Title: Hypermetabolic syndrome as a consequence of repeated psychological stress in mice

Short title:

Repeated stress and hypermetabolism

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Disclosure statement:

The authors have nothing to disclose

Grants or fellowships supporting the study

This work was supported by grants of the DFG (Deutsche Forschungsgemeinschaft), the Land Mecklenburg-Vorpommern and the University of Greifswald as part of the research training group GRK840 “Host-pathogen interactions in generalized bacterial infections”.

Abstract

Stress is a powerful modulator of neuroendocrine, behavioral and immunological functions. After 4.5 days of repeated combined acoustic and restraint stress as a murine model of chronic psychological stress severe metabolic dysregulations became detectable in female BALB/c mice. Stress-induced alterations of metabolic processes that were found in a hepatic mRNA expression profiling were verified by in vivo analyses. Repeatedly stressed mice developed a hypermetabolic syndrome with severe loss of lean body mass, hyperglycemia, dyslipidemia, increased amino acid turn-over, and acidosis. This was associated with hypercortisolism, hyperleptinemia, insulin resistance, and hypothyroidism. In contrast, after a single acute stress exposure changes in expression of metabolic genes were much less pronounced and predominantly confined to gluconeogenesis, probably indicating that metabolic disturbances might be initiated already early but will only manifest in repeatedly stressed mice .

Thus, in our murine model, repeated stress caused severe metabolic dysregulations leading to a drastic reduction of the individual's energy reserves. Under such circumstances stress may further reduce the ability to cope with new stressors such as infection or cancer.

CATABOLIC PROCESSES for energy mobilization and anabolic functions such as growth or healing need to be balanced to sustain health (1-5). Psychological and physiological stressors can disturb neuroendocrine, immunological, behavioral, and metabolic functions (1, 4, 6-8) and adaptive physiological processes aim to reconstitute a dynamic equilibrium (1, 9). These stress responses are normally short lasting and physiologically important for survival to cope with a changing environment or to deal with potentially life-threatening situations (1, 9). In modern society, however, individuals are continuously confronted with stressful stimuli, and prolonged neuroendocrine responses probably harm rather than protect (1, 3, 10, 11). Stress-induced neuroendocrine alterations include activation of the sympathetic nervous system (SNS) with increased secretion of catecholamines, and stimulation of the hypothalamus-pituitary-adrenal (HPA) axis with heightened release of glucocorticoids (GCs) (1, 8, 9). Prolonged and increased release of catecholamines is associated with cardiovascular diseases such as hypertension, myocardial infarction or stroke (1, 5, 12). Excessive secretion of glucocorticoids was found to be linked to diabetes, dyslipidemia, cardiovascular alterations, immunosuppression and mood disorders (1, 5, 13).

Recently, we showed in a murine model of psychological stress, that BALB/c mice develop severe systemic immunosuppression due to 4.5 days of intermittent stress exposure that we rated as chronic stress.

This immunodeficient state was associated with lymphocytopenia, T cell anergy, high rates of lymphocyte apoptosis in lymphoid organs, impaired phagocytic and oxidative burst responses, and induction of an anti-inflammatory cytokine bias. Stress-induced immunosuppression, on the one hand was accompanied with attenuation of a hyperinflammatory septic shock but, on the other hand, with a reduced clearance of experimental infections in the long-term (8, 14). Furthermore, we documented behavioral alterations with increased depression-like behavior and neuroendocrine alterations such as prolonged activation of the HPA axis and increased turnover of catecholamines. Finally, a prominent stress-induced loss of body mass without significant changes of food and water intake during the observation period became detectable (8). It is known that stress exposure is linked to changes of body weight. There is evidence that hypothalamic control of food intake is influenced by stress, which in consequence alters metabolism. In such situation some people lose and others gain weight in response. However, the molecular mechanisms of the stress-body weight connection remain to be elucidated.

Several authors propose chronic stress to be a main feature in the pathogenesis of the metabolic syndrome which is associated with obesity, type II diabetes/insulin resistance, dyslipidemia and hypertension (3, 5, 7). On the other hand, stressors like injury, infection, traumatic events or prolonged sleep deprivation capably induce hypercatabolism, and therefore, may cause cachexia (2, 3, 10, 15-20). Such metabolic driven wasting may result from pain, depression or anxiety, causing malabsorption and maldigestion or morphological and functional alterations of the gastrointestinal system (3, 16, 21-24) and is typically seen during repeated inflammatory processes or during sepsis (2, 15-17).

In the literature, two main pathways leading to massive loss of body mass are discussed: the response to starvation and the hypermetabolic response. Starvation is induced by inadequate calorie intake and causes the utilization of body′s own tissue (3, 16, 25). At first, carbohydrate stores are emptied for energy production. Secondarily, the body switches to usage of lipid and protein stores for gluconeogenesis. Finally, fatty acids are absorbed into the liver where ketone bodies are produced which for example neurons can use as energy source to restore functional homeostasis. In a feedback loop, efferent neurons signal to the periphery so that gluconeogenesis is lowered and protein breakdown is diminished which causes adaptation to starvation (3, 16). The supply for basal energy production then comes from the calories of adipose stores (3, 26).

In contrast, a hypermetabolic response which is often seen during critical illness is predominantly mediated by hormones and inflammatory mediators (2, 6, 7, 15-17, 27). As

during the initial phase of starvation gluconeogenesis is accelerated by usage of lipids and amino acids (6, 17, 27, 28). Protein breakdown is massively increased due to glucagon and glucocorticoid effects, and can be accelerated by proinflammatory cytokines such as TNF or IL1 (16, 29, 30). Amino acids, along with fatty acids and glycerol, are used for gluconeogenesis in the liver causing hyperglycemia (6, 31). This process is mainly mediated by catecholamines and corticosteroids and finally results in a rapid loss of lean body mass without sufficient metabolic adaptation to diminish tissue breakdown (2, 16, 18, 32).

To get further clues which metabolic alterations account for the pronounced loss of body weight in our repeated stress model, we decided for a global screening of gene expression in the liver.

Based on documented changes of the expression profile of hepatic genes involved in carbohydrate, lipid and amino acid metabolism we started to characterize metabolic disturbances and found biological relevant alterations of glucose metabolism, dyslipidemia and changes in amino acid turnover in repeatedly stressed BALB/c mice.

Materials and Methods

Animal experiments

Female BALB/c mice aged 6-8 weeks were randomly grouped into the experimental and control groups starting at least 4 weeks before being used in experiments. The group size in different experiments differed from 6-12 mice per cage. Animals stayed in their group until the end of the experiments and were not mixed up to avoid social stress. All animals were maintained with sterilized food (ssniff R-Z, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water ad libitum for adaptation under minimal stress conditions. Influences of irregularities of the estrous cycles of unisexually grouped female mice (33) were not analyzed selectively and may cause higher standard deviations in the statistical analyses.

Animal rooms were on 12:12 light/dark cycle and were maintained at a constant environment prior to the experiment. In order to avoid any additional effect (for example acoustic or olfactory effects) the handling of mice during the adaptation period and during the experiments was restricted to one investigator. All animal procedures were carried out as approved by the Ethics Committee for Animal Care of Mecklenburg-Vorpommern, Germany.

Repeated stress model

Mice were exposed to combined acoustic and restraint stress on four successive days, for 2 h twice a day during the physiological recovery phase of rodents (0800-1000 a.m. and 0400-0600 p.m.). On day 5 only one stress session was performed in the morning. For immobilization mice were placed in 50-ml conical centrifuge tubes with multiple ventilation holes without penning the tail. Acoustic stress was induced by a randomized ultrasound emission device between 19 and 25 kHz with 0-35 dB waves in attacks (SiXiS; Pat.NO.109977, Taiwan) allowing the mice no adaptation to the stressor (8, 14). Between the stress sessions mice stayed in their home cages and had free access to food and tap water. Control mice were kept isolated from stressed animals during the 4.5 days of stress exposure to avoid any acoustic or olfactory communication between the groups. Therefore, the non-stressed group stayed in the incubator where the animals were adapted. The stressed mice remained outside the incubator in the same animal laboratory during the whole period of the stress model. All successive experiments and analyses were performed starting at 1000 a.m. after the ninth stress exposure. Different *in vivo* analyses were performed with n=6-12 mice/group in at least two experiments according to the experimental protocol to ensure reproducibility. For array analysis 2 independent stress experiments were performed with n=9 mice/group (first experiment) and n=8 mice/group (second experiment).

Acute stress model

Mice were exposed to a single 2 h combined acoustic and restraint stress cycle in the morning (0800-1000 a.m.). *In vivo* analyses were performed immediately after or 6 h after the stress session with n=6-9 mice/group. Two acute stress experiments for array-analysis were

composed of n=8 animals for each control group and n=9 animals/stress group in the first and n=8 animals/stress group in the second experiment.

Organ harvesting for RNA preparation
Organs of unnarcotized mice were explanted immediately after cervical dislocation in order to avoid RNA degradation. For liver samples a small piece of tissue was immediately homogenized with a micropestle in 350 µl Buffer RLT/ 1% β-Mercapoethanol (QIAGEN GmbH, Hilden, Germany/Sigma-Aldrich Chemie GmbH, München, Germany). The liver lysates were shock frozen in liquid nitrogen. All samples were stored at -70°C.

RNA preparation
Liver sample lysates were thawed and processed at room temperature for RNA preparation with a RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. After ethanol precipitation the RNA was quantified spectrophotometrically and its quality was verified using an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies Inc., Santa Clara, CA, USA).

DNA array analysis
For each group pools containing equal amounts of RNA from each individual animal were prepared and used for subsequent microarray analysis. 5 µg of pooled total RNA were used for the synthesis of double-stranded cDNA and this solution then served as template for an in-vitro-transcription reaction (IVT) using GeneChip Expression 3' Amplification Reagents (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After spin-column based cleanup concentration and quality of cRNA were measured as described above.
cRNA was fragmented, added to the hybridization cocktail, denatured and hybridized with Affymetrix GeneChip Mouse Expression Arrays 430A/ 430A 2.0 according to the manufacturer's instructions. Washing, staining and scanning were performed using Affymetrix GeneChip FluidicsStation and scanners according to standard protocols.

DNA array data analysis
The Affymetrix expression analysis was performed for livers of repeatedly stressed and healthy control mice with technical duplicates of two independent biological experimental series each. For the analysis of the effects of acute stress array hybridizations were also performed of two independent biological experiment for both groups (control and acute stress). Affymetrix array image data generated with MAS 5.0 (repeated stress) were analyzed using the GeneChip Operating Software (GCOS) 1.2 (Affymetrix, Santa Clara, CA, USA) with default values for parameter settings. For normalization, a scaling procedure with a target value of 150 was employed. Image data of the acute stress experiment were directly analyzed in GCOS 1.4 with default settings and normalized by scaling to the target value 500. After data transfer to the GeneSpring software package (Agilent Technologies Inc., Santa Clara, CA, USA) genes displaying differential regulation in response to repeated and acute stress were identified based on the following criteria:
i) The signal of probe sets had to be present in the arrays at least in the control (for repressed genes) or in stressed mice (for induced genes) in both biological experiments, ii) the difference of mean signals between control and stressed mice had to equal or exceed 100 and iii) the fold change factors calculated from the signal values in each experimental replicate had to exceed a cutoff of ≥ 1.5 or ≤ -1.5 in both biological experiments.
Lists of probe sets displaying differential regulation in both acute and repeated stress or specifically after acute or repeated stress were then uploaded as Excel spreadsheets into the Ingenuity Pathway Analysis Version 5.5 (Ingenuity® Systems http://www.ingenuity.com) and used for the interpretation of the array data in the context of already published knowledge. Biological functions were assigned to the networks based on the content of the Ingenuity Pathway Knowledge Base (IPKB).

Real-time PCR
DNA was removed by DNase-treatment and subsequent to purification using a RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany) and ethanol precipitation, concentration and quality

of RNA samples was assayed as described above. Validation of expression data by real-time PCR was separately carried out for all individual RNA preparations (n=9 plus n=8 mice/group) of the two biological experiments with repeated stress exposure. For real-time PCR analysis 1 µg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit in the presence of SUPERase•In RNase inhibitor (Ambion/ Applied Biosystems, Foster City, CA, USA). 20 ng of cDNA served as template for real-time PCR using the following 20x TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): Asl (Mm00467107_m1), Srebf1 (Mm00550338_m1), Pck1 (Mm00440636_m1), Gadd45b (Mm00435123_m1), Sds (Mm00455126_m1), and Actb (Mm00607939_s1). Differential regulation in repeatedly stressed and control mice was confirmed by comparing the △Ct values (Ct value of the target gene – Ct value of the reference gene Actb in identical cDNA samples) of all control mice and repeatedly stressed mice with a Mann-Whitney test, requiring a p-value of $p \leq 0.05$.

Assessment of body weight, food intake and water consumption

Body mass was determined for stressed and control mice prior to the first and immediately after the 9th stress session. Average food and water intake per cage of stressed and non-stressed groups were monitored during these 4.5 days of stress exposure (incl. spillage) by measuring weight of food pellets (g) and volume of drinking water (ml) for cages with 9 equally aged mice.

Harvesting of blood and organ samples

Immediately after the single (acute stress) or in the repeated stress model after the 9th stress session, mice were anesthetized with 75 µg/g BW Ketamin Curamed® (CuraMED Pharma GmbH, Karlsruhe, Germany) and 16 µg/g BW Rompun® (Bayer AG, Leverkusen, Germany) diluted in pyrogen-free 0.9% sodium chloride (Braun, Melsungen, Germany). Blood was harvested by retroorbital puncture and collected in K2E-EDTA vacutainer tubes (BD Vacutainer™, Plymouth, UK). Plasma samples were either analyzed immediately or stored at -20°C until use. Samples of adrenal glands, liver and spleen for histological staining were immediately put into liquid nitrogen and stored at -80°C until further analysis.

Measurement of plasma hormones

Plasma levels of corticosterone, insulin, resistin, growth hormone, total triiodthyronine (T3), total thyroxine (T4) and leptin were quantified by ELISA according to the instructions of the supplier (OCTEIA Corticosterone EIA, IDS, Boldon, UK; Mouse insulin ELISA kit, Mercodia AB, Sweden; Mouse Resistin ELISA, BioVendor, Germany; Diagnostic Systems Lab Inc., Webster, USA; Total T3 and Total T4ELISA kits, Alpha Diagnostic Int., USA; MD Bioscience Inc., St. Paul, USA, mouse leptin ELISA, IBL, Germany).

Measurement of blood glucose levels

A drop of plasma sample was picked up on a test strip and glucose level was immediately measured using the ACCU Chek® compact system (Roche Diagnostics, Germany). Glucose concentrations were analyzed in anesthetized mice immediately after a single or the ninth stress session, and 30 min to 2 h after the termination of the last repeated stress session.

Measurement of plasma pH levels

Freshly prepared plasma samples were analyzed by pH indicator strips immediately after the last repeated stress session.

Measurement of plasma lipid composition

Total triglycerides and total cholesterol content, high density (HDL)-, low density (LDL)- and very low density (VLDL)-cholesterol content were analyzed by lipid-electrophoresis in the Department of Clinical Chemistry at the university hospital of Greifswald.

Measurement of plasma amino acid composition

EDTA-plasma samples were harvested and specimens were generated by pipetting 30 µl of fresh plasma onto filter papers (No. 903, Whatman plc, UK), and were left to dry at room temperature. The amino acids were analyzed by tandem mass spectroscopy (Refurb Wallac MS2 tandem mass spectrometer, Perkin Elmer,

Rodgau, Germany) using a modified method of Nagy et al. (34) and a commercial CE marked kit (Perkin Elmer, Neogram amino acids and acylcarnitines non-derivatized TMS kit).

Histological staining

To analyze lipid vacuole formation 8 µm thick kryrosections of the liver were stained by hematoxylin-eosin (HE) staining.

Lipid in cryosections of the adrenal glands (8 µm thick) was stained by the lipid soluble dye Sudan III. Non-cholesteryl esters are stained orange. Tissues were counter-stained with hematoxylin.

The content of carbohydrates in the liver was analyzed in cryosections (8 µm) using a Periodic Acid Schiff (PAS) staining protocol. Aldehyde-groups of carbohydrates cause a purple staining.

To investigate the content of fibrotic tissue cryosections (8 µm) were analyzed by Azan staining (Heidenhain). Collagen and reticular fibrotic tissue is stained sharp blue, cell granula are stained yellow to red, and chromatin is stained red.

Statistical analysis

Statistical analysis exclusive of DNA array data analysis (see above) was carried out with GraphPad Prism Version 3.02 for Windows (GraphPad Software Inc., San Diego, USA). Differences between samples of stressed and non-stressed mice were analyzed by Mann-Whitney test not assuming Gaussian distribution. All data in this study were expressed as mean ± SD; $p<0.05$ was considered statistically significant.

Results

Repeated stress-induced cachexia accompanied by hypercortisolism, hyperleptinemia and hypothyroidism

Repeated psychological stress caused a severe loss of body mass in BALB/c mice while mean food intake and water consumption were unaltered during 4.5 days of stress exposure: Food intake was 95.1 ± 19.9 g/cage in the repeatedly stressed vs. 91.9 ± 17.4 g/cage in the non-stressed groups and water consumption was 250 ± 40 ml/cage in stressed vs. 240 ± 40 ml/cage in the control mice (n=3 independent experiments with 9 mice/cage). Given that we consistently found normal food and water intake we questioned whether the severe loss of body mass after repeated stress exposure depends on hormonal changes. First we found, that repeatedly stressed mice showed increased corticosterone concentrations in the peripheral blood (Fig. 1A) along with a hypertrophy of the adrenal cortex with decreased size of lipid storage vesicles in the glucocorticoid-producing zona fasciculata (Fig. 1B,C). In fact, the rise in circulating GCs was less pronounced than after acute stress (Suppl. Mat. 1), but the continuing HPA axis response might contribute to the reduction of body weight of up to 20 % during the time course of repeated stress exposure (Fig. 2A). This loss of body mass was not associated with significantly altered growth hormone concentrations in the blood of stressed (13.6 ± 7.6 ng/ml) vs. non-stressed mice (10.8 ± 5.4 ng/ml). However, stress-induced hyperleptinemia was measured (Fig. 2B). Total triiodthyronine (Fig. 2C) and total thyroxine levels (Fig. 2D) were reduced in the plasma of stressed animals whereas thyreoglobuline concentrations remained unchanged (data not shown).

Repeated stress-induced changes of global hepatic gene expression

In an approach to a more comprehensive characterization of the metabolic changes that occur as a result of repeated acoustic and restraint stress we recorded the expression signatures of liver from repeatedly stressed BALB/c mice and compared them with those of non-stressed controls. The Affymetrix-based mRNA expression profiling of the liver of repeatedly stressed vs. non-stressed animals revealed induction and repression, respectively, of 120 and 50 genes in both independent stress experiments performed (for complete listing see Suppl. Mat. 2). To discriminate effects of repeated stress from those of acute stress we additionally analyzed the changes in the hepatic gene expression that occurred as a result of a single stress exposure. In this model of acute stress 192 and 123 genes displayed stress-

mediated induction or repression of expression (for complete listing see Suppl. Mat. 3).
Comparatively analyzing the effects of acute and repeated stress it became clear that both types of stress target a common set of 94 genes. 221 and 76 genes were predominantly regulated by acute and repeated stress, respectively (Fig. 3A).
In order to analyze the changes in gene expression within the framework of already accumulated knowledge, the lists of genes differentially expressed after acute and repeated stress or both were subjected to an analysis employing the Ingenuity Pathway Analysis (IPA) software. This software allowed for an intuitive mining of the data of the 391 differentially expressed genes to gather an impression of the biological rationale of the expression changes experimentally observed within the context of published data.
When the IPA software was utilized to analyze the molecular and cellular functions targeted by stress an influence on broad categories such as "cell growth and proliferation" and "cell death" was noted (Suppl. Mat. 4). However, it was also apparent that genes related to metabolic diseases were most significantly influenced by the repeated stress exposure (Fig. 3B). This finding was in line with the metabolic disturbances observed before. Supporting this notion of a major impact of repeated stress on metabolism highly significant changes were also noted for more specific categories such as amino acid metabolism and lipid metabolism. Some of these influences on metabolism were already noted during acute stress because changes related to metabolic disease ranked at number six when genes commonly influenced by acute and repeated stress were analyzed. Genes involved in more specific categories of metabolism such as lipid and amino acid metabolism were only moderately influenced by acute stress. Thus, the gene expression profiling favors the idea that acute stress sets into a motion a gene regulation cascade that is then manifested during repeated stress exposure finally leading to the observed metabolic disturbances.
To elucidate the reasons for stress-induced cachexia we decided to selectively concentrate on changes of expression of genes whose products are involved in metabolic processes and regulation of metabolic pathways.
Several genes that were regulated in the liver of repeatedly stressed animals could be linked to hypercatabolism (Fig. 3 and Suppl. Mat. 4). Genes relevant for amino acid metabolism, especially amino acid transporters and enzymes metabolizing glucogenic amino acids (Slc15a4, Slc25a15, Slc3a1, Asl, Got1) were mostly induced (Suppl. Mat. 2 and 4). Moreover, the gene expression profiling of the liver of repeatedly stressed mice indicated increased metabolism of lipids (Adh4, Apoa4, Cd74, Chpt1, Cyp17a1, Cyp2b10, Cyp3a13, Cyp4a10, Cyp8b1, Hsd17b2, Hsd3b2, 4632417N05Rik (Hspc105), Saa2, Slco1a1, Xbp1).
To validate the array data we performed real time RT-PCR focusing on chronic stress-induced dysregulation and its pathophysiological effects. Therefore, we chose genes that were associated with repeated stress-influenced metabolic processes of carbohydrate metabolism (Pck1), fat metabolism (Srebf1), and amino acid metabolism (Asl, Sds) and with stress-induced apoptosis (Gadd45b). For all selected genes the regulation found with the Affymetrix-based expression profiling was confirmed (Tab. 1).

Induction of gluconeogenesis in repeatedly stressed mice

Stimulated by the observed loss in total body mass and the suspected involvement of carbohydrate metabolism we specifically investigated the expression profiles for relevant genes even if this category was not part of the first most significant biological functions according to the IPA categorization. Stress-induced increased expression of Foxo1, Igfbp1, Irs1, and Pck1, as well as reduced mRNA levels of Srebf1 can induce hyperglycemia because of activation of gluconeogenic pathways. In contrast, the gene products of Cebpb, Igfbp1, St3gal5, Tnfrsf1b are associated with hypoglycemia and may indicate counter-regulatory processes to decrease blood glucose levels.
In singularly stressed mice, *in vivo* analysis did not reveal significant changes of carbohydrate regulation pathways e.g. of leptin concentrations in the plasma, blood glucose levels or liver histology (Suppl. Mat. 1).
In contrast, repeated stress induced pathophysiological relevant alterations of protein

and lipid metabolism to provide fuel for gluconeogenesis. Only in chronically stressed mice disturbances of the carbohydrate metabolism became detectable also *in vivo*. This included a transient hypoglycemic period immediately after the termination of the ninth stress session. However, after resuming food intake in the home cage blood glucose levels increased rapidly and resulted in a prolonged hyperglycemia that still was detectable 2 h later (Fig. 4A). In the liver of repeatedly stressed mice an increased usage of carbohydrate-reservoirs was assessed by reduced PAS-staining that stains aldehyde-groups of carbohydrates in tissue and revealed reduced storage of carbohydrates in the liver of repeatedly stressed mice compared to healthy control mice (Fig. 4B,C). Moreover, after repeated stress, insulin concentrations in the plasma were slightly increased (272.5 ± 131.4 pg/ml) compared with control mice (170 ± 149 pg/ml). Resistin, an insulin-resistance inducing adipokine, was significantly increased in the plasma of repeatedly stressed mice when compared with non-stressed animals (Fig. 3D). Last, but not least, analysis of pH in EDTA plasma samples revealed stress-induced acidosis (Fig. 4E).

Hypercholesteremia after repeated stress exposure

Global gene expression analysis of the liver of repeatedly stressed mice revealed stress-induced changes of the gene expression profile of lipid metabolism (Fig. 3 and Suppl. Mat. 2 and 4). Therefore, we started to analyze the lipid turnover of these mice. After repeated stress exposure a hepatic steatosis was observed (Fig. 5A) whereas, no significant numbers of lipid vesicles were detected in the liver of control mice (Fig. 4B). A Sudan III staining, which selectively stains triglycerids but not cholesterol esters, did not indicate any differences between stressed vs. non-stressed mice (data not shown). Therefore, the lipids which were accumulated in the liver were presumably not triglycerides but steroids or their precursor molecules. This is supported by the array data that showed up-regulation of genes for steroid metabolism (Cyp17a1, Cyp2b10, Cyp39a1, Cyp4a14, Por; see Suppl. Mat. 2).

Analysis of plasma lipid composition in repeatedly stressed mice showed reduced triglyceride levels (Fig. 5C) but increased total cholesterol concentrations (Fig. 5D). Among lipoproteins the HDL-fraction was increased (Fig. 5E) whereas VLDL-concentrations were strongly decreased (Fig. 5F). LDL-cholesterol levels did not change (Fig. 5G).

In contrast to the repeated stress model, we did not reveal differences in plasma lipid composition or histological alterations in the liver when comparing acutely stressed and control mice (Suppl. Mat. 1). Also the expression profiling of acutely stressed mice did not reveal major changes in genes involved in lipid metabolism, probably indicating that hepatocytes of stressed mice, started an anticipatory gene expression program during the repeated stress sessions (Suppl. Mat. 2 and 3) to face the stressful situation whose physiological impact did not become detectable until stress exposure was repeated.

Loss of essential amino acids in repeatedly stressed mice

The gene expression analysis of the liver of repeatedly stressed animals also showed altered expression profiles of genes whose products are involved in amino acid metabolism (e.g. Asl, Got1, Prodh, Slc15a4, Slc25a15, Slc3a1, Tdo see Suppl. Mat. 2 and 4). Despite the small group size of analyzed animals, the amino acid composition of fresh plasma samples revealed significantly reduced concentrations of several essential amino acids, e.g. arginine, threonine, methionine, and tryptophan, whereas non-essential amino acids showed fewer alterations in repeatedly stressed mice (Suppl. Mat. 5). Additionally, gene expression profiling of the liver of repeatedly stressed mice showed an induction of genes for amino acid transporters and amino acid metabolizing enzymes (Sds, Slc15a4, Slc25a15, Slc3a1, Got1, Tat) and provided hints for increased activation of amino acid degradation pathways (Aass, Ahcy, Asl, Prodh, Tdo2). The induction of Asl expression (Suppl. Mat. 2 B) along with a loss of arginine and citrulline in the plasma (Suppl. Mat. 5) provided hints for altered urea cycle activity. This substantiates the observations of systemic usage of the body's protein stores in repeatedly

stressed BALB/c mice. In contrast, after a single acute stress session, we found induction of mRNA expression of only a few glucogenic amino acid transporters and metabolizing enzymes in the liver (Sds, Slc38a2, Tat) which did not result in altered amino acid levels in the periphery (data not shown).

Additional expression data

Finally, we found a number of other stress-induced alterations of hepatic gene expression. Several genes that were regulated in the liver of repeatedly stressed mice provided hints for cell death (e.g. Gadd45b, Cdkn1a, Ccnd1, Tab.1, Suppl. Mat. 2, Suppl. Mat 4 A, B). These alterations are currently studied in our laboratory in more detail.

Among the genes that were selectively regulated in repeatedly stressed mice but not after a single stress exposure we found evidence for increased local steroidogenesis in the liver (e.g. Cyp17a1, Hsd17b2, Hsd3b2) and heightened bile acid production (Cyp2b10, Cyp8b1). Plasma taurine levels in these animals showed a slight tendency to be reduced (553.57 ± 91.24 nM in repeatedly stressed vs. non-stressed mice 631.66 ± 33.13 nM). The gene of the taurine transporter Slc6a6 in turn was repressed in liver tissue after a single stress session and remained down-regulated also in repeatedly stressed animals (Suppl. Mat. 2 and 3).

In addition, we found stress-induced changes in mRNA expression of genes related to detoxification of radicals, acute phase response and immune regulation. The biological relevance of these data is currently analyzed in further *in vivo* experiments.

Discussion

BALB/c mice which are highly susceptible to psychological stress recently were shown to suffer from an impaired antibacterial defence and from depression-like behavior within a period of 4.5 days of combined acoustic and restraint stress (8, 14).

Here, we show that these mice also developed a hypermetabolic syndrome resulting in severe loss of total body mass.

Our data reveal that already a single acute stress exposure caused profound changes in hepatic gene expression. Genes important for metabolic pathways regulating the carbohydrate turnover showed stress-induced alterations of mRNA expression in hepatic tissue. An acute stress response is essential for energy mobilization to "fight or flight" in a potentially harmful situation and to sustain or reconstitute allostasis (1). Inititally catecholamines activate glycogenolysis, gluconeogenesis and accelerate lipolysis which subsequently is assisted by catabolic glucocorticoid (GC)-induced pathways (4, 5, 12, 32, 35). Acute psychological stress in our model was associated with activation of the stress axes and an induction of the expression of gluconeogenic genes and of transporters for glucogenic amino acids (Pck1, G6pc, Slc37a4, Slc15a4, Slc25a15, Slc38a2, Slc3a1, Sds, Slc6a6, Tat).

When stress remained a singular event we did not find significant changes in metabolic parameters (Suppl. Fig. 1), but when stress was repeated female BALB/c mice developed severe systemic neuroendocrine and metabolic alterations. Several researchers found, that repeated acute restraint stress causes a loss of body weight in rodents (36, 37, 38) mainly mediated by initially increased energy expenditure and reduced food intake which normalized or even heightened within few days after starting repeated stress due to neuroendocrine adaptations (36, 38) Others showed prolonged lowered food intake during 5-days of repeated restraint stress due to prolonged neuroendocrine dysregulation (37). In our study, we found no changes of total food consumption during 4.5-days in repeatedly stressed animals compared with non-stressed controls which may result from an initial reduced food intake after the first stress session and increased food intake in later phase of repeated stress exposure where we additionally found manifested neuroendocrine and metabolic dysregulation. Repeatedly stressed mice suffered from an increase in metabolic rate in excess of the normal metabolic response. Such a hypermetabolic response leads to a marked increase in energy demands. Protein inappropriately becomes an energy source and increased use of protein rapidly depletes lean

body mass. A hypercatabolic response is a typical feature in infection, cancer and prolonged critical illness and goes along with fever, dysregulations of the cardiovascular system, hyperglycemia, dyslipidemia, accelerated proteolysis, tissue damage/cell death, perfusion disturbances and invasion by microorganisms (2, 3, 6, 15-18, 30, 39). In contrast, starvation is connected with diminished food intake, hyperthyroidism and reduced protein catabolism (3, 16, 40). During a hypercatabolic response as in our repeated stress model food intake is often normal (3, 16, 40) and associated with hypothyroidism (2). Recently it was shown, that prolonged sleep deprivation also can cause such a hypermetabolic response in rats (19, 20). The stress experiments in our model were performed in the recovery phase of the animals and sleep deprivation may have affected metabolic functions. Both repeatedly stressed mice and long-term sleep deprived rats showed lowered total T3 and T4 levels which did not depend on altered TSH concentrations (20). Koban et al. propose a reciprocal relationship of catecholamines, which progressively increase during sleep-deprivation and thyroid hormone concentrations that decline continuously in prolonged reduction of sleep time (20). However, in contrast to our repeatedly stressed mice which showed unaltered food intake, sleep-deprived rats were hyperphagic while body mass was massively consumed (19, 20). They did not find altered GCs levels whereas chronic psychological stress characteristically was associated with persistently high corticosterone concentrations in the plasma.

The activation of the central nervous system (CNS) can profoundly affect metabolic regulation such as shown for the thyroid hormone release (20). Target organ of metabolic regulatory pathways is predominantly the liver (1, 4, 41). The activation of the HPA axis with increased GC levels can stimulate food intake and activate carbohydrate, fat and protein catabolism (1, 4, 41). In turn, the brain receives signals such as actual glucose and lipid concentrations or increased energy demand (1, 5, 42). In consequence, CNS activation induces regulatory pathways that equilibrate metabolism to supply the needed energy e.g. increasing glucose formation in the liver (1, 4, 42).

Hypercortisolism was shown to shift metabolic functions towards carbohydrate, lipid and protein catabolism (23-25, 37-43) to sustain energy supply by replenishing glucose which is the main energy source of the body. Glucose can be released after glycogenolysis or induction of gluconeogenesis when supply with food is insufficient. Primarily alanine and glutamate are precursor molecules for gluconeogenesis (23-25, 43-46). Hepatic induction of alanine aminotransferase 2 (Gpt2) and of glutamate oxaloacetate transaminase 1 (Got1) may supply the metabolites for glucose synthesis (23-25, 44). Thereafter, alanine and glutamate can be reconstituted by biotransformation whereas essential amino acids cannot be replenished by biosynthesis in repeatedly stressed mice (45,46). Deamination of amino acids results in the production of ammonia which is detoxified in the liver by the urea cycle (43, 46, 47). Increased expression of the urea cycle enzyme argininosuccinate lyase (Asl) in the liver of repeatedly stressed mice along with usage of arginine and citrulline as intermediate products of the urea cycle indicates heightened stress-induced ammonia detoxification to provide C-bodies of amino acids for metabolic pathways such as gluconeogenesis. Lactate, which alternatively can serve as substrate for hepatic gluconeogenesis, was shown to be produced in high amounts in peripheral tissues during hypermetabolism such as during sepsis and can cause lactic acidosis (6, 23, 25, 28, 48, 49). Acidosis which was detectable in repeatedly stressed mice is often paralleled with insulin resistance and hyperglycemia (2, 17, 48). In fact, in repeatedly stressed mice we found increased concentrations of the adipokine resistin which is inducible by GCs, prolactin and growth hormone and has been identified to lower insulin sensitivity (45). Prolonged hyperglycemia especially in critical ill patients increases the risk of infectious complications, neuronal damage and multiorgan dysfunction syndrome (17, 18, 36, 39, 49). In line with this, repeatedly stressed mice suffered from a reduced antimicrobial response (8, 14). Lam et al. showed that increased glucose sensing by the brain and elevated intracerebral concentrations of lactate

reduced the hepatic secretion of VLDL-cholesterol and caused a decline of plasma triglyceride levels in rodents (42). Also Ricart-Jané et al. found that repeated restaint stress caused loss of plasmatic triacylglycerols with low VLDL levels which was accompanied by a reduced food intake (37) Heightened triglyceride clearance, and increased lipolysis to assemble C3-bodies for gluconeogenesis can result in essential fatty acid deficiency (50-52).

In our stress model, such hypotriglyceremia went along with hypercholesteremia and up-regulation of GC-sensitive cytochrome genes in the liver (51-53). Cyp4a enzymes are involved in removal of fatty acids and can counter-regulate hepatic steatosis which we found to be a typical consequence of repeated stress exposure in mice. Increased expression of Cyp17a1 gives hints for hepatic induction of steroidogenesis (52) in repeatedly stressed mice, and up-regulation of expression of Cyp39a1 and Cyp2b10 indicate increased removal of steroids by bile acid production (53). Importantly, the cholesterol metabolism of rodents and of humans is difficult to compare. In mice HDL is the main lipoprotein present in the blood and essentially required for steroidogenesis (54, 37), whereas humans use LDL for steroid synthesis and have lower HDL concentrations (54, 55). Furthermore, HDL is able to scavenge endotoxins from the plasma (56). The relevance of stress-induced elevation of plasma HDL levels in mice e.g. for steroid synthesis or for scavenging the bacterial compound lipopolysaccharide (LPS) remains to be elucidated. Other authors found that LPS or TNF challenges particularly increase the catabolic rate (57, 58). Such effects seem to be mediated via TNF-mediated neuroendocrine stimulation e.g. activation of the sympathetic nervous system that primarily causes glucose formation (4, 5, 12, 32, 35, 58-61). In addition, release of catecholamines potentially induces the expression of the leptin gene in adipose and hepatic tissue (61-63). Leptin then afferently can signal to the brain to sustain inhibitory effects on food intake. In this relation, one should expect reduced food consumption in the hyperleptinemic repeatedly stressed mice. However, we did not find altered food intake whereas total body mass furthermore was lost. One possible explanation for the drastic loss of body weight along with hyperleptinemia is that leptin potentially can increase the energy expenditure such as enhancing the activation of the respiratory chain and therefore can increase energy consumption (64, 65). In this study, we showed that already acute stress can induce drastic changes of hepatic gene expression which did not significantly disrupt allostatic regulation of metabolism in mice. In turn, repeated psychological stress in BALB/c mice along with systemic immunodeficiency that was reported previously (8, 66), induced a hypermetabolic stress syndrome.

It is not clear why some individuals during prolonged stressful situations lose weight while others gain body mass (2, 3, 7, 10, 15-24). Since there is an increased number of patients suffering from metabolic syndrome and clinical relevant associated illness, many publications show that chronic stress is promoting the development of a metabolic syndrome which is associated with gain of fat mass (obesity), type II diabetes, hyperlipidemia and hypertension (3, 5, 7). In contrast, in our animal experiments with BALB/c mice as a mouse strain with high stress susceptibility (8), we found metabolically driven wasting because of a hypercatabolic stress response. We assume that the genetic predisposition influence the development of either stress-induced metabolic syndrome or loss of body weight phenotype. Moreover, it is shown that beside genetic predisposition environmental factors influence prenatal and postnatal neuronal and neuroendocrine differentiation resulting in different coping styles in the adult (67). They showed that proactive/aggressive animals develop stress-induced hypertension, cardiac arrhythmias and inflammation whereas reactive/passive individuals are more susceptible to anxiety disorders, metabolic syndrome, depression and infection (67). However, the neurobiology and endocrine regulation of these different coping styles is not well understood, yet. A loss of biological reserves as in our model of repeated stress exposure is as fatal as the development of a metabolic syndrome because of losing the ability to fight infection and cancer (3, 16, 21-24).

In the clinical setting it is now clear that catabolic response will become autodestructive

if not contained. The severity of complications will occur in proportion of lost body protein. In our model, particularly arginine deficiency became evident. Interestingly, alimentation with arginine and omega-3-fatty acids-enriched enteral feeds decreased hospital days and infectious complications in critically ill patients (68) which commonly show a loss of about 10% of lean body mass (63-65). Healthy adults require about 0.8 g protein/kg BW/d to maintain homeostasis. Stressful events such as traumatic injury or infection increase the body's protein requirement up to 1.5-2 g protein/kg BW/d or even more. However, humans cannot metabolize more than 2 g/kg BW/d. This often results in a fatal negative nitrogen balance in severely ill patients (69). Finally, amplified protein breakdown with a loss of more than 40% of lean body mass leads to irreversible cell damage (68-71).

Here, we show that highly demanding psychological stress in the absence of injury or infection is able to induce a severe hypermetabolic syndrome in mice. Such overwhelming wasting condition can reduce the individual's resistance to further stressful stimuli, such as injury or infection.

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Table 1. Real-time PCR validation of array data in repeated stress experiments.

Target gene	control group[1] △Ct (target-reference Actb)	repeated stress group[1] △Ct (target-reference Actb)	p-value[2]
Asl	2.90 ± 0.33	1.50 ± 0.23	< 0.0001
Gadd45b	9.66 ± 0.64	6.83 ± 1.55	< 0.0001
Pck1	2.53 ± 0.52	0.02 ±0.72	< 0.0001
Sds	5.66 ± 0.41	3.70 ± 0.64	< 0.0001
Srebf1	8.01 ±0.18	8.36 ± 0.13	< 0.0001

[1]Validation of expression data by real-time PCR was carried out for all individual RNA preparations of the two biological experiments (n=9 plus n=8 mice/group) of the two experiments focusing on effects of repeated stress exposures. [2]Differences of △Ct values were analyzed by Mann-Whitney test.

Fig. 1: Repeated stress-induced activation of the HPA axis in BALB/c mice. A. Increased plasma corticosterone levels in repeatedly stressed mice (black box plot) compared with non-stressed mice (white box plot) n=9 mice/group. B, C. Hypertrophy of the zona fasciculata of the adrenal cortex (marked by white line) in repeatedly stressed mice (B) compared with non-stressed controls (C); HE staining (x100); each picture representative for n=9 mice/group. **p<0.05 Mann-Whitney U test; data reproduced in at least 3 independent experiments*

Fig. 2: Repeated psychological stress-induced loss of body weight, increase of plasma leptin levels, and hypothyreoidism in mice. A. Loss of body mass during the period of 5 days intermittend stress (black box plots) compared with non-stressed control mice (white box plots), n=12 mice/group. B. Plasma leptin levels after nine stress cycles compared with non-stressed mice, n=12 mice/group. C, D. Triiodthyronine, T3 (C) and thyroxine, T4 (D) concentrations in the plasma of repeatedly stressed and control mice, n=12 mice/group. **p<0.05; **p<0.01 Mann-Whitney U test; data representative for 2 independent experiments*

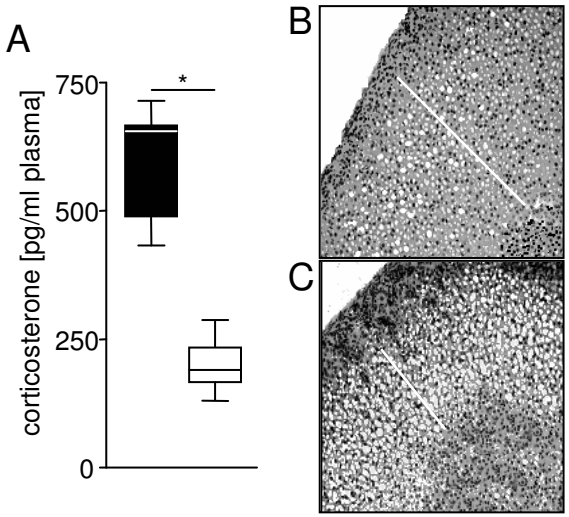
Fig. 3: Impact of acute and repeated stress on metabolic genes and genes associated with metabolic disease. A. Graphical display of genes differentially expressed after acute or repeated stress or both stress types. The numbers given in the Venn-diagram include cDNAs, that are not functionally annotated: 29 of 221 genes regulated specifically in acute stress, 9 of 94 genes regulated in both acute and repeated stress, and 2 of 76 genes regulated specifically for repeated stress. B. Impact of acute and repeated stress on metabolic functions. The lists of genes differentially expressed either only after acute stress and repeated stress or after both acute and repeated stress were loaded into Ingenuity Pathway Analysis version 5.5 (Ingenuity Systems, www.ingenuity.com) in order to interpret the affected genes within the context of the published literature. Metabolism seemed to be a major target of gene regulation after repeated stress exposure and thus the influence of acute and repeated stress on metabolism associated genes was analyzed. The impact of acute or repeated stress alone as well as both stress types is displayed by showing the rankings within the list of statistically significantly over-represented functional groups for genes related to metabolic disease, amino acid metabolism and lipid metabolism. *For complete listing of top functions that are regulated see Suppl. Mat. 4*

Fig. 4: Disturbances of murine carbohydrate metabolism after repeated psychological stress. Kinetics of blood glucose levels immediately after termination of the 9th stress cycle (black box plots) compared with controls (white box plot); n=9 mice/group. B,C. Reduced storage of carbohydrates in the liver of repeatedly stressed mice (B) compared with non-stressed mice (C); PAS staining (x200); each picture is representative for n=9 mice/group. D. plasma resistin levels, and E. pH of EDTA-plasma of stressed and non-stressed mice; n=12 mice/group. **p<0.05 Mann-Whitney U test; data representative for 2 independent experiments*

Fig. 5: Disturbances of fat metabolism in repeatedly stressed mice. A, B. Hepatic steatosis in repeatedly stressed mice (A) compared with non-stressed mice (B); HE staining (x20); each picture representative for n=9 mice/group. C-G. Plasma fat composition of repeatedly stressed mice (black box plots) and non-stressed controls (white box plots): triglyceride levels (C), total cholesterol (D), HDL-cholesterol (E), VLDL-cholesterol (F) and LDL-cholesterol (G) were measured immediately after the 9th stress session, n=12 mice/ group;**p<0.01, ***p <0.001 Mann-Whitney U test*

Suppl. Mat. 1: Acute stress-induced alterations in BALB/c mice. A. Increased plasma corticosterone levels in acutely stressed mice (grey box plot) compared with non-stressed mice (white box plot) n=9 mice/group. B. Plasma leptin levels after a single stress session compared with non-stressed mice, n=9 mice/group. C. Plasma glucose concentrations immediately after acute stress exposure compared with controls ; n=6 mice/group. D. No histologically detectable alterations in the liver of stressed and non-stressed mice. HE-staining, each picture representative for n=9 mice/group. ***p<0.01 Mann-Whitney U test, n=9 mice/ group; data representative for at least 2 independent experiments*

Suppl. Mat. 4: Impact of acute and repeated stress on metabolic genes and genes associated with metabolic disease. A. Graphical display of genes differentially expressed after acute or repeated stress or both stress types. The numbers given in the Venn-diagram include cDNAs, that are not functionally annotated: 29 of 221 genes regulated specifically in acute stress, 9 of 94 genes regulated in both acute and repeated stress, and 2 of 76 genes regulated specifically for repeated stress. B. List of top functions that are regulated in the liver selectively after acute stress, after both acute and repeated stress and selectively after repeated stress exposure. The lists of genes differentially expressed either only after acute stress and repeated stress or after both acute and repeated stress were loaded into Ingenuity Pathway Analysis version 5.5 (Ingenuity Systems, www.ingenuity.com) in order to interpret the affected genes within the context of the published literature. C. Impact of acute and repeated stress on metabolic functions. Metabolism seemed to be a major target of gene regulation after repeated stress exposure and thus the influence of acute and repeated stress on metabolism associated genes was analyzed. The impact of acute or repeated stress alone as well as both stress types is displayed by showing the rankings within the list of statistically significantly over-represented functional groups for genes related to metabolic disease, amino acid metabolism and lipid metabolism.


A
B
C
corticosterone [pg/ml plasma]
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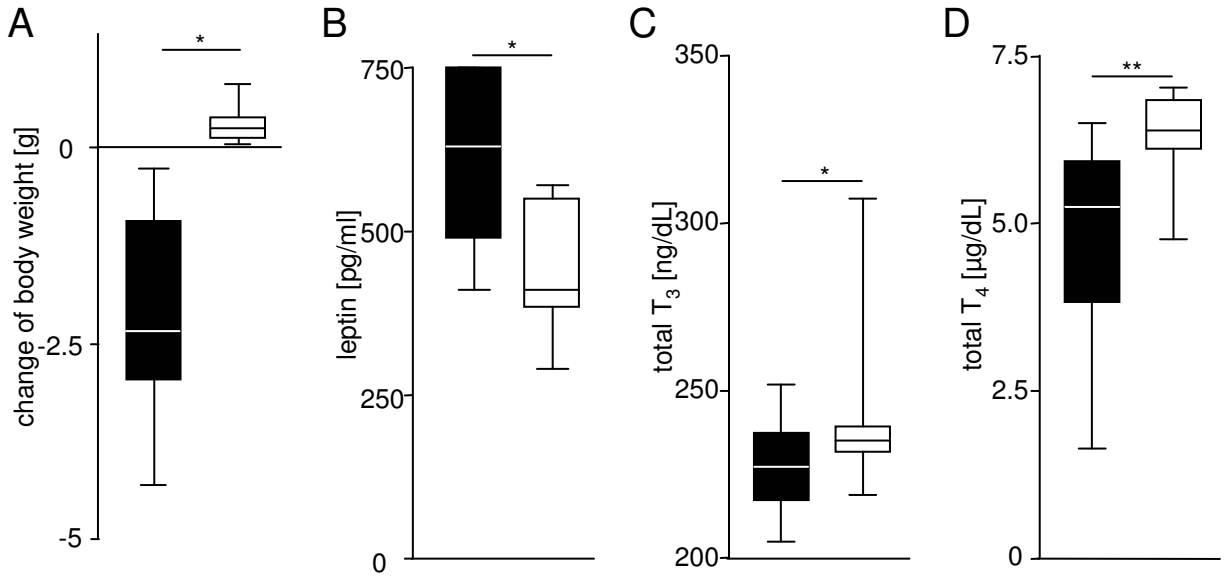
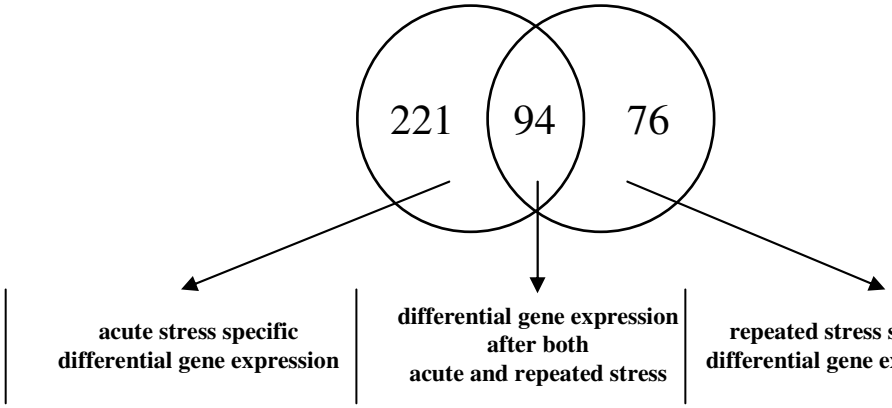
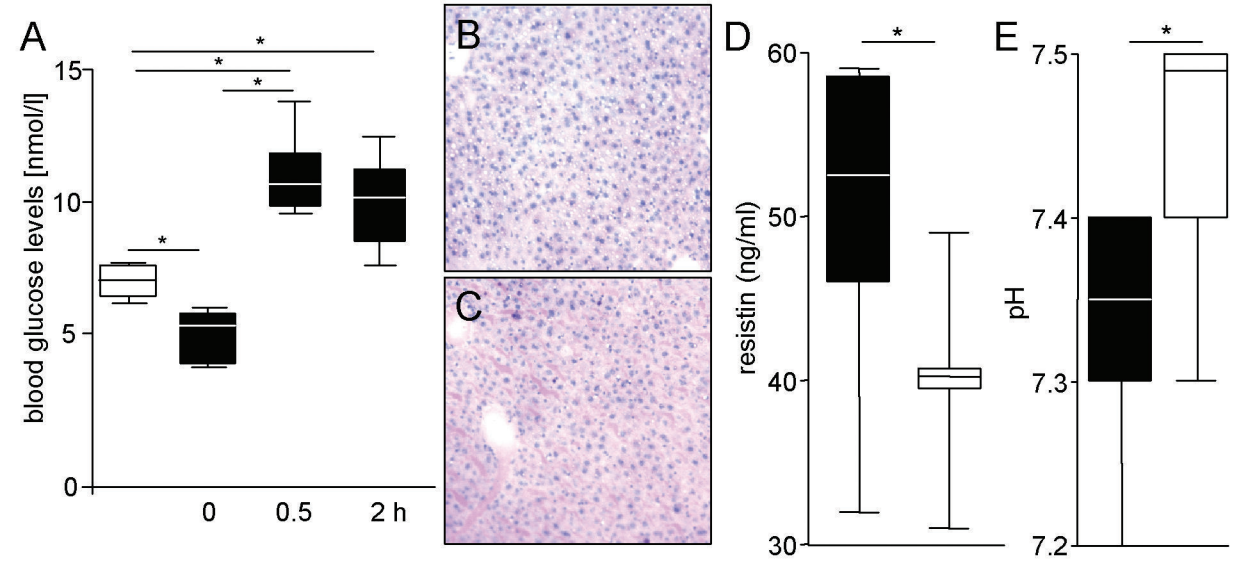

A
B
C
D
change of body weight [g]
0
-2.5
-5
*
leptin [pg/ml]
750
500
250
0
*
total T_3 [ng/dL]
350
300
250
200
*
total T_4 [µg/dL]
7.5
5.0
2.5
0
**

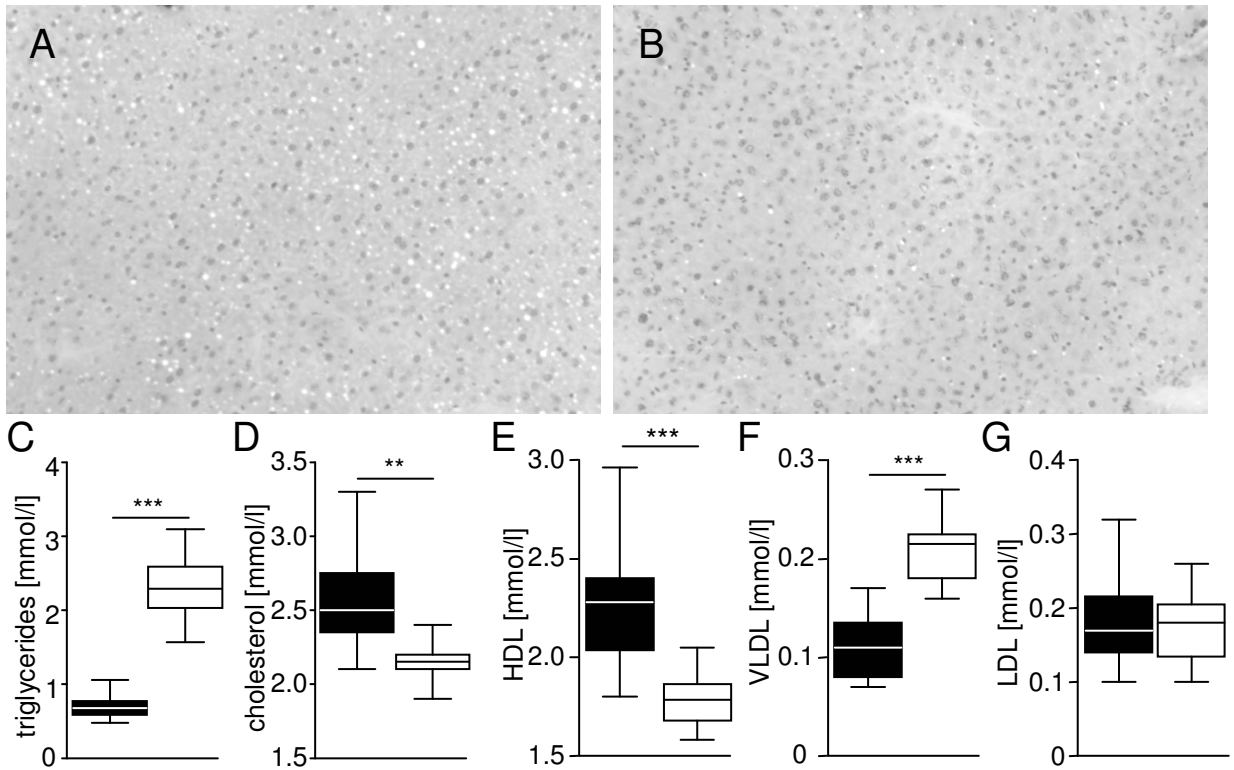
A. Numbers of differentially expressed genes after acute and chronic stress or in both models



B. Selected over-represented metabolic functions in the lists of differentially expressed genes

Function	Rank	Rank	Rank
Metabolic Disease	26	6	1
Amino Acid Metabolism	24	62	6
Lipid Metabolism	16	16	5


A
B
C
D
E
blood glucose levels [nmol/l]
15
10
5
0
0
0.5
2 h
*
*
*
*
*
*
resistin (ng/ml)
60
50
40
30
pH
7.5
7.4
7.3
7.2


A
B
C
triglycerides [mmol/l]
***
D
cholesterol [mmol/l]
**
E
HDL [mmol/l]
***
F
VLDL [mmol/l]
***
G
LDL [mmol/l]