The anorectic anx/anx mouse displays signs of astrogliosis in the hypothalamus

*Introduction:* Anorexia Nervosa is a serious psychiatric condition that has been suggested to have a neuroimmunological mechanism as a part of the pathophysiology. Hypothalamic inflammation occurs in an anorectic animal model called the anx/anx mouse in the form of up-regulation of inflammatory markers and activated microglia. However, neuroinflammation based on astrogliosis has not been studied in this model. *Aims:* This study aimed at establishing if astrogliosis occurs in the hypothalamus of the anx/anx mouse model. *Material and Methods:* A total of 8 animals - 4 of each genotype (anx/anx and +/+) at postnatal day 21 were employed. The brains were dissected, the hypothalamus sectioned and immunohistochemistry was performed with tyramide signal amplification method using glial fibrillary-acidic protein (GFAP) as a marker for astrogliosis. The GFAP immunofluorescence in anx/anx and +/+ mice was measured in different hypothalamic areas involved in the regulation of food intake. *Results:* A statistically significant increase of GFAP-marked astrocytes was present in the paraventricular nucleus (p=0.04) and arcuate nucleus (p=0.03) of the anx/anx mouse in comparison to +/+ mice. There was no significant increase of GFAP-marked astrocytes in the medial preoptic area (p=0.07). *Conclusions:* The anx/anx mouse displays signs of astrogliosis in the hypothalamus suggesting an important role for astrocytes in anorexia. The underlying cause for the astrogliosis present in the hypothalamic regions in the anx/anx mouse is not known and needs to be further studied.

*Keywords:* Astrogliosis; Hypothalamus; Anorexia; Eating disorder; Neuroinflammation
**Abbreviations**

AGRP – Agouti-gene Related Protein

AN – Anorexia Nervosa

Arc – Arcuate Nucleus

BBB – Blood Brain Barrier

CART – Cocaine- and amphetamine regulated transcript

CNS – Central Nervous System

GFAP – Glial Fibrillary Acidic Protein

IHC – ImmunoHistoChemistry

LHA – Lateral Hypothalamic Area

NPY – Neuropeptide Y

MPA – Medial Preoptic Area

POMC – Pro-OpioMelanoCortin

PCR – Polymerase Chain Reaction

PVN – ParaVentricular Nucleus

ROS – Reactive Oxygen Species

TSA – Tyramide Signal Amplification
Introduction

Anorexia and related diseases

Anorexia (poor appetite) is a life-threatening condition, which has been linked to several neuroinflammatory mechanisms as part of the pathophysiology. (1-3). The most well-known disease with anorexia as main symptom is the psychiatric diagnosis anorexia nervosa (AN), in which restrictive eating and self-starvation frequently lead to an extremely emaciated state (4). The Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders gives the following diagnostic criteria for AN: “persistent restriction of energy intake leading to significantly low body weight, intense fear of gaining weight or persistent behavior that interferes with weight gain (even though significantly low weight) (...) disturbance in the way one’s body weight or shape is experienced or persistent lack of recognition of the seriousness of the current low body weight” (5). In 2012 the prevalence of AN was estimated to be 0.9 % among adult females and about 0.01 % among males (6). In contrast to this number, 20 % of the females in a Canadian study were reported to display disturbances in their eating patterns (7). AN is the most lethal of all psychiatric disorders and has a mortality rate of 10%, where the causes of death are starvation, heart failure and suicide (6). It is a common perception that AN is caused by socio-cultural factors. However, twin studies have revealed a strong genetic contribution (48-76%) to the development of this disease (8). Thus, genetics and environmental factors seem to interact and determine the risk of developing AN. Additionally autistic traits are present at a higher rate in AN patients than in control groups (9). Anorexia may also occur in patients with cancer or infectious diseases and is then known as “cachexia”, or “secondary anorexia” (10). The term “cachexia” includes not only a poor appetite but also a high energy expenditure, which results in a catabolic state of the body – meaning that energy is used to a higher rate than produced (11). Approximately 80 % of end stage cancer patients suffer from cachexia (11). Another illness related to anorexia is failure to thrive which is a specific anorectic condition that occurs in young children (12). The main symptom of failure to thrive is poor appetite, which results in a low caloric intake which in turn leads to inadequate growth and development for the child’s age (13).

Current treatments of anorexia and related diseases

No effective treatments for AN are currently available and the rates of relapse, chronicity and death among patients with AN are high (4). Family therapy consisting of sessions focusing on the familial dynamics with a psychiatrist is the only treatment that has been shown to improve
the weight and menstruation pattern of AN patients (14). The rate of re-hospitalizations has however been reported to be continuously as high in the groups with family therapy as in the control groups (14). The treatment of cachexia consists of two parts; pharmaceutical and nutritional (15). Neither of these are however satisfying since cachexia frequently is the actual terminal cause of death in end stage cancer patients (10). Failure to thrive is treated differently depending on the underlying cause of developing the symptoms, but the main feature is the nutritional part (12). However, one critical condition that may follow the treatment of patients with failure to thrive is the re-feeding syndrome which is a possibly fatal state with not yet fully understood pathological mechanisms (12). It is clear that development of effective treatments for this group of severe diseases is necessary, and thus further understanding of the biological pathogenic mechanisms of anorexia is needed.

**Regulation of food intake**

The poor food intake and thereby likely a disturbed regulation of hunger and satiety are central parts of the pathophysiology of anorexia (15, 16). A neural network crucial in this regulation is formed by several nuclei, among them the arcuate nucleus (Arc), the medial preoptic area (MPA) and the paraventricular nucleus (PVN), which resides in the hypothalamus (17). The PVN and the lateral hypothalamic area (LHA) may be considered to be “subordinated centers” of the Arc. Furthermore, the PVN traditionally has been perceived as the center for “satiety”, and the lateral hypothalamus the center of “hunger” (18). This network controls three main features of the energy balance of the body: appetite/food intake, energy expenditure, and energy storage (which consists mainly of the deposition of fat) (17). Several peripheral signals aid this neural network to monitor the energy status in the body (17). These signals consist of circulating metabolites such as glucose and free fatty acids, and circulating hormones secreted from peripheral tissues such as insulin, leptin and ghrelin (17). Leptin is released from adipocytes and induces satiety via receptors on neurons in the Arc. Further, the concentration of leptin decreases as the fat deposits of the body decrease and *vice versa* (18). Thus a severely starved individual, such as an AN patient, have low levels of leptin circulating and subsequently should have a lowered satiety signaling. Activation of leptin receptors of the Arc induces satiety and inhibits hunger by release of different peptides (10, 19) (figure 1). Anorexigenic peptides (pro-opiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART)) inhibit food intake and signal satiety (19, 20).
Orexigenic peptides (agouti-related protein (AGRP) and neuropeptide Y (NPY)) are inhibited by high levels of leptin and insulin, and induce food intake and signal hunger (19, 20).

Figure 1. Hypothalamic regulation of food intake. The neurons in the Arc are central in the hypothalamic regulation of food intake, in particular the AGRP/NPY- and POMC/CART expressing neurons. The activation of AGRP/NPY neurons leads to food intake stimulating signaling (+), while POMC/CART activation leads to food intake inhibiting signaling (-). The activity of these Arc neurons are affected by peripheral hormones e.g. leptin and insulin, which can enter the brain parenchyma from the blood stream due to a more permeable BBB in the mediobasal hypothalamus proximal to the third ventricle. By binding to respective receptor on the Arc neurons, leptin and insulin will stimulate the POMC/CART neurons and inhibit the AGRP/NPY neurons. Leptin and insulin is released in proportion to the amount of body fat. This means that an underweight person will have low levels of leptin and insulin binding to their receptors and the effect should then be lowered food intake inhibiting signaling and increased food intake stimulating signaling. However, there seems to be a dysfunction of this system in anorectic subjects. Figure adapted with courtesy of Ida Nilsson (10). Arc = Arcuate nucleus, AGRP = Agouti-related protein, BBB = blood-brain barrier, NPY = Neuropeptide Y, POMC = Pro-opiomelanocortin, CART = Cocaine- and amphetamine regulated transcript.
The different neurons of the Arc expressing the aforementioned peptides are connected to neurons of several parts of the hypothalamus (the MPA, LHA and PVN amongst others) and other parts of the brain (21). NPY is one of the most potent inducers of food intake and this peptide also inhibits the signaling of satiety (21). NPY has been shown to be pathologically up-regulated in the hypothalamus of AN patients (20). Zhang et al. (20) have suggested that this is a sign of a dysfunction in the signaling of NPY. Dysregulation of this network as well as neuroinflammation has been revealed in the hypothalamus of anorectic animal models (i.e. the anx/anx mouse) (1, 22-29).

**Neuroinflammation**

An immunological reaction called “neuroinflammation” occurs after injury or infection of the central nervous system (CNS) (30). Neuroinflammation is a recurrent phenomenon in psychiatric diseases such as schizophrenia, bipolar disorder and autism (31-33). The blood-brain barrier (BBB) is the most important protection against micro-organisms and other pro-inflammatory macromolecules that circulate in the blood stream and the main purpose of the BBB is to minimize the passage of cells and other macromolecules into the neural parenchyma (30). Cells in the CNS are also involved in the immunological response, including resident cells such as astrocytes, microglia and endothelial cells and infiltrating cells such as T-cells and macrophages (30). Astrocytes and microglia are considered to be the effector cells of the immune system (30) and they differ in that microglia are activated rapidly while astrocytes react with a delay (34). Microglia are of myeloid origin and are described as the “macrophages of the brain” while astrocytes are derived from ectodermal cells and are the most abundant cell type found in the brain (35). Microglia and astrocytes have an intimate connection as they may activate each other in pro-inflammatory environments by excreting cytokines and other molecules (36). The main purpose of the astrocytes has historically been described to keep the environment viable and act as support for neurons (37). Studies have however shown that astrocytes also play an important role in the development of neurons and by controlling neurotransmitter homeostasis (glutamate, GABA and adenosine) and in modulating synaptic plasticity (35, 37). When any form of neural injury occurs, astrocytes can form a glial scar, which is the most extreme consequence of a process called astrogliosis (38).
Astrogliosis

Hyperplasia and hypertrophy of astrocytes are typical signs of a phenomenon known as “astrogliosis”, which is a feature of several neurological diseases, such as Alzheimer’s disease, stroke and traumatic brain injury (39). Astrogliosis can range from a mild to a severe degree, where the latter involves formation of glial scars (39). The function of glial scar formation is to limit the spread of inflammatory molecules and restrict the spread of damage in the CNS, however the scar formation also leads to reduced axonal growth and thus restricted function of the CNS (38). Up-regulated expression of glial fibrillary-acidic protein (GFAP) and other intermediate filament proteins (vimentin and nestin) is considered the hallmark of astrogliosis (36). Studies with knock-out mice have shown that in absence of these proteins related to astrogliosis no restriction of damage is possible and the injury of the CNS is significantly more spread (38). As Sofroniew et al. extensively reviews, the function of the “reactive” (activated) astrocytes is not yet known (39). The function of astrogliosis has been suggested to be to narrow down the area of cytotoxic and inflammatory cells and mediators, but as Sofroniew et al. further discuss, what happens if the astrocytes are activated incorrectly? Is it possible that the main role of astrocytes in the CNS is not protective? Genomic analysis of reactive astrogliosis has shown that reactive astrocytes can have either a protective or a detrimental effect, depending on the type of injury that has caused astrogliosis (40). Some of the molecules that have been shown to be able to trigger astrogliosis are different cytokines (TNFα and IL6 among others), neurotransmitters (glutamate and noradrenalin) and reactive oxygen species (ROS) (38, 41). ROS has been found to be abundant in the hypothalamic area of the anorectic mouse model called anx/anx (42).

The anx/anx mouse

The anorectic anx/anx mouse is a unique animal model, which displays several traits common in anorectic diseases of humans such as emaciation and self-starvation (10, 11, 13, 16). It arose after a spontaneous mutation at the Jackson laboratory in 1976 (43). The mutation is recessive and the most prominent trait in mice that are homozygous for the anx mutation is that they significantly reduce their food intake and bodyweight (figure 2), and die within 3-5 weeks (postnatal day 21-35) from starvation (43). Neurological problems such as tremors, as well as hyperactivity are sometimes also displayed (43). The anx/anx mice have several alterations in neurotransmitter systems and neuropeptidergic systems that originate in the hypothalamic area (44). For example, NPY and AGRP are accumulated in the cell bodies of
the Arc in the *anx/anx* mouse (19). Gene expression profiling performed by Lachuer et al. has revealed that up-regulated and down-regulated genes in the *anx/anx* mouse are related to hypothalamic inflammation, and this pattern is similar to the hallmarks of multiple sclerosis (MS), a well-known neuroinflammatory disease (23). Lachuer et al. further suggested that the anorectic phenotype of the *anx/anx* mouse is based upon an inflammatory processes occurring in the hypothalamus (23). Neuroinflammation based upon activated microglia have been shown in the *anx/anx* (1), but no research has been made on the presence of astrogliosis in this anorectic model. Several studies have additionally revealed signs of neurodegeneration and mitochondrial dysfunction in the hypothalamus of the *anx/anx* mouse (1, 42, 45).

![Figure 2. The anorectic *anx/anx* mouse.](image)

Figure 2. The anorectic *anx/anx* mouse. The anorectic mouse model is shown to the right and is notably smaller than the healthy +/+ mouse seen to the left. The picture was taken at p21. Adapted picture with courtesy of Jeanette Johansson (46). p21 = postnatal day 21.

**Hypothalamic inflammation in eating disorders**

Several pro-inflammatory cytokines induce weight loss when administered, either peripherally or centrally (2), and inflammation of the hypothalamus can thus be related to weight loss. An autoimmune component has been found in patients with AN which suggests that an inflammatory process is part of the neuropathology of this type of eating disorder (3). In addition, microglia in the hypothalamus are activated in the anorectic *anx/anx* mouse (10). This is also the case in the hypothalamus of both obese mice and humans, where both astrogliosis and activation of microglia has been shown to take place (25, 47). These findings indeed show a paradox in the research field, because inflammation of the hypothalamus seems to be present in states of both positive and negative energy balance (2, 47). While microglial activation in the brains of the anorectic mice has been previously studied, the potential role of astrogliosis has been poorly characterized.
Aim
The aim of this study was to establish if astrogliosis occurs in the hypothalamus of the anorectic anx/anx mouse.

Materials and Methods
All chemicals were from Sigma ALDRICH (St. Louis, MO) unless otherwise specified.

Animals employed
The anorectic mouse model anx/anx was used to investigate the role of astrogliosis in anorexia. A total of 8 animals were studied: 4 anx/anx mice and 4 wild type (+/+) littermates. The mice were housed at 25 °C with a 12-h light-dark cycle and had continuous unlimited access to food. The animals were genotyped and phenotypically characterized based on their body weight (+/+ mice weighed 8-9 g while anx/anx mice weighed 4-5 g at p21). Before sacrifice, the mice were anesthetized with isoflurane (3-5%, Forene, Abbott Scandinavia AB, Solna, Sweden) at postnatal day 20-21 (p20-p21), and their tissues fixed through perfusion with paraformaldehyde. The brains were subsequently dissected for further analyzed by immunohistochemistry (IHC).

Genotyping
In order to determine which mice were homozygotes (anx/anx or +/-) and which mice were heterozygotes (anx/+), genotyping was performed using simple sequence length polymorphism markers (D2Mit133F and D2Mit133R) mapped to the subchromosomal region (proximal to the agouti-locus, on chromosome 2) where the anx mutation is situated (43).

DNA extraction
Small pieces of tail tips were collected for DNA extraction. The tail tips were dissolved in 500 µl lysis buffer (100 mM Tris-HCl at ph 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl and 100 ng/ml proteinase K) and 1.5 µl proteinase K (Qiagen, Washington DC, Maryland) in a water bath at 55°C for approximately 3 hours, or until the samples were completely dissolved. The samples were then centrifuged (5417C Eppendorf, Hamburg, Germany) at 14,000 rpm for 10 minutes at 4 °C. The supernatant from the centrifugation was added to Eppendorf tubes with 500 µl isopropanolol. The samples were then again centrifuged at 14000 rpm for 10 minutes at 4 °C; the pellets were thereafter dried for 30 minutes at room temperature after
pouring out the supernatant. After this procedure, the pellets were dissolved in 100 µl of sterile water. The DNA was thereafter vortexed and diluted with sterile water.

**DNA amplification**

The master mix was prepared for the PCR by 148 µl sterile water, 10 µl f-primer (Thermo Electron GmbH, Ulm, Germany), 10 µl R-primer (Thermo Electron GmbH), 5 µl dNTP (Laroca, Rockland, ME), 25 µl 10x buffer (Finnzyme/Thermo scientific, Gothenburg, Sweden) and 2 µl dynazyme (Finnzyme/Thermo scientific) and subsequently 20 µl of this mixture was added to each diluted DNA sample. Sequence of the primers and PCR programs are presented in table 1.

**Table 1. Primers and PCR program used for genotyping.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2Mit133F</td>
<td>CTC ATA ACC CCC CAC TCT CT</td>
<td>95°C, 3 min followed by 30 cycles (55°C, 30 s; 72°C, 30 s; 95°C, 30 s); 72°C, 10 minutes; 4°C remaining time.</td>
</tr>
<tr>
<td>D2Mit133R</td>
<td>TAG CAA AAT AAC TTA GGC GGC</td>
<td></td>
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</tbody>
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PCR = Polymerase Chain Reaction, min = minutes.

**Gel electrophoresis**

Metaphor gel was prepared by mixing 50 ml of Tris/Borate/EDTA (TBE) (Nationaldiagnostics, Atlanta, Georgia) with 1.5 g metaphore agarose (Lonza, Stockholm, Sweden) and by allowing to mix for 15 minutes. The mix was then heated in a microwave for 2 minutes on defrost and about 2 minutes on 300 W to make sure that the metaphor agarose was properly dissolved in the TBE. 6 µl of Gelred (Biotium, Hayward, CA) was added and the mixture was then poured into a taped container and left to set for 30 minutes. When the PCR was ready and the gel set, the gel was placed in an electrophoresis container which was filled with TBE until it fully covered the gel. 5 µl of loading dye (Fermentas/Thermo scientific, Gothenburg, Sweden) was added to each DNA sample and 5 µl of ladder (Generuler/Thermo scientific, Gothenburg, Sweden) was added to the first well. Thereafter 20 µl of each sample was added to different wells (different pipette tips were used in order to prevent contamination), 6 µl of Gelred (Biotium Hayward, CA) was added to the bottom of the container in order to hinder diffusion of DNA. The electrophoresis container was then plugged in and set to 90 V for approximately 2.5 hours. The gel was then studied in Trans
UV-light (Molecular Image, Gel DocxR, BioRad, Hercules, CA) to determine which DNA samples were heterozygote and which where homozygote for the \( anx \) or ++ alleles (figure 3).

**Figure 3. Gel electrophoresis of representative PCR samples.** All mice included were genotyped using simple sequence length polymorphism markers mapped to the subchromosomal region where the \( anx \) mutation is situated. This region is longer in the \( anx/anx \) genotype than in the +/- genotype, which was used to determine the genotype of the mice (the longer the region is, the slower it will wander through the gel). The PCR sample from heterozygote mice displayed duplicate bands, as seen to the right in the figure. PCR = Polymerase Chain Reaction.

**Immunohistochemistry**

In order to be able to compare the rate of astrogliosis between the two animal groups, GFAP-expressing astrocytes were visualized in the brain tissue using IHC.

**Tissue preparation**

After the dissection, the brains were snap-frozen with carbon dioxide and coronal brain sections (14 \( \mu \)m) cut on a cryostat (Microm, Heidelberg, Germany) and collected at room temperature on gelatin coated microscope slides. Sections were collected throughout the hypothalamus with 8x14 \( \mu \)m distance (4x14 \( \mu \)m distance at the Arc) with focus on specific hypothalamic areas connected to regulation of food intake (Arc, MPA and PVN).

**GFAP detection**

To detect GFAP, tyramide signal amplification (TSA) (PerkinElmer Life Science Inc., Boston, MA) IHC technique (48) was employed. More in detail, the sections on microscope slides were stored in a -20°C freezer to keep the tissues intact. Before starting IHC, the sections were dried for 30 minutes at room temperature (RT) in order to fixate the tissues on the slides. When the sections were dried and marked, they were washed in small glass tanks in 0.01 M PBS buffer for 10 minutes at RT. After this, the slides were put in PBS and 0.03 % hydrogen peroxide in order to eliminate endogenous peroxidase which otherwise might interfere with the Horse Radish Peroxidase-conjugated (HRP-conjugated) secondary antibody used later on. Subsequently the slides were washed in PBS buffer in 3 rounds, 5 minutes each.
The primary antiserum against GFAP (Sigma #3148) was added 1:8000 in 0.3 % PBS buffer with Triton, Bacitracin and NaAzide by dropping 200 µl of this mix onto each glass which then were incubated in PBS-wet box (box with PBS-covered filter paper at the bottom) overnight at 4 °C. The borders were sealed with tape in order to prevent any evaporation. The following day the slides were washed in PBS buffer for 10 minutes in a winnow at room temperature and subsequently washed in Tris-NaCl-Tween (TNT) buffer (999 ml TBS + 500 µl Tween 20) for 20 minutes at RT, and then incubated on slide with TNB buffer (50 ml TBS + 0.25 g blocking reagent (PerkinElmer Life Science Inc., Boston, MA) for 60 minutes at room temperature. In order to identify the correctly bound antibodies, the slides were thereafter incubated with a HRP-labeled secondary antiserum (polyclonal Swine Anti-Rabbit Immunoglobulin HRP, DAKO ab, Copenhagen, Denmark) 1:200 in TNB on slide (200 µl/slide) for 30 minutes. Afterwards the slides were washed in TNT for 30 minutes at RT which was followed by enhancement with Biotinyl Tyramide conjugated with green fluorochrome (PerkinElmer Life Science Inc., Boston, MA) (1:200 in amplification diluent (PerkinElmer Life Science Inc., Boston, MA)) for 10 minutes in RT. Subsequently the slides were washed in TNT for 30 minutes in RT and mounted in a 3:1 mix of glycerol and phosphate buffered saline containing DABCO (1,4-diazabicyclo [2.2.2] octane). The slides were stored in -20°C before analyzing in a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) with 2x, 4x, 10x and 20x objectives. Thereafter the pictures from the microscope were analyzed in Image J; a software that quantifies pixels and thus makes it possible to measure the amount of fluorescence seen in the pictures. The fluorescence was measured using integrated density (the product of area and mean gray value), which in short may be described as the sum of the values of the pixels in the selected area.

**Statistics**

By using Image J the fluorescence density of GFAP was determined. The mean value and standard deviation of fluorescence density was determined for each group (anx/anx and +/+ ) at each of the three different hypothalamic sites (MPA, PVN and Arc). Since there were two groups that we wanted to compare and the number of animals employed was quite small (under 10 in total), student t-test was employed. This was possible since the animals compared are inbred siblings, which makes their genetic background equal except for the anx interval. Standard deviation was calculated for each group and site. Data are presented as means +/- standard deviation (SD). Significant value was set at p<0.05.
Ethical considerations

To preserve the brain tissue optimally for GFAP analysis, in vivo perfusion was performed. This may be an ethical dilemma since this could cause suffering of the animals. In order to minimize this possible suffering, the animals were anesthetized with isoflurane (prior to the perfusion). The advantage of doing this study on animals is the possibility to use brain tissue for immunohistochemistry, which would be almost impossible to do on humans due to the lack of clinical samples. The disadvantage of doing the study on mice is the dilemma whether the findings are possible to translate to human tissue, and of course the possible suffering the mice may be experiencing. This study may provide important information on the neurobiological processes occurring in the brain at a state of negative energy balance, which is a research area that has not been explored enough thorough. The study has been reviewed and received ethical approval by Stockholm norras djurförsöksetiska nämnd number/submission date: N404/10.

Results

The quantitative IHC analysis of astrocytes with GFAP as a marker revealed significantly increased immunohistochemical labeling in the hypothalamus of the anx/anx mice, i.e. in the PVN and Arc (anx/anx n=4, +/+ n=4), in comparison with +/+ (wild type) mice at p21. No statistically significant difference was seen in the MPA (anx/anx n=4, +/+ n=3). No apparent signs of glial scar formation were observed in any of the areas.

Paraventricular Nucleus

An apparent hypertrophy and hyperplasia of GFAP positive astrocytes was seen in the PVN (Figure 4) of the anx/anx mouse. When quantifying the GFAP-labeling with Image J a statistically significant increase (p=0.04) of GFAP-positive cells was revealed in the anx/anx compared to the +/- mice (Figure 5).
Figure 4. *anx/anx* mice displayed an astrogliosis-like pattern in the PVN. A representative picture of PVN after GFAP IHC revealed increased GFAP-immunoreactivity in the *anx/anx* (B) compared to the +/+ mice (A) at p21. The somas of the GFAP-stained astrocytes were bigger and the branching of their axons covered larger areas in the PVN of the *anx/anx* than the +/+ . The marked triangular area indicates the area of the PVN. GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, p21 = postnatal day 21, PVN = Paraventricular nucleus.

**Figure 5. Increase of GFAP-positive cells in the PVN of the *anx/anx* mice.** The *anx/anx* mice displayed significantly more GFAP immunolabeling than the +/+ mice at p21 after quantification with Image J. Data are presented as means +/- SD. Error bars indicate SD and * indicates p-value <0.05. GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, PVN = Paraventricular Nucleus, p21 = postnatal day 21, SD= Standard deviation.

**Arcuate Nucleus**

An apparent hypertrophy and hyperplasia of GFAP positive astrocytes were seen in the Arc region of the *anx/anx* mice (Figure 6). After quantifying the GFAP-labeling with Image J, a statistically significant increase of GFAP in the Arc region of the *anx/anx* mice was detected when compared to the +/+ mice (p=0.03) (Figure 7).
**Figure 6.** *anx/anx* mice displayed an astrogliosis-like pattern in the Arc. A representative picture of Arc after GFAP IHC revealed increased GFAP-immunoreactivity in the *anx/anx* (B) compared to the +/+ mice (A) at p21. The somas of the GFAP-stained astrocytes were bigger and the branching of their axons covered larger areas in the Arc of the *anx/anx* than the same area of the +/+. The marked triangular areas indicate the area of the Arc. Arc = Arcuate Nucleus, GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, p21 = postnatal day 21.

**Figure 7.** Increase of GFAP-positive cells in the Arc of the *anx/anx* mice. The *anx/anx* mice displayed significantly more GFAP immunolabeling than the +/+ mice at p21 following quantification with Image J. Data are presented as means +/- SD. Error bars indicate SD and * indicates p-value <0.05. GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, Arc = Arcuate Nucleus, p21 = postnatal day 21, SD = Standard deviation.

**Medial preoptic area**

An apparent hypertrophy and hyperplasia of GFAP positive astrocytes was seen to some degree in the MPA of the *anx/anx* mice (Figure 8). No statistically significant difference in GFAP immunolabeled astrocytes in the MPA region of the *anx/anx* mouse could be shown.
after quantifying the fluorescence with Image J (p=0.14). However, a trend towards an increase of GFAP-stained astrocytes was recorded in the anx/anx mice (Figure 9).

**Figure 8.** anx/anx mice displayed an astrogliosis-like pattern in the MPA. A representative picture of MPA after GFAP IHC revealed increased GFAP-immunoreactivity in the anx/anx (B) compared to the +/- (A) at p21. The somas of the GFAP-stained astrocytes were bigger and the branching of their axons covered larger areas in the MPA of the anx/anx than the same area of the +/- . The marked triangular area indicates the area of the MPA. GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, MPA = Medial preoptic area, p21 = postnatal day 21.

**Figure 9.** Increase of GFAP-positive cells in the MPA of anx/anx mice. The anx/anx mice displayed more GFAP immunolabeling than the +/- mice at p21 after quantification with Image J. This increase in immunolabeling was however not statistically significant. Data are presented as means +/- SD (indicated by error bars). GFAP = Glial fibrillary-acidic protein, IHC = Immunohistochemistry, MPA = Medial preoptic area, p21 = postnatal day 21, SD = Standard deviation.
Discussion

This study was aimed to determine whether astrogliosis occurs to a higher degree in the hypothalamus of anx/anx mice compared to +/+ mice by using GFAP as a marker in IHC experiments. To our knowledge, this is the first study that has determined the presence of astrogliosis in the hypothalamus of the anx/anx mouse model. The results showed a significant increase of astrocytes at p21 in the PVN and the Arc of the hypothalamus, while no statistical significance could be shown in the MPA, which might be due to low sample size or methodological issues.

Astrogliosis in the anx/anx mouse model

Neuroinflammation has been documented in animal models and humans with eating disorders, e.g. obesity and AN (25, 42, 47). Astrogliosis is a crucial component in neuroinflammation with complex and heterogeneous physiological mechanisms (38) which has been poorly studied in the anorectic brain. Neurodegeneration could be either the cause or effect of the revealed astrogliosis in the anx/anx hypothalamus, which in turn may lead to a malfunction of the hunger and satiety centers and thus cause the self-starvation observed in this animal model.

Possible causes and effects of astrogliosis in the anx/anx mouse

The astrogliosis occurring in the anx/anx mouse may be attributed to protective or detrimental processes. This dilemma occurs since the activated astrocytes seen in astrogliosis are heterogeneous in their gene expression patterns and may act differently depending on environmental factors (39). Microglia are one important controller of these environmental factors and earlier studies have shown that activated microglia, and hypothalamic degeneration, is present in the hypothalamus of the anx/anx mouse (1). One possible theory is that the activated microglia create a pro-inflammatory environment which in turn activates the astrocytes leading to an accelerated degeneration of the axons and neurons of the hypothalamus. Another possible hypothetic outcome of this scenario is that the astrogliosis occurs in order to protect the neurons from detrimental effects caused by the microglia. A dysfunction of mitochondrial complex I has been observed in the brain of anx/anx mouse which in turn leads to increased oxidative stress (42). Oxidative stress has been shown to increase the rate of activated astrocytes (41) and may also trigger activation of microglia that in turn, as earlier mentioned, may initiate astrogliosis.
Astrogliosis, anorexia and autism

Patients with autism have been reported to show signs of astrogliosis (49, 50) and interestingly autism is a diagnosis occurring more frequently in patients with AN than in the general population (9). In addition, autistic individuals often report problems with feeding behavior (51). Since we have provided evidence for astrogliosis in an anorectic model, mechanisms that are triggered by reactive astrocytes could be one potential link between these two diseases. One theory may be that the reactive astrocytes release molecules or modify the synaptic activity of certain neurons, which could predispose for autistic behavior and anorectic feeding patterns.

Astrogliosis, anorexia and diabetic phenotypes

The fact that similar neuroinflammatory activity has been shown in models for both positive (e.g. diabetes and obesity) and negative (e.g. anorexia) energy balance has been a dilemma within the field the last decade. Recent findings which show that the anx/anx mouse is hyperglycemic (Nilsson et al, not unpublished data) could however open up for further understanding of the state of the hypothalamus and the possible defense mechanisms against hyperglycemia occurring in this brain area. However, high sucrose diet, and thus high blood glucose, has earlier been shown by Fuente-Martin et al to decrease the rate of astrogliosis in the hypothalamus of mice (51). A possible explanation for this dissimilarity is that high blood glucose caused by inadequate insulin signaling may involve a different mechanism in the hypothalamic area compared to high blood glucose caused by a high sucrose diet as in Fuente-Martin et al study. As high glucose levels have been observed in the anx/anx mouse one possible hypothesis could be that this high flow of glucose activates the astrocytes of the Arc area. This might be theoretically possible since astrocytes are the cells responsible for glucose transport into the hypothalamus of the brain, and an abnormally high activation of their insulin receptors might cause a reactivity of the astrocytes that in turn might lead to astrogliosis. Additionally this area is where the BBB is most permeable and thus hypothetically most sensitive for high glucose concentrations in the peripheral blood.

Astrogliosis and free fatty acids

Several studies have provided evidence of a connection between obesity and astrogliosis in the hypothalamus e.g. (25, 47). The findings of this study could seem contradictory to these
previous conclusions. However, the rate of free fatty acids (FFA) as an underlying factor to the development of astrogliosis could provide an explanation. FFAs are able to cross the BBB and a high level of these polar molecules may be present due to excessive breakdown of fat deposits (i.e. anorectic condition) but also due to high levels of fat deposits (i.e. obese and diabetic conditions). Patients with AN have been shown to have a higher rate of unsaturated FFAs and total cholesterol in the blood compared to healthy control groups (53, 54). Additionally, lipoproteins obtained from AN patients have been shown to induce higher oxidative stress in astrocytes (55), which supports the hypothesis that FFAs could be an underlying factor for the development of astrogliosis. If the anx/anx mouse would also display increased rates of FFAs this could explain the earlier mentioned hyperglycemia of this animal model, as high rates of FFAs is known to play a role in the risk of developing diabetes (56). Future studies of the rate of FFAs in the anx/anx mouse compared to the rate of FFAs in obese mice may provide further information to be able to confirm this hypothesis.

**Strengths and limitations of the study**

The methods used in the present study are well known and extensively used, and the anx/anx mouse is a well-established model for anorectic conditions. The usage of GFAP as a marker for astrocytes, as well as the large variation within the sample groups may however be discussed.

**Strengths**

The strengths of the study are the usage of a well-known and validated method and additionally the unique mutation of the employed animals (anx/anx), since no external factors are needed to induce the phenotypic traits of anorectic conditions. The TSA method is widely used for amplification of weak signals in IHC. However it is important to follow a well-designed protocol and use the correct antibodies as well as the correct concentrations in order to not get incorrect binding and thus incorrect results (i.e. background fluorescence). The protocol followed in this study (57) has been developed, revised and optimized for many years in the lab of Prof. T. Hökfelt, and may thus be confirmed as valid.

**Limitations**

The primary limitation of this study is the low number of animals employed. Even though the usage of inbred mice enables a low number of mice, inclusion of a larger amount of animals
would likely give more power and less fluctuating results. Maybe a statistical increase of astrogliosis in the MPA also could have been detected if there were more animals used in each group. The large variation within each sample group is the “Achilles heel” of the results of the study as it could mean that the differences seen may not be as significant in the populations as in the samples. This limitation could however also potentially be eliminated with the usage of a larger amount of animals. The usage of GFAP as a marker has been widely discussed, as it has been shown to not be specific enough for activated astrocytes (36). GFAP is a sensitive marker but not all activated astrocytes express GFAP (41). However, so far no other marker has been scientifically proven to be superior. The results of this study are not less valid despite the lacking specificity of GFAP as a marker since there are two different groups of the same species and breed being compared. So even if the GFAP marker is not as specific as expected, the difference between the +/+ and the anx/anx group is still significant as the lack of specificity is equal for the two groups. It would however be interesting to perform another study with the same layout and use other markers for astrocytes (e.g. vimentin and/or nestin), to see if the patterns of astrogliosis would differ significantly from the findings of this study. Another important aspect of the usage of GFAP as a marker is the varying expression of the protein under the circadian light-dark cycle (58). Even though the majority of the samples came from tissues from mice that were sacrificed during midday, all samples cannot be accounted for. Methodological issues occurred during the implementation of this study, as the precise area of the MPA was difficult to identify in the microscope. In some of the tissues the exact area of the MPA had not been sectioned on to the glasses, and thus areas nearby the center of the MPA were used. This might be a contributing factor to the lacking significance of astrogliosis in the MPA.

**Significance**

This is the first study, as earlier mentioned, which has been able to show that the anx/anx mouse displays signs of astrogliosis in the hypothalamus at postnatal day 21. This is indeed an important discovery, as astrogliosis of the hypothalamus seems to be a part of the pathophysiology of both obese and anorectic subjects. However, an important limitation of the findings of this study is the large variation of astrogliosis within the sample groups, which significantly restricts the impact of the results. The main importance of the results of this study is that a new possible path of the neurobiological mechanism behind anorexia has been
found, and further understanding of it may in the future aid in the search for new therapeutics, based on the modulation of astrocytes, for the treatment of anorexia-related diseases.

**Future studies**

Further studies are needed to evaluate what the cause and the effect of our results are. We are currently performing IHC experiments on younger brain tissues (postnatal day 0-15) in order to determine if it is the astrocytes or the microglia that are activated first in the hypothalamus of the *anx/anx*. The levels of FFAs and lipoproteins in the *anx/anx* are also an important feature to perform further studies on in order to understand the mechanism behind astrogliosis. Measurement of the rate of GFAP micro-RNA (mRNA) in the hypothalamic area of the *anx/anx* mouse as well as usage of other markers for astrogliosis would also be desirable to further determine the rate of astrogliosis in this animal model for anorexia. Human fMRI and/or IHC studies of the hypothalamus of AN patients are additionally needed to establish whether the findings of this study are significant also in human anorectic conditions.

**Conclusions**

Hypothalamic inflammation has been reported to be related to eating disorders (1, 4, 10, 23). Our results showed that the *anx/anx* mouse displays signs of astrogliosis (an indication of neuroinflammation) in the hypothalamic region. By providing evidence that astrogliosis occurs in this model for anorexia, an additional role for glial cells in eating disorders, including anorexia and obesity, has been revealed. Further studies of younger *anx/anx* mice and e.g. the lipid profile of this animal model as well as the rate of GFAP mRNA are needed to explore the cause and effect of astrogliosis occurring in the hypothalamic region.

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References


