GENE EXPRESSION AFTER METABOLIC STRESS AND ANTIPSYCHOTIC TREATMENT IN HUMAN CELL CULTURES

Summary of PhD Thesis

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INTRODUCTION

Diathesis–stress in dermal fibroblasts

Mental disorders are exclusively human conditions and cannot be studied directly in the dysfunctioning organ; thus, modeling them raises several problems and necessitates compromises. Patient derived cellular functional assays enable a system-oriented view, in which mental disorders are the manifestation of gene * environment and gene * gene interactions.

In 2012, major depression (MD) was the leading cause of disability worldwide affecting almost every 5th person in the population. According to the polygenic and diathesis–stress aspects of MD, the constellation of certain genetic factors results in increased vulnerability against environmental stressors, maladaptation and the development or remission of clinical symptoms. MD is a complex metabolic–immune disorder. Stress hormones, vasopressin, enteroendocrine factors, cytokins, and immune functions show significant alterations in MD patients. These suggest that the genetic load manifests on a cellular level after stress in the central nervous system and periphery also.

MicroRNAs ensure quick, accurate, coordinated gene expression modulation which is essential in stress response. These small, single-stranded, non-coding RNAs are implicated in multiple processes associated with cell cycle, apoptosis, differentiation, regeneration, metabolic adaptation and synaptic plasticity. Postmortem and animal studies revealed microRNA alterations in the brain, blood and cerebrospinal fluid (CSF) in MD, anxiety, bipolar disorder, and schizophrenia. Furthermore, microRNAs are involved in the gene expression and behavior modulatory effects of stress, psychotropic and antidepressant drugs.

In the recent 5 years, there has been growing literature on human dermal fibroblasts (HDFs) as potential model systems or biomarker resources in psychiatric disorders. They are relatively easy to obtain, maintain and propagate. The cultures are homogenous, free of pre-sampling *in vivo* effects, and retain genetic stability for 15-20 passages. Furthermore, the gene expression, receptor profile and signaling pathways of fibroblasts are similar to cells of neuro-ectodermal origin. They were used to investigate disease-related metabolism, redox homeostasis, membrane transport, apoptotic susceptibility, circadian rhythm or pharmacology in affective, neurodevelopmental and neurodegenerative disorders.

Psychopharmacological assay with iPSC-derived neurons

Since Yamanaka and Takahashi showed that human postmitotic somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs), iPSC-research is one of the most prosperous fields of bioscience with exceptional possibilities in *in vitro* disease modeling, regenerative medicine and drug development. The differentiation of iPSCs is thought to follow *in vivo* spatio-temporal developmental pathways, and a combination of growth factors/small molecules is sufficient to evoke high neurotransmitter specificity: GABAergic cortical interneurons, dopaminergic midbrain neurons, or glutamatergic cortical neurons.

In 2014, Yu et al. produced hippocampal glutamatergic granule neurons from iPSCs using key developmental cues specific to the dentate gyrus identity and opened the doors to model adult CNS neurogenesis *in vitro*. The neurogenesis in the hippocampal subgranular zone continues lifelong: new, immature neurons migrate into the inner layers, differentiate into glutamatergic dentate granule cells, and integrate into the neural circuitries. Adult neurogenesis, modulated by various intrinsic and extrinsic stimuli from neural, glial and somatic cells, plays inevitable role in learning and memory what makes it so ambitious subject of *in vitro* researches. Dysfunctional neurogenic patterns are indicated in several psychiatric disorders (recently reviewed by Apple et al., 2016). For instance, the neurogenic rate is reduced in stress, MD or schizophrenia which might be reversed by antidepressant and atypical antipsychotic (AAP) medication.

Study objectives

We aimed to study the diathesis−stress model of MD in patient and control-derived HDFs. We addressed to answer the following questions: (1) How metabolic stress alters the mRNA and microRNA expression of HDFs? (2) Are there any differences between the transcriptome of fibroblasts gained from MD and control subjects? (3) Are there any differences in the stress response of MD and control HDFs?

In Study 2, we aimed to investigate how various antipsychotics (APs) affect the gene expression of differentiating and maturing hippocampal granule cells *in vitro*. Thus, we set up an *in vitro* pharmacological assay using neural cells originated from human iPSCs and treating them with typical AP haloperidol (HL) or with atypical olanzapine (OL) and risperidone (RP) at two different concentrations.

MATERIALS and METHODS

Fibroblast cultures and metabolic stress treatment

Skin biopsy was obtained from 16 patients with MD (12 females and 4 males) and 16 healthy control subjects (CNT) matched in age, race and gender. The diagnosis of current MD episode was established according to the DSM-IV-TR. The Vanderbilt University Institutional Review Board (IRB, Nashville, TN, USA) approved the study and written informed consent was obtained from all study participants before any procedures were conducted.

Primary fibroblast cultures, obtained from the skin biopsies, were grown in standard conditions, the medium was changed 3 times per week. The samples were subcultured for 5-10 passages to minimize *in vivo* effects. After 2-3 weeks, three plates were initiated from each single cell line (1.2 x 10^6 cells per plate), and the medium was changed with two different metabolic stress conditions: (1) glucose deprived, galactose-containing (GAL); (2) reduced lipid (RL); and (3) standard (STD) medium. All cultures were grown for seven days; the medium was changed 3 times per week. Cell growth and proliferation were not affected by GAL or RL treatment. After one week the cells were washed with ice-cold PBS twice, trypsinized, pelleted and stored at -80 °C until RNA isolation.

mRNA and microRNA level determination, pathway analysis

Total cell RNA and small RNA fractions were isolated from the frozen samples using the mirVana miRNA isolation Kit according to manufacturer's instructions. Agilent 2100 Bioanalyzer was used to determine the quality and size distribution of the RNA. cDNA synthesis, amplification, and labeling were performed using the Enzo Life Sciences Single-Round RNA Amplification and Biotin Labeling System. The level of gene expression was assessed with Affymetrix GeneChip HT HG-U133_PM Array Plate. The magnitude of expression change was determined by the average logarithmic ratio (ALR) for each group, for each gene probe. A gene was considered to be differentially expressed when ALR >0.585 and both pairwise and groupwise p-value ≤ 0.05 (Student's test). In order to validate our microarray data, we measured relative mRNA levels of selected genes by quantitative polymerase chain reaction (qPCR) with custom-designed RT2 Profiler PCR Arrays.

After cDNA preparation (miScript II RT Kit), the individual samples were pooled into four groups based on the gender and age (n=4/group). The relative amounts of 1008 microRNAs were measured with Human miRNome miRNA PCR Arrays using miScript SYBR Green PCR Kit according to the manufacturer's instructions. For microRNA

expression quantification comparative Ct method was used with a dual criterion: $|\Delta\Delta Ct| \geq 0.3785$ and *p*-value of ≤ 0.05. Twenty-two microRNAs were selected for follow up on the 16 individual, not-pooled samples using the same assays.

Gene set enrichment analysis (GSEA) was carried out for the significantly changed genes with the GenePattern software based on the BioCarta defined molecular pathways. To reveal the correlations between the mRNome and microRNome alterations, we searched the miRDB online database (based on miTarget2 dataset). Pathways potentially altered by the significantly changed microRNAs were identified with DIANA-mirPath software (based on Targetscan 5.1 and KEGG pathways).

iPSC differentiation and antipsychotic treatment

Human iPSCs were differentiated into Prox1 positive hippocampal granule neurons according to the protocol published by Yu et al. (2014). All the cell cultures were grown under standard conditions. Briefly: hiPSCs were cultured feeder-free with mTeSR media on matrigel coated plates, under standard conditions. The media was changed daily. On day 1, whole colonies were transferred onto ultra-low adherence dishes for free-floating embryoid body formation and treated with differentiating media. After 20 days, EBs were plated onto poly-L-ornithine and laminin (PORN/L) coated plates in N2B27 media. Between days 27-30, adhered, neural rosette-containing EBs were manually collected and dissociated with accutase. Cells were plated onto PORN/L dishes with NPC media (N2B27 media plus bFGF2 and laminin). Finally, P2 neural progenitor cells (NPCs) were subcultured onto PORN/L 6-well plates (1.7×10^5 cells/well; 3 wells/treatment) and confocal imaging chambers. On the following day, NPC medium was replaced with differentiating medium, containing APs at two different concentrations.

Seven treatment groups were distinguished: HL_{low} and HL_{high} (haloperidol 10 ng/ml and 100 ng/ml); OL_{low} and OL_{high} (olanzapine 50 ng/ml and 500 ng/ml); RP_{low} and RP_{high} (risperidone 100 ng/ml and 1000 ng/ml). Since APs were solved in dimethyl sulfoxide (DMSO), the control cells were treated with the same differentiating medium containing DMSO (0,2 μl/ml). The neurons were differentiated for 19 days.

To investigate the pluripotency hallmarks of 6/2/F hiPSCs, we labeled the cell suspensions with anti-human SSEA4-PE and used fluorescence activated cell sorting (FACS) with Attune™ NxT flow cytometer. For cell typisation, iPSC and differentiated neural cultures were immunostained with stem cell markers (NANOG, OCT3/4) and neural markers (PROX-1, MAP2), respectively.

Real-time quantitative PCR and data analysis

After 19 days of differentiation and AP-treatment, total cell RNA was isolated with Trizol following the manufacturer's protocol. The quality of the RNA samples were assessed with Nanodrop 2000 Spectrophotometer. cDNA synthesis was carried out using the Promega Reverse Transcription System.

For real-time quantitative polymerase chain reaction (rt-qPCR), we used TaqMan PCR probes for metabotropic glutamate receptor 2 and 7 (mGluR2, mGluR7), vesicular glutamate transporter 1 (VGLUT1), microtubule-associated protein 2 (MAP2), neuronal differentiation 1 (NeuroD1) and glial fibrillary acidic protein (GFAP). We chose large ribosomal protein P0 (RPLP0) for endogenous control. We calculated the normalized gene expression level with the ΔΔCt method; a gene was considered differentially expressed if $|\Delta\Delta Ct| \geq 0.3785$ and *p*-value ≤ 0.05.

RESULTS and DISCUSSION

How fibroblasts adapt on transcriptome-level?

GAL-treatment, a widely used oxidative stress, affected 2063 genes compared to STD condition. Pathway analysis revealed enrichment of 19 gene sets, including DNA repair, checkpoint, and cell-cycle related pathways. The RL-treatment means metabolic overload for the cells. It altered the transcription of 984 genes (65% increased) and 15 mainly immune, protein or cellular regeneration related pathways. The overlap between the stresses were 4 upregulated pathways indicated in oxidative stress and inflammation (PPARA); metabolism (CHREBP2); cell cycle progression (RACCYCD); and apoptosis, stress regulation (HSP27).

GAL-treatment modulated 45 microRNAs and RL-condition changed the expression of 34 microRNAs compared to STD culturing. The overlap were 4 microRNAs: hsa-miR-146b-5p, hsa-miR-129-3p, hsa-miR-543, and miR-550a. The pathway analysis suggested that these 4 microRNAs participate in the regulation of 57 gene sets. Twenty-two of these were predicted to be controlled by all 4 microRNAs, 16 of them were intracellular signaling pathways. When we measured 22 microRNAs in the individual, not-pooled samples, our data suggested that pooling of the samples did not skew artificially the microRNA pattern observed in GAL and RL.

We found significant correlation between the GAL- and RL-induced mRNA and microRNA profiles suggesting that although, the most significantly changed gene products showed variation, the overall transcriptome changes across the two metabolic stress conditions showed considerable similarity. Further, the changes in microRNA expression

contributed considerably to altered mRNA expression profile in both the GAL and RL conditions.

Manifestation of MD diathesis in fibroblasts

We compared the mRNome of fibroblasts from MD patients and CNT subjects in STD conditions: 162 genes were differentially expressed in the MD samples, 114 transcripts were downregulated which indicate rather loss-of-function genetic dysregulation than gene induction in the disorder. The most prominent decreases were observed in genes and pathways associated with cell-to-cell communication, adhesion, and immune functions. Our data are aligned with peripheral and postmortem studies which suggested that MD genetics might manifest in immune and (neuro)development related gene set disturbances.

Interestingly, 43% of the MET/HGF intracellular signaling pathway's genes showed MD-specific expression pattern. Previous results suggest that the gene set is actived during development, stress and regeneration. MET receptor promotes motility, proliferation, and protects cells against hypoxia and serum deprivation; however, it can exert proapoptotic effect also depending on the cell-type. Previously, serum HGF level was found to correlate with the presence, severity and progression of symptoms in MD patients. Our hypothesis, that the deficit of the HGF/MET signaling pathway in peripheral cells might preclude the beneficial effects of this compensatory elevated HGF, might be the subject of further researches.

Thirty-eight microRNAs were differentially expressed in MD cells (17 downregulated and 21 increased). Noteworthy, at least 8 of them have been previously associated with neuropsychiatric disorders. The pathway analysis performed with the microRNA data revealed universal intracellular signaling pathways, gene sets related to cell cycle, cell communication, adhesion, immune, and neural functions. Remarkably, Fan et al. identified 22 neural function associated pathways on the basis of blood cell microRNome of MD patients. Although, our diseased HDFs showed different microRNA pattern, 18 of these were also indicated in our samples. These observations remind us that MD is a multidimensional disorder and molecular anomalies are detectable in peripheral tissues.

Comparing the mRNome and microRNome profile, the MD-specific microRNA and mRNA profile appeared to be functionally connected, as 50% of the gene expression changes could be explain by the microRNome alterations. However, considering that 64% of the differentially expressed mRNAs are targeted by more than one microRNA, and microRNAs can either up- or downregulate the same gene depending on the cellular and external environment, we can only establish hypotheses on the exact cause-effect relationships.

Stress vulnerability of MD-fibroblasts

On the basis of the diathesis–stress model of MD, we presumed that the gene expression studies could be more informative if we challenge the MD fibroblasts. Both GAL and RL stress resulted in robust transcriptome changes in the MD fibroblasts: 1196 and 312 mRNAs were affected, respectively. Approximately one third of the stress response proved to be MD-specific (26% and 33% of the genes; 48% and 52% of the pathways). Some of the pathways were already identified in the STD condition; however, metabolic stress uncovered other MD-associated impairments in the control of metabolism, energy production, cell migration, cell cycle and apoptosis.

Interestingly, microRNA stress response demonstrated even more MD-specificity: 81% of the GAL-modified and 90% of the RL-affected microRNAs was detected only in MD-samples. Thirty-eight percent of the stress-induced microRNAs are related to metabolism control, 60% have role in proliferation and apoptosis, and 30% participate in cell motility. It is noteworthy, that significant proportion of the changed microRNAs is regulated by tumor suppressor p53 (GAL: 13%, RL: 33%). The overlap between the GAL and RL-induced miRNome stress response were 23 microRNAs.

The RL-environment showed to be especially effective in exaggerating the adaptational deficits of MD cells. It induced primarily negative changes in the microRNome which refers to appearance of new enzyme sets e.g. for lipid-synthesis or anaerob energy production. RL-environment also revealed an insufficient hypoxamir-response in MD samples. HypoxamiRs are essential in the mitochondrial adaptation to oxidative stress. In our study, CNT fibroblasts upregulated six hypoxamirs in response to RL, whereas MD fibroblasts downregulated them. This particular difference further supports the notion of mitochondrial dysfunction in MD.

These data suggest anomalies of cell proliferation and survival regulation in MD fibroblasts when exposed to metabolic challenges; and underpin that stress can pinpoint molecular clues of genetically determined stress vulnerability.

Characterizing iPSCs and iNCs

In our 2nd study, we differentiated hippocampal granule cells from iPSCs. Pluripotent and differentiated cells were characterized by morphology, FACS and ICC. More than 95% of the 6/2/F iPSCs showed stem cell like phenotype in their morphology, surface marker (SSEA4) and pluripotent-specific transcription factor (Oct3/4 and NANOG) expression and were able to form EBs and further develop into NPCs. Three weeks after differentiation induction cells exhibited Prox1 and MAP2 positivity, underlying that approximately 80% of the cells are hippocampal granule cells.

Antipsychotic treatment induced gene expression alterations

To examine the effect of APs on differentiating neurons, we treated developing hippocampal granule cells with HL, OL and RP at two different concentrations for 19 days. HL is a typical antipsychotic drug acting primarily on dopamine D2 receptors with lower activity at D1, D3, D4, serotonin 5-HT2A, and α1 adrenergic receptors and exerting a hyperglutamatergic effect. In contrast, OL and RP are atypical antipsychotics (AAPs) which operate primarily as 5HT2A antagonists and relatively weak D2 antagonists. They block with moderate affinity several other serotonin, dopamine, histamine and alfa-adrenergic receptors.

NeuroD1, key regulator of vertebral neurodevelopment, was overexpressed in HL_{low}, OL_{high}, RP_{low} and RP_{high} treated cells compared to CNT neurons, but HL had reverse effect at higher concentration. Previously, agents inducing *NeuroD1* proved to promote neural differentiation and the beneficial effect of APs have been attributed partially to their capacity to increase neurogenesis/survival; however, the results are often inconsistent especially in case of HL. Our results suggest that APs might facilitate cell survival only at certain concentrations.

Similarly, *MAP2* transcription was augmented by HL_{low} treatment compared to CNT, while HL_{high} exerted opposite effect. *MAP2* upregulation is usually evaluated as increased synaptic plasticity, and thought to reflect functional and/or structural changes in the dendritic tree. Accordingly, our findings suggest that HL_{low}, but not OL and RP-treated cells exhibited active synaptic dynamics.

In contrast, OL and RP affected the *GFAP* expression in a concentration-dependent manner: the AAPs enhanced it at low concentrations, while OL_{high} and RP_{high} depressed it. Importantly, astrocyte and GFAP-alterations were reported in several neuropsychiatric conditions, but little is known about the effect of psychopharmacons on these cells which play key role in neural functions.

vGLUT transcription was decreased by HL_{low}, RP_{low} and RP_{high} treatments, but not altered by OL. This protein, encoded by the *SLC17A7* gene, orchestrates the synaptic vesicle cargos: its expression pattern directly influences glutamate release (i.e. glutamate quantal size) and correlates with learning and memory processes. Accordingly, VGLUT1 plays a pivotal role in glutamate release, synaptic plasticity, postnatal neurodevelopment and learning. Downregulation is implicated in the cognitive symptoms of affective and neurodegenerative disorders.

Finally, we measured the expression levels of two metabotropic glutamate receptors. *mGluR2* was underexpressed in OL_{high} but upregulated in HL_{low}. HL_{high}-treated cells exhibited significantly lower *mGluR2* expression than HL_{low}. Presynaptic mGluR2s are key regulators of glutamaterg neurotransmission and agonists represent potentially new AP medications. Animal and postmortem data indicates that AAPs downregulate *mGluR2* through serotonin 2A receptor (5HT2A) transmitted histon modification. This suggests that HL might activate *mGluR2* through an other, currently unknown mechanism in a concentration-dependent manner what could be interesting subject for further investigations.

mGluR7 was also upregulated in HL_{low} but suppressed in HL_{high} and RP_{low}. Lower expression of mGluR7 may increase risk of developing schizophrenia, have potential therapeutic implication in cognitive impairment and extrapyramidal movement disorders. Thus, investigating the effect of current and potential APs on mGluR7 might be an important view in the future.

CONCLUSIONS

On the basis of our observations, we argue that *in vitro* model systems have potency in psychiatric research. First, we worked with the classic, but still valid patient-derived HDFs which have been utilized in psychiatric studies since the 1970s to study signal transduction alterations, oxidative stress, etc. Up to now, we are the first, who used these peripheral cells to investigate the (epi)genetic regulation of stress reaction and MD. We suggest that the above presented results correspond with the hypothesis that MD is a genetically determined, systemic maladaptational disorder.

First, we wanted to know how metabolic stress-response manifests on the level of mRNAs and microRNAs in HDFs? Our results suggest that metabolic stresses resulted in similar, but not identical, robust transcriptional changes affecting gene sets implicated in cell cycle, apoptosis, inflammation, and metabolic adaptation. The microRNA signature contributed considerably to the mRNA stress-response. Next, we examined if there are any

differences between the MD and CNT samples in the mRNA and microRNA profile? We found a functionally interconnected microRNA–mRNA network disturbance in MD cells: namely, a loss-of-function genetic dysregulation with most prominent decreases in genes related to cell communication, adhesion, immune functions and apoptosis. Finally, we compared the stress response of MD and control HDFs. These data underpin our hypothesis that the dysregulated microRNA profile in MD contributes to the maladaptation. We can also assume that MD cells can reach the required metabolic changes *via* non-physiological ways what result in increased vulnerability. Contrary to CNT cells, RL-medium showed to be greater challenge for MD cultures. Understanding this phenomena and investigating the MD-specific stress response might help to get closer to the genetic background of maladaptation.

In study 2, we used one of the most promising models in current neuropsychiatric research: iPSC derived neurons. We established an *in vitro* pharmacological assay treating differentiating hippocampal granule cells with APs. It is widely accepted that exogen factors impairing the hippocampal neurogenesis have detrimental effect on cognition and mood. Hence, studying neurogenesis during AP treatment have implications for the large community of patients who take these medication for years or decades. However, as far as we know, we are the first who used this *in vitro* experimental paradigm.

RP and OL increased the expression of *NeuroD1,* indicative of accelerated neurogenesis and/or augmented neural survival, but did not affected *MAP2* transcription, a marker of functional–structural synaptic changes. Our results call attention to the existing, but varying effects of APs on the expression of *mGluR*s which are actively studied new therapeutic targets in psychiatric disorders and implied in hippocampal LTP. Similarly, the RP and HL_{low}-attenuated *VGLUT1* expression might contribute to the understanding of cognitive aspects of long-term AP-treatment. Probably, the most interesting finding was AAP-modulated GFAP expression that can be remarkable subject of future research.

Considering our and previous results, we have to acknowledge that APs exert complex, poorly understood modulatory effect on the gene expression and cell pathophysiology in a brain region- and cell type-specific manner. Understanding these mechanisms might have direct consequences in clinical and experimental pharmacology.

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