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# HYPOTHALAMIC REGULATION OF FOOD INTAKE – FOCUS ON THE *ANX/ANX* MOUSE

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When my sister was suffering the worst from anorexia nervosa during the mid 90´s, I started to develop a huge frustration against the theories fluctuating in society, trying to explain the disease. It was back then, and might still so be, common to hear justifications related to “a controlling mum” and an “absent dad”. Even though we all in the family knew the incorrectness in such rationalizations, I guess we all felt a fragment of guilt. This made me want to search for other explanations. Still today, I believe, that many people think about disorders such as anorexia nervosa and bulimia nervosa as purely concerning dieting and an obsession of being slim, conditions that can be easily alleviated by “getting oneself together”.

In general, disorders of the brain, in particular mental disorders, are often perceived as something shameful, which rarely happens when dealing with other vital organs such as a kidney or a heart. To be remembered is that in mental disorders the pathology is simply centered round another organ, and in this case is manifested in changes of mood, thought or behavior instead of in blood pressure.

Adding to this, the notion that so little research is being conducted in the field of anorexia, especially in relation to the vast amounts of research conducted on obesity, and the often inadequate treatment strategies of eating disorders strongly motivated me to write this thesis.

Johanna, Mathilda & Mamma, this one’s for you.

# ABSTRACT

The main goal of this thesis is to increase the knowledge about one of the most important tasks of the brain, the hypothalamic regulation of food intake. The hypothalamus is considered to be the brain's main center for regulation of food intake and it is integrating signals regarding energy status, from the body, to initiate a proper behavioral response. A malfunctioning of this sensitive system can cause disturbed eating behavior, and have serious consequences for the organism's well being. Disturbed eating behavior is not only part of the traditional eating disorders, such as anorexia nervosa and bulimia nervosa, but also contributes to overweight and obesity, thereby increasing the risk for several severe disorders and conditions. In addition, anorexia/cachexia is a frequent complication of failure to thrive in infants, malignant tumors and inflammatory diseases, and is contributing significantly to the mortality of these disorders.

We use the unique anorectic *anx/anx* mouse as a model system for regulation of food intake. In **Paper I,** we studied the normal development of the projections from NPY/AGRP expressing neurons in the arcuate nucleus (Arc), in normal mouse, and were able to conclude that the first three postnatal weeks appear to be critical for the development of this hypothalamic food intake-regulating system. Previous studies have shown several neurochemical abberances in the hypothalamus of the *anx/anx* mouse, in particular in the distribution of neurotransmitters and -peptides known to have a potent regulatory role in the control of food intake, such as NPY, AGRP, CART and POMC. In order to evaluate when these aberrances first appear, we compared the development of the NPY/AGRP system in *anx/anx* with +/+ mice in **Paper II**. We concluded that the NPY/AGRP system in *anx/anx* mice develop as in +/+ mice until P12, after which it appears as if the normal gradual increase in fibers cease and even decrease. In addition, we detected a region specific activation of microglia in several hypothalamic, as well as extra hypothalamic areas, in *anx/anx* mice from P12 and onwards. Interestingly, these were all areas in which we previously detected a reduced density of NPY/AGRP-ir fibers in *anx/anx* mice, indicating that the aberrant hypothalamic neurochemistry in the *anx/anx* mice could be related to an inflammatory/neurodegenerative process. To further investigate this possibility we analyzed the expression of MCH class I. In **Paper III** we show expression of MHC class I mRNA and protein in the projection areas of the Arc neurons, to a large extent attributed to microglia, but remarkably also in a few arcuate-neuron , in the *anx/anx* mice,. We also found evidence for hypothalamic degeneration in the *anx/anx* mouse, by showing co-labeling of NPY and active caspase 6 in Arc, DMH, amygdala and zona incerta. Caspase 6 is required for axonal degeneration, and has been implicated in the pathology of neurodegenerative disorders. Taken together, this provides evidence of a neurodegenerative process in hypothalamus of the *anx/anx* mice. In **Paper IV**, we aimed to identify the *anx* gene and mutation, as well as the underlying mechanism causing the anorectic phenotype of the *anx/anx* mouse. We concluded that the anorexia and premature death of the *anx/anx* mouse is realated to hypothalamic mitochondrial dysfunction and that the *anx* mutation leads to lower levels of the *Ndufaf1* gene and protein. This leads to less fully assembled complex I in the mitochondrial oxidative phosphorylation system, as well as accumulation of sub-complexes resulting and increased production of reactive oxygen species. The increased levels of reactive oxygen species can initially act as a signaling molecule affecting hypothalamic neurons, leading to reduced food intake, oxidative stress and in the long run to inactivation and degeneration of Arc food intake-regulating neurons in *anx/anx* mice.

## LIST OF PUBLICATIONS

I. **Nilsson I**, Johansen JE, Schalling M, Hökfelt T, Fetissov S. Maturation of the hypothalamic arcuate agouti-related protein system during postnatal development in the mouse. Brain Res Dev Brain Res. 2005; 155: 147-54

II. **Nilsson I**, Lindfors C, Fetissov SO, Hökfelt T, Johansen JE. Aberrant agouti-related protein system in the hypothalamus of the *anx/anx* mouse is associated with activation of microglia. J Comp Neurol. 2008; 507: 1128-40

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II. Fetissov SO, Hallman J, **Nilsson I,** Lefvert AK, Oreland L, Hökfelt T. Aggressive behavior linked to corticotrophin-reactive autoantibodies. Biol Psychiatry. 2006; 60:799-802

III. Johansen JE, Fetissov SO, Bergström U, **Nilsson I**, Faÿ C, Ranscht B, Hökfelt T, Schalling M. Evidence for hypothalamic dysregulation in mouse models of anorexia as well as in humans. Physiol Behav. 2007; 92:278-82

IV. Hökfelt T, Stanic D, Sanford SD, Gatlin JC, **Nilsson I**, Paratcha G, Ledda F, Fetissov S, Lindfors C, Herzog H, Johansen JE, Ubink R, Pfenninger KH. NPY and its involvement in axon guidance, neurogenesis, and feeding. Nutrition. 2008; 24:860-8

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocorticotropin |
| AGRP | agouti-gene related protein |
| α-MSH | α-melanocyte-stimulating hormone |
| AMPK | adenosine monophosphate kinase |
| AN | anorexia nervosa |
| *anx* | the anorexia gene/mutation |
| Arc | arcuate nucleus |
| BAC | bacterial artificial chromosome |
| BN | bulimia nervosa |
| BN PAGE | blue native polyacrylamide gel electrophoresis |
| BNST | bed nucleus of stria terminalis |
| BrdU | bromodeoxyuridine |
| C(I-V) | complex (I-V) in the oxidative phosphorylation system |
| CART | cocaine- and amphetamine-regulated transcript |
| cM | centimorgan |
| CNS | central nervous system |
| *db* | diabetes mutation |
| DHE | dihydroethidium |
| DMH | dorsomedial hypothalamic area |
| DSM | diagnostic and statistical manual of mental disorders |
| E | embryonic day |
| FTT | failure to thrive |
| GABA | γ-aminobutyric acid |
| -GFP | green fluorescent protein |
| GI | gastrointestinal |
| GSHR | growth hormone secretagogue receptor |
| HRP | horseradish preoxidase |
| Iba | ionized calcium binding adapter |
| i.c.v. | intracerebroventricular |
| IFTT | idiopathic failure to thrive |
| IHC | immunohistochemistry |
| ir | immunoreactive |
| IR | insulin receptor |
| ISH | in situ hybridization |
| KO | knockout |
| Lep | leptin |
| Lepr | leptin receptor |
| LHA | lateral hypothalamic area |
| -LI | -like immunoreactivity |
| MC 1-5 | melanocortin receptor 1-5 |
| MCH | melanin-concentrating hormone |
| ME | median eminence |
| MHC | major histocompatibility complex |
| MPOA | medial preoptic area |
| MSG | monosodium glutamate |
| mTOR | the mammalian target of rapamycin |
| Ndufaf1 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1 |
| NOFTT | non-organic failure to thrive |

| | |
|---|---|
| NTS | nucleus tractus solitarii |
| NPY | neuropeptide Y |
| *ob* | obese mutation |
| OFTT | organic failure to thrive |
| OXPHOS | oxidative phosphorylation system |
| P | postnatal day |
| P1 | bacteriophage P1 |
| PAC | P1 artificial chromosome |
| PBN | parabrachial nucleus |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| POMC | pro-opiomelanocortin |
| PVN | paraventricular nucleus |
| ROS | reactive oxygen species |
| RT | room temperature |
| SDS PAGE | sodium dodecyl sulfate polyacrylamide gelelectrophoresis |
| SMR | standardized mortality ratios |
| SN | substantia nigra |
| SSLP | simple sequence length polymorphism |
| STS | sequence tagged sites |
| TSA | tyramide signal amplification |
| TUNEL | terminal dUTP nick end labeling |
| wt | wildtype |
| Y 1-5 | neuropeptide Y receptor 1-5 |

# 1 BACKGROUND

The regulation of food intake is a most important task of the brain, and a dysregualtion of the systems involved can have fatal consequences. The hypothalamus is considered to be the major feeding center of the brain (Schwartz 2006), and it is integrating nutritional status signals from the body to initiate a proper response - to eat or not to eat. When this sensitive system is malfunctioning it will lead to disturbed eating behavior and/or appetite, which will have serious consequences with great impact on the organism´s well being. The focus of this thesis is to shed further light on hypothalamic regulation of food intake.

Eating disorders, such as anorexia nervosa (AN) and bulimia nervosa (BN), represent a complex group of poorly understood disorders that currently lack effective pharmacological or other biological, target-directed therapy. The chronicity and mortality of eating disorders are among the highest of all psychiatric illnesses (Hoek 2006; Nielsen et al. 1998; Sullivan et al. 1998). AN is believed to be the deadliest of all psychiatric disorders, but standardized mortality ratios[1] (SMR) vary considerably, from 0-17.8 (Papadopoulos et al. 2009). The same study, based on a Swedish nationwide cohort, concluded an overall SMR of 6.2 (95% CI 5.5-7.0) for AN (Papadopoulos et al. 2009), and in Sweden the lifetime prevalence[2] of AN is estimated to be 1.20% of females and 0.29% of males (Bulik et al. 2006).

AN is a multifaceted disease likely involving multiple organs and interactions between environmental, psychosocial and genetic risk factors. Growing evidence suggests that neurobiological variabilities make a substantial contribution to the pathogenesis (Kaye 2008). According to the diagnostic and statistical manual of mental disorders V (DSM-V) the following criteria needs to be fulfilled for diagnosis with AN: an intense fear of gaining weight, refusal to maintain adequate nutrition, often associated with an erroneous image of the self as fat, loss of original body weight to < 85% of normal, disturbance of body image and negative self-evaluation and absence of at least three consecutive menstrual periods (DSM-IV, 1994). Other common aspects of AN are hyperactivity which occurs in 31-80% of the cases (Hedebrand *et al*., 2004) and osteoporosis in 20-50% of the patients (Legroux-Gérot *et al*., 2007; Munoz *et al*., 2002; Herzog *et al*., 1993; Zipfel *et al*., 2000). Both AN and BN are characterized by anomalous patterns of feeding behavior, and deviant attitudes and perceptions relating to body weight and shape. Anorectics rarely stop eating completely and are resistant to feeding drives rather than having a complete suppression of appetite. BN is commonly associated with binge eating followed by some type of compensatory action to get rid of the excess of food ingested, commonly self-induced vomiting or extreme exercise. BN is not necessarily associated with reduced body weight.

[1] a ratio between the observed number of deaths in a study population and the number of deaths that would be expected, based on the age- and sex-specific rates in a standard population and the age and sex distribution of the study population

[2] the total number of cases in the population that up to the point of assessment has experienced the condition, compared to the total number of individuals

According to DSM-V the following criteria are required for diagnosis with BN: negative self-evaluation influenced by body shape and weight, no anorexia nervosa, frequent occurrence of binge eating episodes accompanied by a sense of loss of control and recurrent inappropriate behavior (i.e. vomiting, use of laxatives, fasting, or excessive exercise) intended to prevent weight gain. Both of the last mentioned behaviors, need to occur at least twice a week, on average for three months (DSM-IV, 1994). Transitions between syndromes occur in many cases and 25-30% of bulimics have a prior history of AN. Individuals with AN and BN are also more frequently diagnosed with anxiety, depressive and obsessive compulsive disorders at some time in life (Kaye 2008). It is suggested that additive genetic factors account for 50-80% of the liability of AN and BN, estimates similar to those for schizophrenia and bipolar disorder (Bulik et al. 2006; Kaye 2008).

The management of AN treatment requires a multidisciplinary approach combining appropriate refeeding scheme back to normal weight, the ceasing of weight losing behavior, and a range of psychological therapies and techniques that help the patient to cope with disordered thoughts and dysphoric mood while achieving the treatment goals (Kaye 2008). However, the lack of knowledge of physiopathology and etiology of AN has seriously impeded the clinical efforts to develop and adjust the care and treatment regimes. For that purpose it appears crucial to gain deeper insight into the behavioral, psychological and metabolic changes and alterations of hormones, cytokines and neurotransmitter/neuropeptide synthesis and release encountered during the different phases of AN.

A lot of attention has, in the past decades, been drawn to the other end of the weight spectrum, overweight/obesity, and its detrimental effects on humans as well as the enormous costs for society. Overweight and obesity increase the risk of severe diseases such as cardiovascular disease, type II diabetes and some types of cancers. Two to seven percent of the total healthcare costs have been estimated to be related to obesity in several developed countries (Low et al. 2009). The prevalence of obesity and overweight in Sweden in 2006 was approximately 52% of males and 37% of females, and the same values for USA, in 2006, were 73% and 61% , respectively (numbers from SCB, OECD and Folkhälsoinstitutet). A *Pubmed* search using the keyword “obesity” generated in November 2009 more than 125 000 hits, whereas a search on “anorexia” generated a bit over 21 000 hits, to a great extent reflecting the fact that much less time, money and effort are spent on understanding underweight/anorexia compared to overweight/obesity. In addition to AN, one can also add cachexia and failure to thrive (FTT), to the group of conditions that are related to underweight.

Cachexia is a syndrome with decreased appetite in combination with an increased metabolism leading to weight loss, loss of muscle and adipose tissue and weakness. HIV, cancer, inflammatory disorders, chronic kidney disease and chronic heart failure are all associated with cachexia (Tan and Fearon 2008). During such conditions, when the body struggles to cope with the disease, it is deleterious to further push the body into a catabolic state. In advanced stage cancer, 80% of patients suffer from cachexia, and it is one of the most frequent causes of death in these cases (Ramos et al. 2004).

FTT is a term used to describe an infant or a child whose growth is inadequate for its gender and age. FTT may be of either organic (OFTT) with an identifiable medical cause, such as chromosomal abnormalities, gastro-intestinal (GI) or endocrine disorders, or of non-organic (NOFTT) origin (Schwartz and Abegglen 1996). The majority of cases in developed countries are of non-organic origin. These cases can be associated with psychosocial problems, however, more frequently no organic or psychosocial etiology is found. If no organic problems are found and all psychosocial factors have been excluded, the condition is termed idiopathic FTT (IFTT). In many of these cases, the infant has a decreased appetite resulting in a decreased caloric intake. It appears as if the hypothalamus is not synchronized to the caloric requirements of the infant. One to five percent of all pediatric hospital admissions are estimated to be associated with FTT (Kasese-Hara et al. 2002; Shaoul et al. 2003).

I believe that everyone, who ever tried to diet or simply had to wait for supper, a while longer than expected, can easily recognize the powerful forces of hunger. In fact, hunger is one of our strongest drives. It thereby seems paradoxical that a severely underweight person, such as an AN patient, can ignore hunger and refuse to eat, likewise that a cachectic patient or a growing child, in high need of energy, do not feel hunger.

With this said, studies concerning energy homeostasis in general, relating to both the obesity and anorexia parts of the weight spectrum, are obviously of great importance. This thesis focuses in particular on gaining new knowledge regarding the molecular mechanism(s) of food intake-regulation centered in the hypothalamus, relating them to anorexia and starvation, but is to a large extent also significant for the area of obesity and overweight as well as food intake-regulation in general.

## 1.1 ENERGY HOMEOSTASIS

Few tasks of the brain are as critical for the survival of the species, as the insurance of adequate intake of energy and nutrients. Therefore, intricate systems have developed to ensure that these requirements are fullfilled, i.e. the energy homeostasis, a complex system of signals regulating food intake and energy utilization, with the goal of keeping the energy stores sufficient and relatively constant (Figure 1). Usually the signals are involved in short- and long-term regulation. The former, also called the meal-related regulation, is coupled to the initiation and termination of meals. These signals mainly originate in the GI tract and project via the vagus nerve to the nucleus tractus solitarii (NTS) of the brainstem. When food reaches the stomach and intestine, distention of the wall gives rise to signaling via the vagus nerve. A similar mechanism operates when specific nutrients are sensed by receptors located in the GI tract.

The long-term regulation has its center in the hypothalamus, aiming at stabilizing the energy stores, which are mainly located in adipose tissue, and also to a small extent as glycogen in muscle and liver, for longer periods such as years (Schwartz 2006).

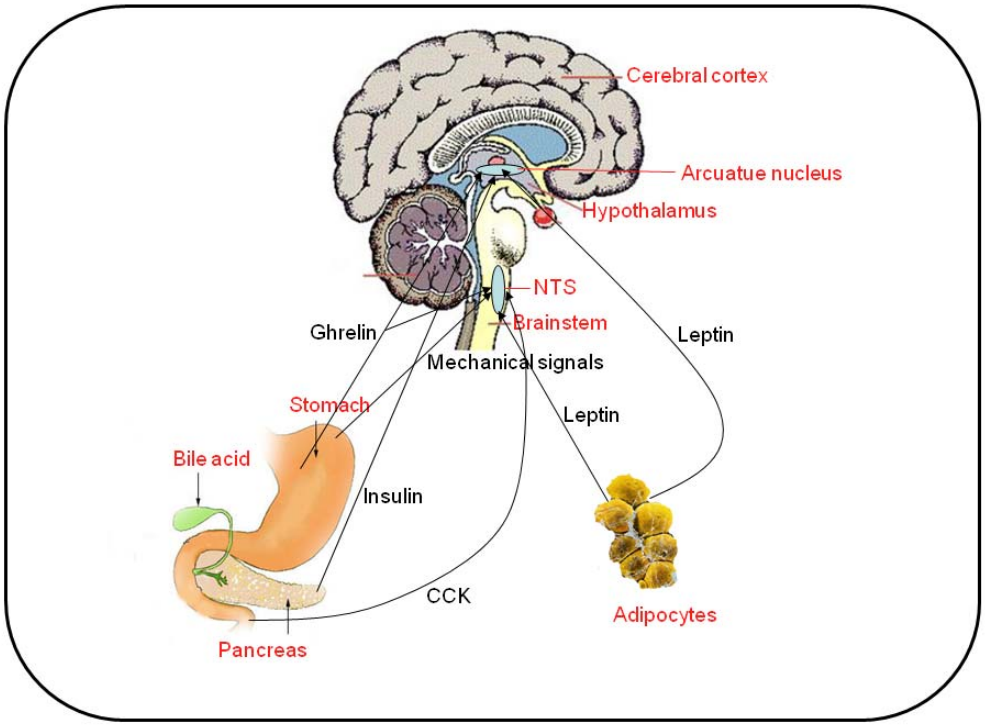


**Figure 1**. Overview of the central regulation of energy homeostasis. Modified from (Johansen 2006).

### 1.1.1 Hypothalamus – center for long-term regulation of food intake

The center of long-term regulation of food intake originates in neurons in the mediobasal part of hypothalamus, the arcuate nucleus (Arc). In this area the blood-brain-barrier is partly permeable, allowing entrance of peripheral hormones, binding to receptors on neurons in Arc, thereby affecting their activity and controlling the energy status (Figure 2). Two groups of neurons in Arc are vital for the regulation of food intake. One group expresses the anorexigenic, food intake-inhibiting neuropeptide pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), the other group expressing the orexigenic, food intake-stimulating neuropeptide Y (NPY) and agouti-gene related protein (AGRP).

The POMC/CART and NPY/AGRP neurons make contact with each other to balance and coordinate their activity and both groups of neurons send extensive projections to other areas within hypothalamus, e.g. the dorsomedial hypothalamus (DMH), the lateral hypothalamic area (LHA), the medial preoptic area (MPO) and the paraventricular nucleus (PVN). They also innervate extrahypothalamic regions, e.g. bed nuclei of stria terminalis (BNST), thalamus, NTS, the parabrachial nucleus (PBN), the ventral tegmental area and amygdala, the BNST and PBN being far rostral and far caudal projection areas, respectively (Broberger et al. 1998).

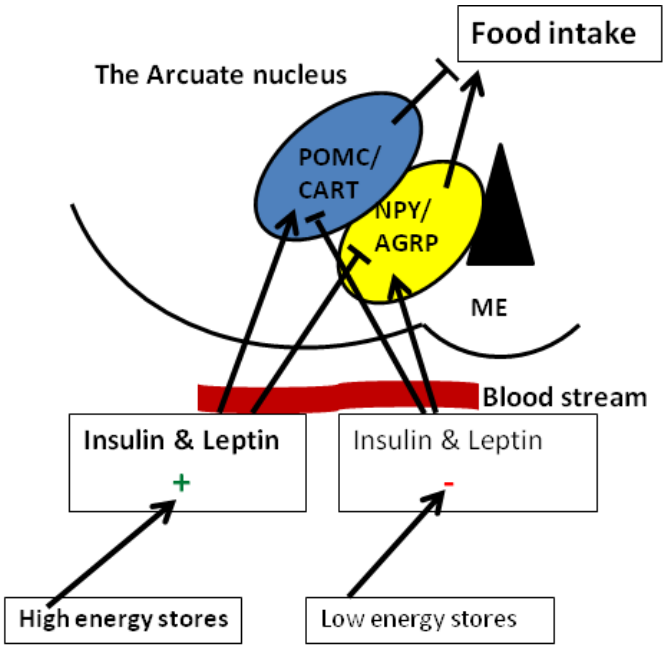


**Figure 2.** Schematic overview of how leptin and insulin regulate food intake via the NPY/AGRP and POMC/CART neurons in Arc during low and high energy stores.

#### *1.1.1.1 Peripheral monitoring of energy homeostasis*

A number of peripheral signals regulate the bodily energy stores, i.e. mainly fatty acids in adipose tissue, but also to a small extent glycogen in muscle and liver. In addition to the humoral signals, leptin and insulin, other substances also appear to be involved. These are e.g. GI hormones such as peptide YY and ghrelin and the female sex hormone estrogen (Kalra and Kalra 2004; Morton et al. 2006; Morton and Schwartz 2001).

**Leptin** is a critical humoral signal, regulating food intake, and the product of the obese (*ob)*-gene, cloned in 1994 (Zhang et al. 1994). Already in the 1950´ies Kennedy proposed a lipostatic mechanism for the purpose of maintaining energy homeostasis (Kennedy 1953). Thus, he postulated that the size of the fat stores is sensed by a 'lipostat' that subsequently regulates energy homeostasis, i.e. food intake and energy utilization, to maintain body weight at a specific set point. A few years later Hervey could demonstrate the prescence of a hormone, that later turned out to be leptin, acting as such a lipostatic signal (Hervey 1959).

Leptin is expressed by adipose tissue and circulates in the blood stream in levels corresponding to the amounts of adipose tissue. Leptin enters the brain via the median eminence located in the mediobasal part of the hypothalamus, close to Arc and binds to receptors expressed on the food intake-regulating POMC/CART and NPY/AGRP neurons. The binding of leptin to the anorexigenic POMC/CART neurons is increasing their activity. The opposite is true for the orexigenic NPY/AGRP neurons, i.e. decreased activity. Thus, the net effect of leptin on Arc neurons is decreased food intake (Figure 2) (Schwartz 2006). In addition to the direct effects via the NPY/AGRP and POMC/CART neurons, leptin has been suggested to be an important factor for the

postnatal development of these neuronal projections from Arc to its target areas in and outside the hypothalamus (Bouret et al. 2004).

The leptin receptor is encoded by the diabetes *(db)*-gene and exists in several splice variants, of which the long form has a cytoplasmic region required for signal transduction (Barash et al. 1996; Lee et al. 1996).

**Insulin** is primarily familiar for regulating the blood glucose levels, but has also been suggested to be involved in neuronal growth and differentiation (Heidenreich 1993), as well as neurotransmitter release and synaptic plasticity (Jonas et al. 1997; Wan et al. 1997). In addition, insulin is together with leptin critical in the regulation of energy homeostasis via the arcuate neurons (Baskin et al. 1999). Like leptin, insulin is circulating in the blood stream reflecting the energy status in the body, though being secreted from pancreas instead of adipose tissue. The receptors for insulin (IRs) are distributed in most tissues of the body and are broadly expressed in the central nervous system (CNS) (Bruning et al. 2000). IRs are expressed in the arcuate food intake-regulating neurons, and insulin binding stimulates the activity of the POMC/CART neurons, while inhibiting the NPY/AGRP neurons (Schwartz 2006). Neuron-specific deletion of the insulin receptor gene results in increased food intake and obesity (Bruning et al. 2000).

**Ghrelin** is the only known hormone with orexigenic effect produced in the periphery, primarily in the stomach. In addition to being a powerful stimulator of food intake, ghrelin is suggested to function in GI motility, gastric acid secretion and various immunological and reproductive processes (Nogueiras et al. 2008). The plasma levels of ghrelin are stringently correlated with recent food intake and are, in contrast to insulin, increased before and decreased after a meal (Morton and Schwartz 2001). Ghrelin binds to its receptor, growth hormone secretagogue receptor 1 (GHSR-1), expressed on NPY/AGRP neurons, and stimulates the neuronal activity. Expression of GHSR-1 has, up till now, not been seen on POMC/CART neurons. However, since the NPY/AGRP neurons project onto the POMC/CART neurons, a secondary inhibitory effect of ghrelin can therefore be sensed by the POMC/CART neurons. Thus, the net effect of ghrelin on food intake-regulating arcuate neurons is to increase food intake (Andrews et al. 2008; Nogueiras et al. 2008).

#### *1.1.1.2 Neuropeptides central in regulation of food intake*

**NPY** is a 36 amino acid peptide (Tatemoto 1982; Tatemoto et al. 1982), which in 1984 was found to be involved in the regulation of food intake (Morton and Schwartz 2001; Stanley et al. 1992). In addition, NPY is involved in many other functions, including modulation of mood and anxiety, as well as in sympathetic functioning, pain and circadian rhythms (Kalra and Kalra 2004). It exerts its effects via six G-protein-coupled receptors, whereby the Y1 and Y5 appear to be most important for the regulation of food intake (Kalra and Kalra 2004; Morton and Schwartz 2001).

NPY is abundantly expressed throughout the peripheral and CNS but the population in Arc appears to be the most important one for the regulation of food intake. More than 90% of the NPY neurons in Arc co-express AGRP (Broberger et al. 1998; Hahn et al. 1998). In addition, these neurons also synthesize the inhibitory transmitter γ-aminobutyric acid (GABA) and the steroid hormone estrogen (Ovesjo et al. 2001).

The expression of NPY is up regulated during negative energy balance, e.g. in fasted animal and in models of starvation (Fetissov et al. 2005; Morton and Schwartz 2001), and NPY is one of the most potent orexigenic transmitters known. Thus, intracerebroventricular (i.c.v.) administration of NPY requires only a single injection to potently stimulate feeding in rodents (Morton and Schwartz 2001).

**The melanocortin system** of the hypothalamus constitutes a unique food intake-regulating system, including both the anorexigenic **POMC peptides** and the orexigenic **AGRP**, both exerting their action via melanocortin receptors (MCs), as agonists and inverse agonists/endogenous antagonist, respectively.

POMC is a precursor molecule cleaved post-translationally to generate two different classes of peptides, the melanocortins such as α-, β- and γ-MSH, adrenocorticotropin (ACTH) and the β-endorphins (Coll and Loraine Tung 2009; Cone 2005; Watson et al. 1978). POMC is expressed both peripherally, e.g. in skin, pituitary and hair follicles and centrally, e.g in Arc and NTS (Cone 2005). In Arc, POMC is co-expressed with CART (Vrang et al. 1999). The documented effects of melanocortins are far further than just regulation of food intake and energy expenditure, also being involved in such varied processes as the regulation of blood pressure, heart rate, inflammation and erectile function (Cone 2005). The effects of POMC peptides are mediated via five G-protein-coupled receptors, MC1-5 (Cone 2005). α-MSH exerts the anorexigenic effects via agonistic action at MC3 and -4, the receptors also acted upon by AGRP, however as an inverse agonist/endogenous antagonist. The POMC neurons are stimulated by binding of leptin and/or insulin to the corresponding receptors (Cone 2005; Cowley et al. 2001; Grill and Kaplan 2002). These neurons can, in contrast, be inhibited by the neighboring NPY/AGRP neurons (Cone 2005; Morton and Schwartz 2001).

AGRP is an orexigenic polypeptide, composed of 132 amino acids, exclusively expressed by the arcuate NPY neurons (Broberger et al. 1998; Hahn et al. 1998). AGRP, as the name implies, has 25% homology with the agouti protein, solely expressed in hair-follicles. Agouti acts in a paracrine fashion to inhibit deposition of the black pigment eumelanin. By antagonistic action at MC1, agouti favors the production of the yellow pigment phaeomelanin (Morton and Schwartz 2001). Like agouti, AGRP exerts its effects via the melanocortin system by antagonism/inverse agonism on MC3 and -4.

The orexigenic effects of AGRP have been revealed by central administration or genetic over-expression of the peptide resulting in hyperphagia, anabolism and obesity. AGRP appears to have more long-term effects than NPY. In fact, a single i.c.v. injection can have effects lasting up to a week, in comparison to NPY´s effect lasting for hours rather than days (Morton and Schwartz 2001).

Both fasted rodents and mice deficient in leptin express lower levels of POMC mRNA in hypothalamus, which can be alleviated by administration of leptin (Mizuno et al. 1998). In addition, expression of AGRP is increased in two mouse models of obesity, the leptin deficient *Lep$^{ob/ob}$* and leptin receptor lacking *Lepr$^{db/db}$* mice, as well as in fasting mice (Flier 2006)

**CART peptides** exert anorexigenic effects and are in addition involved in a wide range of processes in the brain, e.g. reward and endocrine functions (Rogge et al. 2008). In the brain, CART is expressed by neurons in nucleus accumbens, LHA, PVN and Arc, as well as other parts of the brain and periphery (Douglass et al. 1995; Rogge et al. 2008). In Arc CART is co-expressed with POMC (Vrang et al. 1999).

I.c.v. administration of CART peptide fragments in rats was shown to decrease food intake, and i.c.v. injections of antibodies directed against CART peptides had the opposite effect (Lambert et al. 1998). CART mRNA was found to be decreased in Arc of food-deprived rats, as well as in rats and mice with disrupted leptin signaling, while intraperitoneal leptin administration (to mice) increased the CART mRNA levels in Arc (Kristensen et al. 1998).

#### *1.1.1.3 Additional transmitter substances in energy homeostasis*

In addition to the peptides mentioned above, there are additional neuropeptides, transmitters and messenger molecules, with well-documented food intake-regulating properties. I have chosen to mention them in just a few sentences, since they are not the focus of this thesis.

**Galanin** is a 29 amino acid peptide (Tatemoto et al. 1983) that is produced by hypothalamic neurons located in e.g. Arc, DMH, PVN and LHA. Galanin stimulates food intake, with a preference for fat (Leibowitz and Kim 1992), but is in comparison to other orexigenic peptides, such as NPY, less potent. Thus, the food intake stimulating effect is less notable and lasts for a shorter period (Gil-Campos et al. 2006; Meister 2007).

**The endocannabinoid system** modulates the rewarding aspects of food, in synergy with dopamine, and exerts orexigenic effects. In the hypothalamus, endocannabinoids have been suggested to interact with, among others, the NPY/AGRP and POMC/CART neurons (Bellocchio et al. 2008).

**Dopamine** is another important food intake-regulating neurotransmitter, together with noradrenaline and serotonin often called classical neurotransmitters. Orexigenic effects of dopamine were first indicated by the reduced food intake as a consequence of 6-hydroxydopamine lesions in rats (Ungerstedt 1971). However, the role of dopamine in food intake-regulation is not as straight forward as initially suggested. Rather the effects of dopamine on food intake appear to be dependent on the region, where it is released, e.g. regulating meal number and duration when released in the

hypothalamus, and being involved in the reward aspect of food intake when released in the nucleus accumbens (Meguid et al. 2000; Meister 2007).

**Noradrenaline** was initially suggested to exert orexigenic effects (Grossman 1962), while more recent studies have suggested the opposite. Thus, noradrenaline may, like dopamine, either stimulate or inhibit food intake, depending on the brain regions into where it is released (Meister 2007; Wellman 2000).

A third classical transmitter, **serotonin**, is synthesized by neurons in the dorsal raphe, sending wide, ascending projections, including to the hypothalamus (Meister 2007). Disturbances of the serotonergic systems results in increased appetite and body weight (Waldbillig et al. 1981). Release of serotonin is enhanced in the hypothalamus in response to food intake, mostly reflecting carbohydrate intake, promoting satiety (Meguid et al. 2000).

**Melanin-concentrating hormone (MCH)** (Kawauchi et al. 1983) and **hypocretin** (de Lecea et al. 1998), also called **orexin** (Sakurai et al. 1998), are both expressed in LHA and exert orexigenic effects (Hervieu 2006; Matsuki and Sakurai 2008).

Furthermore, fast amino acid transmitters, such as **GABA** and **glutamate** are highly relevant in the aspect of regulation of food intake, even though they often have been neglected. GABA is the main inhibitory transmitter in the CNS and is co-expressed with AGRP and NPY in Arc (Broberger et al. 1998; Hahn et al. 1998) and has been suggested to stimulate food intake (Meister 2007; Stratford and Kelley 1997). Glutamate is an excitatory transmitter expressed in the hypothalamus by at least a proportion of the arcuate POMC/CART neurons and probably also by the majority of hypocretin/orexin neurons in LHA. Injection of glutamate into LHA stimulates food intake (Meister 2007).

#### *1.1.1.4 Other factors involved in energy homeostasis*

Besides the array of messenger molecules, other mechanisms have been shown to be of special importance in the regulation of food intake and energy availability. One such mechanism is **the adenosine monophosphate kinase (AMPK)** activity, which responds to the ratio of AMP/ATP, i.e. AMPK activity increases in response to increased AMP/ATP ratio. The activation of AMPK stimulates mechanisms aiming at increasing cellular ATP levels, e.g. increased uptake of glucose (Horvath et al. 2009). Both NPY/AGRP and POMC/CART neurons in Arc express AMPK (Minokoshi et al. 2002).

**The mammalian target of rapamycin (mTOR)** is another molecule documented to sense the energy availability and the activity appears, as for AMPK, to be dependent on the ratio between AMP/ATP, although in an inverse fashion compared to AMPKA (Dennis et al. 2001).

**Reactive oxygen species (ROS)** are generated as a byproduct of substrate oxidation, but recent studies argue that ROS also have a crucial role in regulating neuronal responses in a substrate dependent manner (Horvath et al. 2009), both in sensing

glucose and lipids (Benani et al. 2007; Leloup et al. 2006). It has even been shown that uncontrolled generation of ROS in NPY/AGRP neurons impairs the firing of these neurons (Andrews et al. 2008).

**ATP-sensitive potassium channels ($K_{ATP}$ channels)** in VMH and Arc have been suggested to be another player in energy homeostasis, in particular in glucose sensing. $K_{ATP}$ channels are expressed by 70% of the arcuate neurons, including NPY/AGRP and POMC/CART neurons (Gyte et al. 2007; van den Top et al. 2007). It is becoming increasingly evident that the $K_{ATP}$ channels are involved in insulin and leptin signaling pathways, as well as in neuropeptide expression in Arc (Gyte et al. 2007; Harvey et al. 1997; Spanswick et al. 2000).

## 1.2 DEVELOPMENT OF ARCUATE CIRCUITS CONTROLING FOOD INTAKE

The development of the arcuate circuits regulating food intake is, as neurodevelopment in general, divided into three major steps: neurogenesis, neuronal migration and axon projection and synaptogenesis. Neurogenesis of arcuate neurons, and other hypothalamic neurons, is initiated in the neuroepithelium in the wall of the third ventricle. Neurons destined for the Arc are generated between embryonic day (E) 12 and 17 (Bouret 2009; Bouret 2010), but the development of neuronal projections from these neurons to their target areas takes place primarily postnatally. Bouret and collegues performed axonal tract labeling in mice, using DiI, and concluded that innervation of DMH occurs at postnatal day (P)6, of PVN by P8-10 and innervation of LHA somewhat later, at P12 (Bouret et al. 2004). A similar temporal pattern was shown in rats when studying the development of arcuate NPY/AGRP projections (Grove and Smith 2003). In accordance with this, responsiveness of arcuate neurons to leptin, studied by expression of cFOS, is seen as early as by P6 in Arc, but no earlier than at P10 and P16 in PVN and LHA, respectively (Bouret 2009; Bouret 2010).

## 1.3 THE ANORECTIC *ANX/ANX* MOUSE

This thesis is based on studies of the anorectic *anx/anx* mouse. Genetic animal models of disturbed energy homeostasis have been of great importance in elucidating the mechanisms behind regulation of food intake and energy expenditure. Most commonly these have been models of obesity, while studies of models of anorexia have been limited. One reason for this could be that genetic animal models of anorexia/reduced food intake are less common, for various reasons. The anorectic *anx/anx* mouse is thus a valuable tool when studying regulation of food intake and energy expenditure.

The recessive *anorexia anx* mutation was found at the Jackson laboratory in 1976 (Maltais et al. 1984). The *anx* mutation arose spontaneously in the F2 generation of a cross between DW/J and an inbred strain derived from a cross between M.m.poschiavinus and an inbred Swiss strain.

**Figure 3**. An *anx/anx* mouse to the left, and +/+ mouse to the right, age P21.

The male *anx* carrier was crossed to B6C3H-a/a F1 female, and the mutation has been maintained on this background at the Jackson Laboratory.

Mice homozygous for the mutation, *anx/anx* mice, are characterized by reduced food intake and an emaciated appearance. From around P5, and possibly even earlier, *anx/anx* mice eat less than normal littermates (+/+), although it is noteworthy that the day-to-day variations in food intake resemble the pattern seen in +/+ mice. From around P9 they start to deviate from the normal growth curve, and by P21 they weigh half as much as the +/+ mice, approximately four vs. eight grams (Figure 3). They also display neurological problems, such as head weaving, tremors and uncoordinated gate. *anx/anx* mice die around P21, supposedly due to the severe starvation (Maltais et al. 1984).

### 1.3.1 Neurochemical aberrances in the *anx/anx* mice

Histochemical studies of the anorectic *anx/anx* mice have revealed aberrant transmitter and neuropeptidergic systems, in particular in systems important for the regulation of food intake and energy metabolism (summarized in Table 1) (Broberger et al. 1999a; Broberger et al. 1999b; Broberger et al. 1998; Broberger et al. 1997; Fetissov et al. 2005; Jahng et al. 1998; Johansen et al. 2000; Johansen et al. 2007; Son et al. 1994). Immunohistochemistry (IHC) with antibodies raised against the orexigenic peptides NPY and AGRP show reduced density of fibers in all projection areas studied (PVH, LHA, DMH and Arc) when comparing *anx/anx* mice and +/+ mice at P21. In addition both peptides show an increased intensity of staining in cell bodies in Arc, resembling what is seen with colchicine injections. In situ hybridization (ISH) studies of AGRP or NPY mRNA levels have been conflicting. Broberger et al. (1997) concluded that there was no difference in mRNA levels of NPY in *anx/anx* when compared with +/+ mice at P21, which was confirmed by Jahng et al. (1998). But a later paper showed an increased expression of both AGRP and NPY mRNA in *anx/anx* mice of the same age as in the previous study (Fetissov et al. 2005).

IHC with antibodies against α-MSH, one of the POMC peptides, showed markedly attenuated ir fibers, and staining for ACTH, another POMC-peptide, showed reduced numbers of ir-cell bodies. The NPY receptor Y1 which decorates the soma and dendrites of POMC/CART neurons (Kopp et al. 2002) confirmed these results by showing a reduced density of both ir dendrites and cell bodies (Broberger et al. 1999b).

In addition, two studies have demonstrated serotonergic hyperinnervation of Arc, as well as the olfactory bulb, frontal cortex, hippocampus and cerebellum in *anx/anx* mice (Jahng et al. 1998; Son et al. 1994). BrdU- and TUNEL-labeling studies have shown increased cell proliferation and apoptosis in dentate gyrus of these mice (Kim et al. 2001). A differential display analysis have identified several genes that can be related to the phenotypes of the *anx/anx* mice, among them apoptotic protease activating factor 1 (Chun et al. 2003). Increased hypothalamic expression of neurotrophin tyrosine kinase receptor 3 has also been documented in the *anx/anx* mice, a receptor which also is associated with eating disorders in humans (Mercader et al. 2008b). A gene expression study on hypothalamus indicated inflammatory processes in the hypothalamus of the *anx/anx* mice (Lachuer et al. 2005), another showed enrichment of deregulated genes involved in cell death, cell morphology and cancer, as well as an alteration of several signaling circuits involved in regulation of energy homeostasis (Mercader et al. 2008a).

**Table 1.**

**Neurohistochemical aberrances in hypothalamus of the *anx/anx* mice, at P21**

| MARKER | IMMUNOHISTOCHEMISTRY | | IN SITU HYBRIDIZATION |
|---|---|---|---|
| NPY | ↑cell number | ↓ fiber density | ↑/no change mRNA |
| AGRP | ↑ cell number | ↓ fiber density | |
| ACTH | ↓ cell number | | |
| α-MSH | | ↓ fiber density | |
| CART | ↓ cell number | ↓ fiber density | ↓ mRNA |
| POMC | | | ↓ mRNA |
| Y1 | ↓ cell number | ↓ dendrite density | ↓ mRNA |
| Y2 | | | ↓ mRNA |
| Y5 | | | ↓ mRNA |

## 1.4 ADDITIONAL GENETIC MOUSE MODELS OF DISTURBED ENERGY HOMEOSTASIS

In addition to the mutant *anx/anx* mice several other models, in particular knock-out (KO) mice, have been extensively studied, and important information about the transmitters and pathways critical for the regulation of food intake has been obtained from these mice (summarized in Table 2). The most familiar animal models of obesity are likely the mutant mice *Lep*$^{ob/ob}$ and *Lepr*$^{db/db}$, with spontaneous mutations in the gene coding for leptin and its receptor, respectively (Coleman 1973).

**Table 2.**

| Selected Mouse Models of disturbed energy homostasis | | |
|---|---|---|
| **MODEL** | **EFFECTS ON ENERGY HOMEOSTASIS** | **REFERENCES** |
| *anx/anx* | starvation, reduced food intake, emaciation | (Maltais et al. 1984) |
| *Lep*$^{ob/ob}$ | obesity, | (Coleman 1973; Zhang et al. 1994) |
| *Lep*$^{db/db}$ | obesity, diabetes | (Chen et al. 1996; Coleman 1973; Tartaglia et al. 1995) |
| NPY-KO | none | (Erickson et al. 1997) |
| AGRP-KO | none | (Qian et al. 2002) |
| AGRP-ablation, neonatal | none | (Luquet et al. 2005) |
| AGRP-ablation, adult | inhibits feeding, starvation | (Gropp et al. 2005; Luquet et al. 2005) |
| Overexpression AGRP | obesity, increased food intake, hyperinsulinemia | (Ollmann et al. 1997) |
| Agouti yellow ($A^y$) | obesity, resistance to satiety | (Huszar et al. 1997; Lu et al. 1994). |
| POMC-KO | obesity, decreased energy expenditure, hyperphagia | (Challis et al. 2004) |
| CART-KO | increased feeding, obesity | (Moffett et al. 2006) |
| MC3-KO | moderate obesity, reduced energy expenditure | (Butler et al. 2000) |
| MC4-KO | obesity, hyperphagia, hyperinsulinemia | (Huszar et al. 1997) |
| *tub/tub* | obesity, insulin resistance | (Kleyn et al. 1996) |
| *Cpe*$^{fat/fat}$ | obesity, hyperinsulinemia | (Naggert et al. 1995) |

Considering the potent effect of NPY on food intake it was surprising that the NPY-KO mice did not show any phenotype with respect to energy balance (Bannon et al. 2000; Erickson et al. 1997). Likewise, AGRP-KO mice do not have an obvious phenotype related to body weight, food intake or adiposity (Qian et al. 2002), and neonatal ablation of AGRP has no effect on food intake nor body weight (Luquet et al. 2005). However, adult AGRP ablation inhibits feeding and results in starvation (Gropp et al. 2005; Luquet et al. 2005), thus suggesting that some compensatory mechanism(s) isactive during the young ages. The starvation caused by adult ablation of AGRP neurons was originally hypothesized to be due to the loss of GABA-ergic inhibitory input from the NPY/AGRP neurons on the POMC/CART neurons, thereby causing increased anorexigenic signaling (Gropp et al. 2005). Wu and collegues showed that the starvation resulting from adult ablation of AGRP neurons is independent of melanocortin signaling (Wu et al. 2008) and can instead be attributed to a loss of GABA-ergic signaling, primarily to PBN (Wu et al. 2009). In addition, both CART- , POMC-, MC3- and MC4-KO mice, as well as transgenic overexpression of AGRP results in obesity (Butler et al. 2000; Challis et al. 2004; Coll and Loraine Tung 2009; Cone 2005; Huszar et al. 1997; Meister 2007; Ollmann et al. 1997). The agouti-yellow mice have ectopical expression of the agouti peptide, resulting in blockade of the MC4 receptor and obesity (Huszar et al. 1997; Lu et al. 1994).

## 1.5 NEUROINFLAMMATION AND IMMUNE REACTIONS IN THE BRAIN

The CNS was for long held as an immune privileged organ, a view primarily based on the restricted access of immune cells, since the blood-brain-barrier prevents entrance of cells and macromolecules (Hickey 2001; Thaler et al. 2010). However, it is now becoming increasingly evident that the CNS is an immunocompetent organ, where T-cells and endogenous glia cells can be activated to participate in the immune response of the brain (Hickey 2001).

### 1.5.1 Activation of microglia

Microglia become rapidly activated in response to minor pathological changes within the CNS, and represent key players in the immune defence of the brain, clearing the tissue from cellular debris. During both the embryonic and postnatal development of the brain, microglia enforce the programmed elimination of excess neurons, while in the mature brain microglia are activated in response to e.g. neurodegeneration, trauma, ischemia, inflammatory and immunological stimuli (Beyer et al. 2000; Milligan et al. 1991b; Streit et al. 2005; Streit et al. 1988). Activated microglia secrete several pro-inflammatory molecules, e.g. cytokines, chemokines and proteases as part of the clearing process. Excessive and sustained secretion, can however aggravate acute or chronic degeneration. Microglia can also have a pure supporting function, increasing the neuronal survival by secreting anti-inflammatory substances and neurotrophic factors (Nakajima and Kohsaka 2004; Streit et al. 2005). In addition, microglia may support generation of new neurons, neurogenesis, in both intact and injured brain. Acquiring of either a detrimental or supportive role by activated microglia seem to be dependent on the state of activation and thereby the types and levels of cytokines released (Ekdahl et al. 2009). An important factor in the activation of microglia is the induction of antigen-presenting capacity by expression of major histocompatibility complex (MHC) class I and II (Neumann 2001).

### 1.5.2 Expression of MHC in CNS

Microglia cells express MHC molecules, under certain conditions, e.g. during abnormal neuronal activation, after axotomy, cytokine treatment and viral infection; in fact even neurons can express MHC class I (Corriveau et al. 1998; Foster et al. 2002; Linda et al. 1998; Neumann et al. 1995; Schultzberg et al. 1989; Thams et al. 2008). Several studies state that neuronal MHC class I expression is induced in response to the activity of the neuron and that silent/inactive neurons are more prone to express MHC class I than active ones. Thus, neurons exposed to tetrodotoxin, resulting in ‘paralysis’, show a dramatic induction of MHC class I-related genes and proteins (Neumann et al. 1997). On the contrary, others have shown that MHC class I expression is increased in hippocampal neurons following kainic acid-induced-seizures (Corriveau et al. 1998). Interestingly, electrically active neurons have been reported to control the immune function of surrounding glia cells by suppressing the glial MHC class I expression, a process suggested involving neurotrophic factors (Neumann 2001; Wekerle 2005). Thus, inactive neurons may not only have the potential to express MHC class I proteins themselves but could also give rise to an up regulation of MHC class I proteins in microglia cells in the vicinity.

### 1.5.3 Hypothalamic inflammation – impact on energy homeostasis

Inflammatory signaling in the hypothalamus, by e.g. cytokines, appears to be a crucial mediator in the so called sickness response, that includes anorexia, cachexia, fever, inactivity, lethargy, anhedonia and adipsia (Thaler et al. 2010). Several of the inflammatory cytokines secreted by activated microglia, e.g. interleukin-6 and tumour necrosis factor-α have been shown to promote negative energy balance. One example being that injection of cytokines into the third ventricle results in decreased food intake. The mechanism appears to involve binding of the cytokines to receptors on NPY/AGRP and α-MSH/CART neurons, thus affecting the anorexigenic and orexigenic signaling of these neurons, respectively (Deboer and Marks 2006; Enriori et al. 2007).

In addition, obesity is linked to increased levels of circulating cytokines which together with an excess of nutrients, e.g. glucose or fatty acids, trigger inflammatory pathways in a variety of tissues, causing resistance to both leptin and insulin. In fact, hypothalamic inflammation appears to favor weight gain by a mechanism linked to resistance to leptin, insulin and other humoral factors (Enriori et al. 2007; Thaler et al. 2010).

## 1.6 NEURODEGENERATION AND NEURONAL CELL DEATH

The selective and progressive dysfunction and death of neurons occurring in neurodegenerative diseases, such as Parkinson, Huntington, Alzheimer and multiple sclerosis, can be due to many factors. Oxidative stress, free radical formation, impaired bioenergetics, mitochondrial dysfunction and neuroinflammatory processes are among the most frequently mentioned causes of neurodegeneration (Jellinger 2009). However, death of neurons/degeneration does not only occur in pathological states. During development, excess synapses and neurons are eliminated through a selective and well coordinated process, a process in which microglia appear to play an important role (Milligan et al. 1991a).

The electrical excitability and structural and synaptic complexity of neurons put an extraordinarily large demand on energy production (ATP) by the oxidative phosphorylation system (OXPHOS) in the mitochondria. In particular the axons and synaptic terminals are sensitive, since they require high amounts of ATP to rapidly restore ion gradients, via ion pumps, following depolarization and neurotransmitter release. The high energy demands of synapses make them highly susceptible for degeneration during conditions of increased stress, ischemia and dramatic reduction in energy availability (Mattson and Liu 2002).

### 1.6.1 Hypothalamic degeneration – relation to food intake and energy homeostasis

Neonatal ablation of hypothalamic AGRP neurons has no effect on food intake or body weight (Luquet et al. 2005), while adult ablation is shown to result in decreased food intake and starvation (Gropp et al. 2005; Luquet et al. 2005; Wu et al. 2008; Xu et al. 2005). This indicates that some compensatory mechanism is acting during the ages.

Likewise, acute ablation of AGRP-neurons, over a few days, gives rise to a more severe phenotype than progressive ablation over months. This, once again, indicates compensatory mechanisms and possibly neuronal plasticity in the case of a less acute loss of neurons. In fact, Kokoeva et al. (2005) have shown that neurogenesis occurs in hypothalamic feeding centers in adult mice and suggested it to play a role in development of obesity by changing the energy-balance set point (Kokoeva et al. 2005; Ryu et al. 2008). Furthermore, hypothalamic neurons have been shown to regenerate in adult mice following neurodegeneration, and some of them even differentiated into AGRP-neurons, as well as leptin-responsive neurons (Pierce and Xu 2010).

Ablation of hypothalamic POMC neurons in mice causes obesity, though the obesity phenotype is less dramatic than in the POMC-KOs. Interestingly, simultaneously ablating both the AGRP and POMC neurons leads to an even less pronounced obese phenotype (Xu et al. 2005).

Other examples of the relation between food intake and neurodegeneration is the finding that reduced caloric intake or periodic fasting increases the resistance of neurons to metabolic and oxidative injury, and thereby enhances survival and plasticity of neurons (Mattson and Liu 2002). In addition, monosodium glutamate, a neurotoxin selectively destroying 80-90% of the neurons in Arc including neurons expressing galanin och NPY, cause several endocrinological dysfunctions, e.g. stunted growth and obesity, when given to rats (Meister et al. 1989).

## 1.7 MITOCHONDRIA – RELATION TO NEURODEGENERATION

The mitochondria play a vital role in the metabolism of the cell by being the major producer of ATP through the OXPHOS system. Mitochondria also play a critical role in apoptosis and contribute to the complexity of genetics by having its own genome (Leonard and Schapira 2000a).

### 1.7.1 The oxidative phosphorylation system

OXPHOS, located in the inner membrane of the mitochondria, is the major site for ATP production in cells. In short, oxidation of NADH and $FADH_2$, generated by the metabolism of amino acids, sugars and fatty acids, produces electrons that are shuttled along the respiratory chain, giving rise to an electrochemical gradient, and in the end generation of ATP (Leonard and Schapira 2000a). OXPHOS is made up of five multisubunit complexes (I-V), out of which complex (CI) is the largest. At CI, NADH generated via the metabolism of e.g. sugars, is oxidized, thereby releasing electrons that are transferred via electron carriers (ubiquinone and cytochrome *c*) as well as CIII and CIV to the final acceptor, molecular oxygen. At the same time protons are pumped from the matrix to the intermembranal space, thereby generating an electrochemical gradient across the membrane which is used to drive the generation of ATP by CV, also called ATP synthase (Andrews et al. 2005a).

CI is made up of 45 or 46 different subunits (depending on article cited), out of which seven are encoded by the mitochondrial genome and the rest are encoded by nuclear genes (Dunning et al. 2007; Lazarou et al. 2009; Sheftel et al. 2009; Ugalde et al. 2004). With respect to the large number of subunits included in CI, it can be expected that many factors are required for the proper assembly of this complex of proteins. So far, at least eight putative assembly factors for CI have been documented, NDUFAF1, NDUFA12L (B17.2L, NDUFAF2), Ecsit, huInd1, C6ORF66 (NDUFAF4), C8ORF38, C20ORF7 and AIF (Ogilvie et al. 2005; Saada et al. 2008; Saada et al. 2009; Sheftel et al. 2009; Vogel et al. 2005; Vogel et al. 2007). The failure to assemble properly functioning CI is a major contributor to mitochondrial disease and frequently results in early childhood death (Smeitink et al. 2001).

### 1.7.2 Mitochondrial dysfunction

Mitochondrial dysfunction have been implicated in a variety of neurodegenerative diseases, e.g. Parkinson, Huntington and Alzheimer (Ekstrand et al. 2007; Leonard and Schapira 2000b). ROS appear to be key factors in the degenerative process by causing oxidative stress and cell death. The majority of ROS are products of the OXPHOS system, in particular CI, even under normal conditions, but levels increase substantially during dysfunction of CI (Ott et al. 2007).

Mitochondrial disorders present very different clinical manifestations, e.g. limb weakness, encephalomyopathies, myopathy, seizures and lactic acidosis (Leonard and Schapira 2000a). CI deficiency is the most common cause of respiratory chain dysfunctions and often occurs as a result of impaired assembly of the 45/46 proteins building up CI. Defects in humans are implicated in energy generation deficiencies, neurodegeneration and altered apoptotic signaling (Lazarou et al. 2009), and have, in fact also, been related to the neuronal degeneration in Parkinson´s disease (Schapira 2006).

Leigh syndrome and Leigh-like syndromes are a group of mitochondrial disorders with onset in early childhood, caused by dysfunction of the OXPHOS system. In particular deficits in CI, II, IV or V, coenzyme Q or of the pyruvate dehydrogenase complex have been documented. Most frequently, disease-causing mutations are in genes coding for subunits or assembly factors of the respiratory chain complexes, and mutations in all 14 genes coding for core subunits of CI have been described. Leigh syndrome is also known as subacute, necrotizing encephalopathy, and is a severe neurodegenerative disease. Despite large variations in the clinical and genetic background of the disease, a common theme is a progressive decline of the CNS due to focal, necrotizing lesions of several brain regions, including diencephalon, cerebellum or brainstem. Clinical hallmarks include weakness, hypotonia, tremor, FTT, dysphagia, ptosis and squinting (Chol et al. 2003; Finsterer 2008; Kruse et al. 2008).

A study of synaptic mitochondria, in which CI, III or IV was inhibited, showed that CIII and IV could be inhibited by 70-80% before major changes in oxygen consumption occurred, whereas inhibition of CI by only 25% was sufficient to cause marked reduction in oxygen consumption (Mattson and Liu 2002), indicating that CI is the most sensitive of these complexes.

# 2 AIMS OF THE STUDY

The over all goal of this thesis is to increase the knowledge about the development and function of molecular mechanisms involved in hypothalamic regulation of food intake. A second focus of the project is to study how inflammatory mechanisms and oxidative stress interact with hypothalamus to produce anorectic conditions.

Specific aims are:

1. To identify the *anx* gene and mutation.
2. To elucidate the role of the *anx* product in food intake-regulating pathways, particularly in the hypothalamus.
3. To determine the general gene expression pattern in the hypothalamic arcuate nucleus of the *anx/anx* and normal mouse by microarray studies.
4. To determine when in postnatal development the characteristic appearance of the NPY/AGRP and POMC/CART system in hypothalamus first become apparent in the *anx/anx* mouse.
5. To elucidate what is causing the aberrant appearance of NPY/AGRP and POMC/CART systems in the *anx/anx* mouse.

# 3 MATERIALS & METHODS

This section includes only a brief summary of the material and methods included in the thesis. Detailed protocols can be found in the corresponding papers.

## 3.1 ANIMALS (PAPER I-IV)

*anx* breeding pairs (B6C3-*a/a-a* +/+ *anx*) were originally obtained from the Jackson laboratory (Bar Harbor, ME), and a breeding colony was established at Karolinska Institutet in 1995. The animals were kept in an animal facility at 12h light-dark cycle and a temperature of 25°C. Pups were allowed to suckle ad libitum. Handling was minimized and all experiments were approved by the local ethical committee (Stockholms norra djurförsöksetiska nämnd).

### 3.1.1 Genotyping (Paper I-IV):

All mice included in this thesis were genotyped using simple sequence length polymorphism (SSLP) markers mapped to the sub-chromosomal region, where the *anx* mutation is located. The primer pairs were designed around simple repeats, usually $CA_n$ located in the *anx* interval. Due to different genetic backgrounds, the *anx*-product and the +/+ product deviate in size, making separation of the products by gel electrophoresis possible. Markers were purchased from Research Genetics, Inc. (Huntsville, AL).

In addition, all mice included were phenotypically characterized based on emaciation and neurological abnormalities. Occasionally small and emaciated mice were found, which by genotyping turned out not carry the *anx*-mutation and where therefore not included in the studies. This emphasizes the importance of genotyping when working with mutant mice.

## 3.2 MAPPING OF THE *ANX*-GENE (PAPER IV)

The mapping of the *anx*-gene was done during the thesis work of my supervisor, Jeanette Johansen, ten to fifteen years ago, and was performed with methods that were standard at that time. I will only briefly explain how this was done.

To begin with, the difference between a linkage and a physical map should be noted. A linkage map positions genes or genetic markers in relation to each other on an interval, given in centimorgan[3] (cM) and is based on the recombination frequencies during cross-over of homologous chromosomes. The greater the frequency of recombination is, the farther apart the two genetic markers are assumed to be. A physical map gives the distance between two loci in base pairs.

[3] 1cM is equivalent to 1% crossover rate

### 3.2.1 Creating a linkage map of the *anx*-gene (Paper IV)

The first step in mapping a gene is to localize the locus of interest to a sub-chromosomal region, and the second step is focused on identifying closely linked markers. When working with mutant mice with a deviant phenotype, such as the *anx/anx* mice, a panel of mice is created in which segregation of the mutant and wild type (wt) alleles can be followed by expression of the phenotype(s). One usually starts by crossing one strain carrying the mutation with another strain. The greater the genetic distance between the strains, the better the chance of finding polymorphisms at marker loci. Regarding strains, it is also important to choose the other strain so that the phenotype of the mutant allele can be easily observed, e.g. the choice of black-coated mice when the mutation is related to brown fur color.

Already when this project was first initiated in our laboratory, the *anx*-interval had been mapped to an interval around 20 cM proximal to the agouti-locus, on mouse Chromosome 2 (Maltais et al. 1984). To further narrow down the interval and to create a linkage map of the *anx* interval, two different intercrosses were set up. In addition to intercrossing, backcrossing can be used. Both types of breeding schemes start with a cross between two parental strains. Intercross continues with a cross between two F1 animals of the same heterozygous genotype at the loci of interest, often two siblings, while backcrossing continues with mating between a heterozygous F1 animal with a homozygous animal for either allele, usually from a parental strain. Intercrossing was choosen, since it has two main advantages over the backcross. First, intercross is the only choice when dealing with lethal recessive mutations, such as the *anx*-mutation, since both the heterozygous F1 parents will be normal. The second advantage is that informative meiotic events occur in both parents, thereby rendering twice as much information per animal compared to the backcross. However the data obtained are also more complex and difficult to analyze.

Cross 1 (B6C3Fe-*anx A/+ a* x B6C3H F1 intercross) resulted in 2,050 F2 progeny (4,100 meioses). Cross 2 was set up between B6C3Fe-*anx A/+ a* and CAST/Ei, and 372 F2 progeny (744 meioses) from this cross were analyzed. DNA was prepared from tail tips according to standard salting out procedures. All mice were genotyped with SSLP markers mapped to this subchromosomal region. *D2Mit* markers were purchased from Research Genetics, Inc.. The markers were chosen based on the chromosomal map position reported to the linked interval on Chromosome 2.
Additional markers, the *D2Jojo* primer pairs, were designed around simple sequence repeats located in the *anx* interval.

Recombination frequencies and standard errors were calculated as described (Green 1981).

### 3.2.2 Creating a physical map of the *anx*-gene (Paper IV)

Once the gene of interest has been closely linked to one or more DNA markers, it is possible to clone the region containing the gene. In general, linkage should be closer than 1cM prior to starting physical mapping.

Prior to the full genome sequencing project one had to start building the physical maps by screening genomic libraries, which is ideally done with markers that show absolute linkage to the gene and closely flanking markers on both sides of the gene. Three mouse libraries were used to screen the *anx*-interval. The P1 library (derived from E14 [129/Ola] ES cells) was screened by successive rounds of PCR to identify clones that carried the markers as recommended above. The other two libraries, PAC (RPCI-21) and BAC (RPCI-23, segment two), were screened to fill gaps and/or to provide additional coverage. DNA from the clones was isolated and used to generate sequence tagged sites (STS) to serve as probes when screening for overlapping clones. A subset of the overlapping P1, PAC and BAC clones spanning the *anx*-interval was shotgun sequenced. In the first step DNA from the genomic clones is mechanically sheared to generate random fragments, typically 1-3 kilobases in length. The fragments are then cloned into a vector with common promoter/primer sites. The sequence data obtained from each end of the random clones were assembled to a contig of the original genomic clone using the gap4 program of the Staden package (Staden 1994). Later, when genomic databases such as Ensembl were made available, the sequence was compared to the published sequences.

## 3.3 IN SITU HYBRIDIZATION (PAPER I & III):

ISH is a method used to localize regional cellular expression of mRNA in tissues. We used radioactive ISH with oligoprobes, typically encompassing 48 bases, to study expression of AGRP during development in +/+ mice, and MHC class I related genes in *anx/anx* and +/+ mice. An alternative is to use riboprobes which are cDNA clones spanning a large proportion of the transcript. Oligoprobes results in weaker signals than riboprobes, but this is usually not a problem when studying neuropeptides such as AGRP, since they are relatively abundant in the brain.

The ISH technique has been described thoroughly (Schalling et al. 1991) and can be viewed in more detail in **Paper I and III**. In brief, frozen brain tissue was cut on a cryostat and thaw-mounted on glass slides. Anti-sense oligoprobes were synthesized by Cybergene AB (Huddinge, Sweden) and isotopic-labeling of these at the 3' end was done enzymatically. Labeled probes were hybridized to sections in a cocktail for 16h at 42°C. The sections were, after washing and air-drying, dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY), exposed and developed with D-19 developer (Kodak). The sections were analyzed using a microscope equipped with a dark-field condenser and a digital camera.

The validity of results obtained with ISH is to a large extent dependent on the specificity of the probe, which can be controlled for in various ways. To begin with the probe sequence is compared with nucleotide databases to verify that it is unique for the mRNA of interest. If possible the distribution pattern of labeling can be compared with the pattern found with IHC. Another common way to test specificity is to

incubate slides in a mix including both labeled probe and excess of unlabeled probe, which should result in loss of signal. This is the method we adapted.

## 3.4 IMMUNOHISTOCHEMISTRY (PAPER I-IV):

IHC is used to localize regional expression of protein in tissues or cell culture. A thorough protocol can be found in **Paper I-IV**. Briefly, anesthetized mice were perfused via the ascending aorta with Tyrode's $Ca^{2+}$-free solution, followed by fixation with a mixture of para-formaldehyde and picric acid (Pease 1962; Zamboni and DeMartino 1967). Brains were rapidly dissected out and immersed in the same fixative for 90 min prior to leaving them over night in sucrose solution. On some occasions, in **Paper III**, only immersion fixation was used. Commonly 14 µm thick sections were cut on a cryostat and thaw-mounted on gelatine-coated glass slides. Sections were processed either with the indirect immunofluroescence technique (Coons 1958) or according to the tyramide signal amplification (TSA)-plus System (PerkinElmer Life Science, Inc Boston, MA).

For the indirect method, incubation with primary antisera, over night, was followed by incubation with secondary antibodies conjugated with fluorescent marker for 30 min at room temperature (RT) and rinsing in PBS.

For TSA, incubation with primary antisera, over night, was followed by incubation with horseradish peroxidase (HRP)-conjugated antibodies and subsequently with biotinyl tyramide (Perkin Elmer). HRP catalyzes the deposition of numerous activated tyramide molecules, which bind to electron dense areas in the vicinity. A great advantage of the TSA technique is that the additional steps amplify the signal and thereby allows an approximate 10X reduction in the concentration of antiserum.

After mounting, the samples were analyzed with a fluorescence microscope and/or a confocal scanning microscope, equipped with appropriate lasers.

One distinct advantage with IHC is the possibility to look at the cellular localization of a protein, and with a confocal microscope an even higher resolution can be achieved. An additional advantage is the ability to do double/triple-labeling with two or three markers, by which co-localization or close apposition of proteins/peptides can be studied.

The validity of results obtained with IHC is directly related to the specificity of antibody/antisera, which can be controlled for by preadsorption of the antibody/antiserum with the corresponding immunogen, resulting in loss of signal. Another possibility is Western blot, where a single band indicates high specificity of the antisera. It is also possible to compare the expression pattern obtained with those from previous studies by others. Even though the antibody/antiserum specificity has been tested, some uncertainty will still remain, which is the reason for using terms like "immunoreactive" (-IR) and "like-immunoreactivity" (-LI) being used.

### 3.4.1 TUNEL- and active caspase 6-staining (Paper III)

Terminal dUTP Nick End Labeling (TUNEL) is an assay commonly used to detect apoptotic cells, and we used it to study such cells in hypothalamus of the *anx/anx* mice. The method is based on enzymatical labeling of free 3´OH DNA ends in apoptotic cells by terminal transferase (Gavrieli et al. 1992).

In brief, tissues were fixed and prepared as reported in the previous 'immunohistochemistry' section, and treated with 2% $H_2O_2$ in methanol to block endogenous peroxidases. Permeabilization solution was applied to sections before applying TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche Diagnostics, Mannheim, Germany). Sections were washed, mounted and analyzed in a fluorescence microscope.

Mean values for each animal (including 3-4 sections/animal, spanning approximately bregma -1.22 to -2.06 mm) and mean group values were calcualted. Students T-test was used to evaluate possible statistically significant differences between *anx/anx* and +/+ mice.

The use of TUNEL-labeling as an apoptotic marker has been debated (Gold et al. 1994; Kanoh et al. 1999). Criticism has been raised, since the terminal transferase adds labeled nucleotides to a variety of DNA ends, and since DNA damage is not a feature unique for apoptosis, but can occur in necrosis and during repair of DNA as well. The TUNEL-method is also dependent on the staining reagent's accessibility to the DNA strand breaks (Saraste and Pulkki 2000). In addition, the duration of apoptotic cell death has been estimated to be approximately 6-24hrs, resulting in a relatively short window for detection by for example TUNEL-labeling. Thus, only a small detectable proportion of apoptotic cells is present at a single timepoint.

Activation of cysteine proteases, called caspases, is a critical step in apoptosis increasingly often used as a marker for this type of cell death. We used IHC to study activation of caspases in hypothalamus of the *anx/anx* mice (see 'immunohistochemistry' section in 'materials and methods'). The caspases cleave cellular substrates, such as the nuclear lamina and proteins related to the stability of the chromatin structure, giving rise to the apoptotic morphology and fragmentation of DNA. It is suggested that different types of cell death share common mechanisms in the early phase, whereas the sort of caspase determines the type of cell death occurring (Gavrieli et al. 1992; Saraste and Pulkki 2000). For example, caspase 3 appears to be involved in neuronal cell body apoptosis, whereas caspase 6 is required for axonal degeneration (Nikolaev et al. 2009). Thus, for studying cell death in brain tissue, a combination of TUNEL-labeling and caspase staining appears to be better than either of them alone. By also adding morphological studies of neurons and supporting cells, e.g. nuclear condensation and/or shrinkage of neurons, as well as activation of glia cells, one can strengthen the cell death studies further. In **Paper III** we performed such morphological IHC studies with markers of activated microglia (Iba1 and OX42) and markers for the arcuate neurons of interest in the *anx/anx* mice (NPY and Y1).

### 3.4.2 Dihydroethidium injections (Paper IV)

Injections of dihydroethidium (DHE) can be used to visualize local in situ production of ROS, since it is oxidized to fluorescent ethidium when reacting with endogenous superoxide ($O_2^{*-}$) (Andrews et al. 2005b). Intravenous injection of DHE was given once via the femoral vein to *anx/anx* and +/+ mice which were subsequently sacrificed by perfusion with fixative (as above) two hours after the injection. Sections, cut on a cryostat, were processed according to indirect IHC method (Coons, 1958) to visualize arcuate neurons and microglia.

## 3.5 MICROARRAY (PAPER IV)

Microarray expression analysis is a high-throughput method and a common way to detect differences in expression in a large set of genes, between e.g. patients and controls, as well as mutated and normal animals.

We used the Affymetrix Mouse Genome 430 2.0 Array containing 45,000 probe sets. Total RNA was made from Arc of 3 *anx/anx* and 3 +/+ mice. Comparisons were performed between all *anx/anx* mouse samples and all +/+ samples giving a total of 9 comparisons. Results were included for pathway analysis, if they showed increased or decreased expression in at least 7 out of 9 comparisons. In addition, a second inclusion criterion was a signal, as defined by the Affymetrix MAS 5.0 software, present in at least 3 out of 6 arrays.

### 3.5.1 Ingenuity pathway analysis (Paper IV)

Genes from the microarray analysis, identified as being differentially expressed in *anx/anx* mice, were used to generate canonical pathways prominent in Arc of *anx/anx* mice, by Ingenuity Pathway Analysis (IPA; Ingenuity Systems®, www.ingenuity.com). Canonical Pathways Analysis gives the most relevant cell signaling and metabolic pathways in the dataset.

Genes from the microarray dataset that met a cutoff of 1.4 fold change (up or down), and were associated with any of the canonical pathways in the Ingenuity knowledge base, were included in the statistical analysis. The significance of the association between the dataset and the canonical pathway was calculated by Fisher's exact test, determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. The final canonical pathways included in **Paper IV** were those that had a p-value below 0.05.

## 3.6 REAL-TIME PCR (PAPER IV)

Real-time PCR is a convenient way to quantitatively analyze expression levels of genes. We used it to study the expression of *Ndufaf1*, the candidate gene for the *anx*-mutation, in several tissues. In short, total RNA from six samples per genotype and tissue (brain, pancreas, heart, lung, kidney, liver and skeletal muscle) was prepared. cDNA was synthesized by reverse transcription of total RNA. cDNA was mixed with

primers, probes and Master Mix and the assay was run on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Gene amplifications were performed in duplicates and data were obtained as threshold cycle ($C_T$) values. Relative gene expression was calculated with the standard curve method. Quantity of target genes was calculated by the use of mean values of duplicates relative to those for well known house-keeping genes, which were chosen based on literature search. β-actin and TATAbp was used for liver, pancreas and lung, PPIA and GAPDH for heart, β-actin and GAPDH for brain, β-actin and PPIA for kidney and TATAbp and PPIA for skeletal muscle.

## 3.7 SDS PAGE (PAPER IV)

Western blot is a method used to quantify the levels of a specific protein in a sample of tissue, either homogenate or extract. The most common type of Western blot is based on electrophoresis of proteins in a buffer containing sodium dodecyl sulfate (SDS) and on polyacrylamide gels (PAGE). SDS maintains the protein sample in a denaturated state, once it has been treated with reducing agents to remove secondary and tertiary structures, and gives a negative charge to the proteins, which thereby will move through the gel, towards the positive electrode. Smaller proteins will move faster through the gel and a separation of proteins is created based on molecular size. Gel electrophoresis is followed by transfer of the proteins to a membrane, where they are accessible for detection, by the use of antibodies specific for the protein of interest. This transfer can be based on either capillary forces or electric current pulling the proteins from the gel to the membrane.

We used Western blot to study the protein levels of NDUFAF1, in brain from *anx/anx* and +/+ mice. Brain lysates were diluted with loading buffer and applied on a gel, transferred by wet transfer to a membrane which was blocked with milk, followed by incubation with primary antiserum against NDUFAF1, and secondary antibody conjugated with horseradish peroxidase. The signal was detected by chemiluminescence and exposure on film.

## 3.8 BLUE NATIVE PAGE (PAPER IV)

Native PAGE is a non-denaturating blot which keeps protein complexes associated and folded as they would be inside the cell. Blue Native PAGE (BN PAGE) is based on the Coomassie blue dye, which provides negative charges to the complexes, allowing electrophoretic separation.

We used BN PAGE to study levels of complex I and subcomplexes in the OXPHOS system of *anx/anx* mice. Briefly, hypothalamus from *anx/anx* and +/+ mice were dissected out and mitochondria were isolated as described (Lapidus and Sokolove 1993). Hypothalamic tissue was homogenized in preparation buffer and, by centrifugation at low speed, all unbroken cells and cell nuclei were removed. Further, the mitochondrial pellet, obtained by centrifugation at high speed, was washed and resuspended in buffer. Mitochondrial proteins were prepared for BN PAGE using NativePAGE™ Sample Prep Kit and ran on NativePAGE™ gradient gels (Invitrogen,

Carlsbad, CA). After electrophoresis, gels were further processed for Western blotting by transferring the proteins to membranes and immunodetection was performed using antibodies against subunits of CI and CII. The signal was, as for SDS PAGE, detected with chemiluminescence.

## 3.9 SEQUENCING (PAPER IV)

To search for sequence alterations in our candidate gene, *Ndufaf1*, genomic DNA and total RNA/cDNA from *anx/anx* and +/+ mice were sequenced. Genomic DNA was prepared from tail-tips using standard salting out procedures. For RNA preparation, mice were decapitated, and whole brain was rapidly dissected out and frozen in ice-cold isopentane. Total RNA was extracted using TRIZOL Reagent and manufacturer´s protocol. After quality check of the extracted RNA, cDNA was synthesized by reverse transcription of total RNA with oligo-dT (SuperScript™ III First Strand Synthesis System for RT-PCR, Invitrogen). In order to identify sequence alterations between *anx/anx* and +/+ of *Ndufaf1*, genomic DNA and cDNA were PCR amplified. Sequencing of the PCR product was performed on an ABI 3730 DNA Analyzer and the BigDye Terminator v3.1 Cycle Sequencing kit was used (Applied Biosystems Inc, Foster City, CA).

## 3.10 ALLELE SPECIFICITY (PAPER IV)

To evaluate if the down regulation of *Ndufaf1* is directly related to the mutation, and not a secondary effect of the starvation, we performed an allele specificity analysis. Sequencing of the *Ndufaf1* gene in *anx/anx* and +/+ mice revealed a silent T/C SNP (Ensembl gene ID ENSMUSG00000027305 *Ndufaf1* +87 T/C) that could be used to separate the *anx*-allele from the wt-allele. Total brain tissue from four heterozygous (*anx*/+) mice were used. RNA and DNA were isolated using Trizol, and cDNA was prepared in triplicates using the same procedure as for real-time PCR and sequencing. Duplicates of the DNA and cDNA were PCR amplified over the T/C SNP and quantified using PyroSequencing. Imbalanced expression of the two alleles was assessed by comparing the ratio of the mean peak heights from the two alleles in cDNA to the corresponding peak heights in genomic DNA [peak height$_{\text{wt allele}}$ (cDNA)/ peak height$_{\textit{anx}\ \text{allele}}$ (cDNA)]/[ peak height$_{\text{wt allele}}$ (gDNA)/ peak height$_{\textit{anx}\ \text{allele}}$ (gDNA)].

## 3.11 MITOCHONDRIA COUNT (PAPER IV)

To make sure that the documented defects in the oxidative system of *anx/anx* mice was not due to a reduced number of mitochondria, we performed real-time PCR with SYBR green. Two probes directed against mitochondrial DNA, i.e. cytochrome B and cox 1, and a marker for nuclear DNA, cyclophyllin, were used to evaluate the proportion of mitochondrial vs nuclear DNA in *anx/anx* compared with the proportions in +/+ mice. cDNA from four *anx/anx* and four +/+ hypothalamus were prepared with DNeasy Qiagen according to manufacturer´s protocol (Qiagen, Washington DC, Maryland), and the assay was run on the Sequence detector system ABI-prism 7000 (Applied Biosystems Inc)

## 3.12 RESPIROMETRY (PAPER IV)

A respirometer/oxygraph is a device used to measure the rate of respiration by measuring oxygen consumption, either at the level of whole animal or at the cellular level (Haller et al. 1994).

In order to evaluate the functionality of the respiratory chain, in particular of CI and CII, in the *anx/anx* mice, we used the respirometer Oxygraph 2K equipped with polarographic oxygen sensor (Oroboros Instruments, Innsbruck, Austria). In short, hypothalamus from *anx/anx* and +/+ mice was homogenized in a buffer denominated MIR05 containing EGTA, $MgCl_2$, $KH_2PO_4$, HEPES, sucrose, taurine, BSA and K-lactobionate and was added to the oxygraph chamber together with buffer. After equilibrating the respiration medium with air, the substrates were added to measure different states of respiration. Thus, to measure state 4 respiration of CI, which reflects activities of complexes and proton leakage across inner mitochondrial membrane, pyruvate and malate were added. By addition of ADP, state 3 respiration, the respiration that is coupled to ATP synthesis, was measured and followed by adding glutamate to increase the respiratory capacity. Succinate was added to study the combined respiration via CI and CII. The integrity of the mitochondrial membrane was verified by addition of cytochrome C and to measure respiration in the absence of proton gradient the un-coupler FCCP was added. Finally, CI and CII were inhibited by addition of rotenone and antimycin, respectively.

# 4 RESULTS & DISCUSSION

## 4.1 DEVELOPMENT OF ARCUATE FOOD INTAKE-REGULATING SYSTEMS IN *ANX/ANX* AND +/+ MICE (PAPER I, II & III)

In **Paper I**, we studied the expression of AGRP mRNA by ISH in normal mice and were able to conclude that AGRP appear to be exclusively expressed in Arc during the developmental ages studied, i.e. P1, 5, 10, 15 and 21. Low levels were found already by birth (P1), although detection required longer exposure time than for the other ages. Throughout the postnatal development the hybridization signal for AGRP gradually increased, although the difference between P15 and 21 was only minor. By IHC we were also able to show that a few AGRP-ir fibers are present in Arc at birth, followed by a gradual increased density, reaching the levels resembling thos of adult by P21. AGRP-ir cell bodies could only rarely be detected, at all ages studied.

Regarding the projection areas of the arcuate AGRP (NPY) neurons, at birth, a small proportion of AGRP-ir fibers was present in BNST, MPOA, PVN, LHA, DMH, amygdala, the paraventricular nucleus of thalamus and dorsal raphe. The areas surrounding LC (medial parabrachial- and Barringtons nucleus) were the only areas in which AGRP-ir fibers were devoid at birth, and were found at the earliest by P5. An innervation pattern resembling the adult was reached around P15 in DMH and LHA, and at P21 in PVN (**Paper I**). This is in accordance with earlier studies using axonal tracing in mice (Bouret et al. 2004) and IHC against NPY in rat (Grove and Smith 2003). Thus, the three first postnatal weeks appear to be critical for the development of hypothalamic food intake-regulating systems and represent a window in time, during which different environmental factors, such as low/high nutrient availability or levels of humoral factors, might influence this development.

In **Paper II**, we compared the development of the AGRP (NPY) system in *anx/anx* with +/+ mice by IHC with antibodies directed against AGRP. The ages P0, 5, 10, 12, 15 and 21 were included. We were able to conclude that the AGRP (NPY) system in *anx/anx* mice develops as in +/+ mice until P12, after which the normal gradual increase in fibers apparently ceases. When comparing the AGRP-ir fiber density in *anx/anx* mice at P21 with P15, it even seemed as if fibers were disappearing (Figure 4 A, B, E and F).

In **Paper III**, we used IHC with antibodies directed against the NPY receptor Y1 which decorates the soma and dendrites of POMC neurons, to study the development of the POMC/CART system in *anx/anx* and +/+ mice. Here we demonstrated that the POMC/CART system in *anx/anx* mice does not deviate from the +/+ pattern until P21 (exemple from PVN in Figure 4 C, D, G and H). Thus, we concluded that the abnormal histochemical labeling pattern of the POMC/CART system in *anx/anx* mice appears later during the postnatal development in comparison to aberrances in the NPY/AGRP system.

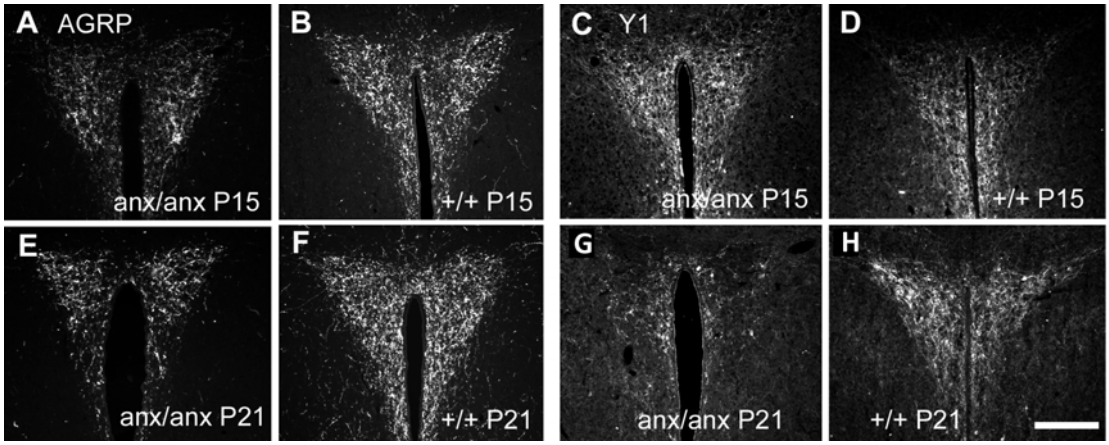


**Figure 4.** Micrographs of AGRP- (A, B, E and F) and Y1-IHC (C, D, G and H) in PVN of *anx/anx* (A, E, C and G) and +/+ mice (B, F, D and H) at P15 (A-D) and P21 (E-H). Note that a reduced density of AGRP-ir fibers in the *anx/anx* mice is present already at P15, while such a reduction in Y1-ir processes is present first by P21. Scalebar = 200µm

## 4.2 INFLAMMATORY AND DEGENERATIVE MARKERS IN HYPOTHALAMUS OF THE *ANX/ANX* MOUSE (PAPER II & III)

### 4.2.1 Activated microglia

In **Paper II**, we used antiserum directed against ionized calcium-binding adapter one (Iba1) to study activated microglia, since the expression of this molecule is strongly up regulated in microglia under this condition. Activation of microglia occurs as a response to various injuries in the nervous system, e.g. stroke, virus infection or degeneration. We were able to detect a region-specific activation of microglia in several hypothalamic as well as extra-hypothalamic areas in *anx/anx* mice from P12, with a stronger activation at P15 and 21 (exemplified by P21 in Figure 5). Interestingly, these were all areas in which we previously had detected a reduced density of NPY/AGRP immunoreactive fibers in *anx/anx* mice. This was the first indication in my thesis work that the aberrant hypothalamic neurochemistry in the *anx/anx* mice could be related to a neurodegenerative process.

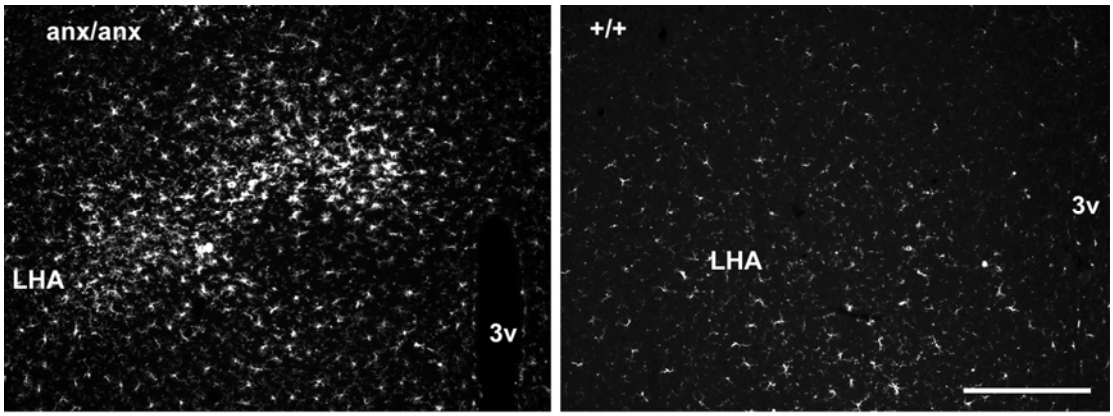


**Figure 5.** Activated microglia cells, visualized by Iba1-IHC, in hypothalamus of an *anx/anx* mouse. LHA, lateral hypothalamic are; 3V, the third ventricle. Scalebar = 200 µm

In **Paper II**, we also concluded that activated microglia cells were occasionally found in close apposition to the arcuate NPY expressing neurons. We studied this relationship further in **Paper III**. By double-labeling IHC for either NPY and Iba1 or Y1 and OX42 (an additional marker for activated microglia) combined with confocal microscopy, we were able to detect several activated microglia, tightly surrounding, almost embracing NPY/AGRP- and POMC/CART- expressing neurons (examplified by NPY-ir neuron in Figure 6 A), resembling the pattern seen in a previous study and described as a new type of cell death (Ribak et al. 2009). Ribak and collegues suggested that this cell death deviates from both apoptosis and necrosis and instead, involves a novel, microglia-associated mechanism characterized by plasma membrane lysis, neural edema and nuclear phagocytosis (Ribak et al. 2009).

### 4.2.2 Hypothalamic expression of MHC class I

In **Paper III**, we found further indications for a neurodegeneration in the *anx/anx* mouse. Using ISH and IHC we detected strong and region specific expression of MHC class I and related molecules, in all areas previously showing activation of microglia. This was perhaps not very surprising since activated microglia are well known to express MHC class I. The interesting finding was the detection of the MHC class I co-subunit β2m in Arc NPY/AGRP and POMC/CART neurons. For long the CNS was seen as an immune privileged site, being devoid of expression of MHC class I and other immunerelated molecules, but several studies have, during the last decades, reported expression of MHC class I by both neurons and glia cells during pathological conditions, such as multiple sclerosis, viral encephalitis and brain injury (Moffett and Paden 1994; Neumann 2001; Xiao and Link 1998), but also in relation to changes in electrical activity (Corriveau et al. 1998; Neumann 2001; Neumann et al. 1995). Thus, 'paralysis' of neurons with tetrodotoxin was shown to cause a dramatic induction of MHC class I-related genes and proteins in all neurons (Neumann et al., 1997). In contrast, others have shown that MHC class I expression is increased by induced seizures in hippocampal neurons, i.e. during increased neuronal activity (Corriveau et al., 1998). However, both these conditions have an abnormal neuronal activation in common, which could explain the apparently conflicting results. Most of the β2m-expressing neurons in Arc of the *anx/anx* mice were absent of or had low expression of FosB (**Paper III**), a marker for cellular activity (Frenois et al. 2007), suggesting that the neurons expressing the MHC class I subunit are silenced or have a low activity. Taking into account a study showing that MHC class I is crucial for the selective maintenance of synapses and regeneration after injury (Oliveira et al. 2004), one can speculate that MHC class I is expressed to spare the abnormally active neuron from synaptic elimination.

### 4.2.3 Markers of cell death

In **Paper III**, we show further support for a degenerative process in hypothalamus of the *anx/anx* mice, by TUNEL-labeling as a marker for apoptotic cells. When merely counting labeled cells in Arc, no significant difference was found between *anx/anx* and +/+ mice, but when including VMH, LHA and DMH, spanning approximately bregma

-1.22 to -2.06 mm, significantly more TUNEL-labeled cells where found in *anx/anx* mice at P21.

Strong support for hypothalamic degeneration in the *anx/anx* mouse was provided by studying active caspase 6 by IHC in **Paper III**. Active caspase 6 is required for axonal degeneration, in contrast to caspase 3 which is required for cell body apoptosis (Nikolaev et al. 2009). We detected active caspase 6 in NPY-positive fibers in hypothalamus, in several in an apparent continuation of the NPY-ir fiber. There was in some instances even coexistence of NPY and active caspase 6 in between parts of the fiber expressing only one of the two markers (Figure 6B). It is likely that neuropeptide expression lacks in the part of the fiber in which the active caspase 6 dependent degenerative process has started. Regarding double-labeling of active caspase 6 and α-MSH, no clear co-localization was detected. However, the two markers were often expressed in close vicinity to each other, not definitely ruling out expression in the same fiber. Also, degeneration of the POMC neurons mainly seems to affect cell bodies and dendrites (Broberger et al. 1999b) (**Paper III**).

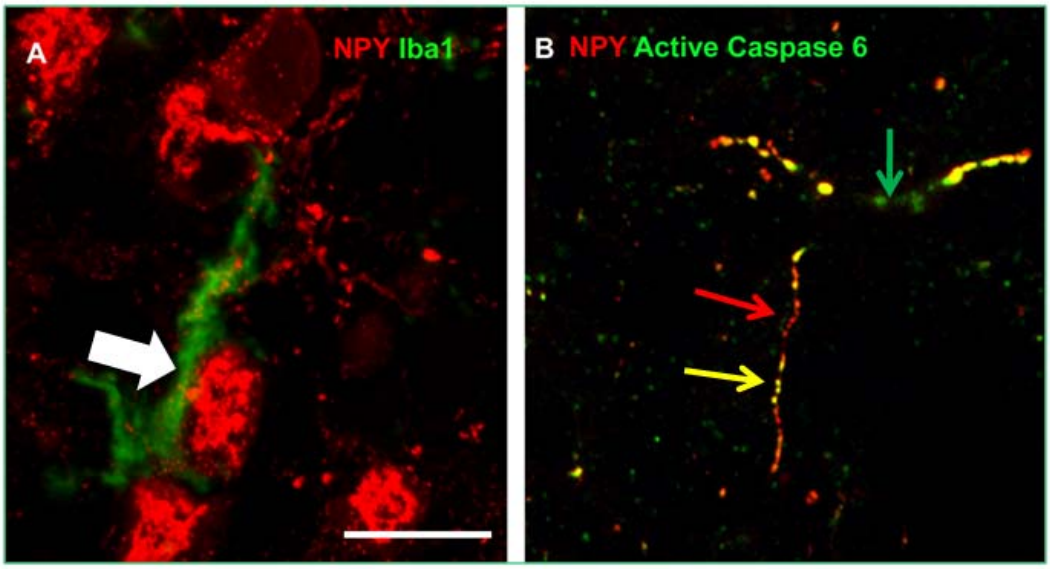


**Figure 6.** A. Activated microglia, expressing Iba1, in close apposition to a NPY-ir neuron in Arc of an *anx/anx* mouse, indicated by a thick arrow. B. NPY-fiber expressing active caspase 6 in hypothalamus of an *anx/anx* mouse. Green arrow points toward a section of the fiber expressing only active caspase 6, red arrow a section expressing only NPY, and yellow arrow a section with coexpression of NPY and active caspase 6. NPY, neuropeptide Y. Scalebar = 20µm

In addition to the previously mentioned markers of apoptosis/degeneration we have also studied FluoroJade, active caspase 3 and leukemia inhibitory factor 1 without gaining any conclusive results. Whether this is because these markers are involved in a type of cell death/degeneration that does not occur in the *anx/anx* mice, or if it is because of technical restraints remains to be determined. It should though be mentioned that the microglia-associated cell death studied by Ribak et al (2009) did not show labeling with these classical markers of apoptosis either.

## 4.3 MICROARRAY AND PATHWAY ANALYSIS (PAPER IV)

The microarray analysis of basal hypothalamus, mainly Arc, from *anx/anx* and +/+ mice, included in **Paper IV**, revealed 132 up regulated and 73 down regulated genes in *anx/anx* mice, the later ones including *Ndufaf1*. By an Ingenuity Pathway Analysis (IPA) of the genes with altered expression, we identified the canonical pathways associated with the genes. These were centered round mitochondrial dysfunction and the OXPHOS system. The results, thus, provided the first evidence that the *anx* mutation is leading to defects in the mitochondrial OXPHOS system.

## 4.4 THE *ANX*-MUTATION AND GENE (PAPER IV)

In **Paper IV**, we performed studies to elucidate the anx-gene and -mutation and its biological function. One of the approximately 40 genes in the *anx*-interval, *Ndufaf1*, was, based on the microarray study and its biological function, of particular interest to us. We verified the down regulation of the *Ndufaf1* gene by Taqman real time expression analysis in total brain, pancreas, liver and lung, and showed a down regulation at the protein level by Western blot of total brain. In addition, we also studied the mRNA expression levels in kidney and heart, but no significant differences were found in these tissues.

No unique sequence alteration was found in coding sequences when sequencing the *Ndufaf1* gene, neither in genomic DNA (exon) nor cDNA (**Paper IV**). A possible explanation is that the mutation is located in regulatory sequences such as a promoter region, which was also indicated by the differential expression in various tissues.

An allele-specificity analysis was performed in **Paper IV,** to make sure that the down regulation of *Ndufaf1* was caused by the *anx*-mutation and not a secondary response to the starvation in these mice. This was done by comparing the genomic and cDNA levels of *the anx*-allele with the wt-allele in heterozygous (*anx*/+) mice. A ratio (cDNA versus genomic DNA) of 1 indicates no difference in allelic expression, whereas a ratio above one indicates a higher expression of the wt-allele. The ratio was, in all four cases studied, higher than one (**Paper IV**), this suggesting that the down regulation of *Ndufaf1* expression in *anx/anx* mice is indeed associated with the *anx* allele of *Ndufaf1,* rather than a secondary effect of the phenotype. All based on the fact that the *anx*/+ mice do not show any body weight or neurological phenotype.

The product of the *Ndufaf1* gene, NDUFAF1, is a factor important for the assembly of CI in the OXPHOS system (Vogel et al. 2005). CI is made up of 45/46 different proteins and needs to be properly assembled for optimal function, i.e. oxidation of NADH and transport of electrons, which in the end is leading to generation of ATP. In **Paper IV**, BN PAGE was used to assess the assembly of CI in *anx/anx* and +/+ mice and indicated lower levels of fully assembled CI and presence of subcomplexes of CI in *anx/anx* hypothalamus.

After injection of dihydroethidium, which creates a red fluorescent staining in the tissue when reacting with endogenous ROS, we have seen increased levels of ROS in *anx/anx* hypothalamus. Furthermore, we detected increased levels of superoxide

dismutase 2, a mitochondrial scavenger of ROS (**Paper IV**). In addition, the Affymetrix microarray study, performed on Arc from *anx/anx* and +/+ mice, revealed several genes involved in oxidative stress that were up regulated in *anx/anx* mice, e.g. SOD1 and peroxiredoxin 1. The subsequent IPA analysis revealed that the most prominently affected pathways in the anorectic mice are centered round mitochondrial dysfunction and the OXPHOS system (**Paper IV**).

In addition, in **Paper IV**, we assessed the mitochondrial respiration in hypothalamus of *anx/anx* mice in comparison to +/+ mice. We were able to conclude that the coupled respiration via CI is approximately 30% less in *anx/anx*. No significant difference was detected in mitochondrial respiration via CII in *anx/anx* compared with +/+ hypothalamus.

Lastly, by studying the mitochondria count in *anx/anx* and +/+ hypothalamus, in **Paper IV**, we found no difference between genotypes. We could thereby conclude that the aberrances in OXPHOS system are not due to a lower mitochondria count in *anx/anx* mice.

# 5 CONCLUSIONS & HYPOTHESES

The studies included in this thesis provide strong evidence that the characteristic phenotypes of the *anx/anx* mouse, e.g. starvation, emaciation and neurochemical aberrances in food intake-regulating systems, are associated with a down regulation of *Ndufaf1* and consequently a deficiency of fully assembled CI in OXPHOS. *Ndufaf1* is coding for one out of eight known assembly factors for CI (Ogilvie et al. 2005; Saada et al. 2008; Saada et al. 2009; Sheftel et al. 2009; Vogel et al. 2005; Vogel et al. 2007). CI consists of at least 45 proteins, that need to be assembled properly for optimal function, i.e. oxidation of substrates and initiation of the transport of electrons in the OXPHOS chain, which in the end leads to generation of ATP, by ATP synthase. In addition, CI is the main intracellular source of ROS in the mammalian cell, also during normal conditions, i.e. when solely fully assembled CI is present. Non-accurate/reduced assembly of CI, e.g. by reduced levels of one of the assembly factors, often leads to increased levels of ROS and reduced levels of ATP.

ROS could hypothetically be generated by the leakage of electrons from subcomplexes made up of a few of the 45/46 proteins in CI. In *anx/anx* hypothalamus we show CI-deficiency with lower levels of fully assembled CI and increased levels of subcomplexes. Some of these subcomplexes are likely to still have oxidative capacity, but might not be able to transport the electrons further and thereby leak electrons into the matrix of the mitochondria and thereby contribute to the increased ROS production in the *anx/anx* mouse. ROS have been implicated in a number of pathological states such as apoptosis, cancer, atherosclerosis, inflammation and neurodegenerative disorders (Orrenius 2007). However, ROS do not only cause oxidative stress, but can also act as a food intake-regulating signal affecting the arcuate neurons, monitoring glucose and lipid levels. Increased levels of ROS inhibit food intake (Benani et al. 2007; Leloup et al. 2006). Our believe is, thus, that the food intake-suppressing effect of ROS could contribute to the initial starvation in the *anx/anx* mice, during the first postnatal days (Maltais et al. 1984).

Most of the phenotypes of the *anx/anx* mouse, such as starvation and emaciation, closing of eyelid, abnormal gait and tremors can be attributed to abnormalities in brain and/or muscle. These are the organs most commonly affected by human conditions with defects in CI, such as Leigh syndrome, which are related to the high energy requirements of these tissues.

Several studies have indicated a neurodegenerative process in the hypothalamus of the *anx/anx* mice. We have shown reduced AGRP-LI in all the projection areas of the NPY/AGRP neurons in *anx/anx* mice from around P12-15 in correlation with an activation of microglia both spatially and temporally (**Paper II**). In addition we have shown that Y1-LI, a marker for the POMC/CART neurons, is reduced in *anx/anx* mice from around P15- 21 (**Paper III**). Interestingly, activated microglia were often seen forming a curve tightly around the AGRP/NPY- and POMC-neuron in a pattern resembling what Ribak et al.(2009) described as a new type of cell death, microglia-associated cell death. Using electron microscopy Ribak and co-authors show that the microglia is apposed to openings in the plasma membrane of the surrounded dentate

gyrus granule cell. When larger openings occurred, the neuron displayed a watery cytoplasm and nucleoplasm and damaged organelles (Ribak et al. 2009). This type of cell death differs from both necrosis and apoptosis and might thereby escape detection by classical degenerative/apoptosis markers (Ribak et al. 2009). In fact, we have performed studies with such classical markers, e.g. FluoroJade, caspase 3 and leukemia inhibitory factor, in the hypothalamus of the *anx/anx* mice, without detecting any signs of apoptosis/degeneration.

We have also shown expression of MHC class I mRNA and protein in projection areas of arcuate neurons, to a large extent attributed to microglia, but remarkably also in a few arcuate-neurons expressing Y1 or AGRP (**Paper III**). By TUNEL-labeling we detected significantly increased number of apoptotic cells in hypothalamus of the *anx/anx* mice compared with +/+ mice (**Paper III**). But perhaps the most striking evidence for hypothalamic degeneration in these anorectic mice is the co-labeling of NPY and active caspase 6 in Arc, DMH, amygdala and zona incerta. Caspase 6 is required for axonal degeneration, in contrast to neuronal cell body apoptosis which involves caspase 3 (Nikolaev et al. 2009) and has been implicated in the pathology of degenerative disorders such as Alzheimer's (Nikolaev et al. 2009) and Huntington's (Graham et al. 2006) diseases. All these findings, in combination, strongly support a neurodegenerative process in the hypothalamus of the *anx/anx* mice.

The phenotypes of the *anx/anx* mice are centered round the brain and possibly the pancreas and muscles. According to our real-time expression analysis, *Ndufaf1* is down regulated in brain, pancreas, muscle, lung and liver but not in kidney and heart. This discrepancy between tissues might indicate that the *anx* mutation is located in a tissue specific promoter and explains why the core *anx*-phenotypes are related to brain functions, as well as why we do not detect any unique sequence alteration in coding regions of *Ndufaf1*. In addition, most of the studies so far have been indicative of aberrances, in particular neurodegeneration, only in the hypothalamus of the *anx/anx* mice, except for one study showing increased apoptosis also in hippocampus of the these mice (Kim et al. 2001).

So how could one explain that apparently only specific organs or brain regions and even specific neurons appear to be affected by the mutation in *Ndufaf1*? It is unlikely that the *anx* mutation is affecting a promoter specific for certain types of neurons, since we detect a 50% reduction in *Ndufaf1* expression also in mRNA from total *anx/anx* brain. If the *anx* mutation would only affect certain types of neurons, such as the arcuate neurons, this reduction would be less pronounced when mRNA from total brain was analyzed. A much more attractive explanation is that other factor(s) makes some neurons more sensitive to CI deficiency, increased levels of ROS and/or oxidative stress. One potential explanation is related to the ATP- and sulphonylurea-sensitive $K^{+}_{\text{-ATP}}$ channels. These channels are well characterized and their electrophysiological and pharmacological role in skeletal-, cardiac- and smooth muscles, pancreatic $\beta$-cells and peripheral as well as central nervous system, including the AGRP/NPY- and POMC/CART-neurons, are well documented (Dunn-Meynell et al. 1998; Ibrahim et al. 2003; Thomzig et al. 2005). As the name implies, this group of potassium channels is regulated by intracellular ATP, which closes the channel leading to membrane depolarization, thereby linking cell excitability to metabolic status (Ashcroft and

Ashcroft 1990). An important function suggested for the neuronal $K^+_{-ATP}$ channels is neuroprotection (Blondeau et al. 2000). During conditions of low intracellular ATP, e.g. during metabolic stress and ischemia, the $K^+_{-ATP}$ channels are open and the membrane is hyperpolarized. This reduces neuronal activity and neurotransmitter release, in order to save ATP, and could protect against excitotoxicity and calcium overload, which is of importance in situations of metabolic stress (Ballanyi 2004; Blondeau et al. 2000). However, if the neuronal activity is chronically reduced, the expression of genes and proteins important for neuronal survival will be reduced, and neurodegeneration is likely to occur (Chergui et al. 1997; Liss and Roeper 2001). Thus, a transient activation of the $K^+_{-ATP}$ channels may be a short-term neuroprotective response to metabolic stress, whereas chronic $K^+_{-ATP}$ channel-activation could have fatal consequences for the cell. One can speculate that the longer time needed to build up the electrochemical gradient across the mitochondrial membrane when CI is not functioning optimally, as in the *anx/anx* mice, leads to lower levels of ATP generated by ATP synthase.

Several studies state that neuronal MHC class I expression is activity dependent, in such a way that silent/inactive neurons are more prone to express MHC class I than active ones (Neumann et al. 1995; Neumann et al. 1997). Interestingly, we have detected expression of MHC class I in arcuate neurons of the *anx/anx* mice (**Paper III**). This would indicate silencing/inactivation of arcuate NPY/AGRP and POMC/CART neurons, potentially as an initial response to save these neurons from ATP-deficiency. In addition, MHC class I molecules have been shown to be crucial for the selective maintenance of synapses and regeneration after injury (Oliveira et al. 2004) . A possibility is that, in the *anx/anx* mice, up regulation of neuronal MHC class I expression, in discrete neuron populations in Arc, aims at sparing these neurons from synaptic elimination. Thus, during a hypothetical recovery phase, regeneration of the MHC class I expressing neurons would be facilitated.

$K^+_{-ATP}$ channels consist of two types of subunits, the pore-forming inwardly rectifying potassium channel (Kir6-) family members and the regulatory sulphonylurea receptor subunit (SUR) (Ashcroft 1988; Liss et al. 1999). The complete channel consists of four subunits of each type joined together. The most likely combination of $K^+_{-ATP}$ channels in Arc (both NPY/AGRP and POMC neurons) is Kir6.2/SUR1 (Dunn-Meynell et al. 1998; Ibrahim et al. 2003; Thomzig et al. 2005). Interestingly, the same combination is also found in a subpopulation of dopaminergic neurons in the SN, where it has been shown to be highly sensitive to inhibition of CI by rotenone administration (Liss et al. 1999). CI inhibition has for long been implicated in the pathogenesis and degeneration of dopaminergic neurons in Parkinson's disease (Beal 1996; Hanna and Bhatia 1997; Hirsch et al. 1997). Furthermore, chronic infusion of low doses of the CI inhibitor rotenone has been shown to induce Parkinsonism as well as selectively cause neurodegeneration of dopaminergic neurons in rats, even though the infusions caused a uniform CI inhibition throughout the brain (Betarbet et al. 2000). It is thus possible that the reduced *Ndufaf1* expression and CI deficiency observed in *anx/anx* mice leads to hyperpolarization and inactivation of the more vulnerable neuronal subpopulations expressing the Kir6.2/SUR1 $K^+_{-ATP}$ channels, like the arcuate neurons and the dopaminergic subpopulation in substantia nigra (SN) and striatum. This could in turn explain the selective degeneration of arcuate neurons seen in the *anx/anx*

mice. Based on this theory we studied SN of *anx/anx* and +/+ mice by IHC, but no apparent difference was detected with any of the three markers used, i.e. Iba1, CCK and TH. We cannot, however, exclude the possibility of detecting differences in SN by the use of other markers and/or more sensitive techniques, e.g. stereology. In fact, altered dopaminergic transmission has been detected in striatum of the *anx/anx* mice (Johansen et al. 2001). Another possibility is that the arcuate neurons, which are highly active, perhaps especially during the first three weeks, during weaning, are more severely affected by ATP-deficiency and increased ROS levels.

In conclusion, this thesis provides strong evidence that the anorexia and premature death of the *anx/anx* mouse is associated with hypothalamic mitochondrial dysfunction. The *anx* mutation leads to lower levels of *Ndufaf1* as well as of fully assembled CI in the OXPHOS system and accumulation of sub-complexes resulting in increased production of ROS. The increased ROS levels could initially act as signaling molecules affecting hypothalamic neurons, leading to decreased appetite, oxidative stress and in the long run inactivation and degeneration of arcuate neurons in *anx/anx* mice.

# 6 FUTURE PERSPECTIVES

In the near future, the focus of the *anx/anx* project will be genetic studies, primarily to find the sequence alteration of the *anx*-gene, but also to study regulatory motifs in the sequence. Additional studies will center round the degenerative process in the *anx/anx* mice, likely by studying the projections of the NPY/AGRP- and POMC/CART neurons with electron microscopy, possibly combined with IHC, and by crossing the *anx/anx* mice with POMC- and NPY-GFP mice. By electron microscopy we would like to further study microglia-associated cell death as discussed in this thesis. Based on the results of this thesis we intend to treat the *anx/anx* mice with anti-inflammatory, anti-oxidative and antibiotic compounds.

In the longer aspect, a dream project would be to take the conclusions and hypothesis generated in this thesis into the clinic, e.g. to study the activity of hypothalamic neurons and microglia activation in AN and cachectic patients, as well as children with FTT.

# 7 PERSONAL REFLECTIONS

When presenting our data in public I am often, and not surprisingly, asked in what way our studies relate to AN. My belief is that the process of deranged food intake-regulating neurons and subsequent degeneration is a likely explanation to the lost hunger and satiety that many AN-patients describe, as well as to the problems of gaining them back, once these signals are lost. In AN there is often in the beginning, a voluntary decision to initiate dieting, in contrast to the *anx/anx* mice were the initial starvation is related to the mutation, but in both cases a vicious cycle is started, where increasing numbers of the food intake-regulating neurons are affected. In addition, there is a genetic component taking part in the process of developing AN, probably making some people more prone to fall into this vicious cycle. Based on this reasoning, my conviction is that strategies to treat AN should focus on 're-learning' to eat, by scheduling the daily food intake. At the same time arcuate neurons should be 're-educated' when and how they should be active, as well as which neuronal contacts should be generated, eliminated and strengthened.

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