Evaluation of Phenotypic Changes of Acyl-CoA Binding Protein / Diazepam Binding Inhibitor Overexpression in Transgenic Mice and Rats

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L1, Canthia building, University of Kuopio, on Friday 3rd October 2008, at 1 p.m.

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio
ABSTRACT

Obesity and metabolic syndrome are growing health problems worldwide and are closely linked to fatty acid metabolism. Elevated free fatty acid levels are observed in both disorders. Recent studies have shown that fatty acids or their metabolites are linked to the pathophysiology of various diseases associated with obesity and metabolic syndrome. In the cytosol, fatty acids are converted to acyl-CoA. Acyl-CoA are important intermediates of lipid and glucose metabolism and they have been shown to function as regulators of many metabolic processes, therefore their intracellular availability is tightly controlled. This is achieved by the presence of binding proteins e.g. fatty acid binding proteins (FABPs), acyl-CoA binding protein (ACBP) and sterol carrier protein-2. Of these proteins, ACBP has the highest affinity and specificity towards medium and long chain acyl-CoAs. ACBP is a 10 kDa protein that is ubiquitously expressed throughout the body and it has been shown to be able to function as an intracellular pool former and transporter of acyl-CoAs. In addition, various other cellular functions have been proposed for ACBP, including inhibition of diazepam binding to benzodiazepine receptors. Therefore, ACBP has also been named as diazepam binding inhibitor (DBI). Although, ACBP has been associated with several biochemical processes at the cellular level, its physiological significance in mammalians is less clear. In order to reveal the physiological effects of this interesting protein we have created a mouse and rat lines overexpressing the endogenous mouse ACBP gene under the gene’s own promoter region.

Our results show that ACBP overexpressing mice display an enlargement of the lateral ventricles, decreased plasticity of excitatory synapses and impairment of hippocampus-dependent form of learning and memory. In contrast to previous results, our animals did not show any signs of anxiety or pro-conflict behaviour nor did the ACBP overexpression influence the kainate or pentylenetetrazole induced seizure activity of transgenic mice. Furthermore, it was shown that in rats ACBP increased the liver and adipose tissue acyl-CoA pool size. The ACBP induced elevation was dependent on the acyl-CoA form and on the fed-fasted state of the animals. On the contrary, high levels of ACBP did not influence serum or plasma levels of other lipid forms. In addition, constant overexpression affected the glucose tolerance of transgenic animals only if these animals were challenged with a high fat diet enriched with medium chain fatty acids for four weeks. To further investigate the possible mechanisms behind these physiological changes we studied ACBPs influence on the mRNA levels of transcription factors involved in the regulation of lipid metabolism and on the protein levels of AMP-activated protein kinase (AMPK). Our results demonstrated that ACBP influences PPARγ, PPARδ and SREBP-1 expression of the transgenic animals in a nutrition state dependent manner and that the regulation of SREBP-1 is mediated through increased AMPK protein levels. Furthermore, our results indicated that the constantly elevated levels of ACBP does not influence food intake or the body weight of the transgenic animals. ACBP regulates the hypothalamic mRNA levels of two key enzymes of fatty acid metabolism: fatty acid synthase is down-regulated and carnitine palmitoyltransferase 1 up-regulated. In the hypothalamus, inhibition of these enzymes is associated with reduced food intake. In addition, ACBP down-regulates the hypothalamic expression levels of PPARδ and SIRT-1, transcription factors regulating lipid and glucose metabolism.

These results demonstrate that long-term overexpression ACBP has a limited effect on the phenotype of transgenic mice and rats, since it does change the expression pattern of particular genes involved in regulation of glucose and lipid metabolism. Our findings provide new information on the physiological role of ACBP, especially in the regulation of transcriptions factors.
ACKNOWLEDGEMENTS

This thesis work was carried out at the A.I. Virtanen Institute for Molecular Sciences during the years 2002-2008. I wish to express my profound gratitude to Professor Karl-Heinz Herzig, M.D., Ph.D. for introducing me to this fascinating research subject and for all the support and guidance during these years. You have though me a valuable way to look at science in wider perspective.

I wish to thank also Prof. Paul Trayhurn, D.Sc., FRSE and Prof. Kalervo Hiltunen, M.D., Ph.D. for the review of this thesis, your expert comments and suggestions were of great help. Ewen Macdonald, Ph.D. is gratefully acknowledged for the language revision.

My sincere thanks belong to all co-authors of the publications. Prof. Leena Alhonen, Ph.D. and now deceased Prof. Juhani Jänne, M.D., Ph.D. are acknowledged for creation of the animal lines and Veli-Pekka Korhonen, Ph.D. and Tiina Wahlfors, Ph.D. for the isolation of ACBP gene. My sincere thanks belong for Hanna Siiskonen, M.Sc., B.Med. for her great contribution for the mouse paper. I’m deeply grateful also for Tiia Ahtialansaaari, M.Sc., B.Med. for all your help and stimulating conversations during the rat studies. I wish to thank Miika Heinonen, M.Sc., B.Med. and Anne Huotari, M.Sc. for your valuable help, especially in animal handling and for the moments shared during these years. Prof. Seppo Auriola, Ph.D., Prof. Asla Pitkänen, M.D., Ph.D., Prof. Siegfried Wolffram, D.V.M., Ulrich Fölsch, M.D., Juhana Hakumäki, M.D., Selma Kaasinen, Ph.D., Mikko Kettunen, Ph.D., Karlheinz Kiehne, M.D., Timo Mauriala, Ph.D., Markku Penttonen, Ph.D., Raimo Pussinen, Ph.D., Vootele Voikar, M.D., are acknowledged for their contribution to the publications.

My sincere thanks are owned to all the members of Molecular Physiology Research Group. I’m deeply grateful to Riitta Kauppinen, Medical Laboratory Technologist for all the help in lab issues and arranging things. My sincere thanks to Anna-Kaisa Purhonen, Ph.D., B.Med. for all the help and refreshing conversations on science and life in general. Thanks also to Miia Kilpeläinen, M.Sc. (Pharm), Katja Klausz, M.Sc. Kari Mäkelä, M.Sc., Maria Vlasova, Ph.D., for help and moments shared during these years.

Many thanks also to Mrs Kaija Pekkarinen, Mrs. Riitta Laitinen and Mrs. Helena Pernu for secretarial help, you were always willing to assist in arranging things. Pekka Alakuijala, Phil. Lic. and Jari Nissinen, Ph.D. are acknowledged for all the help in solving practical things.

From the world outside science, I would like to thank all my relatives and friends. I’m deeply grateful for my parents Raili Pääkkönen and Reino Uotila for all your love, encouragement and support. Many thanks also to Karin and Seppo Oikari for your help and to Virpi, Mika, Anne, Teemu, Sirpa and Petri for your friendship and great times during the years.

Finally, my warmest thanks go to my loving husband Sami and to our children Laura and Arttu. Thank you for being there for me whatever comes and for all the support and patience during these years. Hopefully the years to come are as good as they have been.

This study was supported in part by the Finnish Cultural Foundation of Northern Savo.

In Kuopio, August 2008
ABBREVIATIONS

ACBP       acyl-CoA binding protein
ACC        acyl-CoA carboxylase
ACS        acyl-CoA synthetase
AMPK       AMP-activated protein kinase
ANOVA      analysis of variance
ANT        adenine nucleotide translocase
BMI        body mass index
CCK        cholecystokinin
cDNA       complementary DNA
CNS        central nervous system
CPT-1       carnitine palmitoyltransferase 1
CSF         cerebrospinal fluid
DBI        diazepam binding inhibitor
ER         endoplasmic reticulum
FABP       fatty acid binding protein
FAS        fatty acid synthase
FFA        free fatty acid
GABA       γ-aminobutyric acid
GABA_A     type A γ-aminobutyrate receptor
GTT        glucose tolerance test
HNF-4α      hepatocyte nuclear factor-4α
i.c.v       intracerebroventricularly
i.p.        intraperitoneally
KA         kainate
LC         long chain fatty acids
LTP        long-term potentiation
MC         medium chain fatty acids
MRI        magnetic resonance imaging
mRNA       messenger ribonucleic acid
ODN        octadecaneuropeptide
PCR        polymerase chain reaction
PPAR       peroxisome proliferator-activated receptor
PBR        peripheral benzodiazepine receptor
PTZ        pentylenetetrazole
RNA        ribonucleic acid
sg         syngenic
SIRT       sirtuin
SNP        single nucleotide polymorphism
SREBP      sterol regulatory element-binding protein
tg         transgenic
Thr        threonine
TTN        triakontatetraneuropeptide
LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to by their corresponding Roman numerals:


IV Oikari S, Huotari A, Mauriala T, Auriola S, Purhonen AK, Heinonen MV, Kiehne KH, Fölsch UR, Alhonen L, Herzig KH. Role of ACBP overexpression on candidate genes (PPAR, SREBP-1, Sirt-1, AMPK, FAS and CPT-1c) involved in fatty acid metabolisms in rat hypothalamus. Manuscript.

*Equal contribution
# TABLE OF CONTENTS

1. INTRODUCTION ........................................................................................................ 15

2. REVIEW OF THE LITERATURE ................................................................................. 17
   2.1 Acyl-CoAs ........................................................................................................... 17
   2.2 ACBP .................................................................................................................. 18
       2.2.1 ACBP gene ................................................................................................. 18
       2.2.2 ACBP protein ............................................................................................. 20
       2.2.3 ACBP expression ......................................................................................... 21
       2.2.4 Functions of ACBP ..................................................................................... 23
           2.2.4.1 Functions associated with receptor binding or central nervous system ........................................................................ 23
               2.2.4.1.1 Receptor binding .......................................................................... 23
               2.2.4.1.2 Implications of benzodiazepine receptor binding ..................... 26
               2.2.4.1.3 Other central nervous system related functions ......................... 28
           2.2.4.2 Peripheral and acyl-CoA binding associated functions ................. 28
               2.2.4.2.1 Acyl-CoA binding .................................................................. 28
               2.2.4.2.2 Regulation of transcription factors ........................................... 32
           2.2.4.3 Other described functions of ACBP .................................................. 33
   2.3 Transcription factors ......................................................................................... 35
       2.3.1 Peroxisome proliferator-activated receptors ............................................ 35
       2.3.2 Sterol regulatory element binding proteins ................................................. 36
   2.4 AMP-activated protein kinase .......................................................................... 38

3. AIMS OF THE STUDY .............................................................................................. 40

4. MATERIALS AND METHODS .................................................................................. 41
   4.1 Isolation of mouse ACBP/DBI gene ................................................................. 41
   4.2 Animals .............................................................................................................. 41
   4.3 Western blotting ............................................................................................... 43
   4.4 Real-Time Quantitative PCR ............................................................................ 43
   4.5 Histological and morphological analysis .......................................................... 44
       4.5.1 Immunohistochemistry ............................................................................. 44
       4.5.2 Cellular localization of DBI ..................................................................... 45
       4.5.3 Measurement of ventricle areas ............................................................... 45
   4.6 High resolution magnetic resonance imaging (MRI) ....................................... 45
   4.7 Behavioural testing ............................................................................................ 45
       4.7.1 Fear conditioning (FC) ............................................................................. 46
       4.7.2 Water maze (WM) .................................................................................... 46
   4.8 Synaptic transmission and induction of long-term potentiation (LTP) ............ 47
1. INTRODUCTION

Obesity is a major health problem worldwide. During the last years its prevalence has increased at such an alarming rate that has been described even as an epidemic. According to World Health Organisation (WHO) in year 2005, worldwide approximately 1.6 billion adults (over 15 year old) were overweight (body mass index (BMI) over 25) and of these 400 million were obese (BMI over 30 in Western population or BMI over 27 in East Asian population). An even greater concern is that worldwide at least 20 million children under 5 years old are overweight. Traditionally obesity has been considered as a problem only in the Western countries, but recent development has been the dramatic increase in the numbers of obese people also in the low- and middle-income countries. In Finland, recent report revealed that approximately 57% of men and 43% of women are overweight and that about 15% of both men and women are obese (Helakorpi et al., 2008). Obesity has been associated with various diseases such as type II diabetes, heart disease, stroke, hypertension and some cancers, to mention only a few. In addition to obesity, these diseases can also be considered as a part of metabolic syndrome, a condition characterized by a group of metabolic risk factors accumulating in one person. These include abdominal obesity, high blood triglyceride levels, low blood HDL cholesterol levels, elevated blood pressure and insulin resistance or glucose intolerance. The prevalence of metabolic syndrome and obesity go hand in hand, although a person with metabolic syndrome is not necessarily obese. Central obesity is more important than BMI in the definition of metabolic syndrome (The IDF consensus worldwide definition of the Metabolic syndrome, 2006).

Both obesity and metabolic syndrome are closely linked to fatty acid metabolism. Hence it is not surprising that various enzymes and other factors regulating the metabolism of fatty acids have been associated with the pathology of these diseases. The fact that fatty acids and their derivates have been shown to be involved in the regulation of cellular metabolism makes them extremely interesting in understanding the cellular changes in several diseases (Jump et al., 2005). In the cytosol, free fatty acids are converted to acyl-CoAs. Acyl-CoAs are important intermediates of lipid and glucose metabolism, but in addition they have been shown to function as regulators of various metabolic processes (Corkey et al., 2000; Faergeman and Knudsen, 1997). Especially the role of fatty acids and acyl-CoAs in the regulation of gene expression via their influence on different transcription factors has lately been associated with the pathophysiology of various diseases. In the cytosol, acyl-CoAs bind to proteins e.g. fatty acid binding proteins (FABPs), acyl-CoA binding protein (ACBP) and sterol carrier protein-2. These proteins act as pool formers and transporters of intracellular fatty acids and acyl-CoAs. The binding properties and their ability to influence metabolic and regulatory functions of fatty acids and acyl-CoAs are different for each of these proteins. The role of FABPs in the regulation of gene expression has gained much interest. Not only FABPs but also ACBP has been shown to modulate the regulatory functions of acyl-CoAs (Knudsen et al., 2000; Schroeder et al., 2008).
Acyl-CoA binding protein (ACBP) is a 10 kDa protein that binds acyl-CoAs with high affinity and specificity. It is believed to function as an intracellular acyl-CoA pool former and transporter (Knudsen et al., 1993). In addition, ACBP can influence the regulatory functions of acyl-CoAs. It can release acyl-CoA induced inhibition of certain enzymes and moreover can influence acyl-CoA mediated regulation of gene expression via peroxisome proliferator-activated receptors (reviewed by Schroeder et al., 2008). A recent study has also indicated that ACBP can influence transcriptional regulation of lipid metabolism through hepatocyte nuclear factor-4α (HNF-4α)(Petrescu et al., 2003). In addition to its important role in lipid metabolism, ACBP has been shown to displace diazepam from its binding sites on the type A γ-aminobutyrate receptors (GABA_A) and therefore the protein has also been named as diazepam binding inhibitor (DBI) (Guidotti et al., 1983). Although there is extensive data on the role of ACBP in many different processes, the detailed physiological importance and effects of the endogenously expressed form of this versatile protein are still unclear. This study aimed to investigate the different physiological aspects of ACBP as well as the molecular mechanism behind them.
2. REVIEW OF THE LITERATURE

2.1 Acyl-CoAs

Fatty acids are normally an important source of metabolic energy, but they also act as substrates for membrane biogenesis and as storage forms of energy. Free fatty acids enter the cells either by diffusion or by protein-mediated transport and are esterified to acyl-CoAs by Acyl-CoA synthetase (ACS). Acyl-CoAs are then bound and transported by intracellular proteins such as fatty acid binding proteins (FABPs), acyl-CoA binding protein (ACBP) or sterol carrier protein-2. Acyl-CoAs are the active forms of fatty acids that can further be utilized in metabolic pathways. In addition to the extracellular import of free fatty acids combined to esterification, acyl-CoAs can be formed in biosynthesis from citrate through acetyl-CoA and malonyl-CoA. The main metabolic pathways that utilize acyl-CoAs include the formation of energy in mitochondrial β-oxidation, back-conversion to free fatty acids and usage as glycerolipids like triacylglycerols and phospholipids in membrane formation. In addition, intracellular acyl-CoA concentrations are also regulated by the activity of acyl-CoA hydrolases found in most subcellular locations. The concentration of acyl-CoAs is reported to be in the range of 5-160 μM, depending on tissue and metabolic state (reviewed by Faergeman and Knudsen, 1997). There are several factors e.g. fasting, diets, diseases such as diabetes and some drugs, which have been reported to influence the intracellular acyl-CoA levels. There are substantial differences also in the subcellular concentrations. In addition to being intermediates of cellular metabolism, acyl-CoAs have been shown to regulate processes like lipid and energy metabolism and signal transduction. In metabolism, acyl-CoAs have been shown to either inhibit or to stimulate many enzymes such as acetyl-CoA carboxylase, AMP-activated protein kinase and acyl-CoA synthetase. In signal transduction, acyl-CoAs are indicated to regulate membrane trafficking, ion channels and ion pumps and dependent on the subtype either stimulate or inhibit the function of protein kinase C (reviewed by Corkey et al., 2000; Faergeman and Knudsen, 1997). Recent data has also shown that acyl-CoAs are involved in the regulation of gene expression through transcription factors like peroxisome proliferator-activated receptors and HNF-4α (Faergeman and Knudsen, 1997; Schroeder et al., 2008).

![Figure 1. Acyl-CoAs: their role in cellular metabolism and processes that are regulated by acyl-CoAs. FFA = free fatty acids, TG = triglycerides, FAS = fatty acid synthase, CPT-1 = carnitine palmitoyltransferase 1.]
2.2 ACBP

ACBP is a 10 kDa protein that binds acyl-CoA esters (C14 to C22) with high specificity and affinity (Rasmussen et al., 1990; Rosendal et al., 1993). The same, identical protein was also isolated from rat brain by its ability to displace diazepam from its binding sites on type A γ-aminobutyrate receptors (GABA<sub>A</sub> receptors) and consequently named as diazepam binding inhibitor (DBI) (Guidotti et al., 1983). ACBP homologues have been identified from all eukaryotics studied, including animals, plants, protozoa and algae. Even though ACBP homologues have not been identified in most of the bacteria and archaea species, some pathogenic eubacterial species have genes that resemble ACBP (Burton et al., 2005). The ACBP protein is highly conserved across species; the rat and mouse proteins are 98.8% identical, while there is 79.3% similarity between rat and human proteins. In addition, ACBP has a broad tissue distribution, it has been found in all mammalian tissues investigated. This suggests that ACBP is associated with one or more basic cellular functions.

2.2.1 ACBP gene

The mouse, rat and human ACBP genes consists of four exon regions and three introns (Fig. 3), but the size of the gene varies from 5.6 kb in humans to 8.7 kb in rats. Also the chromosomal location of functional ACBP gene varies from species to species. In rats, ACBP is located in chromosome 13, while in humans it is in chromosome 2 and in mouse in chromosome 1. The rat ACBP gene family consists of one expressed, functional gene and four additional processed pseudogenes of which one was shown to exist in two allelic forms (Mandrup et al., 1992). Although multiple transcription initiation sites were observed in the rat ACBP gene, there is no evidence for alternative splicing and hence only one variant coding for an 87 amino acid protein is expressed (Mandrup et al., 1992). Unlike rats, both humans and mice have multiple variants of the ACBP gene. The mouse ACBP gene can give rise to two splice variants that are reported to be due to alternative usage of exon 1. The two variants are 87 and 135 amino acid residues long and display differences in the N-terminal part of the protein (Nitz et al., 2005). In mouse, there are no reported pseudogenes, although there is a gene coding for endozepine-like peptide (ELP) that has over 50% protein similarity with ACBP. In addition, the ligand binding motifs of these two proteins are highly conserved. The ELP gene has only one intron in the 5' untranslated region and hence it has been suggested that ELP gene might have evolved by retroposon-mediated gene duplication of the ancient ACBP gene (Valentin et al., 2000). In humans, there are three variants of the ACBP gene. They encode for proteins of 86, 88 or 104 amino acid residues (Kolmer et al., 1995; Nitz et al., 2005). The differences are in the N-terminal part of the proteins and do not interfere with the functional acyl-CoA binding motifs. These alternative variants arise from differential usage of exon 1, pointing to the possibility of differential promoter usage.

The promoter region of the rat ACBP gene displays all the key features of a housekeeping gene (Mandrup et al., 1992). Nevertheless, it has been demonstrated that ACBP expression levels vary from
tissue to tissue, with the highest levels being found from tissues, which have a high lipid turn-over. Furthermore, hormones, like insulin and androgens, fasting, feeding and state of cell differentiation can influence the ACBP gene expression (Bhuiyan et al., 1995; Hansen et al., 1991; Swinnen et al., 1996). The rat and human ACBP genes are regulated by transcription factors that are involved in lipid metabolism. Intron 1 of the rat ACBP gene contains a peroxisome proliferator activated receptor (PPAR) response element (PPRE) that is conserved also in human and mouse genes (Helledie et al., 2002a). This PPRE binds and is activated by both PPARα and PPARγ, but not PPARδ (Neess et al., 2006; Sandberg et al., 2005). PPRE in the intron 1 deviates from the classical PPARα response element by having a guanine as a spacer between the two repeats instead of an adenine, this reduces the binding affinity of PPARα (Helledie et al., 2002a). ACBP is a PPARα regulated gene, which displays a discrepancy in its response to fasting. PPARα is involved in the fasting response by up-regulating the gene expression. In contrast to the other PPARα regulated genes, ACBP’s expression tends to be reduced by fasting (Bhuiyan et al., 1995). From rat ACBP gene, a second PPRE was located from the promoter region, but transient transfection studies revealed that it is not functional (Helledie et al., 2002a). In addition to PPARs, the involvement of CCAAT/enhancer binding protein (C/EBP) has been proposed, as there is a response element in the rat promoter region, but a more detailed investigation revealed that the rat ACBP gene is not activated by C/EBPα (Helledie et al., 2002a). Analysis of the human and rat ACBP promoter revealed the presence of a conserved sterol regulatory element (SRE)-like sequence and further studies confirmed that ACBP is activated by sterol regulatory element binding protein (SREBP) isoforms (Neess et al., 2006; Swinnen et al., 1998). The function of SREBP isoforms is enhanced by the auxiliary transcription factors SP-1 and nuclear factor-Y (NF-Y), whose binding sites are located upstream of the functional SRE element (Neess et al., 2006). The co-regulation with SREBP and PPARα might be a possible explanation for the reduction in ACBP transcription after fasting, since SREBP is known to be involved in the down-regulation of genes in response to fasting.

The human ACBP gene has been considered as a candidate gene for several diseases and the occurrence and association of single nucleotide polymorphism (SNP) of the ACBP gene has been investigated. Multiple missense mutations in the ACBP gene have been found in patients suffering from schizophrenia. A total of 18 novel SNPs were found, three of which caused missense changes in conserved amino acids. The case-control association analyses of the three major SNPs showed no significant association with the disease (Niu et al., 2004). SNPs of the ACBP gene have also been investigated from patients with anxiety disorders. The exonic SNP rs8192506 was found to be associated with anxiety disorders with panic attacks. The rare allele guanine in the SNP was overrepresented in the control group. The resulting Val88Met change was determined to have a protective role against anxiety disorders with panic attacks (Thoeringer et al., 2007). In addition, a significant association with type 2 diabetes and an SNP of the ACBP gene has been reported in two independent German study populations. A minor allele of SNP rs2084202 of the human ACBP gene was shown to be associated with a reduced
risk of the disease. Since the SNP is located in the promoter region of the ACBP slice variant 1-c, it was proposed that the SNP causing the adenine to guanine substitution could alter a site important for transcription regulation, thus affecting ACBP expression levels (Fisher et al., 2007).

2.2.2 ACBP protein

The ACBP gene codes for an approximately 10 kDa protein, depending on species and possible transcription variants. The protein is monomeric and forms a four-helix bundle structure. Either NMR or crystal structures have been published from bovine, *Plasmodium falciparum* and human ACBP (Andersen et al., 1991; Taskinen et al., 2007; van Aalten et al., 2001). All the known structures have the same four-helix bundle folding, with a topology of up-down-down-up. The helices A2 and A3 form a parallel helix pair with a 13 residue loop between them, also helices A1 and A3 are assembled as two parallel pairs (Fig. 2) The binding of acyl-CoA molecule induced only a few structural changes near to the binding pocket in all the structures studied (Kragelund et al., 1993; Taskinen et al., 2007; van Aalten et al., 2001). The acyl-CoA binding pocket can be divided into three parts, 1) the adenine ring binding pocket formed by Tyr 32 (human) and the acyl moiety of the acyl-CoA molecule. 2) The 3'-phosphate binding site formed by Tyr 29, Lys 33 and Lys 55 (human). 3) A hydrophobic groove for the acyl moiety binding formed by multiple amino acids (Taskinen et al., 2007). In all, 40% of the total binding affinity is accounted for by the interactions with the 3'-phosphate of the CoA part of the ligand (Faergeman et al., 1996; Kragelund et al., 1999). The most recent data on the structure of human ACBP protein revealed that in addition to a monomeric binding of acyl-CoA, ACBP can also bind to acyl-CoAs in a dimeric form where one ACBP molecule will bind the adenine part of the acyl-CoA and the ω-end of the acyl chain will be bound by a second ACBP molecule (Taskinen et al., 2007). This model might help to explain the mechanism by which ACBP is able to accept and donate acyl-CoA molecules from various targets such as membrane structures and enzymes.

In addition to the full-length ACBP protein, several processing products of the protein have been identified from rat brain and shown to have functional significance. These peptide fragments, which contain the same C-terminal structure originating from amino acids 33-50 of the native ACBP protein, are formed by posttranslational processing by endopeptidases and possess differential biological activities (Slobodyansky et al., 1989). Further studies have indicated that these peptides, commonly called endozepines, can also be found outside central nervous system. The most abundant and longest of these peptide fragments is triakontatetraneuropeptide, TTN which consists of amino acids 17-50 of native rat ACBP protein. In a hydrophobic environment, this peptide can adopt an α-helical structure unlike the other peptide fragments (Berkovich et al., 1990). A second processing product, which has been intensively studied, is called octadecaneuropeptide, ODN which consists of amino acids 33-50 of native rat ACBP protein. The least studied fragment is the eicosapentaneuropeptide, EPN which consist of amino acids 26-50 of the full-length ACBP protein (Ferrero et al., 1986b; Slobodyansky et al., 1989).
The occurrence of shorter fragments originating from these three processing products and their biological function has also been investigated.

### 2.2.3 ACBP expression

ACBP is ubiquitously expressed throughout the whole body, although there are significant differences in the expression levels from tissue to tissue. The highest levels are found from tissues with either high lipid metabolism or steroid synthesis. In certain tissues, ACBP expression is located in specialized cells. In the brain, the highest levels of either ACBP mRNA or ACBP-like immunoreactivity can be found in cerebellum, hypothalamus and reticular thalamic nucleus (Alho et al., 1985; Ball et al., 1989; Ferrarese et al., 1989). The ACBP expression is also localized in brain areas, in cerebellum the highest levels of ACBP are found in Bergmann glia, whereas levels are low in Purkinje and Golgi neurons. In the hypothalamus, the highest ACBP concentrations are found in the nerve terminals of the arcuate nucleus and median eminence (Alho et al., 1985; Alho et al., 1988). The ACBP protein and its processing products, ODN and TTN, have been detected in both neurons and in glial cells, although some suggest predominantly glial expression (Tong et al., 1991; Alho et al., 1995; Tonon et al., 1990). The amino acid sequence of rat ACBP does not have a apparent signal sequence for transmembrane passage (Mocchetti et al., 1986), nevertheless ACBP has been located in synaptic vesicles and it is released after nerve depolarisation in both rat brain tissue and in primary neuronal cultures (Costa and Guidotti, 1991). Recent data has shown that horizontal optokinetic stimulation of rabbit retina evokes an increased expression of ACBP from Müller cells. Furthermore, it was shown that threonine phosphorylated ACBP is also secreted from these cells after potassium chloride or phorbol myristic acetate stimulation. It is
likely that protein kinase C is responsible for the phosphorylation of ACBP (Qian et al., 2008). Unlike the situation in rats and humans, in Drosophila melanogaster ACBP is not expressed in the adult nervous system, only the larval and pupal brains of the species had detectable levels of ACBP (Kolmer et al., 1994). In the periphery, the highest ACBP levels have been found from liver, kidney, adrenal gland, intestine and adipose tissue. In liver, ACBP levels are high throughout the tissue, while in kidneys and adrenal gland there are specialised cells containing higher levels of ACBP expression compared to the rest of the tissue. In adrenal cortex, high concentrations of ACBP-like immunoreactivity were found in the cells of zona glomerulosa, while the levels in the zona fasciculata and reticulata were considerably lower (Bovolin et al., 1990). In kidneys, ACBP expression is concentrated in the epithelial cells of the convoluted tubules and the ascending limb of the loop of Henle (Bovolin et al., 1990). High levels of ACBP were also found in the Leydig cells of testis (Schultz et al., 1992). In the muscle tissue, which has a relatively low expression of ACBP, it was observed that ACBP levels are different, depending on the muscle fiber type. The highest levels were found in slow-twitch oxidative soleus muscle and lowest from fast-twitch glycolytic white gastrocnemius. The ACBP expression in muscle correlated with the expression of carnitine palmitoyltransferase-1 (CPT-1) expression levels and it was found to be increased in obese Zucker rats (Franch et al., 2002).

**Figure 3.** Main characteristics of the rat ACBP gene (A) and protein (B and C). The rat ACBP gene consists of four exons as indicated by numbered boxes (A). Gray boxes indicate known transcription regulation sites. The rat ACBP protein is a 87 amino acid peptide with three known processing products, the length and relative location in the native peptide being shown (B). Section C summarizes the structural and functional differences between the native ACBP protein and the two processing products. PPRE= peroxisome proliferator activated receptor (PPAR) response element, SRE= sterol regulatory element, NF-Y= nuclear factor-Y, TTN= triakontatetrapetide, ODN= octadecaneuropeptide, EPN= eicosapentaneuropeptide, PBR= peripheral benzodiazepine receptor.
Traditionally ACBP is considered as a cytosolic protein, although it has been shown to be secreted after nerve depolarisation in both rat brain tissue and in primary neuronal cultures (Costa and Guidotti, 1991). In the cytosol of mammalian cells, fluorescently labelled ACBP was shown localize mainly to endoplasmic reticulum (ER) and Golgi (Hansen et al., 2007). This localization was found to be ligand-binding dependent; a mutated ACBP unable to bind acyl-CoAs did not show a similar localization as the normal protein. Furthermore, depletion of fatty acids from cells significantly reduced the ACBP localization to Golgi while fatty acid overloading enhanced the association (Hansen et al., 2007). In addition to cytosolic localization, it has been shown that ACBP as well as its ligands, the acyl-CoAs, are present in the nucleus of rat liver and hepatoma cells (Elholm et al., 2000). Further experiments confirmed nuclear localization of ACBP also in 3T3L1 adipocytes and showed that the expression of ACBP in CV-1 cells resulted in substantial accumulation in nucleus (Helledie et al., 2000).

2.2.4 Functions of ACBP

Originally ACBP was identified as a putative modulator of type A γ-aminobutyrate (GABA_\text{A}) receptors by Guidotti et al. (1983). Further studies revealed that ACBP can modulate also the function of peripheral-type benzodiazepine receptors (Besman et al., 1989; Guidotti et al., 1983; Yanagibashi et al., 1988) and metabotropic receptors that are positively coupled to phospholipase C via a pertussis toxin-sensitive G protein (do Rego et al., 2007). An identical protein was independently isolated from bovine liver by Morgensen et al. (1987) and was shown to bind long-chain acyl-CoA esters. Over the years, further effects have been added to the list of putative functions, e.g. inhibition of glucose induced insulin release from pancreas (Chen et al., 1988), cholecystokinin (CCK) release in intestine (Herzig et al., 1996) and calpain activation in cell death (Shulga and Pastorino, 2006).

2.2.4.1 Functions associated with receptor binding or central nervous system

2.2.4.1.1 Receptor binding

Type A γ-aminobutyrate (GABA_\text{A}) receptors are heteropentameric membrane proteins that form ligand-gated ion channels that respond to gamma-aminobutyric acid (GABA). Binding of GABA to the synaptic recognition site on the receptors leads to opening of anionic channels, allowing the influx of Cl⁻ ions (reviewed by Bormann 1988). In addition to the recognition site for GABA, these receptors have multiple modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol (Bormann, 1988). The vast majority of the GABA_\text{A} receptors are characterized by their sensitivity to benzodiazepines, although this response is dependent on the subunit structure of the receptor, as this heteropentameric peptide can be formed from several classes of subunits (\(\alpha_1-6, \beta 1-3, \gamma 1-3, \delta, \varepsilon, \pi\) and \(\rho 1-3\)) with different modulatory properties (reviewed by Rudolph and Mohler, 2006). Early studies identified at least two different allosteric modulatory sites for benzodiazepines in GABA_\text{A} receptors that are relevant for the action of ACBP. One site binds the anxiolytic benzodiazepines (diazepam) and the
anxiogenic β-carboline carboxylate esters. In addition, it can bind the benzodiazepine antagonist flumazenil (reviewed by Costa and Guidotti, 1991). The second site can bind a convulsant benzodiazepine, 4'-chlorodiazepam. The function of this modulatory site is resistant to flumazenil inhibition (Costa and Guidotti, 1991). Originally ACBP was shown to inhibit binding of \[^{3}\text{H}]\text{diazepam}\) to GABA\(_A\) receptors, thus it was named diazepam binding inhibitor (DBI) (Guidotti et al., 1983). In subsequent studies, ACBP was shown to inhibit also \[^{3}\text{H}]\text{methyl-beta-carboline-3-carboxylate},\[^{3}\text{H}]\text{flumazenil}\) and \[^{3}\text{H}]\text{PK 11195}\) binding to various allosteric modulatory centres of GABA\(_A\) receptors (Barbaccia et al., 1988). ACBP did not affect the gating of the GABA\(_A\) receptor in the absence of GABA, but was demonstrated to be a negative modulator of GABA induced Cl\(^-\) ion conductance in primary cultures of spinal cord neurons and in neurons prepared from mouse embryos. The effect of ACBP was shown to be dose dependent (at dosages 1 to 10 μM) and was inhibited by flumazenil (Bormann et al., 1985; Bormann, 1988). From further pharmacological profiling with recombinant rat GABA\(_A\) receptors expressed in kidney cells, it was concluded that ACBP could function as a partial intrinsic negative modulator of GABA\(_A\) receptors (Costa and Guidotti, 1991).

In addition to GABA\(_A\), the peripheral benzodiazepine receptor (PBR, also known as translocator protein and mitochondrial benzodiazepine receptor) has been found to bind benzodiazepines. PBR is wildly expressed throughout the body, the highest levels are found in steroid producing tissues. In the central nervous system, PBR expression is restricted to ependymal and glial cells. Unlike GABA\(_A\), PBRs are intracellular receptors located mainly at mitochondrial outer membrane. The described functions of this receptor include regulation of steroidogenesis and apoptosis (reviewed by Casellas et al., 2002). PBR transports cholesterol to the inner mitochondrial membrane and therefore has been suggested to be a rate-limiting step in the steroid and bile acid synthesis (reviewed by Lacapere and Papadopoulos, 2003). Binding of the benzodiazepines to PBR was found to stimulate cholesterol transport and the formation of steroids, although chronic administration did not have the same effect as acute treatments (Lacapere and

![Figure 4. GABA\(_A\) receptor. Besides GABA recognition site this Cl-ionophore has multiple modulatory binding sites for benzodiazepines, barbiturates and neurosteroids.](image-url)
In addition to porphyrins, ACBP has been identified as an endogenous ligand for PBR. Early studies revealed that ACBP lacking two carboxyterminal amino acids (Gly-Ile) could stimulate steroidogenesis in bovine adrenals (Besman et al., 1989; Guidotti et al., 1983). Further studies have confirmed that des-(Gly-Ile)-ACBP purified from rat or bovine brain and testis stimulates the cholesterol transport in isolated adrenocortical and Leydig cells (reviewed by Papadopoulos and Brown, 1995). Moreover, it was shown that both native and recombinant ACBP are able to stimulate cholesterol loading of Cyp11a1 (first enzyme involved in synthesis steroids) in an *in vitro* reconstituted enzyme system (Brown and Hall, 1991). In addition, high affinity PBR drug ligands are displaced by ACBP (Bovolin et al., 1990; Garnier et al., 1993; Papadopoulos et al., 1992) and ACBP and PBRs are able to form a protein complex in Leydig cells (Garnier et al., 1994). The endogenous effect of ACBP has been studied by using ACBP knock-down with antisense oligonucleotides. Experiments in hormone responsive MA-10 cells and constitutively steroid producing R2C Leydig cells indicated that suppression of ACBP terminated the steroid production in these cells (Boujrad et al., 1993; Garnier et al., 1994).

In addition to the native ACBP protein, also its processing products ODN and TTN bind and modulate the function of benzodiazepine receptors. The affinities and the effects of the processing products are different from each other and from the native ACBP protein. Differences in the affinity and effects of ODN and TTN can be explained by their sequence length (34 amino acids versus 18) and by differences in the secondary structures. ODN is able to displace $[^3]$Hflumazenil from rat brain membranes, but is unable to effect binding of $[^3]$HPK 11195. On the contrary, TTN can actively displace $[^3]$HPK 11195, but inactive against $[^3]$Hflumazenil binding. TTN can also enhance the inhibitory effect of picrotoxin on GABA-dependent $[^3]$Hflunitrazepam binding in a similar manner as 4'-chlorodiazepem (Slobodyansky et al., 1989). ODN prefers the benzodiazepine sites of GABA$_A$ receptors that bind also anxiolytic bendodiazepines and anxiogenic β-carboline carboxylate esters and are sensitive to flumazenil, while TTN prefers sites that bind the convulsant benzodiazepine, 4'-chlorodiazepam, and is insensitive to flumazenil (reviewed by Barbaccia et al., 1990). The two processing products prefer separate binding sites and have a different ability to replace drug ligands from the benzodiazepine binding sites, also distinct from the unprocessed, native ACBP. ODN and TTN have an effect at lower concentrations compared to whole ACBP (Costa and Guidotti, 1991). ACBP can also be secreted in a threonine phosphorylated form from retinal Müller cells. It was shown that threonine phosphorylated ODN has a higher affinity towards the GABA$_A$ receptor compared to unphosphorylated ODN or unphosphorylated ACBP (Qian et al., 2008). The affinities of the ACBP, ODN and TTN towards PBRs are also different. ODN shows no significant affinity towards PBRs and is not able to stimulate steroid synthesis, as does native ACBP. On the contrary, the affinity of TTN towards PBRs is higher than that of the unprocessed ACBP and it is able to stimulate the steroid synthesis in cell lines in a similar fashion as native ACBP (Berkovich et al., 1990; Papadopoulos et al., 1991; Papadopoulos et al., 1992).
2.2.4.1.2 Implications of benzodiazepine receptor binding

GABAergic neurotransmission is one of the best known inhibitory mechanisms operating in the central nervous system. Enhancement of GABAergic neural inhibition and hence the activation of its receptors represents the basis of the therapeutic treatment of many disorders and diseases like generalized anxiety, panic anxiety, sleep disturbances and epilepsy. ACBP can affect the function of GABA_A receptors either directly or via neurosteroid synthesis by regulating PBR activity in glial cells. Neurosteroids are shown to bind and influence the activity of GABA_A receptors, although the effect is dependent on the steroid form (Bormann, 1988). A modified version of the Vogel punishment test, a test that measures the anti-conflict and pro-conflict activity of drugs that act at GABA_A receptors, was originally utilized to investigate biological actions of intracerebroventricular (i.c.v.) injection of ACBP (Ferrero et al., 1986a; Ferrero et al., 1986b). These studies showed that ACBP could reverse the anti-conflict effects of diazepam and that the pro-conflict effect of ACBP was antagonised by the pre-treatment with flumazenil. In addition to native ACBP, also the effect of ODN and TTN has been investigated. Both processing products were even more potent in producing a pro-conflict action than the unprocessed protein. In drug ligand displacing studies, the action of ODN was competitively prevented by flumazenil, whereas TTN was resistant to this drug (Slobodyansky et al., 1989). It was noted that i.c.v injections of ODN in male mice led to increased aggressive behaviour in a dose dependent manner. This effect was reduced by the benzodiazepine receptor antagonist (Kavaliers and Hirst, 1986). Furthermore, i.c.v injections of ODN to both mouse and rats induced anxiety (De Mateos-Verchere et al., 1998). These effects were antagonised by both diazepam and flumazenil, indicating that the effect of ODN was being mediated through GABA_A receptors. Interestingly, i.c.v injections of high doses of ODN did not induce tonic and/or clonic convulsion in rats. In this way ODN differs from native ACBP (Ferrero et al., 1986b). Furthermore, in mice low doses of ODN reduced convulsions and mortality induced by pentylenetetrazole (Garcia de Mateos-Verchere et al., 1999). Pentylenetetrazol can block GABA_A receptors chloride channels and it has been used to evaluate the effect of anticonvulsant drugs acting on GABA_A receptors.

Since ACBP is involved in anxiety and pro-conflict behaviour in animals, ACBP levels have been determined from patients suffering from various diseases linked to either GABA_A receptor function or neurosteroid synthesis. The level of ACBP like immunoreactivity was increased in the cerebrospinal fluid (CSF) of individuals with major depression with a severe anxiety component (Barbaccia et al., 1986; Ferrero et al., 1988; Roy et al., 1988). The role of ACBP in anxiety has been further studied in rodents. In a study utilizing acute noise stress in rats, it was found that both ACBP and ODN protein levels were increased in the hippocampus of stressed animals (Ferrarese et al., 1991). Furthermore, psychological stress increased cerebral ACBP mRNA expression in mice, while physical stress did not have the same effect (Katsura et al., 2002). ACBP levels has been measured from individuals defined as pathological gamblers, it was found that there were increased levels of ACBP in a subgroup of patients showing clear signs of depression (Roy et al., 1988). Reports on patients with multi-infarct dementia or
dementia with Parkinson's disease indicated that there is no change in CSF ACBP-like immunoreactivity (Barbaccia et al., 1986; Ferrero et al., 1988). Interestingly, a subsequent study showed that Parkinsonian subjects with dementia have elevated ACBP levels in CSF (Ferrarese et al., 1990). There are conflicting results also from patients with Alzheimer's disease. Studies by Barbaccia et al. (1986) and Ferrero et al. (1988) reported no significant difference in ACBP like immunoreactivity between Alzheimer patients and controls, while Ferrarese et al. (1990) showed increased CSF ACBP levels. Furthermore, it has been demonstrated that beta-amyloid peptides can stimulate the ACBP mRNA expression and ACBP related peptide release in cultured rat astrocytes (Tokay et al., 2005). In addition, somatostatin, a peptide which levels have been shown to be reduced in Alzheimer's disease (Cervia and Bagnoli, 2007), can influence the ACBP levels. Somatostatin can decrease the expression and release of ACBP from rat astrocytes (Masmoudi et al., 2005). ACBP levels have been measured also from CSF of patients with various other diseases. In schizophrenic patients there was no change observed in ACBP levels (Barbaccia et al., 1986), while patients with Huntington's chorea exhibited decreased ACBP levels (Ferrarese et al., 1990). Patients with hepatic encephalopathy showed increased cerebrospinal fluid ACBP levels. Normalization of the mental status of these patients reduced the ACBP levels back to normal levels (Rothstein et al., 1989). Significantly elevated plasma ACBP levels have been measured from adult patients with epilepsy. Plasma levels of pediatric epilepsy patients were also elevated, although not to the same extent as seen in adults (Ferrarese et al., 1998). Strikingly, when the patients were divided into subgroups, it was found that the highest increments of ACBP levels were found from adult patients with generalized epilepsy and from drug-resistant adult and pediatric patients (Ferrarese et al., 1998). Previous studies with intrahippocampal injection ACBP peptide fragments (amino acids 42 to 50 and 43 to 50) have provided further evidence for a possible role of ACBP in epilepsy. Injections of these fragments to rats evoked limbic seizures typical of epilepsy. These seizures could be inhibited by administration of PK 11195 (selective antagonist of the benzodiazepine receptor subtype of GABA<sub>A</sub> receptor) (Vezzani et al., 1991).

Anxiety is considered to be one of the clinical features commonly found in the withdrawal syndromes caused by alcohol (ethanol), nicotine and morphine. A single dose of ethanol did not affect mouse cerebral cortex ACBP mRNA expression or the protein content, but induction of alcohol dependence by inhalation of alcohol vapour for 8 days caused a significant increase in both ACBP mRNA and protein levels in mouse cerebral cortex (Katsura et al., 1995). Furthermore, withdrawal of ethanol from these animals caused a further enhancement of ACBP expression (Katsura et al., 1995; Katsura et al., 1998a). Similar results have been obtained also from ethanol incubations of primary cultures of cerebral cortical neurons (Katsura et al., 1998a). In addition, long-term administration of both morphine and nicotine increased ACBP levels of cerebral cortex and withdrawal further increased the expression (reviewed by Ohkuma et al., 2001). Further studies demonstrated that the effect of nicotine to ACBP expression was mediated through nicotinic acetylcholine receptors (Katsura et al., 1998b), while the effect of morphine
occurred through activation of mu-opioid receptors, but not by kappa- or delta-opioid receptors (Katsura et al., 1998b; Shibasaki et al., 2007).

### 2.2.4.1.3 Other central nervous system related functions

The lateral area of hypothalamus displays high ACBP expression levels (Alho et al., 1985). This region has been shown to play a major role in the control of food intake. In addition, agonists of benzodiazepine receptors increased the palatability and evoked hyperphagic responses (Cooper, 1989). Injection of ACBP into the fourth ventricle of mouse suppressed the intake of 5% sucrose, water and 0.9 mM quinine-HCl and inhibited the preference of the animals to drink 0.05% saccharin instead of water (Manabe et al., 2001). Furthermore, i.c.v injection of ODN to rodents deprived of food for 10 hours dose-dependently reduced food intake during the 12h follow-up period (de Mateos-Verchere et al., 2001). In rats, continuous i.c.v. infusion of ODN for 15 days reduced the food intake during the first days of its administration. The appetite reducing effect of ODN was not influenced by pre-treatment with diazepam (de Mateos-Verchere et al., 2001). Moreover, i.c.v. administration of the ODN peptide to rats resulted in increased expression corticotropin-releasing hormone mRNA and decreased expression of neuropeptide Y, both peptides known to be involved in the regulation of food intake (Compere et al., 2005). Since diazepam, a GABA<sub>A</sub> receptor and PBR agonist does not affect the suppression of food intake caused by ODN, the mechanism of its function was further investigated. Recent results indicate that the effect of ODN is mediated through the metabotropic receptor positively coupled to phospholipase C via a pertussis toxin-sensitive G protein (do Rego et al., 2007). The anorexigenic effect of ODN has been recently demonstrated also in goldfish (Matsuda et al., 2007). In addition to the receptor mediated role in the regulation of food intake, ACBP might influence appetite also through acyl-CoAs, as these esterified fatty acids have been found to play an important role in hypothalamic control of food intake (reviewed by Lopez et al., 2007).

### 2.2.4.2 Peripheral and acyl-CoA binding associated functions

ACBP has been shown to have important functions outside the central nervous system that are not related to the receptor binding ability of this protein. Consequently, ACBP was independently characterized as an impurity in fatty acid binding protein preparations and was shown to induce synthesis of medium chain acyl-CoA esters by goat mammary gland fatty acid synthetase (Mogensen et al., 1987). Further analysis showed that this protein is identical to ACBP/DBI isolated from rat brain.

#### 2.2.4.2.1 Acyl-CoA binding
ACBP is able to bind saturated acyl-CoA esters with a carbon chain length over 8 recidues, the acyl-CoAs with a shorter chain length (2 to 4 carbons) did not show any significant binding (Knudsen et al., 1989; Mikkelsen and Knudsen, 1987). The acyl-CoA binding affinity of ACBP is highest towards chain
lengths of 14 to 22 carbons, while ability to bind shorter and longer acyl-CoA esters is considerably lower. ACBP binds specifically acyl-CoAs, as it exhibited no affinity towards nonesterified fatty acids and only low affinity towards free CoA ($K_d$ 2 $\mu$M). ACBP showed no binding to acylcarnitines, cholesterol, or to a number of nucleotides (Rosendal et al., 1993). The $K_d$ of ACBP towards acyl-CoAs ranged from 0.6 to 7 nM, depending on the acyl-CoA form and the method of determination (Faergeman et al., 1996; Schroeder et al., 2008). More detailed studies revealed that ACBPs binding affinity is influenced by the degree of saturation, the relative affinities being as follows: saturated $>$ monounsaturated $>$ polyunsaturated (Frolov and Schroeder, 1998; Huang et al., 2005). Evidence on the physiological role of ACBPs ability to bind acyl-CoA has been obtained from yeast, rat hepatoma cell lines and from mouse liver. In the yeast, *Saccharomyces Carlsbergensis*, overexpression of yeast ACBP gene resulted in a significant increase of intracellular acyl-CoA pool (Knudsen et al., 1994). In a rat hepatoma cell line, McA-RH 777, stable integration of rat ACBP cDNA led to elevated ACBP levels and to increased incorporation of palmitic acid when the cells were incubated with it (Yang et al., 2001). The most compelling data on the physiological role of ACBP in acyl-CoA pool size regulation has thus far been obtained from liver of a transgenic mouse line overexpressing mouse ACBP cDNA under the control of phosphoglycerate kinase promoter (Huang et al., 2005). These animals display a 33% elevation in their liver ACBP protein level, resulting in an approximately 69% increase in liver acyl-CoA pool. The liver acyl-CoA pool was increased in carbon chain dependent manner; mainly the levels of saturated and polyunsaturated acyl-CoA were increased while there was no change in the levels of monounsaturated acyl-CoAs. The ACBP effected also the intracellular location of the acyl-CoAs, with the highest increment of acyl-CoA levels found in the liver membrane/organelle fraction (Huang et al., 2005).

*In vitro* investigations on the effects of ACBP on the cellular function demonstrate in part the mechanisms by which ACBP can influence the intracellular acyl-CoA pool size and also reveal new intracellular effects of the protein. The ability of ACBP to extract membrane bound acyl-CoAs (Rasmussen et al., 1994) can increase the soluble fraction of acyl-CoAs. By binding and extracting acyl-CoAs from membrane-associated enzyme acyl-coA synthetase (ACS), an enzyme that converts fatty acids to acyl-CoAs, it was found that ACBP could release ACS of the end-product inhibition and enhanced its function (Rasmussen et al., 1993). In addition, the activities of acyl-CoA carboxylase (ACC) and adenine nucleotide translocase (ANT), two proteins involved in lipid metabolism whose function is inhibited by acyl-CoA, were influenced by ACBP. It has been shown that ACBP can efficiently release these proteins from acyl-CoA inhibition at acyl-CoA/ACBP ratios under 0.9 (Rasmussen et al., 1993). Furthermore, ACBP was able to protect acyl-CoA esters from hydrolysis by microsomal hydrolases (Rasmussen et al., 1993). This *in vitro* data indicate that ACBP is capable of both increasing acyl-CoA production and decreasing its hydrolysis and hence it can increase the overall
intracellular acyl-CoA pool, as was proven in two cell lines and also more recently in vivo in mouse liver.

In addition to being able to function as a pool former of acyl-CoAs, in vitro data suggests that ACBP can also act as an intracellular transporter of acyl-CoAs. In addition to extracting acyl-CoAs from membranes, ACBP can also transport the extracted acyl-CoAs to mitochondria or ER and donate them for either β-oxidation or glycerolipid synthesis (Rasmussen et al., 1994). The role of ACBP in the β-oxidation was further supported by studies showing that carnitine palmitoyltransferase 1 (CPT-1), an enzyme mediating the transport of acyl-CoAs to mitochondrial β-oxidation, is capable of interacting with the acyl-CoA/ACBP complex. The activity of CPT-1 is correlated with the concentration of ACBP bound acyl-CoAs, but not with levels of free acyl-CoAs (Abo-Hashema et al., 2001). Also other enzymes have a similar preference for the acyl-CoA/ACBP complex over free acyl-CoAs. It has been claimed that in isolated macrophage microsomes any elevation in the ACBP concentration can inhibit the activity of acyl-CoA:cholesterol acyltransferase if the acyl-CoA pool is held constant (Kerkhoff et al., 1997). On the contrary, a recent study has shown that in isolated liver microsomes, ACBP's influence is dependent on the presence of exogenous cholesterol as is the case with the other fatty acyl-CoA binding proteins (fatty acid binding protein and sterol carrier protein-2) involved in the regulation of acyl-CoA:cholesterol acyltransferase activity. This study demonstrated that in the presence of exogenous cholesterol, ACBP is a strong stimulator of acyl-CoA:cholesterol acyltransferase activity, whereas in the absence of exogenous cholesterol, ACBP functioned as an inhibitor of this enzyme (Chao et al., 2003). Similarly to CPT-1 and acyl-CoA:cholesterol acyltransferase, also acyl-CoA:lysophospholipid acyltransferase from human red blood cells preferred the acyl-CoA/ACBP complex over free acyl-CoAs (Fyrst et al., 1995). These results demonstrate that ACBP is able to function as an intracellular transporter of acyl-CoAs and possibly it can create a pool of acyl-CoAs to be directed for specific cellular purposes. In addition, ACBP can influence also other regulatory functions of acyl-CoAs. Peptides with S-acylation sites have been demonstrated to be able to be acylated at significant rates in the presence of acyl-CoA. Addition of ACBP under physiological molar ratios with respect to acyl-CoAs can inhibit this process (Leventis et al., 1997). It is assumed that ACBP sequesters the acyl-CoAs in a complex, which is unable to serve as an S-acyl donor, and as a consequence, ACBP may be able to prevent the spontaneous nonenzymatic S-acylation caused by acyl-CoAs (Leventis et al., 1997). ACBP was also demonstrated to be able to enhance the acyl-CoA induced activation of Ca\(^{2+}\) release channel of skeletal muscle sarcoplasmic reticulum, indicating that the ACBP/acyl-CoA complex either interacted directly with components of terminal cisternae membranes or indirectly through intermediates able to donate acyl-CoA to binding sites on the membranes (Fulceri et al., 1997).

Besides results emerging from ACBP overexpression experiments, data on the physiological function of ACBP has also been obtained from knock-out and knock-down studies in yeast, Trypanosoma brucei and
different mammalian cell lines. In addition, there is a mouse line with a spontaneous deletion of 400 kb area in chromosome 1 containing the ACBP gene (Lee et al., 2007; Ohgami et al., 2005). Disruption of the ACB1 gene, a gene that codes for the yeast homolog of ACBP, from *Saccharomyces cerevisiae* resulted in slight reduction of the growth rate when the yeast cells were grown on ethanol, but no effect was observed if they were grown on glucose (Schjerling et al., 1996). Despite of the fact that ACBP disruption had only a limited effect on growth rates, this altered strain could not compete with wild type cells when they were cultivated on the same plate. Interestingly, the knock-out of ACBP in yeast resulted in 1.5 to 2.5 fold increase in overall intracellular acyl-CoA levels, although the increase in the acyl-CoA level was caused solely by *de novo* synthesized stearoyl-CoA (C18:0). Further experiments revealed that in the absence of ACBP, yeast fatty acid synthetase tended to form mainly long chain acyl-CoAs. Addition of ACBP resulted in a decrease in the chain length of synthesized acyl-CoAs, suggesting that ACBP efficiently transported the newly synthesized acyl-CoAs for further utilization (Schjerling et al., 1996). In a more recent study utilizing the ACB1 gene depleted *S. cerevisiae* strain, it was noted that the deletion originally caused a slower growing phenotype that adapted into a faster growing phenotype (Gaigg et al., 2001). Further experiments with a strain having a conditional knock-out of the ACB1 gene detected no change in the total acyl-CoA pool, but a significant increase in stearoyl-CoA (C18:0) amount, as found also in the previous study. Depletion of ACBP did not affect the general glycerolipid synthesis; there was no change in the phospholipid pattern, rate of synthesis or in the turnover of phospholipid classes. In contrast, depletion of ACBP caused a reduction in total fatty acids with a chain length of C26:0 and also a 50-70% reduction in the sphingolipid synthesis. As a consequence the mutated strain accumulated 50-60 nm vesicles and autophagocytotic-like bodies in the cytosol and displayed perturbed plasma membrane structures. These results suggest that ACBP is involved in yeast membrane assembly and organization by creating a specific pool of acyl-CoAs required in membrane trafficking (Gaigg et al., 2001).

Several mammalian cell lines have been used to study the effects of reduced ACBP expression. Antisense RNA or siRNA constructs have been utilized to knock-down the ACBP protein levels in mouse 3T3-L1 cells and in human HeLa, HepG2 and Chang cells. The ACBP antisense RNA used in 3T3-L1 cells caused inhibition of the adipocyte differentiation process. The treated cells showed a significant reduction in the lipid accumulation and the morphological differentiation level of the cells was accordingly reduced. The inhibition of differentiation was correlated with the expression levels of ACBP antisense RNA (Mandrup et al., 1998). Further investigation on the effects of ACBP knock-down revealed that it caused a reduction in the expression of two transcription factors involved in the terminal stages of adipocyte differentiation, PPARγ and C/EBPα. On the other hand levels of transcription factors C/EBPβ and C/EBPδ, both believed to be involved in the initiation of adipocyte differentiation, were slightly elevated. Although knock-down of ACBP altered the differentiation of the adipocytes, it did not affect the clonal expansion or the growth rate of the antisense transfected 3T3-L1 cells (Mandrup et al.,
In human HeLa, HepG2 and Chang cells, the siRNA technique was utilized to knock-down the ACBP protein expression. Targeted knock-down resulted in visual growth arrest and detachment of cells in all three cell lines. ACBP depletion in HeLa cell induced apoptosis (Faergeman and Knudsen, 2002). Similar results have also been obtained from T. brucei in which attempts to create a total knock-out ACBP were unsuccessful. A conditional knock-out of ACBP in the same species showed that after 48 h of ACBP depletion, the cells were unable to divide and underwent cell death over the next 4 days (Milne et al., 2001). In contrast to the results obtained by Faergeman and Knudsen, a more recent study by Shulga and Pastorino (2006) found that ACBP knock-down did not cause cell apoptosis in HeLa cells, rather the opposite. In their study, ACBP was found to influence the components of calpain-induced cell death and that the knock-down of ACBP actually inhibited cell damage (Shulga and Pastorino, 2006).

A mouse line with a 400 kb deletion in chromosome 1 (nm1054 mice) has also been utilized to investigate the physiological effects of ACBP in vivo. Originally this mutation nm1054 arose in the CBA/J mouse line but it has been subsequently transferred also to C57BL/6J and 129S6/SvEvTac lines (Ohgami et al., 2005). The deleted region contains multiple areas that code for functional or predicted genes, with ACBP being one of them. With respect to ACBP, one of the most interesting phenotypes of the mutated mouse line is abnormal skin and hair development. The animals have sebocyte hyperplasia and sparse, matted hair with a greasy appearance (Lee et al., 2007). In addition, these mice show altered fatty acid metabolism with changes in hair triacylglycerol levels. To study in detail which of the genes deleted are involved in this phenotype, Lee et al. (2007) have utilized nm1054 mice with additional transgenic expression of specific regions originally deleted from these mice. These studies indicated that transgenic expression of the region containing the ACBP gene and a novel predicted gene 3110009E18Rik (BAB29121) could reverse the skin phenotype of the mutated nm1054 mouse back to normal. As the authors could detect only mRNA expression of ACBP from these transgenic nm1054 mice, they concluded that knock-out of ACBP was most likely responsible for the deranged skin and hair phenotype of the mouse line (Lee et al., 2007).

2.2.4.2.2 Regulation of transcription factors

ACBP is located in the cytosol as well as in the nucleus of cells. Using real-time imaging and fluorescently labelled recombinant ACBP, it was shown that about 22% of the protein is located inside the nucleus (Schroeder et al., 2008). In addition, acyl-CoAs have been found to modulate regulation of gene expression (reviewed by Black et al., 2000; Jump et al., 2005; Schroeder et al., 2008), indicating that ACBP might have an additional role in these processes. Studies utilizing ACBP knock-out yeast strains detected changes in the gene expression (Knudsen et al., 2000; Schjerling et al., 1996). Further studies investigating the effects of ACBP on PPAR activation have shown that ACBP could inhibit the trans-activation of PPAR subtypes (Helledie et al., 2000; Helledie et al., 2002b). These experiments utilized a monkey kidney cell line (CV-1) transfected with a reporter construct and the vector expressing
one of the PPAR subtypes. Tetradecylthioacetic acid (TTA), a ligand that activates all PPAR subtypes and is metabolized to CoA ester, or a PPARγ specific activator BRL49653 were used as activating ligands. Results from these studies demonstrated that ACBP attenuated the ligand induced activation of all three PPAR subtypes when TTA was utilized as activating agent, whereas ACBP was unable to influence the function of BRL49653. Furthermore, these studies showed that the effect of ACBP seemed to be mediated through the ligand-binding part of PPARs. These data indicate that the effect of ACBP is mediated through binding of the ligand that activates PPARs, not by a direct interaction with the transcription factor (Helledie et al., 2000; Helledie et al., 2002b).

Hepatocyte nuclear factor-4α (HNF-4α) is a transcription factor regulating genes involved in both lipid and glucose metabolism. It has been shown that acyl-CoAs are able to either activate or inhibit the function of HNF-4α, depending on the chain length and degree of saturation (Hertz et al., 1998; Petrescu et al., 2002). Both in vitro and in vivo experiments showed that ACBP is able to physically and functionally interact with HNF-4α. The first line of evidence about the ability of ACBP to influence HNF-4α came from the fact that ACBP and HNF-4α were co-immunoprecipitated by antibodies to either one of these proteins. The double immunolabeling and laser scanning confocal microscopy results confirmed the colocalization in the nucleus and revealed that the intermolecular distance between these two proteins is only 53 Å. The close distance between ACBP and HNF-4α was further confirmed by immunogold electron microscopy, demonstrating that this colocalization is indeed specific. ACBP exhibited no interactions with the other nuclear proteins studied (Petrescu et al., 2003). In addition, direct interaction with ACBP elicits changes in the secondary structure of HNF-4α enhancing the transactivation (Petrescu et al., 2003). Interestingly, the ability of ACBP to increase the activity of HNF-4α is dependent on the ACBP/HNF-4α ratio. When the ratio is below 0.7 there is a clear correlation in the ACBP amount and HNF-4α activation, but with ratios higher than 0.7 the correlation disappears. This might be due to competition for the ligand, namely acyl-CoAs, as both proteins can bind these compounds with high affinities. This protein level dependent influence might be important factor determining the influence of ACBP on HNF-4α activity (Petrescu et al., 2003).

2.2.4.3 Other described functions of ACBP

ACBP has been associated with several other functions. In the duodenum, ACBP has been shown to be involved in trypsin sensitive cholecystokinin (CCK) release. ACBP was isolated from porcine duodenum in the search for a peptide responsible of proteases sensitive CCK releasing factors. Further investigations demonstrated that in rats ACBP is involved in the feedback regulation of pancreatic secretion and the postprandial release of CCK (Li et al., 2000). By utilizing STC-1 cells, a murine tumor cell line known to express CCK, it was shown that ACBP could elicit Ca²⁺ oscillations via the voltage-dependent L-type Ca²⁺ channels. This change in the intracellular Ca²⁺ content resulted in increased secretion of CCK from these cells (Yoshida et al., 1999).
ACBP can alter also the endocrine secretion of pancreas. ACBP is expressed in δ- and α-cells of islets and in the epithelial cells of exocrine pancreas (Chen et al., 1988; Ostenson et al., 1991). The effect of ACBP on pancreatic insulin secretion has been studied in several models. Utilizing isolated rat islets and porcine ACBP, Ostenson et al. (1990) demonstrated that ACBP did not influence the basal insulin secretion at 3.3 mM glucose or arginine stimulated insulin secretion, but inhibited glucose or 3-isobutyl-1-methylxanthine stimulated insulin release. Furthermore, the negative effect of ACBP on insulin secretion is specific and does not change glucose stimulated release of somatostatin or the glucagon release (Ostenson et al., 1990; Ostenson et al., 1991). Similar results were obtained using rat ACBP in isolated rat islets in either static or perifusion experiments. In addition, the processing product, ODN, had similar effects on insulin secretion as native peptide, whereas TTN had no effect on either glucose stimulated or on the basal insulin secretion (Borboni et al., 1991). Further studies with the processing products ODN and TTN in both isolated rat islets and hamster insulinoma (HIT-T15) cells confirmed previous results, showing that the effects of ODN on glucose and glibenclamide stimulated insulin release are most likely mediated by cytoplasmic Ca$^{2+}$ levels (De Stefanis et al., 1995). In contrast to these short term experiments, cultivation of fetal rat pancreatic islets enriched in beta-cells for three days in a medium with different concentration of porcine ACBP exhibited no effects on islet insulin and polyamine levels or in the insulin secretion. This long term incubation of pancreatic cells with ACBP resulted only in a dose-dependent inhibition of beta-cell DNA synthesis which could be overcome by glucose stimulation (Sjoholm et al., 1991). Intravenous infusion of ACBP to rats caused a moderate and transient reduction in plasma insulin levels in glucose stimulated animals. Further investigation with perifused rat islets showed that ACBP was mainly affecting the acute-phase of glucose stimulated insulin release (Ostenson et al., 1994). These results indicate that ACBP can function as a short term modulatory effector of glucose stimulated insulin release. This can be either through the abilities of ACBP or ODN to alter the signalling pathways that influence cytoplasmic Ca$^{2+}$ levels or through changes in the acyl-CoA pool.

ACBP has also been shown to influence cell death through calpain activation. Calpains are proteases that have been claimed to have an important role in apoptosis and necrosis (review by Harwood et al., 2005). ACBP has been proposed to function as an m- and μ-calpain activator. Activation of both calpains requires Ca$^{2+}$ ions. ACBP has been demonstrated to drastically lower the Ca$^{2+}$ concentrations needed to activate m-calpain, while the effect on μ-calpain is similar but less drastic. The activating effect of ACBP was shown to be dependent on calcium, but not to be affected by the acyl-CoA binding state of ACBP (Melloni et al., 2000). Further experiments have shown that ACBP has a critical role in Bid (a proapoptotic protein that is activated by proteolytic activation by caspase-8 or calpains) induced activation of calpains (Shulga and Pastorino, 2006). In isolated mitochondria, activated Bid can increase the ACBP content of supernatant without altering the mRNA expression of the protein. Furthermore,
knock-out of ACBP by siRNA was shown to inhibit Bid induced cell death and that ACBP required the presence of PBR to be able to influence the function of Bid protein. It was proposed that Bid protein can mediate its calpain activating and hence apoptosis activating effects through the ACBP/PBR complex, enabling further activation of additional Bid proteins and other apoptosis inducing factors (Shulga and Pastorino, 2006).

2.3 Transcription factors

2.3.1 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the steroid hormone nuclear receptor super family, which comprises a large group of ligand dependent transcription factors. PPARs have been claimed to have a wide range of functions in the regulation of lipid metabolism, energy balance and inflammation. In addition, PPARs have been shown to be involved in various diseases like obesity, diabetes and atherosclerosis. Like other family members, PPARs have both a DNA binding domain and a ligand binding domain. The C-terminal ligand binding domain directs the specific binding of the ligands needed for activation of the PPARs. In addition to ligand binding, this domain is also required for heterodimerization with the retinoid X receptor (RXR) and for undergoing an interaction with other transcriptional co-factors. The central DNA binding domain facilitates the binding of ligand activated PPARs to specific regions called PPAR response elements (PPREs) located in the promoters of regulated genes. Since they are ligand dependent transcription factors, activation of PPARs starts with binding of a specific ligand, causing a conformation change that facilitates the formation of the heterodimer with RXR. This complex is then able to bind to PPREs. In the absence of ligand, PPAR-RXR heterodimers exhibit active repression by co-repressors, histone deacetylases and chromatin-modifying factors preventing the function of PPARs. Ligand binding also facilitates the binding and release of accessory factors that can have a profound effect on the functionality of the complex. The known co-factors of PPARs include repressors like N-CoR and SMRT and activator proteins like PPARγ co-activator-1 (PGC-1) and CREB (reviewed by Ahmed et al., 2007; Seedorf and Aberle, 2007; Zoete et al., 2007). Furthermore, the activity of PPARs is regulated by various protein kinases (reviewed by Burns and Vanden Heuvel, 2007). The function of factors regulating PPARs is cell and tissue specific and although normally the PPAR complex activates gene expression, in some circumstances the effect may be opposite (reviewed by Ahmed et al., 2007). The PPAR family consists of three isotypes PPARα, PPARγ and PPARδ, which is also named PPARβ. They all share the same basic structure and method of activation, but have differences in their preferred ligands, tissue distribution and in the cellular processes that they regulate. In addition to functional differences, these proteins are encoded by separate genes.

PPARα was the first member of the PPAR transcription factor family to be characterized. Originally it was isolated from mice and found to interact with drugs that caused peroxisomal proliferation in liver.
(Issemann and Green, 1990), consequently all isoforms were named as peroxisome proliferator-activated receptors. PPARα is expressed in liver, heart, kidney, skeletal muscle, intestine and pancreas. Lower levels have also been detected in many other tissues. In general, PPARα is mainly expressed in tissues with high oxidative capacities. In accordance to its tissue distribution, PPARα has been associated with regulation of fatty acid oxidation, lipid metabolism and inflammation, thus far approximately 80-100 genes have been identified as being regulated by PPARα (Ahmed et al., 2007). In addition to synthetic drug ligands, i.e. the fibrates, PPARα ligands include both saturated and unsaturated long chain fatty acids, branched chain fatty acids and eicosanoids. In addition to fatty acids, also long chain acyl-CoAs are able to bind PPARα with high affinities. The role of acyl-CoAs on PPARα function is controversial; some studies have reported an inhibitory effect on gene expression through recruitment of co-repressors while others have shown that acyl-CoAs have activating effects on PPARα regulated genes (Reviewed by Gilde and Van Bilsen, 2003; Schroeder et al., 2008).

The highest levels of PPARγ isoform are found in the adipose tissues. Other sites of expression include skeletal muscle, liver, heart, intestine and the cells of the vascular and immune system. PPARγ ligands include long chain polyunsaturated fatty acids, nitrated forms of some fatty acids and some prostaglandins. Thiazolidinedione drugs like pioglitazone and rosiglitazone serve as synthetic ligand activators of PPARγ. PPARγ has an essential role in adipocyte differentiation and lipid storage. In addition, it is involved in the regulation of glucose homeostasis and inflammation (Reviewed by Ahmed et al., 2007).

PPARδ, is the least extensively studied isoform of the PPAR family. Compared to the other two isoforms, its expression is more ubiquitous, including the adipose tissue, skeletal muscle and heart. It seems that especially in muscle tissue, PPARδ has an important role, as its expression is 10 to 50 times higher than that of the other isoforms. PPARδ has been found to alter gene expression in a rather similar manner as PPARα, it enhances fatty acid catabolism and energy uncoupling. The main difference between the two isoforms is the tissues involved, with PPARα mainly affecting liver while PPARδ functions in muscle. PPARδ has also been indicated to regulate membrane lipid synthesis and turnover, fat storage and lipid accumulation in macrophages (Reviewed by Ahmed et al., 2007; Seedorf and Aberle, 2007). Natural ligands of PPARδ seem to include saturated fatty acids of chain length 14 to 18 and polyunsaturated fatty acids of chain length 16 to 20. In addition, some eicosanoids have been shown to be able to bind PPARδ. GW501516, derivate of phenoxyacetic acid, is a specific synthetic ligand for PPARδ (Seedorf and Aberle, 2007).

2.3.2 Sterol regulatory element binding proteins
Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors regulating genes involved in cholesterol and lipid metabolism. They have been shown to play important roles for
example in adipocyte differentiation and insulin-dependent gene regulation. Separate genes code for SREBP-1 and SREBP-2. SREBP-1 is further divided to SREBP-1a and SREBP-1c; these originate from the presence of a differential transcription start sites. SREBP proteins consist of an N-terminal transcription factor domain that is linked by two transmembrane segments to the C-terminal regulatory domain. SREBPs are synthesized as large precursor proteins that are inserted into the ER. Precursors are transported from ER to the Golgi apparatus and activated by cleavage of the transcriptional domain, which then can enter the nucleus. The main control of the SREBP activity occurs in the ER where the protein is bound by the SREBP-cleavage-activating protein (Scap), which acts as a sterol sensor. In sterol depleted cells, the Scap/SREBP complex is able to interact with the COPII coat proteins, enabling SREBP to be incorporated into vesicles and transported to the Golgi where two proteases (site 1 (S1p) and site 2 (S2p) proteases) cleave the peptide and release the active domain of SREBP. The released transcriptional domain of SREBP is transported into nucleus as a dimer by importin β, a protein required for the transfer of the complex across the nuclear envelope. In the presence of sterols, Scap binds also the sterols and hence the complex is no longer able to interact with COPII coat proteins, but rather undergoes interactions with Insig-1 and Insig-2 (insulin-induced genes). The binding of the Scap/SREBP complex to Insig proteins retains them on the ER and prevents the activation of SREBPs (reviewed by Bengoechea-Alonso and Ericsson, 2007; Eberle et al., 2004; Espenshade, 2006; Shimano, 2001). The active fragment of SREBP forms a basic helix-loop-helix leucine zipper transcription factor that is able to bind to both classical E-boxes and sterol regulatory elements (SREs). Maximal activation of genes by SREBP requires the presence of additional DNA-binding proteins, namely NF-Y, CREBP or Sp1, depending on the gene. The nuclear SREBPs are targets of various posttranslational modifications that can affect their functions; these include phosphorylation, acetylation, sumoylation and ubiquitination. A number of co-factors like p300, CBP and PGC-1β have also been shown to alter the regulatory functions of SREBPs. SREBPs are in part also regulated at the transcriptional level, for example insulin and other nutritional status related factors have been shown to regulate SREBP expression in various tissues. There is also evidence that liver X receptor (LXR) can regulate the expression of SREBP. Variation in binding affinities, co-regulator usage and other modifications affect differentially the functions of the three SREBP forms. SREBP-2 is mainly involved in the regulation of cholesterol metabolism, whereas SREBP-1s are involved general energy metabolism including fatty acid and glucose/insulin metabolism. There are further differences between SREBP-1a and SREBP-1c, with SREBP-1a being the more potent regulator due to its larger transcriptional domain. Overall more than 30 genes have thus far been identified as SREBP target genes. There are also differences in the tissue distribution between SREBP-1c and SREBP-1a. SREBP-1c is expressed in most tissues with high levels in liver, adipose tissue, skeletal muscle and brain, whereas SREBP-1a is highly expressed in certain tissues like spleen and intestine (reviewed by Bengoechea-Alonso and Ericsson, 2007; Espenshade, 2006; Shimano, 2001).
2.4 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is one of the main regulators of energy balance, switching cells from an anabolic state to a catabolic state and it can influence hypothalamic regulation of food intake. AMPK is a heterotrimeric protein consisting of the catalytic α subunit and the regulatory β and γ subunits. There are multiple genes coding for each subunit, two for subunits α and β and three for subunit γ, making 12 possible heterotrimeric combinations (reviewed by Hardie, 2008). The α subunit contains a kinase domain and a C-terminal domain that interacts with the β-subunit. The β-subunit has domains that interact with both α- and γ-subunits and a carbohydrate binding domain that is believed to function in fuel sensing. The γ-subunit has multiple domains involved in the activation of the complex (reviewed by McGee and Hargreaves, 2008).

The main activator of AMPK is 5′-AMP. AMPK is activated both by a direct allosteric effect and by 5′-AMP promoted phosphorylation of the critical threonine 172 on the α-subunit. In total, 5′-AMP is able to induce 1000-fold increase in the kinase activity. In addition to 5′-AMP, upstream kinases have been shown to activate AMPK. The most common kinase is tumor suppressor kinase LKB1 and its two accessory proteins STRAD and MO25 (Woods et al., 2003). A second upstream kinase believed to be able to phosphorylate the critical Thr 172 is calmodulin-dependent protein kinase kinase (CaMKK)-α and –β (Hardie, 2008). At the whole body level, certain hormones such as leptin and adiponectin have been found to influence the activity of AMPK in various tissues.

AMPK activation promotes catabolic pathways that generate ATP and at the same time it down-regulates anabolic pathways that consume ATP. A key pathway involved is the glucose metabolism: AMPK is increasing the glucose uptake of muscle tissue both by increasing translocation of glucose transporter-4 (Glut-4) to plasma membranes and by enhancing its gene expression. In other tissues AMPK is influencing the glucose uptake by increasing the activity of glucose transporter-1 (Glut-1) (Hardie, 2008). In addition, AMPK can also induce glycolysis and inhibit gluconeogenesis. In addition, AMPK

![Figure 5](image.png)

Figure 5. Pathways relevant for ACBP related processes that are activated (→) or inhibited (─┤) by AMPK.
regulates the fatty acid metabolism through acyl-CoA carboxylase (ACC). Phosphorylation of ACC by AMPK has been shown to inactivate it. This results in inhibition of fatty acid synthesis and through the reduced malonyl-CoA levels in increased oxidation of fatty acids. Malonyl-CoA can regulate the rate-limiting step of fatty acid oxidation by inhibiting the function of CPT-1. In addition to direct effects on enzymes regulating metabolism, it has been shown that AMPK is involved in transcriptional regulation of genes. Phosphorylation by AMPK has been found to influence the function of multiple transcription factors and other regulators of gene expression, including PPARs and HNF-4γ (reviewed by McGee and Hargreaves, 2008). In addition to influencing cellular energy balance, AMPK can also alter the whole body energy status by influencing hypothalamic regulation of food intake (Lopez et al., 2007). The ability of AMPK to modify a multitude of metabolic processes in many different tissues means that it is one of the main regulators of energy homeostasis.
3. AIMS OF THE STUDY

The aim of this study was to investigate the physiological role of acyl-CoA binding protein (ACBP) / diazepam binding protein (DBI) by utilizing transgenic mouse and rat lines overexpressing mouse ACBP gene under the control of endogenous promoter. In order to generate the animal models, the mouse ACBP gene was isolated and characterized.

Specific aims of this thesis were

1. To investigate the long-term effects of endogenously expressed ACBP in mouse central nervous system, in particular the role of ACBP in the behaviour of the animals. (I)

2. To investigate the effects of long-term overexpression of ACBP on physiological parameters of rats and the molecular mechanisms behind possible changes, with a special focus on lipid and glucose metabolism. (II)

3. To study the influence of different dietary conditions on glucose and fat metabolism in transgenic rats overexpressing ACBP. (III)

4. To characterize the role of ACBP on gene expression regulation in the hypothalamus. (IV)
4. MATERIALS AND METHODS

4.1 Isolation of mouse ACBP/DBI gene

Mouse DBI gene was isolated from a genomic library of mouse strain 129/SvJ (Stratagene, La Jolla, CA, USA) by PCR screening method using two primer pairs (Israel, 1993). The first pair amplified a 255 bp region of the first intron of mouse DBI gene (GenBank acc. L76367). For the second primer pair, rat DBI gene was compared to a mouse EST sequence database in GenBank using BLAST. One EST containing sequence originating from intron three of mouse DBI gene was found and primers were designed to amplify the region from intron three to exon four of the DBI gene. The genomic library was plated into 96 well cell culture plate at density 1000 pfu/well. Samples were analysed by PCR and secondary screening was performed. After tertiary screening, phages from positive wells were plated on LB-agar and individual plaques were picked up for PCR analysis. Positive clones were selected and subcloned into Bluescript KS (Stratagene). One positive clone was sequenced on both strands by primer walking and transposon-mediated DNA sequencing according to the manufacturer's instructions (Template Generation System, Finnzymes, Finland).

4.2 Animals

The isolated gene (GenBank AF220221) was microinjected using standard techniques into the pronuclei of zygotes derived from Balb/c x DBA/2 mice or from Wistar rats. In mice studies, both female and male adult transgenic (tg) and syngenic (sg) mice were utilized. In rat studies, adult male tg and sg animals were used. The weight of the rats was measured at the age of 6 weeks and 1 year. Food intake of the 6 week old rats was measured by weighing the food pellets once a day for a period of four days. To study effects of changes in the nutritional status, rats were either ad libitum fed or 16 h fasted. The effect of high fat diets with low energy content (energy content of 13.1-13.4 KJ/kg and 44-49% of energy from fat) was studied with four month old male rats (average weight 380 g) that were divided into two groups: I. Fed with high fat diet enriched with medium chain fatty acids (MC) (mainly octanoic (C8) and decanoic acid (C10), Tables 1 and 2) for 4 weeks and fasted overnight (16 h) before sample collection. II. Fed with high fat diet enriched with long chain fatty acids (LC) (mainly hexadecanoic (C16) and octadecanoic acid (C18), Tables 1 and 2) for 4 weeks and fasted as the MC diet group. The high fat diets were formulated (Institute of Animal Nutrition and Physiology, Kiel University) to have low energy content compared to commercial high fat diets (19.8 kJ kg⁻¹; Table 1) in order to investigate the effects of different fatty acid chain lengths without interference of high energy content. To study the involvement of AMPK in the regulation of the transcription factors, AMPK inhibitor ((compound C (6-[4-(2-piperidin-1-yloxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), Sigma) at a dose of 11 mg/kg body weight or control injections with dimethylsulphoxide were administrated i.p. to fed tg rats. Animals
Table 1. Composition of high fat diets used.

<table>
<thead>
<tr>
<th>Component</th>
<th>LC diet</th>
<th>MC diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (LC = palm oil, MC = synthetic triglycerides)</td>
<td>128.8 g/kg diet</td>
<td>145.6 g/kg diet</td>
</tr>
<tr>
<td>Soy bean oil</td>
<td>30.7 g/kg diet</td>
<td>30.1 g/kg diet</td>
</tr>
<tr>
<td>Lecithin</td>
<td>25.8 g/kg diet</td>
<td>25.3 g/kg diet</td>
</tr>
<tr>
<td>Corn starch</td>
<td>178.5 g/kg diet</td>
<td>175.1 g/kg diet</td>
</tr>
<tr>
<td>Casein</td>
<td>155.8 g/kg diet</td>
<td>152.8 g/kg diet</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8 g/kg diet</td>
<td>1.8 g/kg diet</td>
</tr>
<tr>
<td>Gelatine</td>
<td>11.7 g/kg diet</td>
<td>11.4 g/kg diet</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>31.3 g/kg diet</td>
<td>30.7 g/kg diet</td>
</tr>
<tr>
<td>Water</td>
<td>386.5 g/kg diet</td>
<td>379.1 g/kg diet</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>8.0 g/kg diet</td>
<td>7.8 g/kg diet</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>41.1 g/kg diet</td>
<td>40.3 g/kg diet</td>
</tr>
<tr>
<td>Energy content*</td>
<td>13.4 kJ kg⁻¹</td>
<td>13.14 kJ kg⁻¹</td>
</tr>
</tbody>
</table>

*For comparision: High fat diet from Research Diets (New Brunswick, NJ, USA) D12451: 19.80 kJ/kg diet (45 kcal% from fat)

Table 2. Fatty diet composition of MC and LC diet

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>LC diet</th>
<th>MC diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8:0</td>
<td>n.n</td>
<td>85.5 g/kg diet</td>
</tr>
<tr>
<td>C10:0</td>
<td>n.n</td>
<td>31.6 g/kg diet</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.4</td>
<td>0.5 g/kg diet</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.5</td>
<td>n.n</td>
</tr>
<tr>
<td>C16:0</td>
<td>50.8</td>
<td>4.9 g/kg diet</td>
</tr>
<tr>
<td>C16:1</td>
<td>n.n</td>
<td>n.n</td>
</tr>
<tr>
<td>C18:0</td>
<td>59.5</td>
<td>1.6 g/kg diet</td>
</tr>
<tr>
<td>C18:1</td>
<td>7.6</td>
<td>7.1 g/kg diet</td>
</tr>
<tr>
<td>C18:2</td>
<td>21.7</td>
<td>21.8 g/kg diet</td>
</tr>
<tr>
<td>C18:3</td>
<td>2.9</td>
<td>2.9 g/kg diet</td>
</tr>
<tr>
<td>Others</td>
<td>1.0</td>
<td>n.n</td>
</tr>
</tbody>
</table>

n.n. = Not detectable
were sacrificed by decapitation, tissues were collected in either liquid nitrogen or RNAlater solution (Qiagen, Hilden, Germany) and stored at -70°C until further analysis unless otherwise stated. All animals were housed in groups of two to six (two to three for rats or three to six for mice) animals per cage under a controlled environment (temperature 20 ± 1°C, humidity 50-60%, lights on 07.00-19.00), unless otherwise stated. The experiments were approved by Institutional Animal Care Committee of the University of Kuopio and by the Provincial Government. Procedures were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC.

4.3 Western blotting

Tissues were homogenized with Ultra-Turrax (IKA, Staufen, Germany) in buffer (25mM TRIS, 0.1 mM EDTA, 1 mM DTT and protease and phosphate inhibitor cocktails if required (Sigma, St Luis, MO, USA)). Equal amounts of protein were separated in SDS-PAGE and electrophoretically transferred onto 0.2 μm nitrocellulose or PVDF membranes. Nonspecific binding was blocked by 5% powdered milk in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). ACBP, AMPKα and Thr 172 phosphorylated AMPKα were detected with incubation in specific antibody (ACBP 1:1000 dilution; Peninsula Laboratories, Bachem, Bubendorf, Switzerland or 1:400 dilution Santa Cruz Biotechnology, Santa Cruz, CA, USA, AMPK and Phospho AMPKα 1:1100 dilution, Cell Signaling Technology, Danvers, MA, USA) followed by HRP labelled secondary antibody (1:17 000 dilution, Zymed Laboratories, San Francisco, CA, USA). Antibody-antigen complexes were visualized with ECL Plus (GE Healthcare, Chalfont St. Giles, UK). Detection was done with either Typhoon 9400 Imager (GE Healthcare) or Storm 860 Imager (GE Healthcare). Band densities were analyzed with ImageQuant™ TL program (GE Healthcare). Equal loading of the proteins was controlled with simultaneous detection of β-actin or α-tubulin (Cell signaling technology, 1:1500 dilution).

4.4 Real-Time Quantitative PCR

Total RNA was isolated from tissues by RNeasy mini kit (Qiagen, Hilden, Germany). Genomic DNA was digested by Dnase I (Qiagen) and RNA was reverse transcribed to cDNA (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA).

Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems) using SYBR Green technology and 18s RNA as a control gene. PCR primers (Table 3) were designed with eprimer3 program or taken from the literature (Hoekstra et al., 2003; Rodgers et al., 2005; Schmittgen and Zakrjsek, 2000). Reactions contained 2 μl sample cDNA (40-60 ng), 1* SYBR Green master mix (Applied Biosystems), 2-15 pmol of primers in a total volume of 30 μl. All samples were handled as duplicates in the following conditions: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each assay included a relative standard curve of three serial dilutions of cDNA from control sample and no template controls.
TABLE 3: Primers used in quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse ACBP/DBI gene</td>
<td>CACTCAGGCAACCTACATC</td>
<td>GAACAGCATCTCTTCATCAG</td>
</tr>
<tr>
<td>Mouse and rat ACBP mRNA</td>
<td>GAAAGCGCCTCAAGACTCAAGC</td>
<td>TTCAGCTTTGTTCCACGAGTCC</td>
</tr>
<tr>
<td>Rat PPAR α</td>
<td>TGAACAAAGACGGGATG</td>
<td>TCAAACCTTGGGTCCATGAT</td>
</tr>
<tr>
<td>Rat PPAR δ</td>
<td>GAGGGGTGCAAGGGCTTTT</td>
<td>CACTTGTTGCGGTTCCTCTTCTG</td>
</tr>
<tr>
<td>Rat PPAR γ</td>
<td>CATGCTTTGGAAGGATGCAG</td>
<td>TTCTGAAACCGACAGACTGACT</td>
</tr>
<tr>
<td>Rat SREBP-1</td>
<td>AGCGCTACCGTTCTCTATC</td>
<td>GCGCAAGACAGACAGATTAT</td>
</tr>
<tr>
<td>Mouse and rat 18s</td>
<td>GTAACCCCGTTGAACCCATT</td>
<td>CCATCCAATCGGTAGAGCG</td>
</tr>
<tr>
<td>Rat SIRT-1</td>
<td>CAGTGTCATGTGTTCTTTGC</td>
<td>CACCGAGGAACTACCTG</td>
</tr>
<tr>
<td>Rat PGC-1α</td>
<td>ACTGAGCTACCTTGGGATG</td>
<td>TAAGGATTCGTTGTTGAC</td>
</tr>
<tr>
<td>Rat FAS</td>
<td>CAGTTCCTCGTGACTCATC</td>
<td>TCATCAAAGGTGTTGC</td>
</tr>
<tr>
<td>Rat CPT-1c</td>
<td>AATCCCTCACCTCATGC</td>
<td>ATCCCAACTGGAAGCACTCT</td>
</tr>
</tbody>
</table>

4.5 Histological and morphological analysis

Mice were perfused intracardially with 20 ml of PBS and then 3x20 ml of 4% PFA. Brains were immerse-fixed in 4% PFA for 4 hours and moved to PBS. Cryoprotection was carried out using 20% sucrose in PBS for 24 hours. Brains were frozen in liquid nitrogen-isopentanol solution for 20 minutes. Brain slices of 16 μm were cut using cryotome. The whole brain was cut and every third slice was taken for staining.

4.5.1 Immunohistochemistry

DBI-immunoreactive neurons were stained utilizing the biotin-avidin technique (Lewis et al., 1986). Briefly, the sections were treated with 1% hydrogen peroxide to remove endogenous peroxidase, and washed in potassium phosphate–buffered saline (KPBS), pH 7.4 before blocking in 10% normal goat serum (NGS) and 0.5% Triton X-100 in KPBS. Thereafter, sections were incubated for 3 days (+4°C) in DBI antibody (1:16 000, Paesel-Lorei, Hanau, Germany) in 1% NGS, 0.5% Triton X-100 in KPBS. The sections were washed with KPBS containing 2% NGS before incubation for 1h with biotinylated goat anti-rabbit immunoglobulin G (1:1500 Vector, Burlingame, CA, USA). After washing in KPBS with 2% NGS, sections were incubated in avidin-biotin solution (Vectastain Elite ABC kit (Standard), Vector) for 45 min and washed in KPBS with 2% NGS. Incubations in the secondary antibody and in the avidin–biotin solution were repeated. The DBI-antibody complex was visualized with 0.05% 3',3'-diaminobenzidine (DAB; Pierce Chemical, Rockford, IL, USA), and 0.05% hydrogen peroxide in KPBS. Sections were mounted onto gelatin-coated slides, dried overnight at 37°C, and intensified with osmium tetroxide and thiocarbohydrazide.
4.5.2 Cellular localization of DBI

To examine the cellular localization of DBI, double immunostaining with neuronal (neuronal nuclei, NeuN), astrocytic (glial fibrillary acidic protein, GFAP) was performed. In short, sections were rinsed in KPBS, blocked in 10% normal horse serum (NHS) and incubated for 3 days at 4°C in a primary antibody mix containing DBI antibody (1:1000, Peninsula Laboratories) and NeuN antibody (1:15 000, Chemicon International, Temecula, CA, USA) or GFAP antibody (1:2000, Roche, Mannheim, Germany). After washing, the sections were incubated overnight at 4°C with the secondary antibody mix containing Cy2-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and biotinylated horse anti-mouse IgG (1:200, BA-2000, Vector, Burlingame, CA, USA) followed by an incubation of Cy5-conjugated streptavidin (2 μg/ml, Jackson ImmunoResearch Laboratories). Sections were mounted on non-gelatinized slides with GelMount and dried overnight before analysis. Photographs were taken from the granule cell layer and the CA3 layer of hippocampus with Nikon Eclipse/Ultra WIEW (Tokyo, Japan) confocal microscope.

4.5.3 Measurement of ventricle areas

The ventricle areas were measured from Nissl stained slides at 48 μm intervals using Stereo Investigator 2000 (MicroBrightField, Williston, VT, USA) program in a system comprising of Olympus BX50 microscope linked to a personal computer. Both ventricles from each section were measured blinded regarding to phenotype and the means were calculated.

4.6 High resolution magnetic resonance imaging (MRI)

Adult mice (tg n=5 and sg n=5) were anesthetized with an i.p. injection (50 μl/10 g body weight) of a mixture of fentanyl-fluanisone and midazolam, and externally fixed to a custom-built animal holder for high resolution magnetic resonance imaging (MRI). Warm air was blown through the magnet bore during MRI. A s.m.i.s. console (Surrey Medical Imaging Systems, Guildford, UK) interfaced to a 9.4 T vertical magnet (Oxford Instruments, Oxford, UK) was used for MRI with a single loop surface coil (diameter 27 mm) in the transmit/receive mode. Multi-slice T2-weighted images were acquired using a single-echo spin-echo method (time-to-repetition 3000 ms, time-to-echo 45 ms, 4 scans/line, field of view 25.6x12.8 mm2 (transversal) or 25.6x25.6 mm2 (coronal), matrix size 256x64 and slice thickness 0.75 mm).

4.7 Behavioural testing

Five sg and 6 tg male, 12 sg and 12 tg female mice (6 months old at the time of testing) were used for behavioural analysis. Behavioural test battery included the tests for exploratory activity (elevated plus maze, light-dark box, Y-maze, open field), nociception (hot plate), coordination (beam walking, rotarod),
and learning and memory (fear conditioning, water maze). All tests were performed essentially as described by Voikar et al. (2004).

4.7.1 Fear conditioning (FC)

This classical conditioning analyses two memory components – association of unconditioned stimulus (foot-shock, US) with a particular compartment (contextual memory) and simple association of conditioned stimulus (tone, CS) with shock. The experiments were carried out employing a computer-controlled fear conditioning system (TSE, Bad Homburg, Germany). Training was performed in a clear acrylic cage (35 x 20 x 20 cm) within a constantly illuminated (550 lx) fear conditioning box. A loudspeaker provided a constant, white background noise (68 dB) for 120 seconds followed by 10 kHz tone (CS, 75 dB, pulsed 5 Hz) for 30 seconds. The tone was terminated by a foot shock (US, 0.7 mA, 2 seconds, constant current) delivered through a grid floor. Two CS-US pairings were separated by a 30-second pause.

Contextual memory was tested 24 h after the training. The animals were returned to the conditioning box and total time of freezing (defined as an absence of any movements for more than 3 seconds) was measured by infrared light barriers scanned continuously with a frequency of 10 Hz. The CS was not used during this time. Memory for CS (tone) was tested 2 h later in a novel context. New context was a similarly sized acrylic box. The light intensity was reduced to 100 lx, the floor was plain (without a shock grid) and the background colour was black (as opposed to the white colour used in training context). After 120 seconds of free exploration in the novel context, the CS was applied for an additional 120 seconds and freezing was measured as above. In addition, the activity was registered in all phases of training and testing. Freezing behaviour and activity were later corrected for the baseline measures (preconditioning for context test and new context for cue test) and expressed as a change from the respective baselines.

4.7.2 Water maze (WM)

The WM test was introduced for testing spatial learning and memory in rodents (Morris, 1981). The system used here consisted of a black circular swimming pool (diameter 120 cm), escape platform (diameter 10 cm) submerged 0.5 cm under the water surface in the centre of one of four imaginary quadrants and computer interfaced video tracking system (EthoVision, Noldus, The Netherlands). The animals were released to swim in random positions facing the wall and the time to reach the escape platform was measured in every trial. Two training blocks consisting of three trials each were conducted daily. The interval between trials was about 5 min and between training blocks about 5 h. The platform remained in a constant location for 3 days (6 sessions) and was thereafter moved to the opposite quadrant for 2 days (4 sessions). The transfer tests were conducted approximately 18 h after the 4th, 6th and 10th training sessions. The spatial memory was estimated by the time spent in the zone around the platform.
(covering 6.25% of the total area of the water tank) and in the corresponding zones of the three remaining quadrants. In addition, the swimming distance and the thigmotaxis were measured. Thigmotaxis was defined as the time spent swimming within the outermost ring of the water maze (10 cm from the wall).

4.8 Synaptic transmission and induction of long-term potentiation (LTP)

Tg mice (n=17) and their sg littermates (n=18), 5–8 months old, were used. Both females (n=20) and males (n=15) were investigated to see whether a sex difference causes changes in synaptic neurotransmission and/or plasticity. Mice were anesthetized with halothane and brains rapidly dissected. Slices were obtained from the middle third of hippocampi in both hemispheres and stored in a holding chamber at room temperature (~24°C). The holding chamber was continuously gassed with 95% O₂-5% CO₂. Individual hippocampal slices were transferred for recording into a submerged chamber held at 32 ± 1°C with a bathing solution containing (in mM) NaCl 124, KCl 3, KH₂PO₄ 1.25, CaCl₂ 3.4, MgSO₄ 1.0, NaHCO₃ 26, D-glucose 10 and L-ascorbate 2. Slices were bubbled with 95% O₂-5% CO₂ to prevent calcium precipitation and perfused with a rate of 2.0 ml/min.

Electrophysiological recordings were made from the apical dendritic field of CA1 area in the hippocampal slice using a glass microelectrode filled with 2 M NaCl. A Bipolar electrode made of twisted tungsten wire (50 μm) for stimulation was placed in the stratum radiatum of CA1 at a lateral distance of minimum 200 μm from the recording electrode. The stimulation intensity was adjusted to obtain about 50% of the maximal excitatory postsynaptic potential (EPSP) amplitude and input was stimulated (0.1 ms pulse duration) every 30 seconds. If the baseline of EPSP responses did not stabilize, the experiment was discontinued. Paired pulse facilitation (PPF) was assessed by measuring the percent change of the second response relative to the first response to a pair of stimulation pulses separated by 75 msec. After a stable baseline period (20 min), LTP was elicited in the pathway by two applications of 10 bursts (inter-burst interval 30 s) of high frequency stimulation in a ‘theta burst’ pattern (TBS), i.e. 100 Hz trains of 4 pulses separated by 200 ms (Larson and Lynch, 1986; Pussinen and Sirvio, 1998). For maximal induction of LTP, the stimulation intensity was increased by setting the pulse duration to 0.3 ms. Monosynaptic field EPSPs were band-pass filtered from 0.1Hz to 3 kHz and digitized at 10 kHz. Data were recorded and analyzed by a microcomputer using the pCLAMP8.0 program (Axon Instruments, Foster City, CA, USA).

4.9 Seizure induction

4.9.1 Kainic acid

26–30 mg/kg of kainate (KA) (in 0.9% NaCl, 26 – 30 mg/kg, Opika-™ Kainic Acid, Ocean Produce International, Nova Scotia, Canada) was administered intraperitoneally (i.p.) to either 5 or 8 month old tg
and sg mice. One observer, blind to the genotype of the animals, determined the occurrence of behavioural seizures in mice visually for four hours after KA injection. The time to the first behavioural manifestations of seizures, number and severity of behavioural seizures during the 4 h follow-up period were scored according to a modified Racine’s scale (Lahteinen et al., 2003; Racine, 1972)

4.9.2 Pentyleneetetrazole (PTZ)

(Sigma). Eight sg and 8 tg male mice (5 months old, weight 27.0-38.5 g) were used for studying the seizure threshold with PTZ. PTZ was dissolved in 0.9% NaCl and 60 mg/kg was injected i.p. Two observers, blind to the genotype, monitored the animals for one hour after the injection. Time to the first behavioural manifestations of seizures, number and severity of the seizures during the 1 hour follow-up were recorded.

4.9.3 Histological evaluation

Mice, which had been injected with 28 mg/kg KA were perfused as described above 48 hours after injection. Brain sections were made in the coronal plane (30 μm, one-in-five series). The amount of degenerating neurons after KA injection was evaluated using Fluoro-Jade B staining. After incubation in decreasing alcohol concentrations, the slides were bathed in a solution containing 0.06% potassium permanganate for 15 min, washed and incubated in Fluoro-Jade B solution containing 0.001% Fluoro Jade stain in acetic acid. After washing, the slides were dried, dehydrated in an alcohol series and xylene and mounted with DePeX. The amount of degenerating neurons in granule, hilus, CA3a, CA3b, CA3a and CA1 areas were visually evaluated as triplicates and scored. Evaluation started at the section where the suprapyramidal and infrapyramidal blades connected for the first time and continued for the six following sections.

4.10 Acyl-CoA extraction and measurement

Acyl-CoAs were extracted and measured from 6 independent samples of visceral adipose tissue and liver of transgenic and syngenic rats as described by Mauriala et al. (2004). In brief, tissue samples were powdered under liquid nitrogen and heptadecanoyl-CoA (C17:0) was added as an internal standard. Powdered tissues were homogenized in 5 ml ice-cold isopropanol-K2HPO4 and acidified with glacial acetic acid and washed with hexane-isopropanol and hexane. The hexane phase was discarded and proteins in lower phase were precipitated with saturated ammonium sulphate and methanol-chloroform solution. After centrifugation, the protein pellet was washed, centrifuged and supernatants combined. After addition of water the upper aqueous phase was washed with chloroform. The extracted chloroform phases were combined with the lower phase, washed with water and the aqueous phases from the extractions were then pooled. Prior to the analysis samples were dried under nitrogen and re-dissolved in
5% methanol-water. Acyl-CoAs were detected using on-line HPLC-ESI-MS/MS. This method was validated in the range of 0.1-15.0 pmol/μl of acyl-CoAs and recovery was 60±5%.

4.11 Triglyceride, free fatty acid and cholesterol measurement

Serum free fatty acids were measured from fed tg and sg rats using an enzymatic colorimetric method by NEFA C kit (Wako, Neuss, Germany). Plasma triglycerides and cholesterol were determined with Ecoline S reagents (DiaSyS, Holzheim, Germany).

4.12 Glucose tolerance test (GTT)

A glucose dose, 1 g/kg body weight, was administered i.p. to unanaesthetized rats, blood samples were collected from saphenous vein at 0, 10, 20, 60 and 120 min after injection. Plasma glucose was measured using EPOS 5060 (Eppendorf, Hamburg, Germany) and serum insulin by rat insulin ELISA (Crystal Chem, Downers Grove, IL, USA).

4.13 Statistical analysis of data

Results of the GTT and insulin levels were analyzed with two-way repeated measurements ANOVA and Bonferroni post-tests. Significance of differences in mean values of synaptic responses and LTP were tested by analysis of variance (ANOVA, MANOVA). Otherwise differences between groups were assessed by Student’s t – Test or Mann-Whitney U test when appropriate. Statistical significance was defined as p value < 0.05. All statistical tests were done using Graph Pad Prism (Graph Pad software, San Diego, CA, USA) or SPSS 11.5 software.
5. RESULTS

5.1 Gene
In order to create the transgenic animal lines, mouse ACBP/DBI gene was isolated from a genomic library. The ACBP gene (11.1 kb) consisted of 1.8 kb promoter, 4 exon regions including the recently found alternative exon 1 (Nitz et al., 2005) and 1.9 kb 3' flanking region. The promoter region contained various potential binding sites for transcription factors including peroxisome proliferator activated receptors (PPARs) that have been previously reported to take part in the regulation of ACBP/DBI expression (Helledie et al., 2002b). The exon/intron structure was similar to that of the rat and human genes. Comparison of the exon sequences with mouse ACBP/DBI cDNA revealed no base differences in the protein coding region. The nucleotide sequence of the isolated mouse ACBP/DBI gene has been submitted to GenBank database under accession number AF220221.

5.2 ACBP expression

5.2.1 Mouse line
Microinjections created two mouse lines carrying the ACBP/DBI transgene. The line designated UKU302F was chosen for further studies on basis of higher expression levels. Transgenic (tg) mice were viable and fertile. Quantitative real-time PCR from genomic DNA of the tg mice confirmed that there is a 30 times increase in the gene copy number and the brain ACBP/DBI mRNA levels were 6.2 times higher in the tg animals than in their sg littermates (Paper I, Fig.1). Western blots confirmed that the tg animals had 37 times more ACBP/DBI protein than the sg littermates. Tg animals had elevated mRNA and protein levels also in the other tissues studied.

The sites of transgenic expression of ACBP in the central nervous system were studied in more detail. Staining with ACBP/DBI antibody showed excessive expression in the infragranular region of the dentate gyrus in tg animals (Paper I, Fig.2). Double staining with neuron specific NeuN and glial cell marker GFAP demonstrated that both in the infragranular region as well as in the CA3 field of the hippocampus DBI was mainly expressed in glial cells (Paper I, Fig. 3).

5.2.2 Rat line
The isolated endogenous mouse ACBP gene (AF220221) including the promoter region was used to create a rat line overexpressing ACBP. Transgenic rats overexpressing ACBP were viable and fertile. Quantitative real-time PCR results from liver, adipose tissue and isolated hypothalamus showed that ACBP mRNA was about 6-27 times higher in tg animals than in sg controls (Paper II, Fig.1 and Paper IV, Fig.1) Especially in adipose tissue there was a considerable increase (27 times) in ACBP mRNA
levels. Western blot results confirmed the elevated ACBP levels. Furthermore, quantitative real-time PCR results revealed that in liver of transgenic (tg) animals, fasting doubled the ACBP mRNA levels (Paper II, Fig.1).

The effects of the high fat diets on the expression of ACBP mRNA were investigated in liver and in visceral adipose tissue. In liver, both sg and tg rats showed a similar trend to increased ACBP expression with the LC diet compared to the MC diet fed rats (Paper III, Fig.1). On the contrary, in adipose tissue, the MC diet-fed tg rats had significantly elevated levels of ACBP compared to the LC diet fed tg rats (Paper III, Fig.1). There was no difference between diets in the sg controls.

5.3 Phenotype of the mouse line

5.3.1 Ventricle size

Basic histology revealed enlarged ventricles in the tg animals, therefore more detailed investigations were performed. Mean ventricle areas were calculated for each mouse and compared between tg and sg animals by planimetric analysis. Adult tg mice exhibited a significantly larger mean ventricle area than sg controls. In addition, the difference in ventricle size was confirmed by MRI study showing that the adult tg animals had a five times larger mean ventricle area than their sg littermates (Paper I, Fig. 4). MRI studies with newborn mice revealed that the differences were already evident at birth and evaluation of the rat line (Fig. 6) confirmed that the hydrocephalus is specific effect of increased ACBP levels and not caused by an insertional mutagenesis.

5.3.2 Behavioural testing

ACBP has been indicated to induce pro-conflict behaviour and anxiety (De Mateos-Varchere et al., 1998; Ferrero et al., 1986a; Ferrero et al., 1986b). Evaluation of the behaviour of transgenic animals showed that there was no major difference between the groups in motor and exploratory activity as assessed by open field, elevated plus maze, light-dark and Y-maze tests. Furthermore, the pain sensitivity (hot plate) and coordination (rotarod, beam balancing) were not affected by the overexpression of the ACBP/DBI transgene. Nonetheless, in the fear conditioning experiments tg males displayed significantly reduced

Figure 6. Hydrocephalus in rats. MRI pictures shown enlarged vesicles in tg rats. Mean ventricle volume in sg 15.4±5.16 mm³ and in tg 53.2±37.14 mm³.
freezing in conditioning context (a hippocampus-dependent form of memory) as compared with sg males (Paper I, Fig 5). However, there was no difference between females (effect of genotype F(1,31)=3.6, p=0.067; interaction of genotype and sex F(1,31)=6.8, p=0.014). The extent of freezing in response to the conditioned stimulus (tone; amygdala-dependent) did not differ between the sg and tg mice.

All groups learned to find the hidden escape platform in the water maze experiment (Paper I, Fig.6a). However, in the first transfer test (after 4 learning blocks), the sg mice displayed a clear preference for the trained quadrant whereas the tg mice did not show any spatial selectivity (Paper I, Fig. 6b). After prolonged training (2 additional blocks of trials) tg mice also learned to search in the trained quadrant (Paper I, Fig. 6c). Both groups were able to learn to find the platform in the new (opposite) location and showed a preference for the new zone in the third transfer test (Paper I, Fig. 6d). All mice rapidly learned to swim to the platform when it was made visible, confirming that they possessed sufficient motor and motivational abilities to perform the swimming navigation task.

5.3.3 Long term potentiation (LPT)

The effect of ACBP on spatial and emotional learning and memory was further analysed with LPT. Field EPSP waveforms evoked by Schaffer-collateral stimulation were measured in CA1 during the baseline recording period. Comparable stimulation currents elicited equivalent amplitude responses in tg and sg slices and no differences in responses were observed between the groups in any parameter tested (amplitude, slope, half-width; n.s)(Paper I, Table 1).

Theta burst stimulation led to a transient post tetanic potentiation up to three minutes in both sg and tg mice. In contrast, it evokes to a long-term potentiation of synaptic strength only in sg but not in tg mice. In sg mice, Schaffer-collateral synapses exhibited approximately 100% increase in EPSP amplitude measured 10-45 minutes after high frequency stimulation, whereas the potentiation was approximately 20% in DBI tg mice during this time period (Paper I, Fig. 7). There was no difference between the sexes (for gender effect F=0.06, n.s.). Thus, there were significant differences in the long term potentiation of EPSP amplitude between female tg and sg mice (for group effect F=5.20, p<0.05) and between male tg and sg mice (for group effect F=6.13, p<0.05). In all mice, there were significant differences in the long term potentiation of EPSP amplitude between tg and sg mice (for group effect F=11.21, p<0.01)(Paper I, Fig.7).

5.3.4 Seizure induction

In order to investigate in more detail the possible effect of ACBP on GABA_A receptor function, i.c.v injections of KA and PTZ were performed. All mice, except one sg female treated with 30 mg/kg of KA, developed seizures during the four hour observation period (Paper I, Table 2). The highest mortality (38.5%) in tg mice occurred at 28 mg/kg. In sg mice, the highest mortality (58.3%) was seen at 30
mg/kg. The time period from injection to death was longest at the lowest dose 26 mg/kg used in both tg and sg animals. The time became shorter as the dose was increased in tg mice but this was not observed in sg animals, however the difference was not statistically significant. Furthermore, there were no significant differences between sg and tg mice in the total numbers of seizures or in the latency to first seizure during the 4-hr observation period (Paper I, Table 3). There was no difference in the amount of degenerating neurons in granule, hilus, CA3a, CA3b, CA3a and CA1 areas (Paper I, Table 4).

All mice injected with 60 mg/kg PTZ developed seizures. Sg animals had even higher mortality (37%) than tg mice (0%) but there were no statistical differences in either the number or the time period to the first seizure.

5.4 Phenotype of the rat line

5.4.1 Weight

It has been shown that ODN, a processing product of ACBP can influence the weight and food intake of rodents (de Mateos-Verchere et al., 2001). In our tg animals, no weight difference was observed when these rats were compared sg littermates at the age 6 weeks (188±25 g for sg versus 169±35 g for tg) or at the age of 1 year (515±29 g for sg versus 524±25 g for tg). During the four week period on the MC or LC high fat diets, the weight of the animals was monitored. There was no significant difference between tg and sg rats within the same diet group.

5.4.2 Food intake

The daily food consumption was measured for four days. The mean daily food intake (in grams) was not affected by the increased ACBP expression (19.8 ± 1.7 g for sg versus 18.8±2.5 g for tg). Taking into account the weight of the animals did not influence the results.

5.4.3 Acyl-CoA levels

It has been shown that overexpression of ACBP increases the acyl-CoA content of yeast and mouse liver (Huang et al., 2005; Knudsen et al., 1994). To ensure that mouse ACBP gene was functionally active in rats and to study whether overexpression of ACBP could affect acyl-CoA contents, we measured acyl-CoA levels from visceral adipose tissue, liver and brain of fed and fasted animals. Our results demonstrate that the mouse ACBP gene is functionally active in rats: liver C16 acyl-CoA levels were increased (C16:0 2.8 times and C16:1 2.3 times higher) as well as the C20 levels (C20:0 1.4 times and 20:4 1.3 times higher) in fed animals. There was no difference in any of the liver acyl-CoAs measured from fasted animals (Paper II, Fig.2, note the different scale). In adipose tissue, there was a 5 to 11 times increase in C16:0, C16:1 and C18:1 in the fed state. In fasted animals, levels of C16:0, C16:1, C18:0 and
C18:1 were 2 to 4 times higher in tg animals (Paper II, Fig.2). In brain there was an increase of C20:4 in the fed tg rats. Interestingly, the fasted tg rat had an decrease in C20:0 levels compared to sg littermates (Paper IV, Fig.2).

5.4.4 Free fatty acids, triglycerides and cholesterol

Overexpression of ACBP did not influence serum free fatty acid, plasma triglyceride and cholesterol levels of tg rats fed with standard diet (Paper II, Table 2). In addition, high ACBP expression did not affect the free fatty acid levels of tg rats fed with either medium or long chain high fat diets.

5.4.5 Glucose tolerance

GTT was performed to test whether overexpression of ACBP and/or the subsequent changes with increased levels of cellular acyl-CoAs affect plasma glucose levels or insulin secretion. There was no significant difference between tg rats and their sg controls in the glucose tolerance or in the insulin levels when fed with normal diet (Paper II, Fig.3).

GTT was also performed after feeding a MC and LC high fat diet for four weeks. The MC diet fed tg animals had improved glucose tolerance (ANOVA p=0.0036. Paper III, Fig.2), and a significant reduction in overall insulin levels (ANOVA p=0.016). The insulin levels were significantly lower at 20 min and 120 min (p< 0.05. Paper III, Fig.2) indicative of an improved insulin responsiveness. In LC diet fed animals, there was no difference in glucose tolerance or in the insulin levels (Paper III, Fig.2).

5.5 Gene regulation

5.5.1 Peroxisome proliferator-activated receptors

To study the effects of ACBP overexpression on regulators of metabolic responses, mRNA levels of transcription factors PPARα, γ and δ were measured in liver and adipose tissue of tg and sg animals in different metabolic states: fed and fasted state and after feeding high fat diets with MC or LC fatty acids for four weeks. In fed tg animals, expression of liver PPARγ and PPARδ mRNA was down-regulated by 82% and 62% respectively compared to sg littermates (Paper II, Fig.4), whereas there was no significant difference in PPARα mRNA levels. In adipose tissue, there was a decrease in PPARγ by 23% and by 43% in PPARδ levels (Paper II, Fig.4).

In contrast, fasting significantly increased the expression of all liver PPARs by 2 to 7 times in tg rats (Paper II, Fig.4a), while in sg rats there was simply a trend to higher expression of PPARα, but there was no change in the PPARγ and δ levels. In adipose tissue, PPAR γ levels were reduced by 70% in fasted syngenic rats, while expression was increased by 37% in the tg littermates. The expression of
PPARδ was significantly increased (3.3 times) by fasting in tg rats, but remained unchanged in the sg controls (Paper II, Fig.4b). Fasting abolished the difference in liver PPARγ and δ expression levels between tg and sg animals. On the contrary, in adipose tissue, fasting evoked higher PPAR γ and δ expression in tg animals when compared to their sg controls.

Furthermore, dietary factors can influence the PPAR expression. Expression of liver PPARα or PPARγ in sg animals did not change under the MC compared to the LC diet, but liver PPARδ levels were increased in MC diet fed sg rats compared to the LC diet (Paper III, Fig.3). The tg rats fed with the MC diet had decreased expression levels of liver PPARα and PPARγ when compared to the LC diet fed animals and no change in PPARδ. Tg animals fed with the LC diet had 2 to 3 times higher levels of liver PPAR mRNA expression than their sg littermates fed with the same diet. In the MC diet group, tg rats had significantly reduced (by 43%) levels of liver PPARγ when compared to sg controls. In adipose tissue, there were no significant changes in PPARγ or PPARδ expression between MC and LC diet fed tg or sg animals. The MC diet fedtg rats displayed significantly increased (by 31%) levels of PPARγ compared to the sg animals fed with the same diet (Paper III, Fig.3).

5.5.2 Sterol regulatory element-binding proteins

Another metabolic regulator known to be involved in ACBP and PPAR regulation is SREBP (Fajas et al., 1999; Neess et al., 2006; Swinnen et al., 1998). The effects of ACBP overexpression on SREBP-1 mRNA was measured from liver and adipose tissue. Expression of SREBP-1 mRNA was down-regulated by 77% in liver and by 23% in adipose tissue (Paper II, Fig.5). In sg rats, fasting reduced the SREBP-1 mRNA levels in both liver and adipose tissue. In tg animals, fasting reduced SREBP-1 mRNA levels only moderately and only in liver (Paper II, Fig.5).

The differences in SREBP expression were also investigated in response to the diets with the sg and tg animals having a lower liver SREBP-1 expression when fed with the MC diet compared to the LC diet. The tg rats fed with the MC diet exhibited decreased levels (by 35%) of liver SREBP-1 compared to sg controls. On the contrary, after the LC diet tg rats had 1.5 times higher SREBP-1 levels compared to their sg littermates (Paper III, Fig.3). In adipose tissue, both sg and tg animals had similar levels of SREBP-1 expression when on the MC diet. Tg animals fed the LC diet had increased SREBP-1 levels when compared to sg controls (Paper III, Fig.3), in sg rats, SREBP-1 expression was unchanged.

5.5.3 AMP-activated protein kinase

SREBP-1 and PPARs have been demonstrated to be regulated by AMP-activated protein kinase (AMPK) (Leff, 2003; Zhou et al., 2001). Therefore, we determined protein levels of the α subunit of AMPK in the liver of fed rats. The levels of total AMPKα and its active form, Thr 172 phosphorylated AMPKα, were significantly elevated in the liver tissue of tg rats (Paper II, Fig.6). Furthermore, intraperitoneal
### Table 4. Summary of the effects of nutritional state and ACBP on PPAR and SREBP-1 mRNA expression and AMPK protein levels.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tg compared to sg in fed state</td>
<td>tg compared to sg in fasted state</td>
</tr>
<tr>
<td>PPARα</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>PPARγ</td>
<td>-</td>
<td>no change</td>
</tr>
<tr>
<td>PPARδ</td>
<td>-</td>
<td>no change</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>-</td>
<td>no change</td>
</tr>
<tr>
<td>AMPK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><strong>Liver</strong></td>
<td><strong>Adipose tissue</strong></td>
</tr>
<tr>
<td>PPARγ</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PPARδ</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>-</td>
<td>no change</td>
</tr>
</tbody>
</table>

+ up-regulation, - down-regulation

### Table 5. Summary of the effects of high fat diets and ACBP on PPAR and SREBP-1 mRNA levels and AMPK protein levels.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC compared to LC in sg</td>
<td>MC compared to LC in tg</td>
</tr>
<tr>
<td>PPARα</td>
<td>no change</td>
<td>-</td>
</tr>
<tr>
<td>PPARγ</td>
<td>no change</td>
<td>-</td>
</tr>
<tr>
<td>PPARδ</td>
<td>+</td>
<td>no change</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMPK</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Liver</strong></td>
<td><strong>Adipose tissue</strong></td>
</tr>
<tr>
<td>PPARγ</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>PPARδ</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>no change</td>
<td>no change</td>
</tr>
</tbody>
</table>

+: up-regulation, -: down-regulation,
MC: medium chain high fat diet, LC: long chain high fat diet
injections of the AMPK inhibitor, compound C, to tg rats resulted in 17% reduction in protein levels of Thr 172 phosphorylated AMPKα (Paper II, Fig.7a), while quantitative real-time PCR analysis of SREBP-1 and PPARs detected a 2.2 fold increase in SREBP-1 mRNA expression in compound C treated tg animals with no difference in PPARγ nor PPARδ mRNA levels (Paper II, Fig.7b).

The effect of the two high fat diets on the AMPK levels was also investigated. There was no difference in AMPK protein levels between MC and LC diet fed animals. In the LC diet fed group, tg animals had 48% lower (p=0.02) protein levels of AMPKα than their sg littermates (Paper III, Fig.4).

5.5.4 Gene expression in the hypothalamus
The changes in mRNA expression of several transcription factors known to be involved in hypothalamic or peripheral regulation of fatty acid metabolism and two key enzymes participating in hypothalamic fatty acid induced food intake regulation were measured from fed and fasted animals. In the fed state, the tg rats had a reduction in the expression levels of FAS (Paper IV, Fig.3), sirtuin-1 (SIRT-1) and PPARδ (Paper IV, Fig.4) when compared to sg controls. In addition, the tg animals had an increase in the mRNA levels of CPT-1c (Paper IV, Fig.3). Fasting increased the SIRT-1 expression of the tg rats, while it did not influence the expression of the sg animals nor alter the expression of PPARδ, FAS or CPT-1c of the tg animals. On the contrary, the sg rats displayed decreased FAS levels and increased CPT-1c expression after fasting, but no change in PPARδ expression. There were no difference in the expression of SREBP-1 between tg and sg animals in either the fed or fasted state, both tg and sg rats showed a fasting induced reduction in SREBP-1 levels (Paper IV, Fig. 4).

5.5.5 AMP-activated protein kinase in the hypothalamus
The possible role of AMPK, a known regulator of hypothalamic food intake, was investigated. The protein levels of both total AMPKα and the active form, Thr 172 phosphorylated AMPKα were measured from hypothalamus of fed tg rats and sg littermates. There were no significant differences in the total AMPKα or the phosphorylated AMPKα levels between tg and sg animals (Paper IV, Fig.5).
6. DISCUSSION

6.1 ACBP gene and animal models

Acyl-CoA binding protein (ACBP) / diazepam binding protein (DBI) is a 10 kDa protein ubiquitously expressed throughout the whole body. Many different functions have been associated with this protein. ACBP can displace diazepam from type A γ-aminobutyrate receptors (GABA\textsubscript{A} receptors) (Guidotti et al., 1983) and modify the function of peripheral benzodiazepine receptors (PBR) (Bovolin et al., 1990; Garnier et al., 1993; Papadopoulos et al., 1992). In addition to its effects on the benzodiazepine receptors, the ability to bind and act as a pool former and transporter of acyl-CoAs is one of the best recognized functions of ACBP (Rasmussen et al., 1990; Rosendal et al., 1993). Although there are a plethora of reported effects, the precise function of ACBP in the physiology of mammals remains unclear. Previous studies have been conducted mainly with cells, or they have utilized exogenous administration of the protein to investigate the short term effects of ACBP. Huang et. al. (2005) utilized transgenic mice overexpressing ACBP focusing only on changes in the liver fatty acid composition of these mice. Importantly ACBP cDNA expression was controlled by a phosphoglycerate kinase promoter, which does not allow endogenous regulation of ACBP (Huang et al., 2005). Therefore we were interested to investigate the role of ACBP in rodent physiology and regulation gene expression. We utilized a construct containing the entire mouse ACBP gene under the gene’s own 1.8 kb promoter region to allow endogenous expression of the transgene.

We isolated the mouse ACBP/DBI gene from a genomic library of mouse strain 129/SvJ and utilized the isolated gene to create mouse and rat lines with constant overexpression of ACBP. The isolated mouse ACBP gene (AF220221) consisted of a 1.8 kb promoter, 4 exon regions including the recently found alternative exon 1 (Nitz et al., 2005) and a 1.9 kb flanking region. No differences to the published cDNA sequences could be detected. Furthermore, the isolated gene contained all of the so far published regulatory regions of the mouse ACBP gene. The isolated mouse ACBP gene allowed us to create a mouse line expressing high levels of native ACBP. The mouse ACBP gene was additionally utilized to create a rat line with high levels of ACBP. The mouse and rat ACBP protein sequences are 98.8% similar and reports on the regulation of ACBP gene transcription regulation have found no major differences (Helledie et al., 2002a). The basic characterization of both mouse and rats lines demonstrated that the transgene was overexpressed in all tissues studied. In the central nervous system of mice, the transgene was found to be expressed in the same locations as previously reported for the endogenous protein (Costa and Guidotti, 1991; Yanase et al., 2001) The mouse ACBP gene was also functional in rats, since elevated levels of mouse ACBP increased the acyl-CoA pool of tg rats. Both transgenic mouse and rat lines were viable and fertile. We utilized a rat line overexpressing ACBP as rats are more easily subjected to surgical manipulation and tissue preparation. In addition, the genetic background of the
mouse line utilized (F1 cross of Balb/c x DBA/2) was unsuitable for the intended metabolic studies, resulting most likely from the fact that high metabolic rate of DBA/2 strain (Pennycuik, 1967) was randomly mixed within the metabolic background of Balb/c strain.

6.2 ACBP in central nervous system

Our results from a mouse line overexpressing the mouse ACBP gene clearly demonstrate that long-term elevation of ACBP levels does not cause anxiety or any pro-conflict behaviour, in contrast to published data (Guidotti, 1991). Previous studies have investigated mainly the short term effects of ACBP and its processing products (Kavaliers and Hirst, 1986), while in our animals, the expression levels were chronically elevated. Since acute administration of ACBP has been suggested to have a modulatory effect on the GABA\textsubscript{A} receptors and PBRs, it is logical that the long term endogenous expression of ACBP would recruit adaptive processes. GABA\textsubscript{A} receptor signalling is important for neural signalling and therefore its function is under tight regulation. Results from the experiments on benzodiazepine stimulation of peripheral benzodiazepine receptors (PBR) provide further evidence for compensation of the long-term effects. These studies indicate that the acute and chronic effects of PBR ligands are quite different. Acute administration of PBR ligands evoked increased steroid levels in both rats and humans, whereas chronic treatment with PBR ligands did not affect circulating steroid levels of female rats (Lacapere and Papadopoulos, 2003). One possible mechanism to adapt to the chronic effects of ACBP is down-regulation of SREBP-1. SREBP-1 up-regulates the expression of steroidogenic acute regulatory protein (StAR), a protein involved in cholesterol transport to PBR (Lacapere and Papadopoulos, 2003). This indicates that although increased ACBP levels activated PBR it also caused a down-regulation of StAR levels, resulting in reduced cholesterol transport to PBR. In agreement, in our transgenic animals there were no changes in the plasma levels of cholesterol. Interestingly, we detected a reduction only in the liver and adipose tissue SREBP-1 mRNA levels, but not in the hypothalamus, yet this does not rule out the possibility that SREBP-1 could be affected in other brain regions. To further investigate the role of ACBP in neural signalling, we performed seizure threshold experiments with KA and PTZ. There was no difference in the KA induced seizures between tg and sg controls in the number, severity or duration of the seizures and the administration of KA did not evoke any changes between tg and sg mice in the histology preparations performed after the seizures. In the CNS, KA activates kainate receptors, which have been shown to down-regulate GABAergic inhibition (Clarke et al., 1997; Michaelis, 1998). Due to the similarities in the seizure behaviour and pathology evoked by KA and epilepsy, this compound has been utilized as a model of temporal lobe epilepsy. Our results indicate that either ACBP does not influence the mechanism by which KA induces seizures or the continually elevated levels can activate compensatory mechanisms that abolish the effects of ACBP. Elevated plasma ACBP levels have been detected from a subgroup of patients suffering from epilepsy (Ferrarese et al., 1998). Chronic overexpression of ACBP by itself or with KA did not evoke any differences in the seizure activity, indicating that ACBP has a modulatory role. Furthermore, i.c.v injections of PTZ is another well-
established seizure model. The actions of PTZ are mediated by its antagonistic action on the GABA<sub>A</sub> receptors. Similar results were obtained with the PTZ injections as seen in the KA studies, i.e. overexpression of ACBP did not affect the seizures. In contrast to the KA results, there was a difference in the PTZ induced mortality, sg animals had even a higher mortality (37%) than the tg mice (0%). Similar results have been reported by Garcia de Mateos-Verchere et al. (1999), i.e. administration of ODN (100 ng for mice) prior to PTZ (100 mg/kg, i.p.) injections reduced the mortality by 30%. These results suggest that ODN/ACBP influence the function of GABA<sub>A</sub> receptors in a way that reduces the ability of PTZ to block Ca<sup>2+</sup> channels, but do not prevent it completely. The mechanism by which ACBP reduces mortality requires further investigation on the role of ACBP in GABA<sub>A</sub> receptor and PBR regulation.

Behavioural and learning aspects of ACBP overexpressing mouse were studied in behaviour tests and long-term potentiation (LTP). Our results from LTP experiments show that elevated levels of ACBP could affect the development of long-term synaptic plasticity in the CA1 area of hippocampus. The tg mouse exhibited decreased plasticity in the excitatory synapses without any changes in the inhibitory or excitatory synaptic transmission. ACBP has been reported to have an inhibitory effect on the GABA<sub>A</sub> receptors (Costa and Guidotti, 1991), therefore overexpression of ACBP should increase rather than decrease the excitatory response. The molecular reasons for these unexpected findings are unclear and require further investigations. The observed decrease in LTP might be due a reduction in NMDA receptor activity. The excitatory response in LTP is known to be dependent on the activation of NMDA receptors (Larson and Lynch, 1988) and NMDA receptor activation can increase the ACBP mRNA expression in cerebral cortical neurons (Katsura et al., 2001). Behaviour tests confirmed that high expression levels of ACBP did not affect the basic behavioural pattern of the animals. Nevertheless, changes in the LTP were projected on the animals, tg mice had reduced freezing in the conditioning context. These results indicated that ACBP overexpression influences the hippocampus-dependent learning and memory.

In addition, we detected that the overexpression of ACBP lead to enlargement of the lateral ventricles. This phenomenon was evident at birth and in both transgenic mice and rats, indicating that it is specific effect of ACBP overexpression. One possible explanation might be perivascular astrocytosis. This process has been shown to be the cause of hydrocephalus in other models (Crews et al., 2004) and in addition Alzheimer type II astrocytosis has been associated with increased levels of ACBP (Butterworth et al., 1991). The precise influences and the development method of these enlarged lateral ventricles will require further investigation, as well as the possible role of ACBP in the pathology of Alzheimer’s disease.
6.3 ACBP and hypothalamic regulation of food intake

Hypothalamus and particularly the arcuate nucleus in the hypothalamus are considered to be one of the main sites for regulation of energy homeostasis and food intake. The involvement of nutrients and especially the role of fatty acids in the hypothalamic regulation of food intake have attracted considerable attention. Recent studies have shown that fatty acids or more specifically their esters, acyl-CoAs are involved in appetite regulation (Lam et al., 2005a; Obici et al., 2002). The hypothalamic pool of acyl-CoA can be formed either from fatty acid entering the cells or by endogenous synthesis. Peripheral administration of long chain fatty acids elevated the hypothalamic long chain acyl-CoA pool size (Lam et al., 2005a). In addition, all of the components required for the intracellular synthesis and oxidation of acyl-CoAs are expressed in neurons at a relatively high level. Some of the main components of the acyl-CoA synthesis or oxidation, including FAS, CPT-1 and AMPK have been associated with regulation of food intake in the hypothalamus (Lam et al., 2005b). Interestingly, the functions of all three enzymes are closely linked to each other, they all are involved in acyl-CoA pool size regulation. Although fatty acids, especially acyl-CoAs, are associated with hypothalamic regulation of food intake, little is known about the role of proteins that bind acyl-CoAs, namely fatty acid binding proteins (FABPs) and ACBP. The hypothalamic levels of ACBP have been found to be among the highest in central nervous system, and ACBP expression in hypothalamus is concentrated in the nerve terminals of the arcuate nucleus and median eminence (Alho et al., 1985; Alho et al., 1988). In addition, previous studies utilizing the processing product ODN have indicated that it is involved in the regulation of food intake in a receptor mediated manner. Furthermore, i.c.v injection of ODN has been shown to reduce food intake in rodents. The action of ODN was found to be mediated through metabotropic receptors positively coupled to phospholipase C via a pertussis toxin-sensitive G protein (de Mateos-Verchere et al., 2001; do Rego et al., 2007). Furthermore, Compere et al. (2006) has suggested that ACBP might be involved in appetite control caused by androgens. This study demonstrated that androgens and glucocorticoids up-regulated the ACBP mRNA expression in hypothalamus of male mice.

Although these previous results indicate that ACBP might play a role in the regulation of food intake our results from ACBP transgenic rats demonstrated that there was no difference in the daily food intake or in the weight of the tg and sg animals. In addition, there was no difference in the weight of old rats (1 year old) indicating that there are no differences during the life span of the animals that could become manifested as an accumulatory difference in weight. These results suggest that long term overexpression of ACBP does not influence the food intake, but do not exclude the possibility that ACBP can function as a short term modulator. It is likely that the hypothalamic acyl-CoA pool is under tight regulation that does not allow drastic changes. In accordance, further investigation of our ACBP overexpressing rat line demonstrated that there were only slight changes in the brain acyl-CoA pool. Fed tg animals had increased levels of C20:4 and the fasted tg rats had decreased levels of C20:0. Huang et. al. (2005) reported that in liver, ACBP can influence the acyl-CoA pool size in terms of chain length and saturation.
dependent manner. This was suggested to be caused by variation in ACBPs ability to bind different acyl-CoAs forms and changes in the activity of acyl-CoA utilizing enzymes. Our transgenic rats had significant changes in the hypothalamic gene expression pattern: The mRNA levels of two known regulators food intake and acyl-CoA pool were differentially expressed in tg and sg rats. The expression of FAS was reduced and the levels of CPT-1c were elevated in the fed tg animals. The reduction of FAS results into decreased acyl-CoA production and increased accumulation of malonyl-CoA, an important inhibitor of CPT-1. Decrease in function and expression levels of FAS are associated with a reduction in food intake (Lopez et al., 2006; Shimokawa et al., 2002). On the contrary, increased CPT-1c levels have been associated with increased food intake (Obici et al., 2003; Wolfgang et al., 2006). Elevated CPT-1c levels do not influence the acyl-CoA pool considerably, since the protein is unable to transport the acyl-CoAs into mitochondria (Wolfgang et al., 2006). Nevertheless, CPT-1c is able to bind malonyl-CoA and affects the food intake and weight control. Wolfgang et al. (2008) have shown that mouse line with knock-out of CTP-1c exhibit decreased food intake and lower body weight, without any changes in the gene expression or in the malonyl-CoA and acyl-CoA levels. The exact role of CPT-1c in the appetite control remains unclear. Wolfgang et al. (2008) suggest that CPT-1c has rather a regulatory than a metabolic role, as the malonyl-CoA and acyl-CoA levels remained constant, yet a metabolic role cannot be excluded since acyl-CoA levels were measured from hippocampus, not hypothalamus (Wolfgang et al., 2008). Therefore, in hypothalamus CPT-1c might be able to release the other hypothalamic forms of CPT-1 (namely CPT-1a) from inhibition caused by malonyl-CoA. The decrease in FAS expression and increase of CPT-1c expression might provide a possible mechanism for the unchanged food intake of the tg animals. To further investigate these changes we measured the mRNA levels of transcription factors are involved as well as the protein levels of AMPK. Our fed tg animals had reduced levels of PPARδ and SIRT-1 mRNA in the hypothalamus. There was no previous data on the actions of these transcription factors in the food intake regulation. In peripheral tissues, it seems that PPARδ is involved in the regulation of FAS in liver and the liver and muscle isoforms of CPT-1 in muscle tissue (Barish et al., 2006; Lee et al., 2006). These previous data combined with our results suggest that PPARδ is involved in FAS regulation also in hypothalamus. PPARδ up-regulates FAS (Lee et al., 2006), hence a reduction PPARδ levels may contribute to the observed reduction in hypothalamic FAS levels. The increased CPT-1c levels cannot be explained by the reduced PPARδ levels, but one possible mechanism might be the reduced levels of SIRT-1, a transcription factor involved in glucose metabolism. SIRT-1 has been shown to regulate peripheral forkhead box O1 (FOXO1) transcription factor, which in turn increases food intake in rodents (Nakae et al., 2008; Yang et al., 2006). Our results from the ACBP overexpressing rats demonstrate that constantly high levels of ACBP do not affect the food intake. The tight regulation of hypothalamic acyl-CoA pool size evoked changes in the gene expression profile that may provide a possible preventive mechanism. Based on our results, we cannot rule out a short term modulatory effect of ACBP. The results on the actions of ODN injections point to this possibility, acute administration
reduced food intake of mice and rats but over the long term, the effects were diminished (de Mateos-Verchere et al., 2001).

### 6.4 ACBP and physiology of rats

We utilized our rat line overexpressing mouse ACBP to study the physiological effects of constantly high levels of ACBP. In accordance with previous results from yeast, cell lines and mouse liver (Huang et al., 2005; Knudsen et al., 1994; Yang et al., 2001) we observed increased acyl-CoA levels in liver and in addition in adipose tissue of our tg rats. Our results clearly demonstrate that the increased levels of ACBP influenced the acyl-CoA pool in a specific manner. The variation in ACBP's binding affinity toward different acyl-CoA forms and the ability to enhance further utilization of different acyl-CoA forms can, in part, explain the selective nature of the acyl-CoA increase (Faergeman et al., 1996; Huang et al., 2005; Jolly et al., 2000). In addition, our results show that the effects of ACBP on the acyl-CoA pool were dependent on the nutritional state (fed/fasted) of the animals and on the tissue studied. Previous studies have shown that fasting can influence the intracellular acyl-CoA pool (Woldegiorgis et al., 1985) and the differential metabolic usage of fatty acids and acyl-CoAs in different tissues may be another explanation for our results. On the contrary to the acyl-CoAs, we did not observe any changes in the serum free fatty acid levels or in the plasma triglyceride or cholesterol levels. Huang et al. (2005) reported that mice overexpressing ACBP under the control of a phosphoglycerate kinase promoter had increased liver triglycerides and phospholipids while the cholesterol levels were unchanged, but these tissue levels are not comparable with circulating levels. Furthermore, high ACBP levels in combination with either MC or LC diets did not induce any changes in the serum free fatty acid levels between tg and sg rats.

Acyl-CoAs and ACBP have been claimed to be involved in insulin secretion, ACBP is believed to inhibit glucose stimulated insulin release and acyl-CoAs can influence several key players of signal transduction in β-cells (Corkey et al., 2000; Ostenson et al., 1990). Nonetheless, we did not detect any differences in the GTT or in the insulin levels during the test between normally fed sg and tg rats. In accordance, it has been shown that ACBP mainly affected the acute phase of glucose stimulated insulin release and that in long term experiments, it had only limited effects (Ostenson et al., 1994; Sjöholm et al., 1991). On the contrary, when the tg rats were challenged with a MC diet we found that high levels of ACBP improved the glucose tolerance, tg rats displayed lower blood glucose in combination with reduced insulin levels. When the animals were challenged with a LC diet, no differences between tg rats and the controls were observed. Our results indicate that in animals fed with standard rodent diet or LC diet overexpression of ACBP either did not influence the glucose tolerance or its actions were prevented. On the other hand, our results also indicated that ACBP might play a role in the improved insulin sensitivity observed in the medium chain high fat diet versus long chain high fat diet fed animals (Han et al., 2003; Takeuchi et al., 2006), i.e. high levels of ACBP appears to have a beneficial role in the MC diet fed rats. The MC and LC
high fat diets utilized in our study were specially designed to have low energy content allowing the investigation chain length specific effects of fatty acids, therefore our results and results obtained by Han et al. (2003) and Takeuchi et al. are not directly comparable. The mechanisms preventing changes in GTT of standard diet and LC diet fed tg rats and the ACBP induced changes that might improve glucose tolerance in the MC diet fed animals were further characterized by determining the effects of ACBP on gene expression regulation.

6.5 ACBP and regulation of gene expression

The effect of constant ACBP overexpression on the mRNA levels of transcription factors regulating lipid and glucose metabolism were measured from liver and adipose tissue of rats. In addition, the effects of nutrition state (fed/fasted) and diets (MC and LC high fat diets) were investigated. Furthermore, a protein levels of AMPK, a well-known regulator of cellular metabolism, was determined from the livers of the animals fed with either standard diet or high fat diets with either MC or LC fatty acids.

6.5.1 PPARs

ACBP had the least effect on PPARα. Only in the LC diet fed animals there was a difference between tg and sg animals in the expression levels of PPARα, with tg rats having significantly elevated levels compared to sg littermates. In contrast to PPARα, ACBP seems to affect the mRNA expression of the other isoforms of the PPAR transcription factor family. Accordingly, previous studies have shown that ACBP is able to inhibit the trans-activation of PPARs at the protein level (Helledie et al., 2000; Helledie et al., 2002b). In the fed state, the tg rats displayed reduced levels of PPARγ and PPARδ in both liver and adipose tissue. The reduction of the fed state expression of PPARγ and PPARδ points to increased accumulation of fatty acids, as PPARγ is known to stimulate lipid storage in adipose tissue and PPARδ is involved in fatty acid oxidation. Accordingly, our tg animals exhibited increased acyl-CoA levels. Fasting influenced the animal’s response to ACBP, it abolished the differences in PPARγ and PPARδ expression levels in the liver and in adipose tissue it evoked increased expression in tg rats. It seems that the regulatory elements activated by fasting are a stronger stimulus for PPARγ and PPARδ transcription than ACBP, or the consequences of increased levels of ACBP are prevented in such a manner that it is active only in the fasted state. The fact that the acyl-CoA pool of the animals was also affected in a fed/fasted state dependent manner suggests that acyl-CoAs might underlie the influence of ACBP on PPARs. It has been shown that acyl-CoAs can affect the activity of PPARs in a chain length specific manner (Faergeman and Knudsen, 1997; Schroeder et al., 2008). Furthermore, the involvement of other regulatory factors like AMPK cannot be ruled out. In addition to fasting, both MC and LC diets affected the ACBP induced regulation of PPARγ and PPARδ. The LC diet fed tg animals had increased liver PPARγ and PPARδ levels, while the MC diet evoked reduced PPARγ expression in the liver and increased expression in adipose tissue of tg rats. Previous studies have indicated that medium and long
chain high fat diets can control the expression of PPARs and other transcription factors in different ways (Han et al., 2003; Takeuchi et al., 2006), although there have been discrepancies in the results obtained. Han et al. (2003) reported that medium chain high fat diet fed rats had reduced adipose tissue PPARγ levels compared to long chain high fat diet, while Takeuchi et al. (2006) detected an increased expression of PPARγ. Our results demonstrate that there were no difference in adipose tissue PPARγ expression between MC and LC diet fed sg animals, while the tg rats had reduced PPARγ levels compared to sg littermates. In addition to chain length specific effects of the fatty acids used (Faergeman et al., 1996; Huang et al., 2005; Jolly et al., 2000), the fact that our diets energy content is not comparable with the diets used by Takeuchi et al. (2006) and Han et al. (2003) might explain the differences in dietary responses. We utilized high fat diets with low energy content (Table 1) to be able to focus on the effect of fatty acid chain length. Our results from LC diet suggest that tg animals exhibited increased lipid metabolism, as the expression levels of the transcription factors and ACBP were elevated. Results from the MC diet fed animals demonstrate that the effect of ACBP on the PPARγ mRNA levels might be responsible for the improved glucose tolerance of MC diet fed tg rats. The down-regulation of liver PPARγ levels leads to decreased liver lipid accumulation, which is accompanied by elevated lipid storage in adipose tissue resulting from increased PPARγ expression.

6.5.2 SREBP-1

SREBP-1 is another transcription factor known to be involved in the regulation of both ACBP and PPARs (Fajas et al., 1999; Neess et al., 2006; Swinnen et al., 1998). In fed animals, overexpression of ACBP resulted in decreased levels of liver and adipose tissue SREBP-1 mRNA. Fasting abolished the difference between tg and sg animals in both of the tissues studied. These results are in accordance with the results obtained from PPARγ and PPARδ. Changes in the intracellular acyl-CoA contents might be responsible for the decreased SREBP-1 levels as unsaturated fatty acids can reduce SREBP-1 mRNA levels (Jump et al., 2005). The involvement of additional regulatory factors cannot be ruled out, for example liver X receptor (LXR) α, a known regulator of SREBP-1, which has been reported to act in a nutritional state dependent manner (Eberle et al., 2004). SREBP-1 expression was elevated in the LC diet fed and decreased in the liver of MC diet fed tg rats. In contrast to the effect of the diets on PPAR expression, also the adipose tissues expression of SREBP-1 was increased in the tg rats fed with the LC diet.

6.5.3 AMPK

The protein levels of the catalytic α subunit of AMPK were determined to further investigate how ACBP can influence the regulation of transcription factors. AMPK is known to be involved in the phosphorylation mediated regulation of both PPARs and SREBP-1 (Leff, 2003; Zhou et al., 2001). The fed tg rats had elevated levels of both total AMPKα and the active, Thr 172 phosphorylated, form in their
livers. In addition, we detected that in the liver of LC diet fed rats, overexpression of ACBP decreased the levels of total AMPKα. These changes in the AMPKα levels are in accordance with the observed changes in the expression levels of PPARs and SREBP-1, indicating that the regulatory effects of ACBP might be mediated through AMPK. To further investigate this possibility, we utilized intraperitoneal injections of a specific inhibitor of AMPK (compound C). The results demonstrated that injections of compound C significantly elevated the mRNA levels of SREBP-1 in fed tg animals, while there was no change in the expression levels of PPARs. This indicates that regulation of SREBP-1 is mediated through AMPK, while the effects of ACBP on PPARs are independent of the AMPK activity. Further support for a role for AMPK in the effects of ACBP on SREBP-1 expression was obtained in hypothalamus where there were no differences in the AMPKα levels between tg and sg animals. In accordance, also SREBP-1 mRNA expression remained unchanged. Our results also reveal that the acyl-CoA form of palmitate (C16:0) was elevated in both liver and adipose tissue of tg animals, increased levels of palmitate have been shown to activate AMPK (Wang et al., 2007; Watt et al., 2006). In addition, the C16:0 levels are normally elevated by fasting, providing a possible explanation for the nutritional state specific effects. In the brain tissue, there were no changes in the AMPKα levels and the overexpression of ACBP did not influence the C16:0 pool size. This suggests that the elevated acyl-CoA levels of ACBP overexpressing animals affect SREBP-1 expression through an increased activity of AMPK.
7. SUMMARY

The principal aim of this study was to investigate the physiological role of acyl-CoA binding protein (ACBP) / diazepam binding protein (DBI) in two rodent models. ACBP has been associated with various functions, some of which are proposed to be mediated mainly through processing products of the native protein. Therefore, we have utilized a construct that allows endogenous regulation of the gene and natural processing of the ACBP protein. The following results were obtained.

I The mouse ACBP gene was isolated and characterized. There were no observed differences in the coding sequence of the construct and it contained all the regulatory regions published so far. The continually elevated levels of ACBP resulted in enlargement of the lateral ventricles both in mice and rats. In addition, in mice, ACBP overexpression resulted in decreased plasticity of excitatory synapses and impairment of the hippocampus-dependent form of learning and memory. In contrast to previous results, our animals did not show any signs of anxiety or pro-conflict behaviour nor did we find any influence on the kainate or pentylenetetrazole induced seizure activity in the transgenic mice.

II In metabolic phenotyping, our rat line overexpressing the mouse ACBP gene under the genes own promoter region was utilized. The transgenic rats had a significantly increased acyl-CoA pool both in liver and adipose tissue. Changes in the acyl-CoA pool were found to be tissue and acyl-CoA form specific. In addition, the ACBP induced changes were dependent on the nutritional state of the animals. On the contrary, there were no changes in the serum free fatty acid or plasma triglyceride and cholesterol levels and also the glucose tolerance of the transgenic animals remained unchanged. Although the physiological effects of ACBP were limited, the overexpression caused marked changes in the regulation of gene expression. Peroxisome proliferator activated receptors (PPARγ, PPARδ) and sterol regulatory element-binding protein-1 (SREBP-1) mRNA levels were significantly reduced (by 23–82%) in liver and adipose tissue of fed transgenic rats. In addition, adenosine monophosphate-activated protein kinase (AMPK) protein levels were increased (by 60%). These changes were dependent on the nutritional state of the animals, fasting abolished the reduction of PPARs and SREBP-1 in the liver, while evoking up-regulation of PPARs in adipose tissue. Our results indicate that the reduction of SREBP-1 seems to be mediated through AMPK, whereas the regulation of PPARs is AMPK independent. These results demonstrate an important feedback loop; ACBP expression is regulated by both PPARs and SREBP-1.

III To investigate further the role of ACBP in the physiology and regulation of gene expression, we challenged our ACBP overexpressing rat line with two high fat diets enriched with either medium or long chain fatty acids for four weeks. Transgenic animals fed with the medium chain diet had improved glucose tolerance and lower serum insulin levels compared to controls. In addition, their liver PPARγ (by 43%) and SREBP-1 (by 35%) mRNA levels were reduced, while the adipose tissue PPARγ levels were
increased by 37%, pointing to a possible mechanism by which ACBP may improve glucose tolerance. The LC diet fed transgenic rats exhibited no differences in the glucose or insulin levels compared to controls, but they had increased levels of liver PPARs and SREBP-1 (1.5-3.5 times higher) and decreased protein levels of AMPKα (by 48%). This can suggest that the LC diet increased the overall lipid metabolism of the transgenic rats.

**IV** It has been shown that the hypothalamic regulation of food intake is influenced by fatty acids, especially their intracellular active forms, acyl-CoAs. Since there was no previous data on the effects of fatty acid / acyl-CoA binding proteins on these processes, we examined whether constant overexpression of ACBP could influence the hypothalamic regulation of food intake and gene expression. Our results indicated that ACBP did not influence the food intake or the weight of the animals. Further investigations revealed that fed tg rats had significant changes in their gene expression profile: mRNA levels of FAS were reduced while the levels of CPT-1c were increased. Both of these proteins have been previously shown to play important roles in the regulation of food intake. In addition, mRNA levels of transcription factors and protein levels of AMPK were measured. Fed transgenic rats displayed no differences in the AMPK protein levels or SREBP-1 mRNA expression, but had reduced levels of PPARδ and SIRT-1, indicative of a possible regulatory mechanism behind the observed changes in the FAS and CPT-1c expression and revealing possible further regulatory targets of ACBP induced gene expression in hypothalamus.
8. REFERENCES


Kuopio University Publications G. - A.I. Virtanen Institute


G 57. Gurevičius, Kestutis. EEG and evoked potentials as indicators of interneuron pathology in mouse models of neurological diseases. 2007. 76 p. Acad. Diss.


