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**MOLECULAR STUDIES OF HYPOTHALAMIC FOOD INTAKE  
REGULATING SYSTEMS AND CENTRAL MYELINATION IN TWO  
ANORECTIC MOUSE MODELS**

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# Molecular studies of hypothalamic food intake regulating systems and central myelination in two anorectic mouse models

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To  
Ludvig  
&  
Mom  
for being indestructible

In Loving Memory of  
Antonina  
Rose-Marie  
Marit  
Ann-Marie

”Only you can control your future.”

Dr. Seuss

## ABSTRACT

The aim of this thesis is to increase the understanding of the hypothalamic mechanisms that are vital for food intake regulation, in particular as it relates to anorexia, and the molecular mechanisms underlying myelination and nodal/paranodal domain organization. The majority of the thesis concerns the anorectic phenotype of two mouse models for which we have found both great similarities and differences pointing to different mechanisms generating similar symptoms and neuropeptidergic alterations. This thesis starts with immunohistological and electron microscopic investigations of the regulatory effect of the GPI-anchored cell adhesion molecule Contactin-1 in myelination in the central nervous system (CNS) and functional compartmentalization of myelinated nerves (**Paper I**). With the help of the *Cntn1*-KO mouse and a *Cntn1*-KO cross expressing green fluorescent protein in myelin, our analysis revealed several novel functions of Contactin-1 in CNS myelination. These include the demonstration of Contactin-1 expression in oligodendrocytes *in vivo* and its role in regulating neuron-glia interactions required for myelin membrane extension and myelination. Further, we found that Contactin-1 is essential for the domain organization of myelinated nerves by organizing the attachment of the terminal myelin loops to the axon membrane at the paranode. Contactin-1 is thus a key molecule for forming functional fast propagating high conduction velocity myelinated nerves in the CNS. We continue to explore the roles of Contactin-1 in the CNS by investigating the anorectic and hypothalamic phenotype of the *Cntn1*-KO in comparison with the anorectic *anx/anx* mouse (**Paper II and IV**). In these two studies involving immunohistochemistry and *in situ* hybridization techniques, we show similarities between the two models in the deviation from the wild type expression levels and location of hypothalamic neuropeptides (NPY, AgRP,  $\alpha$ -MSH/POMC and MCH) important for food intake regulation (**Paper II**). However, further analysis revealed apparent differences with regard to the expression of astroglial and microglial markers in the hypothalamic system, as well as in the hippocampus (**Paper IV**). A significant upregulation of markers of astroglial and of microglial activation (previously published) was found in the *anx/anx* hypothalamus, indicating an inflammatory reaction. In contrast, the *Cntn1*-KO mouse displays no such glial responses in the hypothalamus. We did however detect increased expression of the microglia marker in the hippocampal dentate gyrus of the *Cntn1*-KO mouse, which we did not see in the *anx/anx* mouse. Based on previous findings associating the *anx/anx* mouse with a mitochondrial dysfunction, we explored the possibility of a reduced metabolic rate of hypothalamic neurons (**Paper III**). Enzymatic assays, *ex vivo* autoradiography and Western Blot of the *anx/anx* hypothalamus revealed reduced glucose uptake, reduced cellular metabolic rate both in basal and ischemic conditions and reduced ATP-turnover. The ratio of the metabolic master regulator, AMPK-P/AMPK, was reduced in the anorectic *anx/anx* hypothalamus. Taken together this is indicative of a hypometabolic state in the hypothalamus of the *anx/anx* mouse resembling what is seen during hibernation. The two anorectic mouse models have many similarities and many differences making them valuable to further understand the food intake regulating systems. By elucidating molecular pathways the data in this thesis may in the future yield improved understanding of disorders such as Anorexia Nervosa and Multiple Sclerosis.

## LIST OF SCIENTIFIC PAPERS

- I. Contactin-1 regulates myelination and nodal/paranodal domain organization in the central nervous system.  
Çolakoğlu G\*, **Bergström-Tyrberg U\***, Berglund EO, Ranscht B  
*PNAS*, 2014, 111(3):E394-403 \* Equal contribution to the experiments.
- II. Alterations of arcuate nucleus neuropeptidergic development in contactin-deficient mice: comparison with anorexia and food-deprived mice.  
Fetissov SO, **Bergström U**, Johansen JE, Hökfelt T, Schalling M, Ranscht B  
*European Journal of Neuroscience*, 2005, 22(12):3217-28
- III. Reduced metabolism in the hypothalamus of the anorectic *anx/anx* mouse.  
**Bergström U\***, Lindfors C\*, Svedberg M, Johansen JE, Häggkvist J, Schalling M, Wibom R, Katz A, Nilsson IAK. \* Equal contribution  
*Manuscript*.
- IV. Glia cells in the hypothalamus and the hippocampus of two anorectic animal models: the Contactin-1 null mutant and the *anx/anx* mouse.  
**Bergström U**, Jonsson Axelsson L, Schalling M, Hökfelt T, Ranscht B, Nilsson IAK  
*Manuscript*.

## ADDITIONAL PAPERS

- I. Evidence for hypothalamic dysregulation in mouse models of anorexia as well as in humans.  
Johansen JE, Fetisov SO, **Bergström U**, Nilsson I, Fay C, Ranscht B, Hökfelt T, Schalling M.  
*Physiol Behav.* 2007, 10;92(1-2):278-82
  
- II. Genetic Control of  $\beta$ -Cell Mass Homeostasis.  
Soundarapandian MM, Nieves ML, Pasquier R, **Bergström U**, Atkinson MA and Tyrberg B  
*The Open Endocrinology Journal*, 2010, 4, 11-24



# CONTENTS

1	BACKGROUND.....	13
1.1	Anorexia Nervosa .....	13
1.2	Animal models of Anorexia Nervosa.....	14
1.2.1	The <i>anx/anx</i> mouse .....	16
1.2.2	The <i>Cntn1</i> -KO mouse .....	17
1.2.3	Contactin.....	17
1.2.4	Other animal models of anorexia.....	18
1.3	Hypothalamic regulation of food intake.....	19
1.3.1	Anatomy and neurochemistry of the hypothalamus .....	19
1.3.2	Postnatal development of the AgRP/NPY system .....	20
1.3.3	Food intake regulatory hormones from the periphery .....	21
1.4	Glia cells.....	22
1.4.1	Astrocytes .....	22
1.4.2	Oligodendrocytes .....	23
1.4.3	Microglia .....	24
2	Thesis objectives.....	25
3	MATERIALS & METHODS .....	26
3.1	Animals (paper I-IV).....	26
3.2	Immunohistochemistry (paper I, II AND IV) .....	27
3.2.1	Fixation of tissue .....	27
3.3	Immunohistochemical labeling.....	28
3.4	<i>In situ</i> hybridization (paper II).....	29
3.5	Electron microscopy .....	29
3.6	Western blot (paper II & III).....	30
3.7	<i>EX vivo</i> autoradiography (paper III).....	30
3.8	Enzymatic activity assays (paper III) .....	31
3.9	HPLC.....	32
4	RESULTS & DISCUSSION.....	33
4.1	Contactin regulates myelination and nodal/paranodal organization in the central nervous system (paper I) .....	33
4.1.1	Contactin-1 in axon-glia interactions initiating myelination .....	33
4.1.2	Contactin-1 and paranodal assembly in CNS.....	34
4.1.3	The role of Contactin-1 in formation and maturation of the Node of Ranvier .....	34
4.1.4	Summary of Paper I .....	35
4.2	Comparative studies of hypothalamic food intake regulatory systems in animal models of anorexia (paper II & IV) .....	35
4.2.1	Changed hypothalamic NPY and AgRP expression.....	36
4.2.2	Changed hypothalamic $\alpha$ -MSH/ POMC expression.....	36
4.2.3	MCH expression in the LHA .....	37
4.2.4	Contactin-1 expression in the hypothalamus.....	37

4.2.5	GFAP immunohistochemistry in the hypothalamus and hippocampus.....	37
4.2.6	Iba1 immunohistochemistry in the hypothalamus and hippocampus.....	38
4.2.7	Summary of Paper II & IV.....	38
4.3	Reduced hypothalamic metabolism in the <i>anx/anx</i> mouse (paper III).....	39
4.3.1	Enzymatic activity of the respiratory chain subunits in the <i>anx/anx</i> hypothalamus .....	39
4.3.2	Glucose transporters and AMPK activation in the <i>anx/anx</i> hypothalamus .....	40
4.3.3	A hypometabolic state in the <i>anx/anx</i> hypothalamus.....	40
4.3.4	Reduced glucose uptake in the <i>anx/anx</i> hypothalamus.....	41
4.3.5	Summary of Paper III.....	41
5	CONCLUSION AND HYPOTHESIS .....	42
6	FUTURE PERSPECTIVES.....	46
7	ACKNOWLEDGEMENT .....	47
8	REFERENCES.....	49

## LIST OF ABBREVIATIONS

3V	Third ventricle
ADP	Adenosine diphosphate
AGRP	Agouti gene related protein
AMP	Adenosine monophosphate
AMPK, AMPK-P	5' AMP-activated protein kinase (-P = phosphorylated)
AN	Anorexia Nervosa
<i>anx</i>	The anorexia gene/mutation
APC	Adenomatous polyposis coli
Arc	Arcuate nucleus
ATP	Adenosine triphosphate
$\alpha$ -MSH	$\alpha$ - melanocyte- stimulating hormone
BMI	Body mass index
CART	Cocaine- and amphetamine-regulated transcript
Caspr	Contactin associated protein
CI, CII, CIII, CIV	Mitochondrial oxidative phosphorylation system complex 1-4
CNS	Central nervous system
CNTN	Contactin
DAPI	4',6-Diamidino-2-Phenylindole- fluorescent stain binding to A-T rich regions in DNA
DiI	fluorescent lipophilic dye used for neuronal tracing
EM	Electron microscopy
GFAP	Glia fibrillary acidic protein
GLUT1, GLUT4	Glucose transporter 1 and 4
Iba1	Ionized calcium-binding adapter 1
IHC	Immunohistochemistry
ir	Immuno reactivity
ISH	<i>in situ</i> hybridization
Kir6.2	Inward-rectifier potassium ion channel (major component of $K_{ATP}$ )
KO	Knock-out (absent)

Kv1.2	Voltage gated potassium channel 1.2
LHA	Lateral hypothalamic area
MBP	Myelin basic protein
MCH	Melanin concentrating hormone
ME	Median eminence
MOG	Myelin-oligodendrocyte glycoprotein
MPO	Medial preoptic (area)
mRNA	Messenger ribonucleic acid
Nav1.2 and Nav1.6	Voltage gated Sodium channel alpha subunit type 1.2 and type 1.6
NG2	Neuron-glia antigen 2
NPY	Neuropeptide Y
PFA	Paraformaldehyde
PNS	Peripheral nervous system
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RPTP $\beta$	Receptor-type protein tyrosine phosphatase beta
SUR1	Sulfonylurea receptor 1 (regulatory unit on K <sub>ATP</sub> )
WT	Wild type (normal control)

# 1 BACKGROUND

Whether concerning the development/plasticity of the hypothalamic systems involved in regulation of food intake, or myelination and nodal/paranodal assembly of the optic nerve both require a delicate interplay and temporal timing of specific molecular events and interactions. These mechanisms are in the present thesis explored in two animal models displaying the core features of Anorexia Nervosa (AN); emaciation and starvation due to low food intake.

## 1.1 ANOREXIA NERVOSA

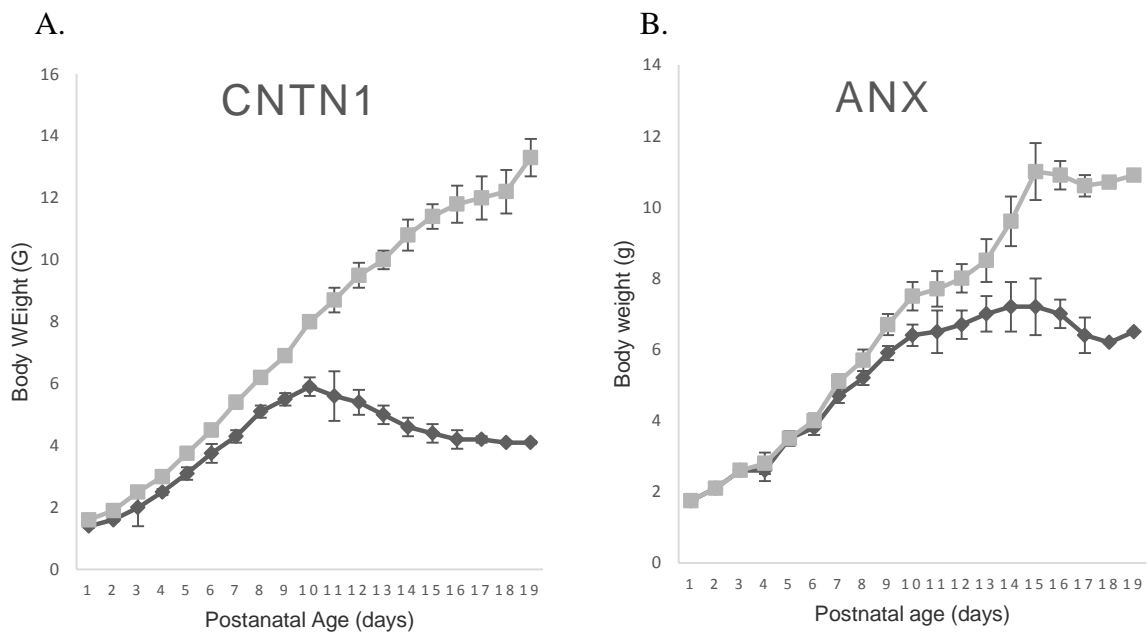
AN is an eating disorder diagnosed, as other psychiatric conditions, according to among others the diagnostic and statistical manual of mental disorders 5th edition, DSM-5, for which the diagnosis criteria includes; restriction of energy intake, fear of weight gain despite being underweight and disturbed perception of body image [1-3] and with a body mass index below 19. The prevalence of AN is highest in females aged 15-19 years which corresponds to about 40% of all AN patients. Overall AN affects around 1,2% of females and 0.3% of males, sometime in life [4]. The incidence rate in females is 109,2 per 100 000 per year and the incidence in males is about 1 per 100 000 persons –years, found by studies in UK and the Netherlands [5-7]. AN has the highest mortality rate among all mental disorders, i.e. approximately 10% of those affected die as a consequence of the disorder due to e.g. heart-, kidney failure or suicide [8-10]. Another frequently occurring symptom of AN is amenorrhea, meaning a loss of menstrual cycle, in women, which is a result of a decrease of reproductive hormones. This usually returns with increased weight but not always and then the result may be linked to hypothalamic dysfunction [11-13]. Complications of the cardiovascular system as a result of decreased contractility and electrolyte imbalances are also common. Besides somatic disturbances comorbidity with other psychiatric diagnoses, like depression, anxiety and obsessive- compulsive disorders are common [14-17].

To date the etiology of AN is to a large extent unknown, there are however strong indications that the disorder is multifactorial with an interplay between environmental/societal factors and a genetic predisposition [18] [19]. The genetic attribution is high, i.e. around 50-70% of the background has been traced to additive genetic factors [20-23]. In patients with AN or Bulimia Nervosa there have been correlations to disturbances in the hypothalamic-pituitary-adrenal axis, characterized by hypersecretion of corticotropin-releasing hormone, CRH which may lead to cortisol resistance [1, 24-26]. Studies of obesity have gained understanding in the mechanisms regulating appetite and stimulation of satiety (see section below on hypothalamic regulation of food intake) [27].

## 1.2 ANIMAL MODELS OF ANOREXIA NERVOSA

While several animal models of obesity are well characterized and used in research such as the leptin deficient  $Lep^{Ob/Ob}$  (commonly called  $Ob/Ob$ ), the leptin receptor deficient  $Lep^{Db/Db}$  (commonly called  $Db/Db$ ) or high-fat diet induced obese mice, much fewer anorectic animal models exist. The complexity of human AN makes preclinical studies of the disorder limited to focus on characteristic traits shared with the human version of the disease, such as emaciation and starvation [28, 29]. However, these models are still able to generate important new information of specific aspects of the disorder such as knowledge about the hypothalamic food intake regulating systems in an anorectic condition. Two anorectic mouse models are used in the present work; the  $anx/anx$  and the *Contactin-1* null mutant (*Cntn1*-KO) mouse.

One diagnosis criteria for AN is as stated above a significant weight loss below 85% of normal weight for age and height or a body mass index below 18 [30]. A significantly reduced body weight is observed from approximately postnatal day (P) 10 for both the  $anx/anx$  and the *Cntn1* mouse, see figure 1.



**Figure 1.** A. Body weight curves for *Cntn1*-KO (black diamonds) vs, WT (grey squares) (modified from Berglund et al. [31]) and B.  $anx/anx$  (black diamonds) vs WT (grey squares) (modified from Maltais et al. [32]).

The reduction in body weight results in a severely emaciated appearance of the mice by P16 and P21, for the *Cntn1*-KO and the  $anx/anx$  mouse, respectively, when compared to their wild type (WT) siblings (Figure 2).

A.



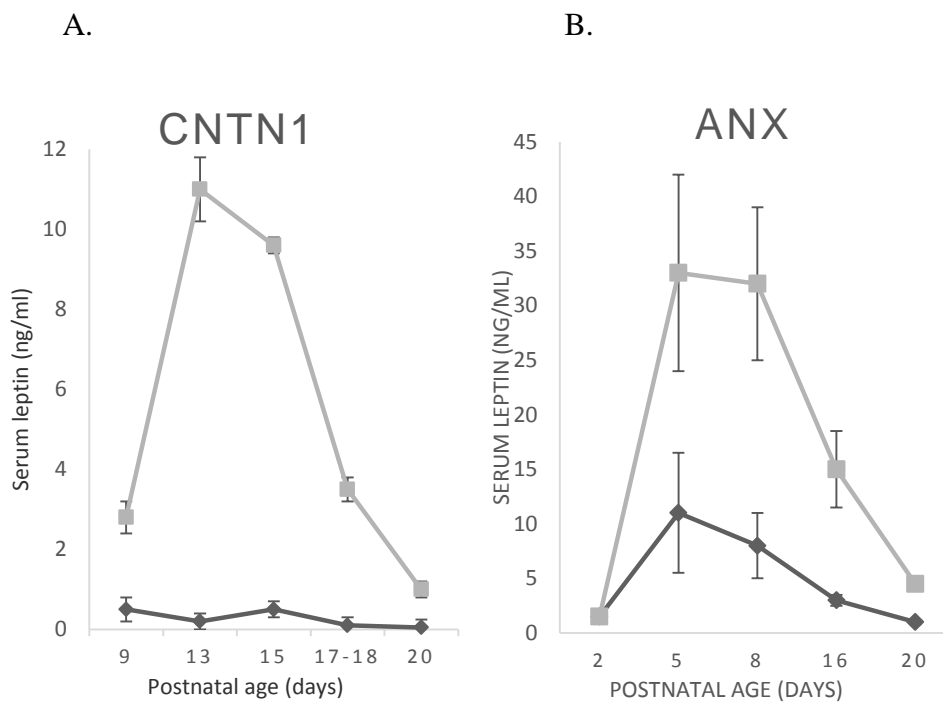
B.



**Figure 2. A.** *Cntn1* (bottom) and WT sibling age P16, **B** *anx/anx* (top) and WT sibling age P21.

The second common nominator between the *Cntn1*-KO, the *anx/anx* mouse and patients with AN is the reduced and insufficient food intake. This despite that they in all cases, animals as well as humans, have full access to food. Maltais et al. showed that the stomach content of the *anx/anx* mouse was significantly reduced already from P5 [32]. The *Cntn1*-KO mouse has a reduced stomach content compared to the WT littermates both for milk and even less with solid food (Bergström U and Ranscht B, unpublished data). This mouse is consequently helped with assisted feeding twice daily.

A third parameter shared between AN and the two animal models is the levels of the adiposity derived hormone leptin (see below). The levels of leptin is reduced in blood from AN patients [33, 34] and is also reduced in the two anorectic animal models in this thesis; the *Cntn1* and the *anx/anx* mouse (Figure 3) [32, 35].



**Figure 3. A.** Serum leptin levels in *Cntn1* vs WT mice (unpublished data, Bergström U and Ranscht B) and **B.** *anx/anx* vs WT mice (modified from Johansen J.E. et al [35]).

### 1.2.1 The *anx/anx* mouse

The *anx/anx* mouse arose spontaneously at the Jackson laboratory (Bar Harbor, ME) back in 1976 [32]. The *anx* mutation is an autosomal recessive mutation that arose in the F2 generation of a cross between an inbred strain derived from crossing *M.m.poschiavinus* and an inbred Swiss strain with DW/J. The mouse was brought to Karolinska Institutet by Prof. M Schalling, in the mid 90'ies and has been bred independent of the Jackson line since then. The mutation is maintained on the B6C3H-a/a F<sub>1</sub> background and was mapped to a 0.2 cM interval on mouse Chromosome 2 [32, 36]. As explained above the *anx/anx* mouse eat significantly less than their WT siblings already from P5. Subsequently the mouse becomes emaciated and dies in a rather lethargic state around postnatal day 21. The *anx/anx* mouse is bred from heterozygote parents as the *anx* mutation is too detrimental and lethal before breeding can begin.

Several papers have shown aberrances in neuropeptidergic and -transmitter systems in the hypothalamus that are central for regulation of food intake [37-50]. Nilsson et al showed up regulation of a marker for microglia selectively in hypothalamic areas involved in food intake regulation [45]. Microarray studies of the *anx/anx* hypothalamus by Mercader et al and Lachuer et al. revealed a cachectic and inflammatory profile, respectively [51, 52]. Up regulation of several microRNAs in the hypothalamus was shown as well as increased expression of the *Ntrk3* gene, coding for a receptor for Neurotrophic Tyrosine Kinase, Receptor, Type 3 [53]. In addition to the hypothalamus, increased proliferation and apoptosis was documented in the dentate gyrus part of the hippocampus of these mice [54]. Lastly, it has been shown that a gene in the interval were the *anx*-mutation is located called NADH:ubiquinone oxidoreductase complex assembly factor 1, (*Ndufaf1*) is down regulated in the *anx/anx* mouse [36]. This gene is an assembly factor for the first complex (C) in the oxidative phosphorylation system in the mitochondria [55] (Figure 4). A down regulation of this gene thus results in a reduced capacity of CI and increased production of reactive oxygen species (ROS) [36].

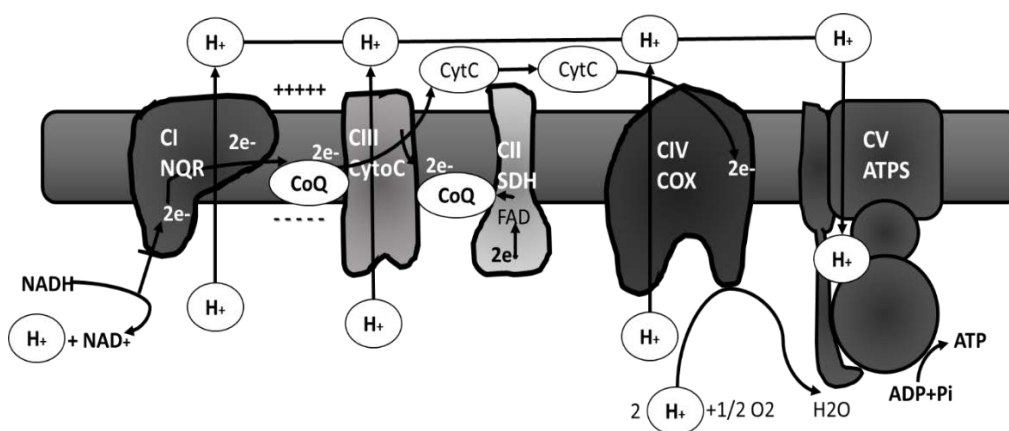


Figure 4. The mitochondrial oxidative phosphorylation system; Complex I to V with the enzymes abbreviated analyzed in Paper III. NADH: ubiquinone oxidoreductases (NQR): succinate dehydrogenase (SDH), succinate: cytochrome c reductase (SCR), cytochrome C oxidase (COX), ATPS: ATP synthase. Adapted from general textbooks.



### 1.2.2 The *Cntn1*-KO mouse

The *Cntn1*-KO mouse was created in the Ranscht laboratory by Dr. Eric O. Berglund through insertion of a neomycin resistant cassette into exon 3 of the *Contactin-1* gene thereby disrupting the normal functions of the protein [31]. The gene construct was then transferred to embryonic stem (ES) cells by homologous recombination. The ES clones were subsequently transferred into blastocysts creating chimeras. The mouse is maintained on either a mixed 129SVJ 3x C57Bl/6 3 x Black Swiss or a pure C57Bl/6 background and no developmental, molecular or phenotypic differences are observed between the lines. The *Cntn1*-KO mouse is born identical to the WT littermates, however by P8 a reduced body weight and tremors start to become apparent. They are fed twice a day from P10 and given subcutaneous saline injections and in addition they had free access to milk and chow. The characteristic phenotypes develop gradually with ataxia and anorexia as the major traits, but also a failure to rectify to upright when turned on to the back. The *Cntn1*-KO mouse dies in a very emaciated state between P16-19, despite given nutritional supplementation twice. The *Cntn1*-KO mouse is born from heterozygote parents, as homozygote breeding is impossible due to the detrimental phenotypes and lack of survival.

In the cerebellum is Contactin-1 localized to the synaptic contacts between Golgi and granule cells and in the *Cntn1*-KO mouse this results in a decrease of both pre- and postsynaptic GABAergic proteins in these cells [56] as well as deformed cerebellar glomeruli (unpublished Bergström, U.). The *Cntn1*-KO mouse also has a reduction in neurite outgrowth from isolated dorsal root ganglion and cerebellar granule neurons, implicating specifically Contactin-1 to be involved in neurite outgrowth [57, 58]. Loss of Contactin-1 in myelinated nerves disrupts the paranodal attachment sites between myelin and the axon and slows down the propagation of compound action potentials along the nerve [59]. Finally, the *Cntn1*-KO has been utilized to analyze the nodal and paranodal complexes of centrally myelinated nerves [60-63].

### 1.2.3 Contactin

The Contactins are a family of immunoglobulin cell adhesion molecules expressed exclusively in the nervous system. The other Contactin family members are Contactin-2 (TAG-1), Contactin-3 (BIG-1), Contactin-4 (BIG-2), Contactin-5 (NB-2) and Contactin-6 (NB-3)[64, 65]. Contactin-1 was first to be named Contactin and occurs in early publications as only Contactin or F11 (chicken) [66] or F3 (mouse) [67] or Gp135 (human) [68]. At the amino acid level, human Contactin-1 is 78% identical to chicken Contactin-1/F11 and 94% to mouse Contactin-1/F3 [69]. Both Contactin-1 and Contactin-2 have been linked to important developmental events involving axon guidance, myelination, neuronal cell adhesion, migration and neurite out growth [70, 71]. The Contactins are characterized as cell surface glycoproteins with six immunoglobulin-like domains and four fibronectin-like regions and

are linked to the cell membrane through a glycosylphosphatidylinositol (GPI)-anchor [72],[73]. In myelinated nerves in the central nervous system (CNS) and the peripheral nervous system (PNS), Contactin-1 is found in at the paranodes located on each side of the node of Ranvier. The axonal Contactin-1 interacting in cis with Contactin associated protein -1 (Caspr-1) on the axonal side and in trans with neurofascin -155 on the glia side [59]. In the CNS, Contactin-1 is in addition localized at the nodes of Ranvier and on the oligodendrocytes where it regulates not only paranodal domain organization but also myelination and nodal structure and function [74]. Contactin-1 regulates the cerebellar granule cell migration, and guidance of granule cell axons and dendritic projections from granule and Golgi cells [31]. The interaction between Contactin-1 and Caspr is essential for the latter's release from the endoplasmic reticulum and transport to the plasma membrane [75]. Caspr is a transmembrane protein that contains a cytoplasmic region that can interact with other molecules and anchor the complex to the cytoskeleton. Contactin-1 is localized to synapses and essential for targeting Caspr to synapses in the hippocampus and affect the proper distribution of receptor-type protein tyrosine phosphatase beta (RPTP $\beta$ ) expressed by neurons and glia cells [76]. However, Contactin-1 role in synaptic plasticity is independent of its association with Caspr [77]. Finally, Contactin-1 has most recently also been associated in tumor invasion and metastasis [78] and was found to interact with Caspr2 [79] which may be linked to autism [80, 81]. Interestingly, genetic studies have found associations between genes related to contactins and AN [82, 83].

#### **1.2.4 Other animal models of anorexia**

In addition to the *Cntn1*-KO and the *anx/anx* mouse models for AN there are a few other rodent models displaying various phenotypes resembling traits in the human condition AN, most commonly the low food intake and the emaciation. The activity-based anorexia (ABA) mouse/rat model possess the key characteristics of AN, mainly hyperactivity and reduced food intake. The anorexia of the ABA rodents is induced by food restriction and introduction of an exercise wheel into the cage. When reinstating unrestricted access to food, when the wheel still is present, they continue to prefer running instead of eating. This leads continued weight loss until death. If the wheel is removed when food is again unrestricted the weight is normalized [84, 85]. Another mouse model that also becomes hypophagic and hyperactive is the dopamine deficient (DD) mice. These mice lack the dopamine-synthesizing enzyme, tyrosine hydroxylase, in dopaminergic neurons. Around postnatal day 34 this mouse dies as a result of starvation [86]. Both mice and rats centrally injected with brain derived neurotrophic factor, BDNF, have been found to induce feeding suppression [87] and weight loss [88]. Interestingly, variants of the BDNF gene have been associated with AN [89]. Adult ablation of AgRP neurons also results in anorexia [90] while neonatal ablation of the same neuronal population has no body weight phenotype [91]. Anorexia is furthermore observed in mice after ablation of Glut4 neurons in the hypothalamus [92] and with hypothalamic inhibition of AMPK food intake is reduced [93, 94].

### **1.3 HYPOTHALAMIC REGULATION OF FOOD INTAKE**

Energy homeostasis refers to the processes that govern the balance between the ingested energy from food, the input, and the cellular energy expenditure, the output, by the process of basal metabolism and physical activity (increased metabolism) [95, 96]. A positive energy balance indicates a higher energy intake than what is consumed by energy expenditure, and will over time result in weight gain when the energy is stored mainly as fat. Negative energy balance is a result of lower energy intake than what is consumed e.g. during fasting or starving. There are several clinical manifestations that affect this delicate balance to maintain a healthy energy balance, such as diabetes, obesity and the reverse malnutrition, anorexia and cachexia [97, 98]. The hypothalamus is the area of the brain that is central in the coordination of the regulation of food intake and thus provides potential therapeutic targets both concerning anorexia and obesity [99-103].

#### **1.3.1 Anatomy and neurochemistry of the hypothalamus**

The hypothalamus is part of the diencephalon and is involved in mediating endocrine, autonomic and behavioral functions. The hypothalamus controls hormone release from the pituitary gland, temperature, food intake, reproduction and the circadian rhythm [104]. The center for regulation the food intake is a cluster of cells located in the mediobasal hypothalamus, the Arcuate nucleus (Arc) [105, 106].

The Arc is located adjacent to the third ventricle and the median eminence where the blood-brain-barrier is more permeable, which makes these neurons susceptible to hormonal variations originating from the periphery. The Arc contains neuroendocrine neurons (ventrolateral Arc) and centrally projecting neurons (ventromedial) [105-108]. This thesis focus on the centrally-projecting neurons projecting to the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA), but the Arc also establish additional connections with other hypothalamic areas well as extra-hypothalamic areas including the medial preoptic area (MPO) and the dorsomedial hypothalamus (DMH). One of the two main Arc neuronal populations projecting centrally is the neuropeptide Y (NPY) and agouti-related protein (AgRP) expressing mostly GABAergic neurons mediating orexigenic signaling (increase food intake) [109]. The second Arc population is the pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) expressing glutamatergic neurons mediating anorexigenic signaling (decrease food intake). In addition to PVN and LHA projections the Arc AgRP/NPY and POMC/CART neurons interconnect with each other to regulate their neuronal activity and downstream effects and to ensure quick feedback in response to energy demand [42, 110-112]. There is at least one other GABAergic neuronal population in the Arc negative of AgRP or POMC, which is mildly involved in post-weaning feeding and projects directly to PVN [113].

The afferent inputs to the PVN include inputs from NPY/ AgRP positive neurons in the Arc regulating energy intake and inputs from the hippocampus regulating stress responses [114, 115]. Some efferent axons from the PVN project to median eminence at the base of the brain where neurosecretory nerves release hormones as part of the hypophyseal portal system (system of blood vessels in the brain connecting the hypothalamus with the anterior pituitary) resulting in hormone release regulation [115]. Other PVN axons project also to other brain regions as well as to the brainstem and spinal cord affecting the sympathetic system maintaining the cardiovascular homeostasis [116].

### **1.3.2 Postnatal development of the AgRP/NPY system**

The Arc AGRP/NPY system involved in food intake regulation develops postnatal in mouse [110] and rat [117]. In mice, the first Arc projections to the DMH are established at postnatal day 6 (P6), while but the projections to the PVN develops significantly later with the first mature connections established around P8 to P10 [110].

Different mouse strains have different developmental timelines and do not 100% correlate to each other molecular expression patterns [118-124] it is thus of importance to reflect on the time factor in developmental studies [125, 126]. In general the brain of newborn mice is more immature at birth and developmentally corresponds to human fetuses at the beginning of the third trimester of pregnancy [127]. However, the projections of human food intake regulatory pathways develop predominantly after birth [128] but the cell clusters representing the different hypothalamic areas are formed during later prenatal development.

The Arc projections are highly modifiable in mice pups and are affected by environmental factors coming from peripheral hormonal changes and genetic factors from within the AgRP/NPY and POMC/CART neurons [129-132]. This is observed in diet- induced obese rats where the Arc projections are permanently damaged [133].

In addition to the direct effect on food intake (see below), leptin is suggested to play an important role during postnatal development by regulating axonal outgrowth of both AgRP/NPY and POMC/CART neurons to target areas in the hypothalamus [110]. The rationale behind this is the leptin level peak during postnatal development that coincides with the Arc axonal outgrowth and the fact that this early the leptin levels doesn't correspond to the levels of adipose tissue. Leptin may during the postnatal development of the food regulatory pathways in the hypothalamus function as a growth factor promoting axonal outgrowth.

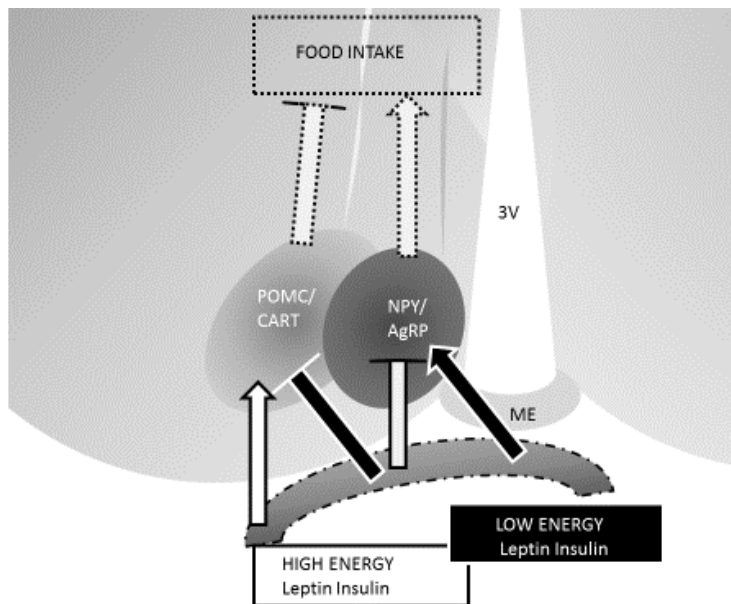
### 1.3.3 Food intake regulatory hormones from the periphery

Energy homeostasis is regulated via peripheral signals from both adipose tissue and the gastrointestinal tract penetrating the more permeable blood-brain-barrier (BBB) next to the median eminence and binding to receptors on the AgRP/NPY and POMC/CART neurons in Arc (Figure 5), and via the vagus nerve projecting to the brain stem.

**Leptin** is a hormone predominantly released from adipose tissue in response to food intake and the levels in blood correlates with the amount of adipose tissue. The hormone is transcribed from the obese *Ob*-gene and actively influences energy homeostasis. Circulating leptin enters the brain via the BBB and its receptor, the Ob-Rb, which is widely expressed in the hypothalamic regions; Arc, VMH, DMH, LHA and MPO [134-136]. Leptin binds to its' receptor which lead to downstream signaling involving activation/phosphorylation of STAT3 and in the orexigenic NPY/AgRP neurons it inhibits the activity, however, leptin activates anorectic POMC/CART neurons [105, 137]. Under conditions with low amounts of circulating leptin, like fasting, the orexigenic pathway is switched on and under the reverse conditions the anorexic pathway is switched on [138].

**Ghrelin** is the only known orexigenic hormone and was first discovered to be produced in the gastrointestinal tract but have been found to be produced in almost all tissues including Arc (NPY/AgRP neurons) in the hypothalamus together with its' receptor, growth hormone secretagogue receptor (GHSR) [139, 140]. Ghrelin can be transported bi-directionally across the BBB and has an antagonistic effect to action of leptin on the NPY receptors and correlates reversely to the levels of insulin, resulting in increased activity of the NPY/AgRP neurons.[141] Ghrelin expression will therefore lead to increased food intake [142]

**Insulin** is secreted from the pancreas and positively correlates with long-term energy balance but is primarily regulating blood glucose levels. Insulin enters the brain via the BBB and the concentration of insulin inside the brain is proportional to the serum levels. In the hypothalamus, insulin acts as an anorectic signal reducing food intake and body weight [143]. Insulin signals via a cell-surface insulin receptor widely expressed in the brain but particularly in hypothalamic Arc nuclei involved in food intake but also in DMH, PVN and PVN [144, 145]. Insulin signaling results in inhibition of the orexigenic NPY/AgRP neurons and activation of POMC/CART neurons [146]. Besides regulating the neuropeptidergic expression in the hypothalamus regulating food intake, both insulin and leptin signaling also includes opening or activation of the ATP dependent potassium channel,  $K_{ATP}$ , resulting in hyperpolarization and reduced metabolic activity of the neuron [147, 148].



**Figure 4.** Schematic illustration of the Arc with POMC/CART and AGRP/NPY neurons and their responses to the peripheral hormones leptin and insulin.

## 1.4 GLIA CELLS

The glia cells of the central nervous system are divided into two types; the macroglia and the microglia. During the development macroglia cells consists of astrocytes and oligodendrocytes but in adult there is a fourth type of glia cell, the adult oligodendrocyte precursor cells expressing NG2 [149]. Astrocytes and oligodendrocytes together with neurons are generated from neuro-epithelial stem cells located in the ventricular zone surrounding the ventricles [150, 151]. The origin of the microglia is myeloid, the same as monocytes but where the microglia precursor cells reside and differentiate into microglia is not fully understood but suggestions are that they are the meningeal macrophages or circulating blood monocytes [152].

### 1.4.1 Astrocytes

Astrocytes contributes to the formation of the BBB [153]. They associate with oligodendrocytes and myelin, and interacts directly with neurons by supporting synapse formation and maintenance [154]. Astrocytes are further implicated in neurotransmitter reuptake [155]. In the hypothalamus, the attaching strength of the excitatory synapses in the PVN is regulated by astrocytes [156], and in the hippocampus they are found to affect both excitatory and inhibitory synapses [157]. Depending on their activity the astrocytes attain different conformation changes the spherically bushy variant (activated), the less bushy form (inactive), and the very elongated, non-excitatory version (Muller cells in the retina and Bergmann glia in the molecular layer of cerebellum) [158]. When compared between species the complexity and size increases with increased complexity of the brain [159].

### 1.4.2 Oligodendrocytes

Oligodendrocytes provide insulation and results in compartmentalization of the axons resulting in rapid impulse propagation [160]. Oligodendrocytes also provide a trophic support to the axons in the paranodal and juxta-paranodal regions [161-164]. The compartmentalization divides the myelinated axons into the node of Ranvier where the sodium channels, Nav1.2 and Nav1.6, are clustered. The node of Ranvier is flanked by two paranodes at which the myelin sheaths are attached through septate-like junctions, which create a tight barrier between the nodes of Ranvier and the juxtapanode underneath the myelin. Paranodes serve as a conduit between the axon and the myelin, and to segregate the sodium channel clusters at the nodes from the potassium channels at the juxtapanodes to enable saltatory nerve conduction. Neural/glial antigen 2, NG2, is commonly used to identify the oligodendrocyte precursor cells which are immature glial precursor cells that have the ability to differentiate into oligodendrocytes [165]. Adenomatous polyposis coli, APC, can be used to identify fully mature oligodendrocytes [166]. Ankyrin-G can be used as a marker of the node of Ranvier [167].

**Myelination** is the process under which the axons of nerve cells are being wrapped and insulated by myelinating glial cells, Schwann cells in the periphery and oligodendrocytes in the central nervous system. The role of axon myelination is to enable saltatory propagation of nerve impulses thereby increasing action potential velocity [168].

Myelin is an extension of the oligodendrocyte plasma membrane, which wraps an axonal segment in a spiral fashion and forms a condensed multi-lamellar sheath, which is typically identified in electron microscopy (EM) cross sections as a dense profile [169, 170]. Myelin oligodendrocyte glycoprotein, MOG, is important for structural integrity of the myelin sheath and expressed late by oligodendrocytes [171]. Myelin basic protein, MBP, and proteolipid protein, PLP are also expressed within the myelin and maintain the correct structure of myelin [172, 173].

Large axons (>1 $\mu$ m) are first myelinated with compact myelin and will receive more layers of myelin compared to thinner axons. The ratio between axonal diameter and myelinated fiber diameter, the g-ratio, has frequently been used as a measure for myelination during development [174]. However, even smaller axons (>0.4 $\mu$ m) in the optic nerve and cortex are myelinated but with thinner sheaths.

The physical myelination as such does not preserve axonal functions and survival but the myelinating oligodendrocytes provide molecular interactions that ensure axonal support. The axonal thickness may also increase with increased myelination possibly due to signals provided by molecular axon-glia interactions. This axon-glia interactive signaling includes axonal spiking activity because ATP release [175].

Multiple sclerosis (MS) is probably the most well known demyelinating disease where the insulating myelin covering the fast conducting axons in the brain and the spinal cord are damaged resulting in disrupted neuronal communications. Myelin-related dysfunction, however, has also been connected to schizophrenia [176, 177], depression, autism, Alzheimer disease and Huntington chorea [178].

### **1.4.3 Microglia**

The function of the microglia is to monitor the well-being of their environment and to respond to changes by protective mechanisms or assist in immune reactions [179]. Activated microglia release cytokines and function as immuno- and neuromodulatory messengers acting on the astrocytes and neurons in the area [180]. The morphology of activated microglia is following a highly characteristic pattern with the base line of the resting non-pathological microglia with small soma and moderately ramified (dendritic processes), activated microglia with large round soma and hyper ramified/ bushy processes, continuing activation lead to retraction of processes which reduces the ramification but still large round soma, then in parallel there is a separate type of activated microglia with rod shaped large soma with retracted processes [181]. Chronic psychological distress has been found to induce microglia activation in affected brain regions [182, 183]. This is particularly observed in Alzheimer's [184] and Parkinson's disease [185] but also in association with depression [186] and autism [82]. Microglia is also contributing to postnatal remodeling of synapses [187, 188].



## 2 THESIS OBJECTIVES

The overall goal of this thesis is to increase the knowledge about the hypothalamic systems regulating food intake in anorectic conditions and to explore the role of Contactin-1 in nodal paranodal assembly and the myelination process.

**The specific aims of the studies included in the thesis are:**

**Paper I** To study the developmental role of Contactin-1 in nodal and paranodal assembly and in the myelination process, using the optic nerve as a model for myelinated nerves in the central nervous system.

**Paper II** To compare neuropeptidergic expression in the hypothalamus of two anorectic mouse models, the *Cntn1*-KO and the *anx/anx* mouse, and food deprived mice.

**Paper III** To investigate the activity of the hypothalamic area in the anorectic *anx/anx* mouse.

**Paper IV** To compare the expression of microglia and astroglia markers in the hypothalamus and hippocampus of two anorectic mouse models, the *Cntn1*-KO and the *anx/anx* mouse.

## 3 MATERIALS & METHODS

### 3.1 ANIMALS (PAPER I-IV)

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of Sanford-Burnham Prebys Medical Discovery Institute (La Jolla, CA) and by the local ethical committee, Stockholms norra djurförsöksetiska nämnd, (Stockholm, Sweden). All the animals were housed in animal facilities on 12hr light to dark cycles at a constant 25°C temperature. Food and water was supplied ad libitum and they free to suckle milk from the mother.

*Cntn1*-KO mice were created in the Ranscht laboratory by Dr. EO Berglund [31]. Contactin exon 3 was disrupted by insertion of a neomycin-resistance cassette the mutated allele was transferred to embryonic stem (ES) cells by homologous recombination. The ES clones were then transferred into blastocysts creating chimeras. The *Cntn1*-KO mouse is born from heterozygote parents, as homozygote breeding is impossible due to the detrimental phenotypes and lack of survival. *Cntn1*-KO mice were genotyped by PCR using forward and reverse primers covering exon3 of the *Cntn1* gene and an additional primer inside the neo cassette. PCR generates two DNA fragments, which were separated by agarose gel electrophoresis for size identification. The mutated fragment appeared as a fragment of 369 nucleotides, while the WT fragment was 166 nucleotides. The backgrounds of the *Cntn1*-KO mouse used for the experiments were a mixed (129SVJ × C57Bl/6 × Black Swiss) and a purebred line (C57Bl/6). Both lines display an identical phenotype and molecular patterns of marker expression. Phenotypic characterization of the *Cntn1*-KO mouse was based on the reduced body weight compared to WT littermates (fig 2a) and the ataxia.

To aid identification of Contactin-1 in the oligodendrocytes during the development (**Paper I**) we crossed the *Cntn1* heterozygotes with the transgenic mice expressing GFP (green fluorescent protein) under the PLP (myelin proteolipid protein) promotor provided by Dr. W. Macklin [189]. PLP is the major intrinsic membrane protein of CNS myelin and is expressed in oligodendrocytes [173].

The anorexia mouse model the *anx/anx* mouse harbors a spontaneous mutation that arose in the Jackson laboratory in 1976 [32]. The mutation is maintained on the B6C3H-a/a F<sub>1</sub> background. The animals used in the thesis were obtained by Dr. M Schalling, Karolinska Institutet and maintained there as a separate colony with sibling breeding. The *anx* gene was mapped by Dr. J Johansen and colleagues to a 0.2 cM interval in the nonagouti (a) locus on mouse chromosome 2 [38]. The *anx/anx* mouse is genotyped using simple sequence length polymorphism (SSLP) markers mapped to the sub-chromosomal region to which the *anx* mutation is located on chromosome 2. The primer pairs were chosen around simple repeats located in the *anx* mutation interval, giving rise to a slightly larger *anx*-product, which subsequently can be differentiated by gel electrophoresis. The *anx/anx* mouse was phenotypically characterized by reduced body weight from postnatal day 8-9 and

deteriorating appearance by age. All animals are housed in a 12hr light to dark cycle with food and water provided ad libitum.

### 3.2 IMMUNOHISTOCHEMISTRY (PAPER I, II AND IV)

Immunohistochemistry (IHC) is a method used to visualize proteins/peptides (antigens) of interest at their natural location within the tissue by the use of antibodies that recognize and bind to specific antigens. Those antigen specific antibodies are called primary antibodies, and the Fab' fragment of these binds to the antigen. To detect this binding a secondary antibody is used in which the Fab' fragment is specific to the Fc' part of the primary antibody which is specific for the species that the primary antibody was generated in, i.e. rabbit, mouse and rat etc. The secondary antibody is of a different species i.e. goat or donkey, and can be labeled with a fluorescent label (i.e. FITC or Cy3) or an enzyme reporter (i.e. horseradish peroxidase [HRP] or alkaline phosphatase [AP]). Immunohistochemical protocols are dependent on several parameters such as tissue fixation, detergent type and concentration, time, temperature and antibody affinity.

#### 3.2.1 Fixation of tissue

The fixation procedure is an important part of IHC and the purpose of fixation is to cross link the molecules so they remain at their natural location or at least as a frozen moment in time. However, this crosslinking can result in hidden or exposed epitopes to which the Fab region of the primary antibody is specifically binding. Choice of fixative is therefore crucial for IHC. This is highlighted in **Paper I**.

For **Paper I** Optic nerves were dissected out fresh from different ages and then placed in different fixative for a variation of times following a carefully established protocol to be able to successfully visualize different locations of the Contactin-1 protein. More specifically see information regarding staining optimization in Table 1 in [74]. However, in general 4% paraformaldehyde (PFA) was used as the primary fixation and methanol was used to extract lipid layers in order to expose Contactin-1 epitopes otherwise hidden.

For immunohistochemical evaluation of other brain regions (**Paper II & IV**) *Cntn1*-KO and WT animals were perfused with 4% PFA in 0.1M phosphate buffer pH 7.4 at 25C. Brains rapidly dissected under a dissection microscope and immersed in ice-cold fix for 4 hrs before changing into 30% sucrose in 0.1 M phosphate buffer pH 7.4. After overnight immersion in sucrose the brains are further dissected, and snap frozen using 2-methyl-butane on dry ice.

For immunohistochemical evaluation of the *anx/anx* brain perfusion was performed with a mixture of PFA and picric acid as described by Pease (1962) [190] and Zamboni and De Martino (1967) [191]. The brains were rapidly dissected out and immersed in fixative for 90 min then put in 10% sucrose in 0.1M phosphate buffer pH 7.4, overnight in 4C. Tissue was

then either snap frozen using CO<sub>2</sub> (gas) or 2-methyl-butane (isopentane) on dry ice and cut in a cryostat at 6-14µm thickness.

### 3.3 IMMUNOHISTOCHEMICAL LABELING

The result of the IHC is only as good as the specificity of the primary and secondary antibodies, and this can vary. If antibodies are constructed against an epitope that is hidden the antibody will not bind, and will therefore require an antigen retrieval protocol. In general, antibodies created in animals usually give stronger signals, as animals generate several different antibodies against the antigen (polyclonal). This is to be compared to antibodies generated as monoclonal by hybridoma cells.

In short all the IHC protocols involved binding of primary antibodies with Fab' specificity to the antigen expressed in the tissue. For some protocols we reduced unspecific binding by adding bulky proteins competing with the unspecific binding sites (from donkey serum, goat serum or bovine serum albumin). The Fc' site on the primary antibody is representing the species specificity in which the antigen was produced. The secondary antibody has an identifiable "tag" at the Fc' side and the Fab' side has specificity to the species presented on the primary Fc fragment. With this method one can identify and localize specific peptide sequences in the tissue. The "tag" on the secondary antibody can be a horse-radish peroxidase (HRP) tag as in the case of tyramide signal amplification where it is used to catalyze the deposition of fluorescent labeled tyramide (TSA technique, **Paper II and IV**). Another "tag" on the secondary antibody can be a directly bound fluorescent molecule (**Paper I and II**). A third one is biotin to which the avidin-biotin-peroxidase complex (ABC) can bound and amplify the 3,3'-diaminobenzidine signal (DAB, a substrate for peroxidase) (**Paper I**). In **Paper I** the different fixation techniques visualized different immunolabeling as the different techniques exposed different epitopes. For example, the GPI anchor of the Contactin-1 protein is easily extracted unless crosslinked, however, when it is covered by myelin as in the paranodes, Contactin-1 is more resilient to extraction by methanol.

To identify myelin in whole brain, by using antiserum against myelin basic protein (MBP), we decided to use free-floating sections in order to increase penetration of the antibody, for all others section they were mounted on glass slide. The other method of myelin detection was the Gallyas histochemical staining which is a silver nitrate stain that specifically binds to myelin under certain pH and chemical solution [192].

To validate IHC protocol and primary antibodies one can use tissue or cells to which the antigen for the primary antibody is absent, e.g. tissue from knockout animals. Another approach is to incubate the primary antibody together with an excess of the corresponding peptide. Performing the protocol in absence of the primary antibody can be used as validation of secondary antibodies. The localization of antibody binding can also be validated with in situ hybridization of the corresponding mRNA.

The main difference in IHC techniques used in **Paper II and IV** compared to **Paper I** is the use of picric acid combined with PFA in the fixation, and the usage of the TSA technique for the labeling [193]. The fixation proved to enhance Iba1 and GFAP staining, and the use of TSA technique enhanced any antibody detection and a lower concentration of both primary and secondary antibodies can be used. In **Paper II and IV** the sections were thicker which yields a higher density of cells and makes penetration more important, which was possibly achieved by the addition of the methanol in the ready to mix PFA solution.

### **3.4 IN SITU HYBRIDIZATION (PAPER II)**

*In situ* hybridization (ISH) in tissue provides a direct visualization of the spatial location of a specific mRNA/DNA/microRNA sequence. In **Paper II** (ISH) was utilized to locate and compare Arc mRNA expression of NPY, AgRP, POMC and a-MSH with antisense oligo probes (complementary to mRNA sequence). The specific method used for this *in situ* hybridization was developed by Schalling et al [194] and shortly includes incubating the probes in an hybridization buffer containing salmon sperm as blocking in a humid 42 degree chamber, and then washed and dried before dipped in a nuclear emulsion containing silver crystals followed by developing in Kodak D19 and fixed by Kodak 3000. The probes were radioactively labeled at the 3'-end and after hybridizing the probes to the brain sections on slides the anatomical location of the radioactivity is detected onto a film. To quantify the density of the silver grains representing the hybridized probe, the images were processed using a 256 grey scale (black= 256 and white=0) creating a mean pixel density. The mean pixel density was converted to relative optic density (ROD) to represent the density of the silver grains on processes brain sections [195].

The pros of using radioactive *in situ* probes over fluorescent labeled probes is the increased sensitivity of the method, i.e. a lower copy number of target mRNA can be detected,. The radioactivity of the labeled probes has however a more limited shelf life, compared to fluorescently labeled probes [196]. Radioactivity detection can be scattered when the radioactivity decline and this can affect the resolution of the technique.

### **3.5 ELECTRON MICROSCOPY**

To be able to study ultra-structures in the nodes of Ranvier, paranodes with their septate like junctions and myelin processes and axon-glia interactions in greater magnification and with higher resolution, we used electron microscopy on optic nerve tissue. Electron microscopy allows recognition of the paranodal septate like junctions that are essential for the establishment of the discreet nodal, paranodal and juxtapanodal in **Paper I**.

The preparation of the tissue for the electron micrographs were following the previously described protocol [197]. In short, transcardial perfusion with a mix of 2% formaldehyde and 2% glutaraldehyde followed by a post fixation procedure involving uranyl acetate to enhance

membrane contrast and to stabilize membrane structures including gap junctions [198] and myelin [199] and the finally embedded in an epoxy embedding compound and the ultra-thin sections were stained with a solution containing lead. The samples are viewed in an electron microscope in vacuum. The cellular details revealed by electron microscopy compared to regular confocal imaging are far more detailed and generating images, electron micrographs with higher magnification up to 10, 000,000 times (regular up to 2000x).

### 3.6 WESTERN BLOT (PAPER II & III)

Western blot (WB) is used to investigate the levels of protein expression in homogenate or extract from tissue/cells. The basis for WB is separation of proteins according to size on, most commonly, denaturing sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE), which subsequently are transferred by to a membrane immobilizing the proteins that then can be further analyzed using immunological detection of specific proteins. Before running the proteins on the SDS-PAGE gel, they are denatured by the use of reducing agent ( $\beta$ -mercaptoethanol) and the anionic detergent, SDS, which maintains the denatured conformation of the proteins by binding to the polypeptides evenly. The bound SDS creates a negatively charged molecule with charge approximately relative to its' size, which can be separated on the SDS-PAGE gel running towards the positive electrode. In **Paper I** WB was performed on whole mouse brains after protein extraction using RIPA buffer to maintain the integrity of the immunoreactivity of the membrane bound proteins, and the method was used on whole brain in order to detect levels of myelin. In **Paper IV** WB was performed on isolated hypothalamus and the lysis buffer used contained HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (standard buffer), to detect protein levels of AMPK unphosphorylated and phosphorylated and two glucose transporters, Glut1 and Glut4.

As WB provides knowledge on protein level in a sample it is just a relative method of protein size as it depends on the amount of bound SDS and if a protein has a greater hydrophobic content, like membrane proteins the amount of bound SDS varies significantly [200].

### 3.7 EX VIVO AUTORADIOGRAPHY (PAPER III)

Because of the small size of various brain regions in the *anx/anx* mouse functional magnetic resonance imaging, fMRI, is not possible. Instead we in **Paper III** used *ex vivo* autoradiography in order to investigate the metabolic activity of in particular the hypothalamus of these mice. Autoradiography as such is just an image on an x-ray film, which has been exposed to radioactive emissions, in our case from radioactive glucose taken up by the brain cells. In short our assay was performed by injections of radioactively labeled Deoxy-D-(DD)-glucose and investigating the uptake as a measure of incorporated radioactivity. To normalize the glucose uptake the animals were fasted prior to injection of radioactive glucose and the injected glucose was taken up by the cells for 45 min before the

brains were rapidly dissected out and frozen. The cryosectioned brain was exposed to phosphor-imaging plates and the results were analyzed as pixels per area in the corresponding regions of interests (ROI), hypothalamus, motor cortex, and thalamus. The ROI data was normalized with the activity of the radioactive glucose, the radioactivity levels in the serum and the levels of blood glucose. The injected glucose makes up only a very small portion of the total blood glucose and will thus not add to these levels. In addition, the form of glucose injected, DD-glucose is not metabolized further in glycolysis, which therefore makes it ideal for studying uptake of glucose into tissues [201].

### **3.8 ENZYMATIC ACTIVITY ASSAYS (PAPER III)**

Enzymatic assays can be used to quantify various substrates in a suspension and are based on measuring the activity of specific enzymes, i.e. how much substrate per unit that is converted over time or the reaction speed of which an amount of substrate is converted. The enzymatic activity of the major complexes of the oxidative phosphorylation (OXPHOS) were in **Paper III** analyzed spectrophotometrically, and the activity was normalized to the activity of citrate synthase (CS) a mitochondrial matrix enzyme often used as a measure of mitochondrial disruption during the experiment [202]. CS acts as pace maker and mitochondrial marker [203]. Enzymatic assays were further used to analyze the levels of metabolites (Cr, PCr, ATP, Glucose-6-P and lactate) in **Paper III** using fluorometry. By adding specific enzymes (pyruvate kinase, creatine phosphokinase, hexokinase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase) and subsequently measuring the fluorescence that is linked to the production of the downstream metabolites NAD(P)H. The methods used for the enzymatic assays for the mitochondrial OXPHOS were developed by Wibom et al [204] for muscle analysis and modified by using a different homogenization buffer containing sucrose, EDTA and BSA (**Paper III**). The method in short involved isolation of mitochondria from homogenized hypothalamic tissue, spun at low speed, pellet was then discarded and the supernatant was spun at high speed. This enabled us to isolate the mitochondrial portion of the tissue homogenate which is the only portion used. The differences in optical density between oxidized and reduced forms of substrate after adding enzymes specific or shared by the different mitochondrial complexes. For specific complex I (CI) activity NADH: ubiquinone oxidoreductases (NQR) was used per units citrate synthase (CS) [205], for complex I versus Complex III NADH: cytochrome c reductase (NCR) [206], for complex II succinate dehydrogenase (SDH), for CII vs CIII succinate: cytochrome c reductase (SCR) [207] and for complex IV (CIV) cytochrome C oxidase (COX) [208]. All values were normalized against citrate synthase (CS). The limitation of this type of assay is the integrity of the samples, which can alter the enzymatic reactions and not reflect on the capacity of the tissue from where the sample originated. Example of lost integrity of the sample would include contamination of cellular material or additives that compromises the enzymatic activity before they are tested in the mitochondrial portion.

### 3.9 HPLC

High-performance liquid chromatography (HPLC), previously called high-pressure liquid chromatography, is a method used to separate, identify and quantify molecules in a solution. We used HPLC in **Paper III** to analyze levels of ATP, ADP, AMP and IMP in hypothalamic tissue in basal condition and after one minute of ischemia as a model of stress. To test this two set of brains were taken one frozen immediately and one after 1 min (the ischemic condition). The metabolites were extracted with the use of perchloric acid and the supernatant containing both mitochondrial and extra-mitochondrial metabolites was analyzed with the use of HPLC. To adjust for blood contamination the value from each metabolite was divided by total creatine. One advantage of HPLC is that several components can be analyzed in the same solution simultaneously.



## 4 RESULTS & DISCUSSION

### 4.1 CONTACTIN REGULATES MYELINATION AND NODAL/PARANODAL ORGANIZATION IN THE CENTRAL NERVOUS SYSTEM (PAPER I)

Previously the Ranscht lab described that the location and function of Contactin-1 expression in the peripheral nervous system (PNS) is restricted to the axonal portion of the paranode. Here it interacts in cis with Caspr, and this complex interacts with NF-155 on the myelinating Schwann cells in trans [59, 209]. In **Paper I** we evaluated the location and function of Contactin-1 in myelinated fast axons in the CNS.

#### 4.1.1 Contactin-1 in axon-glia interactions initiating myelination

Documentations of Contactin-1 expression at paranodes and nodes of Ranvier of myelinated central nerves [60, 210] and by developing oligodendrocytes in culture [211], was published before our additional data was finished. In addition to confirming those findings in **Paper I** we also show Contactin-1 expression by oligodendrocytes in vivo. By using different fixation and extraction methods we exposed the different locations of the Contactin-1 protein and labeled them with antibodies for identification of the different protein locations in the optic nerve at different ages.

The IHC protocols used on the optic nerve were applied at two ages, first at P18 during active myelinogenesis and secondly, at P28 when mature myelin has been established [212, 213]. In short the results can be summarized as follows: The paranodal expression is best observed after a longer fixation time anchoring the molecular membrane complexes and after extracting with methanol. The nodal expression is best observed at P28 as the region has matured but this area needs a longer fixation and can be subjected to methanol treatment without being extracted, possibly being covered by a nodal astrocyte. Finally the surrounding expression representing mainly oligodendrocyte membranes and myelin wraps, are best observed without methanol extraction as Contactin-1 is extracted with this treatment.

Identification of the Contactin-1 expression in myelin was done by triple IHC using markers for oligodendrocyte progenitor cells (NG2) and mature oligodendrocytes (APC). Contactin-1 expression coincided with loose membrane structures on oligodendrocyte precursor cells and downregulated when the myelin matured. At later postnatal ages (P30) only some Contactin-1 expression remains on loose myelin membranes. These data indicated that Contactin-1 delineates migrating, pre-myelinating and myelinating oligodendrocytes but is down regulated when myelin is mature.

The role of Contactin-1 in the formation of central myelin was determined by the use of the *Cntn1*-KO mouse and comparisons of the myelin sheet specific protein myelin basic protein (MBP) and Gallyas myelin stain on whole brain. This revealed that without Contactin-1 there is a 50% reduction of MBP protein expression and a visual loss of myelin in corpus callosum.

This myelin loss observed in the *Cntn1*-KO mouse transpired into a decreased optic nerve diameter visualized by the cross-sections stained with a marker for mature neurofilaments, NF-200. When cross-sections of optic nerve were analyzed in details using electron micrographs a significant reduction of dense myelin profiles were found in the *Cntn1*-KO compared to the WT.

The reduced thickness found in the optic nerve may be due to the significant reduced number of mature (APC positive) oligodendrocytes and the increased amount of thinner axons myelinated (quantified from EM).

#### **4.1.2 Contactin-1 and paranodal assembly in CNS**

As Contactin-1 was found to regulate the paranodal assembly in the peripheral nervous system, PNS [59], the role of paranodal assembly was investigated also in CNS using the optic nerve. In the CNS we saw that the potassium channel, Kv1.2, normally located in the juxtaparanode [214] had lost its' defined boundary and instead relocated adjacent to the nodal expression of sodium channels thereby disrupting the clear separation of the two voltage gated channels needed for fast propagation of action potential [215, 216], the hallmark of myelinated axons.

The importance of Contactin-1 for the paranodal structure was examined using electron micrographs of longitudinal sectioned optic nerves from the *Cntn1*-KO and WT. The WT exhibited the normal pattern with paranodal junctions where the outermost myelin loops closed to the nodes are attached with a junction characterized by transverse electron-dense bands (septate like junctions) [217, 218], however; the paranodal junctions in the optic nerve of the *Cntn1*-KO mouse were severely disrupted with myelin loops displaying overtly and astrocytic processes penetrating the open space between the axon and myelin membranes. The strongest effect from lack of Contactin-1 was a 60% reduction in appearance of bilateral myelin loops (myelin loops present on each side of the node of Ranvier).

The findings of re-localization of the juxta-paranodal Kv1.2 to the intended paranode site and the disrupted septate- like junctions support the importance of the Contactin-1 to form and maintain these structures in CNS as well.

#### **4.1.3 The role of Contactin-1 in formation and maturation of the Node of Ranvier**

The nodal sodium channels consists of two types of subunits where alpha are the pore forming units regulating the electrophysiological properties of the postnatal optic nerve [219, 220]. With the early development of the nodes and in the initial axon segment of (later to be myelinated) nerves sodium channel subtype Nav1.2 is expressed, featuring a lower activation threshold. During myelination and axonal development this subunit is in the nodes exchanged for a higher threshold but faster conduction Nav1.6 subunit with mature channel properties

and conduction characteristics [221, 222]. The effect of Contactin-1 on this developmental switch was investigated with IHC displaying the two populations in the optic nerves of the *Cntn1*-KO mouse vs. WT. Significant reductions were seen in the Contactin-1 mouse both in terms of the number of sodium channels in general and the terms of the number of mature Nav1.6 subunits located to the nodes. These findings can be attributed to the disrupted myelination caused by, the loss of Contactin-1 expression in the nodal and paranodal regions.

#### **4.1.4 Summary of Paper I**

We found strong evidence that Contactin-1 is expressed both by neurons and oligodendrocytes, and the location has expanded to include not only the axonal side of the paranode, but also the axonal node and the myelinating oligodendrocyte. Myelination is a complex but important process requiring many points of interactions between the axon and the myelinating oligodendrocytes. Contactin-1 plays an important role during the early phase of this communication during the postnatal myelination in the central nervous system. The dynamic expression of Contactin-1 is found to be essential for myelination in two ways. First, oligodendrocytes, although apparent in equal numbers and aligned along axons in both genotypes, show reduced capacity to extend membrane sheaths. This shows that Contactin-1 is an important signal between axons and oligodendrocytes to initiate membrane sheath expansion and formation of myelin. Second, Contactin-1 plays an important role in orchestrating the nodal/ paranodal domains in the central nervous system. These data show that Contactin-1 is critical for both myelin formation and function in the CNS.

## **4.2 COMPARATIVE STUDIES OF HYPOTHALAMIC FOOD INTAKE REGULATORY SYSTEMS IN ANIMAL MODELS OF ANOREXIA (PAPER II &IV)**

The phenotypic similarities in body weight and food intake observed between the *Cntn1*-KO and the *anx/anx* mouse were the promoting factors to investigate the food regulatory pathways in the hypothalamus of the *Cntn1*-KO. Previous findings in the *anx/anx* mouse showed marked changes in hypothalamic neurotransmitter and -peptidergic systems related to food intake regulation in these mice [38, 40, 43, 44, 223]. In **Paper II** we compared the neuropeptidergic expression in the hypothalamic systems involved in food intake regulation in the *Cntn1*-KO, the *anx/anx* mouse and 24hr food-deprived three weeks and in some instances three months old mice, by IHC and ISH. Signs of inflammation in the hypothalamic systems have in several studies been associated with syndromes displaying phenotypes that are related do deviant energy homeostatic regulation, obesity and anorexia, which has been visualized with increased expression of glia specific markers and pro-inflammatory molecules [45, 47, 51, 52, 224-233]. The anorectic *anx/anx* mouse has previously been associated with a significant increase of the microglia marker ionized Iba1 in a number of hypothalamic areas [45]. As a follow up study from the comparative

neuropeptidergic analysis in **Paper II** [234] we continued the comparisons by investigating the hypothalamic ir of an astrocytic marker, GFAP, and Iba1, in the *Cntn1*-KO and the *anx/anx* mouse. We also evaluated both markers in the hippocampus of the two animal models (**Paper IV**).

#### 4.2.1 Changed hypothalamic NPY and AgRP expression

The hypothalamic expression pattern of the two orexigenic neuropeptides NPY and AgRP were dramatically changed in the *Cntn1*-KO vs. WT, resembling what is seen in the *anx/anx* mouse [40, 42]. NPY- and AgRP-ir was increased in the Arc soma, while a reduced network of ir-fibers projecting from these neurons to PVN was detected, in both the *Cntn1*-KO and the *anx/anx* mouse. These changes were however not found in the 24h food deprived mouse, indicating that a longer starvation period is required for the changes in distribution of the orexigenic neuropeptides to occur. Alternatively starvation alone will not produce such changes in the absence of a molecular pathology.

ISH with NPY and AgRP mRNA specific probes revealed increased mRNA levels of the two peptides in both the *Cntn1*-KO and the *anx/anx* Arc neurons, correlating with the increase in cell body specific NPY and AgRP-ir. However, in a previous publication from Broberger et al (1997) [40] no differences in NPY mRNA levels in Arc of the *anx/anx* mouse was found. The discrepancy between these results is likely due to a shorter exposure time that was used in **Paper I** as longer time exposure will result in saturation of signal and loss of information. The ISH results of the 24hr food deprived three weeks old mice showed less of an increase, but still a significant increase, in NPY and AgRP mRNA, indicating that even a short period of food-deprivation is enough to give an up-regulation of NPY and AgRP synthesis.

#### 4.2.2 Changed hypothalamic $\alpha$ -MSH/ POMC expression

$\alpha$ -MSH is the active peptide hormone resulting from cleavage of the POMC precursor and is expressed by another population of neurons in Arc [38, 235, 236]. The expression of  $\alpha$ -MSH was diminished in Arc (cell bodies and fibers) and DMH (fibers) of the *Cntn1*-KO compared to WT, and the *anx/anx* mouse showed a resembling but not equally strong decrease. The 24hr food deprived three weeks old mice however had accumulations of  $\alpha$ -MSH-positive granules in the soma, in comparison to WT, while no significant change in the fiber expression was seen. The differences in POMC mRNA observed by ISH mirrored more or less what was seen in  $\alpha$ -MSH peptide expressions, with diminished levels in the *Cntn1*-KO and reduced levels in the *anx/anx* mouse. However, the POMC mRNA level in Arc of the 24hr food deprived mouse was also reduced. This indicates that at least after a longer period of starvation mice respond by reducing the expression of the anorexigenic POMC.

### 4.2.3 MCH expression in the LHA

One innervation target for Arc NPY/AgRP expressing neurons is melanin concentrating hormone (MCH) positive neurons in the LHA [111]. These neurons are involved in the control of energy homeostasis by stimulating feeding [105, 237, 238]. In the *Cntn1*-KO a prominent reduction in MCH-ir fibers in the LHA was observed, while in the *anx/anx* we observed a small reduction in such fibers, and no change in the 24hr food deprived mice. No clear difference was seen in MCH-ir cell bodies in LHA in any of the models when compared to WT. The MCH mRNA transcript levels were however significantly but slightly increased in the *Cntn1*-KO, while no change was seen in the *anx/anx* mouse of the fasted mice.

### 4.2.4 Contactin-1 expression in the hypothalamus

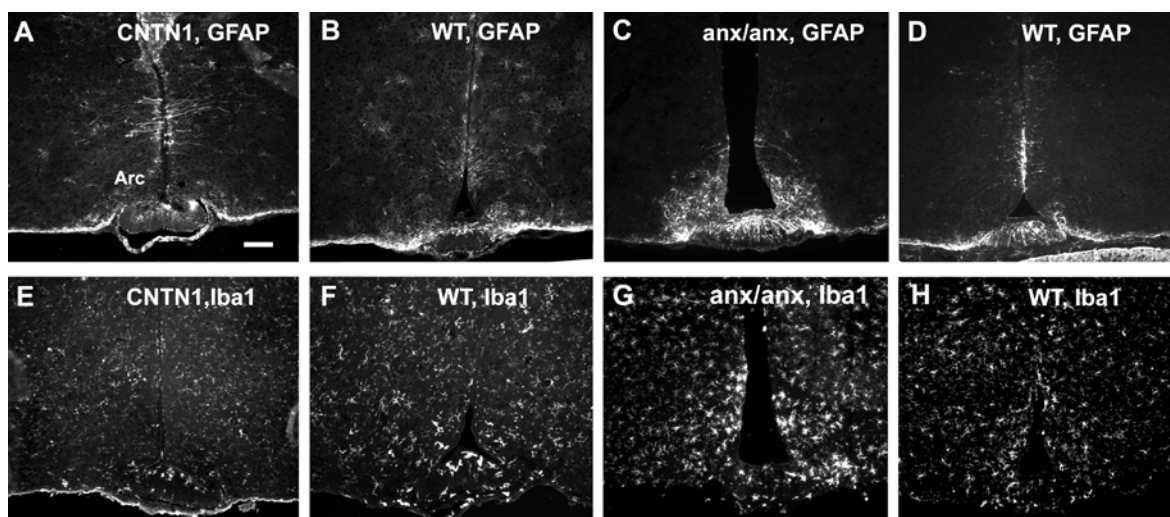
In **Paper I** we also showed that in WT mice, the Contactin-1 protein is expressed in a uniform punctate fashion in neuropil in all the hypothalamic regions evaluated (def. neuropil [239]) including the median eminence and Arc, correlating well with previous findings [240, 241]. More defined, Contactin-1 was in WT found around the soma of the NPY-positive neurons, but was not limited to this population. The same pattern was seen in the *anx/anx* mouse. In the *Cntn1*-KO mouse Contactin-1 immunoreactivity (ir) was not present in neuropil, ir was however observed accumulated in the ER of NPY positive neurons in the Arc. This indicates that the NPY-neurons of the *Cntn1*-KO mouse likely are affected by the lack of functional Contactin-1. The abundant expression in the hypothalamic regions suggests a role for Contactin-1 in the development of these food intake-regulating systems.

### 4.2.5 GFAP immunohistochemistry in the hypothalamus and hippocampus

The expression of the astroglia marker GFAP was quantified by IHC and subsequent ImageJ analysis of the hypothalamic areas; Arc and PVN, and the dentate gyrus of the hippocampus, in the *Cntn1*-ko mouse vs. WT at age P16, and in the *anx/anx* mouse vs. WT at age P21. Image J analysis takes into account the region labeled over the region of interest. The results revealed no differences between the *Cntn1*-KO and corresponding WT in any of the areas (Figure 5A, B). However, the *anx/anx* mouse showed a significant increase of GFAP-ir fibers in the PVN and a trend toward elevated levels in the Arc (Figure 5C, D). The increased GFAP in the *anx/anx* mouse was supported by micro-array data from the Arc-area [36] where it was found up-regulated. Both Arc and PVN are areas known to be importance for regulating food intake [37, 242, 243]. GFAP was primarily identified as a marker for reactive astrocytes found in multiple sclerosis patients [244, 245], however it is not an absolute marker for non-reactive astrocytes but rather associated with CNS lesions [246, 247]. Thus, the two mouse models of anorexia show differences in reactive astrocytes in the hypothalamus, which points to different mechanisms for generating the anorectic phenotype.

#### 4.2.6 Iba1 immunohistochemistry in the hypothalamus and hippocampus

The increased Iba1-ir previously observed at P15 and P21 in the *anx/anx* mouse in both Arc (Figure 5G,H) and PVN [45] was not observed at P16 in the *Cntn1*-KO mouse (Figure 5E, F) indicating that a different mechanism underlies the sub sufficient energy intake in these models. However, the *Cntn1*-KO showed a significant upregulation of Iba1 in the hippocampal dentate gyrus area. These microglia were both bushy and rod-like indicating an advanced state of activation [248]. This was not mirrored in the *anx/anx* mouse.



**Figure 5.** Immunofluorescence micrographs showing GFAP (A-D) and Iba1 (E-H) in Arc of the *Cntn1*-KO mouse (A, E) and WT littermate (B, F) at age P16 and the *anx/anx* mouse (C, G) and WT littermate (D, H) at age P21.

#### 4.2.7 Summary of Paper II & IV

**Paper II** suggests that in comparison with the *anx/anx* mouse, the *Cntn1*-KO mouse in addition to becoming anorectic at a slightly younger age and emaciated faster, also display a stronger neuropeptidergic response to lack of food. This could suggest that different mechanisms are underlying the anorexia of these mice, or it is just an effect of the severity of the condition. This comparative study of two anorectic mouse models highlights the molecular complexity of timing and developmental age as the study involved two separate mouse strains and two different postnatal ages, however, it further solidifies the importance of intact hypothalamic functions as the two models despite large genetic background difference and slight difference in age show great similarities. The study also indicates that the Contactin-1 protein may be involved in the development of the hypothalamic systems involved in food intake. By studying the two models of anorexia in greater details one can get a better understanding of the hypothalamic food intake regulatory system during anorectic conditions, which in the end can aid in improving the treatment of anorexia nervosa.

In addition, despite the similarities between the *anx/anx* and the *Cntn1*-KO, i.e. the anorectic phenotype and similar neuropeptidergic response (**Paper II**), there are some major differences between the two anorectic mouse models. The *Cntn1*-KO mouse has a more

severe phenotype compared to the *anx/anx* mouse, i.e. the weight reduction starts earlier and progresses more rapidly [32, 249] despite given assisted feeding twice daily. Moreover, the *Cntn1*-KO displays a very strong ataxic phenotype, which together with the decreased myelination possibly leads to increased energy needs [127] and significantly reduces the ability to be breastfed. The up-regulation of an astrocyte-marker and a microglia-marker in the hypothalamus of the *anx/anx* mouse (**Paper IV**) [45], could be related to signs of neurodegeneration [47] and mitochondrial dysfunction leading to increased ROS production [46, 47], both documented previously. Another possibility is that the up-regulation of glia markers in *anx/anx* is related to increased serum levels of free fatty acids [250] due to excess breakdown of fat observed in anorectic conditions [251-254]. FFA can stimulate hypothalamic inflammation [255]. The serum levels of FFA have not been analyzed in the *Cntn1*-KO mouse. However, we didn't find up-regulation of neither GFAP nor Iba1 in the hypothalamic regions of Arc and PVN in the *Cntn1*-KO mouse, indicating that the neuropeptidergic changes previously observed in the hypothalamus (**Paper II**) might be a developmental defect rather than degeneration. A developmental defect in the Arc to PVN projection regulated by Contactin-1 could be due to misguided axonal outgrowth [31, 65] or synaptic plasticity [76, 256]. However, Iba1-ir labeling indicating activated microglia was seen both in highly ramified and phagocytic stage was found in the hippocampal region of dentate gyrus in the *Cntn1*-KO which could be related to synaptic plasticity defects previously found in the *Cntn1*-KO mouse [76].

### **4.3 REDUCED HYPOTHALAMIC METABOLISM IN THE ANX/ANX MOUSE (PAPER III)**

We hypothesize that the anorectic *anx/anx* mouse which has a documented mitochondrial dysfunction, leading to increased reactive oxygen species (ROS) production and possibly lower ATP in the hypothalamic Arc, may result in a detrimental effect on the cellular metabolism and resulting reduced neuronal activity. The food regulatory areas of the hypothalamus have an increased expression of a specific subtype of the ATP-sensitive potassium channels,  $K_{ATP}$  required for generating an action potential, making these cells (both neurons and glia cells) more sensitive to changes in energy levels and ROS [148, 257, 258]. In **Paper III** we therefore explored the hypothalamic metabolism of the *anx/anx* mouse.

#### **4.3.1 Enzymatic activity of the respiratory chain subunits in the *anx/anx* hypothalamus**

Evaluation of the enzymatic activity of the complexes in the mitochondrial oxidative phosphorylation system after normalization to citrate synthase revealed significant reductions in activity was seen in complex I (CI), and in the dual assay covering both complex I and complex III (CIII). CIII is linked to CI [259], and its' function is distinguished by measuring CI separate and then measure the combination of CI and CIII and subtracting the CI specific

function. The enzymatic activity observed in Complex II and IV showed no significant changes between the *anx/anx* mouse and WT. CS is a pace maker enzyme regulating the first step of the tricarboxylic acid (TCA) cycle and used as a marker of the amount of mitochondria used. Since no differences was seen in this enzyme in the *anx/anx* mouse compared to the WT mouse the changes seen are not due to a difference in number of mitochondria. These results confirm previous findings showing CI dysfunction being related to the anorexia of the *anx/anx* mouse [36].

#### **4.3.2 Glucose transporters and AMPK activation in the *anx/anx* hypothalamus**

We detected increased protein levels of 5' AMP-activated protein kinase (AMPK) and insulin independent glucose transporter 4 (Glut4) in isolated hypothalamic tissue, from the *anx/anx* mouse vs. WT. AMPK is acting like a metabolic master switch sensing the cellular levels of AMP. AMP levels increases during energy deficiency, like hypoxia, ischemia and glucose deprivation, and this leads to activation of AMPK by phosphorylation into AMPK-P. This activation results in translocating the normally intracellular located Glut4 to the extracellular portion of the membrane, thereby facilitating glucose transport into the cell and increasing cellular energy levels [260, 261]. This pattern of increase is previously observed in tissue as a response to increased metabolic stress such as exercise [262]. To supply for an increased energy demand activated AMPK are involved in the biogenesis of more mitochondria and increased synthesis of Glut4 [263]. The findings at basal state from the *anx/anx* mouse indicates a significant reduction of activated AMPK and when AMPK-P is taken as the ratio of total AMPK the lower value is consistent with a lower metabolic stress and correlates well with some sort of adaptation to starvation or hibernation [264]. This is further observed in Alzheimer's disease where it reflects lower neuronal activity [265, 266].

#### **4.3.3 A hypometabolic state in the *anx/anx* hypothalamus**

Metabolic stress often accompanies conditions involving mitochondrial dysfunction. In order to ensure sufficient ATP levels in such situations, phosphocreatine (PCr) levels are often depleted (since they have donated a phosphate group to ADP), lactate elevated (a by-product from increased glycolysis) and glucose uptake increased [89-91]. By observing the levels of specific metabolites we were able to study the cellular metabolism at basal level and after ischemic stress in the anorectic *anx/anx* mouse vs WT. At basal metabolic level the *anx/anx* mouse and WT had similar metabolic turnover of ATP and glucose-6 phosphate, PCr was however higher indicating that less of this metabolite donated a phosphate group to ADP in order to restore ATP in the *anx/anx* compared to WT. Lactate was lower in *anx/anx* compared to WT hypothalamus, thus indicating lower glycolysis. However, what is interesting is how the cells react under stress in this case ischemia. We found that after ischemia, both ATP and PCr were significantly higher while lactate was still lower in the



*anx/anx* mouse compared to WT. These changes indicate that the *anx/anx* mouse utilizes less energy despite having more energy stored in the forms of phosphorylation donors like the PCr. It also indicates that the *anx/anx* mouse generated more ATP from PCr dephosphorylation and less from glycolysis via lactate production, and overall the ATP turnover was 10% reduced in the anorectic mouse model. The latter was supported by detection of reduced levels of AMP and IMP in ischemia. AMP and IMP can be considered by-products from ATP utilization, and lower levels, thus indicates lower usage of ATP. Taken together the results indicate a hypometabolic state of the *anx/anx* hypothalamus, both during basal and ischemic conditions.

#### **4.3.4 Reduced glucose uptake in the *anx/anx* hypothalamus**

By autoradiography we detected reduced uptake of glucose in the hypothalamus of the *anx/anx* mouse, which would indicate a less metabolically active system. The radioactive <sup>3</sup>H-glucose injected in this experiment is DD-glucose which functions differently from regular glucose in terms of being metabolically inactive, it will thus not play a role in glycolysis and thus not any active role as energy source [201]. It will however be transported inside cells like regular glucose but will remain stationary within the cell. The <sup>3</sup>H-DD-glucose will therefore provide a measurement of glucose uptake, however, only the innate glucose will be utilized by the cells/neurons. Despite normal levels of Glut1 and increased levels of Glut4 in the hypothalamic areas the *anx/anx* mouse had a significantly reduced hypothalamic glucose uptake, which suggest a form of hypometabolism.

#### **4.3.5 Summary of Paper III**

In response to elevated energy demands a normal system usually responds with increased metabolic stress by increased expression of AMPK-total and Glut4 and an increased glucose uptake and increased ATP turnover [262]. However, the *anx/anx* mouse displays a different scenario where elevated levels of AMPK-total and Glut4 are accompanied with hypometabolism and unused energy resources left inside the cells (increased ATP and phosphocreatine after ischemia). The mechanism behind this is not fully understood. However, this reduced response to stress (induced by ischemia) observed in the *anx/anx* mouse may be a result of an adaption to long-term starvation and/or mitochondrial dysfunction and may correspond to a type of metabolic depression observed during hibernation [264, 267].

## 5 CONCLUSION AND HYPOTHESIS

This thesis includes CNS studies that started with investigations of the molecular interactions involved in myelination and functional compartmentalization of nerves (**Paper I**) in relation to the cell adhesion molecule Contactin-1, and continued with the explorations of the anorectic and hypothalamic phenotype of the *Cntn1*-KO mouse in comparison to the *anx/anx* mouse (**Paper II and IV**). These studies show that the two mouse models have many similarities in expression of hypothalamic neuropeptides important for food intake regulation (**Paper II**), but also differences in expression of markers of glia cells in the same hypothalamic system (**Paper IV**). Based on previous findings associating the anorectic phenotype of the *anx/anx* mouse with a mitochondrial dysfunction [36] we explored the possibility that this results in putting the hypothalamic neurons in a hibernation state and in fact found that a reduced hypothalamic metabolic activity was involved (**Paper IV**). We believe that this hibernation could be a contributing factor to the signs of degeneration also documented in this mouse [46, 47], in the sense that a prolonged hibernation/inactivation leads to among others reduced levels of neurotrophic factors and a sign that these neurons or at least some of their projections should be cleared away.

In regard to the functions of Contactin-1 in myelinated axons (**Paper I**), patients with MS are living proofs that the interaction between axons and oligodendrocytes are of essence. A better understanding of the molecular signals regulating the myelination process is beneficial for understanding how myelin can be rebuilt. With the help of the *Cntn1*-KO isolated optic nerve we have highlighted the importance of Contactin-1 in axon-glia communication during later stages of myelination, and also in the formation and maturation of axonal domains. There is a dynamic expression of Contactin-1 on axons and oligodendrocytes essential for CNS myelination. The myelination process starts with precursor cells migrating to selected axons. There they proliferate and mature before the actual myelination or wrapping occurs. Contactin-1 plays a role in the later part of this sequence regulating oligodendrocyte differentiation and expansion of the wraps. A hypothesis regarding how Contactin-1 protein expressed by oligodendrocytes regulates the wrapping involves Src-related kinases that increase the expression of MBP in oligodendrocyte processes [268-270]. In addition, Contactin-1 is involved in the maturation of the axons shown by the significantly thinner optic nerve axons in the *Cntn1*-KO mouse. The maturation of axons depends on their activity, which induces synapse formation and target innervation, and from oligodendrocyte signals and intrinsic factors. Since target innervation occurs in the optic nerve in the *Cntn1*-KO mouse, the intrinsic and oligodendrocyte signals remain to be considered. Contactin-1 interacts with several other cell-surface molecules that may primarily or secondarily regulate the cellular mechanisms needed for axon maturation [60, 271-274]. Another explanation for the reduced axonal thickness, in the *Cntn1*-KO mouse, may be that the smaller diameter axons have a reduced axon-glia signaling needed for maturation of the axons and/or that the glia Contactin-1 protein expression regulate axon maturation [275].

For axons to propagate signals efficiently, the myelin needs to be organized into separate domains so that myelin segments are interspersed by nodes of Ranvier harboring clusters of

sodium channels. The paranodal junctions are segregating the nodal sodium channels from the juxtaparanodal potassium channels underneath the myelin [276, 277]. In the PNS, Contactin-1 is restricted to the axon membrane in the paranodal region [59]. Myelin-forming Schwann cells do not express Contactin-1 and no myelin loss is observed in peripheral nerves of *Cntn1*-KO mice. Similarly, when only the paranodal junction is disrupted in the CNS, as in Caspr-KO mice, the myelin wraps remains intact [278]. These data show that paranodal junctions are not needed for myelin formation but are critical for the domain organization of myelinated nerve in both the CNS and PNS [279-281]. Contactin-1 expression in the paranodal region in both PNS and CNS is important to regulate formation of the paranodal septate-like junctions that are critical for the function but not for the formation of myelin.

The functions of Contactin-1 at the central (but not peripheral) nodes of Ranvier are associated with the level of sodium channel surface expression and the switch from the early Nav1.2 to the mature and faster Nav 1.6 pore forming subunits (**Paper I**). Possible mechanism is direct interactions of Contactin-1 with the non-pore forming sodium channel  $\beta$ 1-subunit [58, 60] that influences the number of sodium channels expressed on axon surface. Numbers of sodium channels, in turn, regulate neuronal electrical activity and a reduced compound action potential was observed in the *Cntn1*-KO [60]. Although such a mechanism could contribute to the observed myelin phenotype, a more likely interpretation is that loss of paranodal septate-like junctions in *Cntn1*-KO mice prevents efficient clustering and maturation of sodium channel clusters and thus lead to their reduction in number and poor maturation

In conclusion, Contactin-1 in myelinated nerves in the CNS (**Paper I**) plays important and separate roles during early and late development of myelinated nerves. As an early signal Contactin-1 regulates axon maturation and initiation of myelination in the CNS. At later stages Contactin-1 orchestrates the formation of nodal and paranodal domains and the formation of the paranodal axon-glia junctions. Knowledge about how a system is generated and functions forms an important basis towards approaches aimed at regeneration after degeneration has occurred. This is highly significant in studies regarding myelin degenerative disease such as multiple sclerosis.

The *Cntn1*-KO mouse exhibit other prominent phenotypes besides the loss of function in fast propagating axons, it also develop severe anorexia and ataxia. The ataxia is related to the cerebellar defect previously described [249]. The anorectic phenotype was explored in this thesis (**Paper II and IV**) by the use of comparative studies with the already established anorectic *anx/anx* mouse and a 24hr fasted mouse. Investigating the expression levels and location of the neuropeptides regulating increase of food intake, the orexigenic NPY/ AgRP neuropeptides originating in Arc, we found that the mRNA levels were upregulated in the *Cntn1*-KO mouse, the *anx/anx* mouse and the food deprived mouse. This can be seen as a normal compensatory response to reduced food intake. However, the most pronounced changes are found in the most emaciated anorectic mouse model, the *Cntn1*-KO mouse. The cellular location of these orexigenic neuropeptides as seen by IHC, was further disrupted with what looked like accumulation of the peptides in the neuronal cell bodies, and reduced levels

in the neuronal fibers projecting to the PVN. To be noted is that the neuronal projections from Arc to PVN target are not fully mature until the third postnatal week [110, 282] and therefore the dramatic cellular changes observed in the *Cntn1*-KO may reflect an inability to regulate axon outgrowth as has been observed in the cerebellum [249]. Interestingly, a similar reduction in axonal outgrowth has been associated with obesity both in leptin deficient mice [283, 284] and mice with monosodium glutamate lesions of the Arc [285]. The outcome, obesity vs anorexia, of hampered axonal Arc-PVN outgrowth, may thus reside within the functionality of the other Arc population, the POMC/CART neurons. It is also possible that the obese vs anorectic phenotype is rather a result of that once the Arc-PVN neurotransmission is weakened, the activity in other regions, such as regions involved in reward aspects of food intake, are allowed to become more prominent.

Another possible explanation to the change in neuropeptidergic expression seen by IHC in the *Cntn1*-KO is that the axons are still present but in the absence of Contactin-1 a transport defect may result in loss of NPY and AgRP-ir in fibers. Contactin-1 has previously been associated with transporting associated proteins to the cell surface as with Caspr [59, 76] and sodium channels [60]. The latter which will further lead to alterations in the electrophysiological activity of the axons which in turn could lead to modifications of the responsiveness to food intake regulatory signaling. However, considering later data from the second comparative study between the *Cntn1*-KO mouse and the *anx/anx* mouse (**Paper IV**) neither the transport hypothesis nor the axonal activity may be valid as inactive axons would be pruned away and degenerative processes would take place. Degenerative processes in the brain are associated with increased expression of GFAP positive astrocytes and Iba1 positive microglia [286, 287]. Increased Iba1-ir cells were found in several hypothalamic regions of the *anx/anx* mouse [45]. Increased GFAP-ir was also seen in the hypothalamus of these mice. The *Cntn1*-KO mouse did not however show any increased GFAP- or Iba1-ir in the hypothalamic areas thus far evaluated; Arc and PVN. More hypothalamic regions will however be analyzed, including the MPO. These data do nevertheless suggest that there is no inflammatory response in the hypothalamus of the *Cntn1*-KO mouse. In contrast to the hypothalamus expression, the dentate gyrus of the hippocampus did exhibit increased expression of the microglia marker, but showed no change for the astrocyte marker. These results indicate that the *Cntn1*-KO may not be compromised by a dysfunctional immune response in the CNS. The reason for the increase Iba1-ir could be related to the synaptic defects previously found in the hippocampal CA1 region of the *Cntn1*-KO [76]. It could also be related to the distorted food intake maybe via the “hunger hormone” ghrelin signaling regulating firing of hippocampal neurons [288] or by secondary hypothalamic projections from the PVN to the different hippocampal areas [289]. No hippocampal increase in glia markers was observed in the *anx/anx* mouse (**Paper IV**), despite previous studies showing increased cell proliferation and apoptosis in the dentate gyrus of the *anx/anx* mouse by Kim et al. [54].

The mitochondrial dysfunction (the main producer of cellular ATP) found in the *anx/anx* mouse may play a crucial role in regulating the activity level of the Arc neurons as they are

expressing a specific subtype of the ATP sensitive potassium channels, Kir6.2/SUR1, regulating the activity levels of the neurons depending on the ATP levels [290, 291]. To test whether the mitochondrial dysfunction in the *anx/anx* mouse results in a reduced activity level of the Arc neurons which in turn would lead to reduced firing potential and a dysfunctional regulation of food intake, the metabolic activity was tested (**Paper IV**). A dysfunction of the mitochondrial ATP producing oxidative phosphorylation system normally generates less ATP and to compensate for this reduction, the cells increase the glucose uptake, increase glycolysis and PCr is reduced since it donates a phosphate to ADP in order to generate ATP. However, in the anorectic *anx/anx* mouse we observed a reduced glucose uptake despite a compensatory up-regulation of the glucose transporter Glut4 but a decreased ratio of the AMPK-P/AMPK indicates no translocation of the glucose transporter. The elevated levels of PCr both at basal level and during stress, indicates that the hypothalamic neurons of the *anx/anx* mouse have an excess of phosphate donors to generate ATP. The decreased levels of lactate indicates that, despite the mitochondrial dysfunction generating less ATP, the anaerobic glycolysis is not utilized, via pyruvate, to generate more energy. The reduced uptake of glucose together with reduced levels of cellular AMP and IMP and increased level of ATP in the *anx/anx* mouse points to that hypothalamic neurons are utilizing less energy and therefore are less active. We did not isolate each cellular type of the hypothalamus when the metabolic activity within the cells were measured and cannot determine the ATP source nor which cell type in the hypothalamus that suffer most from reduced cellular metabolism. The astrocytes regulate synaptic activity of the hypothalamic neurons [292, 293].

To self starve requires extreme determination and most people will start to eat due to very strong hunger senses, as an evolutionary safeguard against too low energy reserves (represented by low levels of leptin and insulin and high levels of ghrelin). However, what if there is genetic errors preventing the neurons to signal normally and starvation can prolong and even feel as a reward or at least some type of satisfaction? What if you are born with reduced hunger feelings long before body appearance is considered? These two questions are central for our studies in the hypothalamus of the two anorectic mouse models. The predisposition to AN is most likely caused by several genes, therefore, reduced cellular metabolic activity and glia activation related to mitochondrial dysfunction in the *anx/anx* mouse and neuropeptidergic changes in the *Cntn-1* KO mouse may be applicatory genetic causes to human adolescents with AN and with kids with failure to thrive. In regards to AN there may be environmental factors that trigger the onset, however, it failure to thrive the environmental factor may not be as strong as the genetic factor as this may start even in infants. The two anorectic mouse models in our studies represent a pure genetic adolescent model of AN.

## 6 FUTURE PERSPECTIVES

In order to further understand the molecular mechanism whereby Contactin-1 modulates hypothalamic NPY/AgRP neurons it would be of value to further explore if/how Contactin-1 affects axonal outgrowth of the Arc projections to the PVN target. This can be done using explant cultures from the Arc and PVN of *Cntn1*-KO and WT mice, and further with DiI labeling of Arc neurons tracing the axonal outgrowth *in situ* in fixed tissue. Another option is to analyze the *Cntn1*-KO mouse crossed with the NPY-GFP mouse to monitor the axons by green fluorescent protein expressed from the NPY promoter. With the analysis of Contactin-1 and Caspr-1 expression levels and location in the *anx/anx* mouse we will be able to understand more of the function of Contactin-1 in the hypothalamus and further tease out the molecular differences between the two anorectic mouse models. Contactin-1 has been found to interact with the chondroitin sulfate proteoglycan phosphacan/receptor-type protein tyrosine phosphatase beta, RPPT- $\beta$ , expressed by astrocytes and on nerve fibers around neurons in the PVN target area. A possible mechanism is that this interaction is involved in modulating the synaptic plasticity of the Arc neurons [294] or to shuttle lactate to neurons for increased ATP production [295]. To investigate if functional synapses are formed in the target area we will use synaptic markers identifying pre- and postsynaptic structures.

Further analyses of glial expression in other hypothalamic areas of the *Cntn1*-KO are needed. This, since the strongest increase in expression of the microglia marker observed in the *anx/anx* mouse was in the MPO [45]. Continuing studies on the *anx/anx* mouse would include anti-inflammatory treatments in order to investigate the role of the inflammatory profile on the anorectic phenotype, i.e. studying possible improvements in survival, body weight, food intake as well as possible modulation of the observed hypothalamic activation of microglia. Since we confirmed that there is reduced cellular metabolic activity in the hypothalamus of the *anx/anx* mouse, we need to investigate which cell type is mostly affected by the mitochondrial dysfunction. This would be done by investigating levels of AMPK-(P) in astrocytes and neurons separately. Cortical astrocytes and neurons have an equal amount of mitochondria but have cell type specific pattern for key glycolytic enzymes as astrocytes favor a higher glycolytic rate and lower oxidative metabolism and neurons favor the opposite [296, 297]. Since astrocytes are involved in the support of normal neuronal process activity it is possible that a lack of this function will promote degeneration of neurons in the hypothalamus.

Finally, the expression pattern of  $K_{ATP}$  in the *anx/anx* mouse should be studied in detail, since we suggest that the expression of  $K_{ATP}$  with the specific pore-forming subunits Kir6.2 and regulatory SUR1 by the Arc neurons makes these neurons extra sensitive to ATP deficiency and increased levels of ROS, and thus put them in a hibernation-like state [148, 257, 258, 298, 299]. The  $K_{ATP}$  expression could be associated with anorexia in the *Cntn1*-KO mouse as well, as the maturation of the NPY/AgRP projections have been associated with initiation of  $K_{ATP}$  (Kir6.2/SUR1) expression and nutritional status [298]. It would thus be worth exploring the channel in these animals as well as in humans with AN.

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