Heroin dependence and methadone therapy: the possible role of the dopaminergic polymorphisms

Application of a fibroblast model to investigate expression changes induced by metabolic stress-treatment

Doctoral thesis

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I. Introduction

Drug dependence is a serious social and medical problem worldwide. Even though the number of first users of heroin decreased in the last years both in Hungary and Europe, dependence is still a crucial problem, further exasperated by other intravenous drugs come into view. Drug dependence, as a psychiatric disorder is proven to be a complex inherited trait, so besides the genetic components, environmental factors and gene-environmental interactions also take place in the development of addiction. The last decades' research suggeststhat instead of some high-impact genes we should rather focus on low-effect polymorphisms, which jointly contribute to the estimated 30-70% heritability. The candidate genes in the genetics of drug dependence comeprimarily from the reward system of the brain and from the pathways of drug metabolism.

In the presented work I investigated the relationship between the genetic polymorphisms of the dopaminergic system and the risk for developing heroin addiction.

A similar approach was used to investigate the therapeutic efficacy of methadone substitution treatment used in heroin dependence. Since the therapeutic outcomes can vary between patients, finding factors contributing to the therapeutic efficiency and thereby creating a more cost-effective treatment is a major goal of studies related to drug dependency.

Along with the genetic approach there is an increasing use of primary cell culture model systems to explore the molecular background of complex traits. During my 1-year scholarship I was able to set up such a model system in which the metabolic stress induced mRNA and microRNA (miRNA) expression changes were examined in a fibroblast model. Our results seem to show promising results for the possible application of the model to study the molecular background of complex diseases, including psychiatric disorders, as well.

II. Objectives

The main course of my PhD work consists of the examination of the association between heroin addiction and dopaminergic genes. The aim was to investigate eleven dopaminergic gene polymorphisms of four possible candidate genes. A few gene variants have been already tested in relation to drug addiction, but the results have been contradictory. Studies carried out in our lab aimed to take sides on the following issues:

1. Are the dopaminergic system genes relevant factors in the genetic background of heroin dependence?

2. Is the widely studied *Taq*IA polymorphism really a primary factor in the hereditary background of addiction?

3. Are there any new information – beyond the results of the conventional statistical calculations – derive from the applied Bayesian bioinformatic analysis (BN BMLA - Bayesian network based Bayesian Multilevel Analysis of relevance)?

The methadone substitution therapy which plays role in the treatment of heroin dependence shows an ambivalent picture of treatment outcomes. Using the samples of patients from the replacement therapy we wanted to find out if genetic variants can play a role in the effectiveness of the treatment. Such studies would allow the development of personalized therapy in the future, therefore, we focused on the following questions:

4. May the investigated dopaminergic gene polymorphisms play a role in predicting the therapeutic efficacy?

Setting up a biological model was also part of my PhD work, which hopefully might be suitable for the exploration of the molecular background of psychiatric disorders. The basic hypothesis of the model was that the fibroblast cell cultures from healthy and diseased (eg. drug addict) persons provide different RNA expression patterns in response to metabolic stress and that these differences may be markers of neurological changes. As a first step we aimed to analyze metabolic stress induced

mRNA and miRNA expression changes from fibroblasts of healthy persons, focusing on the following issues:

1. How similar are the stress induced mRNA and miRNA expression changes in the two types of metabolic stress treatment (glucose and cholesterol depletion)?

2. Is there any connection between the changes of the mRNA and miRNA expression patterns?

3. Are there any relevant molecular pathways changed between the expression patterns of the control and stress-treated fibroblasts, and if so, which pathways are affected by the changes?

III. Methods

Subjects and sample preparation

In the investigation of the genetics of heroin dependence 303 heroin-dependent patients - including 173 methadone-treated patients - and 555 healthy controls were recruited, from whom buccal swab samples were collected after they provided written informed consent. DNA isolation was done in our laboratory.

In the fibroblast study we used cryogenic fibroblast samples from 17 healthy individuals, on which the different stress treatments were performed.After treatment the cells were collected for RNA isolation.

Genotyping

The genotyping of the length polymorphisms were done by PCR and a following gel electrophoresis, while in the case of the single nucleotide polymorphisms PCR-RFLP and allele-specific amplification was performed.

Statistics

In the heroin dependence study the comparison of the control and case groups was done by a traditional chi-square test accompanied by a multivariate analysis of

associations using Bayesian networks in Bayesian multilevel analysis (BN-BMLA).

Expression analysis

The quality control analysis of the isolated fibroblast RNAs was done by the Agilent 2100 Bioanalyzer, the cDNA synthesis, amplification and biotin labeling was performed according to the Affymetrix® protocol with the Enzo Life Sciences' 100 Single-Round Reaction Biotin RNA Amplification and Labeling System kit. 5 mg biotin-labeled fragmented aRNA was hybridized to a GeneChip HT HG-U133 + PM Array Plate chip which step, along with the hybridization, purification steps, the staining and quality analysis was done by the Vanderbilt Microarray core, while assessments of miRNAs were performed by the Mirnics Labor colleagues.

The analysis of the expression data was performed in Microsoft Excel. Hierarchical clustering and pathway analysis also completed the study. The results of the expression study were validated by RT-PCR.

IV. Results

Association of heroin dependence and dopaminergic genes

1. The genetic association analysis was primarily done by conventional (frequentist) statistical methods, where the **genotype frequencies of case (heroin dependent) and control groups were compared (case-control model).**Based on these results the polymorphisms of the DRD2 and DRD4 showed significant association with the development of heroin dependence. However, due to multiple testing **Bonferroni correction** was necessary to be done, whereupon **only the frequency values of DRD2 *Taq*IB polymorphism showed significant difference between the control and case groups**. During the evaluation an additional statistical method called **Bayesian network based Bayesian Multilevel Analysis of relevance (BN-BMLA) was used, which in accordance with conventional case-control analysis confirmed the relevance of *Taq*IB in the development of heroin dependence.**

2. In our study we included the widely studied *Taq*IA (rs1800497) polymorphism as well, which previously showed association according to literature data, but in our study even the initialnominal significance disappeared after Bonferroni correction compared to***Taq*IB, which is the strongest and most relevant result of our study.**

3. Since we have never used the BN-BMLA method formerly, we were curious about how the results of the traditional case-control analysis agree with the results of the network method. We are pleased that the results from the two methods are in line with each other. Apart from the direct effects identified by both analyses**BN-BMLA was able to detect an indirect effect: the interaction of the DRD4 -521 C/T and the DRD4 -615 A/G polymorphisms.** This interaction was confirmed by traditional statistical methods.

4. In the genetics of the effeciency of thesubstitution therapy we found the possible role ofthe dopaminergic system, within it the genetic variants of the DRD4 and DAT may play role in the success of the therapy. **Our results showed association of the therapeutic response**

and the variants of the DRD4 120 bp duplication and the DAT intron 8 polymorphisms, which results are needed to be further confirmed.

The effect of the metabolic stress induction on mRNA and miRNA profiles of human fibroblasts

In the first phase of creating a working fibroblast model 17 healthy fibroblast samples obtained from skin biopsies were cultured in various metabolic stress treatment settings (glucose depleted, galactose-enriched medium (GAL) or lipid reduced, cholesterol free (RL) medium) and induced expression profile changes were measured. Our results can be summarized as follows:

1. The fibroblast model provided well-measured mRNA and miRNA expression changes. **The two types of metabolic stress showed significant similarity on the entire miRNA expression pattern:**the miRNAs which showed changed expression in the glucose depleted, galactose-enriched medium (GAL) treatmentshowed expression changes in the same direction in the lipid reduced, cholesterol-free (RL) medium treatment as well (significant correlation: $r = 0.71$; $p < 0.001$). Similar

situation has been identified during lipid reduced, cholesterol free (RL) treatment, where expression levels of glucose, galactose-enriched medium (GAL) treatment showed a strong correlation with changes in the expression of the other stressor ($r = 0.65$; $p < 0.005$).

2. **Among the individual miRNA expression levels four miRNAs** (miR-129-3p, miR-146b-5p, miR-543 and miR-550A) **showed significant change in either direction in both types of treatments.**

3. miRDB database was used to find the potential mRNA targets of the abovementioned 4 miRNAs. **The expression changes of these targets also showed notable differences** (140 potential target mRNAs out of the total identified 1504, so nearly 10% showed significant expression change in the metabolic stress treatments). **Based on the pathway analysis it seems that the targets of these miRNAs**(miR-129-3p, miR-146b-5p, miR-543 and miR-550A) **are involved in biologically relevant pathways**, such as: cell cycle, apoptosis, inflammatory responses and mRNA production, metabolic adaptation.

Conclusions

In the first part of the work the analysis of the association of the dopaminergic genetic polymorphisms and heroin addiction was performed. The results showed a significant association of the DRD2 *Taq*IB variant, which association remained significant after Bonferroni correction. This result indicates the relevant role of this particular variant in heroin dependence, which role was also confirmed in the BN-BMLA analysis. Previous studies mainly focused on the association of heroin dependence and the DRD2 *Taq*IA variant, despite the fact that it was found not actually in the DRD2 gene, but in the neighbouring ANKK1 kinase gene.

Our results have shown that the DRD2/ANKK1 gene ***Taq*IA polymorphism, compared to the DRD2 *Taq*IB polymorphismwas not significant in association with heroin dependence**.

However the *Taq*IA was not significant after the Bonferroni correction, the nominal significance was detectable in both the conventional and Bayesian analyses. The BN-BMLA method has also shown that the co-occurrence of *Taq*IA and *Taq*IB in the model is

significantly less likely to occur than the occurrence of these variants alone. Thus, the **negligible relevance of *Taq*IA compared to *Taq*IB is due to redundancy** occurs between the two polymorphisms. The BN-BMLA statistical method not only validated the results of the conventional method, but it also detected an **indirect effect of the -615 A/G SNP**, so we can say that the BN-BMLA method is able to analyze interactions and redundancies, and the assessment of results are not only broadened but became more bias-free.

We assumed the role of the dopaminergic system in the therapeutic efficiency as well. In our study, the **dopamine transporter intron 8 and the DRD4 120 bp duplication effects showed a trend effect**, however, due to the low sample size further validation of the results is needed. In any case, we can say that it seems that the genes playing role in the development and in the therapeutic efficiency (termination) are not necessarily the same.

In the second part of the work an examination of the fibroblast model system was carried out. Glucose-deficient, galactose treated and lipid reduced, cholesterol-

deficient treatments caused strong mRNA and miRNA changes in the treated human fibroblast cell cultures. During the evaluation of miRNA expression changes a high degree of similarity in the two metabolic stress treatment was observed and the results suggest that changes in the **expression of these miRNAs contribute significantly to altered mRNA expression** in both galactose-treated and lipid reduced settings. The similar effects caused by the two different stressors suggest that the fibroblast cells provide a **general defense response** to altered homeostasis. Furthermore, our results also suggest that **miRNAs contribute significantly to the mRNA regulation**in case of metabolic stress. In summary it can be said that fibroblast cultures are suitable for well-controlled studies and they are also easily accessible. This would be especially important inpolygenic psychiatric disorders, since there is a limited availability of brain tissue and the already existing animal models are not able to model human disease.

List of publications

Publications related to the thesis:

1. **Vereczkei A**, Demetrovics Z, Szekely A, Sarkozy P, Antal P, Szilagyi A, Sasvari-Szekely M, Barta C. (2013) Multivariate analysis of dopaminergic gene variants as risk factors of heroin dependence. PLoS One, 8: e66592.

2. Kalman S, Garbett KA, **Vereczkei A**, Shelton RC, Korade Z, Mirnics K. (2014) Metabolic stress-induced microRNA and mRNA expression profiles of human fibroblasts. Exp Cell Res, 320: 343-353.

Publications independent from the thesis:

1. Demetrovics Z, Varga G, Szekely A, **Vereczkei A**, Csorba J, Balazs H, Hoffman K, Sasvari-Szekely M, Barta C. (2010) Association between Novelty Seeking of opiate-dependent patients and the catechol-O-methyltransferase Val(158)Met polymorphism. Compr Psychiatry, 51: 510-515.

2. Szekely A, Balota DA, Duchek JM, Nemoda Z, **Vereczkei A**, Sasvari-Szekely M. (2011) Genetic factors of reaction time performance: DRD4 7-repeat allele associated with slower responses. Genes Brain Behav, 10: 129-136.

3. Halmai Z, Dome P, **Vereczkei A**, Abdul-Rahman O, Szekely A, Gonda X, Faludi G, Sasvari-Szekely M, Nemoda Z. (2013) Associations between depression severity and purinergic receptor P2RX7 gene polymorphisms. J Affect Disord, 150: 104-109.

4. Gyollai A, Griffiths MD, Barta C, **Vereczkei A**, Urban R, Kun B, Kokonyei G, Szekely A, Sasvari-Szekely M, Blum K, Demetrovics Z. (2014) The genetics of problem and pathological gambling: a systematic review. Curr Pharm Des, 20: 3993-3999.

5. Garbett KA, **Vereczkei A**, Kalman S, Brown JA, Taylor WD, Faludi G, Korade Z, Shelton RC, Mirnics K. (2015) Coordinated messenger RNA/microRNA changes in fibroblasts of patients with major depression. Biol Psychiatry, 77: 256-265.