Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 894

High-resolution Studies of mRNA Expression in Brain

A Search for Genes Differently Expressed in Schizophrenia

BY

ANJA CASTENSSON

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2003

Dissertation presented at Uppsala University to be publicly examined in Lindahlsalen, EBC, Uppsala, Friday, November 7, 2003 at 09:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract
Castensson, A. 2003. High-resolution Studies of mRNA Expression in Brain. A Search for Genes Differently Expressed in Schizophrenia. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 894. 47pp. Uppsala. ISBN 91-554-5755-X

Gene expression differences between patients and controls can be used to find susceptibility genes and drug targets for a disease. High-resolution strategies are required because the differences between the investigated groups may be small and numerous factors may affect the mRNA quantity. This thesis is based on the use of real-time RT-PCR combined with a new statistical approach, developed to detect small differences between patients and controls and differences due to patient subgroups.

Comparisons between human brain biopsy and autopsy samples showed that post-mortem tissue can be used to make conclusions on the relative mRNA levels in the living brain.

Power analysis based on human brain mRNA expression from 14 genes adjusted with two reference genes, revealed that a sample size of 50 patients and 50 controls was required to detect a 2-fold difference with a power and a confidence of 95%. A similar study in rats revealed that approximately the same sample size was required for rat brain mRNA expression studies.

The mRNA levels of several genes were studied in 55 schizophrenia and 55 control prefrontal brain autopsies, using a novel and more powerful statistical analysis. The serotonin receptor 2C gene (HTR2C) showed a significant 1.5-fold decrease in the patients as compared to controls, and the monoamine oxidase B gene (MAOB) a 1.2-fold increase.

The mechanism behind the decrease of HTR2C mRNA levels was investigated by studying the correlation of drug treatment and HTR2C promoter polymorphisms to the HTR2C expression levels. The observed decrease was present in untreated patients, suggesting that the HTR2C mRNA decrease is correlated with the disease and not the treatment. There was no association between promoter polymorphisms and HTR2C expression levels. Thus, the molecular mechanism for the decreased expression remains unclear. Nevertheless, the results support a role for monoaminergic synapses in schizophrenia.

Keywords: mRNA, gene expression, real-time RT-PCR, schizophrenia, 5-HT (serotonin) receptor 2C, brain, psychiatric genetics

Anja Castensson, Department of Evolutionary Biology, Evolutionary Biology, Norbyv. 18D, Uppsala University, SE-75236 Uppsala, Sweden

ISSN 1104-232X
ISBN 91-554-5755-X
urn:nbn:se:uu:diva-3605 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-3605)

Printed in Sweden by Universitetstryckeriet, Uppsala 2003

How do you define real? If you are talking about what you can feel, what you can smell, what you can taste and see; real is simply electrical impulses interpreted by your brain.

The Matrix

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Castensson, A., Emilsson, L., Preece, P., Jazin, E. (2000) High-resolution quantification of specific mRNA levels in human brain autopsies and biopsies. *Genome Research*, 10(8): 1219-29

II. Alfonso, J., Pollevick, G., Castensson, A., Jazin, E., Frasch, A. (2002) Analysis of gene expression in the rat hippocampus using Real Time PCR reveals high inter-individual variation in mRNA expression levels. *Journal of Neuroscience Research*, 67(2): 225-34

III. Castensson, A., Emilsson, L., Sundberg, R., Jazin, E. (2003) Decrease of serotonin receptor 2C in schizophrenia brains identified by high-resolution mRNA expression analysis. *Biological Psychiatry*. In press.

IV. Sundberg, R., Castensson, A., Jazin, E. Statistical methodology in case-control 5'-nuclease assays. Statistical design, modelling and inference for identification of differentially expressed genes. (manuscript)

V. Castensson, A., Åberg, K., McCarthy, S., Andersson, B., Jazin, E. Serotonin receptor 2C (HTR2C) and schizophrenia: effect of medication and genetic variants on expression levels. (manuscript)

Contents

Abbreviations

ANCOVA	analysis of covariance
ANOVA	analysis of variance
cDNA	copy DNA
CV	coefficient of variation
HTR2C	serotonin receptor 2C
µl, µg	micro (10^{-6}) liter, micro (10^{-6}) gram
mRNA	messenger RNA
N	Number of observations, sample size,
ng	nano (10^{-9}) gram
OMIM	Online Mendelian Inheritance in Man
PCR	polymerase chain reaction
PFC	prefrontal cortex (of brain)
RT	reverse transcription
SNP	single nucleotide polymorphism

Introduction

To find genes involved in schizophrenia and other complex disorders of the brain is a daunting task. It can be approached by performing linkage analysis on affected families, analyzing allele frequencies among populations or by performing microarray screenings (Sawa et al. 2002). Candidate genes are also selected by studying functions related to disease phenotypes, for example in animal models (Seong et al. 2002).

To obtain more information on the function of disease candidate genes, mRNA quantity differences between patients and controls, as well as between animal models and control animals are useful.

However, biologically significant differences in brain mRNA expression between patients and controls can be small, they might be present only in a subgroup of patients, and they might show a considerable inter-individual variability. To be able to detect minute differences in mRNA levels between patients and controls, a strategy was developed using a sensitive real-time RT-PCR method, a large sample size and a powerful statistical model.

The use of mRNA levels to understand brain function

Changes in gene expression can have major effect on brain function. Small shifts in the expression levels of hormones, neurotransmitters, their transporters and receptors as well as other neuron-specific mRNA have been proposed to be related to behavioral differences between individuals, as well as variable susceptibility to drugs (Lesch 2001, Hamer 2002). The description of expression patterns provides clues about regulatory mechanisms, biochemical pathways, and broader cellular functions. It helps to elucidate disease mechanisms and to find drug targets. Therefore, an identification of disease or drug specific differences in expression will add to the understanding of the pathways affected (Bunney et al. 2003). Protein and mRNA expression methods are complementary to achieve these goals.

Advantages of mRNA methods are that they are generally less difficult, more sensitive, and involve more high throughput approaches than protein based methods. The differences found in mRNA levels are often correlated with differences in protein levels. For example, a study of the *C.cresentus*

bacterium cell cycle using a two-dimensional gel method for proteins and DNA microarrays for RNA showed similar expression profiles between protein and RNA (Grunenfelder et al. 2001). Moreover, measuring mRNA levels provides information about gene activity. If one gene is activated, it is possible that other genes in the same pathway are affected as well.

The main disadvantages of measuring mRNA are associated with these molecules not being the final expression product. The mRNA levels may therefore not be correlated with the protein levels or the final protein might be modified and may form complexes with other proteins. In addition, the localization of the final protein is not obtained.

Choice of RNA quantification method

Today there are many different RNA quantification methods available. Selecting a method depends on the aim of the RNA quantification and economical considerations. Microarrays are used to explore thousands of genes while Northern blot, in situ hybridization and real-time RT-PCR are used for more precise analysis of a small number of genes (Fig. 1) (Mirnics et al. 2001, Bunney et al. 2003).

Microarrays, Northern Blot, and in situ hybridization, are hybridization-based detection methods and thus restricted by their requirement for large volumes of samples, by being labor intensive (Bird 1998, Duggan et al. 1999) and by having a smaller dynamic range (Mirnics et al. 2001). The real-time RT-PCR method, on the other hand, requires little sample and can be performed with a high sample throughput (Klein 2002) (Fig. 1). Microarrays are used for large semi-quantitative screenings to search for genes and groups of genes differentially expressed, or co-expressed between sample populations (Bunney et al. 2003). Real-time RT-PCR is used for validation of microarray results and for more precise analysis of selected genes (Chuaqui et al. 2002, Bunney et al. 2003).

Tissue complexity is an additional factor that affects the reliability of the RNA quantity measurement. In situ hybridization (sometimes on tissue microarrays) can be used for information about cellular localization (Chuaqui et al. 2002, Bunney et al. 2003).

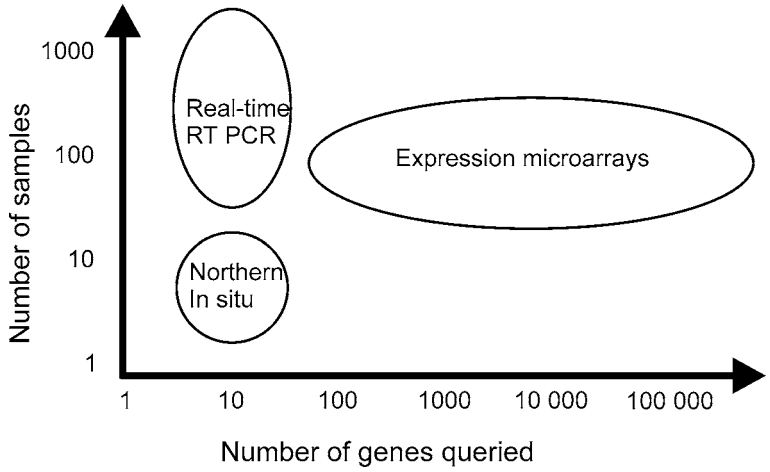

Figure 1. Overview of sample and gene processing ability of different RNA quantification methods.
The high throughput assay for thousands of genes; expression microarrays are complemented by other methods, such as real-time RT-PCR, Northern blot and in situ hybridization. Real-time RT-PCR also has the advantage of being high throughput in sample processing. Figure adapted from Mirnics 2001 (Mirnics et al. 2001).

DNA microarrays

Oligonucleotide and cDNA microarray assays are fluorescence or radioactivity based expression techniques that have revolutionized the detection of gene expression patterns, since they are able to screen mRNA expression of thousands of gene transcripts simultaneously (Yang et al. 2002). The use of microarrays as a genome-wide tools to elucidate the function of genes is increasing (Chuaqui et al. 2002). However, technical difficulties involve the requirement for highly concentrated RNA samples with 50-200 μg RNA or 2-5 μg polyA+ RNA required (Duggan et al. 1999). Additional problems are cross-hybridization (Chuaqui et al. 2002), the reproducibility of assays, and the development of efficient procedures to handle the multitude of data points produced in each individual experiment (Yang et al. 2002). Sensitivity is reported to be 1 copy detected among 100,000, but such high sensitivity will result in a higher rate of false positives and a higher required number of replicates (Duggan et al. 1999,

Lockhart et al. 2000). To accurately detect a difference a sufficient number of replicates is required (Yang et al. 2002).

The technology will evolve further and some solutions to problems associated with microarrays have been proposed. Among the solutions suggested are amplification of the RNA sample (Chuaqui et al. 2002) and using more powerful statistical designs (Yang et al. 2002).

Real-time RT-PCR

Real-time PCR is used to quantify cDNA produced by reverse transcription (RT) of mRNA. The amount of cDNA produced in reverse transcription is proportional to the amount of mRNA in the sample and thus the real-time RT-PCR method is able to quantify mRNA (Gibson et al. 1996).

Reverse transcription (RT)

Reverse transcription is performed using a reverse transcriptase enzyme, primers and nucleotides. The enzyme produces copy DNA (cDNA) from the mRNA template. The reaction can employ gene-specific, random or oligo-dT primers. Gene specific primers increase specificity and decrease background; random and oligo-dT primers, on the other hand, maximize the number of mRNA molecules analyzed (Bustin 2000).

The reverse transcription step is the source of most variability between different reactions in quantitative RT-PCR measurements (Freeman et al. 1999). Therefore, a reference has to be used to adjust the obtained quantities of cDNA. The reference can be an RNA standard with known amount or the measured amount of reference genes present in the RNA sample.

Real-time PCR

Real-time PCR measures the PCR product as it accumulates in the exponential phase of the PCR reaction. The quantity of the template (cDNA) is characterized by the point in time during the amplification (expressed as cycle threshold, Ct) when the amount of amplified PCR product reach above an arbitrary threshold. The larger the quantity of the template (cDNA), the sooner the PCR product reach above the threshold.

This can be compared to an endpoint measurement, which measures the amount of PCR product accumulated after a fixed number of cycles. The amount of PCR product after the reaction is completed is more sensitive to reaction conditions, than the amount during the exponential phase of the reaction. During the exponential phase, the reaction components are not limited. Consequently, Ct values are very reproducible for reactions with the same starting copy number. Real-time RT-PCR show a coefficient of variation (CV) of less than 2% (Klein 2002), as compared to 14 % for

conventional RT-PCR (Zhang et al. 1997a). Furthermore, the real-time RT-PCR method differs from other RT-PCR methods in the following aspects: it can be used in high throughput assays, has less contamination risk, has a larger dynamic range (of 7-8 log), and needs less hands-on time (Klein 2002).

The PCR product is detected by DNA intercalating dyes such as Ethidium Bromide or SYBR Green or by amplicon-specific probes. The probes can be hybridization probes, where the fluorescence is released after hybridization of probe, or hydrolysis probes, where the fluorescence is released after cleavage of probe by the DNA polymerase. Dyes are not sequence-specific and will intercalate in unspecific amplification products and in primer dimers. However, it is now possible to study the melting curve profiles to detect these artifacts (Bustin 2000).

Real-time RT-PCR provides a high-resolution measurement

Any technique needs a sufficient number of individuals and replicas to give reliable measurements (Yang et al. 2002). Due to stochastic effects, reproducibility is reduced for all quantification methods at low mRNA levels (Peccoud et al. 1996). Consequently, even more replicas are required to measure genes that are expressed in small amounts. As most mRNAs are present with 5-15 copies per cell (Alberts et al. 1994) multiple replicas can be a demand for the analysis of many genes.

A small difference in the specific gene cDNA template quantity is amplified using real-time PCR, making this method highly sensitive, and the detection of less than five copies of a template is possible (Klein 2002). Real-time RT-PCR approaches have at least 2-fold resolution (Gibson et al. 1996). The high sensitivity, the high resolution, and the possibility to readily measure many replicas make real-time RT-PCR the preferred choice to measure small differences in mRNA expression.

Statistical analysis of differences between groups

A common approach to understand how genes are affected in a disease is to compare the mRNA levels between patients and controls, or animal models and control animals.

For microarrays, the aim of the statistical analysis is to select genes of interest and there are several strategies to accomplish this goal (Chuaqui et al. 2002, Yang et al. 2002). To confirm that there is a certain difference between two groups, the measurements need to be confirmed with a more precise method such as real-time RT-PCR. In these methods, the difference is tested for statistical significance.

Generally, any test for significant difference calculates the probability (p-value) that the results obtained are due to chance only, given the size of the mean difference, the standard deviation, and the sample size. When an observed difference yield a p-value of 0.05 or less, the difference is considered significant, as only one test out of 20 will have this outcome by chance. If one hundred tests are performed, five of these can show a significant difference by chance. Therefore, a correction due to the number of tests has to be considered. Bonferroni correction corresponds to the division of the p-value with the number of tests (Zhang et al. 1997b). However, the determination of the number of independent tests can be a matter of debate.

Different statistical tests can be applied to compare mean differences but an ANOVA (analysis of variance) test is commonly applied for normal distributed samples (Gaddis 1998). The ANOVA provides the same results as a Students t-test for two groups, however, more than two groups can be compared. The ANCOVA (analysis of covariance) can detect differences between groups while controlling for other variables (Gaddis 1998).

ANCOVA

The ANCOVA model can be formulated as a linear equation between a dependent variable (here: expression of gene Y) and several independent variables (See Formula 1). The independent variables can be categorical, such as diagnosis or quantitative covariates, such as reference gene expression. The expression level of a reference gene is not an independent variable, but can be used as such in ANCOVA.

$$\text{GeneY} = a_{\text{categorical variable}} + b * \text{covariate} + \text{error}$$

Formula 1. The ANCOVA model can be described as a linear equation.
The observed mRNA expression level of a gene (Gene Y) is here fitted to a linear equation including the effects (a) of the different groups within a categorical variable and the slope (b) to the covariate. In reality, more than two variables can be included in the model.

After formulation of the model, the obtained gene Y expression values are fitted to the equation. In other words, the regression coefficients (slope, b) and the categorical effects (a) are calculated from the gene Y data, to make the best fit of the model to the observed data. The equation can then be used to predict the expression for the gene Y. As the model is fitted to the observed data, it will make the mean predicted expression equal the mean

observed expression. The difference in a single sample between the predicted and the observed value constitutes the random error (the residual/prediction error). The model is then used to test differences in group means and/or interaction between variables.

The test between means of groups in the ANCOVA uses the variables in the linear equation to adjust the mean expression for the group factor of interest (here: diagnosis). If the variables included in the model explain a large part of the variability, the estimated error variability will be adjusted to a lower value. Thus, these variables will increase the power to detect a difference in group means.

However, one cannot include an unlimited number of variables, as all or most of the variability in data would then be explained by the model and no data would remain for calculation of the error estimate. In other words, too many variables on a limited sample size would give a rigid model with no or low power to test for any differences.

Schizophrenia

Schizophrenia is a mental disorder with a life time prevalence of 1% in the world population and many of those diagnosed with this disease have to use medication throughout their lifetime. The schizophrenia syndrome is composed by a collection of symptoms, which can vary substantially between patients. However, all schizophrenia patients are characterized by psychotic episodes where patients are not able to perceive reality correctly. Psychotic symptoms include delusions, hallucinations and disordered thoughts (Kandel 2000).

The psychotic symptoms are usually present at the acute phase of the disorder, and are referred to as positive symptoms. Negative symptoms, on the other hand, describe lack of normal function. The negative symptoms, which are more common in the chronic phase, include cognitive deficits and lack of motivation (Kandel 2000). Social and occupational deterioration is often associated with the disease (Andreasen 1995). However, it has been indicated that 50-70% of the patients will improve or recover significantly after the first psychotic episode (Harding et al. 1992). The disorder usually appears between age of 15-25 years for males and 15-30 years for females (Hafner et al. 1998). It is difficult to study the life course of patients because of the changing nature of diagnosis, treatment, and social norms (Schultz et al. 1997). Nevertheless, the suffering of those affected along with that of their relatives, as well as the cost for society, emphasizes the need to study the causes of schizophrenia.

Schizophrenia diagnosis

In addition to being a disorder with a complex etiology, schizophrenia is not as straightforward to diagnose as disorders displaying physical symptoms, such as diabetes or cancer. Today, the diagnosis is often made using the diagnostic criteria DSM-IV or ICD-10 published by the American Psychiatric association and the World Health Organization (Regier et al. 1994, Kandel 2000). The criteria can be summarized as 1-6 months of psychotic symptoms where other mental disorders, such as depression and drug addiction, are ruled out.

The patients diagnosed as schizophrenics belong to a highly heterogeneous group and they suffer from symptoms shared by other psychiatric diseases such as bipolar and unipolar depression. Many investigators agree that there are different underlying etiologies for these psychiatric diseases (Evans et al. 2001). However, bipolar and schizophrenic patients could share susceptibility genes (Evans et al. 2001, Sawa et al. 2002). The arguments for common genes include co-localization of linkage, shared symptoms, co-existence of both diseases in kindreds, and the presence of individuals with a phenotype intermediate between bipolar disorder and schizophrenia (Evans et al. 2001). Moreover, a recent paper show similar changes in the oligodendrocyte gene expression in these two patient groups (Tkachev et al. 2003).

The wide spectrum of schizophrenia symptoms is a problem when searching for genes involved in schizophrenia. This can be addressed by looking at intermediate traits, which are heritable quantitative phenotypes that are thought to be more directly related to disease-causing factors than the diagnosis itself. Intermediate traits are often referred to as endophenotypes but the original meaning of an endophenotype is a biochemical intermediate trait that cannot be measured in an intact organism. A differing mRNA level in a schizophrenia brain can be considered an endophenotype. Other endophenotype examples include dysfunctional eye movement, working memory deficits, and sensory motor gating (Gottesman et al. 2003). Endophenotypes can help to identify specific pathways and genes involved in complex psychiatric diseases (Gottesman et al. 2003). Moreover, endophenotypes can be studied in animal models. It is feasible to check mice for impaired working memory and for social withdrawal while it is not possible to have schizophrenia diagnosis in animals (Seong et al. 2002, Miyakawa et al. 2003).

The prefrontal cortex and schizophrenia

The symptoms of schizophrenia indicate the involvement of various parts of the brain, including the temporal lobe and the prefrontal cortex. The schizophrenia brain does not present any characteristic pathology, such as the neurofibrillary tangles observed in Alzheimer's disease. However, small changes in cytoarchitecture and brain volume have been reported in untreated patients (Torrey 2002). A temporal lobe reduction has been evident in many MRI (Magnetic Resonance Imaging) studies (McCarley et al. 1999).

The prefrontal cortex (PFC) (See Fig. 2) is of specific interest for schizophrenia and has been proposed to have a decreased activity in patients. The PFC is important for motivation, working memory, and making decisions (Weinberger et al. 1994, Kandel 2000). Cognitive deficits related to working memory and motivation are often present in chronic schizophrenia patients. A decrease in prefrontal cortex function may be due to a decrease in frontal lobe volume observed in several studies (McCarley et al. 1999). The volume reduction is less consistently found for frontal than for temporal cortex (McCarley et al. 1999), but a correlation of disease severity and decrease in PFC gray matter is an interesting finding in one recent study (Cannon et al. 2002). In addition to these observations, patients have demonstrated less blood flow in this area of the brain when performing cognitive tasks (Weinberger et al. 1994, Goldman-Rakic et al. 1997, Bunney et al. 2000).

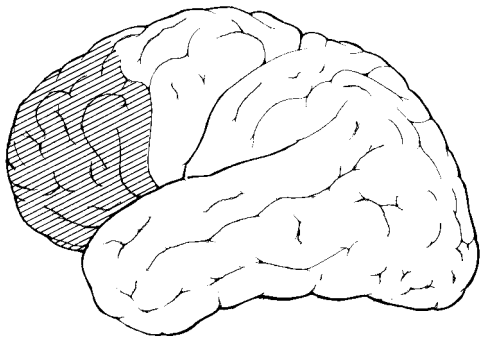

Figure 2. The prefrontal cortex of the brain
The prefrontal cortex, here in gray, is the most frontal part of the brain.

The prefrontal cortex is connected to virtually all sensory and motor systems as well as to a wide range of subcortical structures. The prefrontal cortex and the limbic system to which it is connected matures post adolescence, the period when schizophrenia first becomes apparent in most patients (Spear 2000). The decreased PFC function could either be due to a bad connection to subcortical structures, or to decreased function independent of the connections.

Drug treatment of schizophrenia

The history of drug treatment of schizophrenia starts in the 1950's with chlorpromazine and its derivatives, which have a substantial effect on hallucinations and delusions (Kandel 2000). However, these antipsychotic drugs also give extrapyramidal side effects, such as muscle contractions and problems of movement and gait (Kandel 2000). The extrapyramidal system refers to the basal ganglia and several brain stem nuclei with which they are connected, excluding the pyramidal system.

The next breakthrough came when clozapine was re-introduced in 1989 (Lancet 1989). It had previously been tried in Europe, but withdrawn due to severe agranulocytosis. It is now used with close monitoring of patients in the beginning of treatment to exclude risk group patients (Lancet 1989). This drug showed higher efficacy in treatment resistant patients, was more effective against negative symptoms, and showed less pyramidal side effects (Kane et al. 1988, Kandel 2000, Kapur et al. 2001). However, clozapine and its derivatives have other side effects, the most common being weight gain, and its associated diseases (Wetterling 2001). After the re-introduction of clozapine, several similar substances, with similar treatment effects, were introduced. This group of drugs was referred to as atypical neuroleptics (Seeman 2002). Lately, there has been some debate on whether atypical drugs provide benefits over typical drugs or not (Geddes et al. 2003).

All neuroleptics are antidopaminergic, and their antipsychotic action is correlated with their binding to the dopamine D2 receptor (Kandel 2000). The later atypical variants target other neurotransmitter receptors as well, most notably serotonin. The mechanism that makes atypical drugs different from typical remains unclear, but a loose binding to the dopamine D2 receptor (Seeman 2002), preferential binding to the serotonin receptor 2A (Kapur et al. 2001), or inverse agonism at serotonin receptor 2C have been suggested (Herrick-Davis et al. 2000).

The response to atypical antipsychotic drugs is heterogeneous, with 30-60% of the patients responding to clozapine (Arranz et al. 2000). Pharmacological response variability between individuals is most likely due

to several genes within each individual, where different genes can be involved in drug targets and/or in drug metabolism (Kawanishi et al. 2000).

What causes Schizophrenia?

There are several ideas and theories around the etiology (the cause) of schizophrenia (Sawa et al. 2002). Today, most researchers agree that schizophrenia is a complex disease, the result of several genes and several environmental factors.

There is a tendency of schizophrenia to be more common in some families. Results from adoption and twin studies support the belief that this is partly due to genes. Adoption studies have shown greater rates of schizophrenia in genetic relatives to people diagnosed with schizophrenia compared to adoptive relatives and control adoptees (McGuffin et al. 1995). For example, one study indicates that approximately 15% of biological relatives of schizophrenia adoptees suffer from schizophrenia compared to 1-3% in adoptive relatives and control adoptees (McGuffin et al. 1995).

Twin studies show that monozygotic twins, twins that share 100% of their genes, show a higher concordance for the disease compared to dizygotic twins, twins that share 50% of their genes. The exact schizophrenia concordance numbers have varied from different studies but show an average of 46% concordance rate for monozygotic twins and 14% for dizygotic twins (Tsuang 2000). The heritability (variance in a phenotype due to genes) calculated in these studies vary between 68-89% (Tsuang 2000).

However, it is also possible that shared biological environment in the uterus for twins and, in the early years for adopted siblings, have an effect. Early shared environment could transfer infectious agents, also proposed to be involved in schizophrenia (Fuller Torrey et al. 2000). The fact that the concordance for monozygotic twins is not 100% demonstrates that it is a complex disease caused both by environmental and genetic factors. Place of birth and birth complications are among the environmental factors suggested to be involved in the etiology (Mortensen et al. 1999, Tsuang 2000). The higher proportion of schizophrenics being born in urban areas could be explained by prenatal infection, as there is a higher risk of infection there (Mortensen et al. 1999). Prenatal infection as a cause for schizophrenia is also supported by raised levels of cytokines in patients (Kirch 1993) and findings of retroviral DNA in cerebral spinal fluid from patients (Karlsson et al. 2001). Another environmental factor, emphasized in the 1970's, was the rearing and the role of the mother. Now this view is strongly discredited as little evidence has been found (Sawa et al. 2002). Nevertheless, there is

evidence that a stressful family environment can have a negative effect when there is an underlying genetic susceptibility (Tsuang 2000).

Schizophrenia genes

Linkage regions

The search for genes involved in schizophrenia has been slow. It has been hampered by the absence of biological markers of the syndrome, such as a clear neuropathology, and by the complex non-mendelian pattern of inheritance (Harrison et al. 2003). One approach to find disease genes is to search for linkage of the disease to chromosomal regions. Linkage studies screen DNA from families with a disease for genomic markers that would follow the disease more frequently than expected by chance.

Several areas of the human genome are proposed to be linked to schizophrenia. Loci implicated include 1q21-q22,q42, 5q11-q13, 6q13-q26,p22, 23, 8p21, 11q14-q21, 12q24, 13q32,q34, 15q15, 18p, and 22q11-q13 (See OMIM #181500). Velo-Cardio-Facial Syndrome is a disease where a large number of patients also have schizophrenia. Linkage to chromosome 22 reported in this group is also of interest for schizophrenia (Murphy 2002).

On many occasions, linkages detected have failed to be replicated, which is probably due to the genetic heterogeneity of schizophrenia and that many genes of small and interacting effects are involved in the disease (Evans et al. 2001). Genetic heterogeneity means that genes causing schizophrenia in some cases, such as genes of large effect in one family might not be involved in other cases or in other families (Evans et al. 2001, Kato et al. 2002). Another reason to the absence of replicated linkage might be that epigenetic factors, which alter the gene activity without changing the DNA, are involved in the disease (DeLisi et al. 2002).

Possible schizophrenia genes

During recent years, a substantial number of genes have been suggested to be involved in schizophrenia. Most have been found by linkage analysis and association analysis revealed that one haplotype/gene variant to be significantly more common in patients.

A variant of the gene neuroregulin 1 (NRG1) was found to be associated with schizophrenia both in Icelandic and Scottish populations (Stefansson et al. 2003). Moreover, mice heterozygous for a deleted NRG1 showed hyperactivity, as seen with the hallucinogenic drug PCP. The symptoms were reversed by treatment with clozapine, which is used to treat schizophrenia (Stefansson et al. 2003).

The gene coding for the regulator of G-protein signaling-4 (RGS4) is present in an area linked to schizophrenia. It's mRNA was found to be decreased in a microarray study, and polymorphisms in the gene showed association to schizophrenia (Harrison et al. 2003).

In a Canadian sample the G72 gene and D-aminoacid oxidase (DAAO) gene, whose proteins interact, showed a strong association to schizophrenia when combined (Chumakov et al. 2002).

The proline dehydrogenase gene (PRODH), which showed a complex association to schizophrenia, is present in the region deleted in Velo-Cardio-Facial Syndrome (VCFS). However this result could not be replicated (Harrison et al. 2003). Mice with inactivated PRODH have problems in sensimotor gating, similar to those found in schizophrenia patients (Harrison et al. 2003).

The cathechol-O-methyltransferase gene (COMT) is also present in the VCFS region and an haplotype with decreased expression is associated with schizophrenia (Bray et al. 2003). Moreover, the promoter variant that show the greatest association to schizophrenia decrease COMT expression (Bray et al. 2003).

Two additional possible schizophrenia genes are the Disrupted in schizophrenia genes (DISC1 and DISC2) (Millar et al. 2000), which are disrupted by a translocation that co-segregates with schizophrenia in a Scottish family. The disruption seems to be causing psychosis in the family but not in the population. The polymorphisms in the gene did not show association to schizophrenia or to bipolar depression in Scottish families even though the area (1q) is implicated in linkage studies (Devon et al. 2001).

Other genes include the gene NOTCH4, involved in neurodevelopment, which showed association to schizophrenia (Wei et al. 2000). Another gene, dysbindin (DTNBP1), showed association to the disease in Ireland and Germany (Harrison et al. 2003).

Finally, a new schizophrenia gene, PPP3CC, coding for calcineurin A γ-subunit was recently published (Gerber et al. 2003). The risk haplotype was present in 38% of the patients. Moreover, a mouse with another subunit of the calcineurin protein knocked out in the forebrain, showed antisocial behavior and a defect working memory (Gerber et al. 2003, Miyakawa et al. 2003).

Schizophrenia candidate genes in neurotransmitter systems

In addition to the genes above, there are candidate genes whose function implies a possible role in schizophrenia. Several neurotransmitter systems may be involved in the disease (Sawa et al. 2002). Here follows a brief description of the neurotransmitter systems implicated in schizophrenia.

Ligand binding studies show that schizophrenic patients have higher levels of dopamine binding sites than controls (Mackay et al. 1982). In addition, neuroleptics that are used to treat schizophrenia work as antagonists on dopamine receptors and drugs that enhance dopamine action, such as cocaine and amphetamine, can induce psychotic symptoms. Dopamine is also involved in motivation, which is lacking in chronic schizophrenia patients.

In addition, the serotonergic system might be involved in schizophrenia, as it is targeted by the atypical neuroleptics and the psychotic drug LSD. Moreover, the serotonergic system interact with the dopaminergic system and might exert an inhibitory control over mesocorticolimbic dopamine systems (Di Matteo et al. 2001). Serotonin is involved in mood and emotional behavior.

The glutamate system has also been implicated in schizophrenia, with the NMDA glutamate receptor as the major candidate. NMDA receptors are involved in memory and brain development (Mohn et al. 1999). Mice with a modified GRIN1 gene, which codes for the essential NDMA receptor subunit, show stereotypic behaviors that can be ameliorated by treatment with antipsychotic drugs that antagonize dopaminergic and serotonergic receptors (Mohn et al. 1999). The drug PCP (phenylcyclidine) works as a NMDA antagonist and produce symptoms similar to schizophrenia. PCP also affects GABA expression (Abe et al. 2000).

Nicotinic receptors have also been suggested to be involved in schizophrenia due to the high prevalence of smokers among patients and the relationship of nicotine to dopamine (Breese et al. 2000).

In addition to the neurotransmitter receptors mentioned above, other molecules involved in the activity and plasticity of neurotransmitters can play a role, such as transporters and enzymes involved in the synthesis and degradation.

Messenger RNA expression studies in schizophrenia

To understand how genes are affected in a disease, a common approach is to compare the mRNA levels between patients and controls. A differing mRNA level in a schizophrenia brain can be considered an endophenotype that can help to identify pathways involved in the disease. Since 1990, there has been a wealth of studies published with regards to screening for mRNA differences between schizophrenia patients and controls (See table 1 in paper III). Until the breakthrough of real-time RT-PCR and microarrays, the most common methods for mRNA quantification were in situ hybridization or competitive RT-PCR and usually a few selected candidate genes were screened for differences.

Recently, with the development of microarray technology expression, thousands of genes have been screened for their involvement in schizophrenia. These studies have detected several gene groups involved in diverse functions, particularly presynaptic proteins and myelin related genes (Mirnics et al. 2000, Hakak et al. 2001, Hof et al. 2002, Vawter et al. 2002). A recent study on a custom-made array, composed by schizophrenia candidate genes cDNA, found an up-regulation of several apoliprotein (APOL) genes, important in cholesterol transport (Mimmack et al. 2002). An additional recent paper verified changes of myelin and oligodendrocyte related genes in schizophrenia using differential display PCR, real-time RT-PCR and micro arrays (Tkachev et al. 2003).

Multiple genes have been reported to show significant differences between patients and controls. However, most studies, including microarray screenings, used an average sample size of ten patients compared with ten controls and methods with low dynamic range. Differences between patients and controls on microarrays need to be obtained with a sufficient number of replicate arrays and individuals (Yang et al. 2002) and then the differences should be validated using real-time RT-PCR (Chuaqui et al. 2002).

Finally, the possibility of brain-area specific effects needs to be considered when interpreting expression differences. It has been suggested that contradictory findings of expression differences in schizophrenia brain may be due to that the cortical pathology in schizophrenia is heterogeneous over different areas of the brain (Selemon 2001).

Method Issues

Before the discussion of the results obtained in the papers, here follows a description of some methodological issues related to real-time RT-PCR quantification of mRNA levels. First, the inhibition of the PCR by reverse transcription reagents is mentioned. Second, the choice of a reference to normalize for experimental variability is discussed. Third, the selection of a dilution series with known quantity, a standard series, is considered.

RT-inhibition of the signal in real-time PCR

The measured signal produced in real-time RT-PCR from a mRNA of a specific gene increase when a higher concentration of polyA+ RNA is used in the reverse transcription (RT) reaction. Despite this, the signal in real-time PCR decreases with an excess amount of the RT-reaction added into the real-time PCR reaction. This is due to the PCR inhibitory effect of several reagents in the RT reaction. The optimal dilution of a RT-reaction to be used for PCR was in our hands 1:8-9. Thus, a 20 µl RT-reaction was diluted to 160 µl and then aliquoted with 4 µl per replica plate. One RT-reaction would be enough for 40 replica plates to use in real-time PCR.

Normalization for experimental variability

Normalization is performed to adjust for variability in the total RNA quantity in each sample and for different reverse transcription efficiencies between samples. Measured mRNA quantity of a specific gene is normalized using a reference. The reference can be the total RNA quantity of the sample measured by a spectrophotometer, external synthetic RNA with known amount added in each sample, or reference genes present in the cDNA population of the sample.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD) and the beta-actin (ACTB) mRNA in addition to 12S and 18S rRNA are the classical normalization genes, used since Northern blot was introduced. Both ACTB and GAPD can be inadequate for several types of studies, because they have

been shown to vary between tissues and with treatment (Lee et al. 2002). However, the ACTB and GAPD gene expression are the most invariable housekeeping genes, as revealed in a microarray study comparing four different tissues (Lee et al. 2002). Ribosomal RNA is probably more stable but constitutes 85-90% of the total cellular RNA, thus a completely different order of abundance than mRNA, which constitutes 3-5% (Alberts et al. 1994). Moreover, the use of rRNA is not possible in mRNA enriched samples. Reference genes should always be tested for non-variability between groups or conditions compared. It is important to compare different reference genes and study if any would give unexpected results.

Reference genes can be measured in separate PCR reactions, as we did, or in the same tube as the target gene. The advantage of multiplex PCR is that the variability specific for each PCR will be similar in all amplicons. However, problems involve the need for multiple fluorescent fluorophores and risk of mutual interference of primers. Finally, when reference genes are used the PCR efficiency of the reference gene and candidate genes need to be similar when no DNA standard curve is used.

Selection of standard curve for real-time RT-PCR

The Ct value from real-time measurement can be transformed to starting template copy number, by using samples that contain a diluted series of known amount of target RNA or DNA, a standard curve. The RNA standard curve gives a measure of the efficiency in the RT and the PCR, while a DNA standard curve gives only a measure of the PCR step efficiency.

We used a genomic DNA standard to adjust for differences in PCR efficiency between different genes (primer sets), which is important when using reference genes. Threshold cycle (Ct) values were transformed into target amount using a standard dilution series. The standard dilution series gives the linear equation of the logarithm of the target amount to the Ct value, where the value of the negative slope depends on the PCR amplification efficiency (Ct = slope x logarithm of target amount + intercept). The resulting equation is called a standard curve.

In relative quantification (which focuses on differences between samples) any arbitrary quantity is sufficient for standards, (and thus also for target amount) as long as it reflects the differences between the different standard dilutions. The standard curve can give a rough estimate of the cDNA copy number for the gene measured, knowing the weight of a human genome and assuming that each genome contains one copy of every gene.

One risk of using a genomic DNA as a standard is that it might not amplify in the PCR with the same efficiency as the cDNA. Moreover, there

is a risk of the genomic DNA deteriorating with repeated use. Deterioration affects the starting point for the amplification and may result in a shift in the Ct value with repeated use. A more serious consequence of using a genomic standard is that all the primers have to be designed within exons, to be able to amplify a DNA sample. This increases the risk of DNA contamination and contamination decreases sensitivity of the assay.

One way to avoid the genomic standard in the future is to use a dilution series from a cDNA pool, which is obtained from the same tissue as the samples. The PCR efficiency can be obtained, knowing the relative quantities of the cDNA dilution series standard.

In addition, it is not necessary to include a standard curve in each PCR reaction if the amplification efficiency does not differ between individual PCR reactions for the same amplicon. The efficiency can be measured once for each amplicon and the copy number in subsequent measurements can be calculated from the Ct values using the standard curve equation.

Research Aims

The ultimate aim is to find mRNA expression differences in brains from patients suffering from schizophrenia as compared to brains from normal individuals, and to determine the specific mechanism that causes these changes.

The specific aim is to develop an accurate and powerful strategy to detect even small differences in brain mRNA levels between patients suffering from psychiatric disorders and controls.

Specific aims of the papers included

Paper I: To develop a strategy to detect small differences in mRNA expression in human brain autopsy samples, and to evaluate if mRNA levels in samples obtained post-mortem would reflect levels in the living brain.

Paper II: To evaluate inter-individual variability in mRNA expression in rat brain.

Paper III: To detect differences in mRNA expression levels in prefrontal cortex between schizophrenia patients and controls.

Paper IV: To develop a powerful statistical model to detect expression differences in human brain.

Paper VI: To investigate the mechanism responsible for the decrease in serotonin receptor 2C expression in schizophrenia brain.

Significance of the Present Investigation

Different expression levels of specific gene products can have major effects on brain function. Small shifts in expression levels of hormones, neurotransmitters, their transporters, their receptors and other neuron-specific mRNA have been proposed to be related to behavioral differences between individuals, as well as variable susceptibility to drugs.

Therefore, the identification of group specific differences in mRNA levels will add to the understanding of brain function, disease etiology, and can contribute to the search for new drug targets.

Present Investigation

Paper I. High-resolution quantification of specific mRNA levels in human brain autopsies and biopsies.

In paper I, we evaluated the possibility to detect small differences in mRNA expression in human brain autopsy samples. In addition, we compared mRNA measurements from samples from the living brain and post-mortem samples.

Methods

Expression levels were measured by real-time PCR quantification of reverse transcribed polyA+ RNA. The RNA was obtained from a set of 27 frontal cortex autopsies and from another set of 27 samples where 14 were autopsies and 13 were biopsies. The biopsies were in a few cases also from frontal cortex, but many were from parietal cortex and other cortical areas. Reproducibility of the real-time PCR assay, using the same primer set on the same cDNA, was high: 97-99 %.

The data obtained by real-time RT-PCR were 10-logarithmically transformed before analysis to obtain a normal distribution. Differences between groups were tested using ANCOVA analysis, using two reference genes, beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPD), as covariates.

The inter-individual variability for the power calculations were obtained by calculating the mean and standard deviation of the mRNA expression values, adjusted with the two covariates, for the 27 autopsies. Each individual adjusted mean was obtained in an ANCOVA analysis by assuming each replicate as an independent sample.

Results

The comparison of autopsies to biopsies showed a decrease in absolute quantity in autopsies, but the decrease was similar in all genes, and relative levels did not show any significant differences.

The power analysis yielded the number of individuals required in each group to detect a 1.5-fold to 10-fold difference between two groups given the variability seen in individual adjusted means. The sample size calculations indicated that at a power and a confidence of 95%, 22-221 individuals in each group would be necessary to detect a 1.5-fold difference (9-76 for a 2-fold difference), even after adjusting expression with covariates as ACTB and GAPD.

Discussion

We conclude that autopsies can be used to study the mRNA levels in the living brain. The finding of preserved relative mRNA levels in post-mortem brain is supported by a later study on mouse, using cDNA microarrays (Trotter et al. 2002). The correlation between frontal cortex autopsies and biopsies was present although the biopsies could be from other cortex areas than the frontal. The cortical origin is not highly important in this comparison, as the expression profile is grossly similar in most areas in human brain cortex (Watakabe et al. 2001).

We concluded that least 50 individuals in each group are needed to detect small (twofold) differences between two groups, using the same ANCOVA model. The genes with high inter-individual variability in mRNA levels are of interest when searching for common gene variants and normal expression variability involved in common complex diseases and drug response. Thus, a large sample size is crucial for this type of investigations.

Paper II. Analysis of gene expression in the rat hippocampus using Real Time PCR reveals high inter-individual variation in mRNA expression levels.

As described in the introduction, animal models can be used to study endophenotypes and treatments related to complex diseases such as schizophrenia. Rats are important for searching for mRNA expression and behavioral differences due to different environmental stimuli (Stevens et al. 1997) or drug treatment (Chong et al. 2002). Rats are usually from an inbred strain and are bred in a homogenous environment to reduce inter-individual variability. In paper II, we measured mRNA expression in rat brain to evaluate variability between individuals.

Methods

Whole hippocampi from twenty-nine male Sprague-Dawley rats were obtained and then homogenized. Total RNA and polyA+ RNA was prepared and reverse transcribed as described previously in paper I.

The cDNA levels were quantified using real-time PCR. In the method a gene-specific probe or a DNA intercalator, such as the SYBR green dye can be used to detect accumulation of PCR product. The two detection methods were compared and the results showed a high correlation (r=0.99) for the two genes tested. Thus, the unspecific intercalator is a satisfactory replacement for probe detection as long as there is a single PCR product. Gel pictures and melting profiles of completed PCR reactions indicated a single PCR product for all genes included in this study.

The data obtained by real-time RT-PCR was 10-logarithmically transformed before analysis to obtain a normal distribution. Gene expression values were adjusted using beta-actin (ACTB) expression values as a covariate in an ANCOVA. The adjusted means of individual duplicates was used for determining the sample size required to detect a difference between two groups.

Nine hippocampi were divided into two halves before RNA preparation to study the expression of two genes more closely. One side of the hippocampus was used to compare the results for the two genes between the methods Northern blot and the real-time RT-PCR assay. The two methods gave similar results for the nine individuals. The two sides from each

individual were also compared to each other in the SYBR green assay and showed no difference (not shown in paper).

Expression levels from thirty-seven genes were measured on 20 additional hippocampi samples using the SYBR green real-time RT-PCR. The 37 genes included 23 known genes and 14 Expressed Sequence Tags (ESTs)

Results

The measured mRNA quantities, adjusted with reference gene beta-actin indicated very different levels of expression between genes, with as much as a 28000-fold difference between the most extreme genes.

The variability between individuals was high, even after correction with reference gene ACTB. There could be a 20-fold difference between the extreme individuals in a highly variable gene. A power analysis with 95% power and confidence, indicates a sample size of 5-135 individuals in each group is required to detect a 1.5 fold difference (3-47 if a 2-fold difference) between two groups. Even if the most variable genes are removed, the required sample size still ranged from 3-30 individuals to be able to detect a 2-fold difference.

Discussion

Large differences were found between the normalized expression levels of the tested genes. However, the correction with beta-actin might not be equally optimal for all genes. Moreover, it is not possible to know if the variations in mRNA levels reflect different proportions of different cell types or the amount of gene activity. This makes comparisons between genes less adequate than sample comparisons for the same gene.

We found a large variability between individuals in mRNA levels. It is not likely that the variability observed is due to experimental variability, due to several reasons. The reasons are that the reference gene would normalize experimental differences between samples, there was no difference between two separate brain preparations from the same individual, and two different methods produced similar results.

We conclude that inter-individual variability is a large factor in mRNA measurements of rat brain. This is surprising since the rats are inbred strains and are raised in a homogenous environment. The cause for different mRNA levels between individual rats could be transient events preceding their death or it could be that there are differences in their environment or genes not assessed in this study. The inter-individual variability in mRNA levels is seldom assessed in rats, but a substantial inter-individual variation in mRNA

levels was found for an inbred strain of mice. (Pritchard et al. 2001). More rats than necessary should never be sacrificed, but small differences in mRNA obtained in a small sample size need to be considered cautiously.

Paper III. Decrease of Serotonin Receptor 2C in Schizophrenia Brains Identified by High-Resolution mRNA Expression Analysis

We tested sixteen genes for significantly different expression levels in prefrontal cortex for schizophrenia, as compared to controls.

There has been a wealth of studies published during the last ten years comparing mRNA levels from schizophrenia brains and controls, which are summarized in paper III. The previous studies used in situ hybridization and RT-PCR (in most cases not real-time) to quantify mRNA, with the majority focusing on the hippocampus or the prefrontal cortex. The sample size would normally be ten patients compared to ten controls. The study presented here used a more sensitive method and a larger sample size.

Methods

In the present study, 55 schizophrenia patients and 55 controls were included to study the expression of 16 selected genes. Several of the genes had previously been reported as differently expressed in schizophrenia. The prefrontal brain samples were homogenized, polyA+ RNA were prepared and reverse transcribed. The cDNA was then quantified using the sensitive real-time PCR method.

A novel statistical approach was used to evaluate the mRNA expression for the selected genes. A linear model of analysis of covariance (ANCOVA) was developed and it is described in detail in paper IV. The model allowed additive group effects for gender, brain bank, and replica plate (due to samples being reverse transcribed on three different plates) and linear statistical regression effects for age, post-mortem time, and the two reference genes. The model used logarithmic (base 10) expression data that had been averaged over the two duplicate samples per individual.

Results

Previously reported differences in mRNA expression levels were tested again in the present investigation. The genes were neurotrophic tyrosine kinase receptor, type 3 (NTRK3), reelin (RELN), synapsin II (SYN2),

NMDA ionotropic glutamate receptor (GRIN1), serotonin receptor 2A (HTR2A) and glutamate decarboxylase 1 (GAD1). We noted weak tendencies to expression differences in these genes that point in the same direction as previous studies. However, the differences were far from significant.

Schizophrenia patients showed a highly significant 1.5-fold decrease compared to controls for serotonin receptor 2C gene (HTR2C). The p-value for HTR2C was 0.0003 (and not the p-value 0.001 reported in paper III) using the ANCOVA analysis developed in paper IV. The HTR2C mRNA expression difference was confirmed with a repeat run with the same primers.

A significant 1.2-fold increase in monoamine oxidase B gene (MAOB), a dopamine and phenylethylamine-degrading enzyme was also found. The p-value for MAOB was 0.0005 (and not the p-value 0.001 reported in paper III) using the ANCOVA analysis developed in paper IV.

Two genes showed a less significant change in this study. One gene was the catechol-O-methyltransferase gene (COMT) (corrected p-value: 0.04). This gene codes for a dopamine, epinephrine, and norepinephrine-degrading enzyme. The other gene was the monoamine oxidase A gene (MAOA) (corrected p-value: 0.03) that codes for a serotonin and noradrenaline-degrading enzyme. The MAOA gene actually shows an increase in mRNA as MAOB also does, which could be due to a possible co-regulation of these genes. This hypothesis is further supported by the fact that the MAOA and MAOB genes are situated closely together on the X-chromosome.

Discussion

We could not confirm several previous findings of differential expression in schizophrenia. This could be due to differences in the populations of patients or in the brain areas within PFC studied, but it could also be related to a high inter-individual variability in gene expression. Discoveries of small changes in schizophrenia must be regarded cautiously when sample size is small, semi-quantitative methods are used and there is lack of replication.

To test the power of our strategy, the required sample size to detect a 1.5-fold difference, with a power and a confidence of 95%, was calculated for MAOB and HTR2C, although not included in the paper. The calculations were based on a conventional two-sided Students t-test with the variability estimates obtained from the 55 control residuals. The sample sizes required to detect a 1.5-fold difference were for the HTR2C gene 40 patients and 40 controls and for the MAOB gene 15 patients and 15 controls. The required sample sizes would have been larger if the added variability of the schizophrenia group would have been included in the model. The methods,

statistical analysis and larger sample size used here show promise to find small significant changes of expression between patients and controls.

The genes MAOA, MAOB, COMT and HTR2C that showed a differential mRNA expression levels in schizophrenia, may become stronger candidates as susceptibility genes for the disease and/or for the development of new therapeutic strategies. Linkage to schizophrenia have been reported to MAOB (Dann et al. 1997) while bipolar depression show linkage to Xq24-q27, the area of the HTR2C gene (Pekkarinen et al. 1995).

The HTR2C gene is of interest as several atypical neuroleptics used to treat schizophrenia have affinity for this receptor (Meltzer 1999). Moreover, it is shown to be involved in the regulation of mesocortical and mesolimbic release of dopamine (Di Matteo et al. 2001). These pathways are of great interest for schizophrenia as an increased activity of the mesocortical pathway and a decreased activity of the mesolimbic pathway could explain cognitive difficulties and psychotic symptoms respectively (Kandel 2000). Furthermore, DNA polymorphisms and RNA editing in the HTR2C gene have been associated with schizophrenia subtypes (Segman et al. 1997, Segman et al. 2000, Sodhi et al. 2001, Zhang et al. 2002).

Interestingly, recent papers support COMT, a dopamine-degrading enzyme, as a strong candidate gene for schizophrenia (Harrison et al. 2003) with a variant with decreased activity to be more frequent in schizophrenia patients (Bray et al. 2003). This indicates that the genes that in our analysis demonstrates a difference at the 0.05 significance level also may be of interest for schizophrenia.

The two dopamine-degrading enzymes COMT and MAOB do not show the same direction of change. The results here points to a decreased activity of parts of the serotonin system, with decreased levels of one serotonin receptor and the serotonin degrading enzyme MAOA. Nevertheless, in agreement with other studies (Harrison et al. 2003), these results indicate faulty monoaminergic synapses in schizophrenia.

Paper IV. Statistical methodology in case-control 5'-nuclease assays: Statistical design, modeling and inference for identification of differentially expressed genes

Paper IV describes the mathematical models developed for the mRNA expression data from brain, measured by real-time PCR quantification on reverse transcribed mRNA, followed by a 10-logarithm transformation. This thesis does not aim to cover the mathematical explanation of the formulas, but rather to briefly describe the statistical design.

Data source and formulas used

The data obtained for Paper I was used to determine the optimal statistical analysis to use in paper III. Further analysis was then performed on data obtained for Paper III. During the writing of this thesis, it was found that the formulas used for the analysis in paper III were slightly different from the ANCOVA formulas 7 – 10 and formula 13 in Paper IV. Therefore, all the analysis was corrected in this thesis. However, this does not change the conclusions of paper III. In fact, this resulted in more significant p-values for genes significant, but gave otherwise similar results. The p-values resulting from the corrected SAS formula are indicted in the summary for paper III.

Incomplete Balanced Block design

Before setting up measurement on the schizophrenia samples, statistical analysis and modeling was made on data from Paper I. From the data it was concluded there was much less variability between replicate samples from the same individual than between individuals. Precision between an estimate based on two rather than on three measurements differed negligible, so it was decided that duplicates rather than triplicates could be used.

By using duplicates, it was possible to make an almost completely balanced design of duplicate samples of the 110 individuals over three reverse transcription plates and this was used in Paper III. Patients were allocated to three different blocks with 18 individuals in each block (18x3=54), and a similar design was performed for controls. The two remaining individuals were included in two blocks (18+18+19=55). Each block would be present on two (creating duplicate measurements) of the three reverse transcription plates. Two patient blocks and two control blocks

were on each reverse transcription plate, in a different combination on each plate. The six blocks were equal in the composition with regard to sex and brain bank.

Data handling in paper III and IV

Missing data was replaced by zero, the 10th logarithm of one copy mRNA, which was assumed to be the absolute detection limit. Leaving out data points corresponding to absence of detected expression of analysis would cause left censoring in low-expressing genes.

Correlation of all measurements to reference genes were investigated and gross outliers removed before means of duplicates were calculated. The mean duplicate data without outliers was used for ANCOVA analysis, as this would give a more precise estimate of the individual expression level. To use duplicates instead of means of duplicates in analysis was not useful, as regression on reference genes was not same between individuals (i.e. for the averages) as between samples within individuals. As every individual two duplicates are on different plates, there could be plate effects on individual mean. However, this should not effect the average disease effect as patients and controls are balanced over the three plates.

A different fitting than in a conventional ANCOVA model

In a conventional ANCOVA model, means between groups are compared after removal of variability due to other variables. There is one important difference between a conventional ANCOVA model and the one constructed in paper IV and used in paper III.

The important modification is to fit the model on control data only. In other words, the values of the regression coefficients and the categorical coefficients are calculated from control data only. The patient expression level is then predicted from that model. A conventional model would also include patient data when fitting the model.

The modified ANCOVA assume that that a disease effect could be visible both as a constant disease effect similar in all patients (as in a conventional ANCOVA) or a differing disease effect, due to patient subgroups.

Test for significant different mean expression

The difference between each patient's observed and control-model predicted data is the prediction error. The error is primarily caused by random individual variation, but could also include a systematic disease effect. Individual controls also show random deviation from the control model, but

in this case, these deviations are referred to as residuals, rather than as prediction errors. The mean of the 55 control residuals is zero, as the control observed data is the data set used to fit the model.

A disease effect in a gene can be a mean difference between patients and controls (constant disease effect) and/or a disease effect in subgroups. The first effect is visible as a shift in the mean of patient prediction errors from zero (controls mean) and the second as a greater variability in the patient prediction errors compared to the variability in controls residuals.

In paper III, the mean prediction error in patients (constant disease effect) is tested for significance. A modified t-test for independent samples, where the standard deviation and the degrees of freedom (45) are obtained from the control model, was used to calculate the p-value for constant disease effect. No test for increased variability has been developed but a tendency of this is visible in the HTR2C and MAOB residuals/prediction errors (see paper IV).

Advantages of a control based model

The advantage in using this model is the greater power to detect differences between patients and controls due to subgroups in the patient group. We assumed that subgroups within the patient group would be a result of the heterogeneity of the disease and drug treatment, and therefore we argue that the modified test would be more useful than a conventional ANCOVA.

The papers III and IV test only for the constant disease effect (formula 7 – 10 and formula 13). However, the p-values from these tests will be more sensitive to differences due to subgroups as the variability from those groups are not allowed to influence the inter-individual variability estimate. A conventional ANCOVA was also performed on data for paper III, and the p-values for HTR2C and MAOB were larger, (especially when compared to the corrected p-values: 0.0003 and 0.0005) but still highly significant (p-values: 0.002 and 0.006).

Since the modified model is based on the 55 control individuals rather than the full sample size of 110, the inter-individual variability estimate used to calculate the p-value will be less precise. However, since the control group is large the loss in precision was in practice negligible.

Interaction effects on expression

A disease subgroup effect can sometimes be identified as an interaction effect with other variables in the data set.

Interaction effect is a subgroup effect, where subgroups are identified by another variable in the model (as for example sex or reference gene) will show a different disease effect than other subgroups. One example of

interaction would be if only female patients show a decreased expression compared to controls. Another example would be if only patients with high expression values, i.e. with high measurements of reference gene, would show a disease effect, something that could be caused by limitations of the quantification method.

Interaction effects with other variables were explored for serotonin receptor 2C expression using a conventional ANCOVA model and the results can be found in the discussion of Paper V.

MAOB and HTR2C are significantly different

The two genes monoamine oxidase B (MAOB) and serotonin receptor 2C (HTR2C) were found to be significantly different in their mean expression compared to controls, using the t-test statistics on prediction errors obtained by the ANCOVA type model described above.

The serotonin receptor gene showed a slightly larger mean difference (1.5-fold see paper III) than MAOB (1.2 fold). HTR2C had larger variability than MAOB and lower measured levels of mRNA, with several individuals below the detection limit.

It is likely, however, that the two genes are co-regulated, probably in concert with many other genes, maybe preferentially genes active in monoaminergic synapses. Interestingly, MAOB and HTR2C residuals showed no correlation in controls ($r=-0.02$) but their prediction errors showed a correlation in patients ($r=-0.45$). This indicates that patients that express MAOB mRNA at high levels are expressing HTR2C mRNA at low levels, while controls do not have this correlation.

Paper V. Serotonin receptor 2C (HTR2C) and schizophrenia: effect of medication and genetic variants on expression levels.

We found a 1.5-fold decrease in serotonin 2C expression in schizophrenia. Patients are treated with antipsychotic medication and any found difference between patients and controls in mRNA levels can be due to that treatment. Therefore, we evaluated HTR2C expression levels between different drug-treatment groups, including a group of untreated patients.

Next, we studied single nucleotide polymorphisms in the HTR2C promoter, since some variants can affect expression levels (Yuan et al. 2000). The HTR2C gene is located on the X-chromosome (Xq24). As males only have one allele of X-chromosome genes, it is possible to know which allele is affecting expression for those individuals.

Methods

We measured HTR2C expression in 55 control and 55 schizophrenia prefrontal cortex samples. The mRNA levels were measured by the real-time RT-PCR method and were then 10-logarithmically transformed. The data was then analyzed using a modified ANCOVA, which is described in paper IV. The residuals from this model were used as a measure of individual expression level in paper V. These expression levels are normalized for differences due to several variables, including total mRNA quantity among others.

The patients were divided into four drug-groups; patients treated with atypical neuroleptics, typical neuroleptics, with unknown treatment, and those untreated at death. Eleven patients were untreated at the time of death. The mean expression level of the drug-groups were compared with each other (ANOVA) and to controls (modified ANCOVA model).

Three single nucleotide polymorphisms (SNPs) were genotyped using a DASH-assay and confirmed by sequencing. The three SNPs are located at positions –666, -458, and -292 nucleotides from the major transcription start of the HTR2C gene (Xie et al. 1996). Haplotypes were built by hand. Haplotype frequencies between patients and controls were tested for differences (chi-square). Furthermore, the mean expression levels of the four most common haplotype combinations were compared to each other (ANOVA).

Results

There was no significant (p-value: 0.4) difference between the HTR2C expression levels between the three drug groups; patients treated with typical neuroleptics, atypical neuroleptics or untreated patients. All drug groups showed a significant decrease compared to controls. The untreated group showed a 1.6-fold decrease (p-value: 0.01). The group that received atypical neuroleptics showed a 2-fold decrease (p-value: 0.002) and the group that received typical neuroleptics presented a 1.3-fold decrease (p-value: 0.05).

The HTR2C promoter haplotype frequencies did not reveal any significant differences between patients and controls (p-value: 0.6). Furthermore, the analysis of mean mRNA expression levels in the different haplotype groups failed to indicate significant differences between them (p-value: 0.4).

Discussion

As discussed in paper IV, it is possible that the observed decrease in HTR2C mRNA could be due to subgroups of patients identified by other variables in the data set, i.e. interaction. We analyzed the diagnosis effect in the HTR2C data for possible interaction with other variables in the data set, but did not include the results in paper V. No interaction effects on the HTR2C expression were detected between diagnosis and other variables, including sex, age, brain bank, or reference genes. Therefore, we conclude that subgroups defined by these variables cannot explain the decrease seen in schizophrenia patients. The variable brain bank shows an independent effect on HTR2C gene expression in both patients and controls. However, the bank-effect was accounted for by the ANCOVA-type model used, and will not affect the haplotype and drug effects tested in paper V.

The effect of atypical neuroleptics on HTR2C expression is of interest. These drugs produce different effects on schizophrenia symptoms, they present different side effects and several atypical drugs bind to HTR2C receptors while typical drugs do not (Meltzer 1999, Kapur et al. 2001, Wetterling 2001, Leucht et al. 2003). The results obtained indicate a larger decrease in patients treated with atypical drugs. However, no significant difference was obtained. If such difference exists it could indicate that the HTR2C decrease is a desired response to the changes in the schizophrenia brain absent in typical treated patients or it might indicate that the patients with low HTR2C expression belong to a group that benefits the most from atypical drug treatment.

More importantly, the decrease in HTR2C mRNA was present in patients untreated at death. This suggests that the HTR2C mRNA decrease to be

present in patients before medication and thus it should indicate a pathway affected in schizophrenia patients.

However, whether the HTR2C decrease is a cause to or an effect from the changes in the schizophrenia brain remain unclear. If it is causative, the HTR2C gene should be a schizophrenia gene of small effect as no X-linked inheritance pattern is seen in schizophrenia (See OMIM #181500). Since there was no significant association with the polymorphisms studied here and the disease, we have not obtained any support for the HTR2C mRNA decrease to be a causative change in schizophrenia. The results rather support the hypothesis of the HTR2C mRNA decrease to be an effect. The correlation of HTR2C decrease with MAOB increase in patients, but not controls, also supports this hypothesis, as this indicates that the difference seen is an effect on the monoaminergic system, rather than pointing out a single gene as more important.

However, it is still possible that common variants in the HTR2C gene are involved in the schizophrenia pathology and confer susceptibility to intermediate phenotypes of schizophrenia. These phenotypes could be related to drug response or symptoms, such as cognitive deficits. Previous studies indicate association between such symptoms and schizophrenia (Sodhi et al. 1995, Segman et al. 1997, Segman et al. 2000, Zhang et al. 2002). The HTR2C gene might also play a role in other psychiatric disorders as there is linkage to Xq24-q27 in a study on bipolar depression (Pekkarinen et al. 1995).

In conclusion, the HTR2C gene is of interest to schizophrenia disease etiology and drug treatment, but it is probably a gene of small effect and a co-player with many other genes. The identification of additional co-players could be performed using expression microarrays.

Future Perspectives

Exploration of expression differences

We found small but significant differences in gene expression levels for four genes involved in monoaminergic synapses in paper III. The genes were the serotonin receptor 2C gene (HTR2C), the monoamine oxidase A gene (MAOA), the monoamine oxidase B gene (MAOB), and the gene coding for catechol-O-methyltransferase (COMT). To confirm a difference, replica PCR measurements can be made using the same primers, and in the next step using other primers, to confirm that the change is gene specific. In addition, different splice variants can be explored. Furthermore, local RNA differences can be studied by in-situ hybridization. In addition, protein levels, modifications, activity, and interactions can be investigated (Phizicky et al. 2003).

We evaluated in paper V the possibility that polymorphisms in the HTR2C gene may be associated with HTR2C gene expression and schizophrenia. No such association was found but other genes that demonstrated expression differences could be studied in a similar way. It is also possible that the HTR2C gene promoter contain polymorphisms, not genotyped in our sample, that show association to schizophrenia. Further exploration on the HTR2C gene could involve study of the co-regulation with other genes, especially those involved in the monoaminergic synapses.

Future candidate gene selection on DNA microarray

In this thesis, we used a candidate gene approach where the selection of genes was made from the literature. In the future, such selection will be done using the same samples in a microarray study. This project is underway comparing patient pooled RNA to controls pooled RNA. Pooling was necessary to obtain a sufficient amount RNA to use for the 3 to 5 replicate array hybridizations necessary for adequate precision of measurement. Patient RNA samples are pooled according to the medication received,

including atypical neuroleptic users, typical neuroleptic users, and untreated patients as described in paper V. This strategy allows the evaluation of changes related to drug treatment. Once these changes have been found it can be validated on real-time PCR, using the cDNA replica plates from 110 separate individuals the same way as described in paper III and paper V.

Concluding Remarks

The complex mRNA patterns of the living brain

The assumption that there are long-lasting mRNA levels associated to a disorder may not hold. The mRNA levels might be different from controls only at particular phases of a disorder or only in response to specific stimuli. The mRNA levels also vary between different cells and between different areas of the brain. It is likely that a complex disease, such as schizophrenia, resulting from a complex interplay of genes and environment, will have complex temporal and spatial mRNA expression patterns.

Therefore, there are two specific limitations associated with measuring mRNA levels in a brain tissue sample. The first is that the results needs to be validated with methods such as in situ hybridization, to study the localization of the expression difference in the brain (Chuaqui et al. 2002). The second limitation is that the mRNA levels might not reflect a constant steady-state level, but a temporary level, reflecting the situation of the individual at the time of death.

It is possible to obtain tissue specimens during different phases of disorders that affect non-vital organs, but not for those that affect the brain. An alternative to obtain tissue specimens is to study the brain at work by non-invasive imaging methods, such as fMRI (functional Magnetic Resonance Imaging) or PET scan (Positron Emission Tomography) (Blasberg et al. 2003). These methods can study the blood flow and the sugar metabolism, in addition to reporter gene expression in the living brain (Blasberg et al. 2003). However, monitoring mRNA expression of natural genes in the working human brain is still in the distant future.

We addressed the problem of post-mortem samples by comparing the mRNA levels of biopsies to autopsies. The results showed that the relative levels were well conserved. However, it is worth mentioning that the biopsies showed higher variability than the autopsies. This can be due to mRNA being more variable in a living brain, and/or to the biopsies being taken in a more variable manner than the autopsies, as they are removed during surgery for different conditions such as epilepsy or brain tumors.

The possibility remains that there are steady state differences in mRNA levels between schizophrenia patients and controls. Therefore, given a sensitive and reliable method of measurement, a powerful statistical approach and a sufficient number of samples, these differences might be detected. The strategy developed in paper I and IV has the potential of meeting these standards.

Drug treatment effects on gene expression

One major difference between patients and controls is that patients are treated with antipsychotic medication. These drugs affect the brain, including the mRNA expression. Information on medication and especially on untreated patients, as we had the opportunity to investigate in the final paper, makes it possible to draw conclusions about which expression changes are related to the disease but not to the drug treatment. Moreover, mRNA levels in non-schizophrenic patients treated with neuroleptics can be studied. This information can be complemented by studying mRNA levels in rats treated with neuroleptics.

Inter-individual variability in gene expression

The search for disease genes has, to a large degree, focused on changes in coding sequences that would modify the function of the translated protein. Equally important, the non-coding DNA has gained focus during the last years, when it was realized that the total amount of genes (about 30.000) in the human genome was smaller than previously expected (Venter et al. 2001). The function of most non-coding DNA is unknown, but one possibility is that a major part is involved in the regulation of gene activity. Known cis-regulatory regions (regions that control expression of genes on the same chromosome) in the human genome have been found to be highly polymorphic (Stephens et al. 2001) and in many cases affecting gene expression (Rockman et al. 2002). Regulatory DNA variation might also have a prominent role for psychiatric traits, as exemplified by associations

between regulatory polymorphisms in the serotonin transporter gene and the monoamine oxidase A gene to anxiety and aggression (Hamer 2002).

Interestingly, a recent microarray study on lymphoblastoid cells found that there is a substantial inter-individual variability in human gene expression, and that the expression levels show familial aggregation (Cheung et al. 2003). The presence of a high inter-individual variability in human brain mRNA levels, presented in paper I, support this report. Therefore, the inter-individual variability needs to be considered in searching for expression differences between groups. Finally, it is likely that normal inter-individual expression variability of genes might provide the key to common complex disorders. The brain mRNA expression levels could serve as an endophenotype and thus help to identify the possibly large amount of regulatory polymorphisms involved in the complex diseases of the brain.

Acknowledgements

In particular I wish to thank:

The many people in the USA and the UK that donated their postmortem brains to science and thus made my research projects possible. Thank you for your confidence.

The Beijer foundation for financing my PhD position the first years.
The Evolutionary Biology dept for financing the last year

My supervisor Elena Jazin, for giving me the chance to learn research, psychiatric genetics, statistics, and a lot more. Moreover, thanks for always making me look my best at presentations with an incessant training.

Professor Ulf Landegren for giving me the chance to start at the department of genetics and pathology.
Professor Ulf Pettersson for giving advice and help before and during the break.
Professor Hans Ellergren for accepting me in the final year.
Professor Rolf Sundberg for providing the statistical knowledge and models and for answering statistical questions.

Lina Emilsson, for the work made on shared papers and for being cool; Karolina Åberg, for her kindness and the shared passion for knowledge; HongXing Zhao, for being the one that more than anyone else convinced me to go back; Eva Lindholm, for introducing me into the group and being my email support from NY; to Peter Saetre for help with SAS and statistics; to Julia Lindberg, for fun talks; Fredrik Granberg, for lab music; Jorune Balciuniene, for fun observations; Shane McCarthy for inspirational help; Jenny and Pelle, for fun conversations; Niklas Nordquist, for computer help, Ludmila Elfineh for coffee talks and labsupport.

I also wish to thank, all the numerous nice and helpful people at the department of Genetics and Pathology and dept of Evolution Biology. In particular I wish to thank; Jeanette Backman, Elisabeth Sandberg, Gunilla Tibbling, Eddie Lundh, Tommy, Rose-Marie Andersson, and Nisse. A great thanks to the nice bunch, no one named no one forgotten at our corridor at EBC.

Thank you AnhNhi, Ulrika S, Anette, and Kicki, for sharing the troubles of being a Ph.D. student.
Also special thanks to all the helpful people in the SLE group and the padlock group at Rudbeck as well to the people at the genome sequencing group at KI.

And thanks to William Jiang and Stevey Hammarsten, for technical help with hearing aids. To the old Knivsta gang, Matti, Helene, Minna, and Kattis for being a gang of extraordinary and impressive women. Especially Karin, for good company. All friends for company and support, sometimes by email. Natalie and Fredrik for pub talks and party help.
Mum, for convincing the doctors I wasn't brain damaged but partly deaf when I could not learn to speak as a kid and for everything else. Dad, for always believing me to be very smart. Klara, for being the best little sister;
And Henrik, for making me what I want to be: Happy.

References

Abe, S., T. Suzuki, T. Ito, A. Baba, T. Hori, et al. (2000). "Differential expression of GABA(A) receptor subunit mRNAs and ligand binding sites in rat brain following phencyclidine administration." Synapse **38**(1): 51-60.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, et al. (1994). Chapter 8. The Cell Nucleus: RNA Synthesis and RNA Processing. Molecular Biology of the Cell. R. Adams. New York, Garland Publishing**:** 369.

Andreasen, N. C. (1995). "Symptoms, signs, and diagnosis of schizophrenia." Lancet **346**(8973): 477-81.

Arranz, M. J., J. Munro, J. Birkett, A. Bolonna, D. Mancama, et al. (2000). "Pharmacogenetic prediction of clozapine response." Lancet **355**(9215): 1615-6.

Bird, I. M. (1998). "Size separation and quantification of mRNA by northern analysis." Methods Mol Biol **105**: 325-36.

Blasberg, R. G. and J. G. Tjuvajev (2003). "Molecular-genetic imaging: current and future perspectives." J Clin Invest **111**(11): 1620-9.

Bray, N. J., P. R. Buckland, N. M. Williams, H. J. Williams, N. Norton, et al. (2003). "A haplotype implicated in schizophrenia susceptibility is associated with reduced COMT expression in human brain." Am J Hum Genet **73**(1): 152-61.

Breese, C. R., M. J. Lee, C. E. Adams, B. Sullivan, J. Logel, et al. (2000). "Abnormal regulation of high affinity nicotinic receptors in subjects with schizophrenia." Neuropsychopharmacology **23**(4): 351-64.

Bunney, W. E. and B. G. Bunney (2000). "Evidence for a compromised dorsolateral prefrontal cortical parallel circuit in schizophrenia." Brain Res Brain Res Rev **31**(2-3): 138-46.

Bunney, W. E., B. G. Bunney, M. P. Vawter, H. Tomita, J. Li, et al. (2003). "Microarray technology: a review of new strategies to discover candidate vulnerability genes in psychiatric disorders." Am J Psychiatry **160**(4): 657-66.

Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." J Mol Endocrinol **25**(2): 169-93.

Cannon, T. D., P. M. Thompson, T. G. van Erp, A. W. Toga, V. P. Poutanen, et al. (2002). "Cortex mapping reveals regionally specific patterns of genetic and disease-specific gray-matter deficits in twins discordant for schizophrenia." Proc Natl Acad Sci U S A **99**(5): 3228-33.

Cheung, V. G., L. K. Conlin, T. M. Weber, M. Arcaro, K. Y. Jen, et al. (2003). "Natural variation in human gene expression assessed in lymphoblastoid cells." Nat Genet **33**(3): 422-5.
Chong, V. Z., L. T. Young and R. K. Mishra (2002). "cDNA array reveals differential gene expression following chronic neuroleptic administration: implications of synapsin II in haloperidol treatment." J Neurochem **82**(6): 1533-9.
Chuaqui, R. F., R. F. Bonner, C. J. Best, J. W. Gillespie, M. J. Flaig, et al. (2002). "Post-analysis follow-up and validation of microarray experiments." Nat Genet **32**(6): 509-14.
Chumakov, I., M. Blumenfeld, O. Guerassimenko, L. Cavarec, M. Palicio, et al. (2002). "Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia." Proc Natl Acad Sci U S A **99**(21): 13675-80.
Dann, J., L. E. DeLisi, M. Devoto, S. Laval, D. J. Nancarrow, et al. (1997). "A linkage study of schizophrenia to markers within Xp11 near the MAOB gene." Psychiatry Res **70**(3): 131-43.
DeLisi, L. E., S. H. Shaw, T. J. Crow, G. Shields, A. B. Smith, et al. (2002). "A genome-wide scan for linkage to chromosomal regions in 382 sibling pairs with schizophrenia or schizoaffective disorder." Am J Psychiatry **159**(5): 803-12.
Devon, R. S., S. Anderson, P. W. Teague, P. Burgess, T. M. Kipari, et al. (2001). "Identification of polymorphisms within Disrupted in Schizophrenia 1 and Disrupted in Schizophrenia 2, and an investigation of their association with schizophrenia and bipolar affective disorder." Psychiatr Genet **11**(2): 71-8.
Di Matteo, V., A. De Blasi, C. Di Giulio and E. Esposito (2001). "Role of 5-HT(2C) receptors in the control of central dopamine function." Trends Pharmacol Sci **22**(5): 229-32.
Duggan, D. J., M. Bittner, Y. Chen, P. Meltzer and J. M. Trent (1999). "Expression profiling using cDNA microarrays." Nat Genet **21**(1 Suppl): 10-4.
Evans, K. L., W. J. Muir, D. H. Blackwood and D. J. Porteous (2001). "Nuts and bolts of psychiatric genetics: building on the Human Genome Project." Trends Genet **17**(1): 35-40.
Freeman, W. M., S. J. Walker and K. E. Vrana (1999). "Quantitative RT-PCR: pitfalls and potential." Biotechniques **26**(1): 112-22, 124-5.
Fuller Torrey, E. and R. H. Yolken (2000). "Familial and genetic mechanisms in schizophrenia." Brain Res Brain Res Rev **31**(2-3): 113-7.
Gaddis, M. L. (1998). "Statistical methodology: IV. Analysis of variance, analysis of covariance, and multivariate analysis of variance." Acad Emerg Med **5**(3): 258-65.
Geddes, J., P. Harrison, N. Freemantle and L. D. Selemon (2003). "New generation versus conventional antipsychotics." Lancet **362**(9381): 404-5.

Gerber, D. J., D. Hall, T. Miyakawa, S. Demars, J. A. Gogos, et al. (2003). "Evidence for association of schizophrenia with genetic variation in the 8p21.3 gene, PPP3CC, encoding the calcineurin gamma subunit." Proc Natl Acad Sci U S A **100**(15): 8993-8.
Gibson, U. E., C. A. Heid and P. M. Williams (1996). "A novel method for real time quantitative RT-PCR." Genome Res **6**(10): 995-1001.
Goldman-Rakic, P. S. and L. D. Selemon (1997). "Functional and anatomical aspects of prefrontal pathology in schizophrenia." Schizophr Bull **23**(3): 437-58.
Gottesman, II and T. D. Gould (2003). "The endophenotype concept in psychiatry: etymology and strategic intentions." Am J Psychiatry **160**(4): 636-45.
Grunenfelder, B., G. Rummel, J. Vohradsky, D. Roder, H. Langen, et al. (2001). "Proteomic analysis of the bacterial cell cycle." Proc Natl Acad Sci U S A **98**(8): 4681-6.
Hafner, H., K. Maurer, W. Loffler, W. an der Heiden, P. Munk-Jorgensen, et al. (1998). "The ABC Schizophrenia Study: a preliminary overview of the results." Soc Psychiatry Psychiatr Epidemiol **33**(8): 380-6.
Hakak, Y., J. R. Walker, C. Li, W. H. Wong, K. L. Davis, et al. (2001). "Genome-wide expression analysis reveals dysregulation of myelination- related genes in chronic schizophrenia." Proc Natl Acad Sci U S A **98**(8): 4746-51.
Hamer, D. (2002). "Genetics. Rethinking behavior genetics." Science **298**(5591): 71-2.
Harding, C. M., J. Zubin and J. S. Strauss (1992). "Chronicity in schizophrenia: revisited." Br J Psychiatry Suppl(18): 27-37.
Harrison, P. J. and M. J. Owen (2003). "Genes for schizophrenia? Recent findings and their pathophysiological implications." Lancet **361**(9355): 417-9.
Herrick-Davis, K., E. Grinde and M. Teitler (2000). "Inverse agonist activity of atypical antipsychotic drugs at human 5-hydroxytryptamine2C receptors." J Pharmacol Exp Ther **295**(1): 226-32.
Hof, P. R., V. Haroutunian, C. Copland, K. L. Davis and J. D. Buxbaum (2002). "Molecular and cellular evidence for an oligodendrocyte abnormality in schizophrenia." Neurochem Res **27**(10): 1193-200.
Kandel, E. R. (2000). Disorders of Thought and Volition: Schizophrenia. Principles of Neural Science. E. R. Kandel, J. H. Schwartz and T. M. Jessell. International edition, McGraw-Hill**:** 1206.
Kane, J., G. Honigfeld, J. Singer and H. Meltzer (1988). "Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine." Arch Gen Psychiatry **45**(9): 789-96.
Kapur, S. and G. Remington (2001). "Atypical antipsychotics: new directions and new challenges in the treatment of schizophrenia." Annu Rev Med **52**: 503-17.
Karlsson, H., S. Bachmann, J. Schroder, J. McArthur, E. F. Torrey, et al. (2001). "Retroviral RNA identified in the cerebrospinal fluids and

brains of individuals with schizophrenia." Proc Natl Acad Sci U S A **98**(8): 4634-9.
Kato, C., A. Petronis, Y. Okazaki, M. Tochigi, T. Umekage, et al. (2002). "Molecular genetic studies of schizophrenia: challenges and insights." Neurosci Res **43**(4): 295-304.
Kawanishi, Y., H. Tachikawa and T. Suzuki (2000). "Pharmacogenomics and schizophrenia." Eur J Pharmacol **410**(2-3): 227-241.
Kirch, D. G. (1993). "Infection and autoimmunity as etiologic factors in schizophrenia: a review and reappraisal." Schizophr Bull **19**(2): 355-70.
Klein, D. (2002). "Quantification using real-time PCR technology: applications and limitations." Trends Mol Med **8**(6): 257-60.
Lancet (1989). "Clozapine." Lancet **2**(8677): 1430-2.
Lee, P. D., R. Sladek, C. M. Greenwood and T. J. Hudson (2002). "Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies." Genome Res **12**(2): 292-7.
Lesch, K. P. (2001). "Variation of serotonergic gene expression: neurodevelopment and the complexity of response to psychopharmacologic drugs." Eur Neuropsychopharmacol **11**(6): 457-74.
Leucht, S., K. Wahlbeck, J. Hamann and W. Kissling (2003). "New generation antipsychotics versus low-potency conventional antipsychotics: a systematic review and meta-analysis." Lancet **361**(9369): 1581-9.
Lockhart, D. J. and E. A. Winzeler (2000). "Genomics, gene expression and DNA arrays." Nature **405**(6788): 827-36.
Mackay, A. V., L. L. Iversen, M. Rossor, E. Spokes, E. Bird, et al. (1982). "Increased brain dopamine and dopamine receptors in schizophrenia." Arch Gen Psychiatry **39**(9): 991-7.
McCarley, R. W., C. G. Wible, M. Frumin, Y. Hirayasu, J. J. Levitt, et al. (1999). "MRI anatomy of schizophrenia." Biol Psychiatry **45**(9): 1099-119.
McGuffin, P., M. J. Owen and A. E. Farmer (1995). "Genetic basis of schizophrenia." Lancet **346**(8976): 678-82.
Meltzer, H. Y. (1999). "The role of serotonin in antipsychotic drug action." Neuropsychopharmacology **21**(2 Suppl): 106S-115S.
Millar, J. K., J. C. Wilson-Annan, S. Anderson, S. Christie, M. S. Taylor, et al. (2000). "Disruption of two novel genes by a translocation co-segregating with schizophrenia." Hum Mol Genet **9**(9): 1415-23.
Mimmack, M. L., M. Ryan, H. Baba, J. Navarro-Ruiz, S. Iritani, et al. (2002). "Gene expression analysis in schizophrenia: reproducible up-regulation of several members of the apolipoprotein L family located in a high-susceptibility locus for schizophrenia on chromosome 22." Proc Natl Acad Sci U S A **99**(7): 4680-5.
Mirnics, K., F. A. Middleton, D. A. Lewis and P. Levitt (2001). "Analysis of complex brain disorders with gene expression microarrays:

schizophrenia as a disease of the synapse." Trends Neurosci **24**(8): 479-86.
Mirnics, K., F. A. Middleton, A. Marquez, D. A. Lewis and P. Levitt (2000). "Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex." Neuron **28**(1): 53-67.
Miyakawa, T., L. M. Leiter, D. J. Gerber, R. R. Gainetdinov, T. D. Sotnikova, et al. (2003). "Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia." Proc Natl Acad Sci U S A **100**(15): 8987-92.
Mohn, A. R., R. R. Gainetdinov, M. G. Caron and B. H. Koller (1999). "Mice with reduced NMDA receptor expression display behaviors related to schizophrenia." Cell **98**(4): 427-36.
Mortensen, P. B., C. B. Pedersen, T. Westergaard, J. Wohlfahrt, H. Ewald, et al. (1999). "Effects of family history and place and season of birth on the risk of schizophrenia [see comments]." N Engl J Med **340**(8): 603-8.
Murphy, K. C. (2002). "Schizophrenia and velo-cardio-facial syndrome." Lancet **359**(9304): 426-30.
Peccoud, J. and C. Jacob (1996). "Theoretical uncertainty of measurements using quantitative polymerase chain reaction." Biophys J **71**(1): 101-8.
Pekkarinen, P., J. Terwilliger, P. E. Bredbacka, J. Lonnqvist and L. Peltonen (1995). "Evidence of a predisposing locus to bipolar disorder on Xq24-q27.1 in an extended Finnish pedigree." Genome Res **5**(2): 105-15.
Phizicky, E., P. I. Bastiaens, H. Zhu, M. Snyder and S. Fields (2003). "Protein analysis on a proteomic scale." Nature **422**(6928): 208-15.
Pritchard, C. C., L. Hsu, J. Delrow and P. S. Nelson (2001). "Project normal: defining normal variance in mouse gene expression." Proc Natl Acad Sci U S A **98**(23): 13266-71.
Regier, D. A., C. T. Kaelber, M. T. Roper, D. S. Rae and N. Sartorius (1994). "The ICD-10 clinical field trial for mental and behavioral disorders: results in Canada and the United States." Am J Psychiatry **151**(9): 1340-50.
Rockman, M. V. and G. A. Wray (2002). "Abundant raw material for cis-regulatory evolution in humans." Mol Biol Evol **19**(11): 1991-2004.
Sawa, A. and S. H. Snyder (2002). "Schizophrenia: diverse approaches to a complex disease." Science **296**(5568): 692-5.
Schultz, S. K., D. D. Miller, S. E. Oliver, S. Arndt, M. Flaum, et al. (1997). "The life course of schizophrenia: age and symptom dimensions." Schizophr Res **23**(1): 15-23.
Seeman, P. (2002). "Atypical antipsychotics: mechanism of action." Can J Psychiatry **47**(1): 27-38.

Segman, R. H., R. P. Ebstein, U. Heresco-Levy, M. Gorfine, M. Avnon, et al. (1997). "Schizophrenia, chronic hospitalization and the 5-HT2C receptor gene." Psychiatr Genet **7**(2): 75-8.
Segman, R. H., U. Heresco-Levy, B. Finkel, R. Inbar, T. Neeman, et al. (2000). "Association between the serotonin 2C receptor gene and tardive dyskinesia in chronic schizophrenia: additive contribution of 5-HT2Cser and DRD3gly alleles to susceptibility." Psychopharmacology (Berl) **152**(4): 408-13.
Selemon, L. D. (2001). "Regionally diverse cortical pathology in schizophrenia: clues to the etiology of the disease." Schizophr Bull **27**(3): 349-77.
Seong, E., A. F. Seasholtz and M. Burmeister (2002). "Mouse models for psychiatric disorders." Trends Genet **18**(12): 643-50.
Sodhi, M. S., M. J. Arranz, D. Curtis, D. M. Ball, P. Sham, et al. (1995). "Association between clozapine response and allelic variation in the 5-HT2C receptor gene." Neuroreport **7**(1): 169-72.
Sodhi, M. S., P. W. Burnet, A. J. Makoff, R. W. Kerwin and P. J. Harrison (2001). "RNA editing of the 5-HT(2C) receptor is reduced in schizophrenia." Mol Psychiatry **6**(4): 373-9.
Spear, L. P. (2000). "The adolescent brain and age-related behavioral manifestations." Neurosci Biobehav Rev **24**(4): 417-63.
Stefansson, H., J. Sarginson, A. Kong, P. Yates, V. Steinthorsdottir, et al. (2003). "Association of neuregulin 1 with schizophrenia confirmed in a Scottish population." Am J Hum Genet **72**(1): 83-7.
Stephens, J. C., J. A. Schneider, D. A. Tanguay, J. Choi, T. Acharya, et al. (2001). "Haplotype variation and linkage disequilibrium in 313 human genes." Science **293**(5529): 489-93.
Stevens, K. E., R. G. Johnson and G. M. Rose (1997). "Rats reared in social isolation show schizophrenia-like changes in auditory gating." Pharmacol Biochem Behav **58**(4): 1031-6.
Tkachev, D., M. L. Mimmack, M. M. Ryan, M. Wayland, T. Freeman, et al. (2003). "Oligodendrocyte dysfunction in schizophrenia and bipolar disorder." The Lancet **362**(9386): 798-805.
Torrey, E. F. (2002). "Studies of individuals with schizophrenia never treated with antipsychotic medications: a review." Schizophr Res **58**(2-3): 101-15.
Trotter, S. A., L. B. Brill, 2nd and J. P. Bennett, Jr. (2002). "Stability of gene expression in postmortem brain revealed by cDNA gene array analysis." Brain Res **942**(1-2): 120-3.
Tsuang, M. (2000). "Schizophrenia: genes and environment." Biol Psychiatry **47**(3): 210-20.
Vawter, M. P., J. M. Crook, T. M. Hyde, J. E. Kleinman, D. R. Weinberger, et al. (2002). "Microarray analysis of gene expression in the prefrontal cortex in schizophrenia: a preliminary study." Schizophr Res **58**(1): 11-20.

Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, et al. (2001). "The sequence of the human genome." Science **291**(5507): 1304-51.

Watakabe, A., T. Sugai, N. Nakaya, K. Wakabayashi, H. Takahashi, et al. (2001). "Similarity and variation in gene expression among human cerebral cortical subregions revealed by DNA macroarrays: technical consideration of RNA expression profiling from postmortem samples." Brain Res Mol Brain Res **88**(1-2): 74-82.

Wei, J. and G. P. Hemmings (2000). "The NOTCH4 locus is associated with susceptibility to schizophrenia." Nat Genet **25**(4): 376-7.

Weinberger, D. R., M. S. Aloia, T. E. Goldberg and K. F. Berman (1994). "The frontal lobes and schizophrenia." J Neuropsychiatry Clin Neurosci **6**(4): 419-27.

Wetterling, T. (2001). "Bodyweight gain with atypical antipsychotics. A comparative review." Drug Saf **24**(1): 59-73.

Xie, E., L. Zhu, L. Zhao and L. S. Chang (1996). "The human serotonin 5-HT2C receptor: complete cDNA, genomic structure, and alternatively spliced variant." Genomics **35**(3): 551-61.

Yang, Y. H. and T. Speed (2002). "Design issues for cDNA microarray experiments." Nat Rev Genet **3**(8): 579-88.

Yuan, X., K. Yamada, S. Ishiyama-Shigemoto, W. Koyama and K. Nonaka (2000). "Identification of polymorphic loci in the promoter region of the serotonin 5-HT2C receptor gene and their association with obesity and type II diabetes." Diabetologia **43**(3): 373-6.

Zhang, J., M. Desai, S. E. Ozanne, C. Doherty, C. N. Hales, et al. (1997a). "Two variants of quantitative reverse transcriptase PCR used to show differential expression of alpha-, beta- and gamma-fibrinogen genes in rat liver lobes." Biochem J **321**(Pt 3): 769-75.

Zhang, J., H. Quan, J. Ng and M. E. Stepanavage (1997b). "Some statistical methods for multiple endpoints in clinical trials." Control Clin Trials **18**(3): 204-21.

Zhang, Z. J., X. B. Zhang, W. W. Sha and G. P. Reynolds (2002). "Association of a polymorphism in the promoter region of the serotonin 5-HT2C receptor gene with tardive dyskinesia in patients with schizophrenia." Mol Psychiatry **7**(7): 670-1.

Acta Universitatis Upsaliensis

Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology
Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology.* (Prior to October, 1993, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology".)

Distributor:
Uppsala University Library,
Box 510, SE-751 20 Uppsala, Sweden
www.uu.se,acta@ub.uu.se

ISSN 1104-232X
ISBN 91-554-5755-X

Errata

High-resolution Studies of mRNA Expression in Brain: A Search for Genes Differently Expressed in Schizophrenia

Anja Castensson 2003, Uppsala University

List of papers:
Paper IV: submitted manuscript

Page 3, figure 1.
The scale includes 100,000 genes queried on microarrays, while it should stop at 10,000 genes.

Page 11 piece 3, last sentence
The heritability (variance in a phenotype due to genes)
It should be:
The heritability (proportion of the variance in a phenotype due to genes)