STRESS-INDUCED CHANGES IN THE METABOLISM, GUT MICROBIOME AND BEHAVIOR

PhD thesis

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Introduction

External and internal challenges trigger physiological and behavioral responses, which aim to reinstate homeostasis and adaptation. Stress response is a centrally integrated, complex recruitment of neuroendocrine, autonomic, metabolic and behavioral regulatory circuits, including activation of the sympathetic nervous system and the hypothalamo-pituitary-adrenocortical (HPA) axis. Stress coping is effective, if the response is rapidly activated when necessary, and terminated when it is no longer needed. Inadequate, excessive and prolonged stress responses may lead to stress-related disorders (metabolic, immunological diseases and stress-related brain disorders).

Stressors can be divided into four main categories: physical; psychological; social; physiological. In terms of duration, stressors can be classified into two main categories: acute or chronic exposure. In the stress response, fast-acting agents (such as catecholamines, neuropeptides) and slower, gene-mediated corticosteroid effects contribute to an adequate response to the stressor, which leads to enhanced vigilance, focused attention, and in general, increase gluconeogenesis, glycogenolysis, proteolysis or lipolysis providing energy supply to "crucial" organs. Corticotropin-releasing hormone (CRH), which is produced in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) is essential for initiating the neuroendocrine stress response and regulating the behavioral and metabolic responses to stress. Several afferent inputs trigger CRH neurons in the PVN, including ascending brainstem pathways that convey visceral and sensory stimuli, and a complex limbic pathway, which is activated by psychological stressors. Metabolic- and nutrient-related information is mediated through either local hypothalamic inputs originating in the arcuate nucleus or ascending medullary afferents.

The arcuate nucleus of the hypothalamus contains two major population of neurons that control energy balance; the orexigenic neurons releasing neuropeptide Y and agouti-related protein (NPY/Agrp) and anorexigenic neurons producing pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART). These neurons express receptors for detecting energy-related nutrients (glucose and FFA), hormones (leptin, insulin and ghrelin) and equipped with projections ascending to second-order neurons in the hypothalamic regions such as PVN.

The microbiome is a complex and dynamic mixture of microorganisms. These communities interact with host and can influence health and disease. The composition of gut

microbiome is shaped by the host and can constantly change by different factors. These factors include way of birth, breast-feeding, diet, stress, aging, drugs (antibiotics) etc. The bidirectional crosstalk between host and microbiome occurs through direct and indirect ways. Gut microbiome produces different metabolites like SCFAs, 5-HT precursor and various other neurotransmitters acting locally in the enteric nervous system or systemically. Furthermore, microbiome display indirect actions through vagus nerve activity, via cytokine production.

Normally, a harmonic interaction is maintained, which is beneficial for both side. However, a gut microbial shift can result dysbiosis in microbiome, which can induce the decline of gut barrier. Therefore, gut permeability increases and thus, different pathogen bacteria can penetrate or bigger molecules can enter into the blood circulation such as cell wall of Gram + derived LPS, which can trigger TLR-4 derived inflammatory pathway. Different studies demonstrated stress triggers dysbiosis and results in "leaky gut" syndrome. Gut dysbiosis has been found in association with neurological/psychiatric diseases, such as autism, anxiety, depression, schizophrenia and neurodegenerative disorders Alzheimer's and Parkinson's diseases.

Aims

My **first** aim was to investigate the effect stress on the metabolic system. Therefore, I raised the following specific questions:

- How an acute restraint stress affects metabolic variables and locomotor behavior?
- What is the role of the hypothalamic paraventricular nucleus in the regulation of metabolic- and behavioral changes?
- What are the differences between metabolic changes seen in response to acute and chronic stress?

How the metabolic system recovers after chronic stress?

My **second** aim was to reveal chronic stress-induced changes in mouse gut microbiome and to test the hypothesis, whether rifaximin – non-absorbable antibiotic restores chronic stress-induced gastrointestinal and inflammatory symptoms and changes in microbiome along with stress-induced changes in anxiety-like behaviour.

Third, we challenged the hypothesis if there is a relation between the systemic antibiotic consumption, gut dysbiosis and the prevalence of Parkinson's disease.

Methods

Animals

Experiments were performed on male mice with C57BL/6J genetic background. CRH-Ires-Cre transgenic mouse line was used in chemogenetic experiments.

***Stereotaxic surgery*:**

CRH-IRES-Cre transgenic mice were anesthetized and bilateral stereotaxic injection of a virus vector construct pAAV8/hSyn-DIO-hM3D(Gq)-mCherry was performed into both side of paraventricular nucleus (PVN). After surgery, mice recovered for 3 weeks. Control animals have been injected with pAAV-hSyn-DIO-mCherry.

Stress procedures:

- *Acute psychogenic stress - Restraint*

Restraint stress was performed using transparent ventilated Falcon tubes fitted to the size of the animals. This procedure minimized the space around the animal, prevented them from turning and provided stressful stimulus, without being harmful.

- *Chronic variable stress (CVS) paradigm*

During CVS, experimental animals were stressed for 4 weeks, two times daily, by different psychogenic stressors (social defeat, water avoidance, light/dark changes, forced swimming, soaked bedding, slanted cages, isolation, crowding, shaking, restraint, foot shock, rat feces odor). These stressors were randomly used throughout the procedure.

- *Two-hits stress protocol*

Two-hits stress protocol is a combination of early life adversity (maternal separation, MS) –first hit- followed by chronic variable stress (CVS) paradigm in the adulthood –second hit. During maternal separation, pups were removed from their dams for 3 h daily, in

postnatal days 1–14. Unseparated control (Control) litters were left undisturbed, except for change of bedding once a week.

General procedure in rifaximin experiment

During chronic variable stress, half of the animals received 300 mg/kg bw/day rifaximin, a non-absorbable antibiotic. Rifaximin was dissolved in 5% hypromellose solution in drinking water. The other half of mice (controls) received 5% hypromellose to drink. Fluid intake and body weight of the animals was monitored and rifaximin concentration in the drinking water was adjusted.

Metabolic measurements

Mice were singly housed in TSE Phenomaster metabolic cages and metabolic parameters were measured by indirect calorimetry. Body composition (lean mass, fat mass) was determined by MRI.

Behavior tests

Ethogram, open field test, elevated plus maze test, and sucrose consumption test were performed. Behavioral tests were video recorded and analyzed later with Solomon coder computer based event recorder software or with EthoVision XT video tracking software. Locomotor behavior was automatically detected in TSE Phenomaster system, during metabolic measurements.

Gut permeability test in vivo

After overnight of fasting, animals received FITC-labeled 4kDa Dextran via oral gavage. 2 hours later, blood was collected from heart, centrifuged and serum was collected. Serum samples were diluted with an equal volume of PBS and FITC concentration was measured from 100 µl of diluted serum at excitation 485nm and emission 535nm wavelength using Cytation 5 Cell Imaging Multimode reader.

Epidemiological data collection

Antibiotic consumption data, collected between 1997-2009 by the ESAC project (European Surveillance of Antibiotic Consumption network) and data from the ECDC (European Centre for Disease Prevention and Control) database (2010-2017) were used.

Correlation was calculated between antimicrobial consumption data and changes in PD prevalence.

Plasma analyses:

- To determine plasma endotoxin levels, commercially available limulus amebocyte lysate (LAL) assay was used in accordance of the manufacturer's instructions.
- Plasma glucose level was determined by Glucose Colorimetric Detection Kit from plasma according to the manufacturer's protocol.
- Plasma triglyceride (TG) level was measured by multiparameter diagnostic device for triglycerides
- Plasma adrenocorticotrophin hormone (ACTH) and corticosterone concentrations were measured by *radioimmunoassay* (RIA).

Microbiome analysis

Microbiome analysis was performed quantitative real-time PCR.using taxon- and species-specific primers corresponding to sequences found in the bacterial 16S ribosomal RNA genes.

Gene expression analysis

Gene expression analysis were performed by quantitative real-time PCR.

Histological analyses

Histological analyses were performed on free-floating brain sections and paraffine-embedded colon sections. On brain sections, c-Fos fluorescent *immunohistochemistry* (IHC) was performed and on colon sections, H&E and macrophage marker (F4/80) IHC were done, which was visualized by FITC. Images were analysed by Image J software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (ver. 6.01; San Diego, CA, USA) using one way or two-way ANOVA with the appropriate post hoc test. Repeated measurement was used in the self-control experiments, time and treatment being the repeated measure. To determine the significant differences between the two group means, unpaired, two sided t-test was performed.

Results

Effects of acute psychological stress on locomotor behaviour and metabolic variables

Stressed mice displayed increased locomotor activity in the first hours after acute stress, it remained elevated in the following three hours, however, average of the first four hours were not significantly changed. The average locomotor activity was not changed when the in the entire light phase was analyzed. Nevertheless, locomotor activity of stressed animals was significantly decreased in dark phase compared to the basal measurements. Stressed mice consumed more food in the first four hours after acute stress. After the third hour, significant difference was noticed between basal measurement and the measurements after acute stress. However, this difference was equalized later and the cumulative food intake was not changed neither in the entire light phase nor in the entire dark phase. Energy expenditure (EE) was higher during the first four hours after acute stress and it was also significantly elevated in the entire light phase compared to the basal measurement. However, there was no difference in the dark phase. Respiratory exchange ratio (RER) did not change to acute stress. Body weight and fat mass were decreased 24 hours later of acute stress.

Effect of CRH^{PVN} activation on locomotor behavior and metabolism

Locomotor activity did not change after activation of CRH^{PVN} neurons. The average of cumulative food intake was significantly higher in the first four hours after CNO injection. There was no difference in cumulative food intake in the entire light phase, however, it was significantly decreased in the dark phase. After CNO injection, average of energy expenditure was significantly higher in the first four hours. After the fourth hour, there was no difference in EE between saline and CNO treatment. There was no difference in the respiratory exchange ratio during 23 hours of metabolic measurements after CNO injection compared to RER values after saline injection. Body weight and fat mass were significantly reduced and lean mass did not change at the end of measurements.

Effects of chronic variable stress on metabolism

The average of locomotor activity of chronically stressed mice was significantly higher in the light phase. In the dark phase, locomotor activity was not changed significantly. Food consumption of the stressed mice was higher after CVS procedure but it was elevated significantly only during the active phase. The cumulative food intake remained elevated

during recovery compared to the control mice, however, the difference was not significant. Energy expenditure of chronically stressed mice was continuously higher during the three days of metabolic measurements compared to control mice. However, during recovery, the average energy expenditure was decreased significantly in the active phase. During metabolic measurements, respiratory exchange ratio was continuously higher in the stressed mice after CVS procedure; however, RER of the stressed mice was dropped after two days to the control level only in the light phase. Fat mass was significantly lower and lean mass was higher in chronically stressed animals. During recovery, fat and lean masses were restored to the control level and body weight significantly increased.

Effect of chronic stress on gut microbiome and its restoration after rifaximin treatment

Exposure to "two hits" chronic stress paradigm resulted in significant reduction of DNA in the colon content indicating reduction of total colonic bacteria. Colon microbiome diversity has changed with increased abundance in the phylum of *Bacteriodetes* and *Proteobacteria*.. Chronic stress resulted in increased abundance of *Clostridium* in response to stress. Rifaximin treatment restored the abundance of *Proteobacteria* and decreased *Clostridium sp.* in the chronically stressed mice.

Effect of rifaximin on chronic stress-induced changes on behaviour

To examine the effects of chronic stress and rifaximin on mouse behavior, we observed and quantified four distinct elements: walking, surveying, rearing and grooming during 5 min in novel environment. Compared to no-stress controls, all mice, which have been previously exposed to MS + CVS displayed exaggerated locomotor behavior. The frequencies of all four selected behavioral elements were increased significantly in stressed mice. Similarly total duration of walking, rearing and grooming were increased significantly in stressed mice, however surveying decreased. Increased locomotion was detected during the open field test in MS + CVS mice. In addition, stressed mice spent less time in the center and their first latency to border was shorter than those of controls. In elevated plus maze test, preference for the open arms was significantly lower in case of stressed mice than controls. MS + CVS resulted in a significant decrease of sucrose consumption in stressed mice. In each behavior tests rifaximin treatment had no effect.

Effect of MS+CVS and rifaximin treatment on the gut and gut-related immunity

MS + CVS significantly reduced the thickness of colonic mucosa compared to control. Mucosa thickness was not different in rifaximin-treated groups. Colonic mRNA expression of tight junction proteins, occludin and tight junction proteins TJP1 and TJP2 was increased in both rifaximin treated groups, but remained unchanged in response to stress. Expression of Reg3b, a C-type lectin with antimicrobial activity, was significantly elevated in chronically stressed mice. Rifaximin treatment restored Reg3b mRNA level to that of the controls. We tested gut permeability in vivo, using FITC-labeled 4 kDa dextran. Increased gut permeability has been revealed in chronically stressed mice, while rifaximin treatment restored the normal, non-stressed. Quantitative histological analysis of F4/80 positive (+) areas revealed significant rifaximin effect in the submucosa. In the lamina propria of MS-CVS animals, the area covered by F4/80+ profiles were significantly increased in stressed mice, which was reduced to that of the controls in response to rifaximin treatment. To assess bacterial translocation in chronically stressed mice, we PCR amplified bacterial DNA from mesenteric lymph nodes. Compared to non-stressed controls, chronic stress resulted in 50% elevation of bacterial load in the MLN. Rifaximin administration interfered with stress-induced increase of bacterial load.Plasma LPS levels were measured. Higher LPS level was detected in plasma of stressed mice than in the controls but the increase of plasma LPS was not detected in antibiotic treated mice.

Hypothesis: correlation between Parkinson's disease prevalence, consumption of certain antibiotics and gut microbial dysbiosis

Systemic antibiotics significantly affect the microbiome resulting chronic dysbiosis in the gut, which may contribute to pathogenesis of neurological diseases. For instance, Parkinson's disease (PD) is often associated with gastrointestinal symptoms. Gut dysbiosis in PD favors curli-producing *Enterobacteria*. Curli is a bacterial α-synuclein (αSyn) which is deposited first in the enteric nervous system and amyloid deposits are propagated in a prion like manner to the central nervous system. We tested the hypothesis whether consumption of different groups of antibiotics is associated with the change of Parkinson's disease (PD) prevalence in different European countries. Significant positive correlation was found between the

consumption of narrow spectrum + penicillinase (β-lactamase) resistant penicillin and the increased prevalence of PD.

Conclusions

1. Acut psychogenic stress has profound effect on the metabolic parameters, energy expenditure and food intake as well as on locomotor activity.
2. The hypothalamic paraventruclar nucleus is a part of a neuronal circuit, which mediates acute stress effects on metabolism and activity.
3. Chronic stress results in lasting changes of body composition and metabolism, with increased food intake, energy expenditure and respiratory exchange ratio. These chronic stress-induced alterations recover differently after cessation of the challenge.
4. Chronic stress results in gut dysbiosis, increased gut permeability and recruitment of activated macrophages in the colonic mucosa.
5. Rifaximin -a gut specific antibiotic- restores stress-induced changes in gut microbiome, gut permeability and bacterial load, however these positive changes in the gut are not accompanied by restoration of normal behavior.
6. Gut dysbiosis following systemic antibiotic treatment might be a risk factor in the development certain neurodegenerative disorders, such as Parkinson's disease.

Treatment	Locomotor behavior	Food intake	Energy expenditure	RER	Body-weight	Fat mass	Lean mass
Acute stress	↑	↑	↑	-	↓	↓	-
Potentiation of CRH^{PVN} neurons	-	↑	↑	-	↓	↓	-
Chronic stress	↑	↑	↑	↑	-	↓	↑

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