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DIABESITY

Novel Molecular Drug Targets for Obesity and Type 2 Diabetes

Integrated Project

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FINAL REPORT

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Project coordinator name: Professor Suzanne Dickson

Project coordinator organisation name: University of Gothenburg

Section 1

Publishable final activity report

Project execution

Project Objectives

The ultimate objective of the DIABESITY project is to provide 4 - 5 novel validated molecular drug targets for the prevention and treatment of obesity and diabetes.

The specific project objectives as outlined in the original research plan:
1. To identify genes and processes linked to the pathogenesis of DIABESITY and thereby find novel molecular drug targets.
2. To characterise those potential drug targets, in particular by using molecular physiological studies and integrative human biology.
3. To validate those targets using molecular genetics approaches in conjunction with phenotypic analyses.

Contractors involved

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7	MRC Mammalian Genetics Unit, UK	Dr Roger Cox
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DIABESITY: shaping the future of research in the area of obesity and type 2 diabetes.

DIABESITY has brought together a leading team of researchers in the area of obesity and type 2 diabetes that have made many significant achievements that will influence the future direction of research in these important disease areas. The success of the project can be illustrated by the fact we achieved all of our goals as outlined in our proposals to the EC but also by the very large number of publications. The current number of publications arising from the project is 213, although the final number of articles arising may be substantially more than this. 46 publications have an impact factor of 8 or above. The average impact factor is 6.83 and the total impact is 1455. Importantly, the project will have strengthened European efforts in this area for many years to come as we have become more integrated and aware of possibilities for progressing particular strategies through collaboration and pooled efforts.

We believe the project has the capacity to influence future strategies in a number of ways. We have successfully demonstrated, for example, that the important role of the hypothalamus in the control of appetite and energy balance, highlighting the importance of discrete nerve cell populations, of specific genes and specific mechanisms regulating those genes.
A number of novel diabesity targets have emerged from our work. The next stage of activity in the process leading towards the development of new drugs for treatment of these diseases is to find out much more specific information about the targets in terms of their role in normal physiology and in their pathophysiology leading to disease.

Coordinator's foreword

Below we describe the main findings of the DIABESITY consortium during the 5 years. All of the research described here has been undertaken by the Partners and their research groups. Many successful collaborations have formed and strengthened, giving rise to activities that will extend well beyond the project. For simplicity, I have indicated, in most places, the main Partner responsible for the work outlined.

DIABESITY targets.

The ultimate goal of the DIABESITY project was to identify and validate 4-5 novel targets for obesity and type 2 diabetes, in terms of new knowledge that contributes to the understanding of the processes and mechanisms leading to these disorders. Over the 5 year course of the project, the consortium has explored a large number of potential targets; some promising targets emerged only late and need much more work. Among these, we should highlight three as being of considerable potential importance:

(1) ***FTO*** (Fatso), a gene emerging from genome-wide association studies as being linked to BMI. Exploration of this gene as an important target continues in both clinical and rodent studies (Gerken et al. Science. 2007, 318:1469; Loos et al., Nat Genet. 2008 40:768; Wardle et al., JCEM 2008 93:3640; Willer et al., Nat Genet. 2009 41:25).

(2) ***WFS1***, a gene first associated with type 2 diabetes by members of the consortium using a gene-centric candidate gene approach and confirmed in genome-wide association studies. (Sandhu et al., Nat Genet. 2007 39:951; Franks et al., Diabetologia. 2008, 51:458; Florez et al., Diabetologia. 2008 51:451).

(3) ***MTNR1B*** - A recent GWA study with over 36,000 individuals revealed that variants in ***MTNR1B*** influences fasting glucose levels. (Prokopenko et al., Nat Genet. 2009 41:77).

The DIABESITY project as a whole was conceived around the hypotheses that obesity was primarily a disorder of hypothalamic function, that the hypothalamus contained discrete neuronal populations with key specific roles in regulation of body composition, and that identifying these populations would lead to new targets for intervention. The underlying premises of the Project have been amply borne out by the research conducted by the consortium, and strongly supported by significant new findings from outside. The criteria agreed by our Project Steering Group for explained that "Identification and validation" requires that the target is shown to be linked directly to mechanisms that impact on body weight, appetite, metabolism, insulin production or insulin action, and that there shall be a reasonable expectation that manipulation of the target molecule by agonists, antagonists or antibodies in the future can be used to treat obesity, type 2 diabetes and/or closely-related diseases.

Of the many potential targets that have led to significant publications by members of the Consortium, the following stand out in these regards:

(1) ***Bsx***, a transcription factor that is exclusively expressed in hypothalamic neurones of the arcuate nucleus and dorsomedial hypothalamus and which is an essential element for inducing NPY and AgRP expression in these nuclei.
(2) The central ***ghrelin*** signalling system, that includes the cloned receptor for ***ghrelin***, ***GHS-R1A***. We have identified 2 novel important mechanisms of action: (1) a CNS action on peripheral fat utilization and (2) an action at the level of the pathways controlling reward.
(3) Forkhead genes, especially ***FOXC2***. ***FOXC2*** is a transcription factor expressed in adipocytes and increased expression of this gene leads to a lean and insulin sensitive phenotype.
(4) ***Interleukin-1*** and ***interleukin-6*** signalling systems. Translation work including mouse knockout and polymorphism studies of assocations with fat mass in humans provide a clear indication that that ***IL-6*** and ***IL-1*** function as adipokines, suppressing fat mass by a CNS mechanism, for which studies are ongoing.
(5) ***Rev-erb***α, a nuclear receptor that integrates metabolic and circadian signals. We have shown its role in the development of insulin resistance and that it cross talks with other nuclear receptors to influence the inflammatory response.
(6) ***Cannabinoid receptor 1*** (***CB1***). The first obesity therapy targetting this receptor was withdrawn as a result of adverse effects on mood, but Consortium work shows that targeting this receptor in peripheral tissues may be effective in some patient groups.

Others that have received considerable attention by the Consortium include

(1) ***11β-HSD-1***, an enzyme important for adipogenesis and insulin sensitivity in adipocytes.
(2) The ***pancreatic polypeptide*** (PP) signalling system, that includes the NPY receptors ***Y2*** and ***Y4***. It provides an anorexigenic gut-brain signal that is now being evaluated in human studies.
(3) ***GOAT***, a novel enzyme catalyzing the addition of a diet derived fatty acid side chain to serin 3 of the gastric peptide hormone ghrelin, that is necessary to give ghrelin its appetite and fat mass promoting activities.

WP1 Invertebrate Genetics (Leader: Marcus Geese, Partner 5)

Objectives for WP1: *To find new genes and processes linked to the pathogenesis of obesity and T2D by identifying conserved genes linked to body composition and metabolism from mutagenesis studies in Drosophila melanogaster, and to investigate the relevance of these genes in appropriate mammalian test systems.*

The project objective was to find new genes and processes linked to the pathogenesis of obesity and type 2 diabetes by identifying conserved genes linked to body composition and metabolism from mutagenesis studies in *Drosophila melanogaster*, and to investigate the relevance of these genes in appropriate mammalian test systems. This was carried out by high throughput genetic screens employing transposon-mediated mutagenesis approaches to identify metabolic diseases related phenotypes with corresponding mutations in *Drosophila*. The first screen based on the identification of proteins modifying the activity of uncoupling proteins (UCPs) as energy dissipation is one of the most attractive mechanisms for the treatment of obesity and the metabolic syndrome. Two related screens directly explored changes in the energy storage metabolites triglyceride and glycogen. Mutagenic fly lines showing modifications of UCP activity or highly increased or decreased energy storage metabolites have been selected and the responsible candidate target genes have been identified by polymerase chain reaction (PCR). Altogether, approximately 14000 candidate target genes have been identified. The mammalian, in especially the human homologues, have been identified by database searches.
Identified candidate target genes have been prioritised and selected for becoming a target in pharmacological therapies of obesity and type 2 diabetes based on novelty and drugability. An initial validation of these genes has been performed through expression analysis in adipocytes and animal disease models in selected tissues using real time PCR. The most promising candidates have been further validated in disease relevant cellular and animal models. Patent applications have been filed for selected target genes for the therapy of metabolic disorders.

One of the most promising new targets is Slc25a32 (SOUP) which was identified in two *Drosophila* screens as a modifier of UCP activity and as a modifier of energy storage triglyceride levels. The gain of function of Slc25a32 in adipocytes lead to an increase in energy storage while the loss of function resulted in the opposite phenotype complementing the initial phenotype in the *Drosophila* screen. Furthermore, the gain of function in mice resulted in increased UCP2 expression. A drug discovery program to identify small molecular weight compounds modifying Slc25a32 activity has been started.

WP2 Human Genetics (Leader: Roger Cox)

Additional Partners: 1b (Enerbäck) and 10 (Ingenium)
Objectives for WP2: To find new genes and processes linked to the pathogenesis of obesity and type 2 diabetes by mutagenesis studies in mice.

The overall objective of workblock A was to find new genes and processes linked to the pathogenesis of type 2 diabetes using mutagenesis models in *Drosophila* and mice. The approach in mouse was to use random ENU (a powerful point mutagen) mutagenesis in a phenotype and genotype driven approach. In the phenotype driven approach we (Partner 7, Cox) measured phenotypes such as blood glucose, hormones, body weight, fat and lean mass, oxygen consumption and carbon dioxide output. Further we carried out a sensitized phenotype screen where we introduced null alleles of genes important in the adipocyte, ***FOXC2*** and UCP-1, in order to reveal novel phenotypes. In the genotype approach we screened the Harwell ENU DNA archive for mutations in genes of interest and then generated live mice for phenotyping using corresponding frozen sperm stocks.

We have shown using the *Oed/Sml* ENU mutant model that imprinting of the *Gnas* gene cluster encoding G stimulatory subunit of adenylate cyclase gives rise to opposite metabolic phenotypes of obesity and leanness, and in the latter case resistance to high fat diet. This results from differences in metabolic rate and contributes new knowledge to the role of this gene in metabolism. We have characterized the insulin resistance phenotype of the *Buttermouse* line and identified a mutation in a novel gene involved in histone 3 lysine 4 (H3K4) methylation and potentially is a new process linked to the pathogenesis of DIABESITY. We have successfully carried out new ENU screens generating new models for the mapping and identification of genes involved in DIABESITY. We have shown that a novel gene, Sox4, is involved in insulin secretion and also interacts with insulin resistance caused by a heterozygous insulin receptor knockout mutation. We have also shown that a novel gene *Nnt* accounts for the insulin secretory defect in C57BL/6J mice. We have identified a point mutation in the obesity associated gene ***FTO*** in the mouse by screening the Harwell ENU DNA archive. The mutation cased a dominant maturity onset reduction in body weight and a decreased fat mass. The mice exhibited an increase in oxygen consumption and carbon dioxide output indicating an increased metabolic rate. This indicates that this gene accounts for the BMI association found in human association studies. These studies have met the objective of indentifying new genes and processes linked to the pathogenesis of type 2 diabetes.

ENU mutagenesis in the mouse has identified a number of interesting novel genes including *Sox4* involved in insulin secretion and interacting with the insulin receptor pathway, *Mll2* a gene involved in histone methlyation and in insulin resistance and *Gnas* an imprinted gene involved in obesity and resistance to a high fat diet. An ENU allele of ***FTO***, a gene identified in human association studies for diabetes has demonstrated that this gene is involved in controlling metabolism and body mass. Work on a number of other models has progressed during the project and will lead to identification of further novel genes.

A new ENU mutagenesis screen using ***FOXC2*** and *Ucp-1* null mutations as a sensitised background was carried out and a number of lines identified as a resource for future work aimed at identifying regulators of ***FOXC2***. The ***FOXC2*** gene may be involved in mitochondrial function within the adipose cell and may be a potential target for compounds that increase metabolism.

Similarly work in drosophila to identify genes involved in body composition and metabolism using transposon-mediated mutagenesis has identified large number of candidate genes. These are being exploited by the SME partner responsible for this work, with selection of genes as potential pharmacological targets.

Thus, Partner 1b (Enerbäck) has investigated the role of ***FOXC2*** as a systemic regulator of insulin sensitivity has been strengthen in collaboration with Gerald Shulman at Yale have established that mice with elevated ***FOXC2*** expression in adipose tissue indeed are more insulin sensitive as measure by the golden standard i.e. hyperinsulinemic euglycemic clamp technique. It is interesting to note that both muscle and liver is more insulin sensitive and that the blunting effect on glyconeogenesis in liver, mediated by insulin, is to a large extent preserved in such mice even when challenged with high fat diet. As a consequence of these experiments and as outlined in the research plan, we plan to identify factors secreted from adipose tissue as potential mediators of these "distant effects". Apart from altered holoenzyme composition and elevated levels of beta-adrenergic receptors, we have also noted decreased phosphodiesterase 4B activity in adipocytes with increased ***FOXC2*** expression – all these factors contribute to an increased sensitivity in the beta-adrenergic-PKA signaling pathway in adipocytes. In this way adipocytes can increase their endogenous PKA pathway sensitivity by increasing ***FOXC2*** expression. Data published by others and us suggests that this leads to increased mitochondrogenesis, mitochondrial oxidation and induction of uncoupling protein 1. This implicates ***FOXC2*** as an interesting target for pharmacological intervention aimed to convert some excess triglyceride to heat. Furthermore, human genetic data show association with with a frameshift mutation in ***FOXC2*** and diabetes in a family. The collaboration with Roger Cox and the "sensitized" ENU screen will, in an unbiased manner, explore the possibility to identify regulators of ***FOXC2***.

WP3 Monogenic disease *(Leader: Inês Barroso)*

Additional Partners: 12 (Hebebrand) and 18 (O'Rahilly)

Objectives for WP3: To find new genes and processes linked to the pathogenesis of obesity and T2D by identification of major missense or nonsense mutations in candidate genes causing severe early onset human obesity, or severe human insulin resistance; and subsequently to establish the molecular mechanisms whereby such mutations lead to the phenotype; and to establish the extended phenotype associated with these mutations.

Within the context of this work we used large-scale candidate gene re-sequencing and also association testing to investigate the role of specific genes in syndromes of insulin resistance, type 2 diabetes and obesity.

The contribution of this workpackage to the international effort for discovering new genes linked to metabolic disease, together with studies of the role of specific gene variants in metabolic disease is best exemplified by listing the high number of high impact publications arising.

1) Attribution of function of several genes in metabolic disease:
a. Gerken *et al*., The Obesity-associated ***FTO*** gene encodes a 2-oxoglutarte dependent nucleic acid demethylase. Science 2007; 318:1469-72.
b. Payne *et al*., The human lipodystrophy gene BSCL2/seipin may be essential for normal adipocyte differentiation. Diabetes 2008; 57:2055-60.
c. Farooqi *et al*., Clinical and molecular genetic spectrum of congenital leptin receptor deficiency of the leptin receptor. New Engl J Med 2007; 356:237-47.
d. Agostini *et al*., Non-DNA binding, dominant-negative, human PPARgamma mutations cause lipodystrophic insulin resistance. Cell Metabolism 2006; 4:303-311.
e. Lee *et al*., A POMC variant implicates ß-melanocyte stimulating hormone in the control of human energy balance. Cell Metabolism 2006; 3:135-40.
f. Savage *et al*., A prevalent variant in PPP1R3A impairs glycogen synthesis and reduces muscle glycogen content in humans and mice. PLoS Medicine 2008;5:e27.
g. Farooqi *et al*., Heterozygosity for a POMC-null mutation and increased obesity risk in humans. Diabetes 2006;55:2549-53.
h. Loos *et al*., TCF7L2 polymorphisms modulate proinsulin levels and {beta}-cell function in a British Europid population. Diabetes 2007; 56:1943-7.
i. Nogueiras *et al*., The central melanocortin system directly controls peripheral lipid metabolism. J Clin Invest. 2007; 117:3475-88.

2) We have established a new programme employing functional neuroimaging to gain insights into the molecular control of human appetitive behavior.
a. Farooqi *et al*., Leptin regulates striatal regions and human eating behaviour. Science. 2007;317:1355.

3) We have clarified the role of specific genetic variants, within candidate genes, in metabolic disease:
a. Fawcett et al., Evaluating the role of LPIN1 variation on insulin resistance, body weight and human lipodystrophy in UK populations. Diabetes 2008;57: 2527-33
b. Young et al., The V103I polymorphism of the MC4R gene and obesity: population based studies and meta-analysis of 29 563 individuals. Int J Obes (Lond). 2007; 31:1437-41.
c. Mesa et al., Lamin A/C polymorphisms, type 2 diabetes, and the metabolic syndrome: case-control and quantitative trait studies. Diabetes 2007;56:884-9.
d. Collins et al., Adiponectin receptor genes: mutation screening in syndromes of insulin resistance and association studies for type 2 diabetes and metabolic traits in UK populations. Diabetologia. 2007;50:555-62.
e. Tan et al., Analysis of genetic variation in Akt2/PKB-beta in severe insulin resistance, lipodystrophy, type 2 diabetes, and related metabolic phenotypes. Diabetes. 2007;56:714-9.
f. Wermter et al., Preferential reciprocal transfer of paternal/maternal DLK1 alleles to obese children: first evidence of polar overdominance in humans. Eur J Hum Genet 2007;16: 1126-34

g. Müller et al., No evidence for an involvement of variants in the cannabinoid receptor gene (CNR1) in obesity in German children and adolescents. Mol Genet Metab 2007; 90: 429-34
h. Friedel et al., Mutation screen and association studies in the diacylglycerol O-acyltransferase homolog 2 gene (DGAT2), a positional candidate gene for early onset obesity on chromosome 11q13. BMC Genet 2007; 8:17
i. Fawcett et al., PARL Leu262Val is not associated with fasting insulin levels in UK populations. Diabetologia. 2006;49:2649-52.
j. Harding et al., Polymorphisms in the gene encoding sterol regulatory element-binding factor-1c are associated with type 2 diabetes. Diabetologia. 2006;49:2642-8.

WP4 Case-Control studies (Leader: Inês Barroso, Partner 3)

Additional partners: Partner 18 (O'Rahilly) and 12 (Hebebrand)

Within the context of this work we used state-of-the-art methodologies to test for genetic association between SNPs and BMI, obesity, type 2 diabetes and various related quantitative traits such as fasting glucose and lipid levels amongst others. Initially our approach comprised candidate gene studies, involving re-sequencing, when required to identify genetic variants, followed by SNP tag selection, genotyping and association testing for relevant traits. During the course of DIABESITY, genome-wide association studies (GWAS), which are hypothesis free and aim to test genomic variants throughout the genome for their association with various diseases and traits, became feasible. We hence were early adopters of this new approach and undertook GWAS to test for association between genetic variants and several different DIABESITY related traits. Using a combination of large scale candidate gene and genome-wide approaches we were highly successful in meeting our objectives of identifying new loci impacting DIABESITY and related quantitative traits.

WP4 objective To find new genes and processes linked to the pathogenesis of obesity and T2D by a high-throughput candidate gene/association study strategy.

The contribution of this workpackage is best exemplified by listing the high number of high impact publications arising in the following areas:

1) Identification of novel loci impacting BMI and risk of obesity:
a. Loos et al., Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nat Genet 2008; 40: 768-75.
b. Willer et al., Six New Loci Associated with Body Mass Index Highlight a Neuronal Influence on Body Weight Regulation. Nat. Genet, *in press*.
c. Geller et al., Melanocortin-4 receptor gene variant I103 is negatively associated with obesity. Am J Hum Genet 2004; 74:572-81.
d. Herbert et al., A common genetic variant is associated with adult and childhood obesity. Science 2006;312: 279-83.
e. Loos *et al*., Comment on A common genetic variant is associated with adult and childhood obesity. Science 2007; 315: 187.
f. Lyon et al., The association of a SNP upstream of INSIG2 with body mass index is reproduced in several but not all cohorts. PLoS Genet. 2007;3: e61

g. Hinney et al., Genome wide association (GWA) study for early onset extreme obesity supports the role of fat mass and obesity associated gene (***FTO***) variants. PLoS ONE 2007; 2:e1361.

2) Identification novel loci impacting type 2 diabetes risk and related glycaemic traits:
a. Sandhu et al., Common variants in ***WFS1*** confer risk of type 2 diabetes. Nat Genet 2007*;* 39: 951-953.
b. Prokopenko et al., Variants in the melatonin receptor 1B gene (***MTNR1B***) influence fasting glucose levels and risk of type 2 diabetes. Nat. Genet. *in press.*
c. Barroso et al., Population-specific risk of type 2 diabetes conferred by HNF4A P2 promoter variants: a lesson for replication studies. Diabetes 2008; 57: 3161-5

3) Contribution to the identification of novel loci influencing DIABESITY related traits:
a. Novel locus impacting LDL cholesterol concentrations- Sandhu et al., LDL-cholesterol concentrations: a genome-wide association study. Lancet 2008; 371: 483-91.
b. Newton-Cheh et al., Five blood pressure loci identified by genome-wide association study of 26,644 people. *Submitted*.

The Particular contribution of Partner 12 (Hebebrand) to WPs 3 and 4 is summarised as follows:
During the DIABESITY funding period, we pursued three complementary approaches: (1) genome-wide association (GWA), (2) genome wide linkage and (3) candidate gene studies. (1) GWA: In 2007 we performed a 500k (Affymetrix) GWA for early onset extreme obesity on 487 extremely obese children and adolescents and 442 lean controls (Hinney et al., PLoS ONE 2007). Because only individuals of the opposite ends of the quantitative BMI distribution were included, this study was highly powered to detect obesity genes. In our GWA six SNPs in the 'fat mass and obesity associated gene' ***FTO*** showed the strongest evidence for association with obesity (Hinney et al., PLoS ONE 2007). In a subsequent study we did not detect association between ***FTO*** variant rs9939609 with weight loss in a lifestyle intervention and lipid metabolism markers in German obese children and adolescents (Müller et al., BMC Med Genet 2008). (2) Genome wide linkage: A linkage scan was performed for juvenile obesity in 295 families (1,236 individuals, 10k Affymetrix). By multipoint linkage analyses seven genomic regions of suggestive evidence for linkage were identified by nonparametric (4q34.2, 17q25.1, Xq21.33) and/or parametric methods (6p23, 9p21.3, 11p15.1, 14q32.13, 17q25.1). (3) Finally, traditional candidate gene studies (mutation screens, association and transmission disequilibrium tests) have been performed for several genes. Examples are as follows: (a) We detected the first truly validated polygenic variant (V103I in the melanocortin-4 receptor gene; Geller et al., Am J Hum Genet 2004) and participated in a large meta-analysis to confirm this finding (Young et al., Int J Obes 2007). The I103 variant entails a mean BMI reduction of 0.5 kg/m² and is associated with low serum triglyceride levels (Brönner et al., J Clin Endocrinol Metab 2006). No independent effect was detected on waist circumference (Heid et al., Obesity 2008). We contributed data to two large meta-analysis that (i) identified two common variants near MC4R associated with fat mass, weight and risk of obesity (Loos et al., Nat Genet 2008) and (ii) detected six novel candidate genes associated with increased BMI (Willer et al., Nat Genet in press). (b) We were able to confirm the association of

a risk allele near INSIG2 (insulin induced gene 2) for obesity (Herbert et al., Science 2006) and we additionally replicated the association as part of in an independent meta analysis (Lyon et al, PLoS Genet 2007). (c) In our study investigating the delta-like 1 homolog gene (DLK1) we detected preferential reciprocal transfer of paternal/maternal DLK1 alleles to obese children and therefore, first evidence of polar overdominance in humans (Wermter et al., Eur J Hum Genet 2007). In further candidate gene studies we investigated the role of the ***Cannabinoid receptor 1*** gene (CNR1; Müller et al., Mol Genet Metab 2007), the fatty acid amide hydrolase gene (FAAH, Müller et al., subm.) and the diacylglycerol O-acyltransferase homolog 2 gene (DGAT2, Friedel et al., BMC Genet 2007) in body weight regulation.

WP5 Positional cloning (Leader: Jörg Hager)

Additional Partners: 12 (Hebebrand)

WP5 Objective To find new genes and processes linked to the pathogenesis of obesity and type 2 diabetes using linkage studies to identify genomic regions and genes implicated in obesity.

A new physical identity-by-descent mapping approach (genomic hybrid identity profiling: GenomeHIP^{TM}) was used to identify regions of identity by descent (IBD) that are linked to childhood obesity. Two independent sets of nuclear families with childhood onset of morbid obesity were obtained from partner 11 (Hager) to perform GenomeHIP. Families were included if at least one offspring had an age adjusted BMI >95th percentile and at least one sibling had an age adjusted BMI > 90th percentile. Using these criteria, the first set of families comprised of 91 (95 independent sib-pairs) and the second set included 75 families (83 independent sib-pairs).
Results obtained:
Analysis of GenomeHIP data provided evidence for linkage for regions on chromosomes 7 (pmax=1.2e-07), and 10 (p=1.02e-06) using a p-value of 2.2e-05 for evidence of significant linkage (Kruglyak and Lander, 1995).
The region on chromosome 7 encompasses the CNTNAP2 gene a member of the neurexin family of proteins. An association study of the gene in the initial Marburg families and in an independent set of French obese cases vs normal weight controls showed significant evidence for association for markers in the CNTNAP2 gene and BMI (rs7809716, p= 0.006 and rs1405109, p= 0.0002 in the Marburg and French sample respectively). Moreover we were able to show that B57 mice characterized by diet induced obesity due to over-feeding have a marked decrease in the expression of this gene compared to non-diet induced obese mice and normal weight control strains.
The chromosome 10 region encompasses the ***pancreatic polypeptide*** receptor 1 (PPYR1 or NP***Y4***R). The ligand to this receptor ***pancreatic polypeptide*** (PP) has been shown to mediate a strong satiety signal making this receptor a good target for obesity. Following the linkage results we studied this receptor for variants that might be associated with obesity. Two major findings resulted from this study: 1) we could show for the first time that there are at least two copies/chromosome of the ***NPY4***R gene in the human genome and that both seem to be functional; 2) we identified 11 missense mutations in he receptor. Using functional cell assays we could show that most of these influence receptor function by impacting either ligand binding affinity and/or decreased intracellular signaling. These findings are consistent with a role of

this gene in obesity and has implications for the use of its ligand PP as an anti-obesity agent.

WP6 Neuroanatomy and Neurophysiology (Leader: Björn Meister, Partner 15)

Additional Partners: 13a (Leng), 16 (Mercer), 14 (Liposits), 1a (Dickson) and 1c (Jansson)

WP6 Objective To characterise and validate CNS-produced DIABESITY drug targets, including known candidates as well as novel targets identified by WPs 1, 2 and 5.

The members of WP6 have together published a large number of papers that provides further knowledge regarding the hypothalamic neuronal networks that regulate feeding. Special emphasis has been devoted to the key target ***ghrelin***, but also to other afferent hormones informing the CNS about peripheral energy status such as leptin, PYY and endocannabinoids. Studies on the patterns of expression of the immediate early gene c-fos in schedule-fed rats have showed that the appetite-regulating circuitry of the hypothalamus is activated in anticipation of food presentation, while the satiety circuitry is activated very rapidly beginning with the first food consumption. Neuronal cell types in the ventromedial hypothalamic nucleus (VMH) have been characterized and functionally studied during their responses to several appetite-related stimuli. The neurons of the paraventricular nucleus (PVN) and arcuate nucleus (ARC) have been chemically characterized with regard to neurotransmitters and receptors, thereby clarifying the phenotypes of target neurons for ***ghrelin***, leptin and endocannabinoids. The results show that several key neurons regulating food intake and body weight differentially contain a large number of specific neurotransmitters in complex patterns. Special interest has been focused on the blood-brain barrier, which plays a fundamental role in controlling the access of circulating substances to the target neurons of the hypothalamus. Potential target molecules that do not cross the BBB may be used in order to selectively influence neurons that are located in a subdivision of the ARC. A large number of genes have been analyzed in the Siberian hamster, a model of seasonal body weight regulation, using laser-capture microdissection and species-specific microarray technology as well as *in situ* hybridization to examine differential gene expression in discrete brain regions.

In order to clarify how the brain controls body weight, Partner (15 Meister) has focussed research on the neuronal systems that regulate energy balance and which are located in the hypothalamus and brainstem. Using *in situ* hybridization and immunohistochemistry, we have shown that orexigenic neurons in the hypothalamic arcuate nucleus, apart from containing neuropeptide Y and agouti-related peptide (AgRP), also contain the inhibitory transmitter GABA, whereas anorexigenic neurons synthesizing the precursor pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) in addition contain several transmitters that affect food intake. These include glutamate, acetylcholine, pituitary adenylate cyclase-activating polypeptide (PACAP), dynorphin and orphanin FQ (nociceptin). Nociceptin is present in orexin-, but not melanin-concentrating hormone (MCH),-containing neurons (Figure). In obese *tub/tub* mice, which develop a maturity-onset

obesity, we have demonstrated a down-regulated expression of AgRP and NPY in neurons of the arcuate nucleus combined with hyperphagia. We have demonstrated that *tub/tub* mice show an abnormal cholinergic and GABAergic innervation of certain vessels in the ventromedial aspect of the arcuate nucleus. In order to clarify the cellular localization of the blood-brain barrier (BBB), we have used immunohistochemistry to localize protein components of the BBB in the mediobasal hypothalamus and brainstem. The results suggest the existence of vessels that lack protein components specific for the BBB in the ventromedial aspect of the hypothalamis arcuate nucleus and dorsomedial part of the brainstem nucleus tractus solitarius (NTS). Such vessels may represent key routes of entry of peripheral hormones to reach and influence neurons that regulate energy balance. This knowledge may be used in order to develop drugs that can influence specifically neurons that control body weight without affecting neurons in other parts of the brain, thereby avoiding negative side effects.

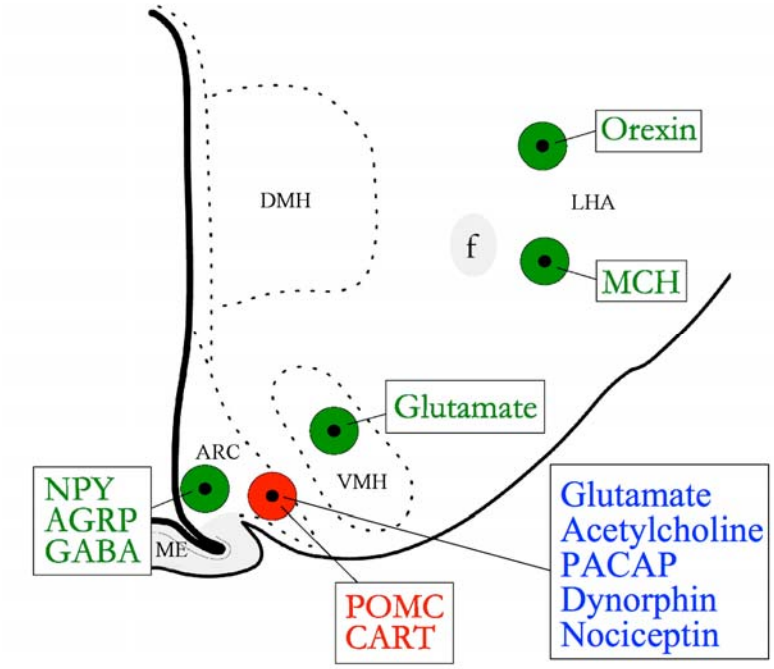

Fig. Summary of chemical content of orexigenic (in green) and anorexigenic (in red) neurons in the hypothalamus. Orexigenic neurons in the arcuate nucleus contain the peptides NPY and AGRP together with the inhibitory transmitter GABA. Anorexigenic neurons in the ventgrolateral part of the arcuate nucleus contain POMC, that gives rise to α-melanocyte stimulating hormone (α-MSH), an anorexigenic peptide, and CART. We have shown that POMC neurons also contain glutamate, acetylcholine, PACAP, dynorphin and nociceptin. The ventromedial nucleus (VMH) contains more than 90% glutamatergic neurons. In the lateral hypothalamic area (LHA), there are orexigenic orexin and MCH neurons. We have recently shown that orexin, but not MCH neurons, contain the orexigenic peptide nociceptin. The gray shaded area in the median eminence (ME) indicated the boundaries of the blood brain-barrier (BBB.

Over the 5 years of this project, the research group of Partner 13a (Leng) has been involved in fundamental studies of the hypothalamic mechanisms involved in appetite regulation, seeking a better understanding of the role of specific pathways to inform rational choice of targets for intervention. The work has involved neuroanatomical studies and studies of the electrical activity of neurones, as well as studies of peptide release in the hypothalamus. The single most important outcome of the work in this

was published in the high impact journal *Cell Metabolism*, and was the subject of extensive media coverage. This was a very large study of the patterns of expression of the immediate early gene c-fos in schedule-fed rats. This study showed that the appetite-regulating circuitry of the hypothalamus is activated in anticipation of food presentation, while the satiety circuitry is activated very rapidly beginning with the first food consumption. This was reported in the media as evidence of "the brain getting ready for dinner."
A second major achievement by Partner 13a (Leng) has been our work in characterising the role of the ventromedial hypothalamus in processing appetite-related stimuli. The VMN has a very important role as a key satiety centre, and emerged as a key focus of interest during the course of the DIABESITY project, but hitherto virtually nothing was known about exactly how it processed such information. We have now completed an electrophysiological classification of neuronal subtypes in the VMN, and a functional analysis of their responses to several appetite related stimuli, including responses to administration of leptin or the key DIABESITY target ***ghrelin***.

The main achievements of Partner 14 (Liposits) concern the nature and functional connectivity of several neuronal circuits that regulate energy homeostasis at the hypothalamic level in both rodents and humans. The scope of the studies has primarily been focused on identifying the communication among neurons residing in the arcuate nucleus, the primary hypothalamic feeding center, and the hypophysiotropic TRH- and CRH- synthesizing neurons, well-known regulators of thyroid and adrenal axes, distributed in the paraventricular nucleus of the hypothalamus.
The Budapest group (Partner 14, Liposits) have published 29 papers. The cumulative impact of these publications was 111.479 and the findings were already cited 140 times although some of these publications came out just months ago. During the time course of the project, the group provided evidence about the involvement of brainstem afferents in the innervation of the hypophysiotropic TRH and CRH neurons, characterized the involvement of leptin, insulin and glucose in the regulation of the feeding-related neuronal groups of the arcuate nucleus and the hypophysiotropic TRH neurons, mapped the hypothalamic ***CB1*** containing system and described its role in the innervation of the hypophysiotropic TRH neurons and in the mediation of the effect of ***ghrelin*** on the food intake and on the electrical properties of the parvocellular neurons in the PVN, described the role of the melanocortin system in the regulation of food intake and neuronal activation during the early phase of re-feeding, characterized the glutamatergic phenotype of the hypophysiotropic neurons, described the connection of feeding-related neuronal groups with the hypophysiotropic LHRH neurons and characterized the feeding-related neuronal circuits of the human hypothalamus. The dissection of these complex circuitries was achieved based on a combination of refined methods including unique triple and quadruple staining for rodent brain slices for several important neuroactivity modulating factors at the same time.

The integrated contributions of the other Partners are given below (WP 7 for Partner 16, WP9 for Partner 1a and WP 10 for Partner 1c)

WP7 Neuroendocrinology (Leader: Julian Mercer, Partner 16)

Additional Partners: 1c (Jansson), 24 (Tschöp), 4 (Bloom), 6 (Broenning) and 19 (Pagotto)

WP7 Objective: To characterise and validate DIABESITY drug targets, including known candidates as well as novel targets identified by WPs 1, 2 and 5, by their physiological actions on body phenotype and by their impact upon neuroendocrine systems.

The withdrawal of the initially promising anti-obesity drug rimonabant (Sanofi) from the clinic due to psychiatric events and the consequent question marks hanging over development programmes for other ***CB1*** antagonists add additional importance to one of our major objectives. To establish whether a drug acting as a ***CB1*** blocker that could not cross the blood brain barrier, i.e. which has a peripheral mode of action, could be as effective as an antagonist that accesses the whole body, including the brain, we compared results from total ***CB1*** KO mice to results from ***CB1*** neuronal conditional KO mice. Our findings of an intermediate phenotype in conditional neuronal KO mice fed a high fat diet when compared to wild type animals and whole KO animals will pave the way to exploring the efficacy of drugs working exclusively at adipocyte (fat) and hepatic (liver) levels. The generation of drugs such as ***CB1*** receptor blockers not able to pass the blood brain barrier may be a novel, sophisticated and safe approach to tacking obesity and associated co-morbidities. Ongoing research into the ***ghrelin*** signalling system focussed on the CNS neuroendocrine network and its interaction with the melanocortinergic system in regulating energy metabolism in adipocytes. ***Ghrelin*** and the synthetic melanocortin receptor antagonist, SHU9119, induced expression and activity of lipogenic enzymes in WAT, while the synthetic melanocortin receptor agonist, MTII, induced lipolysis using the same « remote control » over adipose tissue lipid metabolism gene programs . The results indicated a shift in nutrient partitioning towards preferential oxidation of carbohydrates and consequential effective sparing of lipids. In addition, hypothalamic-pituitary-adrenal as well as hypothalamic-pituitary-thyroid axes play a modifying role by enhancing the lipogenic response to melanocortin receptor anatagonism. We used mice with targeted deletions to test the hypothesis that simultaneous deletion of ***ghrelin*** and the ***ghrelin*** receptor, ***GHS-R1A***, (dKO) would lead to a more significant energy metabolism phenotype than deletion of either ligand or receptor alone. We found that simultaneous deletion of ***ghrelin*** and its receptor enhances the metabolic phenotype compared with single gene-deficient mice, possibly suggesting the existence of additional, as of yet unknown, molecular components of the endogenous ***ghrelin*** system. This finding has triggered a search for additional receptors and ligands for the ghrelin system using gene profiling approaches in several tissues and profile subtraction of wild-type and several loss-of-gene-function ko models. This work is completed by parallel research with the Siberian hamster model of seasonal body weight regulation balanced investigations of candidate genes (including those arising from within the DIABESITY consortium) with a laser capture microdissection (LCM)/microarray combination approach to identify novel molecular targets. ***Ghrelin*** signaling in the hamster was sensitive to food deprivation in terms of both blood levels and hypothalamic ***ghrelin*** receptor (GHSR) mRNA, but there were no effects of daylength. Thus, ***ghrelin*** appears to only play a minor role in modulating long-term seasonal body weight cycles. We had shown previously that POMC mRNA in the hamster hypothalamus is markedly down-regulated in short daylength, and we

examined the prohormone convertases 1 (PC1/3) and 2 (PC2) which are involved in the endoproteolytic processing of the POMC precursor. The results suggested that a major part of seasonal neuroendocrine body weight regulation may involve post-translational processing mediated by the prohormone convertases, PC1/3 and PC2, in addition to regulation of gene expression of energy balance related neuropeptide precursors. We examined differential gene expression by LCM/micoarray in a discrete brain region, the dorsal-medial-posterior arcuate nucleus (dmpARC) and the adjacent ependymal layer. Of particular interest were clones for VGF (nonacronymic) and secretogranin 3, and a clone with sequence homology to glycogen phosphorylase. These genes and a number of additional related or pathway-relevant genes were studied in more detail by in situ hybridization following specific daylength manipulations.

Partner 24 (Tschöp) has established a series of experiments that demonstrated the existence of a neuroendocrine network in the CNS involving ***ghrelin***, its hypothalamic target neurons and the central melanocortinergic system directly regulating energy metabolism in adipocytes. In collaboration with partners 23 (Rohner-Jeanrenaud) and 20 (O'Rahilly) we compared central and peripheral effects of direct and indirect activators or inhibitors of the central melanocortin system on their impact on peripheral lipid metabolism. We were able to show that ***ghrelin*** and the synthetic melanocortin receptor antagonist SHU9119 could induce expression and activity of lipogenic enzymes in WAT, while the synthetic melanocortin receptor agonist MTII induced parameters of lipolysis. Most of these effects appeared to be independent on increased food intake and body weight gain, as fat mass was still increased after ***ghrelin*** or SHU treatment in animals not allowed to overeat by pair-feeding them to vehicle infused control animals. This indicates a shift in nutrient partitioning towards preferential oxidation of carbohydrates and consequential effective sparing of lipids. Our experiments using triple-β-adrenoceptor deficient mice or direct sympathetic nervous system (SNS) fibre recording from WAT nerve fascicles indicated that the SNS plays an important role in the mediation of centrally induced changes onto peripheral lipid metabolising tissues, especially towards WAT. However, we found also evidence that the classical hypothalamic-pituitary-adrenal as well as hypothalamic-pituitary-thyroid axes play a modifying role by enhancing the lipogenic response to melanocortin receptor anatagonism. There here discovered interplay between the efferent autonomic (sympathetic) nervous system as the principal mediator of remote controlled peripheral tissue metabolism and the efferent endocrine hypothalamus-pituitary-hormone axes as amplifying modulators provides a new paradigm for the CNS regulation of metabolic homeostasis that can now be applied to other circuits, end-organs and metabolic processes.

In addition, Partner 24 (Tschöp) further dissected the role of ***ghrelin*** in energy balance regulation. While pharmacological treatment of laboratory animals with ***ghrelin*** induces robust adipogenic effects, mice with targeted deletion of either ***ghrelin*** or its receptor ***GHS-R1A*** have normal energy balance, food intake, and adiposity on a standard diet. We therefore tested the hypothesis that simultaneous deletion of ***ghrelin*** and the ***GHS-R1A*** in mice (dKO mice) would lead to a more significant energy metabolism phenotype than deletion of either the ligand or the receptor alone. The simultaneous genetic disruption of both genes of the ***ghrelin*** system leads to an enhanced energy metabolism phenotype. dKO mice exhibit decreased body weight, increased energy expenditure, and increased locomotor

activity on a standard diet without exposure to a high caloric environment. Mice on the same genetic background lacking either the ***ghrelin*** or the ***GHS-R1A*** gene did not exhibit such a phenotype on standard chow, thereby confirming earlier reports. Furthermore, when maintained on a high-fat diet, dKO mice exhibited deteriorated glucose tolerance, indicating a novel, unexpected and important role for the ghrelin system in the protection of insulin secreting pancreatic beta-cells. Ongoing studies are now examining to which extent systemic ghrelin action or paracrine ghrelin signalling within the islet cells may be involved in this unique phenomenon. In summary, simultaneous deletion of ***ghrelin*** and its receptor enhances the metabolic phenotype of single gene-deficient mice compared with wildtype mice, possibly suggesting the existence of additional, as of yet unknown, molecular components of the endogenous ***ghrelin*** system and revealing an additional important function in the protection of insulin secreting cells from toxic nutrient impact derived from cafeteria diets. In addition, ***ghrelin*** may play a role for proper embryological development of pancreatic β-cell function, especially in the presence of maternal high-fat feeding.

Furthermore, the Tschöp lab and colleagues have over the course of the 5 years of this project intensely scrutinized the mechanistic details of ghrelin activation. Based on those studies, Partner 24 (Tschöp) and EUFP6 collaborators have now established a novel gastric lipid sensor model, in which the recently discovered ghrelin activating acyl transferase GOAT (MBOAT4) is using dietary lipids (preferably medium chain fatty acids) as substrate for ghrelin activation at Serine 3, thereby informing the CNS about specific characteristics of the caloric environment. Recent insight from such studies also shows that active ghrelin is lowered in the absence of dietary lipids and that in such situations gastric GOAT expression is downregulated. This series of discoveries provides a novel peripheral drug target in GOAT, which allows to specifically target the active ghrelin system and its effects on energy balance and body fat without interfering with any effects of other ghrelin gene derived molecules such as des-acyl ghrelin or obestatin.

The many collaborative contributions of the other partners to this workpackage are done in association with other WPs and are described there.

WP8 Gene targetting (Leader: Jens Brüning, Partner 6)

Additional partners involved: 23 (Trier) and 19 (Pagotto)

<u>WP8 objective</u> To use targeted mouse mutagenesis to generate models for validation of CNS-related DIABESITY drug targets (including novel targets identified by WPs 1, 2 and 5).

Over the funding period, Partner 6 (Brüning) has achieved several fundamental novel aspects with respect to the regulation of energy homeostasis particularly via cell type specific modulation of gene activity in the arcuate nucleus of the hypothalamus beyond the state of art in the field. Particularly, they have demonstrated against a dogmatic view in the field, that insulin action in POMC-neurions is not critical for maintenance of energy homeostasis, while we could define a critical role for insulin action in AgRP-neurons in control of peripheral glucose metabolism via regulation of hepasatic glucose production. These experiments have defined novel regulatory principals in the central regulation of peripheral metabolism. Moreover, they have defined the molecular basis for a novel therapeutic intervention in obesity via

application of ciliary neurotrophic factor. They demonstrated that CNTF acts via gp130 receptors specifically in POMC-neurons to achieve weightloss even under conditions of leptin resistance in mice. Accordingly, ablation of the gp130 receptor specifically in POMC-cells abrogated this pharmacological action. Ultimately they have defined the role of intarcellualr signalling components of the insulin and leptin signalling machinery in ARC-neurons in vivo and have developed novel tools to define the neurocircuitry underlying the complex regulation of energy homeostasis. Taken together, all the anticipated deliverables and milestones have been met and partially even exceeded. The importance of the obtained finding is reflected by the publication of numerous f these studies in the most prominent scientific journals in the field, such as Cell Metabolism, JCI and PNAS.

The major scientific achievement of Partner 23 (Trier) was the identification of a novel transcription factor named ***Bsx*** for brain-specific homeobox gene and its functional analysis in mice in a collaborative effort with the group of Matthias Tschoep (contractor 21 in the DIABESITY consortium). This work has been published in Cell Metabolism Sakkou et al. 2007, Vol.5(6) pp. 450-463 which has drawn a lot of press coverage including the Cordis website: http://cordis.europa.eu/search/index.cfm?fuseaction=news.simpledocumentLucene&RCN=27808..
In this work ***Bsx*** has been identified as a molecular link between feeling hungry and fidgetiness that could serve as a target for combating obesity.
Bsx was shown to regulate in mice both the level of spontaneous physical activity and two brain peptides, NPY and AgRP that regulate how much the mice eat.
Mice lacking ***Bsx*** moved less and showed different eating behaviours. The ***Bsx*** gene is conserved across species, so may also be involved in energy balance in humans.
It is thus valid to speculate that mutations in human ***BSX*** may explain why some over-eaters become obese whereas others do not: people with normal copies of the gene may be better at matching their activity levels to their food intake.

WP9 Afferent signalling (Leader: Carlos Dieguez, Partner 8)

Additional Partners involved: 1c (Jansson), 21 (Rohner-Jeanrenaud), 4 (Bloom) and 1a (Dickson).

WP9 Objective To validate CNS-related DIABESITY drug targets, particularly novel targets identified by WPs 1, 2 and 5 by investigating how they are influenced by afferent signals from the periphery to the hypothalamus in relation to energy balance.

Partner 8 (Dieguez) has been heavily involved in uncovering new drug targets for obesity. The work has been carried out in most instances in collaboration with other members of the consortium led to the following accomplishments:
-*The **ghrelin** system as a drug target.* From a conceptual point of view targeting of this orexigenic pathway could be done at several levels: a) Inhibiting ***ghrelin*** secretion from their major source, ***ghrelin***-producing cells in the stomach. We managed to do so through a compound that was considered an GLP-1 agonist, exendine-4. The work (Perez-Tilve et al Diabetes) showed that this was a feasible approach and furthermore that the effect was likely to be mediated by a yet uncharacterized receptor. We postulate that this drug could be of use in particular for

Prader-Willi syndrome, a disease that is associated with hyper***ghrelin***aemia-driven hyperphagia and obesity. B) By new ***ghrelin*** antagonists. This approach was undertaken by other members of the consortium (Partner 1a, Dickson) in collaboration with an DIABESITY-associated partner from industry (AeternaZentaris). C) By uncovering the postreceptor mechanisms through which ***ghrelin*** exerts its orexigenic effect. In collaboration with the groups of Berlin (MT) and Heildelberg (M.Treier) we documented that ***ghrelin*** acts by increasing ***Bsx***-gene transcription (Nogueiras Endocrinology). In addition we further documentented in collaboration with the University of Cambridge and Berlin that ***ghrelin*** acts through a pathway involving AMPK-ACC-FAS and CPT-1 (Lopez et al Cell Metabolism). Taken together these studies uncover several new hypothalamic targets related to the ***ghrelin*** system for the treatment of obesity.
-*Resistin.* In work carry out by Partner 8 (Dieguez) in collaboration with parter 1a (Dickson) we have shown that this adipokine can behave as an anorexigenic agent at hypothalamic level (Tovar et al EJE). Furthermore we shown that it acts at central level influencing peripheral lipid metabolism.(Vazquez et al Endocrinology). Further work is being carry out in order to uncover the receptor through which resistin exert these effects in order to develop appropriate agonist/antagonist.

The main contribution of Partner 1a (Dickson) to the project has been further elucidation of the site and mechanism of action of ***ghrelin***. Moreover, we have explored actions of ***ghrelin*** that extend beyond energy homeostasis, namely its impact on the reward pathways and on thos controlling mood. We have explored, in particular, 2 models of suppressed ***ghrelin*** signalling: gastrectomized rats (that have a 60-80% reduction in circulating ***ghrelin***) and rats treated centrally with ***ghrelin*** antagonists (JMV2959). We have described the impact of ***ghrelin*** replacement on the expression of key hypothalamic genes involved in energy balance regulation (Egecioglu et al, 2007). We also found surgical depletion of gastric hormones is associated with reduced meal size as food intake and meal size appears to be independent of the food reservoir function of the stomach (Furnes et al, 2008). In 2 manuscripts (one submitted and one in press), we will describe the impact of ***ghrelin*** antagonists on (1) food intake (***ghrelin***- and fasting-induced) and electrical activity of arcuate nucleus cells and (2) body fat accumulation. Collectively the studies suggest that ***ghrelin***-induced food intake and fat accumulation can be suppressed by these agents and that these effects are mediated by ***GHS-R1A***. In 3 articles (Jerlhag et al, 2006; Jerlhag et al, 2007; Jerlhag et al, 2008), we report that ***ghrelin*** also targets a key mesolimbic pathway involved in mediating the rewarding properties of both natural (eg. Food) and artificial rewards (eg alcohol). The pathways is referred to as the cholinergic-dopaminergic reward link. The results suggest that ***ghrelin*** may increase food intake, at least in part, via stimulating this pathway. Moreover, the data raise the possibility that the physiological role of ***ghrelin*** extends beyond hunger to include the search for rewarding substances. We are now preparing a further 2 manuscripts describing the effects of gastrectomy (article 1) and ***ghrelin*** treatment (article 2) on emotional rectivity in rats. We found that ***ghrelin*** exerts effects that are pro-anxiety and pro-depression and have explored the molecular mechanisms underlying these effects by investigating expression of many genes involved in anxiety and depression in relevant brain areas.
We have also collaaborated in projects with other targets, for example, showing (1) the anorexigenic action of resistin (with Partner 8, Dieguez; Torvar et al, 2007), (2) the distribution and colocalization of ***IL-6*** receptors at key CNS sites (with Partner

1c, Jansson, manuscript in preparation) and (2) the VMN as a target for ***ghrelin*** (collaboration with Partner 13a, Leng)

The main role of Partner 21 (Rohner-Jeanrenaud) has been to extend our understanding on the neuroendocrine regulation of glucose and lipid metabolism. For this purpose, we infused various neuropeptides or hormones in normal or obese/insulin resistant type 2 diabetic rodents to determine the impact of such manipulations on fat storage, lipid utilization, glucose metabolism and insulin sensitivity. In such studies, we tried to delineate whether the effects observed are elicited centrally or peripherally and to what extend they depend from changes in food intake. In most instances, we also determined the mechanisms mediating the metabolic effects of the neuroendocrine factors studied. As a whole, our data contributed to improve our knowledge on the neuroendocrine interactions required for the maintenance of metabolic homeostasis, as well as on some alterations responsible for the development of obesity/type 2 diabetes. Among the deliverable proposed, 75% of the data were published as original articles. Twenty-five % of these articles were obtained through joined collaborations within the frame of the "DIABESITY" consortium.

The most important finding of Partner 1c (Jansson) during the 5 years of the DIABESITY project is that the inflammation regulating ***interleukin-1*** (***IL-1***) system seems to affect body fat in both experimental animals and humans. Before the start of the Project, we had found that endogenous ***IL-6***, another pro-inflammatory cytokine like ***IL-1***, suppresses obesity in mice (Wallenius et al Nat Genet 8:75-79, 2002). We now found that mice with a knockout of the gene coding for the only functional ***IL-1 receptor*** (***IL-1R1***) get mature onset obesity (Garcia M et al Diabetes 55:1205-1213, 2006). Other researchers in Japan and Europe found that that IL-1/IL-6 double knockout mice get severely obese, in line with our observations of moderate obesity in ***IL-6*** and ***IL-1R1*** gene knockout mice. Conversely, mice lacking the natural ***IL-1R1*** antagonist ***IL-1Rα*** get lean and obesity resistant. Thus, ***IL-1R1*** agonists like ***IL-1β*** seem to decrease obesity in mice, while the natural antagonist ***IL-1Rα*** may promote obesity by suppressing ***IL-1*** effects.

In translational studies we (Partner 1c) have been able to show evidence that genetic variations in the ***IL-1*** system are associated with fat mass. We first investigated the association between well known, common and functional polymorphisms of the ***IL-1*** system, mostly studied before by immunologists, nd the regulation of body fat. We found several polymorphisms that were associated with different measures of global and regional fat mass in two populations of younger and older Swedish men (Strandberg L et al J Clin Endo Metab, 91:2749-2754; Strandberg L et al Obesity 16:710-713, 2008). In a very recent study, we expanded our search to include four important genes in the ***IL-1*** system. In a systematic manner, using several tagging SNPs in each gene, we analyzed the major genetic variations of each gene. We found that the ***IL-1Rα*** gene have not previously described gene polymorphisms and haplotypes that are associated with fat, but not lean, mass (Andersson N et al Int J Obes, in press 2009).

WP10 Regulation of adipose tissue mass (Leader: Daniel Ricquier, Partner 20)

Other Partners involved: 22 (Staels), 1b (Enerbäck) and 13b (Seckl)

*<u>WP10 objective</u> To characterise and validate DIABESITY drug targets by studies of key factors in the regulation of adipose tissues, which are important target organs for the hypothalamic regulation of energy homeostasis. We will continue to study components of adipose tissue such as nuclear receptors (**Rev-erb**–α, PPARα and glucocorticoid receptors), the corticoid metabolism enzyme, 11ß- hydroxysteroid dehydrogenase type 1 (11ß-HSD1) and uncoupling protein 2 (UCP2).*

Excessive glucocorticoid action in humans causes obesity, diabetes and cardiovascular disease. Mice over- or underexpressing glucocorticoid receptor (GR) in adipose tissue as well as mice with a global reduction in GR levels (GR+/- mice) were generated to test whether adipose GR density regulates metabolic phenotype. Whereas increased adipose GR density increased fat mass in female transgenic mice, reduced GR density in adipose tissue conferred resistance to diet-induced obesity in female, but not in male mice. Globally reduced GR expression did not affect body weight or response to high fat diet, presumably due to compensatory hypothalamic-pituitary-adrenal axis activation that maintains normal energy homeostasis. 11b-hydroxysteroid dehydrogenase type 1 (11b-HSD1) amplifies intracellular glucocorticoid action. Previously we have shown the insulin-sensitising and beneficient metabolic effects of 11b-HSD1 deficiency. We have now shown that 11b-HSD1-deficient mice show an exaggerated acute inflammatory response in 3 different models of inflammation and have shown the key role played by C/EBPb, a transcription factor that regulates many aspects of the inflammatory response, in the auto-regulation of 11b-HSD1 by glucocorticoids. C/EBPb, a glucocorticoid regulated gene itself, may be a pivotal factor at the crossroads of metabolism, inflammation and glucocorticoid action.

We have also demonstrated that ***Rev-erb***α plays a crucial role in adipogenesis. Indeed, embryonic fibroblasts isolated from ***Rev-erb***α-KO mice show delayed differentiation into adipocytes and decreased expression of mature adipocyte markers, whereas ***Rev-erb***α over-expression stimulated adipocyte differentiation and intracellular lipid accumulation. In contrast, ***Rev-erb***α-KO mice display a massive adipose tissue accumulation with enlarged adipocytes. We have also demonstrated that RORα influences the adipogenic process in an opposite manner, in agreement with its well known opposite effect on transcription of common RORα and ***Rev-erb***α target genes. In addition, glucose clamps and glucose/insulin tolerance tests show that ***Rev-erb***α-deficient mice are slightly less sensitive to insulin and have a decreased glucose metabolic clearance rate. Finally, we have shown that ***Rev-erb***α plays a role in fatty acid and bile acid metabolism, again clearly indicating that ***Rev-erb***α participates in the control of energy homeostasis.

Besides hormone receptors, we also studied mitochondrial transporters of the UCP (uncoupling protein) family that are potentially able to regulate lipid storage, energy expenditure, inflammation and insulin secretion. Antibodies against UCP1 and UCP2 were isolated and were usable, however it was non possible to isolate antibodies against UCP3 non cross-reacting with UCP2. A MALDI-TOF Mass Spectrometry

analysis of UCP1 extracted from tissues was set up. We studied Ucp2-KO mice as well as patients bearing UCP2 mutations. Ucp2-/- mice are more susceptible to experimental encephalomyelitis and type 1- diabetes. These data, explained by its abundance in macrophages, highlight a protective role for UCP2 against auto-immune diseases. In other respects, studies of mouse embryonic fibroblasts devoid of UCP2 proved that UCP2 favors fatty acid oxidation and participates to sparing of glucose. Identification and functional analysis of mutations in patients presenting congenital hyperinsulinism, confirmed the role for UCP2 in regulating negatively insulin secretion. UCP2 appears as a new regulator of fatty acid/glucose oxidation and insulin secretion.

WP11 Human Physiology (Leader: Arne Astrup, Partner 2)

Additional partners involved: 18 (O'Rahilly), 9 (Ghigo), 19 (Pagotto), 4 (Bloom) and 17 (Nolan)

WP11 objective To validate DIABESITY drug targets, particularly novel targets identified by WPs 1, 2 and 5 by in vivo studies in humans.

The role of Partner 9 (Ghigo) was to describe: a) the nutritional and metabolic control of ***ghrelin*** secretion in humans in vivo; b) the metabolic impact of ***ghrelin*** administration in humans, both in its acylated and unacylated form, in normal subjects and in patients with type 2 Diabetes Mellitus; c) direct peripheral actions of ***ghrelin*** on pancreatic beta cell function and viability, and to identify synthetic ***ghrelin*** agonists or antagonists as potential candidate drugs for conditions of altered glucose metabolism.
The results allowed to describe that:
.a. in normal healthy humans, short-term fasting does not modify mean ***ghrelin*** concentration although it abolishes its circadian variations and, among nutrients, only carbohydrates represent a potent inhibitory signal on ***ghrelin*** secretion in humans. In fact, lipids and proteins do not play a major role in the control of ***ghrelin*** secretion independently of the route of administration.
.b. both in normal subjects and in patients with type 2 Diabetes Mellitus both acylated and unacylated ***ghrelin*** influence glucose metabolism. In particular, acylated ***ghrelin*** (AG) seems to impair glucose metabolism potentially inhibiting insulin sensitivity and likely increasing the hepatic glucose output. On the contrary, unacylated ***ghrelin*** (UAG), likely acting via specific but still uncloned receptors, seems to exert an insulin sensitizing action, suggesting a promising anti-diabetogenic action through an original mechanism of action.
.c. both AG and UAG potently promote beta cell growth and survival under severe death conditions in vitro. Moreover, in vivo, in newborn STZ-treated rats, both UAG and selected synthetic UAG fragments have been shown to preserve islet cell mass and to reduce diabetes at adult age. Moreover, UAG has been found to promote the expression of the main genes that regulate β-cell survival and function and glucose metabolism, granting UAG and its analogs a therapeutic potential in medical conditions associated with impaired β-cell function.

Partner 17 (Nolan) joined the DIABESITY project at the beginning of Year 3 (January 2006), as originally planned at the outset of the project. The main focus of this group has been the extreme phenotype of early onset Type 2 diabetes, associated

with early onset obesity. In collaboration with Prof Steve O'Rahilly's group, we have also characterized extreme insulin resistance in a patient with Majewski primordial dwarfism. This latter study is ongoing, with genetic mechanistic characterization in progress with a wider collaborative consortium

Exercise-related studies. Partner 17 (Nolan) has demonstrated that despite undergoing identical aerobic exercise training for three months, obese young patients with early onset type 2 diabetes failed to improve their insulin sensitivity, free fatty acid concentrations or maximal oxygen uptake following the exercise intervention (Burns et al, Diabetologia 2007). Matched obese non-diabetic subjects, in contrast, had a 20% increase in oxygen uptake and a reduction in free fatty acid concentrations. These findings in whole-body physiology have led us to investigate the potential cellular mechanisms underlying this combination of severe insulin resistance and exercise resistance (details of preliminary results are summarised above).
We demonstrated that there was a dissociation between the lack of response following exercise in terms of VO2max and glucose disposal and a clear reduction in the concentration of plasma visfatin – a novel adipokine. The exact role of visfatin in intermediary metabolism will require further study.

Partner 17 (Nolan) has also studied acute (1 bout of one hour) and short term (one week, one hour per day) aerobic exercise intervention study in similar cohorts of subjects to those described above. Following acute exercise, non-diabetic subjects show a clear acute increase in skeletal muscle PGC-1-α, whereas those with early onset Type 2 diabetes show no response. This may be yet another important contributor to the observed lack of whole body response to exercise of the type and intensity that we have studied to date in these severely insulin resistant patients. This work has been conducted by two clinical research fellows (one part-funded by the DIABESITY project) and a PhD student (also partly funded by the DIABESITY project). The results of these studies will help promote better understanding in the pathogenesis of insulin resistance in skeletal muscle and hopefully towards the development of novel therapeutic targets in the future.

Partner 17 (Nolan) has initiated a more complex intervention protocol at the end of 2008, involving both a dietary and an exercise intervention in subjects with early onset Type 2 diabetes. Both diet and aerobic exercise will be used in random order for three month periods of supervised intervention, and all subjects will undergo metabolic characterisation and muscle biopsy before and after each intervention.

Partner 4 (Bloom) has used a tranlation approach involving both clinical work (This workpackage) but also complimentary rodent studies that have formed part of the contribution to WPs 7 and 9. All of the major achievements are described here. The overall aims of the activities of Partner 4 (Bloom) have been:
1. To investigate the postprandial dynamic peptide PYY3-36 (PYY(3-36)) response.
2. To investigate if PYY(3-36) and glucagon-like peptide-1 (GLP-1) inhibit food intake additively in rodents and man.
3. To investigate the effects of ***ghrelin*** on food intake in human volunteers and patients with anorexia
4. To investigate the effect of combined gut hormone (***PP*** and PYY) infusion on appetite in human volunteers.

To address these aims, the following tasks have been performed: by Partner 4 (Bloom):
1. Postprandial satiety and plasma PYY responses were assessed in twenty lean and twenty obese humans in response to six test meals of varying calorie content.
2. We studied the effects of peripheral co administration of PYY(3-36) with GLP-1 in rodents and man.
3. We studied the effects of ***ghrelin*** in human lean and obese volunteers and also in patients with anorexia due to cancer or renal failure
4. We studied the effect of combined gut hormone (***PP*** and PYY) infusion on appetite in human volunteers.

The major findings of Partner 4 (Bloom) can be summarised as follows:
1. Human volunteers had a graded rise in plasma PYY ($R2 = 0.96$; $P < 0.001$) during increasing calorific meals, but the obese subjects had a lower endogenous PYY response at each meal size ($P < 0.05$ at all levels).
2. Whereas high-dose PYY(3-36) (100 nmol/kg) and high-dose GLP-1(7-36) (100 nmol/kg) inhibited feeding individually, their combination led to significantly greater feeding inhibition.
3. ***Ghrelin*** potently increased food intake in human lean and obese volunteers and also in patients with anorexia due to cancer or renal failure
4. We have shown that ***PP*** and PYY(3-36) inhibit food intake in humans but this effect is not additive.

Thus Partner 4 (Bloom) has shown that gut hormones represent novel targets for the development of novel agents for the development of anti-obesity drugs. The work described has demonstrated the efficacy and safety of gut hormone administration in humans. This work provides the rationale for the development of gut hormones as potential anti-obesity agents for the treatment of obesity.

The DIABESITY logo

The DIABESITY website can be found at: www.diabesity.eu