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LIVER-BRAIN AXIS IN THE REGULATION OF ENERGY METABOLISM:

ROLE OF THE HEPATIC ESTROGEN A RECEPTOR IN REGULATING THE INTERFACE BETWEEN THE BLOOD AND THE HYPOTHALAMIC ARCUATE NUCLEUS.

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Short Abstract

The hepatic estrogen receptor α (hERα) has been implicated in the regulation of energy metabolism. In this thesis, we investigate the role of hERα in accessing peripheral signals to the hypothalamic arcuate nucleus (ARH) to regulate energy metabolism. Our results show that the absence of hERα alters the increase in permeability of the blood/NA interface normally induced by fasting to regulate energy homeostasis. This alteration is associated with increased transcripts and plasma levels of the hepatokine LECT2 in the liver. Furthermore, we demonstrate the inhibitory effect of LECT2 on NA vascular permeability after fasting. Collectively, our results suggest that hepatic estrogen signaling, via the control of LECT2 secretion, has a permissive effect on the regulation of the blood/NA interface and on the adaptation of the hypothalamic response to metabolic stress.

French short Abstract

Le récepteur des œstrogènes α hépatique (hERα) est reconnu comme un acteur important dans la régulation du métabolisme énergétique. Dans cette thèse, nous étudions le rôle de l'hERα dans l'accès des signaux périphériques au noyau arqué hypothalamique (NA) pour réguler le métabolisme énergétique. Nos résultats montrent que l'absence de hERα altère l'augmentation de la perméabilité de l'interface sang/NA normalement induite par le jeûne pour réguler l'homéostasie énergétique. Cette altération est associée à des niveaux élevés de transcrits et de concentrations plasmatiques de l'hépatokine LECT2 au niveau hépatique. De plus, nous démontrons l'effet inhibiteur de LECT2 sur la perméabilisation des vaisseaux du NA après le jeûne. Dans l'ensemble, nos résultats suggèrent que la signalisation œstrogénique hépatique, via le contrôle de la sécrétion de LECT2, a un effet permissif sur la régulation de l'interface sang/NA et sur l'adaptation de la réponse hypothalamique au stress métabolique.

Summary

Energy metabolism is regulated at different levels of the body, with a constant need for the organs to integrate and send messages to communicate with each other. This dialogue takes place in part via the endocrine system, where hormones and their receptors network the various organs. The aim of the thesis project was to establish the link between the liver and the brain, two organs involved in this network.

The brain, and more specifically the hypothalamus, plays a central role in regulating energy metabolism, having to integrate and respond to messages from peripheral organs. However, dialogue with cerebral structures can only take place through various interfaces such as the blood-brain barrier, the choroid plexuses and the circumventricular organs; interfaces that protect the brain by regulating the transport of signals between the cerebral and blood compartments.

Work in the host laboratory has shown the role of the median eminence, a circumventricular organ located in the tuberal region of the hypothalamus, as an interface that regulates exchanges between the blood compartment and the arcuate nucleus, a first-order hypothalamic nucleus that integrates peripheral signals involved in energy metabolism. The permeability of this interface varies according to the animal's energy status and the oestrus cycle in females, regulating to a greater or lesser extent the access of peripheral signals such as hormonal messages (leptin and ghrelin) to the arcuate nucleus. In this way, the blood-arcuate nucleus interface contributes to the regulation of metabolism.

Among the hormones involved in regulating energy metabolism, we were interested in estrogens. Well known for their role in reproduction, estrogens are also involved in regulating energy metabolism. The effects of estrogen metabolism are mainly associated with the receptor α (ERα), whose invalidated expression throughout the body causes hyperphagia and

a reduction in energy metabolism. In the liver, the female mouse model in which ERα expression is specifically abolished in hepatocytes has established the role of these hepatic receptors in preventing obesity and steatosis (accumulation of lipids in the liver). Therefore, the liver, via its α receptors, plays a role in regulating energy metabolism.

The aim of the thesis project was to establish the links between the liver and the blood/arcuate nucleus interface by testing the hypothesis of the involvement of estrogens and their hepatic α receptor in this dialogue.

Our results show that the absence of hERα alters the increase in permeability of the blood/NA interface normally induced by fasting to regulate energy homeostasis. This alteration is associated with elevated transcripts and plasma levels of the hepatokine LECT2 in the liver. Furthermore, we demonstrate the inhibitory effect of LECT2 on ARH vessel permeabilization after fasting. Overall, our results suggest that hepatic estrogen signaling, via the control of LECT2 secretion, has a permissive effect on the regulation of the blood/NA interface and on the adaptation of the hypothalamic response to metabolic stress.

French summary

La régulation du métabolisme énergétique se fait à différents niveaux de notre organisme avec un besoin constant pour les organes d'intégrer et d'envoyer des messages pour communiquer entre eux. Ce dialogue se fait en partie via le système endocrinien où les hormones ainsi que leurs récepteurs mettent en réseau les différents organes. Le projet de thèse visait à établir le lien entre le foie et le cerveau, deux organes impliqués dans ce réseau.

Le cerveau, et plus particulièrement l'hypothalamus a un rôle central dans la régulation du métabolisme énergétique, devant intégrer et répondre aux messages provenant des organes périphériques. Cependant, le dialogue avec les structures cérébrales ne peut se faire qu'au travers des différentes interfaces telles que la barrière hémato-encéphalique, les plexus choroïdes et les organes circumventriculaires ; interfaces qui protègent le cerveau en régulant le transport des signaux entre les compartiments cérébral et sanguin.

Les travaux du laboratoire d'accueil ont montré le rôle de l'éminence médiane, organe circumventriculaire situé dans la région tubérale de l'hypothalamus, en tant qu'interface qui régule les échanges entre le compartiment sanguin et le noyau arqué, noyau hypothalamique de premier ordre qui intègre les signaux périphériques impliqués dans le métabolisme énergétique. La perméabilité de cette interface varie suivant le statut énergétique de l'animal et le cycle œstral chez la femelle, régulant plus ou moins l'accès des signaux périphériques tels que les messages hormonaux (la leptine et la ghréline) au noyau arqué. Ainsi l'interface sang/noyau arqué contribue à la régulation du métabolisme.

Parmi les hormones impliquées dans la régulation du métabolisme énergétique, nous nous sommes intéressés aux œstrogènes. En effet, bien connus pour leurs rôles dans la fonction de reproduction, les œstrogènes sont aussi impliqués dans la régulation du métabolisme

énergétique. Les effets des œstrogènes sur le métabolisme sont principalement associés au récepteur α (REα), dont l'invalidation de l'expression dans l'ensemble de l'organisme provoque une hyperphagie et une diminution du métabolisme énergétique. Au niveau hépatique le modèle de souris femelles où l'expression des REα sont abolis spécifiquement au niveau des hépatocytes a permis d'établir le rôle de ces récepteurs hépatiques et des œstrogènes dans la prévention de l'obésité et de la stéatose (accumulation de lipides dans le foie). Ainsi le foie via ses récepteurs α joue un rôle dans la régulation du métabolisme énergétique.

Le projet de thèse proposait d'établir les liens entre le foie et l'interface sang/noyau arqué en testant hypothèse de l'implication des œstrogènes et de leur récepteur α hépatique dans ce dialogue.

Nos résultats montrent que l'absence de hERα altère l'augmentation de la perméabilité de l'interface sang/NA normalement induite par le jeûne pour réguler l'homéostasie énergétique. Cette altération est associée à des niveaux élevés de transcrits et de concentrations plasmatiques de l'hépatokine LECT2 au niveau hépatique. De plus, nous démontrons l'effet inhibiteur de LECT2 sur la perméabilisation des vaisseaux du NA après le jeûne. Dans l'ensemble, nos résultats suggèrent que la signalisation œstrogénique hépatique, via le contrôle de la sécrétion de LECT2, a un effet permissif sur la régulation de l'interface sang/NA et sur l'adaptation de la réponse hypothalamique au stress métabolique.

Content table

LIVER-BRAIN AXIS IN THE REGULATION OF ENERGY METABOLISM: 1

INTRODUCTION.....23

I. HYPOTHALAMIC REGULATION OF THE ENERGY HOMEOSTASIS.....24

A. THE HYPOTHALAMUS: MASTER OF HOMEOSTASIS 24

1. The arcuate nucleus.....25

2. Ventromedial nucleus.....26

3. Dorsomedial nucleus.....28

4. Lateral hypothalamic nucleus 29

5. Paraventricular nucleus.....30

6. The suprachiasmatic nucleus 30

B. PLAYERS IN THE REGULATION.....31

1. Gastrointestinal tract.....31

2. Adipose tissue.....35

3. Pancreas.....36

4. Ovaries and tests.....37

C. THE HYPOTHALAMIC REGULATION OF THE DIFFERENT NUTRITIONAL STATES.....38

1. Fed state 39

2. Fasted state.....39

3. Refeeding state 40

II. THE PERIPHERY-BRAIN INTERFACES 42

A. DIFFERENT INTERFACES BETWEEN THE PERIPHERY AND BRAIN.....42

1. Blood-brain barrier.....43

2. Choroid plexuses.....46

3. CIrcumventricular organ 47

B. THE INTERFACES' PROPERTIES.....51

1. Diffusion properties *51*

2. Barrier properties *54*

C. THE ME-ARH INTERFACE 58

1. MBH vascular complex *59*

2. Tanycytes *60*

D. THE ME-ARH REGION AS A FIRST STOP IN THE HYPOTHALAMUS REGULATION 64

1. Peripheral routes *64*

2. A fasting state induces the ME-ARH plasticity *65*

III. THE ESTROGEN AND ITS HEPATIC RECEPTOR ESTROGEN A 69

A. ESTROGEN 69

1. The root of the sexual dimorphism: feminization and masculinization *70*

2. Physiology of the Menstrual Cycle *72*

3. The end of estrogen's protective effect: the menopause *73*

B. STRUCTURE OF THE ESTROGEN RECEPTOR A 74

C. THE LIVER ANATOMY AND FUNCTION 78

1. The liver's anatomy *78*

2. Liver's functions *81*

D. FUNCTION: THROUGH THE LERKO STORY 84

1. The LERKO model, a constitutive mice model to study the liver ERα *86*

2. hERα, a bridge for the metabolic and reproductive functions *86*

3. NAFLD worsened by the lack of hERα *88*

4. The estradiol is not the only hERα ligand *89*

IV. THE LIVER, THE HYPOTHALAMUS, AND THE ESTROGEN 92

A. THE HYPOTHALAMUS AND LIVER ACT TOGETHER TO REGULATE THE METABOLISM 92

1. Innervation connection *92*

2. Glucose sensing *93*

3. Lipid metabolism.....94

B. THE RELATION OF THE HYPOTHALAMUS AND ESTROGEN IN METABOLISM 94

C. THE BRIDGE BETWEEN THE HYPOTHALAMUS AND LIVER: HEPATOKINES 96

1. Growth Differentiation Factor-15 (GDF15) 96

2. Fibroblast Growth Factor 21 (FGF21) 97

3. Tsukushi (TSK).....98

4. Hepatocyte Growth Factor (HGF): 98

5. Dipeptidyl peptidase 4 (DDP4).....99

6. Fetuin-A.....100

7. Insulin-like growth factor (IGF-1).....100

8. Angiotensinogen.....101

9. Follistatin (FST): 101

10. Adropin.....102

11. Leukocyte cell-derived chemotaxin-2 (LECT2) 102

RESULTS 109

I. THE HEPATIC ERA'S SEXUAL DIMORPHIC ROLE ON THE HYPOTHALAMUS PLASTICITY 110

A. ME-ARH BARRIER ADAPTATION TO FASTING IS SIMILAR IN FEMALE AND MALE WILD-TYPE ADULT MICE 110

B. LERKO MICE SHOW IMPAIRED FASTING-INDUCED PLASTICITY OF THE BLOOD/ARH INTERFACE IN FEMALE MICE 114

*1. The hepatic ERα impact on the MBH nutritional-dependent plasticity of the **female** mice.....114*

2. The fasted male LERKO mice 117

1. Diffusion studies: the diffusion of peripheral signals into the ME-ARH region 118

II. METABOLIC CONSEQUENCES OF THE INVALIDATION OF THE HEPATIC ESTROGEN RECEPTOR A 121

A. UNCHANGED BODY COMPOSITION 122

B. MILD CHANGES IN METABOLIC PARAMETERS 124

1. LERKO female mice have mild increased ambulatory activity during the active phase and changed food intake 124

2. The males LERKO have an increase in energy expenditure not explained by ambulatory activity 127

III. FINDING THE POTENTIAL HEPATOKINE-LIGAND CANDIDATES TO EXPLAIN THE ME-ARH PHENOTYPE IN FEMALE LERKO MICE 128

A. GLYCEMIA AND HEPATIC VEGFA FALL SHORT OF EXPLAINING THE HERA-DEPENDANT ME-ARH IMPAIRMENT 129

B. NARROWING THE LIST OF CANDIDATES: HEPATOKINES RECEPTORS FOUND IN THE HYPOTHALAMUS 131

C. REGULATION OF BOTH LECT2 HEPATIC SECRETION AND ITS RECEPTOR TIE1 EXPRESSION ARE LOST IN LERKO MICE 133

IV. INJECTION OF LECT2 PROTEIN INHIBITS THE FASTING-INDUCED PLASTICITY OF THE BLOOD/ARH AND IMPAIRS THE REFEEDING BEHAVIOR OF WT MICE. 136

DISCUSSION 142

MATERIALS AND METHODS 154

APPENDIX 162

BIBLIOGRAPHY 170

Figures index

Figure 1: Anatomy of the hypothalamus and its nuclei and the primary pathway involved in energy regulation. *.....28*

Figure 2: Scheme of organs connecting with the hypothalamus through their respective hormones *.....36*

Figure 3: OVC localization in the mouse and human brain *.....49*

Figure 4: Organization of continuous and fenestrated endothelium and diaphragm structure. *.....53*

Figure 5: Tight junction complex and repartition in the Medio basal hypothalamus *....56*

Figure 6: Tanycytes repartition in the hypothalamus and anteroposterior zones of the MBH *.....60*

Figure 7: ME-ARH region nutritional-dependent plasticity in the fenestrated vessels and tight junction *.....67*

Figure 8: ERα structural organization *.....75*

Figure 9 : Liver basic anatomy of human and mouse *.....79*

Figure 10: Nerve pathway between the hypothalamus and the liver *.....93*

Figure 11: Experimental fasting protocol *.....110*

Figure 12: ME-ARH barrier adaptation to a fasting period is similar in female and male adult mice *. .....* ***Erreur ! Signet non défini.***

Figure 13: Fasting-induced plasticity of the blood-ARH interface is impaired in female LERKO mice *.....116*

Figure 14: Fasting-induced plasticity of the blood-ARH interface is unchanged in male LERKO mice. *.....117*

Figure 15: Blue Evans dye diffusion studies in the ME-ARH region *.....119*

Figure 16: LERKO female and male body composition change. *.....121*

Figure 17: Basal Metabolic Metabolic Studies of LERKO mice*.....123*

Figure 18: Fasting metabolism of LERKO female and male*.....126*

Figure 19: Glycemia and hepatic VEGFa fall short to explain the hERα-dependant ME-ARH impairment.*.....128*

Figure 20: Associated receptors of the hepatokines secreted by the liver*.....130*

Figure 21: Confirmation of the hepatokines' receptors mRNA presence in the hypothalamus through in situ hybridization followed by an immunostaining.*.....131*

Figure 22: Fasting-induced regulation of both LECT2 hepatic secretion and its receptor Tie1 expression in fenestrated vessels are impaired in LERKO female mice. *.....134*

Figure 23: histag-conjugated recombinant LECT2 injection (rLECT2) inhibits the fasting-induced plasticity of the blood-ARH *.....137*

Figure 24: Blue Evans dye diffusion studies after rLECT2 injection.*.....138*

Figure 25: Food intake studies on rLECT2 injected female mice.*.....139*

Figure 26: Hepatic Estrogen Receptor α controls fasting-induced blood-hypothalamus barrier plasticity via LECT2/Tie1 signaling in female mice*.....143*

Figure 27: 3D acquisition of anti-MECA-32 signal or fenestrated vessels of the ME-ARH region of the hypothalamus*.....164*

Figure 28: Immunostaining of the fenestrated vessels and vessels following a clearing technique of an entire hypothalamus *.....165*

Figure 29: Anti-ZO-1 immunostaining of the ME-ARH region after a clearing technique of an entire hypothalamus*.....166*

Tables index

Table 1: Non-exhaustive list of peripheral hormones involved in energy homeostasis *.....32*

Table 2: Estrogen receptors family *.....77*

Table 3: List of hepatokines and associated roles in metabolism *.....99*

Table 4: LECT2 correlation with metabolic parameters *.....106*

Abbreviations

AA	Amino acid
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic Hormone
AF-1	Activating function domain
AFP	α-fetoprotein
AgRP	Agouti-related peptide
AKT	Protein kinase B
ANGPT	Angiopoietin
AP	Area postrema
ApN	Adiponectin
ARH	Arcuate nucleus hypothalamic
AT1R	Angiopoietin receptor 1
AVP	Arginine-vasopressin
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BCSFB	Blood cerebrospinal fluid barrier
BE	Blue Evans dye
BMI	Body mass index
BrdU	Bromodeoxyuridine
CART	Cocaine and amphetamine-regulated transcript
CH	Cholesterol
ChR2	Channelrhodopsin-2
CKK	Cholecystokinin
CKK1R	Cholecystokinin 1 receptor
CP	Choroid plexus
CVO	Circumventricular organ
CSF	Cerebrospinal fluid
DBD	DNA binding domain
DHA	Dorsal hypothalamic area
DMA-1	Dendrite-Morphogenesis-Abnormal
dmARH	Ventromedial arcuate nucleus hypothalamic

DMH	Dorsomedial nucleus
DPP4	Dipeptidyl peptidase 4
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
EC	Endothelial cells
EC	Endothelial cell
ENHO	Energy Homeostasis associated
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ERα	Estrogen receptor α
ESR1	Estrogen receptor gene
FA	Fatty acid
FAS	Fatty acid synthesis
FGF21	Fibroblast growth factor 21
FGFR1	Fibroblast growth factor receptor 1
FI	Food intake
FSH	Follicle-stimulating hormone
FST	Follistatin
FV	Fenestrated vessel
G-6-P	Glucose 6 phosphate
GABA	gamma-aminobutyric acid
GCGR	G-protein coupled glucagon receptor
GDF15	Growth differentiation factor 15
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFRAL	GDNF family receptor α-like
GLP-1	Glucagon-like peptide 1
GLP1R	Glucagon-like peptide 1 receptor
GLUT	Glucose transporter
GnRH	Gonadotropin-releasing hormone
GPR19	G protein-coupled receptor 19
GTP	Gamma-Glutamyl Transferase

HDL	High-density lipoprotein
HFD	High-fat diet
HGF	Hepatic growth factor
HIF-α	Hypoxia-inducible factor 1
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor 1
IGF-1	Insulin Growth Factor 1
IL	Interleukin
IR	Insulin receptor
JNK	Jun N-terminal Kinase
KO	Knockout
LatA	Latrunculin-A
LBD	Ligand binding domain
LDL	Low-density lipoprotein
LECT2	Leukocyte cell-derived chemotaxin-2
LepR	Leptin receptor
LERKO	Liver estrogen receptor knockout
LH	Luteinizing hormone
LHA	Lateral hypothalamic nucleus
LPS	Lipopolysaccharides
LSEC	Liver sinusoidal endothelial cells
LSEC	Liver sinusoidal endothelial cells
MAFLD	Metabolic-associated fatty liver disease
MAPK	Mitogen-activated protein kinases
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MBH	Medio-basal hypothalamus
MC4R	Melanocortin-4 receptor
MCH	Melanin-concentrating hormone
ME	Median eminence
MSH	Melanocyte stimulating hormone
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NH	Neurohypophysis

NKT	Natural killer T cells
NPY	Neuropeptide Y
OVLT	Organum vasculosum of the lamina terminalis
OVX	Ovariectomy
PCOS	Polycystic ovarian syndrome
PFA	Perifornical area
PG	Pineal gland
PI3K	Phosphoinositide 3-kinase
PLVAP	Plasmalemma vesicle-associated protein
PNN	Perineuronal net
POA	Preoptic area
POMC	Pro-opiomelanocortin
PPARα	Peroxisome proliferator-activated receptor α
PVD	Polymodal ventral dendrites
PVN	Paraventricular nucleus
PYY	Peptide YY
RAS	Renin-angiotensin system
Rbp4	Retinol-binding protein
RER	Respiratory exchange ratio
SCN	Suprachiasmatic nucleus
SCO	Subcommisural organ
SERM	Selective estrogen receptor modulators
SF-1	Steroidogenic Factor 1
SFO	Subfornical organ
SHBG	Sex hormone-binding globulin
SREBP-1C	Sterol regulatory element-binding protein 1
TAMP	Tight junction-associated MARVEL domain-containing proteins
TEER	Transendothelial electrical resistance
TG	Triglycerides
Tie	Tyrosine Kinase Immunoglobulin-like EGF-like Domains
TJ	Tight junction
TMN	Tuberomammillary nucleus
TNF-α	Tumour necrosis factor α

UCP-1	uncoupling protein 1
VEFG	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHM	Ventromedial nucleus
VIP	Vasoactive intestinal polypeptide
VLDL	Very low-density lipoprotein
vmARH	Dorsomedial arcuate nucleus hypothalamic
WT	Wild-type
ZO	Zonula Occludens

INTRODUCTION

I. Hypothalamic regulation of the energy homeostasis

A. The Hypothalamus: Master of Homeostasis

Nestled in the brain, the hypothalamus coined and observed by the scientist Wilhelm His in 1893, it is a master of metabolic and reproductive functions notably. In humans, the hypothalamus is located just above the brainstem and below the thalamus and is part of the diencephalon, a region within the forebrain. It plays a central role in maintaining the body's equilibrium, orchestrating various bodily functions, and serves as a bridge between the nervous and endocrine systems. This connection ensures the body's proper functioning. (Kreier & Swaab, 2021). The hypothalamus is composed of numerous nuclei located in pairs on each side of third ventricle, a testament to the complexity and importance of its role in maintaining homeostasis. These nuclei are classified as follows:

- ✓ Periventricular hypothalamic nuclei: the arcuate nucleus (ARH), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMH), the paraventricular nucleus (PVN)
- ✓ The nuclei at the border of the 3d ventricle / the medial part of the hypothalamus: the lateral hypothalamic nucleus (LHA), perifornical area (PFA), dorsal hypothalamic area (DHA), tuberomammillary nucleus (TMN)
- ✓ Peri-hypothalamic nuclei: preoptic area (POA), suprachiasmatic nucleus (SCN)

The hypothalamic nuclei could be described according to their function. The arcuate nucleus is a “first-order nucleus” , as a first integrator of peripheral cues, and projects to the “second-order” nuclei, the VMH, DMH, and LHA that will elaborate and transmit responses to the periphery (Luquet & Cruciani-Guglielmacci, 2009).

The hypothalamus significantly influences vital functions, including temperature regulation, hunger, thirst, sleep, and hormonal balance. Here, we will focus primarily on the arcuate nucleus, its neuronal populations, and functions relating to the food intake balance.

1. The arcuate nucleus

At the base of the hypothalamus and third ventricle (3V) are the arcuate nuclei (ARH), a pair of dense population of neurons separated/mirrored by the third ventricle. The ARH is divided into the ventromedial region (vmARH) and the dorsomedial region (dmARH). The ARH localization allows a first access to external cues coming from the median eminence (the hypothalamus' gate to the periphery) giving the ARH title of first order nucleus. The dense population of the ARH comprises neurons regulating homeostasis by integrating the peripheral signals and giving orders to subsequent nuclei. The ARH's primary purpose is to maintain an energy homeostasis and it possesses a circuitry allowing it to take decision on food intake and energy expenditure. This circuitry is composed of neurons containing orexigenic (hunger-related) peptides: agouti-related peptides (AgRP) and the neuropeptides Y (NPY); and anorexigenic (satiety-related) peptides: proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) (Deem et al., 2022). The expression of these peptides in their specific neurons forms distinct neuronal populations. The AGrP neuron population can induce food intake by releasing AgRP and NPY (Horvath *et al.*, 1997; Andermann & Lowell, 2017). On top of inciting food intake, AgRP's negative valence can influence meal and place preference for food intake (Betley *et al.*, 2015). AgRP can also regulate the locomotor activity in mice, maybe as a strategy to seek food (Mesaros *et al.*, 2008). The ablation of the NPY/AgRP neurons in neonatal mice has no impact on their energy balance, however, ablation of these neurons in adult mice results in severe starvation (Luquet *et al.*, 2005). The anorexigenic population of neurons expressing POMC and CART is the

antagonist of the previously described AgRP and NPY population. In mice, virus-mediated deletion of the POMC neurons triggers obesity in males and females. POMC knock-out (KO) male and female mice are overweight due to increased lean and fat mass (Xu *et al.*, 2005).

Interestingly, POMC mutations have been found in humans, resulting in obesity and adrenocorticotropic Hormone (ACTH) deficiency (a product of POMC and proprotein convertase 1 (PC1) enzyme) (Krude *et al.*, 1998). The CART neuron invalidation showed a role in glucose metabolism and body weight in males (Wierup *et al.*, 2005). The basal CART effect on female mice has been studied less, but it is known that CART can increase alcohol consumption in female mice (Maddern *et al.*, 2024).

The ARH is subject to sexual dimorphism, for example, male rats seem to have a higher neuron density than females and less dendritic branching (Leal *et al.*, 1998). In AgRP knockout mice, only females will have a decreased fat mass. The NPY neuron invalidation in mice highlights a role for NPY in body weight in both male and female and higher bone mass (shown in males) (Baldock *et al.*, 2009).

The arcuate nucleus, as the first-order nucleus, will orchestrate with its orexigenic and anorexigenic neuronal populations a system that integrates external energy cues to “decide” whether or not to seek and eat food. The ARH will communicate with the other, second-order nuclei (VMH, DMH, LHA, and PVH) to carry out the appropriate physiological and behavioural response.

2. Ventromedial nucleus

The Ventromedial nucleus (VMH), a second-order hypothalamic nucleus, is above the ARH and borders the third ventricle. The primary neuronal population of the VMH is glutamatergic and complemented with neurons expressing leptin receptor (LepR), the estrogen receptor α (ERα), and steroidogenic factor 1 (SF-1) (Ziegler et al., 2002; Khodai & Luckman, 2021).

The role of the VMH in hypothalamic homeostasis regulation was investigated in 1942, by the lesion of the nuclei in rats, resulting in an increase in body weight due to an increase of fat mass (Hetherington & Ranson, 1942). Recent studies showed that the anorexigenic peripheral hormone leptin can regulate body weight by activating the VMH's SF1 neurons (Dhillon *et al.*, 2006). The ARH neurons project onto the VMH to regulate feeding through the Melanocortin 4 receptor (MC4R) and NPY receptors, making the VMH a satiety center (Xu *et al.*, 2003; Zhang *et al.*, 2020). The VMH is also a key player in locomotor activity, as its lesion disturbs the patterns of circadian locomotor activity, as well as the adaptation of this activity to fasting conditions (Challet *et al.*, 1996). VMH has a connection with peripheral organs like the liver, where an electrical stimulation in the rodents VMH's increased the hepatic glycogen breakdown and the release of glucose into the bloodstream (Shimazu et al., 1966; Khodai & Luckman, 2021).

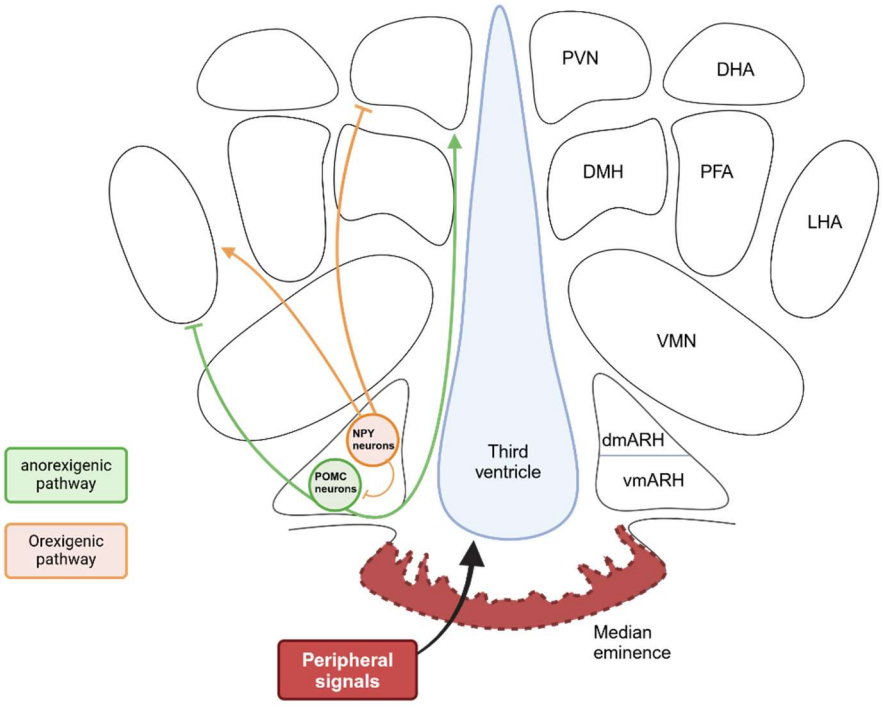


Figure 1: Anatomy of the hypothalamus and its nuclei, illustrating the primary pathway involved in energy regulation.

Metabolic cues arrive from the periphery to the hypothalamus; they reach the first order nuclei, the arcuate nucleus (ARH), where they trigger the anorexigenic pathway through the NPY/AgRP neurons or the orexigenic pathway through the POMC/CART neurons. From the ARH, these neurons will project and carry out their messages towards the other hypothalamic nuclei. DMH: Dorsomedial nucleus, PVN: Paraventricular nucleus, VHM: Ventromedial nucleus, LHA: Lateral hypothalamic nucleus, PFA: Perifornical area, DHA: Dorsal hypothalamic area. Green/Orange arrows show stimulatory (normal arrow) or inhibitory (flat arrows) communication.

3. Dorsomedial nucleus

The dorsomedial nucleus (DMH) is a second-order nucleus close to the third ventricle, more dorsal than the VMH. In mice, it is known to be involved in meal anticipation and initiation as C-fos activity (a widely used marker of neuronal activity) in the DMH increases when mice are

trained for scheduled meals (Gooley et al., 2006). The inhibition of DMH neurons can alter the thermoregulation in mice (DiMicco & Zaretsky, 2007) and trigger the autonomic response to stress in rodents (Brasil *et al.*, 2020). The ARH neurons establish their projections to the DMH during perinatal life, strengthening the communication between the two nuclei (Bouret et al., 2004). During an overnight fasting, the DMH cholinergic neurons will sense the lack of nutrients and reduce the GABA outputs to the VMH, LHA, and ARH (Groessl *et al.*, 2013).

4. Lateral hypothalamic nucleus

The lateral hypothalamic nucleus (LHA) is located further apart from the third ventricle. LHA is a hypothalamic nucleus that projects onto several other parts of the brain to carry out and exchange information. Like the other hypothalamic nuclei, the LHA helps regulate food intake, additionally to sleep-wake states, and motivation through the dopaminergic reward system (Bonnavion *et al.*, 2016). A distinct population of neurons emerged from the LHA is the orexin expressing neurons. These neurons are activated during a wake state and inhibited during the sleep state, and help the ARH to further regulate the timing of the feeding (Sakurai, 2007). As a result of their role, their ablation in mice was used to develop a model for narcolepsy and hypophagia (Hara *et al.*, 2001). Another population of the LHA modulates the orexin neurons, namely the neurotensin neurons, involved in the regulation of the dopaminergic system (Brown *et al.*, 2018). In addition, a population of melanin-concentrating hormone (MCH) neurons, of which half express the CART peptides and have glucose-sensing capabilities (Vrang *et al.*, 1999) is also located in the LHA. Similar to other nuclei, a sexual dimorphism exists in the LHA: for example, while a 48 hours fasting will activate the MCH neurons in female and male rats at the same rate, a glucose injection in these fasted mice will inhibit the female MCH neurons faster (Funabashi *et al.*, 2009).

5. Paraventricular nucleus

In the rostrocaudal part of the hypothalamus lies the paraventricular nucleus (PVN), it receives afferent neurons from the ARH and the LHA (Larsen et al., 1994). The PVN lesion leads to obesity and insulin resistance, classifying the PVN's overall activity as anorexigenic (Sims & Lorden, 1986). The PVN contains parvocellular and magnocellular neurons, which project onto the Solitary tract nucleus (NTS), and on the acting on peripheral organs such as the adipose tissue and liver through parasympathetic regulation (Hill, 2012). The PVN can act on the feeding intake by projecting to the NTS and modulating gastric distention signal. Another key PVN subpopulation is the oxytocin-expressing neurons that control maternal behavior and penile erection (Da Costa *et al.*, 1996; Wen *et al.*, 2023).

6. The suprachiasmatic nucleus

The suprachiasmatic nucleus adds to the importance of the hypothalamus in vital functions. Indeed, the SCN is the center of circadian rhythm regulation. Light, whether natural or artificial, is received by the eyes, which transmit the information through the nerve tract to the vasoactive intestinal polypeptide (VIP) expressing neurons, the circadian pacemakers, which will communicate with vasopressin (AVP) neurons to carry out appropriate response across the brain. The SCN will be considered active when there is light, representing the active phase for diurnal humans characterized by increased food intake and locomotor activity. For the nocturnal rodents, it represents the resting phase of the animal. Inversely, once the light is off and the "sleep hormone" melatonin is released, the SCN will be considered inactive in the resting phase of humans and the active phase in rodents. The cities' artificial lights can desynchronize the SCN and lead night-shift workers to develop metabolic imbalances (S. Shi *et al.*, 2013; Buijs *et al.*, 2017; Bahinipati *et al.*, 2022).

B. Players in the regulation

The communication between the periphery and hypothalamus involves different organs central to energy homeostasis. These organs release hormones, factors, or energy substrates into circulation to relay information on their current state or redistribute energy resources **(table.1)**. The hypothalamus heavily relies on these cues to regulate metabolism.

1. Gastrointestinal tract

The gastrointestinal tract, or GI tract, comprises the mouth, stomach, intestine, and rectum. GI is central to metabolism as it receives and processes new energy sources. These energy sources - glucose, lipids, and proteins - will be sensed, used, or stored after digestion. Enteroendocrine cells are found all along the GI, which secrete signals depicting satiety or hunger to maintain homeostasis.

a) Stomach

After being masticated, food will descend into the stomach and continue the digestion. The stomach sends out information depending on the nutritional status. In an empty stomach, the endocrine cells will convert preproghrelin to ghrelin. **Ghrelin** is a 4 KDa hormone discovered first for its potential role in the growth hormone release (Kojima *et al.*, 1999) and then described as a “hunger hormone” in the stomach, as it triggers the gastric tract mobility as well as acid secretion, preparing the stomach for digestion (Masuda *et al.*, 2000). The stomach can modulate other organs’ metabolism through ghrelin, such as increasing the storage of lipids through adipogenesis in the adipose tissue in response to a lack of an energy source from a meal. Ghrelin can reach the hypothalamus and access the ARH to promote the orexigenic pathways *via* its receptor, the growth hormone secretagogue receptor (GHS-R), thus increasing food intake (Tschöp, Smiley and Heiman, 2000; Abizaid and Horvath, 2012; Vivot *et al.*, 2023).

Table 1: Non-exhaustive list of peripheral hormones involved in energy homeostasis

Hormone	Tissue	The main effect on food intake	Effect on hypothalamus	Sexual dimorphism
Ghrelin	Stomach	Orexigenic	Inhibition of POMC neurons and increase the NPY/AGRP neuron activity	In ♀, higher ghrelin is secreted in the follicular phase (Barkan *et al.*, 2003)
Gastrin	Stomach	Anorexigenic	Inhibition of food intake (Zhang et al., 2005; Cowan et al., 2016)	In ♀, higher gastrin release (Feldman et al., 1983)
GLP-1	Small intestine	Anorexigenic, increase insulin	Decrease food intake by acting on the GLP-1R in the PVN	GLP-1 treatment yields better result in ♀ (Rentzeperi *et al.*, 2022)
CKK	intestine	Anorexigenic	Activates CCK1R in the NTS and activates appetite suppression through PVN (D'Agostino *et al.*, 2016)	/
Obestatin	Stomach	Orexigenic	Modulates ARH neurons (Szlis et al., 2018).	Higher obestatin plasma level in ♀(Lippl *et al.*, 2008)
Adiponectin	Adipose tissue	Both	Increase or decrease POMC activity (Tang et al., 2021).	More adiponectin plasma level in ♀, (but not equalize by OVX) (Gui, Silha and Murphy, 2004)
PYY	Intestine	Orexigenic	Act on NPY and POMC activity to regulate food intake (Batterham et al., 2002)	/
Insulin	Pancreas	Anorexigenic	Glucose metabolism regulation at the hypothalamus (Paranjape *et al.*, 2011)	Higher insulin fating level in female mice (Vital, Larrieta and Hiriart, 2006)
Glucagon	Pancreas	Orexigenic	Receptors GCGR in the hypothalamus (Cunningham *et al.*, 2021)	/
Leptin	Adipose tissue	Anorexigenic	LepR in the hypothalamus, controls feeding and substrate metabolism (Duquenne *et al.*, 2021)	Higher leptin in ♀ (Saad *et al.*, 1997)

Derived from preproghrelin, **obestatin** is a small peptide (23AA) with a short lifespan. It is sometimes called anti-ghrelin as it has opposite effects such as inhibiting food intake through

the G protein-coupled receptor 39 (GPR39) (Zhang *et al.*, 2005; Cowan *et al.*, 2016). In sheep, obestatin acts on food intake by modulating the orexigenic/anorexigenic neuron populations of the ARH (Szlis *et al.*, 2018).

After the mastication of a meal, the stomach will start the post-prandial digestion process. The stomach will trigger not only the release of hormones to communicate the change in nutritional status, but also by its distentionwill send a signal through the vagal nerve to reach the hypothalamus and signal satiety (Wang *et al.*, 2008).

b) Intestine

The different parts of the intestine will be stimulated differently according to the nutritional status, releasing specific hormones in response to physiological needs. The first part of the intestine is the duodenum, where the enzymes produced from the bile are released to digest the meal. It is continued by the jejunum, where nutrients and vitamins are absorbed until the meal reaches the ileum to absorb the remaining vitamins, such as B12 (Collins et al., 2024).

One of these intestinal hormones stimulated and secreted following a meal in the GI tract is **Cholecystokinin**. Located in the duodenal and ileum cells of the intestine, the CKK is also found in neurons of the suprachiasmatic nucleus (Nässel & Wu, 2022). The CCK is on the anorexigenic spectrum of the metabolism. Once a meal triggers these cells, the CCK will exert a dual action by releasing more bile enzymes and reducing food intake (Antin *et al.*, 1975; Liddle, 1997; Cawthon & De La Serre, 2021). The CCK receptor, CKK1R, is located in the hypothalamus nuclei and the solitary tract (Mercer & Beart, 2004). Solicitation of the CCK receptor in the NTS *via* an optogenetic approach can reduce food intake in mice, and this mechanism involves the paraventricular nucleus (D'Agostino *et al.*, 2016). Another anorexigenic peptide of the GI is **enterostatin**, which suppresses food intake, specifically lipid

intake and insulin secretion, (Berger *et al.*, 2004; Park *et al.*, 2009). This lipid-specific action might involve the melanocortin system through the MC4R receptor (Lin et al., 2007).

In addition, food intake triggers the **glucagon-like peptide 1** or GLP-1 release which has anorexigenic effects on metabolism. GLP-1 is an incretin hormone, meaning it will stimulate insulin secretion by reaching the β cells of the pancreas. At the level of the hypothalamus, GLP-1 will act through its receptor GLP-1-R, which is found in the PVN. The postnatal deletion of the receptor will have metabolic consequences by increasing food intake and body weight (Liu *et al.*, 2017; Diz-Chaves *et al.*, 2020). Incretin was first used as a diabetes treatment and has recently been repurposed as a weight loss treatment. Coherent with the observation that obese people have lower levels of circulating GLP-1 than leaner people (Anandhakrishnan & Korbonits, 2016).

Peptide YY (PYY) is another peptide released from the more distal part of the GI tract. It stems from the same family as NPY. Like GLP-1, the circulating levels of PYY increase following a meal and reduce food intake in rodents. The peptide acts *via* the Y2 receptor located in the ARH, inhibiting NPY and activating POMC neurons (Batterham *et al.*, 2002).

Importantly in recent decades, research has highlighted a significant component in metabolism, the **microbiota**, and its implication in metabolism and health. The composition of the microbiota results in modulating the different GI-hormones. Germ-free mice display less circulating GLP-1, CKK, and PYY levels, overall hinting at a lesser sensibility of the GI tract to food cues (Batterham *et al.*, 2002; Duca *et al.*, 2012). This loss of sensitivity is reproduced in fasted germ-free mice where mRNA expression of AgRP and NPY were reduced (Hamamah & Covasa, 2022).

2. Adipose tissue

When the energy homeostasis is too far off balance, by overeating or prolonged fasting, the white adipose tissue will act to store or release the free fatty acid to restore the balance. The white adipose tissue will regulate the homeostasis by releasing factors to communicate its status. The white adipose tissue corresponds to the majority of the adipose tissue and is distributed across the body, especially in the subcutaneous and visceral areas. The brown adipose tissue is located near the kidney and in the interscapular area in humans. The brown adipose tissue will regulate the thermogenesis (Park, Kim and Bae, 2014).

Adipose tissue produces and secretes the satiety hormone **leptin**. The 16KDa molecule expression is triggered in the adipose tissue by factors related to food intake, such as insulin (Tsai *et al.*, 2012).

Leptin can access the hypothalamus and trigger a central regulation of food behavior and homeostasis. Leptin will increase proportionally to the adipose tissue content and with a food intake, reach the vmARH and will inhibit the AgRP neurons and activate the POMC/CART neurons (Takahashi & Cone, 2005; Mesaros *et al.*, 2008). Leptin will hyperpolarize the NPY neurons, shunting the GABA release that inhibits the anorexigenic POMC neurons (Cowley *et al.*, 2001). In return, the hypothalamus regulates the white adipose tissue by lipolysis-inducing neurons influencing lipolysis activity (Geller *et al.*, 2019). The hypothalamus also acts on the brown adipose tissue, the AgRP neurons regulate the BAT glucose uptake, and the NPY neurons act on the PVN to regulate UCP1 activity through the sympathetic output and reduce the brown adipose tissue thermogenesis (Y.-C. Shi *et al.*, 2013; Steculorum *et al.*, 2016).

Adiponectin or ApN is an adipokine with a behavior opposite to that of leptin. ApN levels are inversely proportional to body fat (Arita *et al.*, 1999). ApN has two receptors, AdipoR1 and AdipoR2, found in endothelial cells, brain tissue, B cells, and muscles. This adipokine's

influence on food behavior is paradoxical as it can be anorexigenic and orexigenic. The ApN can bind to AdipoR1 in the ARH and stimulate the Phosphoinositide 3-kinase (PI3K) pathway and subsequent POMC activity to reduce food intake, or conversely activate the AMP-activated protein kinase (AMPK) pathway and inhibit POMC activity (Tang *et al.*, 2021).

3. Pancreas

The pancreas is an endocrine and exocrine organ; it produces many enzymes to digest nutrients and is the hub for glucose metabolism regulation, with insulin and glucagon keeping homeostasis. **Insulin** is a 6KDa hormone secreted into the circulation by β cells following a

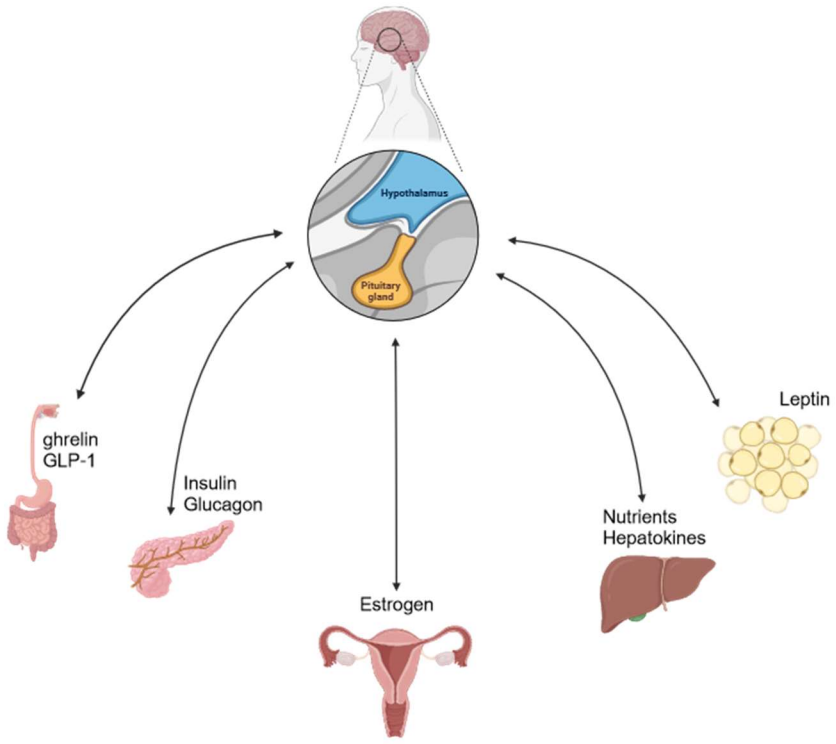


Figure 2: Scheme of organs connecting with the hypothalamus through their respective hormones

The central nervous system, notably the hypothalamus, will integrate peripheral messages sent by different organs. The hypothalamus will sense energy substrates such as glucose, fatty acids, amino acids, hormones, adipokines, and hepatokines… Once received, the hypothalamus will adapt its response to maintain energy homeostasis. Created with Biorender.

meal and a spike in glucose in order to return the glucose to basal levels by stimulating 1) its storage as glycogen in the liver or 2) the synthesis of lipids and their storage in the adipose tissue. Insulin insensitivity and hyperglycemia are signs of insulin resistance, the cause of type 2 diabetes. A factor in the insulin resistance is the leptin resistance: in a physiological setting, leptin can inhibit insulin expression, and leptin resistance can desensitize this regulation negatively (Amitani *et al.*, 2013).

The insulin receptors found in the ventromedial hypothalamus and their deletion in rodents suffice to replicate the physiological manifestation of type 2 diabetes, such as glucose dysregulation and islet dysfunction (Paranjape *et al.*, 2011). Hypothalamic response to insulin is also conveyed through Glucose transporter type 4 (GLUT 4) receptors; its hypothalamic-specific ablation in rodents is similar to an anorexia mouse model (Ren, Lu et al., 2014; Ren, Yan, *et al.*, 2014).

Upon meal ingestion, the pancreas releases **pancreatic peptides**, or PP, that send a satiety signal to the hypothalamus and decrease the activity of the anorexigenic neuronal populations. To counterbalance the insulin in the homeostasis of glycemia, the other pancreatic hormone, **glucagon,** is stimulated by hypoglycemia (Asakawa *et al.*, 2003).

4. Ovaries and tests

The ovaries and testis are endocrine organs indirectly involved in homeostasis regulation. They both have a distinct hormonal profile that influences and shapes metabolism and is the source of sexual dimorphism seen across the different organs' functions. They produce steroid hormones like testosterone, which is more potent in males, and estrogen, which is more potent in females.

The hypothalamus indirectly triggers the sex steroid hormone expression by releasing the Gonadotropin-releasing hormone (GnRH) into the median eminence to reach the anterior pituitary gland, where it stimulates the production and secretion of two gonadotrophins - the luteinizing hormone and follicle-stimulating hormone (LH and FSH). LH will stimulate Leydig cells of the testis to increase testosterone levels in males, while FSH will promote spermatogenesis. The testosterone produced in the testis will reach the hypothalamus for negative feedback once released into the circulation. In females, LH and FSH stimulate the ovaries for estrogen, progesterone production, and egg maturation. More than a staple for reproductive health, estrogen is a player in metabolism regulation as an anorexigenic factor. Ovariectomized mice (OVX) lacking gonadal sex steroids will increase their food intake and body weight when food is *Ad libitum*. However, after the end of fasting, the OVX mice will eat less than their SHAM counterparts (Yu *et al.*, 2020). In post-menopausal persons, the decrease in estrogen content will lead to similar behavior as the OVX mice: hyperphagia and reduced energy expenditure (Dragano et al., 2020). This has been mostly corrected with the help of HRT, hormone replacement therapy. The estrogens act on reproduction and metabolism through its receptors, the estrogen receptors α and β (ERα, ERβ) the G-protein coupled receptor 30 (GPR30). The ERα and ERβ are found in the hypothalamus, ERα is expressed in the ARH, PVH, and VMH (Kruijver *et al.*, 2002). This will be covered more extensively in chapters III and IV.

C. The hypothalamic regulation of the different nutritional states

The hypothalamus is the central regulator of food intake and adapts its response to peripheral cues (Kinzig *et al.*, 2007; Power and Iremonger, 2021). The hypothalamus adaptation to the nutritional state is translated by activating or inhibiting the anorexigenic and orexigenic neurons to maintain energy homeostasis.

1. Fed state

The fed state represents the period when there are enough energy substrates. It will be signalled to the hypothalamus by activating the anorexigenic signal and inhibiting the orexigenic signal. In a basal state, the mice will consume 20-30% of their daily food intake during the resting phase and 70-80% in the active phase (Ayala *et al.*, 2006). Once fed, the organism will reduce the food intake and increase anorexigenic signals such as POMC, a precursor for melanocyte-stimulating hormone (α-MSH and B-MSH) from the melanotropin family. Once triggered, POMC will be converted to α-MSH and released through synapse contact into the melanocortin neurons to bind to the post-synaptic receptors melatonin-4-receptors (MC4R) (Kim *et al.*, 2000; Balthasar *et al.*, 2005). A disruption at this level, like the ablation of the MC4R in mice, would result in obesity (Huszar *et al.*, 1997). The fed state can be replicated by an intraperitoneal glucose injection in fasted sheep, increasing the POMC mRNA and decreasing orexigenic peptides mRNAs (Archer *et al.*, 2004). Leptin release from the adipose tissue depolarizes POMC neurons via a cation channel and inhibits surrounding GABA neurons to allow POMC activity (Cowley *et al.*, 2001).

2. Fasted state

During fasting, as the energy from a previous meal is depleted, the metabolism transitions into a fasted profile or a post-absorptive state. After the glucose and glycogen pools in the liver are emptied, the organism must turn to other energy sources, such as ketone bodies or free fatty acids. However, these energy sources are temporary, and the organism cannot rely on stored resources indefinitely. The need for nutrients triggers the hypothalamus to increase locomotor activity and food intake. In mice studies, the physiological consequences vary depending on the fasting period, ranging from 4 hours to 72 hours (Jensen et al., 2013).

A restriction of food will accentuate the activation of the NPY/AgRP neuron population in the arcuate nucleus, which will release the peptides through their projections in the PVN and LHA. Once AgRP is released, it will stunt the anorexigenic α-MSH effect by inhibiting the MC3R/M4CR receptors and thus increase food intake (Betley *et al.*, 2013). An optogenetic approach highlighted the connection between the AgRP neurons and the POMC neurons, using Channelrhodopsin-2-assisted circuit mapping (here, AgRP and POMC neurons expressed the ChR2 to allow photoactivation): the AgRP population released the neurotransmitter GABA, and inhibited the POMC and MC4R pathway. However, no synapse activity was observed in the connections from POMC to AgRP. The AgRP partner, NPY, can regulate food intake in several ways. NPY orexigenic function passes by receptors Y1 and Y5 in the hypothalamic nuclei (Ladenheim *et al.*, 2007; Nguyen *et al.*, 2012). NPY will also modulate the metabolism by reaching the PVN. Indeed, injecting NPY directly into the PVN of rats is enough to increase the food intake (Stanley & Leibowitz, 1984) and reduce mice's energy expenditure.

Another parameter under the hypothalamus control during fasting is locomotor activity: when food resources are limited, animals need to use their stored energy to hunt/gather a meal. The VMH nucleus will be solicited as it is a regulator for locomotor activity (Gladfelter & Brobeck, 1962; Challet *et al.*, 1996).

3. Refeeding state

A meal ends the fasting state, triggering a spike in glycemia and an increase in lipids in the circulation, followed by a rise in leptin and insulin. These signals reach the hypothalamus, which transitions back to a fed state. The level of c-fos activity of the ARH neurons in mice is higher in a fasted state compared to a fed state. After one hour of refeeding, the hypothalamus sends anorexigenic signals to avoid overeating. The DMH could be the hypothalamic nucleus

that controls food intake during refeeding. Indeed, DMH neuronal activity was increased one hour after refeeding. Inactivation of DMH neurons during refeeding resulted in increased food intake compared to the control mice (Imoto *et al.*, 2021).

In humans, the refeeding period requires the mobilization of fluid and electrolytes. Long-term fasting due to anorexia, malnutrition, and renal failure can lead to a refeeding syndrome. The longer the fasting, the more the energy substrates, vitamins, and electrolytes will be depleted. The first meal will drive the expected increase in glucose and insulin, which will trigger the electrolytes (phosphorus and magnesium) to exit the cellular spaces and start the metabolism processes. This syndrome, if too strong, could lead to hypophosphatemia, hypomagnesemia, and respiratory failure (Persaud-Sharma et al., 2024). This is a potential problem as eating disorders thrive, and “diets” suggest day-long fasting periods to reduce body weight. Strengthening the knowledge of the refeeding period could help understand and further help prevent instances of maladaptive refeeding.

The hypothalamus is the master of metabolism and reproduction functions and operates thanks to the feedback of the peripheral organs. The next chapter will explain how the communication with the brain is possible. The blood-brain barrier highly protects the brain; however, interfaces exist in specific parts of the brain, allowing a more permissive but not total diffusion of metabolites or signaling cues. The hypothalamus heavily depends on a key interface, the median eminence to communicate with the periphery and function correctly.

II. The periphery-brain interfaces

A. Different interfaces between the periphery and brain

The brain needs to be protected, from circulating toxins and pathogens that are not filtered by the liver. However, the brain is also dependent on bidirectional communication with the periphery 1) to sustain its energy levels, 2) to receive signals essential to the monitoring of physiological functions, and 3) to send back hormones for the proper maintenance of these functions.

This is why highly specialized interfaces are put in place to allow communication without compromising the brain integrity.

The communication between the periphery and the brain is possible *via* nerve inputs/outputs and blood circulation (Reese & Karnovsky, 1967). The blood circulation will reach the brain vasculature, where vessels harbor specific barrier properties depending on their localization. The primary type of vessels in the brain is constitutive of the blood-brain barrier (BBB) interface, composed of continuous endothelial cells surrounded by a basement membrane, astrocytes, and pericytes. These components work together to seal the vessels, preventing the passive diffusion of large or complex molecules. BBB is not the only interface with the periphery; the choroid plexus (CP) and the circumventricular organs (CVO) are both located in specific parts of the brain, always connected to ventricles (Strazielle & Ghersi-Egea, 2000). The main difference with the BBB is that both CP and CVO have fenestrated vessels (FV) instead of continuous, allowing a different regulation and communication with the periphery. The choroid plexus primarily produce cerebrospinal fluid (CSF) and release it in the ventricle circulation. CVOs include the subfornical organ, area postrema, subcommissural organ, vascular organ of lamina terminalis (OVLT), and the median eminence. CVOs are named after

their location, their structures are near the third or fourth ventricles, and all possess FV. Importantly, to counteract the lost barrier function due to the fenestrations, they possess ependymoglial cells or tanycytes, that will assume the barrier role and regulate the communication between the brain and the periphery. CVOs can be sensory or secretory organs; the median eminence of the hypothalamus is considered to be both.

1. Blood-brain barrier

The concept of the blood-brain barrier (BBB) was introduced to the scientific community in the 19th century by Ehrlich. Ehrlich's experiment involved injecting trypan blue dye in mice, wherein he observed that the dye did not diffuse into the brain parenchyma but halted at the endothelial cells of brain capillaries (Ehrlich, 1898). The blood-brain barrier anatomy was further described in 1967 with the observation of the diffusion of horseradish peroxidase (HRP), a 44KDa enzyme. After a peritoneal injection, the peroxidase was washed out through perfusion of the rodents and was only found in endothelial cell vesicles, not further into the parenchyma of the mice (Reese & Karnovsky, 1967). This contrasts with results in other tissues, such as the liver, where the HRP could reach the parenchyma (Lowe *et al.*, 1985). More recent studies use Evan's Blue (EB) dye, a 1Kda molecule, to study the diffusion. While the diffusion of EB is usually restricted by the BBB, a diffusion in the brain parenchyma indicates leakage, and a barrier disruption that can be due to conditions like small vessel diseases and lupus (Kamintsky *et al.*, 2020; Chowdhury *et al.*, 2021). Many molecules with relatively larger sizes cannot cross the BBB (like the 150KDa dextran), while very small molecules, such as gaseous molecules (O2, CO2), can pass through the lipid membranes and passive diffusion (Armulik *et al.*, 2010).

The barrier function of the BBB, also called a neurovascular unit, is due to (1) the tight junctions sealing the endothelial lumen, (2) the pericytes wrapping the endothelial cells, and (3) the astrocytes end-feet. The complex is embedded within a perivascular extracellular matrix produced by endothelial cells and pericytes (Abbott et al., 2006; Chowdhury *et al.*, 2021).

a) BBB Endothelial cells

BBB endothelial cells form a "continuous endothelium" characterized by tight junctions forming a complex organization fusing the endothelial cells and blocking paracellular diffusion **(fig.4)**. Tight junctions (TJ) are protein complexes sealing cell membranes together and partially blocking paracellular diffusion. TJ can block ions (sodium, chloric) to maintain high trans-endothelial electrical resistance (TEER), which is essential in blocking diffusion and indicative of the barrier strength (Butt et al., 1990). The BBB tight junctions are primarily composed of occludin, claudins 3 and 5, and Zonula occludens (ZO) proteins (Stevenson et al., 1986; Furuse et al., 1993; Morita et al., 1999; Mullier et al., 2010). The endothelial cell's TJ regulation and strength can be influenced by factors such as NF-κB (Brown et al., 2003), leptin (modulates transcytosis) (Pan et al., 2004), and Vascular endothelial growth factor (VEGF), which has been shown to accentuate BBB leakage (Zhang et al., 2000). These BBB vessels are characterized by their carriers/receptors, which create easy access for the energy substrates to the brain. For example, specific transporters allow nutrients to access the brain, such as glucose through GLUT-1 (De Vivo *et al.*, 1991), amino-acids through SLC7A5 (Tărlungeanu *et al.*, 2016), and lipid transport through LDL receptor (Dehouck *et al.*, 1997).

b) Pericytes

Pericytes are mural cells found primarily in the brain, retina, and near endothelial cells (EC) (Frank et al., 1987). Pericytes are located in the basement membrane surrounding the endothelial cells and are fixed to the EC by adhesion plaques (Rouget, 1873; Xu et al., 2019).

The pericyte's primary role in the BBB is to support the endothelial cells, modulate the blood flow, and support the permeability regulation (Liu *et al.*, 2012). They are recruited *via* the PDGFB/PDGFBR (Platelet-derived growth factor subunit) pathway, expressed in the BBB endothelium (Lindahl *et al.*, 1997). Once the pericytes are recruited, they can act on the EC maturation *via* the angiopoietin-Tie receptor axis (Sato *et al.*, 1995; Fiedler *et al.*, 2006). *In vivo* studies in pericytes-deficient mice demonstrated a malfunction of the BBB permeability and, therefore, the pericytes' importance (Armulik *et al.*, 2010).

c) Astrocytes

On the outer part of the BBB, there is a layer of astrocytes. Astrocytes have a plethora of functions; here, they elongate their endfeet over 99% of the pericytes-EC complex, completing the neurovascular unit (Kacem *et al.*, 1998). Astrocytes also participate in the permeability of the BBB vessels by secreting factors such as VEGF, angiopoietins Ang1&2 towards the endothelial cells to modulate the tight junction regulation (Alvarez et al., 2013). On the other hand, astrocytes can uptake and distribute molecules to the brain parenchyma. They are known to uptake glucose to redistribute it to all brain cells (Jakoby *et al.*, 2014) and even perform glycogen storage *in vivo* (Staricha *et al.*, 2020).

Both barrier and diffusion properties are indispensable for the brain to function properly. Dysfunction of the BBB can be associated with neurological diseases such as Parkinson's and Alzheimer's. A lack of permeability regulation can leak unwanted blood-borne factors into the brain parenchyma (Weiss *et al.*, 2009).

2. Choroid plexuses

Similarly, to the blood-brain barrier, an interface between blood and cerebrospinal fluid (CSF) exists, the Blood-CSF barrier (BCSFB), also named the choroid plexuses (CP). There are 4 CPs bound to the different brain ventricles. Coherent with their location, the CPs produce the CSF, making them secretory organs. The CSF carries the energy substrates and factors by moving through the ventricles. From the CPs in the lateral and third ventricles, going through the Sylvius aqueducts to the fourth ventricle to exit toward the arachnoid granulation (Damkier et al., 2013).

The choroid plexus is a capsule composed of an ependymal cell layer delimiting the CP in the ventricle, and an epithelial layer resting on a basement membrane, within which there is a core of endothelial cells. Unlike the BBB, the CP endothelium does not fully serve as a barrier for the brain. The vessels in the CP are fenestrated, they possess diaphragms in the cells described as windows between the vessel lumen and the periphery. Electron microscopy on rabbits showed that the fenestrated vessels comprise a diaphragm called fenestrae, allowing molecule passage through the FV (Pappas & Tennyson, 1962). The fenestrated capillaries form a plexus that “floats “ in the CSF ventricles (Redzic, 2011). The CP’s fenestrated vessels are more permissive to molecules entering the CSF. To balance the FV diffusion, the epithelium layer assumes the barrier function. Indeed, the epithelial cells separate CSF-blood diffusion by being sealed by a tight junction complex and restricting paracellular diffusion (van Deurs & Koehler, 1979). This epithelium barrier properties were demonstrated by (Brightman & Reese, 1969) via horseradish peroxidase injection into mice showing leakage from the FV. The BBB and CP tight junctions are similar in composition, except for the type of Claudin they expressed. They share the expression of Claudins 1 and 3; CP express Claudins 2 and 11; the BBB express Claudins 5 and 12 (Wolburg *et al.*, 2001; Nitta *et al.*, 2003; Hawkins & Davis,

2005). The difference in Claudin expression could change the TEER of these interfaces (Greene et al., 2019). Indeed, the TEER is higher in BBB (less permissive) than the CP (Crone & Christensen, 1981; Saito & Wright, 1984). The TEER being relevant in the barrier exchange would mean different regulations of molecule diffusion in the BBB and BCSFB.

3. CIrcumventricular organ

A more complex blood-brain barrier interface is the circumventricular organs, termed by Dr. Helmut O. Hofer, describing organs lining the third and fourth ventricles **(fig.3)**. These specialized organs share a characteristic with the CP: they possess fenestrated vessels (Gross et al., 1987; Ciofi *et al.*, 2009). A significant change from the CP is that the CVOs barrier function does not rely on an epithelium but on an ependymoglial cell layer located specifically in the CVOs, the tanycytes. CVOs are separated into 2 categories:

- sensory organs: Subfornical organ (SFO), Area postrema (AP), organum vasculum of the lamina terminalis (OVLT)
- secretory organs: Pineal gland (PG), neurohypophysis (NH) and the Subcommissural organ (SCO)

The exception being the median eminence (ME) being both sensory and secretory (Cottrell & Ferguson, 2004; ‘The circumventricular organs’, 2017). OVLT, ME, SFO, and AP possess fenestrated vessels in their external part that can access the peripheral signal or secrete hormones. Within the CVO, a specialized cell type, an ependymoglial-derived called the tanycyte (or tanycyte-like cell), assumes the barrier function. Other CVOs, like the pineal gland, neurohypophysis and SCO, behave as CVOs but differ in their structures.

a) Secretory CVO

The pineal gland (PG) is located in the posterior part of the third ventricle (close to the SCO) and is described as a secretory CVO. It is a major player in the circadian rhythm and sleep pattern, producing and secreting melatonin from tryptophan into the CSF. (Wurtman, Axelrod and Phillips, 1963; Arendt and Aulinas, 2000; Tan *et al.*, 2018).

The neurohypophysis (NH) is a highly vascularized CVO located below the Median eminence and forms with the adenohypophysis the Hypophysis. The NH releases vasopressin and oxytocin hormones into the blood circulation (Van Leeuwen & Swaab, 1977; Falke, 1991). The Magnocellular neurosecretory cells, starting in the hypothalamus (supraoptic and paraventricular nuclei), possess axons ending in the NH, allowing the secretion of the 2 hormones as well as some neurotransmitters (Meeker *et al.*, 1991; Brown *et al.*, 2020).

The Subcommisural organ (SCO) is found in the dorso-caudal part of the third ventricle at the beginning of the Silvius aqueduct (the bridge between the third and fourth ventricle). This CVO is peculiar due to its lack of fenestrated vessels and, therefore, shunts any external diffusion into the SCO parenchyma. The SCO consists of a layer of ependymoglial cells lining a part of the third ventricle. These ependymoglial cells are surrounded by an organized tight junction (Duvernoy & Risold, 2007; Langlet, Mullier, *et al.*, 2013). The SCO secrete glycoproteins into the CSF that form the Reisner's fibers, reaching into the fourth ventricle and being involved in the morphogenesis (Cantaut-Belarif *et al.*, 2018).

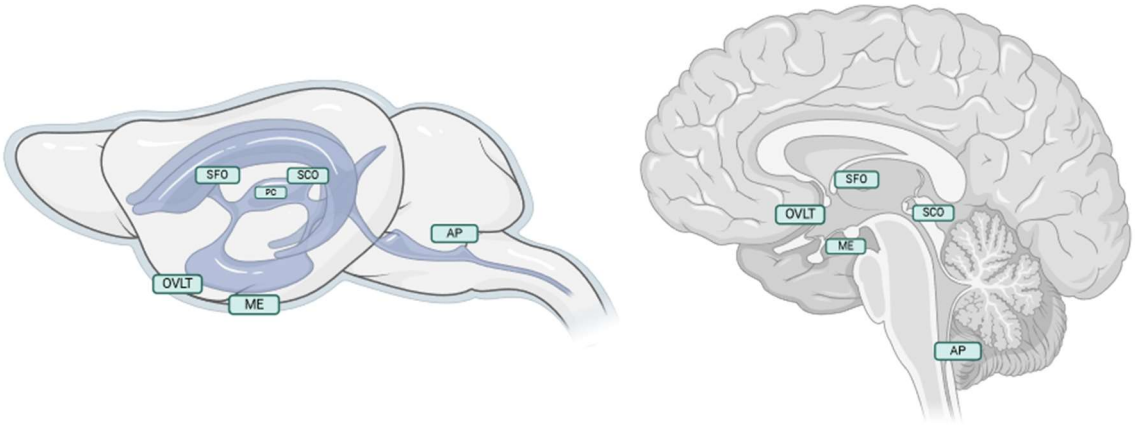


Figure 4: OVC localization in the mouse and human brain

The different circumventricular organs are spread across the brain's ventricles. Sagittal view of a (A) Mouse brain and its ventricle in blue. And a (B) human brain. OVLT, organum vasculum of the lamina terminalis; SFO, Subfornical organ; ME, Median eminence; SCO, Subcommissural organ; AP, Area postrema. Created with Biorender

b) Sensory CVO

The subfornical organs (SFO) are located in the lateral ventricles near the choroid plexuses. The SFO's tanycytes form a shell encapsulating the fenestrated vessels in a ventromedial core within the SFO. The SFO is also responsible for salt and water intake behavior via sodium sensing (Noda & Hiyama, 2015). SFO interacts with a range of molecules able to regulate its activity and metabolism. Like angiotensin-II, which increases c-fos expression of the SFO and regulates thirst (Fitts, 1994; Jeong et al., 2021).

The area postrema (AP) is the most posterior of the CVOs, located in the fourth ventricle, and is defined by a perivascular zone enclosing the AP, a central zone containing the capillaries, and the lateral zone containing tanycyte-like cells surrounding the fourth ventricle

(Langlet, Mullier, *et al.*, 2013). The first known function of the AP was related to vomiting, it is a “trigger” zone by being a part of the dorsal vagal complex with the nucleus tractus solitary (Miller & Leslie, 1994; Babic & Browning, 2014) The AP is involved in food intake by sensing the anorexigenic factor GDF15, expressed by the liver, through its receptor GFRAL (Breit *et al.*, 2023; Zhang *et al.*, 2023).

The organum vasculum of the lamina terminalis (OVLT) is the most anterior CVO, located in the anteroventral part of the third ventricle. It possesses a capillary plexus and an outer lateral zone (Morita *et al.*, 2016). Like the SFO, it has a role in fluid regulation and vasopressin secretion (Naganawa *et al.*, 2017; Jeong et al., 2021; Pestana-Oliveira *et al.*, 2021). There is a GABA neuron pathway between the OVLT and SFO to control the water intake behavior (Matsuda *et al.*, 2017).

On top of their regulation of the metabolism, the sensory CVOs report to the master regulator of the organism, the hypothalamus, through a neuron network. The SFO neurons project to the PVN, allowing the regulation of the vasopressin secretion from the neurohypophysis. The OVLT receives fibers from GnRH neurons, the regulators of reproduction, and GnRH neurons also send projections into the eminence median to release GnRH into the circulation (Kawano & Masuko, 2010).

The median eminence is a sensory and secretory C**VO** and the entryway to the hypothalamus. While each CVO described above is currently known as either a sensory or secretory organ, the median eminence (ME) is the first to be described as both, possibly because it is the most studied CVO (around 6000 results in pubmed and around 1000 for all the others CVOs combined), and for a good reason: the localization of the median eminence, at the median basal part of the hypothalamus which is the critical regulator of the metabolic and reproductive functions, makes this CVO the very center of this communication.

Historically, it was first described as a secretory organ for liberating neurohormones in the adenohyphysis *via* its capillary bed, and later as a sensory organ *via* a retrograde blood flow towards the ME (Page et al., 1978; Ciofi *et al.*, 2009).

B. The interfaces' properties

The endothelial cells and tight junction complex embody the diffusion and barrier properties to ensure the function of the different interfaces. The fenestrated endothelial cells will allow partial diffusion into the brain parenchyma, and the tight junction will become the major actor in the barrier properties.

1. Diffusion properties

a) Endothelial cells

Endothelial cells (EC) line all vessels and allow blood supply throughout the body. They form the endothelial layer with a lumen at its center. The endothelium is surrounded by a basal membrane, elastin membrane, and smooth muscle cells to control the blood flow. To help the vessel adapt to the environment, invaginations in the plasma membrane of endothelial cells, called plasmalemma vesicle (or caveolae), can help the signaling in the endothelium (Shaul & Anderson, 1998). EC can also propagate from an existing vessel where there is a need for a process called angiogenesis. Vascular endothelial growth factor (VEGF) is an important factor in inducing angiogenesis, it is notably triggered by a lack of oxygen (Alberts et al., 2002). On top of the angiogenesis, VEGF is associated with higher permeability, which can be undesirable for some vessels, like the BBB vessels, where it can lead to leakage and inflammation. However, it is beneficial for the vessels located in CPs or CVOs when the context demands it (i.e., fasting for the ME).

b) The fenestrated endothelium

Fenestrated vessels are not specific to the brain and are found across the organs. In 1979, FV were found in skeletal muscles in humans, in rare instances but possibly related to pathological conditions (Ghatak & Nochlin, 1979). Fenestrations can also be found in lymphatic vessels of muscles and the thyroid (Korneliussen, 1975; Luciano & Koch, 1975). In 1993, electron microscopy studies found fenestrations in capillaries of the nasal mucosa near the basal layer of the epithelium of rabbits and were discussed to be helpful in endonasal fluid secretion (Grevers, 1993). An hypothesis is that the stomatal diaphragm (calveolae with a diaphragm) and transendothelial channels (fusion of calveolae forming a tunnel in the EC with diaphragm at both ends) are precursors for endothelial fenestrations (Ichimura *et al.*, 2008; Denzer *et al.*, 2023).

c) Plasmalemma vesicle-associated proteins (PLVAP)

The endothelium's fenestration resembles, under electronic microscopy, a circular dip into the endothelial cell and a "knob" at the center of the lumen (Rhodin, 1962). In 1990, the culture of bovine endothelial cells showed the ultrastructure of the diaphragm. It has a diameter of around 60-80 nm and contains plasmalemma vesicles and transendothelial channels (Furuya, 1990). These Plasmalemma vesicle-associated proteins (PLVAP or PV-1) are necessary to build a diaphragm in the fenestrations. PLVAP is a glycoprotein (60KDa) with an intracellular domain (caveolin 1 binding site), a transmembrane domain, and an extracellular domain (N-glycosylation sites, coiled-coil domains). PLVAP is found in smaller capillaries, mostly in endocrine organs, lungs, spleen, and kidneys (Guo *et al.*, 2016). Dimerization of PLVAP protein will form diaphragm fibrils, α-helical 3D structures. The fibrils will cross each other and create the diaphragm, intertwined at the center by disulfide bonds and forming a "knob/knot". The fibrils will end with an N-glycosyl segment that determines the diaphragm's outer part. To

seal the structure together, scaffolding protein located in the endothelial cell will hook the fibril into the membrane of the pore or “rim” of the fenestration (Stan, 2007; Chang *et al.*, 2023).

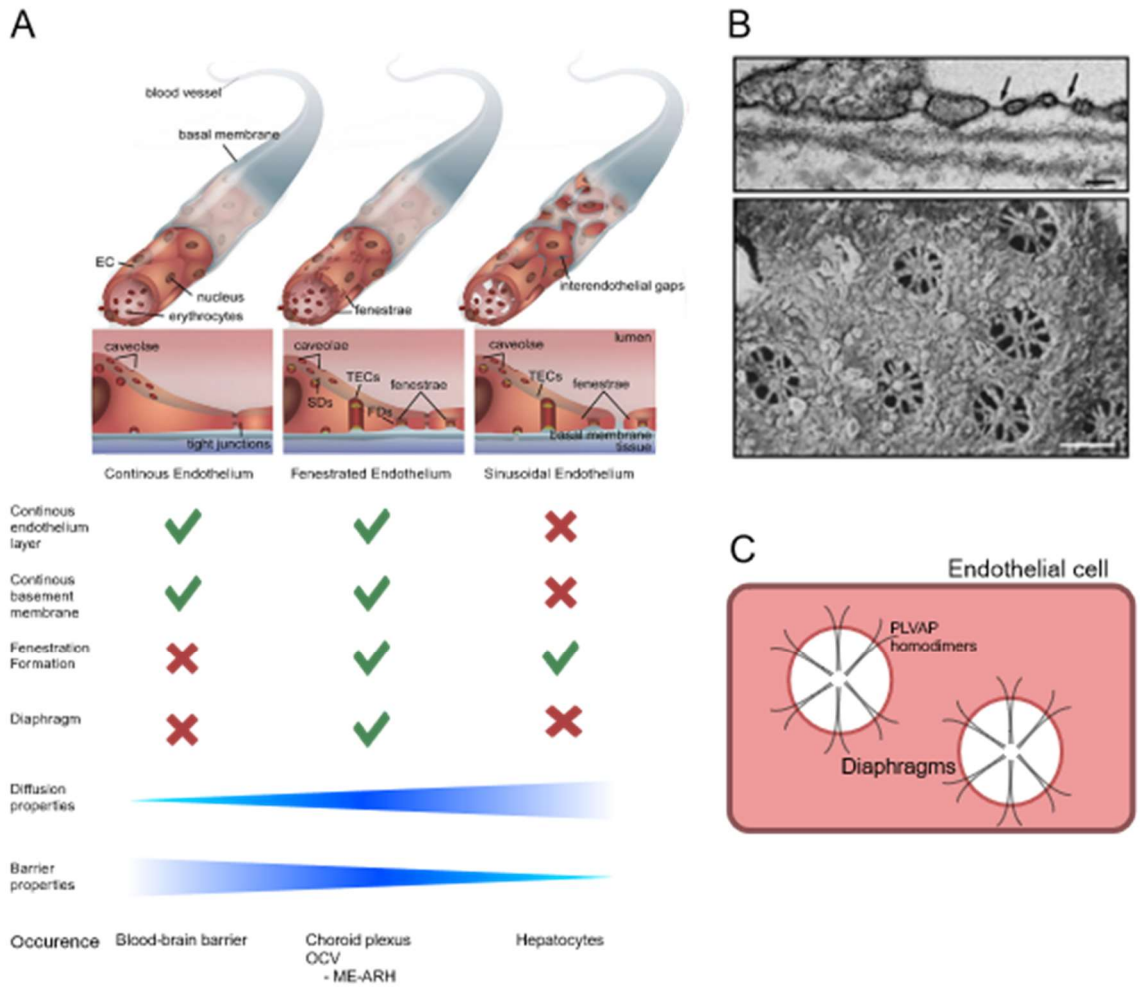


Figure 5: Organization of continuous and fenestrated endothelium and diaphragm structure.

(A) Scheme of the different types of endothelium: continuous, fenestrated, and sinusoidal. The fenestrated and sinusoidal vessels possess fenestrations or pores, but only the fenestrated vessels have a diaphragm. Adapted from Denzer et al.*, 2023. (B) Electronic microscopy representation of the diaphragm of the fenestrated vessels. Adapted from Chang al 2023. (C) scheme of the PLVAP dimers forming the diaphragm. Created with Biorender.*

PLAVP-null mice have a high lethality at birth and delayed growth, showing the importance of PLVAP in development (Herrnberger *et al.*, 2014; Auvinen *et al.*, 2019). Lack of PLVAP in adult mice has an impact on processing triglycerides, the mice also develop a protein deficiency (Stan *et al.*, 2012).

In some organs, the presence of fenestrated vessels can indicate a pathology. For example, it is encountered in diabetes, which in the retina increases VEGF and fenestrated vessels, causing a leakage in the blood-retinal barrier (Klaassen et al., 2013; Tomita *et al.*, 2020).

The lack of PLVAP will lead to the formation of fenestration without a diaphragm. In the zebrafish, the lack of PLVAP in the hypophysis showed no diaphragm and more fenestrations, increasing the permeability (Gordon *et al.*, 2019). A similar phenomenon is observed in PLVAP knockout mice; Evan's Blue diffusion studies showed a higher permeability and leakage in the organs (Herrnberger *et al.*, 2012; Stan *et al.*, 2012; Auvinen *et al.*, 2019). Highlighting that the presence of the diaphragm in the fenestration does not allow complete free diffusion, but rather a controlled diffusion.

2. Barrier properties

Continuous endothelial cells and tanycytes have something in common: they are joined together (respectively) by tight junctions. Tight junctions are complexes designed to seal the pericellular space between cells. They help the tanycytes forming the CVO structure to take over the role of barrier in the ME-ARH region when it is lacking in fenestrated ECs.

Tight junction proteins form a complex between the cells and regulate paracellular transport. The claudins, occludins, and JAM proteins from two cells will bind together. Proteins such as Zonula Occludens 1 (ZO-1) will bind to the proteins to bundle them and reinforce the tight junction by implicating the cytoskeleton (Fanning and Anderson, 2009). This will form strands

of tight junctions running in the apical section of cells to create points of tightness between the cells, thus joining them.

a) Scaffolding protein - Claudins

Claudins are transmembrane proteins whose primary goal is to restrain the paracellular transport by adhesion cell to cell and are a component of all (observed) tight junctions. There are 27 claudin types that are differentially located throughout organs; many knockout mice models of the claudins exist, highlighting their differences (Mineta *et al.*, 2011). Some claudins are essential. Indeed, their knockout is lethal for pups and mice. This is the case for Claudin 7 (thermal dysregulation) and Claudin 1 (wrinkled skin, no movement) (Furuse *et al.*, 2002; C. Xu *et al.*, 2019).

The deficiency of other claudins leads to milder effects, Claudin 12 knockout where

renal reabsorption is limited (Plain *et al.*, 2020); Invalidating Claudin 3, highly expressed in the liver, is not enough to disturb the tight junction's overall integrity but enough to disrupt the passage of bile in the blood vessels (Baier *et al.*, 2021). The tight junction integrity is seemingly unchanged in a Claudin 5 knockout mice model. However, the Claudine 5 deficiency impairs the flux/ exchange of information by allowing smaller molecules (an 800Da tracer) to pass through the vessels of the brain (Nitta *et al.*, 2003). Claudins formed by 4 transmembrane domains are the core of tight junctions by polymerizing into strands possessing binding sites for the other TJ pieces (Günzel & Yu, 2013; Suzuki *et al.*, 2014).

b) Junction-associated protein - TAMP family

The tight junction-associated MARVEL domain-containing proteins, or TAMP family, are a tight junction-associated family (Occludin, Tricellulin, Marvel3D). The most known TAMPS is the occludin, which was one of the first tight junctions proteins to be identified after isolating the TJ from hepatocyte cells (Tsukita & Tsukita, 1989; Furuse *et al.*, 1993). An occludin

knockout mouse model showed no lethality in pups. However, male mice failed to reproduce with wild-type mice. Oppositely, females could produce litter but had altered maternal behavior (Saitou *et al.*, 2000). When treated with VEGF *in vitro*, the tight junctions are altered, and paracellular transport is increased, a phenomenon shunted by overexpression of occludin, attributing to the occludin a role in the stability of the tight junctions (Murakami et al., 2009).

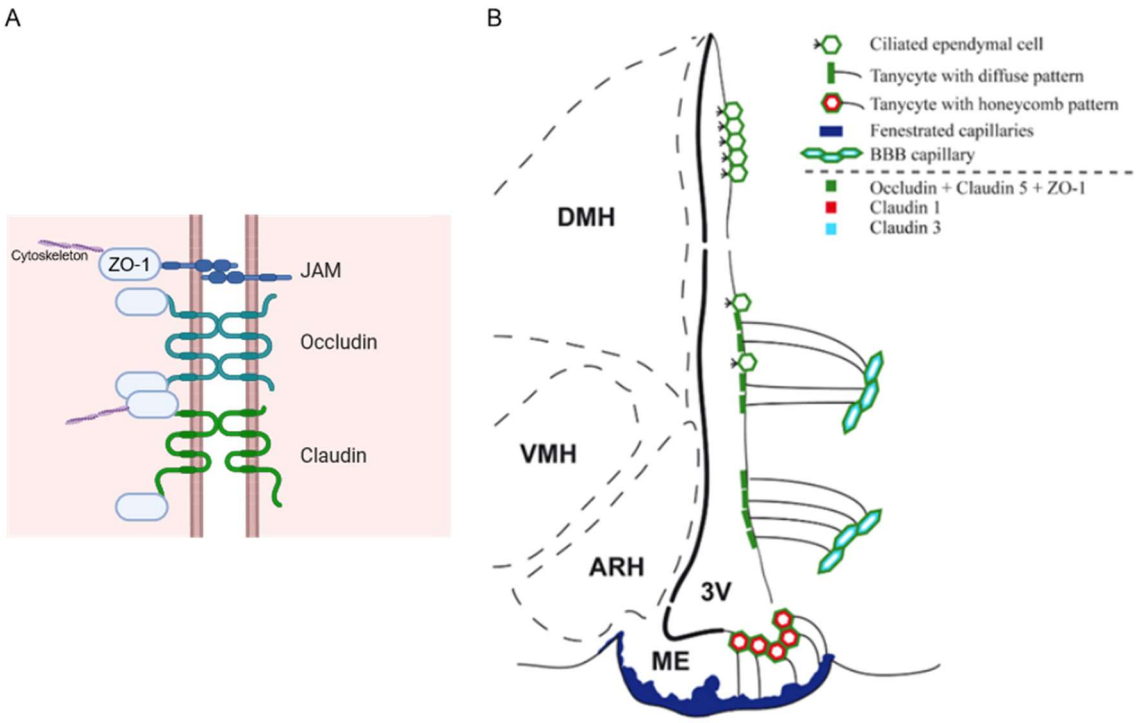


Figure 6: Tight junction complex and repartition in the Medio basal hypothalamus

(A) Scheme of the main tight junction families forming a tight junction complex between two cells. (B) The mediobasal hypothalamus is represented with the tight junction mapping throughout the tanycytes and the vessels. At the base of the hypothalamus, there is the fenestrated vessels dark blue) where there are little tight junction complexes. The bundle of protein: occludin, claudin 5 and ZO-1 are more ubiquitous in the vessels and tanycytes as cell as the ependymal cells. Claudin 3 is found in the BBB vessels, whereas claudin A is mostly found at the ME tanycytes and partially at the vmARH tanycytes. Adapted from Mullier & al 2010 and created with biorender.

Tricellulin is found in a complex called a tricellular tight junction, which is the point of the adhesion of three cells together. Tricellulin knockout results in a more diffuse pattern of the tight junction in the epithelium (Ikenouchi *et al.*, 2005; Raleigh *et al.*, 2010).

c) Tight junction adhesion - JAM family

Supporting the TJ complex, the adhesion molecules, or JAM proteins, are part of the immunoglobulin family (Itoh *et al.*, 2001). Along the claudins, the JAM-A regulates the cell polarity of epithelial cells and endothelial cells, helping shape the cell membrane, and may be involved in the apical location of the tight junction (Otani *et al.*, 2019). The JAM possesses a PDZ, a binding domain allowing the JAM to interact with other TJ proteins owning a PDZ domain (ZO-1) (Hartmann *et al.*, 2020).

The VE-cadherin is another JAM protein. VE-cadherin has a transmembrane domain that forms a bridge between the endothelial cells. It is known to partly regulate the permeability of paracellular transport by being internalized in the cell (Gavard & Gutkind, 2006).

d) Zonula occludens

The Zonula occludens tight junctions proteins are ZO-1,2 and 3, scaffolding proteins from the MAGUK family (membrane-associated guanylate kinase family). The ZOs will bind to transmembrane and cytosolic tight junctions to bind them together. The ZO proteins all have 3 PDZ domains to bind with the JAMs and connexins, an SH3 domain to bind with ZONAB (ZO-1 associated nucleic acid binding), and a GUK (guanylate kinase-like) domain to bind with occludin (Bauer *et al.*, 2010). The knockout mice models of the ZOs can help us discern their importance; the ZO-2 knockout model has weaker TJ and higher permeability, leading to a lethality in the model. The ZO-1 knockout model has insufficient angiogenesis in the yolk sac, leading to lethality. However, the ZO-3 knockout mice survive (Katsuno *et al.*, 2008; Xu *et al.*,

2008). Their distribution differs as well, ZO-3 is expressed in the epithelial cells, in the liver, the lungs, and the kidney. ZO-1 is more expressed in the brain and blood vessels (Inoko *et al.*, 2003). It is the ZO-1 that is primarily implicated in endothelial TJ regulations by recruiting the myosin fibers to the TJ structure. ZO-1 recruits and binds to the actin filaments to be incorporated into the TJ complexes (Fanning *et al.*, 1998). Using an inhibitor of actin polymerization, the Latrunculin-A (LatA) forces the disassembly of ZO-1-TJ after 25-35 minutes *in vivo*. Removing the LatA from the dish allows TJ to reassemble in under two hours, giving a window into the TJ dynamics (Beutel *et al.*, 2019).

The tight junctions in the brain are mainly found in the endothelial cells. Claudin 5 is the most expressed in the BBB, followed by claudin 3, 1, and 12. The BBB TJ contains occludin and ZO-1/2 (Stamatovic *et al.*, 2016).

C. The ME-ARH interface

In the next part of this chapter, we will describe the median eminence's anatomy, functions, and regulation, its particular relationship with the hypothalamic arcuate nucleus, sometimes described as CVO itself, and the ME-ARH's importance in metabolic homeostasis. The Medio basal part of the hypothalamus (MBH) is tailored to secrete and receive information from and to the hypothalamic nuclei controlling the metabolic functions. The ME-ARH or blood-ARH interface is region is divided into (1) an external zone, (2) an internal zone, and (3) an ependymal zone (Shaver *et al.*, 1992):

(1) A bed of fenestrated vessels at the very base of the hypothalamus forming capillary loops capable of reaching the vmARH, called the median eminence (ME)

(2) A parenchyma, composed of neurons, glial cells, and the end-feet of the tanycytes.

(3) A layer of tanycytes defining the medio-basal part of the third ventricle. These tanycytes are surrounded by tight junction complexes restraining the paracellular transport route. Tanycytes are located at the basement of the third ventricle facing the ME, at the lateral parts of the third ventricle facing the ARH.

Together, these components will create a crossroad where molecules coming from the periphery will be filtered through the ME fenestrated vessels and tanycytes and possibly reach the hypothalamus.

The ME-ARH is divided by an anteroposterior classification of the ME-ARH region and its tanycytes. There are 4 zones **(fig.5 B)**, zone 1 is the anterior part of the ME, where appear fenestrated vessels, and the ARH starts to face the basal part of the 3V. The zones 2 and 3, are where the ME has the most fenestrated vessels, sometimes reaching between the vmARH and the third ventricle. Zone 4 is characterized by the ME capillary plexus folding onto itself and the pituitary stalk.

1. MBH vascular complex

The beginning of the hypothalamic vasculature studies dates to the 1930s when Popa G.T, MD, and Fielding M.B observed a capillary complex in the pituitary stalk (Popa and Fielding, 1933). A few decades later, the vasculature of the mediobasal hypothalamus was found to be different from the rest of the hypothalamus (Page, Leure-Dupree and Bergland, 1978). Then, the ME was found to be responsible for the liberation of neurohormones and for receiving external cues to be processed by the hypothalamus. The MBH blood supply comes from the hypophysis arteries, the rest of the hypothalamus is vascularized by the Willy's circle. The arteries divide into smaller capillaries reaching the ME and leaving the ME to drain the blood supply as a part of the pituitary's portal vein. As the capillaries reach the ME they form loops

that can reach up the ARH and create a surface exchange between the blood from the loops/capillaries and the facing cells, the tanycytes (Prevot *et al.*, 2018).

2. Tanycytes

Tanycytes are bipolar ependymoglial cells boarding the basal part of the third ventricle. They were described by the scientist Ramon y Cajal in 1909 and named by Ernst Horstmman in 1954 as stretched cells, and were first involved in the secretory function of the ME by the observations made by (Guldner & Wolff, 1973) on the localization of synapse contact between the stretched cells and neurons. They were also shown to regulate the GnRH secretion towards the neurohypophysis (Prevot *et al.*, 1999). The tanycytes' uptake of insulin-like growth factor 1 (IGF-1) highlights its sensory capacity (Dueñas *et al.*, 1994).

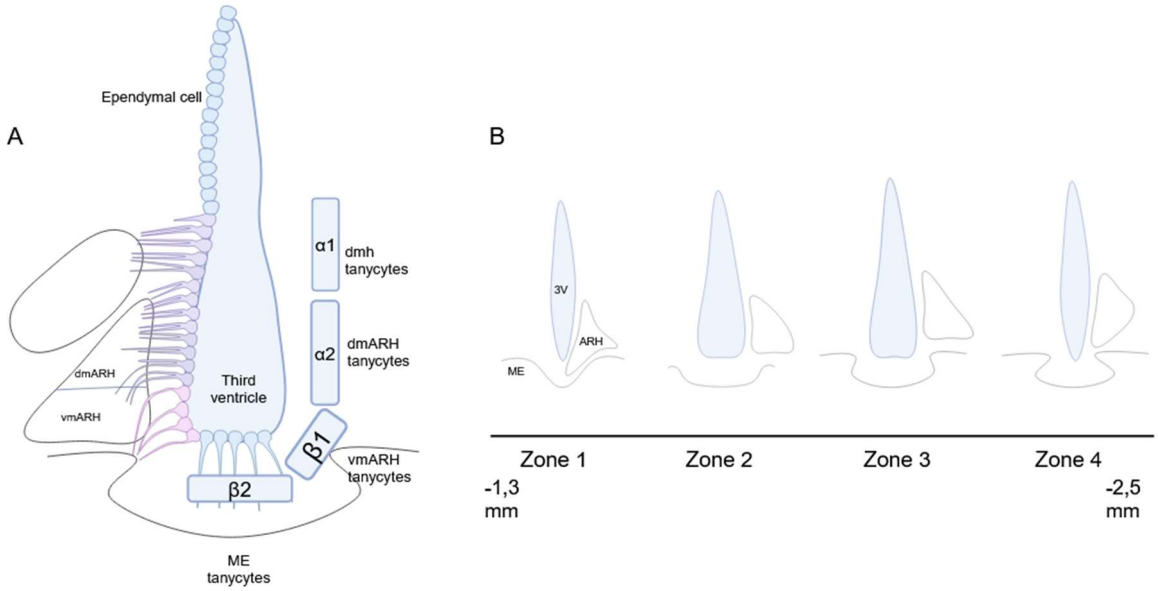


Figure 7: Tanycytes repartition in the hypothalamus and anteroposterior zones of the MBH

A: Representation of the tanycytes localization around the third ventricle, α 1 or DMH tanycytes facing the DMH, α 2 or dmARH tanycytes facing the dorsomedial part of the ARH, β 1 or vmARH tanycytes facing the ventromedial part of the ARH and the β 2 or ME tanycytes facing the median eminence. B: anteroposterior zones of the ME-ARH region in the MBH, Zone 1 (from bregma −1.3 to −1.6 mm), Zone 2 (from bregma −1.6 to −1.8 mm), Zone 3 (from bregma −1.8 to −2.1 mm) and Zone 4 (from bregma −2.1 to −2.5 mm). dmARH: dorsomedial arcuate nuclei,

vmARH: ventromedial arcuate nuclei, 3V: third ventricle, ME: Median Eminence, DMH, Dorsomedial hypothalamic nuclei

As stated in earlier paragraphs, the tanycytes can be found in the CVOs as a part of the barrier between the periphery and the brain. In the ME, they are mostly located in the basal part of the third ventricle, and their presence is scarce in the more dorsal part of 3V. Indeed, they form a cell gradient with cuboid ependymocytes, microvilli-bearing cells that help the CSF movement (Flament-Durand & Brion, 1985). The tanycytes' origin hypothesizes that they are residual radial glial cells (Neural stem cells), with a similar morphology of elongated processes emerging from their cell body (Goodman & Hajihosseini, 2015). Like the radial glial cells, tanycytes could display stem cell functions, as BrdU injection labeled proliferating tanycytes (Bennett *et al.*, 2009; Kano *et al.*, 2019). The radial glial cells majorly differentiate as astrocytes, so it makes sense that tanycytes harbor mature astrocyte markers like GFAP (Goodman *et al.*, 2020).

a) Heterogeneity

In 1973, (Akmayev *et al.* 1973) proposed a tanycytes classification that was used for decades. It is divided into 4 sub-types: the tanycytes B 2, β 1, α 2, and α 1 based on the tanycytes localization in the hypothalamus **(fig.5 A)**. However, recent studies highlighted the heterogeneity and complexity of tanycytes that might reach beyond their strict localization. For example, divergence in their origin as lateral tanycytes derived from progenitor expressing Shh and differ in morphology are they are either unciliated or biciliate (Mirzadeh *et al.*, 2017). In the ME-ARH, tanycytes can be divided into groups according to their relationship with fenestrated vessels: some tanycytes are always in proximity/contact with FV, other tanycytes are only close to FV depending on the environment (i.e., fasting), and some are only in contact with BBB vessels.

Tanycytes can also vary according to their contact with specific cell populations, such as the hypothalamic NPY, POMC neurons, and astrocytes. These contacts can be protrusions in the distal part of the endfeet of the tanycytes. Throughout the ME-ARH "zone," the protrusions/contacts are found in similar proportions (Pasquettaz *et al.*, 2021).

b) Types of tanycytes

In 2018 an updated classification was proposed by (Prevot *et al.*, 2018) and will be used in this manuscript. They classify tanycytes according to their origin, cilia, relation to FV, cell markers, and associated neurons.

The **ME tanycytes**, previously known as the β2 tanycytes, are on the frontline to receive the external cues that manage to pass the FV. ME tanycytes constitute the floor of the third ventricle and are unciliated (Mirzadeh *et al.*, 2017). Their prolongation reaches into the ME parenchyma and contacts the ME-fenestrated vessels. At their apical part, they have a heavy endocytosis-transcytosis complex and a dense population of microtubules (Rodríguez, González, and Delannoy, 1979). This aligns with a study showing leptin transport within the tanycytes (Balland *et al.*, 2014). The **vmARH unciliated tanycytes** (β1-α2) are at the frontier between the Arcuate nucleus and the median eminence. Their prolongations resemble an arch starting from the body of the tanycytes nestled in the 3V layer reaching for 300µm into the vmARH and then into the lateral part of the median eminence to end in a "bundle" of end-feet in the lateral limits part of the ME (Rodríguez *et al.*, 2019). They are the bridge between the 3V basal part and its lateral walls. These tanycytes can still be in contact with fenestrated vessels forming loops close to the vmARH. The former α2 tanycytes, now called **dmARH tanycytes,** are biciliated and more dorsal tanycytes further away from the ME-ARH region. They are not known to be in contact with FV but only with BBB vessels (Mirzadeh *et al.*, 2017).

The **vmh/dmh tanycytes** (α1 tanycytes) are scarce and intertwined with ependymal cells that will constitute most of the 3V apical parts.

c) Tanycytes connections to another cell type

Aside from the relation between the TJ and FV, the tanycytes also are making direct contact with both BBB vessels and FV; around 70 % of tanycytes contact ARH vessels, and around 30% of tanycytes have a contacting protrusion with neurons. In the characterization study, a research team laid out the tanycytes' network of connection with neurons (Pasquettaz *et al.*, 2021). They discovered that the number of tanycytes' prolongations are similar in the ARH, VMH, and DMH hypothalamic nuclei. However, the connection between and the DMH was primarily established in zones 3 and 4 of the ME-ARH.

Looking at the connection between the tanycytes and the ARH, the tanycytes' prolongations in the ARH are more prone to make a protrusion reaching NPY anorexigenic neurons (23.9%) than the POMC orexigenic neurons (10.5%). The tanycytes' protrusions reach further than vessels and neurons; in the ARH, they connect with the glial cells, notably the astrocyte fibers (55%) but also microglial cells and NG2 (Neurogenic cells) positive cells (KOMITOVA *et al.*, 2009).

a) The other barrier, the PNN barrier

The perineuronal net, or PNN, is an extracellular matrix (ECM) composed of proteoglycans and located at the basis of the vmARH arching towards the ME extremities. In the hypothalamus, the PNN was described as crucial in the lactation period for developing hypothalamic neurons by (Mirzadeh *et al.*, 2019). The PNN is submitted to regulation by the nutritional status, indeed, a 17-hour fasting period reduces the PNN area in the ME-ARH region (Kohnke *et al.*, 2021). This opens windows to possible biregulation through a multibarrier system in the hypothalamus that is worth exploring in the future.

D. The ME-ARH region as a first stop in the hypothalamus regulation

1. Peripheral routes

a) Permeability

To study the possible diffusion or leakage in the brain, an injection of a dye *in vivo* is a standard practice, the Evans' Blue dye (EB), a 1Kda molecule is widely used in such studies. The Evan's Blue diffusion study showed the barrier of diffusion of the ME-ARH region (Mullier *et al.*, 2010). Injecting the dye in the third ventricular allowed the 1KDa molecules to diffuse into the ARH nucleus and the tanycytes but no further. However, when injected in the periphery, the dye was found in the ME-ARH fenestrated vessels and the BBB vessels. At the BBB vessels, there was little to no diffusion outside of the vessels. Inversely, at the FV, the EB diffused throughout the ME parenchyma, reaching the ME tanycytes. Later, another study demonstrated that the IP-injected EB could reach the ARH when subjected to strenuous contexts such as a fasting state (Langlet, Levin, *et al.*, 2013a).

In 1992, an intravenous injection of radioactive aminobutyric acid (GABA) in rats showed the diffusion of GABA across the plexus choroid and median eminence. Notably, they showed a diffusion in the ARH, pushing the fact that the nucleus is a part of the ME-CVO complex (Shaver *et al.*, 1992)..

a) Paracellular route: distribution of tight junction in the tanycytes

Although all tanycytes and ependymal cells are surrounded by a tight junction belt formed by occludin, claudin 5, and ZO-1 **(fig.5 A)**, divergence in TJ composition is found in various parts of the hypothalamic tanycytes. In the ME-ARH tanycytes, their apical pole is surrounded by another claudin, the claudin 1 (Mullier *et al.*, 2010). A paper found that after a BBB leakage, claudin 1 was upregulated and colocalized with ZO-1, leading to a reduced permeability in the

BBB (Sladojevic *et al.*, 2019). The claudin 1 could serve in the ME-ARH as a first responder to form with ZO-1 a stronger paracellular barrier when blood efflux increases through the fenestrated vessels.

b) Tanycytic transcellular transport

On top of the discussed paracellular route, another transport pathway has emerged by attributing not only a barrier function to tanycytes but also a carrier one. It started in 1974 with an injection of horseradish peroxidase, staining the tanycytes and showing the tanycytes' capacity to uptake molecules (Wagner & Pilgrim, 1974). The tanycytes route is a hypothesis that has been strengthening during this past decade. Fluorescent leptin IP injection showed its presence in the ME-ARH region and in the tanycytes. The leptin uptake in the tanycytes is possible because of the expression of LepR, the leptin receptor, and the inhibition of the tanycytic transport of leptin is sufficient to alter lipid metabolism, showing the central role of the tanycytic transcellular transport in the hypothalamic regulation of the metabolism.

2. A fasting state induces the ME-ARH plasticity

An essential function of the hypothalamus is regulating energy balance and food intake. The hypothalamic nuclei are populated with many anorexigenic or orexigenic neurons. Therefore, it makes sense that the ME-ARH plays a role in communicating energy status to the hypothalamus. In male mice, 24-hour starvation increases the number of fenestrated vessels in the ME and in the space between the 3V and the vmARH, resulting in more direct access for the peripheral molecules to the ARH's neurons **(fig.7)**. The more permissive environment will be met with a response from the tanycytes' tight junctions to reinforce their barrier and avoid unwanted paracellular transport. The ZO-1 and Claudin staining increases in the ARH, and the ZO-1 in the vmARH presents a more organized pattern (Langlet, Levin, *et al.*, 2013a).

The fasting adaptation of the ME-ARH is explained by a blood glycemia dip, a non-processable 2-deoxyglucose peripheral injection inhibiting glucose will increase the VEGFa content in the tanycytes, and a VEGF injection in fed mice increases the fenestrated vessels presence. Therefore, tanycytes will sense the glucose levels and regulate the fenestration of the endothelial cells in the ME and vmARH region. In this region, 70% of tanycytes protrusions reach the fenestrated vessels. This mechanism helps the hypothalamus to switch to a hypothalamic fasting profile, stimulating the orexigenic neurons and inhibiting the anorexigenic neurons (Langlet, Levin, *et al.*, 2013a). VEGF is also modulated by central clues like the activation of MCH neurons which increase the VEGF signal and leptin sensitivity (Jiang *et al.*, 2020).

The relationship between glucose and the ependymoglial cells is also important, the glucose transporters, GLUT1 and GLUT2 receptors are found in the tanycytes. Tanycytes-GLUT2 are sufficient to regulate the beginning of food intake in the active phase and ghrelin secretion in rodents (Barahona *et al.*, 2022). The circadian-dependant regulation also regulates the GLUT1 located in the tanycytes, lesions of the SCN showing that tanycytes-GLUT1 are necessary to regulate peripheral glycemia (Rodríguez-Cortés *et al.*, 2022).

Fasting is not the only context in which the hypothalamus vascular system will be impacted. A high-fat diet in rodents increases VEGF expression and increases angiogenesis i in all hypothalamus nuclei and the ME-ARH region (Gruber *et al.*, 2021).

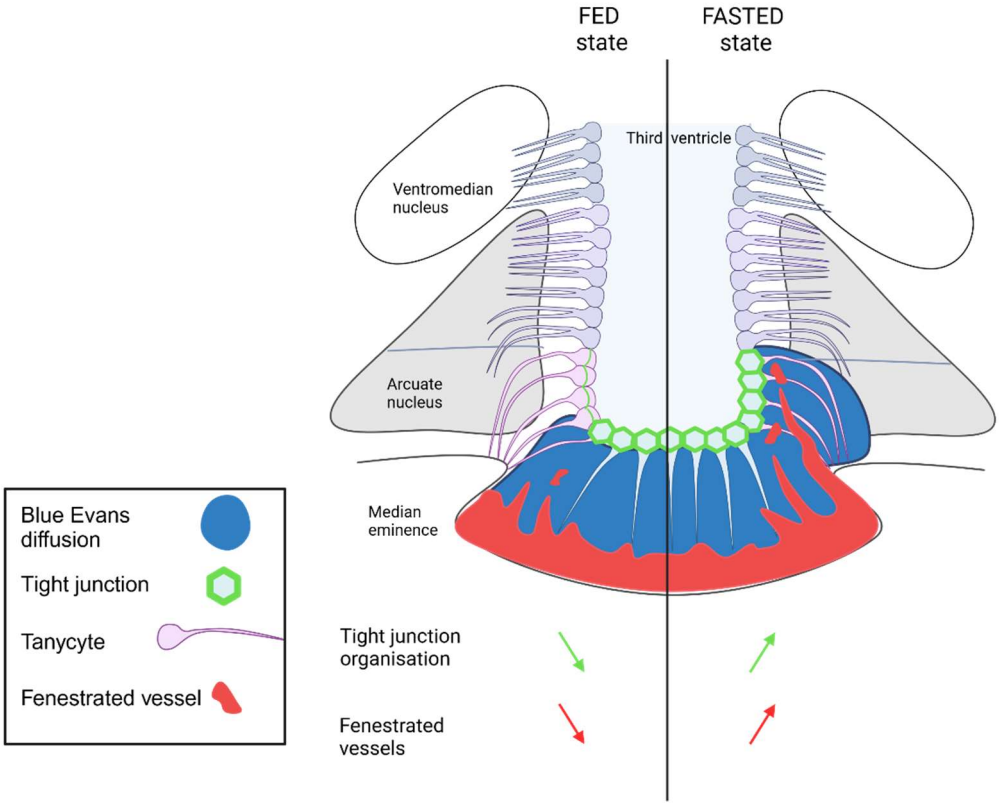


Figure 8: ME-ARH region nutritional-dependent plasticity in the fenestrated vessels and tight junction

As uncovered by the paper of Langlet in 2013, the MBH of mice possesses a nutritional-dependent plasticity. During the fed state, the tight junction surrounding the ME tanycytes will be organized in a "honeycomb pattern," the rest of the tanycytes' TJ, notably facing the vmARH, have a diffuse pattern. The fenestrated vessels will be primarily localized in the ME region. This will be complemented with Evan's Blue injection, showing the diffusion of the dye coming from the fenestrated vessels, into the ME parenchyma and not fully reaching the ARH. A fasted state will increase the number of fenestrated vessels; the FV can reach the vmARH. Accordingly, the tanycytes' tight junction will reorganize in response to the FV, and the honeycomb pattern will be seen in the vmARH tanycytes. Food restriction will affect the MBH plasticity, allowing an increased diffusion area in the parenchyma; the Evan's Blue will reach the vmARH. This can allow a fasted vmARH to have a direct access to the peripheral signal coming from the circulation. ARH: arcuate hypothalamic nucleus, ME: median eminence, 3V: third ventricle, TJ: tight junction, FV: fenestrated vessels. Created with biorender.

The brain interfaces, such as the choroid plexus and circumventricular organs, create opportunities for communication through the fenestrated vessels. This chapter established that structures like tanycytes and tight junctions are essential to secure a barrier function to the brain and control communication. The hypothalamus and the median eminence work together to receive information and regulate homeostasis. However, the ME-ARH is a plastic organ and can be regulated by metabolic signals. These signals can stem from several peripheral organs: in this thesis, we chose to study the impact of the liver on the hypothalamic regulation of metabolism, precisely focusing on the role of the hepatic estrogen receptor α. The next chapter will thus lay out the basics on estrogens and the hepatic estrogen receptor α to understand their role in metabolism.

III. The estrogen and its hepatic receptor estrogen α

The goal of this chapter is to introduce the importance of the hERα. This chapter will recapitulate the estrogen role in physiology, the basis of liver anatomy and function, and then dive into one of the liver's most potent receptors, and quite representative of the liver's function as it bridges the reproduction and metabolism, the estrogen α receptor. The hERα structure will be detailed, as well as its roles through the description of the well-described mouse model, the LERKO.

A. Estrogen

Estrogen is a steroid hormone that, alongside progesterone, characterizes female metabolism and the reproductive system. Estrogen shapes female development, reproductive years, and menopause. The leading members of this family are estradiol (E2), estrone (E1), estriol (E3), and estetrol (E4) during pregnancy.

- Estrone (E1) is a precursor of estradiol and can bind to the ERα and ERβ receptors. Notably, E1 continues to be produced after menopause and is the estrogen that can cause breast cancer after menopause (Qureshi et al., 2022).
- Estradiol (E2) is the primary estrogen. It is produced by the ovaries and also by adipose tissue after menopause. E2 is crucial in the development of sexual and metabolic characteristics.
- Estriol (E3) is a metabolite of E1 and E2. It has low basal levels that rise during pregnancy (Katagiri et al., 1976).
- Estetrol (E4) is an estrogen exclusively produced during pregnancy by the fetus's liver, reaching maternal blood circulation. Like the other estrogens, E4 binds to the estrogen receptors but with a lower affinity (Tseng & Gurpide, 1978).

Estrogen production in females starts with the release of LH and FSH. LH triggers steroidogenesis in the ovaries' theca cells to produce androgens: androstenedione and testosterone. The aromatase enzyme converts these androgens in the granulosa cells into E1, E2, and E3.

In females, estrogen plays a central role in reproductive and non-reproductive functions. During the menstrual cycle, estrogen levels fluctuate, influencing the development and shedding of the uterine lining. Estrogen is vital for the maturation of ovarian follicles, ovulation, and the maintenance of pregnancy. Beyond reproduction, estrogen contributes to the development of secondary sexual characteristics. It is essential for maintaining bone density, body fat distribution, cardiovascular health, and cognitive function. Estrogen decline during menopause is associated with an increased risk of atherosclerosis, metabolic disorders, and osteoporosis. Hormone Replacement Therapy (HRT) is often used to manage symptoms and maintain health during the menopausal transition.

While present in lower concentrations in males than in females, estrogen is produced primarily through the conversion of testosterone by the enzyme aromatase. In men, estrogen contributes to the regulation of bone density, fat distribution, cardiovascular health, and cognitive function. Imbalances in estrogen levels in men can lead to conditions such as gynecomastia (enlargement of breast tissue) and may impact fertility.

1. The root of the sexual dimorphism: feminization and masculinization

Sexual dimorphism refers to the differences between female and male phenotypes. It starts with the presence of the Y chromosome and its SRY gene, which directs the gonads to differentiate into testes, where testosterone and estrogen will be produced. In the absence of the SRY gene, as with two X chromosomes, the gonads differentiate into ovaries, leading

to distinct steroid hormone profiles: females are estrogen-dominant, while males rely on androgens.

The first stage of sex-specific development occurs during gestation when steroid hormone production begins in the gonads and influences the hypothalamus. At this time, the hypothalamic-pituitary-gonadal (HPG) axis becomes active, with the first release of LH and FSH peaking in the fetus, followed by inhibition until mini puberty (Becker & Hesse, 2020; McCarthy, 2020). The second stage of differentiation starts around six months of age in human males and around two years in females. In rodents, this second stage occurs late in gestation. This critical period, known as mini puberty, is when the HPG axis becomes active in a sex-specific manner, adjusting hormone levels (testosterone and estrogen). The third stage is puberty, where physical and sexual characteristics develop, and reproductive functions become viable (Kuiri-Hänninen et al., 2014; Becker & Hesse, 2020). Neonatal exposure to testosterone impacts the hypothalamus and the GHRH-GH axis, affecting the liver by shifting its gene expression profile toward a male pattern (Ramirez et al., 2010). Masculinization and feminization refer to the processes leading to the development of male or female phenotypes. A pioneering study by Flores et al. (1973) using immature rhesus male monkeys showed that aromatase converts testosterone into estradiol in the hypothalamus, supporting the aromatase hypothesis that male brain masculinization is estrogen-driven. Estrogen increases aromatase activity in the male brain, with higher levels found in areas such as the hypothalamus, amygdala, and nucleus of the stria terminalis. Treating female pups with estrogen results in aromatase distribution similar to that of males (Roselli et al., 1985; Wu et al., 2009). In adult males, aromatase and estrogen drive sexual behavior, as brain-specific aromatase knockout in mice reduces mating behavior, and lack of estrogen receptors leads to reproductive deficiencies (Ogawa et al., 2000; Brooks et al., 2020). In female rodents, brain feminization can block masculinization by acting on the

preoptic area, increasing DNA methylation and dendritic spine density, and reducing male mating behavior (Nugent et al., 2015).

Estrogen can alter the feminization process, an 11-day estrogen treatment in female pups is sufficient to render the rodent sterile (possessing anovulatory follicles) through the impaired hypothalamic Kisspeptin expression (Park *et al.*, 2023). Blocking feminization by neonatal exposition to testosterone also has a masculinization effect (DHT-dependent) by increasing food intake and decreasing POMC mRNA expression and leptin sensitivity in adulthood (Nohara *et al.*, 2011).

All of these studies suggest that a delicate balance of hormones is needed to achieve the female or male phenotype.

2. Physiology of the Menstrual Cycle

a) The menstrual cycle in Humans

The menstrual cycle in humans is a complex, regulated process that typically spans around 28 days, although variations are common. The cycle is divided into two main phases: the follicular and luteal phases, with ovulation occurring when the hormonal levels of E2 are the highest. The cycle begins with the menstrual phase, characterized by the shedding of the uterine lining. In the follicular phase, rising levels of follicle-stimulating hormone (FSH) stimulate the development of ovarian follicles. As these follicles mature, estrogen levels increase, reaching a peak just before ovulation. Ovulation is triggered by a luteinizing hormone (LH) surge, which releases the mature egg from the ovary. The luteal phase follows, marked by the corpus luteum formation, which produces progesterone. If fertilization does not occur, the corpus luteum degenerates, leading to a drop-in progesterone levels and the onset of menstruation. In coordination with the ovaries, this cyclical process is tightly

regulated by the hypothalamus and pituitary gland, orchestrating hormonal changes that prepare the body for potential pregnancy.

b) The estrous Cycle in Mice

Unlike humans, mice's cycle is called the estrous cycle, with estrus representing the fertility period. The cycle is typically shorter than in humans, lasting around 4-5 days. The estrous cycle consists of four stages: proestrus, estrus, metestrus, and diestrus. During proestrus, estrogen levels increase, leading to the maturation of ovarian follicles. Estrus is marked by a surge in luteinizing hormone (LH), which triggers ovulation. Metestrus and diestrus involve the preparation of the uterus for potential pregnancy and the return to baseline hormonal levels. The hypothalamus and pituitary gland regulate the estrous cycle, with estrogen and progesterone playing critical roles in orchestrating these cyclical changes. Understanding the estrous cycle in mice is crucial for reproductive research and the study of female reproductive physiology.

3. The end of estrogen’s protective effect: the menopause

Once the oocyte pools are depleted, estrogen production ceases, causing significant changes and marking the end of reproductive functions. The post-menopausal state involves more than infertility; it reshapes the metabolic landscape and increases the risk for cardiovascular and liver disease. Menopause begins due to a lower number of follicles and a decrease in estradiol (E2), which reduces the negative feedback on pituitary gonadotropins. This leads to a compensatory rise in LH and FSH levels, inevitably resulting in irregular menstrual cycles (Weiss et al., 2004; Shaw et al., 2010). Estrogen deficiency in the hypothalamus can reduce the number of neurons in the hypothalamus. For example, Kisspeptin neurons regulate the release of GnRH and, subsequently, LH/FSH and are also involved in thermoregulation (Santoro et al., 2021).

The duration of menopause can last several years, during which a transient decline in cognitive function may occur (Grummisch et al., 2023). Once the ovarian reserve is depleted,

the menstrual cycle ends, and the post-menopausal state begins. At this time, estrone becomes the dominant estrogen, produced in adipose tissue and the liver by converting androstenedione (Brady, 2015). After menopause, the risk of certain diseases increases, such as non-alcoholic fatty liver disease, both in healthy individuals and those with diabetes (Hamaguchi, 2012; Raverdy et al., 2023).

B. Structure of the Estrogen Receptor α

The primary endogenous ligands for the estrogen receptor α (ERα) are estrogen hormones, with 17β-estradiol being the most potent and biologically active form. Estrogen receptors, including ERα, contain several isoforms and can be nuclear receptors or membrane receptors. 17β-estradiol is the main ligand for the receptor; other endogenous estrogens, like estrone and estriol, also bind to ERα with varying affinities.

ERα is part of the steroid hormone receptor family, which includes nuclear receptors, protein G-coupled protein receptors (e.g., GPR30, GPRC6A), ion channel receptors (e.g., GABA-R), and receptors associated with the sex hormone-binding globulin (SHBG/SHBG-R) complex. ERα was first described as part of the nuclear receptor sub-family, which includes ERβ, the glucocorticoid receptor, the mineralocorticoid receptor, the progesterone receptor, and the androgen receptors (Giguère et al., 1988; Mangelsdorf et al., 1995; Vickers & Lucier, 1996).

Steroid hormone receptors share a common ancestor dating back 600 million years (Pearson, 2012; Arnal et al., 2017), explaining the wide range of functions attributed to these receptors. Structurally, they consist of six domains containing two zinc fingers with a DNA binding domain (DBD), a ligand binding domain (LBD), and a hinge domain between the DBD and LBD (Green et al., 1986; Krust et al., 1986).

Estrogen receptors, including ERα, contain estrogen response elements (EREs), which are DNA sequences with a high affinity for estrogen, making these receptors estrogen-

dependent and vital for transcriptional activity. ERα has no transcriptional activity without ligands (Green et al., 1986; Berry et al., 1990a; Liu et al., 2003). There are two nuclear estrogen receptors, α and β, and membrane receptors such as GPR30. ERα is encoded by the ESR1 gene located on chromosome 6 in humans (6q25.1-q25.2) and chromosome 10 in mice (10 A1; 10 2.03 cM).

The ESR1 gene has eight exons transcribed and translated into six domains (A to F), forming ERα. These domains enable ERα to respond to estrogen-ligand signals, bind to specific DNA sequences, and regulate the transcription of target genes **(fig.8)**. Ligand binding induces conformational changes in the receptor, facilitating its interaction with coactivators and initiating gene expression.



Figure 9: ERα structural organization

The ERα is composed of 595 amino acid, they form 6 domains and 8 exons. Starting from the N terminal there is the AF1, activating function domain; NTD, amino terminal domain; DBD, DNA binding domain; LBD, ligand binding domain and the AF-2, the activating function domain 2 ending at the C terminal. Adapted from Fuentes & Silveyra, 2019.

- *A domain*

The A domain is located at the NH2- terminal, this is the variable part within the ERs family indeed, with shorter ERα isoforms of 46KDa as well as a long isoform of 66KDa. As well as the ERβ. Deleting the A domain (hERα1-37) in vivo shows a vital role in ERα-dependent ligand-independent transcription. then to function as linked to the E domains (Griffin *et al.*, 1999; Métivier *et al.*, 2002).

- *B/E domains: AF-1 / AF-2*

The B domain contains a transcriptional activating function called AF-1, while the E domain contains AF-2, working together to achieve tissue-specific transcription. When the B domain is excised from mice ERα and treated with E2 in vivo, it highlights a ligand-independent function (Lees et al., 1989). Literature and reviews (Arnal et al., 2017) note that ERα is primarily a ligand-dependent receptor. *In vitro*, AF-1 seems unstructured, changing its binding properties (Wärnmark et al., 2001). AF-1 activation requires phosphorylation of the 118-serine residue (Kato et al., 1995), altering its capacity to bind coactivators (Kumar et al., 2004).

AF-1 allows the receptor to interact with cellular signaling pathways and co-regulatory proteins, even in the absence of estrogen. However, full transcriptional activity of ERα often requires a ligand, inducing conformational changes that allow efficient interaction with coactivators, especially in the LBD where AF-2 is located (Tora et al., 1989).

AF-2 is ligand-dependent, specifically on estrogen. Ligand binding induces conformational changes in the receptor, allowing AF-2 to interact with coactivator proteins, crucial for full transcriptional activation and regulation of target gene expression.

The ERα activity can depend on the cell type, being more AF-1 “permissive” (e.g., HepG2, yeast) or AF-2 “permissive” (e.g., Hela cells) (Métivier et al., 2002). Thus, the cell type studied should be considered as it might result in different actions.

- C domain: DNA Binding Domain (DBD)

This domain contains the two zinc finger motifs common to the SNR family. It binds to specific DNA sequences known as estrogen response elements (EREs) in the target genes.

- Linker Domain (D)

Connects the DNA-binding domain to the ligand-binding domain, and contains a nuclear localization signal (NLS).

- Ligand-Binding Domain (LBD):

Located at the C-terminus, binds to estrogen and other ligands and mediates the interaction with coactivators or corepressors. LBD is responsible for the conformational changes that allow the receptor to activate transcription upon ligand binding (Fuentes and Silveyra, 2019).

- The activation of the nuclear ERα by estrogen

In the cell, estrogen binds to ERα, causing two E2-ERα units to dimerize. Once the ligand and receptor are anchored, the nuclear localization sequence on domain D translocates the ligand-receptor to the nucleus, activating ERE-dependent or independent mechanisms (Gambino *et al.*, 2012; Yaşar *et al.*, 2017).

Table 2: Estrogen receptors family

	Function on Metabolism	**Function on liver**
ER β	Total knockout: body weight gain, increased fat mass. No change in food intake and energy expenditure (Foryst-Ludwig *et al.*, 2008)	In hepatic stellate cells (Wang *et al.*, 2022)
GPR30 / GPER	Obesity, insulin resistance (Sharma and Prossnitz, 2016)	Hepatocyte (Ikeda *et al.*, 2012) Hepatic stellate cells (Cortes *et al.*, 2019)
Membrane ERα	Total knockout: Insulin resistance (Allard, Morford, *et al.*, 2019)	Cell growth, ERK pathway activation (Adlanmerini *et al.*, 2015)

C. The liver anatomy and function

1. The liver's anatomy

The liver, situated beneath the ribcage, is a vital organ weighing approximately 1.4 kilograms in an adult human and 2-3 grams in an adult mouse. Its structure is intricate and well-organized, consisting of two primary lobes separated by the falciform ligament, which further divides into smaller lobes and segments. The liver's internal architecture comprises a network of blood vessels essential for its functions:

- Hepatic Artery: This artery is the primary supplier of oxygenated blood to the liver.
- Portal Vein: Transport blood from the digestive system to the liver.
- Hepatic Duct: Functions to transport bile produced by the liver.

These three components collectively form the portal triad, serving as a central hub for the liver's exchange with the periphery. The liver is shielded by connective tissue known as Glisson's capsule and by the peritoneum.

The liver's structural unit is the lobule. Each lobule consists of six portal triads, forming a hexagonal shape. Within this hexagon, hepatocytes are arranged in lines extending toward a central vein located at the center of the lobule.

a) Hepatocytes and their supporting system

The hepatocytes represent 60% of the hepatic cells in humans (Racanelli and Rehermann, 2006), and around 90% in mice, the remaining cells are stellate cells, endothelial cells, and Kupffer cells. These cells form the liver's functional unit in the lobules: the hepatic acinus. Among these acini, hepatocytes have a zonation depending on metabolic parameters ranging from glucose uptake and release, Bile acid synthesis, and fatty acid oxidation (Kietzmann, 2017).

In the outer hepatocytes (Zone 1) the B-oxidation regulation is prone to periportal injury. The hepatocytes in zone 2 are most attributed role proliferative under normal homeostasis. The inner hepatocytes (Zone 3) are more prone to regulate carbohydrate metabolism.

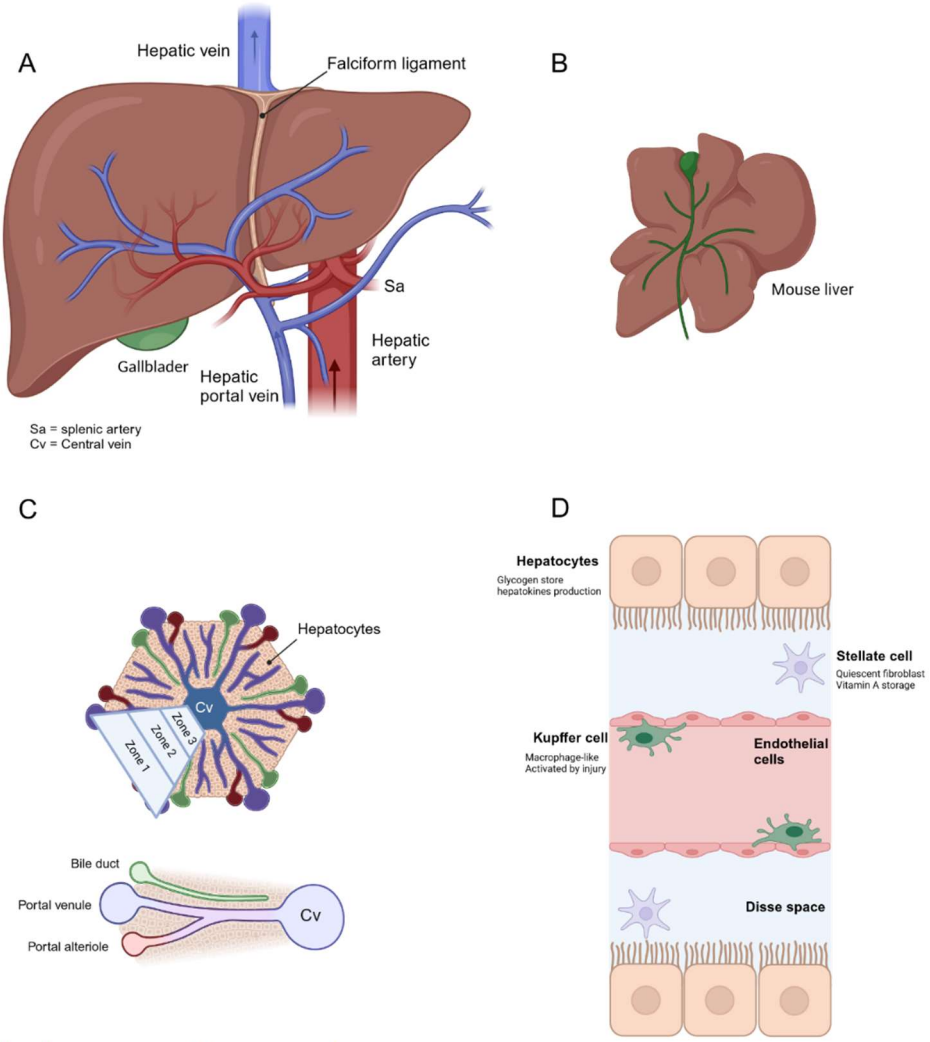


Figure 10 : Liver basic anatomy of human and mouse

(A) Human liver with two lobes and (B) mouse liver with four lobes. (C) The hepatic unit has rows of hepatocytes with the triad: bile duct, portal venule, and arteriole. The zone 1,2 and 3 represent the 3 zones where the liver metabolism is different. (D) a close of the reparation of the hepatic cells in the liver. Created with Biorender.

a) Endothelial cells

Portal vessels of the liver comprise a network of blood vessels that transport blood from the gastrointestinal tract, including the stomach, intestines, and spleen, to the liver for processing. These vessels are critical for the liver's functions, including detoxification, nutrient processing, and metabolic regulation. The portal vessels include the portal vein and hepatic portal system. Blood carried by the portal vessels is rich in nutrients and substances absorbed from the digestive system, flowing into the liver, where it is filtered and processed before entering the general circulation. This unique vascular arrangement enables the liver to play a central role in maintaining metabolic and homeostatic functions within the body.

The **Liver sinusoidal endothelial cells (LSEC)** are a specialized type of endothelial cell lining the hepatic sinusoids, the smallest blood vessels within the liver. These cells form a porous and fenestrated lining that allows the exchange of molecules like lipoproteins between the blood in the sinusoids and hepatocytes. Their diameter ranges from 50 to 300nm (Szafranska et al., 2021). Sinusoidal endothelial cells also play a role in regulating blood flow within the liver. They have unique characteristics that enable the efficient exchange of oxygen, nutrients, and metabolic waste products (Braet and Wisse, 2002). LSECs are set through development; indeed, in perinatal mouse liver, the LSECs possess diaphragm and PVALP-dependent fenestrae. This particularity disappears in adulthood as their PVALP-dependent formation (Auvinen et al., 2019). Additionally, they contribute to the liver's immune functions by acting as a barrier between the blood and the liver tissue, helping to filter out potentially harmful substances and pathogens. Sinusoidal endothelial cells are an integral part of the hepatic microcirculation, supporting the liver's diverse metabolic and regulatory functions. Capillarization of the LSECs is a consequence of liver disease as well as a catalyzer as they change their profile as inflammatory and lack of information shunt proper liver communication and function (Gracia-Sancho et al., 2021).

b) Stellate cells

First called “fat storing” cells, Stellate cells or Ito cells are pericytes found in between the hepatocytes and the sinusoidal endothelial cells of the liver, in the space of Diss. They are known for containing lipid droplets capable of storing vitamin A. In a healthy liver, the cells are in a quiescent state and move into an activated state when the liver is damaged and become proliferative with a fibroblast phenotype (Friedman, 2008; Hou & Syn, 2018). They are primarily associated with fibrosis or cirrhosis where they heavily produce extracellular matrix protein like collagen, a marker for scaring in hepatic diseases. In a more positive light, they possess stem capabilities, and their release of the HGF (hepatic growth factor) could help the hepatocyte's proliferation (Kordes *et al.*, 2021).

c) Kupffer cells

Named after Karl Wilhelm von Kupffer in 1876, these cells are found near the endothelial cells. They are known as hepatic macrophages and can be triggered by liver injury, to start an inflammatory response to protect from further infections and phagocytes' infectious component (Dixon *et al.*, 2013; Nguyen-Lefebvre & Horuzsko, 2015). An initiator of the Kupffer cell response is endotoxin (lipopolysaccharides, LPS), potential coming from the digestive tract blood flow through the portal hepatic vein. The Kupffer response might be sex-specific as estrogen injection in mice renders them more sensitive to the Kupffer cell response (Ikejima *et al.*, 1998). The Kupffer cells have a protective role toward LSEC against apoptosis (Hutchins *et al.*, 2013).

2. Liver’s functions

The liver is an indispensable metabolic and regulatory center with a repertoire of functions that impact virtually every aspect of human physiology. The liver is the central hub for metabolizing nutrients, it is responsible for processing carbohydrates, proteins, and fats, converting them into energy or storing them for future use. This metabolic versatility helps to maintain stable blood sugar levels and provides a reservoir of energy in times of need.

a) The intertwined lipid and carbohydrate metabolisms

Carbohydrates, notably glucose, are the primary energy source used to fuel the organism's functions. After a meal, glucose travels into the bloodstream and is dispersed to the organs. There, it is converted into pyruvate and subsequently transformed into ATP through the glycolysis process. High levels of glucose (hyperglycemia) have deleterious effects. Organs cope with and avoid this problem by storing the surplus glucose as glycogen (or as fatty acids) through glycogenesis. The pancreas releases insulin, which binds to its tyrosine kinase receptor, activating the enzyme glycogen synthase and associated pathways. Glycogen stores are mainly found in the cytoplasm of hepatocytes (but also in muscles) and are composed of β glycogen and its aggregated form called α glycogen (Roach, 2002). In addition to triggering glycogen storage, insulin response inhibits gluconeogenesis and glycogenolysis in hepatocytes.

As blood glucose levels decrease, the pancreas releases the hunger hormone glucagon, which solicits glycogen stores in the liver to perform glycogenolysis and release glucose content. After a prolonged fasting period, glycogen stores become depleted, and the liver turns to an alternate energy source, transforming it into glucose to maintain homeostasis through an enzyme pathway called gluconeogenesis (Han et al., 2016). The GLUT2 transporter facilitates the release of glucose from hepatocytes into the bloodstream and also mediates external glucose entry into hepatocytes.

Another pathway glucose can take is de novo lipogenesis, where it is transformed and stored in triglyceride form with the enzyme FAS, fatty acid synthase. Constant upregulation of this enzyme pathway can contribute to the process of non-alcoholic fatty liver disease (NAFLD). Excess triglycerides are stored in hepatocytes in very low-density lipoprotein (VLDL).

After a meal, caloric restriction forces lipolysis of triglycerides in adipose tissue into fatty acids that travel to the liver. In instances where glucose availability is low, fatty acids can undergo β-oxidation to produce ketone bodies (Jensen-Urstad and Semenkovich, 2012).

PPARα, a transcription factor predominantly found in the liver but also present in muscles and adipose tissues, is one of the factors influencing lipid and carbohydrate metabolism. PPARα modulates metabolic factors such as FGF21, a hepatokine fasting-induced stress factor. It plays a role in the onset of liver diseases such as NAFLD.

b) Protein metabolism

Amino acids are the building blocks of proteins and are essential for various biological processes, roles in the synthesis of proteins, enzyme function, and as precursors for molecules involved in various metabolic pathways. The amino acids come from the gastrointestinal tract to the liver where they can be uptake by the liver or distributed to the peripheral organs. The muscle, intestine, and adipose tissue can produce amino acids. After the depletion of glucose and glycogen stores by a fasting period, the liver will utilize the amino acids coming from the muscles and adipose tissue like glutamine or alanine in the gluconeogenesis pathway to maintain the glycemia level and the ketogenesis pathway to produce ketone bodies (Paulusma *et al.*, 2022).

c) Fasting in the liver

Like the hypothalamus, the liver is a hub for the regulation of the nutrient-absorptive (fed) and post-absorptive state (fasting). After a meal, energy expenditure primarily utilizes glucose as a primary source of energy. If not replenished by another meal, the organism will first turn to the liver's glycogen stores via glycogenolysis. The enzymes for glycogenolysis will target the B first as they are easier to access and allow a slow burning of the glycogen stores (Jiang et al., 2016). In rats, glycogen stores will be depleted by 80% after a 24-hour fast (Remesar and Alemany, 1980) and close to null after 36 hours of fasting (López-Soldado et al., 2020). The energy source utilized in a fasting state will depend on sex. Male mice will preferentially reserve amino acids and use lipids, while females will dip into the amino acid pool to maintain the ATP source (Della Torre et al., 2018).

d) Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD), or more recently called metabolic associated fatty liver disease (MAFLD), is a condition characterized by excessive lipid accumulation in hepatocytes without significant alcohol consumption. Lipids originate from both exogenous sources through meals and endogenous sources through de novo lipogenesis or lipolysis in adipose tissue. The accumulation is partly due to VLDL not keeping up with fatty acid entry into the liver. NAFLD encompasses a spectrum of symptoms and disease severity, ranging from steatosis (fatty liver accumulation) to cirrhosis (advanced liver scarring), passing through non-alcoholic steatohepatitis (NASH) and liver fibrosis. NASH is characterized by liver inflammation and damage in addition to fat accumulation. NAFLD is often associated with metabolic risk factors such as obesity, type 2 diabetes, insulin resistance, and liver cancer (Nassir, 2022). Relevantly, in the early stage of NAFLD, rodents showed increased capillarization of LSEC, indicating poorer communication between hepatocytes and blood circulation (Pasarín et al., 2012).

D. Function: Through the LERKO story

As is the case with most health and human-related sciences, Mus musculus has been the animal model of choice for studying estrogen receptors. A multitude of transgenic mouse models were created to study the precise functions and impact of ERs within an in vivo model. Starting with an ERα total knockout, using cre-lox technology, mice with floxed sequences surrounding the ERα exon 3 and a cre mouse (Lubahn et al., 1993). Both male and female infertility mice were unable to produce pups, had atrophied ovaries, and had no cycle. A limitation of this model was that only the AF-1 domain (which is not present in all ERα isoforms) was deleted, meaning there was residual ERα activity and described by some not to be a complete ERα knockout. Another model targets the third exon, representing the DNA-binding domain, where the estrogen response element (ERE) binds and is present

throughout the ERα isoforms. In this model, female ERαKO mice display a polycystic ovary syndrome (PCOS)-like phenotype, with increased LH content, ovarian cysts, and the uterus lining cycling according to the estrus cycle. Gonadotropin stimulation increases oocytes in wild type (WT) mice but has no impact on aERKO as their number of oocytes was 0. aERKO mice have stunted fertility, and female mice failed to produce oocytes and, therefore, to start a pregnancy (Dupont et al., 2000). Male aERKO mice also have an impacted phenotype as a loss of germ cells is observed, and lower sperm motility results in infertility (Goulding et al., 2010). Both male and female aERKO mice have metabolic alterations such as insulin resistance and adipocyte hyperplasia. Male mice display hyperphagia-independent obesity, as their energy expenditure is lower.

Preventing DNA binding on ERα is enough for mice to develop a severe phenotype by altering vital functions such as reproduction and metabolism. However, the ER being ubiquitous, there is a need to detangle the impact of each tissue-specific ERα.

The liver is a significant player in reproduction and metabolism, and ERα being its main ER, it is necessary to understand the role of hepatic ERs and it's the complex regulation/physiology. To achieve this, a model derived from the aERKO was generated in 2011 by (Della Torre *et al.*, 2011), the liver estrogen receptor α knockout mice, LERKO using the CRE-Lox system (Gu et al., 1993). The LERKO is a cross between an ERαflox/lox mouse where the loxP sequences surround the third exon like aERKO and a C57BL/6-Tg (Alb-cre) 21Mgn/J mice. LERKO is hepatocyte-specific due to the CRE recombination of the LoxP sites being conditioned by the albumin promoter. Albumin RNA is found only in hepatocytes (Feldmann *et al.*, 1972; Della Torre *et al.*, 2011). Moreover, while ERα is principally expressed in hepatocytes, (Zhou et al., 2001).

1. The LERKO model, a constitutive mice model to study the liver ERα

The albumin promoter is used in these models to specify the invalidation in the hepatocytes. The LERKO model is designed to be constitutive model. The albumin promoter's activity in mice and the promoter's potency correlated with rodents' age. Mice with an alb-cre gene bred with a lox-glucokinase mouse will result in offspring recombining the gk gene. At birth, recombination of the gene seems to be only 40 % to rise to 80 % at six weeks slowly (Postic & Magnuson, 2000). Going against this observation, (Della Torre *et al.*, 2018) looked at the ERα hepatic expression in the LERKO at age E18.5, P0, P1, P8, P20, P40, and P50, and levels of mRNA stayed flat across the ages. As the control littermate showed similar expression at E18.5 and P1, it increases for males and females at P8 to remain higher in females through adulthood and decreases in males. This shows that the mice model LERKO is functional at an early age, and developmental compensation or redirection can occur. Interestingly, in males at P1, a peak of the ESR1 mRNA is seen and does not reproduce in LERKO and in females, linking to the masculinization role of hERα. Albeit milder than the ERaKO model, the LERKO pups have a slightly lower weight growth in the first 30 days of their life before catching their littermates. Female LERKO started to cycle five days earlier than the wild-type mice, but their cycles were regular.

2. hERα, a bridge for the metabolic and reproductive functions

(1) Basal metabolism in LERKO

The study of the metabolic parameters found that LERKO, either male or female mice, had no changes in overall body weight and glucose metabolism (Matic *et al.*, 2013). No change in body weight or cumulative food intake was seen in female LERKO mice as well (Meda et al., 2020). But cumulative food intake was lower in LERKO males. In this study, the lack of hepatic ERα change shifts toward the utilization of lipids more than carbohydrates, which may be due to reduced cumulative food intake during the dark period (Qiu et al., 2017).

The hepatic ERα function on food intake was highlighted in other contexts, such as a high-fat diet. Mice, male and female, under HFD ate more than their wild-type counterparts and changed lipid profiles.

(2) Calorie restriction leads to fertility impairment through hERα in females

In humans, females store more fatty acids, making lipids their preferred energy source during exercise compared to males in a fed state. In a fasted state, females will have higher levels of free fatty acids and lower glycemia than males (Horton et al., 1998; Soeters et al., 2007). Some reports depict a decrease in estradiol levels in females following fasting (Solianik et al., 2024), although this is not observed in every human study (Cienfuegos et al., 2022).

A decrease in nutrients and calorie intake can stunt vital aspects of any species, such as reproduction. In female physiology, a proper nutritional state is required to prepare a healthy environment for pregnancy. Such deficiencies lead to alterations in the estrous cycle. hERα proves to be a regulator, as a 40% calorie restriction is sufficient for hERα to alter the estrous cycle, with mice spending less time in the proestrus phase (Della Torre et al., 2011).

Transcriptomic analysis of LERKO mice after a six-hour fasting period revealed a sex-specific response to calorie deficit through hERα. It is noteworthy that muscles and adipose tissue were used as controls and did not demonstrate sexual dimorphism, further contrasting with the liver and hepatic ERα's impact on sex-dependent metabolic regulation.

After a short fast, female mice upregulated lipogenesis-related genes via hERα, synthesizing new lipids using amino acids. This strategy preserves stored lipids and supports reproductive needs, as suggested by the study. The wild-type male response was to use the carbohydrate route by upregulating genes related to gluconeogenesis and sparing amino acids, whereas the LERKO male strategy following fasting was closer to that of female wild-

type by using amino acids (Della Torre et al., 2018). In males, glycemia at 6 and 16 hours of fasting remains higher in LERKO. This glycemic dysfunction is accompanied by an increase in pyruvate and Glucose-6-phosphatase, indicating a spike in gluconeogenesis during fasting (Qiu et al., 2017).

3. NAFLD worsened by the lack of hERα

After examining the general metabolic parameters of LERKO mice, it was observed that they did not exhibit markers of non-alcoholic fatty liver disease (NAFLD), such as lipid accumulation (oil red staining) or collagen accumulation (H&E staining) in hepatocytes. However, Della Torre et al. (2016) reported different results in older mice, as the oil red test showed increased lipid accumulation in both 10-month-old male and female LERKO mice.

Imposing a high-fat diet exacerbated hepatic triglyceride accumulation in female LERKO mice, while female controls remained at basal levels observed with normal chow diet, highlighting the protective effect of ERα. This protective effect was not recapitulated in males, as both LERKO and wild-type male mice exhibited an increase in NAFLD-like phenotype (Meda et al., 2020).

Overexpression of ERα through injection of human ERα with an adenovirus into obese mice showed prevention of NAFLD by decreasing SREBP-1C transcription, aiding in relocating liver triglycerides (Wang et al., 2015).

The different stages of the estrous cycle are linked to estradiol levels, and hepatic ERα expression follows this trend, along with its effect on metabolism (Villa et al., 2012). The LERKO model helped in understanding that lipid metabolism follows the E2/ERα cyclicity, especially cholesterol transport and reverse cholesterol transport (RCT). Cholesterol, a lipid, is transported to the liver by high-density lipoprotein or exported from the liver to the circulation and periphery through low-density lipoproteins. In female wild-type mice, ERα helps increase the number and efficiency of HDL at the proestrus stage compared to the

lower estrogen phase, where more LDL is observed. Hepatic ERα ablation reduces HDL efficiency in bringing cholesterol to the liver (Della Torre et al., 2016, p. 216). This is a protective mechanism for females to prevent cholesterol build-up in the periphery and blood vessels. In post-menopausal women, LDL content increases alongside triglycerides, but HDL remains unchanged (Li et al., 2021). Previous studies assessed HDL levels but not their efficiency.

4. The estradiol is not the only hERα ligand

Estradiol is the ligand associated with the estrogen receptors. This pairing allows the E2 to exert its effect on metabolism and reproduction. Studies combining ERα-deprived mouse model, estradiol treatment, and ovariectomies confirmed this.

(1) Estetrol (E4)

The estetrol is the estrogen produced by the fetus during pregnancy, it was presented as a potential treatment for menopause. The E4 is partially independent of the hepatic ERα, indeed, E4 treatment in OVX mice can prevent insulin resistance, body weight gain, and steatosis. However, removing the hERα stunted only the steatosis protective effect as the obesity parameters remained protected (Buscato *et al.*, 2021).

(2) Amino acid

An *in vivo* experiment suggested that amino acids (AA) could potentially act as ER ligands. Using hepatocyte cells derived from the ERE-luciferase mice model, capable of activating the receptor, supplementation with amino acids was sufficient to increase hepatic ERα activity (Della Torre et al., 2011). A diet rich in amino acids binds to hepatic ERα to prevent metabolic impairments resulting from ovary ablation in mice. This estrogen-independent activation of hepatic ERα, as suggested by the authors, could lead to new strategies concerning menopause (Della Torre et al., 2021).

b) Boosting the ERα action: tamoxifen

Synthetic compounds known as selective estrogen receptor modulators (SERMs) can also act as ERα ligands. Examples of SERMs include tamoxifen and raloxifene, which are used clinically for purposes such as breast cancer treatment and osteoporosis prevention.

In the liver, ERα is primarily found in the hepatocytes; the receptor is also present in the liver sinusoidal endothelial cells (LSECs) and Kupffer cells. In the liver, ERα mediates estrogen's effects on various liver functions and metabolic processes.

As observed in the LERKO model, the ablation of hepatic ERα exacerbates or induces NAFLD-like symptoms on both standard and high-fat diets. These findings suggest that hepatic estrogen receptor α could be a therapeutic target for treating NAFLD. Tamoxifen, a selective modulator of estrogen receptors or SERM, initially utilized in breast cancer treatment (Ward, 1973), can act as either an agonist or antagonist to ERα depending on the cell type and target genes of the activation domains AF1 and AF2 (Berry, Metzger, and Chambon, 1990a).

In the case of hepatic ERα, tamoxifen treatment had a more pronounced impact on hepatic ERα than estradiol treatment in OVX mice with NAFLD-like symptoms and obesity. Tamoxifen, acting through ERα, can mitigate weight gain and lipid accumulation in adipose tissue and liver induced by a high-fat diet. Although estradiol treatment can reduce these parameters, its effects are less pronounced (Guillaume et al., 2019), indicating that tamoxifen could enhance the protective role of hepatic ERα against metabolic diseases.

This chapter established a common goal between the hypothalamus, estrogen, and the liver. This common goal is to maintain the proper function of both reproductive function and metabolism homeostasis. The next chapter will dive into the known facts about the Liver-hypothalamus axis. This chapter will also introduce the several hepatokines and possible links with the hypothalamus or brain. Indeed, the hepatokines being secreted by the liver into the blood circulation, like energy substrate, might be potentially significant in the hypothalamus regulation, as we will see in the results part of the thesis).

IV. The Liver, the hypothalamus, and the estrogen

This last chapter reunites all of the parts talked in the previous chapters. We will first describe some established mechanisms between the liver and the hypothalamus. In the second part of the chapter, we will focus on the hepatokines secreted from the liver, which can act as a communicator for the liver. We will look at their connection with hypothalamus but also with the estrogen and its receptor.

A. The hypothalamus and liver act together to regulate the metabolism

1. Innervation connection

In the previous chapters, we emphasized communication dependent on blood circulation and periphery-brain interfaces. Innervation by the autonomic nervous system is another essential communication tool. The innervated organs report to the inputs sent out by the brain. The liver's hepatocytes and sinusoidal cells are innervated by sympathetic and parasympathetic nerves connecting them to the hypothalamus. Notably, a connection between the liver and the hypothalamic PVN was established. The pathway liver–PVN goes one step further as it reaches other hypothalamic nuclei (LHA, ARH, NTS) (Stanley *et al.*, 2010). The PVN sympathetic efferent, stimulated by an electrical stimulus, triggered hepatic glycogenolysis, resulting in hyperglycemia **(fig.10)**. Parasympathetic stimulation, however, will result in glucose storage, indicating the role of innervation in liver glucose metabolism (Ban, 1967; La Fleur et al., 2000).

The hypothalamus innervates the liver, impacting lipid metabolism. Many studies have observed a connection between vagal signaling and VLDL synthesis and release, reverse cholesterol transport and lipogenesis (Taher, Farr and Adeli, 2017).

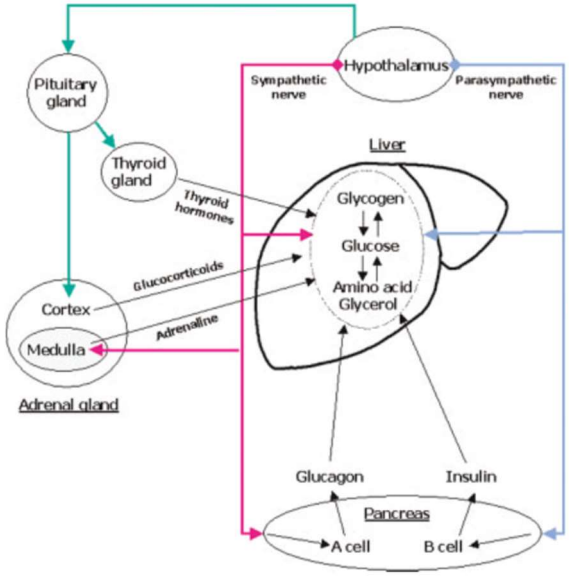


Figure 11: Nerve pathway between the hypothalamus and the liver

Schematic of the connection between the liver and the hypothalamus. Green lines represent the hypothalamic-pituitary pathway; pink represents the Sympathetic nerve pathway; blue represents the parasympathetic nerve pathway. The hypothalamus nerve pathway will directly act on the liver on the hepatic glucose metabolism or indirectly through the pancreas. Adapted from Uyama et al, 2004.

2. Glucose sensing

he brain's energy source is glucose, but it cannot store it. Humans can consume around 150g of glucose per day. Glucose uptake in the brain is facilitated by the GLUT1 and GLUT3 receptors. To modulate glucose levels, neurons can be either glucose-inhibited (AgRP, orexin) or glucose-excited (POMC, MCH). These neurons are primarily located in the hypothalamic nuclei, particularly the ARH and the VMH (Pozo and Claret, 2018). Fasting will increase the GLUT1 mRNA within the MBH of mice (Langlet, Levin, *et al.*, 2013a). The liver and hypothalamus collaborate in regulating blood glucose levels. The hypothalamus can stimulate the liver to release glucose when blood sugar levels are low or inhibit glucose production when they are elevated. Leptin I.C.V. injection in rats modulates hepatic glucose metabolism by suppressing hepatic glycogenesis and increasing gluconeogenesis (Rossetti

et al., 1997; Shi *et al.*, 1998). Additionally, insulin receptors located in orexigenic AgRP neurons in the MBH regulate hepatic glucose production, potentially conserving energy and increasing glycogen stores until the next meal (Könner *et al.*, 2007).

3. Lipid metabolism

Like glucose metabolism, lipid metabolism is central to the liver's function and is either stored, produced, or released by the liver. The released lipids in the circulation can be sensed by the hypothalamus (López *et al.*, 2005). In the other direction, the hypothalamus, specifically the arcuate nucleus, can control the hepatic lipid pathways. The ARH controls lipid utilization and triglyceride synthesis through the melanocortin system and MC3 and 4 receptors (Nogueiras *et al.*, 2007). The ARH could regulate the hepatic lipid distribution to other organs according to the nutritional status. Indeed, a central injection of NPY mimicking fasting, The ARH orexigenic peptide, was sufficient to trigger a VLDL release from the liver (Bruinstroop, Fliers and Kalsbeek, 2014; Taher, Farr and Adeli, 2017).

B. The relation of the hypothalamus and estrogen in metabolism

Estrogen link to the hypothalamus was observed in rodents where ovariectomy led to body weight gain and supplementation with estradiol led to reduced food intake, the hypothalamus being the metabolic control center, estrogen must pass by it (Roy & Wade, 1977). The estrogen reduces the food intake, in females, the food intake is therefore synchronized with the estrous cycle eating less in the proestrus-estrus and eating more at the diestrus stage (Asarian & Geary, 2002). The estrogen being more dominant in females, it makes sense that males' food intake is higher (Asarian & Geary, 2013).

Estrogen controls female metabolism differently than males; males are more sensitive to insulin than leptin (opposite for females). Indeed, high estrogen levels in females or castrated males supplemented with estradiol reduced their food intake after a leptin injection

compared to SHAM males. However, an insulin injection failed to reduce food intake in castrated males treated with E2 (Clegg *et al.*, 2006).

The estrogen receptors are found notably in the hypothalamus in the VMH, PVH, and ARH figures (Chakraborty *et al.*, 2003; Liu & Shi, 2015). Through the ERa, the estradiol will act on the POMC neuron population, by increasing its anorexigenic activity. However, a more extended estradiol treatment of OVX can decrease the POMC mRNA expression (Treiser & Wardlaw, 1992; Pelletier *et al.*, 2007). The opposite goes for the NPY neurons activity a shorter estradiol exposition will increase the peptide expression and OVX + E2 decrease the NPY mRNA in the PVN and ARH (Bonavera *et al.*, 1994; Shimizu *et al.*, 1996; Roepke, 2009). These results convey a separate response to a temporary, more physiological estrogen peak or a steady estrogen stream.

The estrogen receptor, ERα is expressed in tanycytes and vessels of the ME-ARH (Langub and Watson, 1992). While ERα is the main isoform, ERβ is also found in tanycytes. The presence of these receptors is indicating that the hypothalamus is sensitive to the estrogen. This is confirmed *in vitro* on endothelial and tanycytes coculture by (De Seranno *et al.*, 2010), where in a previous paper (De Seranno *et al.*, 2004) they showed the capability of endothelial cell to modulate the tanycytes endfeet retraction through the cytoskeleton (actin) to then precise that this mechanism be regulated in by an oestradiol treatment triggering endothelial nitric oxide secretion leading to the tanycytes retraction. This mechanism is enough to disrupt the estrous cycle in mice in vivo. The retraction of the tanycytes will have a specific purpose in reproduction function, the endfeet of tanycytes surround the terminal of GnRH neurons, and at the proestrus phase / pre-ovulatory phase where the estrogen is higher, the retraction of the tanycytes will allow GnRH release to the adenohypophysis and the LH /FSH. This morphological reshaping is reversed in the dioestrus (low E2) phase (Prevot *et al.*, 1999). Notably, higher levels of LH in female mice correlated with higher PLVAP-stained vessels, a marker for fenestrated vessels (Ciofi *et al.*, 2009).

In women, leakage within the BBB can be found years after a pre-eclampsia episode (high arterial tension due to a placenta defect) (Canjels *et al.*, 2022). Oestrogen can also be linked to the BBB as estrogenic treatment decreases the BBB leakage (via Blue Evans injection) induced by telencephalic injuries in zebrafish (Pellegrini *et al.*, 2023). In mice, an LPS challenge, triggering an inflammatory response leading to loosening the BBB in OVX mice, was restored by estrogen treatment. In this study mice, males were not as protected from the BBB rupture as the females, which might be due to lower levels of estrogen (Maggioli *et al.*, 2016). The VMH is also involved in sexual behavior and aggression behavior and seems to differ between males and females, the dichotomy being driven by Estrogen receptor α (ERα) expressing neurons (Hashikawa *et al.*, 2017; Khodai and Luckman, 2021).

C. The bridge between the hypothalamus and liver: Hepatokines

Hepatokines are molecules secreted mainly or exclusively by the liver into the circulation to carry out messages of the liver's metabolic status and trigger adaptation in other organs. Hepatokines represent a vast study topic, some of them more studied than others, but to comprehend the role of the liver in communication with its periphery, it is vital to understand the functions of the hepatokines and the environment in which they operate. Here is an overview of a non-exhaustive list of hepatokines important to metabolic health and their link with the brain or the hypothalamus and the estrogen **(table 3)**.

1. Growth Differentiation Factor-15 (GDF15)

The growth differentiation factor – 15 (GDF15) is highly expressed in the mice liver and adipose tissue but less in human livers under the basal state. It seems the GDF15 is a stress marker and an unbalanced metabolism marker in humans as it is positively correlated with aging, HIV, exercise, and anorexia (Agarwal *et al.*, 2022; Conte *et al.*, 2022; Plomgaard *et al.*, 2022). The hepatokine has attracted much attention in the last years in the neuroendocrinology fields through its role in anorexia and its ties with one of the CVOs (cf

chapter ME-ARH), the area postrema. With its receptor GFRAL, the hepatokines induce an anorexic effect in mice (Breit *et al.*, 2023; Zhang *et al.*, 2023). However, its increases during a fasting period trigger B oxidation to fuel ketogenesis in mice (Zhang *et al.*, 2017). GDF15 is considered a therapeutic approach to prevent/reverse obesity and NAFLD symptoms. As tested in female mice, selective activation of the hepatic ERα by tamoxifen acts by GDF15 to induce weight loss and adipose tissue accumulation (Guillaume et al., 2019). This paper further hints at the communication between the liver and the hypothalamus.

2. Fibroblast Growth Factor 21 (FGF21)

From the FGF family, Fibroblast Growth Factor 21 is primarily synthesized in the liver alongside FGF15 and FGF19. This particular FGF is mainly studied because of its high implication in metabolism. It is considered like GDF15 as a metabolic stress hepatokine, increased in fasting periods and obesity. In humans with a healthy body mass index (BMI), the FGF21 will stay put during a 60-hour fasting; another experiment described that a 10-day fasting was needed to increase FGF21 in healthy participants. By dissecting the FGF21 levels in the first three days of fasting in female patients, a study found that the FGF21 levels remained unchanged during the day compared to the fed state, and the FGF21 peaked during the night (Andersen, Beck-Nielsen, and Højlund, 2011). Inversely, in obese patients, FGF21 positively correlated with BMI and decreased after 12h-36h of fasting (Fazeli *et al.*, 2015; Nygaard *et al.*, 2018). In mice, hepatic FGF21 is more reactive, and an overnight fasting period is sufficient to increase the FGF21 serum levels (Archer *et al.*, 2004). Fasting modification of the FGF21 levels can lead to the question of the hepatokines involvement in the hypothalamus regulation of metabolism. In addition, the mice knockout increased weight, lean, and fat mass (at 24 weeks), but KO fasted mice had less ambulatory activity and overall energy expenditure, but no changes were observed in food intake. Furthermore, FGF21 seems to be controlled in female mice by the hERα by having ERE sequences and FGF21

levels not rising in LERKO-OVX mice repleted with E2 treatment. Tying to the ovariectomized female patient, a hormone replacement therapy (estrogenic compound) was associated with lower FGF21 (Badman *et al.*, 2009; Allard, Bonnet, *et al.*, 2019). Fluorescent-FGF21 peripheral injection reaches the median eminence and its fenestrated vessels, showing the ability to reach the hypothalamus (Xu *et al.*, 2017). Its potential as a messenger was confirmed in the (Pena-Leon *et al.*, 2022) project, where hepatic FGF21 activates GABA/dopamine receptor 2 neurons in the LHA at weaning stages to set up a protective environment from obesity. In adult mice, hepatic FGF21 is sufficient to trigger glutamatergic neurons in the hypothalamic nuclei VMH and reduce sugar intake (Jensen-Cody *et al.*, 2020).

Of note, FGF21 is found to be expressed in the tanycytes and is active in a fasted state to activate neurons capable of increasing lipolysis in the adipose tissue (Geller *et al.*, 2019).

3. Tsukushi (TSK)

The primary liver-expressed protein, Tsukushi (TSK), was the subject of a recent paper highlighting the impact of hepatokine on obesity regulation. The fed-induced hepatokine seems to have deleterious effects on the metabolism. Indeed, the invalidation of TSK in an MC4R deficient obese mice model, is enough to reduce the food intake and body weight gain of these mice (Q. Wang *et al.*, 2021).

4. Hepatocyte Growth Factor (HGF):

Hepatocyte Growth Factor is primarily produced in the liver, mainly in endothelial and Kuppfer cells (Matsumoto & Nakamura, 1992). Moreover, it is known for its liver regenerating capabilities through its c-MET receptor, and *in vitro*, the couple can increase VEGF and its subsequent angiogenesis (Zhang *et al.*, 2003). In another *in vitro* experiment, HGF treatment on GnRH-1 neurons (GN11) cell lines accelerated the migration of neurons (Giacobini *et al.*, 2002).

Table 3: List of hepatokines and associated roles in metabolism

Hepatokine	Metabolic implications	Relation with (h)ERα	Communication with the hypothalamus
GDF15 Receptor: Gfral	Human: Increase with anorexia, aging, and exercise (Agarwal et al., 2022; Conte et al., 2022; Pommard et al., 2022). Mice: Increase β-oxidation in fasted mice (Zhang et al., 2017). Knockout: (M: male) increases weight, and earlier onset obesity in HFD (Tran *et al.*, 2018).	hERα activated by tamoxifen (but not E2) increases GDF15 and prevents obesity in mice (Guillaume *et al.*, 2019)	Activation of the HPA (corticosterone) pathway (Cimino *et al.*, 2021)
FGF21 Receptor: FGFR1; β-klotho	Human: (M+F) Fasting in healthy patient FGF21 ↑ after 10 days; in obese patient: (M) FGF21↓after 36 hours (Nygaard *et al.*, 2018) Mice: (M) fasting: FGF21 ↑ Knockout: BW ↑ and EE ↓	FGF21 possesses ERE sequences and is controlled by hERα (Allard, Bonnet, *et al.*, 2019)	Reaches the hypothalamus (Xu et al., 2017) The protective effect of obesity (Pena-Leon et al., 202)
DPP4 Receptor: GLP1R	Human: Inhibitors are used as type 2 diabetes treatment (Hwang *et al.*, 2015) Mice : (KO) inflammation ↑ (Ghorpade *et al.*, 2018)	/	Decrease Aβ in I/R in OVX mice (Pratchayasakul *et al.*, 2017)
HGh **c-MET** Receptor:	/	Estrogen modulates HGF ERE: yes (Liu, Lin and Zarnegar, 1994)	/
Fetuin-A Receptor: IR	/	/	Protection against brain-related inflammation (Heinen *et al.*, 2018)
Tsukushi	Positive correlation with NAFLD (Lam *et al.*, 2024)	/	Increase obesity parameters (Q. Wang *et al.*, 2021)
Adropin Receptor: GPR19	Water intake (Jasaszwili *et al.*, 2020)	Regulated by hERα (Meda et al., 2022; Stokar et al., 2022	/

5. Dipeptidyl peptidase 4 (DDP4)

DPP4 is a 110KDa transmembrane glycoprotein. Mainly found in the liver and therefore considered hepatokines, DPP4 is also found secreted by the adipose tissue. DPP4 inhibitors are used as a complementary treatment to metformin for type 2 diabetes patients (Deacon, Mannucci and Ahrén, 2012; Hwang *et al.*, 2015). DPP4 hepatocyte-specific knockout mice showed less DDP4 in the circulation, indicating the liver-DPP4 could communicate with other organs. The study also concluded that the deficiency in DPP4 had beneficial effects as it decreased inflammation and reversed glucose intolerance and insulin resistance in obese

knockout mice (Ghorpade *et al.*, 2018). DPP4 has an interesting link with estrogen regarding the cardiac ischemia-reperfusion injury, which is an aftermath effect of tissue reoxygenation leading to increase in β-amyloid (Aβ) in the brain. In OVX mice (estrogen deficient), the insulin resistance will cause higher Aβ production, which is counteracted when these mice are injected with either a DPP4 inhibitor or an estrogen treatment (Pratchayasakul *et al.*, 2017). This paper forms a connection between the hepatokine and a possible relationship with the estrogen receptor and also leads to an insulin resistance treatment specific to menopausal women.

6. Fetuin-A

Fetuin-A is primarily synthesized in the liver in adulthood and embryonic cells and is a member of the cysteine protease inhibitors (cystatin) family (Chekol Abebe *et al.*, 2022). The hepatokines is a 60KDa carrier protein, first observed in the brain at development stages, and now is seen to have a protective role against inflammation in the adult human brain (Heinen *et al.*, 2018). The hypothalamic-pituitary-thyroid axis connects the fetuin-A and hypothalamus. The hepatokine levels in humans are correlated with those of the thyroid-stimulating hormone (TSH), which is a pituitary hormone regulated by the hypothalamic TRH and triggering the thyroid function (Dadej, Szczepanek-Parulska and Ruchała, 2022).

7. Insulin-like growth factor (IGF-1)

Like Fetuin-A, insulin-like growth factor 1 (IGF-1) is a hepatokine involved with the hypothalamus-pituitary axis. The growth hormone-releasing hormone (GHRH) neurons in the hypothalamus trigger growth hormone (GH) production and release in the pituitary gland. Once GH is released into the circulation, it reaches the liver, which then releases IGF-1 into the circulation to act via the IGF-1 receptor (IGF-1R). IGF-1 can regulate its own production via the IGF-1R located in the hypothalamus and pituitary gland (Al-Samerria and Radovick, 2021). It is known that in females, IGF-1 varies across the estrous cycle, and this hepatokine

is essential for the growth of the uterine lining in preparation for a possible gestation. It makes sense that hERα is associated with this pathway, as evidenced by decreased IGF-1 levels and a shortened estrous phase (Della Torre et al., 2011).

8. Angiotensinogen

Angiotensinogen is a peptide expressed in the liver and is part of the renin-angiotensin system (RAS). This system regulates sodium intake and water homeostasis. Angiotensinogen undergoes an enzyme cascade regulated by renin from the kidney and the angiotensin-converting enzyme (ACE), resulting in angiotensin I and II. Angiotensin II acts on the Angiotensin II receptor type 1 (AT1R) to increase thirst, vasoconstriction, and sympathetic tone (Lubel et al., 2008). Angiotensin can cross the blood-brain barrier (BBB). However, it might not entirely originate from the liver, as it can also be produced in astrocytes by increasing glutamate concentration. Angiotensin can modulate the excitatory signaling of hypothalamic PVN sympathetic neurons and can also act on the subfornical organ (SFO), a hub for water intake regulation (Mendelowitz, 2016). Estrogen, through hERα, can impact the renin-angiotensin system by increasing angiotensinogen secretion into circulation. This aligns with menopause in humans, where decreased estrogen levels reduce RAS activity, leading to inflammation (Stavréus-Evers et al., 2001; Gersh et al., 2021).

9. Follistatin (FST):

Follistatin is expressed in the liver and also in the hypothalamus of rodents and primates; however, the primary source in humans is the liver. Follistatin has been found to be positively correlated with glycemia and type 2 diabetes in humans (Hansen and Plomgaard, 2016). In primates, GnRH stimulation of FSH can be stunted by follistatin (from the ovaries) through the inhibition of the protein activin (Meriggiola et al., 1994; MacConell et al., 1998).

10. Adropin

Adropin is a 43-amino-acid peptide hormone encoded by the ENHO gene. It is primarily expressed in the liver but also in the brain, heart, and pancreas. Adropin acts via its receptor GPR19, and this pairing inhibits water intake in rodents. However, adropin appears to have more functions than water homeostasis. Adropin is heavily involved in metabolism; its levels increase in obese mice, it can increase insulin sensitivity, and it has a protective effect against fatty liver disease (Jaswiec et al., 2020). This protective effect is particularly observed in female mice, where estrogen, via hERα, increases adropin levels, and metabolic impairment due to ovariectomy is reversed by adropin treatment (Meda et al., 2022; Stokar et al., 2022). In the brain, adropin could have another protective function for neurons by protecting the BBB integrity through increasing VEGFR2 and angiogenesis (Gunraj et al., 2023).

11. Leukocyte cell-derived chemotaxin-2 (LECT2)

The leucocyte cell-derived hepatokine, or LECT2, was first discovered by Yamagoe et al. (1996) through the purification of a human leukemia T-cell line. The 16 kDa protein was initially described as a neutrophil chemotactic protein in an *in vitro* assay due to its homology with Mim-1, a neutrophil-activating protein in chickens. Independently characterized in the same year, as a growth-promoting factor, chondromodulin II (Chm-II), was discovered from fetal bovine cartilage, exhibiting low heparin affinity and promoting proteoglycan synthesis with a sequence identical to LECT2 (Hiraki et al., 1996).

LECT2 showed less chemotactic activity than known chemotactic proteins such as interleukin-8 or N-Formylmethionyl-leucyl-phenylalanine, suggesting additional functions for LECT2 (Yamagoe et al., 1996). This chemotactic function was further detailed in later papers, elucidating its role in leukocyte migration towards sites of inflammation.

Of note, LECT1 (leukocyte cell-derived chemotaxin-1 or chondromodulin 1), like LECT2, is a chemotactic factor that helps recruit immune cells to sites where their activities are needed to combat infections or respond to tissue damage, guiding leukocytes to sites of inflammation or infection. Another similarity is that LECT-1 plays a role in angiogenesis and osteoarthritis progression (Zhu et al., 2019).

Indirect immunoperoxidase staining of human liver biopsies showed the presence of LECT2 in the cytoplasm of all hepatocytes and the endothelial cells of the arteries and the liver's portal vein. However, no LECT2 protein was found in the sinusoidal endothelial cells (Yamagoe et al., 1997; Nagal et al., 1998). Quantitative transcriptomics analysis (RNA-Seq) in humans shows predominant expression of LECT2 in the liver and sparse expression in the testis, kidney, and adipose tissue (Fagerberg et al., 2014). The Human Protein Atlas shows LECT2 expression mainly in the liver, muscles, and bones but not in the brain (Uhlen et al., 2010).

a) Role of LECT2 in liver's diseases, a friend or a foe?

LECT2 is part of the innate immune system and is involved in the early stages of immune responses to various pathogens, including bacteria and viruses. It contributes to the rapid mobilization of immune cells to the site of infection or tissue damage **(table 4)**.

In human studies, the livers of women and men with acute liver failure were found to have glutamate pyruvate transaminase/glutamic oxaloacetic transaminase or GPT/ GOT (Markers for liver disease) levels inversely correlated to LECT2 serum liver. As the liver failure improved, LECT2 serum content increased, and patients who survived the hepatic acute failure had higher LECT2 levels compared to the deceased patients, suggesting that LECT2 has a role in the regeneration of hepatocytes after injury (Sato *et al.*, 2004). In mice studies, mice injected with concanavalin, a carbohydrate-binding protein known to harm hepatocytes, showed injured liver by their GPT levels rising between 8 and 24 hours after the intravenous injection and LECT2 levels decreased at the same time (Tiegs, Hentschel

and Wendel, 1992; Gantner *et al.*, 1995). These results were confirmed and exacerbated by using LECT2 deficient mice as well as higher content of Il-4 and TNF-α by activating NKT cells (Saito *et al.*, 2004; Okumura *et al.*, 2017). Changes in LECT2 according to hepatocyte damage and a role in regulating immune-adapting show the hepatokines involvement in liver disease. Notably, the change in the plasma LECT2 levels might hint at a role outside the liver.

At the same time, a higher level of LECT2 could indicate a hepatic steatosis when acting on its receptor CD209, activating the JNK pathway and increasing TG accumulation and insulin resistance in mice (Hwang *et al.*, 2015). Similarly, for NAFLD, overexpression of hepatic LECT2 in HFD mice worsens the NAFLD symptoms by regulating the STAT-1 pathway (J. Wang *et al.*, 2021). This harmful aspect could be explained by an increase in endoplasmic reticulum stress, which can lead to the death of hepatocytes and can increase LECT2 expression (Lebeaupin *et al.*, 2018; Park *et al.*, 2021).

Recent studies on liver fibrosis unveiled a new role for the LECT2 in regulating angiogenesis through Tie1, an endothelial-specific receptor. Tie1 is a part of the TIE receptor family with Tie2, ANGPT-1,2 and 4, there are tyrosine kinases receptors. Like the VEGF receptor family, this family is important for blood vascular and lymphatic vessel development and regulation (Saharinen *et al.*, 2015).

b) Metabolic Regulation: insulin resistance to obesity: LECT2, a fast-acting protein

In the last decade, many functions have been attributed to LECT2, notably in the metabolic realm. Indeed, plasma levels of this hepatokine are correlated with the severity of NAFLD and metabolic syndrome (Yoo, Hwang, Choi, Lee, Chung, Ji-A Seo, et al., 2017). A positive correlation is also observed with higher body mass index (BMI), linking LECT2 to obesity and insulin resistance (Lan et al., 2014a; Huang et al., 2018). In mice, LECT2 levels decreased after a fasting period (17h and 24h) in both ICR and C57BL/6J mice (Kikuchi et

al., 2020). These results highlight the sensitivity of LECT2 to metabolic perturbations and energy changes, both short and long-term.

Furthermore, LECT2 seems to have a relatively high turnover. After administering radiolabelled LECT2 recombinant protein and analyzing in vivo kinetics in male mice, its half-life in plasma is around 30 minutes. It is rapidly secreted from the kidney into the urine, where it is detected for a few hours. Notably, LECT2 remains detectable in intestinal content and the testis for up to 3 hours (the experiment cut-off time) (Kikuchi et al., 2020). Changes in body weight alongside LECT2 levels impact physiology and require the body to adapt to constantly changing energy intake, which could have long-term consequences on insulin resistance and weight gain. Mice fed an HFD show an increase in LECT2 levels after one week of the diet, which remains elevated until the mice switch to a chow diet. LECT2 serves as an overfeeding marker along with insulin, liver triglycerides, and glycemia (Lan et al., 2014a; Chikamoto et al., 2016).

LECT2-deficient mice exhibit the same body weight, food intake (when ad libitum), and thermogenesis as wild-type mice but have higher energy expenditure (running wheel) under a basal diet. Under an HFD, wild-type mice develop muscle insulin resistance, which is less pronounced in LECT2 KO mice (Lan et al., 2014a), strengthening the hypothesis that sustained high levels of LECT2 could be harmful.

This makes LECT2 a rapidly adaptive protein to metabolic changes and possibly an actor in homeostasis maintenance. These studies could form the basis for research on whether LECT2 resistance is possible and if it could have consequences on physiology. As with insulin or leptin, high levels of LECT2 should not persist; they are designed to be acute and temporary unless the liver and hepatocytes are targeted and LECT2 expression and secretion are disrupted.

LECT2 seems to have functions beyond hepatic regulation. *In vitro* studies of LECT2 KO mice show that the dendrites of hippocampal neurons appear to be shorter due to LECT2-dependent microtubule regulation. Furthermore, LECT2 regulates the expression of specific neuron markers such as BDNF, a growth factor involved in learning (Koshimizu and Ohtomi, 2010a, 2010b).

Table 4: LECT2 correlation with metabolic parameters

Parameters	LECT2 correlation	Studies
Liver TG	**Positive**	(Chikamoto *et al.*, 2016)
Adipose TG	**No correlation**	(Chikamoto *et al.*, 2016)
Systemic inflammation	**Positive**	(Ando *et al.*, 2012)
Liver failure/hepatocyte lesions	**negative**	(Tiegs, Hentschel and Wendel, 1992; Gantner *et al.*, 1995)**.**
Fibrosis	**Positive**	(M. Xu *et al.*, 2019)
BMI, obesity severity	**Positive**	(Okumura *et al.*, 2013 ; Lan *et al.*, 2014 ; Huang *et al.*, 2018)
Exercice	**Négative**	(Lan *et al.*, 2014a)
Fasting	**Negative**	(Lan *et al.*, 2014a; Kikuchi *et al.*, 2020)

The liver and the hypothalamus are heavily regulated by estrogen, which could lead to the hypothesis that some of these hepatokines are regulated by estrogen and might impact the hypothalamus accordingly.

Objective

The Hypothalamus and liver are key organs in metabolism and reproduction. These two functions, especially in females, coexist to modulate homeostasis depending on reproductive needs. Estrogen, a sexual hormone, is often responsible for their coupling.

This project aims to decipher if, similar to metabolism and reproduction, estrogen could be involved in the communication between the hypothalamus and the liver via the hepatic estrogen receptor α (hERα).

Our objectives were to: (1) investigate if the hepatic estrogen receptor α modulated the ME-ARH region's nutritional-dependent plasticity in the hypothalamus in male and female mice, (2) observe possible metabolic consequences in the mice model, and (3) to investigate the possible pathway between the hepatic estrogen receptor α and the ME-ARH.

To achieve these objectives, we used a mouse model called the LERKO or Liver estrogen receptor KO. We designed *in vivo* experiments and used a fasting protocol followed by immunolabelling of the fenestrated vessels and tight junction to assess the ME-ARH plasticity. To further understand the metabolism of these mice, we used metabolic cages in different nutritional contexts, i.e., fed and fasted states. After the second axis of this project, we focused only on the female mice. To determine how the hERα could act on the ME-ARH's plasticity, we looked at the hepatokines produced and secreted by the liver. We used RNAseq resources and hybridization *In situ* technique to narrow down a list of hepatokine-ligand couples and set our focus on LECT2-Tie1. Lastly, we studied the direct effect of LECT2 in the hypothalamus by injection of recombinant LECT2 in Wild-type female mice after a fasting period and assessed the ME-ARH plasticity.

RESULTS

I. The hepatic ERα's sexual dimorphic role on the hypothalamus plasticity

A. ME-ARH barrier adaptation to fasting is similar in female and male Wild-type adult mice

Fasting-induced plasticity of the interface between the blood and ARH is one of the regulatory mechanisms for maintaining energy homeostasis. This plasticity consists of an increase in the number of fenestrated vessels found in the parenchyma of the ME and vmARH when animals are deprived of food, in contrast to those fed ad libitum (vascular plasticity). A previous paper first described the ME-ARH barrier reorganizing in male mice in response to a prolonged 24h (13h-13h) fasting period (Langlet, Levin, et al., 2013a). The number of fenestrated vessels in the median eminence and near the vmARH increases.

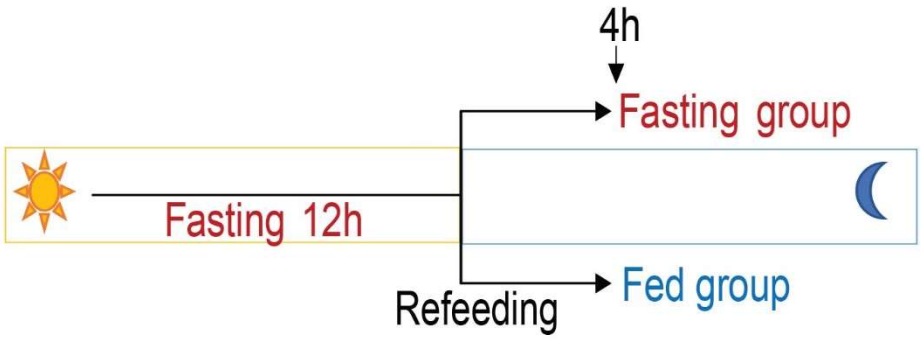


Figure 12: Experimental fasting protocol

To study the plasticity induced by fasting, all the mice (from the FED or FASTED groups) were deprived of food during the resting phase to calibrate the energy state of the animals. At the start of the active phase, the animals are divided into 2 groups: while half of the LERKOs and LERWTs have access to food again (Fed condition) the other half remain fasting (Fasting condition). Animals from both groups (Fed and Fasting) were sacrificed after the first 4 hours of the active phase.

This is accompanied by the reorganization of ZO-1, a protein of the tanycytes' tight junction. However, no publication mentioned whether this phenomenon is replicated in female mice.

In this project, we decided to use a different fasting protocol by using 16 hours fasting **(fig.12)**. All the mice are subjected to food deprivation throughout the resting phase to standardize the energy state of the animals. Then, at the start of the active phase, the mice were separated into two groups: half of the mice were given access to food (Fed condition), while the other half remained in a fasted state (Fasted condition). We chose this particular fasting protocol to study the communication between the liver and the hypothalamus. This particular fasting protocol is more physiological due to the majority of the fasting happening during the resting phase of the mice. This 16h period will allow a better contrast between the fed and fasted group in the liver metabolism (Montagner et al., 2016).

The first point of this project is to assess if (1) A 16 h fast is enough to induce the fasting-dependent plasticity in the ME and the vmARH and (2) if female mice follow a similar pattern as male mice.

To assess the ME-ARH region plasticity, immunochemistry with fluorescence was performed in the hypothalamus slice. The fenestrated vessels will be MECA-32 antibodies and counted in the ME and vmARH region separately. The tight junction organization will be observed with a ZO-1 antibody.

To test our results, we first used a t-test to compare the fed and fasted conditions in the vmARH and the ME in males and females, respectively. These results show that **male and female** mice subjected to fasting have increased fenestrated vessels in the vmARH and ME regions **(fig.13).** However, subsequent tanycytes' tight junction reorganization is only found in the vmARH vicinity in our study. The male and female wild-type mice respond similarly to fasting at the ME-ARH region. This fasting protocol is sufficient to reorganize the fenestrated

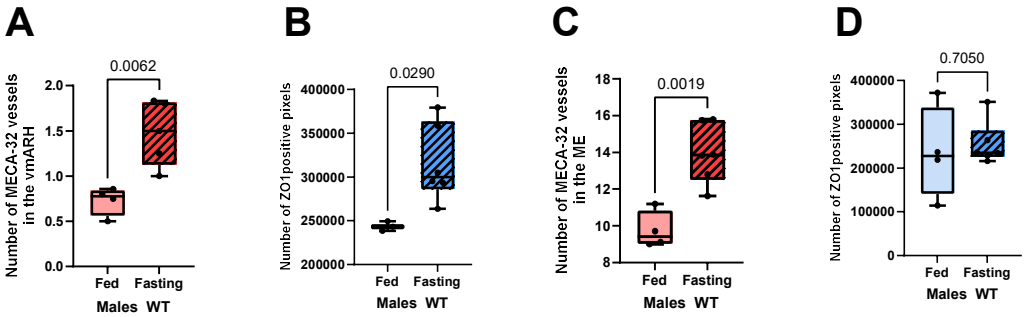
vessels in the vmARH and the ME. However, a limitation of this protocol seems to be the weaker reorganization of the tight junction due to fasting in the ME region.

We then compared the male's and females' plasticity using a Two-way ANOVA test followed by a Fisher's LSD (least significant difference) test **(fig.13 I-L).** In females and males, we still see an increase in FV in the vmARH and ME regions. For the tight junction, only the female's vmARH region seems to respond to the fasting **(fig.13K)**. There is a difference between the male and female' TJ's reorganization in the vmARH region. Indeed, after 16 hours of fasting, the female mice will have a significantly higher level of ZO-1 positive signal than males.

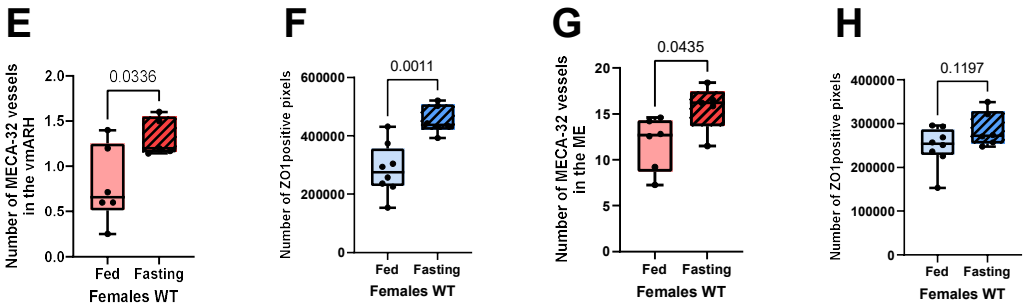
The first objective allowed us to confirm that a 16-hour fast has an impact on ME-ARH fenestrated vessels' plasticity in both males and females

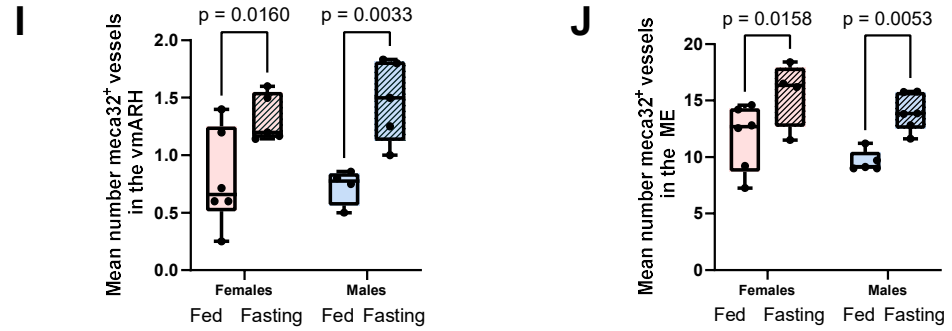
In this project, we investigate the role of the hepatic ERα receptor in regulating the ME-ARH interface using the *LERKO* model established in 2011. This model allowed a more precise approach than a complete ERα knockout by studying the specific role of the ERα hepatic receptor. Thanks to this model, the role of hepatic ERα has been identified in hepatic metabolism in preventing steatosis, metabolic diseases such as obesity and diabetes, and, interestingly, in the hypothalamic control of energy metabolism.

Males adaptation to fasting



Females adaptation to fasting





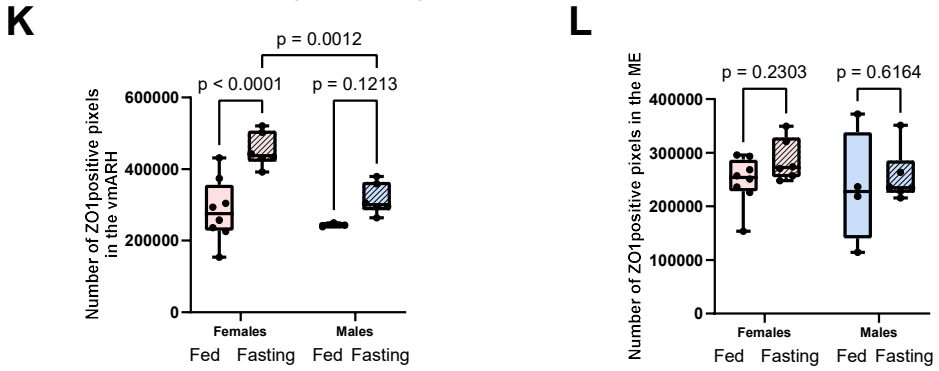


Figure 13: ME-ARH barrier adaptation to a fasting period is similar in female and male adult mice.

Male and female wild-type mice were fasted for 16h following the protocol in fig.12. Fenestrated vessels (MECA-32) and tight junction of tanycytes (ZO1) were analysed in males (A-D) and females (B-H) in the vmARH region (A ,B ,E,F) and ME region (C,D,G,H). The potential for ME-ARH reorganization sexual dimorphism was further appreciated in the vmARH region (I) and ME region (J) for the fenestrated vessels and vmARH (K) and ME (L) for the tight junction. Data were analysed with a t-test or a two-way ANOVA test followed by a fisher's LSD test.

B. LERKO mice show impaired fasting-induced plasticity of the blood/ARH interface in female mice

To investigate the role of hepatic ERα in liver-hypothalamus communication and its possible impact on ME-ARH nutritional-dependent regulation, we compared the vascular plasticity of the hypothalamus of LERKO mice. As stated in chapter 3 of the introduction, the LERKO is a knockout model of the estrogen receptor α, specifically in the hepatocytes. The control of the LERKO will be the LERWT, their wild-type littermates.

Quantifying MECA-32 positive vessels detected in the ME and ARH (in red) and the number of ZO-1 pixels assessing the tanycytes barrier adaptability (in green) in the tuberal region of LERKO and LERWT mice, the plasticity of the blood/ARH barrier was analyzed in both female mice and male mice.

1. The hepatic ERα impact on the MBH nutritional-dependent plasticity of the **female** mice

In response to 16 hours of fasting, the number of MECA-32 positive vessels in the vmARH region and in the ME of the LERWT female mice significantly increased during fasting compared with the fed LERWT female mice **(fig.13 I-J)**. However, for the LERKO female, the number of fenestrated vessels was consistent across the fasted and fed conditions in both regions. Additionally, comparing fasting for the two genotypes, the number of fenestrated vessels was significantly reduced in LERKOs compared to LERWTs in both

regions **(fig.14 B-D)**. These results highlight a role for the hepatic ERα in the ME-ARH plasticity.

These findings indicate that the number of MECA-32 positive fenestrated vessels in LERKO mice is similar regardless of the animal's energy state and comparable to the fed state in LERWT mice.

Then, we looked at the tanycytes barrier plasticity by analyzing ZO-1 expression. At the vmARH level, ZO1 immunostaining in LERWT shows a significant difference in the number of ZO1-positive pixels and a more structured expression pattern in the fasted condition compared to the fed condition. This difference was not observed in LERKO, where ZO1-positive pixel numbers and expression patterns did not differ between the fed and fasted conditions. **(fig.14 C)**. Despite the ZO-1 signal being increased in the fasted LERWT mice (vs. FED LERWT), it was not significantly increased compared to fasted LERKO female mice. Only the fed state showed a significant difference when comparing the two genotypes, with an unexpectedly higher ZO-1 signal in the fed LERKO compared to the fed LERWT **(fig.14 C)**.

Taken together, at the vmARH level in LERWT mice, the rise in fenestrated vessels is linked to a rearrangement of the ZO1 expression pattern during fasting. Neither vascular plasticity nor tanycytic plasticity was observed in the LERKO mice.

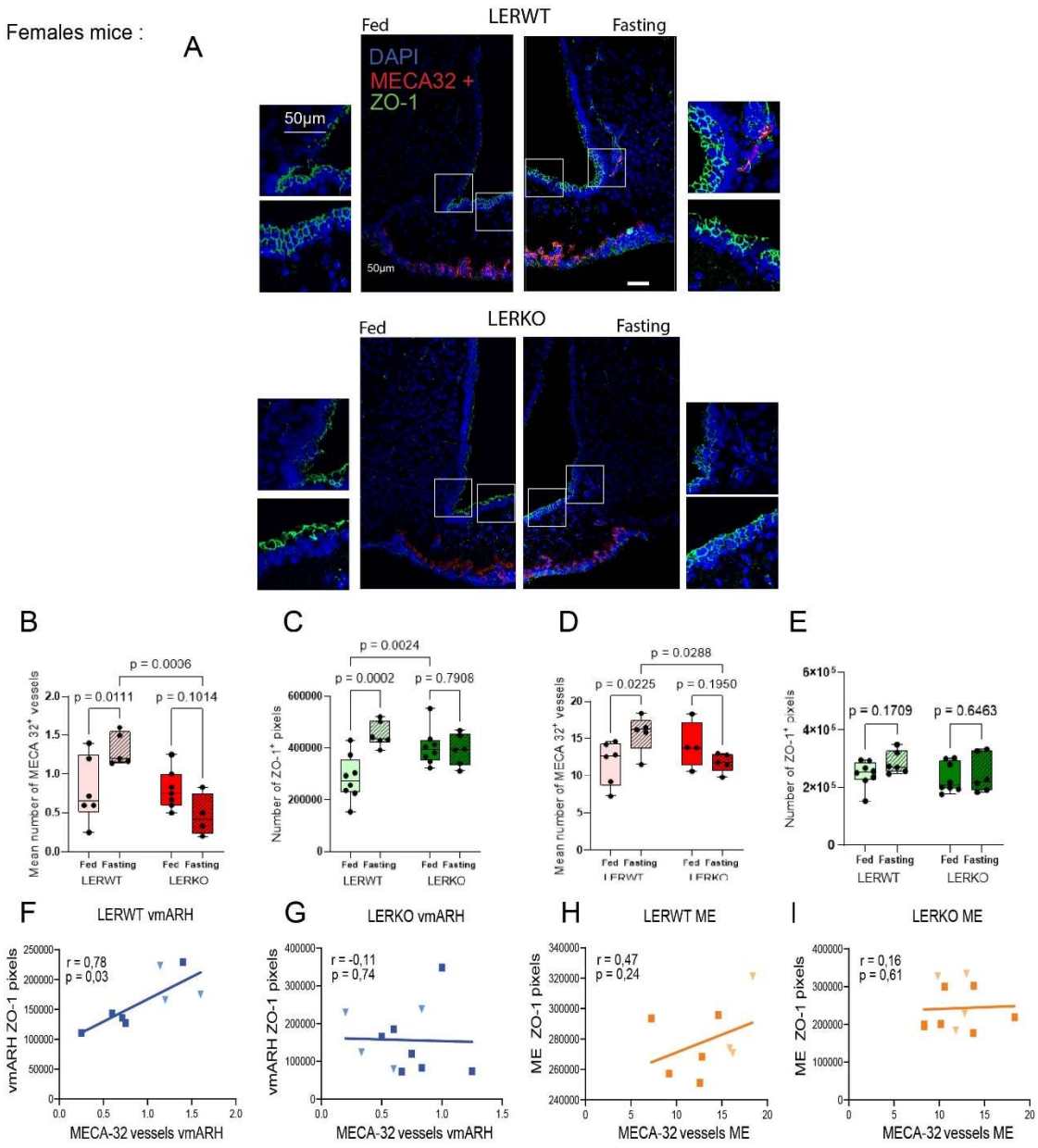


Figure 14: Fasting-induced plasticity of the blood-ARH interface is impaired in female LERKO mice

(A) Meca3-32 (red) and ZO-1 (green) immunoreactivity in the tuberal region in fed and fasting LERWT and LERKO female mice. Diet and genotype effects on the number of Meca-32-positive micro-vessel loops and ZO-1 positive pixels in the vmARH (B-C) and ME (D-E) in LERWT and LERKO female mice. Correlations between the fenestrated vessels and the tight junction positive pixels located in the vmARH (F-G) and ME (H-I) of the LERWT and LERKO female mice, respectively. Fed (•) and fasted (Δ) mice were pooled in the nonparametric Spearman correlations. Data are represented in box and whiskers (min to max) or mean +- SEM. Statistical analysis was done using a two-ANOVA test followed by a Fisher's LSD test comparison analysis and a Spearman correlation test.

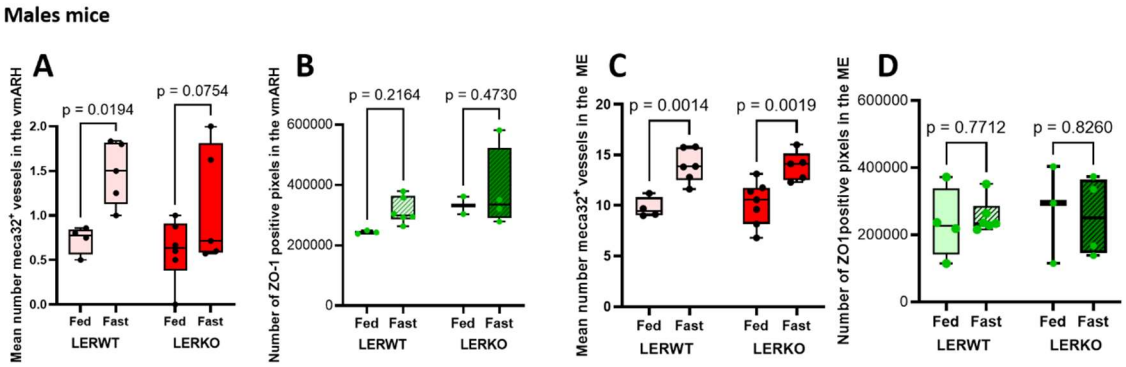


Figure 15: Fasting-induced plasticity of the blood-ARH interface is unchanged in male LERKO mice.

The same experimental protocol was used to study plasticity as the one described in Figure A-2. Diet and genotype effects on the number of Meca-32-positive micro-vessel loops and ZO-1-positive pixels in the vmARH (A-B) and ME (C-D) in LERWT and LERKO male mice. Data are represented in boxes and whiskers. Statistical analysis was done using a two-ANOVA test followed by a fisher's LSD test.

2. The fasted male LERKO mice

When observing the male mice, the invalidation of the hepatic estrogen receptor α tells a different story. A 16-hour fasting increases the fenestrated vessels in the vmARH and ME region (fig.15 A-B). The LERKO male mice had similar results as LERWT in the ME region; however, the FV in the vmARH was not significantly increased, with a p-value of p = 0,0754 **(fig.15 A)**. Strikingly, this fasting seems not to affect the ZO-1 reorganization in the LERWT males and LERKO **(fig.15 B-D)**.

This experiment on males has limitations, as it failed to completely replicate the fasted phenotype seen in Langlet et al. (2013). However, it is interesting to see that there were little differences between the two male genotypes and that the hepatic receptor might not play a role (or a lesser one) in the males' regulation of the plasticity of the ME region.

1. Diffusion studies: the diffusion of peripheral signals into the ME-ARH region

The increase of fenestrated vessels during a fast is thought to increase peripheral signals' direct access to the ME-ARH region. Here, we want to study if the lack of adaptation to fasting is seen in females LERKO's fenestrated capillaries also results in impaired permeability to the periphery. To better characterize the permeability of the interface to molecules from the blood, the diffusion of Evans blue dye in the parenchyma of the ME and vmARH was assessed after intraperitoneal injection in fasted conditions **(fig.16 A).**

After collecting hypothalamus coronal sections, pictures were taken of the unprocessed cuts (The blue Evans is easily washed out). This technique helps assess the opportunity for peripheral molecules to pass through the diaphragm of the fenestrated vessel toward the arcuate nucleus**.** It is established that a fasting period and increased fenestrated vessels can increase the diffusion of blue Evans in the ME-ARH region.

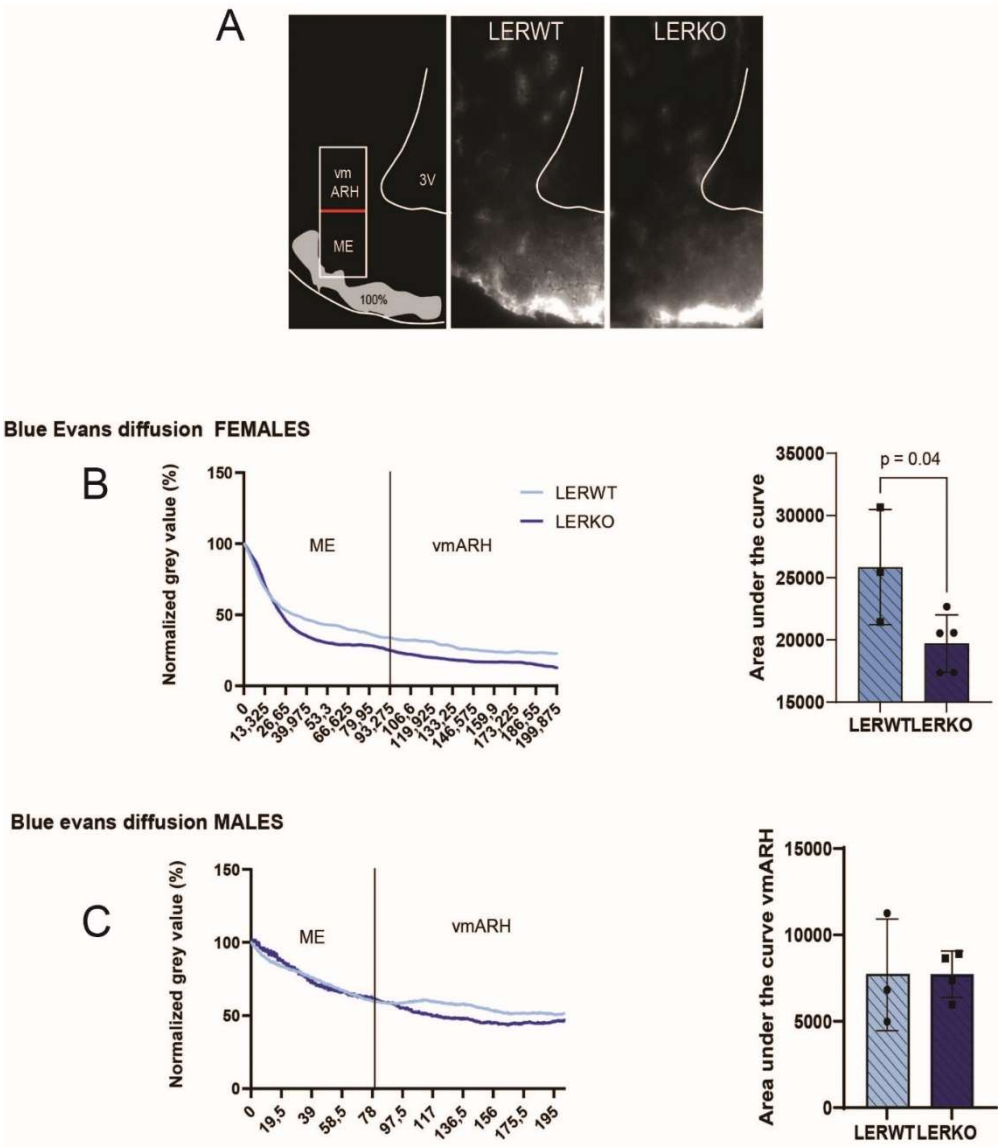


Figure 16: Blue Evans dye diffusion studies in the ME-ARH region

(A) Illustration of the method for analyzing the diffusion gradient of Evans blue dye in the hypothalamic tuberal region and representative sagittal section showing the diffusion of Evans blue dye (in grey) in LERWT and LERKO mice in fasting condition; Quantification (%) and area under the curve (AUC) of Evans blue dye diffusion gradient in the ME and ARH on sagittal sections illustrated in females (B) and males (C). Data are represented in mean +- SEM. Statistical analysis were done by first analyzing the area under the curve values and followed by a t-test.

For the female mice, less dye managed to reach the ARH, 10 % (100% being the dye found at the MBH capillary bed) compared to 17% for the LERWT **(fig.16 B).** LERWT females were found to have greater diffusion into the parenchyma compared to LERKOs, with a significantly higher area under the curve in LERWT mice compared to LERKO mice.

For males (fig.16 C), the injection of blue Evans in LERKO and LERWT fasted mice did not reveal a difference in diffusion throughout the ME-ARH region. This is coherent with the results in **(fig.14)** where the two genotypes had a similar response to the fasting protocol.

These results are a turning point for the project. The impact of the hepatic Erα impairs the vascular and tanycytic plasticity of response to fasting in female mice. The hERα seems to act on the vmARH region and its capacity to diffuse peripheral signals to the hypothalamus. The hERα involvement in females, but not males, is indicative of the receptor's sexual dimorphism regulation. The next part of the project is to see the potential metabolic consequences in the LERKO male and female mice.

II. Metabolic consequences of the invalidation of the hepatic estrogen receptor α

Fasting-induced plasticity of the blood/ARH interface is a mechanism that regulates energy homeostasis by controlling the access of signals to the brain. Therefore, investigating whether this mechanism's alteration in LERKOs results in body composition and changes in food intake and energy expenditure was essential. These parameters were examined in LERWT and LERKO mice fasted and fed ad libitum in a regular chow diet using metabolic cages and MNR imaging for body composition.

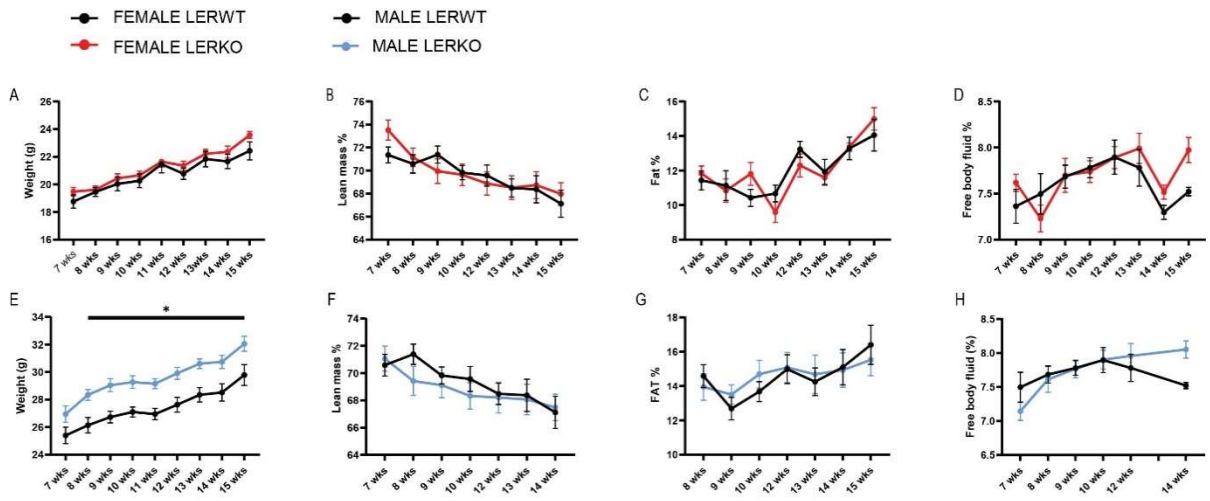


Figure 17: LERKO female and male body composition change.

*Body weight (week 7 to week 15) and body composition (week 7 to week 15) changes: body weight in g followed by the percentage of fat mass, lean mass, and free body fluid in female mice (1-D) and males' mice (E-H) LERWT n=8-7 and LERKO n=8-7. Data are represented in mean +- SEM. Statistical analysis was done using a two-ANOVA test followed by a Fisher's LSD posthoc multiple comparisons * p<0,05*

A. Unchanged body composition

Weekly monitoring of the animals over 12 weeks showed no significant differences in weight between LERWT and LERKO **females**. Moreover, the evolution of parameters, including fat mass, lean mass, and fluid composition concerning weight (%), was identical between LERWT and LERKO females **(fig.17 A-D).**

However, **male** knockout mice have a different body composition than those found in previous studies, which found no weight and body composition differences **(fig.17 E-H)** (Matic *et al.*, 2013). Here, the male LERKO group had a higher weight at eight weeks old, and that increase continued across the experiment (15 weeks old). Body composition analysis singled out lean mass in g as the reason for the higher weight in LERKO, but the percentage of lean mass was similar between the 2 genotypes.

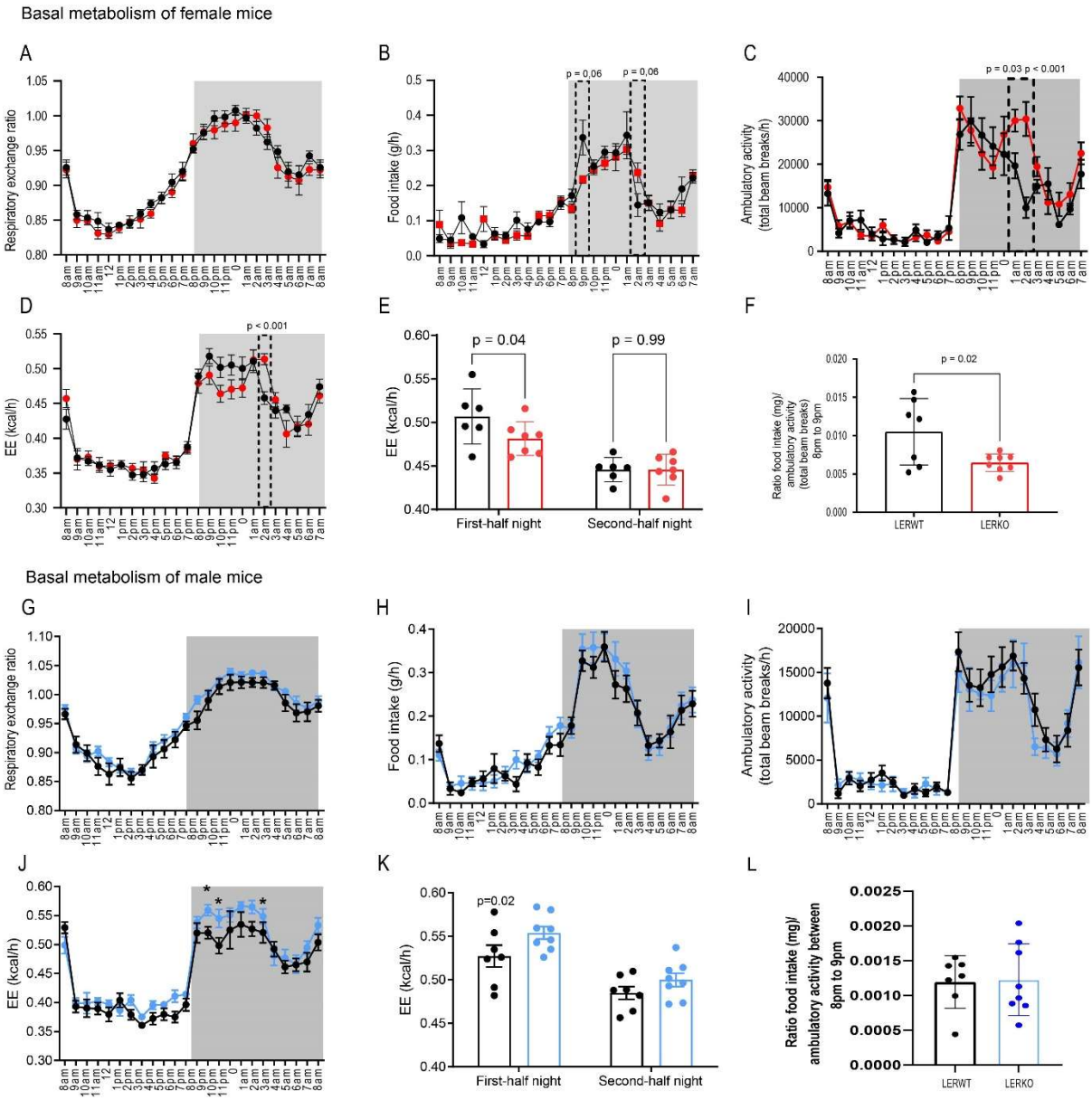


Figure 18: Basal Metabolic Metabolic Studies of LERKO mice

Each time point corresponds to the mean value of time points over several days of recording for each mouse. The resting phase corresponds to 7 am-7 pm (white background), and the active phase is 7 pm to 7 am (grey background). LERKO n=7 and LERWT n=7 Parameters measured are food intake, energy expenditure, RER, and ambulatory activity in females (A-F) and males (G-L). (A, G) Respiratory exchange ratio over 24 hours. (B, H) Non-cumulative food intake over 24 24-hour periods and highlight the two relevant time points: 9 pm and 2 a.m. (C, I) Ambulatory activity per hour over 24 hours in beam breaks (mice crossing one of the laser beams within the cages). (D, J) Energy expenditure over 24 24-hour periods in kilocalories spent per hour and highlight on the two relevant time points: 9 pm and 2 am energy expenditure. (E, K) energy expenditure during the first and second part of the night. (F, L) The ratio of the food intake over the ambulatory activity from 8 pm to 9 pm (the active phase). Data are represented in mean +- SEM. Statistical analysis was done using a two-ANOVA test followed by a Fisher's LSD posthoc multiple comparison

B. Mild changes in metabolic parameters

Diving into the basal metabolic cages results of LERKO and LERWT mice, where the mice were recorded for several days, the graphs represent the mean for each hour of these days. Metabolic parameters were recorded in basal and fasted states to assess the animals' metabolic behavior:

- The food intake was measured to establish a feeding pattern in these animals.
- Respiratory exchange ratio or RER is the ratio of CO2 produced and O2 consumed and indicates the primary type of energy substrate used. The use of carbohydrates will lean the RER towards 1. The lipids utilization will require more oxygen and decrease the RER to 0,7.
- The energy expenditure is the sum of the basal metabolic rate, energy related to temperature, and physical activity. For these results, it is reported by a number of calories spent in an hour.
- Ambulatory activity is a tenant of the energy expenditure, measured with infra-red beams of light, it is the movement of the mouse in the cage.

1. LERKO female mice have mild increased ambulatory activity during the active phase and changed food intake

In female mice, the wild-type females have an increased food intake in the first hours of the active phase (9 pm). A surge that is not noticeable in the LERKO females. Otherwise, the intake was comparable in both groups **(fig.18 B)**. No significant differences in the use of energy substrates were observed between LERWT and LERKO females **(fig.18 A)**. Energy expenditure increased in LERWT and LERKO mice at the beginning of the active phase. However, despite no statistical difference, the two curves separated, with a minor increase in energy expenditure in LERKO compared to their LERWT littermates during the first 4 hours. Furthermore, between 1 am and 2 am (time of 4 hours physiological refeeding), when

energy expenditure decreased in females LERWT, it increased in LERKO, with a significant difference for the 2 am measurement, after which the two curves merged and no longer differed **(fig.18 D)**. During the active phase, the LERKO has increased ambulatory activity **(fig.18 C)**. The increase in ambulatory activity and food intake in LERKO should go hand in hand, as more energy intake leads to more energy spent. Unexpectedly, here, this is not the case when looking at the ratio of food intake over ambulatory activity. Indeed, the LERKO female mice engage in excessive ambulatory activity compared to the food they ingested.

Then, we subjected these same female mice to a **fasting period** in the metabolic cages **(fig.19)**. Here, the fasting is 24 hours, from 1 pm to 1 pm. The beginning of the active phase triggers an increase in ambulatory activity in the LERWT, which is easily associated with a need to search for food. This 9 p.m. ambulatory peak is, again, not replicated in the LERKO. Still, they seem to have a delayed peak at 1 a.m. **(fig.19 E)**, similar to the impairment seen in the basal metabolism **(fig.18 C)**.

These data demonstrate that modification of the plasticity of the blood/ARH interface observed during prolonged fasting in female LERKO mice does not cause any differences in the body composition of the animals in normal physiological conditions. Nevertheless, a delay in the increase in locomotor activity is briefly observed in LERKO female mice compared to LERWT mice during the transition from the resting state to the active phase.

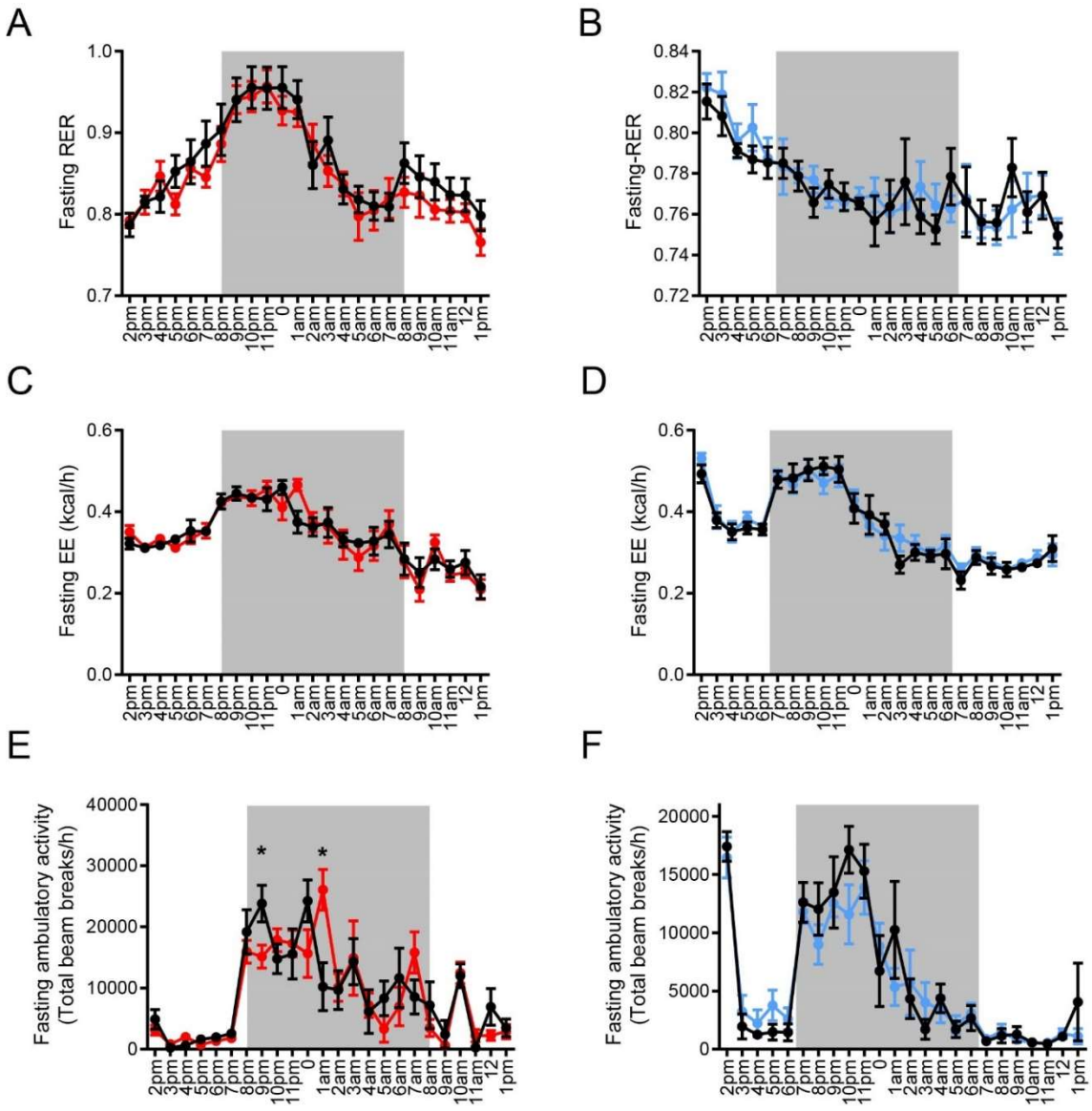


Figure 19: Fasting metabolism of LERKO female and male

*Metabolic recording of the RER (A, B), Energy expenditure (C, D), and ambulatory activity (E, F) after a 24-hour fasting period in females and males LERWT and LERKO mice. Data are represented in mean +- SEM. Statistical analysis was done using a two-ANOVA test followed by a Fisher's LSD posthoc multiple comparison * p<0,05*

2. The males LERKO have an increase in energy expenditure not explained by ambulatory activity

For **male** LERKO food intake pattern and energy substrate use (RER) are similar to the LERWT **(fig.18 G-L)**. However, they do have a higher energy expenditure in the first part of the night than their LERWT counterpart. This increase is steady from the second hour of the active state until 3 a.m. Contrary to the female LERKO, this increase in LERKO males is not explained by the ambulatory activity **(fig.18 I)** or Z-rearing (not shown here). During fasting, the male's LERKO energy expenditure flattened to the level of LERWT **(fig.19)**.

The metabolic changes in LERKO mice, male and female, are subtle. The invalidation of the hepatic ERα seems to impact energy intake, which is explained by the increased energy expenditure for females. However, this increase remains to be explained for males. Interestingly, these results show that the ambulatory activity in female LERKO mice is similar for basal and fasted states. The delayed energy expenditure and food intake could hint at an impairment in communication. The hepatic ERα might be involved in transmitting anorexigenic/orexigenic messages.

In the next part of the project, we will focus on the female mice to understand how the Liver and the hypothalamus communicate through the hERα.

III. Finding the potential hepatokine-ligand candidates to explain the ME-ARH phenotype in female LERKO mice

The first two aims of this study point to the role of hERα in liver-hypothalamus communication in female mice. Albeit its consequences on metabolism are mild and might need further investigation. In this part of the project, the objective is to find how the hepatic ERα can alter the ME-ARH plasticity. We will closely examine the hepatokines, which are produced and secreted primarily by the liver by looking at their expression in hepatocytes. This project aims to see if the receptors of some of these hepatokines are found in the hypothalamus with RNAseq and RNAscope. Lastly, these results will help us narrow down the list of candidates to explain the impairment in the vmARH of the female LERKO mice.

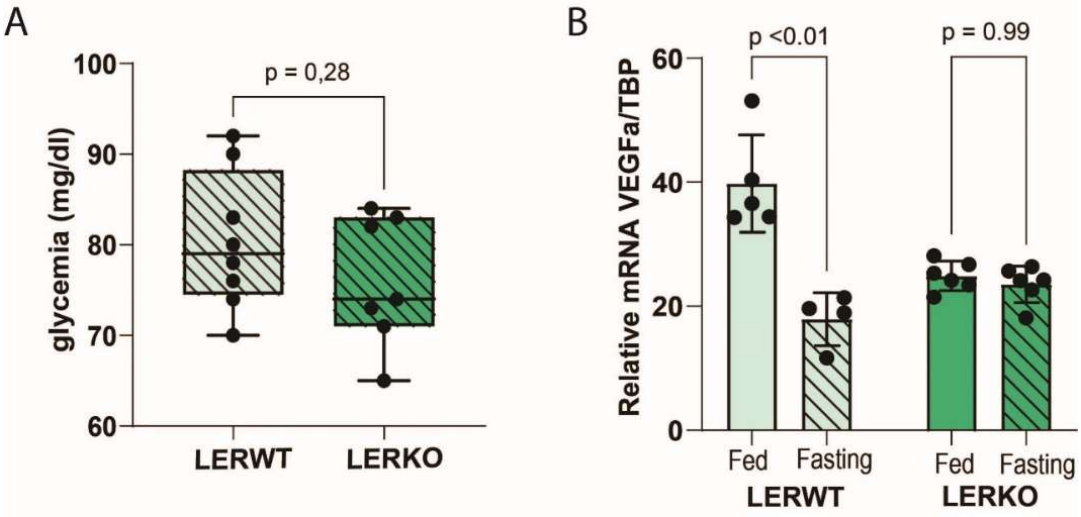


Figure 20: Glycemia and hepatic VEGFa fall short to explain the hERα-dependant ME-ARH impairment.

Fasted glycemia (A) and VEGFa relative mRNA expression in hepatic cells in LERWT and LERKO female mice. Data are represented in box and whiskers (min to max). Statistical analysis was done using a two-anova test followed by a Tukey post-hoc multiple comparison analysis or an unpaired t-test

A. Glycemia and hepatic VEGFa fall short of explaining the hERα-dependant ME-ARH impairment

As described in the introduction (Chapter II), the first discovered mechanism to modulate the ME-ARH fasting-dependent plasticity was hypoglycemia triggering tanycytes-produced VEGF to increase the fenestrated vessels.

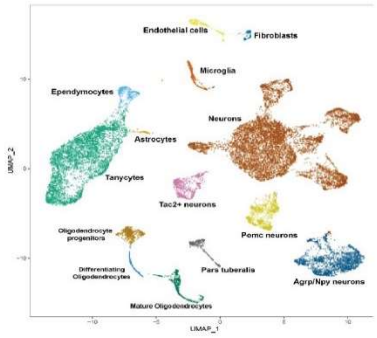
Here, the glycemia during a fast is unchanged in female LERKO compared to LERWT **(fig.20 A).** Moreover, a glucose tolerance test (not shown here) did not show impairment in glucose metabolism. As for the *VEGF* transcripts, the hepatocyte changes are opposite to those expected. In a fasting state, previous studies established that the vascular endothelial growth factor (VEGF) increases in the liver and the hypothalamus, increasing the number of fenestrated vessels in their respective organs (O'Reilly *et al.*, 2010; Langlet, Levin, *et al.*, 2013a). Here in the LERWT, the VEGF is higher in the fed condition compared to the fasted condition. In the LERKO fed, the VEGF transcript level reaches a similar level to the fasted LERKO condition **(fig.20 B).** The previous mechanism to explain ME-ARH plasticity seems to fail in explaining the phenotype here. However, a prospective study could assess the VEGF mRNA level in the tanycytes.

These results indicate that the project must take another route to explain the ME-ARH behavior in LERKO female mice.

A

Hepatokines	Associated receptor		Gene
Lect2	Tie 1	PMID: 31474362	TIE1
FetuinB	TGF-beta receptor	PMID: 32382986	TGFBR2
Postn / periostin	ITGaVb3 ,DDR1	PMID: 32268102 ; PMID: 32330138	ITGAV, DDR1
Fgl1	LAG-3	PMID: 30580966	LAG3
Adropine	GPR19	PMID: 26739651	GPR19
Dpp4	GLP-1R	PMID: 28710180	GLP1R
Igfbp2;1;3 and IGF-1	IGF-1R	PMID: 24292683	IGF1R
Rbp4	STRA6, RXR	PMID: 23959802	STRA6, RXRA
FGF21	FGFR1 and KLB	PMID: 18064602	KLB and FGFR1
Fetuin A	IR (Insulin receptor)	PMID: 30060600	INSR
Follistatin	TGFB1R	PMID: 34440203	TGFB1
GDF15	GFRAL	PMID: 33228454	GFRAL

B



C

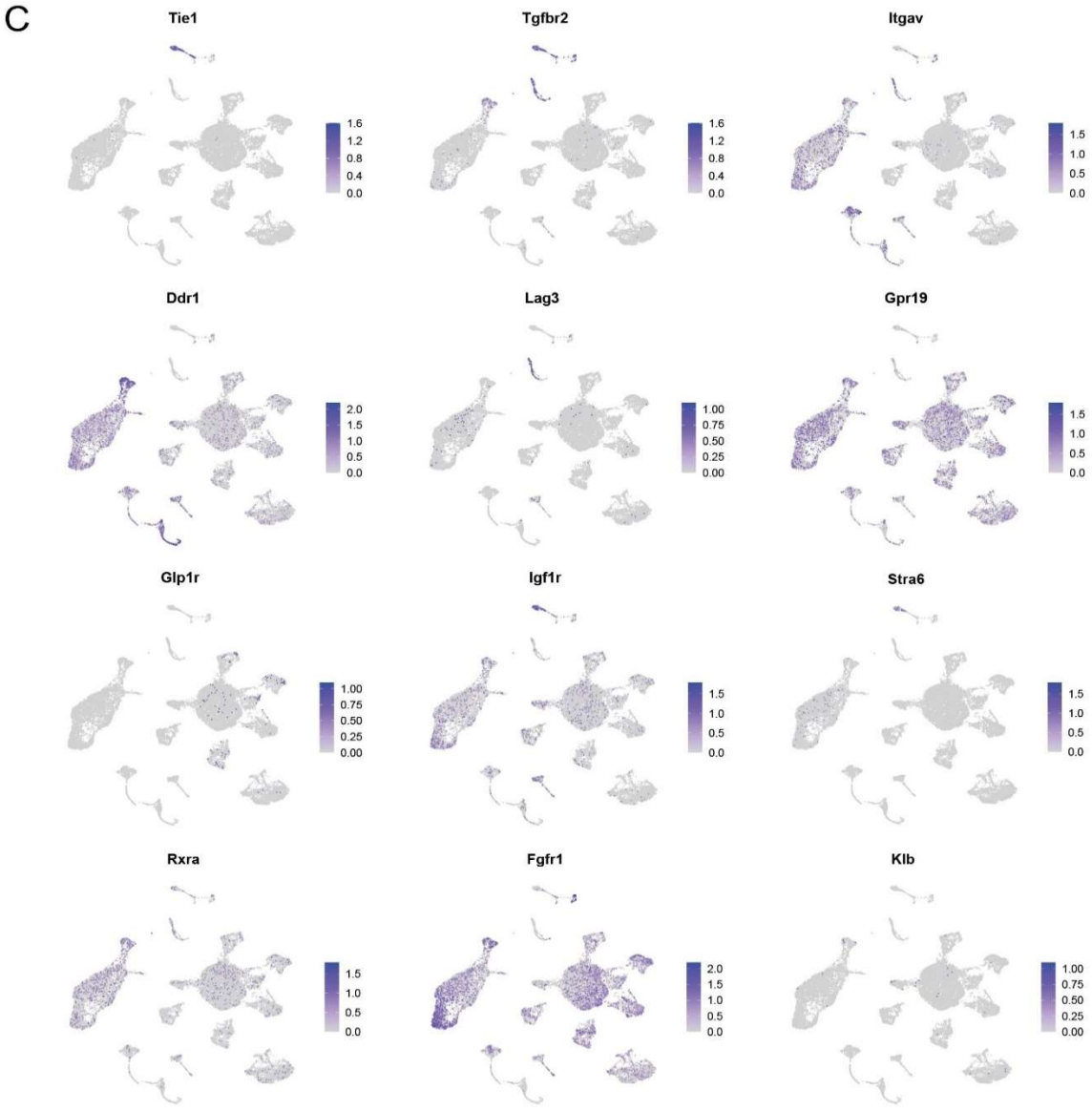


Figure 21: Associated receptors of the hepatokines secreted by the liver

(A) List of common hepatokines and their associated receptors. RNAseq studies: (B) representation of the RNAseq of the hypothalamus and its different cell and subcell types. (C) hepatokine-associated receptors localization in the hypothalmus, purple dots represent the presence and intensity of the RNA.

B. Narrowing the list of candidates: hepatokines receptors found in the hypothalamus

As the liver releases numerous hepatokines involved in communication with other organs, we wanted to determine which hepatokine(s) might alter the plasticity of the blood-ARH / ME-ARH interface. A transcriptomic analysis was conducted on liver tissue from LERWT and LERKO animals under both fed and fasting conditions (data not shown). Hepatokines whose expression profile varied according to the genotype and energy state of the animals were the subject of a literature search to establish their potential role/impact on the blood/ARH interface. In parallel, RNAseq studies were conducted to assess the presence of receptors of these selected hepatokines in cells at the blood-ARH interface **(fig.21 C).**

The mRNA of the receptors is in red, vimentin in red, MECA-32 in green and the nuclear marker dapi un blue.

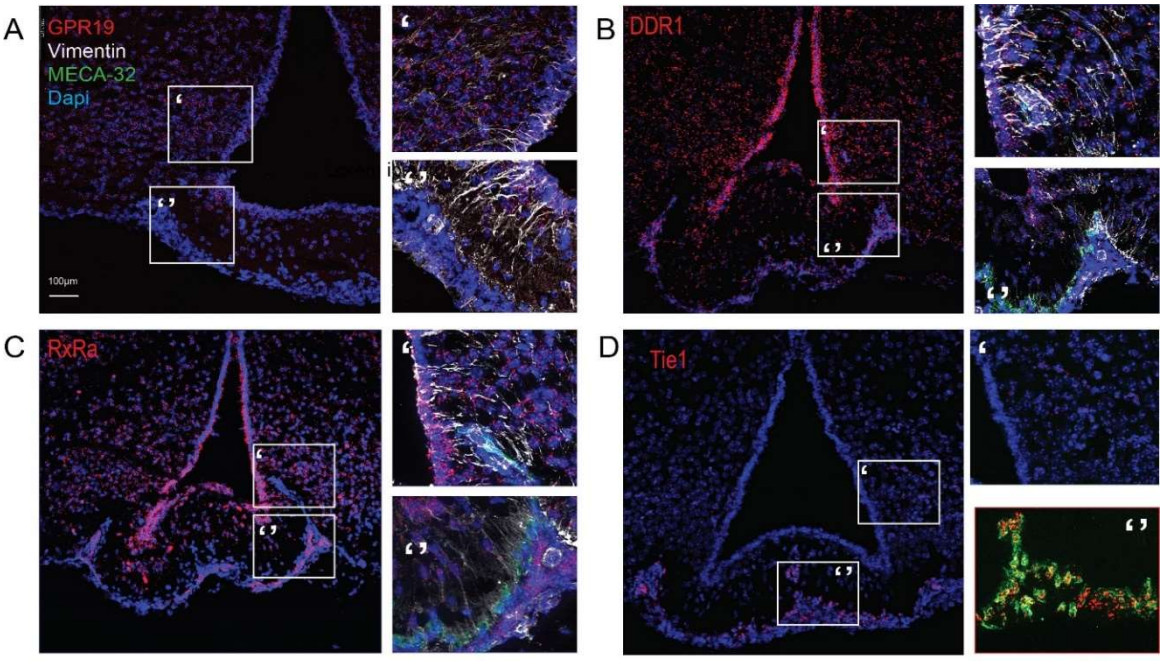


Figure 22: Confirmation of the hepatokines' receptors mRNA presence in the hypothalamus through in situ hybridization followed by an immunostaining.

For each picture there is an inset magnifying the ARH region (') and the ME-ARH region ("). (A) the adropin's receptor GPR19; (B) Periostin's receptor DDR1; (C) RBP4's receptor RxRα and (D) LECT2's receptor Tie1.

This allowed us to highlight several ligand-receptor candidates that are ligands linked to metabolism and possess receptors found in the hypothalamus, such as DDR1 –Periostin, RBP4-RxRaplpha; LECT2-Tie1 and adropin-GPR19. The hypothalamic localization of these receptors was confirmed through RNAscope, an *in situ* hybridization to visualize mRNA on a brain slice **(fig.22).** GPR19 mRNA can be found in most hypothalamus cells except for the vessels, according to the RNAseq results, confirmed by the photograph in **(fig.22 A)** where the mRNA staining seems ubiquitous in the brain parenchyma. The DDR1 receptor is present mainly in tanycytes and mature oligodendrocytes **(fig.21 C; fig.22 B)** in the bulk of the mRNA staining is located at the border of the third ventricle at the apical part of the tanycytes.

RxRα has a similar expression pattern as the DDR1, subtracting the presence in the oligodendrocytes. The last receptor to be confirmed is the Tie1 receptor, which, according to RNAseq, is exclusively found in the vessels of the hypothalamus. The in situ hybridization precisely this finding by highlighting a solid mRNA expression in the fenestrated vessels and, more mildly, in the BBB vessels of the hypothalamus **(fig.22 B)**

The presence of Tie1 in the fenestrated vessels and the previous papers reporting LECT2 as angiogenesis function, notably on the LSEC, make this couple the most likely candidates to explain the first results. Following these results, the couple Tie1-LECT2 became the focus of the next part of the project.

C. Regulation of both LECT2 hepatic secretion and its receptor Tie1 expression are lost in LERKO mice

The hepatokine, LECT2, was described in chapter 4 of the introduction. LECT2 is known to decrease during a fasting period and recently was associated with capillarization capacity in the liver (M. Xu *et al.*, 2019; Kikuchi *et al.*, 2020).

Hepatic transcripts for LECT2 (mRNA) were analyzed using qPCR in LERWT and LERKO under fed and fasted conditions. There was a significant reduction in the number of hepatic transcripts in LERWT under fasted conditions compared to fed conditions.

However, no such difference was observed in LERKO under the same conditions **(fig.23 B).** LERKOs exhibited a significantly higher number of transcripts than WTs in the fasted condition, while no significant genotype effect was observable in the fed condition. This implies that the transcript levels in LERKOs were close to those in the WT-fed condition, indicating the absence of reduced LECT2 transcripts in LERKOs during prolonged fasting. These findings suggest that LERKOs cannot adjust their hepatic LECT2 synthesis rate according to the animal's energy status. Next, we investigated whether these variations in hepatic synthesis led to changes in plasma LECT2 levels **(fig.23 C),** using ELISA in LERWT and LERKO mice under fed and fasted conditions. In LERWT mice, plasma LECT2 levels were significantly lower during fasting compared to the fed condition. However, in LERKO mice, no statistically significant difference was observed between the two conditions, indicating the absence of the impact of the energy status in knockout mice. The effect of genotype during fasting was not observed anymore on the plasma LECT2 levels, probably explained by the dispersion of these values of these measures. Therefore, in LERWT mice, there are alterations of LECT2 hepatic transcripts and plasma LECT2 levels during fasting that are absent in LERKOs.

To complete these results, we examined the expression of LECT2 receptors, *i.e.,* Tie1. Its transcripts were exclusively detected in endothelial cells, with higher expression in cells co-expressing PLVAP transcripts, a marker for endothelial cells with fenestrated

endothelia, as seen in the *in-situ* hybridization studies **(fig.22 D).** The Tie1 transcript was quantified on the LERWT and LERKO female mice fed in fasted conditions.

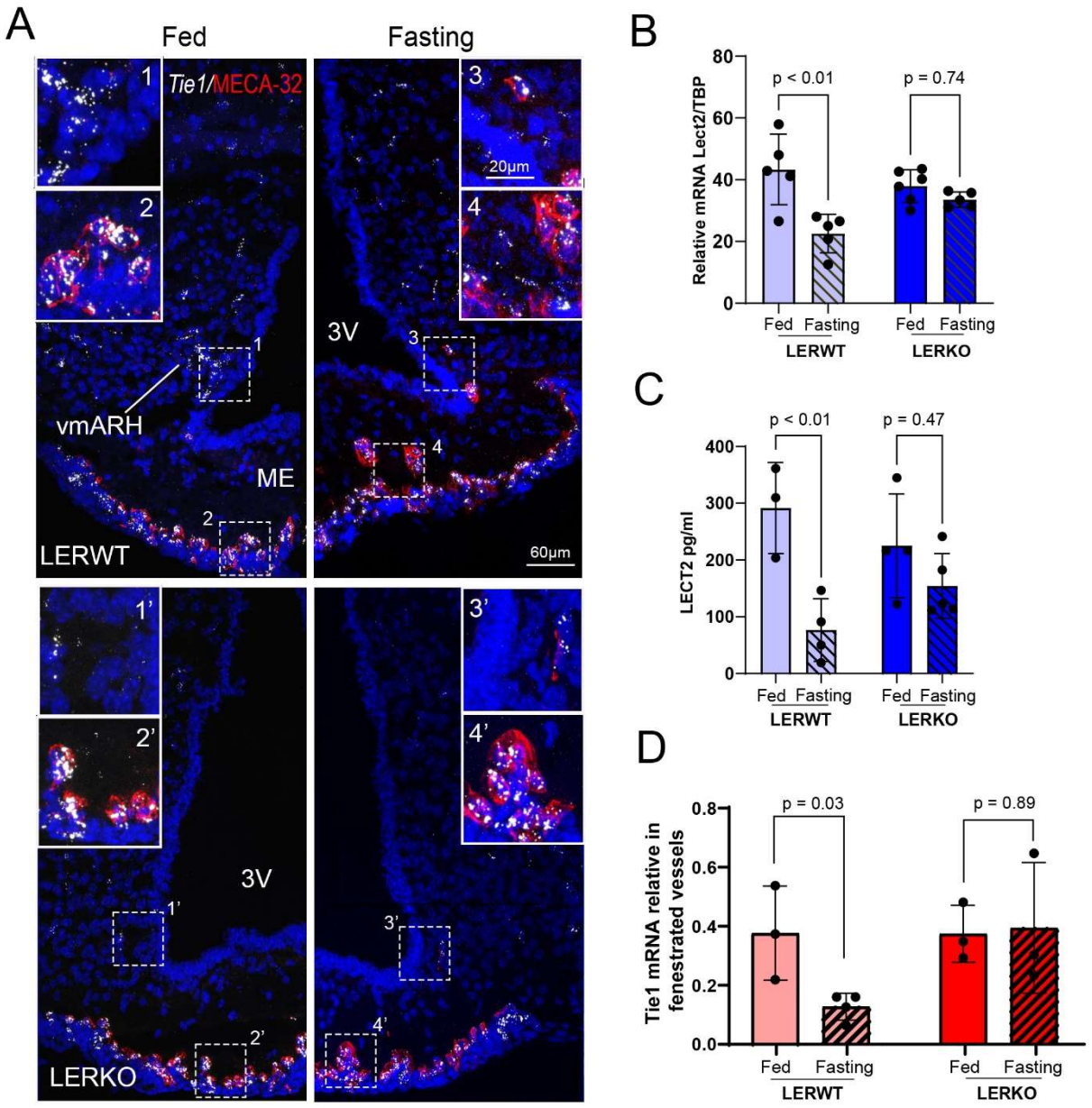


Figure 23: Fasting-induced regulation of both LECT2 hepatic secretion and its receptor Tie1 expression in fenestrated vessels are impaired in LERKO female mice.

(A) Representative images of fluorescence in situ hybridization (FISH) for Tie1 mRNA (red), Meca-32(green) immunoreactivity and DAPI nuclear staining (blue) in the tuberal region in fed and fasting LERWT and LERKO mice. (B) LECT2 relative mRNA expression in hepatic cells and (C) LECT2 plasma level in fed or fast conditions in LERWT or LERKO mice. (D) Quantification of fluorescence in situ hybridization (FISH) for Tie1 mRNA illustrated in (A).

Data are represented in box and whiskers (min to max). Statistical analysis was done using two-anova test followed by a Tukey post-hoc multiple comparison analysis or an unpaired t-test

In LERWT, fasted conditions showed a significantly lower transcript of *LECT2* count than fed conditions. No differences were observed between the two conditions in LERKOs, confirming the previous results showing the absence of the effect of energy status on LECT2 expression in LERKOs **(fig.23 B).**

In summary, these results demonstrate a change in the fasting-induce regulation of the synthesis of hepatic LECT2 and the expression of its receptor tie1 in fenestrated endothelium in LERKO compared to LERWT mice. These findings can be linked to the altered plasticity of the blood/ARH interface in fasted LERKO mice.

IV. Injection of LECT2 protein inhibits the fasting-induced plasticity of the blood/ARH and impairs the refeeding behavior of WT mice.

Given that the decrease in the number of hepatic LECT2 transcripts induced by fasting is related to an increase in the number of fenestrated vessels in the ARH of LERWT mice, while the presence of constant high levels of hepatic LECT2 transcripts is associated with a reduction in the number of fenestrated vessels in the ARH of LERKOs, the effects of LECT2 injection on vessel fenestration and on food intake were tested.

To this end, his tag-recombinant LECT2 (rLECT2) diluted sterile water and 5% glycerol solution versus control solution alone was injected four times, every 40 minutes, to WT mice that had undergone a 24-hour fast (1 pm-1 pm). This condition induced the number of fenestrated vessels. Subsequently, half of the group was sacrificed to perform anatomic and permeability studies of the blood/ARH interface. In contrast, the other half was monitored for food intake 2 hours after refeeding **(fig.24 A)**.

The blood/ARH interface was then examined in both groups: control *versus* rLECT2. Results indicated a decrease in fenestrated vessels in the ARH when rLECT2 was injected, compared to the control group **(fig.24 C)**. A modest trend (P=0.0989) was also observed in the ME **(fig.24 E).** However, the injection of rLECT2 did not affect the ZO1 organization pattern in the 3V wall, ARH, or ME **(fig.24 D-F).** When vessel permeability was assessed by injecting Evans Blue dye simultaneously with the last injection of control or rLECT2, the diffusion gradient was reduced in mice that received rLECT2 as compared to control alone. The area under the curve was significantly less in the presence of rLECT2 **(fig.25).** These findings indicate that the injection of rLECT2 led to a reduction of vessel fenestration, predominantly in the vmARH, resulting in a decline in peripheral molecule diffusion in this area.

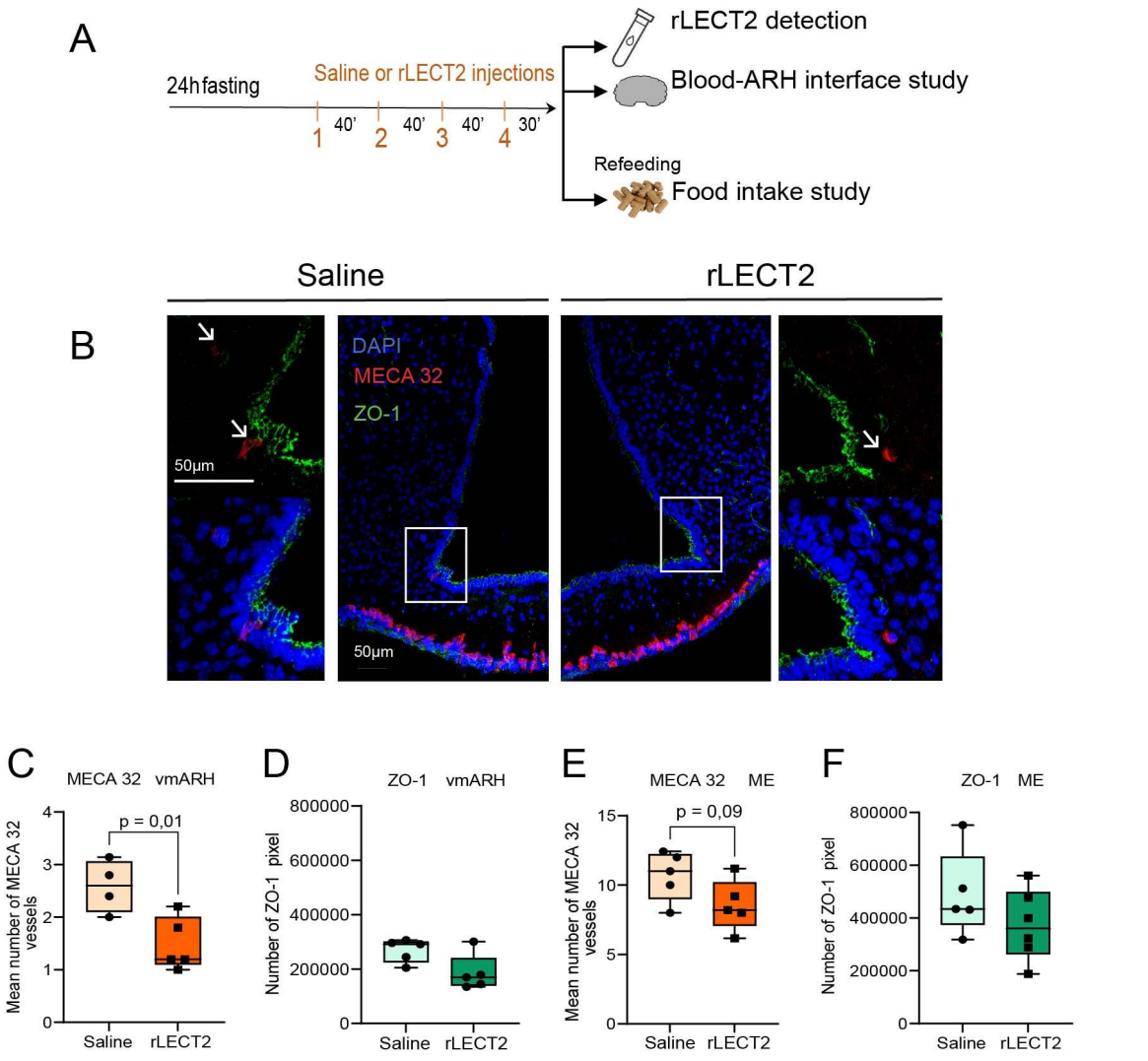


Figure 24: histag-conjugated recombinant LECT2 injection (rLECT2) inhibits the fasting-induced plasticity of the blood-ARH

(A) Experimental protocol: After 22h of fasting, the LERWT and LERKO mice were injected four times, every 40 minutes, with a control solution containing or not 1.2µg/L HR-LECT2. For some of the animals, the last injection of control +/- rLECT2 was combined with an injection of Evans blue solution to study its diffusion in the parenchyma of the ME and ARH. 30 minutes after the last injection, the LERWT and LERKO mice were divided into two groups: one group of mice was sacrificed to collect blood samples and brains to assess plasma HR-LECT2 levels and the study of the blood-ARH interface respectively; the other group was given food back to assess feeding behavior. (B) representative picture of the ME-ARH region of the LERWT and LERKO fasted female mice with the MECA-32 in red and ZO-1 staining in green. (C-F) Several Meca32-positive micro-vessel loops and ZO1 positive pixels in the ARH and ME in fasted mice were injected with control or recombinant LECT2 solution.

Data are represented in boxes and whiskers (min to max). An unpaired t-test was used for statistical analysis.

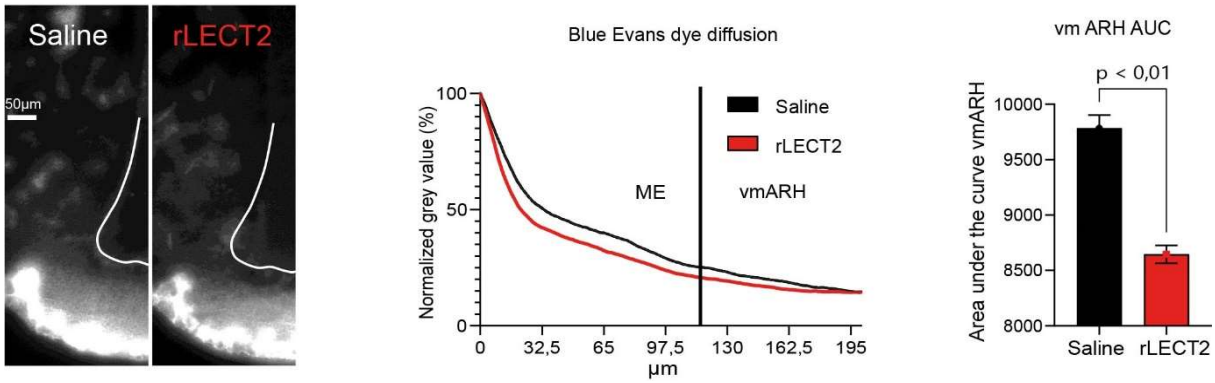


***Figure 25:* Blue Evans dye diffusion studies after rLECT2 injection.**

Illustration of the method for analysing the diffusion gradient of Evans blue dye in the hypothalamic tuberal region and representative sagittal section showing the diffusion of Evans blue dye (in grey) in fasting mice injected with either control or recombinant LECT2 solution; Quantification of the diffusion gradient of Evans blue dye in the ME and ARH on sagittal sections illustrated in fasted mice injected either with control or recombinant LECT2 solution.

Data are represented in mean +—SEM. Statistical analysis was done by first analyzing the area under the curve values and then a t-test.

Finally, we monitored food intake every 15 minutes for 2 hours in these injected WT mice **(fig.26)**. Mice injected with control alone ate as soon as food was provided, while mice injected with LECT2 had access to the food with an average delay of 5 minutes. Although these differences were insignificant, these results are supported by the correlation between time to first meal and plasma LECT2 level for each mouse: the higher the LECT2 level, the greater the delay between food availability and first meal (R2= 0.5) **(fig.26 E)**.

Food intake during the first 15 min was significantly lower in the LECT2 condition than in the control condition **(fig.26 C**). In addition, the amount of cumulative food intake remained lower in mice injected with LECT2, with a significant decrease in the area under the curve in mice injected with LECT2 and a significant decrease in cumulative food intake after 15 minutes of refeeding (p = 0,04) **(fig.26 A-C**). These results indicate that injected rLECT2 induced a delay in refeeding associated with a decrease in food intake, suggesting a reduced adaptation to metabolic changes in the presence of LECT2.

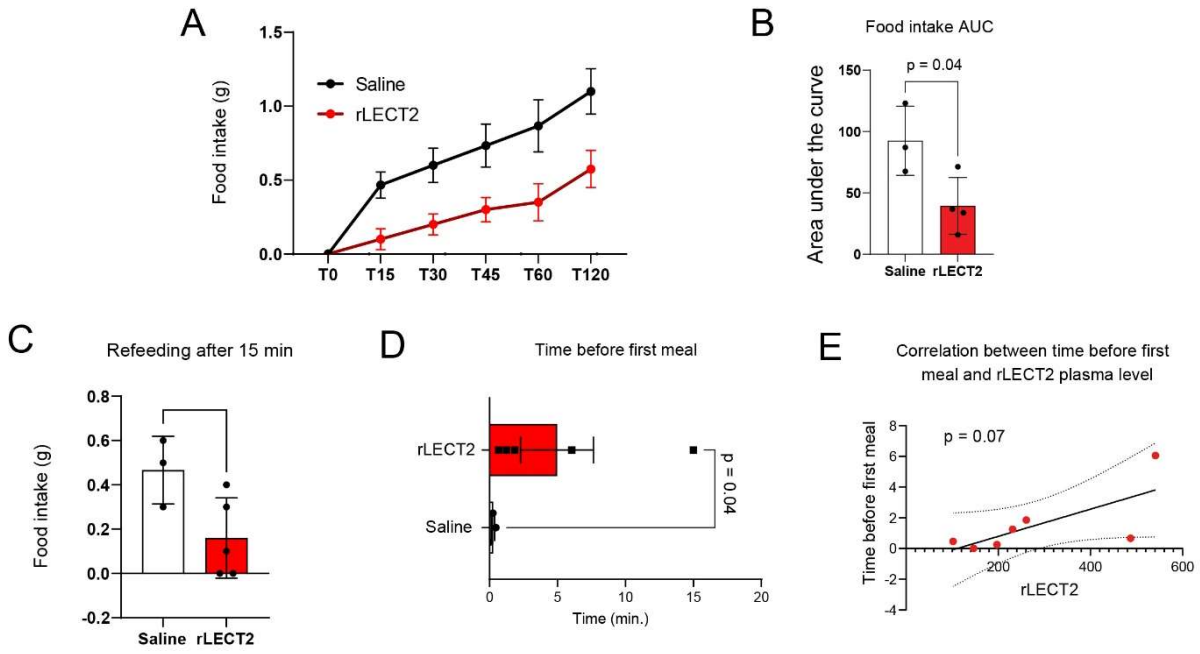


Figure 26: Food intake studies on rLECT2 injected female mice.

Monitoring of food intake in mice fasted for 24 hours and injected with either control (dark) or recombinant LECT2 (red). (A-B) Cumulative food intake after refeeding, the area under the curve of cumulative food intake (C) t-test analysis of cumulative food intake after 15 min refeeding. (D) Time to first meal and correlation between time to first meal and correlation of the time before the first meal and LECT2 plasma level in Wild-type mice injected in control or rLECT2

*Data are represented in mean +- SEM. Statistical analysis was done using a two-ANOVA test followed by an LSD fisher comparison analysis, A spearman correlation test, and a t-test following an area under the curve values. * p<0,05*

In this last part of the project, we demonstrated that LECT2 directly impacts the ME-ARH plasticity and the direct access of the peripheral messages to the ARH. LECT2 seems to impact the hypothalamus regulation, delaying food intake during refeeding.

These findings demonstrate alterations to the plasticity of the blood/ARH interface in LERKOs, resulting in discreet changes in physiological change adaptation, specifically concerning the day/night transition. These changes may be partly attributed to the hepatic synthesis of LECT2, which is persistently high and not regulated by fasting and which, upon administration, has been shown to affect vessel fenestration, subsequently impacting food intake.

Injection of LECT2 protein inhibits the fasting-induced plasticity of the blood/ARH and impairs the refeeding behavior of WT mice.

DISCUSSION

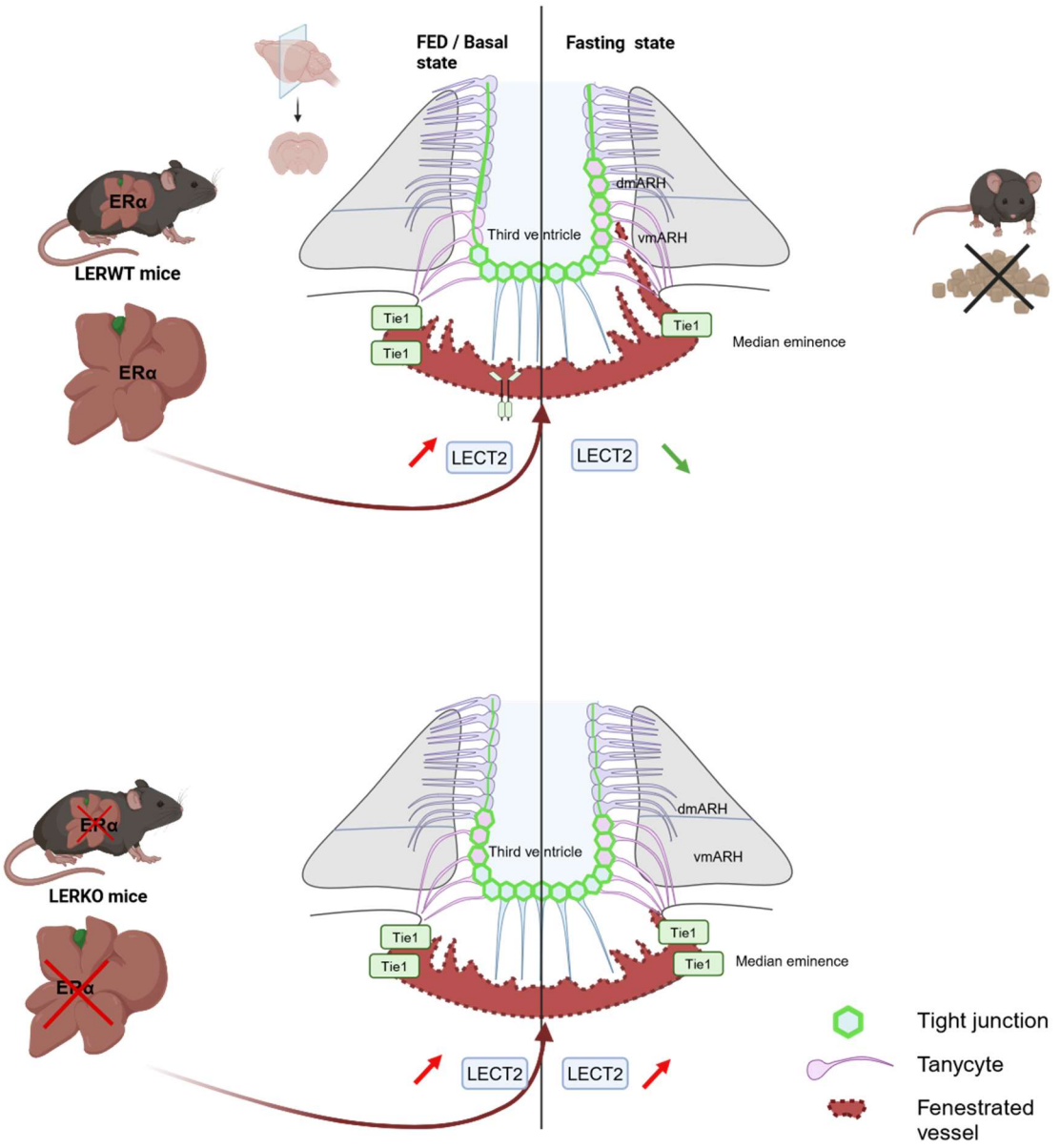


Figure 27: Hepatic Estrogen Receptor α controls fasting-induced blood-hypothalamus barrier plasticity via LECT2/Tie1 signaling in female mice

The hepatic ERα appears to be important in the MBH nutritional plasticity. Indeed, its invalidation leads to an impairment in the fenestrated vessels' reorganization normally triggered by fasting. The hepatokine, LECT2 seems to be involved in this mechanism. As the hepatokine levels are increased, rLECT2 injection replicates the results seen in the LERKO model. Furthermore, the rLECT2 injection delayed the food intake in mice during refeeding. This finding unveils a new link between the liver and the hypothalamus. Through the hERα, the liver will influence the hypothalamus's adaptation to energy availability. Created with Biorender.

Fasting timing matters

The 16 hours of fasting

This research project was designed to understand the hERα action on the hypothalamus, through the fasting paradigm. To study the fasting state, most researchers use rodents as a model; they will deprive them of food for short fasting periods (2h) to more extreme, less physiological fasting periods of 72h. Different fasting periods lead to different conclusions. For example, a 2020 study embarked on finding the optimal fasting period for the insulin intolerance test (ITT) in mice. By comparing several fasting methods, they saw that the glucose remained stable until the 6h-12h fasting period before decreasing, and insulin plasma levels decreased after 2 hours of fasting. They concluded that to avoid stress bias and liver and muscle gene expression changes, a 2 hours depletion of food intake was sufficient to evaluate insulin sensitivity (Carper et al., 2020).

In this project, we chose to subject the mice to a 16-hour fasting. We restricted the food for all mice during the resting phase. Then, the refed group had *ad libitum* access to food for the first 4 hours of the active phase period, and the fasted group remained fasted for 4 hours **(fig. 11)**. This particular fasting time was chosen for several reasons:

1- Given the significant role of circadian rhythm in metabolism, the timing of fasting is crucial. RNAseq studies showed that 22% of hypothalamic mRNA transcripts are circadian-dependant, which is also true for 27% of the hepatic mRNA (Li *et al.*, 2023). A caloric restriction can alter the hepatic clock gene Bmal1, which is known partially for regulating the circadian rhythm according to nutrition. The kinetics of Bmal1 will be altered after 18 hours of fasting (no data for 16 hours) (Patel *et al.*, 2016). Fasting all mice for 12 hours and no longer than 16 hours of fasting will prevent a circadian rhythm bias when focusing on the hepatic ERα influence on the nutritional state and metabolism.

2- The food intake of rodents is lower during the resting phase and higher during the active phase. It is coherent that a 12-hour fasting during the active phase is more

stressful than a 12-hour fasting during a resting period (Jensen et al., 2013). This stress in our mice will be reduced to 4 hours of fasting during the active phase.

3- The 12h fasting during the resting phase will force the refed mice group to eat only during the active phase, strengthening the contrast between the resting and active phases. During the first hours of the active period, the mice tend to move more and eat more. A previous study showed a high contrast between fed and fasted mice's glycemia and free fatty acids plasma levels after 16 hours of fasting (Montagner *et al.*, 2016).

Mild metabolic consequences of the hepatic ERα

Our results using the metabolic cages showed mild alterations in the metabolism of LERKO Mice. In our fasting experience, wild-type females increase their ambulatory activity at the first hour of the active phase, probably to seek food. This increase does not seem to happen for LERKO female mice. These subtle results might suggest an alteration in the transition between the resting phase and the active phase. The impaired plasticity in LERKO female mice could alter the signals controlling energy expenditure and alter the mice's behavior. These results suggest a role for the hepatic ERα receptor in regulating blood-brain communication.

In order to see if the ME-ARH plasticity impacted the ARH neuronal activity, we performed another experiment (not shown here), to assess the c-fos activity in the MBH of fasted female mice. No difference was seen between the female LERKO and LERWT c-fos activity in the neurons located at the ME-ARH or in the vmARH region. However, another study by Benedusi et al in 2017 on LERKO showed an impairment in the AgRP transcript in the ARH. Indeed, the hERα invalidation led to a decrease of AgRP activity during the estrus but not the other stage of the cycle (Benedusi *et al.*, 2017). This decrease in AgRP might explain the trend of female LERKO mice eating less at the beginning of the active phase. Another argument is that the AgRP expression is expected to be higher at the

beginning of the active phase (Lu *et al.*, 2002). The hERα knockout impacts the ME-ARH plasticity, but this impairment could require a longer period or a more stressful environment, a high-fat or high-fructose diet, to see a stronger impact on metabolism, as seen in (Meda *et al.*, 2020).

The hepatic ERα on sexual dimorphism

The hepatic ERα is proven to be a key factor in sexual dimorphism. hERαimpacts the masculinization of the male mice's liver, the receptor is necessary during the postnatal period to shape the male gene expression profiles relating to the metabolism (Della Torre *et al.*, 2018). This can impact how we look at the male LERKO mice model, as it could be considered a mouse model for invalidation of the hepatic receptor and also a feminization model. In the first part of our results, the dimorphism is seen in the plasticity of the ME-ARH. In these first results of the project **(fig.13)**, we performed an analysis of the ME-ARH plasticity in male and female mice. Although, T-test showed plasticity in both ME and ARH regions for wild-type female and male mice, the two-way ANOVA showed that the 16-hour fasting did not trigger the plasticity in the males' vmARH. This could hint at a different regulation between the two sexes but also separate regulation in the ME and vmARH region. It would be interesting to study this further. This is accompanied by the ERα invalidation showing no impact in the males' plasticity response in the ME-ARH region.

The injection of rLECT2 in females reduced the number of fenestrated vessels in the vmARH but not in the ME ($p=0.09$). We could hypothesize that in males, another mechanism could act on their ME region.

The quantification of the ME-ARH plasticity can be a limitation, as this technique use fragile tissue (fresh frozen brain) that can lead to the loss of some information. However, another project is to work on a new way to quantify this barrier (see appendix).

A new mechanism for ME-ARH plasticity

LECT2 influence on the vascular system

Among the hepatokines studied, LECT2 aroused our interest because it had already been described for its effect on energy metabolism (Lan *et al.*, 2014b; Yoo et al., *et al.*, 2017; Huang & Xu, 2021), on vessels (Chen *et al.*, 2016), and on tight Junction (Qin *et al.*, 2022). A paper attributed Tie1 to LECT2 as its ligand and their role in the liver sinusoidal endothelial cells (LSEC). By binding to Tie1, LECT2 interrupts Tie1/Tie2 heterodimerization and increases the homodimerization of Tie2, leading to the inhibition of liver angiogenesis and promoting the capillarization of the LSECs (M. Xu et al., 2019b). These results help to form a parallel between the regulation of the liver's sinusoidal endothelial cells (LSEC) and the ME-ARH region, opening a door to the LECT2/TIE1 regulation in the ME-ARH region.

Interestingly, the number of Tie1 transcripts varied according to the energy status and genotype of the animal in our study. These variations also phenocopied variations in hepatic LECT2 transcripts. In WTs, there was a reduction in hepatic LECT2 transcripts and endothelial Tie1 transcripts following fasting compared to fed animals; in *LERKO*s, LECT2 and Tie1 transcript levels correspond to the fed state of WTs, and no variation as a function of energy status was observed. These results suggest that the effects mediated by LECT2 at the blood/ARH interface involve the Tie1 receptor.

In this project, injection of rLECT2 modifies the fenestrated vessels at the interface, suggesting a direct effect of LECT2 on reducing endothelial fenestration. The effect of rLECT2 on the vascular system was previously investigated by Chen et al. (2016) in the context of hepatocellular carcinoma (Chen *et al.*, 2016). They showed that rLECT2 counteracts the effects induced by VEGFa *via* its VEGFR2 both *in vitro* and *in vivo*. LECT2 selectively suppressed VEGF-induced endothelial cell proliferation, migration, and tube formation. Interestingly, VEGFA and its receptor regulate the blood/ARH interface.

Secreted by tanycytes, VEGFA induces permeabilization of the vascular endothelium *via* VEGFR2 (Langlet, Levin, *et al.*, 2013b). In their study, LECT2 reduced tyrosine phosphorylation of VEGF receptor 2 and phosphorylation of extracellular signal-regulated kinase and downstream AKT (Chen *et al.*, 2016). Further studies are needed to verify the different hypotheses and to identify the mechanisms and receptors involved in mediating the effects of LECT2 on fenestrated vessels.

Thus, in our study, LECT2 could act with Tie1 on angiogenesis or could suppress endothelial permeabilization as described in the precept study.

LECT2 influence on the tight junction

LECT2 could also act on the ZO-1 and tight junction organization. In our study, the tanycytic barrier organization of the basal state (fed condition) of the LERKO female mice is close to the fasting condition in LERWT female mice. The presence of an abnormally well-organized tanycytic barrier independent of an increase in fenestrated vessels may be explained by the high levels of LECT2 in LERKO female mice. LECT2's role in the induction of inter-endothelial junctions has been observed. Of note, the expression of inter-endothelial junction proteins in the retina is greatly reduced in diabetic humans and mice. This decrease is correlated with reduced plasma LECT2 levels. In addition, treatment with rLECT2 attenuated the diabetes-induced reduction in endothelial cell barrier function (Qin et al., 2022; Horton et al, 2010).

However, in our study, rLECT2 did not reproduce this effect on the tanycytes barrier. This may be explained by the fact that LECT2 levels are more constitutively high in LERKOs, and acute injections of LECT2 might not be enough to induce this phenomenon. The role of LECT2 in the induction of the tanycytes barrier remains to be confirmed, and the mechanisms are to be determined.

Hepatokines

In order to identify the hepatic molecules involved in the alteration of the fasting-induced plasticity of blood/ARH interface in LERKOs, we took advantage of a transcriptomic study of hepatokines carried out in parallel by our collaborators as part of an ANR project (NR-18-CE14-0002; *bENEFIt;* 2017-2020).

Other than the LECT2-Tie1 ligand, other ligand-receptor candidates sparked our curiosity, like Adropine-GPR19. Our results showed that GPR19 is found in the hypothalamus, in the neurons and tanycytes, but not in the endothelial cells. As the introduction states, the adropin receptor, GPR19, regulates water intake, a function associated with the CVOs. A 2022 paper showed that hERαdepends partially on the adropin, which is negatively correlated with fatty acid accumulation in the liver and might help to protect female mice from an HFD-dependent NAFLD (Meda *et al.*, 2022). An *in vivo* study observed that adropin can upregulated VEGFR2 and increase angiogenesis (Lovren *et al.*, 2010). Relating this finding to our results, we could hypothesize that the adropin could potentially upregulate the VEGR2 in the tanycytes and increase the fenestrated vessels in the ME-ARH. However, there are contradicting results regarding the adropin plasma level in fasting, it is found to be either increased or decreased (Banerjee *et al.*, 2020).

Hepatic ERα will partially modulate the FGF21 hepatokine, together, they manage to increase the energy expenditure in female mice. However, ovariectomy did not block the FGF21 plasma level increase expected after fasting (Allard, Bonnet, *et al.*, 2019). Pointing at the hepatic Eralpha controls of the FGF21 expression in a nutritional-dependent manner.

This highlights a limitation in our project: the hepatic ERα impacts many hepatokines and liver functions, all activating or inhibiting pathways in return. This complicated the understanding of our mice model.

hERα could alert the hypothalamus of the nutritional status for reproductive reasons in female mice

Studies on hepatic ERα often discuss the receptor's cyclic activity and capacity to prepare for a potential gestation by keeping the store of amino acids or releasing cholesterol into circulation. The hERα role in the hypothalamus could relate to this capacity. Indeed, the cyclicity of the ME-ARH region is already established, for example, with the tanycytes controlling the release of GnRH into circulation according to the estrous cycle. A fasting period is a deleterious sign indicating insufficient energy resources for gestation. It is not only for the metabolism interest but also the reproductive interest that the hERα would alert the hypothalamus by increasing the FV and therefore accentuating access of fasting signal to the vmARH to induce a food intake.

A possible solution to manage menopausal consequences

Fasting is a well-studied condition in mice; in humans, fasting occurs physiologically with mild fasting during the resting phase or even with “intermittent fasting”. Intermittent fasting (IF) in humans is very similar to our fasting model. Indeed, it usually consists of “skipping breakfast,” resulting in overnight fasting and around 4 hours of fasting during the active phase and, therefore, an 8-hour time window to eat. Intermittent fasting's impact on health has been studied this past decade across many fields. In rodents, IF confers neuroprotective effects with effects on memory and cognition (Dong *et al.*, 2024). IF also restores leptin sensitivity in rodents' ARH nucleus loss by a high-fat diet (Oliveira *et al.*, 2022). In humans, IF was helpful in reducing hepatic steatosis lipid accumulation, along with obesity parameters like waist circumference (Ezpeleta *et al.*, 2023; Karimi *et al.*, 2023). For a postmenopausal person, IF reduced the HDL content more than a premenopausal person. This might be explained by the higher HDL levels after

menopause (Lin *et al.*, 2021). Notably, an estradiol treatment during menopause has the same effect as IF on HDL levels (Vaisar *et al.*, 2021).

For our female mice, the invalidation of the hepatic ERα mitigated the fasting plasticity of the ME-ARH region and pointed to dysregulation of fasting and refeeding. This also implied that estrogen might not be the ligand to act on hERα in an IF context about the amino acid. Indeed, a diet rich in AA was sufficient to restore impaired liver metabolism caused by the E2 deficiency in OVX mice (Della Torre *et al.*, 2021).

Even if our study did not uncover exactly how this affects metabolic parameters, we might hypothesize that IF could be an alternative to E2 at menopause to alleviate some of its consequences in the liver and the hypothalamus. In a menopausal context where estrogen is deficient, a protein diet could restore the hERα effect.

Our study, combined with other findings, might point to the influence of liver-hypothalamus communication and hepatic ERα on modulating some of the positive effects of intermittent fasting.

hERalpha could impact another barrier?

This study highlighted the impact of hERα on the ME-ARH plasticity, with notable effects on the fenestrated vessels and tight junctions. The impairment of the plasticity is also seen with the diffusion of Evan's Blue; the hERαinvalidation leads to a decrease in the EB presence in the ME-ARH. This is believed to be due to a decrease in fenestrated vessels that carry out the peripheral blood circulation and messages. A perspective in this study could be to study the impact of hERαon another barrier, the perineuronal net (PNN). The introduction states that the perineural net is an extracellular matrix forming an arch between the eminence median and the hypothalamic nuclei, and that the fasted hypothalamus will have a decreased PNN presence. Indeed, the PNN could also be impacted by the hERαand restrain the diffusion of Evan's Blue , as LERKO mice show that the receptor modulated genes are involved in the extracellular matrix regulation (Fuller *et al.*, 2023).

In conclusion, our results show that (1) the impact of hepatic ERα on the hypothalamus is sexually dimorphic and affects mostly female mice; (2) female *LERKO*s lack the fasting-induced permeability of vmARH vessels and have an abnormal tanycytic barrier in the vmARH; (3) these anatomical modifications of the interface are associated with eating disorders; (4) the anatomical and functional modifications of the interface may be associated with the presence of abnormally high levels of the hepatokine LECT2 in these animals; (5) hepatokine injected into the blood at levels mimicking those found in *LERKO*s mimics the inhibition of vascular plasticity observed in these animals and the eating disorders.

The hepatic receptor estrogen α is pivotal in regulating Leukocyte Cell-Derived Chemotaxin 2. This intricate interplay between ERα and LECT2 within the liver shapes metabolic homeostasis and contributes to the development and progression of metabolic disorders. A comprehensive exploration of the mechanisms and consequences of this regulatory axis is indispensable for a profound understanding of the underlying molecular pathways and their potential for therapeutic interventions in metabolic conditions.

Our results suggest that the absence of hepatic ERα, by altering the regulation of the synthesis and plasma secretion of LECT2, alters the fasting-plasticity of the blood/ARH interface with metabolic consequences.

MATERIALS AND METHODS

All procedures were performed according to the guidelines established by the National Institute of Medical Research and were approved by the local Ethical Committee of Animal Care (APAFIS# 29172-2020121811279767 v5 in Lille and #2020033010572804 in Toulouse) and the French Ministry of National Education, Higher Education and Research.

Animals:

The transgenic mice model LERKO, Liver Estrogen Receptor Knockout mice, was used for most of this project. The LERKO uses the CRE-Lox system on the ESR1 gene (Gu et al., 1993) by breeding an Esr1flox/flox mouse, where the loxP sequences surround the third exon, and a C57BL/6-Tg with an albumin promoter (Alb-cre) mouse. LERKO is hepatocyte-specific due to the albumin promoter. Albumin RNA is found only in hepatocytes (Feldmann et al., 1972; Della Torre et al., 2011). Female and male mice (8-10 weeks) with the deletion of ERα (LERKO) were systematically compared with their Esr1flox/flox littermate controls.

For the experiment regarding the recombinant LECT2 protein, wild-type female mice (C57BL/6J) were used. All mice were bred and raised in an animal facility with a controlled temperature and a 12-hour day/night cycle. The animals had ad libitum access to water and a chow diet.

Fasting Protocol:

During this project, we used two fasting protocols:

A 24-hour fasting protocol: Following the established protocol of Langlet et al. (2013), mice were deprived of food (but not water) from 1 pm to 1 pm the next day.

16-hour fasting protocol: All mice were first food-deprived during the light phase (12 hours) to standardize the energy state of the animals. For each genotype, the fed group was given back food at lights off, while the fasting group remained food-deprived until sacrifice 4 hours after lights off. Two days before the fasting period, litter from a confirmed

stud was added to synchronize all females in estrus, and a vaginal smear was performed to assess the stage of the estrous cycle before euthanasia.

Metabolic Cages and Body Composition:

Mice were individually housed and acclimatized to the cages for five days before experimental measurements in a 12-hour light/dark cycle (lights 7 am to 7 pm) and an ambient temperature of 22 ± 1°C. Mice were analyzed for total energy expenditure, oxygen consumption, carbon dioxide production, and food intake using calorimetric cages (Labmaster, TSE Systems GmbH). Mice were monitored weekly for body weight and body composition. Body mass composition (lean tissue mass, fat mass, free water, and total water content) was analyzed using a nuclear magnetic resonance (NMR) scanner (Minispec mq series, Bruker) according to the manufacturer's instructions.

Tissue Preparation:

Animals were sacrificed by decapitation, and the mice's brains were harvested. The brains were embedded in an optimal cutting temperature (O.C.T) solution to cryoprotect the tissue. Once the brains were completely embedded in O.C.T, they were transferred onto a bed of dry ice to fully freeze before being stored at -80°C.

Immunofluorescence:

Immunofluorescence was performed on 18-µm thick brain sections collected on Superfrost Plus adhesion microscope glass slides. After thawing at room temperature for 10 minutes, sections were washed with PBS 0.1M buffer and treated for 1 hour in blocking solution (PBS 0.1M containing 1% normal goat serum and 0.3% Triton X-100) at 4°C. Sections were next incubated overnight at 4°C with polyclonal rabbit anti-zonula occludens-1. The sections were then subjected to three washes in 0.1M PBS and were incubated for 1 hour and 30 minutes at room temperature with Alexa Fluor-conjugated antibodies as follows:

goat anti-rat 568 (1:500; Thermo Fisher Scientific Cat#A-11077, RRID: AB_2534121) and goat anti-rabbit 488 (1:500; Thermo Fisher Scientific Cat#A-11008, RRID: AB_143165). After a final three washes in PBS, the slides were mounted with a Mowiol and DAPI (1:5000) solution and sealed with cover slips (24x60mm, Epredia). The slides were stored at 4°C until analysis.

List of antibodies:

Zonula occludens 1, tight junction protein 1	ZO-1	Rabbit		polyclonal	Proteintech	AB-23.0003	21773-1-AP	1/1000
Zonula occludens 1, tight junction protein 1	ZO-1	Rabbit		polyclonal	Invitrogen	AB-20.0024	61-7300	1/1000
Platelet endothelial cell adhesion molecule, cluster of differentiation	CD31	Goat		Polyclonal	R&D System	AB-21.0019	AF3628	1/300
Plasmalemma vesicle-associaced protein	MECA-32	Rat		Monoclonal	BD Biosciences	AB-19.2772	550563	1/250
Vimentin	Vimentin	Chicken		Polyclonal	Biolegend	AB-21.0016	919101	1/1000

Quantitative Analyses:

Tight Junction Protein Organization:

A series of adjacent optical sections was collected using the z-stack module of AxioVision at 40X from 18µm-thick coronal sections labeled for ZO-1, through morphologically defined regions of interest corresponding to the ependymal layer bordering the third ventricle in the median eminence and the ARH. Maximal projection images were then binarized to isolate labeled junctional complexes from the background and compensate for differences in fluorescent intensity. These images were skeletonized so that each junctional strand was 1 pixel thick. The integrated density was then calculated for each image, reflecting the

total number of pixels in the skeletonized image and being proportional to the overall level of complexity of junctional strand organization. The values for all sections (8 ± 1 sections/region/animal) were averaged. The resulting value is an accurate index of junctional organization in the area of interest for each animal.

Fenestrated Capillary Loops:

Images (20X objective) were acquired from 18µm-thick coronal sections labeled for MECA-32. MECA-32-immunoreactive vessels of the primary superficial plexus of the median eminence and intrainfundibular capillary loops were counted in the rostrocaudal axis within the coordinates relative to bregma from −1.6 to −2.1 mm. The total number of fenestrated loops of the median eminence and ARH was evaluated in each animal (7 ± 2 sections per animal) by analyzing every other section to avoid over-counting capillary loops.

Permeability Assays- Diffusion studies:

Mice were injected with sterile 100µl 1% Evans Blue dye (Sigma, E21129-10G) in 0.9% saline and killed by decapitation 30 minutes later. After the intraperitoneal injection, the mice were decapitated, and brains were collected, embedded in O.C.T, then frozen in dry ice. Brains were processed for immunofluorescence as described previously (Mullier et al., 2010). Evans Blue staining was directly visualized and imaged with a fluorescent microscope using a 20X objective in the defined region of interest, i.e., the ARH (8 sections/ARH/animal). To determine the Evans Blue penetration into the vmARH, the region of interest starting from the external zone of the median eminence to the vmARH was defined and cropped for each image, and a histogram of the grey value gradient per µm was generated.

rLECT2 Injection:

The recombinant protein LECT2 (rLECT2) (Clinisciences CSB-EP012855MO) was reconstituted with sterile water and 5% glycerol and stored at -20°C. After thawing, rLECT2 was injected by intraperitoneal injection into wild-type female mice. Control mice were injected and subjected to fasting for 24 hours (from 1 pm day 1 to 1 pm day 2) to induce fenestration of the ME-ARH vessels. Before sacrifice, four injections of 1.2µg/50µl/40min of recombinant protein LECT2 (rLECT2) (Clinisciences CSB-EP012855MO) were administered to wild-type mice.

LECT2 ELISA:

Blood samples were taken from the submandibular vein of mice using E.D.T.A coated tubes, centrifuged for 20 minutes at 1500 r.p.m, and stored at -80°C. Plasma LECT2 levels were measured using an ELISA KIT (Cloud-clone corp SEF541Mu) according to the manufacturer's instructions.

Gene Expression Analysis by RT-qPCR:

Total RNA from liver cells was prepared using TRIzol. One thousand nanograms of RNA was reverse transcribed at 25°C for 10 minutes and then at 37°C for 2 hours in 20 µL final volume using the High-Capacity cDNA reverse transcriptase kit (Applied Biosystems). For gene expression, qPCR was performed using SsoFast Eva Green Supermix (Bio-Rad) on the Quant Studio 5 instrument (Applied Biosystems). Primers were validated by testing the PCR efficiency using standard curves (95% < efficiency < 105%) and their sequences were: LECT2 amplification (5' GTGGACAGTACTCTGCTCAAA 3' and 5' TCCCAGTGAATGGTGCATAC 3') and the Tbp reference gene (5' CCTATGACCCCTATCACTCCTG 3' and 5' TGTCCGTGGCTCTCTTATTCTC 3'). Gene expression was quantified using the comparative Ct (threshold cycle) method; the TBP (TATA-box binding protein) gene was used as a reference.

RNASCOPE:

mRNA expression and localization in the hypothalamus cells was observed with *in situ* hybridization was performed on frozen brain sections in the mediobasal hypothalamus of control and LERKO mice with the RNAscope Multiplex Fluorescent Kit v2 according to the manufacturer's protocol (Advanced Cell Diagnostics, Newark, CA, USA). Specific probes were used to detect Tie1, GPR19, DDR1, and RxRα mRNA: RNAscope® Probe- Mm-Tie1-C2(491841-C2); RNAscope® Probe- Mm-Gpr19 (318171); RNAscope® Probe- Mm-Rxra (463121); RNAscope® Probe- Mm-Ddr1 (316671). Following the in situ hybridization (FISH), immunostaining was done using rat anti-MECA-32 antibody (1:500, BDBioscience Cat#AF3628 AB_19.2772), revealed with Goat anti-rat 647 (1:500; Thermo et al. #A-21247, RRID: AB_141778).

Image Acquisition:

Immunohistochemistry was visualized using an Axio Imager Z2 light microscope (Zeiss, RRID: SCR_018856). Brain sections were taken with a 20X objective for the capillaries and a 40X objective for the tight junctions. For FISH experiments and immunofluorescent staining of MECA-32, images were acquired using an inverted confocal microscope (Leica STELLARIS 5 microscope, RRID: SCR_024663).

Statistics:

Results are expressed as the mean ± SEM. Data were analyzed for statistical significance with the Prism software, using a t-test, one-way, or two-way ANOVA, followed by a Tukey post-hoc test when appropriate. Correlations were done using a Spearman test. p-values of less than 0.05 (*) were considered statistically significant

APPENDIX

A new way to Quantify the ME-ARH region

Throughout the primary project, the plasticity of the ME-ARH region was quantified based on classical immunolabeling on 18µm brain slices. The technique used to quantify the endothelial fenestration and ZO-1 organization in the ME-ARH region has been reliable for many years. However, with the advent of new scientific tools, this technique could be improved. For instance, the clearing technique helps visualize the fenestrated vessel area as a whole system.

This project aims to propose an optimized sample processing, immunostaining, tissue clearing, and three-dimensional analysis of the hypothalamic BBB, which can be used to study the mediobasal hypothalamus plasticity under various conditions. This project is still underway.

Optimization of Sample Preparation and Clearing Protocol

Tissue clearing is usually performed on the whole mouse brain using light-sheet microscopy to observe the distribution of certain proteins or the trajectory of neurons. This technique is more appropriate for characterization. This project aims to quantify the MECA-32 and ZO-1 positive signals more easily.

We decided to (1) restrict the clearing protocol to the hypothalamus by cutting a 1mm thick coronal section of the brain using a vibratome (Leica) and (2) use a confocal microscope to enhance the acquisition quality.

Animals were perfused with cold phosphate-buffered saline (PBS) followed by the fixative paraformaldehyde at 4% in PBS before brain dissection. The samples were post-fixed in the fixative overnight, then rinsed and stored in PBS at 4°C. A coronal section was cut from each brain to isolate the hypothalamus.

The samples were subjected to a clearing protocol:

- **Pre-treatment:** Bleaching in 0.3% H_2O_2 for a few hours to reduce autofluorescence. After rinsing with Tris-buffered saline (TBS), the samples were incubated in a solution of 10% 1,2-hexanediol at 37°C to remove the lipids from the brain sections.
- **Immunostaining:** Primary antibody incubation for 3 days at 37°C: Rabbit anti-ZO-1 (Proteintech, #21773-1-AP) 1:1000; Rat anti-MECA-32 (BD Pharmingen, #550563) 1:500; Goat anti-CD31 (R&D Systems, #AF3628) 1:300. Secondary antibody incubation for 3 days at 37°C.
- **Clearing treatment:** Dehydration in ethanol gradually (50%, 60%, 70%, 80%, 90% in TBS 2 x 100% ethanol, 30 minutes at room temperature) and gradual clearing in dibenzyl ether DBE (25%, 50%, 75% in ethanol, 2 x 100% DBE, 1 hour each at 37°C with rotation). Cleared samples were transferred to a fresh DBE solution and kept in the dark at room temperature. Due to their similar refraction index, the sample will be transparent in the DBE solution.

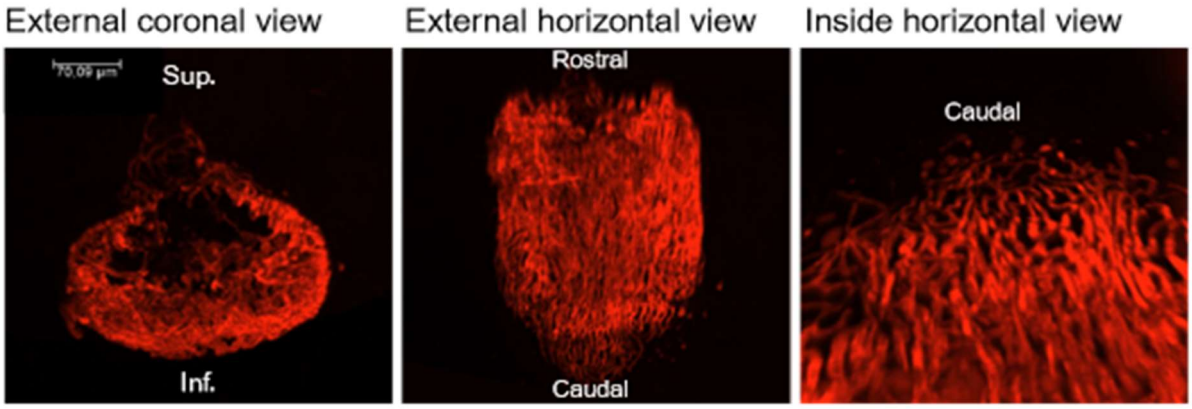


Figure 28: 3D acquisition of anti-MECA-32 signal or fenestrated vessels of the ME-ARH region of the hypothalamus

A 3D acquisition of the fenestrated vessels with the antibody anti-MECAC-32 of the hypothalamus from different view. The 3D acquisition was made with a light sheet microscope. Sup.: superior, Inf.: Inferior

The acquisition of fenestrated vessels using a light-sheet microscope provides a 3D rendering of the ME-ARH fenestrated vessel system, helping us understand its anatomy and better appreciate the direction of each vessel **(fig.27)**. However, the acquisition quality could be improved for better quantification. This is why we attempted acquisition with a confocal microscope. The dehydration with ethanol reduced the sample's thickness, bringing the samples within the depth of field of a Stellaris Leica confocal microscope. The samples were placed on a platform with a glass bottom filled with DBE to keep them cleared.

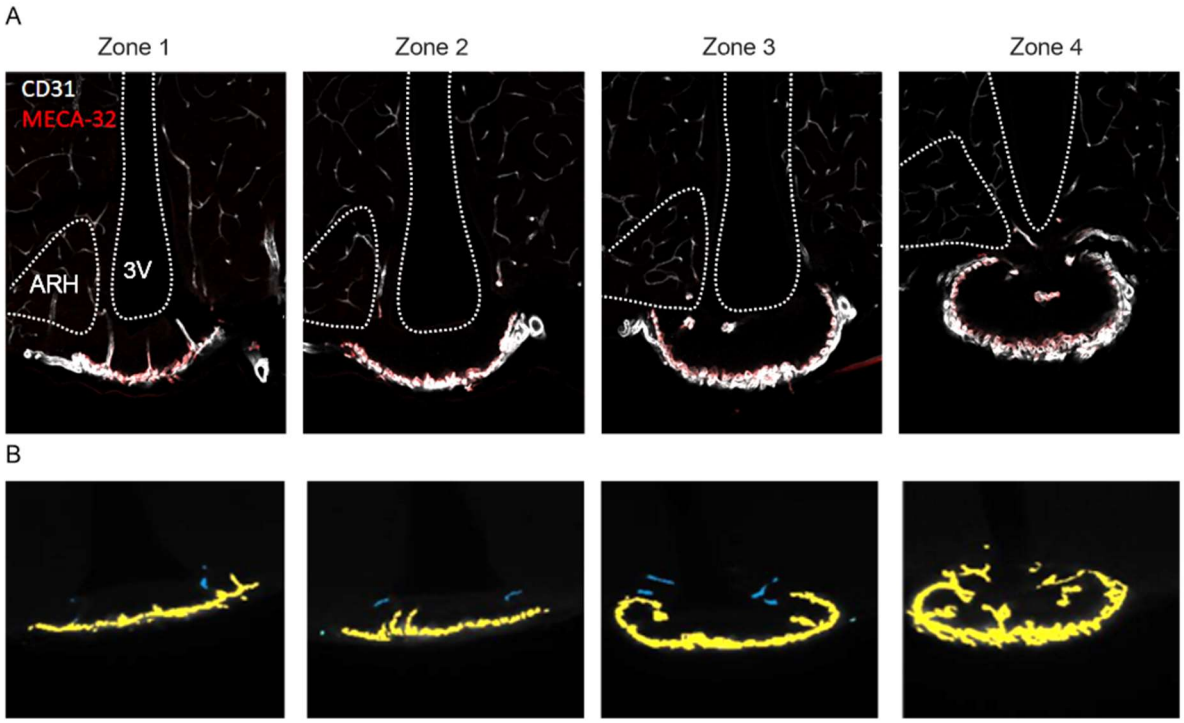


Figure 29: Immunostaining of the fenestrated vessels and vessels following a clearing technique of an entire hypothalamus

(A) Different sections of the acquisition of the hypothalamus' fenestrated vessels (MECA-32, red) and vessels (CD31, white) in Zone 1 to 4 ***(see fig.6)****. ARH: Arcuate hypothalamic nucleus, 3V: Third ventricle. (B) Detection of fenestrated vessels in the median eminence (yellow) and the ARH (blue) by the elastik software.*

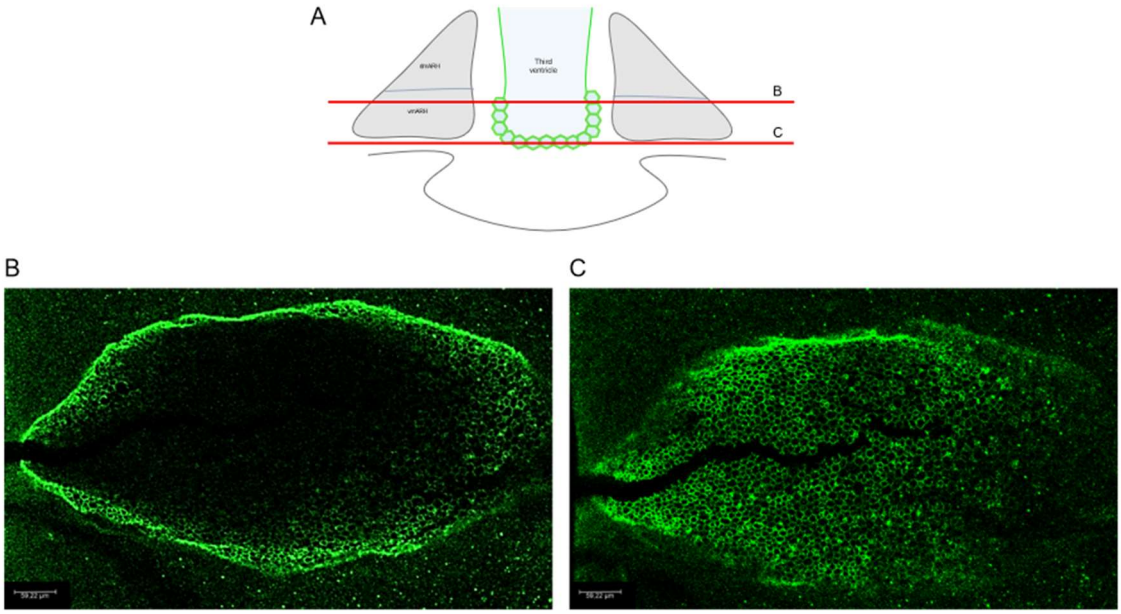


Figure 30: Anti-ZO-1 immunostaining of the ME-ARH region after a clearing technique of an entire hypothalamus

(A) Representation of the mediobasal hypothalamus created with Biorender. The upper red line represents the transversal section in (B), and the lower red line represents the (C) section. B and C are Anti-ZO-1 immunolabelling of the hypothalamus' tanycytic barrier. The acquisition was made with the Stellaris microscope.

A limitation of the 2D technique (hypothalamus section cut with a cryostat) is that the tissue is fragile and can contain defects in individual sections, reducing the number of sections usable for each sample. Here, the 3D sample of the hypothalamus allowed to make an acquisition with the confocal microscope with a Z-stack of 5µm thick. With the confocal acquisition we obtained around 60-70 intact images per hypothalamus. This major improvement will increase the quantity ad quality of the analysis.

Antibody visualization with the confocal acquisition:

The MECA-32 positive signal is clear enough to detect individual fenestrated capillaries, and the immunostaining of CD31 helps visualize all the vessels of the hypothalamus, normalizing the number of fenestrated vessels in each brain **(fig.28 A)**.

The coronal resolution of the ZO-1 antibody positive signal is not optimal, as it fails to properly show the honeycomb pattern surrounding the tanycytes. Therefore, a separate acquisition in

the transverse section was made to observe the bed of the third ventricle, where the ME tanycytes reside. In **(fig.29)**, we see the honeycomb pattern covering the 3V surface.

Towards a new quantification:

A possible technique to quantify the fenestrated vessels is to create a 3D reconstruction of them to extract the area of each zone of the vessels in the ME and the vmARH. The first step is to train Ilastik, a machine learning software, to detect the fenestrated vessels coming from the median eminence or near the vmARH. After that, the software will be able to detect and extract the information from each image forming the acquisition **(fig.28 B)**. With the extracted information, the Imaris software will be able to create a precise 3D reconstruction of the ME-ARH region, allowing us to calculate the area of diffusion of the chosen zone or section.

This technique is underway to become a new method to study fenestrated vessels, but it still needs to be refined, as does the quantification of the ZO-1 signal.

Scientific participation:

- ✓ **Collaboration with Dr. Fanny Langlet, Lausanne, Switzerland September 2021**
    - Fast-Fed paradigm of the ME-ARH hypothalamic region with an exchange Grant from the Society of neuroendocrinology (SNE) awarded in 2021.

- ✓ Participation to **DECLIC** (Dialogues Entre Chercheurs et Lycéens pour les Intéresser à la Construction des Savoirs) in 2019 and 2021
    - meeting with high schoolers to introduce the researchers' life.
- ✓ **Challenge Doc,** Euratechnologie, Lille in October 2022
    - A three-day team work on a research subject proposed by a start-up company
- ✓ Representative of the **EDBSL** PhD candidates 2021-2024

Scientific communication

Poster presentations

- ✓ **FENS congress, July 2022, Paris, France** The Hepatic Estrogen Receptor A Potential Role in The In Mediobasal Hypothalamus Plasticity

  Ophélie Hanot, Blandine Tramunt, Sreekala Nampoothiri, Danièle Mazur, Daniela Fernandois, Pierre Gourdy, Vincent Prevot, Françoise Lenfant, Alexandra Montagner, Bénédicte Dehouck
- ✓ **ICN Congress, August 2022, Glasgow, Scotland** Liver-hypothalamus axis: The role of the hepatic estrogen receptor α in the energy homeostasis

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