). Collectively, these results indicate that short term HFD exposure induces fast microglial cells remodeling in the ARC.

3.6. *Modulation of GFAP-positive cells changes post-prandial hypothalamic neuropeptide and inflammatory-like gene responses to SD and HFD exposure.*

We observed an acute and diet-specific hypothalamic gene response to food exposure for neuropeptides involved in energy balance regulation and pro-inflammatory markers. This response is associated with an increase in GFAP mRNA levels and microglial remodeling in the HT. We subsequently tested if modulating GFAP-positive cells and microglia could change this hypothalamic gene response. In order to modulate GFAP-positive cell activity we used DREADD (Designer Receptors Exclusively Activated by Designer Drugs) technology (Chen et al., 2016; Yang et al., 2015). An Adeno Associated Virus 8 (AAV8) with a GFAP promoter controlling DREADD Gi coupled with mCitrine gene sequence expression in the MBH of mice was used to target GFAP-positive cells. Analysis of mCitrine staining by immunohistochemistry confirmed successful injection (Supplementary Fig. 2A, B and C). Two weeks after virus injection, we treated mice with the specific agonist CNO (Clozapine N-Oxide) at a dose of 1 mg/kg to activate the DREADD Gi and 30 min later we exposed the mice to SD or HFD for 1h. Using this tool, we observed differential inflammatory-like and neuropeptide gene responses to SD and HFD in vehicle (NaCl) group (Fig. 6) compared to our original observation (Fig. 2 and 3). This could be explained by different inflammatory states of animals, as discussed later, but doesn't change the originality of our results. CNO injection did not change 1h food consumption in both SD- and HFD-treated mice (Supplementary Fig. 2D and E). CNO injection did not alter POMC, MCH, AgRP and ORX gene expression response to SD and HFD exposure (Fig. 6B, C, D and F). In contrast, CNO injection, but not vehicle injection, decreased CART hypothalamic gene expression regardless of diet (Fig. 6E). Only NPY gene expression in response to food exposure is changed by the activation of DREADD Gi in GFAP-positive cells in a diet-specific manner (Fig. 6A). Interestingly, CNO injection, but not vehicle injection, decreased IL-1β, TNFα, and CCL2 gene expression in the HT regardless of diet (Fig. 6G, I and J). Moreover, CNO injection, but not vehicle injection, decreased CCL5 and Iba1 gene expression in HFD-treated mice (Fig. 6K and L). However, variability in CCL5 and Iba1 gene expression levels in SD-fed mice prevented statistically robust conclusions. CNO effect seems similar regardless of the diet on CCL5 gene expression (Fig. 6K), but the decrease in Iba1 gene expression is stronger in HFD-fed mice compared to SD-fed mice (Fig. 6L). Finally, IL6 gene expression in response to food exposure is

changed by CNO injection in HFD-treated mice but not in SD-treated mice (Fig. 6H). Collectively, these results show that modulation of GFAP-positive cell activity in the MBH alters hypothalamic neuropeptide and inflammatory-like gene responses to both SD and HFD exposure. Interestingly, our results also demonstrate that specific NPY and IL-6 gene responses to food exposure are altered by the modulation of GFAP-positive cells activity in a diet-dependent manner.

3.7. Modulation of microglial activity changes post-prandial hypothalamic neuropeptide and inflammatory-like gene responses to SD and HFD exposure.

To assess if microglia is involved in the neuropeptide and inflammatory gene responses induced by food exposure, we used PLX5622, which has been previously used to remove microglia from brain (Elmore et al., 2014). PLX5622 is a CSF1R agonist known to regulate microglial density in the brain. Two weeks after PLX5622 treatment, we assessed its efficiency to deplete microglia. PLX5622 almost completely ablated the microglial population as shown by the absence of Iba1 staining in the MBH (Supplementary Fig. 3A and B). We exposed PLX5622-treated and non-treated mice to SD or HFD for 1 h and 3h. Using this tool, we observed differential inflammatory-like and neuropeptide gene responses to SD and HFD in non-treated group (Fig. 7 and Supplementary Fig. 4) compared to our original observation (Fig. 2 and 3). This may be due to animal variability and susceptibility to inflammatory states, which does not undermine our results. SD and HFD intake (Supplementary Fig. 3E, F, H and I) and hypothalamic GFAP gene expression (Supplementary Fig. 3G and J) were not modified by PLX5622-induced microglial depletion. Moreover, PLX5622 treatment did not affect the hypothalamic neuropeptide gene response induced by 1h of food exposure (Supplementary Fig. 4A-F). PLX5622 treatment did not alter CART hypothalamic gene expression response to 3h food exposure neither (Fig. 7E). However, after 3h of food exposure, PLX5622 treatment increased similarly NPY and POMC gene expression regardless the diet (Fig. 7A and B). Concerning the AgRP gene response, result variability in SD-treated mice prevented a statistically robust conclusion, but PLX5622 treatment also seemed to upregulate AgRP gene expression regardless the diet (Fig. 7D). Interestingly, PLX5622 treatment changed MCH and ORX gene expression response to food exposure in a diet-dependent manner (Fig. 7C and F).

With respect to the impact of microglia depletion on inflammatory gene responses the high variance between the samples unfortunately detracted from any robust conclusion (Supplementary Fig. 4G-J and K; Fig. 7 G-K). Nevertheless, PLX5622 treatment increased TNFα mRNA levels in 1h HFD-treated mice (Supplementary Fig. 4I). Moreover, IL-1β and TNFα mRNA levels were decreased in 3h HFD-fed mice and effect seems similar on SD-fed mice (Fig. 7G and I).

Therefore, the results show that microglia depletion affects hypothalamic neuropeptide and inflammatory-like gene responses to both SD and HFD exposure. More interestingly, our results also demonstrate that specific MCH and ORX gene responses to food exposure are affected by the absence of microglial cells in a diet-dependent manner.

4. Discussion

Several studies have shown a link between HFD consumption, inflammation and risk factors associated with obesity. It seems certain that long-term exposure to HFD induces chronic low-grade systemic inflammation, which is responsible for the development of insulin resistance, type 2 diabetes mellitus along with other chronic illnesses such as cardiovascular disease or cancer (Cani et al., 2007; Emerson et al., 2017; Ghanim et al., 2009; Herieka and Erridge, 2014; Kelly et al., 2012; Lumeng and Saltiel, 2011; Thaler and Schwartz, 2010). More recently, hypothalamic inflammation, a process involving neurons, astrocytes and microglia, has been correlated with obesity and chronic HFD consumption (Balland and Cowley, 2017; Baufeld et al., 2016; Buckman et al., 2015; Gao et al., 2014; Guillemot-Legris et al., 2016; Kim et al., 2019; Milanova et al., 2019; Thaler and Schwartz, 2010; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014), underlining the effect of chronic exposure to HFD on the central inflammatory status. Interestingly, brain inflammatory responses in the HT were also observable after only one day of HFD consumption (Buckman et al., 2015) or 24h of lipid perfusion to the brain (Dalvi et al., 2017), well before the development of obesity. Moreover, inhibition of brain inflammatory responses to HFD leads to energy balance dysregulation (Buckman et al., 2015; Fernandez-Gayol et al., 2019). In humans, studies have described acute post-prandial inflammation associated to increased levels of circulating inflammatory markers in response to HFD (Emerson et al., 2017; Herieka and Erridge, 2014). Altogether, these data suggest that HFD-induced inflammation is a physiological response to cope with excess lipids, which could occur at the peripheral or central levels.

In our study, the calories from fat and sugar in HFD were equivalent (40% each), in contrast to numerous studies where fat content accounts for 60% kilocalories (kcal) while carbohydrate content was only 20% kcal. Therefore, the increase in caloric proportion represented by the lipids is more realistic in our model, and comparable to the typical human diet, where the occurrence of lipids in the presence of elevated carbohydrate content contributes to brain inflammation (Andre et al., 2017; Gao et al., 2017; Kim et al., 2019). According to previous studies of systemic post-prandial inflammation, HFD consumption promotes lipopolysacharide trafficking over the intestinal epithelium through trans-cellular and para-cellular transport (Valdearcos et

al., 2014; Buckman et al., 2015). Analysis of endotoxin levels in the serum associated with inflammatory markers in both serum and HT, affirmed that post-prandial inflammation appears first in the HT. Therefore this study established, for the first time, the existence of a specific post-prandial hypothalamic inflammation response to both SD and HFD exposure linked to a unique modulation of mRNA levels of neuropeptides that are known to be involved in the regulation of energy balance (Gao and Horvath, 2008). However, while both SD and HFD consumption increased hypothalamic gene expression of the orexigenic neuropeptides AgRP and ORX, as well as inflammatory markers, IL-1β and TNFα, within the first hours of diet exposure, the amplitude of the response was higher in HFD-fed mice. Moreover, compared to SD exposure in the same time frame, 1h exposure to HFD induced selective expression of MCH orexigenic neuropeptide, as well as pro-inflammatory cytokines, CCL2 and CCL5. A sustained expression of proinflammatory cytokines IL-1β, IL-6, TNFα, chemokine CCL2 and orexigenic neuropeptide MCH was still observable after 3h exposure to food but only in HFD-fed mice. Finally, after 6h of food exposure, the differences observed between SD- and HFD-treated mice tended to disappear, except for the MCH orexigenic neuropeptide mRNA level, which remained higher in the HFD group. This suggests that short-term exposure to a lipid-rich diet triggers hypothalamic gene expression of orexigenic neuropeptides leading to an adaptive behavioral response correlated to increased kilocalorie intake observed 6h post HFD exposure (Gao and Horvath, 2008). It is not yet known how these genetic changes occur in orexigenic neurons, although we believe that they may be the consequence of lipid-induced neuroinflammation. We have yet to define the role of the inflammatory brain cells in this response over such a short time frame in this study. Nevertheless, a recent study demonstrated that one day of lipid perfusion to the brain is sufficient to activate an inflammatory response in NPY/AgRP neurons through TNFα and ER-stress signaling (Dalvi et al., 2017). We could therefore expect such a mechanism to appear even earlier in response to HFD, which could modulate the neuropeptide expression involved in energy balance regulation as we observed in our study.

As previously described, HFD-induced hypothalamic inflammation is associated with glial reactivity (Balland and Cowley, 2017; Baufeld et al., 2016; Buckman et al., 2015; Gao et al., 2014; Guillemot-Legris et al., 2016; Kim et al., 2019; Milanova et al.,

2019; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014; Waise et al., 2015). First, we focused on GFAP-positive cells. Targeting of cell activity decreases the postprandial pro-inflammatory cytokines IL-1β, IL-6, TNFα and the chemokines CCL2 and CCL5 gene response to both SD and HFD exposure. Interestingly AAV8-DREADD-Gi-mCitrine injection exclusively in MBH is powerful enough to downregulate pro-inflammatory marker mRNA levels measured in the whole HT, suggesting that MBH GFAP-positive cells play a critical role in hypothalamic post-prandial inflammation. Moreover, we have provided novel evidence that an acute exposure to HFD specifically increases GFAP mRNA levels in HT within the first hours of food consumption. Another observation worth highlighting is that the IL-6 gene response to food exposure is modulated by GFAP-positive cells in a differential way, depending on diet. In fact, modulation of GFAP-positive cellular activity decreases IL-6 mRNA hypothalamic levels only in HFD-treated mice. This is consistent with recent discoveries demonstrating that IL-6 produced by astrocytes is a major actor in central energy balance regulation and plays a protective role against obesity development during HFD exposure (Fernandez-Gayol et al., 2019; Quintana et al., 2013; Timper et al., 2017). On the other hand, modulation of GFAP-positive cellular activity does not dramatically affect hypothalamic neuropeptide mRNA levels and does not change HFD intake. This is consistent with previously published results and indicates that modulation of NPY/AgRP neuron activity by GFAP-positive cells in response to HFD occurs over a different time frame from our study (Chen et al., 2016; Yang et al., 2015).

Because glial reactivity to HFD consumption is also characterized by microglial reactivity, we then focused on microglia. In accordance with previous studies, we did not observe any effect on HFD intake when microglia was depleted (Waise et al., 2015). We found that 3h of HFD exposure increases hypothalamic mRNA levels of the microglial marker Iba1 compare to SD-fed mice. This rise was associated with soma size enlargement of microglial cells in the ARC of HT only in HFD treated mice. For microglial cells, the association of a specific activity state with morphological aspects remains controversial (Kettenmann et al., 2011; Tremblay et al., 2011). However, what is certain is that soma size enlargement of microglial cells and the modification of Iba1 gene expression initiated by HFD consumption reflects a change in their activity, which might enable protective mechanisms to deal with lipid excess

(Kim et al., 2019; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014). Here we provide novel evidence that microglial reactivity to HFD exposure appears within the first hours of food exposure. Moreover, our approach clearly shows that microglial cells participate in the post-prandial hypothalamic inflammation induced by both SD and HFD, though it is too early to state to what extent and what diet-specificity. In fact, microglial depletion seems to exacerbate hypothalamic inflammatory-like response to 1h HFD exposure, but that amplitude varies between individuals. Because the variability cannot be explained with a correlation with body weight and food intake (data not shown) nor by PLX efficiency (Supplementary Fig. 3) it is tempting to speculate that the involvement of microglia in HFD-induced hypothalamic inflammation differs between subjects and may be involved in the predisposition of an individual to the HFD-induced hypothalamic disturbances involved in obesity. Moreover, our experiments showed that microglial depletion modulated gene response to food exposure of neuropeptides involved in energy balance regulation. Not only did it increase NPY and POMC hypothalamic mRNA levels in both SD- and HFD-treated mice, but it also differentially affected MCH and ORX gene responses to diet type, suggesting that microglial responses to food exposure could specifically act on neurons involved in energy balance regulation in a diet-dependent manner.

Up to now, astrocytic or microglial reactive gliosis in the ARC of the HT in response to chronic HFD exposure and their participation in HFD-induced inflammatory processes and functional impact is still controversial and may depend on the nutritional lipid composition (Harrison et al., 2019; Kim et al., 2019; McLean et al., 2019; Valdearcos et al., 2014). Our study offers new insight on the impact of HFD consumption on astrocytic or microglial populations located in areas involved in energy balance regulation. Both cell types may be activated by short-term food exposure and orchestrate inflammatory-like processes in the ARC modulating neuronal function, but in differential time frames and with a diet specificity. Indeed, during short-term HFD exposure, GFAP gene expression is acute and transient, whereas Iba1 gene expression and microglial reactivity occurs after 3h of diet exposure and lasts over 6h. Moreover, the time frame of microglial reactivity to short-term HFD exposure coincides with the appearance of endotoxin and saturated fatty acid-enriched triglyceride serum content. This supports the hypothesis that saturated

fatty acid-induced hypothalamic inflammation may require microglial activation (Kim et al., 2019; Valdearcos et al., 2014). Therefore, our experiments suggest that the increase of lipid content in diet is primarily sensed by GFAP-positive cells, which initiates the hypothalamic inflammatory response followed by hypothalamic microglial cell activation by central and systemic signals that impact energy balance regulation through the modulation of hypothalamic neuropeptide expression.

Despite the originality of our results, we are aware of study limitations. While we focused on the HT because it is a well-known area involved in energy balance regulation (Gao and Horvath 2008), we do not exclude the possibility that other brain areas may display post-prandial inflammation associated with neuropeptide modulation. Indeed, several studies have shown that chronic HFD consumption leads to hippocampal inflammatory responses and affects behavioral disorders associated with obesity (Waise, Toshinai et al. 2015; Guillemot-Legris, Masquelier et al. 2016; Gzielo, Kielbinski et al. 2017; Tsai, Wu et al. 2018; Spencer, Basri et al. 2019). Furthermore, our methodology prevented a detailed analysis of subtle changes that might occur in the several functionally distinct nuclei of the HT. Moreover, to gain insight on the involvement of each cell type, we used two different tools to manipulate GFAP-positive and microglial cell activity which must be called into question. DREADD technology has already been used to target GFAP-positive cells of the ARC glia in the regulation of neuronal subtype-specific modulation of energy homeostasis (Yang, Qi et al. 2015; Chen, Sugihara et al. 2016). Given this context, we initiated our study using DREADD Gi as this strategy was proven to achieve cellular inhibition - at least in neuronal cell types (Roth 2016). However, a 2019 study has demonstrated that Gi/o protein-coupled receptor may differentially affect brain cell type, inhibiting neurons and activating astrocytes stimulating gliotransmitter release (Durkee, Covelo et al. 2019). Thus, at this stage while it seems difficult to definitely conclude whether GFAP-positive glial cell activation or inhibition mediates HFD-induced feeding behavior changes, our results clearly support GFAP-positive glial cell involvement in this process. Concerning microglial cells, although new tools using DREADD technology to target microglia have been recently reported (Grace, Wang et al. 2018), we chose PLX5622 as it was the only tool available at the time we initiated the study to modulate microglial cells in the brain. PLX5622 has been shown to act mainly on microglia in adult whole brains (Elmore, Najafi et al. 2014; Oosterhof,

Kuil et al. 2018). Thus, in our study we analyzed total brain microglial depletion effects, and recognize that further analysis needs to be done to specify microglial involvement in hypothalamic post-prandial inflammation. Besides, using those two tools we observed differential inflammatory and neuropeptide gene responses in respective control groups for AAV8-DREADD-Gi-mCitrine or AIN76A diet experiments compared to our original observation without cellular targeting. This could be explained by different inflammatory states of animals: sham and non-treated animals vs injected with virus vs treated with an AIN76A diet containing PLX5622. While it is obvious that stereotaxic injection of virus has immunological implications that could affect brain inflammatory status, it is worth noting that 76.9% of carbohydrates contained in AIN76A are sucrose, known to play a critical role in diet-induced hypothalamic inflammation (Andre, Guzman-Quevedo et al. 2017; Gao, Bielohuby et al. 2017).

Overall, while the effect of medium-term and long-term nutritional lipid exposure has been extensively demonstrated to promote hypothalamic inflammation involving astrocytic and microglial cells (Andre et al., 2017; Balland and Cowley, 2017; Baufeld et al., 2016; Gao et al., 2017; Gao et al., 2014; Guillemot-Legris et al., 2016; Nadjar et al., 2017; Thaler et al., 2012; Valdearcos et al., 2017; Valdearcos et al., 2014), little was known about short-term exposure. Here we demonstrate, for the first time, that a moderate increase of nutritional lipid content in diet – representative of current human diets – can exacerbate hypothalamic post-prandial inflammation in a short time and that this response involves hypothalamic GFAP-positive cells and microglial cells. Although this primary response is probably aimed at regulating energy balance, continual nutritional lipid excess may contribute to dysregulating those signals and contribute to the hypothalamic disturbances leading to obesity (Thaler and Schwartz, 2010). Because the amplitude of systemic post-prandial inflammation has been correlated with type 2 diabetes and cardiovascular diseases (Emerson et al., 2017; Milan et al., 2017), our study leads us to believe that exacerbated hypothalamic post-prandial inflammation might predispose individuals to obesity and needs to be characterized in order to address this worldwide crisis.

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Author contributions

C.C., J.L.N., N.B. and C.R. conceived and supervised the study, designed experiments, and wrote the manuscript. CC. performed the majority of the experiments, interpreted results, and generated figures. K.S. performed EIA and multiplex assays. O.L.T. initiated immunohistochemistry experiments and quantitative PCR experiments with CL. J.L. performed most of the quantitative PCR experiments and contributed to data analysis. C.A.M. performed and analyzed results from electrophysiology experiments. S.BF. performed food intake experiments. N.D. participated to management of mice lines. D.D and L. F. performed TG measurements. F.B. participated to the quantification of astrocytes and microglia morphology changes. E.A. and A.B. contributed to data analysis and paper writing. All authors discussed results and/or reviewed the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in this online version.

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Figure Legends:

Figure 1: Short term high-fat diet exposure increases saturated triglyceride and endotoxin levels without affecting cytokine and chemokine levels in serum.

Serum levels of total triglycerides (TG) (A), saturated TG (B), proinflammatory cytokines IL-1β (C) IL-6 (D) TNFα (E), chemokines CCL2 (F) CCL5 (G), endotoxin (3OH C14:00; H) of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either standard diet (SD) or high-fat diet (HFD). Results are expressed in % compared to baseline prior to diet exposure (T0, dashed line = initial level). *p < 0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; ¤p ≤ 0.05 Wilcoxon Signed Rank test SD/HFD ≠ 100; Mean+/- SD. N=6-12.

Figure 2: Short term high-fat diet exposure exacerbated post-prandial inflammatory-like gene response in the hypothalamus..

Quantification of mRNA encoding proinflammatory cytokines IL-1β (A), IL-6 (B), TNFα (C) and chemokines CCL2 (D), CCL5 (E) in hypothalamus of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either standard diet (SD) or high-fat diet (HFD). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative to baseline prior to diet exposure (T0, dashed line = initial level). *p < 0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; ¤p<0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; Mean+/- SD. N=9-12.

Figure 3: Short term high-fat diet exposure induces a specific acute neuropeptide gene expression profile in the hypothalamus.

Quantification of mRNA encoding orexigenic neuropeptides NPY (A), AgRP (D), MCH (C), ORX (F) and anorexigenic neuropeptides POMC (B), CART (E) in hypothalamus of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either standard diet (SD) or high-fat diet (HFD). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold change relative to baseline prior to diet exposure (T0, dashed line = initial level). *p <

0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; ¤p<0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; Mean+/- SD. N=9-12.

Figure 4: Short term high-fat diet exposure induces an acute increase of GFAP gene expression in the hypothalamus.

Quantification of mRNA encoding the astrocyte marker GFAP (A) in hypothalamus of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either standard diet (SD) or high-fat diet (HFD) N=9-12. Quantification of immunohistochemical detection of GFAP protein (stained area, μm^2) (B) in coronal sections of the arcuate nucleus of hypothalamus (ARC; 30μm, -1.70mm relative to Bregma) from mice fed for 1h, 3h and 6h with either SD or HFD N=5-7. Immunohistochemical detection of GFAP protein in ARC of mice fed for 1h, 3h and 6h with either SD or HFD (C). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the $\Delta\Delta$CT method and presented as fold-change relative to baseline prior to diet exposure (in A, T0, dashed line = initial level). Scale bar in (C): 50μm, 3V in (C): third ventricle *p < 0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; ¤p<0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; Mean+/- SD.

Figure 5: Short term high-fat diet exposure induces acute microglial remodeling in the arcuate nucleus of the hypothalamus.

Quantification of mRNA encoding the microglial marker Iba1 (A) in the hypothalamus of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either standard diet (SD) or high fat diet (HFD) N=9-12. Quantification of immunohistochemical detection of Iba1 protein (stained area, μm2) (B) in coronal sections of the arcuate nucleus of the hypothalamus (ARC) (30μm, -1.70mm relative to Bregma) from 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either SD or HFD N=4-7. Quantification of average microglial soma size (C) in ARC of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either SD or HFD N=4-7 n=8-21. Immunohistochemical detection of Iba1 protein in ARC of 8-week-old C57Bl/6J male mice fed for 1h, 3h and 6h with either SD or HFD (D). Electrophysiological recording of current–voltage relationships (E) and membrane capacitance (F) of microglia cells

whole-cell recorded in ARC of 9-10 week-old CX3CR1eGFP/eGFP male mice fed for 3h with either SD (N=3, n=30) or HFD (N=4 n=26). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative to baseline prior to diet exposure (in A, T0, dashed line = initial level). Scale bar in (D): 50μm, 3V in (D): third ventricle. *p < 0.05 Mann–Whitney test or unpaired t test SD *vs* HFD; ¤p<0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; Mean+/- SD.

Figure 6: Modulation of GFAP-positive cells changes post-prandial hypothalamic neuropeptide and inflammatory-like gene responses to 1h SD and HFD exposure.

Quantification of mRNA encoding orexigenic neuropeptides NPY (A), AgRP (D), MCH (C), ORX (F), anorexigenic neuropeptides POMC (B), CART (E), proinflammatory cytokines IL1β (G), IL6 (H), TNFα (I), chemokines CCL2 (J), CCL5 (K) and microglial marker Iba1 (L) in hypothalamus of mice fed for 1h with either standard diet (SD) or high-fat diet (HFD). 2 weeks prior to the experiment, 8-week-old C57Bl/6J male mice were injected with the AAV8-GFAP-DREADD-Gi virus in the mediobasal hypothalamus. To specifically modulate GFAP-positive cell activity, a selective DREADD-Gi receptor agonist, CNO (Clozapine-N-Oxyde), was injected intraperitonealy 30 min before food delivery (CNO). Control mice are injected with vehicle solution (NaCl). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative to NaCl injected-condition prior to diet exposure (T0, dashed line = initial level). *p ≤ 0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; ¤p ≤ 0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; #p ≤ 0.05 Mann–Whitney test or unpaired t-test NaCl-SD *vs* CNO-SD; £p ≤ 0.05 Mann–Whitney test or unpaired t-test NaCl-HFD *vs* CNO-HFD; Two-way ANOVA for multiple comparisons and interaction; Mean+/- SD. N=7-10.

Figure 7: Modulation of microglial activity changes post-prandial hypothalamic neuropeptide and inflammatory-like gene responses to 3h SD and HFD exposure.

Quantification of mRNA encoding orexigenic neuropeptides NPY (A), AgRP (D), MCH (C), ORX (F), anorexigenic neuropeptides POMC (B), CART (E), proinflammatory cytokines IL-1β (G), IL-6 (H), TNFα (I), chemokines CCL2 (J), CCL5 (K) in hypothalamus of mice fed for 3h with either standard diet (SD) or high-fat diet (HFD). Two weeks prior to the experiment, 8-week-old C57Bl/6J male mice were treated with the CSF1R (Colony Stimulating Factor1 Receptor) antagonist, PLX5622, formulated in the AIN76A food to remove microglia in the brain (PLX). Control mice received AIN76A food without PLX5622 for 2 weeks prior to the experiment (∅). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative AIN76A-fed (+/-PLX5622) baseline prior to diet exposure (T0, dashed line = initial level). *p ≤ 0.05 Mann–Whitney test or unpaired t-test CHO *vs* HFD; ¤p ≤ 0.05 Wilcoxon Signed Rank test SD/HFD ≠ 1; #p ≤ 0.05 Mann–Whitney test or unpaired t-test Ø-SD *vs* PLX-SD; £p ≤ 0.05 Mann–Whitney test or unpaired t-test Ø-HFD *vs* PLX-HFD; Two-way ANOVA for multiple comparisons and interaction; Mean+/- SD N=8-9.

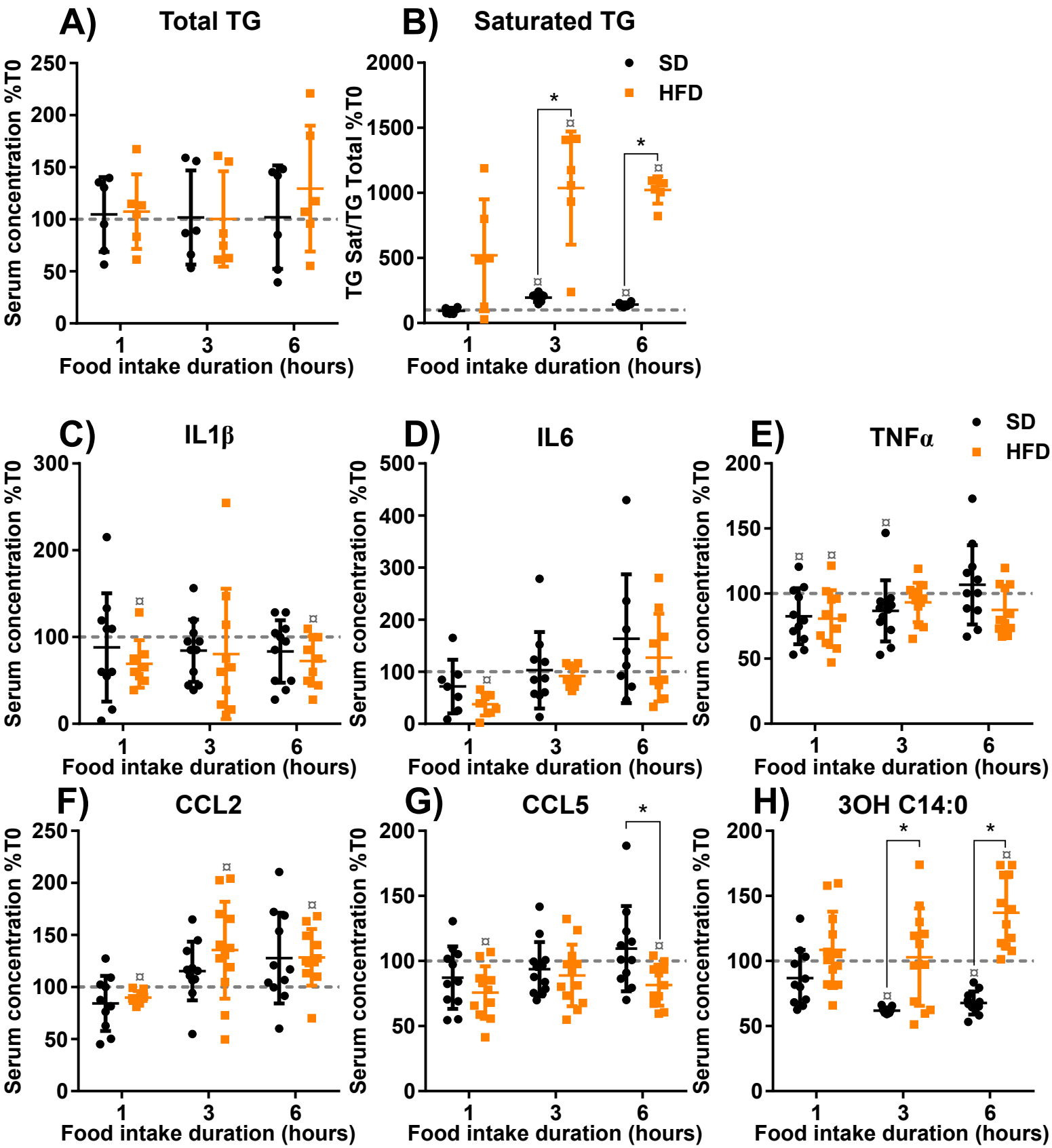

A)
Total TG
Serum concentration %T0
Food intake duration (hours)
B)
Saturated TG
TG Sat/TG Total %T0
SD
HFD
C)
IL1β
D)
IL6
E)
TNFα
F)
CCL2
G)
CCL5
H)
3OH C14:0

Figure 1

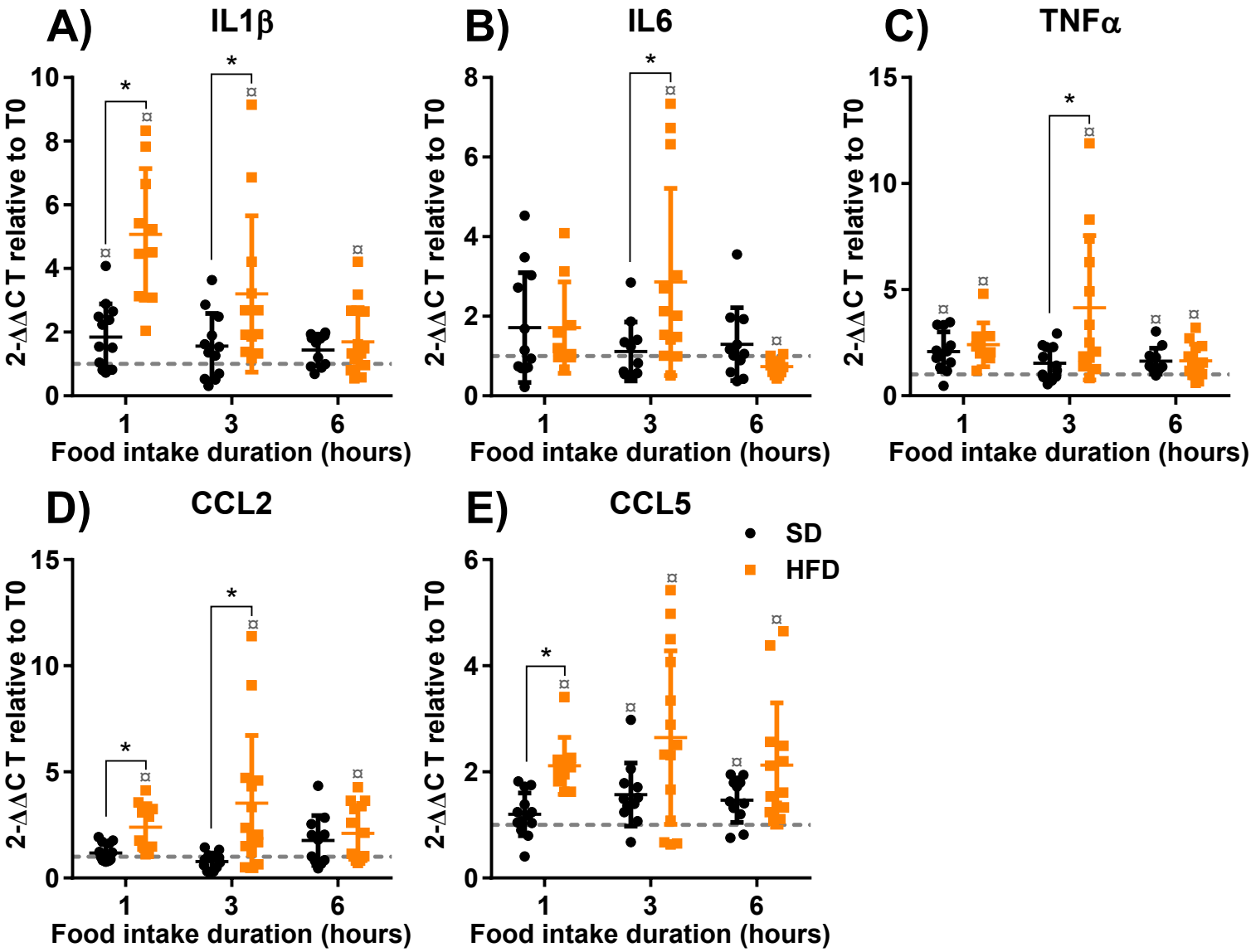

A)
IL1β
B)
IL6
C)
TNFα
D)
CCL2
E)
CCL5
SD
HFD
2-ΔΔCT relative to T0
Food intake duration (hours)
1
3
6
*
¤

Figure 2

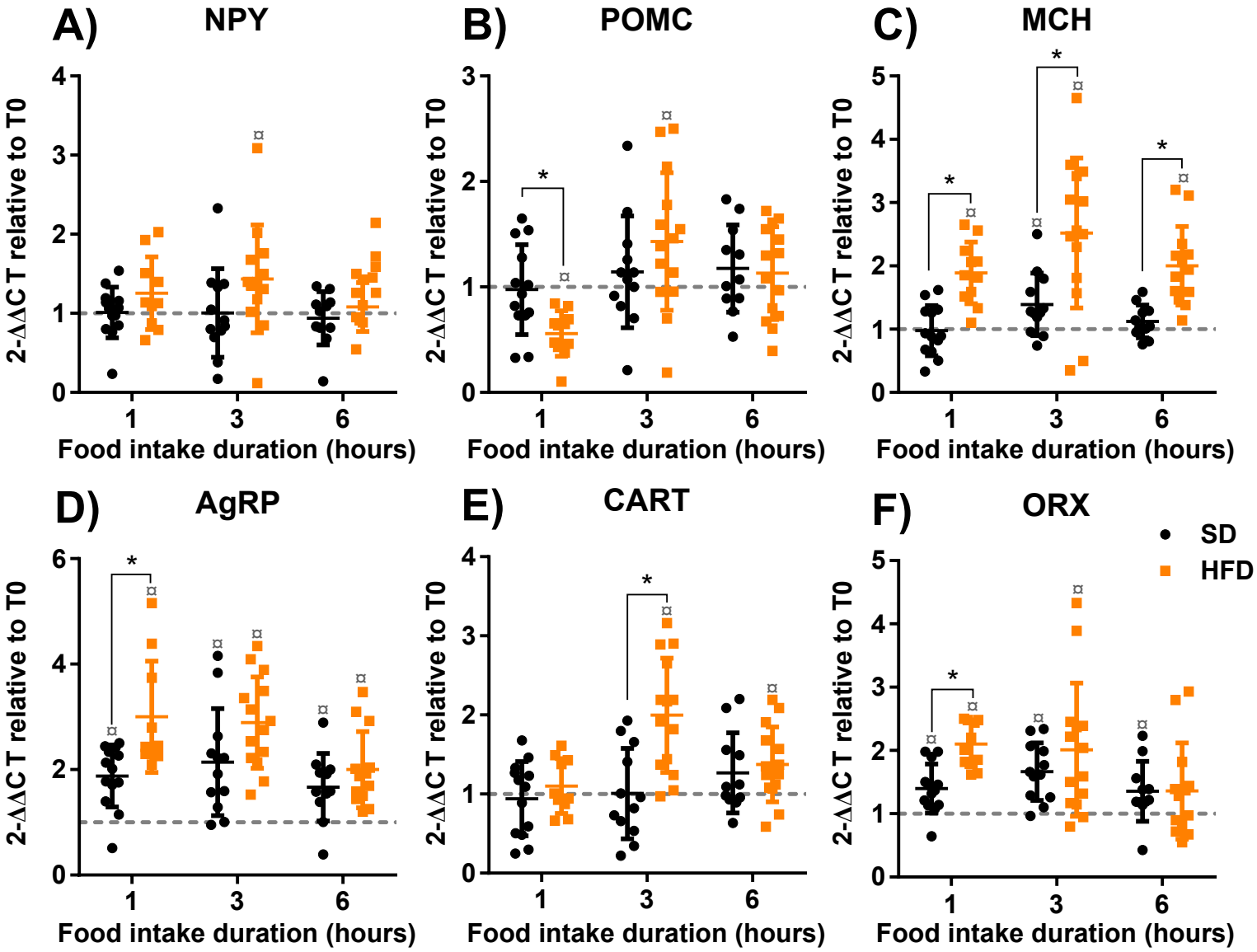

A)
NPY
B)
POMC
C)
MCH
D)
AgRP
E)
CART
F)
ORX
SD
HFD
2-ΔΔCT relative to T0
Food intake duration (hours)
1
3
6
*
¤

Figure 3

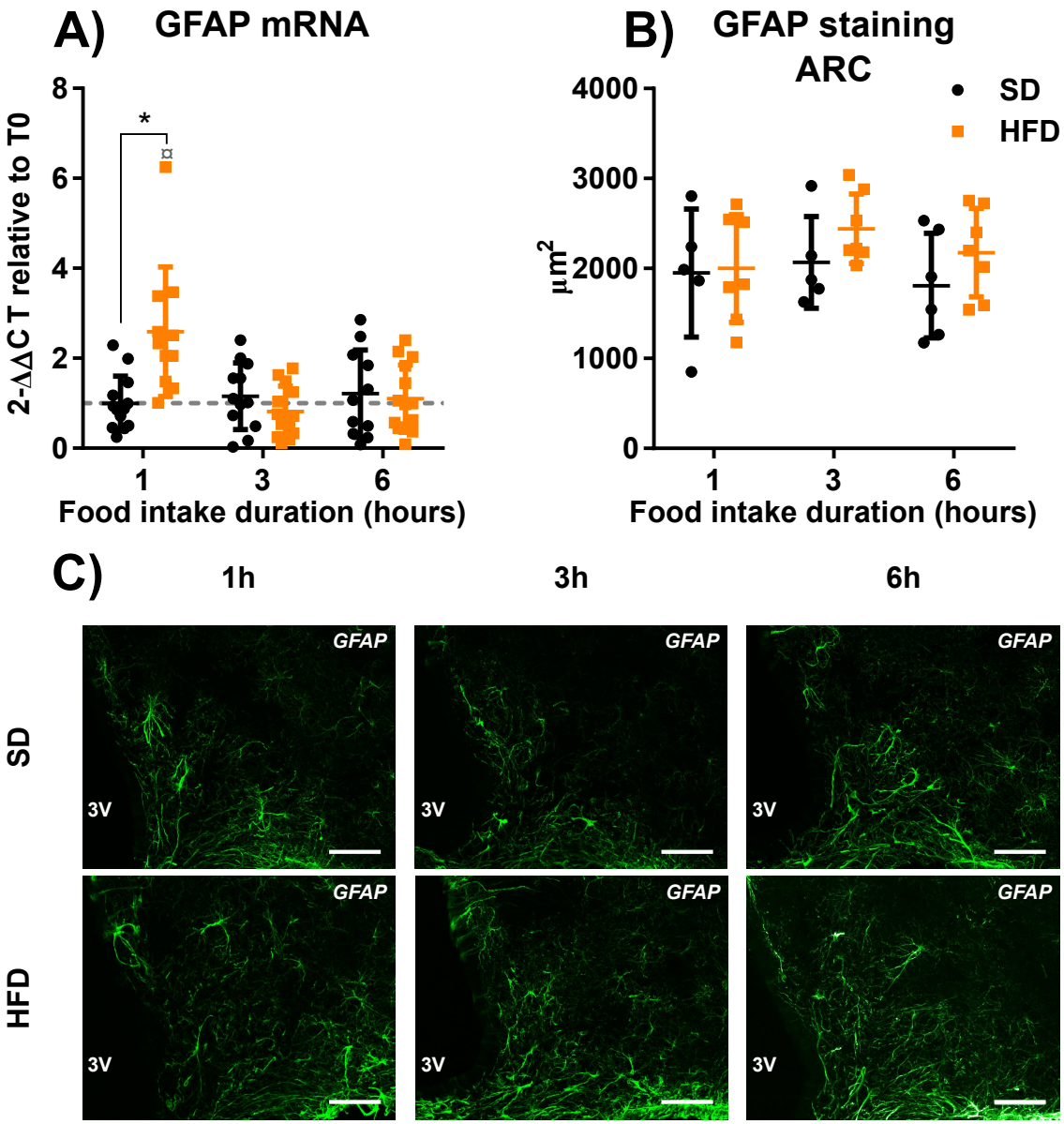

A)
GFAP mRNA
2-ΔΔCT relative to T0
8
6
4
2
0
*
¤
1
3
6
Food intake duration (hours)
B)
GFAP staining
ARC
SD
HFD
4000
3000
2000
1000
0
µm2
1
3
6
Food intake duration (hours)
C)
1h
3h
6h
SD
HFD
GFAP
3V

Figure 4

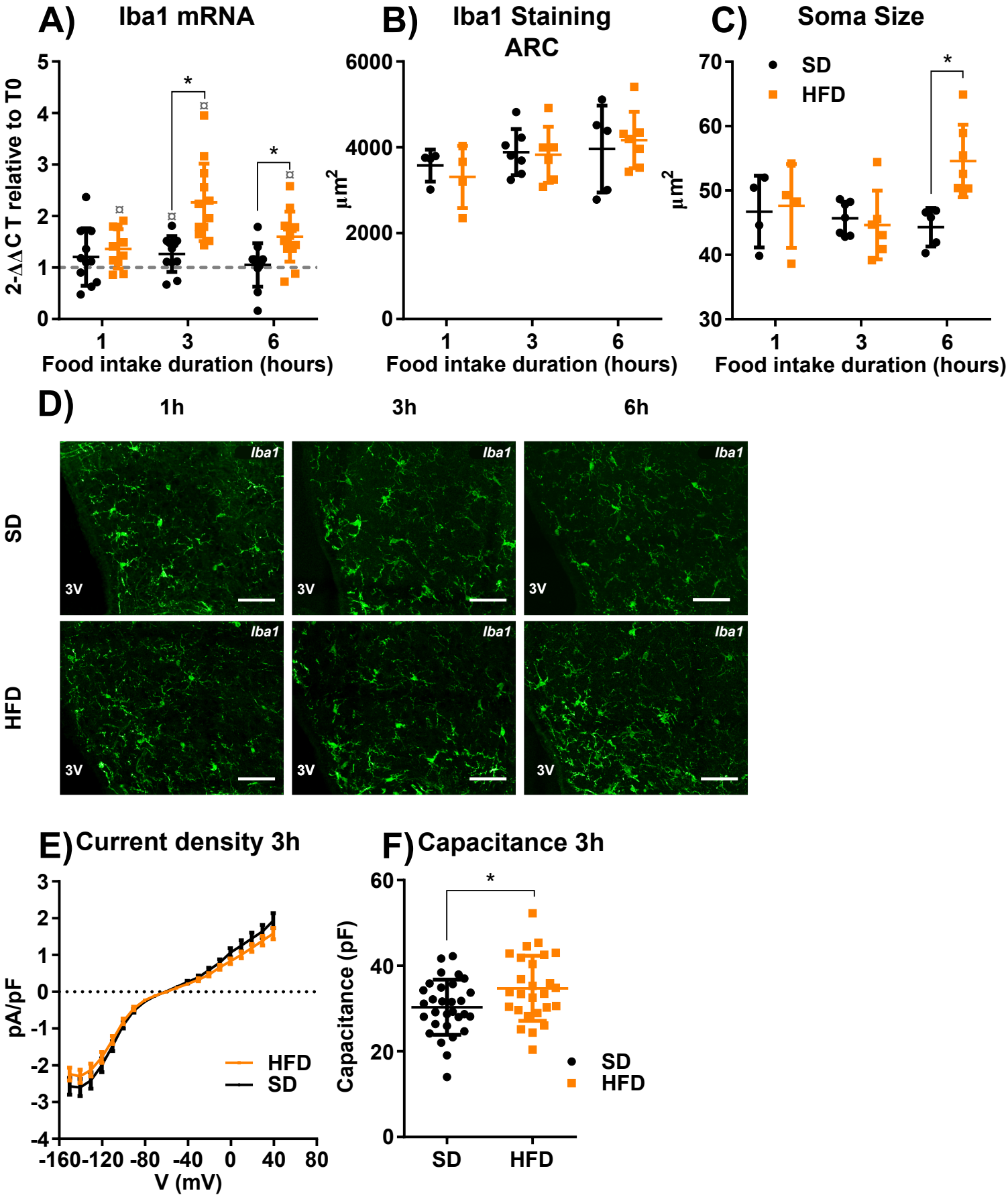

A)
Iba1 mRNA
2-ΔΔCT relative to T0
Food intake duration (hours)
B)
Iba1 Staining
ARC
µm2
Food intake duration (hours)
C)
Soma Size
SD
HFD
µm2
Food intake duration (hours)
D)
1h
3h
6h
SD
HFD
Iba1
3V
E)
Current density 3h
pA/pF
V (mV)
HFD
SD
F)
Capacitance 3h
Capacitance (pF)
SD
HFD

Figure 5

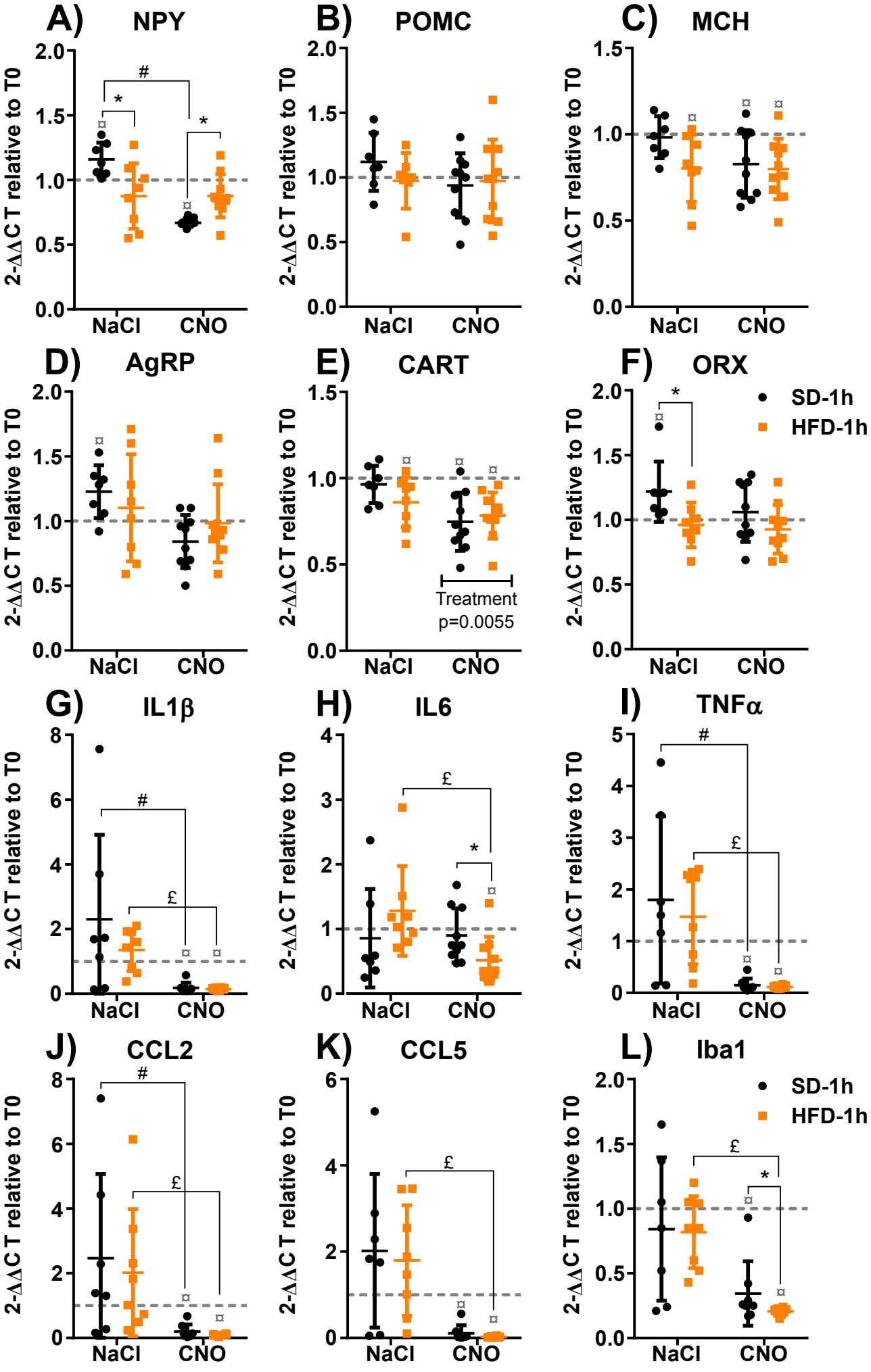
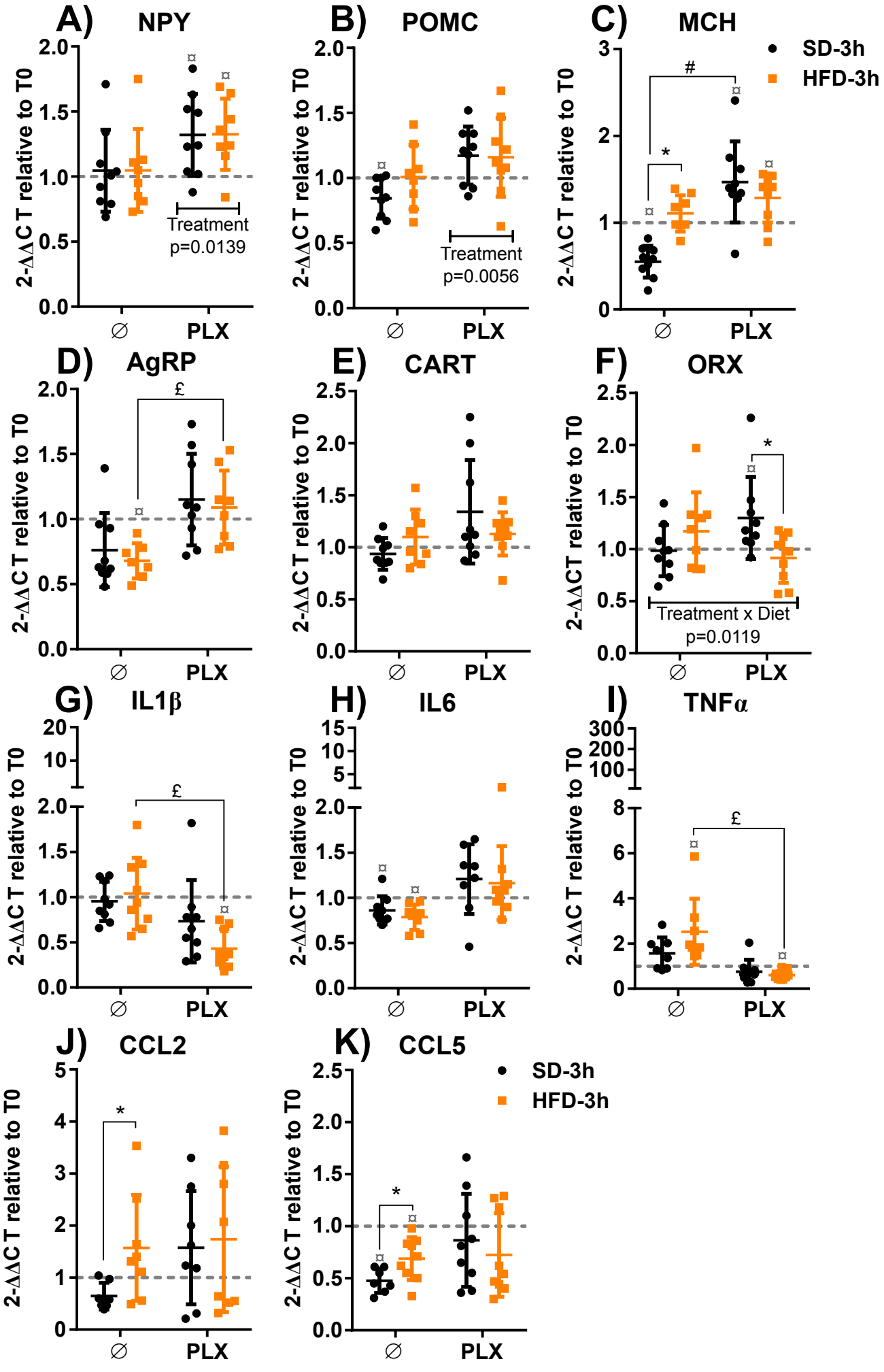

A)
NPY
B)
POMC
C)
MCH
D)
AgRP
E)
CART
F)
ORX
G)
IL1β
H)
IL6
I)
TNFα
J)
CCL2
K)
CCL5
L)
Iba1
2-ΔΔCT relative to T0
NaCl
CNO
SD-1h
HFD-1h
Treatment
p=0.0055

Figure 6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NPY
B)
POMC
C)
MCH
SD-3h
HFD-3h
D)
AgRP
E)
CART
F)
ORX
G)
IL1β
H)
IL6
I)
TNFα
J)
CCL2
K)
CCL5
2-ΔΔCT relative to T0
∅
PLX
Treatment
p=0.0139
Treatment
p=0.0056
Treatment x Diet
p=0.0119

Figure 7

Supplementary data. Supplementary Materials and Methods

Sample Preparation

Reagents:

Acetonitrile (ACN) and isopropropanol (IPA) were purchased from Carlo Erba, France. Chloroform (ChCl3) was purchased from Merck, France. These solvents were LC-MS grade. Methanol (MeOH) was from VWR, France. Formic acid Optima LC-MS quality was purchased from ThermoFisher, France. Ultrapure water was from purelab flex (Veolia Water, France), and formate ammonium (99%) was from Acros Organics, France.

Lipids extraction:

The extraction was performed using 1.5 mL solvent-resistent plastic Eppendorf tubes and 5 mL glass hemolyse tubes to avoid contamination. Methanol, chloroform and water should each be cooled down on wet ice before the lipid extraction.

Lipids were extracted according to a modified Bligh and Dyer protocol.

80 µL of serum was collected in a 1.5 mL Eppendorf tube and 200 µL of methanol was added. After vortexing (30s), the sample was frozen for 20 min at -20°C. The sample was transferred in a glass tube and 100 µL of chloroform was added. The mixture was vortexed for 30s and centrifuged (2500g, 4°C, 10 min). After centrifugation, the supernatant was collected in a new glass tube and 100 µL of chloroform and 100 µL of water were added. The sample was vortexed for 30s and centrifuged (2500g, 4°C, 10 min). 100 µL of the non polar phase was removed, added in a new glass tube and dried under a stream of nitrogen. The dried extract was resuspended in 50 µL of methanol/chloroform 1:1 (v/v) and transferred in an injection vial before liquid chromatography and mass spectrometry analysis.

Instruments and methods

Reverse phase liquid chromatography was selected for separation with an UPLC system (Ultimate 3000, ThermoFisher)**.** Lipid extracts of serum from mice were separated on an Accucore C18 150x2.1, 2.5µm column (ThermoFisher) operated at 400 µl/min flow rate. The injection volume was 3µl of diluted lipid extract. Eluent solutions were ACN/H2O 50/50 (V/V) containing 10mM ammonium formate and 0.1%

formic acid (solvent A) and IPA/ACN/H2O 88/10/2 (V/V) containing 2mM ammonium formate and 0.02% formic acid (solvent B). A step gradient program was used: 0 min 35% B, 0.0–4.0 min 35 to 60% B, 4.0–8.0 min 60 to 75% B, 8.0–16.0 min 97%, 16.0-16.1 100% B This final elution is retained for 6 minutes and finally the column was reconditioned at 35% B for 4.0 min.

The UPLC system was coupled with a Q-exactive orbitrap Mass Spectrometer (Thermofisher, CA); equipped with a heated electrospray ionization (HESI) probe. This spectrometer was controlled by the Xcalibur software and was operated in electrospray positive mode. MS spectra were acquired at a resolution of 70 000 (200 m/z) in a mass range of 250−1200 m/z with an AGC target 1e6 value and a maximum injection time of 250 ms. 15 most intense precursor ions were selected and isolated with a window of 1 m/z and fragmented by HCD (Higher energy C-Trap Dissociation) with normalized collision energy (NCE) of 25 and 30 eV. MS/MS spectra were acquired in the ion trap with an AGC target 1e5 value, in a mass range of 200-2000 m/z, the resolution was set at 35 000 at 200 m/z combined with an injection time of 80 ms.

Data were reprocessed using Lipid Search 4.1.16 (Thermo Fisher). In this study, the product search mode was used and the identification was based on the accurate mass of precursor ions and MS2 spectral pattern. Mass tolerance for precursor and fragments was set to 5 ppm and 8 ppm respectively. m-score threshold was selected at 5 and ID quality filter was fixed at grades A, B and C. $[M+H]^+$, $[M+Na]^+$ and $[M+NH4]^+$ adducts were searched.

Primers used for real-time PCR

Gene	sequence 5'-3'	concentration
mIL-1β-F	TGGTGTGTGACGTTCCCATT	
mIL-1β-R	CGACAGCACGAGGCTTTTTT	600 nM
mIL6-F	CCCAATTTCCAATGCTCTCC	
mIL6-R	TGAATTGGATGGTCTTGGTCC	600 nM
mTNFα-F	TGTACCAGGCTGTCGCTACA	
mTNFα-R	AGGGCAATTACAGTCACGGC	600 nM
mCCL2-F	CCAACTCTCACTGAAGCCAGC	
mCCL2-R	CAGGCCCAGAAGCATGACA	300 nM
mCCL5-F	ACACCACTCCCTGCTGCTTT	
mCCL5-R	AAATACTCCTTGACGTGGGCA	600 nM
mIba1-F	GGATTTGCAGGGAGGAAAA	
mIba1-R	TGGGATCATCGAATTG	600 nM
mGFAP-F	TCGACATCGCCACCTACAG	
mGFAP-R	GTCTGTACAGGAATGGTGATGC	600 nM
mMCH-F	GAAGGAGAGATTTTGACATGCTCA	
mMCH-R	CCAGCAGGTATCAGACTTGCC	600 nM
mORX-F	GCCGTCTCTACGAACTGTTGC	
mORX-R	CGCTTTCCCAGAGTCAGGATA	600 nM
mPOMC-F	AGTGCCAGGACCTCACCA	
mPOMC-R	CAGCGAGAGGTCGAGTTTG	600 nM
mCART-F	CGAGAAGAAGTACGGCCAAG	
mCART-R	CTGGCCCCTTTCCTCACT	600 nM
mNPY-F	CCGCTCTGCGACACTACAT	
mNPY-R	TGTCTCAGGGCTGGATCTCT	600 nM
mAgRP-F	CCCAGAGTTCCCAGGTCTAAGTCT	
mAgRP-R	CACCTCCGCCAAAGCTTCT	300 nM
mGAPDH-F	GAACATCATCCCTGCATCC	
mGAPDH-R	CCAGTGAGCTTCCCGTTCA	300 nM

Supplementary Table 1. Primers used for real time PCR.

Forward and reverse primers were designed using Primer Express software. Genes were analyzed with a SYBR® Green I system.

Supplementary Figures Legends:

Supplementary Figure 1: Short term high fat diet exposure increases 6h kilocalories intake.

Food intake expressed in kilocalories/mouse (A) and grams/mouse (B) of 8-week-old C57Bl/6J male mice exposed for 1h, 3h and 6h to either standard diet (SD) or high-fat diet (HFD) at the onset of the dark period. *p < 0.05 Mann–Whitney test or unpaired t-test SD vs HFD; Mean+/- SD. N=6-12.

Supplementary Figure 2: Effects of GFAP cell activity modulation on food intake.

Immunohistochemical detection of mCitrine (A), GFAP protein (B) and merged mCitrine+GFAP (C) in the arcuate nucleus of the hypothalamus (ARC; 30μm, -1.70mm relative to Bregma). Food intake expressed in kilocalories/mouse (D) and grams/mouse (E). 2 weeks prior to the experiment, 8-week-old C57Bl/6J male mice were injected with AAV8-GFAP-DREADD-Gi virus in the mediobasal hypothalamus. To specifically modulate GFAP-positive cell activity, a selective DREADD-Gi receptor agonist, CNO (Clozapine-N-Oxyde), was injected intraperitonealy 30 min before food delivery (CNO). Control mice are injected with vehicle solution (NaCl). *p ≤ 0.05 Mann–Whitney test or unpaired t-test SD *vs* HFD; Mean+/- SD N=7-10.

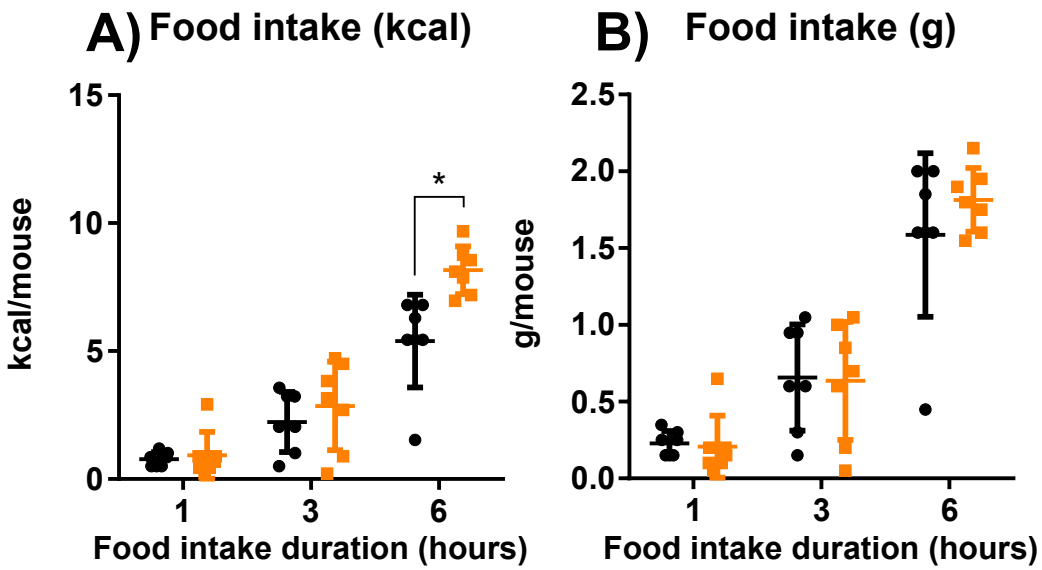
Supplementary Figure 3: Effects of microglial cell activity modulation on hypothalamic inflammatory gene expression, body weight, food intake and GFAP gene response to 1h and 3h SD and HFD exposure.

Immunohistochemical detection of Iba1 (A, B, N=1) in the arcuate nucleus of the hypothalamus (30μm, -1.70mm relative to Bregma), quantification of mRNA encoding inflammatory and neuropeptide genes (C, N=8) and body weight (D, N=45) of 8-week-old C57Bl/6J male mice treated for 2 weeks with the CSF1R (Colony Stimulating Factor1 Receptor) antagonist, PLX5622, formulated in the AIN76A food to remove microglia from the brain (B, PLX). Control mice received AIN76A food without PLX5622 for 2 weeks (A, ∅). Food intake expressed in kilocalories/mouse (E,

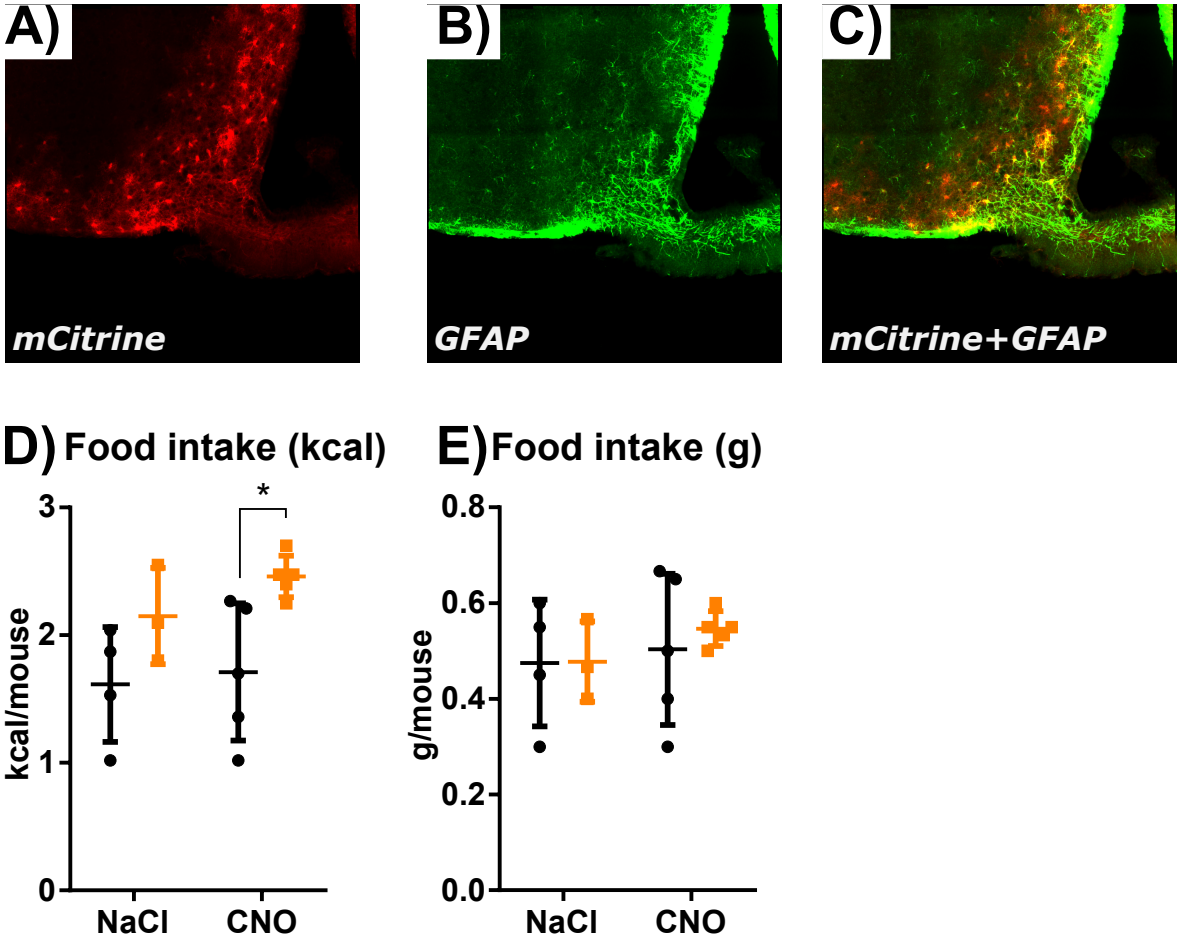
H) and grams/mouse (F, I) and quantification of hypothalamic mRNA encoding astrocyte marker GFAP (G, J) in mice fed for 1h (E, F, G) or 3h (H, I, J) with either standard diet (SD) or high-fat diet (HFD). N=8-9. mRNA are expressed as cycle threshold (CT) or were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative to AIN76A-fed (+/- PLX5622) baseline prior to diet exposure (T0, dashed line = initial level) (G, J). $p ≤ 0.05 Mann–Whitney test or unpaired t-test AIN76A *vs* AIN76A+PLX; *p < 0.05 Mann–Whitney test or unpaired t test SD *vs* HFD; ¤p<0.05 Wilcoxon Signed Rank test SD/HFD *vs* 1; Two-way ANOVA for multiple comparisons and interaction; Mean+/- SD.

Supplementary Figure 4: Effects of microglial cell activity modulation on hypothalamic neuropeptide and inflammatory-like gene responses 1h SD and HFD exposure.

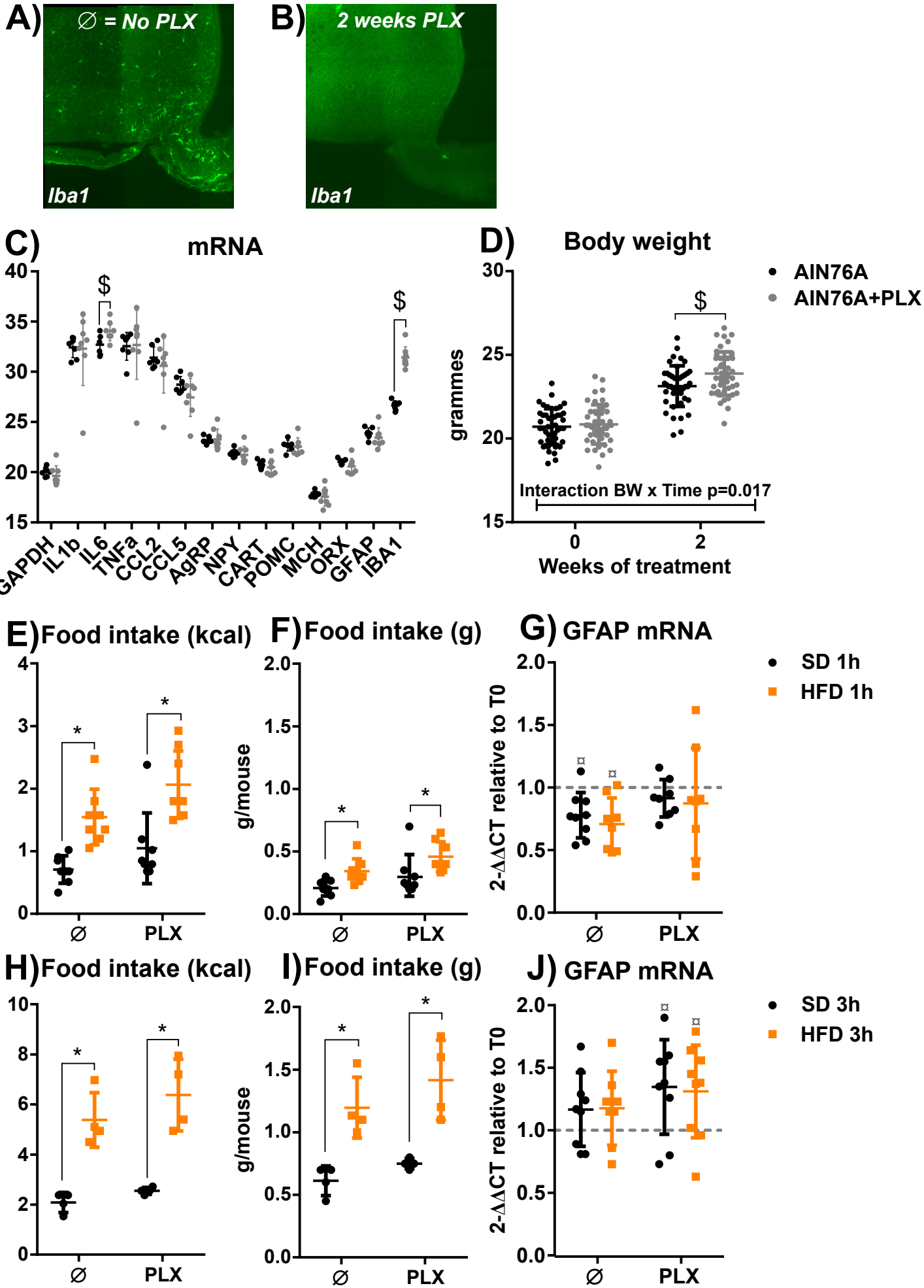
Quantification of mRNA encoding orexigenic neuropeptides NPY (A), AgRP (D), MCH (C), ORX (F), anorexigenic neuropeptides POMC (B) CART (E), proinflammatory cytokines IL1β (G), IL6 (H), TNFα (I), chemokines CCL2 (J), CCL5 (K) and astrocyte marker GFAP (L) in hypothalamus of mice fed for 1h with either standard diet (SD) or high-fat diet (HFD). 2 weeks prior to the experiment, 8-week-old C57Bl/6J male mice were treated with the CSF1R (Colony Stimulating Factor1 Receptor) antagonist, PLX5622, formulated in the AIN76A food to remove microglia in the brain (PLX). Control mice received AIN76A food without PLX5622 for 2 weeks prior to the experiment (∅). All mRNA levels were quantified relative to GAPDH housekeeping gene expression by the ΔΔCT method and presented as fold-change relative to AIN76A-fed (+/-PLX5622) baseline prior to diet exposure (T0, dashed line = initial level). *p ≤ 0.05 Mann–Whitney test or unpaired t-test CHO *vs* HFD; ¤p ≤ 0.05 Wilcoxon Signed Rank test SD/HFD ≠ 1; #p ≤ 0.05 Mann-Whitney test or unpaired t-test Ø-SD *vs* PLX-SD; £p ≤ 0.05 Mann-Whitney test or unpaired t-test Ø-HFD vs PLX-HFD; Two-way ANOVA for multiple comparisons and interaction; Mean+/- SD. N=8-9.



Supplementary figure 1



Supplementary figure 2



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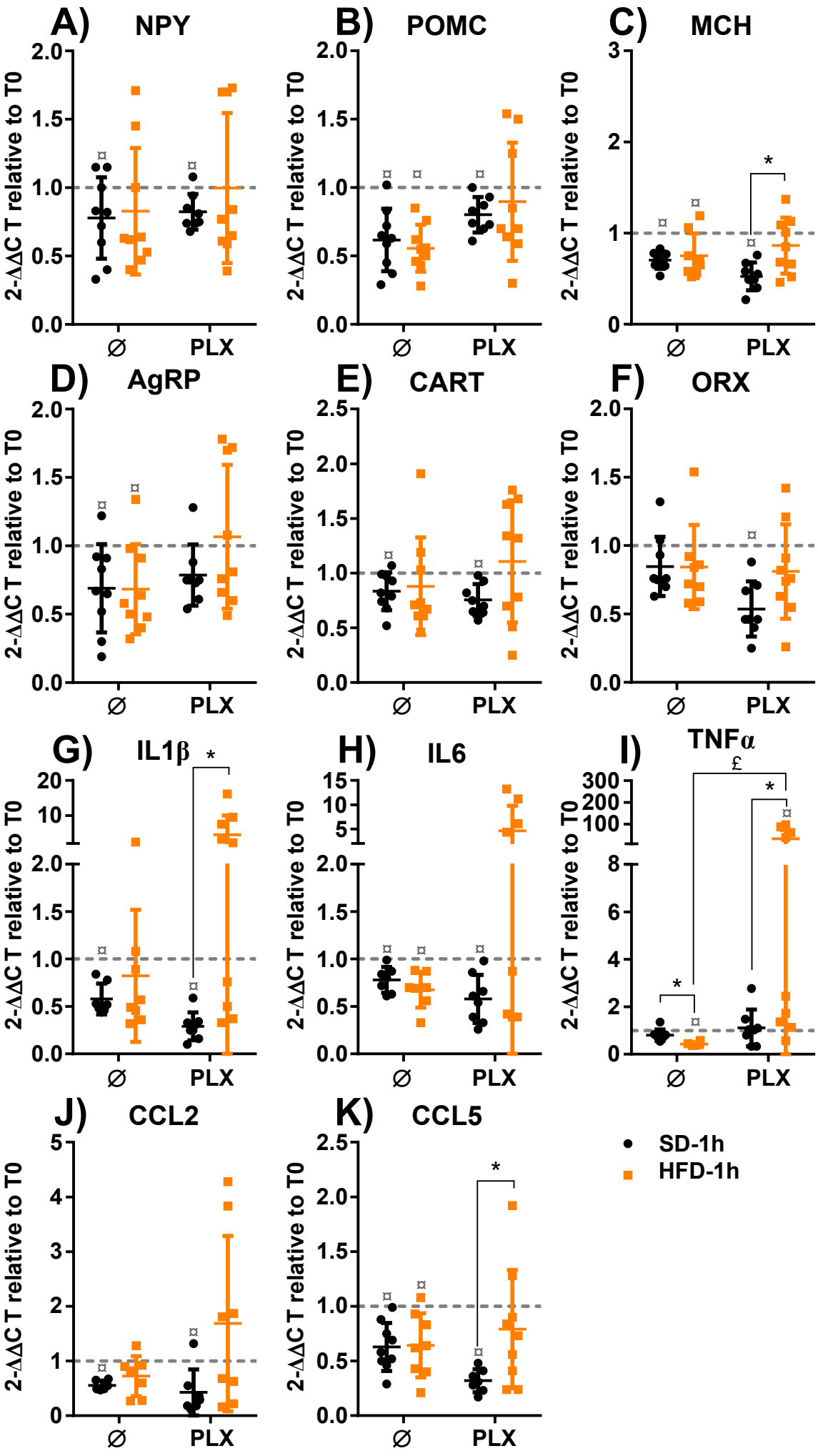

Supplementary figure 4

Scientific Report

SOURCE DATA | TRANSPARENT PROCESS

EMBO reports

Central CCL2 signaling onto MCH neurons mediates metabolic and behavioral adaptation to inflammation

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Abstract

Sickness behavior defines the endocrine, autonomic, behavioral, and metabolic responses associated with infection. While inflammatory responses were suggested to be instrumental in the loss of appetite and body weight, the molecular underpinning remains unknown. Here, we show that systemic or central lipopolysaccharide (LPS) injection results in specific hypothalamic changes characterized by a precocious increase in the chemokine ligand 2 (CCL2) followed by an increase in pro-inflammatory cytokines and a decrease in the orexigenic neuropeptide melanin-concentrating hormone (MCH). We therefore hypothesized that CCL2 could be the central relay for the loss in body weight induced by the inflammatory signal LPS. We find that central delivery of CCL2 promotes neuroinflammation and the decrease in MCH and body weight. MCH neurons express CCL2 receptor and respond to CCL2 by decreasing both electrical activity and MCH release. Pharmacological or genetic inhibition of CCL2 signaling opposes the response to LPS at both molecular and physiologic levels. We conclude that CCL2 signaling onto MCH neurons represents a core mechanism that relays peripheral inflammation to sickness behavior.

Keywords CCL2 chemokine; CCR2 signaling pathway; melanin-concentrating hormone; neuroinflammation; weight loss
Subject Categories Immunology; Metabolism; Neuroscience
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Introduction

Sickness behavior refers to the broad metabolic and behavioral changes that develop over the course of illness in response to inflammatory stimuli. Among these, pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), as well as chemokines, can directly alters mood, food intake, and body weight through local action onto central hypothalamic network regulation energy balance and stress response [1–4].

In visceral injuries, local production of cytokines can rapidly signal to the brain via a direct action onto primary afferent nerves, including the vagus and the trigeminal nerves. Following bacterial infection, activation of resident macrophages of the choroid plexus and of the circumventricular organs, devoid of blood–brain barrier (BBB), induces synthesis of pro-inflammatory cytokines that directly enter the brain. Peripheral cytokines may also cross the BBB using saturable transport systems or transitory local openings. Cytokine activation of perivascular macrophages and brain endothelial cells generates prostaglandins E2 that act on the hypothalamo–pituitary–adrenal axis to regulate stress responses [5]. Finally, the central nervous system (CNS) may synthesize *de novo* cytokines following systemic or central inflammation [6]. However, the cellular and molecular basis by which peripheral inflammation is integrated centrally to adapt food intake and body weight remains largely unknown.

Lipopolysaccharide (LPS), when injected peripherally, triggers all the key features of sickness behavior, that is, peripheral and central inflammation, suppression of appetite, and weight loss [7]. While the molecular underpinnings remain unknown, it has been shown that central, rather than peripheral, inflammation is mediating LPS-induced appetite and weight loss [8], and interestingly, CNS inflammation can be triggered independently from systemic cytokines, by non-hematopoietic cells of the brain expressing the LPS receptor: the Toll-like receptor 4 (TLR4) [9]. Central neural substrate regulating feeding and energy expenditure is composed of several neuropeptidergic circuits primarily located in the hypothalamus and brainstem. Among these, the "first-order" neuronal populations that lie close to circumventricular organs integrate circulating signals of hunger and satiety and represent also a target for peripheral inflammatory signals. They include both orexigenic/anabolic

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producing neuropeptide Y (NPY)/agouti-related polypeptide (AgRP) and anorectic/catabolic neurons that produce pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) in the arcuate nucleus (ARC). Once ARC neurons have integrated peripheral signals, they, in turn, project to "second-order" neurons located in various brain regions, including hypothalamic areas such as the lateral hypothalamic area (LHA), the ventromedial hypothalamic area (VMH), and the paraventricular nucleus (PVN) [10,11]. The hypothalamus–brainstem structure is referred as to the homeostatic circuitry and operates adaptive metabolic and behavioral responses that regulate body weight. [12].

Thus, it was tempting to speculate that central inflammatory signaling may temporarily alter "first-order" and "second-order" neurons to promote LPS-provoked weight loss. While few interleukins receptors were found on hypothalamic neurons [13,14], they are particularly lacking in the LHA neurons, whereas chemokine receptors are widely expressed and functional onto hypothalamic neurons [15], particularly the orexigenic melanin-concentrating hormone (MCH) [16–20] and the hypocretins/orexins (ORX) neurons [21].

We thus hypothesized that chemokines could be crucial intermediates connecting peripheral inflammation to the neuronal substrate mediating metabolic changes and anorexia associated with sickness behavior.

Among chemokines, we identified the monocyte chemoattractant protein 1 MCP-1/C-C motif chemokine ligand 2 (CCL2) as a potential key player because CCL2 is expressed in glial cells and discrete neuronal populations [22], is consistently increased in the CNS following peripheral inflammation [23], and has been shown to be crucially involved in LPS-mediated brain inflammation [24]. Using genetic, electrophysiological, and pharmacological approaches, we demonstrate that CCL2/CCR2 signaling onto MCH neurons is the core mechanism by which peripheral inflammatory response is centrally relayed to operate the behavioral and metabolic changes associated with sickness behavior.

Results

Systemic injection of LPS induces neuroinflammation and activates gene expression of neuropeptides involved in feeding behavior/energy balance

In order to fully characterize the sequence of molecular events that occurs at the central level in response to peripheral inflammation, we first studied the dose–response relationship of peripheral LPS injection on sickness behavior response. Intraperitoneal (ip) injection of 5 μg of LPS per mouse recapitulated most of the characteristic response seen in sickness behavior and was therefore selected for subsequent molecular analyses (Fig EV1A). We next performed a time-course study using targeted transcription profiling by PCR analysis study of pro-inflammatory cytokine genes (IL-1β, IL-6, TNF-α) and selected neuropeptide-encoding genes involved in feeding behavior to precise the molecular events that occur at hypothalamic level after ip LPS injection. As expected [25], ip LPS injection induced a very early (1–3 after post-injection) overexpression of the pro-inflammatory cytokine mRNAs (IL-1β, IL-6, TNF-α), with specific profiles (Fig EV1B). Ip LPS challenge also induced, 1 h after injection, a transitory, yet massive (up to 20-fold), induction of all the energy-related neuropeptide mRNA-encoding genes expressed in the ARC (POMC, CART, NPY, AgRP) (Fig EV1D). Strikingly, ip LPS injection induced a delayed and sustained decrease in MCH and ORX mRNA expression 24 and 48 h after injection (Fig EV1E).

Central inflammation mediated by LPS activates the expression of pro-inflammatory cytokines and CCL2 chemokine family

A dose of 500 ng of LPS per mouse injected centrally was selected based on its ability to recapitulate the time course of weight loss observed after peripheral injection (Fig 1A). Hypothalamic mRNA coding for cytokines and chemokines were quantified in a time window that precedes the first wave of increased cytokine gene expression and before downregulation of MCH or ORX gene expression, that is, 6 h after intracerebroventricular (icv) LPS or saline injection. LPS induced a sixfold to eightfold increase in mRNA-encoding IL-1β, IL-17A, and several other pro-inflammatory cytokines, including TNF-α (Fig 1B). Strikingly, the most robust activation of expression was found for genes encoding the CCL chemokines that bind to the CCR2 and/or CCR5 receptors (CCL2, CCL3, CCL4, CCL5, and CCL7 (Fig 1C)). As CCL2 plays a major role in brain inflammation following peripheral injection of LPS and selectively interacts with CCR2 in rodent brains [24,26,27], we focused on the CCL2/CCR2 signaling as a potential key mechanism relaying centrally the action of peripheral inflammation.

Central CCR2 signaling is required to operate metabolic and behavioral changes induced by LPS

Brain injection of 500 ng of LPS in WT mice induced a long-lasting decrease in body weight compared to saline-injected WT mice (Fig 2A and Appendix Fig S2). Pharmacologic or genetic blockade of CCL2/CCR2 signaling was achieved through either central CCR2 antagonist injection or in the genetic context of CCR2 KO mice. CCR2 antagonist-injected mice and genetic impairment of CCR2 signaling prevented LPS-induced weight loss with a maximum effect at the early times (6–8 h) and a late partial recovery (Fig 2A–C). As shown in the Figs EV2A and B and 2A and B, icv administration of CCR2 antagonist INCB3344 or genetic invalidation of CCR2 expression in mice induced a decrease in the percentage of weight loss of similar magnitude upon peripheral injection of LPS (Fig EV2A and B and Appendix Fig S1) or following icv LPS injection (Fig 2A and B) in mice.

Integrated analysis of energy homeostasis using indirect calorimetry coupled with activity and feeding measurement was used to fully characterize the physiologic implication of CCL2/CCR2 signaling in LPS-induced metabolic changes. Body weight loss following central LPS delivery was associated with a sharp decrease in food intake, energy expenditure, locomotor activity, and a metabolic shift toward lipid oxidation profile as indicated by both fat oxidation and respiratory quotient analysis (Figs 2 and EV3). Pharmacologically opposing CCR2 signaling through central delivery of the selective CCR2 antagonist INCB3344 [28] affected various aspects of LPS-induced weight loss. First, CCR2 antagonist counteracted the anorectic response initiated by LPS (Fig 2D and E) and mitigated the acute decrease in energy expenditure (Fig EV3C and D). Peripheral substrate utilization was calculated based on respiratory exchange ratio (VCO_2/VO_2: RER = 1 indicative of carbohydrate oxidation and RER = 0.7 indicative of lipid oxidation). Icv LPS injection induced a sharp shift in lipid oxidation

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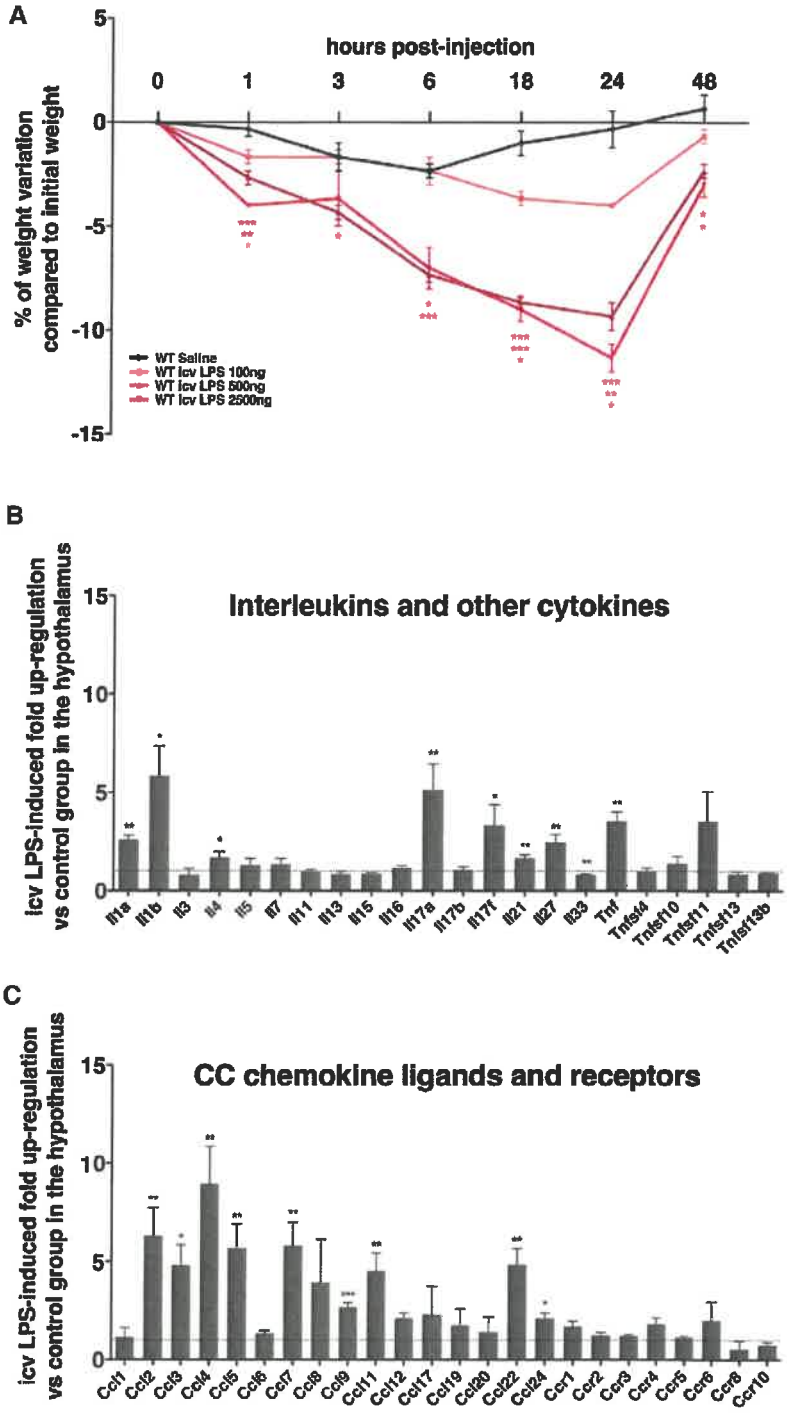

Figure 1. Analysis of inflammatory marker expression in icv LPS-injected mice.
See also Fig EV1.

A Dose–response relationship between icv LPS injection and mice weight loss (n = 3). Data are expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, color-coded asterisks indicate a significant difference between the control saline condition and the experimental condition assigned to the respective color-coded curve.

B, C Analysis by real-time PCR. Arrays of interleukins, cytokines (B), and CC chemokine ligands and receptors (C) gene expression in the hypothalamus 6 h after an acute icv injection of LPS vs. saline in WT mice (n = 4–6 per group). LPS-induced fold upregulation vs. saline condition was calculated using the $\Delta\Delta C_T$ method according to the manufacturer's protocol. Data are expressed as means ± SEM. *P < 0.05; **P < 0.01, and ***P < 0.001.

Data information: Data were analyzed by Student's unpaired two-tailed t-test (A–C).
Source data are available online for this figure.

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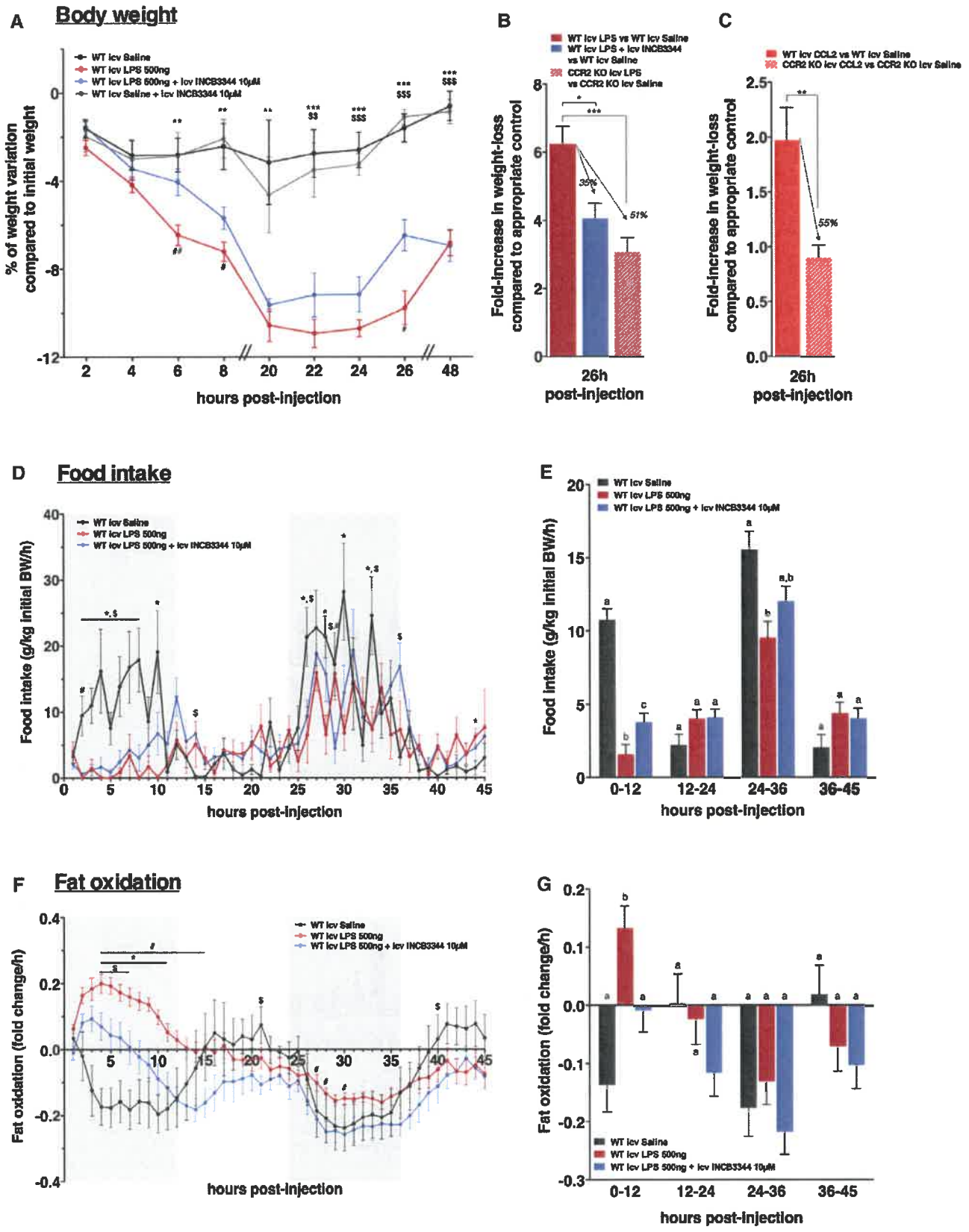

Figure 2.

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Figure 2. LPS decreases body weight and food intake and increases fat oxidation activity through a CCR2-dependent mechanism.
See also Figs EV2 and EV3, Appendix Fig S2 and Appendix Table S2.

A Weight variation (%) compared to initial body weight at different times (from 2 to 48 h) after acute icv injection of saline (black curve), INCB3344 (gray curve), LPS (red curve) or LPS+INCB3344 (blue curve) in WT mice (n = 6–12).

B Fold increase in weight loss compared to appropriate saline-injected control 26 h after acute icv injection of LPS (red bar) or LPS+INCB3344 (blue bar) in WT mice or LPS in CCR2 KO mice (striped bar) (n = 6).

C Fold increase in weight loss compared to appropriate saline-injected control 26 h after acute icv injection of CCL2 in WT mice (orange bar) or in CCR2 KO mice (striped bar) (n = 6).

D Variation of food intake recorded for 45 h during light and dark period (gray area) after acute icv injection of saline (black curve), LPS (red curve), or LPS+INCB3344 (blue curve) in WT mice (n = 5–8).

E Food intake average over four periods of 12 h after acute icv injection of saline (black bars), LPS (red bars), or LPS+INCB3344 (blue bars) in WT mice (n = 5–8).

F Variation of fat oxidation recorded for 45 h during light and dark period (gray area) after acute icv injection of saline (black curve), LPS (red curve), or LPS+INCB3344 (blue curve) in WT mice (n = 5–8).

G Fat oxidation average over four periods of 12 h after acute icv injection of saline (black bars), LPS (red bars), or LPS+INCB3344 (blue bars) in WT mice (n = 5–8).

Data information: Data are expressed as means ± SEM. In (A–D and F), data were analyzed by Student's unpaired two-tailed t-test. $^{*,\$,\#}P < 0.05$; $^{**,\$\$,\#\#}P < 0.01$ and $^{***,\$\$\$,\#\#\#}P < 0.001$. * compares saline and LPS conditions; $^{\$}$ compares saline and LPS+INCB3344 conditions; and $^{\#}$ compares LPS and LPS+INCB3344 conditions. In (E and G), analyses of variances were performed followed by a Tukey's *post hoc* test with the appropriate parameters and their interaction as factor. Data with different superscript letters (a, b, c) differ significantly ($P < 0.05$).
Source data are available online for this figure.

profile (Figs 2F and G, and EV3E and F) and CCR2 signaling blockade protected fat stores while opposing this shift (Figs 2F and G, and EV3E and F). Importantly, the action of both LPS and CCR2 signaling could only be partially correlated to change in food intake and locomotor activity (Figs 2D and E, and EV3A and B). Those results indicate that central inflammation not only affects feeding but also peripheral nutrient partitioning in a CCR2-dependent manner.

Brain-injected LPS reduces MCH mRNA and peptide expression through CCR2 signaling

To study the molecular mechanisms involved in inflammation-induced weight loss and associated changes in energy balance at the brain level, and particularly at the hypothalamic level, we determined levels of mRNA-encoding cytokines (IL-1β, IL-6, TNF-α), 1, 3, 6, 18, and 24 h after icv injection of LPS, to cover primary responses, and 18 and 24 h after injection, to analyze secondary and counteractive responses. As shown in Fig 3A, IL-1β mRNA expression levels displayed two distinct waves that peaked at 3 and 18 h (23-fold) post-injection while IL-6 mRNA (16-fold) and TNF-α mRNA (sevenfold) levels were transitorily elevated at 3 h in the LPS-treated group as compared to the saline controls. We also determined the temporal patterns of CCL2 expression induced by icv injection of LPS and confirmed the strong upregulation of both gene and protein, 3 and 6 h after icv LPS (Fig 3B and C) and ip (Fig EV1C) injection, respectively.

For the neuropeptide-encoding mRNAs, there are differentiable responses following LPS icv injection. Robust (up to 12-fold) and transitory peak of induction was found for POMC (but not CART; Fig EV4A and B), whereas NPY (and also AgRP) mRNA expression was downregulated (twofold) at 1 h post-injection (Fig EV4C and D). In contrast, a late and sustained downregulation occurred for MCH mRNA (Fig 3D) and ORX (Fig EV5A) 18 and 24 h after LPS injection. We therefore characterized the protein levels of MCH and ORX and found that they followed the mRNA patterns observed at 1, 6, 18 h LPS post-injection. MCH (53.5 ± 5.2 ng/mg of proteins) (Fig 3E) and ORX (83.1 ± 2.1 ng/mg of proteins; Fig EV5B) dropped down compared to saline group at 18 h post-LPS. No change was observed for both neuropeptides in the cerebellum extracts used as negative controls (Figs 3E and EV5B).

Finally, we examined the relative importance of the CCR2 signaling pathway in the MCH-expressing neuronal network. Strikingly, the twofold decrease in MCH mRNA expression at 18 h LPS post-injection was reversed in mice treated with the selective CCR2 antagonist INCB3344 [28] and in CCR2 KO mice (at 81 and 77% of the initial MCH mRNA content, respectively) (Fig 3F). Strikingly, the 50% reduction in MCH mRNA expression was also similarly prevented under ip LPS administration (Fig EV2C). Altogether, these results suggest that CCR2 signaling could play a fundamental role in the LPS-based neuroinflammatory response leading to MCH gene downregulation.

Brain-injected CCL2 suppresses MCH mRNA and peptide expression through CCR2 signaling

We tested thereafter the hypothesis that a specific activation of CCR2 signaling may mimic the spectrum of cytokine/chemokine and neuropeptide gene expression variations observed following LPS treatment. We found that icv CCL2 injection increased mRNA coding for IL-1β, IL-6, and TNF-α by a 5.5-, 2.3- and 2.8-fold, respectively, at 6 h post-injection (Fig 4A). A fast (1 h) and long-lasting (up to 18 h) activation of both CCL2 mRNA and protein levels was also found in the hypothalamus (Fig 4B and C). Finally, expression of MCH or ORX-encoding mRNA was downregulated by 2.1- and 2.6-fold at 6 and 18 h, respectively (Figs 4D and EV5C). At the protein levels, CCL2 injection significantly decreased by twofold both MCH and ORX peptide concentrations compared to control group at 6 and 18 h post-injection (Figs 4E and EV5D). No change was observed for both neuropeptides in the cerebellum extracts used as negative controls (Figs 4E and EV5D).

Finally, the twofold decrease in MCH mRNA expression at 18 h LPS post-injection was blunted in CCR2 KO mice and in mice treated with INCB3344 (Fig 4F). This demonstrated that CCL2-induced MCH downregulation in the LHA was fully dependent upon the CCR2 signaling pathway.

CCR2 is expressed by MCH but not ORX neurons in the LHA of normal mice

As the distribution of CCR2 in normal rodent brain was poorly described [23], we characterized CCR2-immunoreactivity (CCR2-IR)

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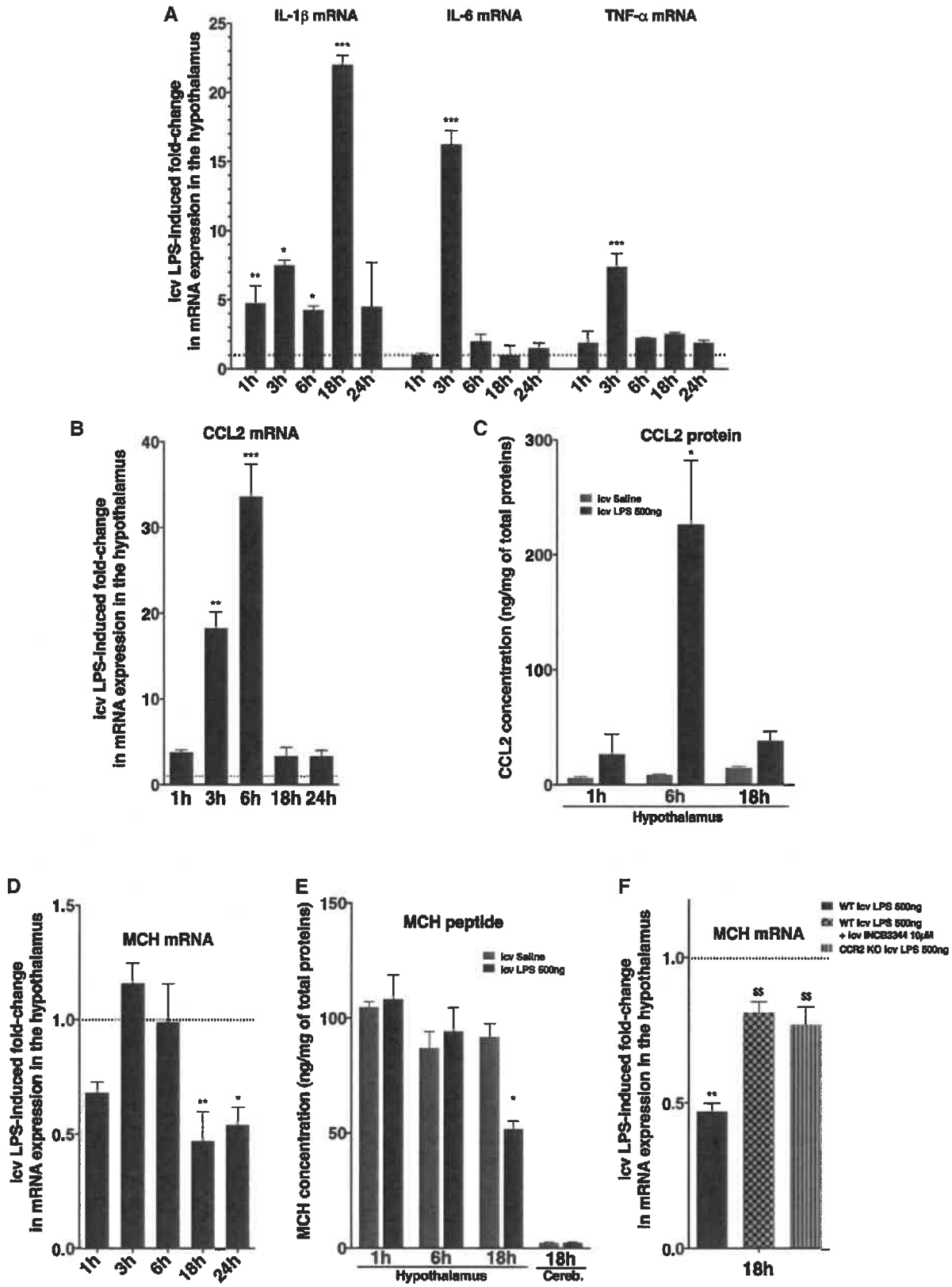

Figure 3.

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Figure 3. Brain-injected LPS induced differential variations in hypothalamic expression of cytokines and of the orexigenic neuropeptide MCH.
See also Figs EV4 and EV5.

A Real-time PCR analysis of the genes coding for the pro-inflammatory cytokines IL-1β, IL-6, TNF-α in the hypothalamus of icv LPS-injected mice at different times after injection (from 1 h to 18 h), normalized to values in icv saline-injected mice (n = 6 per group).

B–E Study of gene and protein hypothalamic expression of the chemokine CCL2 (B, C) and MCH peptide (D, E) in icv LPS-injected mice. Real-time PCR analysis for CCL2 (B) and MCH (D) at different times after injection (from 1 to 24 h), normalized to values in control icv saline-injected mice (n = 6 per group). Measurement of CCL2 (C) and MCH (E) concentrations by EIA after icv LPS injection (black bars) or icv saline injection (gray bars) in mice at 1 h, 6 h and 18 h after injection. Cerebellum was used as negative control (3 independent experiments, n = 6 per group in each experiment).

F The decrease in MCH mRNA levels observed 18 h after icv LPS injection is partly abolished by the CCR2 antagonist INCB3344 (10 μM) and in CCR2 KO mice, as shown by real-time PCR analysis for MCH in the hypothalamus. Results were normalized to values in icv saline-injected WT mice (n = 6 per group). Data are expressed as means ± SEM. $^{**,\$\$}P < 0.01$. * saline vs. icv LPS injection in WT mice. $^{\$}$ icv LPS injection in WT mice vs. LPS+INCB3344 icv injection in WT mice or icv LPS injection in CCR2 KO mice.

Data information: Data in (A–E) are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, icv LPS injection vs. icv saline injection. Data were analyzed by Student's unpaired two-tailed t-test (A–F).

in mice expressing cyan fluorescent protein (CFP) under the control of the MCH promoter (MCH-CFP) [29,30]. MCH (green) and CCR2 (red) immunoreactivities largely overlapped demonstrating that most of the MCH neurons expressed CCR2 receptors (approximately 70%; Fig 5A), whereas CCR2-immunoreactivity was not found within ORX-expressing cells (Fig EV5E).

CCL2 directly hyperpolarizes MCH neurons in mouse brain slices

We performed patch-clamp experiments on fluorescent neurons expressing MCH of the lateral hypothalamic slices prepared from MCH-CFP mice. CCL2 application hyperpolarized MCH neurons (Fig 5B). CCR2 antagonist INCB3344 prevented CCL2-induced hyperpolarization (Fig 5B), and the hyperpolarization amplitude was dependent on CCL2 concentration (Fig 5C). It was of (absolute value in mV) 2.17 ± 0.94 at 0.1 nM CCL2, 3.45 ± 0.56 at 1 nM CCL2 and 5.63 ± 1.08 at 10 nM CCL2.

We investigated whether the hyperpolarization induced by CCR2 activation could be due to G protein activated inward rectifier current (GIRK) activation. The effect inverted around −80 mV that is the equilibrium potential for K^+ ions. We found that 1 nM CCL2 effect was significantly blocked by previous application of 200 μM barium, a concentration known to inhibit specifically KIR channels (Fig 5C). About 200 μM barium alone induced a significant depolarization of MCH neurons (of 10.00 ± 1.67 mV), suggesting that KIR channels are spontaneously opened under control conditions in MCH neurons. We then measured the current induced by CCL2 in voltage-clamp at a holding potential of −60 mV, with a KCl intracellular solution. CCL2 (10 nM) induced a small outward current (+7.5 ± 1.44 pA) associated with a decrease in membrane resistance (Fig EV6A and B). CCL2 (10 nM) also increased by 30% the frequency of spontaneous post-synaptic currents without significant effect on the amplitude of these events (Fig EV6C and D).

Finally, we investigated the consequences of the CCL2 effects on action potential (AP) discharge (Fig 5D). CCL2 induces either delays (Fig 5D, left panel) or failures (Fig 5D, right panel) in action potential emission. This effect was likely a consequence of a decrease in membrane resistance due to GIRK channels and/or receptor channels opening.

CCL2 abolishes KCl-induced MCH release from hypothalamic explants

Based on our observations of decrease in MCH neurons excitability, we evaluated whether CCL2 could modulate MCH release induced by a robust KCl-mediated neuronal depolarization. Perifusion of hypothalamic tissues with 60 mM KCl for 30 min induced a 3.1-fold increase in MCH release (Fig 5E, black line). Strikingly, this increase was fully blunted by co-application of CCL2 (Fig 5E, red line), an inhibitory effect reversed by addition of the CCR2 antagonist INCB3344 (Fig 5E, blue line). In contrast to MCH secretion, application of CCL2 did not prevent ORX release (Fig EV5F).

LPS or CCL2-induced weight loss may be driven by the MCH/MCHR1 signaling

Finally, we tested whether *in vivo* MCH signaling through MCHR1 was an important element to mediate LPS- or CCL2-induced weight loss (Fig 5F). In agreement with previous studies [18,31], the pharmacological inhibition of the MCH receptor by the specific antagonist H6408 blocked the basal orexigenic tone leading to a slight weight loss. LPS but not CCL2 injection doubled this response, suggesting a similar range of action for CCL2 or MCH antagonist on the weight loss. Similar effects were noted in presence or not of H6408 at both 6 and 24 h LPS post-injection. In contrast, an additive effect of the MCH antagonist was noted on the CCL2-driven weight loss at 6 h but not 24 h, highly suggesting that the MCH signaling pathway could participate as a major downstream element at this early time.

Discussion

While a participation of the ARC was established in inflammation-induced anorexia or weight loss [32–34], a contribution of LHA neurons expressing orexigenic neuropeptides like MCH or orexins could also be considered [35,36]. Furthermore, brain CCL2/CCR2 signaling is activated by systemic LPS injection [24,26].

Here, we demonstrated that LPS-induced behavioral and metabolic adaptation resulting in weight loss depends on the integrity of the CCR2 signaling in MCH-expressing neurons and revealed for the first time that locally produced brain chemokines may act directly on appetite-regulating neuronal networks. Brain inflammation was induced by ip and icv injections of LPS, as confirmed by an upregulation of cytokines such as IL-1β, that followed the characteristic two wave patterns observed after peripheral LPS injection [37]. Beneficial and detrimental effects of IL-1β production have been linked to neuroinflammatory conditions and neurodegenerative diseases. The molecular steps leading to IL-1β maturation take place

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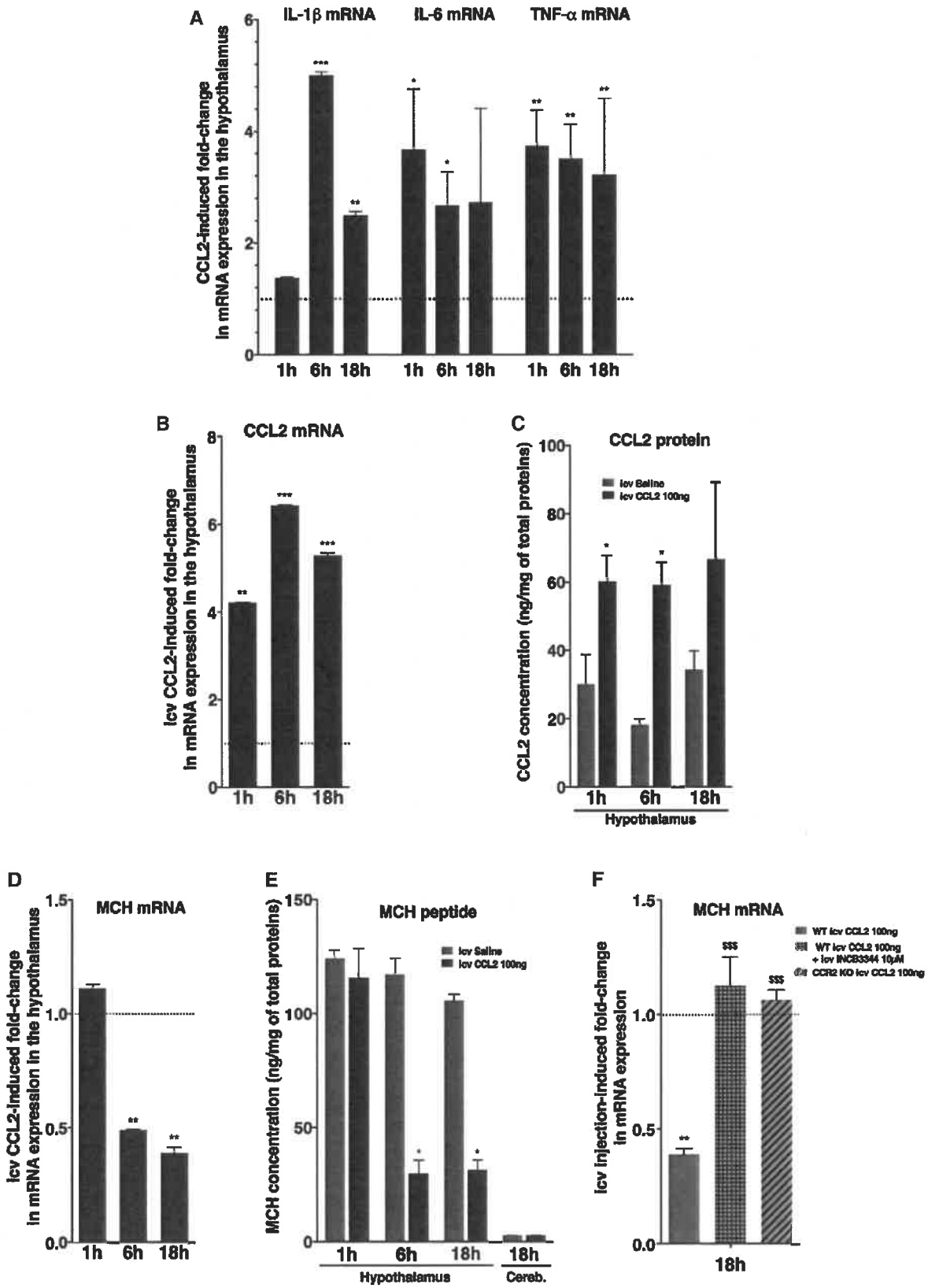
A
IL-1β mRNA
IL-6 mRNA
TNF-α mRNA
CCL2-induced fold-change in mRNA expression in the hypothalamus
1h 6h 18h 1h 6h 18h 1h 6h 18h
B
CCL2 mRNA
Icv CCL2-induced fold-change in mRNA expression in the hypothalamus
1h 6h 18h
C
CCL2 protein
Icv Saline
Icv CCL2 100ng
CCL2 concentration (ng/mg of total proteins)
1h 6h 18h
Hypothalamus
D
MCH mRNA
Icv CCL2-induced fold-change in mRNA expression in the hypothalamus
1h 6h 18h
E
MCH peptide
Icv Saline
Icv CCL2 100ng
MCH concentration (ng/mg of total proteins)
1h 6h 18h 18h
Hypothalamus
Cereb.
F
MCH mRNA
WT Icv CCL2 100ng
WT Icv CCL2 100ng + Icv INCB3344 10µM
CCR2 KO Icv CCL2 100ng
Icv injection-induced fold-change in mRNA expression
18h

Figure 4.

Figure 4. Brain-injected CCL2 altered the expression of pro-inflammatory cytokines and of the orexigenic neuropeptide MCH in the hypothalamus.
See also Fig EV5.

A Real-time PCR analysis of the genes coding for the pro-inflammatory cytokines IL-1β, IL-6, TNF-α in the hypothalamus of icv CCL2-injected mice at different times after injection (from 1 to 18 h), normalized to values in icv saline-injected mice (n = 6 per group).

B–E Study of gene and protein hypothalamic expression of the chemokine CCL2 (B, C) and MCH peptide (D, E) in icv CCL2-injected mice. Real-time PCR analysis for CCL2 (B) and MCH (D) at different times after injection (from 1 h to 24 h), normalized to values in control icv saline-injected mice (n = 6 per group). Measurement of CCL2 (C) and MCH (E) concentration by EIA after icv CCL2 injection (black bars) or icv saline injection (gray bars) in mice at 1, 6, and 18 h after injection. Cerebellum was used as negative control (3 independent experiments, n = 6 per group in each experiment).

F The decrease in MCH mRNA levels observed 18 h after icv CCL2 injection is abolished by the CCR2 antagonist INCB3344 (10 μM) and in CCR2 KO mice, as shown by real-time PCR analysis for MCH in the hypothalamus. Results were normalized to values in icv saline-injected WT mice (n = 6 per group). $^{**}P < 0.01$, $^{\$\$\$}P < 0.001$. * saline vs. icv CCL2 injection in WT mice. $^{\$}$ icv CCL2 injection in WT mice vs. CCL2+INCB3344 icv injection in WT mice or icv CCL2 injection in CCR2 KO mice.

Data information: Data are expressed as means ± SEM. Data in (A–E): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, icv CCL2 injection vs. icv saline injection. Data were analyzed by Student's unpaired two-tailed t-test (A–F).

in an intracellular complex termed the inflammasome [38]. However, the potential role of the NLRP3 inflammasome in the local production of IL-1β in the brain is not well understood [39,40]. In spite of our results linking acute inflammation and weight loss, it is tempting to speculate a role of NLRP3 inflammasome that would be interested to further investigate.

The central inflammation was associated with a striking increase in many CCR2 ligands. Among potential candidates, CCL2 was particularly considered, based on (i) its strong brain expression following systemic LPS challenges [25,26,41], (ii) its key role in the recruitment of monocytes in the brain [42,43], and (iii) the fact that both brain inflammation and weight loss following peripheral LPS injection are markedly reduced in CCL2 KO [24], CCR2 KO, and INCB3344-treated mice (Fig EV2 and Appendix Fig S2). Furthermore, CCL2 and CCR2 are expressed in LHA neurons [22,44], in contrast to sporadic cytokine receptors expression [13,14]. Therefore, we investigated whether CCL2 would act as intermediate between cytokines and neurons in the cascade linking inflammation to alteration of neuronal networks involved in food behavior. Firstly, its particular kinetics of activation following central LPS injection arises between inflammatory cytokines activation and downregulation of orexigenic peptides, such as MCH and ORX. Secondly, its primary receptor, CCR2 is expressed into MCH-producing neurons leading to hypothesize that CCL2 could directly modulate MCH neurons activity. Indeed, in mice with genetic or pharmacological CCR2 disruption, ip and icv LPS injections were inefficient in reducing MCH mRNA. Such mandatory effect of CCL2/CCR2 signaling on MCH gene regulation was confirmed by the full reversion of CCL2-induced MCH mRNA decrease in the CCR2 KO and INCB3344-treated mice. The 50% reduction in MCH mRNA expression was similarly prevented in both paradigms either under ip or under icv LPS administration (Figs EV2C and 3F). The same responses noted under both types of injection indicate that weight loss following icv LPS administration could not result from a general noxious action.

CCL2 generates a hyperpolarization blocked by low barium concentration associated with an increase in membrane conductance, characteristic of activation of a GIRK current. This will tend to shunt somatic excitatory currents and block the propagation of excitatory events. Indeed, CCL2, via the direct stimulation of CCR2, depresses the frequency of discharge of evoked APs. Finally, we addressed the effect of CCL2 on the release of MCH induced by KCl-mediated depolarization [45]. We found a drastic reduction in MCH secretion by adding CCL2, an effect fully blocked by the INCB3344 antagonist.

Intriguingly, further MCH secretion induced by KCl-mediated depolarization was observed in the only presence of the INCB3344 antagonist (data not shown) suggesting that endogenous CCL2 may also contribute to the strong inhibition of MCH release. As a significant proportion of MCH neurons expresses both CCR2 and CCL2 and could be activated by the general depolarization induced by KCl, the locally expressed chemokine would act as a short-loop autocrine modulator amplifying the CCL2 responses driven by astrocyte activation [42,46]. Thus, both sources of brain CCL2 are likely to contribute to the inhibition of MCH secretion.

Among the myriad of mediators involved in the LPS-driven brain inflammation that modifies the activity of neurons [47–50], we demonstrated here that CCL2/CCR2 signaling could directly inhibit a major orexigenic neuronal network, that is, the MCH neuronal pathway.

As CCL2 effects on feeding behavior and body weight were poorly investigated [51], this study is the first demonstrating selective weight loss following icv CCL2 injection in mice, an effect fully abolished in CCR2 KO mice or mice treated with the CCR2 antagonist. Interestingly, extensive analysis of the different metabolic parameters affected by central inflammation also revealed that not only food intake but also peripheral nutrient partitioning is affected by LPS injection and rely onto CCL2 signaling.

Prevention of the weight loss induced by LPS by CCR2 antagonist injection or invalidation of the CCR2 gene in transgenic mice was partial and transitory, indicating that there must be other neuronal pathways involved in this action. Indeed, LepR/γ-aminobutyric acid (GABA)-expressing neurons have been shown to also regulate body weight: leptin mainly acts on GABAergic neurons to reduce body weight and triggers POMC neuronal activity by reducing GABA release onto these neurons. This suggested a body weight-promoting role for GABA released from leptin-inhibited neurons [52–54]. Since ORX neurons do not express CCR2, the few $MCH^{-}/CCR2^{+}$ cells we noticed in the LHA could be related to LepR/GABA neuronal pathway. Importantly, MCH neurons are located in the LHA which is known to be a pre-ganglionic structure whose output directly affects neurons controlling autonomic output, which in turn controls peripheral substrate utilization [55]. Hence, MCH neurons could relay two of the main adaptive responses triggered by central inflammation, that is, reduction in appetite and loss of fat stores through enhanced fat oxidation. Paradoxically, CCR2 signaling is involved in metabolic disorders associated with obesity [56] as underlined by its role in the hyperphagic response to a high-fat diet (HFD) [57], as another chemokine system, the CXCL12/CXCR4 system. Thus, these chemokine systems may have a role in mediating both neuronal and behavioral effects induced by a HFD [58]. Nevertheless, a low-grade inflammation both in the CNS and at the

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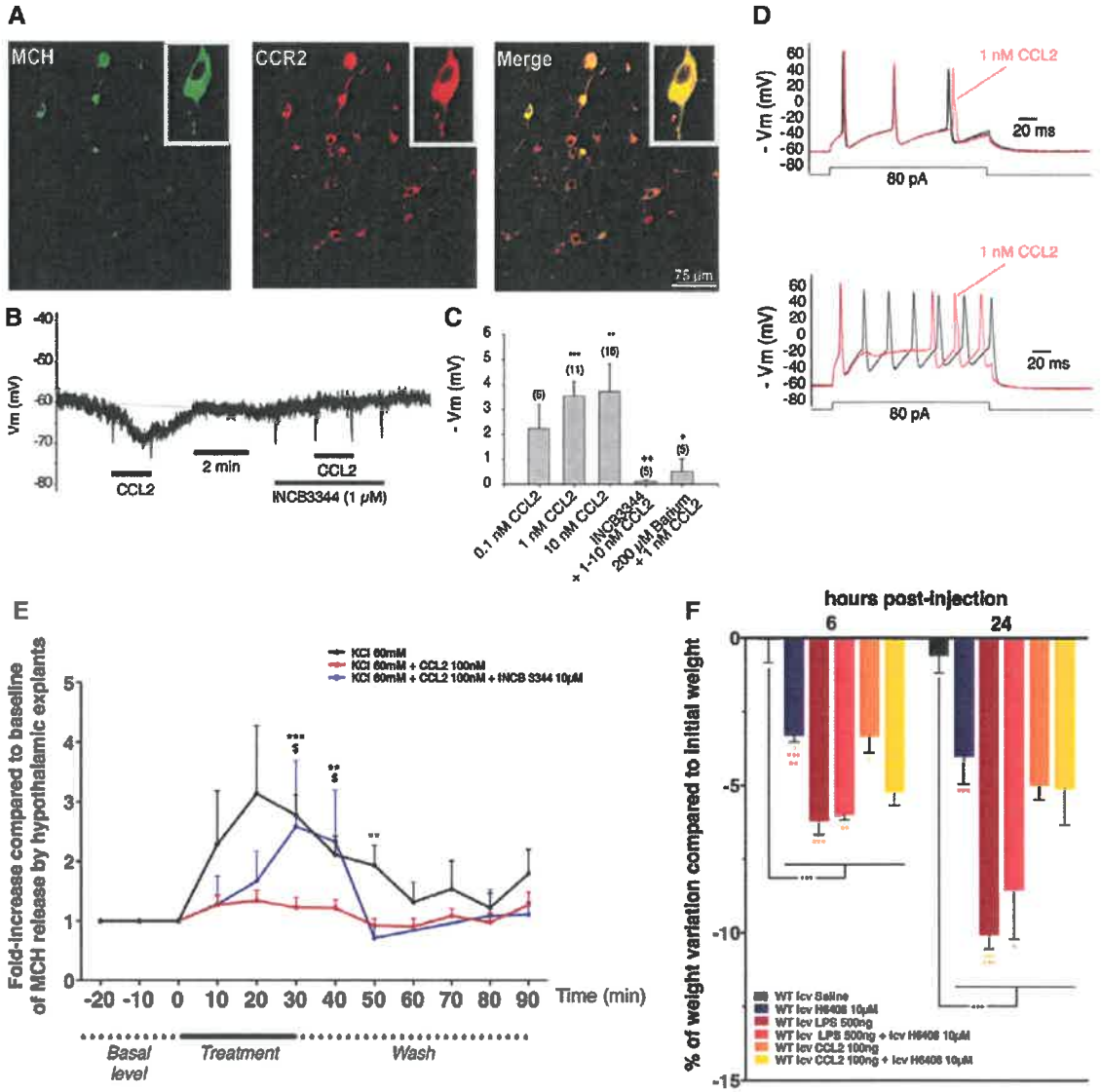

Figure 5. The chemokine CCL2 can directly modulate MCH neurons.
See also Figs EV5 and EV6.

A Co-localization of MCH and the receptor for CCL2 (CCR2) in the LHA of mice expressing CFP under the promoter of MCH. The overlap of MCH and CCR2 immunoreactivity indicates that CCR2 is expressed on MCH neurons in the LHA.

B–D CCL2 effects on MCH neurons of MCH-CFP knock-in mice recorded in current-clamp mode. Representative trace of the effects of CCL2 on MCH neurons (B). The CCR2 antagonist INCB3344 prevents the small hyperpolarization of MCH neurons elicited by CCL2. Effects of CCL2 at various concentrations, alone or in the presence of the INCB3344 (1 µM) or barium (200 µM) on the membrane potential of MCH neurons (n = 5–16) (C). $^{**}P < 0.01$, $^{***}P < 0.001$, t-test after ANOVA; $^{+}P < 0.05$, $^{++}P < 0.01$, paired t-test, against their own control. Data are expressed as means ± SEM. Discharge pattern of two representative MCH neurons in response to a depolarizing current pulse injection (current pulses applied every 10 s) in control condition and in the presence of CCL2 (D). The control trace is recorded just before the application of CCL2 while the CCL2 trace is recorded 1 min after the beginning of CCL2 application. CCL2 delayed the third action potential (left panel) and induced failures in the action potential evoked in the right panel.

E Effect of CCL2 on KCl-induced MCH release. The perifusion of hypothalamic tissues with 60 mM KCl for 30 min induces an up to 3.1-fold increase in MCH release. CCL2 (100 nM) dramatically blunts KCl-induced MCH release, an effect partially by the addition of INCB3344 (10 µM) (3–5 independent experiments, three chambers per condition in each experiment). $^{*,\$}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. * KCl vs. KCl+CCL2 conditions. $^{\$}$ KCl+CCL2 vs. KCl+CCL2+INCB3344 conditions. Data are expressed as mean fold increase in MCH release per chamber vs. basal secretion, ± SEM.

F Effect on MCH-R1 antagonist on LPS- and CCL2-induced weight loss. Data are expressed in mean percentage of weight variation compared to initial body weight at different times (from 2 to 48 h) after acute icv injection of saline (black bar), H6408 (dark blue bar), LPS (red bar), or LPS+H6408 (light red bar), CCL2 (orange bar), CCL2+H6408 (yellow bar) in WT mice (n = 3–12). Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; color-coded asterisks indicate a significant difference from the experimental condition assigned to the respective color-coded bar.

Data information: In (E and F), data were analyzed by Student's unpaired two-tailed t-test.
Source data are available online for this figure.

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periphery is associated with obesity [59,60], while sickness response is associated with high-grade inflammation (explored here), eliciting different inflammatory signaling in the CNS [61]. Therefore, resolving the paradox about the central effects of CCR2 signaling would require evaluating the response of MCH neurons under conditions that promote either positive or negative energy balance.

In summary, our results demonstrate that brain LPS injection drives CCL2 synthesis that could lead to inhibition of MCH neuron activity through selective CCR2 signaling and subsequently decreases body weight/food intake. This represents the first evidence that a chemokine may directly affect a major orexigenic pathway to contribute to modulate a cardinal response of the sickness behavior.

Materials and Methods

Animals

6- to 8-week-old C57Bl/6J male mice (Janvier Labs, France), MCH-CFP transgenic mice (gift from Prof. J.M. Friedman, Rockefeller University, NY, USA), ORX-CFP transgenic mice (gift from Prof. C. Peyron, Lyon Neuroscience Research, France), and CCR2 KO mice (Jackson Laboratories, USA; strain number B6.129S4-Ccr2tm1Ifc/J) were housed in a room maintained at 22 ± 1°C with a 12-h light/12-h dark cycle and were acclimatized for one week before experiments were performed. For indirect calorimetric studies, mice were housed individually in stainless steel cages. Animals had access to water and chow diet *ad libitum* (SAFE; 2,830 kcal/kg protein 21.4%, fat 5.9%, carbohydrate 51.7% #A03). All of the protocols were carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Nice-French Riviera, project agreement no 04464.01).

Drug injections and tissue collection for gene and protein analysis

Drugs were dissolved in saline solution (NaCl 0.9%) for injection. LPS (100 ng to 25 μg; Sigma-Aldrich, France), CCL2 (100 ng; Peprotech, France), INCB3344 (10 μM; MedChem Express, Sweden), and H6408 (10 μM; Sigma-Aldrich) were intraperitoneally and/or stereotaxically injected in WT or CCR2 KO mice, in a total volume of 200 μl (ip) or 5 μl at a rate of 0.5 μl/min (icv) as described in Stereotaxic surgery section. The localization of the icv injection sites was systematically controlled. The INCB3344 was centrally injected since it does not cross the blood–brain barrier. Control mice received saline solution. Experimental groups were injected the same day. Mice body weight was regularly monitored until sacrifice at different times of interest after injection. Hypothalami and cerebella were dissected and processed to study mRNA and protein contents by quantitative RT–PCR and CBA, ELISA or EIA, respectively.

RNA isolation

Total mRNA was isolated according the Chomczynski method as described in Chomczynski *et al* [62] using Fast Prep apparatus (Q-Biogene, France). Two micrograms of total mRNAs was denatured at 65°C for 5 min in the presence of 0.5 mM dNTP and oligodT primers (25 ng/μl; Promega, France).

RT–PCR

Except for RT^2 Profiler PCR arrays (see below) where manufacturer's protocol was followed, reverse transcription of mRNAs was performed using SuperScript® III Reverse Transcriptase (100 U; Life Technologies, France) in a total volume of 20 μl. A negative control lacking RT enzyme was also performed in each assay (NRT). RT and NRT mixtures were diluted 5 times to be used in quantitative real-time PCR experiments.

Polymerase chain reaction array for cytokines and chemokines

RT^2 Profiler Mouse Inflammatory Cytokines and Receptors PCR arrays (PAMM-011Z) ($n = 6$) were used to analyze the expression of a focused panel of genes. Data analysis was performed using the $\Delta\Delta C_T$ method according to the manufacturer's protocol (SABiosciences/Qiagen, France).

Quantitative real-time PCR

Real-time PCR was performed from reverse-transcribed cDNA samples for relative quantitation of mRNA levels for the genes of interest. Quantitative real-time PCR was performed in a LightCycler® 480 apparatus (Roche, France) using LightCycler® 480 SYBR Green I Master (2×) as described by the manufacturer. Primers were designed using Primer Express 1.5 software (Applied Biosystems, USA) and are detailed in Table EV1. Real-time PCR was performed for amplification of mouse IL-1β, IL-6, TNF-α, CCL2, MCH, ORX, POMC, CART, NPY, AgRP, and GAPDH mRNA. In each assay, PCR were performed in duplicate. Relative quantities of target genes were determined by comparison with results for the control housekeeping gene GAPDH.

Chemokine, cytokine, and neuropeptide quantification

Cerebellar and hypothalamic areas were harvested in ice-cold HBSS (Invitrogen, France) containing protease inhibitor cocktail (Roche, France). Extracts were homogenized by polytron and centrifuged. Supernatants were kept frozen until use. The protein content of the extracts was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA). A cytometric bead array (BD™ CBA Mouse Inflammation Kit; BD Biosciences) and a mouse IL-1β ELISA Ready-SET-Go (eBiosciences, France) were used to measure protein levels of IL-6/TNF-α/CCL2 and IL-1β, respectively, according the manufacturers protocols. MCH or ORX peptide levels in tissue lysates or in perifusion samples were measured by enzyme immunoassay kits, as described by manufacturer (refs EK-070-47 and EK-003-30 respectively; Phoenix Pharmaceuticals, USA).

Immunohistochemistry experiments

MCH-CFP and ORX-CFP transgenic mice were perfused with 4% PFA, and brains were collected. Phenotype of MCH-CFP and

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ORX-CFP neurons was determined by immunostaining of CCR2 on 40-μm-thick brain floating sections of MCH-CFP and ORX-CFP mice using the anti-rabbit CCR2 antibodies (1:200, [44]), and secondary antibodies conjugated with Alexa Fluor 594 (1/500; Molecular Probes, USA). Confocal microscopy observations were performed with a Laser Scanning Confocal Microscope (TCS SP5, Leica, Germany). We used high-magnification images (objective: ×63, bar 75 μM) to allow a better visualization of overlap between CCR2 and MCH immunoreactivity and absence of overlap between CCR2 and ORX immunoreactivity. To calculate the % of co-localization, we counted the number of CCR2-positive cells on 10 pictures of six independent brain slices from three different mice.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recording was carried out as previously described [63]. Briefly, hypothalamic slices obtained from brains of 11- to 28-day-old MCH-CFP-KI mice were continuously superfused with a microperfusion system. For current-clamp experiments, we used a K-gluconate internal solution containing (mM): K-gluconate 135, $CaCl_2$ 0.3, $MgCl_2$ 1, HEPES 10, EGTA 1, Mg_2ATP 4, Na_3GTP 0.4, pH adjusted to 7.3 with KOH. Values of access resistance ranged from 12 to 20 MΩ and were left uncompensated. The liquid junction potential between the internal solution (negative) and the standard PBBS solution was 13.2 mV. Membrane potential values given in the text were not corrected for the junction potentials. Measurements were made 2–3 min after obtaining the whole cell to ensure dialysis. MCH neurons were recognized using fluorescence as CFP in these transgenic mice is expressed under the control of MCH promoter thus only in MCH-producing neurons.

Data were digitized at 5–10 kHz using a Digidata interface coupled to a microcomputer running pClamp 9 (Axon Instruments, USA). Current-clamp data were digitized at 0.5 kHz using the same interface. Potentials were digitally filtered at 1–3 kHz. Average data are expressed as mean ± SEM, n = number of neurons.

Perifusion of hypothalamic tissue

Three hypothalamic explants from 8-week-old male C57Bl/6J mice (coordinates: bregma −0.22 to −3.28 mm according to The Mouse Brain in Stereotaxic Coordinates of Paxinos & Franklin) were placed in each perifusion chamber in a controlled environment (37°C, 5% CO_2/95% O_2, constant flow of 0.1 ml/min of MEM (no glutamine, no phenol red, no HEPES; 51200-046 Invitrogen, France) with added 20 μM bacitracin (Sigma-Aldrich), 1 mg/ml BSA (Sigma-Aldrich), 2 mM L-glutamine, and protease inhibitors complete EDTA-free (Roche, France). After a 2-h perifusion to reach equilibrium, the sampling procedure consisted of a 30-min control basal period, a 30-min period during which drugs were added independently or in combination (60 mM KCl, 100 nM CCL2, 10 μM INCB3344) and a 60-min wash with the medium. After each experiment, 60 mM KCl was applied to control the responsiveness of hypothalamic explants. Samples were collected and immediately frozen every 10 min. MCH and ORX concentrations were measured by EIA kit (Phoenix Pharmaceuticals, USA). At least three experiments were carried out for each substance tested (three chambers per group in each experiment).

Stereotaxic surgery

Mice were maintained, anaesthetized through continuous isoflurane (2.5%), then infusion throughout the surgery duration. As soon as the animals were anesthetized, they received an injection of xylazine (Rompun 10 μg/g of BW, Centravet, France). Mice were implanted unilaterally with a chronic 26 stainless steel gauge guide cannula (Plastics One Inc, Roanoke, Virginia, USA) using a Kopf stereotaxic instrument (David Kopf Instruments, Tujunga, USA) to allow icv injection. Unilateral implantation was made into the right lateral ventricle (stereotaxic coordinates relative to Bregma: X: +1 mm; Y: −0.34 mm; Z: −2.5 mm below the surface of the skull) according to The Mouse Brain in Stereotaxic Coordinates of Paxinos & Franklin. Cannulas were maintained in place by dental cement anchored to one stainless steel jewelry screw fixed to the skull. A dummy 33-gauge cannula was inserted to prevent clogging of the guide cannula. At the end of the surgery, mice received an injection of ketoprofen (ketofen 10%, 10 μg/g of BW, Centravet, France). After the surgery, the animals were housed individually for 7 days, during which they were handled, accustomed to the placement of the 33-gauge internal cannula and their body weight monitored daily.

Brain infusion

After 48-h acclimatization in calorimetric cages, non-restrained and conscious mice were weighted and perfused icv by using a catheter connected to the injector inserted into the guide cannula. This catheter was connected to a Hamilton syringe powered by a minipump KDS310 (KD Scientific, Holliston, MA, USA). Mice were accustomed to the operator by frequent handling before the experiment. The first 2 days of the experiment, all mice were perfused with vehicle solution (NaCl, Lavoisier, France) just before the onset of the dark period (18:00 pm; Vt = 2.5 μl; 1 μl/min) for habituation. At the 3rd day of the experiment, mice were divided into three groups and injected either with vehicle solution, LPS (500 ng; Vt: 5 μl), or LPS+INCB3344 (LPS: 500 ng + INCB3344: 10 μM; Vt: 5 μl) at a rate of 1 μl/min just before the onset of the dark period (18:00 pm). The group injected with LPS + INCB3344 was pre-injected with INCB3344 alone (10 μM; Vt: 5 μl; 1 μl/min) one hour before (17:00 pm). Mice were monitored in calorimetric cages for 45 h after those injections.

Indirect calorimetry measurements

Mice were analyzed for whole energy expenditure (EE), oxygen consumption and carbon dioxide production, respiratory exchange rate (RER, CO_2/O_2), food intake (g) and locomotors activity (beam-break/h) using calorimetric cages with bedding, food, and water (Labmaster, TSE Systems GmbH, Bad Homburg, Germany) as described by Joly-Amado *et al* [64]. Fatty acid oxidation was compiled as described by Bruss *et al* [65].

Mice individually housed had free access to food and water *ad libitum* with lights on from 7 am to 7 pm and an ambient temperature of 22 ± 1°C. All animals were acclimated for 48 h in calorimetric cages before experimental measurements.

Whole body composition (fat and lean mass) was measured using an Echo Medical systems' EchoMRI 100 (Whole Body Composition Analyzers, EchoMRI, Houston, USA).

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Statistical analysis

Data obtained following icv injections were analyzed with GraphPad Prism 6 using Student's unpaired two-tailed *t*-test. The threshold for significance was $P < 0.05$. For electrophysiology experiments, ANOVA test was used to analyze the differences between groups, followed by a Student's Newman–Keuls *post hoc* test with a threshold of significance of $P < 0.05$ using a statistical software package (SigmaStat 2.03, Jandel Sci). For indirect calorimetric studies, the results are expressed as mean ± SEM. Variance equality was analyzed by Microsoft Excel's *F*-test, and comparisons between groups were carried out using a Student's *t*-test or by a nonparametric Mann–Whitney *U*-test/Wilcoxon's test. When appropriate, analyses of variances were performed followed by a Tukey's *post hoc* test with the appropriate parameters and their interaction as factor. Data with different superscripts letters (a,b,c) differ significantly ($P < 0.05$).

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Author contributions

CR, NB, and JLN conceived and supervised the study, designed experiments, and wrote the manuscript. CR and NB designed and performed the majority of the experiments, interpreted results, and generated figures and tables. OLT participated to most experiments, developing notably the perifusion set-up with WR, interpreted results, and generated figures and tables. CC performed indirect calorimetric measurements, spontaneous activity, and feeding analyses with RGPD. SL contributed to data analysis of indirect calorimetric experiments and paper writing. MB performed immunohistochemistry experiments; KS and ND participated to qPCR experiments, physiology experiments, and weight measurements of mice. AG performed and analyzed results from electrophysiology experiments. JC contributed to the fax-array analysis. CH discussed results and reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Reviews

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la Lettre

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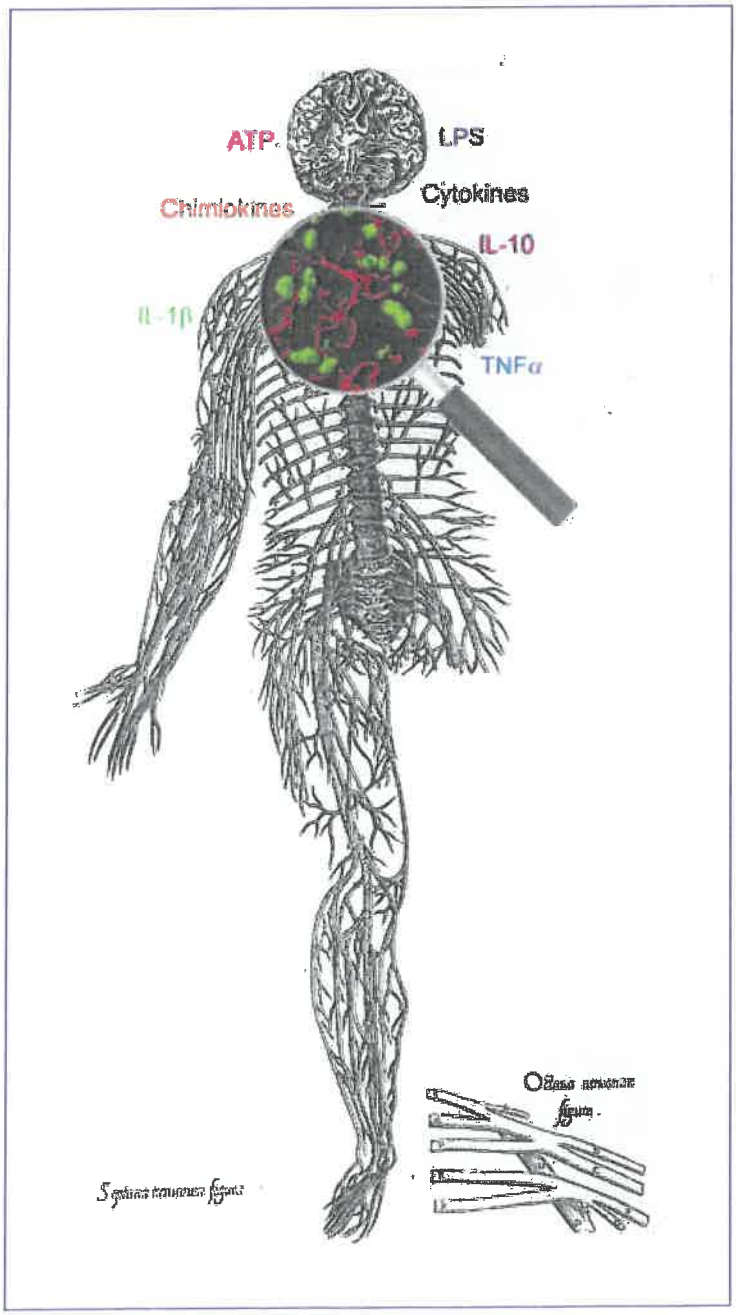

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La neuroinflammation dans tous ses états

| COORDONNÉ PAR STÉPHANE GAILLARD, JOSÉ-LUIS GONZALEZ DE AGUILAR ET ANNABELLE RÉAUX-LE GOAZIGO

Les systèmes nerveux et inflammatoire interagissent étroitement selon des modalités bien particulières au point de constituer une discipline à part entière des neurosciences : la neuroinflammation.

L'inflammation désigne l'ensemble de phénomènes déclenchés par l'organisme en réponse à une agression, telle une infection, une blessure ou un traumatisme. De façon générale, une réaction inflammatoire se manifeste par l'apparition dans la zone affectée de quatre faits marquants : rougeur ou érythème, gonflement ou tuméfaction, sensation de chaleur et douleur. Aux niveaux cellulaire et moléculaire, l'inflammation est un processus dynamique qui fait intervenir de nombreuses cellules immunitaires, ainsi qu'une pléiade de médiateurs chimiques tantôt pro-inflammatoires, destinés à entretenir, tantôt anti-inflammatoires, dont le but est de faire cesser la réponse inflammatoire.

Pendant longtemps, il était admis que le système nerveux central était incapable de développer une réaction inflammatoire, compte tenu principalement de son isolement de la périphérie par la barrière hématoencéphalique. Cependant, nous savons maintenant que les systèmes nerveux et immunitaire interagissent de manière étroite. En effet, le système nerveux central possède sa propre armée de cellules immunitaires résidentes, appelées cellules microgliales, qui sont de véritables meneurs de jeu des réactions inflammatoires. De plus, souvent associées à un processus pathologique, d'autres cellules immunitaires périphériques peuvent infiltrer le parenchyme cérébral. Finalement, même si la réponse inflammatoire ne se manifeste pas dans le système nerveux par la présence des quatre signes cardinaux cités ci-dessus, comme on l'observe dans la périphérie, les cellules immunitaires impliquées, ainsi que neurones, astrocytes et oligodendrocytes, utilisent tous le même langage moléculaire (composé de chemiokines, cytokines et autres molécules), si bien qu'il a fallu créer un nouveau terme, celui de la « neuroinflammation », pour désigner l'ensemble de phénomènes de nature inflammatoire spécifiques au système nerveux.

Le présent Dossier de la Lettre des Neurosciences propose de faire le point sur ce thème. Pour bien commencer, J.-G. Barbara expose l'évolution des concepts liés à l'inflammation et à la neuroinflammation tout au long de l'histoire. Ensuite, le texte rédigé par S. Chalon nous apprend que la neuroinflammation est bel et bien un phénomène observable par des méthodes d'imagerie qui sont en constant développement. Après cela, J.-F. Ghersi-Egea et N. Strazielle soulignent l'importance de l'interface sang-cerveau et du liquide cérébrospinal comme terrain d'entente entre les systèmes nerveux et immunitaire. Carole Rovère et ses collaboratrices nous expliquent comment certains médiateurs chimiques de l'inflammation peuvent moduler l'activité des noyaux hypothalamiques responsables de la régulation de la balance énergétique à l'échelle de l'organisme. Dans un contexte de recherche translationnelle, A. Réaux-Le Goazigo et S. Melik Parsadaniantz abordent la contribution d'agents pro-inflammatoires dans la sensibilisation des neurones cornéens lors de douleur oculaire chronique. G. Dietrich, quant à lui, décrit la modulation que les opioïdes endogènes, en particulier les enképhalines d'origine immunitaire, exercent sur l'intensité de la douleur inflammatoire viscérale. Pour finir, ce dossier présente quelques exemples du rôle fondamental de la neuroinflammation dans des conditions pathologiques de tout âge, qui vont de l'encéphalopathie du prématuré, présentée par J. Van Steenwinckel et P. Gressens, aux maladies neurodégénératives telles que la sclérose latérale amyotrophique, exposée par A. Chiot et S. Boillée, et la maladie de Parkinson, décrite par S. Hunot et J. Fuentealba.

Bonne lecture !

Dans le cas des maladies auto-immunes (telle que la sclérose en plaques), l'interaction des lymphocytes T avec les cellules présentatrices d'antigènes présentes dans les espaces sous-arachnoïdiens et périvasculaires conduit à une exacerbation du passage d'autres lymphocytes et de monocytes à travers les barrières sang-cerveau, et à l'infiltration de ces cellules dans le parenchyme à travers les *glia limitans*. Ces évènements sont des facteurs aggravants de la maladie. Là encore, les plexus choroïdes semblent jouer un rôle particulier, car dans les stades initiaux de la pathologie, ils sont le site privilégié de passage des premiers lymphocytes autoréactifs (5). Les plexus choroïdes sont très sensibles aux stimuli inflammatoires d'origine périphérique ou centrale. Ils agissent comme des sentinelles en charge de détecter les agressions du cerveau, et répondent par une sécrétion épithéliale de molécules inflammatoires dans le stroma choroïdien et le LCS3. Ceci explique probablement que la réponse immunitaire centrale délétère observée dans des modèles animaux de sclérose en plaques semble orchestrée à partir des espaces contenant le LCR, qui communiquent avec les espaces périvasculaires, et à partir desquels l'inflammation diffuse au sein du parenchyme cérébral (6). Lors de pathologies comme le traumatisme cérébral, l'ischémie cérébrale, ou les infections bactériennes, des cellules du système immunitaire inné (monocyte, polynucléaires neutrophiles), sont les premières à infiltrer les zones lésées à travers les barrières enflammées. Ces cellules affluent rapidement au niveau des plexus choroïdes, même si ceux-ci sont éloignés de la zone impactée, indiquant à nouveau le rôle de ces structures dans la détection précoce des stress inflammatoires pouvant altérer le fonctionnement du système nerveux central (3). Enfin, des travaux récents montrent que l'ischémie cérébrale induit l'afflux vers la dure-mère de cellules polynucléaires neutrophiles provenant directement de la moelle des os du crâne, à travers des canaux connectant les zones hématopoïétiques aux vaisseaux sanguins de la dure-mère. Ces cellules alimentent la population des cellules polynucléaires neutrophiles retrouvées dans le tissu cérébral lésé, ce qui montre que le cerveau a la capacité de recruter ces cellules spécifiquement à partir des os du crâne, physiquement proches (7). La pénétration cérébrale de ces cellules pourrait se faire au travers de la BHE activée, ou bien à travers l'arachnoïde après extravasation dans la dure-mère.

Cette vision moderne des voies et mécanismes d'interactions entre le système nerveux central et le système immunitaire prenant en compte la complexité anatomique du cerveau et du système de circulation du LCS offre des perspectives pharmacologiques nouvelles et plus ciblées pour prévenir les troubles neuro-inflammatoires sans compromettre la surveillance immunitaire du cerveau.

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INFLAMMATION HYPOTHALAMIQUE ET DÉRÉGULATIONS DE LA BALANCE ÉNERGÉTIQUE : RÔLE DES CHIMIOKINES

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Introduction

L'équilibre énergétique est finement régulé par une communication bidirectionnelle entre le cerveau et les organes périphériques. L'hypothalamus est l'une des régions du cerveau impliquées dans cette régulation. Il contient plusieurs populations neuronales produisant des peptides orexigènes et anorexigènes. L'activité de ces circuits neuropeptidergiques est modulée par des signaux périphériques tels que les signaux nerveux, les hormones et les nutriments (Figure 1). Une altération du fonctionnement de ces circuits neuropeptidergiques est associée à des dérégulations du comportement alimentaire, qu'il s'agisse d'une perte d'appétit ou d'une surconsommation alimentaire pouvant aboutir à l'obésité. L'inflammation hypothalamique est associée à des dérégulations de l'équilibre énergétique, et une inflammation hypothalamique de haut grade est associée à une perte de poids involontaire et, au contraire, une inflammation hypothalamique de bas grade est associée à l'obésité (1,2). L'obésité, qui ne cesse de se développer depuis la fin du XX^{e} siècle, est fréquemment associée au diabète de type 2, aux maladies cardiovasculaires ou hépatiques. La perte d'appétit consécutive à certaines pathologies inflammatoires comme les cancers, en entraînant un déficit en ressources énergétiques, peut entraver la guérison. Les dérégulations du comportement alimentaire représentent des problèmes de santé publique majeurs. De ce fait, comprendre les mécanismes moléculaires qui lient inflammation hypothalamique et dérégulations du comportement alimentaire pourrait à terme permettre d'identifier des cibles thérapeutiques potentielles. Les études qui ont identifié les médiateurs inflammatoires permettant la communication entre cerveau et périphérie, en particulier en cas d'inflammation, se sont le plus souvent intéressées au rôle des cytokines. Or, dans les dérégulations de la prise alimentaire et de l'équilibre énergétque, il a été suggéré que l'inflammation centrale serait plus importante que l'inflammation périphérique, notamment dans les modèles d'inflammation induite par le lipopolysaccharide (LPS) bactérien et que cette inflammation ne mettrait pas en jeu les cytokines périphériques.

Au niveau central, le rôle primordial des cytokines telles que l'Interleukine-1 béta (IL-1β), l'IL-6 ou le « Tumor Necrosis Factor » alpha (TNF-α), n'est pas exclu, mais des acteurs intermédiaires agissant en aval pourraient lier ces cytokines aux adaptations fonctionnelles et comportementales à l'inflammation. Les chimiokines, une sous-classe de cytokines, des médiateurs pro-inflammatoires protéiques de petite

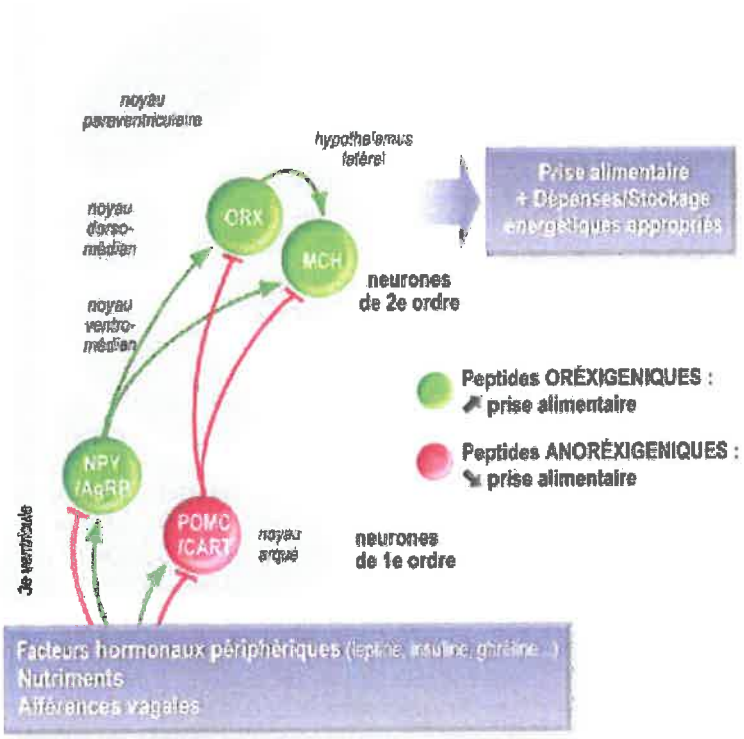

Figure 1 - Régulation homéostatique du comportement alimentaire : schéma simplifié résumant les réseaux neuropeptidergiques hypothalamiques impliqués.

Des signaux donnant des indications sur le statut énergétique d'un organisme sont émis depuis la périphérie et sont intégrés dans un premier temps par des neurones de premier ordre situés dans le noyau arqué, produisant soit des neuropeptides orexigènes, le neuropeptide Y (NPY) et l'« Agouti-related peptide » (AgRP), soit des peptides anorexigènes, la Pro-opiomélanocortine (POMC) et le « Cocaine- and amphetamine-regulated transcript » (CART). Ces neurones projettent vers des neurones de second ordre, tels que les neurones produisant les peptides orexigènes, l'orexine (ORX) et la « melanin-concentrating hormone » (MCH) dans l'hypothalamus latéral. L'intégration des signaux périphériques par ces systèmes neuropeptidergiques participe à la régulation homéostatique du comportement alimentaire et au maintien d'un poids adapté en assurant une prise alimentaire ainsi que des apports et des dépenses énergétiques appropriés. En vert : neurones produisant des peptides orexigènes ; en rouge : neurones produisant des peptides anorexigènes.

taille (8-14 kDa) se liant à l'héparine, pourraient jouer un rôle important dans la dérégulation des circuits hypothalamiques contrôlant la balance énergétique. En effet, les chimiokines et leurs récepteurs sont soupçonnés d'être des médiateurs majeurs des effets de la neuroinflammation, depuis l'attraction des cellules immunitaires, jusqu'aux changements comportementaux. Les chimiokines et leurs récepteurs (ou « système chimiokinergique » sont exprimés constitutivement dans le cerveau, et ce de manière spécifique, tant en termes de régions que de types cellulaires, à l'image des systèmes de neurotransmetteurs ou neuropeptidergiques (3). De plus, le système chimiokinergique a été démontré comme interagissant avec les systèmes de neurotransmetteurs et de neuropeptides, comme l'illustrent les propriétés neuromodulatrices des chimiokines CXCL12 et CCL2 sur la neurotransmission dopaminergique centrale. Par ailleurs, il existe plusieurs interactions entre le système CXCL12/CXCR4 et d'autres systèmes de neurotransmetteurs dans le cerveau (tels que GABA, glutamate, opioïdes et cannabinoïdes) (4,5). Des études suggèrent que le système chimiokinergique est capable de neuromodulation directe, comme par exemple CXCL12 qui pourrait réguler l'activité des neurones à neurones à MCH (Melanin Concentrating Hormone) (6). Ainsi, le système chimiokinergique pourrait être responsable des changements comportementaux associés à l'inflammation.

Inflammation hypothalamique et perte de poids involontaire : rôle de la signalisation centrale de la chimiokine CCL2 et de son récepteur CCR2

Une étude de notre laboratoire s'est intéressée à l'anorexie et à la perte de poids induite par le LPS, avec l'objectif d'identifier éventuellement des chimiokines qui pourraient déréguler les circuits hypothalamiques régulant la prise alimentaire et la balance énergétique de manière plus générale (7).
Tout d'abord, l'expression génique de différents médiateurs pro-inflammatoires dans l'hypothalamus consécutive à une injection intracérébroventriculaire (icv) de LPS chez la souris a révélé une inflammation, comme en atteste la surexpression de divers médiateurs pro-inflammatoires. Parmi ces chimiokines surexprimées, la chimiokine CCL2 a particulièrement retenu notre attention. En effet, celle-ci avait déjà été démontrée comme particulièrement importante dans la neuroinflammation induite par le LPS périphérique (8,9).
La signalisation centrale de CCL2 et de son récepteur CCR2 est effectivement indispensable aux changements adaptatifs métaboliques et comportementaux induits par le LPS. En effet, la perte de poids induite par le LPS injecté en icv chez la souris est réduite de manière significative par la co-injection d'un antagoniste spécifique du CCR2, l'INCB3344, notamment dans les phases précoces (6-8h post-injection). De manière comparable, l'injection de LPS chez des souris déficientes en CCR2 (KO CCR2) induit une perte de poids moindre par rapport à la même injection chez les animaux contrôles. Par exemple, à 26 h post-injection, cette perte de poids est 2 fois moins importante chez les souris KO CCR2 que chez les souris contrôles.
De plus, l'injection icv de LPS induit effectivement une diminution de la prise alimentaire, une diminution de l'activité locomotrice, une augmentation de l'oxydation des lipides et un changement de substrat énergétique en faveur des lipides par rapport aux glucides (ces deux derniers points illustrant vraisemblablement l'utilisation accrue des stocks énergétiques situés dans le tissu adipeux sous forme de lipides), notamment dans les phases précoces (0-12h post-

injection). De la même façon, cette expérience a pu démontrer qu'interférer avec la signalisation centrale du CCR2, par co-injection icv de LPS et d'INCB3344, permet effectivement de réduire significativement ces effets. Ainsi, il apparaît que la signalisation centrale de CCL2/CCR2 est impliquée dans les effets de l'inflammation sur la prise alimentaire mais aussi sur l'utilisation des substrats énergétiques en périphérie.
Pour expliquer les effets de la signalisation CCR2 centrale sur les adaptations métaboliques à l'inflammation, une cible potentielle a été recherchée parmi les systèmes neuropeptidergiques hébergés par l'hypothalamus et participant à la régulation de la balance énergétique. L'expression génique des neuropeptides connus a été étudiée après injection icv de LPS. Les résultats indiquent que dans les phases précoces (1h post-injection) au niveau des peptides produits par les neurones du noyau arqué (ARC), le peptide anorexigène Pro-OpioMélanoCortine (POMC) est surexprimé de manière transitoire, au contraire des peptides orexigènes Neuropeptide Y (NPY) et Agouti-Related Peptide (AgRP), dont l'expression est diminuée de moitié. Par ailleurs, en ce qui concerne les peptides produits par les neurones de l'hypothalamus latéral (LHA), une diminution de l'expression génique et protéique, plus tardive (18-24h post-injection) et durable, des peptides orexigènes MCH et Orexine (ORX) est observée. De plus, l'injection icv de la chimiokine CCL2 est capable, comme le LPS, d'induire une neuroinflammation, comme en atteste une surexpression de cytokines pro-inflammatoires ou encore de CCL2 elle-même, mais aussi, de manière très intéressante, de diminuer l'expression des deux peptides MCH et ORX.

L'étude de l'expression du récepteur CCR2 sur les neurones à MCH et ORX dans l'hypothalamus latéral a permis de détecter l'expression de celui-ci sur environ 70 % des neurones à MCH mais absolument pas sur ceux à ORX, indiquant probablement la spécificité d'un axe CCL2/CCR2-MCH.
Les effets de CCL2/CCR2 sur les neurones à MCH ont été étudiés soit par une approche pharmacologique soit en utilisant des modèles de souris transgéniques. La co-injection de LPS et de l'INCB3344 chez les souris sauvages, de même que l'injection de LPS chez les souris KO CCR2 permet de bloquer en grande partie les effets du LPS sur l'expression de la MCH (à hauteur de 80 % environ à 18h post-injection). Il apparaît donc que l'inhibition du système MCH par le LPS dépende principalement de la signalisation centrale du CCR2. Pour aller plus loin au niveau mécanistique, des expériences d'électrophysiologie ont montré que l'application de CCL2 sur des tranches d'hypothalamus induit une légère hyperpolarisation des neurones à MCH, diminuant alors leur excitabilité. De plus, il semblerait que CCL2 induise des délais et/ou des échecs dans l'émission de potentiels d'action par les neurones à MCH, par diminution de la résistance membranaire. Enfin, l'effet de la signalisation CCL2/CCR2 sur la sécrétion du peptide MCH a été étudié par des expériences de périfusion d'explants hypothalamiques : la sécrétion de MCH induite par un traitement au KCl est totalement inhibée par l'application conjointe de KCl et de CCL2, un effet bloqué par l'INCB3344. L'ensemble de ces études a confirmé encore davantage la spécificité de la voie CCL2/CCR2-MCH puisque CCL2 n'affecte pas la sécrétion d'ORX dans le même paradigme.

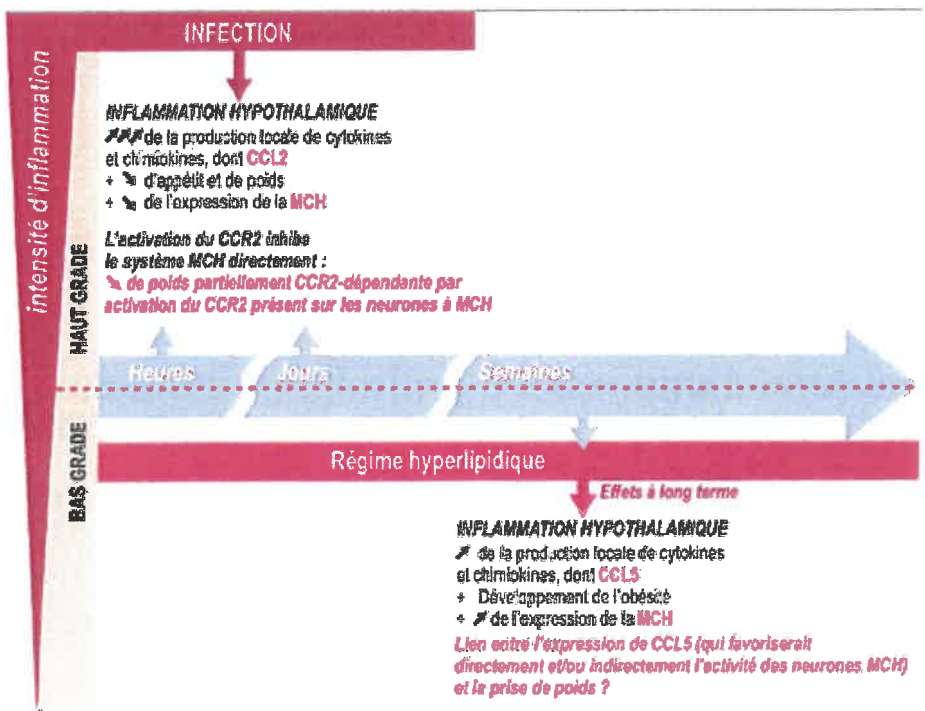

Figure 2 - Liens entre inflammation hypothalamique et dérégulations de poids

En haut : lors d'une inflammation hypothalamique de haut grade induite par le LPS, il y a production locale accrue de cytokines et chimiokines dont la chimiokine CCL2 de manière aiguë (de quelques heures à quelques jours) qui est associée à une perte de poids (une manifestation du comportement de maladie associé à une infection de forte intensité). Cette perte de poids est partiellement dépendante de la voie de signalisation CCL2/CCR2, par inhibition directe des neurones de l'hypothalamus latéral produisant le peptide orexigénique MCH. En bas : La consommation prolongée (plusieurs semaines) d'un régime hyperlipidique est associée à une inflammation hypothalamique chronique de bas-grade qui est associée au développement de l'obésité ainsi qu'à une surexpression de neuropeptides orexigènes comme la MCH. Par ailleurs, la chimiokine CCL5, dont l'expression est augmentée par une consommation excessive et prolongée de lipides, pourrait participer à la prise de poids induite par l'inflammation hypothalamique en activant le système MCH dans l'hypothalamus latéral.

Il apparaît donc que l'axe CCL2/CCR2 au niveau central, en cas d'inflammation de haut grade, soit capable d'agir directement sur les neurones produisant le peptide orexigène MCH en diminuant leur activité, ainsi que la sécrétion et l'expression du peptide. Ceci a pour conséquence de réduire la prise alimentaire des animaux et d'augmenter l'utilisation des réserves énergétiques. Ainsi, l'axe central CCL2/CCR2, par action sur les neurones à MCH, est un acteur majeur de la perte d'appétit et de poids associé à l'inflammation induite par le LPS (Figure 2) (7,10).

Inflammation hypothalamique et obésité : rôle de la chimiokine CCL5

De manière intéressante, l'inflammation est une caractéristique d'un autre trouble de la régulation de la prise alimentaire et du poids corporel : l'obésité.

Une étude réalisée en commun avec l'équipe du Dr Karine Clément a permis d'identifier la chimiokine CCL5 comme la seule dont les niveaux sériques semblent être corrélés à l'apport calorique chez des patients ayant subi une chirurgie bariatrique de type bypass gastrique Roux-en-Y (11). Ces premiers résultats ont justifié des études plus approfondies sur le rôle potentiel de CCL5 comme modulateur de l'activité de neurones hypothalamiques régulant la prise alimentaire, pour éventuellement favoriser la mise en place et/ou le développement de l'obésité.

Ainsi, dans un modèle d'obésité nutritionnelle induite chez la souris par la consommation d'un régime hyperlipidique, une inflammation a été mise en évidence tant au niveau périphérique qu'au niveau hypothalamique, comme en témoigne la surexpression de médiateurs pro-inflammatoires dans le sérum, dans le noyau ARC et le LHA. Le lien entre obésité nutritionnelle et inflammation hypothalamique a bien été décrit (12, 13). Cependant, des résultats récents de notre laboratoire montrent que, parmi les médiateurs pro-inflammatoires surexprimés, les chimiokines comme CCL5, ont un comportement particulier puisque leur expression n'augmente en périphérie, qu'après 12 semaines de régime alors qu'elles sont déjà surexprimées après 8 semaines de régime dans l'ARC et le LHA de l'hypothalamus. Cela suggère un rôle particulier dans la mise en place de l'obésité, par action de celles-ci au niveau central.

Pour confirmer l'hypothèse selon laquelle CCL5 pourrait effectivement perturber les circuits neuropeptidergiques de l'hypothalamus, l'effet de l'injection icv de CCL5 sur l'expression des peptides orexigènes MCH et ORX a été testée. Cette injection aiguë est capable d'augmenter l'expression des deux peptides orexigènes, de manière transitoire pour l'ORX et de manière plus stable et persistante pour la MCH. Dans un deuxième temps, l'effet de CCL5 sur l'activité électrique des neurones à MCH a été étudié. Nos résultats suggèrent que la chimiokine CCL5 est capable de dépolariser ces derniers, facilitant donc leur activation, un effet qui serait médié par une libération présynaptique de glutamate. De plus, des expériences ont montré que la déficience de la chimiokine CCL5 freine significativement le développement d'une obésité nutritionnelle chez la souris adulte, mais pas chez la souris juvénile, puisque la prise de poids de ces souris est significativement inférieure à celle des souris contrôles dès 3 semaines de régime hyperlipidique (données non publiées, Stobbe et al., 2018). Ceci est accompagné par une homéostasie du glucose moins perturbée chez les souris déficientes pour CCL5. Ces résultats suggèrent que la chimiokine CCL5 est importante, au niveau central, dans la mise en place et/ou le développement de l'obésité, probablement par suractivation des neurones hypothalamiques à MCH (Figure 2).

Ces études sont les premières à démontrer le rôle de chimiokines, au niveau central, dans les dérégulations de la balance énergétique, par action directe sur des systèmes neuropeptidergiques hypothalamiques clés tel que le système à MCH.

Conclusion

Ces données soutiennent l'hypothèse selon laquelle les chimiokines sont effectivement capables de déréguler les systèmes neuropeptidergiques de l'hypothalamus qui participent à la régulation de la balance énergétique, pouvant aboutir à une perte ou un gain de poids excessifs. Nous ne sommes qu'aux prémices de l'exploration fonctionnelle des molécules inflammatoires cérébrales responsables des changements comportementaux observés lors d'une stimulation de la réponse immunologique.

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REVIEW
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Hypothalamic Inflammation and Energy Balance Disruptions: Spotlight on Chemokines

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The hypothalamus is a key brain region in the regulation of energy balance as it controls food intake and both energy storage and expenditure through integration of humoral, neural, and nutrient-related signals and cues. Many years of research have focused on the regulation of energy balance by hypothalamic neurons, but the most recent findings suggest that neurons and glial cells, such as microglia and astrocytes, in the hypothalamus actually orchestrate together several metabolic functions. Because glial cells have been described as mediators of inflammatory processes in the brain, the existence of a causal link between hypothalamic inflammation and the deregulations of feeding behavior, leading to involuntary weight loss or obesity for example, has been suggested. Several inflammatory pathways that could impair the hypothalamic control of energy balance have been studied over the years such as, among others, toll-like receptors and canonical cytokines. Yet, less studied so far, chemokines also represent interesting candidates that could link the aforementioned pathways and the activity of hypothalamic neurons. Indeed, chemokines, in addition to their role in attracting immune cells to the inflamed site, have been suggested to be capable of neuromodulation. Thus, they could disrupt cellular activity together with synthesis and/or secretion of multiple neurotransmitters/mediators involved in the maintenance of energy balance. This review discusses the different inflammatory pathways that have been identified so far in the hypothalamus in the context of feeding behavior and body weight control impairments, with a particular focus on chemokines signaling that opens a new avenue in the understanding of the major role played by inflammation in obesity.

Keywords: neuroinflammation, hypothalamus, chemokines, energy balance, metabolic diseases, high-fat diet, obesity, anorexia

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INTRODUCTION

Energy balance is finely regulated *via* a bidirectional communication between the brain and the peripheral organs. One brain area is particularly important in this regulation: the hypothalamus. The hypothalamus shelters, in its different nuclei, several neuronal populations producing peptides that are either orexigenic or anorexigenic. The activity of these neuropeptidergic circuits is, among others, modulated by peripheral signals, of neural or hormonal nature, or by nutrients

themselves (**Figure 1**). Thus, it would make sense that the function of these neuropeptidergic circuits would be impaired in case of feeding behavior deregulation, whether it is a loss of appetite or a food overconsumption. Numerous studies, based either on lesion, pharmacological, or genetic approaches, indeed confirmed this [for review see Ref. (1)]. Interestingly, hypothalamic inflammation has already been linked to energy balance disruptions: high-grade hypothalamic inflammation has been associated to involuntary weight loss and, on the contrary, low-grade hypothalamic inflammation has been associated to obesity (2, 3). Importantly, these feeding behavior deregulations represent major public health issues, especially obesity. Indeed, obesity, which keeps developing since the end of the 20th century, is often associated to potentially deadly comorbidities such as diabetes, cardiovascular diseases, liver diseases, and cancers. Yet, a loss of appetite, consecutive to some inflammatory pathologies such as cancer, can also have severe consequences, as it can impair recovery by inducing a deficit in energy.

Hence, understanding the molecular mechanisms linking hypothalamic inflammation and feeding behavior deregulations could, in the long-term, allow identifying potential therapeutic targets. As previously mentioned, we will focus in this review on hypothalamic inflammation, even though peripheral inflammation is also often associated with energy balance deregulations: in involuntary weight loss, inflammation is rather firstly systemic and a consequence of a primary pathology. In obesity, both systemic and hypothalamic inflammations have been described, and even if this is still debated, recent studies suggest that hypothalamic inflammation precedes systemic inflammation associated to the obese state (4–6).

In this context, in particular when there is systemic inflammation, it is important to understand how the periphery and the brain can communicate, especially regarding inflammatory signals. The most described brain–periphery communication pathways are the neural and the humoral pathways. The neural pathway involves primarily the vagus nerve—which expresses cytokine receptors—and the dorso-vagal complex, whereas the humoral pathway involves circulating cytokines that are overexpressed with inflammation and that can (1) bind to their receptors on the membrane of endothelial cells, (2) get to the brain at the level of the circumventricular organs and of the choroid plexus when the blood–brain barrier (BBB) is incomplete, or

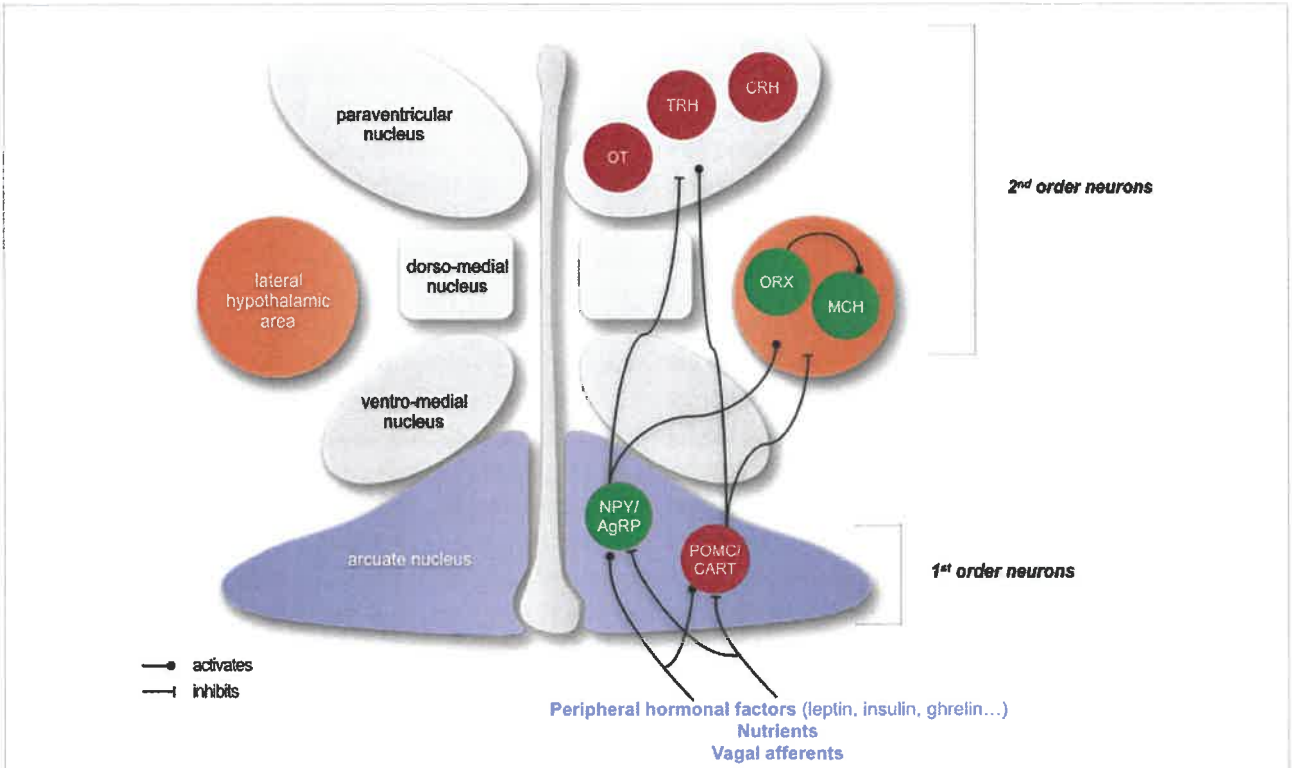

FIGURE 1 | Simplified schematics summarizing the neuropeptidergic hypothalamic networks that are involved in homeostatic regulation of feeding behavior. Signals giving indications about the energy status of an organism are emitted from the periphery and are first integrated by first-order neurons located in the arcuate nucleus, producing either orexigenic neuropeptides (NPY and AgRP) or anorexigenic peptides (POMC and CART). These neurons project to second-order neurons, such as neurons producing the anorexigenic factors oxytocin (OT), thyrotropin-releasing hormone (TRH), and corticotropin-releasing hormone (CRH) in the paraventricular nucleus and neurons producing orexigenic peptides orexin (ORX) and melanin-concentrating hormone (MCH) in the lateral hypothalamus. The integration of peripheral signals by these neuropeptidergic systems participates in the homeostatic regulation of feeding behavior and the maintenance of a suitable weight by ensuring an appropriate food intake as well as appropriate energy intake and expenditure. In green: neurons producing orexigenic peptides; in red: neurons producing anorexigenic peptides. Adapted from Le Thuc and Rovère (7).

(3) reach directly the brain thanks to the increased BBB permeability induced by inflammation (8). However, one must also consider the cellular pathway, which involves the infiltration of immune cells and activation of microglia in the brain parenchyma, and also the microbiota pathway, for example.

Studies that aimed to identify inflammatory mediators allowing bidirectional communication between the periphery and the brain during inflammation mainly focused on the role of peripheral cytokines. Yet, it has been suggested that in the context of disruptions of proper control of food intake and body weight, the central production of inflammatory mediators is more important than the peripheral one, or at least "necessary," in particular in the models of inflammation induced by the bacterial lipopolysaccharide (LPS), where it has been suggested that its effects do not require the signaling from peripheral cytokines (9–11).

Hence, in the context of disruptions of proper energy balance control by hypothalamic circuits, a group of pro-inflammatory mediators might be as relevant as the canonical cytokines, if not more, downstream of the latter: the chemokines. Chemokines are a subgroup of cytokines, small (8–14 kDa) heparin-binding proteins, mainly described for their chemoattractant properties for immune cells to the affected site.

Chemokines and their receptors are suspected to be mediators of the effects of neuroinflammation, from attraction of immune cells to behavioral changes. Beyond participating in the immune response, chemokines and their receptors (the "chemokinergic system") are constitutively expressed in the brain, in a specific manner, both area wise and cell type wise. Interestingly, it is comparable to the neurotransmitters or neuropeptidergic systems (12, 13). Furthermore, the chemokinergic system has been demonstrated to interact with the neurotransmitter and neuropeptidergic systems (14, 15) and some recent studies suggest that the chemokinergic system is able to directly modulate neuronal activity (16–18). Thus, the chemokinergic system could be responsible for the behavioral changes associated to inflammation.

In this review, we discuss how inflammation, with a focus on chemokines, can participate in the deregulation of the hypothalamic control of energy balance and body weight: first, in appetite and weight loss and, second, in the establishment and/or the development of obesity.

INVOLUNTARY WEIGHT LOSS AND INFLAMMATION

Weight loss illustrates an unbalance between energy intake and energy expenditure in favor of expenditure. While both involuntary and voluntary weight losses can be caused by a decrease in food intake or an increase in energy expenditure (increase in basal metabolism, e.g., thermogenesis), only an involuntary weight loss can be explained by an uncontrolled decrease in food intake or by an increase in energy expenditure.

There are multiple possible causes for an involuntary weight loss. This weight loss can be a symptom of a severe primary disease such as cancer, where it is particularly preoccupying when it reaches or exceeds 10% in 1 year. In most cases, one can link an involuntary weight loss to psychic troubles, such as depression, that can induce a sustainable loss of appetite, thus weight loss. Excessive consumption of certain substances (e.g., drugs of abuse and alcohol) can also induce an involuntary weight loss. Yet, a weight loss is only considered "involuntary" when it is not a consequence that can be expected from a specific medical treatment of a known medical condition (19). Moreover, aging is often associated to a loss of appetite which induces, in the long-term, severe malnutrition and, obviously, weight loss. This can also be associated to sarcopenia, a geriatric syndrome characterized by loss of muscle mass and function (19). However, as previously mentioned, an involuntary weight loss can have organic causes such as gastrointestinal diseases [e.g., Crohn's disease, celiac disease (weight loss related to food malabsorption), and digestive ulcer], cardiovascular, endocrine, autoimmune, infectious diseases (e.g., HIV infection, hepatitis, and tuberculosis), neurological diseases (e.g., dementia and Parkinson's disease), or cancer (19, 20). In the case of cancer, weight loss is one of the first symptoms with 50% of patients with cancer reporting weight loss (21). It should be noted that the majority of the abovementioned organic causes are commonly associated with "high-grade"—in other words high-intensity—inflammation. Interestingly, even if benign and time-restricted, numerous pathological states are strongly inflammatory and associated to a reduced appetite or "anorexia." This anorexia is part of a classical defense mechanism of an organism against infection, lesions, etc., that is referred to as "sickness behavior" (22, 23). Sickness behavior involves various behavioral changes that primarily affect mood and energy balance, which develop parallel to infection or to another pathology (22).

Like other strategies adopted by the body to promote healing, anorexia is supposed to be temporary and beneficial. However, in the case of chronic diseases such as cancers, both appetite and weight loss can persist, leading to a further degraded state of the subject (hypoglycemic malaise, amenorrhea in women, decalcification leading to more fragile bones, teeth falling, etc.), comparable, in some aspects only linked to undernutrition, to what is observed with anorexia nervosa (24). Prolonged anorexia, irrespective of its type and its causes, is often a contributory factor to the onset of cachexia. Cachexia is a complex syndrome that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment, i.e., pathological thinness, associated with deep asthenia and organs dysfunction [decreased muscle strength, fatigue, adipose tissue (AT) dystrophy, etc.] (25, 26). Hence, cachexia can be defined as a complex syndrome in which inflammation leads to early satiety and anorexia, decreased fat mass, and weakness (25, 26). Prolonged loss of appetite may even be fatal. One can then assume that inflammation, here high-grade, could disrupt the proper functioning of the hypothalamic systems that are involved, and thus the regulation of feeding behavior. Hence, considering the serious consequences of unresolved anorexia, it is important to better understand the mechanisms linking inflammation and the cerebral centers that control energy homeostasis, because if the link between high-grade inflammation and loss of appetite and weight is established and extensively recognized, the underlying molecular mechanisms have not yet been fully resolved.

Inflammatory Pathways in the Hypothalamus and Involuntary Weight Loss

In the hypothalamus, some studies have been able to identify some inflammatory pathways, involving different cell types, to be relevant for weight loss.

At the molecular level, as previously mentioned, the literature related to the deregulation of the control of energy balance in the anorexia–cachexia syndrome has focused on the role of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α. It has been shown that pro-inflammatory cytokines, whose production can be induced by infectious agents affecting peripheral organs, notably by LPS—the most commonly used model for modeling disease behavior and anorexia associated with inflammation (27)—participate in the induction of sickness behavior.

Some studies deciphering the kinetic aspects of the effect of central or peripheral cytokine injection suggest that pro-inflammatory mediators exert their effect primarily at the central level versus periphery (28, 29). Numerous studies have focused on IL-1β and have shown that its intracerebroventricular (ICV) injection induces profound behavioral changes in rodents (30). Furthermore, the central role of cytokine signaling at the central level has been confirmed, for example, by the fact that the central injection of the IL-1β receptor antagonist prevents the effects induced by the peripheral injection of this cytokine (31, 32). In addition, some studies suggest that inflammatory inducers such as LPS are not capable of directly inducing sickness behavior and require downstream actors such as cytokines. Indeed, for example, unlike the central injection of LPS, central injection of IL-1 is able to induce sickness behavior in mice whose LPS toll-like receptor (TLR) 4 is non-functional (33).

As mentioned earlier, cytokines, involved in the induction and regulation of sickness behavior at the central level, can be released into the circulation by the immune cells (8) or directly produced by neurons and glial cells in the central nervous system (CNS) (34–39). In the context of systemic diseases, the central effects of these cytokines appear to be independent of the place of their secretion. As previously mentioned, when produced in the periphery, cytokines have the following two main pathways to act on the brain: a neural pathway and a humoral pathway. Most cytokines act in a paracrine manner at the site of infection, suggesting that neural afferents may be the target of pro-inflammatory cytokines. Interestingly, the perineural sheath of the vagus nerve contains immune cells that are capable of producing IL-1, in particular in response to LPS (40). Furthermore, the sensory neurons of the vagus nerve express IL-1 receptors and it has been shown that this cytokine stimulates the sensory activity of the vagus nerve (40). Vagotomy experiments have demonstrated the importance of the vagus nerve in the transmission of information from the periphery to the brain: for example, after injection of LPS or IL-1 at the periphery, vagal afferents are involved in the induction of the sickness behavior and in the neural activation of the brainstem, the hypothalamus, and the limbic structures (40).

Chronic administration of cytokines can reproduce the characteristics of anorexia–cachexia syndrome (38, 41–44), while blocking the signaling of one of them, such as TNF, by the use of neutralizing antibodies, inhibits its development (42, 45, 46). Similarly, administration of an IL-1 receptor antagonist prevents anorexia in cancer animal models (47). Some studies have suggested that endogenous brain IL-1 is a mediator of LPS-induced anorexia by acting on the expression of cytokines in the hypothalamus (9). In addition, it appears that IL-1β can act on ARC POMC neurons (48) and that TNF-α indirectly increases energy expenditure *via* β3 adrenergic signaling in the brown AT (thermogenesis) (49). By different approaches, it has been shown that interfering with mediators of inflammation coincides with a reduction in hypothalamic inflammation and prevents weight loss in animal models of anorexia: for example, the inhibition of the "adenosine monophosphate protein kinase" in the hypothalamus reduces hypothalamic inflammation, which is accompanied by an increase in food intake in the case of cancer-associated anorexia, allowing better overall survival (50). Interestingly, in the context of cancer-associated cachexia, the administration of ghrelin, an appetite-stimulating hormone, increases food intake and is accompanied by a decrease in IL-1β (51).

Focusing on the LPS-induced anorexia model, it is interesting to underline that some studies suggest that this loss of appetite is independent from the vagal afferents and that it only depends on central inflammatory mechanisms, where the central effects of peripheral LPS could be, among others, mediated by some cytokines and/or *via* receptors for LPS expressed by some brain cells (11, 52). Indeed, microglia expresses TLR4, through which LPS has been shown to exert some of its effects (53–56). Furthermore, Hines and colleagues have shown that disrupting TLR4 signaling prevents microglial activation, inhibits the production of cytokines, and prevents the development of the sickness behavior that are induced by peripheral LPS (57). In contrast, a recent study by Reis et al. has shown that if microglia and TLR4 are necessary for LPS to acutely induce inhibitory effects on orexigenic agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons, conversely, LPS can actually acutely increase the firing activity of anorexigenic pro-opiomelanocortin (POMC) neurons in a microglia/TLR4-independent manner (58). In addition, the same study presents results which further supports that mediators of inflammation such as microglia and TLR4 can, at least acutely, affect basal food intake and hormonal-dependent modulation of feeding behavior. Indeed, the inhibition of microglia alone, *via* ICV injection of minocycline, leads to an increase in food intake, comparable to the one induced by ICV ghrelin (58). Interestingly, the co-injection of ghrelin and minocycline does not synergistically increase food intake. This implies that the inhibition of microglia itself, as it increases food intake, interferes or prevents ghrelin's orexigenic effects (58).

Changes in gene expression of hypothalamic anorexigenic and orexigenic neuropeptides were assessed in mice or rats that received LPS IP injections (59–61). Generally, peripheral LPS appears, initially, to reduce the expression of the orexigenic peptides and, on the contrary, to increase that of the anorexigenic peptides. In later stages, the expression of orexigenic peptides

increases again, probably to promote food intake and compensate for weight loss.

Some authors have used other models to study to understand links between inflammation in the brain and the induction of sickness behavior.

For example, Jang and colleagues sought to determine the mechanisms behind anorexia and weight loss associated with illness *via* administration, in mice, of either bacterial or viral products: LPS and human immunodeficiency virus-1 transactivator protein (Tat), respectively (62). They found in both cases that pro-inflammatory cytokines such as IL-β, IL-6, and TNF-α were upregulated in the hypothalamus. Furthermore, AtT-20 and SH-SY5Y cells treated with either Tat or LPS exhibit increased POMC transcriptional activity. In addition, the injection of Tat or LPS in the hypothalamus of mice induces in both cases a decrease in food intake and in body weight. They identified POMC as a potential mediator of illness-induced anorexia and as a possible downstream target of NF-κB. Indeed, they showed through different approaches that the NF-κB pathway in the melanocortin system plays an important role in illness-induced anorexia and body weight loss: both administration of AgRP, an endogenous melanocortin antagonist and the inhibition of the NF-κB pathway specifically in the POMC neurons ($Ikk\beta^{\Delta Pomc}$ mice), significantly blunted the effects of Tat and LPS on food intake and body weight. Interestingly, the authors also present results suggesting that leptin-induced anorexia is also dependent on the NF-κB pathway (62).

Another study has shown that a pathway involving TLR2 participates in the induction of sickness behavior through a microglia-POMC neurons axis (63). In this publication, the authors show, by ICV injection of Pam3CSK4, a specific synthetic TLR2 agonist that TLR2 central signaling is able to trigger sickness behavior through activation of microglial cells, which express TLR2, and *via* the NF-κB and COX pathways. The authors also show that activation of central TLR2 is able to modify the synaptic architecture in the ARC, especially at the level of the POMC neurons: it reduces the rate of GABAergic contacts on POMC neurons, whereas it increases the vesicular glutamate transporter 2 (vGLUT2) contacts on POMC soma, translating an increased excitatory state, in correlation with an increased microglial occupancy.

In addition, Murray et al. used the peripheral administration of the viral mimetic poly I:C to induce type-I interferons (IFN-I) overexpression in the brain. Using IFN-I receptor 1 (IFNAR1)-deficient mice, the results of the authors present suggest that IFN-I are involved in the induction of sickness behavior, including anorexia, whereas IL-6 participates in sickness behavior but not in anorexia (64).

Furthermore, a local increase in serotonin levels in the hypothalamus has already been linked to anorexia and cachexia (65–67) and a recent study showed that inflammation induced by peripheral treatment by either IL-6 and/or TNF-α is associated with an impairment in local serotonin turnover in the hypothalamus, a decrease in NPY and AgRP gene expressions, and a decrease in food intake in comparison to control conditions (68). Authors were able to identify upstream inflammatory regulators including interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), IL-6, and IKBKG, an enzyme crucial for the activation of the NF-κB pathway. This study thus suggests that peripheral inflammation reaches the hypothalamus where it impairs serotonin turnover, which is associated to a decrease in food intake.

Taken together, numerous studies support the hypothesis that inflammation at the hypothalamic level is able to disrupt the proper function of neuropeptidergic circuits of the hypothalamus and thus to induce an involuntary weight loss.

However, mediators linking inflammation and its consequences at the level of central systems regulating energy homeostasis, and ultimately on weight, have not been determined with certainty, and one may note that the nature of the mediators responsible for the central effects of IL-1β has been especially poorly described. Thus, chemokines can represent interesting candidates to study further understanding of the underlying mechanisms.

Chemokines in the Modulation of Hypothalamic Neuropeptidergic Circuits in Inflammation-Associated Involuntary Weight Loss

So far, very few studies have sought to determine if chemokines have a role in the induction of involuntary weight loss associated with inflammation by impairing the homeostatic regulation of energy balance by hypothalamic neuropeptidergic circuits.

A study published in 1994 by Plata-Salamán and Borkoski aimed to investigate how chemokines could act on the regulation of feeding. Indeed, as previously mentioned, chemokines are produced in multiple types of cells as a response to pathological conditions such as infection, inflammation, injury, and trauma. Among the stimuli that can induce the release of these chemokines, we can list LPS, and also the IL-1β, TNF-α, and IFN, which have been associated to food intake suppression by direct action on the CNS. Thus, the authors aimed to determine if chemokines could be involved, as downstream mediators, in the decrease in food intake induced by inflammatory signals such as LPS. In order to do so, they used, in rats, ICV microinfusion of different chemokines from two different subfamilies: CXC and CC, also known as the α and β subfamilies. This way, they tested the effect of CXC-motif chemokine ligand (CXCL)-1, 4, 7, 8, 10 and CC-motif chemokine ligand (CCL)-2–4, 5. Even though it is at a lesser extent than the cytokine IL-1β, their results indeed identify certain chemokines as capable of acutely decreasing food intake: CXCL4, CXCL8, CXCL10, CCL2, and CCL5. Interestingly, in their model, these chemokines would affect feeding behavior at different time scales: if they all reduced food intake in the 2 h following injection, only CXCL8, CXCL4, and CCL2 were able to reduce food intake over the whole dark phase and only CXCL8 and CXCL4 were able to decrease the total daily food intake (69). This study is particularly important as it sets chemokines as contributors, in the brain, to the effects of infection/inflammation on feeding behavior. Nevertheless, no mechanistic insights were then provided.

As previously acknowledged, very few studies aimed at better characterizing how chemokines could modulate feeding behavior after this study by Plata-Salamán and Borkoski.

Yet, in a recent study from our laboratory (70), we sought to identify and characterize chemokines that could possibly

deregulate the hypothalamic circuits to alter food intake and energy balance in anorexia and weight loss. We thus assessed the gene expression of several pro-inflammatory mediators in the hypothalamus of mice that had received an acute ICV injection of LPS. After confirmation of LPS-induced inflammation in our mice by observing the overexpression of several pro-inflammatory mediators, we then identified ligands of the chemokine receptors CCR (CC-motif chemokine receptor)-2 and 5 as the most overexpressed. Among them, CCL2 (also known as monocyte chemoattractant protein 1) caught our attention as it has been described as particularly important in the context of LPS-induced neuroinflammation and suggested to be able to reduce food intake (69, 71–73). Thus, we focused on the central signaling of CCL2 and its receptor CCR2 and could demonstrate that it is mandatory for both metabolic and behavioral changes induced by LPS (70). Indeed, inhibiting CCR2 signaling by combined ICV injection of a specific antagonist of CCR2, INCB3344, together with LPS, prevents the weight loss that is induced by ICV injection of LPS alone. Similarly, the weight loss induced by ICV injection of LPS is reduced in mice deficient for CCR2 in comparison to control animals. Experiments in metabolic cages demonstrate that central injection of LPS decreases both food intake and locomotor activity, whereas it increases fat oxidation and induces a shift in used energy substrate in favor of lipids versus carbohydrates. These two last points probably illustrate an increased use of the energy stocked in the AT as lipids. Interestingly, these LPS effects highlighted by the metabolic cages experiments were also reduced when interfering with central CCR2 signaling by co-ICV injection of LPS and INCB3344.

We then identified the neurons, which produce melanin-concentrating hormone (MCH), a peptide known to elicit food intake and to decrease energy consumption, as targets for CCL2 (74). After ICV injection of LPS, we observed a sustained decrease in gene expression and protein levels of MCH, which is blunted/prevented by pharmacological (using INCB3344) or genetic inhibition of CCL2 signaling. Interestingly, we found that, similar to LPS, ICV injection of CCL2 promotes neuroinflammation, together with a decrease in both MCH expression and body weight. Immunostaining experiments showed that 70% of the MCH neurons of the lateral hypothalamus (LHA) express CCL2 receptor. These neurons responded to CCL2 by decreasing both electrical activity and MCH release. Thus, it seems that the inhibition of the MCH system by LPS depends primarily of the central signaling of CCR2. Moreover, in experiments of perifusion of hypothalamic explants treated with KCl and CCL2, MCH secretion is totally inhibited, while co-application of KCl and INCB3344 only, leads to enhanced secretion of MCH. This suggests that endogenous CCL2 could participate, in normal conditions, in modulating MCH system and thus energy balance regulation.

Thus, it appears that the central CCL2/CCR2 axis is able to directly act on neurons producing the orexigenic peptide MCH by reducing their activity and both the expression and secretion of the peptide, leading to a reduced food intake and to an increased use of energy stores. Hence, the central CCL2/CCR2 axis, by acting through MCH neurons, appears as a major actor in appetite and weight loss associated with LPS-induced inflammation (**Figures 2** and **3**). This study was the first to demonstrate that a chemokine can play a role, at the central level, in energy balance deregulation, by acting directly on neuropeptidergic systems in the hypothalamus.

OBESITY AND INFLAMMATION

As previously mentioned, interestingly, inflammation is also a characteristic for an opposite disorder of the energy balance disorder of the regulation of food intake and body weight: obesity. Yet, different from the inflammation associated to involuntary weight loss, this inflammation is of low intensity, also referred to as a "low-grade" inflammation (79). Obesity is characterized by an excessive fat mass distributed throughout the body that can be harmful to health [source: World Health Organization

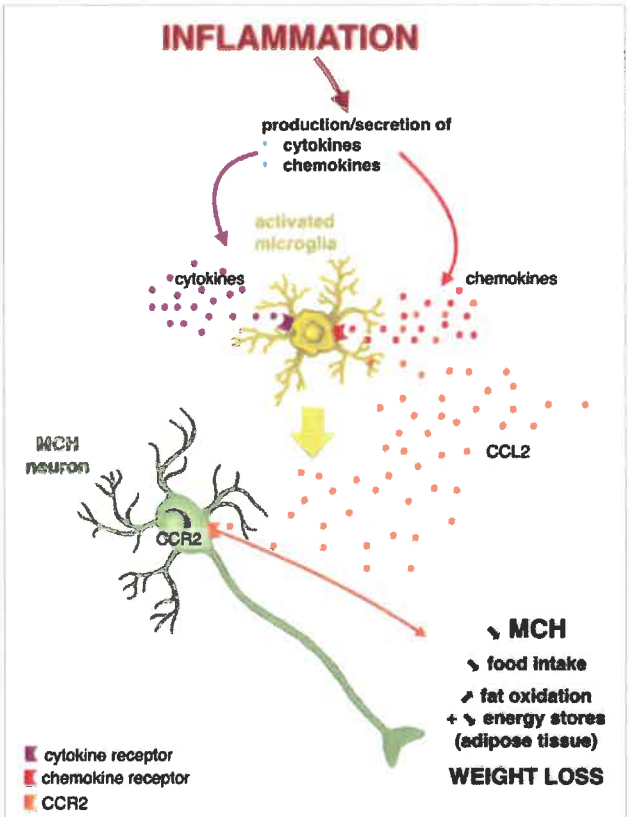

FIGURE 2 | Potential action of the CC-motif chemokine ligand (CCL) 2/CCR2 signaling pathway on melanin-concentrating hormone (MCH) neurons in a weight loss model induced by a central injection of lipopolysaccharide. Hypothalamic inflammation is characterized by overexpression of inflammatory mediators such as cytokines and chemokines. It is possible that these bind to their receptors expressed by glial cells such as microglia, which then activated, can produce even more cytokines and chemokines, including CCL2. However, it is not excluded that CCL2 could act directly on MCH neurons that expressed its receptor in the lateral hypothalamus. This would result in a decreased MCH neuronal activity and in a decreased secretion of this neuropeptide, which is associated to a loss of appetite, an increased fat oxidation, likely reflecting a decrease in energy stores in adipose tissue, and thus a loss of weight. Adapted from Le Thuc and Rovère (7).

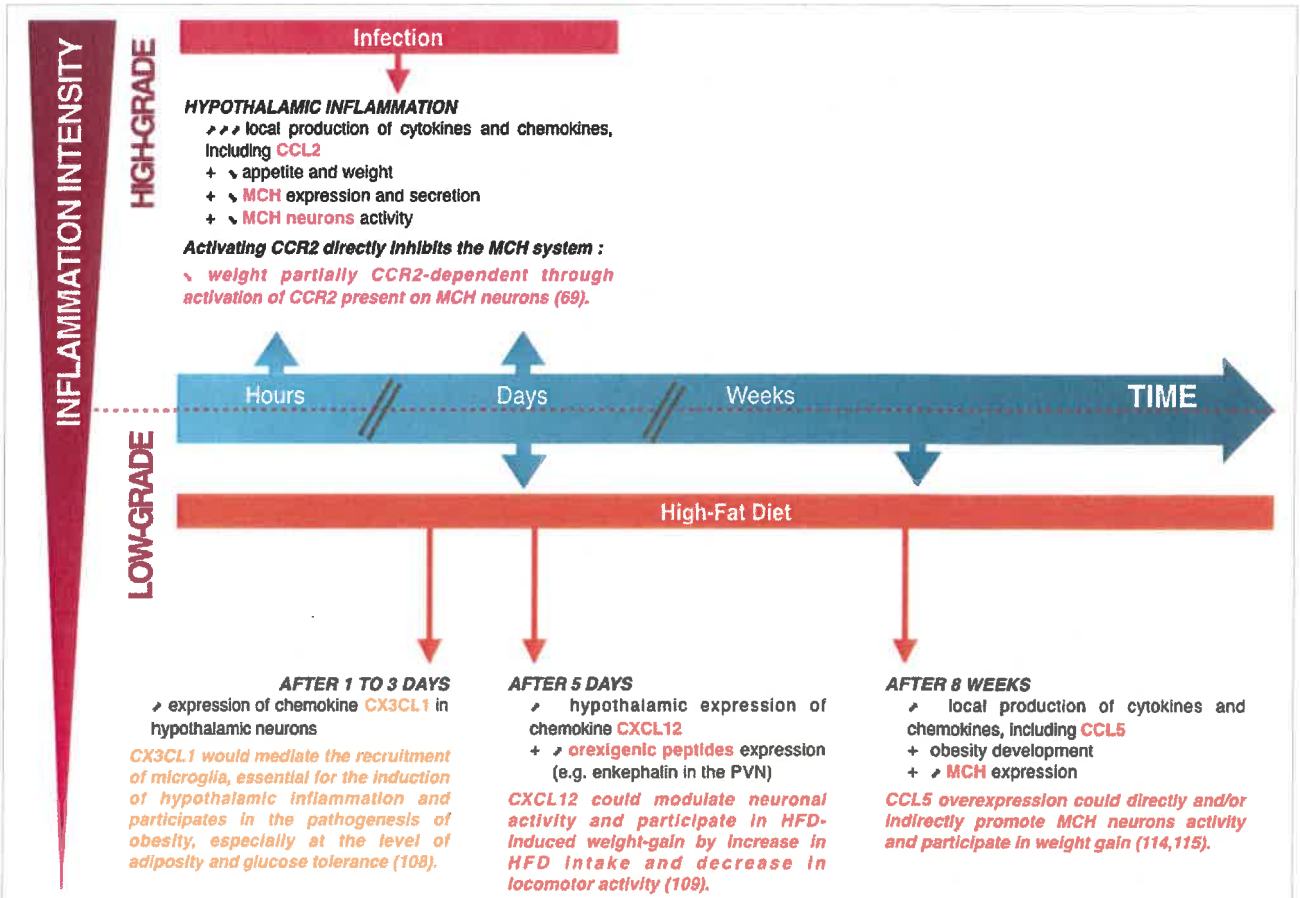

FIGURE 3 | Summary diagram showing the links between hypothalamic inflammation and deregulation of weight. *Upper part*: when lipopolysaccharide induces high-grade hypothalamic inflammation, there is an acute and increase in local production of cytokines and chemokines, including CC-motif chemokine ligand (CCL) 2 (a few hours to a few days) associated with weight loss (a manifestation of the sickness behavior associated with a high-intensity infection). This weight loss is partially dependent on the CCL2/CCR2 signaling pathway, by direct inhibition of the lateral hypothalamus (LHA) neurons producing the orexigenic peptide melanin-concentrating hormone (MCH) (70). *Lower part*: short (1–5 days) or prolonged (several weeks) high-fat diet (HFD) consumption is associated with chronic low-grade hypothalamic inflammation that appears to be linked to the development of obesity, together with an overexpression of orexigenic neuropeptides such as enkephalin in the paraventricular nucleus or MCH in the LHA. The chemokine CX3CL1 appears essential to the induction of hypothalamic inflammation, with an important role in the recruitment of microglia, whereas the chemokines CXC-motif chemokine ligand 12 and CCL5 could modulate neuronal activity and participate in HFD-induced weight loss (75–78). Adapted from Le Thuc and Rovère (7).

(WHO)]. One is considered obese if one's body mass index is greater than or equal to 30 (80, 81). Worldwide, close to 13% of the population were obese in 2014 and the prevalence of obesity nearly doubled between 1980 and 2014 (source: WHO). Obesity is a concern as it represents a risk factor for chronic diseases such as cardiovascular diseases (hypertension, heart disease, and stroke), diabetes, hepatic steatosis, respiratory diseases, musculoskeletal disorders (osteoarthritis, etc.), certain cancers (endometrium, breast, colorectal, etc.), and neurodegenerative disorders (79–81). Furthermore, childhood obesity, which is progressing in an alarming manner, can promote respiratory difficulties, high blood pressure, the emergence of markers of cardiovascular disease, fractures, insulin resistance, and psychological problems. All these aspects in turn increase the risk of adult obesity, premature death, and disability in adulthood (source: WHO).

While the development of obesity may be explained by some genetic aspects or be consecutive to a primary disease and/or its treatment (hormonal and/or psychological factors, drugs, etc.), its most common cause is a change in the population's life-style. This encompasses an increase in sedentariness and hypercaloric diets overconsumption, where the excess in calories intake most often comes from lipids and also carbohydrates (80, 81). As obesity represents a major and growing public health issue, it becomes important to identify and understand its causes and mechanisms. Therefore, understanding the role played by inflammation, both peripheral and central, in the establishment and/or development of obesity and its comorbidities might allow to identify new potential targets in the fight against obesity.

The inflammation associated to obesity exhibits several specificities: first, as mentioned previously, it is low grade and chronic. So far, it has been mostly described in peripheral tissues

(AT, liver, pancreas, etc.). Nevertheless, more recently, it has been shown to also occur in the CNS: a hypercaloric challenge, especially a high-fat diet (HFD), even on the short term, can induce an inflammation in the hypothalamus that is sustained in models of nutritional obesity (82).

Obesity and Peripheral Inflammation

Studies have associated inflammation and obesity for a long time (79). Yet this inflammation is not "typical," as it cannot be associated to the cardinal signs of redness, swelling, heat, and pain. Thus, the inflammation associated to obesity is of different nature. First, it is aseptic: it is caused by the overconsumption of specific nutrients (lipids and carbohydrates); thus, the trigger of this inflammation can be considered to be of metabolic nature. Interestingly, not only the trigger of this inflammation is metabolic, but this inflammation first targets the cells that specialized in metabolism: e.g., adipocytes, hepatocytes, pancreatic β-cells, and myocytes (but also the neurons of the peptidergic systems involved in the regulation of feeding behavior, as detailed further). Hence, this inflammation is often referred to as "metabolic inflammation" or "metainflammation" (79).

The characteristics of the inflammation associated with obesity, in periphery at least, are: (i) being of metabolic nature—it is induced by nutrients and is orchestrated by "metabolic" cells; (ii) being low grade, with moderate and localized overexpression of pro-inflammatory mediators; (iii) creating an impaired milieu where the tissue "composition" in terms of immune cells favors an inflammatory environment in the tissues; and (iv) being sustained in time, without any apparent resolution (79). Furthermore, peripheral metabolic inflammation associated with obesity is deleterious at many levels and, as a consequence of poor eating habits favoring the obese phenotype, inflammation itself promotes tissue dysfunctions, which also contribute to development of obesity, including resistance to insulin and leptin.

Obesity and Hypothalamic Inflammation

In the context of nutritional obesity, inflammatory pathways are not only activated in peripheral tissues but also activated in central areas involved in the control of energy metabolism, especially in the hypothalamus. Several questions then deserve to be asked and answered such as "What are the signals that induce this inflammation?" and "Is inflammation a cause or a consequence of obesity?"

The relationship between nutritional obesity and hypothalamic inflammation was first described by De Souza and his collaborators (83). The authors demonstrated in rats that a 4-month period of HFD feeding activates inflammatory pathways in the mediobasal hypothalamus (MBH), such as JNK and NF-κB, leading to the production of canonical pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 and to deficiencies in insulin and leptin signaling. These observations on hypothalamic inflammation associated to nutritional obesity were confirmed in rats, obese mice, and non-human primates (82, 84–92). Interestingly, some studies have shown that the inflammation induced by the consumption of HFD, likely a response to overnutrition, develops much faster in the hypothalamus than in the peripheral tissues. Indeed, inflammation in the AT, for example, is only observable after several weeks or even months, whereas in the hypothalamus, the consumption of a HFD during a much more restricted period of 24–72 h is enough to induce overexpression of pro-inflammatory cytokines and gliosis—which corresponds to an activation and proliferation of glial cells such as microglia and astrocytes, an inflammation hallmark in the brain (82).

Thus, it appears that the inflammation induced by the excess of nutrients in the hypothalamus precedes the establishment of obesity, as well as peripheral inflammation and metabolic disturbances. It is worth mentioning that the gliosis induced by the consumption of a HFD for a short-period of time (2–3 weeks) is actually reversible, whereas it reappears after longer periods of HFD consumption (8 months) (82). This suggests that the initial short-term gliosis is rather a protective mechanism induced to protect against the "injury" induced by the overload of fat, which is in line with the known roles of these cells, and that with time, the consumption of HFD triggers a durable gliosis and inflammation that would then play a major role in the development of the pathophysiology induced by overnutrition. For example, a study by Dalvi et al. further supports that acute gliosis associated with a moderate period of HFD feeding would actually be protective: as long as the inflammation is restricted to glial cells, the organism seems to attempt to limit brain injury by overexpressing in parallel some protective factors such as the heat shock protein 70 and the ciliary neurotrophic factor. After a longer period of HFD feeding, overexpression of inflammatory mediators within the neurons suggests an exhaustion of neuroprotective mechanism in the hypothalamus, leading to a deregulation of the expression of certain neuropeptides that will favor a positive energy balance (93).

What is the signal that triggers hypothalamic inflammation? It has been suggested that it is the excess of nutrients, which are major physiological regulators of hypothalamic neural networks, that induces the establishment of inflammation at the level of the hypothalamus. Some studies attribute the triggering of hypothalamic inflammation to, more especially, saturated fatty acids (SFA), especially long-chain SFA, due to their accumulation in the hypothalamus when consuming HFD, which could, among others, induce inflammatory signaling by activating the TLR4 pathway. It is interesting to note that other fatty acids, such as long-chain polyunsaturated fatty acids, appear, conversely to long-chain SFA, to be beneficial, especially in the diet-induced obesity (DIO) context, as they have anti-inflammatory properties (94–97). In contrast, not only nutrients but also hormones seem to play a role in the regulation of glial cells function. Indeed, as illustrated by a study by Gao et al., showing that monogenic obese mice with deficient leptin signaling [*ob/ob* (leptin deficient) and *db/db* (leptin receptor mutation)] exhibit less microglial activation than wild-type controls both on chow and on HFD (98). The authors further demonstrated that the lack of leptin signaling affects also the microglial function in the hypothalamus as the expression of several inflammatory mediators is reduced (98). This indicates that the signaling of leptin, already described as a pro-inflammatory adipokine (99), could affect microglial activation. Other mechanisms have been proposed as mediators of the hypothalamic inflammation associated with overnutrition such as the endoplasmic reticulum stress (86, 100, 101). Similarly,

oxidative stress could be an initiating factor and participate in the maintenance of the hypothalamic inflammation induced by nutrition. Indeed, at the periphery, oxidative stress, along with the production of reactive oxygen species (ROS), has been shown to precede severe metabolic disturbances and insulin resistance (102). Yet, the brain alone uses a large amount of the oxygen and calories consumed by the body, making it particularly vulnerable to excessive ROS production and oxidative stress. Indeed, mitochondria naturally produce ROS in physiological conditions, especially during the oxidation of nutrients such as glucose or fatty acids (103), and it has been shown that an excessive production of ROS in the hypothalamus occurs in the obese rat. This impairs not only the local detection of glucose but also the associated physiological response, such as peripheral insulin secretion (104). Autophagy is another possible pathway. It is a cellular process allowing the elimination of damaged cytoplasmic elements and organelles in order to maintain internal homeostasis and structural integrity and also plays a key role in cellular responses to metabolic stress. Under conditions of overnutrition, endoplasmic reticulum stress and oxidative stress can induce autophagy (105, 106). Impairing the pathways of autophagy can increase food intake and weight, in association with overactivation of the IKKβ/NF-κB pathway in the hypothalamus, illustrating the connection between autophagic and inflammatory pathways in the hypothalamus.

Over the recent years, the importance of the role played in hypothalamic inflammation in the onset of metabolic disorders in obesity was indeed underlined and the potential of counteracting inflammation in the hypothalamus as a strategy in the fight against obesity was highlighted.

For example, a recent study by Douglass and colleagues demonstrates that astrocytes in the MBH of HFD-fed mice mediate hypothalamic inflammation together with DIO *via* their own inflammatory signaling (107). Indeed, the authors showed that the inducible and specific deletion of IKKβ in astrocytes, which should blunt astrocytic inflammatory capacity, reduces the mice susceptibility to DIO (reduced weight gain and fat mass associated with a decrease in food intake and an increase in energy expenditure), improves glucose tolerance and insulin sensitivity (according to glucose and insulin tolerance tests), and finally, reduces HFD-induced astrocytosis in the MBH.

Furthermore, it has been shown that subcutaneous application of liraglutide or canagliflozin (an inhibitor of the sodium-glucose cotransporter 2) in obese and insulin-resistant rodents is able to disrupt the activation of microglial cells in the hypothalamus, which is associated with an improvement of insulin and glucose homeostasis. In addition, when the ICV injection of IL-4 in HFD-fed rats increases further HFD-induced inflammation in the hypothalamus and causes excessive weight gain, the ICV injection of an IKKβ/NF-κB blocker allows, on the contrary, to prevent hypothalamic inflammation, which is associated with both a decrease in body weight and fat mass and improvements in glucose metabolism and general energy homeostasis in DIO animals (86, 108–111).

Another study by André et al. shows that overnutrition will induce, in addition to an increase in body weight and in adiposity, an increase in the number of microglial cells in the ARC. The authors show the inhibition of microglia expansion in the ARC, achieved by the central delivery of an antimitotic drug, allows to limit food intake and the increase in body weight and in adiposity and also to restore leptin sensitivity. This is associated with a "predictable" inhibition of the upregulation of inflammatory pathways in the ARC that is normally associated with overnutrition, but also in periphery (112).

Taken together, studies studying the hypothalamic inflammation associated with obesity support that this inflammation plays a key role in the metabolic dysfunctions associated with obesity and represents a very interesting target.

Research aiming to understand how the central regulation of energy balance is altered in the context of overnutrition has mainly focused on neurons so far. Yet, as previously mentioned, since hypothalamic inflammation, especially its gliosis feature, has been associated to overnutrition, a strong interest in the role of glial cells (mainly microglia and astrocytes) emerged more recently. Interestingly, these glial cells, once activated, are capable of overproducing, locally, pro-inflammatory mediators such as cytokines and chemokines that can, in turn, affect the neuropeptidergic systems of the hypothalamus, potentially participating in the onset of obesity.

Interestingly, chemokines and their receptors have been demonstrated to be expressed by glial cells, and also by neurons. Especially, chemokine receptors appear to be more expressed by neurons than cytokine receptors in some nuclei of the hypothalamus, allowing to ask the following question: "Could not chemokines act as one of the latest inflammatory mediator that would link inflammation to the disruption of the proper functioning of the neuropeptidergic systems involved in the regulation of the energy balance to promote weight gain and thus the development of obesity?"

Chemokines in the Modulation of Hypothalamic Neuropeptidergic Circuits in DIO

As it was the case in the context of hypothalamic inflammation and involuntary weight loss, only few studies have aimed to determine and understand if and how chemokines are able to alter the function of hypothalamic neuropeptidergic circuits and thus participate in the development and/or the maintenance of obesity.

A study by Morari and colleagues has identified fractalkine (or CX3CL1) as involved in the early activation of hypothalamic inflammation in a murine model of DIO, likely through recruitment of microglial cells which express CX3CR1, the receptor for CX3CL1 (75). In this study, the authors show that, early after HFD introduction to the mice (1–3 days), CX3CL1 is induced in hypothalamic neurons of obesity-prone mice, unlike what was observed in obesity-resistant mice. Interestingly, the inhibition of CX3CL1 in the MBH by an approach of small interfering RNA allows a reduction in inflammation, glucose intolerance, and diet-induced adiposity (no significant difference in body weight was observed though). It suggests that CX3CL1, mediating early recruitment of microglia induced by HFD and thus participating in the induction of hypothalamic inflammatory response, participates in the pathogenesis of obesity as it impairs glucose tolerance and adiposity (**Figure 3**).

In addition, one recent study highlights the role of the axis CXCL12/CXC-motif chemokine receptor 4 (CXCR4) in the paraventricular nucleus (PVN) of the hypothalamus in mediating both neuronal and behavioral effects of the consumption of a HFD in rats (76). In more details, the authors show that a 5-day consumption of a HFD induces an overexpression of CXCL12 and its receptors CXCR4 and CXCR7 both in the PVN and in the perifornical lateral hypothalamus (PFLH). The authors also show that HFD is able to induce an overexpression of orexigenic neuropeptides enkephalin and galanin in the PVN and orexin (ORX) and MCH in the PFLH. Moreover, HFD is associated with an increase in the number of $CXCR4^+$ cells in the PVN. Conversely, in the arcuate nucleus, the levels of CXCL12 and CXCR4 were too low to be detected. The ICV injection of CXCL12, next to the hypothalamus, is able to recapitulate the effects of HFD consumption: (1) it reduces novelty-induced locomotor activity, as a 5-day HFD feeding period does; (2) it is associated to an increase in gene expression of enkephalin in the PVN; and (3) it induces an acute increase in calorie intake (by overconsumption of the HFD only and no consumption of chow diet, as rats had access to both diets). This last point interestingly suggests that CXCL12 could participate in the control ingestive behavior, especially since some different studies also previously demonstrated that CXCL12 could affect other systems such as the MCH and vasopressin in the hypothalamus or dopamine in the substantia nigra (18, 113, 114). Taken together, these results suggest that CXCL12 could modulate the activity of orexigenic peptide-producing neurons, especially the encephalin neurons in the PVN. This would favor HFD intake and decrease locomotor activity and, thus, participate in HFD-induced weight gain (**Figure 3**).

The chemokine CCL5, also known as RANTES for Regulated on Activation, Normal T Cell Expressed and Secreted, has already been linked to obesity-associated inflammation in periphery. Indeed, obesity is associated with an increase in CCL5 secretion and gene expression in AT in obese human and mice. There, CCL5 is suspected to mediate the increase in the local accumulation of T cells and macrophages, which is involved in the complex genesis of chronic inflammation (115, 116). In addition, our joint study with Dr. Karine Clément's team proposed CCL5 as biomarker of weight evolution in patients undergoing bariatric bypass surgery Roux-en-Y as its levels were nearly eightfold higher in the serum of obese patients than in the one of control patients. CCL5 seems to be the only chemokine of which serum levels appear to be correlated with caloric intake in patients undergoing bariatric bypass surgery Roux-en-Y: they rapidly decrease after surgery, as caloric intake decreases and, later on, when patient's caloric intake and body weight start increasing again, they also increase again (77). This justified further interest in the potential role of CCL5 as a modulator of the activity of hypothalamic neurons regulating food intake and which could possibly promote the establishment and/or development of obesity. Thus, our group characterized its expression profile, both at the peripheral and hypothalamic levels in a model of nutritional obesity in mice. In this model, we found both peripheral and central inflammations, as evidenced by the overexpression of canonical pro-inflammatory mediators in the serum and hypothalamus. CCL5 was also found to be overexpressed in the periphery and in the hypothalamus of obese animals (78). An ICV injection of CCL5 on the expression of orexigenic peptides MCH and ORX seems able to increase the expression of both ORX and MCH, transiently for ORX and in a more stable and persistent manner for MCH. Furthermore, it would appear that CCL5 is capable of depolarizing MCH neurons, thus facilitating their activation (78). These results indeed suggest that CCL5 is acutely able to modify the activity of the hypothalamic orexigenic MCH system. Taken together, these results suggest that the overexpression of CCL5 would promote the overactivation of hypothalamic MCH neurons and thus participate in weight gain (**Figure 3**). Whether CCL5 is a cause or a consequence of obesity, or if it could effectively disrupt the proper function of the neuropeptidergic circuits of the hypothalamus, which regulate energy balance and promote the establishment of obesity, is nevertheless still matter of investigation.

CONCLUSION

As of today, a substantial amount of publications supports that hypothalamic inflammation mediates disruptions in the hypothalamic control of energy homeostasis, especially regarding body weight regulation. If some pathways and cellular actors have been identified, mechanisms are still ill-described and poorly understood.

Chemokines have long been considered as essential mediators of the inflammatory response, but more particularly because of their ability to activate and attract immune cells to the affected site. However, at the central level, the literature has also attributed to chemokines and their receptors, beyond their role in the attraction of leukocytes in the cerebral parenchyma, important roles in neuronal survival as in neurotoxicity, cerebral development, communication between immune and glial cells, and also communication between neurons and glial cells (since all these cell types have been shown to be able to express the actors of the chemokinergic system) and also in neuromodulation (15, 18).

Several studies now support the hypothesis that chemokines are actually able to modulate the activity of certain neurons. We and others were able to demonstrate that their overexpression is able to deregulate the neuropeptidergic systems of the hypothalamus, which participate in the regulation of the energy balance and participate in the development of deregulations of the latter, whether it is an excessive weight loss or gain (**Figure 3**). Moreover, this does not, in any way, exclude that, under physiological conditions, the chemokines are actors of this regulation. These studies on chemokine central signaling now identify chemokines as novel potential therapeutic targets against deregulations of the energy balance.

ETHICS STATEMENT

The protocols were carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Nice-French Riviera, project agreements no. 04042.01 and 04464.01).

AUTHOR CONTRIBUTIONS

OLT conceived and wrote the manuscript. OLT also made the figures. OLT and KS realized the bibliography researches. CC, JLN, NB, and CR critically appraised and revised the manuscript and figures. All authors listed made substantial, direct, and intellectual contribution to the work and gave permission for this manuscript to be published.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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