

Network: Obesity and Related Disorders**Project: Hypothalamic Gene Expression in States of Altered Energy Balance**

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Introduction

Energy balance in humans and animals is maintained by neuroendocrine pathways which process peripheral and central signals and integrate information perceived from the environment. The concentration of two hormones, namely leptin and insulin, in the bloodstream largely reflect the fat mass stored in the body and can be regarded as 'adiposity signals'. Both of these hormones bind to their respective receptors in the brain and trigger downstream signalling cascades which inhibit food intake (anorexigenic) and may propel energy expenditure. The primary neurons responding to leptin and insulin are localised in the *nucleus arcuatus* (ARC) at the base of the medial hypothalamus and form a complex network with other hypothalamic nuclei and the brainstem. Two distinct neuron groups in the ARC have been characterized, producing either anorexigenic (alpha-melanocyte stimulating hormone and cocaine amphetamine regulated transcript) or orexigenic (neuropeptide Y and agouti related peptide) neuropeptides. In states of altered energy balance associated with a rise or a decline of serum leptin and insulin levels the hypothalamic leptin and insulin receptors regulate synthesis and release of these peptides and thereby activate compensatory mechanisms to defend body mass and adiposity. The loss of sensitivity towards peripheral leptin or insulin of this neuronal network appears to be a major cause for disorders in body mass regulation and the development of obesity.



Fig 1: Obesity in a homozygote C57BL/6J-m Lep^{ob} mouse compared to a normal wildtype animal. (Photograph taken by Susanne Keipert).

Project Status**Gene Expression Profiling**

We focus on candidate genes identified in NGFN-1 by microarray based gene expression profiling studies using total RNA extracted from hypothalamus tissue as the starting material. Within the network we provide the analysis of tissue-specific gene expression of candidate genes in animal models under investigation in the research pipeline and offer our expertise in microarray analysis. So far we analysed hypothalamic gene expression patterns associated with different states of energy balance in mouse and rat models using oligo-based microarray technology (Affymetrix GeneChips) and cDNA macroarrays (RZPD) in collaboration

with several partners within the Neuronet Marburg (NGFN-1). Standardised protocols have been established for tissue dissection, RNA preparation and microarray analysis in order to minimize technical variability. In close collaboration with the Institute for Medical Biometry & Epidemiology (Philipps Universität Marburg) the minimal requirements for successful microarray studies were defined and major progress was achieved in the analysis of gene expression raw data using freeware available on the web. We dispose of refined procedures for the comparative analysis of gene expression data, which include a set of quality controls and suitable normalization and standardization procedures prior to the quantitative comparison of gene expression profiles. The replacement of default software solutions by more suitable data analysis methods was essential to detect subtle changes in hypothalamic gene expression associated with different states of energy balance (few genes 2-3fold, most genes <2fold).

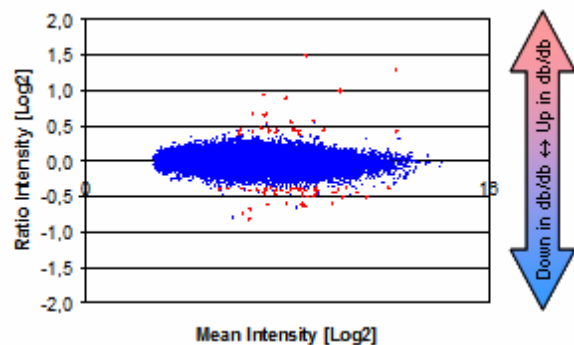


Fig 2: MA-Plot of db/db vs. +/+ gene expression data. Out of the ~39000 transcripts represented on the microarray, 68 candidates (red symbols) were selected by fold-change and statistical evaluation (t-values).

In collaborative studies within the NGFN Neuronet we applied the refined microarray analysis tools to identify candidate genes in mouse models for obesity, including leptin deficient *ob/ob* (Schmidt, MPI Bad Nauheim), leptin receptor deficient *db/db* (Besedovsky and del Rey, Philipps-Universität Marburg) and obese dwarf SMA1 mice (SMP in Munich & Ingenium Pharmaceuticals AG). Furthermore we analysed hypothalamic gene expression in inbred obesity strains resistant [DR] or prone [DIO] to diet-induced obesity and in response to hormonal stimulation (for example acute leptin injection).

By comparison of gene expression patterns in the hypothalamus of these animal models we identified a set of candidate genes which are potentially involved in the regulation of energy balance and body weight. For example microarray analysis of hypothalamic RNA from wildtype and *db/db* mice (n= 5 for each genotype, Fig. 1) revealed that 68 of the ~39000 transcripts represented on the microarrays are most likely to be differentially expressed in the hypothalamus of the *db/db* as compared to the +/+ mouse (Fig. 2). Data mining in the Gene Ontology project revealed that one or more molecular functions and/or involvement in one or more biological processes are known for two thirds of these 68 transcripts. The remaining transcripts represent expressed sequence tags with currently no assigned function.

Validation of Candidate Genes

As the levels of differential expression were rather moderate (1.3 – 2.8 fold; Fig 2), despite a massive phenotypic disturbance of energy balance in *db/db* mice (Fig. 1), validation experiments are currently performed using quantitative real-time RT-PCR with new *db/db* and *+/+* mice not tested previously on microarrays. At the present state we have already validated a subset of selected genes using gene-specific primers in a SYBR-Green based assay. As a prerequisite the corresponding cDNAs have been cloned or retrieved from clone collections (RZPD and other commercial distributors) and sequenced. These candidates include genes encoding for orexigenic neuropeptides, kinases involved in intracellular signalling and proteins involved in the modulation of hormone responses in cells.

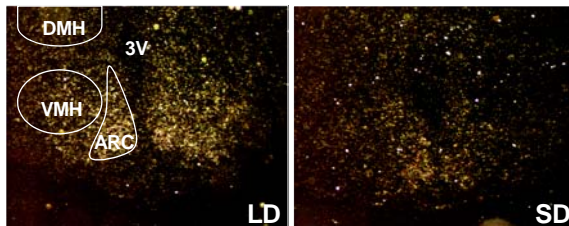


Fig 3: SOCS3 gene expression within the hypothalamus of long (LD) and short day (SD) acclimated Djungarian hamsters (*Phodopus sungorus*), a seasonal model for neuroendocrine mechanisms of body weight regulation¹.

Quantitative In Situ Hybridisation (ISH) methodology established in our laboratory is currently used to localise the neuroanatomical sites of candidate gene expression and to verify differential gene expression in *db/db* and *+/+* mice, as well as in other animal models in states of altered energy balance. Coronal brain cryosections representing the hypothalamic area will be mounted on glass slides and used for quantitative ISH analysis with radiolabeled (35-S) cRNA probes complementary to the target sequence of the identified candidate genes. The quantitative ISH methodology previously has been successfully applied to characterize SOCS3, a suppressor of cytokine signalling, as

an important mediator of leptin sensitivity in the ARC (Fig 3) and the ghrelin receptor in a seasonal model for body weight regulation^{1,2}. Finally, neuroanatomical distribution of differentially expressed candidates will be further investigated by co-localisation with known orexigenic and anorexigenic neuropeptides using ISH and immunohistochemistry³.

Outlook

The applied microarray technology enabled us to identify genes regulated in states of altered energy balance in the hypothalamus of the mouse. Our candidate genes will be evaluated based on mapping of quantitative trait loci (QTL) obtained in animal models for obesity currently under investigation by other partners in our network. This will allow us to distinguish between genes which are generally affected in obesity and others which are differentially expressed only in distinct animal models. Genes with functional annotations relevant to the physiology of energy balance will be further investigated in selected animal models. In this respect, inbred DR and DIO mouse strains are the most promising models regarding the potential to increase our understanding of the polygenic nature of obesity in humans. The most relevant genes should be located in QTL regions associated with the clinical phenotype of obesity. In collaboration with clinical researchers in our network, we initiated the search for single nucleotide polymorphisms in the respective human orthologues and will directly test their possible association with obesity in humans.

Lit: 1. Tups A et al. Photoperiodic regulation of leptin sensitivity in the Siberian hamster, *Phodopus sungorus*, is reflected in arcuate nucleus SOCS-3 (suppressor of cytokine signaling) gene expression. *Endocrinology*. 2004; 145:1185-1193. 2. Tups A et al. Circulating ghrelin levels and central ghrelin receptor expression are elevated in response to food deprivation in a seasonal mammal (*Phodopus sungorus*). *J Neuroendocrinol*. 2004; 16:922-928. 3. Khoroshii RM & Klingenspor M. Neuronal distribution of melanin-concentrating hormone, cocaine- and amphetamine-regulated transcript and orexin B in the brain of the Djungarian hamster (*Phodopus sungorus*). *J Chem Neuroanat*. 2005; 29:137-148.